UNIVERSIDAD NACIONAL AUTÓNOMA

DE MÉXICO

POSGRADO EN CIENCIAS BIOMÉDICAS

INSTITUTO DE ECOLOGÍA

Evolución de sistemas reproductivos y la delimitación de especies en pinos piñoneros

TESIS

QUE PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS BIOMÉDICAS

PRESENTA

Lluvia Hilda Flores Rentería

Director de tesis: Dr. César Augusto Domínguez Pérez-Tejada

Dedico esta tesis a la maravillosa familia que tengo. A TODOS por cuidarme y ofrecerme sus brazos después del accidente del 11 de Noviembre de 2007 en SLP. Especialmente a mis hermanas, Yaahid y Aubanel, por su constante apoyo durante la realización de la tesis en todos los aspectos. A mis padres, César y Yolanda, por su constante amor. A Jean-Guillaume por ser un gran compañero y por aquietar mis crisis del Doctorado. A mi querida amiga la Dra. Judith Márquez por ser un ejemplo académica y personalmente. A todos ellos por creer en mí.

AGRADECIMIENTOS

Al posgrado en Ciencias Biomédicas de la Universidad Nacional Autónoma de México (UNAM).

Al Consejo Superior de Ciencia y Tecnología (CONACyT) por la beca otorgada para la realización de esta investigación (172682).

Al comité tutoral conformados por los Drs. César Domínguez Pérez-Tejada, Judith Márquez Guzmán y Francisco Molina Freaner, quienes me orientaron y apoyaron durante el desarrollo de esta investigación.

A los sinodales Dr. Luis Eguiarte, Dr. Pablo Vinuesa, Dr. Francisco Ornelas, Dr. Alberto Búrquez y Dr. Francisco Molina por todos sus comentarios y sugerencias al documento tesis.

Quiero agradecer al Dr. Daniel Piñero Dalmau por su apoyo, especialmente por su guía y entusiasmo para este estudio.

A los Drs. Amy V. Whipple, Cathering Gehring y Thomas Whitham por sus valiosos consejos y sugerencias durante el escritura de la tesis.

A Rubén Pérez Ishiwara quien contribuyó en el trabajo de campo y de laboratorio.

A mis amigos y colegas que me ayudaron en el trabajo de campo: Paula Sosenski, Artemisa Flores, Verónica Cepeda, Fernanda Baena, Oscar Jiménez, Hugo Carmona y Juan Manuel Hernández.

A Oscar Jiménez por su ayuda durante la extracción de DNA de semillas para calcular la tasa de entrecruzamiento, que por falta de variación en los loci nucleares no fue incluido en este trabajo.

A Alejandra Vázquez y Dra. Judith Márquez por interesarse e impulsar el proyecto "Functional bisporangiate cones in *Pinus johannis* (pinaceae), implications for the evolution of bisexuality in seed plants".

A Andrew Krown quien revisó y mejoró el artículo "A new approach to improve the scoring of mononucleotide microsatellite loci".

A Ana Wegier, Diego del Vecchyo, Alejandra Ortiz por su asesoría en el análisis de datos para el capítulo "Chloroplast markers revels phylogenetic relationships in four mexican pinyon pine species (subsection *cembroides*)".

Al Dr. Gustavo Escobedo por facilitarnos las instalaciones del INAOE en Cananea para hospedarnos durante el periodo de trabajo en Sonora.

A Patricia Martínez por su constante apoyo administrativo.

A Richard Brown del Mississippi Entomological Museum por su ayuda en la identificación de los herbívoros de *Pinus johannis*.

A toda la gente que de alguna manera hicieron aportaciones a esta investigación: Dr. Juan Fornoni, Dra. Karina Jiménez, M. en C. Violeta Méndez, Dra. Alejandra Moreno, M. en C. Lislie Solis, Dra. Jessica Pérez, Dr. César Abarca, Dr. Fernando Rosas, Dr. Rodolfo Salas, Dr. Luke Evans, Dra. Nashelly Meneses, Dr. Gary Allan y Biol. Valeria Alavez.

Quiero brindar un agradecimiento especial a la Familia Valadez de la Amapola, especialmente a la Sra. Anita, Sr. Luis, Sr. Bonifacio, Sra. María y a sus tres hijos Luis, David y Alejandro que nos alojaron durante los meses de trabajo en San Luis Potosí y que colaboraron de alguna forma en este proyecto.

Al Posgrado de Ciencias Biomédicas y al Consejo Nacional de Ciencia y Tecnología por el apoyo económico y las oportunidades brindadas.

A toda la gente que hace de la Universidad Nacional Autónoma de México un lugar de excelencia y crecimiento.

ÍNDICE

Resumen

El género *Pinus* posee un sistema reproductivo monoico, sin embargo existen reportes anecdóticos de individuos unisexuales en algunos pinos piñoneros (subsección *Cembroides*). La recurrencia de unisexualidad en esta subsección sugiere un origen común para la separación sexual en este clado, sin embargo, su clasificación ha sido controvertida. Uno de los objetivos de la tesis fue la delimitación de especies de pinos piñoneros con individuos unisexuales mediante el uso de marcadores de cloroplasto. El estatus de especie fue demostrado para *P. culminicola*, *P. discolor* y *P. johannis*, su relación filogenética sugiere un origen común de la unisexualidad en estas especies.

P. johannis presenta individuos hembra, macho y monoico en simpatría lo que proporciona una oportunidad para poner a prueba hipótesis de la evolución al dioicismo en gimnospermas, las cuales han sido escasamente estudiadas. Para contribuir al conocimiento de la evolución de la separación sexual en pinos se describió el sistema reproductivo y la proporción sexual en algunas poblaciones de *P. johannis* y se comparó con su especie hermana *P. discolor*. Adicionalmente, en la primera se evaluó la estabilidad de los individuos unisexuales y las hipótesis de evolución al dioicismo mediante: la detección de cambios en la expresión sexual durante cinco años, la presencia de características sexuales secundarios y la manipulación de disponibilidad de recursos por medio de la remoción de herbívoros. También, se realizó un estudio comparativo entre *P. johannis* y *P. edulis*, la última siendo la única especie de *Pinus* con labilidad sexual comprobada. Se realizaron cruzas manuales en campo y se comparó el éxito reproductivo así como la intensidad de la herbivoría entre los individuos unisexuales y monoicos. Los individuos de *P. johannis* se agrupan en los que son funcionalmente femeninos y los que son funcionalmente masculinos, por lo tanto *P. johannis* posee un sistema funcionalmente dioico, aunque 1% de los individuos monoicos produjo gran cantidad de megaestróbilos, microstróbilos y estróbilos bisporangiados viables. Se encontró un sesgo en las proporciones sexuales de *P. johannis*. Ésta presenta un patrón bimodal en la expresión sexual mientras que *P. edulis* presenta una transición gradual. Estos resultados en conjunto sugieren la estabilidad de los individuos unisexuales. La depresión por endogamia y las presiones de la herbivoría pueden ser factores importantes en la evolución a la separación sexual en pinos piñoneros. La viabilidad y la distribución especial de los órganos femeninos y masculinos de los estróbilos bisporangiados y su alta frecuencia en diferentes especies de gimnospermas sugiere un mecanismo común para la producción de estructuras bisporangiadas en las plantas con semillas. La carencia de estructuras bisporangiadas como una característica de especies en gimnospermas puede deberse a que la separación sexual se seleccionó debido al grado de depresión por endogamia.

Abstract

Pinus have a monoecious reproductive system, however, there are anecdotic reports of unisexual individuals in some pinyon pines (subsection Cembroides). The recurrence of unisexuality in this subsection suggest a common origin for the sexual separation in this clade, however, its classification has been controversial. One goal of the present thesis was to delimitate the pinyon pine species with unisexual individuals using chloroplast markers. The species status was demonstrated for *P. culminicola*, *P. discolor* and *P. johannis*, its phylogenetic relationship suggests a common origin for the unisexuality in these species.

P. johannis presents female, male and monoecious individuals in sympatry, this represent an opportunity to test evolutionary hypothesis to dioecy in gymnosperms which have been poorly explored. In order to contribute to the knowledge of the evolution to dioecy in pines, the reproductive system and the sexual ratio in *P. johannis* and *P. discolor* was described. In *P. johannis*, the unisexual stability and the hypotheses to dioecy were evaluated by: the detection of sexual expression change through five years, the presence of secondary sexual features and the manipulation of resource availability by the herbivore removal. In addition, a comparative study was done between *P. johannis* and *P. edulis*, the latter being the closest species with studies proving the sexual lability. Manual crosses in the field were done and the reproductive success was compared among unisexual and monoecious individuals. *P. johannis* individuals were grouped in functionally male and functionally female, therefore it has a functional dioecious reproductive system, although 1% of individuals produced high amount of both megastrobili and microstrobili and viable bisporangiate strobili. A female bias was found in the sex ratio in *P. johannis*. It had a bimodal sexual expression pattern whereas *P. edulis* had a gradual transition. Altogether, these results suggest unisexual stability in *P. johannis.* Inbreeding depression along the herbivory pressures can be factors involved in the evolution to sexual separation in pinyon pines. The viability and spatial distribution of female and male organs of bisporangiate cones and their frequent occurrence in gymnosperms suggest a common mechanism in all seed plants for the production of bisporangiate structures. The lack of bisporangiate structure in gymnosperms may be primarily due to selection to avoid inbreeding.

INTRODUCCIÓN

Pinus discolor fue descrita por Bailey y Hawksworth (1983) como la única especie de *Pinus* cercana a ser completamente dioica, aunque de manera anecdótica. Ávila *et al*. (1992) describieron un gradiente en la proporción de individuos unisexuales en esta especie, con poblaciones norteñas, en Chihuahua y Durango, completamente dioicas y al sur, en San Luis Potosí, poblaciones con mayor proporción de individuos monoicos. La presencia de mayor número de individuos monoicos al sur sugiere que esta especie se encuentra en la transición hacia la unisexualidad. Pocas especies de plantas, en particular de gimnospermas, presentan poblaciones con individuos unisexuales e individuos monoicos (Givnish, 1980). La posibilidad de estudiar la evolución del dioicismo en una especie en transición, que además permita la comparación de diferentes hipótesis evolutivas de la unisexualidad (explicadas más adelante), no solo entre poblaciones con diferentes proporciones de individuos unisexuales y monoicos, sino además realizar comparaciones entre individuos unisexuales y monoicos en simpatría, es muy escasa. Todas estas características que posee *P. discolor* brindan la oportunidad de estudiar la evolución a la unisexualidad, no sólo en el género, sino en todas las gimnospermas, que pese a su elevado número de especies con sistemas reproductivos dioicos (52%, Givnish, 1980), pocos estudios han sido generados en relación a la ecología y evolución de sus sistemas reproductivos. Así, el propósito inicial de esta tesis fue estudiar la evolución de la transición del monoicismo hacia el dioicismo. Sin embargo, durante nuestro trabajo en campo observamos que las proporciones sexuales diferían de lo reportado. Esta diferencia pudo deberse a que Ávila *et al.* (1992) realizaron sus observaciones en noviembre, mientras que nosotros realizamos las observaciones en mayo y junio que es el periodo de reproducción para esta especie, por lo que el conteo de las estructuras reproductivas se realizó de manera directa. Así uno de los primeros objetivos que nos planteamos fue la descripción del sistema reproductivo de *P. discolor*.

La segunda complicación que encontramos fue de naturaleza taxonómica, ya que algunos autores consideran a *P. discolor* como sinónimo de *P. johannis* o *P. cembroides,* todas estas especies con reportes de poblaciones con individuos unisexuales (Bailey y Hawksworth, 1983; Rober-Passini, 1978; McCormick y Andresen, 1963). Para delimitar nuestra especie de estudio y conocer el origen y distribución de la unisexualidad, nos propusimos realizar un estudio con aproximaciones taxonómicas con el objetivo de responder si estos taxa son sinónimos o son especies válidas. Sin embargo, todos los marcadores moleculares reportados previamente para especies del género *Pinus* fueron monomórficos o poco variables en este complejo; por ello, fue necesario desarrollar nuevos marcadores polimórficos para estos taxa.

Durante la descripción de los sexos encontramos cinco morfos sexuales y no tres como había sido descrito por Bailey y Hawksworth (1983) y Ávila *et al*. (1992), estos fueron:

Dos puramente unisexuales
- -hembras
- -machos

Tres monoicos
- -monoicos predominantemente hembra,
- -monoicos predominantemente macho y
- -monoicos que producen conos femeninos (megastróbilos), conos masculinos (microstróbilos) y conos bisexuales (estróbilos bisporangiados).

Debido a la incongruencia en los datos previamente reportados y a lo observado en nuestros datos el objetivo de esta tesis fue estudiar de manera comparada la reproducción sexual en algunas especies de la subsección *Cembroides*, en particular la expresión sexual en el complejo *P. johannis-P. discolor*. Con el propósito de entender los patrones de distribución de especies con individuos unisexuales en la subsección *Cembroides* se delimitó el complejo *P. johannis-P. discolor* y sus relativos *P. culminicola* y *P. cembroides*. Se describió el sistema reproductivo de *P. johannis.* La presencia de individuos monoicos con tendencia hacia un sexo nos llevó a evaluar si éstos se comportan funcionalmente como individuos

unisexuales. Por otro lado, evaluamos la estabilidad de los individuos unisexuales de *P. johannis* y comparamos la expresión de la sexualidad de ésta contra *P. edulis* que es una especie con individuos unisexuales lábiles y con un gradiente en la expresión sexual, que va desde completamente hembra hasta completamente macho atravesando por diferentes proporciones sexuales en los individuos monoicos. Se infirió la ruta de evolución al dioicismo en *P. johannis*. Una vez determinada la estabilidad de los individuos unisexuales evaluamos las principales hipótesis de evolución a la unisexualidad. Adicionalmente, la rareza de estructuras bisexuales en gimnospermas nos condujo a describir la viabilidad de los estróbilos bisporangiados en *P. johannis* y se realizó una revisión de la presencia de estróbilos bisporangiados en gimnospermas.

ANTECEDENTES

El género *Pinus* pertenece a la familia Pinaceae, que a su vez forma parte de las 6 familias de coníferas (gimnospermas). Este género posé más de 100 especies y es el género viviente más grande dentro de las coníferas y uno de los más diversos dentro de las gimnospermas (Price & al., 1998; Farjon, 2001). Los pinos son un componente dominante de diversos ambientes (boreal, subalpino, templado, tropical, así como de bosques áridos) (Richardson & Rundel, 1998). Económicamente, los pinos son una fuente importante de madera, papel, resinas, carbón vegetal y alimentos (en especial algunas semillas), ornamentos etcétera (Le Maitre, 1998). La distribución del género *Pinus* se restringe al hemisferio norte, excepto por *P. merkusii*, la cual tiene poblaciones por debajo del ecuador en la región de Malesia (Mirov, 1967). Diferentes factores históricos, ecológicos y genéticos han interactuado para delimitar la distribución de cada especie de pinos.

Durante el Cretácico temprano (alrededor de 130 millones de años) los pinos se diversificaron en dos subgéneros, *Strobus* (haploxylon o pinos blandos), con un haz fibrovascular en las acículas, y *Pinus* (diploxylon o pinos duros), con dos haces fibrovasculares en las acículas (Mirov 1967, Richardson & Rundel 1998). Varias secciones (e.g. *Strobus* y *Pinus*) y subsecciones (e.g. Pinaster y Cembroides) han evolucionado desde la diversificación de estos dos subgéneros (Gernandt *et al*., 2005; ver Tabla 1).

Table 1. Subsections, sections and subgenus into *Pinus,* according with Gernandt *et al*. (2005).

Subgénero	Seccion	Subsección
Pinus	Pinus	Pinaster Pinus
	Trifoliae	Australes Contortae Ponderosae
Strobus	Quinquefoliae	Gerardianae Krempfianae Strobus
	Parrya	Balfourianae Cembroides Nelsoniae

México es una de los centros de diversidad del género *Pinus* con al menos 51 especies (Perry, 1991). Sin embargo, debido a la constante descripción de subespecies o taxa, por otros autores elevados al rango de especies, este número varía dependiendo del autor. La descripción de nuevos taxa se ha dado por el uso de caracteres químicos, como monoterpenos, o caracteres moleculares y poco por el uso de caracteres morfológicos. El carácter morfológico más representativo para la identificación de especies es el cono portador de semillas en etapa madura. Sin embargo, muchos caracteres, sin importar su naturaleza, presentan homoplasia como los granos de polen, número de acículas por fascículo, terpenos, etc. En el caso de los caracteres morfológicos la plasticidad fenotípica puede ser un factor de confusión para identificación de especies (Farjon y Styles, 1997). Así la delimitación de especies ha sido un problema entre taxónomos en lo referente al género *Pinus* (Farjon y Styles, 1997). La mayoría de

"especies conflictivas" pertenece a 3 tres grupos que son mayoritariamente endémicos a México; los pinos de la subsecciones *Cembroides, Ponderosae y Oocarpae* (Richardson, 1998). De acuerdo con Perry (1991) 32 especies son consideradas endémicas a México; y un gran número de ellas se encuentran bajo alguna categoría de tratamiento especial para la conservación y protección de especies como son: *P. culminicola, P. maximartinezii, P. pinceana, P. johannis, P. lagunae, P. rzedowskii, P. nelsonii,* etc.

Reproducción, diversidad de sistemas reproductivos en pinos y la delimitación de especies

Las especies del género *Pinus* producen principalmente dos tipos de conos, conos ovulados (megastróbilos o conos femeninos) y conos productores de polen (microstróbilos o conos masculinos). El tiempo de desarrollo de estos conos depende de la especie. En algunos pinos los conos ovulados son receptivos en mayo o junio y los granos de polen son liberados en el mismo periodo. A este periodo se le conoce como periodo de polinización. La receptividad de los óvulos se puede llegar a visualizar por la secreción que es producida por los óvulos llamada gota de polinización, la cual se ha demostrado está involucrada en la captura de granos de polen por un mecanismo hidráulico (Owens *et al*., 1998; Gelbart y Aderkas, 2002). Una vez que el grano de polen alcanza el micrópilo, el tubo polínico se desarrolla conteniendo las células que darán origen al gameto masculino. El desarrollo del tubo polínico de detiene generalmente antes de entrar el primer invierno. Así la fecundación ocurre un año después al periodo de polinización (Williams, 2009). El desarrollo de las semillas varía de unos meses, como en las especies de la subsección *Cembroides*, o hasta más de un año (Biswas y Johri, 1997). La polinización es estrictamente anemófila. La dispersión de las semillas es principalmente anemófila, sin embargo existen algunas especies en las que la dispersión es por zoocoría, realizada generalmente por aves o roedores (Richardson, 1998).

De acuerdo con Givnish (1980), en gimnospermas, hay una correlación entre el tipo de dispersión de las

semillas y el sistema reproductivo, semillas dispersadas por viento están asociadas al sistema reproductivo monoico (48% de las especies), mientras que semillas dispersadas por animales están asociadas al sistema reproductivo dioico (52% de las especies). El sistema reproductivo de las especies del género *Pinus* está reportado como monoico (Mirov, 1967), es decir, los megaestróbilos y microestróbilos se encuentran dentro de un mismo individuo pero separadas entre sí, lo que corresponde con la dispersión predominantemente anemófila. Sin embargo, existen algunos reportes describiendo especies del género *Pinus* con sistema dioico en el cual los megaestróbilos y los microestróbilos se encuentran en individuos diferentes (individuos unisexuales). Floyd (1983) notó que en algunas especies de pinos piñoneros (subsección *Cembroides*) que presentan individuos unisexuales, también presentan semillas con alas reducidas que son dispersadas por animales, cumpliendo así con las predicciones de Givnish (1980). La distribución de los sistemas reproductivos en *Pinus* se observa en la figura 1. Existe una tendencia de poblaciones con individuos unisexuales en especies de pinos de la subsección *Cembroides* como son: *P. edulis, P. cembroides, P. culminicola, P. discolor* y *P. johannis.* Aunque algunas especies de pinos piñoneros son taxa claramente definidos taxonómicamente como *P. culminicola, P. maximartinezii, P. pinceana, P. rzedowskii, P. nelsonii*, otras son difíciles de distinguir (Richardson, 1998). Por ejemplo, la validez de *P. discolor* y *P. johannis* ha sido disputada entre los taxónomos desde varias décadas. Para algunos autores, *P. discolor* tiene sinonimia con *P. cembroides* subsp. *cembroides* var. *bicolor* Little; o con *P. culminicola* var. *discolor* (Bailey y Hawksworth) Silba (Farjon y Styles, 1997). Otros rechazan la existencia de este taxón, Farjon y Styles (1997) lo consideran, junto con *P. johannis* Robert-Passini, como una variedad de *P. cembroides* (var. *bicolor* Little) mientras que en el otro extremo Perry (1991) y Price *et al* (1998) lo consideran como una especie válida. Passini (1994), considera a *P. discolor* como sinónimo de *P. johannis*. Silba (1986) considera a *P. discolor* y *P. johannis* como variedades de *P. culminicola,* colocándolos más cercanos a *P. culminicola* que a *P. cembroides* un hecho que fue demostrado posteriormente por Malusa (1992). De acuerdo con Zavarin y

Snajberk (1986) existen fuertes diferencias químicas entre *P. discolor* y *P. johannis* que no se reflejan morfológicamente. Tales como la producción de monoterpenos relacionados al sabineno por parte de *P. discolor,* mientras que *P. johannis* produce α-pineno con monoterpenos relacionados al sabineno en cantidades mínimas. Aunque el significado taxonómico de esta investigación ha sido cuestionado por Farjon y Styles (1997), hay quienes establecen que la producción de terpenos puede ser tan útil, o aún más que un marcador molecular. En la tabla 3 se muestran diferencias morfológicas entre *P. discolor, P. johannis* y *P. cembroides*, en el que la característica más evidente para separar al complejo *P. discolor-P. johannis* de *P. cembroides* es la presencia de estomas en la cara abaxial de la acícula en esta última especie. Los trabajos realizados con secuencias de cloroplasto muestran una tricotomia irresuelta entre *P. discolor, P. johannis* y *P. culminicola* (Gernandt *et al,* 2003). Por lo tanto para entender los patrones de unisexualidad en *Pinus*, es necesario primero delimitar el rango de especies, en particular de las especies que presentan dioicismo como en son algunas especies de la subsección *Cembroides*.

Tabla 3. Diferencias morfológicas entre *Pinus discolor, P. johannis* y *P. cembroides.* Tomada de Zavarin y Snajberk, 1986.

Atributo	*Pinus johannis*	*Pinus discolor*	*Pinus cembroides*
Acículas			
• No. x fascículo	3	2.7-3.8	2-3
• Longitud (cm)	3-5	2.5-4	2.5-5.5
• Grosor (mm)	0.9-1.2	0.8-1	0.7-1
• Estomas en cara abaxial	No	No	Si
• Estomas en cara adaxial	Si	Si	Si
• No. canales resiníferos	2	2	2
• No. de cotiledones	6-11	----	10.5-11.2
Conos maduros cerrados			
• Long. pedúnculo (cm)	0.3-0.4	----	----
• Long. del cono (cm)	3-4.4	2-3	----
• Diámetro del cono (cm)	2.2-3.2	2-2.5	----
Semilla			
• Color del endospermo	Blanco	Blanco	Rosa
• Grosor cubierta (mm)	1.04-1.29	0.8-1	0.79-1.15
• Long. de la semilla (mm)	----	10-13	13-14
• Ancho de la semilla (mm)	8-13	7-10	7.5-9
Altura del árbol (m)	1-4	4-9	5-15

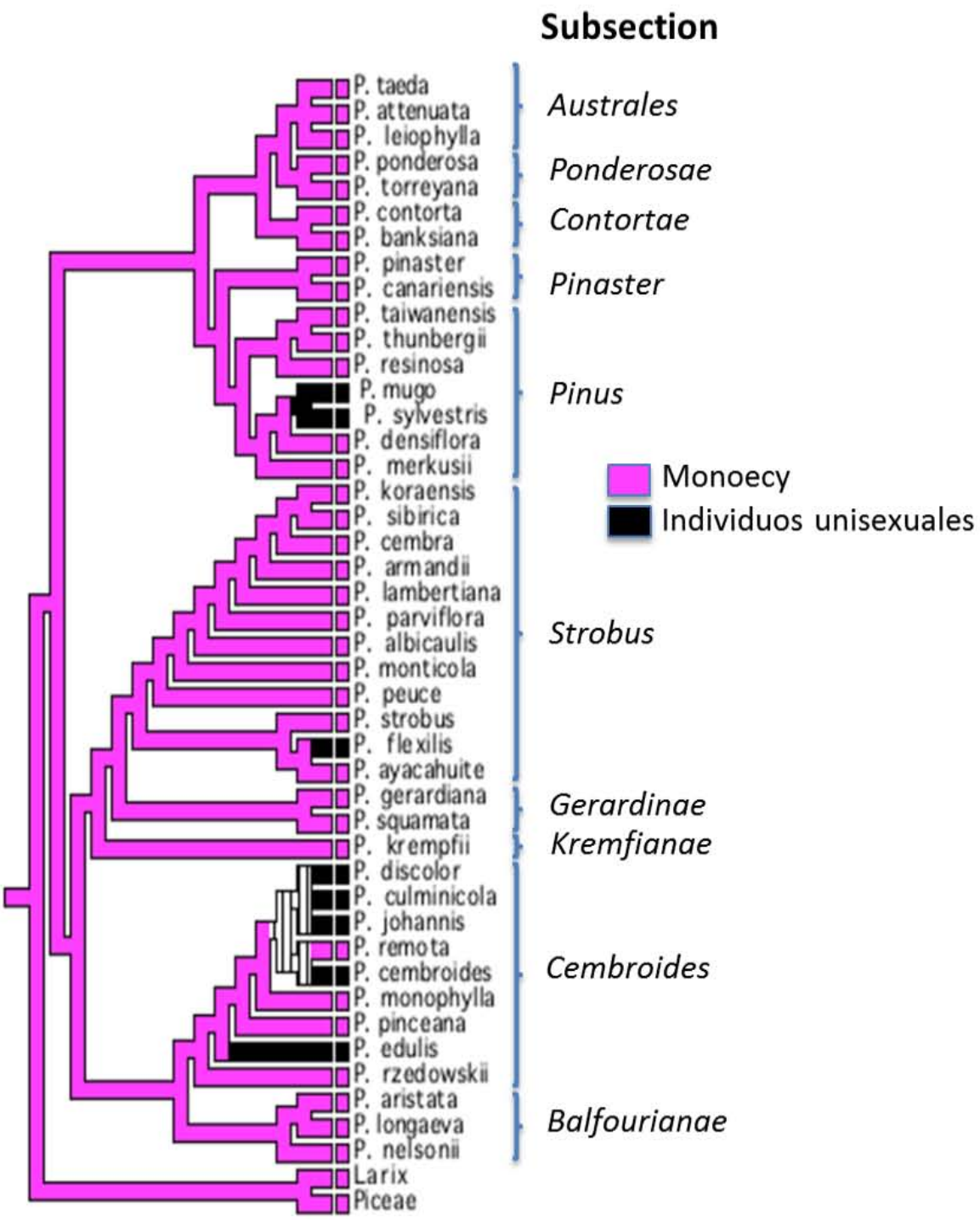


Figura 1. Filogenia compuesta del género *Pinus* basada en Gernandt *et al*. (2005) y Parks *et al*. (2009) representando especies con poblaciones que presentan individuos unisexuales (negro) y monoico (rosa).

Estabilidad de los individuos unisexuales en *Pinus*

La unisexualidad se considera estable si una vez decidido el sexo los individuos no cambian de sexo; en contraste la labilidad sexual se caracteriza por la diferente expresión sexual asociada a factores ambientales. Así un individuo que cambia de sexo en el tiempo se considera secuencialmente hermafrodita o monoico. Debido a que los pinos pueden variar la inversión de los recursos a las diferentes funciones sexuales en relación a cambios en diferentes factores ambientales como el estrés, la edad y la herbivoría generando individuos que producen predominantemente un sexo, los individuos unisexuales se han asumido bajo un sistema sexualmente lábil. Sin embargo, pocos estudios se han realizado para determinar la estabilidad de los morfos unisexuales. Todos ellos en *Pinus edulis*, de acuerdo con Floyd (1985) los morfos unisexuales no son estables y un individuo cambia de sexo con relación a la edad del mismo. De acuerdo con la autora los individuos más jóvenes son machos (sólo producen microestróbilos), posteriormente se convierten en hembras (sólo producen megaestróbilos) y finalmente producen ambas estructuras reproductivas. Estudios más recientes sugieren que la existencia de individuos unisexuales y en particular la presencia de machos en *P. edulis* está mediada por un efecto de la herbivoría, en la que una polilla (*Dioryctria albovittella*) ataca las ramas más gruesas las cuales sostienen a las estructuras reproductivas femeninas; este ataque preferencial tiene como consecuencia la modificación de la expresión sexual de árboles monoicos, reduciendo la función femenina y aumentando la función masculina, pero no explica la presencia de individuos femeninos (Cobb *et al.* 2002). Otros estudios son anecdóticos y especulan las causas que genera la presencia de individuos unisexuales, ellos están basados en correlaciones ambientales que sugieren la unisexualidad de estas especies esta asociada a factores ambientales, sin embargo la estabilidad o labilidad de los sexos no ha sido directamente evaluada. En *P. sylvestris* existen 6 diferentes morfos sexuales: femenino, masculino, predominantemente femenino, predominantemente masculino, monoico y no reproductivo. Tikhovona (2003) determinó que la estructura sexual de la población es muy importante para la

adaptación a condiciones ambientales extremas. Así las condiciones ambientales deterioradas determinan el aumento de árboles machos en *P. sylvestris*, además lo machos tienen acículas más cortas y redondas con muchos estomas en la superficie que sugiere su adaptación a ambientes extremos. Andressen y Beaman (1961) reportaron que *Pinus culminicola* exhibe dioicismo probablemente debido al estrés ambiental al que están sujetos al igual que *P. flexilis* (Kiener, 1935). En otros reportes menos detallados únicamente se describe la presencia de individuos unisexuales. *P. mugo* (sinónimo a *P. montana*) se describió con un sistema reproductivo dioico (Schroeter, 1926 en Kiener, 1935), de acuerdo con Andressen y Beaman (1961) ésta no tiene individuos unisexuales sino predominantemente masculinos o femeninos. *P. cembroides* tiene poblaciones (Montañas Chiricahuas) reportadas como subdioicas, en las cuales coexisten individuos que portan microestróbilos (46.2%), individuos que portan megaestróbilos (51.7%) y una baja proporción (2.1%) de individuos monoicos (McCormick y Andresen, 1963). Sin emgargo, Zavarin y Snajberk (1986) sitúan a *Pinus discolor* en lugar de *P. cembroides* en esta región. Existen otros reportes que proponen que *Pinus discolor* tiene sistema de apareamiento dioico en poblaciones más norteñas, mientras que hacia el sur se encuentran tanto el sistema dioico como el monoico (cuadro I). Es decir, las frecuencias sexuales de *P. discolor* cambian con respecto al gradiente de su distribución (Ávila *et al.* 1992), así esta especie presenta interesantes cualidades para el estudio de la estabilidad y evolución de la unisexualidad en *Pinus.* Sin embargo, estos autores realizaron sus observaciones en otoño, fecha en la que esta especie tiene conos maduros y no durante la formación de los microestróbilos y megaestróbilos (primavera) que es la época más adecuada para poder identificar el sexo de los individuos. Estudios más detallados sobre las frecuencias sexuales y su variación en el tiempo son necesarias para describir el tipo de sistema reproductivo en *P. discolor* (considerada como *P. johannis* o *P. cembroides* dependiendo del autor).

Tabla 2. La distribución de *Pinus discolor* y la frecuencia de los morfos. Tomado y modificado de Ávila *et al.*1992.

Distribución de *Pinus discolor*	Hembra	Macho	Monoico
al suroeste de Nuevo México y sureste de Arizona	51.7	46.2	2.1
al sur de Chihuahua y noroeste de Durango	>	<	0
al suroeste de San Luis Potosí	46.2 50.6 52 52.5	43.6 46.2 48 47.5	10.2 3.2 0 0
en el centro-oriente de Querétaro	55	45	0

Hipótesis de evolución a la separación sexual

Dos hipótesis explican principalmente la evolución a la unisexualidad, una hipótesis versa sobre la asignación diferencial de recursos que optimiza las funciones femeninas o masculinas (Webb, 1999). Por otro lado, para explicar el origen de la especialización sexual se ha postulado que la principal ventaja es evitar la endogamia y los efectos deletéreos de la depresión por endogamia (Charlesworth, 1999). Aunque estás hipótesis no son mutuamente excluyentes pocos trabajos las han evaluado simultáneamente. La primera hipótesis puede ser evaluada comparando la asignación de recursos a las funciones femeninas y masculinas entre los individuos unisexuales y monoicos. Bajo la segunda hipótesis se espera que los individuos monoicos presenten mayor depresión por endogamia debido a los alelos recesivos deletéreos que se expresados en homocigosis bajo autofecundación o cuando ocurre apareamiento con relativos. En *Pinus* ninguna de estas hipótesis ha sido evaluada para determinar

explícitamente las causas que generan la especialización sexual, sin embargo una mezcla de factores ambientales y genéticos parece determinar la expresión de la unisexualidad en *P. edulis* aunque esta posea un sistema sexualmente lábil. La herbivoría crónica en *P. edulis*, causada por la polilla *Dioryctria albovittella*, altera la expresión sexual de un árbol monoico reduciendo la expresión femenina y aumentando la masculina en árboles susceptibles a la polilla, sugiriendo que la asignación de recursos a la defensa decrece la función femenina (Cobb *et al*, 2002). Por otro lado existe evidencia de que la depresión por endogamia en los individuos monoicos opera en diferentes estados de desarrollo. La comparación en la viabilidad de semillas y su germinación ha sido evaluada en *P. edulis* (Floyd, 1983), en ambas características las hembras fueron más exitosas comparadas contra los individuos monoicos. Adicionalmente las semillas de árboles femeninos desarrollaron radículas más largas que las de árboles monoicos. Tales diferencias pueden ser debidas a la endogamia que presentan los árboles monoicos. En *P. edulis* árboles experimentalmente autofecundados produjeron 14.4% semillas viables mientras que los árboles entrecruzados produjeron 90.5% semillas viables (Lanner, 1980). Sin embargo en ningún otra especie existen datos que expliquen las presiones de especialización sexual en el género *Pinus*. Ya que *P. edulis* es la única especies de pinos que cuenta con estudios suficientes con relación a la expresión de la unisexualidad, es necesario comparar la expresión sexual de esta especie contra la expresada en otras especies de pinos con individuos unisexuales, en particular en las especies de la subsección *Cembroides* a la cual pertenece *P. edulis* con el propósito de entender la evolución a la especialización sexual en *Pinus*.

Los pinos están considerados dentro de los organismos genéticamente más variables (Cornelius, 1994; Hamrick *et al.* 1979, Hamrick and Godt 1990; Delgado, 2002). La mayoría presenta altas tasas de entrecruzamiento (Schemske and Lande, 1985). En *Pinus* esto se explica por mecanismos estructurales, temporales o genéticos que evitan la endogamia, tales como monoecismo, dicogamia o incompatibilidad genética (Zinder *et al*, 1977; Wang, 1977; Owens *et al*, 1981; Matziris, 1994; Owens *et al.* 1998) así como

por altos grados de depresión por endogamia actuando en la embriogénesis temprana (Lanner, 1980; Koski 1971, Kärkkäinen & Savolainen 1993). En plantas la depresión por endogamia puede actuar en diferentes etapas del desarrollo o etapas de la vida de un organismo afectando diferentes componentes de la adecuación como la tasa de germinación, el crecimiento y el tamaño de la planta, la cantidad y calidad de las semillas (Charlesworth and Charlesworth 1987; Husband and Schemske 1996).

Estróbilos bisporangiados en *Pinus* y otras gimnospermas

Las gimnospermas están descritas como dioicas o monoicas (Givnish, 1980). Sin embargo algunas gnetales son bien conocidas por la presencia de estructuras bisexuales. La presencia de estructuras como flores (bisexuales) con estructuras femeninas al centro rodeadas por estructuras masculinas, entre otras características, llevó a la agrupación de gnetales, angiospermas, junto con el grupo de fósiles de las bennettitiales, bajo la hipótesis antofita (Doyle y Donoghue, 1986). Posteriores evidencias con datos moleculares revelan que las gnetales pertenecen al grupo de las gimnospermas. Adicionalmente, existen múltiples reportes de especies capaces de producir estróbilos bisporangiados dentro de las coníferas. Sin embargo poca información se ha generado con relación a la viabilidad de estas estructuras.

CAPÍTULO 1

CHLOROPLAST MARKERS REVELS PHYLOGENETIC RELATIONSHIPS IN FOUR MEXICAN PINYON PINE SPECIES (SUBSECTION *CEMBROIDES*)

Chloroplast markers revels phylogenetic relationships in four Mexican pinyon pine species (Subsection *Cembroides*)

Flores-Rentería, L. [a,b*], Wegier A. [a,c], Ortega Del Vechyo D. [a,d], Piñero D. [a], Whipple, A. [b], Molina-Freaner [e], F. and C. A. Domínguez [a]

[a]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, AP 70-275, Coyoacán DF 04510, México.

[b]Current address: Department of Biological Sciences and Merriam-Powell Center for Environmental Research, Northern Arizona University, Flagstaff, AZ 86011, USA.

[c]Current address: CENID-COMEF, Instituto de Investigaciones Forestales, Agrícolas y Pecuarias, Progreso 5, Coyoacán, 04010, DF, México.

[d]Current address: Interdepartamental Program in Bioinformatics, University of California, Los Angeles, USA.

[e]Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México, Apartado Postal 1354, Hermosillo, Sonora 83000, México.

Key words: Chloroplast markers *Pinus cembroides*, *Pinus culminicola*, *Pinus discolor*, *Pinus johannis*, ancestral polymorphisms, phylogeny, haplotype network.

*Author for correspondence (e-mail: lluvia.flores@nau.edu, lluviafloresr@hotmail.com), phone:+ (928) 523-9138

INTRODUCTION

While most recent phylogenetic inferences have been based on molecular data, most only use a few markers and one to few individuals per species. (e.g. Gernandt *et al*. 2005). Organelles have a more recent coalescent time than nuclear markers (Moore, 1995; Yuan and Olmstead, 2008).

Because of this one would expect clear lineages with cpDNA but not with nuclear DNA. Non-recombining DNA sequences have been proposed to be useful in delineating phylogenetic species (Brower 1999). Despite low resolution, this approach can be applied in a preliminary way by determining whether groups in the cpDNA trees corroborate with *a priori* hypotheses of species boundaries in phylogenetic studies. This approach has been successfully tested in numerous studies, especially in differentiating cryptic species, which are morphologically indistinguishable (e.g. Hebert *et al*. 2004; Heinrichs *et al*. 2010). According to Davis (1996) relationships above the species level are of a hierarchical nature (hence traditional phylogenetic methods can be used for tree reconstruction). However, erroneous conclusions can be made when ancestral polymorphisms are shared among species. Thus, relationships below the species level are tokogenetic, and nonhierarchical network-based approaches are more appropriate (Davis 1996; Posada and Crandall 2001).

Pinus is one of the most diverse genera of extant gymnosperms, with approximately 110 species distributed throughout the Northern Hemisphere (Price *et al*. 1998). Resolution of the phylogenetic relationships of this genus has been challenging; *Pinus* contains numerous unresolved groups based on cpDNA (Eckert and Hall, 2006; Gernandt *et al.* 2005; Wang *et al.* 1999) or nuclear genes (Liston *et al.* 1999; Syring *et al.* 2007), especially at low taxonomic levels (Gernandt *et al.* 2003). Phylogenetic hypotheses present discrepancies when using plastid or nuclear DNA (Syring *et al.* 2007; Palmé *et al.* 2009). For example, incomplete lineage sorting, which is the persistence and retention of ancestral polymorphisms through multiple speciation events, has been determined to be the most probable source of widespread allelic nonmonophyly at nuclear loci in species of *Pinus*, especially in subgenus *Strobus* (Syring *et al.* 2007; Willyard *et al*. 2009). High levels of nuclear gene duplication in *Pinus* complicates working with

orthologous genes, as has been shown using nrDNA ITS (internal transcriber spacer) regions (Gernandt *et al.* 2001). Major events of DNA duplication have been probed in *P. taeda,* which revealed copies of retrotransposons that equal the size of the entire *Arabidopsis* genome (Morse *et al*. 2009). In addition, intraspecific hybridization, would distort phylogenetic relations, has been widely documented in *Pinus* (Delgado *et al*. 2007; Liston *et al.* 2007; Willyard *et al*. 2009; Jasinska *et al*. 2010).

One of the groups with more systematic troubles has been Subsection *Cembroides* (Section *Parrya*) in which relationships are poorly resolved (Gernandt *et al.* 2001; Gernandt 2003). Some species in this subsection exhibit less interspecific morphological variation, and as a consequence, taxonomic difficulties in this group often relate to species delineation (Malusa, 1992). The considerable variation among taxa in cone and seed morphology has been largely shunned in taxonomic comparisons because of variability within taxa and overlap between taxa (Bailey and Hawksworth 1987). Molecular phylogenies have been unable to resolve phylogenetic relationship into this group (Liston *et al*. 1999; Gernandt *et al*. 2001; Gernandt *et al*. 2003; Syring *et al*. 2005). One of the most challenging in this group has been the trichotomy of *P. discolor*, *P. johannis* and *P. culminicola* when using the chloroplast markers *matK* and *rbcL* (Gernandt *et al.* 2001; Gernandt 2003). *Pinus discolor* was first classified as *P. cembroides* subs. *cembroides* var. *bicolor* Little (1968) and described as a small pinyon in northern Mexico and the southern U.S. with dark green dorsal leaf surfaces lacking stomata and a bright white ventral surface. It also had smaller seeds and cones than *P. cembroides* (Little 1968). Bailey and Hawksworth (1979) elevated the rank of *P. cembroides* var. *bicolor* to *P. discolor*. Robert-Passini (1978) described *P. johannis* from the mountains above Concepción del Oro, Zacatecas, a population considered by Little (1968) as *P. cembroides* var. *bicolor*. In addition, *P. discolor*

and *P. johannis* have been considered as varieties of *P. culminicola* (Silba, 1985 in Farjon and Styles 1997). According to Eckert and Hall (2006) *P. johannis* and *P. discolor* diverged in the Miocene, approximately 20 MYA. However, morphological differences between these taxa are unclear; the above names have been considered to be synonyms regardless of rank, based on morphological features (Farjon and Styles 1997), but also because they present a dioecious reproductive system whereas other *Pinus* have monoecy (Passini 1994; Flores-Rentería *et al*. 2011 in review). However, Passini (1994) did not evaluate populations from the Sierra Madre Occidental. *P. discolor* and *P. johannis*, are considered different species by others (Perry 1991; Malusa, 1992; Price *et al*. 1998). According to Malusa (1992), *P. johannis* and *P. discolor* have two synapomorphies: small cone size and summer pollen release; their closest relative is *P. culminicola*, forming a monophyletic group. The cpDNA sequence from *P. johannis* collected at its type locality forms an unresolved trichotomy with *P. discolor* and *P. culminicola*, and is distinct from *P. cembroides* (Gernandt *et al* 2003) suggesting it is not a subspecific taxon of *P. cembroides* as suggested by Farjon and Styles (1997). In view of the cpDNA and morphological diversity observed in this group and the absence of sampling of *P. discolor* from the Sierra Madre Occidental or from its type locality in the Santa Rita Mountains, Arizona, more extensive molecular and morphological studies of *P. johannis*-*P. discolor* populations are needed. According to Gernandt *et al*. (2003) *P. culminicola* and *P. johannis* share the same cpDNA lineage, and together with *P. cembroides* should be included in future studies of species limits in *P. johannis* and *P. discolor*. Based on their scattered distribution, lower population density and low regeneration some pinyon species are considered as sensitive taxa based on Farjon and Styles (1997). Because these authors do not regard *P. johannis* as a valid species, the IUCN has not classified it as a sensitive taxon. According to Earle (2011) it certainly warrants such

classification due to *P. Johannis's* rarity, uniqueness, and vulnerability to exploitation and habitat loss associated with development activity.

The present study aims at clarifying systematic and biogeographic relationships of *P. johannis*, *P. discolor* and *P. culminicola*, as well as their relation to *P. cembroides* (subsection *Cembroides*), which have a scattered distribution throughout Mexico, additionally *P. discolor* and *P. cembroides* distribute in southwestern USA. Both, molecular phylogenetic reconstructions from plastid *matK* and *psbA-trnH* spacer sequences, which have been proposed for the plant barcode project providing species discrimination when combined (Kress and Erickson, 2007; CBOL Plant working group, 2009) as well as haplotype networks of chloroplast microsatellites were used. Additionally, in order to determine whether well -defined morphological differences within these subspecies can be linked to specific genotypes, some morphological and ecological features were compared in the *P. johannis-P. discolor* complex, including samples from their type locality and populations from Sierra Madre Oriental and Occidental respectively. Our findings help to delimit the species level in pinyon pines, which are under endangered or risk conservation status (NOM SEMARNAT-059-2010).

