Universidad Autónoma de Querétaro
Facultad de Ciencias Naturales

Diversidad florística y conectividad de humedales temporales de tierras altas en el centro de México

Artículo(s) de investigación

Que como parte de los requisitos para obtener el grado de

Doctor en Ciencias Biológicas

Presenta

Tatiana Lobato de Magalhães

Dirigido por:

Dra. Mahinda Martínez y Díaz de Salas

Querétaro, Qro. a 01 de Agosto de 2019

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Centro Universitario, Querétaro, Qro.
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Para los científicos de humedales que trabajan para conservar este singular ecosistema alrededor del mundo

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RESUMEN

Los humedales temporales se caracterizan por la hidrología efímera y fluctuaciones severas en los niveles de agua, ya que estos ambientes pueden secarse por completo. Este proyecto de investigación doctoral se enfoca en la conservación de la biodiversidad de humedales temporales, un ecosistema singular que desaparece rápidamente en México. Los objetivos fueron, estudiar los patrones de distribución de plantas acuáticas, analizar el flujo génico y la influencia del paisaje en la conectividad de humedales. Entre 2015 y 2016 se visitaron 39 humedales (2,000 - 3,000 metros sobre el nivel del mar) en los estados de Aguascalientes, Guanajuato, Jalisco, Michoacán, Querétaro, San Luis Potosí y Zacatecas. Se recolectaron especímenes botánicos, se registraron datos físico-químicos del agua, del paisaje y se tomaron muestras para estudios de genética de una especie. Se cuantificó la conectividad funcional de humedales temporales mediante el genotipado de siete *loci* con microsatélites de núcleo para 18 poblaciones de *Nymphoides fallax* (Menyanthaceae), una planta acuática tetraploide nativa de humedales de tierras altas en el centro de México. Se evaluó si la conectividad de los humedales junto con variables de estructura del paisaje (e. g. cobertura forestal) se traduce en una mayor diversidad genética y flujo de genes entre los humedales temporales. Se registraron un total de 126 especies de plantas acuáticas (distribuidas en 80 géneros y 38 familias), entre ellas 27 especies amenazadas, 24 con uso económico, 20 nuevos registros y dos especies aún no descritas. Con relación a la distribución de especies se observó que la similitud de las comunidades no aumentó con la proximidad espacial entre los sitios. Los hallazgos del estudio genético sugirieren que *N. fallax* depende en gran medida de la reproducción sexual, se dispersa ampliamente y tiene alta capacidad para adaptarse a variados ambientes. Los humedales más conectados tienen diversidad genética y flujo genético significativamente más altos que las poblaciones de humedales más aislados. La diversidad genética se asoció con distancias de umbral hasta 5 km. Los índices de diferenciación genética FST específicos para cada población se relacionan significativamente con un modelo que incluye el porcentaje de cobertura forestal. Los resultados encontrados permiten explorar aspectos para la determinación de nuevas estrategias de conservación de la biodiversidad en humedales temporales, basadas en el paisaje, la genética y la conectividad.

(**Palabras clave**: genética del paisaje, humedales aislados geográficamente, microsatélites nucleares, *Nymphoides fallax*, plantas acuáticas)

ABSTRACT

Temporary wetlands are characterized by ephemeral hydrology and severe fluctuations in water levels, since these environments can dry out completely. This Ph.D. research project focuses on the biodiversity conservation of temporary wetlands, a unique ecosystem that disappears rapidly in Mexico. The objectives were to study the distribution patterns of aquatic plants and to analyze the gene flow and the influence of the landscape structure on wetland connectivity. Between 2015 and 2016, 39 wetlands (2,000-3,000 m s a.s.l.) were visited in the states of Aguascalientes, Guanajuato, Jalisco, Michoacán, Querétaro, San Luis Potosí and Zacatecas. Botanical specimens were collected, water physical-chemical parameters, and landscape data were recorded and samples were taken for genetic studies of one species. We quantified plant functional connectivity for temporary wetlands by genotyping seven nuclear microsatellite loci for 18 populations of *Nymphoides fallax* (Menyanthaceae), a tetraploid aquatic plant native to highland wetlands in central Mexico. We tested if wetland connectivity indices (e.g., probability of connectivity, PC) and landscape structure variables (e.g., vegetation cover) translates to higher genetic diversity and gene flow among temporary wetlands. A total of 126 species of aquatic plants (distributed in 80 genera and 38 families) were registered, among them 27 threatened species, 24 with economic use, 20 new records and two species not yet described. Regarding the distribution of species, it was observed that the similarity of the communities does not increase with the spatial proximity between the sites. Genetic data suggested that *Nymphoides fallax* depends to a large extent on sexual reproduction, is widely dispersed and has a high capacity to adapt to varied environments. Well-connected populations within the network had significantly higher genetic diversity and gene flow than more isolated wetland populations. Genetic diversity was associated with threshold distances up to 5 km. Population-specific Fst was best explained by a model including forest cover. The results found can better inform new strategies for the conservation of diversity in temporary wetlands, based on landscape, genetics, and connectivity.

(**Keywords**: aquatic plants, geographically isolated wetlands, landscape genetic, nuclear microsatellites, *Nymphoides fallax*)

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CAPÍTULO 1 - Introducción

Humedal temporal, Aguascalientes, México. Fuente: propio autor

1.1 Importancia de los humedales temporales

Los humedales temporales de agua dulce abarcan cerca de 0,81 millones de km^2 de la superficie de la Tierra (Pekel *et al*., 2016). Los humedales temporales se caracterizan por una hidrología efímera, fluctuaciones severas de saturación de agua y períodos secos (Martínez y García, 2001; Calhoun *et al*., 2017). Son unidades geográficamente aisladas, sin conexión hidrológica, y completamente rodeadas de tierras altas a escala local (Mushet *et al*., 2015). Los humedales temporales son dinámicos y pueden cambiar de forma y tamaño (Frohn *et al*., 2009). Los humedales actúan como conectores entre diferentes ecosistemas, ya sea terrestre o acuático (Aavik *et al*., 2013; Ishiyama *et al*., 2014; Uden *et al*., 2014), proporcionan servicios eco sistémicos (Marton *et al*., 2015) y contribuyen sustancialmente al mantenimiento de la biodiversidad (Balian *et al*., 2008).

Los humedales temporales de agua dulce mexicanos albergan 176 especies de plantas acuáticas vasculares (Mora-Olivo *et al*., 2013). Desde una perspectiva florística, los humedales temporales de las tierras altas de México se consideran ecosistemas heterogéneos y muy diversos (Lobato-de Magalhães y Martínez, 2018). De manera alarmante, las estimaciones de las últimas décadas sitúan las pérdidas de humedales en un 62% para México (Landgrave y Moreno-Casasola, 2012).

Se estima que se ha perdido 60% de los humedales en el planeta y la conservación de humedales aún presenta muchas limitaciones en todo el mundo (Cui *et al*., 2012; Davidson, 2014). La degradación y pérdida de humedales continentales es más rápida y expresiva que en humedales costeros (Davidson, 2014), siendo éstos últimos los más estudiados. La pérdida de hábitat y la intensificación del uso de la tierra son las mayores causas de la disminución de la diversidad vegetal (Liira *et al*., 2008). Algunos estudios consideran que los mamíferos y aves son más sensibles a la pérdida de humedales que los reptiles y anfibios (Quesnelle *et al*., 2013). Sin embargo, es escasa la información sobre el efecto de la pérdida de este hábitat sobre las plantas acuáticas.

Para la conservación de humedales es importante establecer redes ecológicas que integren factores hidrológicos y biológicos (Cui *et al*., 2012). En este contexto, el conocimiento de la biodiversidad y de la conectividad de ecosistemas es esencial para establecer procedimientos de restauración y conservación ecológica (Aavik *et al*., 2013). La conectividad de hábitat presenta dos componentes: conectividad estructural y conectividad funcional. Aunque algunos autores prefieren utilizar datos estructurales para inferir la conectividad (Tischendorf y Fahrig, 2000), la genética de paisaje ha demostrado ser una herramienta de gran utilidad para comprender la conectividad funcional (Manel *et al*., 2003).

La conectividad de los humedales tiene una función fundamental en la manutención de la biodiversidad (Ishiyama *et al*., 2014). El tamaño del humedal y su conectividad con otros humedales son factores asociados con la abundancia de las especies (Attum *et al*., 2007). Sin embargo, O'Connell *et al.* (2013) defienden que la proximidad entre los humedales es un factor importante para mantener la riqueza de especies vegetales en ellos. De un modo general, los estudios de conectividad son escasos en ambientes acuáticos (Ayram *et al.*, 2015), sobretodo en humedales temporales aislados geográficamente.

1.2 Diversidad de la vegetación acuática en México

México es uno de los 12 países megadiversos en el mundo, representando uno de los '*hotspots'* de biodiversidad prioritario para la conservación, con un alto grado de endemismo (Myers *et al.*, 2000; Declaración de Cancún, 2002; Martínez-Meyer *et al*., 2014). Las características del clima, relieve, geología, entre otros aspectos, permiten que México tenga una gran diversidad ecológica y de tipos de vegetación (Taroge, 2008). De forma que, México presenta prácticamente todos los tipos de ecosistemas conocidos en el mundo (Rzedowski, 1978). El país presenta 23,314 especies de plantas con flores (División Magnoliophyta), distribuidas en 2,854 géneros y 297 familias (Villaseñor, 2016; Villaseñor y Ortiz, 2014) y un endemismo promedio de 40%, aunque el conocimiento de la biodiversidad se presenta subestimado (Martínez-Meyer *et al*., 2014).

La vegetación acuática comprende parte considerable de la cobertura vegetal de México; así mismo, su conocimiento es incompleto e, incluso, determinados tipos de humedales son desconocidos (Rzedowski, 1978). De acuerdo con el Inventario Nacional de Humedales (Dumac, 2017), cerca del 6% (128,000 km) del territorio mexicano está ocupada por humedales. Hay 142 sitios Ramsar en México, lo que lo convierte en el país neotropical con el mayor incremento de humedales protegidos internacionalmente en las últimas décadas (Mauerhofer *et al*., 2015). Las estimaciones indican que en México se han perdido cerca del 62% de los humedales en las décadas pasadas (Landgrave y Moreno-Casasola, 2012), lo que contribuye a la falta de conocimiento de estos ecosistemas.

México tiene 1,283 angiospermas acuáticas y subacuáticas, de las cuales 157 son endémicas del país (Villaseñor y Ortiz, 2014). En cuanto a las plantas estrictamente acuáticas, hay 240 especies (Mora-Olivo *et al*., 2013). Según Lot et al. (1993) México tiene 747 especies de plantas vasculares. Los humedales temporales albergan casi el 73% de las plantas acuáticas y el 31% de las plantas estrictamente acuáticas en México (Mora-Olivo *et al*., 2013). En particular, en el centro de México hay ecosistemas acuáticos muy diversos. El mayor número de las plantas acuáticas se concentran en altitudes más bajas (Rzedowski, 1978), pero al menos 147 de las plantas estrictamente acuáticas pueblan los humedales ubicados a más de 1.000 m a.s.l. (Mora-Olivo *et al*., 2013). Los estudios científicos de humedales temporales son escasos. En tal contexto, los inventarios florísticos de humedales temporales en tierras altas de México contribuyen al conocimiento y conservación de un ecosistema en rápida desaparición (Calhoun *et al*., 2016). El este proyecto de investigación doctoral se encontró 126 espécies de plantas acuáticas en los humedales temporales de tierras altas en el centro de México (Ver capítulo 2).

1.3 Formas de vida de las plantas acuáticas

La terminología ‘plantas acuáticas’, corresponde a las especies botánicas hidrófilas o macrófitas acuáticas. Las plantas acuáticas pueden pertenecer a los grupos

Charophyta, Briophyta, Pteridophyra, Gimnospermas y Angiospermas (Lot, 2012), y son un componente importante de los ecosistemas acuáticos (Dar *et al*., 2014). Las plantas acuáticas son comúnmente clasificadas en cuanto a su forma de vida (Cook, 1996). Diversas plantas acuáticas presentan distribución cosmopolita; sin embargo, algunas sólo prosperan en ambientes específicos representando elementos endémicos (Rzedowski, 1978).

Existen diferentes propuestas de clasificación para la forma de vida de las plantas acuáticas. Sculthorpe (1985) clasifica a las plantas acuáticas en cinco formas de vida: enraizada emergente, enraizada sumergida, enraizada de hojas flotantes, enraizada de tallos postrados y libres sumergida. Irgang *et al.* (1984) incluyen la terminología 'epifita' y también 'anfibia', para las plantas terrestres que habitan los ecosistemas acuáticos, y consideran siete formas de vida para las plantas acuáticas: anfibia, emergente, epifita, enraizada de hojas flotantes, enraizada sumergida, libre flotante y libre sumergida (Fig. 1.3.1).

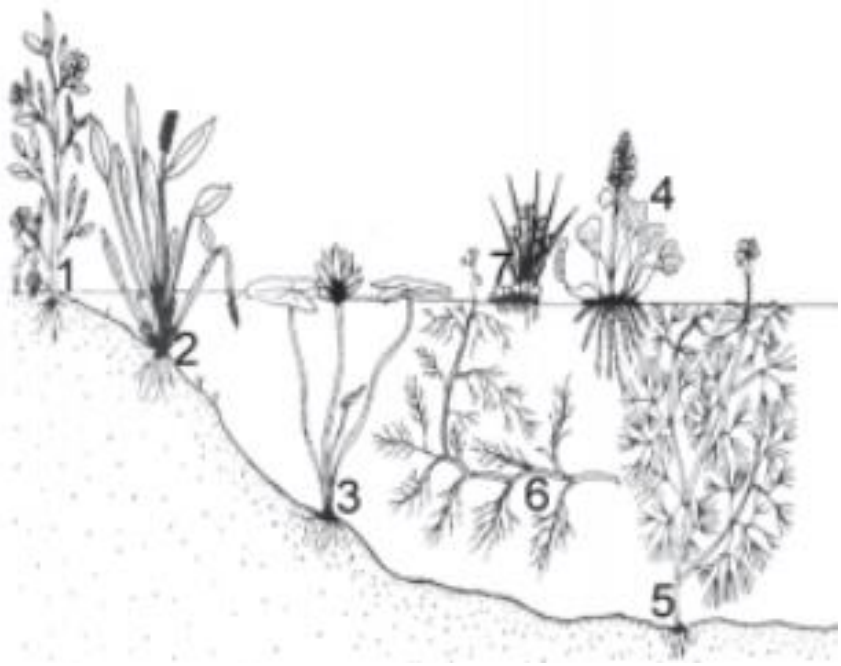

Figura 1.3.1 Formas de vida de las plantas acuáticas: (1) anfibia, (2) emergente, (3) enraizada de hojas flotantes, (4) libre flotante, (5) enraizada sumergida, (6) libre sumergida, (7) epifita. Fuente: Irgang *et al.* (1984)

Lot (2012) divide las plantas acuáticas en tres grupos: estrictas, subacuáticas y tolerantes. La terminología utilizada en la definición de plantas acuáticas y de sus formas de vida es muy importante, dado que influye en el número total de especies consideradas en los inventarios florísticos de humedales y en la clasificación de las plantas acuáticas y sus formas de vida. Por esa razón, comúnmente se observan discrepancias entre los valores de riqueza total de especies botánicas acuáticas para determinadas regiones (Murray-Hudson *et al*., 2012).

En este proyecto de investigación doctoral se encontró cinco de las siete formas de vida propuestas por Irgang *et al.* (1984), son ellas: emergente, enraizada de hojas flotantes, libre flotante, enraizada sumergida y libre sumergida (Ver capítulo 2, Fig. 1.3.2).

Flotante enraizada

Flotante libre

Sumergida enraizada

Sumergida livre

Emergente

Figura 1.3.2 Formas de vida de las plantas acuáticas de los humedales temporales de tierras altas en centro de México. Fuente: propio autor.

1.4 Ecosistemas de humedales temporales

Los humedales se presentan en todas las zonas climáticas y se distribuyen en todos los continentes, contando con una amplia diversidad de tipos (Williams, 2006). Los humedales temporales son ambientes donde ocurren cambios estacionales en la humedad y sequía (Fig. 1.4.1, Martínez y García, 2001). Comúnmente la vegetación de humedales temporales está compuesta por especies tolerantes a la sequía y/o que presentan características que les permiten persistir en el área durante estos periodos (*e.g.*, semillas resistentes a sequía).

Figura 1.4.1 Humedal temporal, Huimilpan, Querétaro, Mexico (agosto, 2015 y mayo, 2016). Fuente: propio autor.

Los humedales son ecosistemas dinámicos y altamente productivos, que pueden alcanzar las mayores tasas de producción primaria entre todo tipo de ecosistemas; por lo que en ellos el reciclaje de nutrientes ocurre de manera muy rápida. La vegetación herbácea de humedales de agua dulce puede producir hasta 100 t/ha/año de materia orgánica, representando uno de los ecosistemas más productivos en el planeta (Dodds y While, 1958; Junk, 1993). Muchos animales terrestres se benefician de los humedales, de modo que los procesos que ocurren en estos ambientes son importantes para soportar la abundancia y diversidad de muchas especies de la flora y fauna, sea terrestre o acuática. Las plantas acuáticas son muy exigentes en cuanto a las condiciones del ambiente; por ejemplo, necesitan de un intervalo especifico de *pH*, temperatura, salinidad y concentración de oxígeno, entre otras. Debido a esto, la composición florística de humedales es tan variada entre los diferentes tipos de ecosistemas acuáticos (Rzedowski, 1978).

1.5 Concepto de humedal aislado geográficamente

La terminología ‘humedales aislados’ ha sido utilizada de manera errónea por la comunidad científica, pues, aunque los humedales no presenten una conexión espacial, estos ambientes pueden presentar conectividad y flujo genético en sus especies (Mushet *et al*., 2015). Para estos autores, el término aislado puede llevar a la confusión en la descripción de este tipo de humedales y no contribuye a la creación de políticas públicas para el uso y la conservación de estos ambientes. En los últimos años gran parte de los investigadores han sustituido el término ‘aislados’ por ‘geográficamente aislados’. De esta manera los humedales aislados geográficamente contribuyen a las funciones del paisaje, y estos ambientes presentan conectividad hidrológica, biogeoquímica y biológica (Cohen *et al*., 2016).

Anteriormente se consideraba que estos ecosistemas eran aislados entre sí y de difícil detección (Hernández, 2005) pues son dinámicos, con variaciones anuales,

presentándose en áreas muy pequeñas. Cuando ocurren de manera aislada, los humedales temporales del altiplano de México funcionan como una 'isla' en un paisaje terrestre, por lo que son importantes para la manutención de la biodiversidad de la flora y fauna - sobre todo de las aves acuáticas (Lopes-Sauti *et al.,* 2014; Reynolds *et al*., 2015). Sin embargo, recientemente se ha resaltado que los humedales temporales pueden no estar aislados para los vectores de biodiversidad (Ver capítulo 4).

1.6 Diversidad y estructura genética

La información molecular sobre la diversidad genética es esencial para comprender las respuestas ecológicas, evolutivas y promover la conservación de especies (Uesugi et al. 2005). Sin embargo, la gran mayoría de las plantas acuáticas todavía no tienen marcadores moleculares específicos. Entre los objetivos de este proyecto de investigación doctoral se puede citar: (*i*) probar la capacidad de transferencia de los marcadores microsatélite de *Nymphoides peltata* (S. G. Gmel.) Kuntze (Uesugi et al. 2005) a *Nymphoides fallax* Ornduff; y (*ii*) analizar la diversidad genética de las poblaciones en humedales temporales de las tierras altas en el centro de México (Ver capítulo 3).

La estructura genética consiste en la distribución diferencial de las frecuencias alélicas entre las poblaciones de una especie (Hedrick, 2011; Rimieri, 2013). La diferencia alélica entre las poblaciones es un resultado de la acción de las fuerzas evolutivas a lo largo del tiempo, como la selección natural, la deriva génica, la mutación y la migración. Generalmente, la selección natural, la deriva génica y la mutación aumentan la diferenciación de frecuencias alélicas entre las poblaciones. Por otro lado, la migración (o flujo génico) es una fuerza evolutiva que contribuye a la homogenización de dichas frecuencias alélicas (Eguiarte *et al*., 2010). El patrón de variabilidad genética entre poblaciones está estructurado en un paisaje geográfico; sin embargo, es posible observar una estructuración genética espacial entre individuos dentro de los fragmentos. El flujo génico puede explicar esta variación de la estructura genética en poblaciones e individuos (Melo, 2012). El flujo génico es considerado una fuerza evolutiva cohesiva,

pues evita que las poblaciones presenten muchas diferencias entre sí para las frecuencias alélicas (Eguiarte *et al*., 2010).

La estructura genética de poblaciones puede ser clasificada en dos tipos: estructura genética alta y estructura genética baja. Cuando existen fuertes diferencias entre las frecuencias alélicas de las poblaciones se considera que la estructura genética es alta; por otro lado, la estructura genética se considera baja cuando las poblaciones presentan pocas o nulas diferencias entre sus frecuencias alélicas (Eguiarte *et al*., 2010).

El análisis de la estructura genética puede ser aplicado para cuantificar las diferencias entre poblaciones predeterminadas o para definir poblaciones a partir de las frecuencias alélicas de individuos (Hedrick, 2011). El conocimiento y comprensión de la estructura genética entre poblaciones son importantes para el manejo y la conservación de los recursos genéticos, así como en la evaluación de los impactos de la fragmentación (Gonçalves *et al*., 2010) y mejoramiento genético (Rimieri, 2013). Determinar la estructura genética de las poblaciones es un aspecto importante en los estudios de genética (Meirmans, 2012), pues la variabilidad genética proporciona a las especies el potencial de adaptarse a los cambios ambientales. La identificación de poblaciones genéticamente distintas es importante para la asignación de unidades de conservación y de unidades evolutivas, así como para proteger especies en riesgo de extinción y proponer la translocación de especies (Godoy, 2009). Además, la estructura genética permite hacer inferencias acerca de la conectividad, de las barreras potenciales al flujo genético y conocer la historia demográfica poblacional de las especies (Piñero *et al*., 2008).

