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Luis García Quintanilla, Profesor Contratado Doctor del Departamento de Biología y Geología de la Universidad Rey Juan Carlos, y Santiago Pajarón Sotomayor, Profesor Titular del Departamento de Biología Vegetal I de la Universidad Complutense de Madrid, AUTORIZAN:

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VºBº Director de Tesis

Dr. Luis García Quintanilla

VºBº Codirector de Tesis

Dr. Santiago Pajarón Sotomayor

Biología reproductiva y genética de poblaciones de *Dryopteris corleyi* y sus especies parentales, un complejo diploide-poliploide

Autor: Ares Jiménez Soria[1]

Director: Luis García Quintanilla[1]
Codirector: Santiago Pajarón Sotomayor[2]

[1]Área de Biodiversidad y Conservación. Departamento de Biología y Geología, Escuela Superior de Ciencias Experimentales y Tecnología, Universidad Rey Juan Carlos.
[2]Departamento de Biología y Geología, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid.

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han descubierto un mono
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RESUMEN

ANTECEDENTES

Poliploidía y evolución

La poliploidía consiste en la presencia de tres o más juegos de cromosomas en las células de un organismo. Este fenómeno es uno de los mecanismos de especiación más frecuentes en la naturaleza. De hecho, se estima que un gran porcentaje de las especies conocidas de plantas y animales tuvo su origen en un evento de poliploidización, seguido o no de una diploidización mediada por mecanismos epigenéticos (Otto and Whitton 2000, Soltis et al. 2009). La duplicación del material genético en las células de un organismo ejerce una profunda influencia en el ciclo de vida de éste, puesto que provoca cambios metabólicos, fisiológicos y de regulación génica (Levin 1983, Wendel 2000). Los organismos poliploides aparecen en el seno de poblaciones diploides como consecuencia de fallos en los mecanismos mitóticos y meióticos de división celular (Jackson 1976, Soltis et al. 2004). Para establecerse una población poliploide, es necesario que sus individuos estén reproductivamente aislados de los parentales diploides, puesto que los retrocruzamientos con éstos darían lugar a híbridos mayormente estériles. Este modelo de evolución poliploide, desarrollado por Levin (1975), es conocido como exclusión del citotipo minoritario.

Existen dos tipos diferentes de poliploidía: autopoliploidía y alopoliploidía. Los organismos autopoliploides poseen tres o más copias del mismo genoma parental, mientras que los alopoliploides poseen, al menos, dos genomas parentales diferentes (Levin 2002). Aunque frecuentemente se habla de poliploidía en su conjunto, los autopoliploides y los alopoliploides poseen comportamientos evolutivos muy diferentes debido a sus distintos tipos de herencia, normalmente polisómica en autopoliploides y disómica en alopoliploides (Levin 2002, Ramsey and Schemske 2002). Estos diferentes tipos de herencia determinan en gran medida la tendencia del poliploide en cuestión a la autofecundación o a la fecundación cruzada (Husband et al. 2008). Además, la presencia de diferentes genomas en los alopoliploides puede proporcionar heterosis o vigor híbrido, es decir, ventajas adaptativas debidas a la interacción entre genomas diferentes típicas de los organismos híbridos (Birchler et al. 2003, Baack and Rieseberg 2007).

Los diferentes eventos de hibridación y poliploidía que pueden tener lugar entre un grupo de especies relacionadas entre sí (normalmente los integrantes de un mismo género) dan lugar a entramados de relaciones entre táxones de diferentes ploidías que comparten algunos genomas. A este proceso se le denomina evolución reticulada (Linder and Rieseberg 2004), y encuentra su máximo exponente en el grupo de los helechos.

