UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE ECOLOGÍA

ECOLOGÍA EVOLUTIVA

GENÉTICA ECOLÓGICA DE *CARICA PAPAYA* EN LAS SELVAS TROPICALES DE MÉXICO

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

MARIANA CHÁVEZ PESQUEIRA

TUTOR PRINCIPAL DE TESIS: Dr. Juan Servando Núñez Farfán
Instituto de Ecología
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Dr. Mauricio Quesada Avendaño
Instituto de Investigaciones en Ecosistemas y Sustentabilidad

MÉXICO, D. F. **JUNIO, 2015**

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Director General de Administración Escolar, UNAM
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Me permito informar a usted, que el Comité Académico, en su sesión ordinaria del día 18 de mayo de 2015, aprobó el jurado para la presentación de su examen para obtener el grado de **DOCTORA EN CIENCIAS**, del Posgrado en Ciencias Biológicas de la alumna **CHÁVEZ PESQUEIRA MARIANA** con número de cuenta **402116440** con la tesis titulada **"Genética Ecológica de *Carica papaya* en las selvas tropicales de México"**, bajo la dirección del **DR. JUAN SERVANDO NÚÑEZ FARFAN:**

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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, D.F. a 04 de junio de 2015

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c.c.p. Expediente del (la) interesado (a).

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Índice

Resumen

México es considerado uno de los principales centros de domesticación de especies en el mundo, y el posible centro de origen y domesticación de la papaya (*Carica papaya*). La importancia de las zonas centros de origen de especies cultivadas radica en que poseen el capital de la diversidad genética necesaria para presentes y futuros planes de mejoramientos de los cultivos. Así mismo, la conservación de tales acervos genéticos es crucial para la conservación de la especie en general. A pesar de esto, poco se sabe sobre la distribución actual y el estado de la diversidad genética de poblaciones silvestres de papaya. En su forma silvestre, la papaya es un árbol raro, de corta vida y rápido crecimiento, asociado a claros o sitios con disturbio. Se distribuye de manera natural en las selvas tropicales y sub-tropicales de México, sin embargo, la conservación de su diversidad genética es amenazada por la fragmentación de su hábitat natural, la cual ha sido altamente fragmentada desde hace aproximadamente sesenta años, principalmente por actividades ganaderas. La fragmentación del hábitat provoca cambios en el ambiente que alteran los procesos biológicos que originan y mantienen la biodiversidad y la dinámica del ecosistema, y reducen la diversidad y abundancia de especies. Además, al reducir la conectividad entre las poblaciones, éstas pierden diversidad genética por deriva génica y endogamia. Asimismo, resulta de gran importancia determinar la historia evolutiva de las especies cultivadas en su zona centro de origen, ya que de esto dependerá el manejo adecuado y la conservación de las poblaciones o parientes silvestres. En la zona del sur de México y Centroamérica muy pocos estudios se han interesado en la historia evolutiva de

plantas tropicales, por lo que se sabe muy poco sobre la historia evolutiva de las especies que ahí habitan de manera natural, como la papaya.

Dada la importancia de conocer el estado de la diversidad genética de poblaciones naturales de *C. papaya* y de entender su historia evolutiva, los objetivos de esta tesis fueron evaluar el efecto de la fragmentación del hábitat en la diversidad y estructura genética de poblaciones silvestres de papaya en la selva fragmentada de Los Tuxtlas (Capítulo 1) y evaluar el estado de la diversidad genética e historia evolutiva en su área de distribución natural en México (Capítulo 2). Los principales resultados de esta tesis indican que la fragmentación del hábitat en la selva de Los Tuxtlas provoca una disminución en la diversidad genética de poblaciones dentro de los fragmentos y que las zonas deforestadas que rodean a dichos fragmentos tienen un efecto negativo en el flujo de genes, aumentando la diferenciación y endogamia de las poblaciones. A una mayor escala, en el Capítulo 2, encontramos una falta de estructuración genética en el pasado, pero una reciente estructuración, sugiriendo que el hábitat natural de la especie no sufrió grandes cambios en su historia, pero que las actividades humanas en la actualidad están teniendo un efecto en la estructura genética de la papaya silvestre.

Esta tesis constituye la primera evaluación del estado de la diversidad genética de poblaciones silvestres de *C. papaya* en México, así como, el primer estudio en evaluar la historia evolutiva de la especie.

Abstract

Mexico is considered as one of the main centers of domestication of species in the world, and possible center of origin and domestication of papaya (*Carica papaya*). The importance of the zones of centers of origin of important crop species, relies in the harboring of genetic resources needed for present and future management plans for crops. Moreover, the conservation of those genetic resources is crucial for the species' conservation. In spite of this, there is a lack of information about the actual distribution and genetic diversity of wild populations of papaya.

In its wild form, papaya is a rare, short-lived, fast growing tree, associated to gaps and disturbed sites. Its natural distribution includes tropical and sub-tropical forests in Mexico. However, the conservation of its genetic diversity is threatened by habitat fragmentation, which has been extremely high since approximately sixty years, mainly due to cattle ranching activities. Habitat fragmentation provokes changes in the environment that alter the biological processes that originate and maintain biodiversity and the ecosystem dynamics, and reduces diversity and species abundance. Furthermore, reductions in connectivity among populations may lead to a loss of genetic diversity due to genetic drift and inbreeding. Also, it is very important to resolve the evolutionary history of crop species in their center of origin, since this will determine an adequate management and conservation of the wild populations. In southern Mexico and Central America, few studies have addressed the evolutionary history of lowland tropical plants, like papaya.

Given the importance of knowing the state of the genetic diversity in natural populations of papaya, and to understand its evolutionary history, the aims of this thesis were to evaluate the effect of habitat fragmentation on the genetic diversity and structure of wild populations of papaya in the fragmented rainforest of Los Tuxtlas (Chapter 1) and to evaluate the state of the genetic diversity and evolutionary history in its natural distribution in Mexico (Chapter 2). The main results of this thesis indicates that habitat fragmentation in Los Tuxtlas rainforest leads to a reduction in genetic diversity of populations inside fragments and that deforested areas surrounding fragments have a negative effect on gene flow increasing differentiation and inbreeding in populations. At a bigger scale, we found a lack of genetic structuring in the past, but a recent one suggesting that the natural habitat of papaya did not suffer important changes in its history, but that recent human activities are changing the genetic structure of wild papaya in Mexico.

This thesis constitutes the first evaluation of the state of the genetic diversity of wild populations of *C. papaya* in Mexico, as well as the first study that evaluates the evolutionary history of the species. Knowing and conserving the actual state of wild populations of important crops is of utmost importance, since they represent the evolutionary potential of species. Their loss represents, not only the loss of the natural genetic diversity of species, but the loss of the genetic resources that can be essential for genetic crop improvement.

INTRODUCCIÓN GENERAL

Genética ecológica de *Carica papaya* en las selvas tropicales de México

Las especies domesticadas juegan un papel fundamental tanto cultural como económico a nivel mundial y son, en muchos casos, pieza indispensable de la seguridad alimentaria. En este contexto, es fundamental acumular información sobre el estado actual de las poblaciones silvestres que dieron lugar a las especies domesticadas, ya que representan los reservorios de diversidad genética de estas especies y pueden jugar un papel relevante en su conservación. De igual manera, es de suma importancia reconocer los principales procesos que podrían afectar dichos reservorios genéticos, tales como la perdida de hábitat, que pueden generar extinciones locales o afectar la conectividad genética entre poblaciones. Desde esta perspectiva es que esta tesis busca aportar información fundamental para la conservación de la papaya en su forma silvestre.

La papaya (*Carica papaya* L.) es un árbol tropical originario del continente americano. Es una especie de la familia Caricaceae, integrada por seis géneros y 35 especies, siendo *C. papaya* el único representante del género *Carica*, desde que Badillo (2000) rehabilitara el grupo *Vasconcella*, anteriormente considerado como una sección dentro del género *Carica*. La familia Caricaceae tiene su origen en el continente africano y posteriormente se dispersó a Centroamérica hace unos 35 millones de años (MA). La especie *C. papaya* y sus especies hermanas *Jarilla chocola* y *Horovitzia cnidoscoloides* divergieron del resto de los géneros sudamericanos, relativamente temprano en la historia de la familia (hace ca. 27 MA), y evolucionaron probablemente en alguna región del sur de México o Centroamérica (Aradhya et al. 1999, Carvalho y Renner 2012), mientras que *C. papaya* divergió de sus parientes más cercanos hace aproximadamente 25 millones de años (Carvalho y Renner 2012). El género *Vasconcella* actualmente contiene 21 especies y

es el género más grande de la familia, seguido por *Jacaratia* con 7 especies. La diversificación del género *Vasconcellea* se relaciona con el pico orogénico del norte de la cordillera de Los Andes, mientras que la diversificación del género *Jacaratia* parece relacionarse con la expansión de la vegetación adaptada a la sequía durante el Mioceno tardío (Carvalho y Renner 2012). Los géneros *Vasconcellea* y *Jacaratia* se distribuyen principalmente en Sudamérica, mientras que los géneros *Carica*, *Jarilla* y *Horovitzia* están distribuidos en México y Centroamérica. El género *Cylicomorpha* se distribuye en África y sólo contiene dos especies (Carvalho y Renner 2012).

La distribución natural actual de *C. papaya* es poco conocida, pero se sugiere que abarca desde el norte de Costa Rica hasta el límite tropical norteño de México (Aradhya et al. 1999, Carvalho y Renner 2012); sin embargo, actualmente *C. papaya* se cultiva ampliamente en muchas regiones tropicales del mundo, siendo una especie de gran importancia económica. Se estima que la papaya empezó a ser consumida por los habitantes antiguos de Mesoamérica alrededor del año 2000 A. C. (Miller 1989). Hoy en día, la papaya es la tercera fruta tropical más producida a nivel mundial, siendo Brasil el principal país productor y México el principal país exportador (Silva-Rosales y González de León 2005). El cultivo de papaya es importante por sus frutos comestibles y por la extracción de la enzima papaína, de diversos usos en la industria farmacéutica y alimenticia. En México, la papaya es la séptima especie cultivada en importancia económica (Molina y Córdova 2006). La principal variedad de *C. papaya* que se cultiva en México es la papaya maradol. Antes de la introducción de esta variedad cubana, en México se cultivaba principalmente la papaya amarilla (o mexicana); sin embargo, tras la

introducción de la variedad maradol, su producción bajó de drásticamente (SAGARPA 2009). Esta variedad es una planta de fácil cultivo, crecimiento acelerado y fructificación temprana. En términos económicos, la tasa interna de retorno es alta, lo que se traduce en que en un corto periodo de tiempo se recupera la inversión; además, tiende a adaptase a una gran diversidad de climas, con excepción de los que presentan heladas. En México, la papaya maradol tiende a presentar una demanda creciente y altos precios, lo que se ha traducido en un importante dinamismo exportador, así como un atractivo margen de rentabilidad. Las perspectivas de mediano plazo en cuanto a producción de la papaya en México son promisorias, dado que el producto muestra una gran aceptación en el mayor mercado del mundo (Estados Unidos) (Silva-Rosales y González de León 2005).

Las principales diferencias entre la papaya silvestre y la papaya cultivada radican en el tamaño del fruto y en la morfología floral. Los frutos de la papaya silvestre tienen un tamaño comparativamente menor a las cultivadas, conteniendo casi sólo semillas y un mesocarpo muy delgado (Manshardt y Zee 1994). Asimismo, las poblaciones naturales de papaya son estrictamente dioicas, mientras que en papayas cultivadas se pueden encontrar individuos femeninos, masculinos y hermafroditas (Carvalho y Renner 2012, Chávez-Pesqueira et al. 2014). En estado natural, *C. papaya* es un árbol raro, de corta vida y rápido crecimiento, asociado a claros o sitios con algún grado de disturbio en selvas tropicales y sub-tropicales (Paz y Vázquez-Yanes 1998). Sus flores son polinizadas principalmente por esfíngidos (Sphingidae: Lepidoptera) (OGTR 2008). La floración y fructificación ocurren a lo largo de todo el año (Chávez-Pesqueira *obs. pers.*) y las plantas pueden reproducirse ocho meses después de su establecimiento (Martínez-Ramos 1985). Los frutos son consumidos y

dispersados por aves y pequeños mamíferos (Chávez-Pesqueira *obs. pers.*). Las semillas germinan entre dos y tres semanas después de caer al suelo con un porcentaje de aproximadamente 30% de éxito para el día 40 (Paz y Vázquez-Yanes 1998). Ya que las semillas pueden permanecer viables por hasta tres años, constituyen un reservorio genético en el banco de semillas de su hábitat natural.

México es considerado uno de los principales centros de domesticación de especies en el mundo, y el posible centro de origen y domesticación de la papaya (Vavilov 1926). La importancia de las zonas centros de origen y diversidad de especies cultivadas radica en que poseen el capital de la diversidad genética necesaria para presentes y futuros planes de mejoramientos de los cultivos (Gepts y Papa 2003). Asimismo, la conservación de tales acervos genéticos es crucial para la conservación de la especie en general. A pesar de esto, existe poca información sobre la distribución actual y diversidad genética de poblaciones naturales de *C. papaya* (Silva-Rosales y González de León 2005), así como, pocos estudios que trabajen con papaya silvestre (Paz y Vázquez-Yanes 1998, Niklas y Marler 2007, Vega-Frutis y Guevara 2009, Brown et al. 2011).

En México, la conservación de la diversidad genética de la papaya silvestre es amenazada por diversos procesos. Por un lado, el hábitat natural de la especie, es decir, la selva tropical, ha sido altamente fragmentado desde hace aproximadamente sesenta años, principalmente por actividades ganaderas (Challenger 1998). La fragmentación del hábitat provoca cambios en el ambiente que alteran los procesos biológicos que originan y mantienen la biodiversidad y la dinámica del ecosistema (Saunders et al. 1991, Didham et al. 1996), y reducen la diversidad y abundancia de especies. Además, al reducir la

conectividad entre las poblaciones, éstas pierden diversidad genética por deriva génica y endogamia (Trakhtenbrot et al. 2005, Aguilar et al. 2008). Por otro lado, el posible flujo génico entre papayas domesticadas y sus parientes silvestres puede tener diversas consecuencias negativas a mediano y largo plazo. El proceso de domesticación de las especies generalmente involucra el manejo de poblaciones con menor diversidad genética debido a la selección de características de interés agronómico, tales como, mayor tamaño de los frutos, germinación sincrónica, reducción de la latencia, entre otros. En consecuencia, el flujo génico entre poblaciones cultivadas y silvestres podría modificar la funcionalidad ecológica y biológica de las plantas silvestres en el ecosistema (Ellstrand 2003) y afectar la capacidad de las poblaciones cultivadas para resistir enfermedades y plagas, así como pérdida de diversidad genética de las poblaciones silvestres (Gepts y Papa 2002). Aunado a esto, existen intenciones de liberar plantas de papayas transgénicas que sean resistentes al virus de la mancha anular, enfermedad que provoca numerosas pérdidas económicas a los productores de papaya (Silva-Rosales et al. 2010). Las consecuencias ecológicas y evolutivas de la introducción de individuos transgénicos en el centro de origen sugerido para la papaya, no han sido evaluadas científicamente.

Por lo anterior, resulta relevante conocer y conservar las poblaciones silvestres de especies cultivadas como reservorios de variación genética (Frankel 1970, Gepts y Papa 2002), e identificar los procesos que amenazan su conservación. Actualmente, la fragmentación del hábitat es considerada como una de las mayores amenazas a la biodiversidad (Wright 2010). Hasta ahora pocos estudios han evaluado los efectos de la fragmentación del hábitat sobre poblaciones silvestres. La papaya silvestre posee

características biológicas que la hacen altamente vulnerable a los efectos de la fragmentación de su hábitat: (i) por ser dioica, su reproducción es altamente dependiente de polinizadores; a su vez, éstos también pueden ser afectados por el aislamiento y distanciamiento de las poblaciones, disminuyendo el éxito reproductivo de las plantas, y eventualmente su diversidad genética (Ghazoul y Mcleish 2001), (ii) su tamaño efectivo puede ser alterado por cambios en la proporción de sexos en poblaciones fragmentadas (Dick et al. 2008), y (iii) su corto ciclo de vida promueve la aparición de los efectos negativos más rápidamente (Matthies et al. 2004). Además, los efectos de la fragmentación del hábitat sobre esta especie pueden ocasionar modificaciones a nivel ecológico ya que la papaya silvestre tiene un papel importante en la regeneración natural de las selvas tropicales debido a que es pionera y está asociada a la regeneración en la dinámica de claros, promoviendo el establecimiento de especies primarias y la sucesión ecológica (Martínez-Ramos 1985). Incluso, *C. papaya* es considerada como una especie con potencial para reforestación en zonas degradadas de selva (OGTR 2003).

Actualmente, no existen estudios en poblaciones silvestres de ninguna especie que sea cultivada bajo un enfoque de la fragmentación del hábitat, a pesar de la importancia de éstas como reservorios de diversidad genética de la especie (Gepts y Papa 2002). Por otro lado, poco se sabe sobre los efectos de fragmentación del hábitat en ecosistemas tropicales, ya que la mayoría de los estudios en plantas se ha realizado principalmente en especies templadas (Young et al. 1996, Fahrig 2003, Aguilar et al. 2008). Sin embargo, dadas las altas tasas de fragmentación y degradación de los ecosistemas tropicales, las investigaciones en especies tropicales son vitales. En el caso de la papaya no existe

ninguna evaluación de la diversidad genética de las poblaciones silvestres , ni estrategias de conservación.

Entender la historia evolutiva de las especies cultivadas en su zona centro de origen es de gran importancia para determinar el manejo adecuado y la conservación de las poblaciones o parientes silvestres. En la zona del sur de México y Centroamérica muy pocos estudios se han interesado en los patrones filogeográficos de plantas del Neotrópico (Gutiérrez-Rodríguez et al. 2011, Dick y Heuertz 2008), por lo que se sabe muy poco sobre la historia evolutiva de las especies que allí habitan de manera natural, como la papaya. El principal enfoque de los estudios filogeográficos de plantas en dicha zona, ha sido examinar eventos históricos tales como las expansiones/contracciones, la fragmentación y las migraciones a partir de refugios durante las fluctuaciones climáticas del Cuaternario de especies templadas (Gutiérrez-Rodríguez et al. 2011).

La distribución y composición de la flora del sur de México y Centroamérica ha sido fuertemente influenciada por eventos geológicos y climáticos, siendo de los más importantes los ocurridos durante el Neógeno, como la formación de los istmos Centroamericano y Tehuantepec, la formación de la Faja Volcánica Transmexicana y de las sierras de la región, así como, los cambios climáticos del Pleistoceno (1.8 millones a 10,000 años antes) (Luna-Vega 2008, Cavers et al. 2003). Sin embargo, se sabe poco sobre cómo las especies tropicales de tierras bajas respondieron a estos eventos. En la zona sureste de México se ha propuesto que dos eventos geográficos pudieron reducir el flujo génico influyendo en la estructuración espacial de la diversidad genética de las especies: el Istmo de Tehuantepec durante el Plioceno y los refugios del Pleistoceno. El Istmo de

Tehuantepec forma actualmente una estrecha franja de tierra baja uniendo las zonas montañosas del centro-sur de México y de Chiapas y de Centroamérica (Ornelas et al. 2010). Se ha sugerido que dicha zona fue un canal marítimo por mucho tiempo en el Plioceno y por lo tanto una barrera al flujo génico durante este periodo (Twyford et al. 2013). En cuanto a los cambios climáticos ocurridos en el Pleistoceno, Toledo (1982) propone que algunas zonas de México, Belice y Guatemala actuaron como refugios de especies tropicales de tierras bajas durante las épocas más desfavorables. Estos refugios son 1) la selva Lacandona, 2) las montañas mayas (Belice), 3) Petén (Guatemala), 4) Izabal (Guatemala), 5) Soconusco (Chiapas), 6) Los Tuxtlas (Veracruz), la Sierra de Juárez (Oaxaca) y 8) Córdoba (Veracruz).

Desafortunadamente hay pocos estudios que hayan evaluado la existencia de estos refugios (Ornelas et al. 2010, Cavers et al. 2003, Gutiérrez-Rodríguez et al. 2011), mientras que otros los han criticado argumentando que las selvas tropicales han permanecido relativamente estables a lo largo de su historia (Colinvaux et al. 2000, revisión en Ramírez-Barahona y Eguiarte 2013). Actualmente, existe más evidencia que avala al Istmo de Tehuantepec como una barrera al flujo génico cuando este estuvo sumergido, provocando diferenciación entre poblaciones a ambos lados en algunas especies tropicales de tierras bajas (Byrson et al. 2011, Gutiérrez-Rodríguez et al. 2011, Ornelas et al. 2013).

Objetivos

Los objetivos generales de esta tesis fueron conocer los efectos de la fragmentación del hábitat sobre poblaciones de papaya silvestre en la selva de Los Tuxtlas (Capítulo 1) y

evaluar el estado de la diversidad genética e historia evolutiva en su área de distribución natural en México (Capítulo 2).

Los objetivos particulares del Capítulo 1 fueron:

- Evaluar los cambios en la variación genética de poblaciones silvestres de *C. papaya* en la selva fragmentada de Los Tuxtlas, así como, su estructura genética.
- Analizar el flujo génico entre plantas de zonas fragmentadas y continuas.
- Analizar modificaciones en la proporción sexual como producto de la fragmentación del hábitat.

Los objetivos particulares del Capítulo 2 fueron:

- Conocer la distribución actual de poblaciones silvestres de papaya en México.
- Evaluar el estado de la diversidad y estructura genética de poblaciones silvestres de *C. papaya* en su distribución natural en México utilizando marcadores nucleares y de ADN de cloroplasto.
- Localizar zonas de mayor diversidad genética para fines de conservación.
- Inferir barreras históricas y recientes al flujo génico, así como, tasas de migración actuales entre poblaciones.
- Dilucidar sobre la historia evolutiva de la especie.

Finalmente, se incluyen como apéndices de la tesis los manuscritos **"Plant-antagonist interactions in fragmented habitats: does plant phylogeny matter?"** y **"Habitat**

fragmentation changes the adaptive value of seed mass on establishment of a tropical canopy tree". Ambos se realizaron durante mis estudios de doctorado y tratan temas relacionados con la fragmentación del hábitat en plantas. El primero es producto del curso *Introducción al metanálisis* impartido por el Dr. Ramiro Aguilar (Universidad de Córdoba, Argentina) en el CIEco, UNAM que tomé en mi segundo semestre de doctorado. Dicho artículo evaluó por medio de un metanálisis tradicional y un metanálisis independiente de la filogenia el efecto de la fragmentación del hábitat sobre la interacción antagonista entra las plantas y sus enemigos naturales. Recientemente, varias revisiones han evaluado el mismo efecto, sin embargo, muestran resultados contrastantes y no corrigen por el efecto de la filogenia de las plantas, presentando resultados poco generalizables. Asimismo, se examinó si ciertas características de la interacción, el ambiente fragmentado y el método utilizado en los estudios, tenían un efecto en la magnitud del efecto de la fragmentación del hábitat en la interacción. Los principales resultados de este trabajo para 98 especies de plantas de 54 familias con el metanálisis tradicional mostraron que las plantas que habitan en los fragmentos sufren menor daño por antagonistas. Este patrón de respuesta fue particularmente fuerte para la interacción de folivoría por insectos como antagonistas. Sin embargo, al efectuar al metanálisis independiente de la filogenia, el efecto global y el efecto particular de los moderadores se perdió. Analizando sólo a los insectos como enemigos naturales de las plantas, se encontró un efecto fuerte y consistente entre el metanálisis tradicional y el independiente de la filogenia, implicando por primera vez efectos genuinos que transcienden la filogenia de las plantas. Este estudio representa el primer metanálisis que incluye una corrección por la

filogenia de las especies sobre los efectos de la fragmentación del hábitat en interacciones planta-antagonista y ofrece una nueva perspectiva sobre la respuesta global de las plantas hacia sus antagonistas en ambientes fragmentados. El artículo se encuentra actualmente en revisión para su posible publicación en la revista Biological Conservation.

El segundo artículo es el trabajo que realicé durante la licenciatura y que escribí durante el doctorado. En este trabajo se evaluó el efecto de la fragmentación del hábitat sobre el establecimiento de plántulas del árbol tropical más abundante en la selva de Los Tuxtlas, *Nectandra ambigens*. En particular, se evaluó si los cambios en el ambiente provocados por la fragmentación del hábitat modificaban el valor adaptativo de la masa de la semilla en cuanto a la supervivencia y vigor de las plántulas, así como, en los ataques por herbívoros y patógenos. Para esto se pesaron las semillas de seis árboles y posteriormente se trasplantaron 1018 plántulas a cuatro sitios experimentales (dos dentro de la selva continua y dos dentro de fragmentos) en la zona de Los Tuxtlas. Durante 540 días se registró para cada plántula su supervivencia, vigor y ataques de herbívoros y patógenos. Los principales resultados de esta investigación indicaron que para *N. ambigens*, la fragmentación del hábitat provoca cambios en las condiciones abióticas y bióticas del sotobosque. En los fragmentos, al haber mayor incidencia de luz, las plántulas sobrevivieron más, y fueron más vigorosas y menos atacadas por sus enemigos naturales, mientras que el sotobosque de la selva continua representa un ambiente más estresante para el establecimiento de *N. ambigens*. Este cambio en el ambiente condiciona el valor adaptativo de la masa de la semilla en relación al establecimiento de plántulas. La selección cambió la composición genética hacia familias con semillas de mayor masa, ya

que éstas determinaron una mayor supervivencia de plántulas en el ambiente más estresante. En contraste, en los fragmentos las plántulas sobrevivieron más, independientemente de la masa de la semilla, y fueron más vigorosas y menos atacadas por sus enemigos naturales. Este trabajo muestra la importancia de evaluar los efectos de la fragmentación del hábitat sobre el establecimiento de árboles tropicales para prevenir o mitigar extinciones locales en ambientes altamente fragmentados. El artículo se encuentra actualmente en revisión para su posible publicación en la revista Biotropica.

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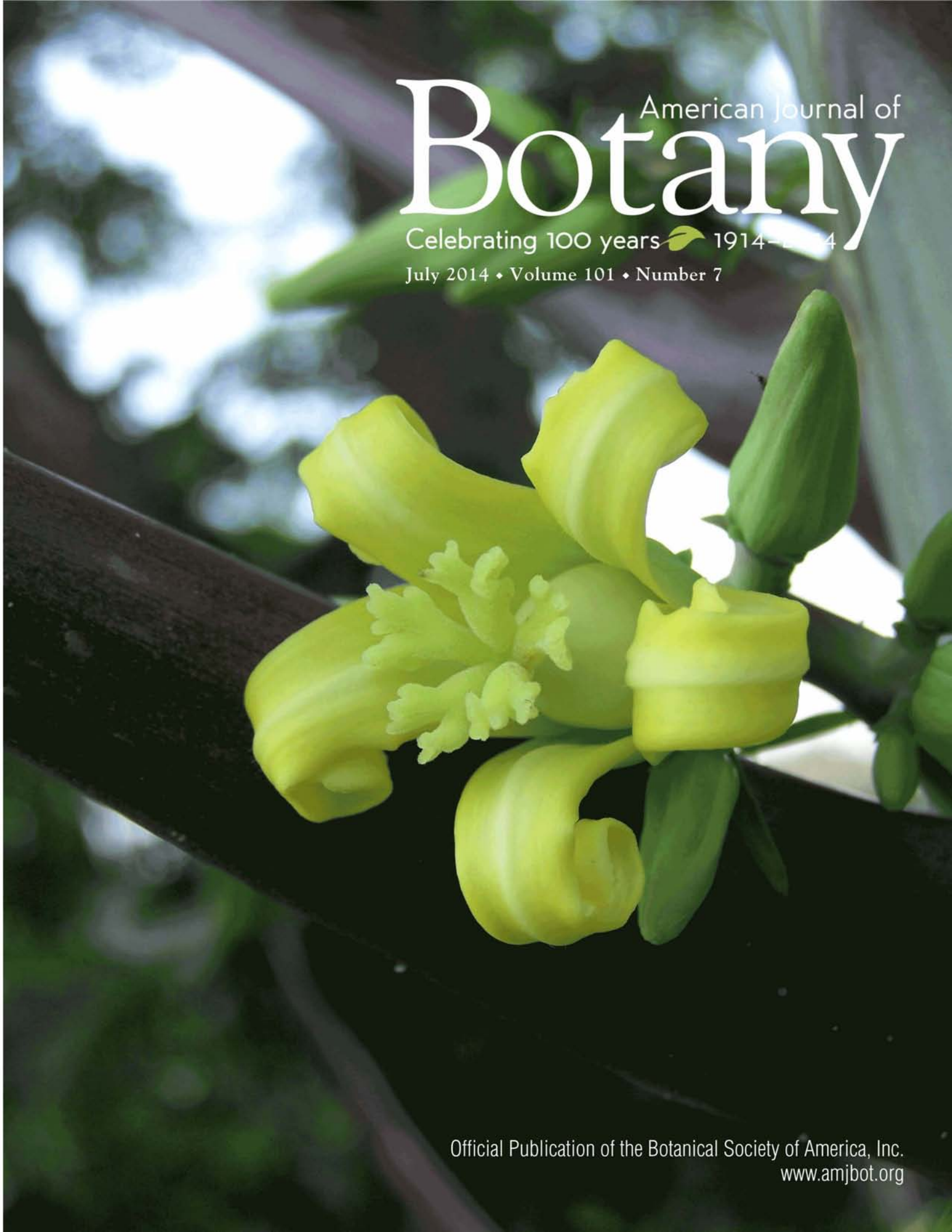
CAPÍTULO I

Habitat fragmentation threatens wild populations of *Carica papaya* (Caricaceae) in a lowland rainforest



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Cover Illustration: Female flower of a wild papaya tree (*Carica papaya;* Caricaceae) from the Los Tuxtlas rainforest, southeastern Mexico. Floral morphology represents one of the main differences between wild and cultivated papaya; natural populations of *C. papaya* are strictly dioecious, whereas cultivated plants can also be hermaphroditic. Wild papayas are mainly pollinated by hawk moths, and their fruits are dispersed by birds and small mammals. Natural populations of *C. papaya* are rare and persist naturally in tropical rainforests from Mexico to North Central America by colonizing large, newly created light gaps. The conservation of the natural habitats of wild populations of important crop plant species such as papaya is of the utmost importance to assure sufficient levels of genetic diversity to maintain the evolutionary potential of these species. In Mexico, the natural habitat of wild papaya has become extensively fragmented over the last 50 years. Due to the dioecy, rareness, and short life of the wild papaya, disturbances in its natural habitat could constrain the reproductive success of populations by altering gene flow and modifiying sex ratios. In this issue in "Habitat fragmentation threatens wild populations of *Carica papaya* (Caricaceae) in a lowland rainforest" on pp. 1092–1101, Chávez-Pesqueira et al. found that genetic variation and high population differentiation in the wild populations of papaya that inhabit forest fragments was lower than in populations in the continuous primeval forest. Moreover, sex-biased populations had reduced effective population sizes. Because agricultural lands and cattle pastures represent important barriers to gene flow of wild papaya in Los Tuxtlas, its mating system, rarity, and short life cycle exacerbate the effects of rainforest fragmentation on the genetic diversity and structure of wild populations, threatening the genetic reservoir and their persistence in papaya's proposed place of origin. *Photo credit*: Juan Núñez-Farfán.

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Abbreviations

Miscellaneous: AFLP, amplified fragment length polymorphisms; a.s.l., above sea level; bp, base pair; BP, before present; BSA, bovine serum albumin; cpDNA, chloroplast DNA; CTAB, hexadecyltrimethylammonium bromide; cv., cultivar; ddH_2O, double-distilled water; dNTP, deoxyribonucleotide E.C., Enzyme Commission; EDTA, ethylene diamine tetra-acetic acid; f. sp., forma specialis; indels, insertions and deletions; ITS, internal transcribed spacer; LM, light microscopy; mya, million years ago; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RAPD, random amplified polymorphic dimorphism; SDS, sodium dodecyl sulfate; SEM, scanning electron microscopy; s.l., sensu lato; s.s., sensu stricto; subsp., subspecies; TEM, transmission electron microscopy

Genetics: A, mean number of alleles per locus; D, mean genetic distance; CI, consistency index; F, fixation index; F_{IT}, total deviation from Hardy-Weinberg expectations; F_{ST}, genetic diversity among populations; F_{IS}, inbreeding within populations; G_{ST}, the proportion of genetic diversity among populations; H_e, Hardy–Weinberg expected heterozygosity; H_o, observed heterozygosity; MP, most parsimonious tree; n, individual chromosome number; Nm, mean number of migrants per generation; P_p, percentage of polymorphic loci; RI, retention index; x, base chromosome number

Statistics and math: ANOVA, analysis of variance; CV, coefficient of variation; df, degrees of freedom; N, number of individuals; p, probability; P, level of significance; PCA, principal components analysis; r, coefficient of correlation; SE, standard error; SD, standard deviation

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HABITAT FRAGMENTATION THREATENS WILD POPULATIONS OF *CARICA PAPAYA* (CARICACEAE) IN A LOWLAND RAINFOREST[1]

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- *Premise of the study:* Wild populations of domesticated species constitute a genetic reservoir and are fundamental to the evolutionary potential of species. Wild papaya (*Carica papaya*) is a rare, short-lived, gap-colonizing, dioecious tree that persists in the forest by continuous dispersal. Theoretically, these life-history characteristics render wild papaya highly susceptible to habitat fragmentation, with anticipated negative effects on its gene pool. Further, species dioecy may cause founder effects to generate local biases in sex ratio, decreasing effective population size.
- *Methods:* We contrasted the genetic diversity and structure of *C. papaya* between wild populations from rainforest fragments and continuous forest at Los Tuxtlas, Mexico. We evaluated recent migration rates among populations as well as landscape resistance to gene flow. Finally, we calculated the sex ratio of the populations in both habitats.
- *Key results:* Populations of wild papaya in rainforest fragments showed lower genetic diversity and higher population differentiation than populations in continuous rainforest. Estimates of recent migration rates showed a higher percentage of migrants moving from the continuous forest to the forest fragments than in the opposite direction. Agricultural land and cattle pasture were found to be the most resistant matrices to gene flow. Finally, biased sex ratios were seen to affect the effective population size in both habitats.
- *Conclusions:* The mating system, rarity, and short life cycle of *C. papaya* are exacerbating the effects of rainforest fragmentation on its genetic diversity, threatening the persistence of its natural populations in the proposed place of origin as well as its genetic reservoir.