1.7 Conectividad, genética del paisaje y marcadores moleculares

La conectividad está definida como la capacidad del paisaje de facilitar o impedir el movimiento de un organismo entre diferentes manchas del hábitat (Tischendorf y Fahrig, 2000). Se puede utilizar la genética para cuantificar la conectividad funcional real directa o indirectamente, y, por lo tanto, proporcionar los medios para probar hipótesis

sobre cómo los aspectos de la matriz del paisaje intervienen en la dispersión y el flujo de genes (DiLeo y Wagner, 2016). El flujo génico es considerado como una medida directa de conectividad, que está definida por la influencia de la estructura, configuración del paisaje y calidad de la matriz en el flujo génico. La conectividad puede también ser descrita como la relación entre la distancia de los ambientes, las características del paisaje y su interacción con la dispersión de las especies y la dinámica de poblaciones (Mushet *et al*., 2015).

La evaluación de la conectividad implica la identificación de sus componentes estructurales y funcionales. Para las plantas, la conectividad funcional implica la dispersión efectiva de propágulos o polen entre los hábitats de recursos, mientras que la conectividad estructural se refiere a la composición de los elementos del paisaje físico y la configuración espacial de los hábitats de recursos (Auffret et al., 2017a). La conectividad funcional de la planta se mide ampliamente mediante observaciones de flujo de polen y dispersión de semillas o por información genética (Auffret et al., 2017a). La mayoría de las plantas que habitan en humedales temporales tienen semillas de larga vida y son capaces de una dispersión frecuente y de larga distancia (Reynolds et al., 2015; Schofield et al., 2018). Los movimientos de plantas acuáticas dentro y entre los ecosistemas de agua dulce pueden conectar humedales a través del paisaje (Schofield et al., 2018). Los estudios de conectividad funcional de las plantas para los humedales pueden centrarse en (i) identificar poblaciones aisladas que están genéticamente desfasadas, (ii) evaluar mecanismos y patrones de dispersión que pueden ser comunes a múltiples especies, y (iii) evaluar los efectos del paisaje en la diversidad genética y el flujo de genes, todo lo cual puede informar las prácticas de conservación (Auffret et al., 2017a, b).

La genética del paisaje es una disciplina interdisciplinaria, que combina métodos y conceptos de la genética de poblaciones con ecología del paisaje y estadística espacial (Balkenhol *et al*., 2009). Esta disciplina fue creada hace menos de dos décadas (Holderegger y Wagner, 2008), y aun es una disciplina que busca por su identidad de sus disciplinas-base. La genética del paisaje integra las interacciones entre especies y el medio ambiente, los vectores de dispersión y las variables de la estructura del paisaje

para comprender las respuestas espaciales de las especies a la fragmentación del hábitat (Dyer, 2015a, b; Auffret et al., 2017a, b). Los estudios del paisaje a nivel genético son importantes porque la estructura genética de las poblaciones naturales está influenciada por la heterogeneidad del paisaje y esta afecta la dispersión de las especies (Luque *et al*., 2012). La comprensión de los procesos de flujo génico requiere un conocimiento detallado de como las características del paisaje estructuran las poblaciones.

En las pasadas décadas los microsatélites se tornaron el marcador molecular más popular en los estudios genéticos (Schlötterer, 2000; 2004) y uno de los más utilizados en genética del paisaje. Existe una gran diversidad de marcadores moleculares, a citar las aloenzimas, los SNPs, los microsatélites, las AFLPs, los RFLPs y los RAPDs. Sin embargo, los marcadores moleculares pueden ser agrupados en dos tipos: (*i*) marcadores neutrales; (*ii*) marcadores adaptativos. Los marcadores neutrales permiten hacer inferencias acerca de cambios recientes (contemporáneos), son apropiados para estudios de genética de paisaje y para evaluar flujo génico, cuellos de botella, endogamia, y deriva génica (Wagner y Fortin, 2013). Los marcadores adaptativos son indicados para los estudios de selección y adaptación, y generalmente son utilizados para evaluar la interacción gene-ambiente, la adaptación local, la adecuación y la viabilidad poblacional (Hedrick, 2011).

Los microsatélites son neutrales, co-dominantes y muy polimórficos y es por ello que son ideales para evaluar patrones de estructura y flujo genético y su relación con el paisaje. Estos marcadores permiten amplificar pequeñas secuencias de ADN, pueden ser identificados por zonas de repeticiones. Ellos son muy variables, evolucionan neutralmente y una vez optimizada la reacción en cadena de polimerasa (PCR) son de fácil procesamiento de cientos de miles de individuos (Holderegger y Wagner, 2008). Con microsatélites es posible identificar alelos de ambos padres (estimación de heterocigosis) y hacer más inferencias y análisis, pues ellos son altamente informativos, presentan gran número de alelos y alta heterocigocidad (Schlötterer, 2004).

En las últimas décadas se ha presentado un notable aumento de los estudios de ecología que incorporan el aspecto de la conectividad. La mayoría de los estudios de conectividad fueron realizados en Europa, seguido de Norte América. Por otro lado, México tiene una pequeña presencia en el número de estudios de conectividad de ecosistemas, sobretodo de ecosistemas acuáticos (Ayram *et al*., 2015). En este proyecto de investigación doctoral se analizó el flujo genético y conectividad funcional en los humedales temporales de las tierras altas en el centro de México (Ver el capítulo 4).

1.8 Modelo de estudio

La metodología de estudio albergó siete etapas (Fig. 1.8.1), son ellas: selección de los sitios de estudio, trabajo de campo y herbario, análisis de la estructura del paisaje, laboratorio de genética para analizar la diversidad y estructura de las poblaciones, análisis estadísticos, análisis de conectividad, escritura de artículos.

Figura 1.8.1 Flujograma del proyecto de investigación doctoral. Fuente: propio autor.

En el proyecto de investigación se eligieron 39 humedales temporales en elevaciones que varían desde 1,900 a 2,700 m a.s.l. en los estados de Aguascalientes, Guanajuato, Jalisco, Michoacán, Querétaro, San Luis Potosí y Zacatecas (Ver mapa en el capítulo 2). Los humedales se ubicaron utilizando referencias bibliográficas, mediante excursiones y a través del apoyo de investigadores locales. Las áreas se encuentran entre las latitudes de 20º y 24º de Norte, y las longitudes 100º y 103º Oeste. El clima es templado semiárido y subhúmedo templado, con las siguientes clasificaciones de Koeppen: "BS1kw", "C (wo)" y "C (w1)". La temperatura media anual oscila entre 12 ºC

y 18 ºC. La precipitación media anual es de 600 a 800 mm, con las precipitaciones más altas en junio o julio.

1.9 Especie focal

Para los estudios de genética del paisaje y conectividad (Ver capítulo 3 y capitulo 4), la planta acuática *Nymphoides fallax* Ornduff (Figura 1.9.1) fue elegida como especie focal considerando los siguientes criterios: (*i*) la especie está presente en la mayoría de las áreas estudiadas previamente en el levantamiento florístico (capitulo 1); (*ii*) la especie es estricta a los ambientes acuáticos; (*iii*) es una especie endémica de tierras altas de México y Guatemala; (*iv*) estudios previos reportan que las poblaciones de la especie están tornándose escasas por la pérdida de hábitat; (*v*) existen marcadores moleculares del tipo microsatelites (SSR) previamente desarrollados para otra especie del género (Uesugi et al., 2005).

Figura 1.9.1 *Nymphoides fallax* Ornduff (Menyanthacea) en un humedal temporal, Huimilpan, Querérato, México. Fuente: propio autor

Nymphoides fallax es una especie tetraploide (36 cromosomas), nativa de humedales temporales, estanques y arroyos en elevaciones superiores a 1,500 m a.s.l. (Ornduff, 1969; Ornduff, 1970). Estudios recientes evidencian que *N. fallax* es una especie alopoliploide que pertenece a un clado neotropical, pero el escenario de hibridación ancestral no es concluyente (Tippery *et al*., 2018). *Nymphoides fallax* tiene reproducción tanto sexual como vegetativa, y una anatomía vegetativa similar a *Nymphoides indica* (L.) Kuntze la otra especie de *Nymphoides* presente en México (Martínez y Gómez-Sánchez, 2006). *Nymphoides fallax* difiere de *N. indica* por tener pétalos amarillos laciniados, anteras amarillas, semillas más grandes y ocurrencia en mayores altitudes (Ornduff, 1969).

Las especies de *Nymphoides* exhiben una variedad de mecanismos reproductivos, como arquitectura de inflorescencias, tipos de morfos florales y morfología de semillas. Distyly es una caracteristica ancestral en *Nymphoides* (Tippery *et al*., 2008, 2018; Tippery y Les, 2011), y promueve el flujo de genes (Barrett, 1980; Barrett y Shore, 2008). Existe información de la variación del tamaño del estambre, el polen y el pistilo entre los morfos de *N. geminata*, *N. humboldtiana*, *N. indica* y *N. peltata* (Ornduff, 1966; Van der Velde y Van der Heijden, 1981; Haddadchi y Fatemi, 2015).

Se considera que la reproducción sexual tiene una pequeña contribución en la reproducción de *Nymphoides* (Larson, 2007; Liao *et al*., 2013; Huang *et al*., 2015). Sin embargo, las estrategias reproductivas está poco estudiada en las especies de *Nymphoides* (Wang et al., 2005). En este proyecto de investigación doctoral no se ha encontrado los clones de *N. fallax* (Ver capítulo 3). *Nymphoides fallax* forma bancos de semillas con un alto número de semillas (~ 1,258 semillas / m2; Zepeda *et al*., 2014) y plántulas, polinizadores y dispersadores están presentes en el campo. Las flores de *N. fallax* tienen heterostilia dimórfica (Fig. 1.9.2 y Fig. 1.9.3; Tippery y Les, 2011), altos coeficientes de consanguinidad y alta diversidad genética dentro de las poblaciones en los humedales temporales de las tierras altas de México (Ver capítulo 3). Por lo tanto, existe una fuerte evidencia que sugiere que la reproducción sexual puede ser igual o

incluso más importante que la reproducción vegetativa en las poblaciones de *N. fallax* (Lobato-de Magalhães y Martínez, dato no publicado).

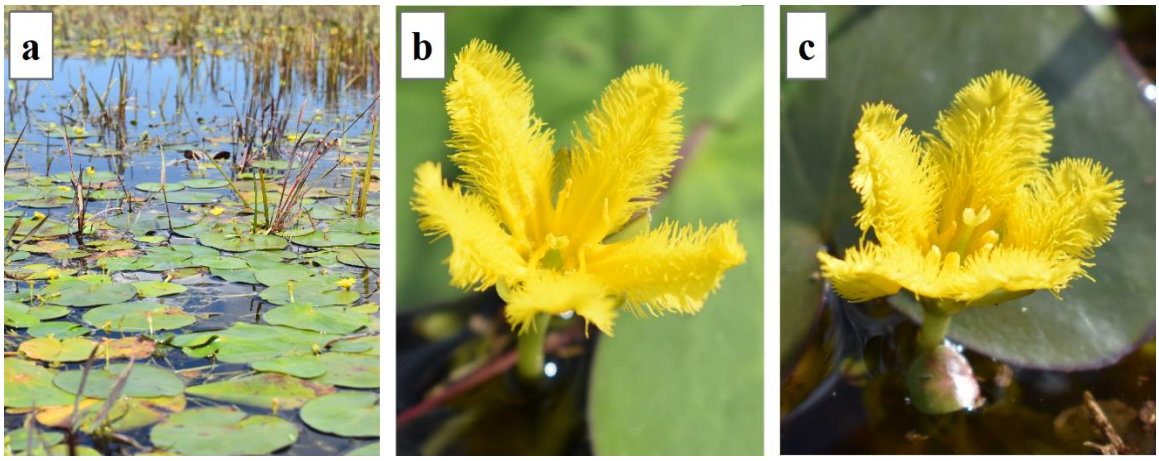

Figura 1.9.2 (a) *Nymphoides fallax* Ornduff (Menyanthaceae) en un humedal temporal, Guanajuato, México; (b) morfo floral de estilo curto, (c) morfo floral de estilo largo. Fuente: Lobato-de Magalhães y Martínez, dato no publicado.

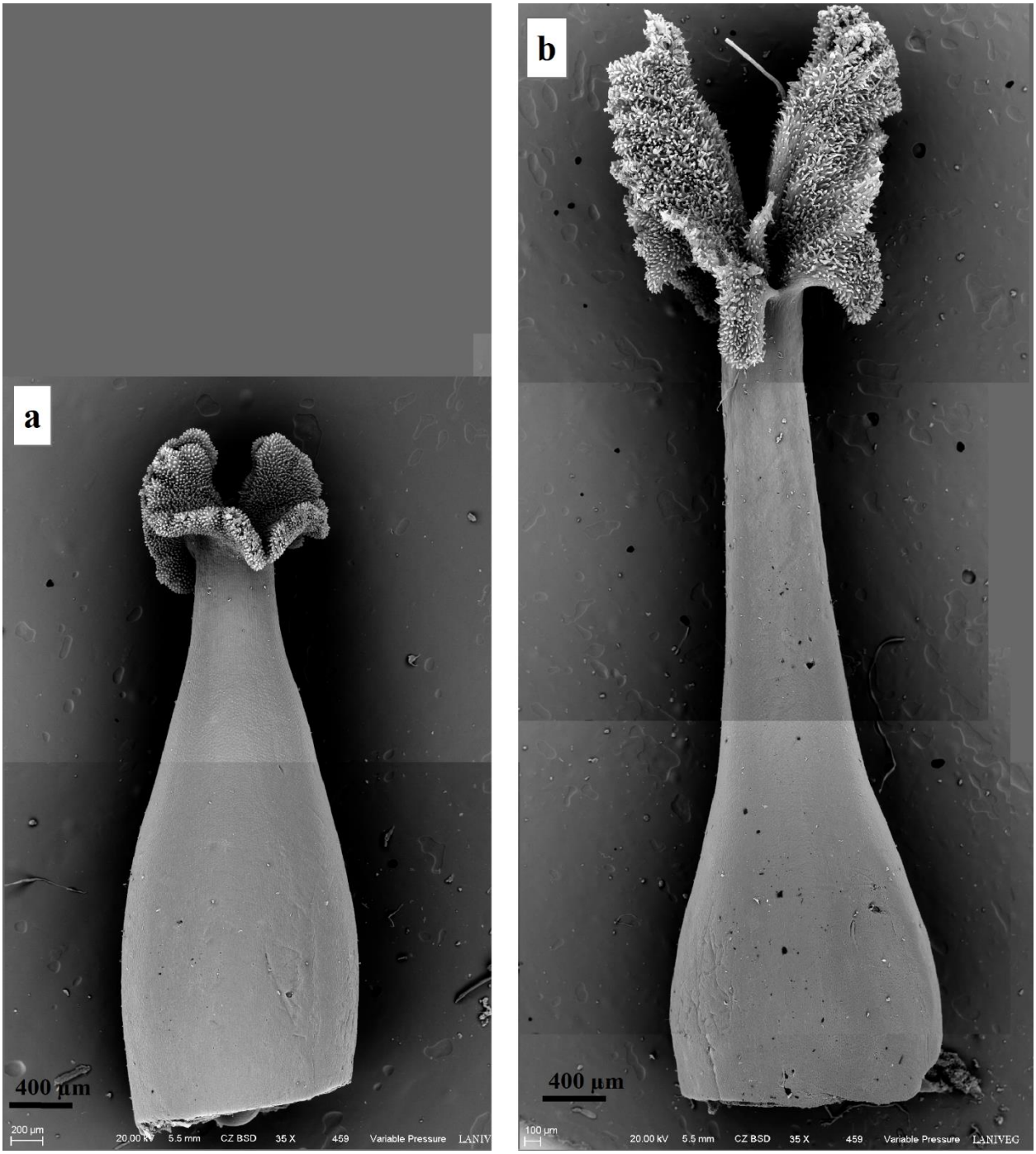

Figura 1.9.3 Foto con microscopia de barrido de la especie *Nymphoides fallax* Ornduff (Menyanthaceae), (a) estilo curto, (b) estilo largo. Fuente: Lobato-de Magalhães y Martínez, dato no publicado.

1.10 Objetivos

El objetivo de la tesis es contribuir al conocimiento de la flora acuática y la conectividad de los humedales temporales de tierras altas en el centro de México.

Los objetivos específicos de la tesis son:

1) Conocer la flora acuática de humedales temporales de tierras altas en el centro de México y determinar la similitud florística entre humedales;
2) Analizar la diversidad y estructura genética de *N. fallax*;
3) Determinar la conectividad funcional de *N. fallax* en humedales temporales de tierras altas;
4) Determinar qué elementos del paisaje funcionan como conductores del flujo genético en humedales temporales de tierras altas.

1.11 Estructura de la tesis

Se presenta la tesis dividida en cinco capítulos: un capítulo introductorio (Capítulo 1), tres capítulos-artículos (Capítulo 2, Capítulo 3, Capítulo 4) y un capítulo de conclusiones generales (Capítulo 5). Cada capítulo-artículo está escrito como un documento independiente para facilitar la publicación del trabajo. Por lo tanto, cada capítulo-artículo comprende un resumen, introducción, materiales y métodos, resultados y sección de discusión. Las referencias del Capítulo 1 siguen al final de la tesis.

En el Capítulo 2 (florística) los principales objetivos fueron evaluar cuántas especies se encuentran en los humedales temporales de altitud y la similitud entre las comunidades y patrones latitudinales de su distribución. Se encontraron 126 especies, 80 géneros y 38 familias. Se observaron cinco formas de vida, todas de hábito herbáceo, 27 especies están amenazadas, 24 tienen uso económico, 48 son endémicas, y son 19 cosmopolitas. Se encontró 20 nuevos registros para algunos estados mexicanos y dos

nuevas especies de *Eleocharis*. Los humedales presentaron similitud entre sí y mayor riqueza a bajas latitudes.

En el Capítulo 3 (diversidad genética) los principales objetivos fueron (*i*) probar la capacidad de transferencia de los marcadores microsatélite de *Nymphoides peltata* (S. G. Gmel.) Kuntze (Uesugi et al. 2005) a *Nymphoides fallax* Ornduff; y (*ii*) analizar la diversidad genética de las poblaciones en humedales temporales de las tierras altas en el centro de México. Siete de 10 loci polimórficos fueron amplificados con éxito y se observó alta diversidad genética (*e.g*. número de alelos y heterocigosidad). Los resultados fueron utilizados para el desarrollo del Capítulo 4.

En el Capítulo 4 (conectividad) los principales objetivos fueron probar hipótesis de conectividad en *N. fallax*: (*i*) la conectividad de humedales, cuantificada por los índices de conectividad del hábitat, se traducirá en una mayor diversidad genética y flujo de genes, ya que las poblaciones que están más cerca geográficamente intercambiarán más flujo de genes que las poblaciones que están geográficamente distantes y (*ii*) las características del paisaje de la matriz intermedia afectarán el flujo de genes entre los humedales temporales. Se encontró que la conectividad de los humedales se asoció con una mayor diversidad genética y que el flujo de genes de *N. fallax* se vio facilitado por la cantidad de bosque en el paisaje.

Finalmente, se presentaron las conclusiones finales en el Capítulo 5, incluyendo una reflexión global acerca de los resultados encontrados en los capítulos anteriores y recomendaciones de manejo y conservación de humedales temporales de tierras altas.

Diversidad florística y conectividad de humedales temporales de tierras altas en el centro de México

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CAPÍTULO 2 - Temporary freshwater wetlands floristics in central Mexico highlands

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***Nymphaea gracilis* Zucc., humedal temporal, Aguascalientes, México. Fuente: propio autor**

Temporary freshwater wetlands floristics in central Mexico highlands

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Abstract

Background: Mexico has a high diversity of aquatic and subaquatic plants that occur between 1,000 and 2,500 m of elevation, although a larger proportion of aquatic plants is concentrated at lower altitudes. Temporary wetlands harbor close to 73 % of the aquatic species in Mexico. These systems are under a strong anthropogenic pressure and suffer constant degradation.
Questions: i) How many species grow in highland temporary wetlands? ii) Are they floristically similar? iii) Is there a latitudinal pattern of species richness?
Studied groups: Charophyta, Pteridophyta, Angiosperms.
Study site and years of study: Central Mexico (39 wetlands) from 2015 to 2016.
Methods: We collected in 39 temporary wetlands for two years. We made a presence/absence list of species per locality, and calculated floristic similarities and correlations between wetlands. We include data characterizing life form, plant use, and conservation status.
Results: We found 126 species belonging to 80 genera and 38 families. The richest families were Cyperaceae, Asteraceae, and Poaceae. As to genera, *Eleocharis*, *Cyperus*, and *Juncus* had more species. Species with the widest distributions were *Persicaria mexicana*, *Marsilea mollis*, *Luziola fluitans*, *Heteranthera peduncularis*, and *Nymphoides fallax*. We found five different life forms – all herbaceous, including 27 threatened species, 24 species with economic use, 48 endemic species, and 19 cosmopolitan species. In addition, we found 20 species recorded for the first time in some states included in our study, and two species of *Eleocharis* that might represent undescribed species. The richest wetland harbors 40 species, the poorest has only five. Wetlands were comparable to each other in species composition, and species richness increases towards the south.
Conclusions: Temporary wetlands harbor a high floristic diversity and are similar to each other. Lower latitudes host higher numbers of species.
Key words: Aquatic plants, floristic similarity, new species records.