MATERIAL AND METHODS

Pinyon pines (*Pinus* subsect. *Cembroides*) are small trees that are broadly and conspicuously distributed throughout semiarid regions from southern Idaho, U.S. to Puebla, Mexico (Malusa, 1992). Seven out of the 11-12 species are recognized from Mexico, four of which are sympatric or parapatric to the widespread Mexican pinyon, *P. cembroides* Zucc. (Bailey and Hawksworth, 1987; Zavarin, 1987). Needles were collected from 8 populations of *P. discolor* D. K. Bailey and Hawksw. (N = 117), 3 populations of *P. johannis* M. F. Robert (N = 57), 3 populations of *P.*

115 *culminicola* D. K. Bailey and Hawksw. (*N* = 21) covering most of the distribution of these
116 species. Additionally, all varieties of 4 populations of *P. cembroides* were collected (*N* = 17):
117 one corresponding to *P. cembroides* var. *lagune*, which is distributed only in a small region of
118 Baja California Sur, one of *P. cembroides* var. *orizabensis*, which has a reduced distribution in
119 Puebla and Tlaxcala, and two populations *P. cembroides* var. *cembroides* (Figure 1 and Table 1).
120 Identification of the samples was carried out based on Price (1998) and Perry (1991). Because
121 the distribution of the complex *Pinus discolor-johannis* is interrupted by the Chihuahuan desert
122 we called all populations located in Sierra Madre Occidental as *P. discolor* and all population in
123 Sierra Madre Oriental as *P. johannis*. In addition, P. discolor has been described as taller tree
124 than P. johannis which looks more arbustive. Thus the population La Amapola, in San Luis
125 Potosi, which has been considered as *P. johannis* or *P. discolor*, was considered to be *P.*
126 *johannis* in this study based on its arbustive appearance and geographic location.
127

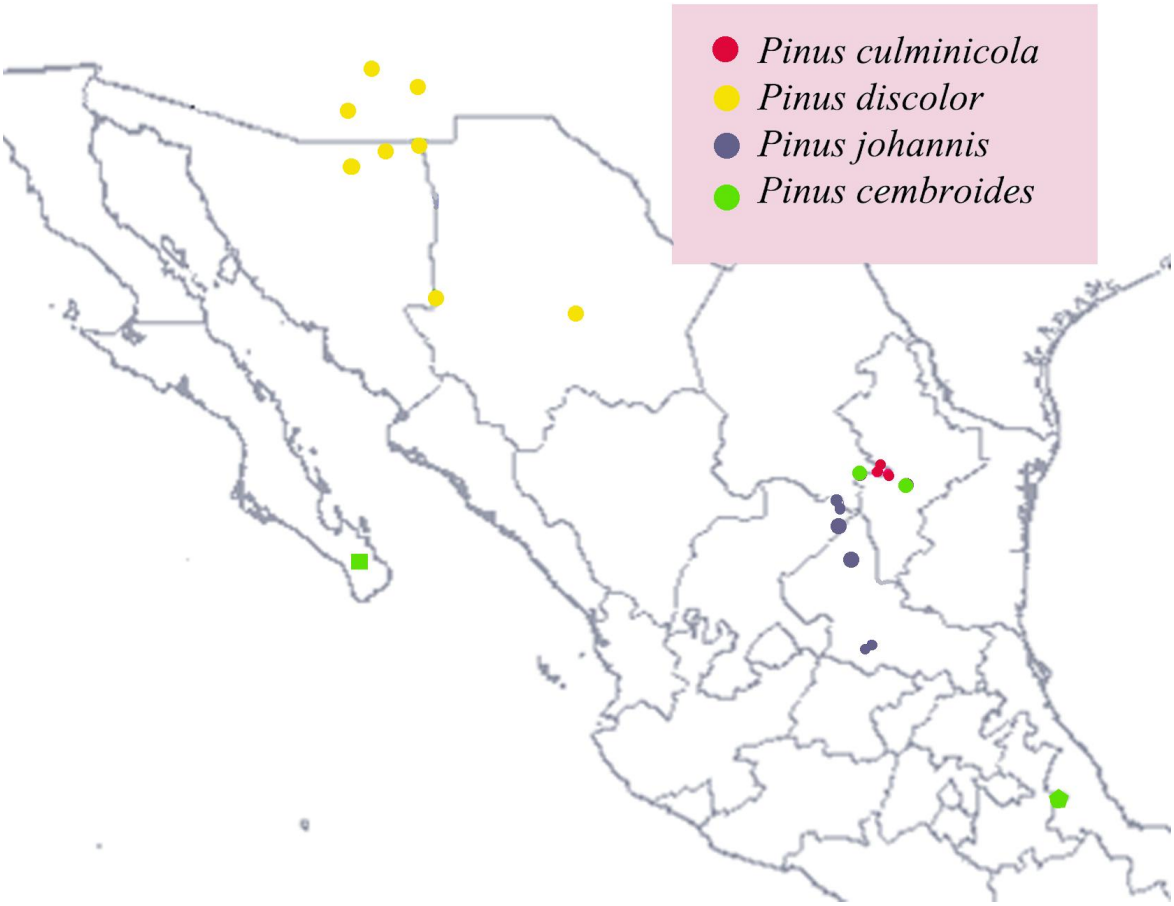


Figure 1. Localities of needle collection for *P. discolor*, *P. johannis*, *P. culminicola*, *P. cembroides* var. *cembroides* (circles), *P. cembroides* var. *orizabensis* (pentagone), and *P. cembroides* var. *lagunae* (square).

Phylogenetic approach and chloroplast markers

DNA extraction and polymorphisms in matK and trnH-psbA spacer —DNA was extracted from needles, using a modified CTAB protocol from Doyle and Doyle (1987), from species in subsection *Cembroides*. *P. cembroides* (*N* = 7), *P. culminicola* D (*N* = 8), *P. discolor* (*N* = 35), and *P. johannis* (*N* = 4). The *matK* region was amplified using two combinations of primers: *matK* orf515-900F (Gadek *et al*. 2000) with *matK*2496R (Gernandt *et al*. 2003) and *matK*1F with

*matK*2R (Wang *et al*. 1999). The *trnH-psbA* spacer markers (Shaw *et al*., 2005) and both combinations of *matK* were amplified in 20 μL PCR reactions using 0.25 U of Taq DNA Polymerase (Invitrogen), 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer and ~20 ng of DNA.

Thermocycler conditions for *matK* were: 94°C for 3 minutes; 30 cycles of 1 minute at 94°C, 50 seconds at 50°C, 5 minutes at 72°C; final extension of 15 minutes at 72°C. Thermocycler conditions for trnH-psbA spacer were: Initial denaturing at 80°C for 5 minutes; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 2 minutes at 72°C; final extension of 5 minutes at 72°C. PCR products were visualized on agarose gels and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California, USA) and a 3730XL Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses—DNA sequences were aligned using the multiple progressive alignment procedure of Clustal W (Thompson, Higgins & Gibson, 1994), with manual corrections. Some *matK* samples were obtained from Genebank (accession numbers Table 1S, see also Gernandt *et al*. 2003**)**. The analyses were carried out with combined data from psbA-trnH plus *matK* for 70 specimens, taking *P. edulis* and *P. californiarum* as outgroups. MrModeltest v2 (Nylander, 2004) was used to select the evolutionary model to be employed in the Bayesian inference (BI). BI was performed with the software MrBayes: Bayesian Inference of phylogeny, version 3.1 (Huelsenbeck & Ronquist, 2001). Uniform, prior probabilities and a random starting tree were used. The Markov Chain Monte-Carlo (MCMC) procedure was run simultaneously and sampled every 100 generations for a total of 1 million generations. The gaps were considered in a binary matrix (Ronquist, Huelsenbeck & Mark, 2005). The majority rule consensus tree was calculated with PAUP 4.10b (Swofford, 2002).

Chloroplast microsatellites, haplotype network and genetic structure

Microsatellites and multiplex design— Chloroplast microsatellite markers (Flores-Rentería and Whipple, 2011) were amplified from DNA of *P. cembroides* (*N* = 17), *P. culminicola* (*N* = 21), *P. discolor* (*N* = 117), *P. johannis* (*N* = 57) (Table 1). Additional markers were developed following Flores-Rentería and Whipple's (2011) protocol. We used an annealing temperature of 56 °C for all combinations in a multiplex design, 0.08 μM of the forward primer, and 0.23 μM of reverse primer, with up to five primer pairs multiplexed at a time. Forward primers were labeled with TAMRA, FAM, and HEX fluorophores at their 5′ end. PCR products were diluted 1:60 with water into a plate for genotyping. Fragment analysis was carried out using 1 μl of the bulk PCR dilution, 0.09 μl Gene-Scan 500 LIZ size standard (Applied Biosystems), and 9.91 μl HiDi Formamide (Applied Biosystems). Fragments were separated on a 3730XL Genetic Analyzer and scored using Genemapper 3.7 (Applied Biosystems). Scoring of each allele was verified by eye for every sample. Because the 79293 microsatellite was associated to a three microsatellites and one substitution, it was sequenced as described previously.

Table 1. Localities of each species included in this study. The number of individuals per population varies for each species. Geographic coordinates are given for each locality. San Luis Potosi (SLP) and Baja California Sur (BCS).

Locality	Coordinates N	Coordinates W	Altitude m.a.s.l	Number of samples
Pinus discolor				
San José, Sonora	31º 15.096	109º 58.336	1902	12
Kipor, Sonora	28º 26.391	108º 31.191	1588	18
San Luis Puerto, Sonora	31º 19.356	108º 45.503	1923	16
Cave Creek, Arizona	31º 54.066	109º 09.394	1494	19
La Mariquita, Sonora	31º 02.420	110º 23.014	1963	14
Galiuro, Arizona	32º 30.919	110º 15.835	1686	11
Colonia Juarez, Chihuahua	30° 16.878	108° 13.425	1975	3
Lemmon, Arizona	32º 21.433	110º 42.542	1668	24
Pinus johannis				
Tocho-Amapola, SLP	22º 01.160	101º 07.706	2391	15
San Miguelito, SLP	22° 01.668	100° 56.387	2145	5
Concepción Del Oro, Zacatecas	24° 37.098	101° 27.140	2120	20
Mazapil, Zacatecas	24° 36.920	101° 27.281	2823	5
Lajas, Slp	23° 18.045	101° 09.797	2368	12
Pinus culminicola				
Cerro Del Potosí, Nuevo León	24° 86.667	100° 21.667	3600	9
La Viga, Coahuila	25° 35.000	100° 51.667	3450	6
Martha, Coahuila	25° 19.027	100° 36.111	3500	6
Pinus cembroides				
var. *cembroides* Galeana, Nuevo Leon	24° 51.906	100° 05.773	1713	5
var. *cembroides* Saltillo, Coahuila	25° 10.394	100° 43.806	1988	5
var. *lagunae* Sierra De La Laguna, BCS	23° 33.21	109° 58.80	~1500	4
var. *orizabensis* Ajalpan, Puebla	18° 45.098	97° 23.335	2387	7

188 *Microsatellite analyses*-- In order to investigate the evolutionary history and relationships among
189 the haplotypes found in this study, a minimum spanning network of haplotypes (Table 2S) was
190 constructed using TCS 1.21 (Clement *et al.* 2000). Inferred insertions or deletions (indels) were
191 treated as one mutation, unordered evolutionary events rather than treating them as missing data
192 or as a fifth state. To break loops (ambiguous connections) within our network, we used the
193 methods described by Templeton and Sing (1993), while using predictions derived from
194 coalescence theory (reviewed in Rosenberg and Nordborg, 2002).
195 The genetic divergence among populations was estimated by partitioning the genetic variance
196 (AMOVA) as described by Weir and Cockerham (1984). For this analysis we included all kind
197 of microsatellites and excluded the sustitution. The genetic distance between pairs of haplotypes
198 was estimated using the programme Arlequin v3.5 (Excoffier and Lischer, 2010) under a
199 stepwise mutation model based on haplotype frequencies (F_{ST} and R_{ST}). The significance of
200 genetic differentiation (deviation from zero) was tested by non-parametric randomization tests
201 using 1,023 random permutations of haplotypes between species were used.
202 ***Allometry and ecological variables***
203 In order to find morphological differences between *P. johannis* and *P. discolor* we measured
204 several features using one to four populations of *P. discolor* (Sierra Madre Oriental) and
205 compared them against samples from two localities at San Miguelito Mountains (Sierra Madre
206 Occidental) where species identification has been controversial. Cone length and pedicel length
207 were measured on 302 cones, 203 from the Amapola locality corresponding to *P. johannis* and
208 99 from *P. discolor* at La Mariquita. Thickness of seed coat was measured on 292 seeds, 112 of
209 *P. discolor* and 180 of *P. johannis*. Length and thickness were measured using a caliper (CD-6,
210 CSX, Mitutoyo Corp.) to the nearest 0.01mm. We measured tree height through a graduated

telescoping pole and calculated the basal area of 336 individuals, 212 from 4 populations of *P. discolor* (Kipor, La Mariquta, San José, San Luis) and 124 from two localities of *P. johannis* (El Tocho and La Amapola). Analysis of covariance (JMP statistical software, SAS 2009) was performed in order to detect differences in tree architecture between *P. johannis* and *P. discolor* by modeling the height with relation to basal area.

RESULTS

Phylogeny in pinyon pines based on chloroplast markers

Combination of both *matK* pair of primers amplifies 1500 bp. No indels were found in this marker into subsection *Cembroides* species, 13 variables sites were recorder among species. *psbA-trnH* spacer presents two indels, 28 bp indel and 4bp repetitions were found in *P. monophylla.* This species also present 15 substitutions, whereas *P. pinceana* and some individuals of *P. johannis* together present three substitutions. Additionally an inversion of 8 bp is share among different species. The concatenated tree using both *matK* and *psbA-trnH* spacer showed low resolution and nonmonophyly of *P. johannis*, *P. discolor* and *P. culminicola* (Figure 2). A monophyletic group of *P. cembroides* individuals including its varieties *orizabensis* and *lagunae* was found in the concatenated phylogeny, they were grouped by three synapomorphic substitutions in *matK* (exclusive to this taxon).

psbA-trnH spacer presents an 8bp inversion which is broadly distributed in different species of subsection *Cembroides*. Therefore a paraphyletic group is found with individuals of *P. discolor*, *P. edulis*, *P. pinceana*, *P. cembroides* var. *lagunae* and *P. culminicola*.

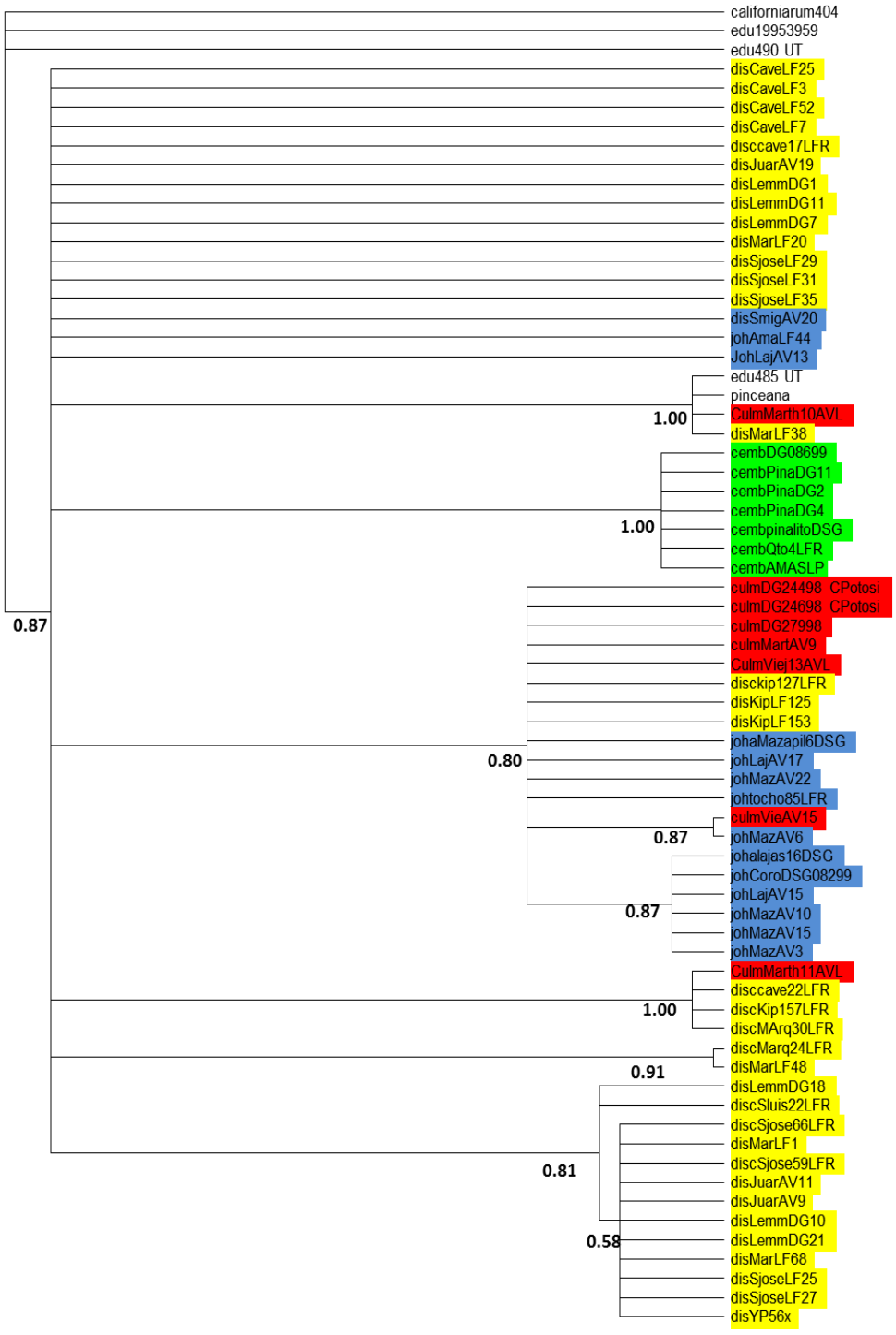


Figure 2. Phylogenetic tree (BI), concatenated *matK* and *psbA-trnH* spacer regions. Posterior probabilities are shown. Individual names are highlighted in green for *P. cembroides*, in red for *P. culminicola*, in yellow for *P. discolor* and in blue for *P. johannis*.

Haplotype network reveals four different species

The chloroplast network shows no shared haplotypes among species, forming a linear structure. Interestingly *P. cembroides* is completely differentiated from the other three species. Based on our sampling, *P. discolor* has a higher number of haplotypes (Figure 3), whereas *P. johannis* and *P. culminicola* have a lower number of haplotypes. This shows that *P. discolor* has intraspecific genetic structure. *P. johannis* is completely differentiated from *P. discolor* which presents lower divergence among the sampled individuals. *P. culminicola* had the fewest number of populations and therefore fewer individuals were included, but it presents important divergent haplotypes, particularly some individuals from Sierra La Marta. Low levels of migration are suggested by one haplotype of *P. discolor* grouped in the extreme of a chain of *P. johannis* in the haplotype network.

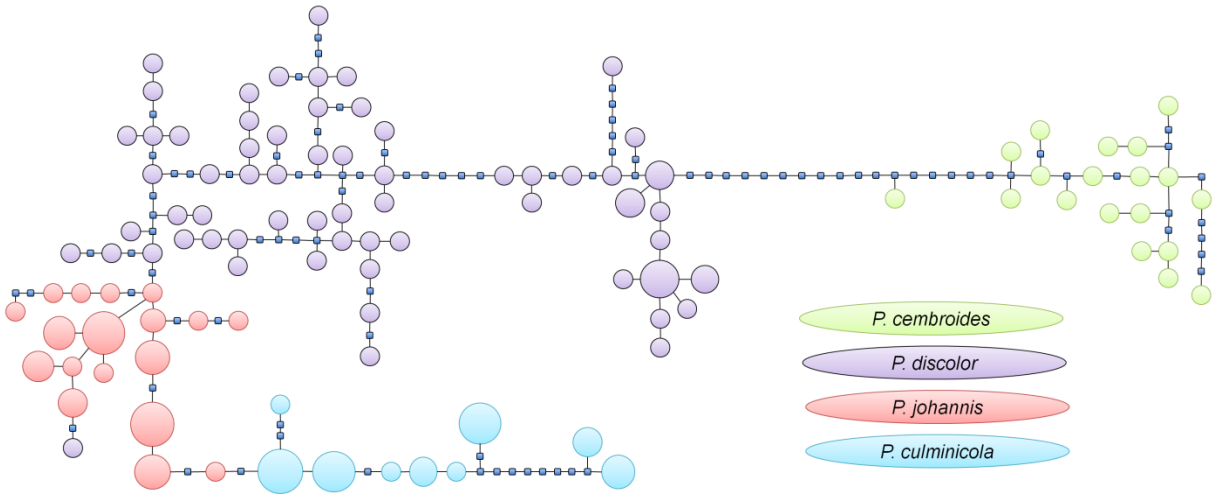


Figure 3. Haplotype network using 18 chloroplast markers of four pinyon pine species. Different colors represent species as codified in table 1. Every haplotype found is represented by a colored circle. Missing data are shown as small squares. The area of the circles is proportional to the haplotype frequency.

Population Structure Analysis separates the four species of Pinus

The analysis of molecular variance (AMOVA) showed most of the genetic variability was accounted for by the within species component (51.33%), followed by variation within population (42.41%) and low genetic variation among populations, (6.24%), the F_{ST} computed among all populations was 0.57. The genetic divergence among species was further investigated by computing a pairwise F_{ST} matrix. F_{ST} varied between species from 0.23 (between *P. culminicola* and *P. johannis*) to 0.63 (between *P. cembroides* and *P. johannis*; Table 2, below diagonal). R_{ST} values varied between 0.16 (*P. discolor* and *P. johannis*) and 0.84 (*P. cembroides* and *P. johannis*, Table 2, above diagonal). Regardless the method all comparisons were statistically significant.

Table 2. F_{ST} and R_{ST} values between pairwise comparisons of species are below and above diagonal respectively. All values of F_{ST} and R_{ST} are significantly different from zero ($P < 0.05$).

	P. cembroides	*P. culminicola*	*P. discolor*	*P. johannis*
P. cembroides	0	0.79	0.72	0.85
P. culminicola	0.58	0	0.19	0.25
P. discolor	0.53	0.33	0	0.17
P. johannis	0.66	0.23	0.38	0

Morphological and ecological differences between P. discolor *and* P. johannis

Morphological features show a significant difference between *P. johannis* and *P. discolor* (Figure 4). Cone length was significantly larger in *P. johannis*: average 2.7 cm, versus 1.9 cm for

P. discolor ($F_{1, 300}$=212.74, *P* <0.0001, R^2=0.41). The pedicel of *P. johannis* was on average longer than that of *P. discolor*: 5.08 and 6.14 mm respectively ($F_{1, 300}$=28.51, *P* <0.0001, R^2=0.1). The seed coat was almost twice as thick in *P. johannis* (1.33 mm) than in *P. discolor* (0.71mm) ($F_{1, 290}$=856.37, *P* <0.0001, R^2=0.74).

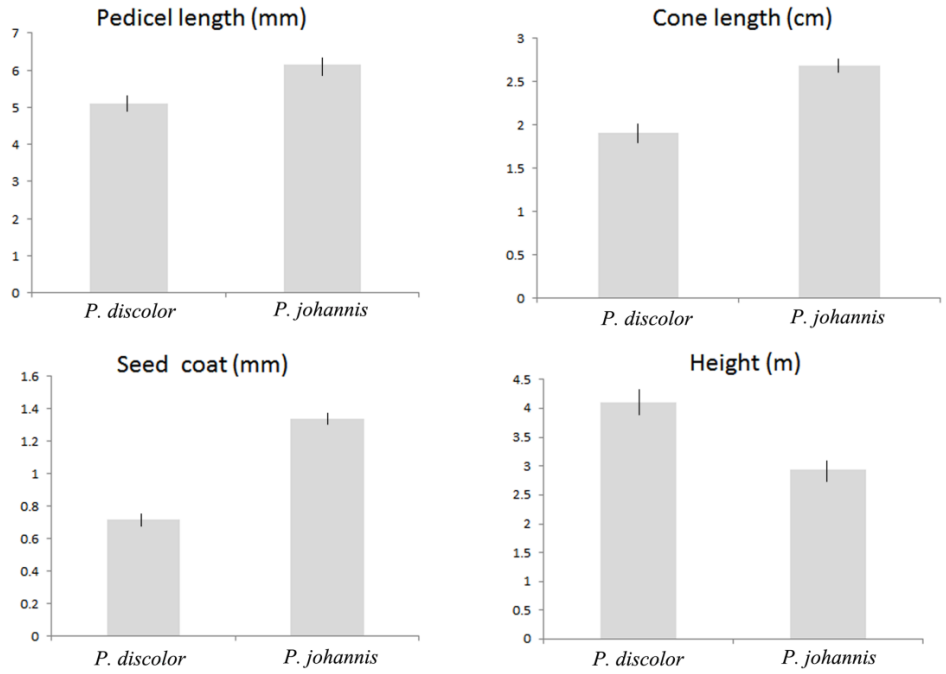


Figure 4. Morphological differences between *P. discolor* and *P. johannis*. Mean and standard error are shown.

The basal area did not differ significant between species, with an average of 235 cm^2 ($F_{1, 334}$ = 0.0001, *p* = 0.99, R^2 = 4.41e-7). The total tree height differs significant between species: on average *P. discolor* was taller than *P. johannis* with 4.1 m and 2.93 m respectively, although *P. discolor* ranged from less than 1 m to 13 m, whereas *P. johannis* ranged from less than 1 m to ~5 m ($F_{1, 334}$ = 25.32, *p* < 0.0001, R^2 = 0.07). Based in the logistic model at certain basal area *P. discolor* was taller than *P. johannis* (Figure 5).

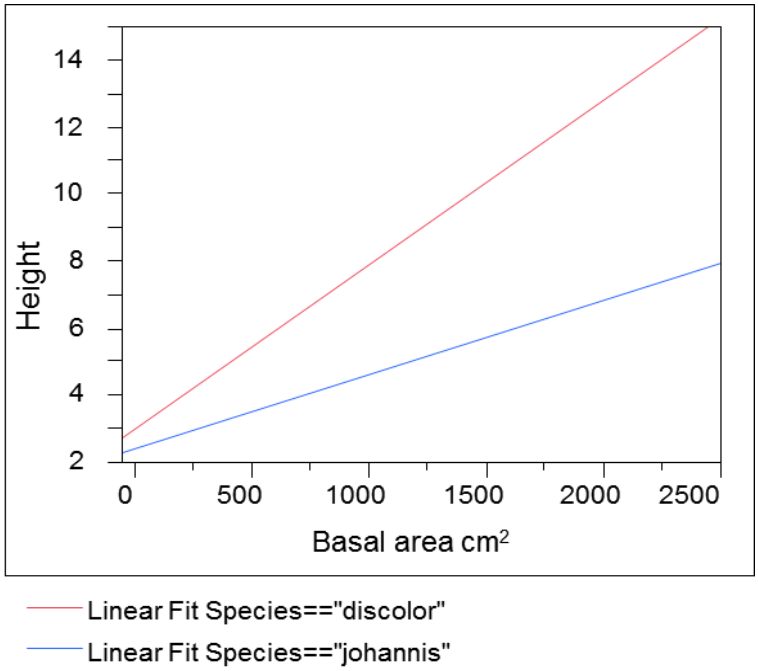


Figure 5. Model of height based on basal area between species. At a given basal area, the predicted height is greater in *P. discolor* than in *P. johannis.*

Discussion

Pinus johannis and *P. discolor* present genetic and morphological differences suggesting they are different species. Genetic significant differences were found in pairwise comparisons (F_{ST} and R_{ST}) and in the haplotype network, which showed no shared haplotype among the four species studied. *P. cembroides*, along with *P. cembroides* var. *orizabensis* and var. *lagunae*, are grouped far apart from *P. discolor*. The same result was observed in the phylogeny using *matK, rpl16, rbcl* (in Gernandt *et al*. 2003) but not when using *matk* and *psbA-trnH* spacer concatenated, because the latter has an inversion shared in several species including *P. cembroides* var. *lagunae*. All approaches corroborate the idea that neither *P. johannis* nor *P. discolor* are varieties of *P. cembroides* as was considered by Little (1968) and Farjon and Styles

(1997). In fact Silba (1985) considered them as varieties of *P. culminicola*; this point of view has some merit in showing that they are more closely related to *P. culminicola* than to *P. cembroides*, as demonstrated by Malusa (1992) using morphological features. Our findings from the haplotype network shows that *P. culminicola* is the sister group of *P. johannis*; in turn *P. johannis* is the sister taxon of *P. discolor*, connected by one mutational step (Figure 3). The 12 mutational steps between *P. discolor* and *P. cembroides* suggest enough time of divergence between them to allow for allele fixation, which was also detected in the phylogeny. However, the classical phylogenetic approach does not differentiate the complex of *P. discolor-P. johannis*. The lack of resolution between *P. culminicola*, *P. discolor* and *P. johannis* using psbA-trnH or *matK* is relevant because its species identity never has been questioned due the presence of 5 needles per fascicule and its small size. In addition, these markers have been proposed to be useful in the Barcoding Project, since in some plant groups they discriminate among species (Kress and Erickson, 2007; CBOL Plant working group, 2009). However, these markers cannot be used universally in plants to identify species (Whitlock *et al*. 2010), particularly in groups where ancestral polymorphism is spread among species, as occurs in *Pinus*. Thus, in species with recent divergence that share ancestral polymorphisms, nonhierarchical network-based approaches (Davis, 1996; Posada and Crandall, 2001) are probably more appropriate. The construction of haplotype networks enables assigning extant haplotypes to an ancestral population or species, while differentiating between ancestral polymorphisms, hybridization and migration (Templeton, 2001). In addition the AMOVA and genetic differentiation suggest strong differences between the four *a priori* defined species. Compared with other studies, the pinyon pines in the present study showed higher values of F_{st}, whereas for different subsections of *Pinus* the F_{st} range from 0.05 in *Contortae* to 0.17 in *Attenuata* (Delgado *et al*. 2002), the total F_{st} for

the four species (*P. cembroides, P. culminicola, P. discolor* and *P. johannis*) was 0.57. Previous F_{st} value for subsection *Cembroides* was 0.15 using isozymes; however, high values were also reported by Escalante (2001) in *P. pinceana* with 0.78. High F_{st} values can be due to the higher number of microsatellites used in the present study, some of them hypervariables as the pentanucleotide 79293 which that condition has been found only in subsection *Cembroides* (Flores-Rentería *et al*. in progress). R_{st} values were higher when comparing *P. cembroides* against all other species. However, pairwise comparisons between all other species were lower compared to the F_{st}.

Are *P. johannis* and *P. discolor* cryptic species?

Although *P. johannis* and *P. discolor* are genetically different as shown by the haplotype network (Figure 3), they share multiple morphological features that complicate their identification, thus they can be considered cryptic species. However, we observed that some quantitative characteristics such as pedicel length, cone length, seed coat thickness, and height discriminate *P. discolor* from *P. johannis* (Figure 4). In our data, measurements of pedicel length, cone length, and height slightly overlap between species. According to Malusa (1992) the features that separate *P. discolor* and *P. johannis* are seed cone scale thickness and pedicel length. In addition, *P. johannis* represents a polymorphism in the amount of resin in the cones from intermediate to very resinous, and in its shrub habit (multiple trunks within 0.4 meters of ground). In contrast, *P. discolor* only has cones with an intermediate amount of resin and presents an arboreous habit. Malusa (1992) did not find differences in the seed coat thickness between *P. discolor* and *P. johannis*, but he did not include samples of *P. johannis* from the San Miguelito Mountains. Samples from that area in our data have a thicker seed coat compared to

four populations of *P. discolor*, and is the only feature that does not overlap between species. Differences in seed coat thickness were also found by Zavarin and Snajberk (1986) and differences in cone length have been described by Perry (1991); according to his description, *P. discolor* usually produces cones 2-3 cm. long, versus 3-4 cm. for *P. johannis*. We support such differences, although our average for *P. discolor* was 1.9 and for *P. johannis* 2.7.

Zavarin and Snajberk (1986), Price (1998), and Perry (1991) describe *P. johannis* as a small tree, usually 2 to 3 m tall, whereas *P. discolor* ranges from 5 to 10 m. We corroborate this difference between species in the sampled populations. Perry describes the diameter of *P. discolor* in the range of 10-50 cm, but has no data for the basal area of *P. johannis*. We did not find significant differences in basal area between species. Because basal area and height depend on the age of the tree, they can overlap between species. Furthermore, they can be plastic features and should therefore not be used to distinguish species individually. The interaction of height and basal area will be a better estimator of species because it reflects the tree architecture, patterns of growing, and allocation resource (Flores-Rentería *et al*. in review). Romero (2001) found a linear relationship between height and age in *P. johannis* and *P. cembroides*. Therefore, if *P. johannis* and *P. discolor* are the same species, a relative increment in the basal area to the height will be expected regardless of population origin. We found that at certain basal area increments, *P. discolor* is taller than *P. johannis*. This agrees with ecological differences between these species. Thus, different growing and allocation resource strategies have evolved in *P. discolor* and *P. johannis*. All morphological differences suggest *P. discolor* and *P. johannis* are different species. In addition, Zavarin and Snajberk (1986) found differences in the production of terpenes between these species, which supports the species status.

The ranks utilized for taxa of pinyon pines vary widely between taxonomic authorities using morphological data. Little (1968) distinguished *Pinus discolor* from *P. cembroides* because of its two-toned needles, smaller cones, and lack of dorsal stomata. Additionally, Bailey and Hawksworth (1979) found differences in the number of needles per fascicle. *P. discolor* has three needles per fascicles on almost an entire given tree, with four occurring occasionally, and two even less so. Passini (1994) showed that the main differences between *P. cembroides* and *P. johannis* are in habit and size, color of the endosperm, and number of cotyledons. This author also noted that the greater similarity appeared to be between *P. johannis* and *P. cembroides* var. *bicolor*. This variety was recognized by Little in 1968; 11 years later Bailey and Hawksworth (1979) elevated its rank to species, modifying the epithet *bicolor* to *discolor*. However, Passini (1994), along with Little, agreed that *P. discolor* and *P. johannis* were the same species, and by the priority principle established the correct name as *P. johannis* Rob.-Pass. with *P. discolor* Bailey and Hawks. and *P. cembroides* var. *bicolor* Little as synonymous.

Interestingly, altitudinal differences appear species-related with *P. culminicola* occupying higher altitude, followed by *P. johannis*, and *P. discolor* distributed in lower areas. This altitudinal variation is associated with the precipitation level, *P. discolor* occupies areas with higher precipitation than *P. johannis*. Geographic distribution between these species supports the idea of different adaptive strategies to drought, as has been proposed for the pinyon pine (Richardson, 1998; Cole *et al*. 2008).

Lack of morphological variation and recurrent misidentifications

In a phenetic morphological analysis, Romero *et al*. (2000) found that the San Luis Potosí populations that they called *P. johannis*, were easily distinguished from *P. johannis* in Zacatecas.

Gernandt *et al*. (2003) found monophyly in samples of *P. cembroides* and the varieties *orizabensis* and *lagunae* when analyzing 1 or 2 individuals per species using *rbcL* and *matK*. However, in that phylogeny one sample of *P. discolor*, from San Luis Potosí, is grouped at the base of the *P. cembroides* clade. Since we included most of the populations of *P. culminicola*, *P. johannis* and *P. discolor* and none of them appears grouped to *P. cembroides*, we think their sample was misidentified in the field, especially since it was collected in San Luis Potosi where *P. cembroides* is sympatric to *P. johannis*. This population in La Amapola, Sierra de San Miguelito, SLP, was first described as a new locality of *P. discolor* by Avila *et al.* (1992). Later Passini (1994) included it in a study showing no significant differences between individuals at La Amapola population and Concepcion del Oro, concluding *P. discolor* and *johannis* are synonymous.

The literature features a constant conflict with the population La Amapola in San Miguelito Mountains, San Luis Potosi. For some authors the species in this area corresponds to *P. discolor* (Zavarin and Snajberk, 1986; Perry, 1991; Ávila *et al*. 1992), while others considered it as *P. johannis* (Passini 1994, Flores-Rentería *et al*. 2011), and few consider the presence of both (Cuenca, 2003; Gernardt *et al*. 2005). Additionally, this taxon is intermixed with *P. cembroides*, which is the dominant species, multiplying the mistakes in identifying these species. We considered this population to be *P. johannis* based on the arbustive appearance and the geographic location.

Our findings contribute to delimit the species level in *P. culminicola*, *P. discolor* and *P. johannis*, the first two of which are under endangered and low risk conservation status, respectively. The IUCN has not classified *P. johannis* as a sensitive taxon because it was not

considered as a valid species. In contrast the Mexican normativity (NOM SEMARNAT-059-2010) do not consider *P. discolor* under any sensitive status, but they consider *P. johannis* under special protection, however they do not mention what classification was used to define the species. We suggest a reevaluation of the conservation status of this species considering the information generated in this study. This group certainly warrants such classification due to its rarity, its uniqueness, and its vulnerability to exploitation and habitat loss associated with development activity.

An integrative approach has to be used when studying species boundaries, especially when there are widespread morphological and genetic ancestral polymorphisms. All of our data suggests that *P. johannis* and *P. discolor* are different taxa. The species status should be considered for both based on their haplotype variation and the genetic structure, as well as morphological differences between *P. discolor-P. johannis* and the accepted species *P. cembroides* and *P. culminicola*.

Acknowledgments

The authors thank to R. Pérez, J. F. Martínez, A. Cuenca, D. Gernandt, F. Nopemuceno, P. de la Garza and R. Salas for their help during sample collection. Important group discussion was undertaken with people of C. Gehring's group to improve this work. We also thank H. Copper and S. Taysor for their comments to this manuscript. This work was conducted by LFR during her Ph.D. dissertation at the Posgrado en Ciencias Biomédicas, Universidad Nacional Autónoma de México with a CONACyT scholarship (172682). Funds were provided by SEMARNAT-CONACyT C01-0201/A-1, SEP-CONACyT 46925 and NSF grant DEB-0816675.

440

441

442

443

444 References

445 **Ávila, N.J., García, M.E., Reyes, A.J. 1992.** Registro de *Pinus discolor* Bailey et Hawksworth
446 en la sierra de monte Grande, San Luis Potosí, México. *Acta Bot. Mex.* 20:9-12.

447 **Bailey DK, Hawksworth FG. 1979.** Pinyons of the Chihuahuan Desert region. *Phytologia* **44**:
448 129-133.

449 **Bailey DK, Snajber K, Zavarin E. 1982.** On the question of natural hybridization between
450 *Pinus discolor* and *Pinus cembroides*. *Biochemical systematics and ecology* **10**: 111-119.

451 **Brower AVZ. 1999.** Delimitation of Phylogenetic Species with DNA Sequences: A Critique of
452 Davis and Nixon's Population Aggregation Analysis. *Systematic Biology* **48**: pp. 199-213.

453 **CBOL Plant Working Group, Hollingsworth PM, Forrest LL, *et al*. 2009.** A DNA barcode
454 for land plants. *Proceedings of the National Academy of Sciences* **106**: 12794-12797.

455 **Cole KL, Fisher J, Arundel ST, Cannella J, Swift S. 2008.** Geographical and climatic limits of
456 needle types of one- and two-needled pinyon pines. *Journal of Biogeography* **35**: 257-269.

457 **Corander J, Tang J. 2007.** Bayesian analysis of population structure based on linked molecular
458 information. Mathematical biosciences, 205:19-31.

459 **Clement M, Posada D, Crandall KA. 2000.** TCS: a computer program to estimate gene
460 genealogies. *Molecular ecology* **9**: 1657-1659.

461 **Cuenca, A. 2003**. Evidencia de dos linajes genéticos en *Pinus cembroides* revelada por
462 microsatélites de cloroplasto. Posgrado en Ciencias Biológicas.

463 **Davis JI. 1996.** Phylogenetics, Molecular Variation, and Species Concepts. *Bioscience* **46**: pp.
464 502-511.

465 **Earle CJ. 2011.** The gymnosperm database [on line]. URL:
466 http://www.conifers.org/topics/sitemap.htm [accessed 20 May 2011].

Eckert AJ, Hall BD. 2006. Phylogeny, historical biogeography, and patterns of diversification for *Pinus* (Pinaceae): Phylogenetic tests of fossil-based hypotheses. *Molecular phylogenetics and evolution* **40**: 166-182.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources.* 10: 564-567.

Delgado P, Cuenca A, Escalante AE, Molina-Freaner F, Piñero D. 2002. Comparative genetic structure in pines: evolutionary and conservation consequences. *Rev Chil Hist Nat.* 75: 27-37.

Farjon A. 1996. Biodiversity of *Pinus* (Pinaceae) in Mexico: speciation and palaeo-endemism. *Botanical Journal of the Linnean Society* **121**: 365-384.

Farjon, A., B.T. Styles. 1997. *Pinus* (Pinaceae). Flora Neotropica Monograph 75. New York, NY: The New York Botanical Garden.

Flores-Rentería, L., Molina-Freaner, F., Whipple, A. and C. A. Domínguez. 2011. The evolution of dioecy in gymnosperms: *Pinus johannis* as a model to understand the evolutionary process leading to sexual separation. Submitted to Journal of Evolutionary Biology.

Flores-Renteria, L., Vazquez-Lobo, A., Whipple, A.V., Pinero, D., Marquez-Guzman, J., Dominguez, C.A. 2011. Functional bisporangiate cones in *Pinus johannis* (Pinaceae): Implications for the evolution of bisexuality in seed plants. *Am. J. Bot.* 98: 130-139.

Flores-Renteria, L., Whipple, A. V. 2011. A new approach to improve the scoring of mononucleotide microsatellite loci. *Am. J. Bot.* 98: e51-53

Gernandt DS, Geada LG, Garcia OS, Liston A. 2005. Phylogeny and classification of *Pinus*. *Taxon* **54**: 29-42.

Gernandt DS, Liston A, Piñero D. 2001. Variation in the nrDNA ITS of Pinus Subsection *Cembroides*: Implications for Molecular Systematic Studies of Pine Species Complexes. *Molecular phylogenetics and evolution* **21**: 449-467.

Gernandt DS, Liston A, Piñero D. 2003. Phylogenetics of *Pinus* subsections *Cembroides* and Nelsoniae inferred from cpDNA sequences. *Systematic Botany* **28**: 17.

Kral, R. 1993. Pinus. Flora of North America Editorial Committee (eds.): Flora of North America North of Mexico, Vol. 2. Oxford University Press.

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 14812-14817.

Heinrichs J, Hentschel J, Bombosch A, *et al*. 2010. One species or at least eight? Delimitation and distribution of *Frullania tamarisci* (L.) Dumort. s. l. (Jungermanniopsida, Porellales) inferred from nuclear and chloroplast DNA markers. *Molecular phylogenetics and evolution* **56**: 1105-1114.

Kress WJ, Erickson DL. 2007. A Two-Locus Global DNA Barcode for Land Plants: The Coding *rbcL* Gene Complements the Non-Coding *trnH-psbA* Spacer Region. *PLoS ONE* **2**: e508.

Lanner, R. M. 1981. The piñon pine. Reno: University of Nevada Press.

Liston A, Parker-Defeniks M, Syring JV, Willyard A, Cronn R. 2007. Interspecific phylogenetic analysis enhances intraspecific phylogeographical inference: a case study in *Pinus lambertiana*. *Molecular ecology* **16**: 3926-3937.

Liston A, Robinson WA, Piñero D, Alvarez-Buylla ER. 1999. Phylogenetics of *Pinus* (Pinaceae) Based on Nuclear Ribosomal DNA Internal Transcribed Spacer Region Sequences. *Molecular phylogenetics and evolution* **11**: 95-109.

Moore, W.S., 1995. Inferring phylogenies from mtDNA variation – Mitochondrialgene trees versus nuclear-gene trees. *Evolution* 49, 718–726.

Morse AM, Peterson DG, Islam-Faridi MN, Smith K E, Magbanua Z, Garcia SA, Kubisiak TL, Amerson HV, Carlson JE, Nelson C, Dana D, John M. 2009. Evolution of genome size and complexity in *Pinus*. *PLoS ONE* **4**: e4332.

NORMA Oficial Mexicana NOM-059-SEMARNAT-2010, Protección ambiental-Especies nativas de México de flora y fauna silvestres-Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio-Lista de especies en riesgo. Diario Oficial, Jueves 30 de diciembre de 2010.

Palmé AE, Pyhäjärvi T, Wachowiak W, Savolainen O. 2009. Selection on Nuclear Genes in a Pinus Phylogeny. *Molecular biology and evolution* **26**: 893-905.

Piercey-Normore DD, Ahti T, Goward T. 2010. Phylogenetic and haplotype analyses of four segregates within Cladonia arbuscula s.l.This paper is one of a selection of papers published as part of the special Schofield Gedenkschrift. *Botany* **88**: 397-408.

Posada D, Crandall KA. 2001. Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology & Evolution* **16**: 37-45.

Robert MF. 1978. Un nouveau pin pignon mexicaine: *Pinus johannis* Robert. *Adansonia,* ser 2 18:365-373.

Romero A, Luna M, Garcia E, Passini MF. 2000. Phenetic analysis of the Mexican midland pinyon pines, *Pinus cembroides* and *Pinus johannis*. *Botanical Journal of the Linnean Society* **133**: 181-194.

Romero, A. 2001. "Historia natural, ecología de poblaciones y fitosociología de Pinus *cembroides* y Pinus johannis (piñoneros) del centro de Mexico", UNAM, Facultad de Ciencias, Doctorado en Ciencias, Biología.

SAS Institute Inc. 2009. *JMP: Version 8.* Cary, NC.

Shaw J, Lickey EB, Beck JT, *et al*. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* **92**: 142-166.

Sinclair E, Bezy R, Bolles K, R. JC, Crandall K, Sites J,Jr. 2004. Testing Species Boundaries in an Ancient Species Complex with Deep Phylogeographic History: Genus Xantusia (Squamata: Xantusiidae). *The American Naturalist* **164**: pp. 396-414.