Biología reproductiva en helechos

Los helechos son plantas vasculares con un ciclo de vida haplo-diploide (Sheffield 2008). En los ciclos de vida de este tipo, el esporófito (diploide) forma esporangios en los que tiene lugar la meiosis. Como resultado de ésta se originan esporas haploides, que son dispersadas y germinan produciendo un gametófito (haploide) de vida independiente del esporófito. En los gametófitos se desarrollan los gametangios u órganos sexuales femeninos (arquegonios) y masculinos (anteridios), en los cuales se generan gametos por mitosis. Los espermatozoides liberados por los anteridios fecundan las ovocélulas que se encuentran en el interior de los arquegonios, y se generan así nuevos esporófitos diploides. El nuevo esporófito se nutre en un principio de los compuestos proporcionados por el gametófito, y posteriormente se independiza del gametófito como consecuencia de la muerte de éste (Sakamaki and Ino 1999, Sheffield 2008).

Los helechos heterósporos producen microsporas, que dan lugar a gametófitos masculinos, y megasporas, que dan lugar a gametófitos femeninos. Por otro lado, los helechos homósporos producen esporas de un único tamaño que generan gametófitos potencialmente hermafroditas. Esta peculiaridad de los helechos homósporos permite que sean capaces de llevar a cabo tres tipos de fecundación (Ranker and Geiger 2008). En el primer tipo, conocido como alogamia o fecundación cruzada ("intergametophytic crossing"), la fecundación se produce entre gametos de diferentes gametófitos que proceden de diferentes esporófitos. En el segundo tipo, conocido como autogamia o autofecundación intergametofítica ("intergametophytic selfing"), la fecundación se produce entre gametos de diferentes gametófitos que proceden del mismo esporófito. Por último, en el tercer modo, conocido como automixis o autofecundación intragametofítica ("intragametophytic selfing"), la fecundación se produce entre gametos generados por el mismo gametófito. Si bien la fecundación cruzada y la autofecundación intergametofítica encuentran su equivalente en las plantas con semillas, la autofecundación intragametofítica, que produce esporófitos diploides totalmente homozigotos, es específica de los helechos homósporos entre las plantas vasculares. Durante muchos años se ha asumido que este último tipo de autofecundación era el habitual en los helechos. Sin embargo, numerosos estudios sobre variación genética en helechos homósporos demuestran que la mayoría de especies se fecunda habitualmente de forma cruzada, produciendo individuos heterozigotos (Ranker and Geiger 2008).

El género de los gametófitos de los helechos homósporos no está determinado por cromosomas sexuales. En su lugar, estos organismos exhiben una expresión sexual lábil,

modificable en función de los estímulos ambientales que reciban (Korpelainen 1998). El estímulo ambiental más estudiado es el grupo de moléculas conocidas como anteridiógenos. Estas moléculas, liberadas por gametófitos arquegoniados (femeninos o hermafroditas), son compuestos estructuralmente asimilables a las giberelinas que reducen la tasa de crecimiento vegetativo y promueven la masculinización en gametófitos jóvenes y asexuales (Yamane 1998, Tanurdzic and Banks 2004). Los anteridiógenos ejercen su influencia incluso en dosis muy pequeñas, con una intensidad proporcional a su concentración en el medio (Näf 1955, Stevens and Werth 1999). Actualmente aún se mantiene el debate sobre si los anteridiógenos funcionan como una hormona determinante del sexo (es decir, como una feromona) o un agente alelopático que promueve la masculinización como consecuencia del estrés (Korpelainen 1994, Chiou and Farrar 1997, Quintanilla et al. 2007). Aunque parece existir una inclinación hacia la expresión sexual masculina en gametófitos estresados y femenina en gametófitos favorecidos (Korpelainen 1994, Guillon and Fievet 2003, DeSoto et al. 2008), parecen necesarios más estudios experimentales para definir las tendencias generales de expresión sexual en un abanico taxonómico, ecológico y de ploidía más amplio.

Diversidad genética.