Resumen

Antecedentes: En altitudes entre 1,000 y 2,500 m ocurre una gran diversidad de plantas acuáticas en México, a pesar de que la mayor diversidad está concentrada en bajas altitudes. Los humedales temporales albergan el 73 % de las especies acuáticas mexicanas. Este ecosistema singular sufre presión de degradación y es de gran interés para la conservación.
Preguntas: i) ¿Cuántas especies se encuentran en los humedales temporales de altitud? ii) ¿La flora de los humedales temporales es similar? iii) ¿Existe un gradiente latitudinal de riqueza de especies?
Grupos en estudio: Charophyta, Pteridophyta y Angiospermas.
Sitio de estudio y año del estudio: Centro de México (39 humedales) en 2015 y 2016.
Métodos: Colectamos en 39 sitios durante dos años. Los datos que se incluyeron fueron: forma de vida, hábito, distribución, uso y riesgo de extinción. Posteriormente, se elaboró un listado de presencia/ausencia de especies por sitio y se calculó la similitud florística por medio del índice de Jaccard y la correlación entre los humedales por medio de Friendly.
Resultados: Se encontraron 126 especies, 80 géneros y 38 familias. Las familias más ricas fueron Cyperaceae, Asteraceae, y Poaceae. Los géneros más ricos fueron *Eleocharis*, *Cyperus* y *Juncus*. Las especies con mayor distribución fueron *Persicaria mexicana*, *Marsilea mollis*, *Luziola fluitans*, *Heteranthera peduncularis* y *Nymphoides fallax*. Se observaron cinco formas de vida, todas de hábito herbáceo, 27 especies están amenazadas, 24 tienen uso económico, 48 son endémicas a MegaMéxico, y son 19 cosmopolitas. Encontramos 20 nuevos registros para algunos estados mexicanos y dos son probablemente nuevas especies de *Eleocharis*. El humedal más rico presentó 40 especies, el más pobre cinco. Los humedales presentaron similitud entre sí y mayor riqueza a bajas latitudes.
Conclusiones: Los humedales temporales son ecosistemas biodiversos que presentan similitud entre sí. En latitudes más bajas se observa mayor número de especies.
Palabras clave: Nuevos registros de especies, plantas acuáticas, similitud florística.

Wetlands are among the most threatened ecosystems of the world (Zedler & Kercher 2005, Cui *et al*. 2012). Estimates over the last decades put wetland loss as high as 60 % worldwide (Davidson 2013), and 62 % for Mexico (Landgrave & Moreno-Casasola 2012). Mexico is a megadiverse country (Declaración de Cancún 2002) and a biodiversity conservation hotspot (Myers *et al*. 2000) that harbors 21,841 flowering plant species (Villaseñor & Ortiz 2014). According to the National Wetland Inventory (Dumac 2017), almost 6 % (128,000 km) of the Mexican territory is occupied by wetlands. There are 139 Ramsar sites in Mexico, which makes it the neotropical country with the highest increase in internationally protected wetlands in recent decades (Mauerhofer *et al*. 2015). Mexico has 1,283 aquatic and subaquatic angiosperms, of which 157 are endemic to the country (Villaseñor & Ortiz 2014). As to strictly aquatic plants, there are 240 species (Mora-Olivo *et al*. 2013). According to Lot *et al*. (1993) Mexico has 747 of vascular aquatic plants. Aquatic plants may belong to the groups Charophyta, Briophyta, Pteridophyra, Gymnosperms and Angiosperms (Lot 2012), and are a major component of aquatic ecosystems (Dar *et al*. 2014).

Temporary wetlands span over approximately 0.81 million km^2 of the Earth's surface (Pekel *et al*. 2016). They undergo severe changes in water saturation levels, and at times can dry completely (Martínez & García 2001). Temporary wetlands are dynamic and can change in shape and size (Frohn *et al*. 2009). They function as a connection among different ecosystems, either terrestrial or aquatic (Aavik *et al*. 2013, Ishiyama *et al*. 2014, Uden *et al*. 2014), provide ecosystem services (Marton *et al*. 2015), and substantially contribute in maintaining biodiversity (Balian *et al*. 2008). Temporary wetlands harbor almost 73 % of the aquatic plants and 31 % of the strictly aquatic plants in Mexico (Mora-Olivo *et al*. 2013). In particular, temporary wetlands in central Mexico are highly diverse ecosystems (Rico-Romero 2015). The largest number of aquatic plants is concentrated at lower altitudes (Rzedowski 1978), but at least 147 of the Mexican strictly aquatic plants populate wetlands located above 1,000 m a.s.l. (Mora-Olivo *et al*. 2013). Scientific studies of temporary wetlands are scarce, which contributes to habitat loss. In such a context, floristic inventories of temporary wetlands contribute to the knowledge and conservation of a rapidly disappearing ecosystem (Calhoun *et al*. 2016). The objectives of our paper are to determine the floristic composition and analyze the level of similarity among temporary highland wetlands in central Mexico.

Materials and methods

The 39 studied temporary wetlands range in elevation from 1,900 to 2,700 m a.s.l. in the states of Aguascalientes (localities 1-12), Guanajuato (13-18), Jalisco (19-22), Michoacán (23), Querétaro (24-32), San Luis Potosí (33, 34), and Zacatecas (35-39, Figure 1, Appendix 1, Figure 2). Wetlands were located using bibliographical references, by field trips, and through the support of local researchers. The areas lie between the latitudes of 20º and 24º North, and longitudes 100º and 103º West (Appendix 1), in the Mexican Transvolcanic Belt and the Mexican Plateau. Weather is semiarid temperate, and temperate subhumid, with the following Koeppen classifications: 'BS1kw', 'C(wo)', and 'C(w1)'. Mean annual temperature ranges from 12 ºC to 18 ºC. Mean annual precipitation is 600 to 800 mm with the highest precipitations in June or July. Natural vegetation surrounding the wetlands is composed of oak forests and grasslands with agricultural activities present (INEGI 2017, CONABIO 2017). Water parameters were as follows (averages): pH 5.98, dissolved oxygen 4.87 mg/L, conductivity 126 µSTm, resistivity 0.027 mΩ, total dissolved solids 62 ppm, and salinity 0.06 PSU (Appendix1).

Plant specimens were collected in the 39 wetlands from August 2015 to November 2016. Collection and herborization followed Lot (1986). Vouchers were deposited at Autonomous University of Queretaro Herbarium "Jerzy Rzedowski", QMEX with duplicates to be distributed in Mexico (CIIDIR, IBUG, MEXU, SLPM, and XAL) and Brazil (LUSC), acronyms according to Thiers (continuously updated). Family classification for ferns followed Smith *et al*. (2006), and Angiosperm Phylogeny Group IV (APG 2016) for angiosperms. Nomenclature followed the International Plant Name Index (IPNI 2017). We used the concept of Lot *et al*. (1986, 1993) to define life form (rooted emergent, rooted submersed, rooted floating, free floating, free submersed), and affinity as aquatic plant, which includes three categories (strictly aquatic,

Author Contributions
Tatiana Lobato de Magalhães conceived, designed, and performed the experiments, analyzed the data, and wrote the paper.
Mahinda Martínez conceived and designed the experiments, analyzed the data, reviewed drafs of the paper.

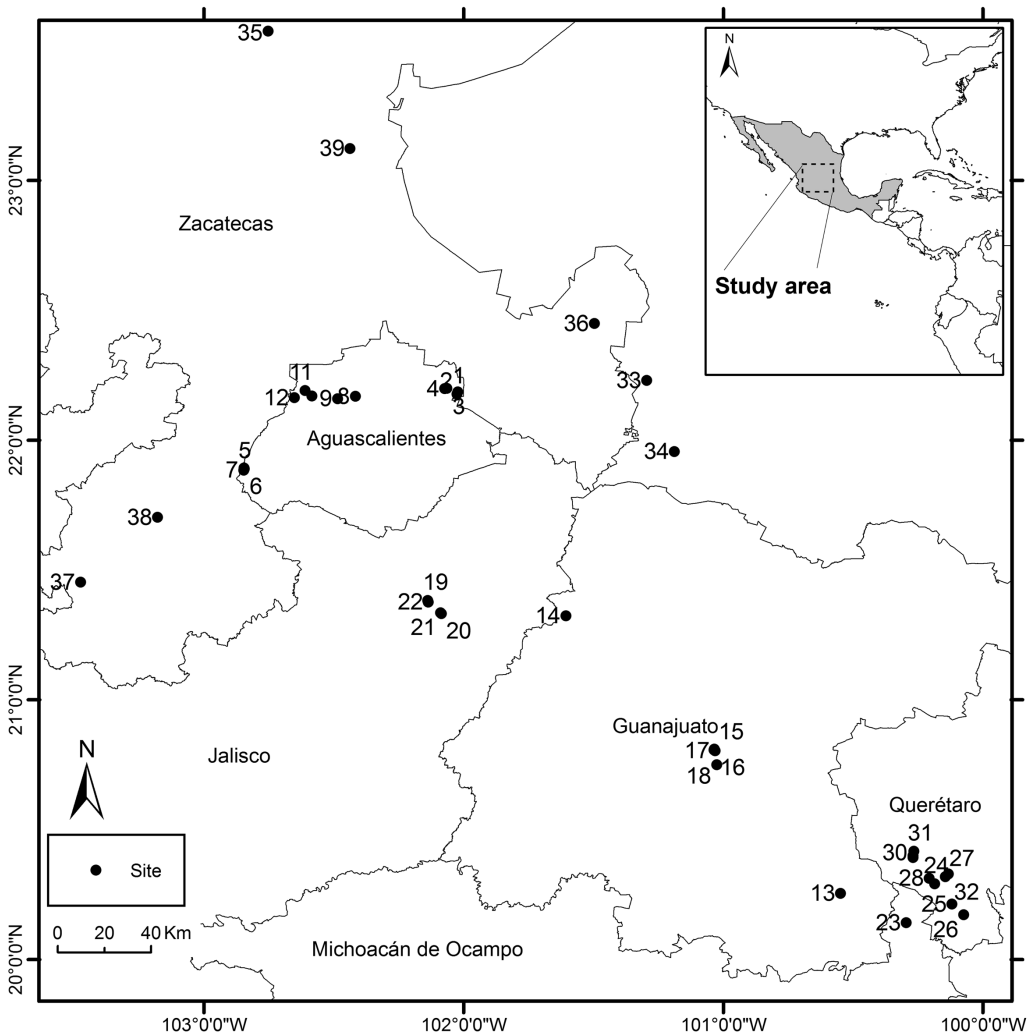

Figure 1. Map of studied temporary wetlands located in the highlands of Aguascalientes, Guanajuato, Jalisco, Michoacán, San Luis Potosí, Querétaro and Zacatecas States, central Mexico.

subaquatic, and tolerant). Strictly aquatic plants definition followed Mora-Olivo *et al*. (2013), subaquatic are according to Lot *et al*. (2013) for monocots and Lot *et al*. (2015).

We elaborated a presence/absence list per site, and calculated the floristic distance (Jaccard 1908, Krebs 1999) and the floristic correlation among wetlands (Friendly 2002). Graphs and maps were elaborated with ArcGIS® version 9.3 and R version 3.31 (R Development Core Team 2017), through *corrplot*, *vegan* packages (Wei y Simko 2017)

We included geographical and altitudinal distribution (Mora-Olivo *et al*. 2013, Lot *et al*. 2013, Lot *et al*. 2015, GBIF 2017, Tropicos 2017), conservation status: either national or international (SEMARNAT 2010, IUCN 2017), use: biofilters, medicinal (Medline 2017), and finally, weeds were defined as such if they are included in Villaseñor & Espinosa-García (1998). Records for the Mexican states were considered as new if the species was not included in Mickel & Smith (2004) for ferns, Lot *et al*. (2013) for monocotyledons, Flora del Bajío (Rzedowski & Rzedowski 2017) for several families, and Martínez *et al*. 2017 for Solanaceae.

Results

We found 126 species of 80 genera and 39 families (Appendix 2). Three were Charophyta, four Pteridophyta, and 119 Angiosperms. The richest family was Cyperaceae (27 species), followed by Asteraceae (17), Poaceae (16), Plantaginaceae (5), Fabaceae (5), and Juncaceae (4, Figure 3-A). The genera with the highest number of species were *Eleocharis* (16 species, Figure 4-A), *Cyperus* (8), and *Juncus* (4). The rest of the genera had three species or less. *Persicaria mexi-*

Figure 2. A) Site 15 in Guanajuato, October 2015. **B)** Site 15 starting to became dry in Guanajuato, December 2016. **C)** Site 7 in Aguascalientes, October 2016. **D)** Site 34 in San Luis Potosí, October 2016. **E)** Site 35 in Zacatecas, October 2015. **F)** Site 32 in Querétaro, October 2016. **G)** Site 29 surrounded by houses in Querétaro, October 2016. **H)** Site 26 it was covered with soil and asphalt during 2016 in Querétaro

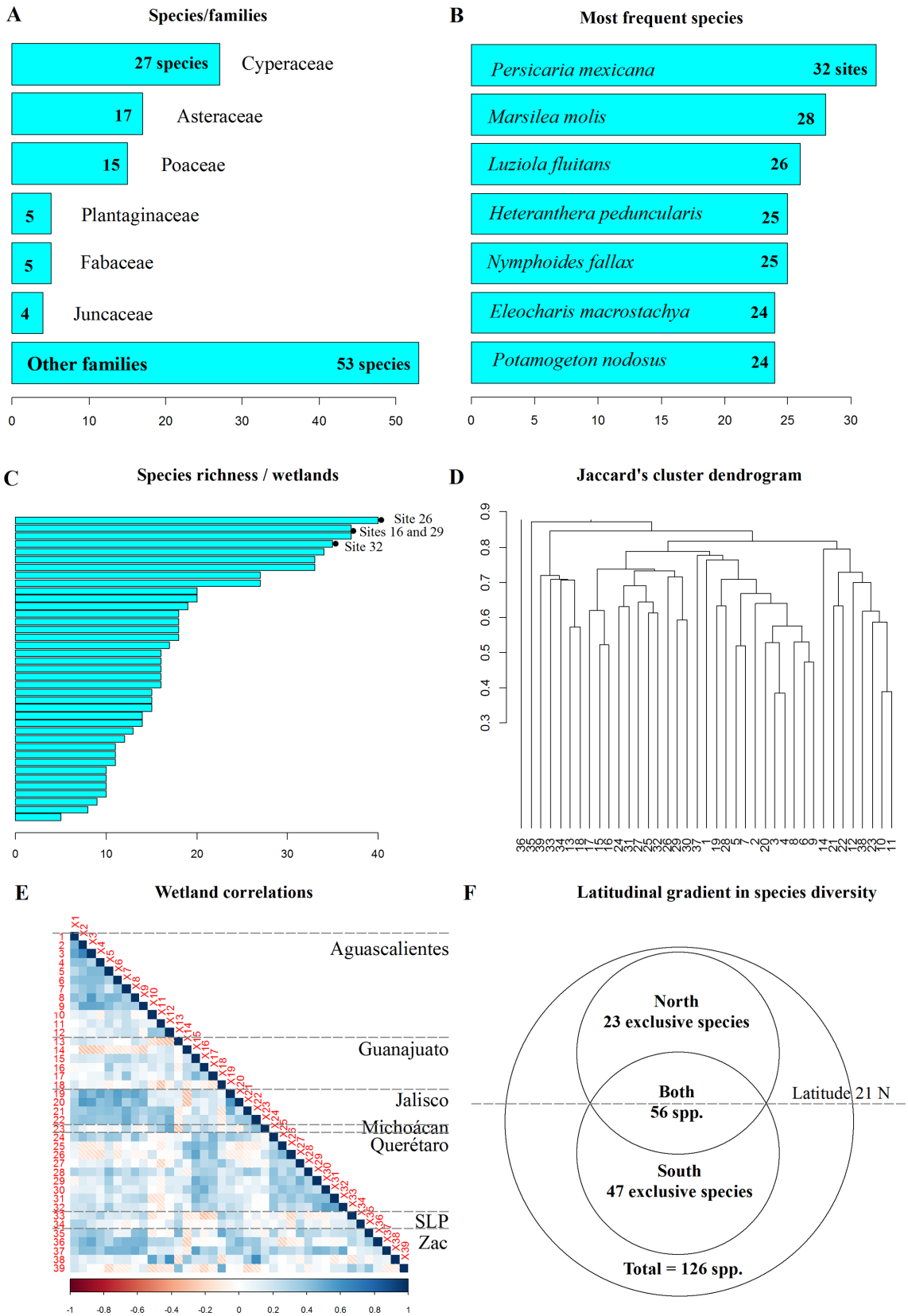

Figure 3. A) Specie richest families; **B)** More frequent species; **C)** Number of species per locality; **D)** Dendrogram cluster by Jaccard; **E)** Floristic correlation between localities; **F)** Specie distribution in higher and lower latitudes from 21° N.

cana had the widest distribution (32 localities, Figure 3-B, Figure 4-B), followed by *Marsilea mollis* (28 localities), *Luziola fluitans* (26 localities, Figure 4-C), *Heteranthera peduncularis* (25 localities, Figure 4-D), *Nymphoides fallax* (25 localities, Figure 4-E), *Eleocharis macrostachya* (24 localities), and *Paspalum distichum* (23 localities). Thirty-six species (28 %) were found in only one locality. Life forms were emergent (93 species), submersed (14 species), rooted floating (13 species), free floating (five species) and free submersed (one species). We found 49 strictly aquatic, 21 subaquatic, 38 tolerant, and 18 with no previous record as aquatic plant. All plants were herbaceous.

The richest wetland had 40 species (site 26 in Querétaro, Figure 3-C), followed by 37 (sites 16 in Guanajuato and 29 in Querétaro). However, one of the wetlands had only five species (site 36 in Zacatecas). The surveyed wetlands had a level of similarity among themselves (Figure 3-D). Wetlands with the highest correlations among them were those from Querétaro (sites 24, 25, 26, 27, 29, 30, 31, and 32, Figure 3-E), followed by those of Aguascalientes (sites 1, 2, 3, 4, 5, 6, 7, 8 and 9, Figure 3-E). Aguascalientes localities also showed a high correlation with Jalisco's Highlands (sites 19, 20, 21, and 22, Figure 3-E) and southern Zacatecas (site 36 and 37, Figure 3-E). In addition, Querétaro localities showed a correlation with Guanajuato (Figure 3-E). We observed the existence of a latitudinal gradient and found 47 species growing only at the lowest latitude wetlands (Figure 3-F). We only sampled 15 of the 39 wetlands at latitudes lower than 21°, but we found 103 of the 126 species (82 %) on these wetlands, 47 of which were exclusive. Wetlands higher than 21° latitude presented 79 species (63 %), and only 23 were exclusive. Forty-eight species are endemic to the MegaMéxico region: *Callitriche heterophylla*, *Eleocharis densa*, *E. ignota*, *E. reznicekii*, *E. tenarum*, *E. yecorensis*, *Eragrostis plumbea*, *Eriocaulon bilobatum*, *Galium proliferum*, *Glandularia teucriifolia*, *Helenium mexicanum*, *Heteranthera peduncularis*, *Heterosperma pinnatum*, *Isoetes mexicana*, *Jaegeria glabra*, *J. purpurascens*, *Karinia mexicana*, *Lobelia fenestralis*, *L. irasuensis* var. *fucata*, *Luziola fluitans*, *Marsilea mollis*, *Nierembergia angustifolia*, *Nymphaea gracilis*, *Nymphoides fallax*, *Plantago linearis*, *Polygala alba*, *P. subalata*, *Potamogeton diversifolius*, *Rorippa mexicana*, *Sagittaria demersa*, *Schkuhria schkuhrioides*, *Sisyrinchium convolutum*, *Sporobolus atrovirens*, *Stevia eupatoria*, *Tagetes lucida*, *T. pringlei*, *Trifolium wormskioldii*, *Tripogandra purpurascens*, *Utricularia perversa*, and *Verbena carolina*. Nineteen species are cosmopolitan. Two species are introduced to Mexico: *Egeria densa*, and *Glyceria fluitans*.

We found 19 species that can occur in low and high elevations: *Diplachne fusca*, *Echinochloa crus-galli*, *Egeria densa*, *E. minima*, *E. montana*, *E. parishii*, *E. schaffneri*, *E. yecorensis*, *Heteranthera limosa*, *H. peduncularis*, *Juncus arcticus*, *Lemna minuta*, *Ludwigia octovalvis*, *L. peploides*, *Najas guadalupensis*, *Paspalum distichum*, *Potamogeton nodosus*, and *Schoenoplectus californicus*, and *Triglochin scilloides*; 24 species occur only above > 1,000 m a.s.l.: *Callitriche heterophylla*, *Echinochloa crus-pavonis*, *E. oplismenoides*, *Eleocharis aciculares*, *E. densa*, *E. ignota*, *E. macrostachya*, *Eriocaulon bilobatum*, *Glyceria fluitans*, *Isoetes Mexicana*, *Jaegeria glabra*, *Juncus dichotomus*, *J. ebracteatus*, *J. microcephalus*, *Karinia mexicana*, *Lemna gibba*, *L. obscura*, *Limosella aquatica*. *Luziola fluitans*, *Nymphoides fallax*, *Potamogeton diversifolius*, *Sagittaria demersa*, *Sisyrinchium convolutum*, and *Tripogandra purpurascens*. We did not find information on altitudinal distribution for the rest of the species collected in this study.

Twenty seven species were listed as threatened, 25 of which are on the international list (IUCN 2017) in the "least concern" category: *Azolla microphylla*, *Callitriche heterophylla*, *Cyperus esculentus*, *Diplachne fusca*, *Distichlis spicata*, *Echinochloa crus-galli*, *Elatine brachysperma*, *Eleocharis aciculares*, *E. atropurpurea*, *E. densa*, *E. macrostachya*, *Glyceria fluitans*, *Hippuris vulgaris*, *Lemna gibba*, *L. minuta*, *Limosella aquatica*, *Ludwigia octovalvis*, *Najas guadalupensis*, *Paspalum distichum*, *Poa annua*, *Polygonum punctatum*, *Potamogeton nodosus* (Figure 4-F), *Setaria parviflora*, *Trifolium amabile*, and *Triglochin scilloides*. Two species, *Nymphaea gracilis* (Figure 4-G), and *Trifolium wormskioldii*, are listed by SEMARNAT (2010) as threatened.