Stech M, Werner O, Gonzalez-Mancebo JM, *et al*. February 2011. Phylogenetic inference in *Leucodon* Schwagr. subg. *Leucodon* (Leucodontaceae, Bryophyta) in the North Atlantic region. *Taxon* **60**: 79-88(10).

Syring J, Farrell K, Businský R, Cronn R, Liston A. 2007. Widespread Genealogical Nonmonophyly in Species of *Pinus* Subgenus *Strobus*. *Systematic Biology* **56**: 163-181.

Templeton AR. 2001. Using phylogeographic analyses of gene trees to test species status and processes. *Molecular ecology* **10**: 779-791.

Weir BS, **Cockerham** CC. **1984**. Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370.

Willyard A, Cronn R, Liston A. 2009. Reticulate evolution and incomplete lineage sorting among the ponderosa pines. *Molecular phylogenetics and evolution* **52**: 498-511.

Whitlock BA, Hale AM, Groff PA. 2010. Intraspecific Inversions Pose a Challenge for the *trnH-psbA* Plant DNA Barcode. *PLoS ONE* 5(7): e11533. doi:10.1371.

Yuan Y, Olmstead RG. 2008. A species-level phylogenetic study of the Verbena complex (Verbenaceae) indicates two independent intergeneric chloroplast transfers/ Molecular *Phylogenetics and Evolution* 48:23–33

Zavarin E, Snajberk K. 1986. Monoterpenoid differentiation in relation to the morphology of *Pinus discolor* and *Pinus johannis*. *Biochemical systematics and ecology* **14**: 1-11.

Zavarin E, Snajberk K. 1986. Monoterpenoid differentiation in relation to the morphology of Pinus discolor and Pinus johannis. *Biochemical systematics and ecology* **14**: 1-11.

Supplementary data

Supplementary table 1. List of accession number of *matK* sequences and the corresponding species name.

Accession number	Species name
AY115765.1	*Pinus edulis*
AY115766.1	*Pinus edulis*
AY115776.1	*Pinus culminicola*
AY115777.1	*Pinus discolor*
AY115778.1	*Pinus johannis*
AY115779.1	*Pinus johannis*
AY115780.1	*Pinus discolor*
AY115781.1	*Pinus cembroides*
AY115782.1	*Pinus cembroides*
AY115783.1	*Pinus cembroides* var. *lagunae*
AY115785.1	*Pinus cembroides* var. *orizabensis*
AY115786.1	*Pinus pinceana*
AY115787.1	*Pinus pinceana*
AY115788.1	*Pinus pinceana*

Table 1S. Haplotype identity per species is shown in the first colum. 18 chloroplast markers were included, 15 were mononucleotide, 1 dinucleotide, 1 pentanucleotidemicrosatellite and 1 substitution. *P. cembroides* (Ce), *P. culminicola* (Cu), *P. discolor* (Di) and *P. johannis* (Jo).

Haplotype	80576	26081	55012	72502	58046	13216	6924	103110	70000	68590	15146	66029	48509	29275	di	penta	mono	G/T
Ce1	161	207	273	195	247	396	110	139	209	304	141	252	267	467	7	10	9	G
Ce2	161	207	273	195	247	396	110	139	209	303	142	252	267	470	7	6	9	G
Ce3	161	207	273	195	247	396	110	139	209	304	142	252	267	467	7	9	9	G
Ce4	161	207	269	195	247	396	110	139	209	304	141	252	267	469	7	9	9	G
Ce5	161	207	273	195	247	395	110	139	209	302	142	252	267	469	7	6	9	G
Ce6	161	207	273	195	247	396	110	139	209	303	141	252	267	469	7	6	9	G
Ce7	161	207	273	195	247	396	110	139	209	303	142	244	267	469	7	6	9	G
Ce8	161	207	274	195	247	396	110	139	209	303	141	251	266	469	7	11	9	G
Ce9	161	207	274	195	247	396	110	139	209	303	141	252	267	469	7	9	9	G
Ce10	161	207	274	195	247	396	111	139	209	304	141	252	267	469	7	11	9	G
Ce11	161	207	274	195	245	391	110	139	209	304	141	252	267	469	7	8	9	G
Ce12	161	207	274	195	247	396	110	140	209	304	141	252	267	469	7	14	9	G
Ce13	161	207	273	195	247	396	110	139	209	304	141	253	268	469	7	10	9	G
Ce14	161	207	273	195	247	396	110	139	209	304	141	253	266	469	7	9	9	G
Ce15	161	207	273	195	246	396	110	139	209	304	141	252	267	467	7	10	9	G
Ce16	161	207	274	195	247	396	110	139	209	304	141	252	266	469	7	7	9	G
Ce17	161	207	272	195	247	396	110	139	209	303	141	251	267	469	7	8	9	G
Ce18	161	207	273	195	247	396	110	139	209	304	141	252	268	469	7	10	9	G
Cu1	165	207	269	195	245	392	108	140	210	303	140	252	267	466	7	3	9	T
Cu2	165	208	269	195	245	391	110	141	210	302	141	251	267	469	7	7	9	T
Cu3	165	208	269	195	245	391	110	141	210	302	141	252	267	469	7	7	9	T
Cu4	165	208	269	195	245	391	110	141	210	302	141	252	267	468	7	6	9	T
Cu5	164	208	269	195	245	391	110	141	210	302	141	252	268	469	7	8	9	T
Cu6	165	207	269	195	245	392	108	140	210	303	140	251	267	466	7	3	10	G
Cu7	165	207	269	195	245	391	110	141	210	302	141	252	268	468	7	7	9	G
Cu8	165	208	269	195	245	391	110	141	210	302	141	251	267	469	7	5	9	T
Cu9	165	208	269	195	245	391	110	141	210	302	141	252	267	469	7	5	9	T
Cu10	164	208	269	195	245	391	110	141	210	302	142	251	270	469	8	7	9	T
Cu11	165	208	269	195	245	391	110	141	210	302	141	251	267	469	7	6	9	T
Cu12	165	208	269	195	245	391	110	141	210	303	141	252	267	469	7	9	9	T
Cu13	164	208	269	195	245	391	110	141	210	302	141	252	269	469	7	9	9	T
Cu14	165	208	269	195	245	391	110	141	210	303	141	252	267	469	7	6	9	T

Cu15	164	208	269	195	245	391	110	141	210	303	142	252	268	469	7	8	9	T
Cu16	165	208	269	195	245	391	110	141	210	303	142	252	268	469	7	5	9	T
Cu17	164	208	269	195	245	391	110	141	210	302	141	252	268	469	7	5	9	T
Cu18	165	208	269	195	245	391	110	141	210	303	141	252	267	469	7	7	9	T
Di1	164	208	269	196	246	391	110	141	210	302	143	252	269	469	7	8	9	T
Di2	164	207	269	196	246	391	109	140	210	302	142	252	267	471	7	9	9	G
Di3	164	208	269	196	246	391	111	140	210	302	142	252	267	468	7	8	9	G
Di4	164	207	269	196	245	391	110	140	210	302	142	252	267	469	7	9	10	G
Di5	164	207	269	196	246	391	111	140	210	302	142	252	267	472	7	5	9	G
Di6	164	208	269	196	245	391	110	141	210	303	142	252	269	466	7	8	9	G
Di7	164	207	269	195	246	391	110	140	210	303	141	252	267	470	7	6	9	G
Di8	164	209	269	195	245	391	110	140	210	302	141	252	269	465	7	9	9	G
Di9	164	207	269	195	246	391	111	140	210	302	141	252	267	471	7	8	9	G
Di10	164	208	269	196	245	391	110	140	210	302	142	252	269	466	7	11	9	G
Di11	164	207	269	195	246	391	111	140	210	302	141	252	269	468	7	5	9	G
Di12	164	208	269	196	245	391	110	141	210	302	143	252	269	469	7	7	9	T
Di13	164	207	269	196	246	391	111	140	209	302	142	252	269	468	7	4	9	G
Di14	164	207	269	196	245	391	111	140	210	302	142	252	269	468	7	3	9	G
Di15	164	207	269	196	247	391	111	140	209	302	142	252	268	466	7	7	9	G
Di16	164	207	269	196	246	391	110	140	210	301	142	252	267	469	7	10	9	G
Di17	164	209	269	196	245	391	110	140	210	302	142	252	269	465	7	8	9	G
Di18	164	207	269	196	246	391	111	140	210	302	142	252	267	471	7	5	9	G
Di19	164	207	269	196	246	391	110	140	210	302	142	252	268	466	7	8	10	G
Di20	164	207	269	195	245	390	110	139	210	302	142	252	267	467	7	7	9	G
Di21	164	207	269	195	245	390	110	140	210	302	142	252	267	466	7	11	9	G
Di22	164	207	269	195	246	391	110	140	210	302	142	252	266	471	7	10	9	G
Di23	164	207	269	196	246	391	111	140	210	302	142	252	269	468	7	8	9	G
Di24	164	207	269	196	246	391	110	140	210	301	142	252	267	469	7	6	9	G
Di25	164	207	268	196	246	391	111	140	210	302	142	252	267	472	7	5	9	G
Di26	164	207	269	196	246	391	110	140	210	302	142	252	268	471	7	8	9	G
Di27	164	207	269	195	246	391	110	140	210	303	141	252	267	471	7	6	9	G
Di28	164	207	269	195	246	391	110	140	210	302	141	252	267	471	7	9	9	G
Di29	164	208	269	196	245	391	110	141	210	302	142	252	268	469	7	8	9	T
Di30	164	208	269	196	245	391	110	141	210	302	142	252	268	468	7	6	9	T
Di31	164	208	269	196	245	391	108	140	210	302	142	252	270	465	7	9	10	G
Di32	164	208	269	196	245	391	110	141	210	302	142	252	268	469	7	7	9	T

Di33	164	208	269	196	245	391	110	141	210	302	142	252	268	470	7	10	9	T
Di34	164	208	269	196	245	391	108	140	210	302	142	252	270	466	7	9	10	G
Di35	164	208	269	196	245	391	110	141	210	302	142	252	268	469	7	10	9	T
Di36	164	208	269	196	245	391	108	141	210	302	142	252	270	465	7	10	10	G
Di37	164	208	269	196	245	391	108	141	210	302	142	252	270	466	7	10	10	G
Di38	164	208	269	195	245	391	110	141	210	303	142	252	268	469	7	7	9	T
Di39	164	208	269	195	245	391	110	141	210	303	142	252	268	469	7	7	9	T
Di40	164	208	269	195	245	391	110	141	210	303	142	252	268	469	7	9	9	T
Di41	164	208	269	195	245	391	110	141	210	303	142	252	268	469	7	10	9	T
Di42	164	207	269	195	246	391	110	140	210	303	142	252	267	471	7	7	9	G
Di43	164	207	269	196	246	391	111	140	210	302	142	252	267	471	7	5	9	G
Di44	164	207	269	196	246	391	111	140	210	302	142	252	267	471	7	5	9	G
Di45	164	207	269	196	246	391	110	140	210	302	142	252	267	470	7	9	9	G
Di46	164	209	269	196	245	391	110	140	210	302	142	252	269	465	7	7	9	G
Di47	164	208	269	196	246	391	110	140	210	300	142	252	267	469	7	10	9	G
Di48	164	209	269	196	245	391	110	140	210	302	142	252	269	465	7	7	9	G
Di49	164	207	269	196	246	391	110	140	210	302	142	252	268	471	7	7	9	G
Di50	164	207	269	195	246	391	111	140	210	303	141	252	267	471	7	5	9	G
Di51	164	207	269	195	246	391	110	140	210	303	141	252	267	471	7	8	9	G
Di52	164	207	269	195	246	391	110	140	210	303	141	252	268	471	7	8	9	G
Di53	164	207	269	195	246	391	110	140	210	302	141	252	267	469	7	8	9	G
Di54	164	207	269	195	246	391	110	140	210	302	141	252	267	469	7	9	9	G
Di55	164	209	269	195	245	391	110	140	210	302	141	252	270	466	7	8	9	G
Di56	164	207	269	195	246	392	111	140	210	302	142	252	268	471	7	7	9	G
Di57	164	207	269	196	246	391	110	140	210	300	142	252	267	469	7	7	9	G
Di58	164	207	269	196	246	391	110	140	210	302	142	252	267	469	7	7	9	G
Di59	164	207	269	195	245	390	110	140	209	302	142	252	267	466	7	8	10	G
Di60	164	207	269	195	245	390	110	140	210	302	142	252	267	466	7	8	10	G
Di61	164	207	269	195	246	391	110	140	210	302	142	252	267	466	7	9	9	G
Di62	164	207	269	195	245	391	111	141	210	302	141	252	268	469	7	8	9	T
Di63	164	208	269	195	245	391	108	141	210	302	142	252	269	466	7	6	9	G
Di64	164	207	269	195	246	391	110	140	210	302	141	252	267	470	7	6	9	G
Di65	164	207	269	195	246	391	111	140	210	302	141	252	267	468	7	8	9	G
Di66	164	207	269	195	245	390	110	140	210	302	141	252	268	466	7	8	10	G
Di67	164	207	269	195	246	391	110	140	210	301	141	252	267	469	7	8	9	G
Di68	164	207	269	195	246	392	115	140	210	302	141	252	267	467	7	6	9	G

Di69	164	207	269	195	246	391	111	140	210	302	142	252	267	469	7	8	9	G
Di70	164	207	269	196	246	391	110	140	210	301	142	252	267	469	7	9	9	G
Di71	164	209	269	196	245	391	110	140	210	302	142	252	269	466	7	8	9	G
Di72	164	207	269	196	245	390	109	140	210	302	142	252	267	469	7	7	9	G
Di73	164	208	269	196	246	391	110	140	210	302	142	252	269	466	7	7	9	G
Di74	164	207	269	196	246	392	110	140	210	302	142	252	268	471	7	7	9	G
Di75	164	207	269	196	246	391	111	140	210	302	142	252	267	471	7	7	9	G
Di76	164	207	269	196	246	391	110	140	210	302	142	252	267	469	7	9	9	G
Di77	164	207	269	196	246	391	110	140	210	302	142	252	266	471	7	8	9	G
Di78	164	207	269	196	246	391	110	140	210	302	142	252	266	471	7	11	9	G
Di79	164	209	269	196	245	391	110	140	210	302	142	252	270	466	7	8	9	G
Di80	164	207	269	196	246	391	110	140	210	302	142	252	268	471	7	7	9	G
Di81	164	207	269	196	246	391	111	140	210	302	142	252	269	468	7	5	9	G
Di82	164	207	269	196	245	391	109	140	210	302	142	252	264	469	7	8	9	G
Di83	164	207	269	196	246	391	109	140	210	302	142	252	267	469	7	7	9	G
Di84	164	207	269	196	246	391	110	140	210	302	142	252	267	468	7	8	9	G
Di85	164	207	269	196	246	391	110	140	210	302	142	252	267	470	7	7	9	G
Di86	164	208	269	196	245	391	110	141	210	302	142	252	268	469	7	6	10	G
Di87	164	207	269	196	247	391	110	140	209	302	143	252	269	466	7	4	9	G
Di88	164	207	269	196	245	391	111	140	210	302	142	252	267	471	7	5	9	G
Di89	164	207	269	196	245	391	109	140	210	302	142	252	267	466	7	8	10	G
Di90	164	207	269	196	245	391	110	141	210	302	142	252	269	466	7	6	9	G
Di91	164	207	269	196	245	391	110	141	210	302	142	252	269	466	7	8	9	G
Di92	164	208	269	196	245	391	110	140	210	302	142	252	269	466	7	9	9	G
Di93	164	207	269	196	245	391	110	141	210	302	142	252	269	466	7	9	9	G
Di94	164	207	269	196	245	391	110	141	210	302	142	252	269	466	7	7	9	G
Jo1	164	208	269	195	245	391	110	141	210	302	141	252	268	469	7	6	9	T
Jo2	164	208	269	195	245	391	110	141	210	302	141	252	269	469	7	7	10	G
Jo3	164	208	269	195	245	391	110	141	210	302	141	252	269	469	7	6	9	T
Jo4	164	208	269	195	245	391	110	141	211	302	141	252	269	469	7	6	10	G
Jo5	164	208	269	195	246	391	110	141	210	302	141	251	267	469	7	7	9	G
Jo6	164	208	269	195	245	391	111	141	210	302	141	252	268	469	7	8	9	G
Jo7	164	208	269	195	246	391	110	141	210	302	141	251	267	469	7	8	9	G
Jo8	164	208	269	195	245	391	111	141	210	302	141	251	268	466	7	8	9	T
Jo9	164	208	269	195	245	391	111	141	209	302	141	252	268	468	7	9	9	T
Jo10	165	208	269	195	245	391	110	141	211	302	141	251	269	469	7	8	9	T

Jo11	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	6	9	T
Jo12	164	208	269	195	246	391	110	141	210	302	141	251	268	470	7	7	9	G
Jo13	165	208	269	195	245	391	110	141	211	302	140	251	269	470	7	6	9	T
Jo14	164	208	269	195	245	391	110	141	210	302	141	251	268	470	7	7	9	G
Jo15	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	7	9	T
Jo16	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	7	9	T
Jo17	164	208	269	195	245	391	111	141	210	302	142	251	268	469	7	7	9	T
Jo18	164	208	269	195	245	391	110	141	210	302	141	251	268	469	7	7	9	T
Jo19	164	208	269	195	245	391	110	141	210	302	141	251	269	470	7	7	9	T
Jo20	164	208	269	195	245	391	110	141	210	303	141	252	269	470	7	7	9	T
Jo21	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	6	9	T
Jo22	164	208	269	195	245	391	111	141	210	302	141	251	268	469	7	7	9	T
Jo23	164	208	269	195	245	391	111	141	210	302	141	252	268	469	7	7	9	T
Jo24	165	208	269	195	245	391	110	141	210	302	141	251	267	469	7	8	9	T
Jo25	165	208	269	195	245	391	110	141	210	302	141	252	267	469	7	8	9	T
Jo26	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	8	9	T
Jo27	164	208	269	195	245	391	110	141	210	303	141	252	269	469	7	6	9	T
Jo28	164	208	269	195	245	391	110	141	210	303	141	252	269	469	7	6	9	T
Jo29	164	208	269	195	245	391	111	141	210	302	141	251	268	469	7	8	9	T
Jo30	164	208	269	195	245	391	110	141	210	302	141	251	268	470	7	7	9	G
Jo31	164	208	269	195	245	391	111	141	210	302	141	251	268	469	7	8	9	T
Jo32	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	5	9	T
Jo33	164	208	269	195	245	391	110	141	210	302	141	252	269	469	7	7	9	T
Jo34	164	208	269	195	245	391	111	141	210	303	141	251	268	469	7	8	9	T
Jo35	164	208	269	195	245	391	111	141	210	302	141	251	268	469	7	9	9	T
Jo36	164	208	269	195	245	391	111	141	210	302	141	251	268	469	7	9	9	T
Jo37	164	208	269	195	245	391	109	141	209	302	141	251	267	469	7	7	9	G
Jo38	164	208	269	195	245	391	111	141	210	302	141	252	268	465	7	8	9	T
Jo39	164	208	269	195	246	391	110	141	210	302	141	251	267	469	7	7	9	G
Jo40	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	8	9	T
Jo41	164	208	269	195	245	391	110	141	210	302	141	251	269	469	7	5	9	T
Jo42	164	208	269	195	245	391	111	141	210	302	141	252	268	469	7	8	9	T

CAPÍTULO 2

SEX RATIO DIFFERS BETWEEN *PINUS JOHANNIS* AND *PINUS DISCOLOR*

Sex ratio differs between *Pinus johannis* and *Pinus discolor*

Lluvia Flores-Rentería

INTRODUCTION

In species with separate sexes (dioecy), unequal adult sex ratios in natural populations are common across a wide range of plant and animal groups (Hardy 2002; Barret *et al*., 2010). By altering the availability or intensity of competition for mates, biased sex ratios can dramatically influence a range of species attributes from genetic diversity (e.g., effective population size; Frankham 1995) to population growth rates and persistence (e.g., Milner-Gulland *et al*. 2003). Among dioecious flowering plants, biased sex ratios are particularly prevalent. A review by Delph (1999) concluded that among published sex ratio estimates only 29% have 1:1 sex ratios, while a majority (57%) has male-biased populations. Female biases are rare. Causes of biased sex ratios fall into two broad categories based on the life history stage at which they arise. Either offspring can be produced with biased ratios (e.g., Taylor 1994, Stehlik and Barrett 2005) or biased sex ratios can develop as a result of mortality differences between the sexes following offspring establishment (reviewed in Delph 1999, Obeso 2002).

Sex ratios may be considered in terms of optimal strategies for populations (e.g., Lewis 1942, Mulcahy 1967, Kaplan 1972), or for individuals (e.g., Fisher 1930, Bodmer & Edwards 1960, Charnov 1975), or they may be explained as the incidental result of chromosomal sex determination mechanisms and ecological factors (e.g., Darwin 1877, Harris 1968, Lloyd 1973, Lloyd & Webb 1977, Opler & Bawa 1978). Biased sex ratios can arise as a result of sex differences in life history and therefore reveal the consequences of trade-offs in resource allocation (Roff 2002).

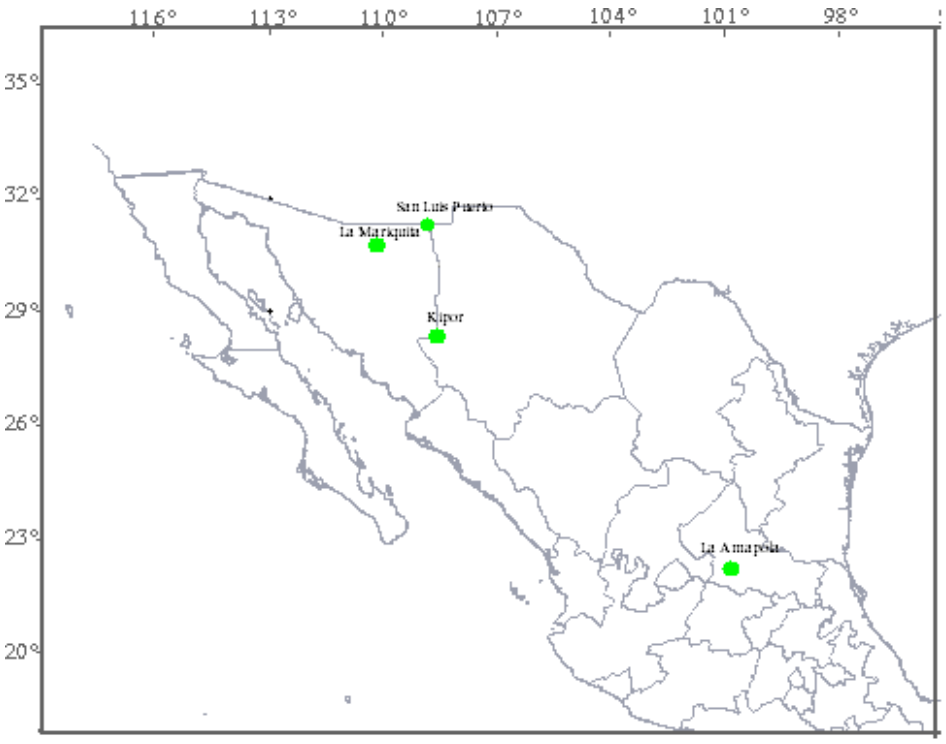
This chapter reports sex ratios for populations of long-lived perennials of two species of *Pinus* which are endemic to Mexico and South of USA. *Pinus* species are considered as monoecious, however, *P. discolor* and *P. johannis* have a dioecious reproductive system. The taxonomy of these species is discussed by Flores-Rentería *et al*. (in progress) and sexual morphs are described in detail (Flores-Rentería *et al*. 2011). Some sex ratios for populations of the two species have been reported previously (Avila *et al*, 1992). However, their observations were done on October instead on May and June that it is their reproductive time.

As monoecious plants produce predominantly one sex, as described in Flores-Rentería *et al*. (2011), the so-called monoecious plants are considered inconstant males, when producing mainly microstrobili, and inconstant female when producing mainly megastrobili (see Lloyd 1974).

METHODS

This study was carried out mainly in Amapola, San Luis Potosí, Mexico (coordinates N 22º 01.160' W 101º 07. 706', 2391 m.a.s.l.) where *P. johannis* along with *P. cembroides* are predominant into vegetation, in this locality *P. johannis* present intense herbivory by a tortrocid that creates a worm nest approximately 10 to 15 cm long. For a comparison of the sex ratio between species we used three additional populations of *P. discolor* (Figure 1): La Mariquita (N31 02.414, W110 23.033, 2063 m.a.s.l., Sonora), San Luis Puerto (31º 19.356' N, 108º 45.503'W, 1923 m.a.s.l. Sonora-Chihuahua -Arizona) and Kipor (N 28° 26.171' W 108° 30.869' 1618 m.a.s.l. Sonora-Chihuahua, south), in the three populations of *P. discolor* no visible worm nest were detected.

As monoecious produce a slight amount of one sex and a big amount of the other, sex ratio was taken considering the functional gender (Lloyd, 1980), therefore we grouped individuals that invest mostly to male function (males and predominantly male monoecious, the latter called inconstant males) and individuals that invest mostly to female function (females and predominantly female monoecious, the latter called inconstant females). Because *P. johannis* (La Amapola) had a female bias, comparisons of sex ratio across the time were made to detect any change during time, whereas in three Sonoran populations we recorded the sex ratio once in 2006. Only in La Amapola sex ratio was calculated during four years, from 2006 to 2009 considering male, female, monoecious predominantly male and monoecious predominantly female. Additionally, functional gender was calculated by year for *P. johannis* (La Amapola).



RESULTS

In La Amapola (San Luis Potosi) *P. johannis* have a proportion of 36.24%, 27.52%, 21.56% and 14.68% to female, male, monoecious predominantly female and monoecious predominantly male respectively (Figure 2).

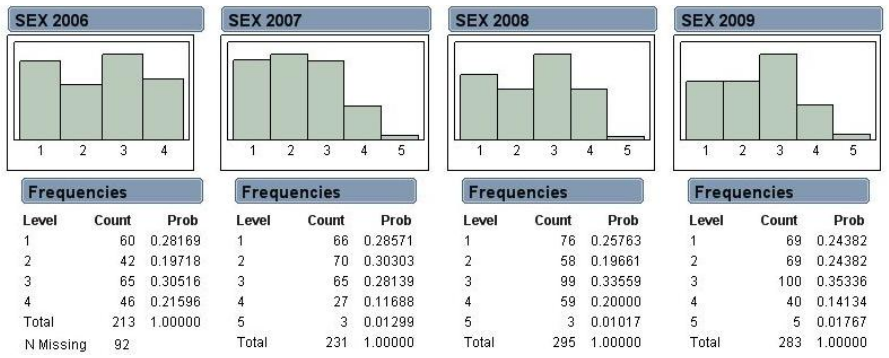


Figure 2. Sexual ratio of *Pinus johannis* in La Amapola. Female (1), Male (2), Predominantly female monoecious (3), predominantly male monoecious (4), monoecious with bisporangiate strobili (5).

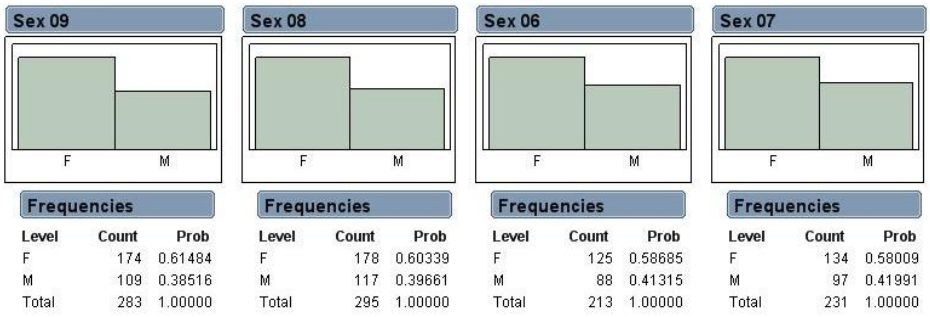


Figure 3. Sexual ratio of *Pinus johannis* in La Amapola. Female and inconstant female (1), Male and inconstant male (2). Monoecious with bisporangiate strobili were excluded because of their lower proportion in the population.

The interesting thing is that when we grouped individuals that invest only and mainly to female function (F) or individuals that invest only or mainly in male function (M) in all years the ratio was near 3:2 (female:male), ergo 60% individuals females and monoecious predominantely female and 40% approximately of males and monoecious predominantely male (Figure 3). During the five years a female bias was detected suggesting the sex proportion is constant between sexes across the time.

In contrast the three populations of *P. discolor* were near the 1:1 sexual ratio. Annual precipitation is higher for all populations in Sierra Madre Occidental (Table 2). Populations of *P. discolor* occur under 2,000 m.a.s.l. whereas *P. johannis* seems higher than this altitude.

Table 2. Sex ratios in Sierra Madre Occidental (*P. discolor*) and Sierra Madre Oriental (*P. johannis*) populations. Annual precipitation values are given in mm.

Population	No. of individuals	Female %	Male %	Annual precipitation
Sierra Madre Occidental				
Kipor	82	52	48	700-800
Mariquita	71	48	52	400-500
San Luis	54	52	48	400-500
Sierra Madre Oriental				
Amapola	268	59	41	300-400

DISCUSSION AND PERSPECTIVES

Dioecious and subdioecious populations have been found in *P. discolor* and *P. johannis* (McCormick & Andresen 1963; Little, 1968; Passini, 1994). Bailey and Hawksworth (1979) describe the dioecious tendency in *P. discolor* "less obvious" toward the southern part of the range that they consider San Miguelito Mountains of southern San Luis Potosi, considered in this study as *P. johannis*. Our findings corroborated latitudinal differences in sex ratio, but we classified sexual expression based on the functional gender and found a ratio near 1:1 in a population of *P. discolor* (Sierra Madre Occidental) and a female-bias in the localities of San Miguelito Mountains (Sierra Madre Oriental). Interestingly, altitudinal differences appear species-related with *P. culminicola* occupying higher altitude, followed by *P. johannis*, and *P. discolor* distributed in lower areas. This altitudinal variation is associated with the precipitation level, *P. discolor* occupies areas with higher precipitation than *P. johannis*. Geographic distribution between these species supports the idea of different adaptive strategies to drought, as has been proposed for the pinyon pine (Richardson, 1998; Cole *et al*. 2008), which can impact the sexual expression.

As we detected intense herbivory in La Amapola by a tortrocid in comparison with the three populations of *P. discolor*, it will be interesting to study the effect of the herbivory on the sexual expression on *P. johannis*. Although the environmental factors as can drive the sexual expression as occurs in *P. edulis* (Floyd, 1983; Cobb *et al*. 2005), more studies are needed to determine the stability of unisexual individuals in *P. johannis*.

Although *P. discolor* and *P. johannis* have been described with unisexual individuals, as showed here, there are some basic questions based on these results. Do unisexual individuals are stable or labile? If they are stable (once the sex is determined it remains stable) what drove the evolution

to the sexual separation? If they are instable or labile (sex change due age, stress, etc.) what would causes the change in *P. johannis*? Are bisporangiate strobili produced constantly in the same individuals? Are these reproductive? All of these questions are explored in the following chapters.

References

Ávila N. J., García M. E. & Reyes A. J. 1992 Registro de Pinus discolor Bailey et Hawksworth en la sierra de monte Grande, San Luis Potosí, México. *Acta Botánica Mexicana* 20:9-12.

Bailey, D.K. & Hawksworth, F.G. 1979. Pinyons of the Chihuahuan Desert Region. *Phytologia* 44:129–133.

Bodmer, W. F., and A. W. F. Edwards. 1960. Natural selection and the sex ratio. Ann. Human Genet. 24:239-244.

Charnov, E. L. 1975. Sex ratio selection in an age structured population. Evolution 29:366-368.

Cole K, Fisher J, Arundel ST, Cannella J, Swift S. 2008. Geographic and climatic limits of needle types of one- and two-needled pinyon pines.

Darwin, C. 1877. The different forms of flowers on plant of the same species. J. Murray, London.

Delph, L. F. 1999. Sexual dimorphism in life history. Pages 149–163 in M. A. Geber, T. E. Dawson, and L. F. Delph, editors. Gender and sexual dimorphism in flowering plants. Springer, Berlin, Germany.

Fisher, R. A. 1930. The genetical theory of natural selection. Oxford University Press, Oxford.

Flores-Rentería, Ll., Vázquez-Lobo, A., Whipple, A.V., Piñero, D., Márquez-Guzmán, J. & Domínguez, C.A. 2011. Functional bisporangiate cones in *Pinus johannis* (Pinaceae): Implications for the evolution of bisexuality in seed plants. *Am. J. Bot*. 98: 130–139.

Frankham, R. 1995. Effective population size/adult population size ratios in wildlife: a review. Genetical Research 66:95–107.

Hardy, I. C. W., editor. 2002. Sex ratios: concepts and research methods. Cambridge University Press, Cambridge, UK.

Harris, W. 1968. Experimental effects on the sex ratio of *Rumex acetosella* L. Proc. New Zeal. Ecol. Soc. 15:51-54.

Journal of Biogeography 35: 257–269.

Kaplan SM. 1972. Seed production and sex ratio in anemophilous plants. Heredity. 28:281–285.

Lewis D. 1942. The evolution of sex in flowering plants. Biol Rev. 17:46–67.

Little. E. 1968 Two new pinyon varieties from Arizona. *Phytologia* 17:329–342.

Lloyd, D.G. 1973. Sex ratios in sexually dimorphic Umbelliferae. Heredity 31:239-249.

Lloyd, D.G. and C.J. Webb. 1977. Secondary sex characters in plants. Bot. Rev. 43:177-216.

McCormick J. & J. W. Andresen. 1963. A subdioecious population of pinus cembroides in southeast Arizona. The Ohio Journal of Science 4:159-163.

Milner-Gulland, E. J., O. M. Bukreeva, T. Coulson, A. A. Lushchekina, M. V. Kholodova, A. B. Bekenov, and I. A.

Mulcahy DL. 1967. Optimal sex ratio in *Silene alba*. Heredity. 22:411–423.

Obeso, J. R. 2002. The costs of reproduction in plants. New Phytologist 155:321–348.

Opler P.A. and K.S. Bawa. 1978. Sex ratios in tropical forest trees. Evolution 32:812-821.

Passini, M-F. 1994. Synonymie entre *Pinus discolor* et *Pinus johannis. Acta Botanica Gallica* 141:387-388.

Richardson DM. 1997. *Ecology* and *biogeography of Pinus*. Cambridge University Press.

Roff, D. A. 2002. Life history evolution. Sinauer, Sunderland, Massachusetts, USA.

Stehlik, I., and S. C. H. Barrett. 2005. Mechanisms governing sex-ratio variation in dioecious Rumex nivalis. Evolution 59: 814–825.

Taylor, D. R. 1994. The genetic basis of sex ratio in Silene alba (=S. latifola). Genetics 136:641–651.

CAPÍTULO 3

PINUS JOHANNIS AS A MODEL TO UNDERSTAND THE EVOLUTIONARY PROCESS LEADING TO SEXUAL SEPARATION IN GYMNOSPERMS

Pinus johannis as a model to understand the evolutionary process leading to sexual separation in gimnosperms

Flores-Rentería, Ll. [a,c*], Molina-Freaner, F.[b], Whipple, A. [c], and C. A. Domínguez [a].

Running title: Unisexuality in *Pinus johannis*

[a]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, A. P. 70-275, México, D. F. 04510 México.

[b]Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México, Apartado Postal 1354, Hermosillo, Sonora 83000 México.

[c]Department of Biological Sciences and Merriam-Powell Center for Environmental Research, Northern Arizona University, Flagstaff, AZ 86011, USA.

Tel: (928) 523-9138, fax: (928) 523-7500.

*E-mail: lluvia.flores@nau.edu

Abstract

Populations of *Pinus johannis* have female, male and monoecious (cosexual) plants, which occasionally produce hermaphroditic structures. Here we investigate gender expression of *P. johannis*, the stability of unisexual individuals and its evolutionary pathway to dioecy.

The reproductive system of *P. johannis* appears to be close to dioecy with 96% of individuals being unisexual or expressing less than 10% function of one gender. In comparison with *P. edulis*, which is the only species of *Pinus* described with unisexual individuals under a labile system, the sexual expression of *P. johannis* is bimodal whereas *P. edulis* possess a gradual transition from male to female. The combination of multi-year surveys and experimental manipulation of resource availability by herbivore removal provides evidence of unisexual stability in *P. johannis*. Detection of stable unisexual individuals and some inconstant male and female individuals, occurring sympatrically in a transitional population to dioecy, suggest this system evolved through a monoecy-paradioecy pathway. This offers a novel illustration of gender variation and the evolutionary pathway to dioecy in gymnosperms which have been poorly studied. *P. johannis* can be used as a model to understand the evolution of dioecy in gymnosperms.

Key words: dioecy, functional gender, gymnosperms, monoecy-paradioecy, secondary dimorphic features, unisexuality.

INTRODUCTION

Angiosperms exhibit extensive breeding systems variation, ranging from hermaphroditism to unisexuality. Dioecy, which represents the extreme sexual separation, is present in only 6% of angiosperms (Renner & Ricklefs, 1995) but has evolved independently from cosexuality in nearly half of angiosperm families through a variety of selective forces and genetic mechanisms (Charlesworth, 2002). In contrast, gymnosperms exhibit a narrow range of variation of breeding systems; 52% of gymnosperms are considered dioecious and 48% monoecious (Givnish, 1980; see Flores-Rentería *et al*., 2011). Despite the high percentage of dioecious species there are very few studies describing the evolutionary pathway to dioecy in gymnosperms. Most theoretical models of dioecy have been developed and tested in angiosperms where dioecy has evolved mainly through the gynodioecy or the monoecy-paradioecy pathway (Geber *et al*., 1999; although see Torices *et al*., 2010). In populations evolving to sexual separation through the gynodioecy pathway individuals bear female or hermaphroditic flowers (Lloyd, 1976). The gynodioecy pathway often involves two mutations of large effect, one conferring male sterility and the second female sterility (Charlesworth & Charlesworth, 1978). In the monoecy-paradioecy pathway, transitional populations exhibit quantitative variation in the ratio of female to male fertility within individuals and are bimodal for gender expression. The monoecy-paradioecy pathway involves gradual changes in sex allocation (female and male fertility) via many mutational steps (Lloyd, 1980).

Recognition of the evolutionary pathway to dioecy is achieved by the detection of inconstant (e.g. the production of ovules by “male” plants) male and female individuals in transitional populations. Lloyd (1980) proposed a way to detect inconstant unisexual individuals by

estimating, quantitatively, the proportion of one plant's genes which are transmitted through pollen (its maleness) or through ovules (its femaleness) relative to the total male and female structures production of one population. This approach describes the functional gender of individuals. Sex inconstancy in males, but not females, would support the gynodioecy pathway (Lloyd, 1980; Dorken & Barret, 2004) because male-sterility usually prevents the production of pollen in females, and therefore sex inconstancy occurs only among male plants, whereas sex inconstancy of both female and male supports a monoecy-paradioecy pathway (Lloyd, 1975, 1980; Freeman *et al*., 1997; Renner & Won, 2001; Dorken & Barret, 2004). It has been assumed that monoecy-paradioecy is the main evolutionary pathway to dioecy from a monoecious ancestor (Lloyd, 1980); however, convincing cases documenting the evolution of dioecy through this pathway are surprisingly rare (Dorken & Barret, 2004; Ehlers & Bataillon, 2007).

The lack of studies in gymnosperms regarding the evolutionary pathway to dioecy is partially due to the fact that most species with unisexual individuals described to date lack of inconstant males or females, suggesting that the evolutionary process of sexual separation is completed and there are no transitional populations to dioecy. In addition, work with long life cycle species is challenging. Conifers are long-lived organisms, retain cones for 1 to 3 years (Williams, 2009), and are susceptible to high cone loss associated to environmental condition (García *et al*., 2000; Ortiz *et al*., 2002; Mueller *et al*., 2005) and high inbreeding depression (Williams 2008, 2009). Also, unpredictable masting events (Crone *et al*., 2011) limit the opportunity to study gender variation. There are few species with monoecious and dioecious populations that allow comparisons between unisexual and monoecious individuals to test evolutionary hypothesis to dioecy; hence it is important to have a gymnosperm model with unisexual and monoecious individuals to understand the process of sexual separation in this

group. *Pinus johannis* (Johann's pine or Zacatecas pinyon) represents a unique opportunity to understand the evolution of unisexuality in gymnosperms because it has male, female and monoecious individuals within the same and different populations (Ávila *et al*., 1992; Flores-Rentería *et al*., 2011), providing a system in which the hypotheses of the mechanism leading the evolution of dioecy in gymnosperms can be evaluated.

The stability of the unisexual individuals has not been determined in *Pinus johannis*. The genus *Pinus* has been considered exclusively monoecious (Mirov, 1967), although there are a few reports of unisexual individuals. In those species where unisexuality has been observed, it is a labile feature within the lifespan of an individual and associated with environmental factors including age, stress and herbivory (McCormick & Andresen, 1963; Floyd, 1983; Cobb *et al*., 2002; Tikhonova, 2003; Mueller *et al.*, 2005). The best characterized system with labile unisexual individuals is *P. edulis*, in which a stem-boring moth (*Dioryctria albovittella*) alters sexual expression by reducing female function and increasing male function (Whitham & Mopper, 1985; Cobb *et al*., 2002). Stable unisexual (truly dioecious) individuals have not been reported in the genus *Pinus.* Therefore, corroboration of the stability of unisexual individuals of *P. johannis* is necessary to understand the evolutionary pathways and the process leading the evolution to dioecy. One way to evaluate sexual stability is through long-term observations to record changes in sexual expression (Ushimaru & Matsui, 2001; Cobb *et al*., 2002; Renner *et al*., 2007; Tikhonova, 2007). Under sexual lability one individual early in life, commonly expresses one sex then the opposite and later in life both sexes, thus detection of age-size classes associated to sexual morphs in a population can suggest sexual lability (Floyd, 1983; Lev-Yadun & Liphschitz, 1987; see, Charnov, 1984). Detection of secondary dimorphic features, which result from different resource use between males and females through time (Lloyd & Webb, 1977),

offers another piece of support for the stability of males and females. In truly dioecious plants, sex expression is often not restricted to reproductive structures but includes sexual dimorphism and secondary sexual characters in the morphology and phenology of the plant (Bullock & Bawa, 1981; Meagher & Antonovics, 1982; Korpelainen, 1998). Sex differences in plant shape is a common dimorphic feature reported in several dioecious gymnosperms as *Ginkgo biloba*, (Grier, 1917), *Juniperus communis* (Lloyd & Webb, 1977), *Zamia skinneri* (Clark & Clark, 1987) and *Taxus baccata* (Iszkuło *et al*., 2009). Differences in the allocation resource by female and male individuals can be reflected not only in the size but in shoot thickness, generating a feature sex-related easy to detect (Harris & Pannell, 2010).

The aim of this study is to characterize the reproductive system of *Pinus johannis,* which apparently has unisexual individuals (Ávila *et al*., 1992; Zavala & Campos, 1993) and determine the stability of unisexual individuals through time. We test if sexual expression of *P. johannis* has a similar environmental component as that observed in the most well-known *Pinus* where unisexuality has been observed. In order to evaluate if individuals of *P. johannis* express one sex as a consequence of resource limitation due high levels of herbivory as has been described in *P. edulis*. We performed a moth removal experiment. We compared the sexual expression of *P. johannis* to *P. edulis*, the latter being the only species of *Pinus* corroborated to have unisexual individuals under a labile system. We investigated the patterns of sex inconstancy in *P. johannis* to determine the evolutionary pathway to dioecy. Finally, we discuss our findings in the context of gymnosperm reproductive systems.

Methods

Study species

Pinus johannis is a pinyon pine, generally multi-stemmed shrub or tree, that produces 3 leaves per fascicle, with stomata present only on the ventral surfaces, The dorsal leaf surface is dark green and the ventral surface is glaucous white (Perry, 1991).

P. johannis has a scattered distribution, ranging from San Luis Potosí, Mexico (La Amapola) in its southern limit to the border between Mexico and the US in Sonora, Arizona and New Mexico. This study was carried out primarily in La Amapola, San Luis Potosí, Mexico (N 22º 01.160' W 101º 07. 706', 2391 m a.s.l.) where *P. cembroides* is dominant in the vegetation and it is associated in some areas to *P. johannis*. In this locality there are five reproductive morphs of *P. johannis*: (1) males that bear only pollen cones, (2) females that bear only ovulate cones, (3) predominantly male monoecious individuals that produce a large number of pollen cones and few ovulate cones, (4) predominantly female monoecious individuals that produce a large number of ovulate cones and few pollen cones, and (5) monoecious individuals that produce bisporangiate cones (Flores-Rentería *et al*., 2011). The sexual categories were described calculating the sexual proportion into individuals as: number of microstrobili / (number of microstrobili + number of megastrobili + green cones.

Sexual change through the time and in relation to the size

Starting in November 2005, when we first visited the population, the sexual identity was determinate by recording the presence of scars that pollen cones left when they dry out and by the presence of ovulate cones in development. From 2006 to 2009, sex was identified during the reproductive period (i.e. May to June). We determined sexual morph by presence or absence of male (microstrobili), female (megastrobili) or bisexual (bisporangiate cones) strobili from 213 to 295 individuals spread in a length of 3.5 km. Sexual morphs were intermixed in the population.

Reproductive trees were labeled and mapped for further sexual morph identification. In 2006 and 2008 we recorded presence or absence of sexual structures of 205 trees during the whole reproductive season resulting in better assignment of sexual expression; therefore we only show results of sexual changes that occurred from 2006 to 2008. We calculated the percentage of individuals that expressed the same sex through this time and the percentage of changes in every direction. All possible directions of change were considered, e.g.: female to monoecious predominantly female, to monoecious predominantly male or to male; etc.