En condiciones de aislamiento reproductivo, tiene lugar en las poblaciones una pérdida progresiva de diversidad genética mediante deriva (Ellstrand and Elam 1993, Young et al. 1996). Paralelamente, la falta de flujo génico entre poblaciones conlleva una diferenciación genética entre ellas proporcional al tiempo que han permanecido incomunicadas (Spieth 1974, Loveless and Hamrick 1984). Ambos fenómenos hacen que la fragmentación de hábitat sea una de las mayores amenazas para la conservación de la diversidad genética y, con ella, de la biodiversidad (Young et al. 1996, Jump and Peñuelas 2006). La capacidad de los helechos de dispersar sus esporas a larga distancia mediante el viento y el potencial de generar un individuo a partir de una sola espora sugieren que estas plantas deberían presentar una alta variación genética intrapoblacional, así como una escasa diferenciación genética entre poblaciones (Soltis and Soltis 1990). Por tanto, ambas características confieren a los helechos una mayor resistencia a la pérdida de diversidad genética que la que poseen otros grupos de plantas.

Existen numerosos estudios sobre variación genética en helechos, la inmensa mayoría de ellos basados en electroforesis de isoenzimas (p. ej. Soltis et al. 1989, Vogel et al. 1999, Quintanilla et al. 2007). En su conjunto, estos trabajos señalan que la mayoría de poblaciones de helechos estudiadas poseen una diversidad genética similar a la detectada en las

poblaciones de angiospermas y que estas poblaciones están poco diferenciadas (Ranker and Geiger 2008). La electroforesis de isoenzimas posee importantes ventajas, entre las que destacan su rapidez, su reducido coste, y la herencia codominante de los alelos enzimáticos. Sin embargo, esta técnica también tiene limitaciones, la más importante de las cuales es la poca variabilidad alélica de las enzimas. Esta carencia restringe su utilidad para estudiar la diversidad genética en comparación con otros marcadores moleculares más variables como los microsatélites (Estoup et al. 1998, Degen et al. 1999). Lamentablemente, los estudios de diversidad genética en helechos realizados con microsatélites son muy escasos, a pesar de su gran potencial para estudiar poblaciones que han atravesado recientemente cuellos de botella genéticos (Pryor et al. 2001, Kang et al. 2008).

El género *Dryopteris*

Dryopteris Adanson (Dryopteridaceae, Pteridophyta) es, con alrededor de 225 especies descritas (Hoshizaki and Wilson 1999), uno de los géneros más diversificados dentro de los helechos homósporos. Este género posee una distribución subcosmopolita y su centro de distribución, así como su mayor abundancia de especies, se halla en el oriente asiático. Las especies de este género muestran un amplio abanico morfológico, pero en general se caracterizan por poseer indusios reniformes y esporas monoletas elipsoidales (Salvo and Arrabal 1986). La hibridación y la alopoliploidía son una constante en *Dryopteris*, que llega incluso a producir híbridos intergenéricos con *Polystichum* (Wagner et al. 1992). Sin embargo, al contrario de lo que sucede en muchos otros géneros, no se conoce ningún caso de autopoliploidía en *Dryopteris*.

Dos especies diploides presentes en la Península Ibérica, *D. aemula* y *D. oreades*, forman parte, respectivamente, de numerosos híbridos interespecíficos y especies alopoliploides (Fraser-Jenkins 1982). Sobre la base de caracteres morfológicos y bioquímicos, se ha sugerido que ambas especies son los diploides parentales del tetraploide *D. corleyi* (Fraser-Jenkins 1982, Fraser-Jenkins and Widén 1993), aunque esta relación aún no ha sido confirmada. *Dryopteris corleyi* es endémico de una pequeña franja costera de unos 60 × 10 km localizada entre la zona oriental de Asturias y la occidental de Cantabria. En dicha zona las dos especies diploides se encuentran geográficamente próximas, aunque a diferentes altitudes y en distintos hábitats: *D. aemula* vive en bosques caducifolios costeros entre los 0 y los 900 m sobre el nivel del mar, mientras que *D. oreades* crece en canchales silíceos a una altitud de entre 600 y 2400 m sobre el nivel del mar. A pesar de estas diferencias de hábitat y altitud, parece perfectamente plausible que, en las zonas de contacto, ambos diploides hibriden entre sí. Sin

embargo, el híbrido entre *D. aemula* y *D. oreades*, *D. ×pseudoabbreviata*, aún no se ha encontrado en la Península Ibérica.