As to the actual or potential uses of the species, 21 have economic importance, 12 as medicinal, and eight as biofilter/ biofuel, and one with both purposes (*Azolla microphylla*, Appendix 2, Figure 4-H). Medicinal: *Azolla* spp. have antibacterial activity (Abraham *et al*. 2015), also *Cosmos bipinnatus* has the same medicinal property (Olajuyigbe & Ashafa 2014, Sohn *et al*. 2013). The oil of *Baccharis salicifolia* is a natural repellent (García *et al*. 2005). *Bidens aurea*

Figure 4. A) *Eleocharis densa* site 27, richest genus. **B)** *Persicaria mexicana* site 13, most frequent specie. **C)** *Luziola fluitans* site 16, recurrent specie. **D)** *Heteranthera peduncularis* site 8, recurrent specie. **E)** *Nymphoides fallax* site 38, recurrent specie. **F)** *Potamogeton nodosus* site 2, endangered. **G)** *Nymphaea gracilis* site 21, endangered. **H)** *Azolla microphylla* site 25, multiple economic use.

acts like omeprazole (De la Lastra *et al*. 1994). *Ludwigia octovalvis* is used against cancer (Chang *et al*. 2004). *Polygonum punctatum* has antibiotic, anti-inflammatory and anti-hyperalgesic properties (Alves *et al*. 2001). *Rumex crispus* has sun protection properties (Demirezer & Uzun 2016). *Schkuhria schkuhrioides* is antimicrobial (Delgado *et al*. 1998). *Stevia eupatoria* has anti-mutagenic and anti-oxidant properties (Cariño-Cortés *et al*. 2007). *Symphyotrichum subulatum* has anti-inflammatory properties (Lee *et al*. 2012). *Tagetes lucida* has medicinal properties as anti-depressive (Bonilla-Jaime *et al*. 2015) and *T. micrantha* has diverse medicinal properties (Linares & Bye 1987). Biofilter/ biofuel: *Azolla filiculoides* and *A. microphylla* are attractive species for the production of renewable biofuels (Miranda *et al*. 2016). *Egeria densa* can remove heavy metals from the environment (Tsuji *et al*. 2017), as can *Eleocharis aciculares*, *E. macrostachya*, and, *E. montana* (Ha *et al*. 2011, Olmos-Márquez *et al*. 2012). *Lemna gibba*, *L. obscura*, *L. minuta* are indicated for phytoremediation of contaminated water (Gallardo-Williams *et al*. 2002, Gür *et al*. 2016, Di-Baccio *et al*. 2017).

Forty four species were recorded as Mexican weeds: *Allium glandulosum*, *Baccharis salicifolia*, *Bahia absinthifolia*, *Bidens aurea*, *Cosmos bipinnatus*, *Cuphea wrightii*, *Cyperus esculentus*, *C. flavescens*, *C. virens*, *Dalea foliolosa*, *Echinochloa crus-galli*, *E. crus-pavonis*, *Egeria densa*, *Eleocharis aciculares*, *E. montana*, *Glandularia teucriifolia*, *Glyceria fluitans*, *Helenium mexicanum*, *Heteranthera limosa*, *Lemna gibba*, *L. minuta*, *L. obscura*, *Ludwigia octovalvis*, *L. peploides*, *Najas guadalupensis*, *Nothoscordum bivalve*, *Paspalum distichum*, *Plantago linearis*, *Poa annua*, *Polygonum punctatum*, *Potamogeton diversifolius*, *P. nodosus*, *Pycreus niger*, *Rorippa mexicana*, *Rumex crispus*, *Schkuhria schkuhrioides*, *Setaria parviflora*, *Sisyrinchium convolutum*, *Sporobolus indicus*, *Tagetes lucida*, *Tagetes micranta*, *Trifolium amabile*, *Tripogandra purpurascens*, and *Verbena carolina*.

Two species probably yet undescribed of the genus *Eleocharis* were found at site 7, Aguascalientes, and site 35, Zacatecas (S. González pers. comm.). New records of 20 species were found for the following Mexican states: Aguascalientes: *Eleocharis parishii*, *E. reznicekii*, *Eriocaulon bilobatum*, *Isoetes mexicana*, *Lemna oscura*, *Potamogeton nodosus*, and *Schoenoplectus californicus*. Guanajuato: *Eleocharis tenarum*, *E. yecorensis*, *Echinocloa oplismenosides*, *Eriocaulon bilobatum*, *Juncus ebracteatus*, and *Lemna minuta*. Michoacán: *Jaegeria purpurascens*. Querétaro: *Azolla microphylla*, *Eleocharis ignota*, and *Lemna oscura*. San Luis Potosí: *Lemna oscura*. Zacatecas: *Elatine brachysperma*, *Eriocaulon bilobatum*, *Heteranthera limosa*, *Isoetes mexicana*, *Juncus arcticus*, *Marsilea mollis*, and *Nierenbergia angustifolia*.

Discussion

In several wetland studies, Asteraceae, Cyperaceae and Poaceae arise as the most important families and emergent species stand out as the most abundant life form in wetlands. (Pott & Pott 2000, Rolon *et al*. 2010, Magalhães *et al*. 2016). Cyperaceae and Poaceae are among the richest aquatic monocotyledons plant families in Mexico (Lot *et al*. 2013). Aquatic Cyperaceae have morphological adaptations that enables them to survive drought spells (Rocha & Martins 2011). As to distributions, aquatics plants frequently are cosmopolitan, but a few only prosper in specific environments and are endemic (Rzedowski 1978). Cyperaceae and Poaceae also have a high endemism among Mexican aquatic plants (Lot *et al*. 2013). *Allium glandulosum*, *Azolla filliculoides*, *Eleocharis ignota*, *Hippuris vulgaris*, *Jaegeria glabra* and *Sagittaria demersa* are some of the 47 species registered only below 21° N. *S. demersa* is endemic to MegaMexico and is considered rare, or even threatened (Lot *et al*. 2002). The Cyperaceae *Eleocharis parashii*, *E. atropurpurea*, *E. coloradoensis*, *E. minima*, *E. reznicekii* and *Schonoplectus californicus* are among the 47 species registered above 21° N latitude. Some of the 56 species are present at both north and south of latitude 21° N were *Callitriche deflexa*, *C. heterophylla*, *Eleocharis aciculares*, *E. densa*, *E. dombeyana*, *E. macrostachya*, *E. montana*, *E. schafenerri*, *E. tenarum*, *E. yecorensis*, *Eriocaulon bilobatum*, *Heteranthera limosa*, *H. peduncularis*, *Najas guadalupensis*, *Nymphoides fallax*, *Triglochin scilloides*, and *Utricularia perversa*. Species with restricted distribution to Mexico or Central America are *Eleocharis reznicekii*, *Eriocaulon bilobatum*, *Sisyrinchium convolutum* (Lot *et al*. 2013), *Utricularia perversa*, and *Nymphoides fallax* (GBIF 2017, Tropicos 2017). Altitudinal distribution presented species which strictly occur at higher

elevations, and others that are able to develop at both low and high elevations. Rzedowski (1978) and Mora-Olivo *et al*. (2013) suggest that there is a pattern of lower species diversity at higher elevation wetlands. However, there are two possible explanations for such a perception: 1) highland wetlands are under-detected and under-collected because many have a temporary water regime, and 2) at higher elevations terrain slopes hinder large water areas and many of the wetlands occupy small areas.

Differences in floristic composition found among sampled wetlands can also be explained by the surrounding vegetation cover and land use, as well as by physical and chemical water characteristics (Declerck *et al*. 2006, Lacoul & Fredman 2006, Ot'ahel'ová *et al*. 2007, Dar *et al*. 2014, Lu *et al*. 2014). Geographically isolated temporary wetlands can contribute to the landscape functions (Cohen *et al*. 2016). Localities 1 (Aguascalientes), 29 (Querétaro), 35 and 39 (Zacatecas) had higher conductivity, dissolved solids and salinity than the rest. Localities 1 and 29 are very close to a town, especially site 29 (Figure 2-G) which is delimited by houses. However, site 29 is among the richest localities, with 37 species. Wetlands 35 and 39 had higher salinity, and had a lower number of species (15 and 10, respectively). Both localities also had pH values above eight. Besides water contamination and nutrient deposition in the water, temporary wetlands are vulnerable to landscape conversion, drainage and obliteration. Site 26 (Querétaro) presented the highest number of species in 2015, however in 2016, it was covered with soil and asphalt, and was completely surrounded by houses (Figure 2-H). We found some aquatic species in 2016, as *Triglochin scilloides* and *Jaegeria purpurascens*, even when the site was already dry. Vegetation restoration of a 100 m belt surrounding a temporary wetland can significantly improve water quality (Bird & Day 2014). Submersed species as *Najas guadalupensis* and *Chara* spp. are important in maintaining ecological processes in wetlands exposed to high level of nutrients (Dierberg *et al*. 2002). Aquatic plants are also economically important as biofilters to remove excess of nutrients (Kostel 2016), as well as to control eutrophication (Fisher & Acreman 2004). *Lemna* spp. acts as a filter and inhibits submersed plants growth by blocking the light (Arroyave 2004, Rai 2008, Cuasquer *et al*. 2016).

A large proportion of the plants listed as weeds (Villaseñor & Espiosa-Garcia 1998), are also aquatic, either strict or subaquatic (Lot *et al*. 2013, Mora-Olivo *et al*. 2013, Lot *et al*. 2015), for example: *Baccharis salicifolia*, *Cyperus esculentus*, *C. flavescens*, *C. virens*, *Echinochloa crus-galli*, *E. crus-pavonis*, *Eleocharis aciculares*, *E. montana*, *Heteranthera limosa*, *Ludwigia octovalvis*, *L. peploides*, *Najas guadalupensis*, *Paspalum distichum*, *Plantago linearis*, *Poa annua*, *Polygonum punctatum*, *Potamogeton diversifolius*, *P. nodosus*, *Pycreus niger*, *Rorippa mexicana*, *Rumex crispus*, *Schkuhria schkuhrioides*, *Sisyrinchium convolutum*, *Sporobolus indicus*, and *Tripogandra purpurascens*. We could not consider them so, since they are in their typical habitat, the temporary wetlands. The concept of weed depends of the moment, place, and conditions where the plant is developing (Lorenzi 1991). In addition, 15 of the 44 species cited as weed are also cited as economically important, such as potentially medicinal (nine species): *Baccharis salicifolia*, *Bidens aurea*, *Cosmos bipinnatus*, *Ludwigia octovalvis*, *Polygonum punctatum*, *Rumex crispus*, *Schkuhria schkuhrioides*, *Tagetes lucida*, and *Tagetes micrantha*, or as biofilters (six species): *Egeria densa*, *Eleocharis aciculares*, *E. montana*, *Lemna gibba*, *L. minuta*, and *L. obscura*. On the other hand, we did not find previous record as aquatic plant for 17 species of Asteraceae (*Acmella repens*, *Aster moranensis*, *Bahia absinthifolia*, *Bidens aurea*, *Cosmos bipinnatus*, *Gnaphalium luteo-album*, *Heterosperma pinnatum*, *Tagetes lucida*, and *T. micrantha*), Fabaceae (*Dalea foliolosa*, *Macroptilium* sp., *Mimosa aculeaticarpa*, *Trifolium amabile*, and *T. wormskioldii*), Rubiaceae (*Galium* cf. *proliferum*), and Verbenaceae (*Glandularia teucriifolia*, *Verbena carolina*). We could consider the above species as weeds for the temporary wetlands where recorded.

In spite of recent compilation studies for Mexican aquatic plants (such as Lot *et al*. 2013, Lot *et al*. 2015), several states (especially Aguascalientes and Zacatecas) need a higher collecting effort. We found seven new records and probably one undescribed species for each state.

Given that temporary wetlands present a high anthropic degradation, but still have a high biodiversity (Pollock *et al*. 1998, Balian *et al*. 2008, Murray-Hudson *et al*. 2012), with economically species (Pott & Pott 2000, Magalhães *et al*. 2016), they should be a conservation priority. Hence, studies of landscape influence on species occurrence are a new challenge to create

strategies for conservation of temporary freshwater wetlands. Knowledge and awareness of the distribution, biodiversity, and economic potential of botanical species in temporary wetlands is the first step to establish conservation policies (Calhoun *et al*. 2016), as they are a very peculiar and highly threatened environment of central Mexico.

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Appendix 1. Temporary wetlands localization of the studied areas and water parameters. * Average of four measurements with Hanna equipment model HI 9829 ** the wetland was dry.

Site	Municipality	State	Latitude	Longitude	Altitude	pH*	ppmDO*	µSTm*	mΩ*	ppmT*	PSU*
1	Asientos	Aguascalientes	22.1769	-102.0252	2,009	6.7	5.90	681.5	0.0015	343.75	0.3375
2	Asientos	Aguascalientes	22.1847	-102.0214	2,004	6.4	7.17	112.5	0.0089	57.50	0.0525
3	Asientos	Aguascalientes	22.1986	-102.064	2,058	6.1	5.75	86.3	0.0143	44.00	0.0400
4	Asientos	Aguascalientes	22.1974	-102.0725	2,078	6.0	5.62	65.0	0.0152	34.00	0.0300
5	Calvillo	Aguascalientes	21.8885	-102.8437	2,342	6.0	4.54	55.0	0.0165	31.00	0.0300
6	Calvillo	Aguascalientes	21.8915	-102.8448	2,345	5.0	4.69	24.5	0.0502	15.00	0.0150
7	Calvillo	Aguascalientes	21.884	-102.8464	2,382	6.0	2.84	73.5	0.0131	39.00	0.0375
8	San José de Gracia	Aguascalientes	22.1681	-102.4166	2,031	5.3	3.71	200.5	0.0050	103.25	0.0975
9	San José de Gracia	Aguascalientes	22.1588	-102.4848	2,047	5.4	5.02	24.0	0.0380	13.75	0.0125
10	San José de Gracia	Aguascalientes	22.1688	-102.5834	2,580	4.8	4.75	6.0	0.0161	3.75	0.0050
11	San José de Gracia	Aguascalientes	22.1905	-102.6102	2,613	4.8	4.31	6.5	0.1260	7.00	0.0050
12	San José de Gracia	Aguascalientes	22.1627	-102.6511	2,646	4.9	6.15	2.0	0.1250	1.00	0.0000
13	Jerécuaro	Guanajuato	20.2542	-100.5477	2,123	5.0	2.67	141.5	0.0065	80.00	0.0800
14	San Felipe	Guanajuato	21.3232	-101.6058	2,669	5.0	4.37	78.5	0.0147	41.00	0.0400
15	San Miguel de Allende	Guanajuato	20.8064	-101.0356	2,303	6.0	2.80	48.8	0.0207	24.50	0.0200
16	San Miguel de Allende	Guanajuato	20.8089	-101.0339	2,307	6.3	7.84	30.5	0.0332	15.25	0.0100
17	San Miguel de Allende	Guanajuato	20.8033	-101.0308	2,276	6.8	5.91	36.0	0.0278	18.00	0.0200
18	Santa Cruz de Juventino Rosas	Guanajuato	20.7492	-101.0244	2,189	6.8	5.88	73.5	0.0136	36.75	0.0300
19	Lagos de Moreno	Jalisco	21.3813	-102.1381	1,992	5.0	5.50	120.8	0.0080	62.25	0.0600
20	Lagos de Moreno	Jalisco	21.334	-102.0883	1,964	5.0	3.39	171.8	0.0060	89.00	0.0850
21	Lagos de Moreno	Jalisco	21.3307	-102.0851	1,975	5.0	3.70	42.8	0.0210	25.75	0.0225
22	Lagos de Moreno	Jalisco	21.3756	-102.1353	2,021	5.0	6.56	8.3	0.1450	5.75	0.0075
23	Epitacio Huerta	Michoacán	20.1405	-100.2947	2,508	5.0	5.58	146.8	0.0069	74.75	0.0725
24	Amealco de Bonfil	Querétato	20.2903	-100.185	2,314	6.7	1.87	85.8	0.0118	43.00	0.0375
25	Amealco de Bonfil	Querétato	20.2122	-100.1186	2,577	6.4	1.85	152.0	0.0066	77.75	0.0725
26	Amealco de Bonfil	Querétato	20.1717	-100.0733	2,614	**	**	**	**	**	**
27	Amealco de Bonfil	Querétato	20.3181	-100.1442	2,245	7.2	4.83	181.8	0.0070	91.75	0.0875
28	Amealco de Bonfil	Querétaro	20.312	-100.2063	2,359	5.0	1.82	111.3	0.0091	60.00	0.0575
29	Huimilpan	Querétato	20.3922	-100.2692	2,318	7.2	2.89	501.8	0.0020	249.75	0.2425
30	Pedro Escobedo	Querétato	20.3994	-100.2686	2,324	7.3	4.83	127.0	0.0079	63.50	0.0600
31	Pedro Escobedo	Querétato	20.4164	-100.2661	2,266	6.8	3.47	40.8	0.0243	20.50	0.0200
32	San Juan del Río	Querétato	20.3283	-100.1328	2,222	7.0	5.71	82.5	0.0135	42.00	0.0375
33	Mexquitic de Carmona	San Luis Potosí	22.2293	-101.2941	2,045	5.0	5.62	39.5	0.0279	22.00	0.0200
34	Villa de Arriaga	San Luis Potosí	21.9556	-101.1876	2,147	5.0	6.56	8.3	0.0700	5.75	0.0075
35	Cañitas de Felipe Pescador	Zacatecas	23.5742	-102.7525	2,032	8.0	4.75	513.0	0.0029	181.50	0.1800
36	Pinos	Zacatecas	22.4481	-101.4958	2,141	9.4	6.88	190.3	0.0053	95.25	0.0900
37	Teúl de González Ortega	Zacatecas	21.4524	-103.4746	1,961	5.0	6.47	6.5	0.1172	4.50	0.0250
38	Tlaltenango de Sánchez Román	Zacatecas	21.7018	-103.1778	2,563	5.0	5.41	121.0	0.0082	68.50	0.0750
39	Villa de Cos	Zacatecas	23.1225	-102.4367	2,008	8.2	7.51	402.0	0.0026	199.50	0.1850

Appendix 2. Species presence/absence in the studied temporary wetlands (X = presence, * = new state record, ** = probably an undescribed specie, *** = introduced specie). V = voucher (T. Lobato collector number, deposited at QMEX); LF = Life form (E = rooted emergent, RS = rooted submersed, RF = rooted floating, FF = free floating, FS = free submersed); Obs = observations: Affinity (A = aquatic, S = subaquatic, T = tolerant), Use (B = biofilter/biofuel, M = medicinal, F = fodder), Endangered (E = endangered in a IUCN list or Mexican list).

Taxon	V	LF	Obs	Wetlands/states																																						
				Aguascalientes												Guanajuato						Jalisco				Mich.	Querétaro									SLP		Zacatecas				
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
CHAROPHYTA																																										
CHARACEAE																																										
Chara cf. *globularis* Thuiller	821	RS	A																																			X				
Chara sp. L.	963	RS	A			X																X																				
Nitella gracilis (J.E. Smith) C.Agardh	855	RS	A							X					X																											
PTERIDOPHYTA																																										
ISOETACEAE																																										
* *Isoetes mexicana* Underw.	1061	RS	A	X*																												X	X					X*				
MARSILEACEAE																																										
* *Marsilea mollis* B. L. Rob. & Fernald	1608	RF	A	X	X		X			X	X		X	X		X	X	X	X		X		X			X	X	X	X	X		X	X	X	X	X	X	X*	X*		X*	X*
SALVINACEAE																																										
Azolla filiculoides Lam.	1459	FF	A, B														X													X	X	X										
* *Azolla microphylla* Kaulf.	1355	FF	A, M/B, E														X											X*							X*							
ANGIOSPERM																																										
ALISMATACEAE																																										
Sagittaria demersa J.G. Sm.	1463	RF	A															X	X												X	X			X							
AMARANTHACEAE																																										
Iresine sp. P. Browne	774	E	T																										X													
AMARYLLIDACEAE																																										
Allium glandulosum Link & Otto	599	E	T																									X	X													
Allium sp. L.	550	E	T															X																								
Nothoscordum bivalve (L.) Britton	1583	E	T														X		X	X	X								X			X			X							
ARACEAE																																										
Lemna gibba L.	812	FF	A, B, E	X																												X										
* *Lemna minuta* Kunth	653	FF	A, B, E													X*																										
* *Lemna obscura* (Austin) Daubs	1460	FF	A, B			X*																										X*				X*						
ASTERACEAE																																										
Acmella repens (Walter) Rich.	1571	E	-																									X	X	X					X							
Aster moranensis Kunth	1305	E	-																														X									X
Baccharis salicifolia (Ruiz & Pav.) Pers.	639	E	S, M																													X										
Bahia absinthifolia Benth.	1368	E	-															X																								
Bidens aurea (Aiton) Sherff	799	E	- , M																								X	X				X	X									
Cosmos bipinnatus Cav.	1036	E	- , M																X								X		X													
Gnaphalium luteo-album L.	1567	E	-								X				X		X												X	X												
Helenium mexicanum Kunth	687	E	S																														X									

Appendix 2. Continuation.

Taxon	V	LF	Obs	Wetlands/states: Aguascalientes 1	2	3	4	5	6	7	8	9	10	11	12	Guanajuato 13	14	15	16	17	18	Jalisco 19	20	21	22	Mich. 23	Querétaro 24	25	26	27	28	29	30	31	32	SLP 33	34	Zacatecas 35	36	37	38	39
Heterosperma pinnatum Cav.	1340	E	-																																							
Jaegeria glabra (S. Watson) B.L. Rob.	1456	RF	A																													X	X									
* *Jaegeria purpurascens* B.L. Rob	1614	RF	A														X	X	X							X*			X	X	X				X						
Schkuhria schkuhrioides Thell.	1367	E	S, M															X	X																							
Stevia eupatoria (Spreng.) Willd.	753	E	T, M																									X								X						
Symphyotrichum subulatum (Michx.) G.L. Nesom	1566	E	T, M		X												X			X		X						X	X		X	X	X	X	X		X		X			
Tagetes lucida Cav.	1037	E	- , M																X																							
Tagetes micrantha Cav.	1477	E	- , M				X				X		X					X	X	X									X					X	X	X						
Tagetes pringlei S. Watson	1457	E	A															X	X								X	X	X			X			X						
BRASSICACEAE																																										
Rorippa mexicana (DC.) Standl. & Steyerm.	1584	E	A																										X				X				X				
CAMPANULACEAE																																										
Lobelia fenestralis Cav.	1025	E	T																														X									
Lobelia irasuensis var. *fucata* McVaugh	1493	E	T															X	X	X													X								
CARYOPHYLLACEAE																																										
Stellaria ovata Willd. ex D.F.K. Schltdl.	1572	RS	A																										X							X					
COMMELINACEAE																																										
Commelina dianthifolia Delile	1095	E	T																									X	X	X			X	X	X						
Tripogandra purpurascens (S. Schauer) Handlos	1496	E	S								X		X					X	X	X								X				X	X	X						
CYPERACEAE																																										
Cyperus aff. *entrerianus* Boeck.	1526	E	T																X				X																		
Cyperus esculentus L.	1315	E	T, E																									X	X	X											X
Cyperus flavescens L. var. *piceus* (Liebm.) Fernald	1482	E	T																	X									X												
Cyperus luzulae (L.) Rottb. ex Retz.	1007	E	T																X				X	X															X		
Cyperus manimae Kunth	662	E	T																	X																					
Cyperus reflexus Vahl	1617	E	T																							X						X									
Cyperus sanguineo-ater Boeck.	1364	E	T																X									X												
Cyperus virens Michx.	1556	E	T																																				X		
Eleocharis aciculares (L.) Roem. & Schult.	1609	RS	A, B, E							X			X	X	X		X	X	X	X					X	X					X	X	X		X			X			
Eleocharis atropurpurea (Retz.) J. Presl & C. Presl	1535	E	T, E																				X	X	X																
Eleocharis coloradoensis (Britton) Gilly	828	E	T																																			X				
Eleocharis densa Benth.	1619	E	A, E					X								X		X		X	X					X	X	X		X			X	X	X						

Appendix 2. Continuation.