In sequential hermaphrodite individuals, the allocation resource theory predicts a relation between sex and size. In some species of *Pinus*, male function is expressed in the earlier reproductive stages, then female function is expressed next, and monoecious individuals are thought to be produced once tree is "reproductively mature" (Floyd, 1983). In order to determine whether sexual morphs are associated with size classes, we measured tree height, which have been correlated with the age in individual of *P. johannis* in La Amapola (Romero, 2001), through a graduated telescoping pole and correlated to sexual proportion. We also calculated the basal area at a height of 15 cm in 214 trees (40-74 of each of the sexual morphs). Differences of basal area among morphs were determined by one-way ANOVA ($P = 0.05$).

Differences in plant architecture and stem mass between sexes

Measurements of height and basal area of 124 trees were used to determine whether there is sex-associated tree architecture, as it has been found in some dioecious gymnosperms (Clark & Clark, 1987; Iszkuło *et al*., 2009). Analysis of covariance was performed including sex as the covariate and basal area as a continuous variable to detect differences in tree architecture among the sexual morphs.

In June of 2009 we sampled shoots in order to evaluate whether sexual dimorphism occurred in shoot mass per unit length (Harris & Pannell, 2010). In order to avoid bias due to differences in stage of growth among shoots, we measured 10 cm length below a recent ramification and took three shoots per tree, one from the bottom, one from the medium part and one from the apex, all of them facing northwest. Since monoecious individuals of *P. johannis* produce many structures of one sex and few of the other one, it was difficult to obtain a sample to compare shoots bearing female and shoots bearing male structures from all different sexual morphs; therefore we combined male and predominantly male monoecious in one group and female and predominantly female monoecious in another. For this analysis 189 individuals were sampled, 92 of the male group and 97 of the female group. The tissue was dehydrated in a conventional drying oven at 65 °C for 24 hours. Mass of dry tissue was measured using an analytical balance to the nearest 0.0001g. Differences among morphs were determined by one-way ANOVA ($P = 0.05$), using dry mass as dependent variables and sexual morph as independent variable (JMP statistical software, SAS 2009).

Sexual expression in *P. johannis* and *P. edulis and the effect of herbivory in the sexual expression*

In order to compare how similar the sexual expression of *P. johannis* is to that of a species with labile unisexual individuals, we chose the best-characterized system, *P. edulis*. *P. edulis* is phylogenetically related (both species belong to subsection *Cembroides*) and share many ecological features (intense herbivory by moths, bird seed dispersal, habit, arid ecosystems, etc.). We collected data for *P. edulis* in Sunset crater (35°39′88″N and 111°42′62″W) and Winona (35°12′0″N and 111°24′0″W), Arizona. We graphed the total number of ovulate and pollen

cones for *P. johannis* and *P. edulis* in 213 and 375 individuals respectively. We chose data from masting years, 1998 for *P. edulis* and 2006 for *P. johannis*.

P. johannis is frequently attacked by *Conophthorus cembroides*, *Leptoglossus occidentalis* and *Eucosma bobana*, insects that damage up to 89% of the cones in some populations (Flores & Díaz, 1988). Intensity of herbivory can be estimated in *P. johannis* by counting the number of webworms and damaged cones, which look brown and smaller than the intact ones. If sexuality depends on resource availability or consumption of one sex by herbivores we expected an increase in the number of monoecious individuals in the population after insect herbivore removal as more trees would have sufficient resources to invest in male and female reproductive structures. To test this hypothesis, we performed an herbivore removal experiment applying cygon (dimethoate, which in *P. edulis* showed results as fast as one year (Whitham & Mopper, 1985) every month for one year (2008-2009) to 115 individuals. Additionally 153 individuals were included as control (no insecticide application). We grouped trees by treatment and by sexual morphotypes to calculate sex frequencies and to compare the changes in sexual expression before and after the insecticide application. In these 268 trees we recorded the sexual expression in 2008 and 2009 and made two categories to detect sexual changes; if they expressed the same sex in the two years they were assigned a 0. If they changed sex from 2008 to 2009, they were assigned a 1 (sex change sex). We use an ANOVA test to detect significant differences in sexual expression between sprayed and control trees. Additionally, to determine if our moth removal was effective, we compared the herbivory intensity between sprayed and control trees.

Functional gender and the evolutionary pathway to dioecy

In order to detect the evolutionary pathway to dioecy by functional gender estimation, we calculated the number of pollen cones or ovulate cones (at receptive stage) for each individual during the whole reproductive season of 2006 (N=124) and 2008 (N=227). *Pinus johannis* typically produces two female cones in the apex of the shoot, while male cones are generally grouped in a cluster of 30 to 50 pollen cones. Direct counts were made of ovulate cones, while pollen cones were counted by clusters. Estimates of functional gender (Lloyd, 1980) were derived from:

$$Gi = \frac{di}{di + liE}$$

Where *Gi* is the functional femaleness of an individual, *di* is its total number of ovulate cones, *li* is its number of cluster of pollen cones and *E* is an equivalence factor that equates the probability of male and female units contributing genes to the next generation. *Gi* ranges from 0 to 1, 0 being purely males and 1 purely females.

$$E = \frac{\sum_i di}{\sum_i li}$$

Results

Sexual expression in *Pinus johannis* and characterization of sexual morphs

Reproductive individuals of *P. johannis* in La Amapola can be grouped in 5 different sexual morphs: female, predominantly female monoecious, male, predominantly male monoecious, and monoecious with a sexual proportion close to 0.5, which eventually produce bisporangiate strobili (Figure 1). Percentages of sexual morphs from 2006 to 2009 are shown in Figure 2. Sexual morphs averages are: female 26.7%, male 23.5%, predominantly female monoecious

31.8%, predominantly male monoecious 16.9%, and monoecious with bisporangiate strobili 1.1%.

Stability of unisexual individuals of **P. johannis** *through the time*

From 2006 to 2008 we followed 205 individuals for the whole reproductive season and recorded the sexual state (change or no change). We found that 65.53 % of the individuals remained with the same sex (Fig. 3, filled black arrows). We noticed that there are two main groups where changes occurred. Changes within the female group occurred from female to predominantly female monoecious or vice versa; and it amounted to 21.36%; changes within the male group occurred from male to predominantly male monoecious or vice versa and it amounted to 10.62%. However, only one change was detected from predominantly male monoecious to predominantly female monoecious. A low frequency of predominantly female monoecious or predominantly male monoecious individuals changed to monoecious individuals producing bisporangiate structures.

We found no differences in the height of trees ($F_{3, 210}$=1.39, ρ =0.24, R^2=0.01), or in the basal area among the sexual morphs ($F_{3, 210}$=2.03, ρ =0.11, R^2=0.02), contrary to the expectation under the allocation resource hypothesis. In a labile system this hypothesis predicts a relationship between sex and size, where typically unisexuality is present in the youngest individuals, and then cosexuality in mature individuals with enough resources to reproduction. Height averages of females, males, predominantly female monoecious and predominantly male monoecious individuals were 2.65, 2.91, 2.85 and 2.91 m, respectively. Basal area averages of females, males, predominantly female monoecious and predominantly male monoecious individuals were 220, 279, 314 and 294 cm^2 respectively. Five sizes classes were found, all of them present sexual

proportions with a tendency to produce only or predominantly one sex into one individual regardless the height-age (Figure 4). Only 2 individuals seem to be close to 0.5 presented two and both fell into the third category, which was the most abundant in La Amapola.

Gender variation in morphology

The relation between height and basal area showed an architectural difference among sexual morphs (ANCOVA: ($F_{7,123}$=12.29, p <0.0001, R^2=0.42; sexual morph: $F_{3,123}$=2.09, p =0.1; basal area $F_{3,123}$=53.97, p <0.0001; interaction: $F_{3,123}$=2.81, p <0.05). For a given basal area, male and predominantly male monoecious trees are taller than female and predominantly female monoecious individuals (Figure 5).

The mass of dried shoots was greater in the group investing mostly in the female function (on average 2.5g) than the group investing mostly in the male function which weighed 1.56g ($F_{1,187}$=38.8, P<0.0001, R_2=0.171863).

Bimodal sexual expression supports stable unisexuality in *P. johannis*

The comparison of male and female expression distributed in individuals of *P. johannis* and *P. edulis* shows a different pattern of sexual expression between species. In *P. johannis* individuals invest exclusively or predominantly in female or male function (Fig. 6A). *P. edulis* has a gradient in sexual expression with many individuals having significant expression of both sexes. A tendency to the maleness is evident in *P. edulis* and only few strictly female individuals were detected (Fig. 6B).

Herbivory does not determine unisexual expression in *P. johannis*

We monitored sexual changes in trees in the herbivore removal experiment, particularly looking for increases in the number of monoecious individuals in sprayed trees. We detected no significant difference in sexual proportion between treatments related to herbivory ($F_{1,267}$=0.40, ρ =0.52, R^2=0.0018) neither an evident increment in the monoecious individuals in the treatments (Table 1). Herbivore removal was confirmed by the reduction of attack in insecticide sprayed trees ($F_{1,267}$=7.59, ρ <0.01, R^2=0.032).

Functional gender in *P. johannis* and the monoecy-paradioecy pathway to dioecy

The measurement of functional gender for 2006 and 2008 shows two main groups: those that are 90-100% male and those that are 90-100% female. A small group is formed by the presence of monoecious considered as inconstant females based on Lloyd's classification ranging from 0.6 to 0.8 *Gi* value (1980). In these trees bisporangiate cones were found (Flores-Rentería *et al.*, 2011). Another smaller group consists of males that produce few ovulate cones, here called inconstant males. In 2006, three individuals were inconstant females and in 2008 only one (Fig. 7). Approximately 95% (43% male and 52 % female) in 2006 and 96% (36% male and 60 % female) in 2008 were unisexual individuals based on the quantitative approach. Inconstancies are showed in both male and female morphs.

Discussion

Our findings suggest *P. johannis* has stable unisexual individuals and some inconstant male and female individuals occurring sympatrically in a transitional population to dioecy. Cosexual individuals, predominantly female and predominantly male monoecious, produce a large amount

of megastrobili and microstrobili respectively, and a low number of strobili of the opposite sexual structure suggesting they behave as female and male in relation to the gamete number they provide to mating. Most trees remained with the same sexual morphotype across time; when changes occurred they were from female to predominantly female monoecious trees (female group) and vs. or from male to predominantly male monoecious (male group) and vs. indicating sexual identity is relatively constant in the trees. Few changes were detected between groups; this can be an environmental effect as it is discussed below. In addition we did not find sexual size classes as predicted by the sex allocation resource theory in a labile system. Detection of secondary dimorphic features between the morphotypes confirms this idea. Herbivory does not seem to be the process behind unisexuality of *P. johannis*. It has different sexual performance compared with other gymnosperms with unisexual individuals under a labile system. The integration of our results indicate that *P. johannis* has a truly dioecious reproductive system. Inconstant male and inconstant females suggest that this system evolved through a monoecy-paradioecy pathway. Therefore *P. johannis* can be used as a model to understand the evolution of dioecy in gymnosperms.

Unisexual stability in plants

Dioecious species are either truly dioecious (if sex expression remains stable) or apparently dioecious (sequentially hermaphroditic, if sex can be reversible; Bierzychudek & Eckhart, 1988). There are different approaches to detect truly dioecious systems; the most direct is genetically (e.g. detection of sexual chromosomes); but it can be hard to detect since there is no universal marker associated to unisexual individuals (discussed in Charlesworth, 2002) and experimental crosses can be difficult to perform, especially in long lived-species. Long-term data on gender variation in natural populations are necessary in studies of the evolution of reproductive systems

using ecological features (e.g. Primack & McCall, 1986; Jordano, 1991). We recorded sex in five consecutive years and showed that most individuals of *P. johannis* remained with the same sex across time, sexual changes were detected mainly within the female group or within the male group supporting a truly dioecious system. However, few individuals changed the sex first to monoecious individuals producing bisexual structures and then to the opposite sex or revert to the original sexual group, and only one change was detected directly from the male to the female group which could result from environmental factors. Sexual systems genetically determined are frequently environmentally influenced (Stelkens & Wedekin, 2010). Freeman *et al*. (1980) reviewed the ability to change the sexual state in response to changes in the environment, size or age in a vast number of dioecious and subdioecious species whose sexual expression is controlled by chromosomes. It has been proposed that changes in floral sex ratio may result from alterations in the architectural arrangement of the inflorescences (e.g. Smith, 1981; Lloyd & Bawa, 1984; Solomon, 1989; Traveset, 1992, 1999); caused by (1) resource allocation constraints or (2) physiological constraints caused by hormones such as auxins, gibberellins and cytokinins. In conifers sexual changes can be induced experimentally by application of exogenous hormones, including the production of bisporangiate strobili (Tosh & Powell, 1986; Owens & Hardev, 1990; Wakushima *et al*., 1997). It has been found that changes in hormonal level can be due environmental factors as light intensity (Alabadí & Blázquez, 2009). Thus, small number of sexual changes between females and males can be detected in individuals with truly dioecious systems as occurs in *P. johannis*.

Secondary dimorphic features have evolved in many dioecious plants (Lloyd & Webb, 1977; Dawson & Geber, 1999). Male and female individuals develop secondary dimorphic features

363 which result from several years of sexual differentiation; these differences are higher in resource-
364 limiting environments (Lloyd & Webb, 1977). Some reports have documented that male and
365 female individuals of conifers exhibit dimorphic features such as trunk diameter, height and
366 branching (Allison, 1991; Gauquelin *et al*., 2002). Evolutionary models based on relative costs
367 and benefits of male and female reproductive structures predict that plants growing under
368 favorable conditions (being larger in size, having a greater resource supply or a greater total
369 reproductive effort) should invest relatively more in female than in male function ("female size
370 advantage hypothesis") (e.g. Freeman *et al*., 1981; Charnov, 1982; Lloyd & Bawa, 1984;
371 Goldman & Willson, 1986). Wind-pollinated plants, however, do not usually follow those
372 theoretical predictions, increasing relative maleness as patch quality improves (e.g. Burd &
373 Allen, 1988; Solomon, 1989; Traveset, 1992; Fox, 1993; Traveset, 1999). Large wind-pollinated
374 plants may benefit from a relatively greater male investment if pollen is carried for longer
375 distances and the local mating competition among sib pollen is lowered ("male height advantage
376 hypothesis") (Burd & Allen, 1988; Ganeshaiah & Shaanker, 1991). More data to test these
377 hypotheses are needed, especially on long-lived trees and shrubs, where the potential for fitness
378 differences related to height or size is greater. In *P. johannis* individuals investing in male
379 function for a given basal area are taller than those investing in female function. The same
380 difference has been reported in *Taxus baccata* (Iszkuło *et al*., 2009). This result supports the
381 "male height advantage hypothesis", however, individuals that invest in female function seem to
382 store more resources in their branches which can be a sink. Thicker branches are more efficient
383 at conducting water (Tyree & Zimmerman, 2002; Slingsby, 2004), females individuals of
384 *Leucadendron laureolum* have thicker branches than males (Harris & Panell, 2010). Evergreen
385 species store large amounts of nitrogen in the foliage, which can be translocated to reproduction

386 or other functions (Millard *et al*., 2001; Millard & Grelet, 2010). Thickness in branches of
387 female individuals *in P. johannis* can be associated to higher resource storage for use during
388 reproductive events, or more efficient water conductance. This suggests that male and female
389 have different strategies to invest and store resource in *P. johannis*.
390
391 **Environmental factors are not responsible of gender variation in *P. johannis***
392 Environmental factor including age, stress (drought) and herbivory can be responsible for
393 unisexuality under a labile system (McCormick & Andresen, 1963; Floyd, 1983; Cobb *et al*.,
394 2002; Tikhonova, 2003; Mueller *et al*., 2005). In this study we did not find evidence for any of
395 these features related to the unisexuality in *P. johannis*.
396 Herbivory can produce apparently unisexual individuals directly, by eating preferentially one sex
397 or indirectly, by subtracting resource to reproduction and transfer them to defense, which can
398 results in allocation of resources to only one sex (Ashman, 2002; Cobb *et al*., 2002). The
399 herbivore removal experiment showed that unisexuality is not related to the attack of herbivores,
400 suggesting *P. johannis* has stable unisexual individuals. The best characterized system with
401 labile unisexual individuals is *P. edulis*, in which a stem-boring moth (*Dioryctria albovittella*)
402 alters sexual expression by reducing female function and increasing male function (Whitham &
403 Mopper, 1985; Cobb *et al*., 2002); in this species, after one year of moth removal, the reversion
404 of the monoecious state was significant and some trees produced 55% of megastrobili (Cobb *et*
405 *al*., 2002).
406 Under the sex allocation resource theory one individual with labile sexuality change its sex
407 through time; expression of the sex requiring fewer resources is expressed first, then the opposite
408 sex and finally both. Detection of sex-related size classes can help to detect labile unisexuality

predicted under the sex allocation resource theory (Floyd, 1983; Charnov, 1984; Lev-Yadun & Liphschitz, 1987). We did not detect any size categories associated by sex in *P. johannis*; in fact we detected male or female individuals in a gradient of different sizes, which suggest this species has stable sexes. In contrast, some conifers with labile reproductive system, as *Abies pinsapo*, *P. edulis* and *P. sylvestris*, male structures are expressed first, then female and in advanced stages they produce both sexes (mixed or truly monoecious) (Fowler, 1964; Floyd, 1983; Allison, 1991; Tikhonova, 2007).

Finally in those studies showing that environmental factors as drought or high radiation are responsible to drive unisexuality, sexes are clumped (Bierzychudek & Eckhart, 1988; Tikhonova, 2003; Nuñez *et al*., 2008). In *P. sylvestris* females are in the center of the population distribution and males in the surrounding areas, which are more limited in resources (Tikhonova, 2003). Although we did not measure stress, in addition to herbivory, we did not detected clumped groups of females or males; in fact we found an intermixed population in relation to size and sex (Figure 1S, supplementary data).

Quantitative gender and the monoecy-paradioecy pathway to dioecy

Although phenotypically we identified five sexual morphotypes (two unisexual and three monoecious morphs) in *Pinus johannis*, this species has a functional dioecious system based on Lloyd's quantitative gender approach. Few studies have been undertaken to characterize the functional gender in angiosperms and gymnosperms since Lloyd proposed his quantitative method (Lloyd, 1976, 1980). Four studies in gymnosperms to date have considered this approach to determine gender in *Taxus canadensis*, *Pinus densiflora*, *Juniperus phoenica* and *Abies pinsapo* (Allison, 1991; Jordano, 1991; Arista & Talavera, 1997; Kang, 2007). However none of

these species showed a strong sexual bimodal distribution as *P. johannis* (Fig. 4). For example, in *A. pinsapo* there is a gradient from female to male, whereas in *P. johannis* >96% of individuals belong to male or female categories and few individuals are considered inconstant males or females (defined as having 10% opposite sex function), suggesting *P. johannis* is in an advanced stage to complete sexual separation.

Because all other pine species are monoecious, and *P. johannis* is a recently evolved taxa (Gernandt *et al*., 2001), it can be assumed that dioecy evolved in *P. johannis* through the monoecy-paradioecy pathway. However, the common assumption that dioecy originates via monoecy-paradioecy pathway in monoecious groups may not always be true, as was demonstrated in *Silene sagittaria* and *Urtica dioica* which evolved through a gynodioecy pathway despite its closest relative has a monoecious system (Dorken & Barret, 2004; Shannon & Holsinger 2007). Thus, detection of inconstant males and females in *P. johannis* supports the monoecy-paradioecy pathway of evolution to dioecy under several mutations of minor effects.

Comparison of sexual expression in some gymnosperms producing unisexual individuals

Givnish (1980) detected a correlation between the breeding systems and the seed dispersal syndromes in gymnosperms. Species with dioecious systems tend to have seeds dispersed by animals. In contrast, species with monoecious systems are wind-dispersed. *Taxus* are mainly dioecious, but, *Taxus canadensis*, which has seed bird-dispersal, was originally considered monoecious and an exception to this "rule". Allison (1991) found that this species has a bimodal quantitative gender distribution, supporting Givnish's hypothesis. According to McCormick & Andresen (1963) in *Pinus*, unisexual individuals are apparently restricted to the wingless-seed

groups and they have zoocory seed dissemination. Some pine species with unisexual individuals are *P. edulis, P. culminicola, P. cembroides*, *P. johannis* and *P. discolor* (Kiener, 1935; McCormick & Andresen, 1963; Floyd, 1983; Ávila *et al*., 1992); almost all belonging to subsection *Cembroides* (Subgenus *Strobus*), and having wingless seeds. One exception is *P. sylvestris* (Subgenus *Pinus*) whose seeds are wind-dispersed (Debain *et al*., 2007). However, none of these species have been demonstrated to have a truly dioecious system. Based on our findings *P. johannis* is the only pine to date to have a truly dioecious system, which fits Givnish's predictions. *P. edulis,* on the other hand, has a labile system in which unisexual expression is associated with age-size and herbivory intensity (Floyd, 1983; Cobb *et al*., 2002). A bimodal pattern from individuals which invest exclusively or predominantly in female or male function was detected in *P. johannis* and no obvious monoecious with a high number of female and male structures was detected. In contrast *P. edulis* has a gradient in sexual expression similar to *P. sylvestris* (Tikhonova, 2007). Additionally, the herbivore removal experiment showed no evident sex lability in *P. johannis* due to differential herbivory as in *P. edulis*. We detected a maleness tendency in *P. edulis*; this phenomenon has been detected in *P. resinosa* in which no strictly female trees were encountered (Fowler, 1964). In xeric habitats monoecious species produce more male structures than female structures (Freeman *et al*., 1981). This pattern has been detected in some pine trees such as *P. resinosa, P. sylvestris, P. edulis and P. culminicola* (Kiener, 1935; Fowler, 1964; Cobb *et al*., 2002; Tikhonova, 2003). Thus a possible relation to stress can drive unisexuality in pines, especially in pinyon which are distributed in semiarid regions of the western United States and Mexico, including a mixture of relictual and more recently evolved taxa (Gernandt *et al*., 2001).

Pine trees can produce predominantly one sex (tendency to maleness or femaleness, see Fowler, 1964). The only species that has been reported to have male, predominantly male, mixed (truly monoecious), predominantly female and female individuals is *Pinus sylvestris* (Tikhonova, 2007), which has a labile system. In contrast, *P. johannis* which apparently has a stable dioecious system, lack of mixed individuals, although those trees which produced bisporangiate strobili (frequency of 1%) tended to produce high amount of both microstrobili and megastrobili. In general, members of the genus *Pinus* have male strobili in the lower portion of the tree and female strobili in the upper portion (Ledig, 1998). In *P. johannis* there is no such sexual regionalization. Female individuals produce female cones in the upper and lower branches and male individuals produce male strobili in upper and lower branches. Therefore the lack of mixed individuals and the common strobili regionalization suggest a specialization of unisexual individuals in *P. johannis*.

Conclusions

Our five year study shows that *Pinus johannis* is evolving to dioecy via the monoecy-paradioecy pathway in the population studied. Monoecious individuals behave as female or male, since they produce few reproductive structures of the opposite sex. In some cases we detected that predominantly male monoecious individuals produced few megastrobili but they did not reach maturity. Predominantly female monoecious individuals apparently contribute viable pollen during the reproductive season (Flores-Rentería *et al*., 2011). However, studies to evaluate the contribution of the monoecious individuals through the minor sex are required. Sexual changes within individuals occur almost exclusively within the female and male groups. Thus, *P. johannis* has stable nearly unisexual individuals in a transitional population to dioecy. More studies are required to determine if the low percentage of changes between the female and male

group are environmentally influenced. This study is the first using a quantitative approach to detect the evolutionary pathway to dioecy in gymnosperms and is consistent with the monoecy-paradioecy pathway expected in a monoecious group, where Pinaceae has a prevalent monoecious reproductive system. Thus, we propose *P. johannis* could be a model to explore the evolution of dioecy in gymnosperms.

Acknowledgements: The authors thank R. Pérez, V. Cepeda, A. Flores and O. Jiménez for their help during the field work. Drs. T. Whitham, and C. Gehring who made useful suggestions that improved this manuscript. This work was conducted by Flores-Rentería during her PhD. studies at the Posgrado en Ciencias Biomédicas (UNAM) with a CONACYT scholarship (172682).

References

Alabadí, D. & Blázquez, M.A. 2009. Molecular interactions between light and hormone signaling to control plant growth. *Plant Mol. Biol.* 69: 409-417.

Ávila, N.J., García, M.E. & Reyes, A.J. 1992. Registro de *Pinus discolor* Bailey et Hawksworth en la sierra de monte Grande, San Luis Potosí, México. *Acta Bot. Mex.* 20:9-12.

Allison, T.D. 1991. Variation in sex expression in Canada yew (*Taxus canadiensis*). *Am. J. Bot.* 78: 569-578.

Arista, M. & Talavera, S. 1997. Gender expression in *Abies pinsapo* Boiss., a Mediterranean Fir. *Ann. Bot.* 79: 337–342.

Ashman, T.L. 2002. The role of herbivores in the evolution of separate sexes from hermaphroditism *Ecology,* 83: 1175–1184.

Bierzychudek, P. & Eckhart, V. 1988. Spatial segregation of the sexes in dioecious plants. *Am. Nat.* 132:34-43.

Burd, M. & Allen, T.F.H. 1988 Sexual allocation strategy in wind-pollinated plants. *Evolution* 42: 403–407.

Bullock, S.H. & Bawa, K.S. 1981. Sexual dimorphism and the annual flowering pattern in *Jacaratia dolichaula* (D. Smith) Woodson (Caricaceae) in a Costa Rican rain forest. *Ecology* 62: 1494-1504

Charlesworth, D. 2002. Plant sex determination and sex chromosomes. *Heredity* 88:94-101.

Charnov, E.L. 1982. *The theory of sex allocation*. Princenton, NJ: Princenton University Press.

Charnov, E.L. 1984. Behavioral ecology of plants. In: *Behavioral Ecology, an evolutionary approach* (J.R. Krebs and N.B. Davies, eds.), pp. 362–379. Sinauer, Sunderland, Massachusetts.

Clark, D.A. & Clark, D.B. 1987. Temporal and environmental patterns of reproduction in *Zamia skinneri*, a tropical rain forest cycad. *J. Ecol.* 75:135-149.

Cobb, N.S., Trotter III, R.T. & Whitham, T.G. 2002. Long-term sexual allocation in herbivore resistant and susceptible pinyon pine (*P. edulis*). *Oecologia* 130:78–87

Crone, E.E., McIntire, E.J.B. & Brodie, J. 2011. What defines mast seeding? Spatio-temporal patterns of cone production by whitebark pine. *J. Ecol.* 99: 438–444.

Dawson, T.E. & Geber, M.A. 1999. Sexual dimorphism in physiology and morphology. In: *Gender and sexual dimorphism in flowering plants* (M.A. Geber, T.E. Dawson, & L.F. Delph, eds) Springer, Berlin Heidelberg New York, pp 175–215.

Debain, S., Chadoeuf, J., Curt, T., Kunstler, G. & Lepart, J. 2007. Comparing effective dispersal of expanding population of *Pinus sylvestris* and *Pinus nigra* in calcareous grassland. *Can. J. Forest Res.* **37**, 705–718.

545 Dorken, M.E. & Barrett, S.C.H. 2004. Sex determination and the evolution of dioecy from
546 monoecy in *Sagittaria latifolia* (Alismataceae). *Proc. R. Soc. Lond. B* 271, 213–219.
547 Ehlers, B.K. & Bataillon, T. 2007. 'Inconstant males' and the maintenance of labile sex
548 expression in subdioecious plants. *New Phytol.* 174, 194–211.
549 Flores-Flores, J.D. & Díaz-Esquivel, D.E. 1988. Tabla de vida y factores de mortalidad para
550 conos y semillas de *Pinus cembroides* Zucco bajo condiciones naturales en el sur de Coahuila.
551 *In: Memorias del II Simposio Nacional sobre Pinos Piñoneros* (M.F. Passini, D. Cibrian-Tovar,
552 T. Eguiluz Piedra, eds.), pp. 123-135. Ciudad de Mexico, Mexico, Universidad Autónoma de
553 Chapingo, 6-8August 1987.
554 Flores-Rentería, Ll., Vázquez-Lobo, A., Whipple, A.V., Piñero, D., Márquez-Guzmán, J. &
555 Domínguez, C.A. 2011. Functional bisporangiate cones in *Pinus johannis* (Pinaceae):
556 Implications for the evolution of bisexuality in seed plants. *Am. J. Bot*. 98: 130–139.
557 Floyd, M.E. 1983. Dioecy in five *Pinus edulis* populations in the southwestern United States.
558 *Am. Mild. Nat.* 110:405-411.
559 Fox, J.F. 1993. Size and sex allocation in monoecious woody plants. *Oecologia* 94:110-113.
560 Freeman, D.C., McArthur, E.D., Harper, K.T. & Blaver, A.C. 1981. Influence of environment on
561 the floral sex ratio of monoecious plants. *Evolution* 35:194-197.
562 Freeman, D.C., Lovett-Doust, J.L., El-Keblawy, A., Miglia, K.J. & McArthur, E.D. 1997. Sexual
563 specialization and inbreeding avoidance in the evolution of dioecy. *Bot. Rev.* 63:65–92.
564 Freeman, D.C., Harper, K.T. & Charnov, E.L. 1980. Sex change in plants: old observations and
565 new hypotheses. *Oecologia* (Berl.) 47:222-232.
566 Fowler, D.P. 1964.) Effects of inbreeding in red pine, *Pinus resinosa.* Ait. *Silvae Genet.* 13: 170–
567 177.

568 Ganeshaiah, K.N. & Shaanker, U.R. 1991. Floral sex ratios in monoecious species- Why are
569 trees more male-biased than herbs?. *Curr. Sci. India* 60:319-321.
570 García, D., Zamora, R., Gómez, J.M., Jordano, P. & Hódar, J.A. 2000. Geographical variation in
571 seed production, predation and abortion in *Juniperus communis* throughout its range in Europe.
572 *J. Ecol.* 88, 436-446.
573 Gauquelin, T., Bertaudière, V., Badri, W. & Montès, N. 2002. Sex ratio and sexual dimorphism
574 in mountain dioecious thuriferous juniper (*Juniperus thurifera* L., Cupressaceae). *Bot. J. Linn*
575 *Soc.* 138: 237–244.
576 Geber, M.A., Dawson, T.E. & Delph, L.F. 1999. *Gender and sexual dimorphism in flowering*
577 *plants*. Springer, Berlin Heidelberg New York.
578 Gernandt, D.S., Liston, A. & Piñero, D. 2001. Variation in the nrDNA ITS of *Pinus* subsection
579 *Cembroides*: implications for molecular systematic studies of pine species complexes. *Mol.*
580 *Phylogenet. Evol.* 21: 449-467.
581 Goldman, D.A., & Willson, M.F. 1986. Sex allocation in functionally hermaphroditic plants: a
582 review and critique. *Bot. Rev.* 52:157-194.
583 Givnish, T.J. 1980. Ecological constraints of the evolution of breeding system in seed plants:
584 dioecy and dispersal in gymnosperms. *Evolution* 34:959-972.
585 Grier, N.M. 1917. Sexual dimorphism and variation in *Ginkgo biloba* L. *Torreya* 17: 225.
586 Harris, M.S. & Pannell, J.R. 2010. Canopy seed storage is associated with sexual dimorphism in
587 the woody dioecious genus *Leucadendron. J. Ecol.* 98:509-515.
588 Iszkuło, G., Jasińska, A.K., Giertych, M., & Boratyński, A. 2009. Do secondary sexual
589 dimorphism and female intolerance to drought influence the sex ratio and extinction risk of
590 *Taxus baccata*? *Plant Ecol.* 200:229–240.

591 Jordano, P. 1991. Gender variation and expression of monoecy in *Juniperus phoenicea* (L.)
592 (Cupressaceae). *Bot. Gaz.* 152: 476–485.
593 Kang, H.S. 2007. Changes in Gender Expression in Korean Populations of *Pinus densiflora* over
594 a Five-Year Period. *J. Plant Biol.* 50: 181-189.
595 Kiener, W. 1935. Unisexual lamber pines. *Science* 82:193.
596 Korpelainen, H. 1998. Labile sex expression in plants. Biol Rev 73:157–180.
597 Ledig, F.T. 1998. Genetic variation in *Pinus*. In: *Ecology and biogeography of* Pinus (D.M.
598 Richardson, ed), pp. 251-280. Cambridge University Press, Cambridge, United Kingdom.
599 Lev-Yadun, S. & Liphschitz, N. 1987. The ontogeny of gender of *Cupressus sempervirens* L.
600 *Bot. Gaz.* 148: 407-412.
601 Lloyd, D.G. 1975. Breeding systems in *Cotula* L. (Compositae, Anthemideae). III. Dioecious
602 populations. *New Phytol.* 71, 109–123.
603 Lloyd, D.G. 1976. The transmission of genes via pollen and ovules in gynodioecious
604 angiosperms. *Theor. Pop. Biol.* 9: 299–316.
605 Lloyd, D.G. 1980. The distribution of gender in four angiosperm species illustrating two
606 evolutionary pathways to dioecy. *Evolution* 34:123-134.
607 Lloyd, D.G. & Bawa, K.S. 1984. Modification of the gender in seed plants in varying conditions.
608 *Evol. Biol.* 17:255-338.
609 Lloyd, D.G. & Webb, C.J. 1977. Secondary sex characters in plants. *Botanical Review* 43: 177-
610 216.
611 McCormick, J. & Andresen, J.W. 1963. A subdioecious population of *Pinus cembroides* in
612 southeast Arizona. *Ohio J. Sci.* 4:159-163.

613 Meagher, T.R. & Antonovics, J. 1982. The population biology of *Chamaelirium luteum*, a
614 dioecious member of the lily family: life history studies. *Ecology* 63: 1690-1700.
615 Millard, P. & Grelet, G.A. 2010. Nitrogen storage and remobilization by trees: ecophysiological
616 relevance in a changing world. *Tree Physiol.* 30, 1083-1095.
617 Millard, P., Hester, A., Wendler, R.& Baillie, G. 2001. Interspecific defoliation responses of
618 trees depend on sites of winter nitrogen storage. *Funct. Ecol.* 15:535-543.
619 Mirov, N.T. 1967. *The genus* Pinus. The Ronald Press Company, New York.
620 Mueller, R.C., Wade, B.D., Gehring, C.A. & Whitham, T.G. 2005. Chronic herbivory negatively
621 impacts cone and seed production, seed quality and seedling growth of suceptible pinyon pines.
622 *Oecologia* 143:558-565.
623 Nuñez, C.I., Nuñez, M.A. & Kitzberger, T. 2006. Sex-related spatial segregation and growth in a
624 dioecious conifer along environmental gradients in northwestern Patagonia. *Ecoscience* 15:73-
625 80.
626 Ortiz, P.L., Arista, M., & Talavera, S. 2002. Sex ratio and reproductive effort in the dioecious
627 *Juniperus communis* subsp. *alpina* (Suter) Čelak. (Cupressaceae) along an altitudinal gradient.
628 *Ann. Bot.* **89**: 205-211.
629 Owens, J.N. & Hardev, V. 1990. Sex expression in gymnosperms. *Crit. Rev.Plant Sci*. 9: 281-
630 294.
631 Perry, J.P. 1991. *The pines of Mexico and Central America*. Timber Press, Portland, OR.
632 Primack, R.B. & McCall, C. 1986. Gender variation in a red maple population (Acer rubrum;
633 Aceraceae): A seven-year study of a "polygamodioecious" species. *Am. J. Bot.* 73: 1239-1248.
634 Renner, S.S. & Ricklefs, R.E. 1995. Dioecy and its correlates in the flowering plants. *Am. J. Bot.*
635 82:596-606.

636 Renner, S.S. & Won, H. 2001. Repeated Evolution of Dioecy from Monoecy in Siparunaceae
637 (Laurales) *Syst. Biol.* 50:700–712.
638 Renner, S.S., Beenken, L.,. Grimm, G.W, Kocyan, A. & Ricklefs, R.E. 2007. The evolution of
639 dioecy, heterodichogamy, and labile sex expression in *Acer*. *Evolution*, 61:2701-2719.
640 Romero Manzanares, A. 2001. Historia natural, ecología de poblaciones y fitosociología de
641 *Pinus cembroides* y *Pinus johannis* (piñoneros) del centro de Mexico", UNAM, Facultad de
642 Ciencias, Doctorado en Ciencias, Biología.
643 SAS Institute Inc. 2009. *JMP: Version 8.* Cary, NC.
644 Shannon, R.K. & Holsinger, K.E. 2007. The genetics of sex determination in stinging nettle
645 (*Urtica* dioica). *Sex. Plant Reprod.* 20:35-43.
646 Slingsby, J. 2004. Branch junction constriction and hydraulic limitation in two species in the
647 cape Proteaceae: a mechanism explaining the trade-off between longevity and the degree of
648 ramification in the cape Proteaceae. Honours, University of Cape Town, Cape Town.
649 Smith, C.C. 1981. The facultative adjustment of sex ratios in lodgepole pine. *Am. Nat*. 118:297-
650 305.
651 Solomon, B.P. 1989. Size-dependent sex ratios in the monoecious, wind-pollinated annual,
652 *Xanthium strumarium*. *Am. Midl. Nat.* 121:209-218.
653 Stelkens, R.B. & Wedekind, C. 2010. Environmental sex reversal, Trojan sex genes, and sex
654 ratio adjustment: conditions and population consequences. *Mol. Ecol.* 19, 627-646.
655 Traveset, A. 1992. Sex expression in a natural population of the monoecious annual, *Ambrosia*
656 *artemisiifolia* (Asteraceae). *Am. Midl. Nat.* 127:309-315.

Traveset, A. 1999. Ecology of plant reproduction: mating systems and pollination. In: *Handbook of Functional Plant Ecology*. (F.I. Pugnaire, & F. Valladares, eds). pp. 545-588. Marcel Dekker, Inc., New York.

Tikhonova, I.V. 2003. Sex estructure of scotch Pine populations in the dry steppe. *Russian J. Ecol.* 6:370-374.

Tikhonova, I.V. 2007. Changes in the sex structure of pine populations related to temperature anomalies. *Russian J. Ecol.* 38:306-310.

Tosh, K.J. & Powell, G.R. 1986. Proliferated, bisporangiate, and other atypical cones occurring on young, plantation-grown *Larix laricina. Can. J. Bot.* 64: 469-475.

Torices, R., Méndez, M. and Gómez, J.M. 2011. Where do monomorphic sexual Systems fit in the evolution of dioecy? Insights from the largest family of angiosperms. *New phytol.* 190: 234-248.

Tyree, M.T. & Zimmerman, M.H. 2002. *Xylem Structure and the Ascent of Sap*. Springer-Verlag, Berlin.

Ushimaru, A. & Matsui, K. 2001. Sex change in tree species: long-term monitoring of sex expression in *Acer rufinerve*. *Nord. J. Bot*. 21:397-399.

Wakushima, S., Yoshioka, H. & Sakurai, N. 1997. Promotion of lateral female strobili production in *Pinus densiflora* by cytokinin application at a specific stage. *J. Forest Res.-JPN* 2: 51-57.

Whitham, T.G. & Mopper, S. 1985. Chronic herbivory: impacts on architecture and sex expression of pinyon pine. *Science* 228:1089-91.

Williams, C.G. 2008. Selfed embryo death in *Pinus taeda*: a phenotypic profile. *New Phytol.* 178: 210-222.

680 Williams, C.G. 2009. Conifer reproductive biology. Springer, New York. Pp. 91-105

681 Zavala, C.F. & Campos, J.L.D. 1993. Una nueva localidad de *Pinus discolor* Bailey &

682 Hawksworth en el centro de Mexico. *Acta Bot. Mex.* 25:21-25.

683

684

685 **Table 1.** Moth removal experiment. Sexual frequencies of trees before insecticide application

686 and control trees in 2008 and one year later (2009). Increment or decrement of sexual

687 frequencies (Δ). Herbivore removal was confirmed by the reduction of attack in insecticide

688 sprayed trees ($F_{1,267}$=7.59, ρ <0.01, R^2=0.032).

689

Insecticide	2008	2009	Δ
♀	0.20	0.23	0.03
♂	0.22	0.25	0.03
⇑♀ monoecious	0.37	0.34	-0.03
⇑♂ monoecious	0.21	0.18	-0.03
Control			
♀	0.32	0.28	-0.04
♂	0.18	0.23	0.05
⇑♀ monoecious	0.32	0.37	0.04
⇑♂ monoecious	0.17	0.12	-0.05

690

691

692

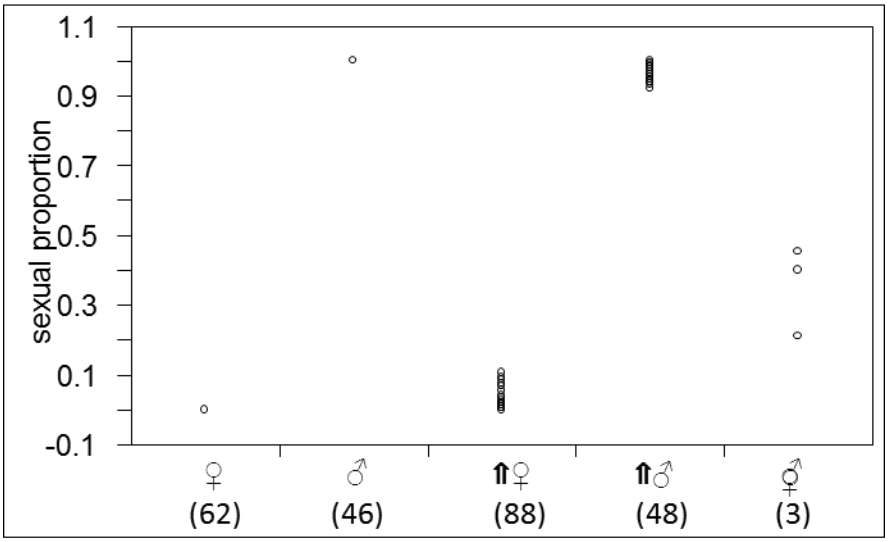


Figure 1. Five sexual morphs were recorded for individuals of *P. johannis.* Sexual categories were characterized by the sexual proportion into individuals. Females (♀) and males (♂) are purely unisexual; three kind of monoecious were found, predominantly female (⇑♀), predominantly male (⇑♂) and a small group of individuals with a sexual proportion close to 0.5. The latter group also can produce bisporangiate strobili (⚥). Sample size is shown in parenthesis.

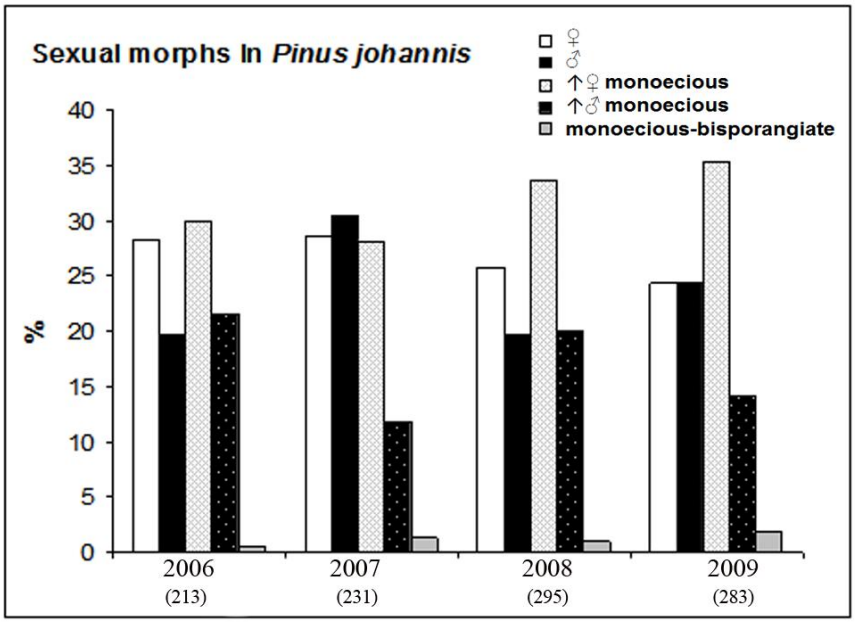


Figure 2. Sexual morphs of *P. johannis* (La Amapola) is based on presence or absence of ovulate, pollen and bisporangiate cones from 2006 to 2009. Sample size per year is indicated in parenthesis.