Numerosos indicios invitan a pensar que *D. corleyi* es un neopoliploide, es decir, un poliploide de origen reciente. Entre dichos indicios se encuentran la presencia de *D. corleyi* en una zona tan delimitada acompañado de los dos supuestos parentales ocupando hábitats antropizados (explotaciones de pinos y eucaliptos), la escasa distancia genética que separa a *D. corleyi* y *D. aemula* (Geiger and Ranker 2005), y la alta variabilidad en el porcentaje de esporas abortivas de *D. corleyi* (Quintanilla and Escudero 2006), hecho que sugiere que esta especie aún está sometida a ajustes en la división celular.

OBJETIVOS

El presunto complejo diploide-poliploide integrado por *D. aemula*, *D. oreades* y *D. corleyi* representa un interesante modelo para estudiar las consecuencias de la alopoliploidía sobre diversas características de los helechos a nivel de genética de poblaciones y de biología reproductiva. Los objetivos concretos planteados en esta tesis doctoral fueron los siguientes:

1.- Dilucidar si *D. corleyi* se originó a partir de *D. aemula* y *D. oreades* (Capítulo 1). Un patrón aditivo para un número suficiente de loci isoenzimáticos confirmaría este origen de *D. corleyi*. Por otro lado, la presencia o ausencia de variación genética en esta especie proporcionaría información sobre la antigüedad de su origen y la posibilidad de que se hubiese originado recurrentemente en diversos eventos de poliploidización.

2.- Describir la diversidad genética y su reparto intra- e interpoblacional en *D. corleyi*, *D. aemula* y *D. oreades* y determinar sus causas (Capítulos 1 y 2). Muestreando un número suficiente de inviduos en varias poblaciones de cada especie y aplicando loci isoenzimáticos se podría determinar cuánta diversidad genética tienen estas especies y cómo está repartida. En caso de ausencia de variación, el desarrollo de microsatélites para las especies invariables permitiría detectar variación genética no revelada por las isoenzimas. Asimismo, se podría comprobar hasta qué punto los microsatélites desarrollados serían utilizables en otras especies de *Dryopteris* y de otros genéros.

3.- Determinar la expresión sexual de gametófitos aislados de las tres especies en condiciones favorables para el crecimiento (Capítulo 3). Conocer la secuencia comparada de aparición de

los órganos sexuales en estas condiciones serviría como línea base para estudios posteriores, así como para observar posibles ventajas competitivas del poliploide. Además, una vez los gametófitos cultivados desarrollasen arquegonios, el sustrato sobre el que crecen podría utilizarse como fuente de anteridiógenos.

4.- Determinar la expresión sexual de gametófitos aislados de las tres especies bajo la influencia de anteridiógenos (Capítulo 3). Una menor tasa de crecimiento y una mayor tasa de masculinidad en comparación con los gametófitos cultivados en condiciones favorables demostraría que estas especies producen anteridiógenos y responden a ellos. Una respuesta de *D. corleyi* distinta a la de los diploides pondría de manifiesto algunas ventajas adaptativas de esta especie que le habrían permitido contrarrestar la exclusión del citotipo minoritario y establecerse en nuevos hábitats.

5.- Determinar la expresión sexual de gametófitos aislados de las tres especies bajo condiciones limitantes de nutrientes y luz (Capítulo 4). Cultivando gametófitos de las tres especies en sustratos con concentraciones variables de nutrientes minerales y con filtros de luz de distinta intensidad se podría determinar qué circunstancias son más favorables para la masculinidad, la feminidad o el hermafroditismo. Adicionalmente, sería posible comparar el efecto masculinizante de las condiciones más limitantes para el crecimiento con el de los anteridiógenos. Por último, se podría comprobar si el tetraploide posee ventajas adaptativas que le permitan crecer y reproducirse mejor que los diploides en condiciones desfavorables.