Key words: *Carica papaya*; dioecy; gap-colonizing species; gene flow; genetic diversity; habitat fragmentation; landscape genetics; Los Tuxtlas; Mexico; population structure; sex ratio; wild papaya.

Land-use change is one of the most important anthropogenic drivers of biodiversity change that largely will determine the future of tropical forests in the 21st century (Sala et al., 2000; Wright, 2010). Habitat fragmentation is a remarkable consequence of land-use change, with tremendous implications for the conservation of tropical biodiversity (Wright, 2010). Changes in biotic interactions between plants and their mutualists, interruption of gene flow, and reduction of genetic variation and inbreeding are some of the negative consequences of habitat fragmentation that may eventually provoke the loss of target plant species. Moreover, wild populations and wild relatives of important crop species inhabiting tropical rainforests represent an important source of genetic diversity for future crop improvement (Jarvis et al., 2008). The present study aimed to study the genetic structure of wild populations of *Carica papaya* in a rainforest of Mexico in the context of habitat fragmentation. Wild populations of *C. papaya*, which naturally occur in tropical rainforests of Mexico, have been reduced due to the loss of large areas of this diverse ecosystem. Rainforests now form a collection of isolated forest fragments, mainly surrounded by cattle pasture (Guevara et al., 2004; Mendoza et al., 2005).

Two factors in particular—distance between fragments and the characteristics of the surrounding land-use matrix—can disrupt interactions between plants and their mutualists, and are expected to affect plant mating systems (Cordeiro and Howe, 2003; Quesada et al., 2004). Reproductive success also can be reduced by fertilization failure, seed abortion, seed viability, seed size, and/or germination capability (Ghazoul and McLeish, 2001). Furthermore, if dispersal among fragments is disrupted, genetic diversity can be reduced, increasing the probability of biparental inbreeding, population homozygosity, and genetic differentiation among adjacent populations (Hamilton, 1999; Ghazoul and McLeish, 2001). In such cases, low effective population sizes are expected, threatening the long-term persistence of plant populations unless dispersal toward isolated populations is restored (Trakhtenbrot et al., 2005).

The strength of negative effects by habitat fragmentation depends on life-history characteristics such as mating system, life span, and rareness. Thus, the negative effects of habitat fragmentation are expected to be more severe for self-incompatible and dioecious species, as well as for short-lived, rare species

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(Leimu et al., 2006). Habitat fragmentation may change mating patterns by increasing self-fertilization in compatible species (Aguilar et al., 2008). Such changes raise concern regarding the future of self-incompatible and dioecious species in fragmented habitats. Although dioecy promotes outcrossing and potentially enhances gene flow (Ellstrand and Elam, 1993; Petit and Hampe, 2006), dioecious species are the most vulnerable to the effects of habitat fragmentation (Murcia, 1996) for different reasons. First, both sexes must be present in the same fragment to achieve reproduction, unless pollen flow between fragments is pervasive (Murcia, 1996). The genetic drift associated with habitat fragmentation can decrease population fitness by changing sex ratios (Byers and Meagher, 2005) and by increasing the level of inbreeding (Dick et al., 2008). In addition, the effective population size of populations is half that of hermaphroditic plants species for the same abundance; since only females are capable of dispersing seeds and only males disperse pollen, effective population sizes can be further reduced if the sex ratio departs from unity (Dick et al., 2008).

The appearance of negative effects on plant populations in connection with habitat fragmentation also is related to species life span. Short-lived species are more prone to negative genetic consequences and are likely to suffer greater effects from genetic drift (Ellstrand and Elam, 1993). Likewise, because the loss of genetic diversity can only become apparent over generations, short-lived plants are expected to express this loss sooner than long-lived plants. By contrast, in long-lived species, the negative effects of habitat fragmentation may not be detected in the adult population, but rather in their progeny (Aguilar et al., 2008) or at the juvenile stage.

Few studies have evaluated the effects of habitat fragmentation on the genetic diversity of dioecious species (Aguilar et al., 2008; Vranckx et al., 2012). Short-lived species have been explored even less (Leimu et al., 2006; Aguilar et al., 2008). Few empirical studies have addressed genetic structure in wild, natural populations of tropical domesticated plants in terms of habitat fragmentation. To fill these gaps, we studied the effects of habitat fragmentation in the wild form of *Carica papaya* (Caricaceae), a dioecious, short-lived, rare species of great economic importance (FAOSTAT, 2012).

The proposed origin and domestication center of *C. papaya* is Mesoamerica, from Mexico to North Central America (Carvalho and Renner, 2012), but today papaya is cultivated in tropical and subtropical areas worldwide (FAOSTAT, 2012). Mexico is the leading exporter of papaya, with 41% of the trade. The main papaya plantations are located in the southeastern part of the country, in the states of Veracruz, Chiapas, Oaxaca, Michoacán, Tabasco, and Yucatán (FAOSTAT, 2012), where wild populations of the species persist and where habitat fragmentation has been extensive over the last 50 yr (Mendoza et al., 2005). *C. papaya* is regarded as a species with the potential to restore degraded rainforest (OGTR, 2008). Its behavior as a typical fast-growing, short-lived pioneer tree, allows it to establish and grow rapidly in recent canopy gaps in the primary forests, as well as in early secondary vegetation (Paz and Vázquez-Yanes, 1998).

In this study, we assessed the effects of habitat fragmentation in the Los Tuxtlas rainforest on the genetic diversity, genetic structure, and sex ratios of natural populations of *C. papaya*. Specifically, we asked (1) whether habitat fragmentation affects the distribution of genetic diversity, (2) whether population differentiation increases among fragmented populations, (3) whether change in land-use restricts gene flow among populations, and (4) to what extent habitat fragmentation affects sex ratios and, consequently, effective population sizes.

MATERIALS AND METHODS

Study system and study site*—Carica papaya* (Caricaceae) is a rare, short-lived arborescent plant native to the neotropics (Fig. 1). Wild populations of *C. papaya* are found in tropical forests from Mexico to Costa Rica (Carvalho and Renner, 2012). In the primeval lowland rainforest of Los Tuxtlas, *C. papaya* only regenerates in large light-gaps in the forest (≥400 m^2; Fig. 1) (Martínez-Ramos, 1985; Núñez-Farfán and Dirzo, 1988) where it can live up to 10 yr. It dies sooner when shaded by other more common, taller, long-lived (up to 30 yr) gap-colonizing trees such as *Cecropia obtusifolia*, *Heliocarpus appendiculatus*, *Omphalea oleifera*, or *Trema micrantha* (M. Chávez-Pesqueira and J. Núñez-Farfán, personal observations). *C. papaya* behaves like a prototypical "nomad" species (van Steenis, 1958), persisting in the forest only by continuous dispersal and the colonization of large, recently created forest gaps (Martínez-Ramos, 1985; Núñez-Farfán and Dirzo, 1988; Paz and Vázquez-Yanes, 1998). In the Los Tuxtlas rainforest, it is a rare species, with small populations only in forest gaps of particular sizes and ages. The wild papaya is a dioecious tree (Fig. 1) pollinated mainly by hawk moths (Sphingidae: Lepidoptera) (OGTR, 2008). Flowering and fructification occur year round (M. Chávez-Pesqueira, personal observation), and plants can reproduce within 8 mo after gap colonization (Martínez-Ramos, 1985). The fleshy fruits of wild papaya are dispersed by birds and small mammals (Fig. 1; M. Chávez-Pesqueira, personal observation). Seeds germinate within 2–3 wk after sowing with approximately 30% germination by day 40 (Paz and Vázquez-Yanes, 1998). Because seeds can remain viable for 3 yr, they constitute a genetic reservoir in the forest seed bank. The main differences besides plant size between wild and cultivated papaya are clearly noticed in fruit size and in floral types (Fig. 1); wild populations are strictly dioecious, while cultivated plants can also exhibit hermaphroditic individuals (Carvalho and Renner, 2012).

Our study was conducted at the Los Tuxtlas Biosphere Reserve in southern Veracruz, Mexico, where natural populations of *C. papaya* occur. Tropical rainforest is the predominant vegetation. The landscape of Los Tuxtlas has been severely altered over the past 50 yr. Estimates indicate that about 75% of the original vegetation has been destroyed (Figs. 2, 4a), while 20% remains as forest fragments and only 5% remains as large, continuous, protected forests (Estrada and Coates-Estrada, 1996).

***Data collection*—**Foliar tissue was collected from 211 trees, from four continuous forest populations (C1: Circuito 1, C2: Vigía, C3: Lyell, and C4: Límite Norte), and from four different forest fragment stands (F1: Dos Amates, F2: Playa, F3: Cerro Borrego, and F4: Ruiz Cortines) (Fig. 2, Table 2). Because of the small size of *C. papaya* populations (~30 individuals), we were able to sample all individuals, and thus our studied populations represent the real population sizes.

Molecular markers and DNA isolation—DNA was extracted using the CTAB method (Doyle and Doyle, 1987) with some modifications, then amplified for six microsatellite loci (Table 1) in a Thermo PX2 thermal cycler (Thermo Electron, Waltham, Massachusetts, USA) following Ocampo et al. (2006) (denaturation at 94°C for 5 min, 35 cycles of 30 s at 94°C; 1 min at 46°C or 50°C; 45 s at 72°C; and a final elongation for 4 min at 72°C). Each PCR reaction (15 μL) contained the following: 7.5 μL of RED Taq ReadyMix PCR Reaction Mix (with 20 mmol/L Tris HCl pH 8.3); 100 mmol/L KCl; 3 mmol/L $MgCl_2$; 0.002% gelatin; 0.4 mmol/L dNTP mix (dATP, dCTP, dGTP, dTTP); stabilizers; 0.06 unit/μL of *Taq* DNA polymerase) (Sigma-Aldrich; St. Louis, Missouri, USA); 0.5 μL of forward and reverse primers (10 μmol/L); 5.5 μL of pure water; and 1 μL of genomic DNA. Amplification products were separated in 8% polyacrylamide gels by electrophoresis at 300 V for 4 h. The gels were stained with silver nitrate.

Of 18 primers from Ocampo et al. (2006) that we tested, six gave good resolution and variation (Appendix S1; see Supplemental Data with the online version of this article). These six primers were then used to obtain the genetic data for the populations of *C. papaya* in both habitats (Table 1).

Individuals of *C. papaya* were sexed using a male-specific, sequence-characterized, amplified region (SCAR) marker, using the original conditions reported by Urasaki et al. (2002). PCR products were separated on 1.5% agarose gels by electrophoresis at 100 V for 50 min. The gels were stained with ethidium bromide. The presence of bands indicated male individuals, and the absence of bands indicated female plants.

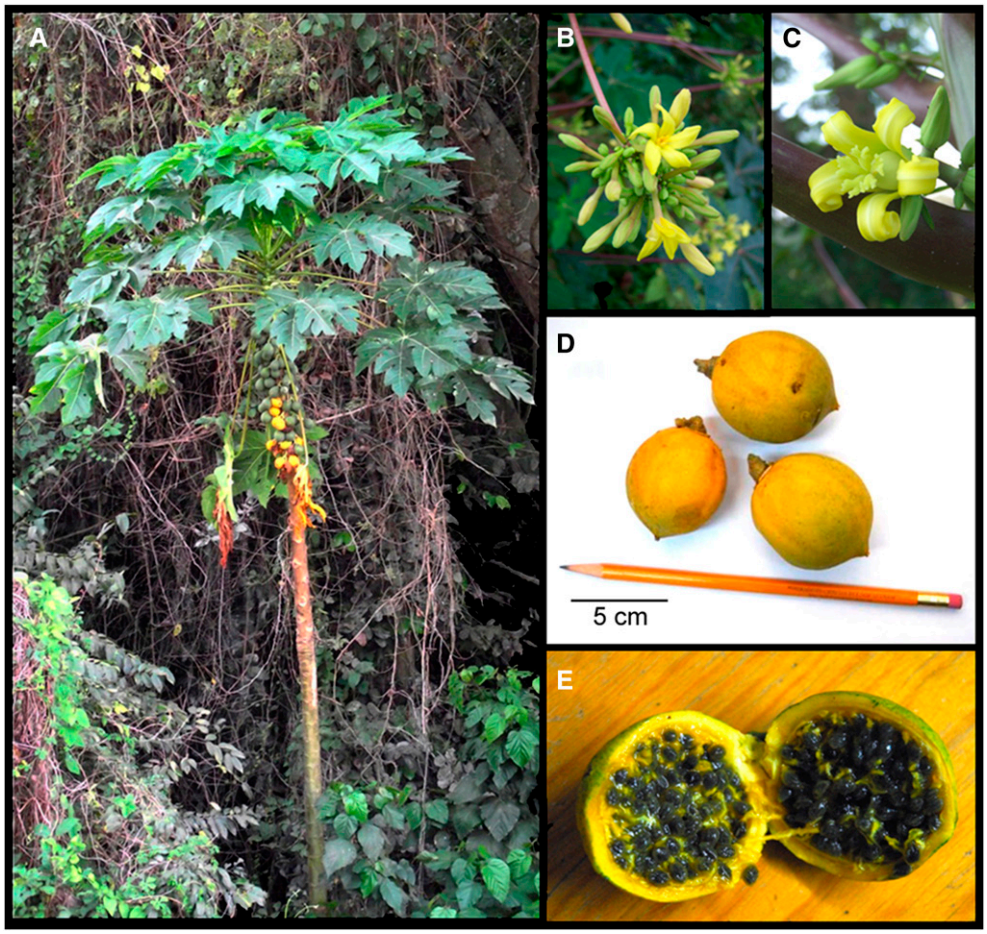

Fig. 1. *Carica papaya*. (A) Female tree in a large forest gap; (B) male and (C) female flowers; (D, E) fruits and seeds.

***Data analyses*—**Standard diversity indices (such as the mean number of alleles per locus [A], mean observed [H_o] and expected [H_E] heterozygosities, allelic richness, and fixation indices [f]) were estimated for each population using FSTAT 2.9.3.2 software (Goudet, 2002). This program was also used to test for differences between groups of populations (continuous forest vs. forest fragments). These tests assumed Hardy–Weinberg equilibrium within samples and used 1000 permutations. Fixation indices were also estimated, together with their 99% confidence intervals, as obtained by bootstrapping (n = 1000).

We further evaluated the genetic differentiation of the populations by assuming a stepwise mutation model with Slatkin's R_{ST} (Slatkin, 1995). In addition, hierarchical genetic structure was determined by AMOVA using ARLEQUIN (Excoffier, Laval, and Schneider, 2005). The program STRUCTURE 2.3.3 (Pritchard et al., 2000) was used to measure levels of genetic admixture and to infer the number of genetic clusters. Thirty iterations were run under an admixture model using correlated frequencies with values of K ranging from 1 to 10. Each run had a burn-in period of 200 000 followed by 1 000 000 Markov chain Monte Carlo replicates. The final K value was determined as done by Evanno et al. (2005).

The program BAYESASS edition 3 (Wilson and Rannala, 2003) was used to estimate short-term migration rates (i.e., after fragmentation of Los Tuxtlas). BAYESASS uses a genetic assignment to estimate short-term dispersal rates, providing an estimate of migration rates over the past two generations. This is in contrast to coalescent methods, which are closer to long-term averages (Paetkau et al., 2004). We performed five runs (each with different starting seed values) of 10 million generations, with a 1 million generation burn-in, and sampled the chain every 2000 generations.

***Landscape genetics*—**We used the program CIRCUITSCAPE 3.4.2 (McRae, 2006) to model landscape conductance to gene flow. The CIRCUITSCAPE algorithm evaluates the total landscape resistance/conductance between sampling sites, based on multiple paths (McRae, 2006). The program generates maps of current (an analogue of gene flow or dispersal density), indicating potentially important areas for maintenance of, or constraints to, functional connectivity. We used land cover to calculate resistance/conductance of the landscape, because dispersers activity, and thus gene flow, for *C. papaya* was anticipated to be highly compromised in cattle pasture and agricultural land and slightly compromised in land with secondary vegetation. As seen in Fig. 4A, each cell of the raster was assigned a categorical conductance value corresponding to the relative probability of dispersal through the habitat type (land cover) encoded by the cell (3 for primary forest, 2 for secondary vegetation, and 1 for cattle pasture and agricultural land). In addition, we used Mantel's tests (Mantel, 1967) for IBD (isolation by distance) and IBR (isolation by resistance) analysis, to determine whether matrices of pairwise population genetic distances [$R_{ST} / (1 - R_{ST})$] were correlated to geographic distance and/or to landscape-derived resistance values. The matrix of resistance distances was obtained from CIRCUITSCAPE. We calculated Mantel's correlation coefficients (r) using the ade4 package (Dray and Dufour, 2007) in the program R (R Development Core Team, 2011). The statistical significance of the estimators was determined with 9999 permutations.

***Sex ratio and effective population size*—**Deviations from a 1:1 sex ratio at both population and habitat levels (i.e., continuous forest or fragment) were assessed with a log-normal model assuming a Poisson-type error, using the program R (R Development Core Team, 2011). In this model, a significant sex × habitat interaction would indicate differences in the sex ratio between the continuous forest and the fragmented forest, whereas differences among populations would be identified by a significant sex × population interaction, regardless of habitat. An ideal population and an ideal habitat, each with a 1:1 sex ratio, were included in the models as an intercept to contrast with the observed data.

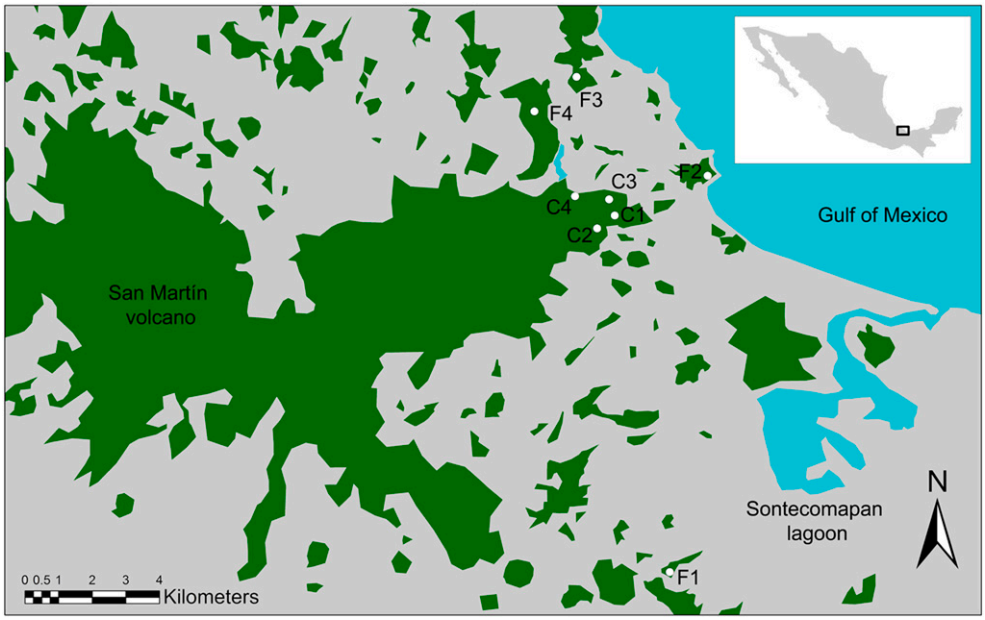

Fig. 2. Populations of *Carica papaya* sampled for the genetic analyses in LosTuxtlas region, southern Mexico. Dark green areas represent the tropical rainforest remnants. White dots represent the sampled populations of *C. papaya*.

Effective population size (N_e) was determined from the sex of each individuals, by assuming that half of the gametes come from individuals of one sex and that N_e depends on the number of individuals of each sex, as follows: $1N = 1/4\ N_f + 1/4N_m$, where N_f and N_m are the number of female and male plants in the total population, respectively (Hedrick, 2011).

RESULTS

***Genetic diversity and structure*—**All loci were polymorphic, and no difference in the mean number of alleles between habitats was detected. Estimates of genetic diversity showed that populations in fragments have signif cantly lower values than populations in the continuous forest. Both genetic diversity (H_E) and observed heterozygosity (H_O) were lower in fragmented populations ($P = 0.011$ and $P = 0.028$, respectively). Likewise, allelic richness was signif cantly lower for populations in fragments ($P = 0.011$; Table 2).

Populations in the continuous forest showed low genetic differentiation among them ($F_{ST} = 0.065$, 95% conf dence interval [CI] = 0.033–0.096); this contrasted strongly with the high and signif cant values of differentiation observed among populations in fragments ($F_{ST} = 0.272$, 95% CI = 0.181–0.377). Across all populations, mean F_{ST} and R_{ST} values were 0.17 and 0.12, respectively. The STRUCTURE analysis showed that populations could be grouped into f ve clusters ($K = 5$). The four continuous forest populations showed genetic admixture (one admixed cluster), while the fragmented populations showed differentiation (four clusters); populations F3 and F4 appeared to be more isolated, while fragments F1 and F2 showed a certain degree of genetic admixture (Fig. 3). The AMOVA showed that most of the variation was contained within populations (87.11%), and that only a negligible fraction (0.51%) was contained within forest types (continuous vs. fragmented) (Table 3). Positive f xation indices indicated a general def ciency of heterozygotes in all populations, and although the average value was higher in fragments, the difference was not statistically signif cant (continuous forest: $f = 0.453$, 99% CI = 0.265–0.656; fragments: $f = 0.579$, 99% CI = 0.317–0.796).

TABLE 1. Microsatellite loci of *Carica papaya*.

Locus name [a]	Motif repeat	Primer sequences (5′-3′)	No. alleles	Allele size
m*Cp*C1R2	$(TC)_{24}$	F: GTCTATCTACCTCCCA R: GAGTGTTATCATAGTCTACA	8	260–284
m*Cp*C1R6	$(TG)_{10}$ $(AG)_7$ $(GA)_{10}$	F: CCAAAACGGAAAACAC R: ATCAAGCTCCCTTTCAC	6	269–281
m*Cp*C1R8	$(CT)_{20}$… $(AC)_5$	F: ATGGCTGAAGACAACTC R: CTCAATAGCCCAATAACA	4	283–293
m*Cp*C1R9	$(CT)_9$	F: TAAAACCCTAACGAGCA R: CAAAGAGCAGACTTGGA	4	130–142
m*Cp*C1R10	$(TA)_4$…$(AG)_{18}$	F: CAGCAGAAAACAAGGG R: GGGTTCCGGTTTAGTT	4	341–349
m*Cp*C1R11	$(GA)_5$ … $(GA)_{13}$ … A… $(AG)_4$	F: GGTGCCCTAATTTTCA R: ACTCGTAAAGAAAACCCA	4	219–229

[a] Ocampo et al. (2006).

TABLE 2. Global and habitat average values of genetic variation estimates of *Carica papaya* (± SD) at Los Tuxtlas rainforest. N = population size; A = mean allele number (± SD); H_O = observed heterozygosity (± SD); H_E = expected heterozygosity (± SD); f = fixation index (99% CI by bootstrap, N = 1000).

Habitat	Population	Coordinates	N	A	H_O	H_E	f
Continuous forest	Circuito 1 (C1)	18°34′53.67″N, 95°4′31.23″W	30	5.00 (1.673)	0.333 (0.203)	0.689 (0.114)	0.517 (0.241–0.825)
	Vigia (C2)	18°34′41.21″N, 95°4′48.13″W	31	4.83 (1.602)	0.403 (0.265)	0.742 (0.068)	0.452 (0.204–0.805)
	Lyell (C3)	18°35′9.19″N, 95°4′36.55″W	15	4.33 (1.506)	0.300 (0.196)	0.606 (0.147)	0.516 (0.302–0.840)
	Límite Norte (C4)	18°35′12.20″N, 95°5′9.38″W	30	4.66 (1.211)	0.483 (0.151)	0.752 (0.047)	0.360 (0.164–0.565)
	Mean		106	5.00 (1.673)	0.391 (0.137)	0.752 (0.068)	0.453 (0.265–0.656)
Fragmented forest	Dos Amates (F1)	18°29′10.13″N, 95°3′38.25″W	31	4.00 (1.095)	0.193 (0.125)	0.527 (0.169)	0.653 (0.213–0.902)
	Playa (F2)	18°35′31.89″N, 95°3′1.88″W	30	4.00 (0.000)	0.211 (0.152)	0.598 (0.056)	0.636 (0.341–0.873)
	Borrego (F3)	18°37′7.16N, 95°5′7.95″W	29	3.00 (0.632)	0.212 (0.136)	0.415 (0.175)	0.474 (0.031–0.821)
	Ruiz Cortines (F4)	18°36′33.98″N, 95°5′48.52″W	15	3.33 (0.816)	0.333 (0.133)	0.518 (0.186)	0.383 (0.047–0.688)
	Mean		105	5.00 (1.673)	0.223 (0.106)	0.663 (0.077)	0.579 (0.317–0.796)
Grand mean			211	5.00 (1.673)	0.308 (0.099)	0.727 (0.061)	0.508 (0.379–0.715)

Recent migration rates among populations, as estimated using BAYESASS, indicate a higher flow from continuous forest to forest fragments (0.0608, SD 0.0160) than in the opposite direction (0.0267, SD 0.0150) (Table 4). Using the migration rate estimates from 15 runs with different starting seed values, we detected significant differences among habitats (t_{28} = 430.43, P < 0.0001).

***Landscape genetics*—**First, current maps of resistance/conductance among populations, as generated by CIRCUITSCAPE, showed several paths of high conductance (i.e., gene flow), mainly between continuous forest populations (Fig. 4B, red). Second, paths of medium conductance (i.e., possible routes of gene flow) among sampled populations of *C. papaya* were detected near the primary forest and secondary vegetation (Fig. 3B, yellow and light blue). Third, areas of low conductance were detected in agricultural land and cattle pasture (Fig. 4B, dark blue). Nonetheless, Mantel's tests demonstrated no correlation between the genetic distance and geographic distance of populations (r = 0.201, P = 0.310), or between the genetic distance and resistance values of populations (r = 0.054, P = 0.439).

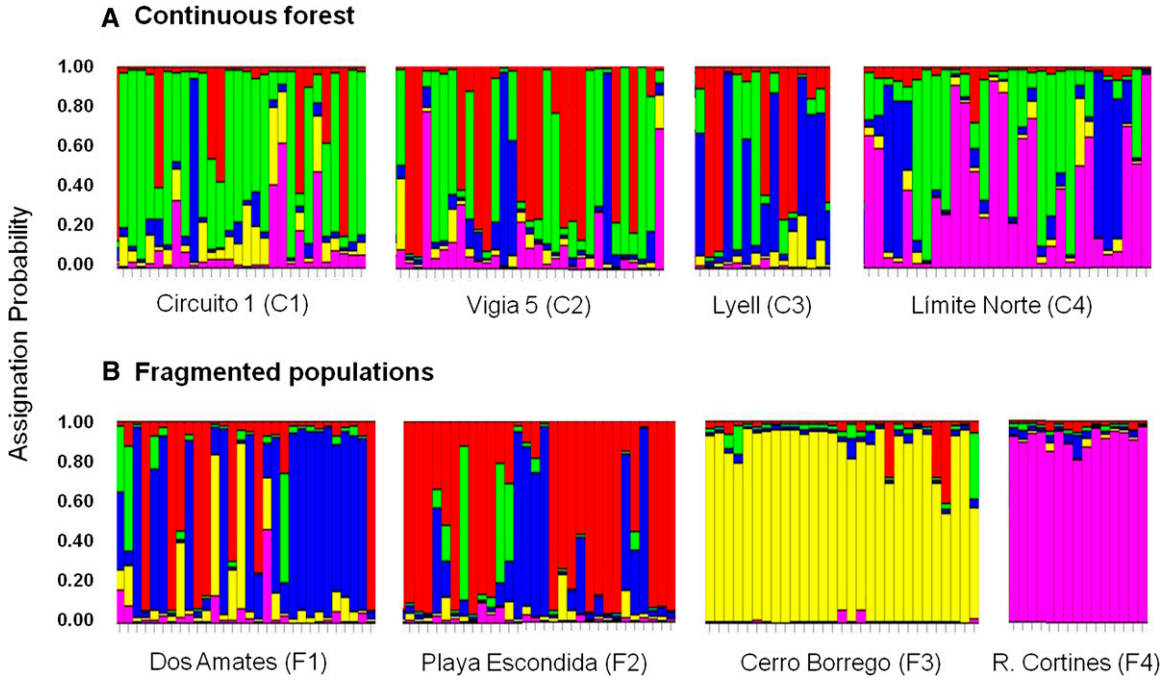

Fig. 3. Colored vertical lines represent the probabilities of individual assignment to each of the five genetic groups (K = 5, indicated by colors), for the (A) continuous and (B) fragmented populations. Names of populations of each habitat are labeled below.

TABLE 3. Analysis of molecular variance (AMOVA) of eight populations of *Carica papaya* at Los Tuxtlas, southern Mexico.

Source of variation	df	Sum of squares	Variance component	% Variation explained
Between habitats	1	874.45	0.65	0.51
Among populations	6	5653.76	16.03	12.39*
Within populations	414	47204.94	114.02	87.11*

Note: *$P < 0.05$.

Sex ratio and effective population size— Generally, both habitats showed the same proportion of male and female plants (χ^2_8 =1.84, P = 0.397) although female plants were slightly more frequent than males in both (133:78). At the population level, however, signif cant differences were found in the sex distribution (χ^2_8 =27.46; P = 0.001). Populations C4 (χ^2 = 6.93; p = 0.008) and F4 (χ^2 = 4.93; P = 0.026) showed the greatest female-biased sex ratios (25 : 5 and 13 : 2, respectively). The effective population size, based on these latter ratios was close to the observed population sizes, but the two populations with a signif cantly biased sex ratio (C4 and F4) showed reduced effective population sizes (Table 5).

DISCUSSION

Land-use change and consequently habitat fragmentation, constitute some of the most important drivers of biodiversity change now and in the future (Sala et al., 2000; Wright, 2010). Besides the relevance of conserving wild populations of *Carica papaya* for their evolutionary importance, this species combines certain ecological characteristics that render it as very vulnerable to tropical rainforest fragmentation. *C. papaya* is a dioecious, short-lived, rare species. For these reasons, wild papaya represents an important study system to address the effects of habitat fragmentation in its natural habitat. Taken as a whole, our results show that for wild papaya trees, the reproductive consequences of habitat fragmentation translate into a loss of genetic diversity and an increase in population differentiation. To our knowledge, this is the first study to measure the genetic diversity of wild populations of a culti vated species in a fragmented landscape (see Brown et al., 2012). Moreover, this is the f rst study on the effects of habitat fragmentation on the genetic structure, genetic diversity, and local sex ratios of a dioecious, short-lived, pioneer tree species in a tropical rainforest.

Although theory predicts that habitat fragmentation and the isolation of populations can lead to genetic drift and inbreeding (Young et al., 1996), some authors argue that only a few studies have reported these effects (Kramer et al., 2008). In contrast, reviews by Aguilar et al. (2008) and Vranckx et al. (2012) have shown that the genetic diversity of plants is strongly and negatively affected by habitat fragmentation. Our f ndings support the latter hypothesis and show that wild populations of *C. papaya* in forest fragments contain less genetic variation than populations located in continuous tropical rainforest. Specif cally, although fragmented and continuous forest populations of *C. papaya* do both contain the same *number* of alleles, local populations in fragmented forests possess particular sets of alleles not shared among populations, as indicated by the higher allelic richness found in the continuous forest populations. This f nding suggests that fragments

TABLE 4. Recent migration rate (SD) between pairs of populations of *Carica papaya* in the rainforest of Los Tuxtlas. Values along the diagonal (in italics) are the self-recipient rates into the source population. The total values (in boldface) represent the sum of values from source populations of the continuous forest and from source populations of fragments to each recipient population (values along the diagonal were not included in the sum).