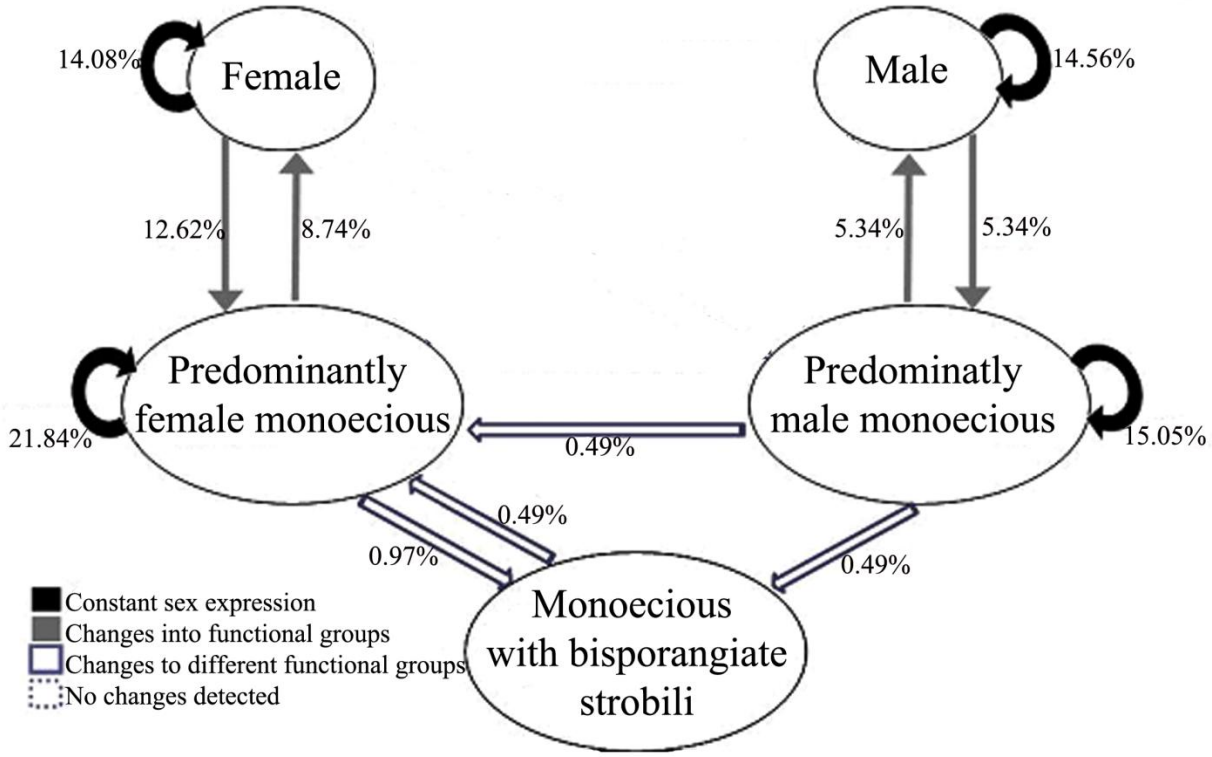


Figure 3. Sex change percentages from 2006 to 2008 among five sexual morphs in *P. johannis*. Filled black arrows show the percentage of individuals that remains with the same sex (total percentage=65.53). Changes into the female group or male group are shown in grey arrows. One transition from a male to a female group was detected (black opened arrow). A low frequency of changes to monoecious individuals producing bisporangiate strobili was found (black opened

arrows). Transitions that were not seen in our data are depicted as dotted lines.

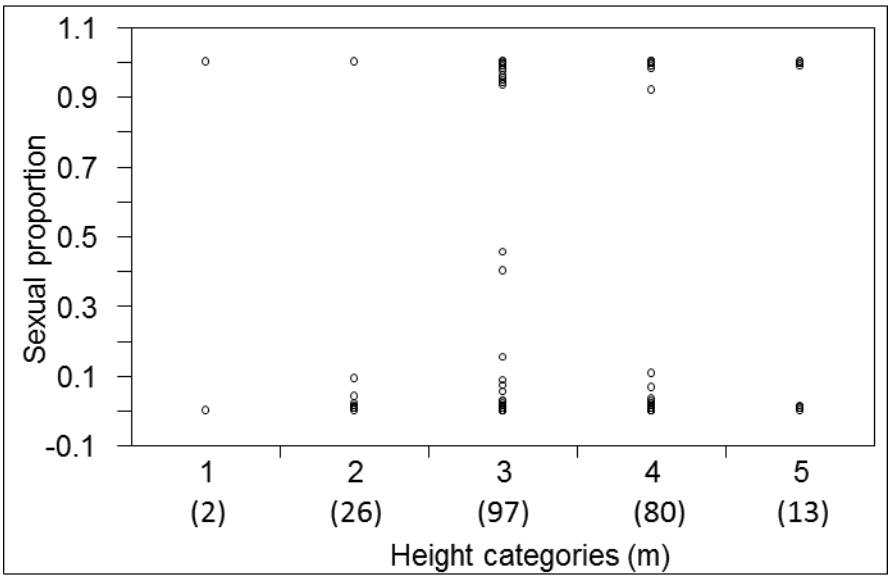


Figure 4. Sexual proportions by height categories show a tendency to produce only or predominantly one sex into one individuals regardless the height or extrapolated age. Only 2 individuals seem to be close to 0.5.

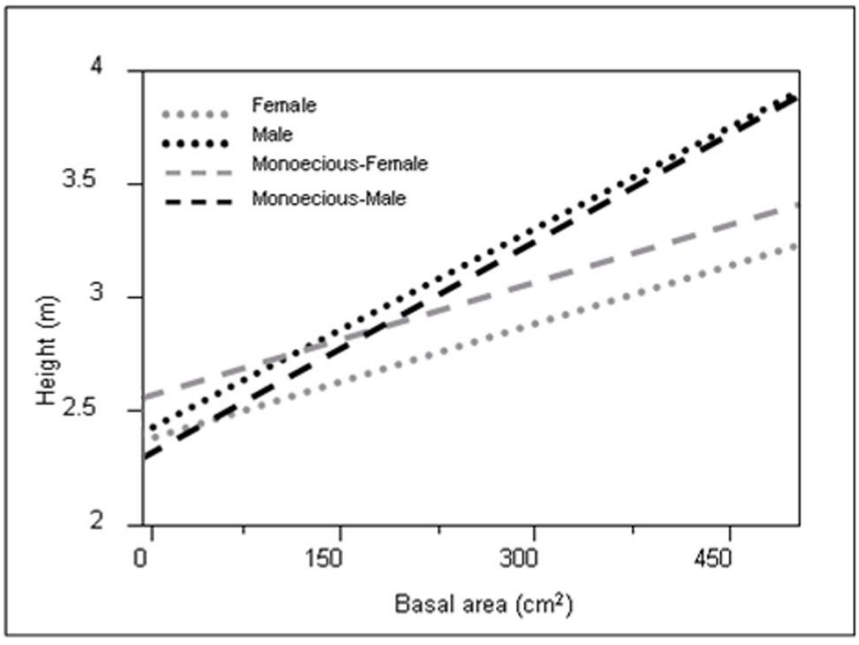


Figure 5. Plant architectural differences among sexual morphs in *P. johannis*. At a given basal area, male and predominantly male monoecious individuals (black lines) are taller than female and predominantly female monoecious individuals (grey lines).

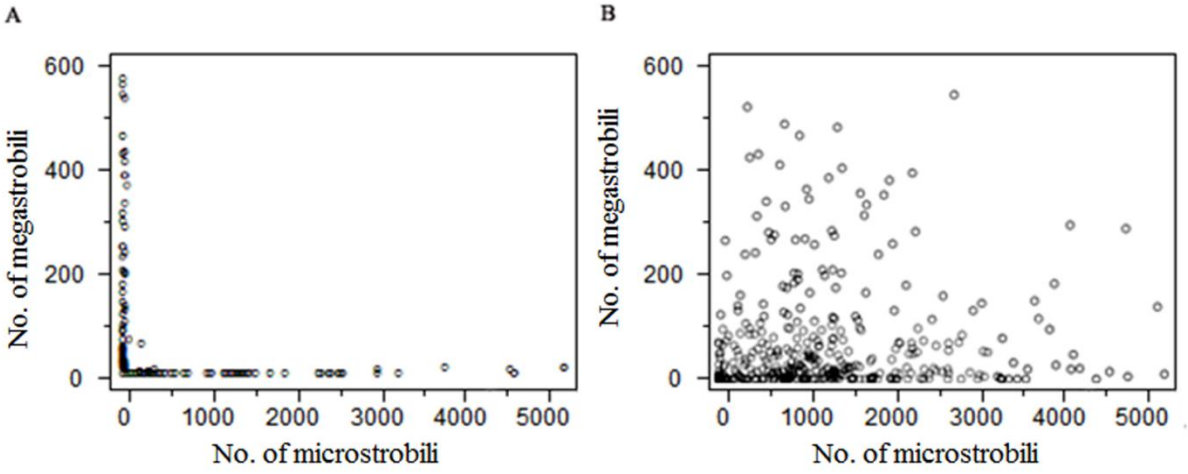


Figure 6. Bivariate distribution of sexual expression in *Pinus johannis* (A) and *P. edulis* (B).

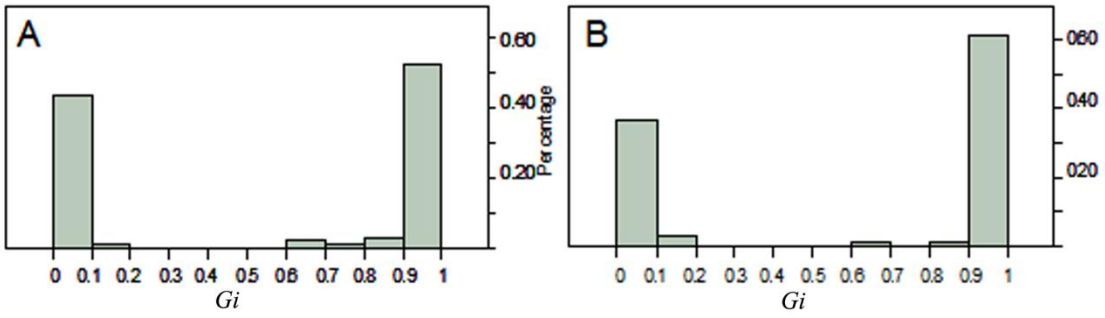


Figure 7. Distributions of functional gender in *P. johannis*. A) Functional gender in 2006. B) Functional gender in 2008. Males=0 and females=1.

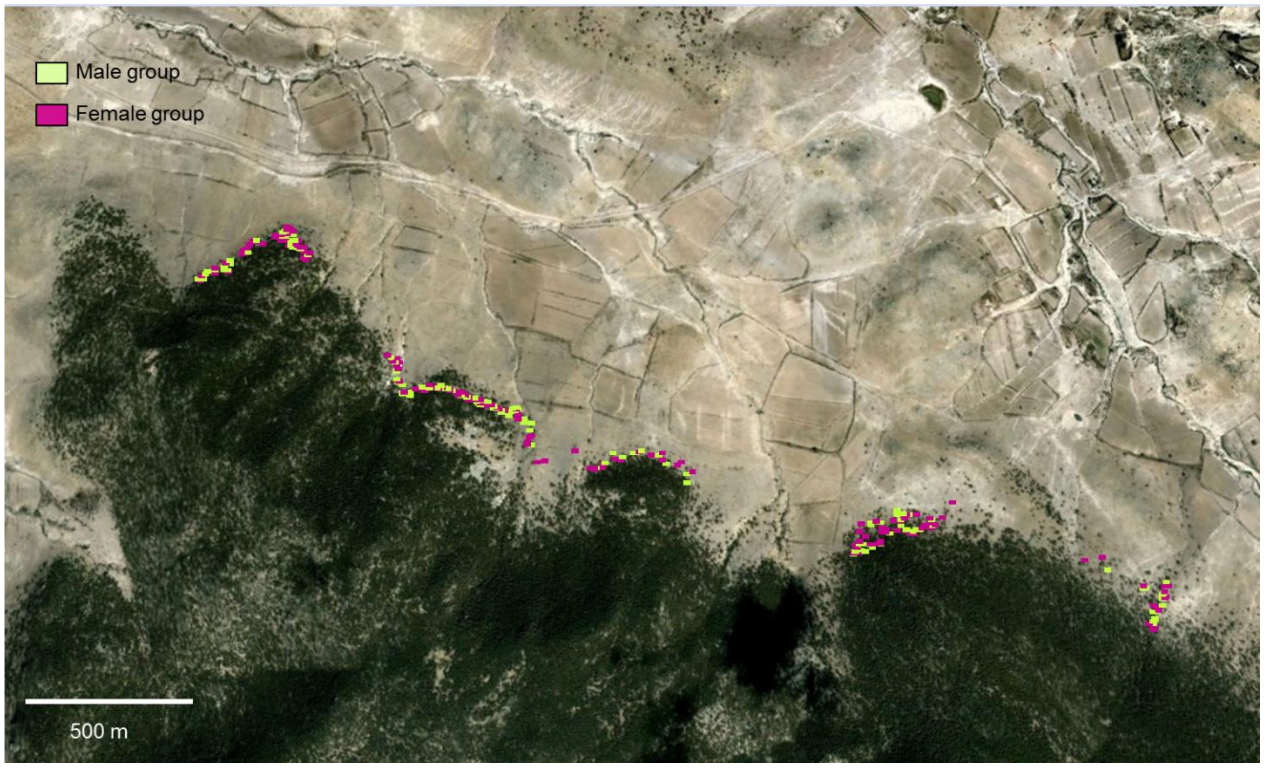


Figure 1S. Sexual morph distribution of *Pinus johannis* in La Amapola, San Luis Potosí. *P. johannis* occurs mainly in the borders of the vegetation area, males and females are intermixed.

CAPÍTULO 4

RESOURCE ALLOCATION AND AVOIDANCE OF INBREEDING AS FORCES DRIVING THE EVOLUTION OF UNISEXUALITY IN *PINUS JOHANNIS*

Resource allocation and avoidance of inbreeding as forces driving the evolution of unisexuality in *Pinus johannis*

Flores-Rentería, Ll. [a], Molina-Freaner, F.[b], Whipple V. A. [c] and C. A. Domínguez [a].

[a]Departamento de Ecología Evolutiva, Instituto de Ecología, UNAM, A. P. 70-275, México, D. F. 04510 México.

[b]Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México, Apartado Postal 1354, Hermosillo, Sonora 83000 México.

[c]Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA fax: 928-523-7500.

Key words: Inbreeding depression, *Pinus*, resource allocation, sexual reproductive system.

INTRODUCTION

Selection for unisexuality can result from a combination of high selfing rates and high inbreeding depression (Charlesworth and Charlesworth, 1979). Alternatively, differences in resource allocation to male and female functions that lead to accelerating fitness gains may also promote separate sexes (Charnov, 1982). These two factors can act simultaneously driving the evolution of separate sexes (Charlesworth, 1999), however few studies have considered these alternatives as potentially concurrent processes.

Species in the process of evolving towards dimorphic sexual systems provide powerful models for investigating the ecology and evolution of gender variation, allowing

comparisons between unisexual and cosexual individuals to test evolutionary hypothesis to dioecy. However few species of plants are known to have these features (reviewed in Case and Barret, 2001; Givnish, 1980). Although 52% of gymnosperms have dimorphic sexual systems, few studies considering evolutionary process to dioecy have been done in this group because of the apparent lack of species with mixed monoecious and dioecious populations. *Pinus johannis* has monoecious (same individual has both female and male reproductive structures in different strobili, megastrobili and microstrobili respectively), female and male individuals sympatrically under a functional dioecy system (Flores *et al.* 2011; in review). This provides an opportunity for examining these two hypotheses of evolution to dioecy in gymnosperms.

The avoidance of inbreeding depression generally has not been hypothesized as a mechanism to promote the evolution of unisexual individuals in *Pinus*, or gymnosperms. Pines are considered as among the most genetically variable of all species (Cornelius, 1994; Hamrick *et al.* 1979, Hamrick and Godt 1990; Delgado, 2002). Pines have high outcrossing rate estimates (Schemske and Lande, 1985), two possible reasons are: a) there is no mating through self-fertilization (possible dichogamy or temporal separation of sexes; Zinder et al, 1977; Wang, 1977; Owens et al, 1981; Matziris, 1994), nor mating among genetically related individuals (possible autoincompatibility postzygotic mechanism, Owens *et al.* 1998) and b) high inbreeding depression acting early in development that purges recessive deleterious alleles (Lanner, 1980; Koski 1971, Kärkkäinen & Savolainen 1993). Inbreeding depression may affect many different components of fitness in plants, e.g., germination rate, plant size and growth, and seed yield (Charlesworth and Charlesworth 1987; Husband and Schemske 1996). If individuals are evolving to unisexuality for avoidance of selfing and inbreeding depression (caused by expression of homozygous recessive deleterious

alleles) we expect higher reproductive success in unisexual individuals than in cosexual individuals, since they reduce inbreeding depression (Webb, 1999).

The resource allocation principle states that if resources are limited, two or more functions compete directly and an increase in resources allocated to one function will result in a decrease in resources allocated to the other (Charnov, 1982?), so higher resources allocated to one sexual function that produces an accelerating fitness gain for increased investment, whatever through male or female function, can drive the evolution of dioecy (Thomson and Brunet, 1990). Then the reproductive success will be higher in unisexual individuals than in cosexual (monoecious) by e.g. increasing the number of reproductive structures or by defending these better from herbivory (Charlesworth, 1999).

The main goal of this study is to test the two hypotheses for the evolution to the dioecy in the unique species in genus *Pinus* with apparently stable unisexual individuals (Flores-Rentería et al in review). We compared the success of self- and out- crossing in the field between unisexual and cosexual individuals by performing manual crosses, to detect mechanisms such as dichogamy or genetic incompatibility systems as well as to answer if unisexuality in *P. johannis* is related to the avoidance of inbreeding depression. Because herbivory can reduce cone production in *P. johannis* by 89% (Flores & Díaz 1988), an herbivore removal experiment was done in order to distinguish cone loss due inbreeding depression versus herbivory. Additionally, we sowed some seeds from cosexual and unisexual plants and compared the germination between sexual morphs. We attempt to know if differences in sex allocation resource to reproduction (number and size of reproductive structures and fruit set) and defense (herbivory attack and cone survival) are driving the unisexual expression in *P. johannis* by characterizing the allocation resource in unisexual and cosexual individuals.

Methods

Plant material

Pinus johannis Rob.-Pass trees produce strobili in May and June and this is the period when pollination occurs; in these months we identified sexual morphs during 2006 to 2009 in the population "La Amapola", San Luis Potosí (101º 07. 706' W, 22º 01.160' N and 2391 m.a.s.l.). We determined sex in 100 to 295 (depending on the year) individuals based on presence/absence female and male reproductive structures. *P. johannis* has four different reproductive sexual morphs: female, male, predominantly female monoecious, predominantly male monoecious and some monoecious individuals that produce bisporangiate strobili. Sexes were determined as described in Flores-Rentería *et al.* (2011).

Sex allocation resource to reproductive structures

In order to measure the investment to the male function we counted the number of microstrobili cluster, measured the microstrobili length and diameter in 149 individuals (males, predominantly male and predominantly female monoecious individuals). In order to test whether unisexual individuals have more resources allocated to reproduction we measured the investment to male and female function in the four sexual morphs in 142 individuals in 2006 and individuals in 254 in2008). We measured the investment to the female function by counting the total number of megastrobili (from May to June) in female, predominantly female monoecious, male and predominantly male monoecious. Differences of total cone production per tree among sexual morphs were evaluated from 2005 to 2009 in late August. We wanted to know if bigger mature cones carry bigger seeds or more seeds than smaller cones, for this purpose we counted number of seeds in 201 cones and measured their length, and did a

bivariate fit. Then we calculated the difference in seed per cone among sexual morphs. In addition, all megastrobili produced in 167 trees were counted in 2008 to examine fruit set of natural crosses (wind pollinated). Mature cones were counted in August 2009 before seed release. Percent fruit set [(number of mature cones/number of megastrobili) × 100], average of ovule number per sexual morph was taken to calculate seed set values [number of mature seeds/number of ovules) X 100]. We used a microscope (Zeiss, Discovery V8 model and Cannon camera Powershot A620, Axion vision program, Carl Zeiss) and a caliper (CD-6, CSX, Mitutoyo Corp.) to the nearest 0.01mm to measure lengths and diameters. Plant material was collected. Differences among morphs were determined by one-way ANOVA ($P = 0.05$), using JMP (JMP statistical software, SAS, 2003). A Tukey-Kramer HSD test in JMP software (SAS, 2003) was used to evaluate comparison differences between morphs.

Herbivory and cone defense

Pinus johannis is frequently attacked by *Conophthorus cembroides, Leptoglossus occidentalis* and *Eucosma bobana*, insects that damage up to 89% of the cones in some populations (Flores & Díaz 1988). Intensity of herbivory can be estimated in *P. johannis* by counting the number of webworms in the shoots near the apex and damaged cones, which look brown and smaller than the intact cones. To detect differences in resource allocation to defense we compared the intensity of herbivory, defined as the percentage shoot attacked and percentage of cones attacked in each tree, between cosexual and unisexual individuals. Three categories of shoot attacked were considered by counting number of webworms present in shoots bearing microstrobili, megastrobili or vegetative shoots without sexual structures

Avoidance of selfing, inbreeding depression and manual crosses

We performed manual crosses in *P. johannis* during May and June of (2006 and 2008) in order to 1) probe whether there are negative effects in the lineage product of self-crosses in monoecious individuals that would suggest evolution to dioecy through avoidance of inbreeding depression and 2) to detect a mechanism that prevent selfing such as incompatibility genetic system or temporal differences in male vs. female expression within individuals (dichogamy) which would reject an evolutionary process to dioecy by avoiding inbreeding depression. In order to detect differences in the reproductive success between unisexual and cosexual mating system, 697 control crosses were done in 2006, corresponding to 602 of outcrossing and 108 of selfing due the low availability of both sexes in monoecious individuals. The buds were covered with waxed bags to avoid natural pollination. Few days after (when was visible a pollination drop) we collected mature pollen from male and both monoecious individuals and pollinated the receptors. Female, predominantly female or male monoecious individuals were receptor, while male, predominantly male or predominantly female monoecious, were used as pollen donor in outcrossing. Selfing was conducted in monoecious individuals acting as pollen donors and receptors. Successful crosses were recorded as a nominal variable at mature cone stage (16 months after pollination).

As inbreeding depression acts during different life stages (Husband and Schemske, 1996; Savolainen, 1996), we evaluated whether seed viability was higher in offspring produced by female individuals compared with cosexual individuals (both kinds of monoecious). Mature cones produced by the manual crosses were collected and we counted number of seeds per cone, and viable and damaged seeds to compare performance of seed viability among sexual morphs. Additionally we collected 681

151 closed cones (natural wind-pollinated) from 83 trees on September 2006 (313 from
152 female, 309 from predominantly female monoecious and 59 from predominantly male
153 monoecious individuals); cones were dry at room temperature to remove mature seeds
154 (4043), seed viability was determined by floatability in water to detect differences in
155 seed viability among sexual morphs. In order to know if inbreeding depression acts
156 differentially among sexual morphs during germination, we sowed 1066 viable seeds
157 (resulting from the floatability test) into jiffy pots filled with a moistened peat. During
158 three months daily observations were done to record germination success. We
159 calculated the germination probability among sexual morphs. An ANOVA test was
160 performed to calculate significant differences.
161
162 During 2008 we conducted 2583 manual crosses with 2392 out-crosses and only 191
163 self-crosses. Selfing crosses in monoecious individuals were used to detect
164 incompatibility system and dichogamy, as well as, to detect receptivity of megastrobili
165 from monoecious individuals at the time of pollen release from the same trees (21
166 predominantly male monoecious and 38 predominantly female monoecious). Three
167 main censuses were made during the cone development (April, June and September,
168 2009). The first census was done 9-10 months after pollination to account cone
169 retention as a successful pollination process, to detect temporal differences in ovule
170 receptivity and pollen release in the self- crosses in both monoecious individuals as well
171 as, to detect an prezygotic incompatibility system; the second census was done after
172 fertilization to detect a post-zygotic incompatibility system as described in other
173 conifers (Owens *et al.* 1998); the last survey was done at the stage of mature cone in
174 order to test differences in outcrossing and selfing during the embryogenesis process.
175 Self- and cross-pollination treatments were performed in 105 selected mothers

(receptor) identified as female and predominantly female or male monoecious individuals, while 153 trees, including male, predominantly male or predominantly female monoecious, were used as pollen donor. All monoecious individuals were considered as donor and pollen receptor to perform self-crosses. One to 60 megastrobili (depending on the production) per tree were labeled and subjected to each pollination treatment. Negative controls were performed using 81 megastrobili, from different individual of all sexual morphs, they were covered with bags before receptivity but no manual pollination was conducted. Morphological study was done to detect viability of pollen donors and aberrant development of pollen grains at time of pollen shed. Sections of ovule and seed were done to detect time of major events on reproduction such as fertilization and seed abortion related to selfing and outcrossing following the methods described in Flores-Rentería *et al.* (2011).

All manual crosses were monitored and revised in the next 16 months until reaching mature cone stage.

Table 1. Number of crosses per sexual morph. Male (♂), female (♀), predominantly male monoecious (⇑♂) and predominantly female monoecious (⇑♀).

Donor sex	Number of crosses	Receptor sex	Number of crosses
♂	980	♀	852
⇑♀	259	⇑♀	1570
⇑♂	1287	⇑♂	104

A nominal logistic model was performed to examine the difference in mating success between, selfing and outcrossing and the effect of donors and receptors. Difference in reproductive values in self-fertilization compared to outcrossing was tested by the pollination effect.

To distinguish the effect of cone loss by herbivory and the inbreeding depression we

sprayed 115 individuals (out 275) with cygon every month during cone development. Thus cone losses of sprayed trees could be related to other factors but herbivory. A contingency analysis was done to detect differences in successful crosses and insecticide application. Analyses were conducted using JMP8.

Results

Allocation resource analysis

Comparison of sex allocation to the male function among sexes

Predominantly male monoecious and males produce more microstrobili cluster than predominantly female monoecious in both years (2006: $F_{2,89}$=17.18, R^2=0.27, P<0.0001 and 2008: $F_{2,188}$=14.97, R^2=0.14, P<0.0001). On average males produced 1159 microstrobili clusters, and predominantly male monoecious 1198 whereas predominantly female monoecious on average produced 6. The number of microstrobili per cluster measurement showed that on average the male morph produces 50.75 microstrobili per cluster, the monoecious predominantly male 49.51, and the monoecious predominantly female 8.96. ($F_{2, 148}$=169.14, ρ =<.0001, R^2=0.82). Using the Tukey-Kramer HSD test was found no significant difference among morphs except that monoecious predominantly female (Q=2.76, α=0.05) trees produced fewer microstrobili per cluster. Microstrobili length measurement showed that monoecious predominantly female microstrobili (average length 75.68 mm) are longer than male, monoecious predominantly male, and the bisporangiate strobili which had an average length of 43, 44 and 48 mm respectively ($F_{3,126}$=87.17, ρ =<.0001, R^2=0.66. Q=2.6, α=0.05).

223 Monoecious predominantly female produced significantly more megastrobili than
224 females, in turn female individuals produced significant more megastrobili than
225 predominantly male monoecious individuals in 2006 ($F_{3,118}$=19.29, R^2=0.20, *P*<0.0001)
226 and 2008 ($F_{2,188}$=25.44, R^2=0.21, *P*<0.0001). Female and predominantly female
227 monoecious individuals produced high amount of megastrobili, on average 134 and
228 434, respectively. In contrast predominantly male monoecious individuals produced on
229 average 9 megastrobili in 2008 and 2.52 in 2006.
230
231 Final cone production did not differ between female and predominantly female
232 monoecious individuals in all years excepting for 2008 when a higher cone production
233 was recorder in comparison to other years (Figure 1). In 2008 female and predominantly
234 female monoecious produced on average 158 and 269 cones per tree respectively.
235 Predominantly male monoecious produced on average 9 cones per tree. Predominantly
236 male monoecious individuals presented the lowest mature cone production in all years
237 (Table 2). However cone production did not differ between predominantly male
238 monoecious and female in 2006 and 2009 that were years of low cone production in the
239 population. During 2009 (the lowest cone production) female and predominantly female
240 monoecious produced on average 29 and 63 cones per tree respectively, whereas
241 predominantly male monoecious produced on average 3 cones per tree.
242 Table 2. Cone production of *P. johannis* from 2005 to 2009.

Year	Statistical values
2005	$F_{2,99}$=4.90, R^2=0.13, P<0.0001
2006	$F_{2,121}$=5.27, R=0.22, P=0.0019
2007	$F_{2,67}$=3.35, R^2=0.09, P<0.04
2008	$F_{2,195}$=38.76, R^2=0.28, P<0.0001
2009	$F_{2,190}$=14.90, R^2=0.13, P<0.0001

243

We found a positive correlation between the cone length and seeds per cone ($F_{1,199}$=111.18, R^2=0.35, P<0.0001). But no significant difference in mature cone length among sexes, it was on average 2.65cm, the range was between 1.62cm and 3.86cm ($F_{2,199}$=2.14, ρ =.11, R^2=0.02).

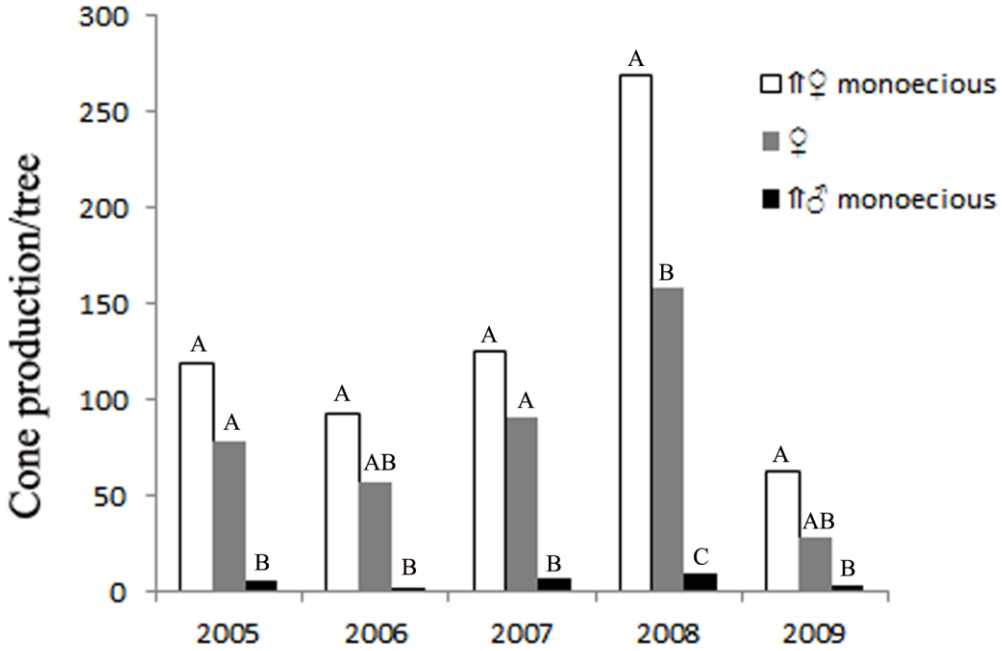


Figure 1. Tree cone production from 2005 to 2009. Significant differences among sexual morphs are represented by letters. During 5 years predominantly female monoecious produced on average higher cones per tree, followed by female and predominantly male monoecious individuals which produce few cones (0-8) per tree.

Fruit and seed set

We followed 167 individuals from 2008 to 2009; we counted the number of megastrobili in 2008 and then recorded the final cone production at the end of August 2009. There was no significant difference between sexual morph in fruit set ($F_{2,164}$=2.48, ρ =0.08, R^2=0.02, Q=2.36, α=0.05). On average fruit set was 17.32 to

predominantly female monoecious, 9.23 to female individuals, and 11.38 to predominantly male monoecious (total mean=13.53%).

Seed set did not differ among sexes ($F_{2, 678}$=2.75, ρ=0.08, R^2=0.008). On average 4.4 % of seed set was recorded.

Herbivory and defense

In order to know how unisexual individuals compared against both kinds of monoecious protect their cones from herbivory, we estimated the intensity of herbivory by tortrocids in shoots bearing microstrobili, megastrobili, as well as vegetative shoots as well as, directly in cones in development. Microstrobili of male and monoecious predominantly male are present nearly the same intensity of herbivory with 3.5% and 3.4% of intensity respectively; however both are more attacked than monoecious predominantly female which present cero attacked shoots bearing microstroibili (Figure 2A; $F_{2,176}$=17.98, ρ =<.0001, R^2=0.16, Q=2.36, α=0.05). The intensity of herbivory of shoot bearing megastrobili did not differ significantly among sexes, female individuals had on average 2.95%, predominantly female monoecious individuals had 4.77% and monoecious predominantly male had on average 3.82% of attacked shoots bearing megastrobili (Figure 2B; $F_{2, 172}$=0.21, ρ =0.8, R^2=0.04). Intensity of herbivory on vegetative shoots differed significant among sexes. Female and predominantly female monoecious individuals differed significant from male and predominantly male individuals in the intensity of herbivory on vegetative shoots. Sexual morphs investing in male function have higher intensity of herbivory compared to sexual morphs investing in female function. Female individuals have on average 2.12% and predominantly female monoecious have 1.96%, whereas male individuals have 13.56%

283 and predominantly male monoecious have on average 11.98% (Figure 2C; $F_{3,243}$=11.7,

284 ρ =<.0001, R^2=0.12, Q=2.58, α=0.05).

285 Cones in development of predominantly female monoecious are significant more

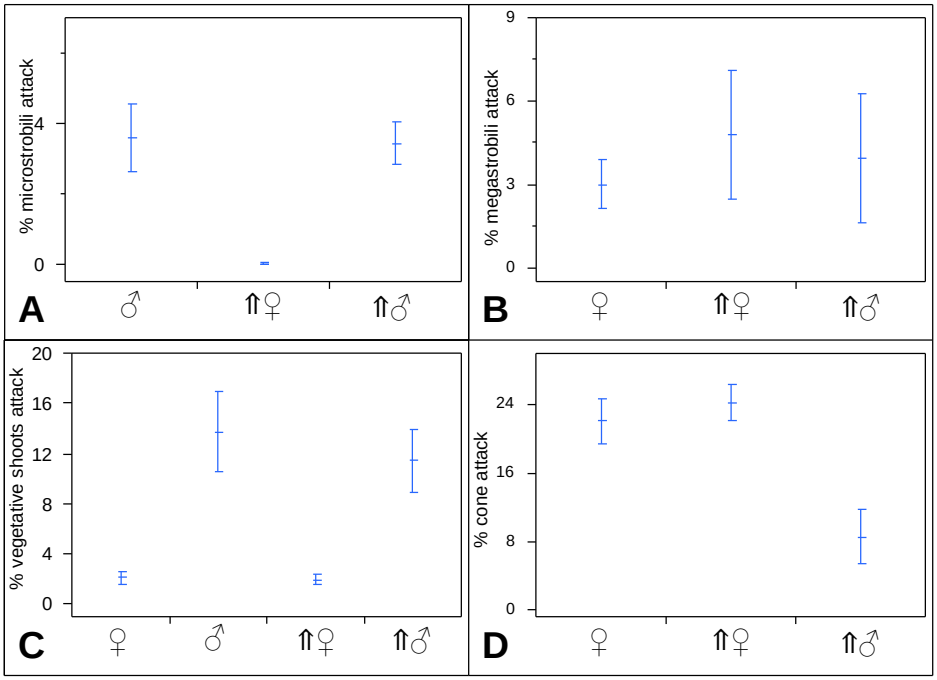
286 attacked than female and predominantly male monoecious and have on average 21.52%,

287 24.67%, and 9.1% of attacked cones (Figure 2D; $F_{2, 183}$=20.91, ρ =<.0001, R^2=0.17,

288 Q=2.36, α=0.05).

289

290



291

292 Figure 2. Patterns of herbivory in *P. johannis*. A) Shoots bearing microstrobili attack in

293 males and monoecious individuals. B) Shoots bearing microstrobili attack in males and

294 monoecious individuals. C) Larvae attack to cones in development. D) Vegetative

295 shoots attack among sexes.

296

297 ***Dichogamy, postzygotic incompatibility system and the avoidance of inbreeding***

A contingency analysis was done to compare differences in the success (1) or failure (0) to reach cone maturity between self- and out-crosses done in 2006. We found that 1.87% of the self- crosses reach maturity and was significant lower than the 7.97% of out-crosses (ρ =0.01, DF=1, R^2=0.019). Thus, 50 seeds were viable from 710 manual crosses, only two as product of selfing. This 50 seeds were used to calculate differences in number of seed per cone and seed viability by sex. No significant differences were found among sexes ($F_{2, 47}$=0.4, p=0.66, R^2=0.017). Predominantly male monoecious did not produce any viable from our crosses. Female and predominantly female monoecious individuals produced on average 0.8 and 0.33 viable seed per cone, respectively.

Seed viability and germination

In order to evaluate reproductive success among sexual morphs in natural crosses (wind-pollinated) 681 cones were collected. Number of seeds per cone did not differed among sexes ($F_{2, 678}$=1.67, ρ =0.18, R^2=0.004), on average 5.57 seeds are produce by cone. Number of viable seeds per cone did not differed among sexes ($F_{2, 678}$=2.75, ρ =0.0.64, R^2=0.0.008), on average 1.7 viable seeds are produced by cone. The percentage of seed viability per cone did not differ among sexual morphs ($F_{2,678}$=2.12, ρ =0.12, R^2=0.06, Table 3).

Table 3. Comparison of number of seeds per cone and viable seeds per cone between manual crosses and natural crosses.

	Manual crosses	Natural crosses
seed number per cone		
♀	7.79	5.48
⇑♀	5.47	6.05
⇑♂	3.66	5.62
viable seeds per cone		
♀	0.8	1.82
⇑♀	0.33	1.99
⇑♂	0	1.41

Regardless the sexual morph germination of the viable seeds (was very low, 25.52 %). Individual mean germination was 3.7, 4.7 and 2 (seedlings per individual) for female, predominantly female monoecious and predominantly male monoecious respectively. Germination probability did not differ among sexes ($F_{2, 70}$=0.64, ρ =0.52, R^2=0.02).

Figure 3. Albino seedling of *P. johannis* showing cotyledons deficient in chlorophyll and green new leaves at the center.

Only three seedlings presented typical features of inbreeding depression, two were albino seedlings and one had yellow pale cotyledons (Figure 3).

Crosses 2008: Megastrobili that worked as a negative control dropped in the first three months. . No temporal separation of the production of microstrobili and megastrobili in monoecious individuals were detected, we use this feature to perform self-crosses and identify incompatibility barriers. In order to find an autoincompatibility system or dichogamy we did the first survey in April to count cone retention as a measure of successful crosses and megastrobili receptivity at the time to pollen release in the same individuals. From 1674 self-crosses performed in May 2008 918 cones were retained in April 2009. The retention of 918 self-crosses suggesting there is neither prezygotic incompatibility system nor dichogamy. The second survey was done after fertilization;

dropping cone increased 50% compared with the survey on April. Interestingly, on the mature cone stage (September) all cones product of selfing dropped and success of out-cross decreased below 10% (Figure 4). However in all surveys success of self-crosses was significant lower than out-crosses (Table 4). No differences in effect of donor or receptor were found in the three surveys (data not shown).

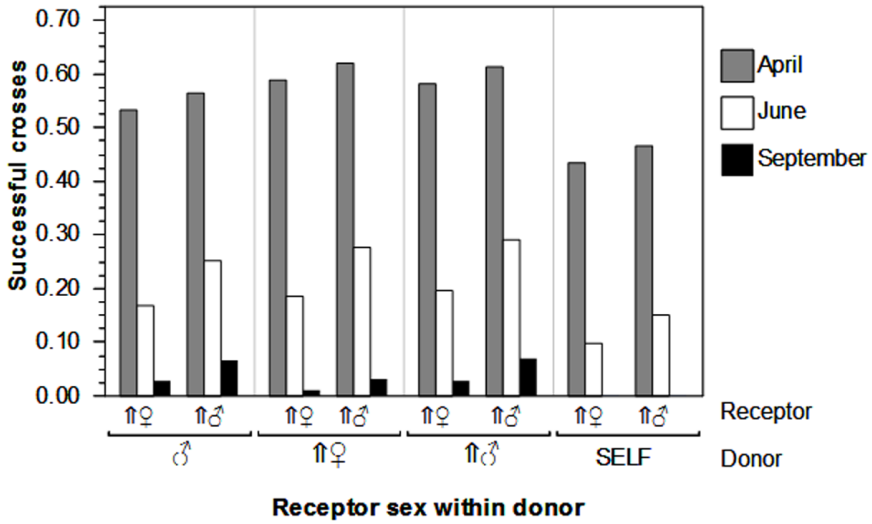


Figure 4. Progress of successful crosses during time of three surveys that correspond to pre-fertilization stage, after fertilization and mature embryo, April in grey, June in white and September in black (2009), respectively. Predominantly female monoecious (⇑♀) and predominantly male monoecious (⇑♂) individuals were used to produce self-lineages. Both kind of monoecious and male (♂) were used to produce outcrossing. None of the cones used in the selfing treatments reach maturity.

Table 4. Significant differences between self- and out-crosses. Statistic values for the nominal logistic fit model per survey.

	p value of significant differences between self and out crosses	Difference -Log likelihood between full and reduce model	DF	X^2	*P* value

April	0.006	5.82	4	11.64	0.02
June	0.01	12.81	8	25.62	0.001
September	0.007	7.37	4	14.75	0.005

Herbivore removal was confirmed by the reduction of webworms and attacked cones in sprayed trees ($F_{1,264}$=7.59, p=0.006, R^2=0.032). After herbivore removal 79% of crosses failed to reach maturity whereas non sprayed trees lost significant higher amount of crosses, 85% (p=0.006, R^2=0.005).

Pollen viability was not significant different among sexual morphs (See Flores-Rentería *et al.* 2011). However abnormal development was evident under the light microscope. The most common abnormalities were pollen with more of two air sacci, or no definition of sacci and pollen conglomerate (Figure 5A-D). In general low frequency of abnormal development of pollen was observed.

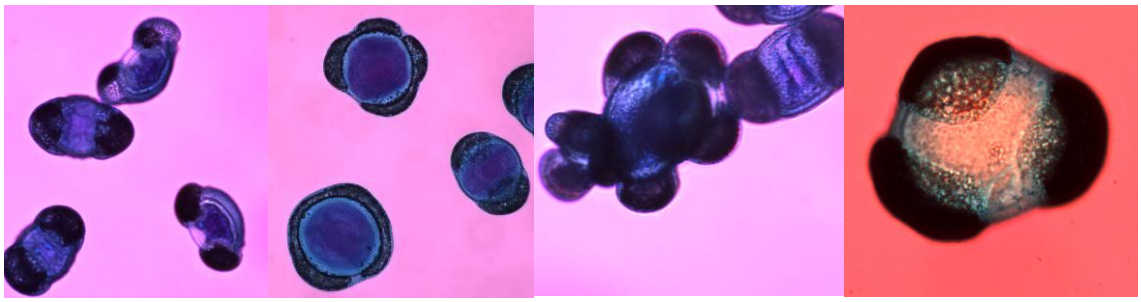


Figure 5. Normal and aberrant (aneuploide) pollen grain at release time. A) Normal pollen with two air sacci. B) Aberrant pollen with abnormal air sacci formation. C) Non tetrad separation creates a conglomerate of pollen grains. D) Pollen grain with three air sacci.

Embryological analysis was done to detect time of fertilization as well as, time of abortion of ovules and seeds. Two ovules are produced by ovuliferous scales. Megastrobili produce more than 30 ovules (Figure 6A). In open pollination, few ovules per cone go beyond pollination (Figure 6B), mainly one ovule per ovuliferous scale,

rarely two (Figure 6C-D). When pollen resumed growth, next spring after pollination, a high number of ovules degenerate even before fertilization (Figure 6E). A second period of abortion occurs soon after fertilization (Figure 6F).

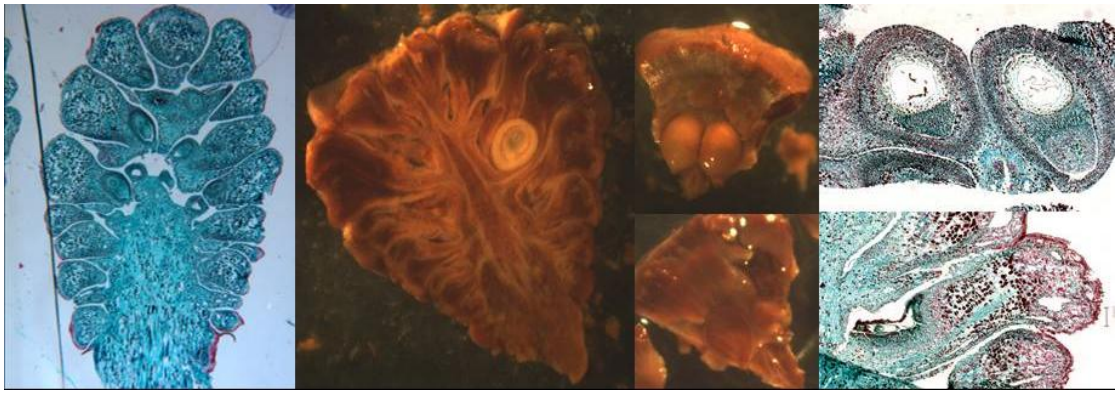


Figure 6. Develop of ovule, seed formation and seed abortion. A) Megastrobili cross section at the time of pollen release, multiple ovules are shown with arrows. B) Few ovules per cone turn into young seeds after fertilization (one year after pollination). Tannins on seed coat and megagametophyte are visible in seed (arrow). C) Occasionally two seed are developed by bract. D) Cross section of C with one ovule (left) showing signs of abortion, as collapsed megagametophyte. Integument (arrow), megagametophy (mg). E) Mostly one seed per bract reach fertilization and then is aborted before or during early embryogenesis. F) Cross section of E. Bract (Br), ovuliferous scale (os), aborted seed (arrow).

Discussion

Gender variation of *Pinus johannis* allows us to investigate two hypothesized selection pressures for sexual separation simultaneously. Our results suggest that high inbreeding depression played a role in the evolution of dioecy in this species. Evidence for influence of resource allocation to reproduction on sexual separation is less clear;

however differences in herbivory intensity among sexual morphs could act as selective pressure driving the evolution of dioecy in *P. johannis*.