Taxon	V	LF	Obs	Wetlands/states: Aguascalientes 1 2 3 4 5 6 7 8 9 10 11 12	Guanajuato 13 14 15 16 17 18	Jalisco 19 20 21 22	Mich. 23	Querétaro 24 25 26 27 28 29 30 31 32	SLP 33 34	Zacatecas 35 36 37 38 39
Eleocharis dombeyana (Kunth) Roem. & Schult.	973	E	T	X X X	X X X					X
* *Eleocharis ignota* S. González & M. Reznicek	1105	E	A		X			X* X*		
Eleocharis macrostachya Britton	1603	E	S, B, E	X X X X X X X X X X X	X X	X X X	X	X X X X	X X	X X
Eleocharis minima Kunth	891	E	A	X X						
Eleocharis montana (Kunth) Roem. & Schult.	1555	E	S, B	X	X X X			X X X		X
* *Eleocharis* aff. *parishii* Britton	1109	E	S	X*						
* *Eleocharis reznicekii* S. González, D.J. Rosen, R. Carter & P.M. Peterson.	1437	E	A	X*						
Eleocharis schaffneri Boeck.	1598	E	S	X X X X X X X	X	X X		X X X X X X	X	X
* *Eleocharis tenarum* S. González & M. González E.	1481	E	T	X	X* X*			X X X X X		X
* *Eleocharis yecorensis* Roalson	1589	E	A		X*	X X				
** *Eleocharis* new specie 1	1313	E	S	X						
** *Eleocharis* new specie 2	1440	E	S							X
Karinia mexicana (C.B. Clarke ex Britton) Reznicek & McVaugh	1575	E	T	X X	X X X			X X		
Pycreus niger (Ruiz & Pav.) Cufod.	1127	E	T	X				X		
* *Schoenoplectus californicus* (C.A. Mey.) Soják	1134	E	S	X* X*						
ELATINACEAE										
* *Elatine brachysperma* A. Gray	1618	RS	A, E	X X X	X	X	X	X		X*
Elatine sp. L.	669	RS	A					X		
ERIOCAULACEAE										
* *Eriocaulon bilobatum* Morong	1550	E	A	X* X*	X*	X				X* X*
FABACEAE										
Dalea foliolosa (Aiton) Barneby	1379	E	-	X	X X			X X		
Macroptilium sp. (Benth.) Urb.	716	E	-					X X		
Mimosa aculeaticarpa Ortega	634	E	-					X		
Trifolium amabile Kunth	742	E	- , E					X		
Trifolium wormskioldii Lehm.	714	E	- , E					X		
HYDROCHARITACEAE										
*** *Egeria densa* Planch.	1115	RS	A, B	X						
Najas guadalupensis (Spreng.) Magnus	1611	RS	A, E	X X X X X X	X X X	X X	X	X X X X	X	X X
HYPERICACEAE										
Hypericum sp. L.	787	E	T					X X X		

Appendix 2. Continuation.

Taxon	V	LF	Obs	Wetlands/states: Aguascalientes 1	2	3	4	5	6	7	8	9	10	11	12	Guanajuato 13	14	15	16	17	18	Jalisco 19	20	21	22	Mich. 23	Querétaro 24	25	26	27	28	29	30	31	32	SLP 33	34	Zacatecas 35	36	37	38	39
IRIDACEAE																																										
Sisyrinchium convolutum Nocca	1243	E	S																																X							
JUNCACEAE																																										
* *Juncus arcticus* Willd.	1580	E	A																										X								X*					
Juncus dichotomus Elliott	1568	E	A	X				X		X			X		X		X	X	X	X							X		X			X	X								
* *Juncus ebracteatus* E. Mey.	1210	E	S	X						X			X	X	X		X*													X												
Juncus microcephalus Kunth	1613	E	A						X				X	X	X				X	X				X	X	X		X	X	X									X	X		
JUNCAGINACEAE																																										
Triglochin scilloides (Poir.) Mering & Kadereit	1607	E	A, E	X									X	X	X		X		X	X						X		X	X		X	X	X						X		
LAMIACEAE																																										
Salvia sp. L.	783	E	T																										X												
LENTIBULARICACEAE																																										
Utricularia perversa P. Taylor	1564	FS	A														X	X	X															X							
LYTHRACEAE																																										
Cuphea wrightii A. Grey	1301	E	T																																							X
MENYANTHACEAE																																										
Nymphoides fallax Ornduff	1586	RF	A		X	X	X	X	X	X	X	X	X	X				X	X	X		X	X	X	X		X	X		X	X	X	X	X	X				X		
NYMPHAEACEAE																																										
Nymphaea gracilis Zucc.	1510	RF	A, E																					X																		
ONAGRACEAE																																										
Ludwigia octovalvis (Jacq.) P.H. Raven	1551	E	S, M, E								X	X							X	X				X			X			X				X					X		
Ludwigia peploides (Kunth) P.H. Raven	1596	RF	A	X								X				X						X	X				X	X		X	X	X	X	X	X	X			X		
PLANTAGINACEAE																																										
Bacopa procumbens (Mill.) Greenm.	1585	E	T																X										X				X								
Callitriche deflexa A. Braun ex Hegelm.	1606	RF	A							X																X		X		X											
Callitriche heterophylla Pursh	1605	RF	A, E										X	X	X		X									X														X	
Hippuris vulgaris L.	1354	RS	A, E																								X							X							
Plantago linearis Kunth	1242	E	S																																X						
POACEAE																																				X						
Bouteloua dactyloides (Nutt.) Columbus	1226	E	S																																						
Diplachne fusca L.) P. Beauv. ex Roem. & Schult.	1592	E	S, E		X			X	X	X	X	X											X	X						X				X			X				
Distichlis spicata (L.) Green	1569	E	T, E														X																					X			
Echinochloa crus-galli (L.) P. Beauv.	926	E	T, F, E					X																																	X
Echinochloa crus-pavonis (Kunth) Schult	1474	E	S	X	X						X	X				X				X	X									X				X		X	X				X

Appendix 2. Continuation.

Taxon	V	LF	Obs	Wetlands/states: Aguascalientes 1	2	3	4	5	6	7	8	9	10	11	12	Guanajuato 13	14	15	16	17	18	Jalisco 19	20	21	22	Mich. 23	Querétaro 24	25	26	27	28	29	30	31	32	SLP 33	34	Zacatecas 35	36	37	38	39
* *Echinochloa oplismenoides* (E. Fourn.) Hitchc.	1602	E	S, F						X			X				X*										X	X	X			X		X	X							
Eragrostis plumbea Scribn. ex Beal	1488	E	T, F															X	X	X																				
*** *Glyceria fluitans* (L.) R. Br.	1601	E	A, E																							X		X													
Luziola fluitans (Michx.) Terrell & H. Rob.	1615	RS	A					X	X	X	X		X	X			X	X	X	X		X		X	X	X	X	X	X	X	X	X	X	X		X			X	X
Panicum aff. *lacustre* Hitchc. & Ekman	1387	E	S												X	X		X											X											
Paspalum distichum L.	1506	E	A, E	X	X		X	X	X	X	X	X			X			X				X	X				X	X	X	X		X	X	X	X			X	X		
Paspalum plicatulum Michx.	1031	E	T																X																					
Poa annua L.	561	E	T, E																											X										
Setaria parviflora (Poir.) Kerguélen	555	E	T, E															X																						
Sporobolus atrovirens (Kunth) Kunth	1498	E	T		X																								X										
Sporobolus indicus (L.) R.Br.	757	E	T																										X			X		X					
POLYGALACEAE																																								
Polygala alba Nutt.	970	E	T																																			X		
Polygala subalata S. Watson	1519	E	T															X	X					X														X		
POLYGONACEAE																																								
Persicaria mexicana (Small) Small	1616	E	S		X	X	X	X	X	X	X	X	X			X	X	X	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X	X		X	
Polygonum punctatum Elliott	1347	E	A, M, E																									X		X	X			X						X
Rumex crispus L.	1570	E	T, M													X	X			X	X							X	X	X		X	X	X			X			X
PONTEDERIACEAE																																								
* *Heteranthera limosa* (Sw.) Willd.	1574	RF	A	X	X			X	X	X	X	X		X						X	X	X		X				X		X	X	X		X	X	X			X*	
Heteranthera peduncularis Benth.	1588	RF	A	X	X	X	X	X	X	X	X	X		X		X		X	X	X	X	X	X				X			X	X	X	X	X	X			X		
POTAMOGETONACEAE																																								
Potamogeton diversifolius Raf.	1542	RF	A		X			X	X	X				X	X				X					X					X										X	
* *Potamogeton nodosus* Poir.	1612	RF	A, E	X*	X*	X*	X*	X*	X*		X*	X*	X*	X*	X*			X		X		X	X	X	X							X			X			X			
RUBIACEAE																																								
Galium cf. *proliferum* A. Gray	1068	E	-															X									X						X							
SCROPHULARIACEAE																																								
Limosella aquatica L.	1604	RS	A, E																							X			X			X								
Limosella australis R. Br.	628	RS	A																													X								
SOLANACEAE																																								
* *Nierembergia angustifolia* Kunth	1312	E	A																																	X*				
VERBENACEAE																																								
Glandularia teucriifolia (M. Martens & Galeotti) Umber	788	E	-																										X											
Verbena carolina L.	789	E	-																									X	X	X		X	X							
Verbena sp. L.	824	E	-																																	X				

CAPÍTULO 3 - Microsatellite loci transferability and genetic diversity of the aquatic plant *Nymphoides fallax* Ornduff (Menyanthaceae), endemic of Mexican and Guatemalan highlands

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***Nymphoides fallax* Ornduff, humedal temporal, Aguascalientes, México. Fuente: propio autor**

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RESEARCH PAPER

Microsatellite loci transferability and genetic diversity of the aquatic plant *Nymphoides fallax* Ornduff (Menyanthaceae), endemic to the Mexican and Guatemalan highlands

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Abstract
Nymphoides species are cosmopolitan aquatics with floating leaves and frequent in freshwater wetlands. *Nymphoides fallax* is restricted to highlands of Mexico and Guatemala. We tested the transferability of 10 microsatellite loci previously developed for *N. peltata* in 18 populations of *N. fallax*. Seven polymorphic loci were successfully amplified. Allele sizes were automatically obtained using 2100 Bioanalyzer Expert software. Genotypes were determined using the allele dosage method. Chromosome counts confirmed that *N. fallax* is a tetraploid. Alleles per locus ranged from 5 to 17; the observed and expected heterozygosities per population ranged from 0.51 to 0.66 and from 0.72 to 0.81, respectively. We observed higher genetic diversity within populations (92%) than among populations (8%). Our results show that cross-amplification is a valid technique for studying *Nymphoides*. No clones were found, indicating that *N. fallax* relies heavily on sexual reproduction. Our work may stimulate further population genetic studies of the genus *Nymphoides* that could be useful for conservation programs, as well as to promote additional research on landscape genetics and reproductive mechanisms in aquatic plants.

Keywords Lab-on-a-chip capillary electrophoresis · Macrophyte · Population genetics · SSR · Temporary wetlands · polyploidy

Introduction

Nymphoides Séq. (Menyanthaceae) harbors 50 species (Tippery and Les 2011), two of which occur in Mexico. All species of *Nymphoides* are aquatics, with floating leaves, and frequent in freshwater wetlands. *Nymphoides* has high seed production, forming seed banks at the bottom of wetlands and lakes; however, only a few seeds are established. Therefore, sexual reproduction constitutes a small contribution to the expansion of *Nymphoides* in their habitat (Huang et al. 2015). Dispersal of *Nymphoides* is usually by zoochory, more specifically by aquatic birds (Cook 1990). *Nymphoides* has a base chromosome number of $x=9$, with several polyploid species (Ornduff 1970; Tippery et al. 2008). Most species of *Nymphoides* are cosmopolitan; however, *Nymphoides fallax* Ornduff (Fig. 1a) has a restricted distribution and is found only in the Mexican and Guatemalan highlands. *Nymphoides fallax* is a tetraploid species (36 chromosomes) native to temporary wetlands (Fig. 1b), ponds, and streams at elevations higher than 1500 m a.s.l. (Ornduff 1969, 1970). Recent studies show evidence that *N. fallax* is an allopolyploid species that belongs to a neotropical clade, but the ancestral hybridization scenario is not conclusive (Tippery et al. 2018). *Nymphoides fallax* has both sexual and vegetative reproduction, and similar vegetative anatomy to *Nymphoides indica* (L.) Kuntze, the other *Nymphoides* species present in Mexico (Martínez and Gómez-Sánchez 2006). *Nymphoides fallax* differs from *N. indica* by having laciniate yellow petals, yellow anthers, larger seeds, and higher altitudinal occurrence (Ornduff 1969).

Temporary freshwater wetlands span close to 0.81 million km^2 of the earth's surface (Pekel et al. 2016). Temporary wetlands are characterized by ephemeral hydrology, severe

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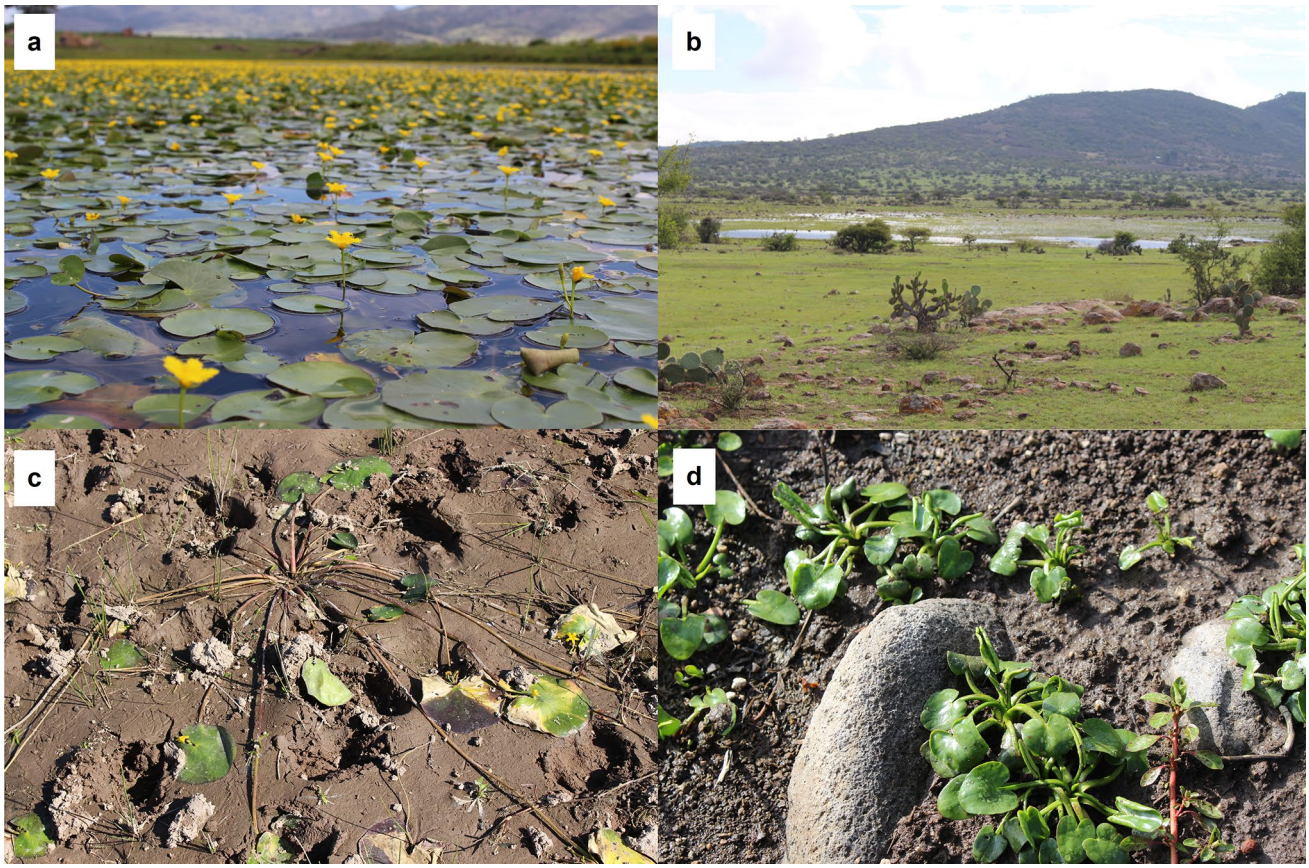

Fig. 1 *Nymphoides fallax* in temporary wetlands of Mexican highlands: **a** flowers, **b** temporary wetland in Querétaro State, Mexico, **c** *Nymphoides fallax* in a temporary wetland in the dry season, **d** *Nymphoides fallax* seedlings

water saturation fluctuations, and dry periods (Fig. 1c; Martínez and García 2001; Calhoun et al. 2017). They are geographically isolated units, without hydrological connection, and completely surrounded by uplands at the local scale (Mushet et al. 2015). Mexican temporary freshwater wetlands harbor 176 vascular aquatic plant species (Mora-Olivo et al. 2013). From a floristic perspective, temporary wetlands of the Mexican highlands are considered heterogeneous and highly diverse ecosystems (Magalhães and Martínez 2018). Alarmingly, estimates over the past decades put wetland losses as high as 62% for Mexico (Landgrave and Moreno-Casasola 2012).

Molecular information about genetic and clonal diversity is essential to understanding ecology, evolution, and species conservation proposals (Uesugi et al. 2005). However, the vast majority of aquatic plants have no specific molecular markers yet. Therefore, scientists should either develop new markers or take advantage of existing ones by focusing on the use of transference as an alternative. Our study aimed (1) to test the transferability of microsatellite markers of *Nymphoides peltata* (S. G. Gmel.) Kuntze (Uesugi et al. 2005) to *N. fallax*, and (2) to analyze the population genetic diversity in temporary wetlands. Microsatellite markers for *N. peltata* have already been successfully transferred to *N. indica* (Shibayama et al. 2006). Molecular markers could be useful in studies of functional connectivity of temporary wetlands in Mexico and Guatemala, having *N. fallax* as a focal species.

Material and methods

Plant material and DNA extraction

We sampled 18 populations (total of 180 individuals) of *Nymphoides fallax* (Fig. 1a) in temporary wetlands of the Mexican highlands (Fig. 1b; Table 1). When present, *N. fallax* typically occurs in relatively high densities in the wetland. In a recent floristic survey carried out by our group in the temporary wetlands of the Mexican highlands, *N. fallax* was abundant, occupying 72% of the entire study area (Magalhães and Martínez 2018). We sampled 10 flowering individuals—at least 8 m apart—evenly distributed across each of the visited wetlands in October and November 2016.

Table 1 Sampled population localization of *Nymphoides fallax* in temporary wetlands of Mexican highlands

Population	State	Latitude	Longitude	Altitude	Area (m^2)	Voucher[a]
1	Aguascalientes	22.1847	−102.0214	2004	12397	888
2	Aguascalientes	22.1986	−102.0640	2058	16108	1149
3	Aguascalientes	21.8840	−102.8464	2382	6226	1147
4	Aguascalientes	22.1681	−102.4166	2031	2362	1160
5	Aguascalientes	22.1688	−102.5834	2580	1200	1194
6	Aguascalientes	22.1905	−102.6102	2613	1075	1177
7	Guanajuato	20.8064	−101.0356	2303	26624	1382
8	Guanajuato	20.8033	−101.0308	2276	9152	1485
9	Jalisco	21.3307	−102.0851	1975	6788	1518
10	Jalisco	21.3813	−102.1381	1992	7761	1502
11	Querétaro	20.3120	−100.2063	2359	6805	1259
12	Querétaro	20.3922	−100.2692	2318	34301	1462
13	Querétaro	20.4164	−100.2661	2266	9424	1282
14	Querétaro	20.3283	−100.1328	2222	23254	1323
15	Querétaro	20.3994	−100.2686	2324	35890	1060
16	Querétaro	20.2903	−100.1850	2314	3294	1395
17	Querétaro	20.2122	−100.1186	2577	1850	1358
18	Zacatecas	21.4524	−103.4746	1961	2713	1549

[a]Collector number of *T. Lobato*, vouchers were deposited at Autonomous University of Querétaro Herbarium "Jerzy Rzedowski" (QMEX)

Leaf tissue samples were immediately dried in silica gel. Total DNA was extracted using a QIAamp® genomic DNA and RNA kit (QIAGEN).

Botanical vouchers were deposited at the Autonomous University of Querétaro Herbarium "Jerzy Rzedowski" (QMEX), with duplicates distributed in Mexico (CIIDIR, IBUG, MEXU, SLPM, and XAL) and Brazil (LUSC); acronyms according to Thiers (2018).

PCR amplification

We tested 10 microsatellites (simple sequence repeats, SSR) loci developed for *Nymphoides peltata* (S.G. Gmel.) Kuntze (Uesugi et al. 2005). The amplification products were analyzed by electrophoresis on 1% agarose gel. The loci were considered successfully amplified when a band close to the expected size was clearly visualized. All polymerase chain reaction (PCR) amplifications were performed in an Esco Healthcare Swift MaxPro Thermal Cycler in a reaction volume of 10 µL containing 5–10 ng of genomic DNA, 0.5 µL of forward primer (10µM), 0.5 µL of reverse primer (10 µM), and 5 µL of master mix (QIAGEN multiplex kit). We amplified the fragment separately for each locus. PCR conditions followed those described in the QIAGEN® Multiplex PCR Kit (QIAGEN) for microsatellites: initial heat activation cycle of 95° C for 15 min; 40 cycles of 94° C for 30 s, annealing temperature for 90 s, and 72° C for 60 s. After the final cycle, one cycle of 60° C for 30 min was added. We did a temperature gradient from 50 to 65° C for each locus, in order to find the best amplification annealing temperature. We included one positive and one negative control for each reaction. DNA concentration on the PCR amplification was measured with a NanoDrop microvolume sample retention system (Thermo Scientific).