Recipient population		Source population										
		Continuous forest					Fragments					
	Population	C1	C2	C3	C4	**Total**	F1	F2	F3	F4	**Total**
Continuous forest	C1	*0.8797 (0.0309)*	0.0203 (0.0184)	0.0412 (0.0230)	0.0124 (0.0117)	**0.0739**	0.0117 (0.0114)	0.0150 (0.0134)	0.0101 (0.0099)	0.0095 (0.0092)	**0.0463**
	C2	0.0736 (0.0383)	*0.7661 (0.0491)*	0.0621 (0.0320)	0.0213 (0.0177)	**0.1571**	0.0157 (0.0144)	0.0399 (0.0268)	0.0115 (0.0110)	0.0098 (0.0098)	**0.0654**
	C3	0.0219 (0.0195)	0.0321 (0.0255)	*0.8059 (0.0437)*	0.0166 (0.0158)	**0.0706**	0.0584 (0.0369)	0.0340 (0.0298)	0.0163 (0.0157)	0.0149 (0.0143)	**0.1236**
	C4	0.0230 (0.0195)	0.0126 (0.0123)	0.0135 (0.0127)	*0.8678 (0.0418)*	**0.0491**	0.0145 (0.0132)	0.0122 (0.0116)	0.0110 (0.0108)	0.0455 (0.0329)	**0.0832**
Fragments	F1	0.0110 (0.0105)	0.0092 (0.0090)	0.0108 (0.0107)	0.0112 (0.0106)	**0.0422**	*0.9209 (0.0262)*	0.0152 (0.0141)	0.0123 (0.0117)	0.0089 (0.0088)	**0.0364**
	F2	0.0106 (0.0101)	0.0253 (0.0196)	0.0175 (0.0152)	0.0108 (0.0102)	**0.0642**	0.0193 (0.0167)	*0.8975 (0.0295)*	0.0100 (0.0099)	0.0089 (0.0087)	**0.0382**
	F3	0.0097 (0.0093)	0.0094 (0.0091)	0.0099 (0.0095)	0.0091 (0.0088)	**0.0381**	0.0107 (0.0100)	0.0096 (0.0093)	*0.9326 (0.0223)*	0.0091 (0.0087)	**0.0294**
	F4	0.0149 (0.0143)	0.0159 (0.0152)	0.0143 (0.0139)	0.0207 (0.0197)	**0.0658**	0.0164 (0.0157)	0.0147 (0.0141)	0.0144 (0.0139)	*0.8887 (0.0346)*	**0.0455**

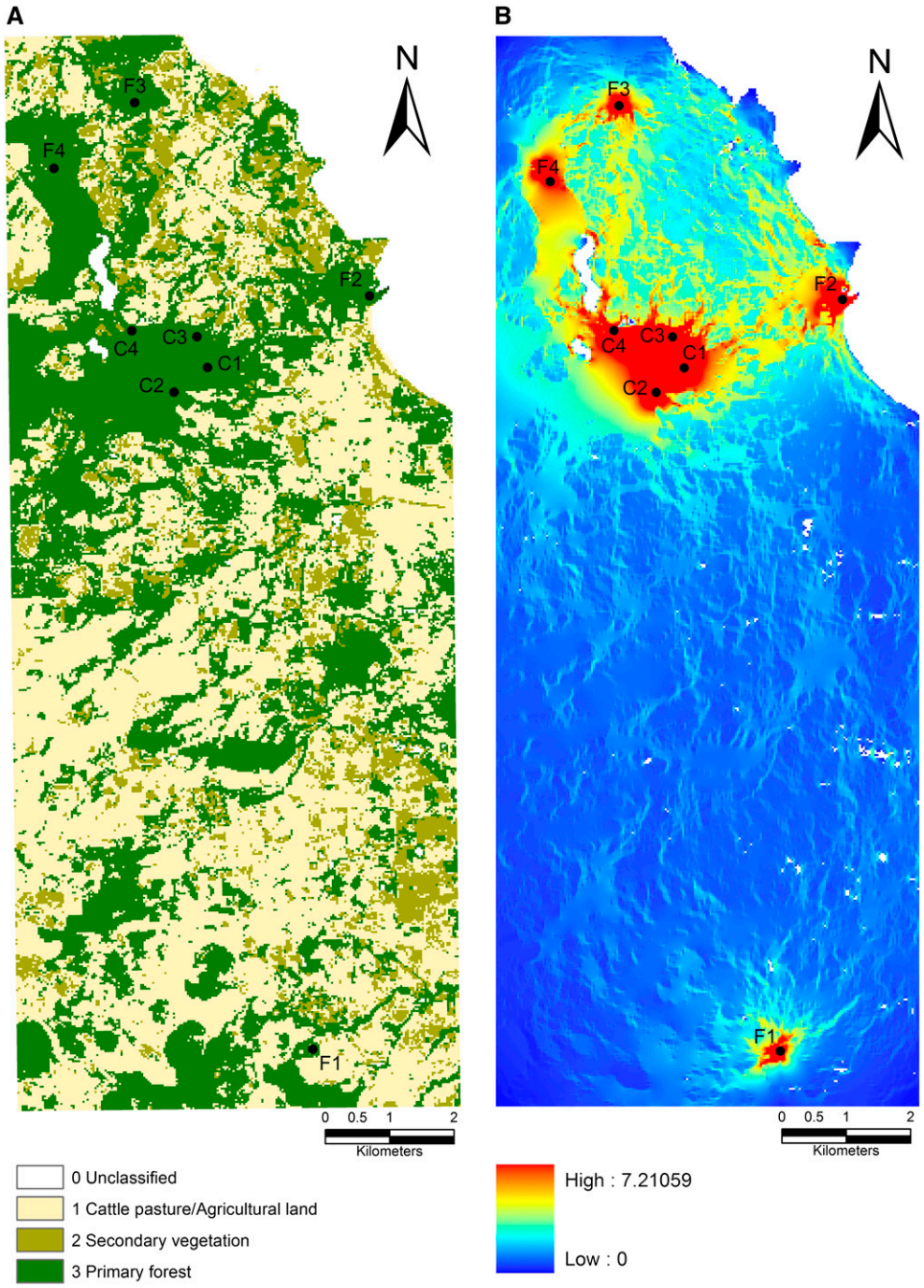

Fig. 4. (A) Map of land cover types in the study area, with conductance values assigned to each type. (B) The isolation by resistance with the highest conductance in red, and low conductance in blue. Black dots represent the sampled populations of *Carica papaya* in Los Tuxtlas region.

receive less gene f ow from adjacent populations, and/or that genetic drift is stronger in these populations. Reductions in gene f ow may arise when pollinator services are constrained by fragmentation, thus increasing mating among relatives within a population (Ellstrand and Elam, 1993). In addition, the regeneration of *C. papaya* in fragments and secondary forests may derive from a reduced contingent of seed donors. In these scenarios, decreased genetic variation in fragmented habitats is not surprising and supports the theoretical predic tions whereby habitat fragmentation negatively affects genetic diversity (Young et al., 1996).

In our study , *C. papaya* showed high le vels of e xpected heterozygosity (H_E = 0.72). These levels fell well within the range of other gap-colonizing species (H_E = 0.57 in *Elaeocarpus grandis* [Rossetto et al., 2004]; H_E = 0.81 in *Jacaranda copaia* [Jones et al., 2005]; H_E = 0.67 in *Vochysia ferruginea* [Davies et al., 2010]), but were higher than those of other dioecious species (H_E = 0.32 in *Cecropia obtusifolia*

TABLE 5. Numbers of female (♀) and male (♂) individuals of *Carica papaya* in the continuous (C) and fragmented (F) populations. Sex ratio, observed population size (N) and effective population size (N_e) are shown.

Population	♀	♂	Sex ratio	N	N_e
C1	11	19	0.366	30	27.8
C2	22	9	0.709	31	25.5
C3	9	6	0.6	15	14.4
C4	25	5	0.833	30	16.6
Total	67	39	0.632	106	98.6
F1	19	12	0.612	31	28.8
F2	13	17	0.433	30	29.4
F3	21	8	0.724	29	23.1
F4	13	2	0.866	15	6.9
Total	66	39	0.628	105	97.5

Note: Sex ratio was obtained as the proportion of females in each population.

[Alvarez-Buylla and Garay, 1994]; $H_E = 0.46$ in *Eurya japonica* [Chung and Kang, 1996]; $H_E = 0.17$ in *Hippophae rhamnoides* [Bartish et al., 1999]). However, when we then compared the genetic diversity of local populations of *C. papaya* at Los Tuxtlas with the estimates obtained for the same species in wider areas in Venezuela, Colombia, and the Antilles (Ocampo et al., 2007) and across Costa Rica (Brown et al., 2012), we found lower H_E values in the latter sites (ranges of 0.54–0.69 and 0.51–0.64, respectively). Likewise, the collection from Costa Rica, which Brown et al. (2012) called operational populations, also exhibited a lower heterozygote values ($f = 0.25$–0.42). Brown et al. collected in open and possibly disturbed habitats, which may be experiencing processes analogous to those in the fragmented forest at Los Tuxtlas ($f = 0.36$–0.65). Moreover, samples from Ocampo et al. (2007) and Brown et al. (2012) may show lower values of genetic diversity as they sampled mainly cultivated and feral plants. The domestication process (or drift) perhaps had an effect in the reduction of genetic variation (Doebley et al., 2006) for those populations. Moreover, gene flow from domesticated populations may cause a reduction in the genetic diversity of wild populations increasing the chances of genetic extinction (Ellstrand et al., 1999). It is interesting to note that Vranckx et al. (2012) did not find any effect of habitat fragmentation on the fixation index of woody plants, and Aguilar et al. (2008) found a negative effect only on plant progenies (i.e., after fragmentation). Thus, both the short life cycle and the mating system of *C. papaya* must be involved in the increase in inbreeding we detected in adult individuals.

A low level of differentiation was found among populations inhabiting the undisturbed rainforest at Los Tuxtlas, which showed high levels of admixture. Similarly, genetic differentiation among populations of *C. papaya* across Costa Rica was very low (Brown et al., 2012). By contrast, populations of *C. papaya* in fragments were highly differentiated despite the short distances separating them. This level of differentiation was even higher ($F_{ST} = 0.33$) when we removed the more distant population (F1) from the analysis. These differences may be due to reduced genetic variation in the fragments (since many of these populations did not share the same alleles), and to a decrease in the extent of gene flow (allowing allele frequencies to drift). Structure analysis inferred five genetic groups and a high degree of genetic admixture in the continuous forest, corresponding to one admixed cluster, as well as a tendency to differentiation in fragment populations, where they form four different genetic clusters. The AMOVA showed that most of the genetic variation is contained within populations (87%), and this may be associated with the particular life history of *C. papaya*—namely, its dependence on light-gaps to persist in the rainforest, promoting strong genetic structuring even at the small scale (Cuartas-Hernández and Núñez-Farfán, 2006; Davies et al., 2010).

Recent migration rates for *C. papaya* showed a higher percentage of migrants moving from the continuous forest to forest fragments rather than in the opposite direction, indicating that populations in the continuous forest act as sources of genes for those populations that remain in the adjacent fragments. By contrast, gene flow coming from forest fragments into the undisturbed forest is less probable, likely due to a scarcity of biotic dispersers. This hypothesis is further supported by our analysis of resistance/conductance through the landscape. The fragmented landscape of Los Tuxtlas rainforest represents a mosaic of suitable and unsuitable habitat for the proper dispersal of *C. papaya* according to our analysis. Populations that remain in primary forest, or in secondary vegetation nearby, stand a higher chance of receiving gene flow from adjacent populations of *C. papaya*. By contrast, cattle pasture and agricultural land do not represent possible routes of gene flow for populations that are surrounded exclusively by pasture. Nonetheless, we did not find a pattern of isolation by resistance for the studied populations of *C. papaya*. It is interesting to note that dispersal is maintained with the farthest population (F1, Dos Amates) even though it is surrounded by few conductance paths. Thus, intermediate, nonsampled populations of *C. papaya* may counteract landscape resistance to gene flow, acting as stepping-stones, as in another bird-dispersed tree (*Dendropanax arboreus*) from Los Tuxtlas region (Figueroa-Esquivel et al., 2009, 2010). Thus, the direction of prevalent gene flow in a fragmented landscape highlights the relevance of maintaining the wild populations of *C. papaya* for their importance in the conservation of genetic diversity and landscape restoration.

Habitat fragmentation did not appear to modify the sex ratio of local populations of *C. papaya*, given that biased sex ratios occur in both habitats. We found that the sex ratio-based effective population sizes for most populations of *C. papaya* were close to the observed census numbers. Nonetheless, two populations did show a significantly biased sex ratio and, consequently, low effective population sizes. These were C4 (Límite Norte) and F4 (Ruiz Cortines); the latter population belongs to a fragment and contained only two male trees (13% of the population). We posit that the nomadic condition of the species promotes random colonization/survival of organisms when a gap is formed, similarly to what may be happening in forest fragments. Yet in isolation, sex-biased populations could have more severe consequences than in a continuous habitat, where populations experience higher possibilities of genetic contact, as indicated by our gene flow estimates.

In fragmented habitats, light-demanding pioneer tree species (i.e., "secondary vegetation") typically are expected to become abundant (van Steenis, 1958; Tabarelli et al., 2010) and to have high genetic diversity and low population differentiation because they are mainly outcrossers and have extensive gene flow via pollen or seed/fruit dispersal. One such prototypical pioneer species at Los Tuxtlas is *Cecropia obtusifolia* (Alvarez-Buylla and Garay, 1994). By contrast, *C. papaya* is a rare species, with nomadic habits that include colonizing only large gaps; it also is dioecious, with a poor seed bank and a relatively short life span (although in disturbed habitats with less shading it might live longer). Thus, to persist, *C. papaya* must occupy recently created gaps before any of the many other gap-colonizer tree species in the region (Martínez-Ramos, 1985; Núñez-Farfán and Dirzo, 1988). In undisturbed

conditions, cross-pollination by insects and seed dispersal into gaps by birds and mammals (the natural condition in the ecology of *C. papaya*) tend to prevent differentiation and the loss of alleles. However, our data show that this was not the case for the studied populations in fragments, which show higher population differentiation and lower genetic diversity. Hence, forest dynamics (gap formation, construction, and mature phases; Whitmore, 1978) and forest conservation are critical for the persistence of rare, "nomadic" pioneer trees and genetic richness.

Wild *C. papaya* plants from fragments have been shown to produce more seeds than those from the primeval forest, although they also showed lower germination rates (M. Chávez-Pesqueira, unpublished data). Although small fragments (<5 ha) at Los Tuxtlas have been considered valuable for maintaining species abundances and diversity (Arroyo-Rodríguez et al., 2009), and although light-demanding trees tend to proliferate and become more abundant in fragmented habitats (Tabarelli et al., 2010), our study shows that the persistence and genetic diversity of *C. papaya* in fragments is threatened, leaving the species with a limited evolutionary potential. Thus, our study demonstrates the importance of evaluating the genetic diversity of species to offer a more realistic assessment on the effects of habitat fragmentation on plants.

In conclusion, we propose that the mating system, rarity and short life cycle of *C. papaya* are further exacerbating the effects of rainforest fragmentation, hence threatening wild populations of this species in its proposed place of origin and genetic reservoir. Conserving the natural habitats of wild populations and the wild relatives of important crop plant species is of utmost importance if we hope to assure sufficient levels of genetic diversity to maintain the evolutionary potential of these species. Indeed, this genetic diversity forms the capital for current and future improvements to crop plants. Moreover, evaluating the genetic diversity of wild populations in the proposed center of origin of their crop species, as in this study, provides interesting and important information on genetic and phenotypic evolution. Efforts to conserve *C. papaya* in the fragmented rainforest of Los Tuxtlas must promote the conservation of its habitat, as well as the conservation of its pollinators and seed dispersers. For the distant and isolated fragments, biological corridors, together with the manual introduction of individuals or reforestation represent viable options to restore or maintain dispersal.

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CAPÍTULO II

Genetic diversity and structure of natural populations of C*arica papaya* in northern Mesoamerica inferred by nuclear microsatellites and chloroplast markers

Genetic diversity and structure of natural populations of *Carica papaya* in northern Mesoamerica inferred by nuclear microsatellites and chloroplast markers

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Abstract

Few studies have evaluated the genetic structure and evolutionary history of wild varieties of important crop species. The wild papaya (*Carica papaya*) is a key element of early successional tropical and sub-tropical forests in Mexico, and constitutes the genetic reservoir for the evolutionary potential of the species. In this study we aimed to determine how diverse and structured is the genetic variability of natural populations of *Carica papaya* in Northern Mesoamerica. Moreover, we evaluated if genetic structure and evolutionary history coincide with pre-Pleistocene events, Pleistocene refugia or recent patterns. Overall, we found a high genetic diversity and gene flow for the species. A lack of phylogeographic structure was found with the plasmid marker, whereas a recent population structure was inferred with the nuclear markers. We did not found evidence that pre-Pleistocene events or refugia played an important role in the genetic structuring of wild papayas. Because of its life history characteristics and lack of an ancient phylogeographic structure, we suggest that *C. papaya* dispersed throughout the lowland rainforests of Mexico (along the coast and foothills of Sierras), thus supporting the hypothesis that tropical forests in Northern Mesoamerica did not

experience important climate fluctuations during the Pleistocene, and the life history of *C. papaya* could have promoted large dispersal and rapid colonization of lowland rainforests. Instead, recent human disturbances, mainly the fragmentation of tropical habitats in Northern Mesoamerica, appear to be the main drivers of genetic structure, and the principal threat to the dispersion and survival of the species in the wild.

Introduction

Mexico is one of the highest ranked countries in terms of species richness and endemism (Myers et al. 2000). Its biological diversity is partially due to its geographical position between the Nearctic and the Neotropics, and to its highly broken topography and diverse ecosystems (Coates & Obando 1996). Southeastern Mexico covers a large part of the territory of Mesoamerica, one of Earth's most important biodiversity 'hotspots' (Myers et al. 2000). Mesoamerica has been recognized as one of the centers of origin of many domesticated plant species (Vavilov 1926); therefore, natural populations of important crops species inhabit this region, as well as many of their wild relative species. However, few studies have addressed the phylogeographic history and genetic structure and diversity of wild populations of important tropical crop species.

The temperate and tropical regions of south-eastern Mexico and Central America have experienced very different historical processes, and posses contrasting barriers to gene flow and climatic changes, with diverse consequences for the phylogeographical structure of plant populations (Cavender-Bares et al. 2011). Although the vast majority of the world's tree diversity lies in the tropics, most of the genetic knowledge of trees comes from temperate and boreal forests, with a marked

emphasis on pines, poplars and oaks (Dick 2010). There is a relative dearth of detailed phylogeographic information on Neotropical lowland plant species (but see: Cavers et al. 2003, Novick et al. 2003, Poelchau & Hamrick 2012, Twyford et al. 2013), even though lowland tropical vegetation covers most part of Mesoamerica. In the northern parts of this region, phylogeographic studies have focused mainly on montane temperate species or tropical species from the cloud forest (Jaramillo Correa et al. 2008, Gutiérrez-Rodríguez et al. 2011, Ornelas et al. 2013, Ruíz-Sánchez & Ornelas 2014).

Genetic structure and genetic divergence within and among plant populations result from a number of contemporary and historical factors acting at various temporal and spatial scales .The historical processes that structure the genetic diversity of trees in tropical latitudes differ in fundamental ways from those of temperate and boreal forests, where the Pleistocene glaciations played a very important role (Dick 2010). For instance, tropical forests a variety of scenarios can be expected. Tropical forests date back to Cretaceous times (Davis et al. 2005) and some authors believe that they have remained relatively stable ever since the climatic fluctuations of the Pleistocene and the Pliocene (Colinvaux et al. 1996, Fine and Ree 2006), which should translate into a total absence of genetic structure of tropical species. However, longer periods of climatic stability in the tropics may reflect range disjunctions around geological barriers that are much older than the Pleistocene vegetation changes that shaped genetic patterns at higher latitudes (Dick 2010). Other authors suggest that actual areas of concentrated species diversity and species endemism could represent zones that acted as tropical refugia during the cool, dry Pleistocene period; these refugia may have received high rainfall and remained continually warm while savannas and tropical dry forests expanded (Haffer 1969, Graham 1973, Toledo 1982, Prance 1987, Pennington et al.

2000), leaving a high genetic structure in such refuges. In addition, because most tropical tree species occur at low population densities, they may be more susceptible to genetic drift, showing inbreeding signs, than the relatively common tree species studied in temperate and boreal forests showing inbreeding signs (Fedorov 1966). Moreover, lowland tropical plants are thought to be physiologically sensitive to mild stress of drought and cold, especially to cool temperatures associated with increasing elevations (Janzen 1967). This could promote a latitudinal gradient with a stronger phylogeographic structure in tropical lowland species than in temperate plants (Dick & Heuertz 2008), as has been found in several studies that document phylogeographic structure of Neotropical trees sampled around geographic barriers (Aide and Rivera 1998; Cavers et al. 2003; Dick et al. 2003; Novick et al. 2003), and higher *FST* in tropical trees than in their temperate zone or boreal forest counterparts (Dick et al. 2008). Finally, current environmental features may drive patterns of gene flow and genetic structure for tropical plants (Poelchau & Hamrick 2012); local patterns of physiographic heterogeneity and habitat fragmentation in more recent times may also have played a role in structuring genetic diversity (Twyford et al 2013).

In southeastern tropical Mexico two geographical barriers are known to have reduced dispersal and generate a spatial structuring of genetic diversity in many species: the Isthmus of Tehuantepec (pre-Pleistocene event) and the Pleistocene glacial refugia (Twyford et al. 2013). The Isthmus of Tehuantepec forms a narrow strip of lowland linking the south-central Mexican highlands to the uplands of Chiapas and Central America (Ornelas et al. 2010). It has been suggested that this area may have been a historical seaway for much of the Pliocene (Morrone 2006), and therefore a major barrier to gene flow during this period (Twyford et al. 2013). Moreover, if the

Pleistocene refugia theory is correct, some regions in Mexico have been suggested to act as Pleistocene refuges (Toledo 1982). In these cases we could expect high levels of population differentiation in tropical species in tropical Mexico by either of these barriers. Moreover, recent anthropogenic disturbances have modified the Mesoamerican landscape. For rare tropical trees, the current rate of deforestation in Mesoamerica is sufficiently high to endanger their continued existence through habitat loss and the potential lack of gene flow as a result of forest fragmentation (Novick et al. 2003), provoking non-continuous habitats that alter their genetic diversity and structure.

The wild papaya (*Carica papaya*) is a tropical nomad tree which occurs naturally in lowland tropical and sub-tropical forests of Mesoamerica. The cultivated varieties of *C. papaya* represent the third most cultivated tropical crop around the world (FAO 2012). In Mexico, natural populations of *C. papaya* are found; however, the wide geographical distribution of wild populations and the lack of collections in many areas of the country have prevented a precise assessment of their diversity and genetic structure for conservation purposes. Few studies have evaluated the genetic structure and evolutionary history of wild varieties of important crops. Phylogeographic techniques can be used to explore the evolutionary diversity of such wild populations, with relevance to conservation and management (Dick 2010). Moreover, Mexico represents one of the regions where Vavilov (1926) suggested that many cultivated species originated, diversified and were domesticated. The aims of the present study were (1) to assess the actual state of natural undomesticated populations of *C. papaya* regarding its distribution and genetic diversity in Northtern Mesoamerica, using nuclear and chloroplas markers, (2) to evaluated the genetic structure and evolutionary history

in its Mesoamerican distribution and whether it is consistent with pre-Pleistocene events, patterns of ancient refugia or with more recent effects, (3) to define the possible barriers to gene flow in recent and ancestral times, and (4) to estimate recent migration rates. Finally, in the light of our findings we discuss some conservation remarks for this important species.

Method

Study system

Carica papaya is a tropical, nomad, short-lived tree (Figure 1) that belongs to the Caricaceae family and is the only member of the genus (Badillo 2000). *C. papaya* is part of a small clade confined to Mexico and Guatemala that also includes three perennial herbs (*Jarilla chocola*, *J. heterophylla* and *J. nana*) and a treelet with spongy thin stems (*Horovitzia cnidoscoloides*) (Carvalho and Renner 2012). There are many cultivated varieties of papaya that differ in traits such as fruit size, color, flavor and tree size. Wild populations of papaya are characterized by a strictly dioecious breeding system (Chávez-Pesqueira et al. 2014) (rather than being trioecious like the cultivated papaya) and have female trees that produce small, seedy fruits with a thin mesocarp (Carvalho and Renner 2012) (Figure 1). Sexual expression in *C. papaya* is genetically determined by a pair of homomorphic sex chromosomes (Yu et al. 2007). *C. papaya* is pollinated by Lepidoptera, principally by sphingid moths, but wind-assisted pollination has also been reported (Vega-Frutis & Guevara 2009). Wild *C. papaya* blooms and fruits all year round (Chávez-Pesqueira and Núñez-Farfán *pers. obs.*).

The geographical distribution of *C. papaya's* clade and the occurrence of wild papayas in Mesoamerica are consistent with a domestication of papaya there (Carvalho and Renner 2012). *C. papaya* diverged from its sister clade some 25 Ma ago. The biogeographic history of Caricaceae involves long distance dispersal from Africa to Central America c. 35 Ma ago and expansion across the Panamanian land bridge sometime between 27 and 19 Ma. Diversification of Vasconcellea, the largest genus of the family, is related to the peak of the northern Andean orogeny, while diversification of Jacaratia appears linked to the expansion of drought-adapted vegetation during the Late Miocene (Carvalho and Renner 2012).

Wild papayas inhabit many parts of southeastern Mexico; they are usually associated to lowland humid and sub-humid tropical forests (Paz & Vázquez-Yanes 1998). Sometimes wild varieties are also found in home gardens of ethnic groups from Yucatan (Terán and Rasmussen 1995) and Veracruz in Mexico (Chávez-Pesqueira and Núñez-Farfán *pers. obs.*), who use wild papaya fruits to make sweet glaze. Wild papaya trees in tropical forests behave like typical fast-growing short-lived pioneer trees; they establish rapidly and grow only in recent and relatively large canopy gaps in mature forest and in early secondary forests (Chávez-Pesqueira et al. 2014). Wild papaya is relatively abundant in recent large man-made clearings that are not plowed. Large natural forest gaps 1-5 years old can sometimes accommodate several wild papaya trees (Paz & Vázquez-Yanes 1998).

Sampling

A total of 355 individuals from 19 populations were sampled, covering the natural distribution of *C. papaya* in Mexico (Figure 2). Between 11 and 35 individuals were sampled in each population (Table 1).

DNA extraction, amplification and sequencing

DNA was extracted using the CTAB method (Doyle and Doyle 1987) with some modifications. Nuclear and chloroplast markers exhibit different modes of inheritance that help elucidate the evolutionary history of species. DNA microsatellites show high variability and have the ability to allow genome-wide information that reflects both pollen and seed dispersal. In contrast, chloroplast DNA (cpDNA) is maternally inherited in many plants (including *C. papaya*) and is dispersed only by seeds, thus providing no information about pollen dispersal and reflecting a different evolutionary history from nuclear DNA because of its small effective population size. Because of this, we used both chloroplast (cpDNA) and nuclear (DNA microsatellites) markers to assess the genetic structure and phylogeographic history of natural populations of *C. papaya*.

Six microsatellite primers from Ocampo et al. (2006) that we had previously used for *C. papaya* (Chávez-Pesqueira et al. 2014) were amplified using a Multiplex approach. Each multiplex (10µl) contained 20 ng of DNA template (2 µl), 0.2 µM fluorescently-labeled forward primers, 0.2 µM reverse primers, RNase/DNase- free water and Reaction Mix (1x). Amplifications reactions were carried on a Veriti® 96-Well Thermal Cycler. The PCRs were performed through touchdown reactions, starting with an initial heat activation at 95° C for 10 min followed by 31 cycles with a denaturation at 94° C for 1 min, an annealing for 1 min, and 1 min of extension at 72° C. Annealing

cycling temperature began at 57° C dropping one degree every cycle until attaining 51° C, this temperature was hold for in 6 cycles, followed by two stages of 12 cycles each one at 55° C and 54° C, respectively. PCR products were run on an ABI Prism 310 and ABI 3730xl automated capillary sequencers, and allele sizes were scored manually using LIZ-500 size standard in GeneMarker v2.4.0 (SoftGenetics, LLC).

Additionally, nine plastid markers (*ndh*J-*trn*F, *trn*Q-5´*rps*16, *ndh*F-*rp*132 y *psb*J-*pet*A*, trn*H-*psb*A, *trn*L-F, *rbc*L800f-600r, *mat*KF1-R1, and r*ps*12-*rpl*20), as well as the internal transcribed spacer (ITS1-2), were tested for amplification and sequence variation in *C. papaya*. *trn*H- *psb*A and r*ps*12-*rpl*20 were chosen because of their high amplification quality and variability among populations of *C. papaya*. The remaining tested markers were poorly amplified or were invariant across populations. PCR reactions were performed in a volume of 15 µl, containing 1µl of DNA, 2 µl of buffer, 1.25 µl of MgCl2 (1.25 mM), 2 µl of dNTP and 1 µl of each primer. The amplification condition for *trnH-psbA* was 94 ° C for 5 min, followed by 35 cycles at 94 ° C for 1 min, 56 ° C for 1 min and 72 ° C for 2 min, and a final extension at 72 ° C for 8 min. For r*ps*12-*rpl*20 the same conditions were used, but the annealing temperature was set to 53° C. PCR products were visualized via 1% agarose gel electrophoresis. All forward and reverse strands were sequenced and then edited in Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA); nucleotide substitutions, indels (*i.e.* insertions or deletions) and inversions were visually checked. Sequences were aligned online with MAFFT version 7 (Katoh and Standley 2013).

Data analyses for neutral microsatellites

Parameters of genetic diversity for each population [polymorphic loci percentage (*%P*), the number of alleles (*A*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), and inbreeding coefficient (*f*)] were calculated using GenAlex 6.5 (Peakall & Smouse 2006, 2012). Comparisons of genetic estimators between different groupings of populations were tested with FSTAT version 2.9.3 (Goudet 2001). We used Microchecker 2.2.3 (van Oosterhout et al. 2004) to detect null alleles.

The genetic structure of populations was inferred using Geneland (version 4.0; Guillot et al. 2008) in R (R Development Core Team 2008). Geneland determines the number of population subdivisions for multilocus genotypic data using a Bayesian procedure, considering spatial proximity when assigning individuals to clusters. Ten independent runs were implemented for each analysis using 1,000,000 MCMC iterations with a thinning value of 100. Uncorrelated and null allele model options were activated. The number of genetic clusters was set to unknown, but the maximum possible number of clusters was limited to 19 to offer a large enough search space for the MCMC algorithm. Geographic coordinates were decimal degrees of the sampling locations with an uncertainty of 400 km (based on the long migration rates found with Bayesass among populations (see Results)). MCMC post-processing was done with a burn-in of 1,000 iterations, and the average posterior probability was used to select the best-suited run.

We further evaluated the genetic differentiation of populations by assuming a stepwise mutation model with Slatkin's *RST* (Slatkin 1995). Partitioning of genetic variation within and among population groups was tested among genetic clusters

derived from Geneland and between populations east and west from the Isthmus of Tehuantepec, separately by analysis of molecular variance (AMOVA; Excoffier et al. 1992) using GenAlex 6.5 (Peakall & Smouse 2012). In addition, the isolation-by-distance model (Wright 1943) was tested using a Mantel test (Mantel 1967), to determine whether matrices of pairwise population genetic distances [RST / (1 − RST)] were correlated to the logarithm of the Euclidean geographical distances. We calculated Mantel's correlation coefficients (*r*) using the ade4 package (Dray and Dufour 2007) in R (R Development Core Team, 2011). The statistical significance of the estimators was determined with 9,999 permutations.

The geographic locations of genetic discontinuities among populations were assessed with the Monmonier´s maximum difference algorithm implemented in BARRIER version 2.2 (Manni et al. 2004). This program first creates a map of the sampling locations from geographical coordinates. From a matrix of pairwise genetic distances (*FST*) between populations, barriers are then represented on the map by identifying the edges of polygons where the maximum distances occur. Population pairwise *FST* comparisons were calculated using ARLEQUIN version 3.5.1.2 (Excoffier et al., 2005). To obtain statistical confidence values for the barriers, 100 replicates of both distance matrices were calculated by resampling individuals within populations.

The program BAYESASS edition 3 (Wilson & Rannala 2003) was used to estimate short-term migration rates. BAYESASS uses a genetic assignment to estimate short-term dispersal rates, providing an estimate of migration rates over the past two generations. This is in contrast to coalescent methods, which are closer to long-term averages. We performed five runs (each with different starting seed value) of 10 million

generations, with a 1 million generation burn-in, and sampled the chain every 2000 generations.

Data analyses of cpDNA data

Haplotype and nucleotide diversities, as well as neutrality test statistics of Tajima's *D* (Tajima 1989) and Fu's *F* (Fu 1997) tests for neutrality with their associated significance values were calculated for each population using ARLEQUIN version 3.5.1.2 (Excoffier et al., 2005). Tajima´s *D* takes into account the genetic diversity and the number of variable sites in a sequence, to test for demographic range expansion. Significant D values can be due to bottlenecks, selective effects, population expansion or heterogeneity of mutation rates (Tajima 1996). Fu's *F* (Fu 1997) uses information of the distribution of haplotypes to test for demographic expansion and it is more sensitive to population growth than Tajima's *D*. Significant large negative *F* values generally indicate sudden population growth.

We were not able to concatenate the two studied regions of cpDNA because inconsistency in the amplification of many individuals, so results will be given separately for both regions. Thus genealogical relationships of haplotypes were estimated independently for *trnH-psbA* and r*ps*12-*rpl*20 sequences using statistical parsimony in TCS 1.2.1 (Clement et al. 2000) using an algorithm for cladograms estimated by maximum parsimony, with insertions–deletions coded as a fifth state.

Spatial structuring of variation in *psbA-trnH* was examined using spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002), to infer population structure without any prior knowledge. SAMOVA attempts to reconstruct groups of locations that are geographically homogeneous and genetically differentiated from each other,

maximizing the proportion of total genetic variance due to differences between groups of populations (*FCT*). We considered values of *K* (group number) between 2 and 19 using 100 initial conditions for each run. Analyses of molecular variation (AMOVA; Excoffier et al. 1992) for *psbA-trnH* were performed to determine how genetic variation is distributed within and among sites, along the groups found by SAMOVA, and between two pre-defined genetic groups: east and west of the Isthmus of the Tehuantepec. A total of 1,000 permutations were performed for each AMOVA. A pattern of isolation by distance (Wright 1943) was evaluated for the chlorplast marker *psba-trnH*, which can indicate restricted seed dispersal, with a Mantel test in R (package ade4; Dray and Dufour 2007). We compared *FST* pairwise genetic distance among populations and the logarithm of the Euclidean geographical distances. The statistical significance of the estimators was determined with 9,999 permutations.

Because nuclear microsatellites reflect a more recent history of populations, we also used BARRIER version 2.2 (Manni et al. 2004) for the *psba-trnH* region to account for more ancient barriers to gene flow.

Genetic differentiation among populations was estimated by computing a distance matrix based on the number of mutational steps between haplotypes (*NST*) and by using haplotype frequencies (*GST*). The occurrence of phylogeographic structures was inferred by testing for significant differences between *GST* and *NST* using PERMUT 2.0 (Pons and Petit, 1996) with 1,000 permutations. Contrary to *GST*, *NST* considers sequence differences between the haplotypes. Thus, a *NST* value higher than a *GST* value indicates that closely related haplotypes are observed more often in a

given geographical area than would be expected by chance (Pons and Petit 1996).

Results

Nuclear microsatellite diversity and population structure

All loci were polymorphic and moderate to high values of neutral genetic diversity in all natural populations of *C. papaya* (Table 2) (*Ho* values from 0.409 - 0.783 and *He* values from 0.634 – 0.806). The populations from Acayucan, Caobas, Matías Romero, Mamantel, Oxtankah, Poza Rica, Tamazunchale and Villa Guadalupe showed *He* values higher than 0.7. In contrast, Cancún, Santiago Astata and Tuxtlas populations, showed the lowest values. Further, *f* values were, in general, close to 0 but with positive and negative values, implying populations with excess (-) and deficiency (+) of heterozygotes Table 2) at microsatellite loci. In particular, the Oxtankah population showed the lowest value (*f* = -0.100) and Tuxtlas population the highest (0.100). We detected null alleles in all loci but their estimated frequency was relatively low (less than 10%) (Microchecker 2.2.3; van Oosterhout et al. 2004)

Six cluster were found by posterior cluster membership (GENELAND; *K* = 6): (1) Cielo, Huasteca, Tamazunchale and Poza Rica; (2) Marquelia and Ventanilla, (3) Tuxtlas, Acayucan, Villa Guadalupe and Palenque; (4) Matías Romero and Sto. Astata; (5) Mamantel and Caobas; and (6) Dzibichaltún, Oxtankah, Río Lagartos, Chichén and Cancún (Figure 3). The inferred clusters contained closely distributed populations, with

exception of Oxtankah (population 15), which was grouped with the northern populations of the Yucatán Peninsula.