Differential resource allocation to defense could play a role in the evolution to dioecy in P. johannis

Under the resource allocation hypothesis, evolution to dioecy could occur if resources have different values to male and female plants (Charnov y Bull, 1977; Charnov, 1982). We did not detect an increment of the reproductive structures in the unisexual individual compare with the most similar type of cosexual (e.g. female vs. predominantly female monoecious). Contrary to our expectations, predominantly female monoecious individuals produced more megastrobili than female. Overall, no significant differences were found in the total cone production between females and predominantly female monoecious. This indicates females have higher cone survival, suggesting an increment of resource allocated to defense. The proportion of juvenile fruits and seeds that mature is dependent upon extrinsic factors, such as weather conditions and seed predation, and the ability of the maternal parent to provide the resources necessary for growth and development (Stephenson, 1981; Mutke et al, 2005; Ordóñez *et al.* 2005). However, no significant differences were detected in the proportion of attack cones between female and predominantly female monoecious individuals. Regardless of the sexual morph, on average the total cone production (73 cones per trees) and fruit set (13%) were very low for the population in comparison with other species of *Pinus* (Stephenson, 1981). Although predominantly female monoecious individuals present slightly higher attack than females no significant differences were found between these two morphs. In addition measurements of herbivory intensity show two patterns, male and predominantly female were more

attacked in the vegetative shoots and shoots bearing microstrobili, whereas female and predominantly female monoecious have higher intensity of herbivory to cones in development. Thus different patterns in herbivory could play a role in the evolution to dioecy in *P. johannis*. Male and predominantly male monoecious had twice higher herbivory. This suggests ecological differences between functional sexes of *P. johannis*. Differential herbivory has been found in other angiosperm and gymnosperm dioecious species. In angiosperms male plants are subject to greater damage than female plants from herbivores feeding in vegetative tissue (reviewed in Agren *et al.* 1999). Male-biased herbivory may be the result of differential resource investment because females allocate more energy to reproduction and defense, whereas males invest more in vegetative growth (Putwain & Harper 1972; Boecklen *et al.* 1990, Cepeda-Cornejo and Dirzo, 2010; Narbona and Dirzo, 2010). Male-biased herbivory is a selective force that can drive the separation of sexes in plants (Verdú *et al.* 2004). Sex-bias herbivory was described in *Ephedra trifurca* (Boecklen and Hoffman, 1993). In *P. edulis* selective herbivory to shoots bearing megastrobili has a positive effect in the microstrobili production and increase female abortion (Cobb *et al*, 2002; Mueller *et al*, 2005). The developing fruits on female plants may experience substantial damage from herbivores feeding on developing seeds (e.g. Hodkinson *et al.* 1979; Ågren 1988; Krischik and Denno, 1990) but this kind of damage does not affect male plants (Ågren *et al.* 1999). This idea was corroborated in *P. johannis* in which cones in development are more attacked in females and predominantly female individuals whereas predominantly male monoecious experienced significant lower herbivory intensity. This suggests differential herbivory among female and male groups in *P. johannis* could play a role in the evolution of sexual separation.

442

443 *Inbreeding depression driving the sexual separation in* Pinus johannis

444 Monoecy is widespread, especially in large wind-pollinated plants such as trees
445 (Richards, 1986). One of the benefits of having separate sexes on the same individual is
446 that plants have the capacity to invest more on one sex or the other, depending upon
447 environmental conditions, in order to maximize the efficiency of both pollen dispersal
448 and pollen capture. Moreover, monoecious plants benefit from a reduction of inbreeding
449 depression, due to the spatial -and often temporal- segregation of sexes (Freeman *et al.*
450 1981). Pines which are mainly monoecious are considered as among the most
451 genetically variable of all species as revealed by measures of quantitative genetic
452 variation (Cornelius, 1994), diversity at allozyme loci and microsatellite markers
453 (Hamrick *et al.* 1979, Hamrick and Godt 1990; Delgado, 2002). Pines have outcrossing
454 rate estimates from morphological markers or from allozymes are between 0.91 and
455 0.98 (Schemske y Lande, 1985). Temporal separation of the sexes and/or
456 incompatibility genetic systems has been documented in some conifer species which
457 can partially explain the high outcrossing rates found in *Pinus* populations. Dichogamy
458 is temporal separation of the sexes in seed plants due to differential maturity of male
459 and female organs in flowers or cones. Dichogamy may take two forms, protandry,
460 when pollen shedding precedes female receptivity, and protogyny, when female
461 receptivity precedes pollen shedding. In contrast homogamy occurs when pollen
462 shedding simultaneously to female receptivity. Temporal separation of the sexes, or
463 dichogamy, is observed in some pine species but not in others. *Pinus palustris* and *P.*
464 *ponderosa* are protandrous (Zinder, Dinos y Derr, 1977; Wang, 1977). *Pinus contorta* is
465 slightly protandrous (Owens, Simpson y Molder, 1981). *Pinus nigra* is weakly
466 protandrous on average but some trees can be protogynous, (Matziris, 1994). Patterns of

pollen shedding and ovule receptivity distributions of the whole orchard suggest *P. contorta* var. *latifolia* present all three temporal sexual expressions depending in the orchard (Owens *et al.* 2005). It has been observed that largest cone loss occurred soon after pollination and resulted primarily from too few ovules being pollinated in the cone. About 80% of the fertile ovules must be pollinated or the cones abort within about 3 weeks after pollination (Owens *et al.* 2005). Thus manual self-crosses in *P. johannis* can help to evaluate patterns of homogamy if female receptivity and pollen shedding produce a cone retained 10 months (before fertilization). The retention of 918 self – crosses suggest there is no temporal separation as dichogamy or genetic incompatibility at pollination time. Our results suggest *P. johannis* has a homogamy system.

Monoecy and protogyny are widespread in wind-pollinated plants (Lloyd and Webb, 1986; Bertin and Newman, 1993; Sargen and Atto, 2004; Friedman and Barret, 2009) and have been considered as mechanism to limit self-fertilization. It has been shown that both mechanism are not particularly effective at restrict selfing (Griffin *et al.* 2000; Friedman and Barret, 2009).

Conifers are generally thought to lack prefertilization self-incompatibility systems (Hagman, 1975 in Williams, 2001). However, Owens *et al.* (1998) suggested that primitive prezygotic incompatibility mechanism might exist in conifers. In *P. johannis* the success of self-crosses prior fertilization was significant lower before fertilization; therefore we did not exclude an incipient prezygotic incompatibility mechanism.

Our second survey showed higher inbreeding depression in self –cross in *P. johannis*. However around 50% of all crosses were lost soon after fertilization. In lodgepole pine self pollinated ovules developed normally for the first year and the early part of the second year, until soon after fertilization. Then the embryos resulting from self-

fertilization and the megagametophyte that contained them aborted and dried, leaving a very uniform brown collapsed, sac-like megaspore membrane within a well-developed seed coat that had an attached wing. Total cone loss was 35-70% and resulted primarily from cone drop soon after pollination. In the cross-, open-, and self-pollination experiments, the greatest loss of cones occurred in self-pollinated followed by cross-pollinated cones (Owens *et al.* 2005). In *P. sylvestris* 95% of the self-fertilized zygotes were eliminated immediately after fertilization (Koski, 1971).

High inbreeding depression acting early in development purges recessive deleterious alleles. Self-fertilization in pines occurs at a low or moderate level (Koski 1971, Muona & Harju 1989, Ledig 1998) and a high level of outcrossing at the mature seed stage is maintained by some mechanism. Lanner (1980) made experimental cross in *Pinus edulis* self-cross produced 14.4% viable seeds while outcross produced 90.5% viable seeds. In *P. taeda,* Williams *et al.* (2001) found 82.7% filled seeds per cone for outcrossing and 19.8% for selfing. However, self-crosses in *P. johannis* produced no viable seeds, suggesting inbreeding depression is higher in this species.

In *P. johannis* monoecious individuals produce a high amount of reproductive structures of one sex and few of the opposite. For example, predominantly female monoecious individual produced on average 1198 microstrobili cluster and 9 megastrobili, thus the proportion of pollen that can contribute to selfing or reproduction its very low; predominantly female monoecious individuals produce on average 6 microstrobili cluster and 434 megastrobili, however none of the mature cones produced viable seeds on our manual crosses of 2006 but natural crosses showed 1.41 viable seeds per cone (they produced 3 cones per tree). In addition, manual crosses performed in 2008 showed no success in self-cross. Therefore probability of primary selfing is reduced in *P.*

517 *johannis*. Regardless the cross type *P. johannis* produce low percentage of fruit set and
518 seed viability. Thus it can be explained by high biparental inbreeding. However poor
519 environmental conditions can reduce maturing seeds. There is evidence of selection at
520 the embryonic stage so that the number of inbreeds is low already at the seedling stage
521 (Koski 1971, Kärkkäinen & Savolainen 1993). For instance, Koski (1973) estimated an
522 average 90 % of the inbred embryos are destroyed before the seed is mature. Selection
523 after the seedling stage is still severe in *Pinus*. Inbred individuals have a higher level of
524 homozygotes, and thereby homozygosity for recessive deleterious alleles is also higher
525 (Charlesworth & Charlesworth 1998). However most of these studies were done using
526 seedlings, thus inbreeding depression operates during seed development. Thus most
527 viable seeds are product of outcrossing. Seed set did not differ among sexes. On average
528 4.4 % of seed set was recorded in *P. johannis*. In contrast *P. cembroides* which is
529 sympatric to *P. johannis* in La Amapola presented 43.34% of seed set (personal
530 observation). On average 2 seeds per cone reach maturity and from them 7.46% are
531 viable. Seed viability and germination did not differ among sexes which suggest
532 inbreeding depression acts strongly during embryonic development.
533 In general, conifers have a mixed mating and selfing rates at the fertilization stage are
534 intermediate (Sorensen, 1994). Most report of inbreeding depression in conifers center
535 on embryonic-stage lethals and other deleterious recessives which affect seedlings. Less
536 is known about how inbreeding depression varies over the course of plant development
537 (Williams and Savolainen, 1996). In plants inbreeding depression appears severe during
538 seed development, typically manifested as empty seeds due embryo mortality (Orr-
539 Ewing, 1965), much less severe during growth and then becomes severe again at the
540 onset of reproduction (Husband and Schemske, 1996). However during embryogenesis,
541 regeneration, and stand development, selection eliminates selfed progeny and mature

reproductive populations usually consist of outcrossed individuals (Muona *et al.* 1988). In Douglas fir, noble fir and ponderosa pine plantations inbreeding depression is very highand no, or very few selfed progeny will contribute to mature reproductive populations (Sorensen, 1999). Koelewijn *et al.* (1999) showed that cumulative inbreeding depression is close to one in *P. sylvestris*, therefore no selfed progeny reach maturity. No significant differences were found in seed viability or percentage germination among sexes in *P. johannis,* this could be explained by intense inbreeding depression during embryogenesis leading mostly viable seed product of outcrossing. However cone loss and high amount of unviable seeds in outcrossing suggest a high biparental inbreeding and high lethal equivalent number in *P. johannis.*

Although most *Pinus* species are characterized for eliminate selectively embryos product of selfing, *P. johannis* presents an extreme inbreeding depression on self-crosses and progeny. High lethal equivalent compared to other conifers is assumed because no self-crosses reach cone maturity. Consequently, this can lead to the evolution of sexual separation in *P. johannis*.

Abnormal development of pollen is known for most *Pinus* species studied. Saylor and Smith (1966) studied pollen development of 21 species and 22 interspecific hybrids, all of them showed some degree of meiotic irregularities, such as precocious chromosomal disjunction and failure of chiasma terminalization. However *P. johannis*, presented low frequencies of aberrant pollen therefore cone losses are not likely to be due to pollen irregularities.

Ecological and reproductive patterns corroborate two sexual groups

Because no significant differences were found between male and predominantly male monoecious individuals in microstrobili production, and the latter did not produce mature cones with viable seeds (manual crosses from 2006), males and mostly all monoecious predominantly male reproduce entirely through the pollen. However in the open pollinated crosses 6 predominantly male monoecious individuals produced viable seeds that germinated, assumed as outcross, based on the breeding experiment in which non self-cross reach maturity. Although a portion of predominantly female monoecious produced more cones per tree than female individuals, 12.62 % of these individuals are able to change their sexual expression from female to monoecious predominantly female and 8.74% in the opposite direction (Flores-Rentería *et al.* 2011, in review), which suggest they belong to the same sexual group. In addition, two herbivory patterns corroborate the idea of two functional sexual morphs, grouping female and predominantly male in one side and male and predominantly male in other.

We found ecological features, as herbivory patterns, that support dioecy in *P. johannis.* Our findings suggest *P. johannis* evolved to dioecy under a combination of ecological (male-biased herbivory) as genetic (intense inbreeding depression) features.

Acknowledgements: The authors thank R. Pérez, F. Baena, V. Cepeda, A. Flores, J. Hernández and O. Jiménez for their help during the field work. We thank to P. Brune for his comments on this manuscript. This work was conducted by Flores-Rentería during her PhD. studies at the Posgrado en Ciencias Biomédicas (UNAM) with a CONACYT scholarship (172682).

591

592

593

594

595 References

596

597 Alabadí D. & M. A. Blázquez. 2009. Molecular interactions between light and hormone
598 signaling to control plant growth. *Plant Molecular Biology* 69: 409-417.
599 Allison TD. 1991. Variation in sex expression in Canada yew (*Taxus canadiensis*).
600 *American Journal of Botany* 78: 569-578.
601 *Ambrosia artemisiifolia* (Asteraceae). American Midland Naturalist 127:309-315.
602 Arista M. & S. Talavera. 1997. Gender expression in *Abies pinsapo* Boiss., a
603 Mediterranean Fir. *Ann. Bot.* 79: 337–342.
604 Ashman TL. 2002. The role of herbivores in the evolution of separate sexes from
605 hermaphroditism *Ecology*, 83: 1175–1184.
606 Ávila N. J., García M. E. & Reyes A. J. 1992 Registro de *Pinus discolor* Bailey et
607 Hawksworth en la sierra de monte Grande, San Luis Potosí, México. *Acta Botánica*
608 *Mexicana* 20:9-12.
609 Ávila N. J., García M. E. y Reyes A. J. 1992 Registro de *Pinus* discolor Bailey et
610 Hawksworth en la sierra de monte Grande, San Luis Potosí, México. *Acta Botánica*
611 *Mexicana* 20:9-12.
612 Bannister MH. 1965. Variation in the breeding system of *Pinus* radiata. In: Baker HG &
613 Stebbins GI (eds) The Genetics of Colonizing Species. Academic Press, New York, p
614 353-372.

615 Beaman J. H. and J. W. Andresen. 1966. The vegetation, Floristics and Phytogeography
616 of the Summit of Cerro Potosi, Mexico. *American Midland Naturalist*, vol. 75, No. 1.
617 Bierzychudek P, & Eckhart V. 1988. Spatial segregation of the sexes in dioecious
618 plants. *American Naturalist* 132:34-43.
619 Biswas C. Y B. M. Johri. 1997. The gymnosperms. Springer-Verlag. pp. 149.
620 Boecklen W. J. and M. T. Hoffman. 1993. Sex-Biased Herbivory in *Ephedra trifurca*:
621 The Importance of Sex-by-Environment Interactions. *Oecologia* 96: pp. 49-55.
622 Bullock S. H. & K. S. Bawa. 1981. Sexual dimorphism and the annual flowering pattern
623 in *Jacaratia dolichaula* (D. Smith) Woodson (Caricaceae) in a Costa Rican rain forest.
624 *Ecology* 62: 1494-1504
625 Burd M. and Allen TFH. 1988. Sexual allocation strategy in wind-pollinated plants.
626 *Evolution* 42: 403–407.
627 Case A. L. and S. C. H. Barrett. 2001. Ecological differentiation of combined and
628 separate sexes of *Wurmbea dioica* (colchicaceae) in sympatry. *Ecology* 82: 2601-2616.
629 Case A. L. and SCH Barret. 2001. Ecological differentiation of combined and separate
630 sexes of *Wurmbea dioica* (Colchicaceae) in sympatry. *Ecology* 82:2601-2616.
631 Chamberlain, C.J. 1935. Gymnosperms Structure and Evolution. Chicago.
632 Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary
633 consequences. *Annu Rev Ecol Syst* 18:237–268
634 Charlesworth D. 1999. Theories of the evolution of dioecy. In: Geber MA, Dawson TE,
635 & Delph LF 1999. (eds) Gender and sexual dimorphism in flowering plants. Springer,
636 Berlin Heidelberg New York.
637 Charlesworth D. and B. Charlesworth. 1979. The Evolutionary Genetics of Sexual
638 Systems in Flowering Plants. *Proceedings of the Royal Society of London. Series B*
639 *Biological Sciences* 205: 513-530.

640 Charlesworth, D. 2002. Plant sex determination and sex chromosomes. *Heredity* 88:94-
641 101.
642 Charnov E. L. & J. Bull. 1977. When is sex environmentally determinated? *Nature*
643 266:828-829.
644 Charnov, E. L. 1984. Behavioral ecology of plants. *In* J.R. Krebs and N.B. Davies
645 [eds.], Behavioral Ecology, an evolutionary approach, 362–379. Sinauer, Sunderland,
646 MA.
647 Clark D. A. & D. B. Clark. 1987. Temporal and environmental patterns of reproduction
648 in *Zamia skinneri*, a tropical rain forest cycad. *Journal of Ecology* 75:135-149.
649 Cobb N. S., R. T. Trotter III & T. G. Whitham. 2002. Long-term sexual allocation in
650 herbivore resistant and susceptible pinyon pine (*P. edulis*). *Oecologia* 130:78–87
651 Cornelius, J. 1994. Heritabilities and additive genetic coefficients of variation in forest
652 trees. *Canadian Journal of Forest Research*. 24: 372-379.
653 Crone, EE., McIntire, EJB & J. Brodie. 2011. What defines mast seeding? Spatio-
654 temporal patterns of cone production by whitebark pine. Journal of Ecology 99: 438–
655 444.
656 Dawson TE, & Geber MA. 1999. Sexual dimorphism in physiology and morphology.
657 In: Geber MA, Dawson TE, & Delph LF 1999. (eds) Gender and sexual dimorphism in
658 flowering plants. Springer, Berlin Heidelberg New York, pp 175–215.
659 Delgado, P., Cuenca, A., Escalante, A. E., Molina-Freaner, F. and D. Piñero. 2002.
660 Comparative genetic structure in pines: evolutionary and conservation consequences.
661 *Rev. chil. hist. nat.*, mar., vol.75, no.1, p.27-37.
662 Dorken, M.E. and S.C.H. Barrett. 2004. Sex determination and the evolution of dioecy
663 from monoecy in *Sagittaria latifolia* (Alismataceae) *Proc. R. Soc. Lond. B* 271, 213–
664 219.

Ehlers, B.K. and *Bataillon*, T. 2007. 'Inconstant males' and the maintenance of labile sex expression in subdioecious plants. *New Phytol*. 174, 194–211.

Farjon, A. and B.T. Styles. 1997. *Pinus* (Pinaceae). Flora Neotropica Monograph 75. New York, NY: The New York Botanical Garden.

Flores Flores, J.D. & Díaz Esquivel, D.E. 1988. Tabla de vida y factores de mortalidad para conos y semillas de *Pinus cembroides* Zucco bajo condiciones naturales en el sur de Coahuila. *In:* Passini, Marie-Francoise; Cibrian Tovar, David; Eguiluz Piedra, Teobaldo, eds. Memorias del II Simposio Nacional sobre Pinos Piñoneros. 1987 August 6-8; Ciudad de Mexico, Mexico., Universidad Autónoma de Chapingo, Centro de Genética Forestal. 123-135.

Flores-Rentería, Ll., Vázquez-Lobo A., Whipple A. V., Piñero D. D., Márquez-Guzmán, J. & Domínguez P-T. 2011. Functional bisporangiate cones in *Pinus johannis* (Pinaceae): Implications for the evolution of bisexuality in seed plants. *American Journal of Botany* 98: 130–139.

Floyd, M. E. 1982. Dioecy in five *Pinus* edulis populations in the southwestern united states. *The American Midland Naturalist* 110:405-411.

Fowler, D.P. 1964. Effects of inbreeding in red pine, *Pinus resinosa.* Ait. *Silvae Genet.* 13: 170–177.

Fox, J. F. 1993. Size and sex allocation in monoecious woody plants. *Oecologia* 94:110-113.

Freeman, D. C., E. D. McArthur, K. T. Harper & A. C. Blaver. 1981. Influence of environment on the floral sex ratio of monoecious plants. *Evolution* 35:194-197.

Freeman, D. C., J. L. Lovett-Doust, A. El-Keblawy, K. J. Miglia, & E. D. McArthur. 1997. Sexual specialization and inbreeding avoidance in the evolution of dioecy. *Bot. Rev*. 63:65–92.

690 Freeman, D. C., K. T. Harper, and E. L. Charnov. 1980. Sex change in plants: old
691 observations and new hypotheses. *Oecologia* (Berl.) 47:222-232.
692 Ganeshaiah, K. N. and U. R. Shaanker. 1991. Floral sex ratios in monoecious species-
693 Why are trees more male-biased than herbs? *Current Science* 60:319-321.
694 García D, Zamora R, Gómez J.M, Jordano P, & Hódar J.A. 2000. Geographical
695 variation in seed production, predation and abortion in *Juniperus communis* throughout
696 its range in Europe. *J. Ecol.* 88, 436–446.
697 Gauquelin T, Bertaudière V, Badri W, & Montès N. 2002. Sex ratio and sexual
698 dimorphism in mountain dioecious thuriferous juniper (*Juniperus thurifera* L.,
699 Cupressaceae). Botanical Journal of the Linnean Society 138: 237–244.
700 Geber MA, Dawson TE, & Delph LF. 1999. (eds) Gender and sexual dimorphism in
701 flowering plants. Springer, Berlin Heidelberg New York.
702 Gernandt, D.S., Liston, A., & D. Piñero. 2001. Variation in the nrDNA ITS of *Pinus*
703 subsection *Cembroides*: implications for molecular systematic studies of pine species
704 complexes. *Molecular Phylogenetics and Evolution* 21: 449-467.
705 Givnish, T. J. 1980. Ecological constraints of the evolution of breeding system in seed
706 plants: dioecy and dispersal in gymnosperms. *Evolution* 34:959-972.
707 Goldman, D. A., & M. F. Willson. 1986. Sex allocation in functionally hermaphroditic
708 plants: a review and critique. *The Botanical Review* 52:157-194.
709 Goubitz S., M. J. A. Werger, A. Shmida, and G. Ne'eman. 2002. Cone Abortion in
710 *Pinus halepensis*: The Role of Pollen Quantity, Tree Size and Cone Location. *Oikos* 97:
711 pp. 125-133.
712 Griffin S. R., K. Mavraganis, and C. G. Eckert. 2000. Experimental analysis of
713 protogyny in Aquilegia canadensis (Ranunculaceae). *American Journal of Botany* 87:
714 1246-1256.

715 Hamrick JL & Godt MJW (1990) Allozyme diversity in plant species. In: Brown AHD,
716 Clegg MT, Kahler al & Weir BS (eds) Plant Population Genetics, Breeding and Genetic
717 Resources. Sinauer Associates Inc., Sunderland, Massachusetts. p 43-63.
718 Hamrick JL, Godt MJ & Sherman-Broyles SL. 1992. Factors influencing levels of of
719 genetic diversity in woody plant species. New For 6: 95-124.
720 Harris, M.S, & Pannell, J.R. (2010) Canopy seed storage is associated with sexual
721 dimorphism in the woody dioecious genus *Leucadendron. Journal of Ecology*. 98 :
722 509-515.
723 Husband BC, Schemske DW (1996) Evolution of the magnitude and timing of
724 inbreeding depression in plants. *Evolution* 50:54–70
725 Iszkuło G., Jasińska A.K., Giertych M., & Boratyński A. 2009 – Do secondary sexual
726 dimorphism and female intolerance to drought influence the sex ratio and extinction risk
727 of *Taxus baccata*? *Plant Ecol*. 200:229–240.
728 Jordano P. 1991. Gender variation and expression of monoecy in *Juniperus phoenicea*
729 (L.) (Cupressaceae). *Botanical Gazette* 152: 476–485.
730 Kang, H. S. 2007. Changes in Gender Expression in Korean Populations of *Pinus*
731 *densiflora* over a Five-Year Period. *Journal of Plant Biology* 50: 181-189
732 Kärkkäinen K & Savolainen O. 1993. The degree of early inbreeding depression
733 determines the selfing rate at the seed stage: model and results from *Pinus* sylvestris
734 (Scots pine). *Heredity* 71: 160-166.
735 Keys RN, Autino A, Edwards J, Fady B, Pichot C & Vendramin GG (2000)
736 Characterization of -nuclear microsatellites in *Pinus* halapensis Mill. and their
737 inheritance in *P. halapensis* and *Pinus brutia* Ten. *Mol Ecol* 9: 2157-2159.
738 Kiener W. 1935. Unisexual lamber pines. *Science* 82:193.
739 Kiener Walter. 1935. Unisexual lamber pines. *Science* 82:193.

740 Koelewijn HP, Koski V & Savolainen O. 1999. Magnitude and timing of inbreeding
741 depression in Scots pine (*Pinus* sylvestris L.). *Evolution* 53: 758-768.
742 *Korpelainen* H. 1998. Labile sex expression in plants. *Biol Rev* 73:157–180.
743 Koski V. 1971. Embryonic lethals of *Picea abies* and *Pinus* sylvestris. *Commun Inst*
744 *For Fenn* 75.3: 1-30.
745 Koski V. 1973. On self-pollination, genetic load, and subsequent inbreeding in some
746 conifers. *Commun Inst For Fenn* 78.10: 1-42.
747 Kral, R. 1993. *Pinus*. Flora of North America Editorial Committee (eds.): Flora of
748 North America North of Mexico, Vol. 2. Oxford University Press.
749 Lanner, R. M. 1980 a self-pollination experiment in *Pinus* edulis. *Great Basin Nat*
750 40:265-267.
751 Lanner, Ronald M. 1981. The piñon pine. Reno: University of Nevada Press.
752 Ledig F.T. 1998. Genetic variation in *Pinus*. In: Richardson DM (ed) Ecology and
753 biogeography of *Pinus*: 251-280. Cambridge University Press, Cambridge, United
754 Kingdom.
755 Ledig FT. 1998. Genetic variation in *Pinus*. In: Richardson DM (ed) Ecology and
756 biogeography of *Pinus*: 251-280. Cambridge University Press, Cambridge, United
757 Kingdom.
758 Ledig FT. 1999. Founder effects and genetic structure in Coulter pine. *Journal of*
759 *Heredity* 91: 307-315.
760 Ledig FT, M Capó-Arteaga, PD Hodgskiss, H Sbay, C Flores-López, MT Conkle & B
761 Bermejo-Velásquez. 2001. Genic diversity and the mating system of a rare mexican
762 piñon, *Pinus pinceana*, and a comparison with *Pinus maximartinezii* (Pinaceae).
763 *American Journal of Botany* 88: 1977-1987.

764 Lev-Yadun S. & N. Liphschitz. 1987. The ontogeny of gender of *Cupressus*
765 *sempervirens* L. *Botanical Gazete* 148: 407-412.
766 Lloyd D. G. & C. J. Webb. 1977. Secondary sex characters in plants. *Botanical Review*
767 43: 177-216.
768 Lloyd, D. G. 1975 Breeding systems in *Cotula* L. (Compositae, Anthemideae). III.
769 Dioecious populations. *New Phytol.* 71, 109–123.
770 *Lloyd*, D. G. *1976*. The transmission of genes via pollen and ovules in gynodioecious
771 angiosperms. *Theor. Pop. Biol.* 9: 299–316.
772 *Lloyd*, D. G. 1980. The distribution of gender in four angiosperm species illustrating
773 two evolutionary pathways to dioecy. *Evolution* 34:123–134.
774 Lloyd, D. G., & K. S. Bawa. 1984. Modification of the gender in seed plants in varying
775 conditions. *Evolutionary Biology* 17:255-338.
776 Lloyd. D. G. 1974. Female-predominant sex ratios in angiosperms. *Heredity* 32: 35-44.
777 Malusa, J. 1992. Phylogeny and biogeography of the pinyon pines (*Pinus* subsect.
778 *Cembroides*). *Systematic Botany* 17(1):42-66.
779 Matheson AC, JC Bell and RD Barnes (1989) Breeding systems and genetic structure in
780 some Central American pine populations. *Silvae Genetica* 38: 107-113.
781 McCormick J. & J. W. Andresen. 1963. A subdioecious population of *Pinus cembroides*
782 in southeast Arizona. *The Ohio Journal of Science* 4:159-163.
783 .
784 Meagher T. R. & J. Antonovics. 1982. The population biology of *Chamaelirium luteum*,
785 a dioecious member of the lily family: life history studies. *Ecology* 63: 1690-1700.
786 Meinke D.W. 1991. Perspectives on genetic analysis of plant embryogenesis, *Plant Cell*
787 3: 857–866

788 Millard P. & G. A. Grelet. 2010. Nitrogen storage and remobilization by trees:
789 ecophysiological relevance in a changing world. *Tree Physiology* 30:1083–1095.
790 Millard, P., A. Hester, R. Wendler & G. Baillie. 2001. Interspecific defoliation
791 responses of trees depend on sites of winter nitrogen storage. *Funct. Ecol.* 15:535–543.
792 Mirov N. T. 1967. The genus Pinus. The Ronald Press Company, New York. Pp.376.
793 Mirov, N. T. 1962 Phenology of tropical pines. *J. Arnold Arbor. Harv. Univ.* 18:218-
794 219.
795 Morgante M, Vendramin GG, Rossi P & Olivieri AM. 1993. Selection against inbreds
796 in early life-cycle phases in *Pinus leucodermis*. *Heredity* 70: 622-627.
797 Morgante M., G. G. Vendramin, P. Rossi, and A. M. Olivieri. 1993. Selection against
798 inbreds in early life-cycle phases in *Pinus leucodermis* Ant. *Heredity* 70: 622-627.
799 Mueller R. C., B. D. Wade, C. A. Gehring & T. G. Whitham. 2005. Chronic herbivory
800 negatively impacts cone and seed production, seed quality and seedling growth of
801 suceptible pinyon pines. *Oecologia* 143:558-565.
802 Muona O & Harju. 1989. Effective population sizes, genetic variability and mating
803 system in natural stands and seed orchards of *Pinus* sylvestris. *Silvae Genet* 38: 221-
804 228.
805 Muona O and AE Szmidt (1991) A multilocus study of natural populations in *Pinus*
806 *sylvestris.* In: Gregorius HE (ed) Population genetics in forestry, Lecture Notes in
807 Biomathematics No. 60: 226-240. Springer-Verlag, Berlin, Germany.
808 Muona O, Yazdani R & Rudin R (1987) Genetic change between life stages in *Pinus*
809 sylvestris: allozymes variation in seeds and planted seedlings. *Silvae Genet* 35: 39-42.
810 Muona O., A. Harju, and K. Kärkkäinen. 1988. Genetic comparison of natural and
811 nursery grown seedlings of *Pinus sylvestris* using allozymes. *Scandinavian Journal of*
812 *Forest Research* 3: 37-46.

Mutke S., Gordo J. Gil, L.2005. Cone Yield Characterization of a Stone Pine (*Pinus pinea* L.) Clone Bank. *Silvae Genetica* 54, 4–5.

Narbona E. and R. Dirzo. 2010. Experimental defoliation affects male but not female reproductive performance of the tropical monoecious plant *Croton suberosus* (Euphorbiaceae). *Annals of Botany* 106: 359-369.

Nuñez C.I., M.A. Nuñez & T. Kitzberger. 2006. Sex-related spatial segregation and growth in a dioecious conifer along environmental gradients in northwestern Patagonia. *Ecoscience* 15:73-80.

Ordóñez J. L., J. Retana, and J. M. Espelta. 2005. Effects of tree size, crown damage, and tree location on post-fire survival and cone production of *Pinus nigra* trees. *Forest Ecology and Management* 206: 109-117.

Ortiz, P.L., Arista, M., & S. Talavera. 2002. Sex ratio and reproductive effort in the dioecious *Juniperus communis* subsp. *alpina* (Suter) Čelak. (Cupressaceae) along an altitudinal gradient. *Ann. Bot.* **89**, 205-211.

Owens J. N., J. Bennett, and S. L'Hirondelle. 2005. Pollination and cone morphology affect cone and seed production in lodgepole pine seed orchards. *Canadian Journal of Forest Research* 35: 383-400.

Owens JN, Takaso T & Runions CJ (1998) Pollination in conifers. *Trends in Plant Sci* 3: 479-485.

Owens, J. N., & V. Hardev. 1990. Sex expression in gymnosperms. *Critical Reviews in Plant Sciences* 9: 281– 294.

Passini M. F. 1994. Synonymie entre *Pinus discolor* Bailey & Hawksworth et *Pinus johannis* M.-F. Robert. *Acta Botanica Gallica*. 141:387-388

Perry, J. P. 1991. The pines of Mexico and Central America. Portland, OR: Timber Press.

838 Price, R.A., A. Liston and S.H. Strauss. 1998. Phylogeny and systematics of *Pinus*.
839 P.49-68 in Richardson, D.M. (ed.), Ecology and Biogeography of *Pinus*. Cambridge
840 University Press.
841 Primack, R.B. and C. McCall. 1986. Gender variation in a red maple population (*Acer*
842 *rubrum*; Aceraceae): A seven-year study of a "polygamodioecious" species. *American*
843 *Journal of Botany* 73: 1239-1248.
844 Renner, S. S, & H. Won. 2001. Repeated Evolution of Dioecy from Monoecy in
845 Siparunaceae (Laurales) *Syst. Biol.* 50(5):700–712.
846 Renner, S. S., and R. E. Ricklefs. 1995. Dioecy and its correlates in the flowering
847 plants. *Am. J. Bot.* 82(5): 596-606.
848 Renner, S. S., L. Beenken, G. W. Grimm, A. Kocyan, & R. E. Ricklefs. 2007. The
849 evolution of dioecy, heterodichogamy, and labile sex expression in *Acer*. *Evolution*,
850 61:2701-2719.
851 Saylor L. C. and B. W. Smith. 1966. Meiotic Irregularity in Species and Interspecific
852 Hybrids of *Pinus*. *American Journal of Botany* 53: pp. 453-468.
853 Schemske D. W. and R. Lande. 1985. The Evolution of Self-Fertilization and
854 Inbreeding Depression in Plants. II. Empirical Observations. *Evolution* 39: pp. 41-52.
855 Slingsby, J. 2004. Branch Junction Constriction and Hydraulic Limitation in Two
856 Species in the Cape Proteaceae: A Mechanism Explaining the Trade-off between
857 Longevity and the Degree of Ramification in the Cape Proteaceae. Honours,University
858 of Cape Town, Cape Town.
859 Smith DN & Devey ME (1994) Occurrence and inheritance of microsatellites in *Pinus*
860 radiata. Genome 37: 977-983.
861 Smith, C. C. 1981. The facultative adjustment of sex ratios in lodgepole pine. Am. Nat.
862 118:297-305.

863 Solomon, B. P. 1989. Size-dependent sex ratios in the monoecious, wind-pollinated
864 annual, *Xanthium strumarium*. American Midland Naturalist 121:209-218.
865 Stephenson A. G. 1981. Flower and Fruit Abortion: Proximate Causes and Ultimate
866 Functions. *Annual Review of Ecology and Systematics* 12: pp. 253-279.
867 Thomas BR, Macdonald SE, Hicks M, Adams DL & Hodgetts RB. 1999. Effects of
868 reforestation methods on genetic diversity of lodgepole pine: and assessment using
869 microsatellite and randomly amplified polymorphic DNA markers. *Theor Appl Genet*
870 98: 793-801
871 Tikhonova I. V. 2003. Sex estructure of scotch Pine populations in the dry steppe.
872 *Russian Journal of Ecology* 6:370-374.
873 Tikhonova I. V. 2007. Changes in the sex structure of pine populations related to
874 temperature anomalies. *Russian Journal of Ecology* 38:306-310.
875 Torices, R., M. Méndez and J. M. Gómez. 2011. Where do monomorphic sexual
876 Systems fit in the evolution of dioecy? Insights from the largest family of angiosperms.
877 *New phytologist* 190(1): 234-248
878 Tosh, K. J. & G. R. Powell. 1986. Proliferated, bisporangiate, and other atypical cones
879 occurring on young, plantation-grown *Larix laricina. Canadian Journal of Botany* 64:
880 469 – 475.
881 Traveset, A. 1992. Sex expression in a natural population of the monoecious annual,
882 Traveset, A. 1999. Ecology of plant reproduction: mating systems and pollination. In:
883 *Handbook of Functional Plant Ecology*. Pugnaire, F.I. and Valladares, F. (editors).
884 Marcel Dekker, Inc., New York. pp. 545-588.
885 Tyree, M.T. & Zimmerman, M.H. 2002. Xylem Structure and the Ascent of Sap.
886 Springer-Verlag, Berlin.

887 Ushimaru, A & K. Matsui. 2001. Sex change in tree species: long-term monitoring of
888 sex expression in *Acer rufinerve*. *Nordic Journal of Botany*, 21:397-399.
889 Wakushima, S. , H. Yoshioka , & N. Sakurai . 1997. Promotion of lateral female strobili
890 production in *Pinus densiflora* by cytokinin application at a specifi c stage. *Journal of*
891 *Forest Research* 2: 51 – 57.
892 Webb CJ. 1999. Empirical studies: Evolution and maintenance of dimorphic breeding
893 systems. In: Geber MA, Dawson TE, & Delph LF 1999. (eds) Gender and sexual
894 dimorphism in flowering plants. Springer, Berlin Heidelberg New York.
895 Whitham TG, & S. Mopper. 1985. Chronic herbivory: impacts on architecture and sex
896 expression of pinyon pine. *Science*. 228:1089-91.
897 Williams CG. 2008. Selfed embryo death in Pinus taeda: a phenotypic profile. New
898 Phytologist 178: 210-222.
899 Williams C. G., Y. Zhou, and S. E. Hall. 2001. A chromosomal region promoting
900 outcrossing in a conifer. *Genetics* 159: 1283-1289.
901 Williams CG. 2009. Conifer reproductive biology. Springer, New York. Pp. 91-105
902 Zavala C. F., & J.L. D. Campos. 1993. Una nueva localidad de *Pinus discolor* Bailey &
903 Hawksworth en el centro de Mexico. *Acta Botanica Mexicana* 25:21-25.
904 Zavarin, E. & Snajberk, K. 1986. Monoterpenoid differentiation in relation to the
905 morphology of *Pinus discolor* and *Pinus johannis. Biochem. Syst. Ecol.* 14: 1-11.
906

CAPÍTULO 5

FUNCTIONAL BISPORANGIATE CONES IN *PINUS JOHANNIS* (PINACEAE), IMPLICATIONS FOR THE EVOLUTION OF BISEXUALITY IN SEED PLANTS

American Journal of Botany

American Journal of Botany 98(1): 130–139. 2011.

Functional bisporangiate cones in *Pinus johannis* (Pinaceae): Implications for the evolution of bisexuality in seed plants[1]

Lluvia Flores-Rentería[2,3,5], Alejandra Vázquez-Lobo[2], Amy V. Whipple[3], Daniel Piñero[2], Judith Márquez-Guzmán[4], and C. A. Domínguez[2]

[2]Departamento de Ecología Evolutiva, Instituto de Ecología, UNAM, A. P. 70-275, México, D. F. 04510 México; [3]Merriam-Powell Center for Environmental Research & Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona 86011 USA; and [4]Departamento de Biología Comparada, Facultad de Ciencias, UNAM, A. P. 70-356, México, D. F. 04510 México

- *Premise of study*: Bisexuality (male and female function in one structure) has been reported as a key innovation of angiosperms. Although there are several reports of "teratological" bisporangiate (bisexual) cones in gymnosperms, there have been none on the viability of their ovules and pollen. Analyses of the development and arrangement of female and male structures on bisporangiate cones of *Pinus johannis* enables us to gain insight on the origin of bisexuality in seed plants, for both angiosperms and gymnosperms.
- *Methods*: Viability of bisporangiate cones was assayed by performing manual crosses and using anatomical and histological methods.
- *Key results*: We determined that bisporangiate cones of *P. johannis* produce functional pollen and ovules. Male and female organs occupy basal and apical positions, respectively, the same positions found in almost all bisporangiate strobili in gymnosperms and bisexual flowers in angiosperms.
- *Conclusions*: The viability and spatial distribution of female and male organs of bisporangiate cones and their frequent occurrence in gymnosperms suggest a common mechanism in all seed plants for the production of bisporangiate structures. This idea is further supported by the presence of homologous genes for sexual organ identity in gymnosperms and angiosperms as reported by other authors. The lack of bisporangiate structure in gymnosperms may be primarily due to selection to avoid inbreeding rather than to genetic constraint.

Key words: bisexuality; bisporangiate cones; breeding systems; dioecy; Pinaceae; *Pinus johannis*; unisexuality; viability.

Bisexuality has been proposed to be an innovation of angiosperms (Theißen and Melzer, 2007; Specht and Bartlett, 2009), but a greater understanding of the expression of bisexuality in other seed plants may show that mechanisms for producing bisexual structures predates the angiosperm–gymnosperm split. Most flowering plants produce bisexual structures (perfect flowers). Only 10–20% of extant angiosperms have a reproductive system with unisexual flowers, and for these species, unisexuality is a derived trait (Richards, 1997; Ainsworth, 2000). Most gymnosperms have unisexual structures: pollen-producing (microsporangiate or male) and ovule-producing (megasporangiate, seed or female) structures (Theißen and Becker, 2004). Generally, gymnosperms are either monoecious (ovule- and pollen-producing structures are produced in different structures within the same individual) or dioecious (ovule- and pollen-producing structures are produced in different individuals) (reviewed by Givnish [1980] and Owens and Hardev [1990]). Bisexuality in gymnosperms occurs only in Gnetales (*Ephedra*, *Gnetum*, and *Welwitschia*) as part of their normal reproductive pattern (Mehra, 1950; Endress, 1996; Haycraft and Carmichael, 2001). Several anecdotal reports indicate that bisporangiate cones (also called bisexual cones or hermaphroditic strobili) occur sporadically in gymnosperms. Such is the case in the families Pinaceae, Araucariaceae, and Cupressaceae (Masters, 1869; Zobel, 1952; Lanner, 1966; Owens and Hardev, 1990; Matziris, 2002). In *Pinus*, bisporangiate cones can form in natural conditions (Fisher, 1905; Rao, 1932; Zobel and Goddard, 1954; Dorman, 1976; Matziris, 2002) or can be stimulated by exogenous hormonal application (Harrison and Slee, 1991; Wakushima et al.., 1996, 1997). In either case, the bisporangiate cones generally have ovuliferous scales associated with bracts at the top and microsporophylls below, thus resembling the arrangement of organs in flowering plants.

There are several cases of the presence of bisporangiate cones in gymnosperms, but most reports correspond to superficial observations and are typically dismissed as abnormal, nonfunctional, deleterious, and even "monstrous", also called "terata" (Zobel and Goddard, 1954; Chamberlain, 1966; Burley, 1976; Dorman, 1976). Although, from a paleobotanical perspective, there is more recent interest in such terata in all seed plants for the insights they might give to the evolution of flowers and for understanding potential neoGoldschmidtian or saltational evolutionary events in plants (see Bateman and Dimichele, 2002; Theißen, 2006). Descriptions evaluating the function of

[1] Manuscript received 27 July 2010; revision accepted 3 November 2010.

The authors thank R. Pérez and O. Jiménez for their help during the field work. A. I. Bieler for technical assistance with microphotographs. Drs. G. W. Rothwell, R. Bateman, T. Whitham, and C. Gehring who made useful suggestions that improved this manuscript. This work was conducted by Flores-Rentería during her PhD. studies at the Posgrado en Ciencias Biomedicas (UNAM) with a CONACYT scholarship (172682).

[5] Author for correspondence (e-mail: lluvia.flores@nau.edu), phone: + (928) 523-9138

doi:10.3732/ajb.1000275

ovules produced in bisporangiate strobili of gymnosperms exist only for Gnetales. Ovules on bisporangiate structures of *Ephedra*, *Gnetum*, and *Welwitschia* are reported to be sterile or abortive (Maheshwari and Vasil, 1961; Endress, 1996; Hufford, 1996; Haycraft and Carmichael, 2001). Gnetales are functionally dioecious, and the ovules in bisporangiate structures serve only to make pollination droplets that attract pollinators (Endress, 1996). Viability of bisporangiate structures in other groups of gymnosperms has not been studied, despite their potential importance in the elucidation of the origin of bisexuality in seed plants. In conifers, occasional bisporangiate structures may remain functional because there has not been selection for sterile structures to serve as pollinator rewards, as in Gnetales. This hypothesis could be tested in wind-pollinated gymnosperm species that occasionally create bisporangiate strobili. We therefore expect that in such species both female and male structures in bisporangiate cones will be functional. Knowledge of the structure and viability of bisporangiate cones in conifers is one important element for interpreting the origin of bisexuality in seed plants. If bisporangiate cones in conifers are viable and develop as bisporangiate flowers in angiosperms do, then these features would support the idea that the developmental genetic mechanisms for bisexuality may predate the divergence of angiosperms and gymnosperms and that the lack of bisporangiate structures may be maintained by selection in conifers. In this paper, we examined the viability of bisporangiate strobili of *Pinus johannis* Rob.-Pass by characterizing the sexes morphologically, performing controlled crosses, and describing ovule and pollen development of bisporangiate cones.