Microsatellite genotyping

The fragment size distribution was determined by running 1 µL of each PCR amplification product on an Agilent Bioanalyzer 2100, using a DNA 7500 chip (Agilent Technologies), microcapillary electrophoresis based on lab-on-a-chip technology. All reagents were stored at 4° C, allowed to reach room temperature for 30 min before use, and prepared following the manufacturers' instructions. Fragment sizes and peak areas for all individuals across the microsatellite loci were determined automatically using 2100 Bioanalyzer Expert software (Agilent Technologies). Allelogram software (Morin et al. 2009) was used to correct genotyping errors between the Lab-on-a-chip capillary electrophoresis runs. Errors in genotypes result from several different sources such as allelic stutter, short allele dominance, and null alleles, affecting the correct alleles identification. In polyploids, genotypes can be homozygotes (e.g., AAAA, BBBB, CCCC), full heterozygotes (e.g., ABCD, ABFG, CDEF), or partial heterozygotes where one or more alleles are present multiple times (Dufresne et al. 2014). We determined the genotypic configurations for all individuals at every locus, considering the following aspects: (1) full

homozygotes and full heterozygotes were a useful reference for determining the allele size range; (2) we defined alleles using histograms, considering allele frequency by zones (allele size in base pairs) and peak patterns (allele dosage in nmol/L); (3) whenever individuals had two or three peaks at a locus (partial heterozygotes), we used the allele dosage method to score the alleles presence (Tani et al. 2005, 2006; Meirmans et al. 2006; Tsuda et al. 2017); (4) dosage effects were evident in most cases. In the few ambiguous cases or where PCR amplification was not enough to reach at least a molarity of 20 nmol/L, the allele was scored as "missing data"; and (5) the method described by Garino et al. (2014) contributed to the electropherogram interpretation.

Data analysis

We calculated genetic diversity parameters for each population and locus, including the number of alleles (NA), effective number of alleles per locus (NAe; Nielsen et al. 2003), allelic richness (A_R), observed (H_O) and expected (H_E) heterozygosity according to the Hardy–Weinberg equilibrium (HWE; Nei 1978), and inbreeding coefficients (F_{IT}, F_{IS}; 20,000 randomizations) using SPAGeDi (Spatial Pattern Analysis of Genetic Diversity) software version 1.5a. The software allows the use of codominant polyploid data (Hardy and Vekemans 2002, 2015). Analysis of molecular variance (AMOVA; Excoffier et al. 1992), multilocus genotypes (MLG), and clone correction (Grünwald et al. 2003) were accessed using Poppr package (Kamvar et al. 2014) in R version 3.4.2 (R Core Team 2017), which allows importing of data for codominant polyploid data. In total, we analyzed 180 individuals from 18 populations.

Chromosomes count

We carried out a chromosome count to identify the number of chromosomes in the sampled *N. fallax* populations and determined the ploidy level. The procedure followed Kirov et al. (2014) and Castro-Castro et al. (2017). Root meristematic cells were obtained from seedlings of five individuals from five populations of *N. fallax*. Ten root tips per individual were collected at 8:00, 10:00, 12:00, and 17:00 h, fixed in 5 mL of 8-hydroxyquinoline at 2 mM for 3 h at room temperature, and preserved in 5 mL of 70% ethanol. The roots were embedded in 3:1 (ethanol: acetic acid) for 15 min at room temperature. We added distilled water and mixed it to clean the roots. Cell suspension was prepared straight away. Next, root tips were stained with aceto-orcein, and plaques were prepared by crushing and heating. Finally, approximately 30 cells were observed for each sample, and photomicrographs of the best-resolution fields were obtained. Slides were examined under an Axio Observer Z.1 microscope (Carl Zeiss Microscopy, Jena, Germany). Selected images were captured using an Axiocam 503 mono (D) microscope camera. Image processing and thresholding were performed using Zen Module 3Dxl software (Carl Zeiss).

Results and discussion

From the ten tested microsatellite markers in this study, seven showed amplification products, were specific, and had strong amplification (Table 2). We found better amplification results in annealing temperatures as follows: *Np152* = 54.4° C, *Np274* = 64.4° C, *Np382* = 53.7° C, *Np641* = 50° C, *Np694* = 50° C, *Np274–2* = 64.4° C, and *Np152–2* = 54.4° (Table 3). The best annealing temperature varied among species. One primer pair amplified two consistent regions with polymorphisms (*Np152* and *Np152–2*). Region pairs had non-overlapping size ranges.

A total of seven nuclear microsatellite loci related to *N. peltata* were polymorphic for *N. fallax*, with five to 17 alleles per locus (74 alleles in total). The observed and expected heterozygosities calculated per locus ranged from 0.295 to 0.822 and 0.574 to 0.927, respectively (Table 3). Microsatellite markers were expected to have sufficient polymorphisms to identify genetic diversity.

Table 2 Microsatellite loci transferred from *Nymphoides peltata* to *Nymphoides fallax*

Locus[a]	Species	Genbank code	Size range (bp)	Repeat motif
Np152	*Nymphoides peltata*	AB185163.1	152–162	$(CA)_{13}$
Np274	*Nymphoides peltata*	AB185165.1	149–173	$(AG)_{22}G(AG)_6$
Np382	*Nymphoides peltata*	AB185168.1	160–174	$(CT)_{13}ATC(CT)_5AT(CT)_4AT(CT)_5$
Np641	*Nymphoides peltata*	AB185170.1	220–234	$(CT)_{15}$
Np694	*Nymphoides peltata*	AB185171.1	147–169	$(TC)_{14}(AC)_{13}$
Np274–2	*Nymphoides peltata*	AB185166.1	135–137	$(AG)_8$
Np152–2	*Nymphoides peltata*	AB185163.1	196–256	$(CA)_{13}$

[a]Uesugi et al. (2005)

Table 3 Genetic diversity per locus of *Nymphoides fallax* in temporary wetlands of Mexican highlands

Locus	A_R	Size range (pb)	H_O	H_E	F_{IT}	F_{IS}	F_{ST}	Annealing temperature
Np152	5	134–166	0.295	0.574	0.3232*	0.2858*	0.0523	54.4° C
Np274	9	130–154	0.822	0.850	0.0433*	0.0481*	−0.0050	64.4° C
Np382	17	133–181	0.727	0.927	0.1785*	0.1604*	0.0215*	53.7° C
Np641	10	197–265	0.315	0.765	0.4066*	0.3751*	0.0503*	50° C
Np694	10	157–211	0.795	0.878	0.0886*	0.0346	0.0560*	50° C
Np274–2	10	196–220	0.761	0.847	0.1013*	0.0575*	0.0465*	64.4° C
Np152–2	13	196–256	0.491	0.757	0.2121*	0.0575*	0.0192	54.4°

A_R allelic richness, H_O observed heterozygosity, H_E expected heterozygosity, F_{IT} total inbreeding, F_{IS} individual inbreeding coefficient, F_{ST} fixation index

*Significant deviation from zero ($P < 0.001$; 20,000 randomization)

The intrapopulation genetic diversity (Table 4) display a population average allele number of 10.57 (ranging from 6.43 to 8.00). Clones were not observed on the sampled individuals. We observed 180 original multilocus genotypes (MLGs), among them one diploid and 179 tetraploid individuals. Lack of clones indicates that sexual reproduction can dominate *N. fallax* populations in temporary wetlands.

We found high and significant inbreeding coefficients at the loci investigated (F_{IS} =0.157; $P < 0.001$; Table 3 and Table 5). Other aquatic plant species have similar findings (Magallán et al. 2009, 2013), including other *Nymphoides* species (Shibayama et al. 2006; Takagawa et al. 2006; Uesugi et al. 2007). However, our inbreeding findings can have alternative explanations: (1) plant populations are small in the temporary wetlands, and this ecosystem is exceptionally dynamic; (2) Wahlund effect occurrence (Hedrick 2011); (3) unknown aspects about the reproductive ecology, such as self-compatibility; (4) presence of undetected null alleles; and (5) we used a small sample size of individuals per population (10 individuals). In a small population, F_{IS} cannot be a reliable indicator of the (partial) disomic inheritance detection (Meirmans and Van Tienderen 2013). Also,

Table 4 Population genetic diversity of *Nymphoides fallax* in temporary wetlands of Mexican highlands

Population	NA	NAe	A_R	H_E	H_O	F_{IS}
1	6.71	5.38	3.01	0.772	0.636	0.128*
2	7.14	5.75	2.94	0.771	0.627	0.166*
3	7.43	5.78	3.02	0.789	0.624	0.141*
4	6.57	4.66	2.80	0.750	0.512	0.252*
5	6.71	5.61	2.95	0.767	0.569	0.218*
6	7.57	5.24	2.99	0.749	0.655	0.107*
7	7.14	5.99	2.88	0.721	0.595	0.145*
8	8.00	6.37	3.07	0.775	0.631	0.146*
9	6.71	4.94	2.92	0.764	0.562	0.219*
10	6.57	4.80	2.86	0.752	0.554	0.214*
11	6.86	5.32	2.86	0.742	0.546	0.223*
12	6.57	5.35	2.95	0.774	0.626	0.128*
13	5.71	5.67	2.91	0.780	0.573	0.228*
14	7.00	4.91	2.83	0.729	0.618	0.115*
15	7.43	6.62	3.08	0.814	0.631	0.222*
16	6.43	5.16	2.83	0.765	0.632	0.190*
17	6.57	5.80	2.92	0.758	0.635	0.149*
18	7.14	5.26	2.88	0.720	0.552	0.180*
All	10.57	6.54	3.08	0.800	0.601	0.184*

NA no. of alleles, *NAe* effective no. of alleles, A_R allelic richness (expected number of alleles among four gene copies), H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} individual inbreeding coefficient

*Significant deviation from zero ($P < 0.001$; 20,000 randomization)

Table 5 Genetic diversity of *Nymphoides*

Species	Ploidy level**	Sample size	Loci number	A_R	NA	H_E	H_O	F_{IS}	F_{ST}	Location	References
N. fallax	Tetraploid	180	7	3.08	10.57	0.80	0.60	0.157*	0.034	Mexico	This study
N. indica	Diploid	26	3	–	4.70	0.32	0.20	0.330	–	Japan	Shibayama et al. (2006)
N. indica	Diploid	157	3	–	16.00	0.50	0.46	0.100	–	Japan	Shibayama et al. (2006)
N. indica	Diploid	124	3	–	11.30	0.38	0.40	−0.060	–	Japan	Shibayama et al. (2006)
N. peltata	Hexaploid	190	9	2.77	3.10	–	0.39	0.170*	–	Japan	Takagawa et al. (2006)
N. peltata	Hexaploid	333	10	2.90	3.50	0.37	0.51	0.283*	–	Japan	Uesugi et al. (2007)
N. peltata	Hexaploid	18	10	3.21	3.50	0.54	0.63	−0.175*	–	Japan	Nishihiro et al. 2009)
N. peltata	Hexaploid	472	10	2.80	8.00	0.725	0.35	–	0.368	China	Liao et al. (2013)

A_R Allelic richness, *NA* no. of alleles, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} individual inbreeding coefficient, F_{ST} fixation index

* Significant deviation from zero ($P < 0.001$; 20,000 randomization). ** Ploidy level follows Ornduff (1970)

sampling a few individuals is a good strategy for detecting variability in many populations—but not to analyze the inbreeding (Hedrick 2013).

Nymphoides species exhibit variation in reproductive mechanisms, inflorescence architecture, floral morphology, and seed morphology (Tippery and Les 2011; Tippery et al. 2018). *Nymphoides fallax* flowers present dimorphic heterostyly (Tippery and Les 2011), which promotes outcrossing (Barrett and Shore 2008). *Nymphoides peltata* can present a significant self-compatibility (Takagawa et al. 2006). Inbreeding by selfing can occur when compatible mates or pollen flow are limited (e.g., in the ecosystems as temporary wetlands) and can promote high inbreeding coefficients. Then, seedlings emerged from the remnant soil seed bank mainly reflect mating among a small number of parents, including selfing. Even though past research has shown that *Nymphoides* has clonal reproduction (Larson 2007; Liao et al. 2013; Huang et al. 2015). We also observed seedling recruitment for *N. fallax* in several Mexican temporary wetlands (Fig. 1d), and our inbreeding findings can indicate that self-compatibility occurs in *N. fallax*. Regrettably, reproduction strategies have been poorly understood in clonal aquatic plants—including *Nymphoides* (Wang et al. 2005).

The estimated F_{ST} value across all populations (F_{ST} =0.034; Table 5) was lower than the value found for several other clonal aquatic plants (Liao et al. 2013). Allelic richness was similar to *N. peltata*, which has distyly—a reproductive mechanism considered to promote genetic diversity (Li et al. 2017). Nonetheless, the expected heterozygosity of *N. fallax* populations was high when compared to *N. peltata* (Table 5; Uesugi et al. 2007; Nishihiro et al. 2009; Liao et al. 2013), even if the ploidy population level was not mentioned. Ornduff (1970) reported *N. peltata* as a hexaploid species, and the assumption of diploidism could underestimate genetic diversity (Dufresne et al. 2014). *Nymphoides fallax* has a similar allele number as *N. indica*, but higher than *N. peltata* (Table 5). The smaller number of alleles for *N. peltata* may reflect the lower genetic variability shown by this species compared with other species in the genus (Larson 2007) or bottleneck effects (Takagawa et al. 2006).

We observed significantly higher genetic diversity within populations than among populations of *N. fallax* (among populations 8.45%, within populations 91.55%, $P < 0.001$, 1000 permutations), as was observed for *N. peltata* (among populations 36.80%, within populations 63.20%, $P < 0.001$, 1000 permutations, Liao et al. 2013; and among populations 20.17%, among subpopulation 24.87%, within populations 54.95%, $P < 0.01$; 1000 permutations, Cao et al. 2017). Sweetman et al. (2013) found similar results for the aquatic Cyperaceae *Schoenoplectus maritimus* L. (among populations 8.00%, within populations 92.00%, $P < 0.001$, 1000 permutations). Magallán et al. (2009, 2013) observed similar genetic results in central Mexican temporary wetlands for the aquatic plants *Eriocaulon bilobatum* Morong (among populations 5%, within populations 95%, $P < 0.001$) and *Triglochin scilloides* (Poir.) Mering & Kadereit (cited as *Lilaea scilloides* (Poir.) Hauman; among populations 15%, within populations 85%, $P < 0.001$), although these authors used different molecular markers. Cao et al. (2017) found higher genetic diversity within populations than among populations for six aquatic plants, using microsatellites molecular markers. They consider that these findings can contribute to explain the connectivity between aquatic ecosystems, migratory bird routes, and species dispersal mechanisms.

We observed 36 chromosomes in all samples of *N. fallax* populations (Fig. 2), as was found by Ornduf (1970), confirming *N. fallax* as a tetraploid species. All sampled times had metaphase chromosomes, which indicates a vigorous vegetative growth of the species.

Polyploidization has significant potential evolutionary consequences for increasing allelic diversity. It is thus one of the major mechanisms of plant speciation (Husband et al. 2013; Alix et al. 2017). Nevertheless, understanding polyploid genetic diversity is difficult, since many population

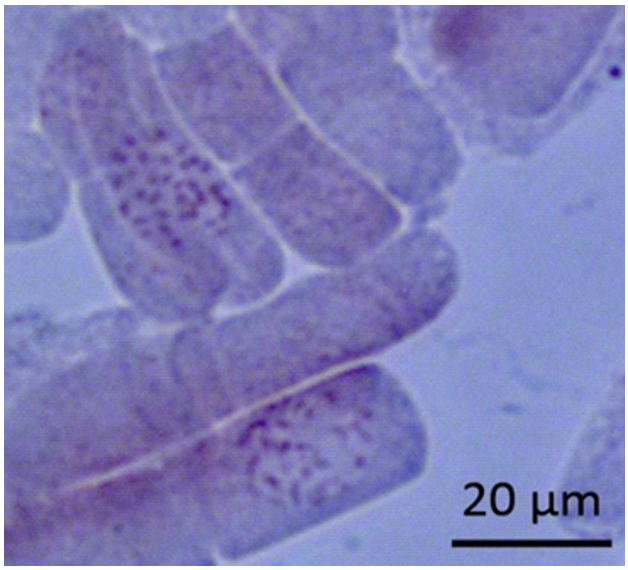

Fig. 2 Chromosomes of *Nymphoides fallax* (2 $n = 4x = 36$ chromosomes)

genetic tools have been developed for diploid species (Dufresne et al. 2014). Also, few researchers have developed an interest in studying polyploid species, even though a large proportion of wild species are polyploid (Soltis et al. 2015). Population genetic analysis for polyploids is more challenging than similar analyses for diploid species (Meirmans and Van Tienderen 2013). Despite the complexity in genotyping codominant polyploids, microsatellites have been used in genetic studies (Dufresne et al. 2014). Many researchers have resorted to coding each allele as a dominant marker (e.g., Moncada and McCouch 2004; Robertson et al. 2010; Assoumane et al. 2013; Sweetman et al. 2013), reducing the content information such as inbreeding levels (Dufresne et al. 2014).

A recent study denoted evidence that *N. fallax* is an allopolyploid species (Tippery et al. 2018). Allopolyploids may show a mixture of disomic and polysomic inheritance patterns, which can make genetic analysis more complex (Dufresne et al. 2014). Meirmans and Van Tienderen (2013) demonstrated that the inheritance type in polyploids affects the study of genetic diversity and population structure. However, these authors consider that it happens when polysomy is assumed, but in fact the species are full disomic. On the other hand, genetic statistics can be unbiased when the species has partial disomy, with a small amount of exchange between subgenomes.

Nymphoides species are found in tropical and temperate wetlands worldwide, and one or more polyploidization events may have occurred in these species (Tippery and Les 2011; Tippery et al. 2018). Liu et al. (2017) found wider geographic distribution patterns in hexaploids than in tetraploids. *N. fallax* presents a restricted distribution compared with *N. peltata* and *N. indica* (Ornduff 1970; Tippery et al. 2018). *Nymphoides peltata* was expected to have higher genetic diversity than *N. fallax* because it is a hexaploid species. Low genetic diversity in *N. peltata* can be explained by (1) bottlenecks or vegetative reproductive effects (Takagawa et al. 2006; Liao et al. 2013), and (2) the way in which the genetic analysis was carried out by authors, who analyzed the species as a diploid.

Nymphoides fallax exhibited genetic homogeneity among populations, high local genetic richness, high genetic diversity among individuals, and no clonal individuals. Polyploid migrant individuals carry more gene copies than a diploid migrant, leading to greater homogenization among populations (Meirmans and Van Tienderen 2013). The findings suggest that *N. fallax* relies heavily on sexual reproduction and disperses pollen and seeds widely, and may also indicate that populations have high connectivity because there is low genetic differentiation between populations.

Hence, studies of molecular markers of aquatic plant species, especially for polyploids, are a new challenge in creating strategies for conservation of temporary freshwater wetlands. There are still few studies on neotropical *Nymphoides* species using microsatellite markers. Knowledge and awareness of the genetic diversity of plants in temporary wetlands is one of the first steps in establishing conservation policies, as they are a very peculiar and highly threatened environment everywhere, and especially in the Mexican highlands.

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Diversidad florística y conectividad de humedales temporales de tierras altas en el centro de México

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CAPÍTULO 4 - Plant functional connectivity of *Nymphoides fallax* in geographically isolated temporary wetlands in Mexican highlands

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Plant functional connectivity of *Nymphoides fallax* in geographically isolated temporary wetlands in Mexican highlands

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Highlights

- Wetlands act as natural connectors among ecosystems
- Information on plant functional connectivity is crucial for wetland conservation
- Connectivity translates to higher genetic diversity of over distances up to 5 km
- Wetland size inclusion in *Si* Hanski connectivity index explain genetic diversity
- Forest habitat surrounding wetlands explain gene flow

ABSTRACT

Wetlands act as natural connectors among terrestrial and aquatic ecosystems, maintaining biodiversity at the landscape and regional scales. Plant functional connectivity integrates species-environment interactions, dispersal vectors, and landscape structure variables to understand species' spatial responses to habitat fragmentation. This study quantified plant functional connectivity among geographically isolated wetlands by genotyping seven nuclear microsatellite loci in 18 populations of *Nymphoides fallax* (Menyanthaceae), a tetraploid aquatic plant native to highland temporary wetlands. We tested if wetland connectivity, quantified with three connectivity indices, translates to higher genetic diversity and if landscape features such as vegetation cover influence gene flow among wetlands. Our results show that wetland connectivity was positively associated with genetic diversity and over threshold distances up to 5 km. Hanski connectivity S_i including wetland size was the best index to explain the effective number of alleles, allelic richness, and expected heterozygosity. Population-specific F_{ST}, as proxy for gene flow, was not explained by geographical distance, but by a model including forest cover. Gene flow of *N. fallax* was facilitated by the amount of forest in the landscape, likely related to the behavior of its dispersal vectors. Our findings allowed us to identify important landscape and wetland features for plant functional connectivity. Landscape genetic studies on aquatic plants evaluating wetland connectivity and landscape features are highly needed to inform conservation strategies for geographically isolated wetlands, a highly threatened ecosystem worldwide.

Keywords: connectivity indices, freshwater wetlands, graph-theory, landscape genetics, microsatellites

1. Introduction

Temporary wetlands are dynamic ecosystems that acts as natural connectors between terrestrial and aquatic systems, maintaining biodiversity at regional and landscape scales (Aavik et al., 2014; Cohen et al., 2016; Calhoun et al., 2017a). Freshwater temporary wetlands span over 0.81 million km^2 of the earth's surface (Pekel et al., 2016). Although total global surface covered by freshwater temporary wetlands may be relatively small, they are valued for the multiple ecosystem functions they provide, such as carbon stocks, nutrient retention, water source, and biodiversity connectivity (Schofield et al., 2018; Davidson et al., 2019).