Genetic differentiation among all populations was moderate (*FST* = 0.148 and *RST* = 0.149) whereas differentiation among Geneland clusters was low (*FST* = 0.112 and *RST* = 0.082). Hierarchical partitioning of molecular variance (AMOVA) for the Geneland groups revealed that the highest proportion of variance was located within populations (84.45%; $P < 0.0001$) and lower proportions among populations within groups (10.64 %; $P < 0.0001$) or among groups (4.91 %; $P = 0.0293$). For the east/west partition, we found the same pattern. Pair-wise genetic differentiation was significantly correlated to geographic distance, according to the Mantel test for all sites ($R = 0.1708$ $P = 0.0189$).

BARRIER suggested that the largest genetic breaks were in many cases concordant with mountainous areas (Figure 3), with the Sierra Madre del Sur, the Sierra Madre Oriental, and the Sierra de Oaxaca being the most prominent barriers for *C. papaya* dispersion. Interestingly, some barriers were identified in the Yucatán Peninsula, between Cancún population and its surroundings and North from Mamantel and Caobas populations, although no visible physical barriers can be identified in such area.

Recent migration rates among populations, as estimated with BAYESASS, showed moderate recent migration rates among populations that were separated as far as *ca*. 420 Km [between Poza Rica (population 4) and Acayucan (population 6) populations] (Table 3). Three populations [Río Lagartos (population 17), Acayucan (population 6) and Tuxtlas (population 5)] were the principal source with higher migrations rates to other populations. Migration rates between Río Lagartos

(population 17) and Chichén (population 18) (migration rate = 0.1955), Río Largartos (population 17) and Oxtankah (population 15) (migration rate = 0.1834), and Río (population 17) Lagartos and Caobas (population 14) (migration rate = 0.1725) were the highest. Some populations like Cancún, Cielo, Marquelia, Río Lagartos and Tuxtlas did not receive significant migration from any other population. In addition, twelve populations did not represent important sources of gene flow (Caobas, Chichén, Dzibichaltún, Huasteca, Mamantel, Marquelia, Matías Romero, Oxtankah, Poza Rica, Santiago Astata, Tamazunchale and Villa Guadalupe).

Chloroplast genetic diversity and structure

We obtained 291 cpDNA sequences for the *psbA-trnH* region and 176 for the *rpl20-rps12* chloroplast region. No individuals of Huasteca population amplified for *rpl20-rps12*. The *psbA-trnH* region was 423 bp long with 30 variable sites, 20 substitutions and 14 indels, while the *rpl20-rps12* region was 721 bp long with 15 variable sites. Indels more than 2 bp long were treated as fifth character state. For *psbA-trnH*, a inversion of 5 bp was also treated as a fifth character state. Haplotype diversity (*h*) for *psbA-trnH* was high for most localities ranging from 0.3072 to 0.9341, reflecting the presence of different haplotypes within each site (Table 4). Nucleotide diversity (π) was moderate (0.0009 – 0.0211) for most populations, indicating some variation between sequences within the same population (Table 4). Within population haplotype diversity (*hs*) was 0.7013 (0.0896) and nucleotide diversity (πs) was 0.0149 (0.0049). For *rpl20-rps12*, *h* and π were 0 for ten out of the18 populations reflecting very low within site diversity and the lack of variation between sequences from the same population.

Only Tamazunchale population showed a moderate haplotype and nucleotide diversity (h = 0.6762, π = 0.0022).

The statistical parsimony network of natural populations of *C. papaya* in Northern Mesoamerica recovered 50 haplotypes for the *psbA-trnH* region and 10 for the *rpl20-rps12* region (Fig. 3 and 4, Table 1S (Supplementary data)). For *psbA-trnH*, 10 out of 50 haplotypes were shared between populations, and 40 were private. The most widespread haplotypes were H1, H2 and H3, which were found in 11, 10 and 10 populations, respectively, and were shared between distant populations. Huasteca (population 2) and Tuxtlas (population 5) were the populations with more private haplotypes, seven (H24, H25, H26, H34, H35, H36, H37) and six (H17, H18, H42, H43, H44, H45), respectively (Figure 4). For *rpl20-rps12*, less variation was found, and most individuals (86%) bore haplotype HA, while 6 out of nine haplotypes were private. We found that Tamazunchale was the populations with the highest number of haplotypes (5), which was also the one with more private haplotypes, together with Chichen (HC and HJ for Tamazunchale and HG and HH for Chichen), and (Figure 5). Due to low variation at r*ps*12-*rpl*20, this marker was not further analyzed.

The analysis of spatial genetic structure for *psbA-trnH* using SAMOVA showed that the highest *FCT* value found was for $K = 2$; however, one group was formed by only one population (population 1). The *K* with the highest *FCT* increment was nine, but again, some groups included only one sampling site. When the SAMOVA was performed between the populations east and west of the Isthmus of Tehuantepec, we found that most variation was contained within stands (51.2 %; $P < 0.0001$), followed by among populations within groups (34.9 % %; $P < 0.0001$), and finally among groups

(13.9%; P = 0.0117). A pattern of isolation by distance was also detected (Mantel test; r = 0.1663, P = 0.0255).

We assessed the potential of genetic breaks that could suggest more ancient barriers among populations, in comparison with the nuclear microsatellite data, using BARRIER for *psbA-trnH* (Figure 3). As for the nuclear markers, the most likely barriers were found in the Yucatán Peninsula separating Chichén and Caobas from all populations nearby. BARRIER also detected two barriers in southern Oaxaca, isolating the Santiago Astata population.

The results from PERMUT showed that the level of *NST* (0.224, SE 0.0410) was not significantly higher (P > 0.05) than *GST* (0.273, SE 0.0571), indicating a lack of phylogeographical structure for *C. papaya*.

Finally, demographic analyses for *psbA-trnH* showed no significant expansion for any of the populations of *C. papaya* (Table4).

Discussion

Evolutionary research in lowland tropical tree species is at an early stage of development (Dick & Heuertz 2008). For *Carica papaya*, we found contrasting results between neutral (nuclear microsatellites) and chloroplast markers (cpDNA). Microsatellite data are known to resolve patterns of recent gene flow and pollen dispersal, due to their high mutational rate, whereas chloroplast markers tend to reflect ancient patterns because lack of recombination, low effective population size and their conservative mutation rate (Powell & Hollingsworth 2001). Our results suggest a lack of

phylogeographic structure for *C. papaya* inferred by chloroplast DNA, but a recent structuring derived from the microsatellite data.

Isthmus of Tehuantepec and Pleistocen refugia hypothesis

We evaluated whether two hypothetical biogeographic events documented for other organisms (Twyford et al. 2013) influenced the spatial structuring of genetic diversity in *C. papaya*: the Isthmus of Tehuantepec and putative Pleistocene glacial refugia. The isthmus has been a barrier due to several sea level oscillations and continental uplifts that occurred throughout the Pliocene and Pleistocene (Lambeck and Chappell 2001). As a result of the isthmus' lower elevation, isolation and reductions to gene flow among populations of lowland tropical species located at each side of the isthmus could be expected. Other authors (Gutiérrez-Rodríguez et al. 2011) have suggested that the Isthmus of Tehuantepec also constituted a barrier to plant dispersal in recent times and not only before Pleistocene. However, we found little evidence of a genetic break corresponding to the Isthmus as indicated by the low *FCT* value in the AMOVA (*FCT* = 0.0491, *P* = 0.0061 for microsatellite data, and *F CT* = 0.1390, *P* = 0.0117 for the *psbA-trnH* plasmid region). Moreover, the genetic relationships depicted by the haplotype network also obscure the role of the Isthmus of Tehuantepec as a biogeographic barrier, since it supported that populations between west and east shared haplotypes. Also, we did not find any significant differences between genetic diversity estimators between our sampled populations east and west of the Isthmus of Tehuantepec, suggesting genetic contact. Finally, with both nuclear and chloroplast genetic data, no genetic break was detected in the Isthmus of Tehuantepec that could suggest an ancient or recent barrier in the zone. Probably, because of the pioneer/nomadic

behavior of wild *C. papaya*, and its short life cycle, populations could have established and expanded rapidly after the Isthmus of Tehuantepec arose, erasing any sign of past genetic breakout. On the other hand, the Pleistocene refugial hypothesis, states that during the glacial maxima of the Northern Hemisphere, the mountainous regions of tropical America served as refugia for rainforest taxa (Haffer 1969, Toledo 1982). We could not test properly this hypothesis given that we did not find natural populations of *C. papaya* in the majority of the proposed refugias in Mexico (La Lacandona, México; Soconusco, Mexico; Los Tuxtlas, Mexico; Sierra de Juárez, Mexico; and Córdoba, México). We only found wild papaya populations in Los Tuxtlas region (Tuxtlas population) and in the La Lacandona rainforest (Palenque population). However, we did find signs of genetic structuring in Tuxtlas population, which bore many private haplotypes for the chloroplast marker, suggesting a long term accumulation of variation and therefore, a possible refugia role of this region. The Palenque population also showed high genetic diversity (Table 4). Despite this, few studies have found evidence when testing the refugial hypothesis, particularly for lowland species. For temperate and tropical cloud forests, evidence has suggested that the great endemism of Mesoamerican highlands is the result of persistence throughout glacial cycles of the relict montane taxa that survived through the inter-glacials rather than the persistence of relict lowland tropical species that migrated to the highlands (Colinvaux et al. 2000). For tropical lowland species like *C. papaya*, responses to historic climatic fluctuations may depend on species-specific adaptations, therefore difficulting the identification of refugia for complex tropical species assemblages (Poelchau & Hamrick 2012). Generalizations about refugia may therefore only hold for species with similar ecological preferences, rather than at broad taxonomic scales (Twyford et al. 2013).

Genetic diversity and structure

Overall, we found high values of genetic diversity. With the microsatellite data, we found values of observed heterozygosity (*Ho)* above 0.7 for many populations across the entire distribution of wild papaya in Mexico (Tamazunchale and Poza Rica in the North, Matías Romero, Acayucan and Villa Guadalupe in the central Tehuantepec Isthmus region, and Caoba, Mamantel and Oxtankah in the Yucatán Peninsula). Three populations showed the lowest values (*Ho* ≤ 0.6): Santiago Astata , Tuxtlas and Cancún. Santiago Astata has the smaller population size and the highest inbreeding coefficient (*f* = 0.387). Wild *C. papaya* combines small population sizes (Chávez-Pesqueira *pers. obs.*) and is strictly dioecious, which compromises their amount and maintenance of genetic diversity in the long term. In the case of the Tuxtlas population, this site lies in a very fragmented rainforest area which could affect genetic contact among sub-populations, thus decreasing genetic diversity. In a previous work in this fragmented region (Chávez-Pesqueira et al. 2014), we found that isolated populations of wild papaya showed lower genetic diversity and higher isolation. This could also be the case for the Cancún population. Many human disturbances have altered many areas of the Yucatán Peninsula, possibly isolating this population. Regarding the plasmid genetic data, we did not detect significant variation for one of the plasmid markers we analyzed (*rpl20-rps12*) and was not informative for our study. In comparison, for *psbA-trnH* we found high variation, probably because this chloroplast region has been recognized to show high levels of variation compared to other cpDNA loci and has been widely used for bar-coding (Kress et al. 2005). We found a high haplotype diversity (*h* = 0.7013) with an average of 5.16 haplotypes per population for *psbA-trnH* for natural populations of *C. papaya*. This level of haplotype diversity is surprising, as single haplotypes may be

expected to be fixed by genetic drift in small populations, typical of *C. papaya*. The most frequent haplotypes (H1, H2 and H3) were widely represented across the *C. papaya* range although 40 haplotypes (80 %) were private. In particular, Huasteca and Tuxtlas were the populations with more private haplotypes, suggesting particular cases of ancestral polymorphisms.

For the neutral microsatellites we found genetic structure associated to 6 groups. All groups were geographically near showing low values of genetic differentiation among them, but high amount of variation within populations. Regarding the chloroplast marker, we found a weak phylogeograhical structure, suggesting ancient seed dispersal across the distribution of *C. papaya* in Northern Mesoamerica. Although wild papayas are mainly pollinated by nocturnal sphingids, which are known to be efficient, and long distance pollinators (Dafni 1992), moving pollen as far as 20 Km (Amorim et al. 2014), fruits may have longer dispersal. Fruits are important resources for frugivores, mainly small mammals and birds (Chávez-Pesqueira et al. 2014), which can be able to cross fragmented habitats in an efficiently way (Diffendorfer et al. 1995; Levey et al. 2005**)**. We could expect that in the past, tropical and sub-tropical forests in Northern Mesoamerica were continuous, allowing an efficient dispersal between wild papaya's populations, but now, the fragmentation of the natural habitat of the wild papaya, may decrease the efficiency of dispersers with low mobility. Moreover, even though we found a great amount of private haplotypes for natural populations of *C. papaya*, which could suggest a high phylogeographic structure, the most common haplotypes were widely represented among populations and covered most of the distribution of this species in Northern Mesoamerica. This could obscure a phylogeographic structure. In the haplotype network (Fig. 3) a different

arrangement of haplotypes can be visually noticed among northern populations (Cielo, Huasteca and Tamazunchale) and the Yucatán's Peninsula populations (Mamantel, Caobas, Oxtankah, Dzibichaltún, Río Lagartos, Chichén and Cancún), reflecting some extent of differentiation. Populations in the center of the distribution, mostly show a mixture of the most common haplotypes. This occurrence of widespread plastid haplotypes in the range of *C. papaya* is consistent with the effective seed dispersal in the past we propose.

Weak population genetic structure spanning tropical America have been reported for pioneer, tolerant to drought trees such as *Ceiba pentandra* (Dick et al. 2007), *Cordia alliodora* (Rymer et al. 2013), *Jacaranda copaia* (Scotti-Saintagne et al. 2013) and *Trema micrantha* (Dick et al. 2013), suggesting an apparent association between drought tolerance and levels of phylogeographical structure (Honorio Coronado et al. 2014). Moreover, Twyford et al. (2013) found a high genetic diversity in *Begonia heracleifolia*, a tropical drought-adapted species distributed in Mesoamerica that did not show genetic signatures of the Pleistocene dry conditions. They argue that the most likely explanations for its high genetic diversity is that their populations survived *in situ* through historical climatic fluctuations, having enough time to differentiate by drift and selection for local adaptation, and limited dispersal of the accumulated new mutations across the range of the species. Although *C. papaya* is not a tolerant drought species, it is a light-demanding tree that tolerates sub-tropical forests conditions, and could tolerate to some extent drought, and also showed high genetic diversity. This could explain, in part, the lack of phlylogeographic structure we found for the wild papaya.

Barriers to gene flow and migration rates

Barriers were highly consistent for the nuclear microsatellites. Important mountain chains were recognized like the most likely barriers to gene flow. In fact, Sierra Madre del Sur, Sierra Madre Oriental, and Sierra de Oaxaca greatly exceed the current elevational limits of lowland rain forest trees (1000 m a.s.l. for *C. papaya*). For Cancún the inferred barrier may be due to its low genetic diversity and anthropic fragmentation rather to a geographic feature preventing gene flow. For the chloroplast marker, no geographical features such as mountain chains were recognized as barriers. The barriers inferred were mainly in the Yucatán Peninsula suggesting the isolation of Chichén and Caobas populations; although there is no actual climatic or geographic reason to explain this, paleobotanical studies have documented dramatic changes in the vegetation cover of the peninsula along its biogeographic history. For instance, extensive savannas existed in what is now covered with tropical rainforest (Vázquez-Domínguez & Arita 2010).

Migration rates obtained from microsatellite data showed that nowadays, the plain Peninsula of Yucatán does not represent a barrier and that Río Lagartos population, the most northern population of the Yucatán Península is an important source of variation for the region. In general, considerable high migration rates were found between populations separated by as far as 420 Km, suggesting that wild *C. papaya* has long dispersal. However, we did not find significant migration rates between populations separated by mountainous chains, thus validating the barrier analysis.

Conservation remarks

Wild *C. papaya* is a key element of early successional tropical and sub-tropical forests in Mexico, and represents the genetic reservoir for the evolutionary potential of the species (Chávez-Pesqueira et al. 2014). Even though, we found overall high levels of genetic variation, we also found that natural populations of this species are becoming structured, probably because of the action of human disturbances on its natural habitat. This result is extremely important to generate adequate conservation strategies for this important crop species. Moreover, the centre of origin for papaya has not been appropriately defined. It has been largely suggested that papaya originated somewhere in Mesoamerica (Vavilov 1926, Storey 1976). We found high genetic diversity in the Isthums of Tehuantepec zone for the plasmid marker, as well as the most represented haplotypes, suggesting a hotspot of diversity in that region, we could propose based on this data a possible origin of the species in that region. In addition, papaya is most closely related to four species from southern Mexico and Guatemala (Carvalho and Renner 2012), suggesting that *C. papaya* could in fact have originated in southern Mexico. Moreover, this plant has been proposed to have been subsequently domesticated in this area by the Mayans (Carvalho and Renner 2012). Finally, our results provide an important warning for the intention of cultivating transgenic papayas in Mexico. Although mating between wild and cultivated papayas have not been assessed, possible "hybrids" have been seen in tropical zones of Mexico (Chávez-Pesqueira and Núñez-Farfán, *pers. obs*). Gene flow between cultivated and wild papayas endangers the genetic pool and evolutionary potential of the species (Gepts & Papa 2003). While no transgenic papayas have been liberated in Mexico, attempts exist (Silva-Rosales et al. 2010). The high migration rates found between natural populations

of *C. papaya* in our study raise concerns about the ecological and evolutionary effects of contamination in wild populations.

Conclusions

Our study showed contrasting stories depending on the genetic marker used. Using neutral and plasmid markers when assessing the genetic structure of species is important for more detailed conclusions. Because of the life history characteristics of wild *C. papaya*, and the lack of an ancient phylogeographic structure, we suggest that tropical forests in Northern Mesoamerica did not suffer very important climate fluctuations, as detractors of the refugial hypothesis have previously suggested (Colinvaux et al. 1996, Colinvaux et al. 2000, Fine and Ree 2006). Our results also suggest that the life history of *C. papaya* promoted its long dispersal and rapid colonization of lowland rainforests thus maintaining genetic diversity throughout its range. However, recent human disturbances, mainly the fragmentation of tropical habitats in Northern Mesoamerica, appear to represent a threat to its dispersion and therefore, to its genetic diversity and structure.

Further research in lowland tropical species of Mesoamerica is necessary to understand the present distribution of genetic variation of species inhabiting this ecosystem, and their phylogeographic history. Moreover, assessments of the actual situation of wild varieties and relatives of crop species are fundamental to assure the maintenance of their genetic reservoirs and evolutionary potential.

Supplementary material

Table 1S. List of the 50 haplotypes for psbA-trnH and 10 haplotypes for rps12-rpl20 generated in TCS XX for wild individuals of *Carica papaya* in Mexico.

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Tables

Table 1. Population number, sample sites, geographic location and sample size (*n*) of wild populations of *Carica papaya* in its natural distribution in Mexico.

Population number	**Sample site**	**Geographic location**	***n***
1	Cielo, Tamaulipas	23°01′01.60′′N -99°07′31.50′′W	35
2	Huasteca, San Luis Potosí	21°50′35.20′′N -99°09′06.50′′W	20
3	Tamazunchale, San Luis Potosí	21°14´36.80´´N -98°44´36.90´´W	30
4	Poza Rica, Veracruz	20°28´49.61´´N -97°39´22.21´´W	16
5	Tuxtlas, Veracruz	18°34´56.94´´N -95°04´44.00´´W	30
6	Acayucan, Veracruz	17°30′51.25′′N -95°05′12.75′′W	25
7	Matías Romero, Oaxaca	16°54´24.42´´N -95°00´35.64´´W	30
8	Santiago Astata, Oaxaca	15°59´01.10´´N -95°39´02.17´´W	11
9	Ventanilla, Oaxaca	15°40´43.15´´N -96°34´10.81´´W	30
10	Marquelia, Guerrero	16°34´51.26´´N -98°47´17.21´´W	23
11	Villa Guadalupe, Tabasco	17°22´10.78´´N -93°37´13.35´´W	30
12	Palenque, Chiapas	17°29´39.98´´N -92°01´09.13´´W	26
13	Mamantel, Campeche	18°32´49.58´´N -91°05´09.00´´W	30
14	Caobas, Campeche	18°30′47.65′′N -89°28′15.21′′W	32
15	Oxtankah, Quintana Roo	18°34´58.40´´N -88°14´40.06´´W	25
16	Dzibichaltún, Yucatán	21°05′28.34′′N -89°35′51.00′′W	28
17	Río Lagartos, Yucatán	21°31´02.66´´N -88°08´25.62´´W	27
18	Chichén Itzá, Yucatán	20°40′56.17′′N -88°34′06.53′′W	26
19	Cancún, Quintana Roo	20°54′33.61′′N -87°25′51.62′′W	18

Table 2. Genetic variation at DNA microsatellite loci in 19 populations of *Carica papaya* (± S. D.) along its natural distribution in Mexico. *n* = simple size; %*P* = polymorphic loci percentage; *A* = allele number; *Ho* = observed heterozygosity; *He* = expected heterozygosity; *f* = inbreeding coefficient.

No.	**Population**	***n***	***%P***	***A***	***Ho***	***He***	***f***
1	Cielo	21	100	6.167 (0.946)	0.667 (0.082)	0.655 (0.076)	-0.017 (0.039)
2	Huasteca	16	100	6.167 (0.703)	0.698 (0.030)	0.710 (0.450)	0.006 (0.044)
3	Tamazunchale	15	100	5.500 (0.617)	0.744 (0.036)	0.720 (0.047)	-0.041 (0.028)
4	Poza Rica	17	100	7.667 (0.760)	0.735 (0.062)	0.806 (0.009)	0.089 (0.073)
5	Tuxtlas	20	100	6.167 (0.477)	0.600 (0.058)	0.671 (0.018)	0.100 (0.090)
6	Acayucan	20	100	7.167 (0.601)	0.742 (0.024)	0.726 (0.026)	-0.025 (0.037)
7	Matías Romero	19	100	7.667 (0.989)	0.711 (0.033)	0.740 (0.030)	0.034 (0.050)
8	Santiago Astata	11	100	4.167 (0.307)	0.409 (0.080)	0.654 (0.029)	0.387 (0.110)
9	Ventanilla	18	100	6.167 (0.401)	0.630 (0.031)	0.688 (0.028)	0.083 (0.030)
10	Marquelia	19	100	4.500 (0.428)	0.693 (0.066)	0.656 (0.036)	-0.064 (0.093)
11	Villa Guadalupe	18	100	7.167 (0.792)	0.750 (0.087)	0.762 (0.036)	0.035 (0.083)
12	Palenque	18	100	6.667 (0.333)	0.667 (0.059)	0.705 (0.046)	0.059 (0.042)
13	Mamantel	20	100	8.000 (1.095)	0.783 (0.069)	0.788 (0.021)	0.013 (0.070)
14	Caobas	20	100	7.833 (0.792)	0.758 (0.033)	0.748 (0.030)	-0.019 (0.050)
15	Oxtankah	20	100	6.667 (0.715)	0.733 (0.063)	0.669 (0.046)	-0.100 (0.070)
16	Dzibichaltún	20	100	5.500 (0.563)	0.692 (0.054)	0.655 (0.018)	-0.054 (0.074)
17	Río Lagartos	20	100	5.667 (0.615)	0.658 (0.082)	0.652 (0.052)	-0.004 (0.100)
18	Chichén	23	100	6.617 (0.477)	0.692 (0.046)	0.690 (0.039)	-0.005 (0.041)
19	Cancún	20	100	5.167 (0.601)	0.583 (0.076)	0.634 (0.077)	0.075 (0.055)
	Mean	**355**	**100**	**6.325 (0.175)**	**0.681 (0.015)**	**0.701 (0.010)**	**0.029 (0.017)**

Table 3. Recent migration rates (± S. D.) and distance (km) between recipient and source populations of *Carica papaya* in its natural distribution in Mexico. Only migration rates higher than 0.05 are reported.

Recipient population	**Source population**	**Migration rate (SD)**	**Distance (km)**
Acayucan	Tuxtlas	0.1439 (0.0311)	112.48
Caoba	Río Lagartos	0.1725 (0.0286)	359.10
Chichén	Río Lagartos	0.1955 (0.0247)	98.64
Dzibichaltún	Cancún	0.1657 (0.0282)	228.13
Huasteca	Cielo	0.1640 (0.0283)	132.34
Mamantel	Palenque	0.0574 (0.0644)	158.15
Matías Romero	Acayucan	0.1079 (0.0538)	74.11
Oxtankah	Río Lagartos	0.1834 (0.0272)	323.60
Palenque	Tuxtlas	0.1340 (0.0391)	341.62
Poza Rica	Acayucan	0.0568 (0.0329)	423.50
PozaRica	Cielo	0.0674 (0.0301)	322.11
Santiago Astata	Ventanilla	0.0556 (0.0254)	107.16
Tamazunchale	Cielo	0.1601 (0.0307)	205.57
Ventanilla	Acayucan	0.1299 (0.0329)	251.69
Villa Guadalupe	Tuxtlas	0.1696 (0.0287)	204.20

Table 4. Population size (n), haplotype number, polymorphic sites, haplotype diversity (*h*) ± SD, nucleotide diversity (π) ± SD, Tajima´s *D* and Fu´s *F* for the plastid *trnH-psbA* marker in 19 wild *Carica papaya* populations in Northern Mesoamerica. Tajima´s *D* and Fu´s *F P*-values are not shown since in all cases *P* > 0.1.

Population	**n**	**Haplotype number**	**Polymorphic sites**	***h***	**π**	***D***	***F***
1.Cielo	18	3	13	0.3072 ±0.1316	0.0062 ±0.0038	-1.1157	4.0306
2.Huasteca	14	9	13	0.9341 ±0.0448	0.0152 ±0.0086	2.2854	-0.6617
3.Tamazunchale	15	4	14	0.7333 ±0.0669	0.0159 ±0.0089	2.1334	5.9607
4. Poza Rica	13	4	13	0.7692 ±0.0724	0.0164 ±0.0092	2.3307	5.3750
5. Tuxtlas	11	6	7	0.8364 ±0.0887	0.0052 ±0.0035	-1.0291	-1.2888
6. Acayucan	17	8	21	0.8382 ±0.0675	0.0211 ±0.0115	0.6628	2.1509
7. Matías Romero	12	4	16	0.7121 ±0.1053	0.0128 ±0.0075	-0.2247	3.9722
8. Santiago Astata	10	3	2	0.5111 ±0.1643	0.0013 ±0.0013	-1.1117	-0.5938
9. Ventanilla	15	9	18	0.9048 ±0.0544	0.0192 ±0.0106	1.2114	0.3064
10. Marquelia	20	5	17	0.7526 ±0.0615	0.0091 ±0.0053	-0.2945	2.8275
11. Villa Guadalupe	14	6	7	0.7912 ±0.0894	0.0061 ±0.0039	0.9683	-0.2864
12. Palenque	19	8	18	0.8304 ±0.0657	0.0113 ±0.0064	-0.5600	0.4672
13. Mamantel	19	4	5	0.5556 ±0.1030	0.0047 ±0.0031	0.3456	1.7388
14. Caobas	15	5	6	0.8095 ±0.0589	0.0050 ±0.0033	-0.0290	0.3408
15. Oxtankah	18	5	6	0.7190 ±0.0910	0.0068 ±0.0042	1.5282	1.5639
16. Dzibichaltún	16	3	2	0.4250 ±0.1326	0.0012 ±0.0012	-0.3301	-0.2898
17. Río Lagartos	16	6	6	0.8417 ±0.0534	0.0071 ±0.0043	2.0978	0.4179
18. Chichén	10	4	5	0.6444 ±0.1518	0.0031 ±0.0023	-0.8222	-0.3120
19.Cancún	19	2	1	0.4094 ±0.1002	0.0009 ±0.0010	0.7937	1.0079

Mean		**5.16**	**10**	**0.7013 ±0.0896**	**0.0149 ±0.0049**	**0.4654 ±1.206**	**1.4067 ±2.1249**
Total	**291**	**50**	**30**				

Figures

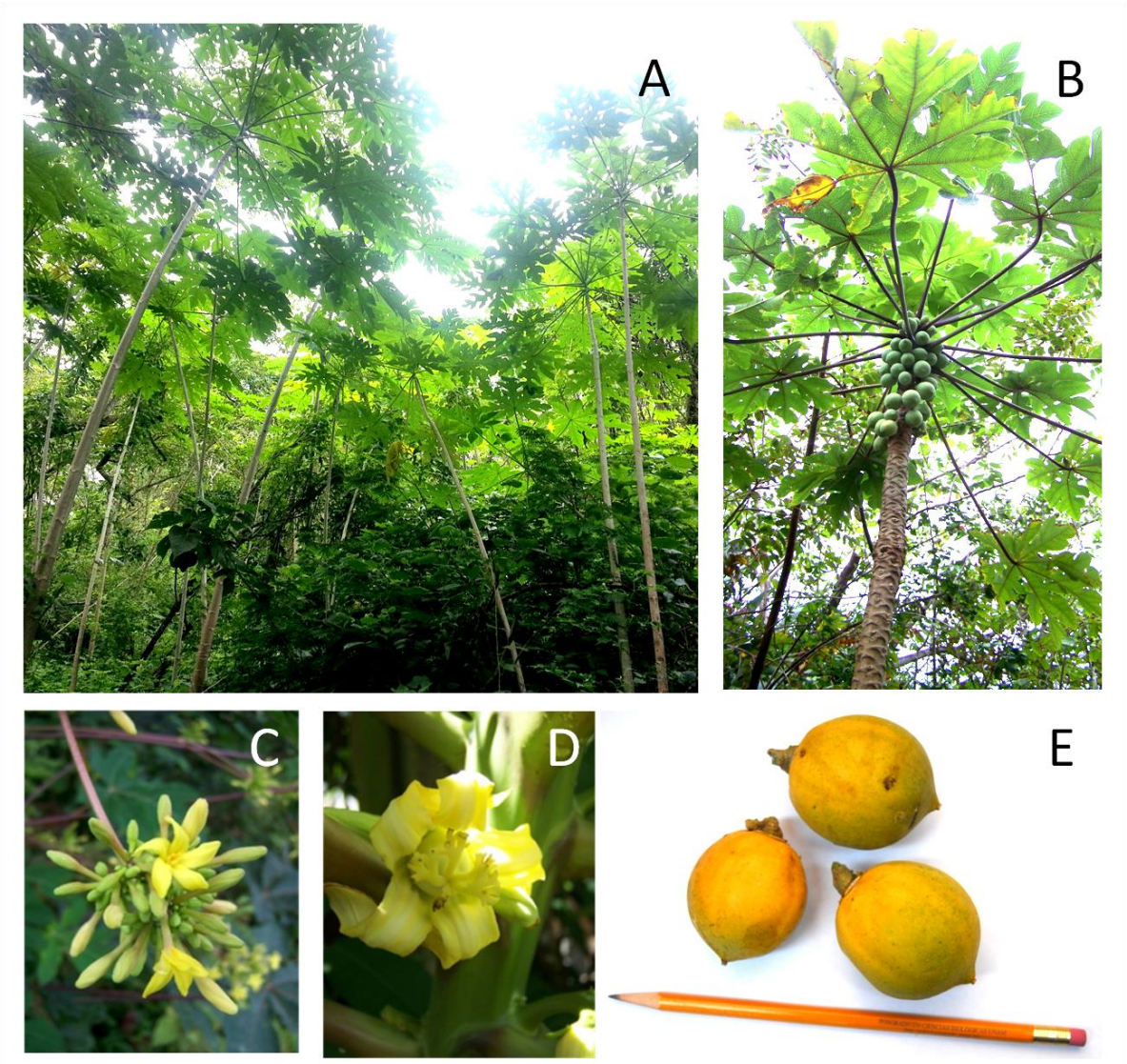

Figure 1. **A**. Natural population of *Carica papaya* at El Cielo, Tamaulipas, Mexico. **B**. Female individual of *C. papaya* with fruits. **C**. Male inflorescence of a wild *C. papaya* plant. **D**. Female flower of a wild *C. papaya* plant. **E**. Mature fruits of a wild *C. papaya* plant.