MATERIALS AND METHODS

Plant material—*Pinus johannis* trees produce pollen and ovulate cones in May and June. During these months, we identified sexual morphs from 2005 to 2009 in the population "La Amapola", San Luis Potosí (101°07.706′ W, 22°01.160′ N and 2391 m a.s.l.). Sexual identification was based on the presence of ovulate cones, pollen cones, and bisporangiate cones. Seed, pollen, and bisporangiate cones were visually distinguished. Young ovulate cones (formed by complexes of ovuliferous scales associated with bracts) are white to green, then turn purple and are 0.7–1.2 cm long. The mature cone is woody, brown, 3–4 cm long, and 2–3 cm wide. Young pollen cones (compound by microsporophyls) are slender and green; they turn yellow before releasing pollen and grow to 4.3–7.5 cm long. Bisporangiate cones have features of ovulate cones toward the apex and of pollen cones toward the base, only until the release of pollen. There are five reproductive morphs of *P. johannis*: (1) males that bear only pollen cones, (2) females that bear only ovulate cones, (3) predominantly male monoecious individuals that produce a large number of pollen cones and few ovulate cones, (4) predominantly female monoecious individuals that produce a large number of ovulate cones and few pollen cones, and (5) monoecious individuals that produce bisporangiate cones.

Embryological analysis—Ovulate, pollen, and bisporangiate cones were collected at different stages of development from all five sexual morphs. Cones were fixed in FAA (formaldehyde, acetic acid, 96% ethanol, water 2 : 1 : 10 : 7) or in 4% paraformaldehyde (v/v) in phosphate-buffered saline (PBS 1×). The fixed tissues were dehydrated with an ethanol series (50%, 70%, 80%, 90%, and 100%) of 60 min each. Samples fixed in FAA were infiltrated with a xylene/Paraplast (Sigma-Aldrich, St. Louis, Missouri, USA) mixture and then embedded in Paraplast. Sections 10 μm thick were cut on a rotary microtome and mounted on slides. The material fixed in paraformaldehyde was rinsed with PBS 1×, dehydrated as described above, embedded in LR White Resin (medium grade; Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA), and then sectioned at 0.80–1.5 μm with an ultramicrotome. Paraffin-embedded material was stained with 1% safranin-fast green in 96% ethanol. Resin embedded material was stained with 0.05% toluidine blue in dH20 and visualized by light microscopy.

Pollen viability—Pollen viability of pollen cones and bisporangiate cones were compared after covering the strobili with wax bags and collecting the released pollen. Pollen was collected in late May and early June and processed with Alexander's stain (Alexander, 1969). Slides containing pollen grains were examined from 15 individuals per common morph (male, predominantly male monoecious, predominantly female monoecious) and from five individuals bearing bisporangiate cones. We made three slides per individual. Pollen grains with violet cytoplasm were considered viable. Images were taken using a microscope (Zeiss, Discovery V8 model) with a digital camera (Canon, Powershot A620). Pollen grains were counted to obtain percentage and mean pollen viability by morph. Differences among morphs were determined by one-way ANOVA ($P = 0.05$).

Controlled crosses—To determine the viability of gametes in bisporangiate cones, we performed manual crosses in May and June. We crossed five individuals that produced bisporangiate cones in 2006 or in 2008. A total of 118 crosses were performed. To test ovule viability, we conducted 36 crosses using bisporangiate cones as the receptor of pollen from male or monoecious individuals. To test pollen viability, we conducted 74 crosses using bisporangiate cones as pollen donors using ovulate cones of female and monoecious individuals as receptors. To avoid cross contamination, we covered immature ovulate and bisporangiate cones with wax bags and waited for 8 d until they were receptive to do manual crosses. Fresh pollen was collected in wax bags, which were then attached to the shoots bearing ovulate or bisporangiate cones. The crosses were checked three times during development. Crosses in 2006 and 2008 were deemed successful if mature cones were present in September 2007 and 2009, respectively.

RESULTS

Bisporangiate cone distribution in trees—In the La Amapola population, bisporangiate cones were detected on six trees from 2006 to 2009, but this trait was not constant. For example, some individuals were predominantly male monoecious in 2006, but in 2007 and 2008, they had 15–50 bisporangiate structures. Then in 2009, they produced purely monosporangiate structures again. Individuals that were initially predominantly male monoecious trees (Fig. 1A), bore bisporangiate cones interspersed within pollen cone clusters (Fig. 1C). Individuals that were initially predominantly female monoecious trees (Fig. 1B), bore bisporangiate cones in the shoot apex where two ovulate cones would normally develop (Fig. 1D). In bisporangiate cones, the ratios of microsporophylls to ovuliferous scales varied; some trees had a higher proportion of male structures to female or vice versa.

***Development of bisporangiate structures in* Pinus johannis**—Ovulate cone development in *P. johannis* has a 16-month cycle in the population studied. Development initiates in April with the ovulate cone primordium, which becomes receptive at the end of May to early June (Fig. 2A). At this time, ovules develop in the ovulate cones (Fig. 2B) and pollen is shed from pollen cones (Fig. 2C). Microsporophylls develop helicoidally around an axis, and on the underside of each microsporophyll, two microsporangia are found (Fig. 2D). During receptivity, the ovules produce pollination drops, including ovules in bisporangiate cones (Fig. 2E). In the bisporangiate cones, microsporophylls develop earlier than ovuliferous scales. Microsporophylls develop at the bottom of the cone, and ovuliferous scales (each one associated with a bract) are developed at the top (Fig. 2F). Ovulate cones enter dormancy around August and remain in this condition until the following spring (mid-April), when growth is resumed and ovulate cones turn green. Fertilization occurs 1 month later. Cones reach their maturity in late August, at which time mature cones turn brown and have woody scales.

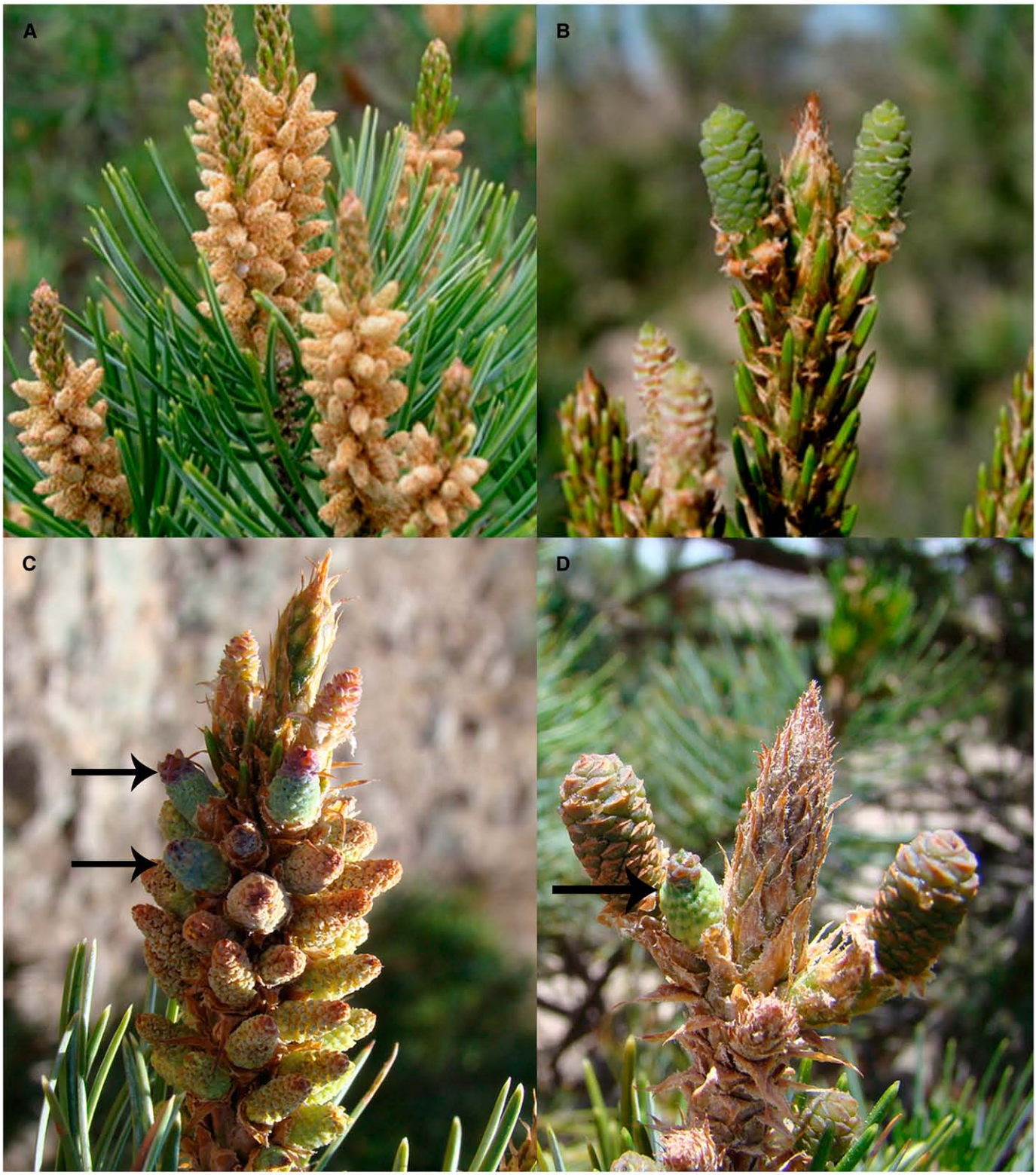


Fig. 1. Disposition of pollen, ovulate, and bisporangiate cones of *Pinus johannis*. (A) Cluster of pollen cones. (B) Ovulate cones develop on the tip of whorled lateral shoots. (C) Bisporangiate cones (arrows) interspersed with cluster of microstrobili of an individual initially monoecious predominately male. (D) Bisexual cone (arrow) originated at the top of the shoot alongside megastrobili in individual initially monoecious predominately female.

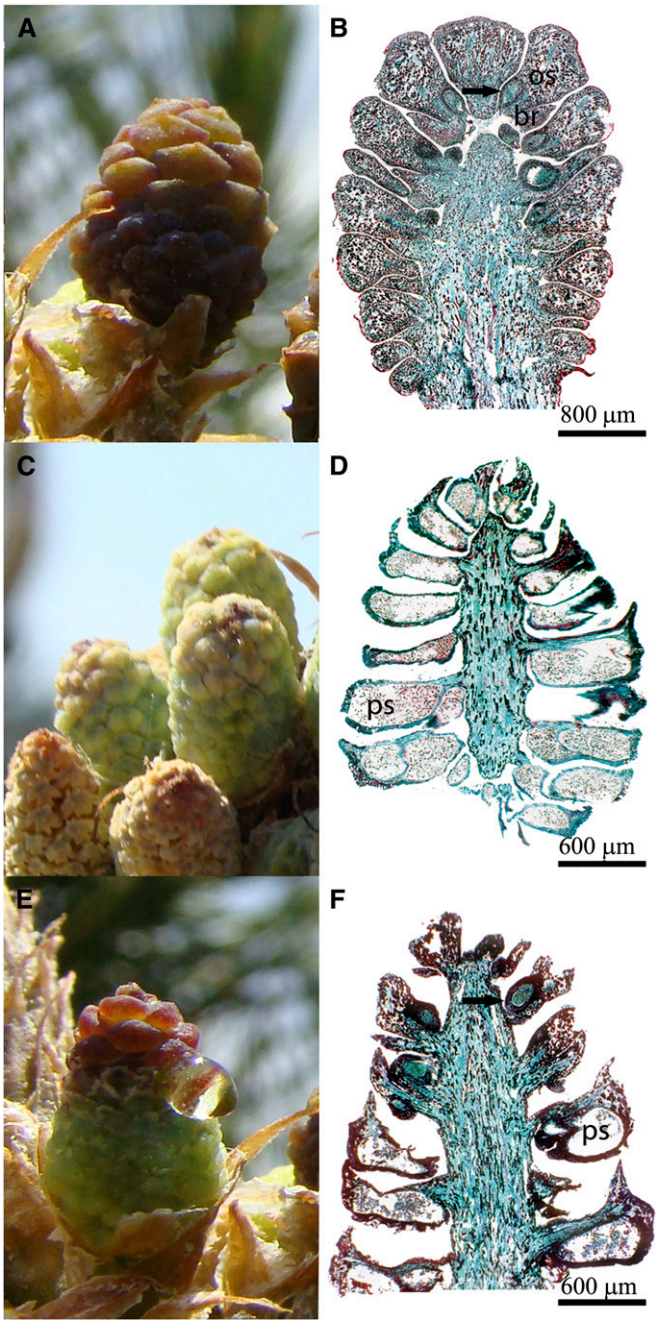


Fig. 2. Ovulate, pollen, and bisporangiate cones of *P. johannis*: photographs of cones in natural conditions (left) and light micrographs of longitudinal sections (right). (A) Ovulate cone; (B) section of ovulate cone; ovules (arrow) are shown in the base of ovuliferous scale (os) and the bract (br). (C) Pollen cone; (D) section of pollen cone with pollen sacs (ps) containing pollen grains. (E) Bisexual cone; (F) section of bisexual cone with ovules (arrow) at the top and pollen sacs (ps) at the bottom. Sections were stained with safranin-green.

Development of microsporangium and megasporangium in bisporangiate and monosporangiate cones—Microgametogenesis starts with the meiosis of the microspore mother cell, resulting in four microspores. The first mitotic division of the microspore forms one prothallial cell and the central cell. The latter divides into one antheridial initial and the second prothallial cell (Fig. 3A). The antheridial initial divides into an antheridial cell and a tube cell, which forms the pollen tube during pollination (Fig. 3B). Pollen grains store starch (Fig. 3C). The antheridial cell forms one generative cell (Fig. 3D) and one sterile cell. The spermatic cells form after mitotic division of a generative cell. However, the pollen grains are released before antheridial cell division, and subsequent mitotic divisions take place during pollination.

Dorsiventrally to each ovuliferous scale, two unitegmic and crassinucellate ovules develop. The integument forms the mycropilar tube. A hypodermal archesporial cell forms at the end of the broad nucellus and divides periclinally to form a primary parietal cell and a primary sporogenous cell. The latter is the megaspore mother cell (Fig. 3E). The megaspore mother cells develop at the time of pollen release. The megaspore mother cell undergoes meiosis and forms a tetrad of megaspores. Only one megaspore is functional; it enlarges considerably, and its nucleus divides mitotically to give rise to the free nuclear gametophyte (Fig. 3F). At this stage, empty pollen grains are observed in the micropyl and near the nucellus. In the next stage, the pollen tube, in the base of the nucellus, grows irregularly (Fig. 3B, 3F). Development of the megagametophyte stops before winter and resumes the following spring. The megagametophyte undergoes several mitotic divisions before cell wall formation, which occurs in late April. Archegonia are formed in May. Embryos mature from the end of August to early September.

There are no differences in microgametogenesis among reproductive morphs. All sexual morphs, including bisporangiate cones, develop mature pollen with prothallial cells, antheridial cells, tube cells, and air sacs. Bisporangiate cones with microsporophylls to ovuliferous scale ratio greater than one do not go through megagametogenesis because the microsporophylls dry up and fall at the end of June along with the pollen cone. However, the bisporangiate cones with microsporophylls to ovuliferous scale ratios less than one behave like the ovulate strobili and go through the free nuclear megagametophyte stage to reach archegonia formation.

Pollen viability—Alexander's stain, which reveals living cells purple and dead cells green (Fig. 4) showed no significant differences in pollen viability among reproductive morphs ($F_{3, 43} = 0.80$, $P = 0.499$). Viability averaged 96.47%.

Controlled crosses—Controlled crosses were monitored over time with three different censuses. During the coenocytic megagametophyte stage, the pollen tube reached the nucellus in bisporangiate cones (Fig. 3F). Almost all crosses remained successful after pollination (Table 1, April). However, when the growth resumed, and especially when fertilization took place, many cones were aborted (Table 1, June and September). Percentage successful maturation using bisporangiate donors or receptors was 6.36%, which is similar to the 3% outcrossing success found for *P. johannis* using monosporangiate donors and receptors. Bisporangiate cones used as pollen donors produced three mature cones (Table 1). Two different patterns were obtained when bisporangiate cones were used as receptors, one or two mature cones were produced per cross when we used bisporangiate cones of predominantly female individuals, whereas a cluster of mature cones was produced when we used bisporangiate cones of predominantly male monoecious individuals (Fig. 5).

DISCUSSION

Functionality of bisporangiate cones—The bisporangiate structures in gymnosperms have been overlooked and considered

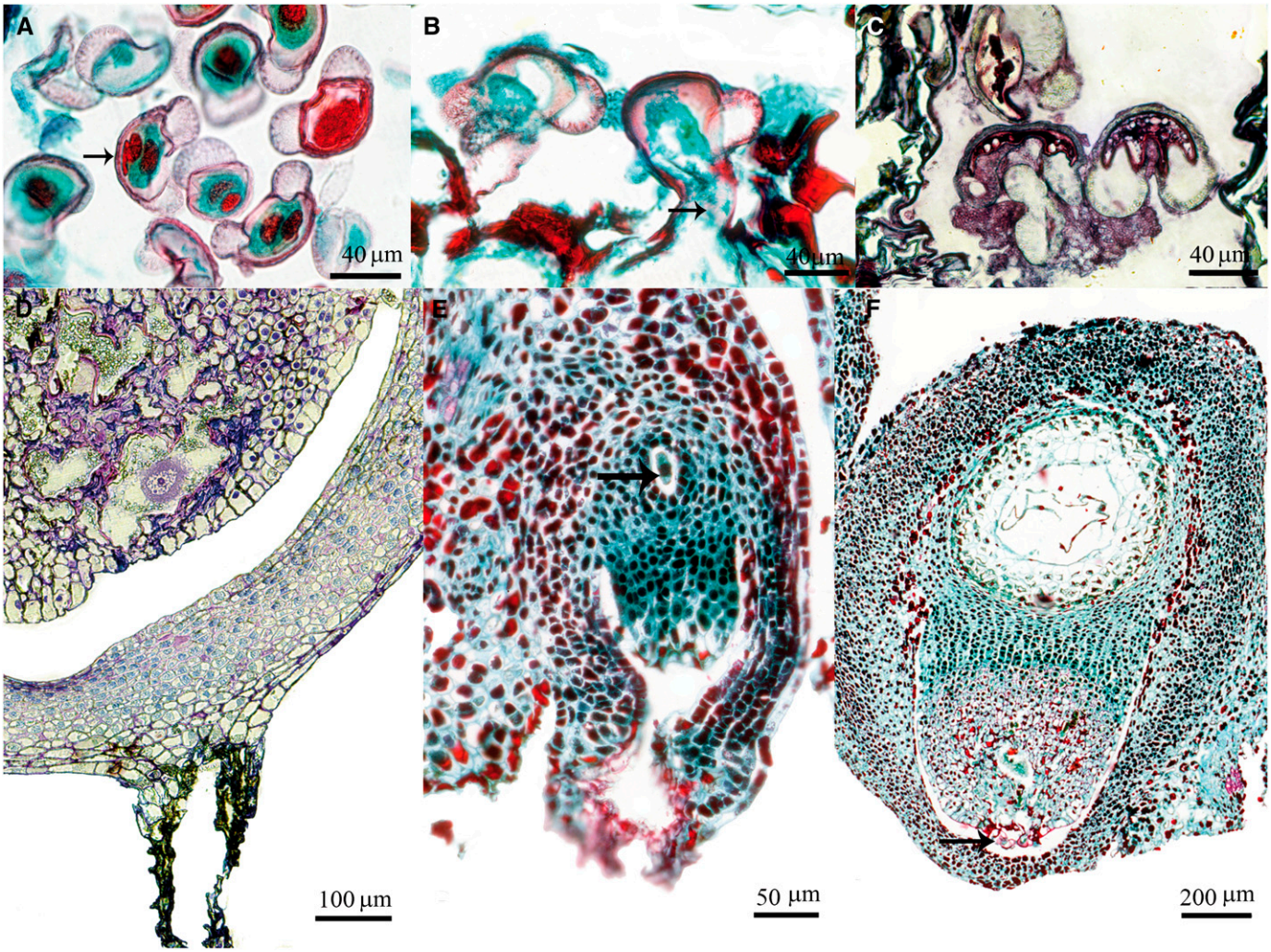


Fig. 3. Light micrographs of normal pollen and ovule development in bisporangiate cones of *Pinus johannis*. (A) Division of antheridial cell and prothallial cell as pollen grain develops (arrow). (B) Pollen tube (arrow). (C) Pollen grain sections with stored starch before tube growth. (D) Generative cell in the nucellus before spermatic cell formation. (E) Unitegmic and crassinucellated ovule and megaspore mother cell (arrow). (F) Developing megagametophyte with pollen tubes in nucellus (arrow). Sections were stained with safranin-green and toluidine blue.

teratological without studies to demonstrate their viability. This lack is attributable to the fact that most trees produce only one or very few bisporangiate structures, making them difficult to study. In addition, bisporangiate strobili with a low proportion of ovuliferous scales naturally dry out and fall from the shoots after anthesis. This process is normal for the pollen cone, because the basal male region dries after shedding pollen, causing the abortion of the distal region too, as shown in the present study. Such phenomena could explain why, in several reports, bisporangiate structures produced apparently normal pollen while ovules did not reach maturity (e.g., *Picea mariana*, *Pseudotsuga taxifolia*, *Agathis brownii*; Littlefield, 1931; Lanner, 1966; Weidlich and Teeri, 1976). Conversely, in *Pinus johannis*, bisporangiate strobili with more female structure remained on the shoots and developed to maturity. The same was observed in *Abies balsamea* (Schooley, 1967), *P. densiflora* (Wakushima et al., 1997), *L. laricina* (Tosh and Powell, 1986), and *P. griffithii* × *P. strobus* (Mergen, 1963).

Bisporangiate strobili in gymnosperms have been found mainly in botanical gardens, greenhouses, and cultivated plants (Shaw, 1896; Littlefield, 1931; Chamberlain, 1935; Haycraft and Carmichael, 2001); they have been described as abnormal and associated with stress under artificial conditions (Zobel and Goddard, 1954; Chamberlain, 1966; Burley, 1976; Dorman, 1976). However, in *P. johannis,* bisporangiate strobili develop in natural populations. Although we do not discount that some stress may be associated with the production of bisporangiate structures, we do discount any restriction of bisexuality to cultivated plants. The fact that most reports of bisexuality in gymnosperms concern cultivated plants likely reflects that they are under closer scrutiny. Because these structures are viable in *P. johannis*, as shown by the histological analysis and the manual crosses, they are likely to be viable in many of the untested taxa as well.

Our study is the first that describes the development of bisporangiate cones in natural conditions. The developmental patterns were not only identical among morphs of *P. johannis*, but they are also similar to other conifers (McWilliam and Mergen, 1958; Biswas and Johri, 1997). In addition, pollen viability of *P. johannis* is high compared with other reports in pines (e.g.,

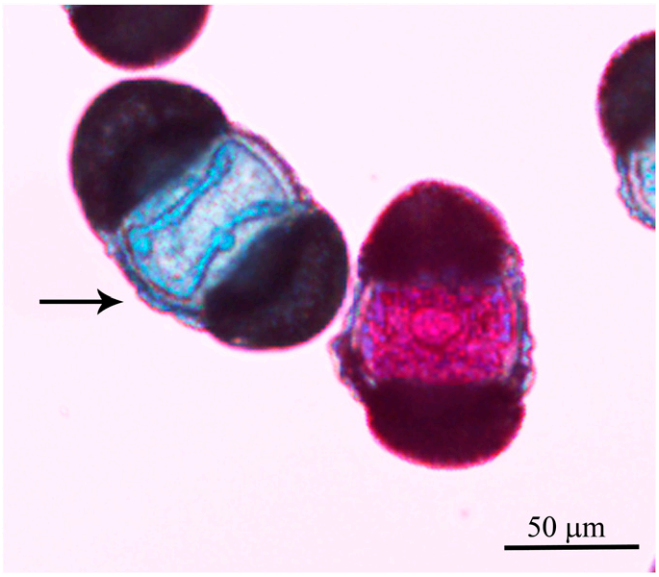


Fig. 4. Viable pollen grains from bisporangiate cones of *Pinus johannis*, stained with Alexander's stain are purple, dead pollen grains are completely green (arrow).

pollen viability of *P. banksiana* range from 54 to 98%; Caron and Powell, 1995).

Three percent of the female cones from controlled crosses in *P. johannis* at the La Amapola locality reached maturity. Thus the 6.3% success rate in crosses, using bisporangiate cones with more ovuliferous scales than microsporophylls, suggests that male and female functions are not altered in bisporangiate structures. Similarly, controlled pollination in bisporangiate structures of *P. griffithii* × *P. strobus* progressed far enough to provide viable seed only when ovuliferous scales comprised at least half of the strobili (Mergen, 1963).

TABLE 1. List of manual crosses using bisporangiate cones as receptors or as pollen donors.

Bisporangiate (*N*)	April	June	September
Receptor (55)	38	7	4
Donor (78)	55	18	3

Pollen at the bottom and ovules at the top—Almost all gymnosperms with bisporangiate strobili have the pollen sacs at the bottom (base) and the ovule-producing structures at the top. As Endress (1996) suggests, this tendency is certainly of interest given that the same pattern occurs in angiosperms. Additionally, Christianson and Jernstedt (2009) found that dioecious *Ginkgo biloba* develops microsporangiate strobili associated with basal bracts and megasporangiate strobili associated with apical bracts, which is the same sequence found in angiosperm organs. In *P. johannis*, all the bisporangiate cones we observed had ovules in the apex and pollen sacs at the bottom. This evidence suggests a strong common pattern of organ disposition between angiosperms and gymnosperms.

Origin of bisexuality and the widespread occurrence of bisporangiate structures in gymnosperms—Understanding similarities of sexual expression between angiosperms and gymnosperms has been considered key to reconstruct the sexual state of the ancestral angiosperm (Endress and Doyle, 2009). However, little progress has been made in understanding the mechanism of the reproductive systems in gymnosperms, especially in species able to express a bisexual condition. Nevertheless, bisporangiate cones have been used to reconstruct hypothetical intermediate states in the evolution of the flower; Theißen and colleagues (Theißen and Becker, 2004; Theißen and Melzer, 2007) offer two proposals: (1) the "out of male" hypothesis assumes that the hermaphrodite flower originated from the male strobilus by the reduction of B-class gene expression in the upper region of the pollen cone, which led to

Fig. 5. Successive development of bisporangiate cones that reached maturity. (A) Cluster of bisporangiate cones developed in June. Few bisporangiate cones had more microsporophylls than ovuliferous scales (bottom left). (B) Same cluster a few months later. Conelets that crossed successfully remain in the shoot. (C) Some of the conelets in (B) reached maturity and produced seed when they were outcrossed.

the development of female rather than male reproductive units; (2) the "out of female" hypothesis considers that the hermaphrodite flowers originated from ovulate strobilus by ectopic expression of B-class genes, in the basal region of the ovulate cone, leading to the development of male rather than female reproductive units. Regardless of the mechanism, the proposed result is the same: ovule-bearing structures at the top and pollen-bearing structures at the bottom. In most of the descriptive studies of conifer species (i.e., *Pseudotsuga taxifolia* and *Pinus nigra*; Fischer, 1905; Littlefield, 1931; Matziris, 2002), the bisporangiate cones are in the position of a male strobili and interspersed with "normal" pollen cones, supporting the "out of male" scenario, where the male strobili gain the female function. However, some individuals of *P. johannis* can produce bisporangiate cones in the shoot apex (also in *Picea mariana*; Weidlich and Teeri, 1976) where the ovulate cones are produced. Thus our investigation of *P. Johannis* suggests that these hypotheses are not mutually exclusive and that the origin of bisexual flowers could be due to the regulation (both up and down) of B-class genes.

According to Theißen and Becker (2004) and Theißen and Melzer (2007), a typical feature that distinguishes flowers from the reproductive cones of gymnosperms is the fact that male (stamens) and female (carpels) reproductive organs are usually united in the flowers (or secondarily separated, as in the unisexual flowers of monoecious and dioecious angiosperms), while they are primarily separated on different structures in gymnosperms. According to Chamberlain (1966), bisporangiate cones exist in all former families of conifers (Abietaceae, Taxodiaceae, Cupressaceae, Araucariaceae, Podocarpaceae in Chamberlain's classification) except in Taxaceae. For most Pinaceae genera, there are reports of individuals that carry bisporangiate cones. No studies have reported such anomalies for *Cathaya*, *Keteleeria*, *Nothotsuga*, or *Pseudolarix* genera. Apart from Pinaceae, bisporangiate structures have been found in other conifers such as *Agathis*, *Araucaria*, *Cedrus*, *Cryptomeria*, *Cupressus*, *Cunninghamia*, *Juniperus*, *Phyllocladus*, *Platycladus*, *Saxegothea*, *Sequoia*, and *Thuja* (see Table 2). Moreover, species of *Gnetum*, *Welwitschia*, and *Ephedra* have a typical bisexual expression in their strobili. The fact that most extant groups of gymnosperms produce bisporangiate structures with the ovule-bearing structures in the apical position and the pollen-bearing structures in the distal position suggests a common mechanism to produce bisporangiate structures. Bisporangiate structures are unknown in Cycadales, which are strictly dioecious; however, they can change sex, which reflects their ability to produce both sexes (Osborne and Gorelick, 2002). Additionally, the fossil record shows that cycadeoidales bore numerous bisporangiate cones (Crepet and Delevoryas, 1972; Crepet, 1974; Owens and Hardev, 1990; Rothwell and Stockey, 2002), suggesting that bisporangiate structures were present before the origin of extant lineages of angiosperms.

The extended presence of bisporangiate cones throughout the gymnosperms reflects the possible existence of a genetic mechanism similar to that of angiosperms. This idea is also supported by the morphological patterning in *Ginkgo biloba* discussed earlier (Christianson and Jernstedt, 2009). Moreover, orthologs of floral B-class (involved in petal and stamen differentiation) and C-class (involved in stamen and carpel differentiation) genes have been found in gymnosperms and are expressed differentially in their reproductive structures. While B-class gene expression is restricted to pollen cones (Mouradov et al., 1999; Fukui et al., 2001; Sundström and Engstrom, 2002), messengers of C-class genes are found in both female and male structures (Rutledge et al., 1998; reviewed in Melzer et al. 2010). Furthermore, orthologues of *LEAFY* (*LFY*), a key gene in the switch between the vegetative to reproductive phases in angiosperms, has been found in different lineages of gymnosperms, and its expression patterns are similar to those reported for angiosperms (Shindo et al., 2001; Dornelas and Rodriguez, 2005; Guo et al., 2005; Vázquez-Lobo et al., 2007). These results together suggest that the common ancestor of seed plants had a regulatory network for differentiation of reproductive structures involving LFY and BC-type MADS-box proteins (Moyroud et al., 2010). Therefore, it is likely that the genetic principles of sex determination of reproductive structures in gymnosperms—as well as its regulatory mechanisms—were present in the common ancestor of gymnosperms and angiosperms and are still conserved today.

Research on the genetic mechanisms underlying flower origin have focused on gymnosperms because they are the closest extant relatives of the angiosperms. However, angiosperms and gymnosperms are estimated to have diverged 325 million years ago (Beck, 1966), and lineages sharing a more recent common ancestor with the angiosperms have become extinct (Doyle, 2008). Persistent uncertainty in the phylogenetic relationships among the four monophyletic groups of extant spermatophytes (cycads, conifers, gnetophytes, and angiosperms; reviewed in Mathews, 2009) has led to ambiguity in inferences on the sexual traits of their ancestors. Particularly controversial has been whether flowers have evolved through condensation of a compound structure (the pseudoanthial theory) or not (see Bateman et al., 2006; Rudall and Bateman, 2010). In agreement with paleontological evidence, seed cones and some pollen cones in extant conifers have compound strobili or are at least derived from compound reproductive structures (Florin, 1951; Wilde, 1975; Mundry and Mundry, 2001, Rudall and Bateman, 2010). Therefore, homology between bisporiangiate cones and bisexual perfect flowers would be supported under the pseudoanthial scenario, where flowers are hypothesized to originate through a condensation of a multiaxial structure (Bateman et al., 2006).

Causes of unisexuality in the reproductive structures of gymnosperms—In self-compatible species, selfing could be detrimental because of significant inbreeding depression (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). Selection would then favor mechanisms that promote outcrossing, such as dichogamy or unisexual flowers, which might explain, in part, the high incidence of dioecy in wind-pollinated species (Culley et al., 2002). The unisexual specialization of gymnosperms may be a way to promote outcrossing because gymnosperms seem to lack autoincompatibility barriers (although a postzygotic mechanism has been reported: Williams et al., 2003).

In conclusion, the numerous occurrences of bisporangiate structures in gymnosperms, along with their viability and spatial disposition (male structures at the bottom and female structures at the top), suggest that this group has the ability to produce bisporangiate structures similar to those of angiosperms. Homologous genes that regulate sex expression in angiosperms have been observed in gymnosperms; such evidence supports a common ancestral mechanism for producing bisexual structures in seed plants. In addition, recent molecular studies of signaling pathways and processes indicate that many developmental pathways, embryogenesis, programmed cell death, and

TABLE 2. List of reports of bisporangiate structures in extant gymnosperms. Most of these reports correspond to single morphological entities (cones) as described by Christianson and Jernstedt (2009), although misinterpretations of the bisexual state can be found in literature when male and ovulate cones develop close to each other. Present names are in parentheses.

Species name	Clade	Reference
Abies balsamea	Pinaceae	Schooley, 1967
Abies excelsa (*Picea excelsa*)	Pinaceae	Dickson, 1860
Abies grandis	Pinaceae	Eis, 1970
Abies lasiocarpa	Pinaceae	Eis, 1970
Abies spp.	Pinaceae	Coulter and Chamberlain, 1901
Agathis brownii	Araucariaceae	Lanner, 1966
Araucaria bidwillii	Araucariaceae	Orwa et al., 2009
Cedrus odorata	Cupressaceae	GardenWeb, 2010
Cryptomeria japonica	Cupressaceae	Hashizume, 1961
Cryptomeria japonica	Cupressaceae	Hashizume, 1973
Cryptomeria japonica	Cupressaceae	Lemoine-Sebastian, 1968
Cunninghamia lanceolata	Cupressaceae	Lemoine-Sebastian, 1968
Ephedra campylopoda	Gnetales	Strasburger, 1872
Ephedra intermedia	Gnetales	Mehra, 1950
Ephedra trifurca	Gnetales	Land, 1904
Gnetum gnemon	Gnetales	Thompson, 1916
Gnetum gnemon	Gnetales	Haycraft and Carmichael, 2001
Gnetum scandens	Gnetales	Lignier and Tison, 1912
Juniperus communis	Cupressaceae	Renner, 1904
Larix europaea (*L. decidua*)	Pinaceae	Bartlett, 1913
Larix laricina	Pinaceae	Tosh and Powell, 1986
Larix microcarpa (*L. laricina*)	Pinaceae	Meyer, 1850 in Masters, 1869
Larix occidentalis	Pinaceae	Kirkwood, 1916
Phyllocladus alpinus	Podocarpaceae	Robertson, 1906
Picea abies	Pinaceae	Flandung et al., 1999 in Theißen and Melzer, 2007
Picea abies	Pinaceae	Tabor, 1990
Picea abies	Pinaceae	Dickson, 1860
Picea spp.	Pinaceae	Santamour, 1959
Picea alba (*P. glauca*)	Pinaceae	Holmes, 1932
Picea canadensis (*P. glauca*)	Pinaceae	Jack, 1895
Picea glauca	Pinaceae	Zasada, et al., 1978
Picea glauca	Pinaceae	Pauley, 1942
Picea glauca	Pinaceae	Marquard and Hanover, 1984
Picea mariana	Pinaceae	Weidlich and Teeri, 1976
Picea mariana	Pinaceae	Caron and Powell, 1990
Picea mariana	Pinaceae	Caron and Powell, 1991
Picea mariana	Pinaceae	Elliot, 1979
Picea morinda (*P. smithiana*)	Pinaceae	Rao, 1931
Pinus caribaea	Pinaceae	Harrison and Slee, 1991
Pinus contorta	Pinaceae	Black, 1961
Pinus densiflora	Pinaceae	Matsuda, 1892
Pinus densiflora	Pinaceae	Righter, 1932
Pinus densiflora	Pinaceae	Wakushima et al., 1996
Pinus densiflora var. *umbraculifera*	Pinaceae	Mergen, 1963
Pinus elliottii	Pinaceae	Mergen and Koerting, 1957
Pinus elliottii	Pinaceae	Hoekstra and Mergen, 1957
Pinus elliottii	Pinaceae	Dorman, 1976
Pinus griffithii (*P. wallichiana*) ×*Pinus strobus*	Pinaceae	Mergen, 1963
Pinus heterophylla (*P. elliottii*)	Pinaceae	Jack, 1895
Pinus longifolia (*P. roxburghii*)	Pinaceae	Rao, 1932
Pinus maritima (*P. pinaster*)	Pinaceae	Goebel, 1905
Pinus massoniana	Pinaceae	Righter, 1932
Pinus montana (*P. cembra*)	Pinaceae	Steil, 1918
Pinus nigra	Pinaceae	Matziris, 2002
Pinus laricio (*P. nigra*)	Pinaceae	Fisher, 1905
Pinus palustris	Pinaceae	Zobel and Goddard, 1954
Pinus taeda	Pinaceae	Zobel and Goddard, 1954
Pinus thunbergii	Pinaceae	Mergen, 1963
Pinus thunbergii	Pinaceae	Saito, 1957
Pseudotsuga douglasii (*P. menziesii*)	Pinaceae	Hill and De Fraine, 1909
Pseudotsuga taxifolia (*P. menziesii*)	Pinaceae	Littlefield, 1931
Saxegothea conspicua	Podocarpaceae	Chamberlain, 1966
Sequoia sempervirens	Cupressaceae	Lemoine-Sebastian, 1968
Sequoia sempervirens	Cupressaceae	Shaw, 1896
Thuja plicata	Cupressaceae	Ross and Pharis, 1987
Tsuga canadensis	Pinaceae	Holmes, 1932
Tsuga heterophylla	Pinaceae	Ross and Pharis, 1987
Welwitschia mirabilis	Gnetales	Land, 1904
Welwitschia mirabilis	Gnetales	Hufford, 1996
Welwitschia mirabilis	Gnetales	Chamberlain, 1921

others are conserved between angiosperms and gymnosperms (Cairney and Pullman, 2007). If we assume that a shared genetic mechanism produces bisporangiate structures in seed plants, it is tempting to consider the possibility of a bisexual ancestor, which might suggest the following: (1) gymnosperms, in the absence of an incompatibility system, evolved to unisexuality to avoid inbreeding depression; (2) the incompatibility system was one of the first innovations in flowering plants; (3), the ancestor of angiosperms may also have been bisexual. Further research is needed to elucidate the shared mechanism regulating sexual reproduction in seed plants.

LITERATURE CITED

AINSWORTH, C. 2000. Boys and girls come out to play: The molecular biology of dioecious plants. *Annals of Botany* 86: 211–221.

ALEXANDER, M. P. 1969. Differential staining of aborted and non aborted pollen. *Biotechnic & Histochemistry* 44: 117–122.

BARTLETT, A. W. 1913. Note on the occurrence of abnormal bisporangiate cones of *Larix europea. Annals of Botany* 27: 575–576.

BATEMAN, R. M., AND W. A. DIMICHELE. 2002. Generating and filtering major phenotypic novelties: neoGoldshmidtian saltation revisited. *In* Q. C. B. Cronk, R. M. Bateman, and J. A. Hawkins [eds.], Developmental genetics and plant evolution, 109–159. Taylor and Francis, London, UK.

BATEMAN, R. M., H. JASON, AND P. J. RUDALL. 2006. Morphological and molecular phylogenetic context of the angiosperms: Contrasting the 'top-down' and 'bottom-up' approaches used to infer the likely characteristics of the first flowers. *Journal of Experimental Botany* 57: 3471–3503.

BECK, C. B. 1966. On the origin of gymnosperms. *Taxon* 15: 337–339.

BISWAS, C., AND B. M. JOHRI. 1997. The gymnosperms. Springer-Verlag, Berlin, Germany.

BLACK, T. M. 1961. Abnormalities of the reproductive system of *Pinus contorta* Loudon. *Annals of Botany* 25: 21–28.

BURLEY, J. 1976. Genetic system and genetic conservation in tropical trees. *In* S. Burley and B. T. Styles [eds.], Tropical trees: Variation, breeding and conservation, 85–99. Academic Press, London, UK.

CAIRNEY, J., AND G. S. PULLMAN. 2007. The cellular and molecular biology of conifer embryogenesis. *New Phytologist* 176: 511–536.

CARON, G. E., AND G. R. POWELL. 1990. Morphological variation, frequency, and distribution of bisporangiate strobili in *Picea mariana. Canadian Journal of Botany* 68: 1826–1830.

CARON, G. E., AND G. R. POWELL. 1991. Proliferated seed cones and pollen cones in young black spruce. *Trees. Structure and Function* 5: 65–74.

CARON, G. E., AND G. R. POWELL. 1995. Pollen sizing in jack pine (*Pinus banksiana* Lamb.) with a hemocytomer. *Silvae Genetica* 44: 96–103.

CHAMBERLAIN, C. J. 1921. *Welwitschia mirabilis*. *Botanical Gazzete* 71: 471–472.

CHAMBERLAIN, C. J. 1935. The gymnosperms. *Botanical Review* 1: 183–209.

CHAMBERLAIN, C. J. 1966. Gymnosperms, structure and evolution. Dover Publications, Mineola, New York, USA.

CHARLESWORTH, D., AND B. CHARLESWORTH. 1987. Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics* 18: 237–268.

CHRISTIANSON, M. L., AND J. A. JERNSTEDT. 2009. Reproductive short-shoots of *Ginkgo biloba*: A quantitative analysis of the disposition of axillary structures. *American Journal of Botany* 96: 1957–1966.

COULTER, J. M., AND C. J. CHAMBERLAIN. 1901. Morphology of spermatophytes. Part I (Gymnosperms). New York, USA.

CREPET, W. L., AND T. DELEVORYAS. 1972. Investigations of North American cycadeoids: Early ovule ontogeny. *American Journal of Botany* 59: 209–215.

CREPET, W. L. 1974. Investigations of North American cycadeoids: The reproductive biology of Cycadeoidea. *Palaeontographica* 148B: 144–169, pl. 52–72.

CULLEY, T. M., S. G. WELLER, AND A. K. SAKAI. 2002. The evolution of wind pollination in angiosperms. *Trends in Ecology & Evolution* 17: 361–369.

DICKSON, A. 1860. Observations on some bisexual cones occurring in *Abies excelsa. Transactions of the Botanical Society of Edinburgh* 6: 418–422.

DORMAN, K. 1976. Genetic and breeding of southern pines. U.S. Department of Agriculture, Forest Service, Agricultural Handbook 471, Washington, D.C., USA.

DORNELAS, M. C., AND A. P. M. RODRIGUEZ. 2005. A *FLORICAULA/LEAFY* gene homolog is preferentially expressed in developing female cones of the tropical pine *Pinus caribaea* var. *caribaea. Genetics and Molecular Biology* 28: 299–307.

DOYLE, J. A. 2008. Integrating molecular phylogenetic and paleobotanical evidence on the origin of the flower. *International Journal of Plant Sciences* 169: 816–843.

EIS, S. 1970. Reproduction and reproductive irregularities of *Abies lasiocarpa* and *A. grandis. Canadian Journal of Botany* 48: 141–143.

ELLIOT, D. L. 1979. The ocurrence of bisexual strobiles on black spruce (*Picea mariana* [Mill.] B.S.P.) in the forest-tundra ecotone: Keewatin Northwest Territories. *Canadian Journal of Forest Research* 9: 284.

ENDRESS, P. 1996. Structure and function of female and bisexual organ complexes in Gnetales. *International Journal of Plant Sciences* 157: S113–S125.

ENDRESS, P. K., AND J. A. DOYLE. 2009. Reconstructing the ancestral angiosperm flower and its initial specializations. *American Journal of Botany* 96: 22–66.

FISCHER, W. 1905. An abnormal cone of *Pinus laricio. Ohio Naturalist* 7: 369–397.

FLORIN, R. 1951. Evolution of cordaites and conifers. *Acta Horti Bergiani* 15: 285–388.

FUKUI, M., N. FUTAMURA, Y. MUKAI, Y. WANG, A. NAGAO, AND K. SHINOHARA. 2001. Ancestral MADS box genes in sugi, *Cryptomeria japonica* D. Don (Taxodiaceae), homologous to the B function genes in angiosperms. *Plant & Cell Physiology* 42: 566–575.

GARDENWEB. 2010. Conifers forum [online]. Website http://forums.gardenweb.com/forums/load/conif/msg05193328526.html [accessed 19 November 2010].

GIVNISH, T. J. 1980. Ecological constraints on the evolution of breeding systems in seed plants: dioecy and dispersal in gymnosperms. *Evolution* 34: 959–972.

GOEBEL, K. 1905. Organography of plants, especially of the archegoniatae and spermophyta. Oxford at Clarendon Press, London, UK.

GUO, C. L., L. G. CHEN, X. H. HE, Z. DAI, AND H. Y. YUAN. 2005. Expressions of *LEAFY* homologous genes in different organs and stages of *Ginkgo biloba. Yi Chuan* 27: 241–244.

HARRISON, D. L. S., AND M. U. SLEE. 1991. Gibberellin A4/7 enhanced flowering in *Pinus caribaea* var. *hondurensis. Canadian Journal of Forest Research* 21: 788–793.

HASHIZUME, H. 1961. The effect of gibberellin in sex differentiation in *Cryptomeria japonica* strobiles. II. *Journal of Japanese Forest Society* 43: 47–49.

HASHIZUME, H. 1973. Studies on flower bud formation, flower sex differentiation and their control in conifers. *Bulletin of Tottori University Forest* 7: 1–139.