Maintaining and increasing wetland connectivity is considered a global priority for preserving biodiversity and ecosystem functioning (IUCN, 2017). Tropical regions harbor most of the world wetland biodiversity, however, landscape effects on gene flow in aquatic plants is unknown, especially using microsatellite molecular markers (Monteiro et al., 2019). The latter fact is the case of the Mexican highland temporary wetlands, a species-rich ecosystem (Lobato-de Magalhães and Martínez, 2018) that is increasingly threatened by land-use transformation and degradation (Calhoun et al., 2017b). Temporary wetlands of Mexican highlands are geographically isolated units, without hydrological connection, and completely surrounded by uplands at the local scale (Lobato-de Magalhães et al., 2019). Moreover, these temporary wetlands occur within a semi-arid environment extremely susceptible to climate change; wetland loss is predicted to occur with increasing temperatures and rainfall decline (Walck et al., 2011). Yet, connectivity for temporary wetland plants has rarely been evaluated (Ayram et al., 2015; Auffret et al., 2017a), not only in Mexico but for the entire tropical region (Monteiro et al., 2019).

Evaluating connectivity involves identifying its structural and functional components. For plants, functional connectivity implies the effective dispersal of propagules or pollen among resource habitats, while structural connectivity refers to the composition of physical landscape elements and the spatial configuration of resource habitats (Auffret et al., 2017a). Plant functional connectivity can be measured by field observations of pollen flow and seed dispersal or through genetic information (Auffret et al., 2017a). Most plants inhabiting temporary wetlands have long-lived seeds capable of frequent long-distance dispersal (Reynolds et al., 2015; Schofield et al., 2018). Aquatic plant dispersal within and among freshwater ecosystems can

connect wetlands across the landscape (Schofield et al., 2018). Plant functional connectivity studies for wetlands can focus on (*i*) identifying isolated populations that are genetically depauperate, (*ii)* evaluating mechanisms and patterns of dispersal that may be common to multiple species, and (*iii*) testing landscape effects on genetic diversity and gene flow, all of which can inform conservation practices (Auffret et al., 2017a, b).

Geographically isolated wetlands are structurally isolated, but they are not necessarily functionally isolated (Cohen et al., 2016). Traditionally, the island biogeography approach was used to assess habitat connectivity using habitat and non-habitat binary maps and the geographical distance between habitat patches as a proxy for evaluating species dispersal and gene flow between habitat patches (Rico et al., 2012). However, recent evidence has shown that geographical distance alone plays a modest role in shaping dispersal and gene flow in wetland animals, while landscape features, such as the nature of the matrix, may be more relevant (Uden et al., 2014; Uroy et al., 2019). Here, we aimed to investigate functional connectivity of geographically isolated temporary wetlands through a landscape genetics approach by combining graph theory and landscape data for a native aquatic plant.

We selected *Nymphoides fallax* Ornduff (Menyanthaceae, 'yellow floating-heart') as a focal species because it is endemic to highland temporary wetlands, is strictly aquatic, and is becoming endangered in some areas (Lot, 2018). Microsatellite molecular markers developed for *N. peltata* (Uesugi et al., 2005) were successfully transferred to *N. fallax* (Lobato-de Magalhães et al., 2019). Here, we test two hypotheses of plant functional connectivity in *N. fallax* populations: (1) High wetland connectivity, quantified by habitat connectivity indices, will translate to higher genetic diversity and gene flow as populations that are geographically closer will exchange more gene flow than populations that are geographically distant within the wetland network. We also expected that the size of source wetlands, as a proxy for population size, will better explain genetic diversity of focal wetlands. Source wetlands that have bigger populations are assumed to be larger sources of gene flow than smaller wetlands within the network. Moreover, (2) landscape features of the intervening matrix will affect gene flow among geographically isolated wetlands. *Nymphoides* seeds are dispersed by animals and water (Smits et al., 1989), and for the second hypothesis we tested if forest and water habitats facilitated gene flow. Estimating plant functional connectivity is critical for decision-making in conservation (Luque et al., 2012; Neel

et al., 2014). Furthermore, estimating landscape effects on genetic diversity and gene flow are an important step to preserve geographically isolated wetlands.

2. Material and Methods

2.1 Study species and data collection

Our focal species – *Nymphoides fallax* – is a distylous tetraploid aquatic herb with floating leaves (Fig. 1), endemic to Mexican and Guatemalan highlands, and occurring in freshwater ponds and temporary wetlands (Lot, 2018; Lobato-de Magalhães et al., 2019). In Mexico, *N. fallax* has become scarce in some areas due to habitat loss (Lot, 2018), but still widespread in our study area (Lobato-de Magalhães and Martínez, 2018). *Nymphoides fallax* has yellow flowers, insect-pollinated, and is dispersed by hydrochory and epizoochory on water birds (Smits et al., 1989). *Nymphoides* reproduction has been considered mostly clonal (Larson, 2007; Liao et al., 2013), however new evidence suggests that sexual reproduction can be equally or even more important in *N. fallax* populations (Lobato-de Magalhães, et al. 2019).

The study landscape has an extension of 380 km × 320 km distributed in five Mexican states: Aguascalientes, Guanajuato, Jalisco, Querétaro, and Zacatecas. We registered the occurrence of *N. fallax* in 25 from 39 temporary and geographically isolated wetlands embedded in agriculture, forest, native grassland, xerophytic shrub, and superficial water matrix (Fig. 1; Table S1; Lobato-de Magalhães and Martínez, 2018). We mapped the wetlands using a Map 64 GPS device (Garmin Deutschland GmbH, Garching, Germany) and using the Datum NAD83.

From September to October 2016, we collected leaves from 10 flowering plants (separated by 8–30m) in 18 temporary wetlands (n = 180) at elevations ranging from 1,961 to 2,613 m a.s.l (Table 1). Geographical distance between pairs of the wetlands ranged from 0.6 to 374 km. Leaves were preserved in silica gel until DNA extraction.

2.2 Laboratory procedures and genetic analysis

We used seven microsatellite markers developed to *N. peltata* (S.G. Gmel.) Kuntze (Uesugi et al., 2005), and which were transferred to *N. fallax*. Detailed information on laboratory procedures and microsatellite genotyping can be found in Lobato-de Magalhães et al. (2019).

We estimated alleles number (*NA*), effective alleles number (*Ne*), allelic richness (A_R), expected (H_E) and observed (H_O) heterozygosity, inbreeding coefficient (F_{IS}), pairwise fixation index (F_{ST}), and genetic distances using SPAGeDi V.1.5a (Hardy and Vekemans, 2015). The number of different multilocus genotypes (*GML*) and clone correction were found using *Poppr* package (Kamvar et al., 2013) in R V.3.4.2 (R Core Team, 2019). In addition, we calculated population-specific F_{ST} (50,0000 burns) with Geste V.2.0 (Foll and Gaggiotti, 2006). Population-specific F_{ST} represents how genetic differentiated each population is from others in the sample and indirectly measures effective dispersal (gene flow) (Foll and Gaggiotti, 2006; DiLeo et al., 2017).

2.3 Genetic structure

Population genetic structure was evaluated by cross-validating population assignments with discriminant analysis of principal components (DAPC; Jombart et al., 2010), using *adegenet* in R V.3.4.2 (R Core Team, 2019). In DAPC, data are first transformed using principal components analysis and clusters are identified using discriminant analysis to maximizes between-group genetic variation, while minimizing the within-group variation. DAPC requires *a priori* selection of the number of retained principal components (PCs) in order to depict population genetic structure. Cross-validation analyses were performed to determine the best trade-off between retaining too few or too many principal components.

We also analyzed genetic structure with Structure V.2.3.4 (Pritchard et al. 2000). We ran the analyses using the admixture model and correlated frequencies (burn-in and run the length of 250,000 and 1,000,000 respectively, $k = 1$ to 18, and ten iterations for each k). To detect the number of genetic clusters that best fit the data we used Delta K in Structure Harvester (Earl & von Holdt 2012).

2.4 Wetland connectivity and genetic diversity

We used a population graph approach to estimate wetland connectivity through comparing three structural connectivity indices: Hanski's index, Integral index of connectivity, and the Probability of connectivity index. The Hanski's index (S_i) calculates patch connectivity by summing distances between focal wetland *i* and all source wetlands *j* using the following equation (Hanski, 1994):

$$S_i = \sum_{i \neq j} exp(-\alpha d_{ij})$$

where d_{ij} is the Euclidean distance between wetland *i* and wetland *j*, α is a constant scaling parameter accounting for dispersal capacity, which we fitted through optimization ($\alpha = 1$). The S_i index assumes that all wetlands are connected, and wetlands with higher S_i values are better connected wetlands within the network. The S_i index was calculated in R V.3.4.2 (R Core Team, 2019).

To identify the threshold distance that determines functional connectivity, we calculated wetland connectivity for each wetland using the Integral index of connectivity (IIC) and the probabilistic connectivity index (PC), using threshold dispersal distances ranging from 0.5 to 400 km in Conefor V.2.6 The IIC and PC indices have shown to perform best than other connectivity indices (Saura and Rubio, 2010). We supposed that *N. fallax* pollen dispersal occurs over short distances by insects, while seed dispersal occurs at larger distances by epizoochory, most probably by water birds (Smits et al., 1989).

We calculated the integral index of connectivity (IIC) using the following equation:

$$IIC = \sum_{i=1}^{n} \sum_{j=1}^{n} \frac{a_i a_j}{1 + nl_{ij}}$$

where *n* is the total number of wetlands in the landscape, a_i, and a_j are nodes attributes *i* and *j*, and nl_{ij} is the number of links (distances) between nodes *i* and *j*. IIC is based on a binary connection model that means that two patches considered are either connected or not depending on the actual distance between them in relation to the predefined distance threshold. IIC ranges between 0 and 1, with highest IIC values representing higher connectivity between wetlands.

We calculate the probability of connectivity (PC) using the following equation:

$$PC = \sum_{i=1}^{n} \sum_{j=1}^{n} a_i a_j p_{ij}^*$$

where p_{ij}* is defined as the maximum product probability of all possible paths between nodes *i* and *j*. PC values are within the same range and interpretation as IIC. PC is based on a probabilistic connection model, where a certain probability of dispersal between the two patches considered (p_{ij}) characterizes the links between nodes *i* and *j* in the graph.

To estimate the three types of wetland connectivity indices we included seven wetlands where the species occurred, but which we had no genetic data (Table 1). We also incorporated the log-transformed wetland area (*A*) as a proxy for population size of source wetlands $_j$ in the calculations of the three indices (denoted here as $S_{i(Aj)}$, $IIC_{(Aj)}$, and $PC_{(Aj)}$). Area was measured with the ArcGIS V.9.2 in m^2. We correlated each wetland connectivity index and wetland area with estimates of genetic diversity (effective number of alleles, allelic richness, expected and observed heterozygosity), inbreeding coefficient (F_{IS}), and population-specific F_{ST} using Pearson linear regressions in Vegan package, R version 3.4.2 (R Core Team, 2019). We selected the best model based on AIC_C using *MuMln* in R V.3.4.2 (R Core Team, 2019). For the best performing connectivity index, we performed significance test, residual analysis, and assessed model fit with adjusted-R^2.

2.5 Landscape data on gene flow

Evaluation of landscape features on gene flow were performed in Geste V.2.0 (Foll and Gaggiotti, 2006), which calculates population-specific F_{ST}. Geste uses a Bayesian approach that includes environmental information a priori, and models associations between the environmental predictors and population-specific F_{ST} with generalized linear models. The latter approach has low type I error rates and has performed well in recovering landscape drivers of gene flow in simulations and empirical data (Foll and Gaggiotti, 2006; DiLeo et al., 2017). We included as predictors the $S_{i(Aj)}$ connectivity index to include a predictor modeling isolation by geographical distance, while for the landscape features, we included slope, proportion of forest and surface water covers (wetlands) in a distance of 1 km from the wetland edge. In order to determine the proportion of each landscape feature, we manually pulled spatial data from the National Institute

of Statistic and Geography and the National Wetlands Inventory at a scale 1: 250,000 with spatial resolution of 50 m (CONAGUA, 2019; INEGI 2019). Run parameters in GESTE were a value of α = 0.2, 250,000 interactions, and 50,000 for burn in. To obtain positive or negative associations, we correlated the landscape features with gene flow estimates using Pearson linear regressions in Vegan package, R version 3.4.2 (R Core Team, 2019). For the best model, we performed significance test, residual analysis, and model fit with adjusted-R^2.

Additionally, we calculated Mantel's test (10,000 permutations) using a matrix of pairwise genetic distances (F_{ST}) among sampled wetlands and a corresponding geographical distance matrix (Rousset, 1997) using *vegan* in R V.3.4.2 (R Core Team, 2019). Genetic distance matrix was transformed to F_{ST} / (1 - F_{ST}), while geographical distances were log-transformed to improve linear assumptions.

3. Results

3.1 Genetic structure

Global pairwise F_{ST} over all loci was 0.034 and population-specific F_{ST} ranged from 0.028 to 0.109 (average = 0.058) (Table 1). Plotting the two first DAPC components showed low genetic structuring (Fig. 2a), where only wetland 18, located at Zacatecas state was the most differentiated along the second DAPC axis. Wetland 18 had also the lowest S_i connectivity index and is the westernmost site (Table 1, Fig. 1). Results from Structure showed that the most probable number of *k*-clusters was three as indicated by Delta K in Structure Harvester, however, admixture was high among clusters with no clear genetic structure (Fig. S1, S2).

3.2 Relationship between wetland connectivity and genetic diversity

We found higher positive associations with genetic diversity (regression coefficients) by incorporating wetland size A_j to the calculation of *Si* connectivity index. In contrast, including A_j in the calculation of IIC and PC indices did not increase the percentage of variance explained by the models, although regression coefficients remained significant. We did not observe any significant correlations (*p-value* < 0.05) in distance threshold higher than 10 km (Table S2).

Results from AIC_C model selection showed that $S_{i(Aj)}$ is the best wetland connectivity index to explain the effective number of alleles (*Ne*, *p-value* = 0.005, r^2 = 0.40), allelic richness (A_R, *p-value* = 0.030, r^2 = 0.30), and expected heterozygosity (H_E, *p-value* = 0.006, r^2 = 0.24), followed by IIC and PC at threshold distances up to 5km (IIC_{5km} and PC_{5km}). Correlations between wetland connectivity with observed heterozygosity (H_O), inbreeding coefficient (F_{IS}), and population-specific F_{ST} were not significant across all connectivity indices including or not wetland size A_j. Correlations between wetland area with genetic diversity were not significant. Thus, we just showed results for the best five top models (Fig. 2b, c, Table 2).

3.3 The effect of landscape features on gene flow

The highest probability model (P = 0.216) included forest cover (Table 3). The latter model had the highest support, and it was evident that wetland connectivity $S_{i(Aj)}$, slope, and water cover did not influence genetic differentiation (pairwise F_{ST}) since models that included these factors had low posterior probabilities (between 0 and 0.08). The estimated σ^2 was 0.327 (HPDI = [0.115; 0.604]). We found a negative association with genetic differentiation and forest cover (*p-value* = 0.032, r^2 = 0.25, Fig. S3), but we did not observe significant associations with $S_{i(Aj)}$, water cover, and slope. F_{ST} pairwise genetic distances were weakly and positively associated with geographical distances (*p-value* = 0.001, r^2 = 0.10).

4. Discussion

4.1 Genetic structure and wetland connectivity

Our genetic data show a weak genetic structure among geographically isolated wetlands, as has been reported in other aquatic plant studies worldwide (Liao et al., 2013; Sweetman et al., 2013; Cao et al., 2017; Kettenring et al., 2018). Results suggest ongoing gene flow among *N. fallax* populations in the region. The weak degree of genetic structuring could be the result of long-distance seed dispersal or the absence graphic barriers to dispersal (Santamaría, 2002; Auffret et al., 2017a; Kettenring et al., 2018). Mexican highland temporary wetlands have a weak genetic structure as demonstrated in our study, and show high genetic diversity (Lobato-de

Magalhães et al., 2019). Potential explanations for the high levels of genetic diversity may be the predominantly outcrossing mating system as in other heterostylous plant, high gene flow levels, persistent seed bank, and ability to adapt to extremely dynamic environments, which fits with previous research in *N. fallax* (Zepeda et al., 2014; Lobato-de Magalhães et al., 2019).

4.2 Relationship between wetland connectivity and genetic diversity

The $S_{i(Aj)}$ wetland connectivity index was the best predictor of genetic diversity. The S_i index in contrast to the IIC and PC indices considers all wetlands connected, an assumption that is consistent with the lack of genetic structure we found. Therefore, it is a totally connected network, where gene flow is exchanged among all geographically isolated wetlands, but closer wetlands to others are more connected within the network. Previous studies on connectivity effects on genetic diversity for wetland plants (Aavik et al., 2014) and other herbs (DiLeo et al., 2017) have similar findings.

Decreasing habitat area can impact the population genetics of plants. We found a better correlation with genetic diversity when the source area was included as a proxy for population size, which suggests that large wetlands may function as a better source for gene flow, either by pollen or seeds, thus maintaining genetic diversity within the wetland network. In addition, IIC is an overall similar predictor for genetic diversity as PC, as observed by Aavik et al. (2014).

4.3 The effect of landscape features on gene flow and potential dispersers

We found a high gene flow scenario. Field observations of *N. fallax* flowers visitors include several Hymenoptera, Odonata, Diptera, and Lepidoptera species. Pollinators promote genetic diversity at short distances (Aavik et al., 2014; Liu et al., 2015). A variety of local dispersal agents for *N. fallax* seeds such as aquatic and terrestrial animals (e.g., turtles, water birds, cattle) can be hypothesized. Aquatic turtles (*Kinosternon* species) were observed in several Mexican highland temporary wetlands (Lobato-de Magalhães, 2019) and might disperse *N. fallax* seeds between wetlands, considering that *Kinosternon* reproduction period (summer rain) coincides with the inundation of wetlands. Based on our and some author's observations, these turtles have a similar geographical distribution of *N. fallax,* occur at the same ecosystem types, and can

disperse extensively overland connecting different temporary and permanent water bodies across the landscape (Enríquez-Mercado et al., 2018, Lobato-de Magalhães, 2019).

The landscape feature associated with gene flow was forested habitat. We observed that geographical distance *per se* did not contribute to gene flow among geographically isolated wetlands, although our results suggest that genetic diversity can be enhanced to up to 5 km. We expected that surface water cover affect genetic differentiation among wetlands, assuming a stepping stone model (Mwaniki et al., 2019). However, we did not observe such associations.

4.4 Conclusions and implications to wetland connectivity conservation

Given the substantial ecological implications of conserving wetland functional connectivity, the analyses we presented here are important for guiding priority conservation areas and seed source for geographically isolated wetland restoration (e.g., high levels of genetic diversity and connectivity). Our comparative approach with three connectivity indices and landscape features allowed us to identify important landscape and wetland features explain genetic diversity and gene flow. Wetland connectivity at the landscape scale is crucial to maintain gene flow and genetic diversity of aquatic plant species inhabiting geographically isolated wetland. For wetland conservation, it is important to preserve the wetlands at a local scale, but also to identify key landscape elements that maintain gene flow and genetic diversity. Specifically, conserving forest at local and landscape scales can improve wetland connectivity. We document the utility of applying wetland connectivity indices as a potential predictors of genetic diversity as these indices can be used to identify priority wetlands for conservation. Wetland size seems to be a key feature to consider for conservation management, since larger and well-connected wetlands could support higher genetic diversity. We should also consider that genetic data obtained by microsatellite markers reflect recent gene flow but, not necessarily current gene flow. That is, land-use change dynamic may occur at faster rates, not detected by microsatellite markers, and time-lag effects may occur in this ephemeral system. In conclusion, landscape genetic and graph-theory approaches are helpful for evaluating functional connectivity of wetland plants and can be useful to plan geographically isolated wetland conservation.

Conflict of interest

The authors declare no conflicts of interest.

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Author Contributions

Conceived and designed field work: TLdM, DCT, MM. Performed the experiments: TLdM. Analyzed the genetic data and wetlands connectivity: TLdM, YR, DCT. Wrote the paper: TLdM, Reviewed drafts of the paper: YR, DCT, MM. All authors provided substantial review and comments to the written manuscript.

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Table 1. Genetic diversity of *Nymphoides fallax* in geographically isolated temporary wetlands in Mexican highlands (*Ne* = effective number of alleles, A_R = Allelic richness, H_E = expected heterozygosity, H_O = observed heterozygosity, F_{IS} = individual inbreeding coefficient), HPDI = 95% highest probability density interval, and wetland connectivity ($S_{i(Aj)}$ = Hanski's index including the size of source wetlands *j*).