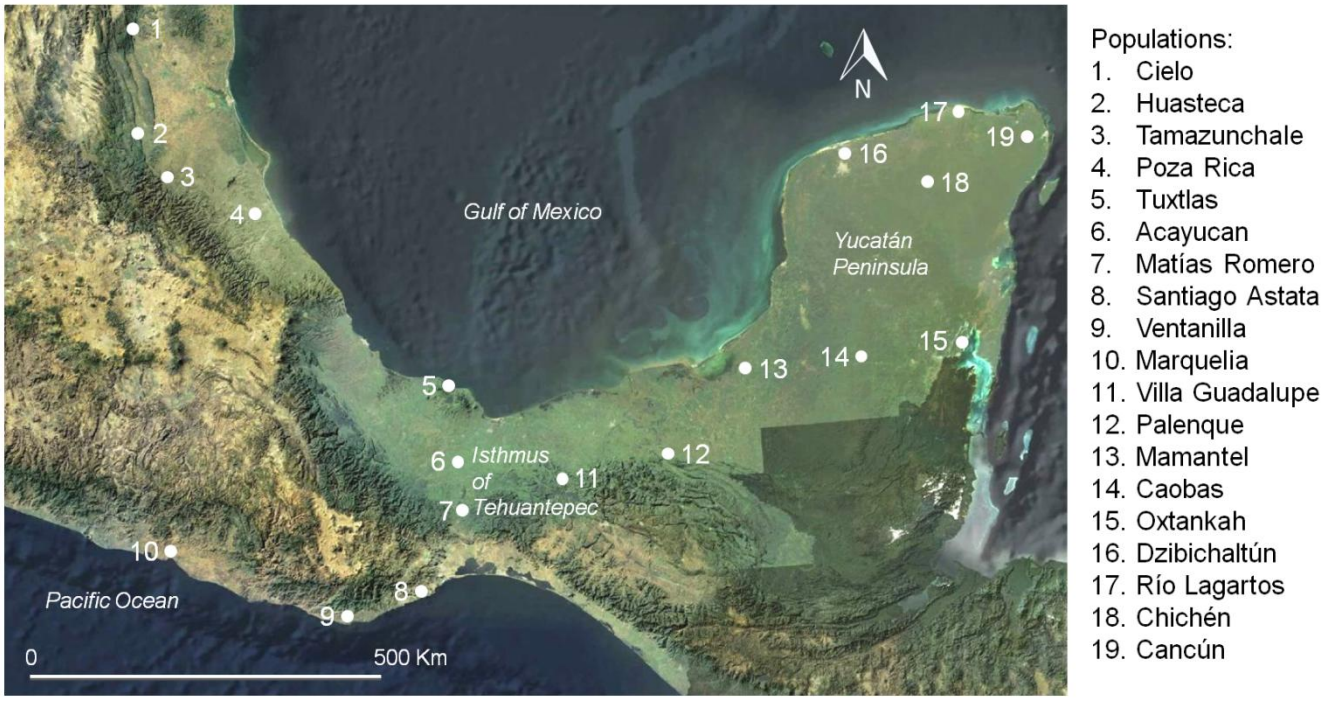

Figure 2. Distribution of the 19 sample locations in southeastern Mexico of natural population of *Carica papaya*.

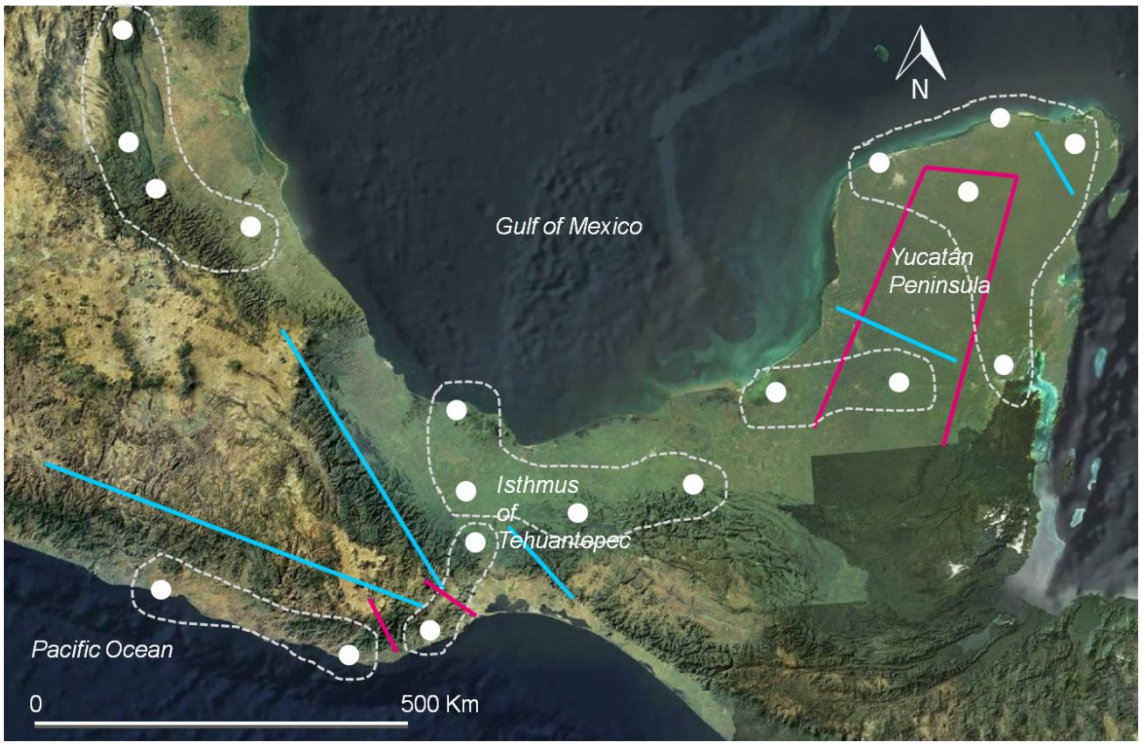

Figure 3. Six genetic clusters inferred from GENELAND using DNA microsatellite loci of natural populations of *Carica papaya* in Northern Mesoamerica. Blue lines represent the location of the most probable barriers obtained with BARRIER for DNA microsatellite loci, and pink lines for the *psbA-trnH* plasmid region.

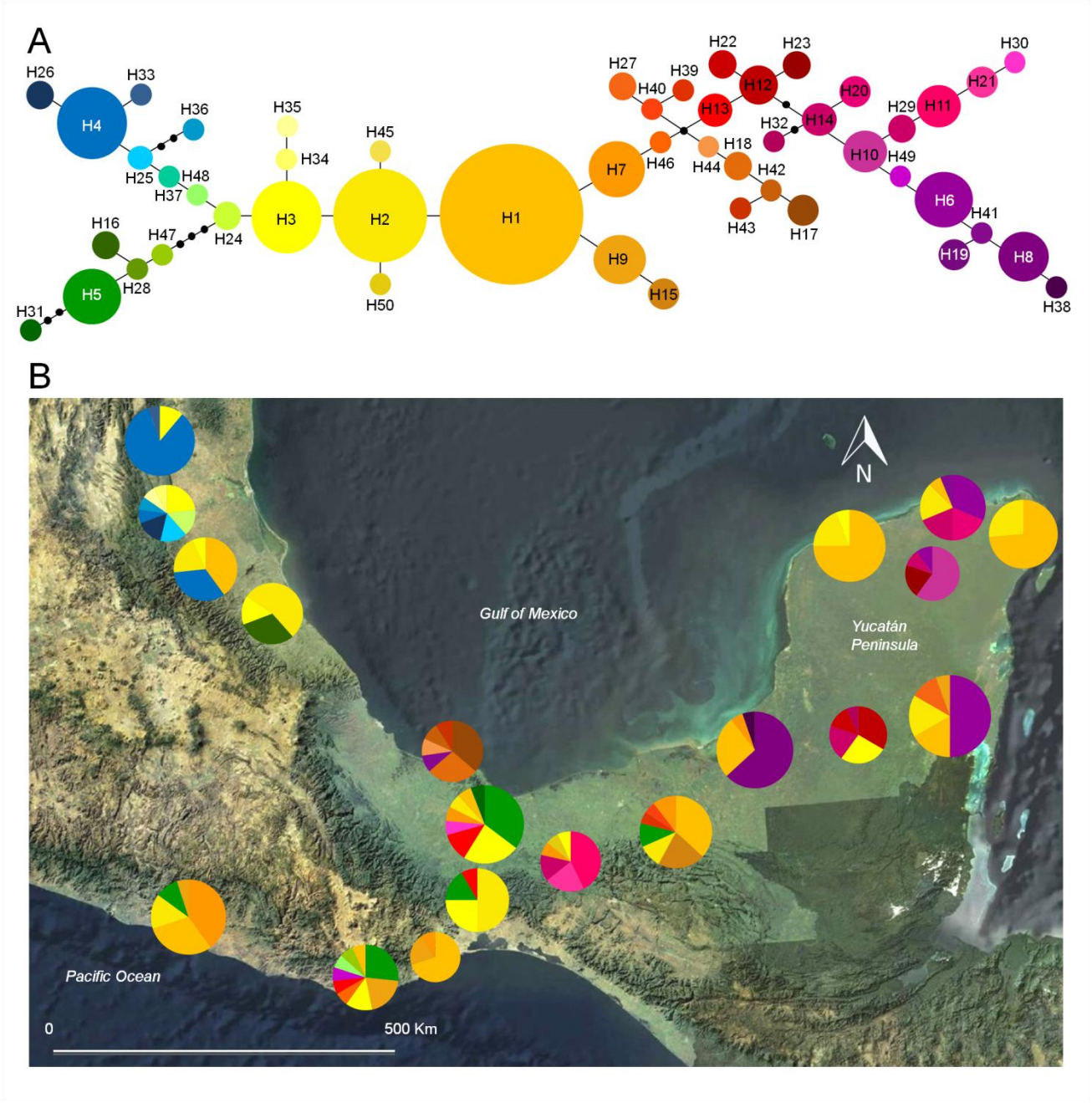

Figure 4. A. Statistical parsimony haplotype network for the *psbA-trnH* chloroplast region of *Carica papaya*. Haplotype designations in the network correspond to those in Table 1S. The size of circles is proportional to the frequency of each haplotype, and small black circles represent nonsmapled haplotypes. B. Geographic distribution of 19 natural populations of C. *papaya* cpDNA haplotypes in southeastern Mexico. Pie charts represent the haplotypes found in each sampling locality. The area of the pie chart

represents the size of the population and the size of sections is proportional to the haplotypic frequency.

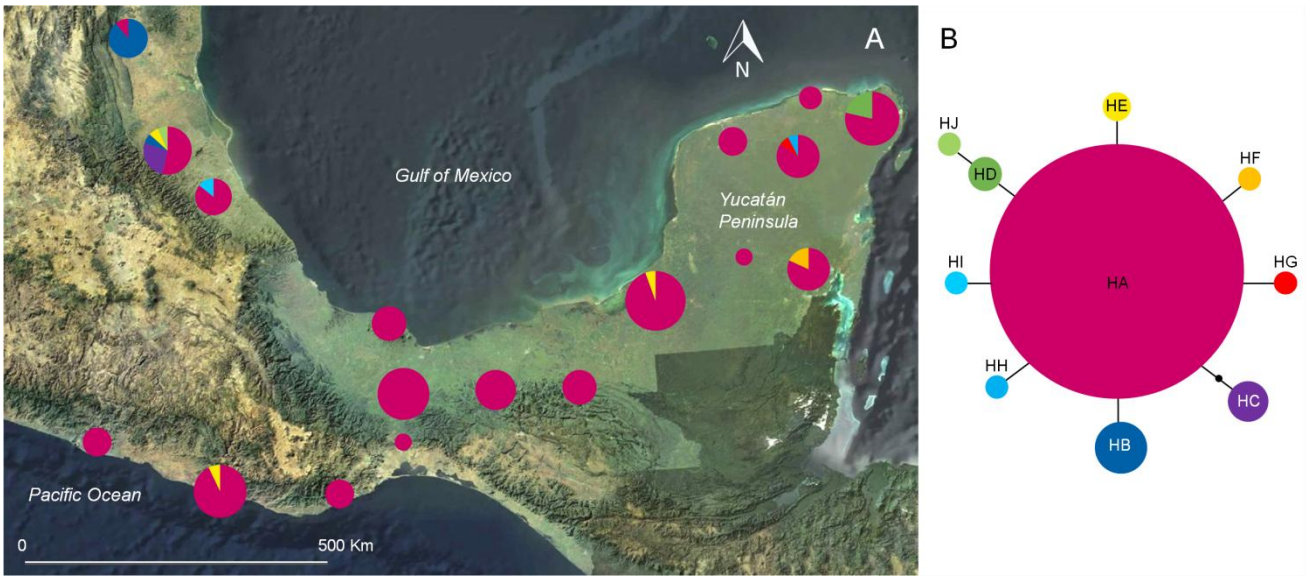

Figure 5. A. Statistical parsimony haplotype network for the *rpl20-rps12* chloroplast region of *Carica papaya*. Haplotype designations in the network correspond to those in Table 1S. The size of circles is proportional to the frequency of each haplotype, and small black circles represent nonsmapled haplotypes. B. Geographic distribution of 19 natural populations of *C. papaya* cpDNA haplotypes in southeastern Mexico. Pie charts represent the haplotypes found in each sampling locality. The area of the pie chart represents the size of the population and the size of sections is proportional to the haplotypic frequency.

DISCUSIÓN GENERAL

Carica papaya es una especie de gran importancia para México. Por un lado, representa una de las frutas más producidas y exportadas al extranjero, con una derrama económica de 580 millones de dólares y la generación de 68 mil empleos directos (SAGARPA 2014). Por otro lado, México ha sido sugerido como el posible centro de origen y domesticación de la papaya (Vavilov 1926), y por lo tanto, el sitio donde las poblaciones silvestres de esta especie habitan de forma natural. A pesar de esto, ha existido poco interés en investigaciones sobre la papaya silvestre.

La presente tesis constituye la primera evaluación del estado de la diversidad genética de poblaciones silvestres de *C. papaya* en México, así como el primer estudio en dilucidar su historia evolutiva. En conjunto, los resultados encontrados en ambos capítulos de esta tesis, nos muestran escenarios distintos sobre la diversidad genética en poblaciones silvestres de papaya. Por un lado, el capítulo I nos muestra un estudio en tiempo ecológico, mientras que en el capítulo II vemos un escenario en tiempo evolutivo. Ambos enfoques nos permiten una visión más integrada del estado de las poblaciones actuales de papaya silvestre en México. Asimismo, los resultados de esta tesis contribuyen al conocimiento del estado actual de la diversidad y estructuración genética de la papaya silvestre y dan la base para presentes y futuros planes de conservación y manejo de esta importante especie.

Estimar la diversidad genética resulta de gran importancia para saber el estado de las poblaciones, ya que a mayor diversidad los individuos tienen mayores probabilidades de sobrevivir a cambios en su ambiente. Por lo tanto, conocer y conservar las poblaciones o parientes silvestres de especies cultivadas es imperativo en países considerados centros de origen y domesticación, como México, ya que

representan el potencial evolutivo de las especies. Su pérdida no sólo representaría la pérdida de la diversidad genética natural de la especie, sino la pérdida de los recursos genéticos que pueden ser esenciales para el mejoramiento genético de las variedades cultivadas (Ellstrand 2003, Gepts y Papa 2003). Hasta la fecha, ningún estudio había evaluado el estado de la diversidad genética de poblaciones silvestres de *C. papaya* en su rango de distribución natural en México. Asimismo, los resultados sobre la historia evolutiva de la papaya resultan de gran importancia ya que existe muy poca información sobre especies tropicales de tierras bajas en la zona de México, por lo que esta tesis representa un antecedente para especies con características y distribución similar al de la papaya en su estado silvestre.

En el primer capítulo de esta tesis, se evaluó el efecto de la fragmentación del hábitat, una de las principales amenazas a la biodiversidad mundial (Wright 2010), sobre la diversidad y estructura genética de poblaciones silvestres de papaya en la selva fragmentada de Los Tuxtlas en el estado de Veracruz, México. Dada la particular historia de vida de la papaya silvestre, se esperaba que resultara una especie altamente vulnerable a la fragmentación de su hábitat natural. Por un lado, dada su corta vida, se podía esperar que los efectos genéticos de la fragmentación aparecieran más rápido en comparación a especies de larga vida. Por otro lado, sus tamaños poblacionales pequeños podrían aumentar las posibilidades de expresar una diversidad genética baja por efectos de deriva génica y endogamia. Finalmente, su sistema de apareamiento, el dioicismo, exige que individuos de ambos sexos se encuentren en el mismo fragmento para asegurar el éxito reproductivo, o que el flujo de genes entre fragmentos sea suficiente para compensar la ausencia de uno de los sexos. Los resultados de este capítulo señalan a *C. papaya* como uno de los pocos ejemplos donde se cumplen las

predicciones teóricas de la fragmentación del hábitat (Young et al. 1996, Aguilar et al. 2008) y un caso único para la selva de Los Tuxtlas. Se mostró que las poblaciones de papaya silvestre que habitan en fragmentos de selva tienen una menor diversidad genética y una mayor diferenciación que las poblaciones que están dentro de la selva continua. También se encontró que las estimaciones de tasas de migración reciente muestran un mayor porcentaje de genes que se mueven de la selva continua hacia los fragmentos que en la dirección contraria, y que la matriz que rodea a los fragmentos tiene un efecto importante en la reducción de flujo génico. En particular, se encontró que las zonas de potrero y zonas de cultivo que se encuentran en la zona de Los Tuxtlas representan ambientes poco favorables para el flujo de genes, aumentando la probabilidad de aislamiento de las poblaciones que quedan en los fragmentos, provocando valores de endogamia más altos en estas poblaciones. Estos resultados en conjunto indican que la fragmentación del hábitat en la selva de Los Tuxtlas representa una amenaza para la permanencia de poblaciones silvestres de *C. papaya,* disminuyendo los niveles de diversidad genética y modificando el importante papel ecológico que cumple en su hábitat natural.

Dado el poco conocimiento de la distribución puntual de papaya silvestre en México y el nulo conocimiento del estado de su diversidad genética, así como la poca información sobre la historia evolutiva de las especies tropicales de tierras bajas que habitan en México, en el segundo capítulo de esta tesis se evaluó por medio de marcadores nucleares y de cloroplasto la diversidad y estructura genética de poblaciones silvestres de *C. papaya* a lo largo de su distribución natural en el país, así como diferentes hipótesis sobre su historia evolutiva. Los resultados más relevantes de este capítulo mostraron una falta de estructuración genética para el marcador de

cloroplasto y una incipiente estructuración para los marcadores nucleares. Ya que los marcadores de ADN de cloroplasto nos hablan de una historia más antigua por su baja tasa mutacional, y nos dan información sobre dispersión por semillas (dada su herencia materna), podemos sugerir que las poblaciones de papaya silvestre estuvieron en contacto genético en el pasado y que muy probablemente su hábitat natural no se encontraba tan fragmentado como en la actualidad, por lo que la dispersión por semilla era eficiente. Por otro lado, los marcadores nucleares (microsatélites de ADN) reflejan información más reciente por su alta tasa mutacional, así como información sobre la dispersión del polen. Con estos marcadores encontramos que las poblaciones actuales de papaya silvestre que se estudiaron muestran cierta estructuración genética, lo cual sugiere que las recientes modificaciones al hábitat natural de esta especie, han tenido un impacto en la conectividad genética entre poblaciones (como se vio en el primer capítulo), provocando esta reciente estructuración. Esto puede ocasionar que eventualmente la diversidad genética disminuya poniendo en riesgo el potencial evolutivo de la especie en su centro de origen sugerido. A pesar de estos resultados, se encontró una alta diversidad genética en la mayoría de las poblaciones silvestres de papaya en su rango de distribución natural en México. Tanto los marcadores neutrales (microsatélites) como los de ADN de cloroplasto, mostraron altos niveles de diversidad genética y dicha diversidad estuvo contenida principalmente dentro de las poblaciones. Sin embargo, los resultados de esta tesis indican que esta diversidad se encuentra amenazada por las recientes modificaciones del hábitat natural de la papaya silvestre.

En cuanto a la historia evolutiva de *C. papaya*, se evaluaron dos posibles escenarios sugeridos para especies tropicales de tierras bajas, como la papaya, en la zona de Mesoamérica. Primero se evaluó si el istmo de Tehuantepec, el cual estuvo

sumergido por grandes periodos de tiempo en el Plioceno (Morrone 2006), pudo actuar como una barrera al flujo génico dejando señales genéticas y diferenciando a las poblaciones al este y oeste de él. La segunda hipótesis fue la de los refugios pleistocénicos (Toledo 1982), donde se argumenta que varias zonas del sur de México y Centroamérica que actualmente presentan altos niveles de diversidad y endemismos, pudieron actuar como refugios de especies tropicales al mantener condiciones ambientales estables, mientras que sus alrededores sufrieron cambios drásticos y no representaban zonas aptas para mantener estas especies. Para el caso de la papaya silvestre, lamentablemente no se pudo probar de manera adecuada la hipótesis de los refugios ya que no se encontraron poblaciones de papaya silvestre en varios de los refugios propuestos. En cuanto a la otra hipótesis, no se encontró evidencia de que el Istmo de Tehuantepec dejara alguna señal genética en la historia evolutiva de la papaya, lo que sugiere que el hábitat natural de *C. papaya* se mantuvo estable a lo largo de su historia y que la dispersión por semilla era eficiente y mantuvo en contacto a las poblaciones. Dado el poco conocimiento sobre la historia evolutiva de especies tropicales de tierras bajas en el norte de Mesoamérica, este resultado es relevante y puede sugerir patrones similares en especies con características parecidas a *C. papaya*.

Otro resultado interesante que se encontró en el segundo capítulo fue que las poblaciones con mayor diversidad, que podrían representar las poblaciones más ancestrales, se ubicaron en el sur de México. Altos niveles de diversidad genética suelen encontrarse en los centros de origen de las especies. En general, en los centros de origen se encuentran la base y reserva de la diversidad genética de la especie, por lo que son prioritarios para la conservación de la diversidad genética. En ocasiones, estos centros de origen coinciden con los centros de domesticación, donde la especie

comenzó a ser manipulada por el hombre. Para la papaya, los haplotipos más representados y diversos del gen de cloroplasto se encontraron en el sur de México, en la zona del Istmo de Tehuantepec, sugiriendo un hotspot de diversidad en esa región y un posible origen de la especie en esa zona. Sin embargo, aún falta una evaluación con poblaciones de todo el rango de distribución natural de la especie para dilucidar su centro de origen. No obstante, dado el estado actual del hábitat natural de la papaya silvestre, y sus altas tasas de deforestación, se sugiere que se conserven las poblaciones con mayor diversidad genética y que se investigue a fondo el centro de origen de la especie, así como tratar de mantener conectividad entre poblaciones silvestres y conservar a sus polinizadores y dispersores bióticos.

Otro aspecto de suma importancia en cuanto a la conservación de la diversidad genética de la papaya silvestre, es la intención de introducir individuos transgénicos. El cultivo de papaya es afectado por varias enfermedades, siendo el virus de la mancha anular (ringspot virus en inglés) la que genera más pérdidas económicas alrededor del mundo. Entre las opciones para mitigarlo se ha propuesto la introducción de individuos transgénicos resitentes al virus de la mancha anular (Silva-Rosales et al. 2010). Desde 1999 se han liberado papayas transgénicas para la resistencia al al virus de la mancha anular en Hawaii (Silva-Rosales et al. 2010); sin embargo, su producción decayó después de esta liberación debido a su baja aceptación por los consumidores, aunque aún, se siguen cultivando. En el caso de México, desde 1995 se han realizado experimentos transformando genéticamente a la papaya. Tres atributos nuevos se han introducido experimentalmente a la planta de papaya: el primero fue resistencia a herbicidas (Cabrera-Ponce et al. 1995), el segundo fue la sobreexpresión en la raíces de la citrato-sintetasa de la bacteria *Pseudomonas aeruginosa*, como medio para

desarrollar el sistema modelo para el estudio de la tolerancia al aluminio (de la Fuente et al. 1997), y el tercero fue la resistencia al VMA utilizando el gen *CP* de plantas enfermas de Veracruz (Silva-Rosales et al. 2010). Estos experimentos se llevaron a cabo en el Cinvestav-Unidad Irapuato. Sin embargo, para 1999 el interés decayó, y no se continuó la investigación. Actualmente, existe una colaboración entre la Universidad de Colima y el Cinvestav-Unidad Irapuato donde se continua experimentando con papaya transgénica resistentes al virus de la mancha anular (Silva-Rosales, et al. 2010). En el caso de que se aceptara el cultivo de plantas transgénica de papaya en México se podría provocar pérdida de diversidad genética de las poblaciones silvestres si existe contaminación de transgenes (Gepts y Papa 2003). Los resultados de esta tesis, en particular las altas tasas de migración que se encontraron entre individuos silvestres, advierten que el polen puede desplazarse hasta 400 kilómetros de distancia. A pesar de que no se han estudiado los efectos ecológicos y evolutivos de la entrecruza entre plantas cultivadas y silvestres de papaya, durante las salidas de campo para la realización de esta tesis, se observaron individuos de papaya con frutos de tamaños medianos y alargados, sugiriendo una posible cruza entre individuos silvestres y cultivados. Esto sugiere que la cruza entre individuos transgénicos y silvestres es posible, y que los reservorios de diversidad genética de la especie pueden contaminarse con transgenes.

En conclusión, esta tesis señala la importancia de conocer y evaluar el estado actual de poblaciones o parientes silvestres de especies con importancia económica. México es considerado como uno de los principales centros de origen y domesticación del mundo para varias especies de plantas, entre las que destacan el maíz, la calabaza, el chayote, el chile, el frijol, el maíz, la vainilla y el algodón, entre otros, y aún falta

mucha información para varias de estas especies. En el caso particular de *C. papaya*, se encontró que esta especie en su forma silvestre es vulnerable a la fragmentación de su hábitat y que el mantenimiento de su diversidad genética depende en gran parte del conocimiento de su historia de vida (especie dioica, de corta vida, con tamaños poblacionales bajos y que depende de dispersores bióticos para mover su polen y sus semillas). Dichas características deben ser tomadas en cuenta en los planes de manejo y conservación para asegurar que estos sean exitosos.

Además de la importancia de las poblaciones de papaya silvestre como reservorios de diversidad genética, esta especie tiene un papel importante en su hábitat natural. Esta tesis también representa uno de los pocos acercamientos que existen a entender y conocer la ecología de la papaya silvestre. *C. papaya* es una especie de corta vida, dioica y con tamaños poblacionales pequeños. Es, además, considerada una especie nómada (Van Steenis 1958), lo que significa que persiste por la continua dispersión y colonización de claros o zonas de disturbio recientes (Martínez-Ramos 1985, Dirzo y Núñez-Farfán 1988), representando un papel importante en la regeneración natural de selvas tropicales y sub-tropicales donde habita. Muy poco era sabido sobre su distribución natural, en parte por la falta de registros o porque en estos rara vez se distingue entre la forma silvestre y la cultivada. En este trabajo se encontraron poblaciones de *C. papaya* silvestre a lo largo de las zonas tropicales y subtropicales del sureste de México. En general, las poblaciones silvestres de papaya se encontraron asociadas a claros dentro de las selvas o a zonas de disturbio o bordes. Se encontró que las poblaciones son, en general, pequeñas con no más de 35 individuos y que también es posible encontrar individuos aislados. Por otro lado, sus flores y frutos representan un importante recurso para la fauna con la

comparte su hábitat. Sobre sus polinizadores, se pudieron observar esfíngidos como se ha reportado en la literatura (OGTR 2008), pero también se observaron mariposas y abejas visitando las flores. Sin embargo, se requiere de un estudio sobre qué tan frecuentes y efectivos son estos polinizadores para las plantas silvestres. Nunca se observaron dispersores de semillas *in situ*, sin embargo, se observaron marcas en los frutos y troncos, que sugieren un consumo de frutos por parte de aves y mamíferos pequeños. En cuanto a la relación de la gente con esta especie en su forma silvestre, encontramos que en varias partes la gente las conoce como papayas de monte y no les dan mucho uso, solo las utilizan en algunos lugares para hacer dulce o mermelada (por ejemplo, en Huejutla San Luis Potosí y en Los Tuxtlas, Veracruz).

Queda mucho por hacer en cuanto a estudios biológicos de *Carica papaya* silvestre. Con base a esta tesis, surgió el interés de seguir estudiando el origen de la diversidad genética de esta importante especie. Es por esto que se propuso a la Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO) el proyecto "Análisis para la determinación de los centros de origen y diversidad genética de *C. papaya* (Caricaceae)". Este proyecto responde a la convocatoria de la CONABIO para generar elementos para la Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT) respecto a la información existente de las especies de las que México sea centro de origen y de diversidad genética; así como las áreas geográficas en las que se localicen. Esto permitirá proponer propuestas de medidas para su protección, de acuerdo a lo establecido en los artículos 86 y 87 de la Ley de Bioseguridad de los Organismos Genéticamente Modificados (LBOGM) y al artículo 23, fracción XXIX del Reglamento Interior de SEMARNAT. El principal objetivo de este proyecto será conocer la distribución puntual de *C. papaya* silvestre en México y completar las colectas en

toda su distribución. Para esto, se utilizó la localización de todas las poblaciones que se colectaron para esta tesis y se realizó un mapa con la distribución potencial (Figura 1), y se obtuvieron zonas donde la probabilidad de que *C. papaya* habite de manera natural, y que no han sido registradas o colectadas. Se pretende validar estas zonas y colectar las poblaciones faltantes para completar los análisis de diversidad genética abarcando todo el rango de distribución natural de la papaya. Los resultados de este proyecto serán de gran importancia para presentes y futuras tomas de decisiones en cuanto a la conservación y manejar los reservorios de diversidad genética de esta importante especie.

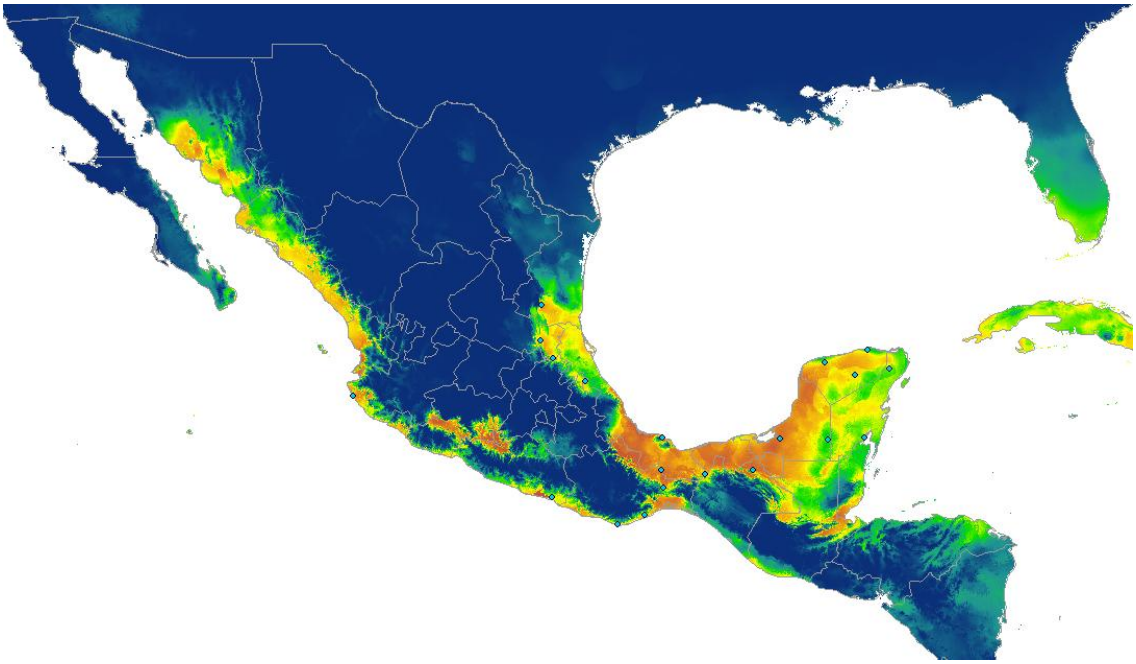

Figura 1. Mapa con la distribución potencial de poblaciones silvestres de *C. papaya*. Los puntos representan localidades colectadas o donde se han visto individuos silvestres. El color rojo indica zonas de alta probabilidad de encontrar individuos silvestres de papaya. El color azul indica zonas donde no es probable encontrar a *C. papaya* de manera natural.

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APÉNDICE I

Plant-antagonist interactions in fragmented habitats: does plant phylogeny matter?

Plant-antagonist interactions in fragmented habitats: does plant phylogeny matter?

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Abstract

Plant-antagonist interactions shape the structure, composition and dynamics of plant communities and ecosystems. Due to their key importance, much research has been advocated to evaluate anthropogenic habitat loss and fragmentation effects on plant-antagonist interactions but no clear response patterns have arisen. Even recent quantitative reviews have failed to provide consistent generalizations. Here we conduct the first phylogenetically independent meta-analysis along with a traditional meta-analytical approach to explore for pseudoreplication problems that might be leading to wrong conclusions. We examined whether characteristics of the interaction, the fragmented landscape, and methodological approaches modulate the magnitude of effects. Traditional meta-analysis showed that plants within habitat fragments suffer on average less damage from antagonists. However, when incorporating the phylogenetic relationships among plants, the overall effect and the particular effects of moderators became non-significant. Interestingly, we found a strong and consistent trend between both meta-analytical approaches in the overall effect of habitat fragmentation on folivory elicited by insects, implying for the first time a genuine effect that transcends the phylogeny of plants and is not undermined by statistical problems of pseudoreplication. Here, we provide robust conclusions to gain a new perspective of global responses of plants by antagonists in fragmented habitats.

Key words: habitat fragmentation, traditional meta-analysis, phylogenetically independent meta-analysis, antagonists, plant damage, herbivory, seed predation.

1. Introduction

Antagonistic plant-animal interactions, the most common and ancient interactions in nature (Scott 1983; Labadeira 1998), involve the direct and indirect damage of plants by animals (or viruses and pathogens) for food or housing (Southwood 1973; García y Chacoff 2007). Antagonistic interactions include principally folivores (leaf consumers), florivores (flower consumers), frugivores (fruit consumers) and seed predators (seed consumers), which collectively are called herbivores. The interaction between plants and their natural enemies influences the dynamics and structure of ecosystems and vice versa. Emerging causal effects from the individual level to the population-level processes can potentially affect forest regeneration and maintenance of plant diversity (Faveri et al. 2008). For instance, plant demography can be altered if the impact of herbivory changes due to plant ontogenetic stage or to the type of tissue that is consumed (Crawley 1997, Simonetti et al. 2006). This may also impact the community level if herbivores modify seedling recruitment altering the number or composition of plant species in the seed rain and seed bank (Hoffmeister et al. 2005, del Val 2012). Therefore, plant-antagonist interactions represent primary conservation targets because of their pivotal role in plant regeneration processes, plant community structure, ecosystem functioning, and biodiversity evolution (García & Chacoff 2007). Interestingly, such antagonistic interactions are also affected by modifications at community and ecosystem levels in a feedback fashion.