HAYCRAFT, C., AND J. CARMICHAEL. 2001. Development of sterile ovules on bisexual cones of *Gnetum gnemon* (Gnetaceae). *American Journal of Botany* 88: 1326–1330.

HILL, T. G., AND E. DE FRAINE. 1909. On the seedling structure of gymnosperms. II. *Annals of Botany* 23: 189–228.

HOEKSTRA, P. E., AND F. MERGEN. 1957. Experimental induction of female flowers on young slash pine. *Journal of Forestry* 55: 827–831.

HOLMES, S. 1932. A bisporangiate cone of *Tsuga canadensis. Botanical Gazette* 93: 100–102.

HUFFORD, L. 1996. The origin and early evolution of angiosperms stamens. *In* W. G. D'Arcy, and R. C. Keating [eds.], The anther form, function, and phylogeny, 58–91. Cambridge University Press, Cambridge, UK.

JACK, J. G. 1895. Some unusual androgynous flower clusters. *Garden and Forest* 5: 222–223.

KIRKWOOD, J. E. 1916. Bisporangiate cones of *Larix. Botanical Gazette* 3: 256.

LAND, W. J. G. 1904. Spermatogenesis and oogenesis in *Ephedra trifurca. Botanical Gazette* 38: 1–18.

LANDE, R., AND D. W. SCHEMSKE. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* 39: 24–40.

LANNER, R. M. 1966. Notes. An unusual bisexual *Agathis* cone. *Pacific Science* 20: 382–383.

LEMOINE-SEBASTIAN, C. 1968. Sexualite, strobiles proliferes et hermaphrodites. *Botanica Rhedonica, Nouveau série, Revue de Biologie végétale* 5: 1–19.

LIGNIER, O., AND A. TISON. 1912. Les Gnetales leur fleurs et leur position systematique. *Annales des Sciences Naturelles*. *Botanique IX.* I6: 55–185.

LITTLEFIELD, E. W. 1931. Bisporangiate inflorescences in *Pseudotsuga. Ohio Journal of Science* 31: 416–417.

MAHESHWARI, P., AND V. VASIL. 1961. *Gnetum*. Botanical Monograph No. 1. Council of Scientific & Industrial Research, New Delhi, India.

MARQUARD, R. D., AND J. W. HANOVER. 1984. The effect of shade on flowering of *Picea glauca. Canadian Journal of Forest Research* 14: 830–832.

MASTERS, M. T. 1869. Vegetable teratology, an account of the principal deviations from the usual construction of plants. Ray Society, London, UK.

MATHEWS, S. 2009. Phylogenetic relationships among seed plants: Persistent questions and the limits of molecular data. *American Journal of Botany* 96: 228–236.

MATSUDA, S. 1892. Bisexual cones of *Pinus densiflora. Botanical Magazíne, Tokyo* 6: 238–239.

MATZIRIS, D. 2002. Short note: Hermaphroditism in black pine. *Silvae Genetica* 51: 130–131.

MCWILLIAM, J. R., AND F. MERGEN. 1958. Cytology of fertilization in *Pinus. Botanical Gazette* 119: 246–249.

MEHRA, P. N. 1950. Ocurrence of hermaphrodite flowers and the development of female gametophyte in *Ephedra intermedia* Shrenk et Mey. *Annals of Botany* 14: 165.

MELZER, R., W. YONG-QIANG, AND G. THEISSEN. 2010. The naked and the dead: The ABCs of gymnosperm reproduction and the origin of the angiosperm flower. *Seminars in Cell & Developmental Biology* 21: 118–128.

MERGEN, F. 1963. Sex transformation in pine hybrids. *Forest Science* 9: 258–262.

MERGEN, F., AND L. E. KOERTING. 1957. Initiation and development of flower primordia in slash pine. *Forest Science* 3: 145–155.

MOURADOV, A., B. HAMDORF, R. D. TEASDALE, J. T. KIM, K. U. WINTER, AND G. THEISSEN. 1999. A DEF/GLO-like MADS-box gene from a gymnosperm: *Pinus radiata* contains an ortholog of angiosperm B class floral homeotic genes. *Developmental Genetics* 25: 245–252.

MOYROUD, E., E. KUSTERS, M. MONNIAUX, R. KOES, AND F. PARCY. 2010. LEAFY blossoms. *Trends in Plant Science* 15: 346–352.

MUNDRY, I., AND M. MUNDRY. 2001. Male cones in Taxaceae *s.l.*—An example of Wettstein's pseudanthium concept. *Plant Biology* 3: 405–416.

ORWA, C., A. MUTUA, R. KINDT, R. JAMNADASS, AND A. SIMONS. 2009. *Araucaria bidwillii. In* Agroforestree database: A tree reference and selection guide, version 4.0 [online]. World Agroforestry Centre, Nairobi, Kenya. Website http://www.worldagroforestry.org/treedb2/AFTPDFS/Araucaria_bidwillii.pdf [accessed 3 November 2010] [accessed 3 November 2010].

OSBORNE, R., AND R. GORELICK. 2002. Sex change in cycads. *Palms and Cycads* 76: 10–15.

OWENS, J. N., AND V. HARDEV. 1990. Sex expression in gymnosperms. *Critical Reviews in Plant Sciences* 9: 281–294.

PAULEY, S. S. 1942. A bisexual cone of white spruce. *Journal of Forestry* 40: 62–63.

RAO, L. N. 1931. Bisporangiate cones of *Pinus longifolia* and *Picea morinda. Journal of Indian Botanical Society* 10: 3.

RAO, L. N. 1932. Peculiar bisexual cones of *Pinus longifolia. Current Science* 1: 103.

RENNER, O. 1904. Über Zwitterblüthen bei *Juniperus communis. Flora* 93: 297–300.

RICHARDS, A. J. 1997. Plant breeding systems, 2nd ed. Chapman and Hall, New York, New York, USA.

RIGHTER, F. I. 1932. Bisexual flowers among the pines. *Journal of Forestry* 30: 873.

ROBERTSON, A. 1906. Some points in the morphology of *Phyllocladus alpinus. Annals of Botany* 20: 259–265.

ROSS, S. D., AND R. P. PHARIS. 1987. Control of sex expression in conifers. *Plant Growth Regulation* 6: 37–60.

ROTHWELL, G. W., AND R. A. STOCKEY. 2002. Anatomical preserved Cycadeoidea (Cycadeoidaceae) with a reevaluation of systematic characters for the seed cones of Bennettitales. *American Journal of Botany* 89: 1447–1458.

RUDALL, P. J., AND R. M. BATEMAN. 2010. Defining the limits of flowers: The challenge of distinguishing between the evolutionary products of simple versus compound strobili. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 365: 397–409.

RUTLEDGE, R., S. REGAN, O. NICOLAS, P. FORBERT, C. COTE, W. BOSNICH, C. KAUFFELDT, ET AL. 1998. Characterization of an *AGAMOUS* homologue from the conifer black spruce (*Picea mariana*) that produces floral homeotic conversions when expressed in *Arabidopsis. Plant Journal* 15: 625–634.

SAITO, Y. 1957. Artificial control of sex differentiation in Japanese red pine and black pine strobilus. *Journal of the Faculty of Agriculture, Tottori University* 3: 1–29.

SANTAMOUR, F. S. 1959. Bisexual conelets in spruce. *Morris Arboretum Bulletin* 10: 10–11.

SCHOOLEY, H. O. 1967. Aberrant ovulate cones in balsam fir. *Forest Science* 13: 102–104.

SHAW, W. R. 1896. Contribution to the life-history of *Sequoia sempervirens. Botanical Gazette* 21: 332–339.

SHINDO, S., K. SAKAKIBARA, R. SANO, K. UEDA, AND M. HASEBE. 2001. Characterization of a *FLORICAULA/LEAFY* homologue of *Gnetum parvifolium* and its implications for the evolution of reproductive organs in seed plants. *International Journal of Plant Sciences* 162: 1199–1209.

SPECHT, C. D., AND M. E. BARTLETT. 2009. Flower evolution: The origin and subsequent diversification of the angiosperm flower. *Annual Review of Ecology Evolution and Systematics* 40: 217–243.

STEIL, W. N. 1918. Bisporangiate cones of *Pinus montana. Botanical Gazette* 66: 68.

STRASBURGER, E. 1872. Die coniferen und Gnetaceen. [Publisher unknown], Jena, Germany.

SUNDSTRÖM, J. F., AND P. ENGSTROM. 2002. Conifer reproductive development involves B-type MADS-box genes with distinct and different activities in male organ primordia. *Plant Journal* 31: 161–169.

TABOR, C. A. 1990. Recurrent appearance of bisporangiate strobili with proliferation on Picea abies. *Rhodora* 92: 257–263.

THEISSEN, G. 2006. The proper place of hopeful monsters in evolutionary biology. *Theory in Biosciences* 124: 349–369.

THEISSEN, G., AND A. BECKER. 2004. Gymnosperm orthologues of class B floral homeotic genes and their impact on understanding flower origin. *Critical Reviews in Plant Sciences* 23: 129–148.

THEISSEN, G., AND R. MELZER. 2007. Molecular mechanisms underlying origin and diversification of the angiosperm flower. *Annals of Botany* 100: 603–619.

THOMPSON, W. P. 1916. The morphology and affinities of *Gnetum. American Journal of Botany* 3: 135–184.

TOSH, K. J., AND G. R. POWELL. 1986. Proliferated, bisporangiate, and other atypical cones occurring on young, plantation-grown *Larix laricina. Canadian Journal of Botany* 64: 469–475.

VÁZQUEZ-LOBO, A., A. CARLSBECKER, F. VERGARA-SILVA, E. R. ALVAREZ-BUYLLA, D. PIÑERO, AND P. ENGSTRÖM. 2007. Characterization of the expression patterns of *LEAFY/FLORICAULA* and *NEEDLY* orthologs in female and male cones of the conifer genera *Picea, Podocarpus,* and *Taxus*: Implications for current evo-devo hypotheses for gymnosperms. *Evolution & Development* 9: 446–459.

WAKUSHIMA, S., H. YOSHIOKA, AND N. SAKURAI. 1996. Lateral female strobili production in a Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) clone by exogenous cytokinin application. *Journal of Forest Research* 1: 143–148.

WAKUSHIMA, S., H. YOSHIOKA, AND N. SAKURAI. 1997. Promotion of lateral female strobili production in *Pinus densiflora* by cytokinin application at a specific stage. *Journal of Forest Research* 2: 51–57.

WEIDLICH, W. H., AND J. A. TEERI. 1976. The occurrence of bisporangiate strobili in subalpine black spruce. *Rhodora* 78: 6–16.

WILDE, M. H. 1975. A new interpretation of microsporangiate cones in Cephalotaxaceae and Taxaceae. *Phytomorphology* 25: 434–450.

WILLIAMS, C. G., L. D. AUCKLAND, M. M. REYNOLDS, AND K. A. LEACH. 2003. Overdominant lethals as part of the conifer embryo lethal system. *Heredity* 91: 584–592.

ZASADA, J. C., M. J. FOOTE, F. J. DENEKE, AND R. H. PARKERSON. 1978. Case history of an excellent, white spruce cone and seed crop in interior Alaska: Cone and seed production, germination, and seedling survival. U.S. Department of Agriculture, Forest Service, Pacific Northwest Forest and Range Experiment Station General Technical Report PNW-65 Portland, Oregon, USA.

ZOBEL, B. J. 1952. Abnormal cone formation in pines. *Texas Journal of Science* 4: 517–520.

ZOBEL, B., AND R. GODDARD. 1954. Pine flowering and seed ripening in Texas. *Texas Forest Service Research* 8: 10.

DISCUSIÓN Y PERSPECTIVAS

Aunque existen reportes de poblaciones con individuos unisexuales para varias de las especies de las subsección Cembroides, e.g. *P. culminicola*, *P. discolor* y *P. johannis*, (Andresen y Beaman, 1961; Ávila et al. 1992; Bailey y Hawksworth, 1983; Rober-Passini, 1978). Sin embargo la validez de estos taxa y sus relaciones filogenéticas han sido ampliamente discutidas (Malusa, 1980; Gernandt, 2003). La subsección *Cembroides* representa un reto para distinguir sus especies genética o morfólogicamente (Malusa, 1980; Price et al. 1998; Gernandt et al. 2001, 2003, 2005). Para México, el grupo más difícil ha sido la tricotomía de *P. culminicola*, *P. discolor* y *P. johannis* (Gernandt et al. 2003; 2005). Al parecer la falta de datos para la Sierra Madre Occidental fue un factor importante para la falta de resolución entre estas tres especies y en particular entre *P. discolor* y *P. johannis*. Un punto clave que generó conflicto fue la identificación de *P. discolor* en La Amapola, San Luis Potosí, ya que estudios posteriores mostraron que se trataba de *P. johannis* (Passini, 1994; Romero-Manzanares, 2000) y con ello se asumió que *P. discolor* y *P. johannis* eran la misma especie sin considerar la localidad tipo de *P. discolor* (Arizona) u otras poblaciones de la Sierra Madre Occidental. La inclusión de múltiples poblaciones de Sonora y Arizona en este estudio, así como el uso de múltiples marcadores de cloroplasto, caracteres morfológicos y variables ambientales, revelaron una separación entre *P. johannis*, *P. discolor* y *P. culminicola*. Nuestros datos sugieren que ninguna de estas especies es variedad de *P. cembroides* como se había propuesto. Sin embargo, sería interesante incluir más individuos de las variedades *P. cembroides* var. *lagunae* y var. *orizabensis* para tener una referencia de la estructura de las variedades aceptadas para *P. cembroides* y distinguir los límites entre especies y variedades de la subsección *Cembroides*. Debido a que el tiempo de coalescencia del cloroplasto es más reciente que la del núcleo, se esperaría mayor resolución en los linajes usando marcadores de cloroplasto, por este motivo se realizó la búsqueda de marcadores polimórficos para la subsección Cembroides, la que carecía de suficientes marcadores informativos (Flores-Rentería et al. 2011). Aunque los marcadores nucleares han sido

utilizados para la delimitación de especies, la alta duplicación génica fue un problema durante la selección de marcadores nucleares polimórficos ya que de los ~25 marcadores muestreados todos presentaron duplicación. El caso más dramático fue bHLH-like que tuvo al menos 8 duplicaciones (datos sin publicar), así la búsqueda de marcadores nucleares ortólogos en especies de la subsección *Cembroides* es necesaria para corroborar los resultados con marcadores de cloroplasto. Sin embargo la integración de datos genéticos, morfológicos y ecológicos es consistente con la separación de las especies *P. cembroides*, *P. culminicola*, *P. discolor* y *P. johannis*.

La presencia de individuos totalmente o predominantemente unisexuales en *P. johannis* y *P. discolor* sugiere que la unisexualidad se originó antes de la separación de estas especies. Andresen y Beaman (1961) reportaron, de manera anecdótica, la presencia de individuos unisexuales en *P. culminicola* y sugirieron una asociación a ambientes pobres. Es necesario incluir las pocas poblaciones de *P. culminicola* en un estudio comparativo sobre la expresión sexual, para determinar si el origen de la unisexualidad precede la separación de la tricotomía de *P. discolor*, *P. johannis* y *P. culminicola*. *P. edulis*, que pertenece a esta subsección presenta unisexualidad lábil, así cuatro de once especies de la subsección *Cembroides* presentan individuos unisexuales, sin embargo el gradiente en la proporción sexual dentro de los individuos de *P. edulis* y la distribución bimodal encontrada en *P. johannis* sugiere que estas especies evolucionaron a la unisexualidad por diferentes vías o que *P. johannis* se encuentra hacia la etapa final de la separación sexual. Observaciones preliminares sugieren que *P. cembroides* presenta individuos que producen estructuras femeninas y masculinas en abundancia en La Amapola. Esto es relevante ya que en esta población se encuentran *P. johannis* y *P. cembroides* en simpatría, así ambas se encuentran bajo condiciones ambientales similares pero la expresión sexual es diferente. La carencia de estudios con relación a la expresión sexual en las otras especies de la subsección *Cembroides* imposibilita rastrear el origen del dioicismo para este grupo, por lo que más estudios son necesarios para

conocer el origen y las posibles causas que generan la evolución a la unisexualidad en un contexto filogenético.

P. johannis presenta cinco morfos sexuales en la población La Amapola (Flores-Rentería et al. 2011). Individuos puramente unisexuales, femeninos o masculinos, e individuos monoicos; interesantemente estos presentan mayoritariamente un sexo, por lo tanto los llamamos monoicos predominantemente femeninos o masculinos; la última categoría corresponde a individuos monoicos que producen de manera atípica muchas estructuras femeninas y masculinas así como estructuras bisexuales (estróbilos bisporangiados). La presencia de esta diversidad sexual es común en angiospermas que con su 5% de especies dioicas la ruta de evolución a la unisexualidad ha sido ampliamente estudiada (Geber et al. 1999; Barret, 2002) sin embargo, no existe a la fecha algún trabajo que señale la ruta de evolución para ninguna especie del 52% de gimnospermas con sistema reproductivo dioico. La estabilidad de los individuos unisexuales en *P. johannis* fue demostrada desde diferentes aproximaciones. Así la presente tesis contribuye al entendimiento de la evolución de la unisexualidad en gimnospermas y sugiere que las mismas presiones que dirigen la unisexualidad están presentes en angiospermas y gimnospermas.

Aunque la separación sexual se ha propuesto como un mecanismo para reducir la endogamia y la consecuente expresión de alelos recesivos deletéreos (Charlesworth y Charlesworth, 1987; Charlesworth, 1999, 2002), interesantemente en *P. johannis* la presencia de individuos unisexuales está asociada a bajos valores de seed set y fruit set. Esto podría sugerir una carga genética muy alta o carencia de recurso. Sin embargo como se comentó anteriormente en la población La Amapola *P. johannis* y *P. cembroides* se encuentran en simpatría y la última produce más conos y más semillas viables por cono. Así la deficiencia en nutrientes parece improbable, aunque diferentes estrategias en el uso de recursos podrían explicar esta hipótesis, en la que *P. cembroides* es más exitosa o resistente al déficit de recursos. Además en la misma localidad *P. johannis* presenta más ataque de

herbívoros con respecto a *P. cembroides*, basado en observaciones superficiales. Altos valores en la carga genética podría haber sido un factor importante en la evolución a la unisexualidad como lo sugieren nuestros datos sobre cruzas manuales. Adicionalmente la diversidad haplotípica en *P. johannis* es menor de la encontrada en *P. discolor*, pero mayor de lo encontrado en *P. culminicola*. Sin embargo la inclusión de múltiples poblaciones para el experimento de cruzas manuales es necesaria para hacer conclusiones generales para la especie, especialmente porque La Amapola se encuentra en el extremo sur de la distribución de *P. johannis*. Determinar si la asignación de recursos es un factor involucrado en la presencia de individuos unisexuales requerirá de futuras mediciones incorporando la tasa de crecimiento y/o la manipulación experimental de suplemento de nutrientes.

La presencia de individuos con múltiples estructuras reproductivas femeninas, masculinas y bisexuales en *P. johannis* (Flores-Rentería et al. 2011) sugiere que estos árboles están desregulados hormonalmente ya que la adición de hormonas en pinos produce el mismo efecto (Wakushima et al. 1996, 1997). Cambios en el sexo de los pocos individuos de *P. johannis* en la población de La Amapola podría estar relacionado a cambios ambientales como ocurre en especies de angiospermas dioicas. Así, menos del 1% de individuos que transita del grupo de individuos únicamente o totalmente masculinos hacia el grupo de individuos únicamente o totalmente femeninos podría deberse a cambios hormonales. La presencia de estructuras bisexuales se consideró exclusiva a las angiospermas también conocidas como plantas con flores, así la flor perfecta o hermafrodita fue una característica que, entre otras, agrupó a las angiospermas con las gnetales y las Bennettitales bajo la hipótesis antofita que ha dado lugar a opiniones contrapuestas (ver discusión en Rottwell et al. 2009), sin embargo la inclusión de las gnetales dentro de las gimnospermas (Winter et al. 1999) y la amplia distribución de casos con estructuras bisexuales en especies de coníferas (Flores-Rentería et al. 2011) sugiere que el mecanismo para la producción de estructuras bisexuales precede la separación de los dos grupos de plantas con semillas.

Pese a que las gimnospermas han sido ampliamente estudiadas desde un punto de vista forestal poca información se ha generado con respecto a la expresión sexual. El estudio de la expresión sexual y su posible manipulación son algo muy importante de considerar, ya que se sabe que muchas especies susceptibles al estrés incrementan la proporción de individuos macho. Nuestros datos sugieren un sesgo a la masculinidad en *P. edulis* y hacia la feminidad en *P. johannis* (La Amapola) ambos asociados al ataque diferencial de tortrocidos (polillas). Así un posible factor ambiental en la evolución de la unisexualidad en las especies de la subsección *Cembroides* podría ser la herbivoría.

Los datos producidos en este estudio para las especies de la subsección *Cembroides* muestran que angiospermas y gimnospermas presentan estrategias similares para la separación sexual.

Referencias

Andresen, J.W. & Beaman, J.H. 1961. A new species of *Pinus* from Mexico. *Journal Arnold Arboretum* 42:437–441.

Ávila N. J., García M. E. & Reyes A. J. 1992 Registro de Pinus discolor Bailey et Hawksworth en la sierra de monte Grande, San Luis Potosí, México. *Acta Botánica Mexicana* 20:9-12.

Bailey, D.K. & Hawksworth, F.G. 1979. Pinyons of the Chihuahuan Desert Region. *Phytologia* 44:129–133.

Bailey, D.K. 1979. New pinyon records for Northern Mexico. *Southwestern Naturalist* 24:389–390.

Barrett, S. 1998. The evolution of mating strategies in flowering plants. *Trends in Plant Science* 3: 335-341.

Barrett, S. 2002. The Evolution on plant sexual diversity. *Nature* 3:274-284

Bawa, K. S. & Beach, J.H. 1981. Evolution of sexual systems in flowering plants. *Annals of the Missouri Botanical Garden* 68: 254-274.

Bawa, K.S. 1980. Evolution of dioecy in flowering plants. *Annual Review of Ecology and Systematics* 11: 15-39.

Biswas C. & B. M. Johri. 1997. The gymnosperms. Springer-Verlag. pp. 149.

Chamberlain, C.J. 1935. Gymnosperms structure and evolution. Chicago.

Charlesworth D. 1999. Theories of the evolution of dioecy. In: Geber MA, Dawson T.E. & Delph L.F. 1999. (eds) Gender and sexual dimorphism in flowering plants. Springer, Berlin Heidelberg New York.

Charlesworth, B. & Charlesworth, D. 1979. Population genetics of partial male sterility and the evolution of monoecy and dioecy. *Heredity* 41:137-154.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annu Rev Ecol Syst* 18:237–268

Charlesworth, D. 2002. Plant sex determination and sex chromosomes. *Heredity* 88:94-639 101.

Charnov E. L. & J. Bull. 1977. When is sex environmentally determinated? Nature 266:828-829.

Cobb N. S., R. T. Trotter III & T. G. Whitham. 2002. Long-term sexual allocation in herbivore resistant and suceptible pinyon pine (P. Edulis).

Cobb, N.S., Trotter III, R.T. & Whitham, T.G. 2002. Long-term sexual allocation in herbivore resistant and susceptible pinyon pine (*Pinus edulis*). *Oecologia* 130:78-87.

dioecy and dispersal in gymnosperms. *Evolution* 34:959-972.

Farjon, A., & Styles, B.T. 1997. *Pinus* (Pinaceae). Flora Neotropica Monograph 75. New York, NY: The New York Botanical Garden.

Flores-Rentería, Ll., Vázquez-Lobo, A., Whipple, A.V., Piñero, D., Márquez-Guzmán, J. & Domínguez, C.A. 2011. Functional bisporangiate cones in *Pinus johannis* (Pinaceae): Implications for the evolution of bisexuality in seed plants. *Am. J. Bot*. 98: 130–139.

Floyd, M. 1983. Dioecy in five *Pinus edulis* populations in the southwestern United States. *American Midland Naturalist* 110:405-411.

Floyd, M. E. 1982. Dioecy in five Pinus edulis populations in the southwestern united states. The American Midland Naturalist

Freeman, D. C., E. D. McArthur, K. T. Harper & A. C. Blaver. 1981. Influence of environment on the floral sex ratio of monoecious plants. Evolution 35:194-197.

Geber, M.A., Dawson, T.E. & Delph, L.F. 1999. *Gender and sexual dimorphism in flowering plants*. Springer, Berlin Heidelberg New York.

Gernandt, D., Liston, A. & Piñero, D. 2003. Phylogenetics of *Pinus* Subsections *Cembroides* and *Nelsoniae* inferred from cpDNA sequences. *Systematic Botany* 28:657–673

Givnish, T. J. 1980. Ecological constraints of the evolution of breeding system in seed plants: dioecy and dispersal in gymnosperms. Evolution 34:959-972.

Givnish, T.J. 1980. Ecological constraints of the evolution of breeding system in seed plants:

Kiener. 1935. Unisexual limber pine. *Science* 82:193

Kline, D. 1960 Giant Dwarf of the Mesa Lands. *Morris Arboretum Bulletin* 21:16–19.

Lanner, R. M. 1980 a self-pollination experiment in Pinus edulis. Great Basin Nat 40:265-267.

Lanner, R.M. 1981. *The piñon pine*. Reno: University of Nevada Press.

Ledig, F. T., Bermejo-Velazquez, B., Hodgskiss, P.D., Johnson, D.R., Flores-Lopez, C. & Jacob-Cervantes, V. 2000. The mating system and genic diversity in Martinez spruce, an extremely rare endemic of Mexico's Sierra Madre Oriental: an example of facultative selfing and survival in interglacial refugia. *Canadian Journal of Forest Research* 30:1156–1164.

Ledig, F.T., Conkle, M.T., Bermejo, B., Eguiluz, T., Hodgskiss, P., Johnson, D.R. & Dvorak, W.S. 1999. Evidence for an extreme bottleneck in a rare Mexican pinyon: Genetic diversity, disequilibrium and the mating system in *P. maximartinezii*. *Evolution* 53:91-99.

Little, E. 1966 A new pinyon variety from Texas. *Wrightia* 3:181–185.

Little. E. 1968 Two new pinyon varieties from Arizona. *Phytologia* 17:329–342.

Malusa, J. 1992. Phylogeny and biogeography of the Pinyon Pines (*Pinus* subsect. *Cembroides*). *Systematic Botany* 17:42-66.

McCormick J. & J. W. Andresen. 1963. A subdioecious population of pinus cembroides in southeast Arizona. The Ohio Journal of Science 4:159-163.

McCormick, J. & Andressen, J. A subdioecious population of *Pinus cembroides* in southeast Arizona. *The Ohio Journal of Science*. 63:159-163.

Mirov N. T. 1967. The genus Pinus. The Ronald Press Company, New York. Pp.376.

Mirov, N. T. 1962 Phenology of tropical pines. J. Arnold Arbor. Harv. Univ. 18:218-219. ??????

Mirov, N.T. 1967. *The genus* Pinus. New York: The Ronald Press.

Mueller R. C., B. D. Wade, C. A. Gehring & T. G. Whitham. 2005. Chronic herbivory negatively impacts cone and seed production, seed quality and seedling growth of suceptible pinyon pines. Oecologia 143:558-565.

Passini, M-F. 1994. Synonymie entre *Pinus discolor* et *Pinus johannis. Acta Botanica Gallica* 141:387-388.

Perry J. 1991. The Pines of Mexico and Central America. Timber Press. Oregon.

Price, R.A., Liston A. & Strauss, S.H. 1998. Phylogeny and systematics of *Pinus*. In: Richardson, D.M. (ed.), *Ecology and Biogeography of* Pinus. Cambridge University Press. ISBN 0-521-55176-5.

Robert, M.F. 1978. Un nouveau pin pignon mexicain: *Pinus johannis* M.F. Robert, sp. *Adansonia*, série 2, 18:365–373.

Romero A, Luna M, Garcia E, Passini MF. 2000. Phenetic analysis of the Mexican midland pinyon pines, *Pinus cembroides* and *Pinus johannis*. *Botanical Journal of the Linnean Society* **133**: 181-194.

Silba, J. 1986. An international census of the Coniferae. *Phytologia* memoir no. 8. 1:217.

Smith, C. C. 1981. The facultative adjustment of sex ratios in lodgepole pine. Am. Nat. 118:297-305.

Stewart, W. 1983. *Paleobotany and the evolution of plants*. Cambridge University Press. Cambridge.

Tikhonova I. V. 2003. Sex estructure of scotch Pine populations in the dry steppe. Russian Journal of Ecology 6:370-374.

Wakushima, S. , H. Yoshioka , & N. Sakurai . 1997. Promotion of lateral female strobili production in *Pinus densiflora* by cytokinin application at a specifi c stage. *Journal of Forest Research* 2: 51 – 57.

Webb, C.J. 1999. Empirical studies: Evolution and maintenance of dimorphic breeding systems. In: Geber, M.A., Dawson, T.E. & Delph, L.F. 1999. (eds) *Gender and sexual dimorphism in flowering plants.* Springer, Berlin Heidelberg, New York.

Winter, K.U.; Becker, A.; Munster, T.; Kim, J.T.; Saedler, H.; Theissen, G. 1999. MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. *Proceedings of the National Academy of Sciences* 96(13):7342-7347.

Zavarin, E. & Snajberk, K. 1986. Monoterpenoid differentiation in relation to the morphology of *Pinus discolor* and *Pinus johannis*. *Biochemical Systematics and Ecology* 14:1-11.

APÉNDICE

A NEW APPROACH TO IMPROVE THE SCORING OF MONONUCLEOTIDE MICROSATELLITE LOCI

American Journal of Botany

American Journal of Botany: e51–e53. 2011.

AJB Primer Notes & Protocols in the Plant Sciences

A new approach to improve the scoring of mononucleotide microsatellite loci[1]

Lluvia Flores-Rentería[2,3,4] and Amy V. Whipple[2]

[2]Department of Biological Sciences and Merriam-Powell Center for Environmental Research, Northern Arizona University, Flagstaff, Arizona 86011 USA; and [3]Departamento de Ecología Evolutiva, Instituto de Ecología, UNAM, A. P. 70-275, México, D. F. 04510 México

- *Premise of the study:* Mononucleotide microsatellites markers are useful for detecting genetic variation among individuals; however, scoring can be error-prone. We developed a new approach to improve the accuracy of allele scoring.
- *Methods and Results:* A set of 14 mononucleotide microsatellite primers of the chloroplast were developed based on published *Pinus* spp. chloroplast genomes. Due to substantial scoring error for mononucleotide repeats ≥ 10 bp, we included part of the microsatellite in the reverse primer to reduce slippage and improve the scoring of these polymorphic markers. For markers 10–20 bp, the error rate in scoring with this method has a binomial 95% confidence interval of 0.7–2.1%.
- *Conclusions:* These new primers provide variable chloroplast markers in species of subsection *Cembroides* and other *Pinus* spp. with more accurate assignment of the alleles. This approach can be used to improve the allele scoring of mononucleotide or dinucleotide repeats from nuclear and chloroplast genomes.

Key words: error rates; mononucleotide microsatellite; *Pinus*; *Taq* polymerase slippage.

Variation in the number of repeats in microsatellites or Simple Sequence Repeats (SSRs) is primarily due to polymerase slippage during replication of DNA (Weber and Wong, 1993). Slippage can also be generated during PCR reactions causing high error rates in scoring (Clarke et al., 2001). The error rate in allele calling for dinucleotide SSRs is ~5% with samples amplified by *Taq* polymerase (Ginot et al., 1996), and it could be higher in mononucleotide repeats, while tetranucleotides have a lower error rate. Polymerase slippage is positively correlated with the length of the microsatellite (Kelkar et al., 2010; Jakobsson et al., 2007), making scoring of mononucleotide SSRs >11 bp highly error-prone (Clarke et al., 2001). Mononucleotide repeats are the most common SSRs in the plant chloroplast genome, and due to their high mutation rate, they represent the most variable markers in this organelle (Provan et al., 2001). We developed a new method to increase the accuracy of scoring alleles by designing primers that include part of the microsatellite to reduce the slippage. We tested this method using new primers developed to amplify mononucleotide repeats in the chloroplast of *Pinus* spp. subsect. *Cembroides*. Microsatellite repeats (≥10 bp) were detected by comparison of eight pine chloroplast genomes (Cronn et al., 2008), and primers were designed to amplify these polymorphic regions in piñon pines. Though these trees possess economic and ecological value, few polymorphic markers have been described. Accurate use of polymorphic mononucleotide loci will be useful for assessing paternity, genetic diversity, gene flow, and hybridization.

[1] Manuscript received 26 October 2010; revision accepted 8 December 2010.

The authors thank A. Krohn for his comments to this manuscript. This study was supported by NSF grant DEB-0816675.

[4] Author for correspondence: lluvia.flores@nau.edu

doi:10.3732/ajb.1000428

METHODS AND RESULTS

Complete chloroplast genomes of *Pinus monophylla* Torr. & Frém., *P. nelsonii* Shaw, *P. longaeva* D. K. Bailey, *P. gerardiana* Wall. ex D. Don, *P. lambertiana* Douglas, and *P. krempfii* Lecomte (Genbank accession numbers EU998745, EU998746, EU998744, EU998741, EU998743, and EU998742, respectively) were aligned to detect microsatellites shared across species. We emphasized loci polymorphic among *P. monophylla* (belonging to subsection *Cembroides*), *P. nelsonii*, and *P. longaeva* (the closest relatives to subsection *Cembroides*) to identify regions likely to be polymorphic in subsection *Cembroides*. Fourteen primers pairs were designed under standard methods to amplify regions >200 bp containing microsatellites (Table 1, Fig. 1A). A few dinucleotide microsatellite loci were present, all TA motifs, and no tri- or tetra-nucleotide loci were found. We observed little variation among species and no variation within species in any dinucleotide SSRs (data not shown).

DNA extraction and detection of cpDNA polymorphisms—DNA was extracted from needles or megagametophytes, using a modified CTAB protocol from Doyle and Doyle (1987) from species in subsection *Cembroides* (*P. californiarum* D. K. Bailey ($N = 6$), *P. cembroides* Zucc. ($N = 10$), *P. culminicola* D. K. Bailey & Hawksw. ($N = 11$), *P. discolor* D. K. Bailey & Hawksw. ($N = 105$), *P. edulis* Engelm. ($N = 280$), *P. johannis* M. F. Robert ($N = 44$), *P. monophylla* Torr. & Frém. ($N = 2$), and *P. pinceana* Gordon & Glend. ($N = 5$)). Ten μL PCR reactions were carried out using 0.25 U of HotStarTaq Plus DNA Polymerase (Qiagen, Valencia, California, USA), 1× CoralLoad PCR buffer, 200 μM of each dNTP, 0.2 μM of each primer and ~20 ng of DNA. Thermocycler conditions were: 5 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 50–56°C and 1 min at 72°C; 8 min at 72°C. PCR products were visualized on agarose gels and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California, USA) and a 3730XL Genetic Analyzer (Applied Biosystems).

Improvement of mononucleotide amplification using internal primers—We improved our marker assays by designing primers to include part of the microsatellite in the reverse primer. First we screened eight individuals across species with the original primers to find the range in length of the repeat. Then a new reverse primer was designed, which included the region flanking the microsatellite and part of the microsatellite and was compatible

TABLE 1. *Pinus edulis* chloroplast mononucleotide microsatellites of ≥ 10 repeats and their primer sequences. Allele number and error rate are based on 96 samples of *P. edulis*.

Marker name	Direction	Primer sequence (5′–3′)	Repeat	GenBank Accession Number and Reverse internal primer (5′–3′)	Size (bp)	No. of alleles	Error rate (%)
80576	forward	[FAM] CGGGAGAGATGGCCGAGTGGT	(A)10	HQ433339			
	reverse	GTGGCATGCGGGAAGGGCTC		GCTATATACAGCCGAGCTATTCTCTTTTTT	162	4	0
6924	forward	[FAM] CCTTCCAACCCCAAGTCCGGT	(C)11	HQ433340			
	reverse	AGCCCGATCCGAAGTGAGCG		GGCATTTTTCTTCTCCAATAAAAATTAGGGGGG	112	3	1.13
102213	forward	[TAMRA] TGATGGGAGTCGATCCGCGA	(A)10(T)11	HQ433341			
	reverse	TGCTGGGTAAGTCTTAGGACCCGA		GAACTAATGGACAAATTCTTTACCATAAAAAAAAAA	290	5	0
108297	forward	[FAM] CCCCAACAAAAGCACGAAAGCC	(A)12	HQ433342			
	reverse	TCTGGCAACTTTACGTAAGGCAGAG		GCACCTCATACGGCTTCTCGTTCAATTTTTTTTT	455	4	0
70000	forward	[HEX] ACGATCGTTCGTCGGGTCCGT	(T)12	HQ433343			
	reverse	GCAATTGTCGCTCCGTTAGTCCGT		CTCCTTTTTCAATTAACAGATAGTGCTAGAAAAAAAAAA	212	3	1.27
103118	forward	[TAMRA] GCGGTGTGAATCCGCTTGTCCA	(T)13	HQ433344			
	reverse	CCCGCAGATACAGGCAAACCCA		CAAGTATGGGTTTTTATCAGTGGATAAAAAAAAAA	140	4	0
66029	forward	[HEX] TTCCTCCTTTTTCAGGGAGGCGA	(T)14	HQ433345			
	reverse	TCAACAGCGGCTAGATCCAGAGGA		CCGATATAATAATAGCTACAGGCTTTACGGGAAAAAAA	255	4	1.18
108909	forward	[TAMRA] CGAGGAACCCTAGATGCTGCCG	(A)15	HQ433346			
	reverse	TCACTCTCGATTGAGTATCCGTTCCCT		TCCAAATTTCCTGAAAAATAAGATCGTTTTTTTTTTT	230	5	2.4
61350	forward	[FAM] ACGGCTGCATTGTCATCAGATCGT	(A)15	HQ433347			
	reverse	TGGGCGGAGTATCCGAAACCGT		GAAGATTACTAGTTCGTAAGAACTCTTCCTTTTTTTTTT	336	6	2.5
58046	forward	[TAMRA] CCCGCGGGATCATTGACGGT	(T)17	HQ433348			
	reverse	ACACCTCGGGAAGGAATCTGTCGAA		GCAACTACTCCCCAGATATATGAAAAAAAAAAAAA	250	4	1.15
68590	forward	[FAM] ATCCCGGCTCTTCCCTGTGGA	(A)17	HQ433349			
	reverse	TGCAGTAGGAGGAAAATCCGTTGGC		CTAATTTATCGATTCTTTTACCTCGCTATTTTTTTTTTTT	301	6	2.35
13216	forward	[FAM] CCGAAACCCCCAGCAGAAGGC	(T)19	HQ433350			
	reverse	CGTTGGCCAGGGCACTGCT		TGGGAATCCCTTTGTTTAATTTTGAAAAAAAAAAAAA	390	7	2.44
55012	forward	[HEX] ACCACGCCAAGAGAAACCCGTG	(A)23	HQ433351			
	reverse	TCAACAAGTCGCACACCCATATTCCA		GTCTGGGTTTGAATCCCTCAGTTCTTTTTTTTTTT	273	6	6.44
29275	forward	[HEX] CCATTCATTTGGAATTGGGCATCTACG	(T)25	HQ433352			
	reverse	GATCGATCTTTTGTCCAACCAACCCA		TTCTCGATAGGCAAGTTTATTGAAAAAAAAAAAAAAA	470	10	9.3

with the original forward primer (Fig. 1B). The repeat length in the primer was equal to the smallest microsatellite detected, minus one or two bases. When using the internal primers we performed the PCR as described previously. However, to allow a multiplex assay, we used an annealing temperature of 56°C, 0.08 μM of the forward primer, and 0.23 μM of reverse primer. Multiplex primer combinations did not mix A and T repeats to avoid primer-dimer formation. Up to five primer pairs were multiplexed. Forward primers were labeled with TAMRA, FAM, and HEX fluorophores at their 5′ end. PCR products were diluted 1:60 with water into a plate for genotyping. Fragment analysis was carried out using 1 μL of the bulk PCR dilution, 0.09 μl GeneScan 500 LIZ size standard (Applied Biosystems), and 9.91 μl HiDi Formamide (Applied Biosystems). Fragments were separated on a 3730XL Genetic Analyzer and scored using Genemapper 3.7 (Applied Biosystems). Scoring of each allele was verified by eye for every sample. To know if this new method improved the reproducibility, we calculated the error rate. We selected 96 samples of *P. edulis* and amplified all markers twice to calculate the percentage of samples with the same allele scored in both runs. Presumably due to competition between primers in the multiplex reactions, some samples did not amplify in both runs (0–8% depending on the marker) and we excluded such samples to calculate the error rate between samples that had evident peaks.

All primers amplified DNA in each of the nine species of subsection *Cembroides*. Most of the markers showed variation; however, variation depends on the microsatellite length. For example, in *P. discolor* marker 61350 has only one allele of (A)10 (Table 2). The improved primer design produces a single peak (Fig. 1B), which results in more reproducible scoring of alleles. Our method improves the scoring of long mononucleotide repeats less than 20 bp. In our investigation, the error rate in scoring mononucleotide repeats from 10 to 20 bp in length has a binomial 95% confidence interval of 0.7–2.1%.

Two markers with lengths up to 26 and 23 bp had error rates of 6.44% and 9.3% (CI of error rate over both alleles: 4.7–13%), which was mainly due to polymerase stuttering. For marker 29275, with a length of up to 26 bp, in *P. californiarum* and *P. monophylla* we designed the reverse primer with 3′ (A)15. However, this optimization worked only for samples with a length less than 20 repeats. In those samples with length >20 bp we were unable to optimize the scoring. A similar case was detected in the primer 55012, which has up to (A) 23 bp. An alternative strategy for these loci would be a 2-step assay with primers having an even longer number of bp of the mononucleotide repeat in the reverse primer for the second step.

This new approach can be applied to any mononucleotide repeat, and to dinucleotide repeats subject to slippage during amplification. The use of the longer primers described here may allow development of more successful multiplex designs.

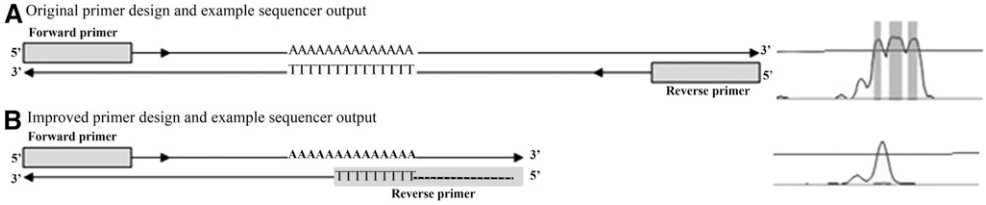


Fig. 1. Original primer design (A) and improved primer design (B) for locus 61350. The upper panel, prior to optimization, shows multiple peaks caused by polymerase slippage. The lower panel, after optimization using internal primers containing a tail of poly-(T), shows a cleaner profile (B).

TABLE 2. Number of alleles found in 280 samples of *P. edulis,* 105 samples of *P. discolor*, and the total number of alleles found in 9 species of subsection *Cembroides* (2–280 samples per species).

Marker name	Number of alleles		Total allele no. in subsection *Cembroides*
	In 280 samples of *P. edulis*	In 105 samples of *P. discolor*	
80576	4	4	4
6924	3	6	6
102213	6	2	9
108297	4	5	6
70000	3	5	5
103118	4	3	4
66029	5	3	6
108909	5	2	5
61350	6	1	7
58046	4	3	6
68590	6	4	7
13216	8	2	10
55012	6	3	10
29275	11	8	16

CONCLUSIONS

Incorporation of part of the microsatellite in one primer improves the scoring and error rates of error-prone microsatellites (specifically mononucleotides longer than 10 bp). The microsatellite loci developed here provide a powerful tool for assessing population structure, paternity, genetic diversity, gene flow, and hybrid zones in *Pinus* spp., especially those in the subsection *Cembroides* where there has been limited success in using polymorphic markers from other *Pinus* spp.

LITERATURE CITED

CLARKE, L. A., C. S. REBELO, J. GONCALVES, M. G. BOAVIDA, AND P. JORDAN. 2001. PCR amplification introduces errors into mononucleotide and dinucleotide repeat sequences. *Molecular Pathology* 54: 351–353.

CRONN, R., A. LISTON, M. PARKS, D. S. GERNANDT, R. SHEN, AND T. MOCKLER. 2008. Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology. *Nucleic Acids Research* 36: e122.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

GINOT, F., I. BORDELAIS, S. NGUYEN, AND G. GYAPAY. 1996. Correction of some genotyping errors in automated fluorescent microsatellite analysis by enzymatic removal of one base overhangs. *Nucleic Acids Research* 24: 540–541.

JAKOBSSON, M., T. SÄLL, C. LIND-HALLDÉN, AND C. HALLDÉN. 2006. Evolution of chloroplast mononucleotide microsatellites in *Arabidopsis thaliana. Theoretical and Applied Genetics* 114: 223–235.

KELKAR, Y. D., N. STRUBCZEWSKI, S. E. HILE, F. CHIAROMONTE, K. A. ECKERT, AND K. D. MAKOVA. 2010. What is a microsatellite: A computational and experimental definition based upon repeat mutational behavior at A/T and GT/AC repeats. *Genome Biology and Evolution* 2: 620–635.

PROVAN, J., W. POWELL, AND P. M. HOLLINGSWORTH. 2001. Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution* 16: 142–147.

WEBER, J. L., AND C. WONG. 1993. Mutation of human short tandem repeats. *Human Molecular Genetics* 2: 1123–1128.