Wetland	*Ne* [a]	A_R [a]	H_E [a]	H_O [a]	F_{IS} [a]	Population specific-*Fst*	Population specific-*Fst* (95% HPDI)	$S_{i(Aj)}$
1	6.71	3.01	0.77	0.64	0.128*	0.058	[0.031; 0.091]	182.78
2	7.14	2.94	0.77	0.63	0.166*	0.065	[0.037; 0.098]	2322.47
3	7.43	3.02	0.79	0.62	0.141*	0.042	[0.020; 0.066]	1730.14
4	6.57	2.80	0.75	0.51	0.252*	0.074	[0.043; 0.115]	2.40
5	6.71	2.95	0.77	0.57	0.218*	0.048	[0.019; 0.074]	27.84
6	7.57	2.99	0.75	0.66	0.107*	0.038	[0.017; 0.061]	30.96
7	7.14	2.88	0.72	0.60	0.145*	0.055	[0.027; 0.081]	6670.95
8	8.00	3.07	0.78	0.63	0.146*	0.028	[0.011; 0.045]	15688.47
9	6.71	2.92	0.76	0.56	0.219*	0.059	[0.033; 0.094]	1209.55
10	6.57	2.86	0.75	0.55	0.214*	0.083	[0.048; 0.120]	2554.31
11	6.86	2.86	0.74	0.55	0.223*	0.060	[0.031; 0.090]	134.33
12	6.57	2.95	0.77	0.63	0.128*	0.060	[0.032; 0.091]	16740.28
13	5.71	2.91	0.78	0.57	0.228*	0.109	[0.065; 0.158]	7645.06
14	7.00	2.83	0.73	0.62	0.115*	0.066	[0.037; 0.099]	5.98
15	7.43	3.08	0.81	0.63	0.222*	0.035	[0.015; 0.056]	16800.42
16	6.43	2.83	0.77	0.63	0.190*	0.059	[0.031; 0.089]	281.32
17	6.57	2.92	0.76	0.64	0.149*	0.058	[0.031; 0.090]	0.11
18	7.14	2.88	0.72	0.55	0.180*	0.040	[0.018; 0.065]	0.00

[a] Source: Lobato-de Magalhães et al. (2019), * = *p*-value < 0.001 (20,000 randomization)

Table 2. Results of the top five models selection testing the effect of wetland connectivity ($S_{i(Aj)}$= Hanski's index including the size of source wetlands, IIC = integral index of connectivity, PC = probability of connectivity) using different threshold distances on genetic diversity (*Ne* = effective number of alleles, A_R = Allelic richness, H_E = expected heterozygosity) of *Nymphoides fallax* in geographically isolated temporary wetlands in Mexican highlands

Models	AIC_C	ΔAICc	*wi*	r^2
Ne				
$S_{i(Aj)}$	25.42	0.00	0.58	0.40
IIC_{5km}	30.21	4.79	0.05	0.37
PC_{1km}	30.30	4.88	0.05	0.31
PC_{3km}	30.42	5.00	0.05	0.29
IIC_{3km}	30.45	5.03	0.05	0.35
A_R				
$S_{i(Aj)}$	-38.94	0.00	0.44	0.30
IIC_{5km}	-35.20	3.74	0.07	0.36
PC_{1km}	-35.13	3.81	0.07	0.14
PC_{3km}	-35.13	3.81	0.07	0.14
IIC_{1km}	-34.95	3.99	0.06	0.14
H_E				
$S_{i(Aj)}$	-82.10	0.00	0.27	0.24
IIC_{5km}	-80.12	1.98	0.10	0.31
PC_{3km}	-80.10	2.00	0.10	0.20
PC_{5km}	-79.95	2.15	0.09	0.24
IIC_{3km}	-79.87	2.23	0.09	0.20

Table 3. Top five probability models in Geste describing the effects of landscape features (forest and water cover) and wetland connectivity $S_{i(Aj)}$ on population-specific F_{ST} of *Nymphoides fallax* in geographically isolated temporary wetlands in Mexican highlands.

Model	Probability
Population-specific F_{ST} ~ forest cover	**0.22**
Population- specific F_{ST} ~ $S_{i(Aj)}$	0.08
Population- specific F_{ST} ~ slope	0.07
Population- specific F_{ST} ~ water cover	0.06
Population- specific F_{ST} ~ forest cover + $S_{i(Aj)}$	0.02

Figure 1. Geographic distribution of geographically isolated temporary wetlands with *Nymphoides fallax* populations in Mexican highlands.

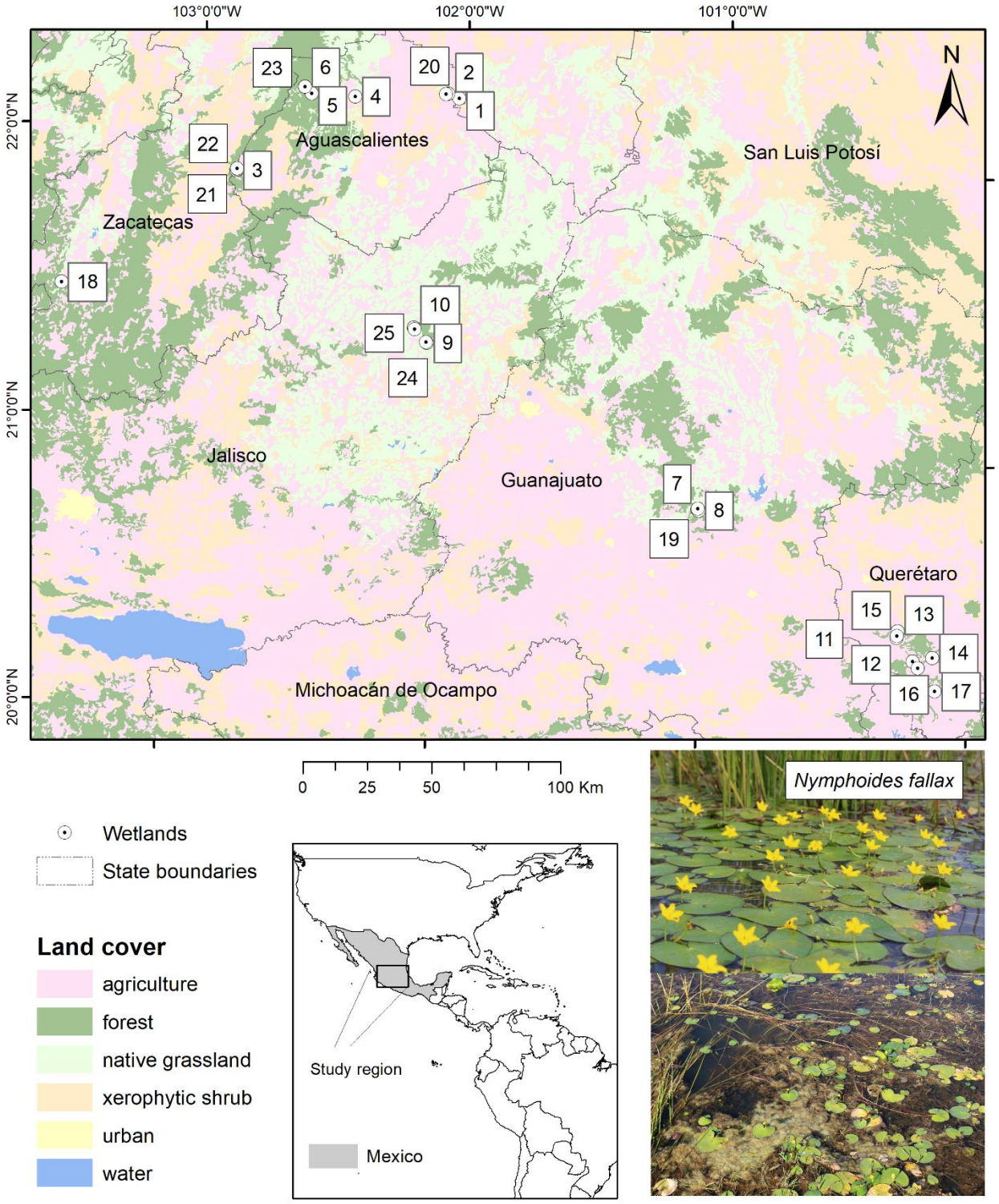
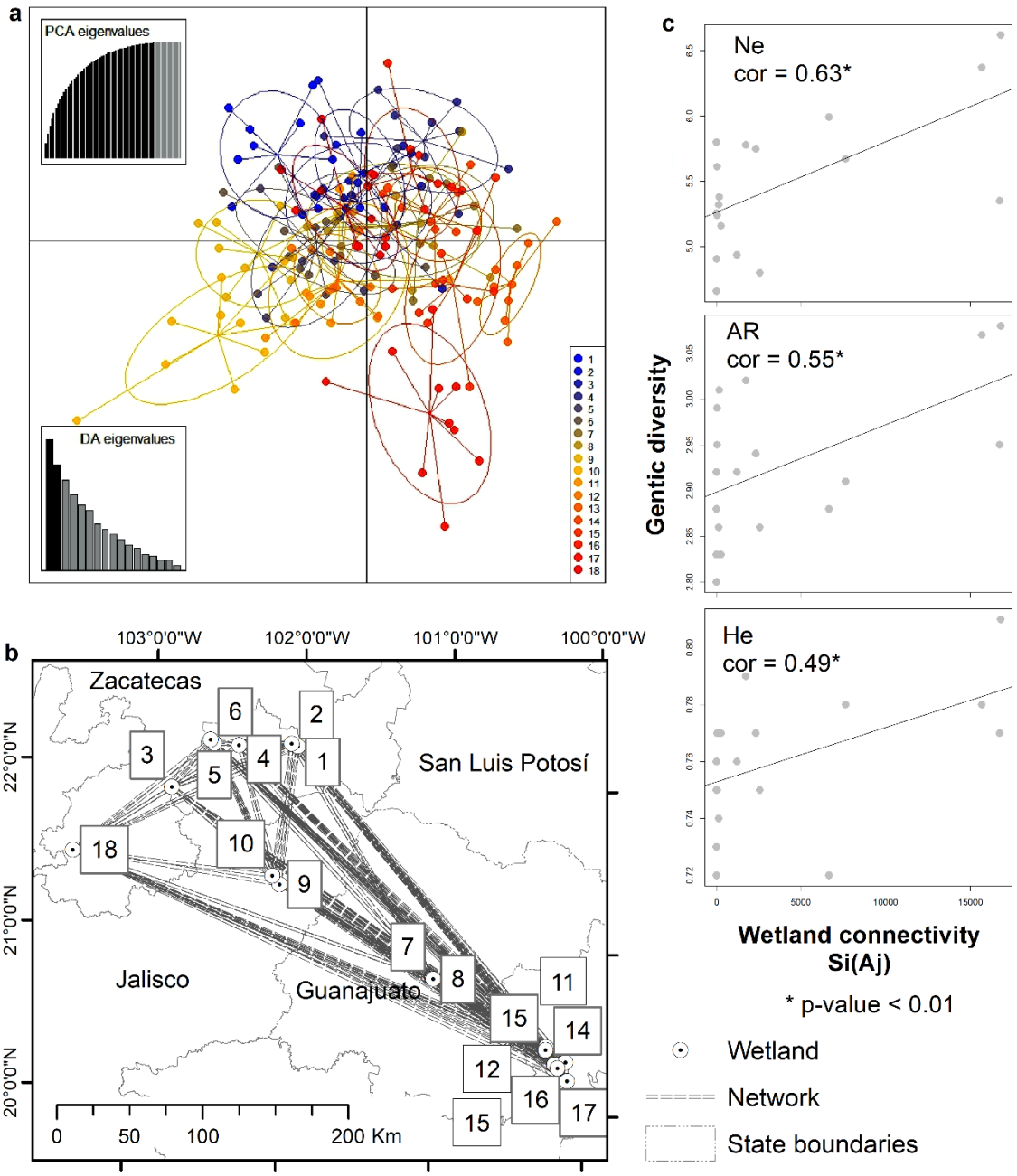


Figure 2. (a) Discriminant analysis of principal components *DAPC* of *Nymphoides fallax* populations in geographically isolated temporary wetlands in Mexican highlands, **(b)** Wetland network, **(c)** Correlations between genetic diversity (*Ne* = effective number of alleles, A_R = Allelic richness, H_E = expected heterozygosity) and wetland connectivity ($S_{i(Aj)}$ = Hanski's index including the size of source wetlands) of *Nymphoides fallax* populations in geographically isolated temporary wetlands in Mexican highlands.



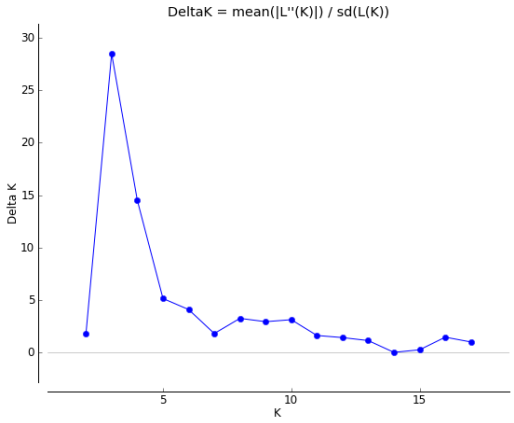


Figure S1. Details on Bayesian clustering analysis using Structure. Summaries of Delta K and L(K) ± 1 standard deviation produced in Structure Harvester. Results are based on a Structure analysis using (a) a burnin of 250,000 iterations and 1,000,000 MCMC iterations.

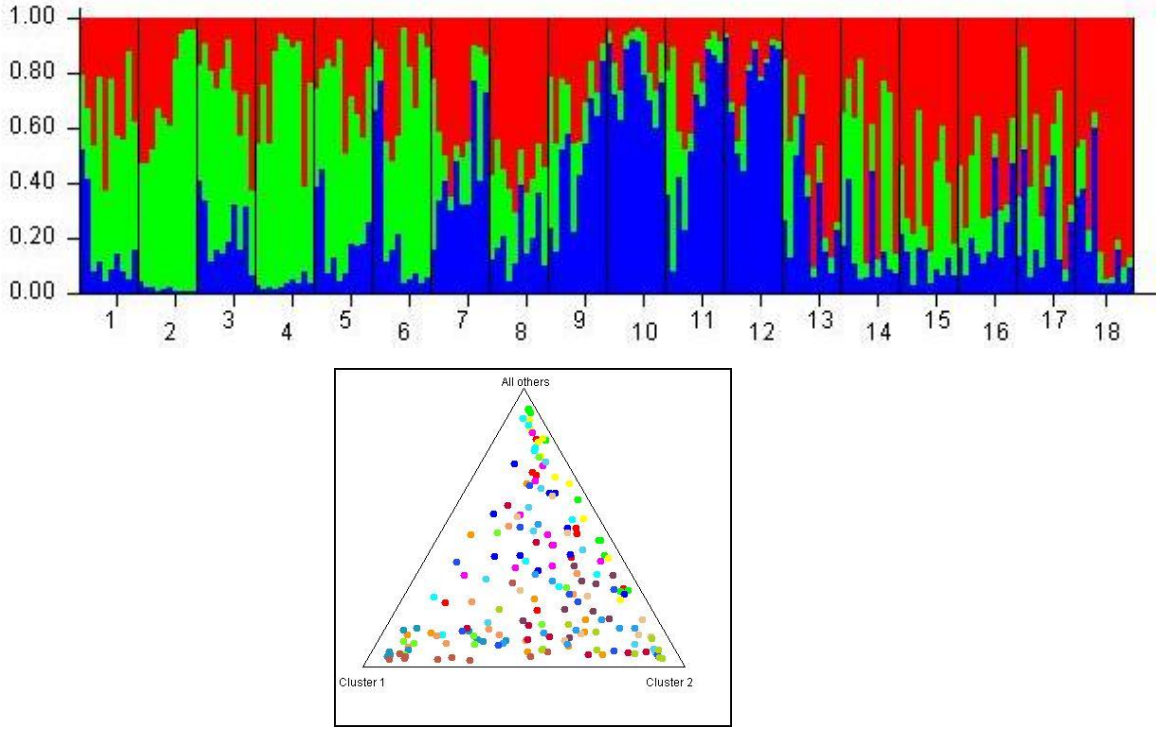


Figure S2. Bayesian clustering analysis results for *Nymphoides fallax* (K=3) in geographically isolated temporary wetlands of Mexican highlands. The sampled sites are ordered from 1 to 18 wetland. Colors represent assignment groups (K), and vertical bars represent individuals. The proportion of vertical bars represented by different colors indicate the probability of assignment of individuals to particular assignment groups (K).

Figure S3. Correlation between population-specific F_{ST} and forest cover for *Nymphoides fallax* in geographically isolated wetlands in Mexican highlands.

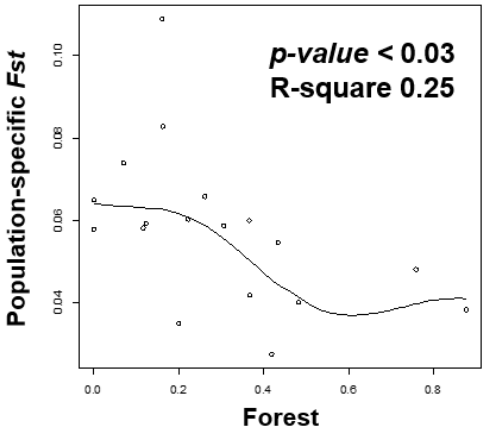


Table S1. *Nymphoides fallax* sampled in geographically isolated temporary wetlands in Mexican highlands, and landscape features (forest and water proportion).

Wetland	Genetic data	Plant species richness [a]	Latitude	Longitude	Elevation (m a.s.l)	Area (m^2)	Max depth (cm) [b]	Forest cover [c]	Water cover [d]
1	yes	36	22.1847	-102.0214	2004	12397	140	0.00	0.005
2	yes	18	22.1986	-102.0640	2058	16108	47	0.00	0.004
3	yes	35	21.8840	-102.8464	2382	6226	68	0.37	0.003
4	yes	34	22.1681	-102.4166	2031	2362	78	0.07	0.072
5	yes	37	22.1688	-102.5834	2580	1200	49	0.76	0.001
6	yes	27	22.1905	-102.6102	2613	1075	29	0.88	0.001
7	yes	33	20.8064	-101.0356	2303	26624	51	0.43	0.000
8	yes	19	20.8033	-101.0308	2276	9152	150	0.42	0.000
9	yes	33	21.3307	-102.0851	1975	6788	83	0.12	0.010
10	yes	17	21.3813	-102.1381	1992	7761	82	0.16	0.007
11	yes	10	20.3120	-100.2063	2359	6805	19	0.37	0.005
12	yes	11	20.3922	-100.2692	2318	34301	59	0.22	0.007
13	yes	18	20.4164	-100.2661	2266	9424	100	0.16	0.005
14	yes	16	20.3283	-100.1328	2222	23254	53	0.26	0.007
15	yes	19	20.3994	-100.2686	2324	35890	62	0.20	0.007
16	yes	20	20.2903	-100.1850	2314	3294	54	0.31	0.003
17	yes	13	20.2122	-100.1186	2577	1850	150	0.12	0.003
18	yes	15	21.4524	-103.4746	1961	2713	99	0.48	0.004
19	no	14	20.8089	-101.0339	2307	2328	40	0.44	0.000
20	no	10	22.1974	-102.0725	2078	5293	41	0.00	0.004
21	no	16	21.8885	-102.8437	2342	1981	113	0.38	0.003
22	no	16	21.8915	-102.8448	2345	1442	120	0.39	0.002
23	no	18	22.1588	-102.4848	2047	2740	49	0.25	0.067
24	no	16	21.3340	-102.0883	1964	1971	49	0.13	0.010
25	no	8	21.3756	-102.1353	2021	5124	54	0.16	0.007

[a] Source: Lobato-de Magalhães and Martínez (2018), [b] Data collected in the field using two perpendicular transects across the wetland, [c] Source: Inegi (2019), [d] Source: National Wetlands Inventory (Conagua 2019).

Table S2. Correlation coefficients between estimates of genetic diversity (N_e = effective number of alleles, A_R = Allelic richness, H_E = expected heterozygosity, H_O = observed heterozygosity) of *Nymphoides fallax* and wetland connectivity (IIC = integral index of connectivity, PC = probability of connectivity) using different threshold distances in geographically isolated temporary wetlands in Mexican highlands.

Distance threshold (km)	IIC				PC			
	N_e	A_R	H_E	H_O	N_e	A_R	H_E	H_O
0.5	0.40	0.27	0.26	0.30	0.49*	0.35	0.36	0.31
1	0.50*	0.36	0.38	0.31	0.56*	0.37	0.41	0.30
3	0.59*	0.49*	0.46*	0.28	0.54*	0.37	0.45	0.29
5	0.61*	0.60*	0.56*	0.31	0.43	0.35	0.49*	0.29
10	0.61*	0.60*	0.56*	0.29	0.37	0.29	0.49*	0.29
25	0.29	0.21	0.32	0.29	0.34	0.24	0.35	0.29
50	0.30	0.22	0.34	0.31	0.36	0.23	0.30	0.29
100	0.41	0.23	0.23	0.29	0.38	0.23	0.25	0.30
150	0.40	0.23	0.20	0.29	0.39	0.23	0.24	0.30
200	0.40	0.24	0.20	0.31	0.39	0.23	0.23	0.30
300	0.39	0.24	0.23	0.31	0.39	0.24	0.23	0.30
400	0.33	0.24	0.23	0.31	0.39	0.24	0.23	0.30

* *p-value* < 0.01

Diversidad florística y conectividad de humedales temporales de tierras altas en el centro de México

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CAPÍTULO 5 - Conclusiones

Humedal temporal, Querétaro, México. Fuente: propio autor

5 Conclusiones

Este proyecto de investigación doctoral acerca de la “Diversidad florística y conectividad de humedales temporales de tierras altas en el centro de México” presenta un aporte importante al conocimiento de la flora acuática y la conectividad de los humedales temporales de tierras altas en el centro de México.

A partir de los resultados encontrados fue posible conocer la flora acuática de humedales temporales de tierras altas en el centro de México, inclusive descubrir 20 nuevos registros de plantas acuáticas para estados mexicanos y dos especies de plantas acuáticas aún no descritas por la ciencia. Estos hallazgos denotan la falta de información para este ecosistema tan amenazado no solamente en México, sino a nivel global. De manera importante se demostró que la distancia geográfica no representa un factor relevante para la similitud de las comunidades de plantas en los humedales temporales, ni para explicar el flujo genético en poblaciones de *N. fallax*, una planta acuática típica de estos humedales temporales.

Los altos índices de diversidad genética y flujo de genes fueron resultados sorprendentes y que corroboraron las expectativas previas. En este proyecto de investigación doctoral se demostró que los humedales temporales, también caracterizados como ecosistemas aislados geográficamente, están funcionalmente conectados y que ciertos aspectos del paisaje (*e.g.* la cobertura de bosque) explican mejor la conectividad que la distancia geográfica.

Los índices de conectividad fueron considerados buenos indicadores de la diversidad genética para la especie estudiada, de manera que se recomienda considerar la conectividad estructural de los humedales como punto clave en los programas de conservación de ecosistemas acuáticos. Resalto también la importancia de expandir estudios de genética del paisaje en los humedales temporales considerando a otras especies, ya que la respuesta a los efectos del paisaje y conectividad puede ser diferente

para los diferentes organismos que habitan o utilizan los humedales temporales para su sobrevivencia.

En conclusión, los estudios de la conectividad de los humedales temporales y su diversidad de especies de plantas acuáticas son prioritarios para informar estrategias de conservación. Todavía hay pocos estudios en ecosistemas acuáticos neotropicales, especialmente con el uso de marcadores microsatélites nucleares y abordando el contexto del paisaje. El conocimiento y la conciencia de la diversidad genética de plantas en humedales temporales es uno de los primeros pasos para establecer políticas de conservación en estes ecosistemas tan peculiares y altamente amenazados en todas partes, y especialmente en las tierras altas mexicanas.

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