The current rates of defaunation and habitat fragmentation are dramatically affecting the interactions between plants and their natural enemies (Galetti et al. 2003; Galetti and Dirzo 2013). The transformation of continuous habitats into mosaics of

isolated forest fragments exposes organisms surviving in the fragments to a modified surrounding environment, where decreasing population size and connectivity often disrupts biotic interactions (Murcia 1995; Tscharntke & Brandl 2004). Only 10% of recent publications referring to ecology of fragmented habitats evaluate interactions and focus mostly on mutualistic interactions, such as pollination and seed dispersal (Ghazoul 2005, Aguilar et al. 2006, 2009). Much less attention is given to antagonistic interactions. Existent literature from the last decades show no clear response patterns on whether damage by antagonists decrease, increase or remain unaltered in fragmented landscapes. Some studies support the hypothesis of lower levels of damage in fragments, (Bersciano et al. 1999; Benítez-Malvido 2001; Arnold and Asquith 2002; Ledergerber et al. 2002; Vásquez et al 2007; Simonetti et al 2007, Faveri et al 2008, Ruiz Guerra et al. 2010), while others suggest increased damage in fragmented habitats (Kruess & Tscharntke 1994; Lienert et al 2001; Elzinga et al. 2005; Stoll 2006; Christie and Hochuli 2005). Moreover, the amount and quality of food resources for antagonists may also change with habitat fragmentation affecting plant productivity and leaf chemistry (Yamasaki & Kikuzawa 2003).

Interestingly, three recent reviews have addressed the effects of habitat fragmentation on plant-animal interactions, including the antagonistic relations between plants and herbivores using different scopes, and they have also shown contrasting outcomes (De Carvalho-Guimarães et al. 2014; Magrach et al. 2014; Martinson and Fagan 2014). While Martinson & Fagan (2014) found lower herbivory in habitat fragments than in continuous habitats, De Carvalho-Guimarães et al. (2014) found the inverse pattern: plants in habitat edges suffered more damage than plants inside habitats. Finally, Magrach et al. (2013) suggested that antagonistic interactions

are more robust to habitat fragmentation since they did not find any effect of habitat fragmentation. Such disparity of general response patterns in the three reviews is quite surprising. Systematic quantitative reviews such as meta-analysis are powerful objective statistical tools that allow estimating an overall effect size of a common factor by combining the results of independent studies addressing similar research questions (Gurevitch & Hedges 2001). Such contradictory overall effects among reviews may be ascribed to different approaches of effect size calculations, criteria of study inclusion, as well as the scopes and databases used by the different reviewers. Despite the reasons, these important attempts to summarize the existent empirical evidence have failed to find a consistent clear response pattern of habitat fragmentation effects on plant-antagonistic interactions.

Moreover, none of these three reviews accounted for phylogenetic non-independence in their overall effect size estimations. Meta-analytic data in ecology and evolutionary biology can seriously violate statistical assumptions of independence, especially when effect sizes are calculated from individual species, as is the case in these reviews. Common shared ancestry of taxonomically related species introduces a correlated error structure that needs to be accounted for in order to avoid misleading conclusions in meta-analyses (Lajeunesse 2009, Chamberlain et al. 2012). Additionally, phylogenetically independent meta-analyses can also allow us to unravel the relative importance of evolutionary phylogenetic relationships over the ecological effects of habitat fragmentation.

The effects of habitat fragmentation on antagonistic interactions can be influenced by sources of variability related to the interaction and/or to external

landscape features. Yet, these factors have not been thoroughly analyzed for multiple species. For instance, responses to habitat fragmentation may differ depending on the type of interaction and degree of specialization, where more specialized plant-antagonist interactions may be more susceptible to be lost in fragmented habitats compared to more generalist interactions. On the other hand, the identity of the interacting partner may also show differential response. For example, if we only consider the mobility of natural enemies we could expected that the higher mobility of birds and mammals may render less susceptibility to fragmentation effects compared to insects, which have comparatively lower mobility. Also, certain types of antagonist interactions may be more susceptible than others. If seed predation is mostly performed by birds and mammals (as in the tropics), then it may be less negatively affected by habitat loss compared to folivory, which is mainly accomplished by insects. Moreover, external landscape features of the fragmented habitats can also influence the magnitude of fragmentation effects on plant-antagonist interactions. The matrix surrounding the fragments may affect plant's susceptibility to antagonist animals by conditioning their dispersion and mobility capacity throughout the landscape (Driscoll et al. 2013). Also, the time elapsed since the onset of fragmentation can determine when biotic interactions would show a change promoted by habitat fragmentation. Because local extinction of species can occur with a considerable delay after the event of habitat loss (i.e., undergo extinction debts; sensu Tilman 1994), recently fragmented habitats may not show significant changes in biotic interactions relative to continuous, undisturbed original habitats. Finally, methodological approaches of published research may also influence the sensibility to find habitat fragmentation effects; experimental studies that deliberately create fragmented environments or place individuals within

certain arrangements may have different ability to detect effects compared to observational studies. Despite the fact that experimental approaches are a key tool for disentangling causation, they may have a cost in terms of loosing external validity when facing complex and dynamic processes such as habitat fragmentation (Sagarin and Pauchard 2010). In fact, the multiples approaches and definitions used by experimental studies may be introducing an important amount of artificial variance that dilutes important effects when studying habitat fragmentation. For instance, experimental approaches could mask the effect of factors such as number of generations since fragmentation, the spatial arrangement of fragments, and the degree of isolation of habitat fragments, among others.

In the present study we conduct phylogenetically independent and traditional meta-analyses to assess the overall magnitude and direction of habitat fragmentation effects on plant-antagonist interactions. We also examine whether certain characteristics of the plant-antagonist interaction (type of interaction, degree of specialization, and identity of antagonist), the approach used by authors (experimental vs. observational studies), and features of the fragmented habitats (type of habitat, time elapsed in fragmentation condition and matrix type surrounding fragments), modulate the magnitude of effects on antagonistic interactions.

2. Material and Methods

We conducted extensive surveys in electronic bibliographic data bases (Scopus and Google Scholar) and searched for studies evaluating habitat fragmentation effects on the interaction of plant species and their antagonists. To attain this we used the

following keyword combination for searching the literature: fragment* AND (herbivor* OR folivor* OR frugivor* OR florivor* OR "seed predat*" OR parasit* OR pathogen* OR antagonist*). Therefore, the antagonistic interactions included here were: folivory, frugivory, florivory, seed predation, parasitism, and damage by pathogens. For a study to be included in our analysis, quantitative data of plant damage inflicted by the antagonist had to be reported. Due to the large amount of approaches to the study of habitat fragmentation, we included studies that compared plant damage in (1) continuous habitat vs. habitat fragments, (2) plant populations with different degrees of isolation, and (3) plants evaluated in the interior and edges of habitats. Despite the approaches used by authors, all studies included here were explicitly aimed at measuring habitat fragmentation effects. For studies that included multiple species, we incorporated each of the species as independent studies (Gurevitch & Hedges 1999). For studies with repeated measures in time, we consistently chose the last measure. Many studies included comparisons of continuous habitat with several habitat fragments of different sizes; in these cases we selected the medium size fragment to have a conservative estimation. When comparisons of continuous habitat were made against two fragments we chose the smallest one. Furthermore, we went through the metadata used by the three recent reviews (De Carvalho-Guimarães et al. 2014; Magrach et al. 2014; Martinson and Fagan 2014), to check for studies that did not appear in our initial search but that matched our selection criteria for inclusion. We were able to incorporate and use 41 effect sizes from these reviews. All non-fragmentation studies from these databases were not included here.

Based on the information given in articles, we classified the plant species according to the identity of the antagonist partner (birds, fungus, insects, mammals and

mixed), and the degree of specialization of the antagonist interaction (generalist vs. specialist). Furthermore, we obtained information about the fragmentation context of every study such as the type of natural habitat (tropical forest, temperate forest, desert/xeric shrubland, grassland and wet meadow (sensu Olsen et al. 2001)), the type of matrix around fragments (pastures, cultivated land, forestry plantations, urban areas, water (in the case of wet meadows) or mixed) and time since fragmentation, which included three broad categories reflecting the most frequent time periods of fragmented habitats assessed by authors (≤ 30 years, 31-60 years and ≥ 60 years). We also recorded the methodological approach used by the studies as to whether the evaluation was experimental or observational. If some information was not given in the article, we either contacted the authors or looked it up in other articles (in the case of species or study sites). All this context-dependent information was used to detect attributes in the studies that could influence the magnitude of habitat fragmentation effects on antagonist interactions.

2.1 Data analysis

We performed all meta-analyses using Hedges´ unbiased standardized mean differences (Hedges´ *d*) as the effect size estimator. Hedges´ *d* expresses the difference in plant damage inflicted by antagonists between fragmented and continuous habitats. To calculate Hedges´ *d* for each species, we obtained the mean values, sample sizes and standard deviations of damage values for each of the two landscape conditions. We used Datathief III software (http://datathief.org/) to obtain the data form graphs. If some of the data were not provided, we either contacted the authors or excluded it. In a few studies using correlational approaches (e.g., plant damage by antagonists along

gradients of habitat isolation) we used Pearson correlation coefficients and sample sizes to calculate Hedges´ *d* through arithmetical transformations (Borenstein et al. 2009). Because variation in effect sizes can be due not only to sampling error but also due to a true random component (Raudenbush 1994; Gurevitch & Hedges 1999) we used random-effects models for all the analyses.

We used metafor (Viechtbauer 2010) to run traditional meta-analyses. Fragmentation effect was considered significant if the 95% biased-corrected bootstrap confidence interval (CI) of the effect size (*d*) did not overlap zero (Rosenberg et al. 2000). Positive values of the effect size (*d*) imply that habitat fragmentation increases plant damage (i.e., higher plant damage in habitat fragments) whereas negative *d* values imply that habitat fragmentation decreases plant damage. In order to test the explanatory power of moderator variables we used between groups Chi-squared test $Q_{between}$ (Hedges and Olkin 1985). Significant $Q_{between}$ indicates that a particular categorical moderator variable (e.g., type of antagonist partner, habitat type, time since fragmentation, etc.) explains part of the heterogeneity among effect sizes. Finally, the possibility of publication bias was evaluated graphically using a funnel plot and statistically calculating a rank correlation test for the asymmetry in the funnel plot. Both examine the relationship between effect sizes and sample sizes across studies. If publication bias exists then studies with small or null effect sizes are missing and the funnel shape is asymmetric and correlation tests are significant. We also calculated a weighted fail-safe number, which indicates whether results are robust regardless of any publication bias (Rosenberg 2005). The weighted fail-safe number value indicates the number of non-significant, unpublished or missing studies that would need to be added to a meta-analysis in order to nullify the overall effect sizes (Rosenthal 1979). If

the calculated fail-safe number is greater than 5*n*+10, where *n* is the number of studies, then publication bias may be safely ignored (Rosenberg 2005).

When considering several effect sizes from different plant species the statistical assumption of independent samples can be violated due to the correlated error structure associated to the intrinsic evolutionary relationship among species, thus potentially affecting general conclusions about response patterns in quantitative reviews (Lajeunesse 2009, Chamberlain et al. 2012). The inclusion of phylogenetic information within meta-analysis allows for correct phylogenetically independent estimations of overall effects. To run a phylogenetically independent meta-analysis we constructed a phylogenetic tree of all the plant species included in our review with PHYLOMATIC (Webb & Donoghue 2004). The hypothesized tree sets the phylogenetic relationships among all the species considered in this review using a megatree based on APG III. The internal branch-length was estimated according to Wikstrom et al. (2001) using PHYLOCOM (Webb et al 2011) (Fig. A1). Six of nine polytomies were solved based on published information (Rohwer 2000; Chanderbali et al. 2001; Swenson & Anderberg 2005; Asmussen et al 2006; Tokuoka 2007; Kahn 2008; Baker et al. 2011; Coutinho et al. 2014; Fine et al. 2014) (families Arecaceae, Lauraceae, Fagaceae, Burseraceae, Euphorbiaceae, Sapotaceae). Phylogenetic trees were converted into ultrametric trees. This procedure satisfies the assumptions of the relationship between the phylogenetic correlations and the time since divergence (Lajeunesse 2009) and also makes effect sizes comparable with the traditional meta-analysis. Lajeunesse´s (2009) method was performed with PHYLOMETA that uses a weighted GLS approach to account for phylogenetic correlations among species. In the case where multiple effect sizes were available for the same species (this happened for18 species), we pooled

them prior to performing the phylogenetically independent meta-analysis. To do this, the overall effect size for each repeated species was estimated using a traditional meta-analysis with a fixed- effect model (Lajeunesse et al 2013). We built a global phylogenetic tree with the entire sample of species for overall fragmentation effect estimations. When analyzing each predictor or moderator variable, we constructed a subset phylogenetic tree for each moderator (e.g., type of antagonist interaction, identity of antagonist, etc.), which contained only the species present in that particular comparison and retains all the branch length information found in the original hypothesized tree. Finally, we estimated Blomberg´s *K* (Blomberg et al. 2003), which measures the strength of the phylogenetic signal in phylogenetic trees, in order to understand the potential of contrasting results between traditional and phylogenetically independent meta-analyses (Chamberlain 2012). In the context of a meta-analysis, *K* values approaching zero imply that closely related species do not share similar effect sizes, whereas values of *K* near or larger than one suggest that closely related species do share similar effect sizes (i.e., effect sizes are conserved). This parameter was obtained using the R package "phytools" and the function phylosig (Revell 2012).

3. Results

The literature search comprised the period 1999 – 2013 of published studies related to habitat fragmentation and antagonistic interactions. A careful screening was performed to determine their suitability for inclusion in our synthesis. We ended up with 77 published papers and two PhD theses that evaluated the effects of habitat

fragmentation on different plant-antagonist interactions. These studies yielded 141 data points from 98 plant species from 54 plant families (Table A1). In Fig. 1 we summarize the number of species within some of the categories examined. In general, tropical forest, as habitat, and insects, as antagonist partners where the most represented within our review dataset.

We found no evidence for publication bias in our data. The funnel plot of effect size vs. sample size showed no skewness suggesting no bias in reporting results from the studies included in this review (Fig. A2). This result was supported by the non significant rank correlation test for funnel plot asymmetry (Kendall´s Tau = -0.484, P = 0.4008), as well as by the calculated weighted fail-safe number (2598), which resulted greater than the expected without publication bias ($5n + 10 = 715$)

The overall weighted mean effect size of habitat fragmentation on the interactions of plants with antagonists across all studies was negative and significantly different from zero when evaluated with the traditional meta-analysis (d_+ = -0.3557, df = 140, P = 0.0012, CI 95% -0.5705 to -0.1409) indicating that on average, plants in habitat fragments suffer significantly less attacks from antagonists compared to plants in continuous habitats. Nevertheless, this effect became non-significant when the phylogenetically independent meta-analysis was performed (d^P = 0.333, df = 97, CI 95% -0.187 to 0.853) (Fig. 2). The total heterogeneity of effect sizes was large and statistically significant in both meta-analyses (traditional meta-analysis: Q_{total} = 790.744, df = 140, P < 0.0001; phylogenetically independent within-study heterogeneity test assuming a fixed-effect model: Q^P_{total} = 2724.54, df^P = 97, P^P < 0.0001) meaning that effect sizes do not share a common effect. The contrasting outcomes between the traditional and the

phylogenetically independent meta-analyses were generally consistent as we examined the different moderators. Moderators with sample size smaller than 10 are not reported in our results nor discussed, but are reported in tables

Among the type of antagonistic interactions, folivory was the only interaction with a significantly negative habitat fragmentation effect (d = -0.471$^{\text{significant} = *}$), but as observed for the overall effect, this was only detected in the traditional meta-analysis. None of the other types of antagonist interactions showed significant fragmentation effects in both meta-analyses (i.e., effect sizes overlapping zero Hedges´ d value; Table 1, Fig. 2). Similarly, within the type of antagonist, only insects (d = -0.434*) showed significant negative fragmentation effects (Table 1, Fig. 2), which also became non-significant in the phylogenetically independent meta-analysis. No difference was observed between generalist and specialist antagonist interactions (Table 1, Fig 2) in neither of the two types of meta-analyses.

When exploring features of the fragmented landscape we found that studies assessing fragmentation effects with cultivated lands as surrounding matrices showed significantly stronger negative effects, implying lower damage by antagonists in fragmented habitats than in continuous habitats (d = -0.524*; Table 1; Fig. 3). In terms of habitat type, significantly lower levels of damage were found in habitat fragments of temperate and tropical forests (d = -0.399* and d = -0.397*, respectively) (Table 1; Fig. 3). When assessing the time since fragmentation we found that plants from fragmented habitats of more than 60 years showed less attacks compared to continuous habitats (d = -0.432*) (Table 1; Fig. 3). As observed before, all these effects become non significant when incorporating the phylogeny in effect size calculations. The only exception was

the effect observed in tropical forests, which remained significant in the phylogenetically independent meta-analysis.

With regards to the methodological approaches used in fragmentation studies assessing antagonistic interactions, only observational approaches showed a negative and significant mean effect size (d = -0.499*) whereas experimental studies showed no significant effects. Again, effect size of observational studies became non –significant when controlling for phylogenetic relationships among plant species.

All the estimated phylogenetic signals ($K_{Blomberg}$, Blomberg et al. 2003), either for the phylogenetic tree constructed with the entire set of species ($K_{Blomberg}$ = 0.249, P = 0.23) as well as for each tree built for each moderator variable (range 0.26-0.38; see Table 1), were all relatively low and always non-significant, implying that effect sizes are not conserved across the phylogeny.

3.1 The case of insects

Insects were by far the most represented antagonist group (Fig. 1) and they showed a strong negative mean effect size in the traditional meta-analysis Table 2; Fig. 4). Because of the large sample size of the insect group, we run further analyses using the same moderators and methods described above. As previously, many effects with a significant effect of habitat fragmentation found in the traditional meta-analysis were lost when we performed the phylogenetically independent meta-analysis. This was the case for damage by generalist interactions was lower in habitat fragments in the traditional meta-analysis (d = -0.685*) but this trend was not consistent between meta-analyses (d^p = -0.753). Similarly, significant decreased damage by insects in tropical forest fragments was not consistent in both meta-analyses (d = -0.463*, d^p = -0.212*),

nor in the temperate forests (d = -0.534*, d^p = -0.416), which showed a non-significant effect in the phylogenetically independent meta-analysis (Table 2). Furthermore, habitat fragments immersed in cultivated land matrices showed negative effects only in the traditional meta-analysis (Table 2). However, and very interestingly, we found that plants in fragmented habitats presented significantly less damage due to insect folivory compared to continuous habitats, and this result was consistent in the phylogenetically independent meta-analysis (d = -0.621*; d^p = -0.986*). Also interesting, when evaluating the effects of habitat fragmentation depending on the time since it occurred, we detected a contrasting result: the traditional meta-analysis indicates a negative mean effect sizes in plants from fragmented habitats of >60 years old (d = -0.511; i.e. less attacks on fragments than continuous habitats) while phylogenetically independent meta-analysis detected the same trend but on fragmented habitats of 30-60 years old (d^p = -1.095*) (Table 2; Fig. 4). Phylogenetic trees built with data of only insects showed in general larger phylogenetic signals (i.e., larger values for Blomberg's K). These phylogenetic signals were statistically significant every time the phylogenetically independent meta-analyses switched to a non-significant effect sizes, except in the case of the level of specialization (Table 2).

4. Discussion

Detrimental effects of antagonists on plants represent a central ecological interaction that affects plant fitness, shaping the structure, composition and dynamics of plant communities and ecosystems (Scott 1983; Crawley 1997). Due to its key importance, much research has been advocated over the past decades to evaluate how current

anthropogenic habitat loss and fragmentation affects plant-antagonist interactions (e.g. Benítez-Malvido et al. 1999; Groppe et al. 2001; Arnold and Asquith 2002; Farwig et al. 2008; Herrerías-Diego et al. 2008; González-Varo 2010; Ruíz-Guerra et al. 2010; De Crop et al. 2012; among others). Individual studies have mainly focused on evaluating fragmentation effects on single plant species or single plant-antagonist interactions, showing varied and contrasting response patterns. Due to such species-specific responses, the sample of species and interactions studied so far can condition the generality of literature syntheses. Within the studied antagonist interactions found in this review, we observed some biases in the selection criteria of researchers (i.e., research bias), where folivory and seed predation in tropical and temperate habitats were the interactions most frequently evaluated. Likewise, insects have been the most studied antagonist group, and most studies evaluated generalist interactions. The particular overrepresentation of generalist insects, however, may be reflecting their normal prevalence in plant-animal interactions in nature (e.g., Waser et al. 1996). In spite of such natural dominance, there are still many other groups of antagonists (pathogens such as fungi and virus, galls, mammals and birds) currently underrepresented in habitat fragmentation research, and they may show a higher degree of plant resource specialization. Such gap of understudied plant-antagonist interactions needs to be filled in future studies for a thorough and robust diagnosis of fragmentation effects. Moreover, we suggest to explore plant families poorly studied and to include abundance data of plants and their interactuants. This will help, for example, to understand the effect of habitat fragmentation over antagonists' populations and explain the decrease of plant damage in habitat fragments. Also,

including fitness evaluations of attacked plants will help recognize to what extent antagonists compromise the persistence of plant populations in fragmented habitats.

While it is not expected to attain any generalization from single species studies, it is especially intriguing that the three quantitative reviews recently conducted (De Carvalho-Guimarães et al. 2014; Magrach et al. 2014; Martinson and Fagan 2014) do not provide consistent global response patterns of fragmentation effects on plant-antagonist interactions. Here, we conducted the first phylogenetically independent meta-analysis of habitat fragmentation effects on plant-antagonist interactions, which also included metadata from these three recent reviews. Our results offer supported conclusions that must be considered to gain a new perspective of global responses of plants by antagonists in fragmented habitats, which may help understand the contrasting findings in previous systematic reviews.

4.1 Traditional meta-analysis

The results of the traditional meta-analysis for 141 data points from 98 plant species from 54 families showed an overall negative effect of fragmentation on the interaction of plants and their natural enemies, indicating that plants within habitat fragments suffer on average less damage from antagonists than in continuous habitats. This response pattern was particularly clear and strong for the interaction of folivory (44% of the studies), with insects as the main antagonists (80% of folivory studies), concurring with the results obtained by the traditional meta-analysis of Martinson and Fagan (2014). Habitat loss and fragmentation can disrupt plant-animal interactions as a result of the structural changes occurring at the landscape level (decreased area of habitat remnants and increased isolation among them; e.g., Didham et al. 1996, Fahrig 2003). Such changes and the presence of inhospitable anthropogenic matrices among habitat

remnants impose physical barriers for interacting animal partners. Insect fauna in particular has shown to be highly susceptible to habitat fragmentation effects, which has been ascribed to their limited dispersal ability and shorter generation times compared to vertebrates, declining in abundance and species richness (Didham 1996; Ewers & Didham 2006; Martinson & Fagan 2014). The results found here agree with this expectation, as insects were the only group of antagonists that showed a negative and significant fragmentation effect, decreasing insect plant damage in habitat fragments. On the contrary, the absence of fragmentation effects on birds and mammals may be due to their higher mobility between fragments (Andren 1994; Bayne & Hobson 1998; Pardini et al. 2005), being less susceptible to habitat fragmentation than insects. In fact, seed predation was not significantly affected by habitat fragmentation, and such result may be linked to the higher number of studies where seed predators were mainly birds and mammals; thus counteracting or diluting the negative effect of insect seed predators. As expected, higher specialization in plant-antagonist interactions showed a stronger negative magnitude of fragmentation effects than generalist interactions, but this difference was not statistically significant. Such result may be due to the low sample size of specialists, which generates large confidence intervals for this group and thus low power for testing the null hypothesis.

Some of the landscape features assessed here showed significant influences in shaping the magnitude of fragmentation effects within the traditional meta-analysis. Regarding the matrix type, we found that plants in habitat fragments surrounded by cultivated land had significant lower levels of damage than in continuous habitats, therefore representing barriers for some natural enemies of plants. The use of chemical products such as insecticides, herbicides, and pesticides in cultivated land matrices may

have an additional negative impact, especially on insect populations (e.g., Winfree et al. 2009). Also, studies conducted in tropical and temperate habitats showed a significant decrease of antagonist attacks in plants inhabiting fragments whereas the rest of habitat types showed non-significant effects. However, such results are most likely the consequence of higher statistical power of these two habitat types, which comprised 78% of the studies. Finally, we found that plants surviving in fragmented habitats of more than 60 years showed a significant decrease from antagonist attacks compared to more recently fragmented landscapes. This result agrees with the theoretical expectation that the time elapsed in fragmentation conditions can determine whether extinction debts are paid or not (Tilman 1994). Our results imply that older fragmented systems have already paid extinction debts of antagonists; therefore showing decreased abundance and diversity of natural enemies of plants. Considering the time scale of habitat fragmentation effects is important to understand the response of plants' interactions with their natural enemies in fragmented habitats (Aguilar et al. 2008, Rivera-Ortiz et al. 2014).

4.2 Phylogenetically independent meta-analysis

Incorporating the phylogenetic relationships in meta-analyses initially addresses the non-independence of effect sizes from species with shared evolutionary history, thus solving a clear violation of statistical assumptions (Adams 2008; Lajeunesse 2009). Here, when we incorporated the phylogenetic relationships among the plant species included in this review, the overall fragmentation effect as well as the particular effects of each moderator variable became non-significant. Such nullifying influence of phylogeny on meta-analytical global effects has been recently put in a broad context by Chamberlain et al. (2012), who re-analyzed 30 published ecological meta-analyses after

incorporating the correlated error structure of phylogenetic relationships among species. They found that accounting for phylogeny reduced overall effect size significance in 40% of the random-effects models meta-analyses. More specifically, decreases in pooled effect sizes after incorporating phylogenetic information were associated with larger phylogenies and those with stronger phylogenetic signal (Chamberlain et al. 2012). Thus, phylogeny can act as an explanatory variable and its relative influence in meta-analytical syntheses will depend on the particular assemblage of the species included in the review, which should be closely linked to the sample of species globally studied so far, after a thorough literature search.

In accordance, when effect sizes are not conserved within the phylogeny (i.e., there is weak or null phylogenetic signal) any phylogenetic correction may have a trivial effect on meta-analytical results, as effect sizes would be fundamentally independent across the phylogeny (Chamberlain et al. 2012). Surprisingly, the phylogeny built with 98 unique species from 54 plant families included in our review showed a low and non-significant phylogenetic signal ($K_{Blomberg}$ = 0.25, P = 0.231; Blomberg et al. 2003), but still the overall effects were nullified when running the phylogenetically independent meta-analysis. Thus, the results for the entire dataset would indicate that it is not precisely the phylogeny per se that is nullifying the overall effects, but simply the decreased power of the omnibus test as a result of decreased sample size from the traditional (N=141) to the phylogenetically independent (N=98) meta-analyses. This asseveration is supported by results of a traditional meta-analysis with the 98 data from each species, which produced identical non-significant results as the phylogenetically independent meta-analysis (Table A2).

With the aim of comparing our results with previous reviews, we attempted to introduce the phylogenetic information and re-analyzed the previous traditional meta-analyses (Magrach et al. 2014; Martinson & Fagan 2014; De Carvalho-Guimarães et al. 2014) but they all failed to provide and report the correct data to do so. Martison and Fagan (2014) were the only ones providing a complete list of plant species studied in their review but surprisingly (similar to Magrach et al. 2014) they only presented effect size values of each species but did not report their variances, precluding the possibility of repeating straightforward their meta-analyses. Thus we do not know for certain how phylogenetic information would affect their overall conclusions. Moreover, their database also includes some plant species assemblages (community level) as well as many studies assessing naturally fragmented spatial structure rather than anthropogenic habitat fragmentation. Thus, their contrasting outcomes may be the result of (i) singular assemblages of species included in their review due to different inclusion criteria, (ii) not focusing strictly on anthropogenic habitat fragmentation, and/or (iii) not controlling for phylogenetic information.

4.3 The case of insects

When assessing only the group of insects, there was a strong and consistent trend for the folivory interaction. Folivory by insects is a well-studied antagonist interaction and of recognized importance on plant fitness, community structure and ecosystems processes (Southwood 1973; Crawley 1997; Del Val 2012). This interaction showed decreased damage in habitat fragments and this effect was consistent with both meta-analytical approaches. This result is important as it implies for the first time a genuine fragmentation effect that transcends the phylogenetic background of plant species sampled and that is not undermined by statistical problems of pseudoreplication.

Furthermore, contrary to the global database, within the insect subsample of studies there were certain phylogenies built for the particular moderator contrasts that showed significant phylogenetic signals (matrix type: $K_{Blomberg}$ = 0.44, P = 0.028, time since fragmentation: $K_{Blomberg}$ = 0.55, P = 0.01, and methodological approach: $K_{Blomberg}$ = 0.44, P = 0.02; Table 2), and the mean effects from these moderators were nullified when incorporating the phylogenies. Thus in these cases, decreased mean effects in phylogenetically independent meta-analyses are the result of conserved effect sizes of the species within these partial phylogenies.

Significant, phylogenetic-independent negative overall fragmentation effects on insect folivory imply that plant populations surviving in fragmented habitats are subjected to less insect damage. Interestingly, previous quantitative reviews assessing fragmentation effects on bee pollinators (Winfree et al. 2009) and on plant pollination and reproduction (Aguilar et al. 2006) have shown significant negative global effects. By integrating these syntheses outcomes we may envision a compensative fragmentation effects whereby overall reductions in richness and abundance of pollinator and herbivore insects negatively affects sexual plant reproduction but positively reduces plant mortality and performance by less insect herbivores. The integrated, simultaneous research of mutualist and antagonist interactions within the same plant species will help disentangle the net fragmentation effects on long term plant population viability (Aguilar et al. 2009), with key implications for plant community structure, ecosystem functioning, and biodiversity evolution in current ubiquitous fragmented landscapes.

4.4 Final considerations

Our review highlights the importance of not just simply incorporating the phylogenetic relationships among sampled species, which resolves the problem of non-

independence of effect sizes, but also of analyzing the phylogenetic signal of each phylogeny, as they can affect overall conclusions (Chamberlain et al. 2012). Any attempt to generalize across a broad range of taxa without evaluating the phylogenetic information can seriously undermine the validity of conclusions drawn from such reviews. Moreover, meta-analyses intended to synthesize on plant-animal interactions in particular, should also attempt to generate phylogenies from both the plant and the animal sides of the interactions. Unfortunately, studies currently tend to focus on one side of the interaction, and thus do not provide sufficient taxonomic information on the interacting counterpart. In this regard, incipient studies focusing on interaction web analyses should help to begin informing on both sides of the interacting web structure, allowing building and analyzing simultaneously plant and animal phylogenies in meta-analytical contexts.

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Figures

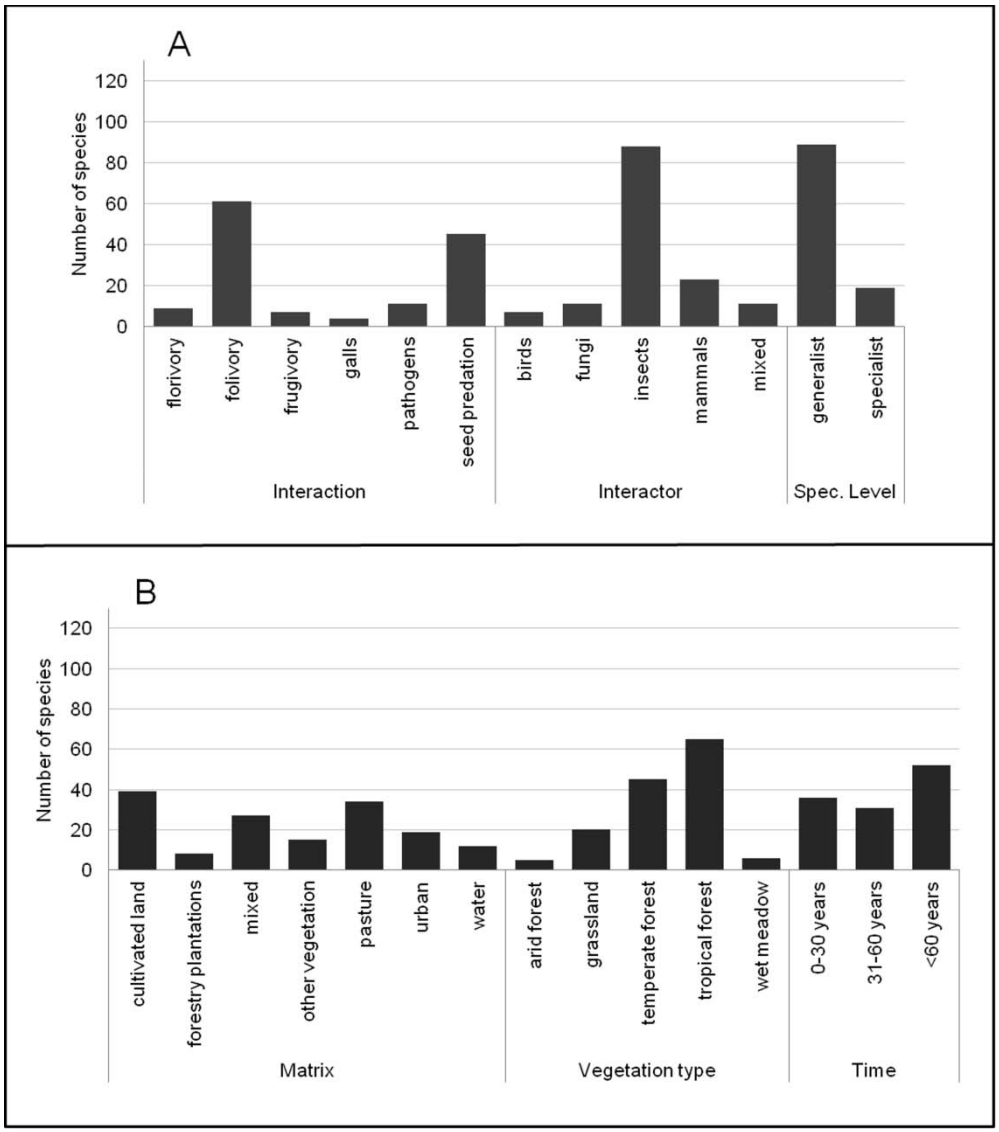

Figure 1. Number of species analyzed within each moderator variable included in the review. (A) Type of the antagonistic interaction, and (B) Landscape features of the fragmented habitat.

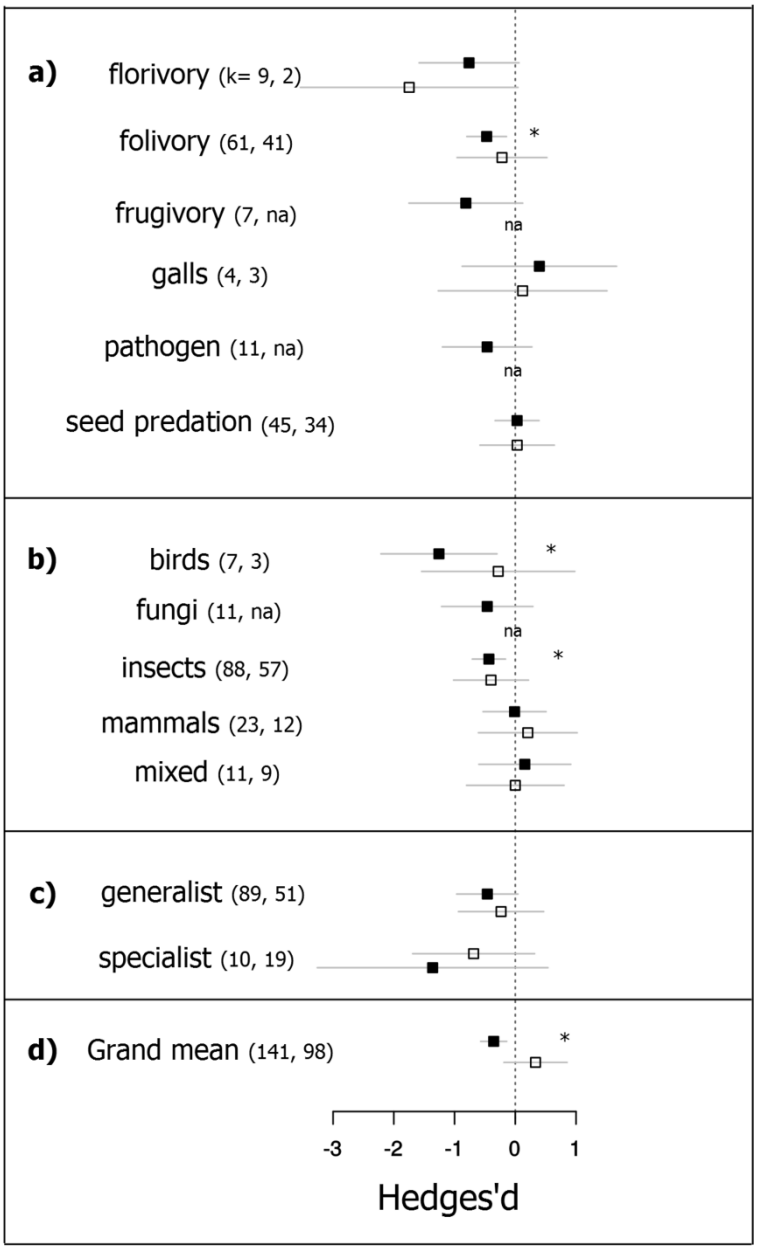

Figure 2. Weighted mean effect sizes and 95% bias-corrected CI of habitat fragmentation on plant-antagonist interactions by the type of interaction (a), type of antagonist (b), specialization of the interaction (c), and for all species (i.e. the grand mean; d). Black squares represent the results for the traditional meta-analysis; white squares represent the results for the phylogenetically-independent meta-analysis. The vertical line represents Hedge´s *d* = 0. Values within a parenthesis indicates the sample sizes (k) for traditional and phylogenetically-independent meta-analysis.* denotes a significant effect.

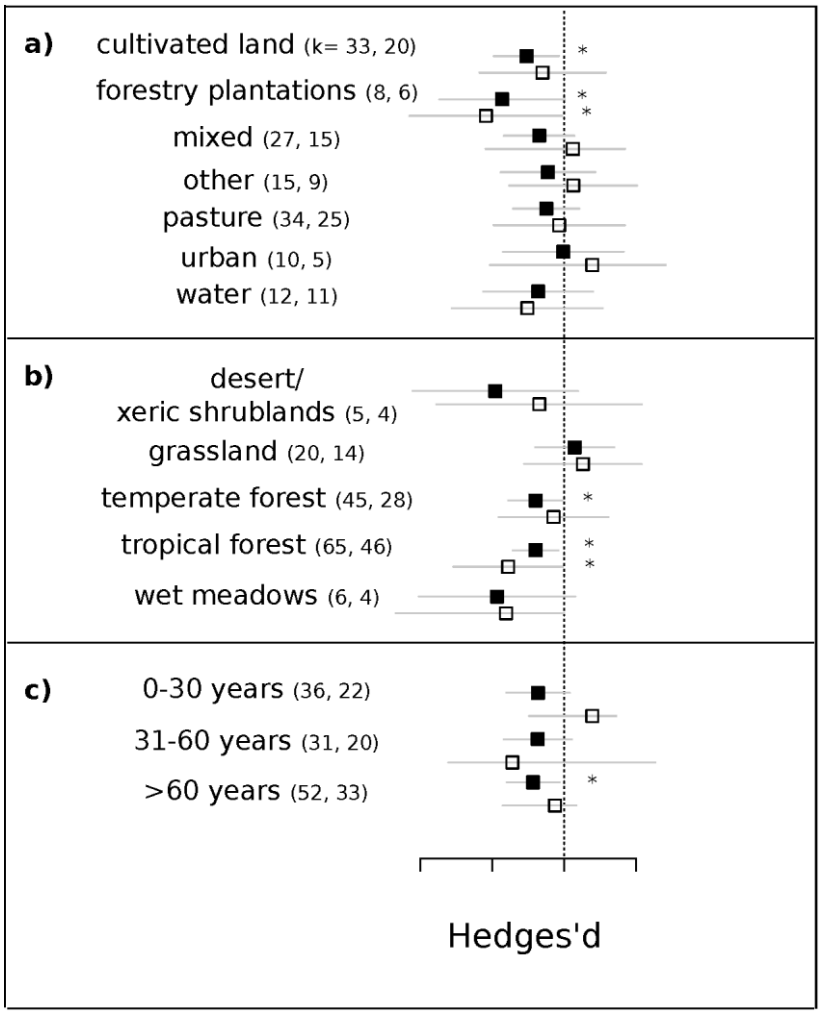

Figure 3. Weighted mean effect sizes and 95% bias-corrected CI of habitat fragmentation over the interactions of plants with their antagonists and categorized by type of matrix (a), natural habitat (b), time since fragmentation (c). Black squares represent the results for the traditional meta-analysis; white squares represent the results for the phylogenetically-independent meta-analysis. The vertical line represents Hedge´s *d* = 0. Values within a parenthesis indicates the sample sizes (k) for traditional and phylogenetically-independent meta-analysis.* denotes a significant effect.

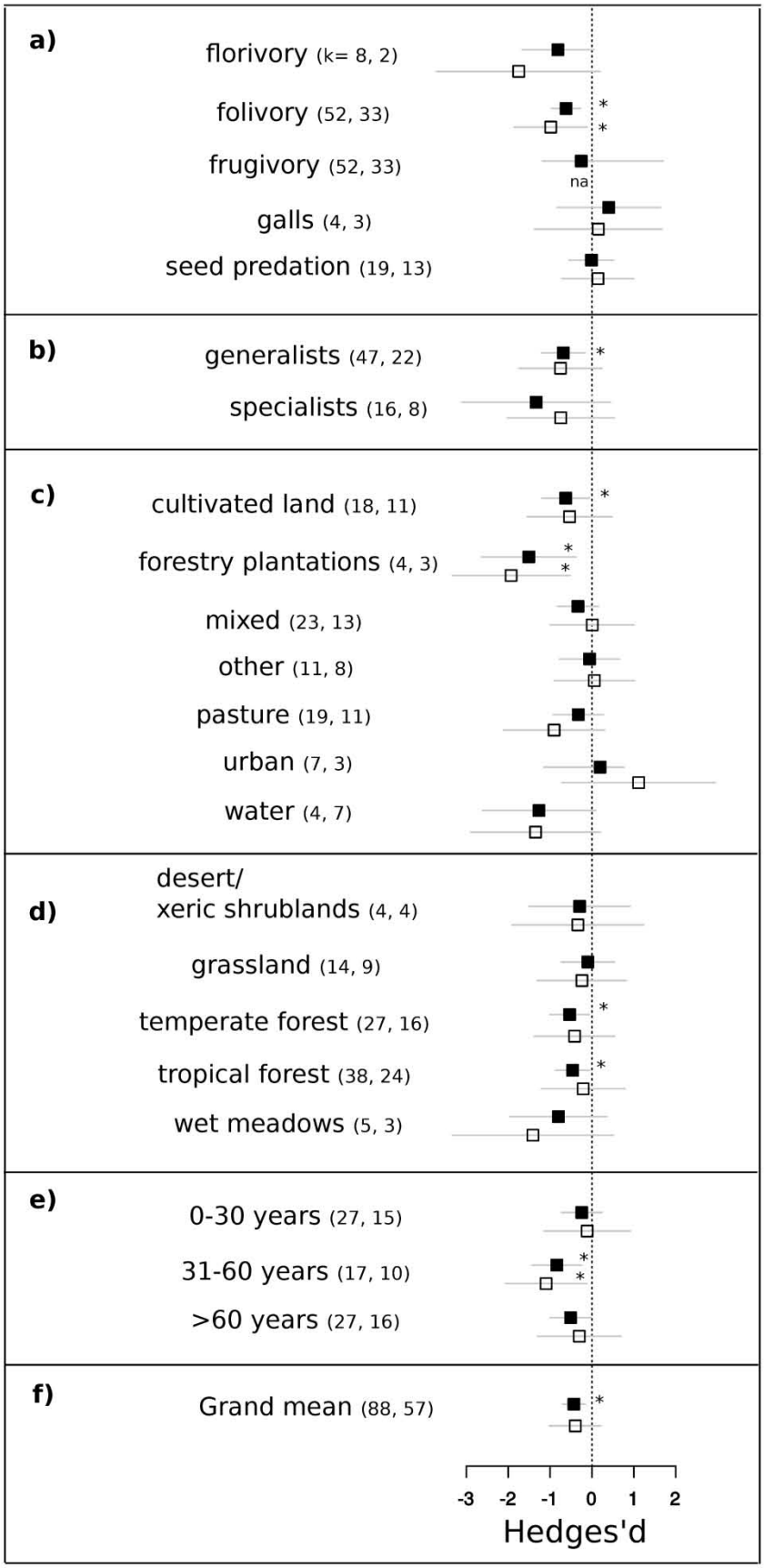

Figure 4. Weighted mean effect sizes and 95% bias-corrected CI of habitat fragmentation over the interactions of plants with only insect as antagonists categorized by type of interaction (a), time since fragmentation (b), and for all species

(c). Black squares represent the results for the traditional meta-analysis; white squares represent the results for the phylogenetically-independent meta-analysis. The vertical line represents Hedge´s d = 0. Values within a parenthesis indicates the sample sizes (k) for traditional and phylogenetically-independent meta-analysis.* denotes a significant effect.

Tables

Table 1. Traditional and phylogenetically independent meta-analyses of habitat fragmentation effects on plant-antagonist interactions. Q_b test evaluating between-group differences are reported by each moderator evaluated. Sample sizes (*K*), the weighted mean effect sizes (Hedges' d_+) and 95% confidence intervals shown. Superscript ^{p} refers to phylogenetically independent meta-analyses. $K_{Blomberg}$ are shown for each partial phylogeny built with the species included in each moderator contrast.

	Traditional meta-analysis				**Phylogenetically-independent meta-analysis**			
Grouping categories	***K***	d_+	**LCI**	**UCI**	K^p	d_+^p	**LCI**	**UCI**
Interaction	Q_b = *7.945*, d.f. = 5, *P* = 0.1592				Q_b^p **= *9.75*, d.f. = 1, *P* = 0.0018** $K_{Blomberg}$ = 0.30, P = 0.129			
Florivory	9	-0.761	-1.584	0.061	2	-1.747	-3.538	0.044
Folivory	61	**-0.471**	**-0.797**	**-0.145**	41	-0.219	-0.959	0.521
Frugivory	7	-0.814	-1.750	0.122	NA	NA	NA	NA
Galls	4	0.396	-0.874	1.667	3	0.121	-1.268	1.510
Pathogens	11	-0.463	-1.200	0.273	NA	NA	NA	NA
Seed predation	45	0.029	-0.332	0.391	34	0.031	-0.581	0.643
Antagonist	Q_b = *7.258*, d.f. = 4, *P* = 0.1228				Q_b^p **= *2.51*, d.f. = 1, *P* = 0.0113** $K_{Blomberg}$ = 0.27, *P* = 0.201			
Birds	7	**-1.256**	**-2.212**	**-0.301**	3	-0.281	-1.542	0.980
Fungi	11	-0.462	-1.214	0.290	NA	NA	NA	NA
Insects	88	**-0.434**	**-0.707**	**-0.161**	57	-0.400	-1.016	0.217
Mammals	23	-0.012	-0.531	0.507	12	0.206	-0.607	1.019
Mixed	11	0.156	-0.600	0.913	9	0.000	-0.803	0.803
Level of specialization	Q_b = *0.8066*, d.f. = 1, *P* = 0.3691				Q_b^p = *0.02*, d.f. = 1, *P* = 0.899 $K_{Blomberg}$ = 0.38, *P* = 0.195			
Generalist	89	-0.460	-0.965	0.044	51	-0.234	-0.936	0.468
Specialist	19	-1.361	-3.259	0.538	10	-0.686	-1.690	0.318

Matrix	Q_b = *2.843*, d.f. = 6, *P* = 0.8282				Q_b^P = *5.9*, d.f. = 1, *P* = 0.0151			
					***K*Blomberg = 0.29, *P* = 0.183**			
Cultivated land	33	**-0.524**	**-0.976**	**-0.071**	20	-0.301	-1.179	0.578
Forestry plantation	8	**-0.864**	**-1.735**	**-0.006**	6	**-1.088**	**-2.153**	**-0.024**
Mixed	27	-0.347	-0.838	0.143	15	-0.121	-1.092	0.850
Other types of vegetation	15	-0.224	-0.883	0.434	9	0.126	-0.763	1.015
Pasture	34	-0.248	-0.709	0.212	25	-0.069	-0.983	0.846
Urban	10	0.012	-0.851	0.826	5	0.392	-1.033	1.818
Water	12	-0.036	-1.124	0.400	11	-0.512	-1.562	0.538
Habitat type	Q_b = *5.463* , d.f. = 4, *P* = 0.2429				Q_b^P = ***4.82*, d.f. = 1, *P* = 0.0281**			
					***K*Blomberg = 0.26, *P* = 0.185**			
Desert/xeric shrubland	5	-0.956	-2.105	0.193	4	-0.347	-1.773	1.079
Grassland	20	0.146	-0.402	0.694	14	0.261	-0.556	1.077
Temperate forest	45	**-0.399**	**-0.774**	**-0.024**	28	-0.147	-0.908	0.613
Tropical forest	65	**-0.397**	**-0.715**	**-0.079**	46	**-0.779**	**-1.541**	**-0.018**
Wet meadow	6	-0.932	-2.021	0.155	4	-0.808	-2.343	0.726
Time since fragmentation	Q_b = *0.071*, d.f. = 2, *P* = 0.9653				Q_b^P = *1.10*, d.f. = 1, *P* = 0.295			
					***K*Blomberg = 0.33, *P* = 0.184**			
< 30 years	36	-0.364	-0.806	0.076	22	0.393	-0.485	1.271
30-60 years	31	-0.367	-0.841	0.106	20	-0.721	-1.611	0.170
> 60 years	52	**-0.432**	**-0.797**	**-0.066**	33	-0.127	-0.852	0.598
Approach	Q_b = *1.461*, d.f. = 1, *P* = 0.2268				Q_b = *1.49*, ***d.f.* = 1, *P* = 0.0222**			
					***K*Blomberg = 0.29, *P* = 0.167**			
Experimental	74	-0.234	-0.525	0.058	41	0.270	-0.314	0.853
Observational	**67**	**-0.499**	**-0.817**	**-0.182**	42	-0.230	-0.967	0.507

Table 2. Traditional and phylogenetically independent meta-analyses of habitat fragmentation effects on only plant-insect interactions. Q_b test evaluating between-group differences are reported by each moderator evaluated. Sample sizes (*K*), the weighted mean effect sizes (Hedges' d_+) and 95% confidence intervals are shown. Superscript ^{p} refers to phylogenetically independent meta-analysis. $K_{Blomberg}$ are shown for each partial phylogeny built with the species included in each moderator contrast.

	Traditional meta-analysis				**Phylogenetically-independent meta-analysis**			
Grouping categories	***K***		**LCI**	**UCI**			**LCI**	**UCI**
Interaction	= *7.945*, d.f. = 5, *P* = 0.1592				**= *8.14*, d.f. = 1, *P* = 0.0043** $K_{Blomberg}$ = 0.36, *P* = 0.090			
Florivory	8	-0.815	-1.671	0.040	2	-1.751	-3.694	0.192
Folivory	52	**-0.621**	**-0.968**	**-0.274**	33	**-0.986**	**-1.861**	**-0.110**
Frugivory	3	-0.257	-1.187	1.702	NA	NA	NA	NA
Galls	4	0.400	-0.839	1.640	3	0.151	-1.370	1.671
Pathogen	NA	NA	NA	NA	NA	NA	NA	NA
Seed predation	19	-0.013	-0.548	0.522	13	0.141	-0.720	1.002
Level of specialization	Q_b = *0.4705*, d.f. = 1, *P* = 0.4928				Q_b^P = *0.14*, d.f. = 1, *P* = 0.7056 $K_{Blomberg}$ = 0.53, *P* = 0.217			
Generalist	47	**-0.685**	**-1.205**	**-0.166**	22	-0.753	-1.749	0.243
Specialist	16	-1.333	-3.112	0.445	8	-0.745	-2.024	0.534
Matrix	Q_b **=** *6.911*, d.f. = 6, *P* = 0.3219				Q_b^P = *14.85,* d.f. = 1, *P* = 0.0001 $K_{Blomberg}$ = 0.44, *P* = 0.028			
Cultivated land	18	**-0.628**	**-1.197**	**-0.058**	11	-0.536	-1.551	0.478
Forestry plantation	4	**-1.508**	**-2.643**	**-0.374**	3	**-1.933**	**-3.337**	**-0.528**
Mixed	23	-0.335	-0.828	0.157	13	0.004	-0.998	1.007

Other type of vegetation	11	-0.058	-0.772	0.655	8	0.057	-0.906	1.019
Pasture	19	-0.326	-0.927	0.274	11	-0.905	-2.115	0.305
Urban	7	0.192	-1.150	0.764	3	1.114	-0.719	2.948
Water	4	-1.266	-2.623	0.090	7	-1.348	-2.895	0.199
Habitat type	Q_b = *1.7092*, d.f. = 4, *P* = 0.7898				Q_b^P **= *0.51*, d.f. = 1, *P* = 0.475** $K_{Blomberg}$ = 0.37, *P* = 0.053			
Desert/xeric shrubland	4	-0.296	-1.507	0.913	4	-0.336	-1.908	1.235
Grassland	14	-0.098	-0.732	0.536	9	-0.241	-1.303	0.821
Temperate forest	27	**-0.534**	**-1.005**	**-0.064**	16	-0.416	-1.380	0.548
Tropical forest	38	**-0.463**	**-0.872**	**-0.055**	24	-0.212	-1.215	0.790
Wet meadow	5	-0.801	-1.962	0.359	3	-1.412	-3.338	0.
Time since fragmentation	= *2.328*, d.f. = 2, *P* = 0.3122				= *1.18*, d.f. = 1, *P* = 0.2778 $K_{Blomberg}$ = 0.55, *P* = 0.010			
< 30 years	27	-0.246	-0.731	0.236	15	-0.114	-1.147	0.919
30-60 years	17	-0.839	-1.427	-0.251	10	**-1.095**	**-2.066**	**-0.125**
> 60 years	27	**-0.511**	**-1.000**	**-0.022**	16	-0.303	-1.300	0.694
Approach	= *1.178*, d.f. = 1, *P* = 0.2776				= *0.06*, ***d.f.* = 1, *P* = 0.8050** $K_{Blomberg}$ = 0.44, *P* = 0.020			
Experimental	36	-0.263	-0.662	0.135	19	-0.233	-1.043	0.577
Observational	**52**	**-0.554**	**-0.895**	**-0.213**	36	-0.621	-1.404	0.163

Appendix A.

Supplementary figures

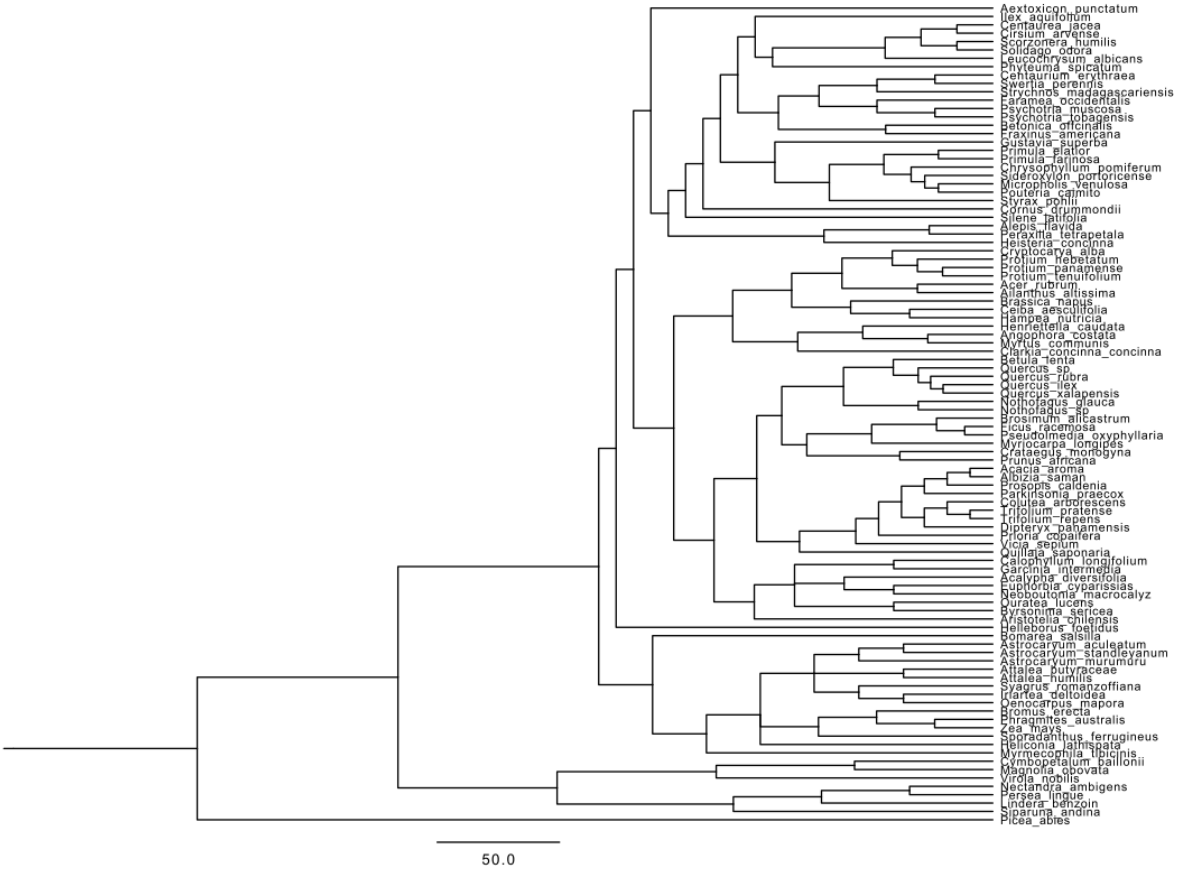

Figure A1. The hypothesized phylogenetic relationships among 98 species of 54 families considered in this review using a megatree based on APG III. Scale number represents millions of years.

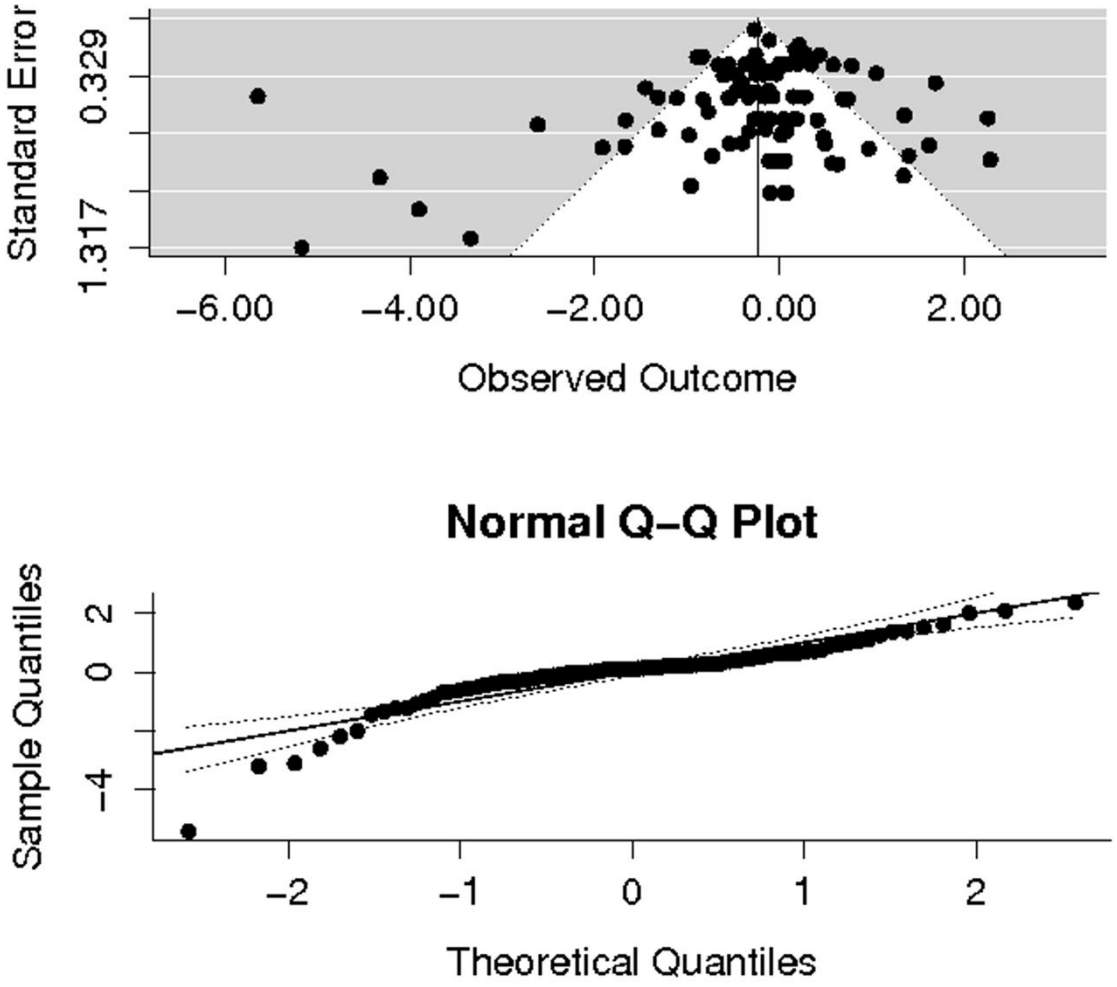

Figure A2. From the traditional meta-analysis: (a) Funnel plot of Z-transformed effect sizes against their within-study sample size (K = 100). (b) Normal quantile plot testing normal distribution.

Supplementary tables

Table A1. Additional Excel file.

Table A2. Traditional meta-analysis with 98 unique plant species. Q_b test evaluating between-group differences are reported by each moderator evaluated. Sample sizes (*K*), the weighted mean effect sizes (Hedges' d_+) and 95% confidence intervals shown.

	Traditional meta-analysis			
	K	d_+	**LCI**	**UCI**
Overall	98	-0.2487	-0.5064	0.0126
Grouping categories	***K***	d_+	**LCI**	**UCI**
Interaction	Q_b = *8.832*, d.f. = 3, *P* = 0.112			
Florivory	2	-0.7954	-1.919	2.120
Folivory	41	-0.4735	-1.035	0.054
Galls	3	0.4613	-1.020	1.076
Seed predation	34	0.0204	-0.211	0.216
Antagonist	Q_b = *4.375*, d.f. = 3, *P* = 0.383			
Birds	3	-0.4803	-1.205	0.960
Insects	57	**-0.3871**	**-0.816**	**-0.054**
Mammals	12	0.1744	0.077	0.540
Mixed	9	0.0272	-0.794	0.848
Level of specialization	Q_b = *0.081*, d.f. = 1, *P* = 0.968			
Generalist	51	-0.289	-0.693	0.086
Specialist	10	-0.514	-1.336	0.942
Matrix	Q_b = *4.148*, d.f. = 6, *P* = 0.753			
Cultivated land	20	**-0.4685**	**-0.959**	**-0.062**
Forestry plantation	6	-0.9371	-2.242	0.367
Mixed	15	-0.2149	-0.621	0.199

Other types of vegetation	9	0.1266	-0.843	0.675
Pasture	25	-0.1346	-0.787	0.464
Urban	5	0.2587	-0.893	1.463
Water	11	-0.4126	-1.299	0.474
Habitat type	Q_b = *4.899* , d.f. = 4, *P* = 0.418			
Desert/xeric shrubland	4	-0.5145	-1.541	1.103
Grassland	14	0.2360	-0.464	0.987
Temperate forest	28	-0.2286	-0.853	0.281
Tropical forest	46	**-0.3415**	**-0.588**	**-0.078**
Wet meadow	4	-0.874	-2.247	0.885
Time since fragmentation	Q_b = *1.338*, d.f. = 2, *P* = 0.737			
< 30 years	22	-0.0795	-0.710	0.229
30-60 years	20	-0.5305	-1.427	-0.095
> 60 years	33	-0.2237	-0.542	0.1106
Approach	Q_b = *1.461*, d.f. = 1, *P* = 0.2268			
Experimental	41	0.2114	-0.413	0.796
Observational	42	-0.2621	-0.836	0.521

APÉNDICE II

Habitat fragmentation changes the adaptive value of seed mass on the establishment of a tropical canopy tree

Habitat fragmentation changes the adaptive value of seed mass on establishment of a tropical canopy tree

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Received _____; revision accepted _____ .

LRH: Chávez-Pesqueira and Núñez-Farfán

RRH: Seed size affects survival of *Nectandra ambigens*

Abstract

Seedling establishment, one of the most critical events of life history of long-lived tropical rainforest trees that occur at low densities, can be altered by habitat fragmentation. Due to the very limiting light conditions in the rainforest understory many species of tropical trees have evolved large seeds that enable the emerging seedlings to use nutritional reserves to withstand the low light conditions. Since fragmentation is known to modify the biotic and physical conditions of forest understory it might change the adaptive value of seed size. We experimentally assessed the potential of forest fragmentation to alter the adaptive value of seed mass on seedling survival, vigour, and attack by natural enemies of the tropical tree *Nectandra ambigens*. Seeds of different families were individually weighed and sowed in experimental sites established in continuous forest and forest fragments. Seedling survival, vigour, as well as damage by herbivores and pathogens were recorded periodically. Overall, seedlings derived from larger seeds had a higher likelihood of survival in both habitats; however, seedling survival and vigour were significantly greater in forest fragments than in continuous forest sites. Moreover, seedlings were less attacked by natural enemies in fragments. We found genetic variance for seed mass among families (h^2 = 0.62), and selection on seed size. Average seed size differed between dead and alive seedlings in three sites. In one fragment, seed size was selectively neutral in relation to survival. Thus seed size distribution is changed by selection (since it promoted survival). The maintenance of genetic variance could be related to the stochastic nature of light-gaps formation. Our results highlight the importance of studies that evaluate the adaptive value of traits susceptible to environmental change for conservation purposes.

Key words

Habitat fragmentation; herbivory; Los Tuxtlas; natural selection; *Nectandra ambigens*; seed mass; seedling establishment; survival.

INTRODUCTION

SEED MASS PLAYS AN IMPORTANT ROLE IN SEEDLING ESTABLISHMENT, ESPECIALLY FOR PLANTS IN RESOURCE-LIMITED ENVIRONMENTS (Grubb & Coomes 1997), SINCE IT PROVIDES NUTRIENTS TO SEEDLINGS THUS PROMOTING SURVIVAL (Metcalfe & Grubb 1995). In tropical rainforests, seed mass has been suggested to constitute an adaptive character selected to allow seedlings of some species to withstand the limiting light conditions of the understory (Foster 1986; Hewitt 1998; Paz & Martínez-Ramos 2003), while "waiting" for light gap formation.

Despite the great variability in seed size among plant species (Foster 1986), an emerging pattern points that species that establish in the deep shade of the rainforest tend to have larger seeds (Westoby *et al*. 1996). Thus, the longer survival gained in dense shade is likely to result from the metabolizable reserves in the seed at the cotyledon stage (Saverimuttu &Westoby 1996b). Paz & Martínez-Ramos *et al*. (2003) experimentally augmented light levels for several species of rainforest *Psychotria* and demonstrated that seed mass is more important in the shade than in gaps where seedlings survived more and grew faster. This evidence suggests that the selective advantage of seed mass could be altered in fragmented environments where light incidence increases due to edge effects (Scariot 2000).

Another potential advantage of large seed reserves relates to a higher tolerance to herbivory by providing energy and material for tissue replacement (Foster 1986). The capacity to tolerate herbivory and physical damage may be especially important in tropical forests (Harms & Dalling 1997) where seedlings of tropical tree species suffer high damage and mortality due to pathogens, insects, and mechanical damage to the shoot (Denslow 1980; Coley 1983; Clark & Clark 1985, 1989; Auspurger 1984).

Tropical rain forests are the most diverse and complex terrestrial ecosystems, harbouring more than 100 tree species per ha that occur at very low densities (Turner 2001). The maintenance of such a high biodiversity in tropical forests has become threatened by habitat fragmentation – the transformation of continuous, undisturbed, habitat into multiple smaller and spatially isolated remnants (Young *et al*. 1996). Changes in tropical forest's structure induced by fragmentation may cause the disruption of biological processes that maintain biodiversity and ecosystem functioning (Saunders *et al*. 1991, Didham 1996).

For long-lived, tropical rainforest trees, germination and establishment are the most critical events in their life cycle (Córdova 1985, Kitajima & Fenner 2000), and habitat fragmentation has the potential to alter these. Disruption of these life-history stages can constrain population persistence as well as plant species´ capability to colonize suitable sites (Herrera & García 2009). For instance, some evidence points that habitat fragmentation reduces the probability of seed germination, and hence seedling establishment, in forest fragments (Bruna 1999). Likewise, for rainforest species that regenerate in the understory and form a seedling bank, seed mass may determine the

likelihood survival and establishment in the face of limiting resources (Paz & Martínez-Ramos et al. 2003), and damage by plants' natural enemies.

Habitat fragmentation exposes remnant organisms in the forest fragments to a modified, frequently altered, environment (Murcia 1995, Tabarelli *et al.* 2004), like increments in air temperature and light incidence and reductions in air and soil relative humidity due to proximity to pasture lands and secondary vegetation zones (Scariot 2000). Moreover, the abundance and distribution of organisms, and the establishment and development of biotic interactions can be modified in fragmented habitats (Murcia 1995). Poor representation of mutualists, or increased effects of predators or pathogens, may increase the likelihood of local loss of tree species (Cordeiro *et al.* 2009). Although many studies have evaluated the effects of habitat fragmentation on mutualistic interactions, such as pollination and seed dispersion (see review by Aguilar *et al*. 2006), less information accounts for antagonistic interactions, like plant-herbivore and plant-pathogen (Kolb 2008). Damage by herbivores and pathogens can modify seedling recruitment altering the number or composition of plant species in the seed rain and seed bank (del Val 2012).

Loss of tree species is one important factor, and is of crucial concern in tropical forests conservation. Many or even most tree species do not occur in small tropical forest fragments because they exist at very low densities (Cordeiro *et al.* 2009). Understanding the threats that tropical trees face as seedlings in fragmented forests will help prevent, reduce, or mitigate potential extinctions. The present study aimed to determine to what extent seed mass affects seedling establishment in the most common tropical rainforest tree *Nectandra ambigens*, at the Los Tuxtlas region; and if

this effect is altered by habitat fragmentation. Moreover, we examined the effects of habitat fragmentation on plant vigour (measured as seedling height, leaf number, and leaf area), and herbivore and pathogens attacks. We hypothesized that the adaptive value of seed size would be more important in the undisturbed, continuous forest, than on fragments where altered light conditions may favour seedling establishment.

METHODS

STUDY SITE

We carried out this study in the rainforest of the Los Tuxtlas Biosphere Reserve, Veracruz, Mexico (18°35.240´N, 95°04.62´W). The region has an elevation gradient from sea level up to 1720 m, with two different climates according to Köppen's climate classification: humid tropical (type A) at low and middle elevations, and moist with mild winters (type C) at high elevations (Gutiérrez-García & Ricker 2011). The climate in the region is hot and humid; in the last 30 years, the annual mean temperature varied from 24.1 to 27.2 ºC, and the average annual precipitation ranged from 1272 to 4201 mm (Gutiérrez-García & Ricker 2011). The dominant vegetation in the region is lowland tropical rainforest (Dirzo *et al*. 1997), characterized by evergreen vegetation and trees exceeding 30 m height (Ibarra *et al*. 1997). The forest understory is carpeted with seedlings of a large number of trees and liana species, as well as several species of ferns and herbs (Piñero *et al*. 1977, Dirzo *et al*. 1997). The most abundant species are *Astrocaryum mexicanum* (Arecaeae) in the low stratum, *Pseudolmedia oxyphyllaria* (Moraceae) in the medium stratum, and *Nectandra ambigens* (Lauraceae) in the upper canopy (Bongers *et al*. 1998). Los Tuxtlas' landscape has been severely altered during

the last 50 years. Estimates indicate that *ca*. 75% of the original vegetation has disappeared, 20% remains as forest fragments, and only 5% remains as large and continuous, intact forests (Estrada & Coates-Estrada 1996).

We established four experimental sites, two in the continuous, intact, rainforest, and two in remnant fragments of rainforest ["Cerro Borrego" (18°39.958´N, 95°05.164´W) and "Cola de Pescado" (18°39.792´N, 95°08.754´W)]. In each site we established two plots, for a total of eight plots. In a previous work (Toledo-Chelala 2010), differences in light conditions were detected for these sites, showing a significant increase in forest fragments.

STUDY SYSTEM

Nectandra ambigens (Blake) C. K. Allen (Lauraceae) is a tropical canopy tree species 20-40 m tall that inhabits lowland tropical rainforests of Mexico, Honduras, and Guatemala. At the Los Tuxtlas rainforest it is one of most common tree species in the forest canopy (Piñero *et al*. 1977, Dirzo *et al*. 1997, Bongers *et al*. 1998). The tree bears hermaphroditic flowers and little is known about its pollination biology (Dirzo *et al*. 1997). Fruiting occurs from September to November every two or three years i.e., "masting years" (Dirzo *et al*. 1997; Carabias & Guevara 1985). Seedling emergence occurs within one month after fruits fall producing a dense carpet of seedlings with densities *ca*. 100 seedlings per square meter under the mother tree (Martínez-Ramos 1991). Like other species with recalcitrant seeds, it does not form a seed bank, but a 'seedling bank'. However, survival of seedlings of the same cohort after 3 years is very low, and few survive to the sapling stage (probability <1%; Córdova-Casillas 1985), probably due to

attacks from herbivores (mainly orthopteran and lepidopteran larvae) (Dirzo *et al*. 1997b), and/or pathogens (García-Guzmán & Dirzo 2001).

EXPERIMENTAL DESIGN

Fruits of six adult mother trees (families) of *N. ambigens* were collected at Los Tuxtlas Tropical Research Station. Seeds were individually weighed using an electronic balance, and sown in numbered trays in a greenhouse. After two months, the emerged seedlings were tagged and transplanted randomly into the four sites (two in the continuous forest, and two in forest fragments) following a complete randomized block design (Steel & Torrie 1981). Each site had two blocks. The emerged seedlings were transplanted and the six families were equally and randomly allocated to all sites and blocks. During 15 months, survival was recorded periodically. We also measured in each census seedling height, number of leaves, leaf area, and presence of leaf damage from herbivores and pathogens.

DATA ANALYSIS

To evaluate the effect of seed mass over families we performed an ANOVA of seed mass considering the family as a random effect. This analysis allowed us to partition the phenotypic variance in seed mass into its genetic (family) and environmental components, and to estimate broad-sense heritability (the transmission of the phenotypic variability within a population from generation to generation; Falconer and MacCay 1996, Lawrence 1984). Assuming that seeds from one mother are related as half-sibs, heritability equals: $h^2 = 4\sigma_g^2 / (\sigma_e^2 / \sigma_g^2) = 4\sigma_g^2/\sigma_p^2$, where σ_g^2 is the genetic variance and σ_p^2 the total phenotypic variance.

Assuming survival as a fitness component of seedlings ($\overline{w}$), we estimated the gradient of natural selection on seed size by means of linear regression (Lande & Arnold 1983). Selection analysis of seed size was performed at three periods: 3 (119 days), 10 (290 days), and 18 months (540 days). Prior to analyses, individual seed mass (z_i) was standardized as $z_i' = \left(z_i - \bar{z}\right) / s$, where $\bar{z}$ and s are the mean and standard deviation of seed size, respectively. This allows measuring the intensity of selection in units of phenotypic standard deviation (Lande & Arnold 1983, Falconer and MacCay 1996). To evaluate differences in survival between habitats, survival was recorded during each census as a binomial variable (alive 1, dead 0) and relativized as $\chi_{it} / \overline{x}$, where χ_{it} is the individual survival at time *t*, and $\overline{x}$ is the population average survival. Hence $\overline{w}$ = 1 (Lande & Arnold 1983). Moreover, we performed an ANCOVA to explore the relationship between seed mass and survival of seedlings (as a measure of fitness) (*seed mass* + *site* + *seed mass* × *site*). A significant effect of seed mass in the model would imply that seedling survival is related to seed mass, whereas, a significant effect of site will mean that sites (fragmented and continuous forest) have an effect on seedling survival. Likewise, a significant effect of the interaction *seed mass* × *site* implies that the effect of seed mass on seedling survival differs among sites.

The time-course survival of seedlings considering the effect of habitat and family was analyzed by means of a proportional hazards survival analysis (Cox 1972), keeping constant the effect of seed mass that was considered as a covariate.

We analysed plant vigour (height, leaf number, and leaf area of each individual plant) using a MANOVA of repeated measures in relation to habitat, family, and seed mass as a covariate.

Logistic regressions were performed to detect differences in the presence (1) or absence (0) of damage by herbivores, as well as damage by pathogens to leaves of *N. ambigens* between sites and habitats. Differences in pathogen incidence due to habitat or herbivory were analysed by means of a generalized linear model (GLM), using a quasi-likelihood binomial distribution with a logit link function. A first-level interaction between predictor variables was initially included in the model but was excluded from the analysis, as it was not significant ($P > 0.05$).

All analyses were performed with JMP v. 10 (SAS 2010) and R statistical software v. 2.13.0 (R Development Core Team, 2011).

RESULTS

A total of 2,195 seeds from six trees (natural progenies) of *Nectandra ambigens* were collected. From this sample, 1018 (46.37%) seeds produced a seedling two months later. These seedlings were used for the field transplant experiment. Average seed mass of emerged seedlings (4.23g) deviated positive and significantly from the average seed mass of all seeds sampled (4.01g; $t = 6.36$, *d. f.* = 1017, $P < 0.0001$).

Average seed mass differed significantly among families (ANOVA: $F = 25.39$; *d. f.* = 5; $P < 0.0001$). The term *family* explained 14.65% of the total phenotypic variance, setting an upper limit to broad-sense heritability of seed mass of $h^2 = 0.663$.

Overall, average seedling survival was higher in forest fragments (58.9%) than in the continuous forests (36.5%) ($\chi^2 = 50.26$; $P < 0.001$) (Figure 1A). Seed mass affected survivorship among families ($\chi^2 = 28.46$; $P < 0.001$); seedlings belonging to families with higher average of seed mass, showed a higher number of survivor seedlings. Moreover, independently of the family or habitat, seedlings derived from high mass seeds survived more ($\chi^2 = 36.30$; $P < 0.001$) (Figure 1B).

Selection for larger seed size was detected early after establishment (3 months) in one site of continuous forest (Table 1). However, 10 months after transplanting selection was detected in three sites (the two in continuous forest and one in fragments). One year and a half after transplanting, selection on seed size continued to affect plant survival in both continuous rainforest and increased its intensity in a fragment (F2; Table 1). No selection on seed size was detected in fragment 2 during the entire study period (Table 1; Fig. 2).

ANCOVA of relative survival (*i.e.*, relative fitness) as a function of site and seed mass (covariate) showed differences in survival among sites as early as 3 months after transplanting, and no effect of seed mass on survival was detected. However, all effects were significant at 8 (290 days) and 18 months (540 days) after transplant (Table 2). The significant interaction pointed that the effect of seed mass (selection) on seedling survival differed among sites (*cf*. Table 1, 2).

We found a difference between the average seed mass of surviving and dead seedlings in both continuous forest sites and one fragment site (Fig. 2). The positive effect of a larger seed size on survival occurred earlier in continuous forest sites than in fragments. In fragment 2 (F2), seed size averages of live and dead seedlings diverged

after 10 months (290 days) (Fig. 2). In fragment 1 (F1), seed size was random in relation to seedling survival and thus average seed size of dead and alive seedlings was statistically similar at the end of the study (Fig. 2). Between fragmented *vs*. intact habitat, there was a change in the average seed size and its phenotypic distribution between live and dead seedlings (Fig. 3).

The number of leaves per seedling was significantly different between habitats ($F = 58.95$; $d.f. = 1$; $P < 0.001$). After 540 days, seedlings in forest fragments had an average of five leaves, and seedlings in continuous forest had less than 3 leaves. Likewise, fragment seedlings showed higher average height (22.3 cm) than continuous forest seedlings (13.5 cm) ($F = 3.86$; $d.f. = 1$; $P < 0.05$). Seed mass and family had a significant effect on the variation for leaf number and height (Table 3). No significant differences in leaf area measures were detected (Table 3).

Seedlings with insect herbivores and foliar damage by pathogens were significantly higher in the continuous forest throughout the study ($F = 35.49$; $d.f. = 1$; $P < 0.001$, for herbivores; $F = 94.80$; f. d. = 1; $P < 0.001$, for pathogens) (Fig. 4A and 4B). We found a significant association of herbivory and pathogen infection on seedlings (GLM Wald $\chi^2 = 2.061$, P = < 0.001). The proportion of seedlings with presence of herbivory and pathogen damage doubled those only attacked solely by insect herbivores or fungal pathogens (Fig.4C).

DISCUSSION

Understanding the response of tropical trees to human disturbance is relevant to better manage and restore fragmented rainforests. *N. ambigens* in the Los Tuxtlas rainforest is the most common tree species. However, since some decades ago the species has been cut down for its valuable timber, making it difficult to find it in forest fragments, and raising concern for its persistence in forest fragments of the region. Information regarding the effects of habitat fragmentation on the establishment of tropical trees species with human use is vital given the extent of tropical fragmented habitats nowadays.

For *N. ambigens*, we found that seed size affected seedling survival and establishment, as we expected. The intensity of phenotypic selection on seed size was higher in the continuous forest understory than in fragments, especially early after transplant. We did not expect, however, that survival and establishment of seedlings of *Nectandra ambigens* would be enhanced in forest fragments. At the end of the study 36% *vs*. 69% of seedlings survived in the continuous forests as compared to the fragments, respectively. Genetic variation (among families) in survival resulted from differences between families in average seed size. Families with a higher mean seed size had higher survival. These results suggest that the genetic composition of seedlings among sites and habitats changed according to family and seed mass. Positive deviations of individual seed size from its family mean, due to environmental deviations or to genetic effects of the sire parent (or their interaction), significantly increased its likelihood of survival. Selection on seed size may be nil (F1) or to act late (F2) in fragments.

Seed mass has been suggested as an important character of plant species whose seedlings establish in forest understory (Foster 1986). In the undisturbed forest (canopy intact) surviving seedlings of *N. ambigens* derived mainly from big seeds. In forest fragments, seedlings showed a higher survival, independent of seed mass. It has been generally considered that larger seeds enhance seedling survivorship at low light intensities, such as the understory of the undisturbed rainforest (Foster 1986, Leishman & Westoby 1994). Thus, the higher light availability found in the studied fragments (Toledo-Chelala 2010) is likely related to the higher seedling establishment and higher seedling growth (seed mass is known to affect positively leaf production and height in plants) (Metcalfe & Grubb 1995, Bonfil 1998, Hewitt 1998, Paz & Martínez-Ramos 2003). The more vigorous seedlings found in the forest fragments, suggest that different selective intensities are acting in the different habitats (continuous *vs.* fragments). The adaptive value of seed mass measured in the continuous forest could be reduced in fragmented habitats. In this case, seeds moving from forest fragments to continuous forest could result in low fitness and demographic modifications for *N. ambigens* populations.

Nectandra ambigens trees produce big seeds (4 g on average) with variation between families, suggesting genetic variance and/or non-nuclear maternal effects in this species. Thus, seed mass could be subject of selection in natural conditions (continuous forest). For *N. ambigens*, we found positive selection in seed mass in relation to survival of seedlings in both habitats. The presence of genetic variance could anticipate a response to selection in limiting environments (as the understory of intact forests), but probably not, or lower, in less stressing environments, like fragments. In fragmented habitats, genetic variance could not represent an advantage because the

environment allows favourable conditions to grow for this species. So, why is genetic variance for seed mass maintained in this species? This could be related to the heterogeneous environment for establishment. The stochasticity of rainforest gaps (*N. ambigens* need not to be within the gap but to receive higher light levels) may favour seedlings derived from smaller seeds, thus maintaining genetic variance. Also, seedlings derived from larger seeds have a higher probability of being present when the 'lucky strike' occurs. Moreover, seed mass may be an important trait not only for survivorship in the shade, but for other biotic factors such as competition and resistance to pathogens and herbivores. Thus, if smaller seeds are benefited in fragmented habitats, establishment of seedlings could also be threatened in a major way by antagonists.

The environmental changes produced by habitat fragmentation are favouring the establishment of *N. ambigens* in the fragments' understory. Light incidence in the understory seems to be the most advantageous resource because it might mimic gaps or forest edges conditions. The light increase in the studied forest fragments (Toledo-Chelala 2010) are turning them into an adequate environment for the survival and growth of *N. ambigens* seedlings in Los Tuxtlas region. In fact, our results show that the number of surviving seedlings in forest fragments doubled the number of those in the continuous forest. Likewise, our results showed that seedling vigour is favoured in forest fragments, probably also as consequence of the environmental change in light availability, promoting taller seedlings with more leaves (photosynthetic area). It is interesting to note that *N. ambigens*, despite being classified as a shade tolerant species (del Amo 1985), reflects an increase in height and leaf production when present in increased light conditions.

Habitat fragmentation also modified the interactions of *N. ambigens* seedlings with insect herbivores and pathogen microorganisms. Seedlings in fragments were less attacked by herbivores and pathogens, probably due to the modified light availability brought about by habitat fragmentation. There is evidence that pathogens and herbivores effect on seedling survivorship may be greater in the shaded habitat than in light gaps (Foster 1986). For the case of pathogen microorganisms, modifications in the understory environment may turn them in a less humid and shaded site for their activity; in contrast to the conditions in the understory of the continuous forest that favour the presence and activity of pathogen microorganisms (Auspurger 1984, Foster 1986, Benítez-Malvido & Martínez-Ramos 2003). Additionally, isolation of populations due to habitat fragmentation, may reduce the diversity and abundance of herbivore insects, and alter their foraging behaviour (Didham 1996). This was reflected in a higher incidence of pathogen and herbivore damage in leaves of *N. ambigens* seedlings in continuous forest. Moreover, our results showed a relation between pathogen and herbivores attack. We found that seedlings with herbivory damage had a higher probability of showing pathogen infection simultaneously, probably because herbivory wounds facilitate pathogen infection (García-Guzmán & Dirzo 2001), or because herbivores act as disease vectors (these two possibilities are not mutually exclusive). This relation was found in both habitats. Others studies have shown similar results. For instance, Ruíz-Guerra *et al*. (2010) found that although fragmentation reduces insect herbivory for many rainforest plant species, this depends on their regeneration strategy; light-demanding species were more attacked than shade-tolerants. In other study, Benítez-Malvido *et al*. (1999), found no differences between leaf fungal infection and herbivory after six years for seedlings of three tropical tree species in the

Amazonian rainforest; however they found an association of fungal infection with damage inflicted by herbivory in two species, and that this association was higher in continuous forest than fragments. Moreover, position of plants in forest fragments may define the levels of attack and infection. For example, Benítez-Malvido & Lemus-Albor (2005) found that the community of seedlings present in the forest edges showed a greater incidence of foliar disease than did those in forest interior, although herbivores attack was similar between edges and interior.

In this sense, decreased attacks from antagonists in habitat fragments suggest a beneficial effect of habitat fragmentation for tropical trees. However, even id natural enemies attack less, seedling abundance and diversity are likely to decrease due to habitat fragmentation (Benítez-Malvido 1998). Moreover, modifications in any ecological process would have consequences at the community and ecosystem level (Lewis & Gripenberg 2008) and should be thoroughly explored in fragmented habitats.

The high survivorship rate, the fewer attacks from herbivores and pathogens, the more vigorous plants in fragments, shows that for *N. ambigens*, forest fragmentation has an effect in the understory conditions which allows a higher probability of seedling establishment and survival. Taken as a whole, our results suggest *N. ambigens* as an important tree species to consider in restoration and regeneration plans in fragmented sites of the region, since it is one of the most abundant components in the Los Tuxtlas rainforest structure.

N. ambigens produces dense carpets of seedlings beneath the mother tree. These constitute its genetic reservoir since it does not have seed bank. This renders this species, and species alike, prone to extinction if the 'seedling bank' disappears. We

experimentally showed that the adaptive value of seed size increases the likelihood of seedling survival wile “waiting” for a light gap. Selection changed the genetic composition towards large seeded families; however, selection, its onset and intensity on seed size, differed among sites. We underscore this finding in relation to restoration of fragmented sites.

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Tables

Table 1. Adaptive value (selective gradient, *β*) of seed mass in relation to survival of seedlings of *Nectandra ambigens* in continuous forest and fragments in three different time spans. Significant values of *P* are shown in bold type.

Time		119 days			290 days			540 days		
Habitat	Site	*β*	*t*	*P*	*β*	*t*	*P*	*β*	*t*	*P*
Continuous	C1	0.0348 (0.0323)	1.08	0.2827	0.2298 (0.0414)	5.55	**<0.0001**	0.2071 (0.0377)	5.48	**<0.0001**
	C2	0.0435 (0.0194)	2.23	**0.0264**	0.1523 (0.0354)	4.29	**<0.0001**	0.1360 (0.0448)	3.03	**0.0027**
Fragments	F1	-0.0309 (0.0271)	-0.77	0.4397	-0.0359 (0.0427)	-0.84	0.4020	0.0077 (0.0443)	0.18	0.8612
	F2	-0.0048 (0.0292)	-0.17	0.8673	0.0801 (0.0346)	2.31	**0.0215**	0.2548 (0.0479)	5.32	**<0.0001**

Table 2. Analysis of covariance of survival of seedlings of *Nectandra ambigens* at three different time spans after seedling emergence. Seed mass was used as a covariate. Significant values of *P* are shown in bold type. *Models for different dates were statistically significant (119 days: F=9.9329 $_{d.f.=7;\ P<0.0001}$, R^2=0.0644; 290 days: F=19.235$_{d.f.=7;\ P<0.0001}$, R^2=0.1176; F=19.488$_{d.f.=\ 7;\ P<0.0001}$, R^2=0.1189).

Time	119 days				290 days				540 days			
Source	*d. f.*	*s. s.*	*F*	*P*	*d. f.*	*s. s.*	*F*	*P*	*d. f.*	*s. s.*	*F*	*P*
Site	3	16.682	21.934	**<0.0001**	3	30.271	25.865	**<0.0001**	3	34.734	24.017	**<0.0001**
Seed mass	1	0.113	0.4454	0.504	1	11.388	29.192	**<0.0001**	1	22.989	47.688	**<0.0001**
Site × Seed mass	3	0.964	1.268	0.284	3	10.447	8.927	**<0.0001**	3	8.709	6.022	**0.0005**
Error	1010	256.054			1010	394.009			1010	486.894		

Table 3. Repeated measures MANOVA of leaf number, plant height, and total leaf area of seedlings of *Nectandra ambigens* (Lauraceae) in continuous and fragmented rainforests along time (540 days). Significant values of *P* are shown in bold type. (T = time, H = habitat, S = site, B = block, SM = seed mass).

	Leaf number			Plant height			Total leaf area		
Effect	*d. f.*	*F*	*P*	*d. f.*	*F*	*P*	*d. f.*	*F*	*P*
Habitat	1	58.954	**<0.0001**	1	3.857	**0.050**	1	2.137	0.1443
Site (habitat)	2	1.960	0.1420	2	2.662	0.070	2	0.687	0.5030
Block (site, habitat)	4	1.884	0.1120	4	2.123	0.076	4	4.137	**0.0026**
Family	5	5.267	**<0.0001**	5	18.021	**<0.0001**	1	3.734	**<0.0001**
Seed mass	1	10.029	**0.0016**	1	43.014	**<0.0001**	5	10.830	**0.0500**
Error	473			473			630		
Time	8	4.764	**<0.0001**	8	8.291	**<0.0001**	4	8.075	**<0.0001**
T × H	8	31.134	**<0.0001**	8	9.108	**<0.0001**	4	7.222	**<0.0001**
T × S (H)	16	7.539	**<0.0001**	16	2.798	**0.0002**	8	1.790	0.0749
T × B (S, H)	32	2.662	**<0.0001**	32	1.687	**0.0097**	16	1.043	0.4065
T × F	40	1.796	**<0.0001**	40	2.372	**<0.0001**	4	2.028	**0.0003**
T × SM	8	5.275	**0.0017**	8	6.856	**<0.0001**	20	2.475	0.0888
Error	466			466			627		

Figure legends

FIGURE 1. *Nectandra ambigens* seedling survivorship in the continuous forest (C1 and C2) and fragments (F1 and F2) (±S.E) sites for 540 days. B. *N. ambigens* seedling survivorship in relation to seed mass (±S.E) for 540 days.

FIGURE 2. Seed mass (±S.E) of surviving (alive) and dead seedlings of *Nectandra ambigens* in continuous forest (C1 and C2) and fragments (F1 and F2) sites. (0 = 0 days; 1 = 119 days; 2 = 189 days; 3 = 290 days; 4 = 341 days; 5= 388 days; 6 = 444 days; 7 = 490 days; 8 = 540 days).

FIGURE 3. Distribution of standardized seed mass of live (black) and dead (grey) seedlings between continuous forest and fragments in three different time spans (A = 119 days; B = 290 days; C = 540 days). Dark dotted lines represent the mean of seed mass for live seedlings; light dotted lines represent the mean of seed mass for dead seedlings.

FIGURE 4. A. Proportion of *N. ambigens* seedlings with presence of leaf herbivory in both habitats. B. Proportion of *N. ambigens* seedlings with presence of leaf damage from pathogens in both habitats. (F = fragments, C = continuous forest) (±S.E). Data from the last census. C. Comparison of the proportion of *N. ambigens* seedlings with pathogen damage with the presence and absence of herbivory in the fragments and continuous habitat (±S.E). Data from the last census.

FIGURE 1

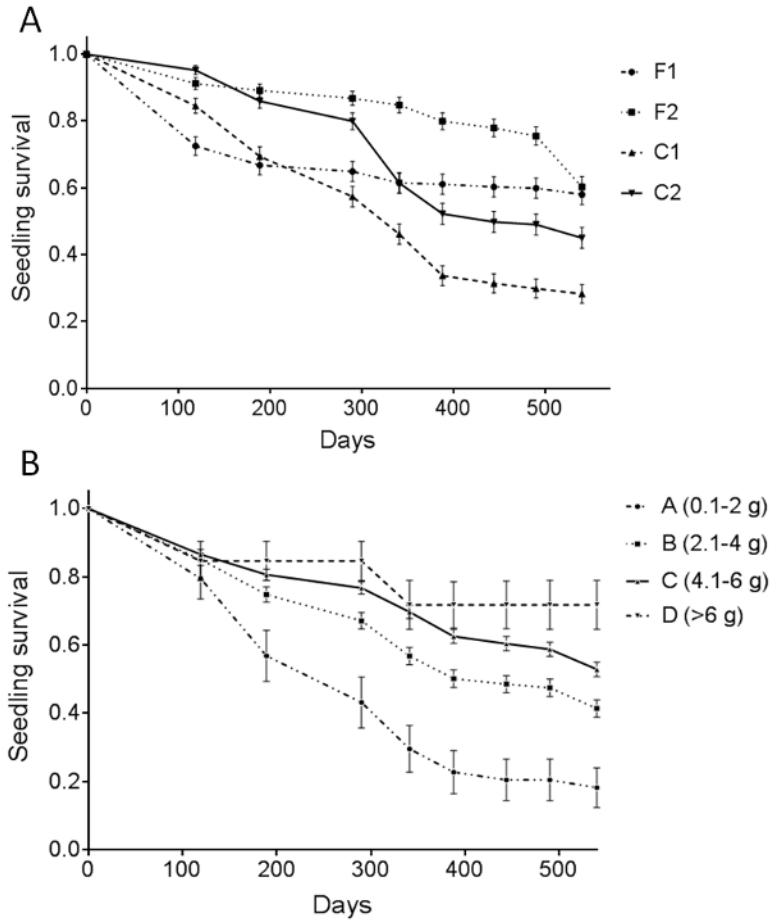

A
Seedling survival
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
Days
F1
F2
C1
C2
B
Seedling survival
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
Days
A (0.1-2 g)
B (2.1-4 g)
C (4.1-6 g)
D (>6 g)

FIGURE 2

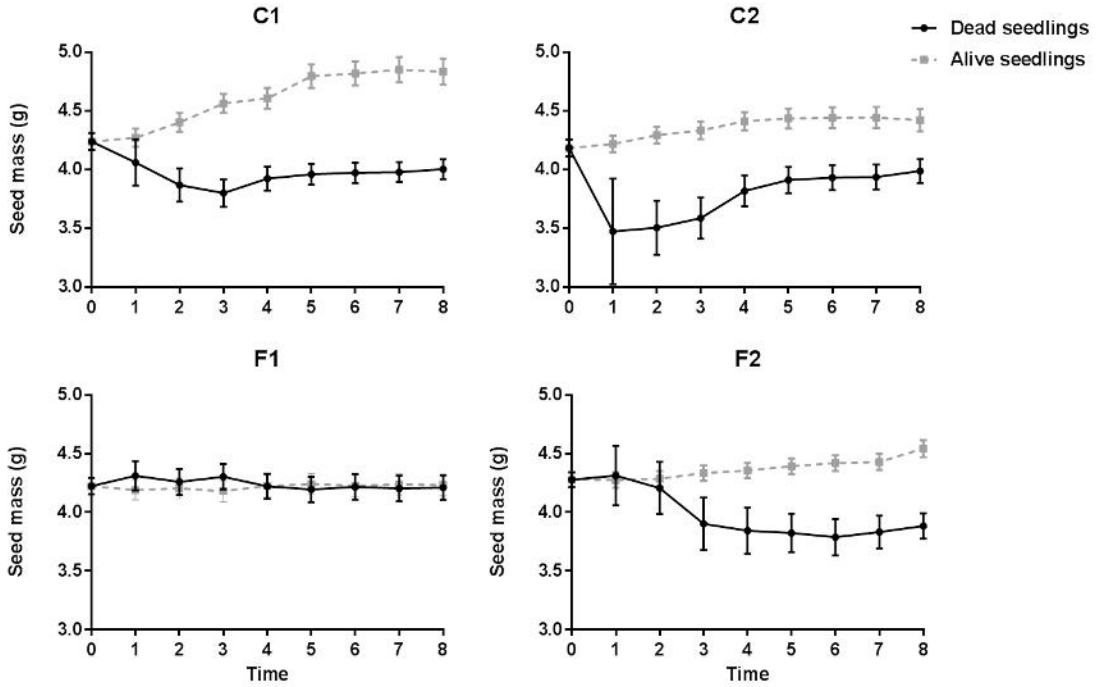


FIGURE 3

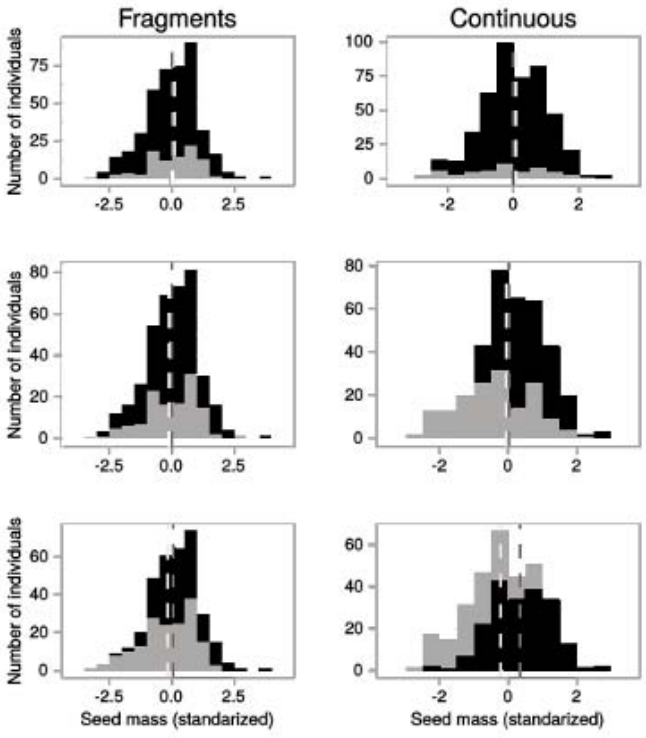
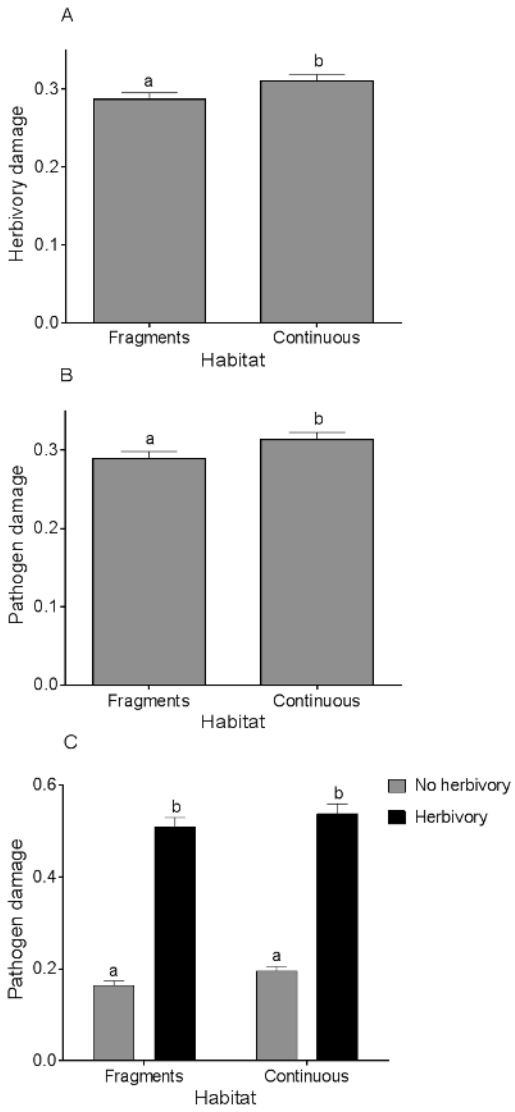


FIGURE 4


A
Herbivory damage
0.0
0.1
0.2
0.3
a
b
Fragments
Continuous
Habitat
B
Pathogen damage
0.0
0.1
0.2
0.3
a
b
Fragments
Continuous
Habitat
C
Pathogen damage
0.0
0.2
0.4
0.6
a
b
a
b
No herbivory
Herbivory
Fragments
Continuous
Habitat