6.- Deducir el sistema reproductivo de estas especies (Capítulos 1, 2 y 3). La expresión sexual de los gametófitos en condiciones de cultivo podría ser un reflejo de lo que ocurre en condiciones naturales. Una maduración sexual más temprana de los órganos femeninos, especialmente si vieniese acompañada de la liberación de anteridiógenos, favorecería la fecundación cruzada. Por otro lado, una maduración de los órganos masculinos previa o simultánea a la de los femeninos favorecería la autofecundación. El ajuste de las frecuencias alélicas de cada especie a las frecuencias esperadas en caso de flujo génico aleatorio proporcionaría también evidencias sólidas sobre los sistemas reproductivos de estas especies en la naturaleza.

METODOLOGÍA GENERAL

Las siguientes metodologías, expuestas aquí de manera abreviada, se explican detalladamente en sus capítulos correspondientes:

Capítulo 1. Se muestrearon poblaciones ibéricas de *D. corleyi*, *D. aemula* y *D. oreades*, tres por especie, recogiendo en cada una un fragmento fresco de hoja de cada individuo para extraer las actividades enzimáticas. Con estos extractos se realizaron electroforesis en geles de almidón. Una vez revelados estos geles, se genotiparon todos los individuos para cada enzima y se calcularon diversos estadísticos de genética de poblaciones que describen la diversidad genética en cada población y su reparto intra- e interpoblacional.

Capítulo 2. Se desarrollaron microsatélites para *D. aemula* a partir de un individuo de esta especie. Una vez diseñados los cebadores que amplificaban cada microsatélite en PCR, se muestrearon individuos de tres poblaciones ibéricas y una azoreña de *D. aemula*. Se extrajo el ADN de cada uno de los individuos muestreados, se amplificaron los microsatélites en PCR, y se genotiparon con un secuenciador. Posteriormente se calcularon diversos estadísticos de genética de poblaciones que describen la diversidad genética en cada población y su reparto intra- e interpoblacional. Adicionalmente, se comprobó la transferibilidad de los microsatélites desarrollados para *D. aemula* a otros 18 táxones de helechos.

Capítulo 3. Se cultivaron gametófitos de *D. corleyi*, *D. aemula* y *D. oreades*. En un primer experimento, los gametófitos de estas especies se cultivaron en condiciones favorables para el crecimiento a fin de comparar el momento de maduración sexual de cada especie. Los sustratos sobre los que crecieron estos gametófitos se utilizaron como fuente de anteridiógenos en un segundo experimento, en el que las tres especies se combinaron como fuente y diana de anteridiógenos. En este nuevo experimento se determinaron el momento de maduración sexual de cada especie y el tamaño de los gametófitos en dos momentos de su crecimiento.

Capítulo 4. Se cultivaron gametófitos de *D. aemula*, *D. oreades* y *D. corleyi* en dos experimentos paralelos, uno sobre sustratos con diferentes concentraciones de nutrientes y otro bajo diferentes intensidades de luz. Para cada uno de los experimentos se calcularon en tres tiempos diferentes el tamaño de cada gametófito, su sexo, y el número de gametangios masculinos y femeninos.

CONCLUSIONES GENERALES

Integrando los resultados obtenidos en los estudios de genética de poblaciones y en los de biología reproductiva, se pueden extraer las siguientes conclusiones:

1.- *Dryopteris corleyi* se originó recientemente a partir de *D. aemula* y *D. oreades*, como demuestran un patrón isoenzimático resultante de combinar los de las dos especies diploides y la ausencia en *D. corleyi* de alelos no compartidos con *D. aemula* y *D. oreades*. La presencia de dos genotipos isoenzimáticos diferentes de *D. corleyi* sugiere la posibilidad de al menos dos orígenes diferentes de esta especie a partir de eventos de poliploidización independientes.

2.- *Dryopteris corleyi* posee una diversidad genética reducida, con tan solo un locus isoenzimático variable. El reparto desigual de los diferentes genotipos entre las poblaciones muestreadas sugiere o bien que uno de los genotipos es de reciente aparición y no ha tenido tiempo de expandirse, o bien que estas poblaciones fueron fundadas por individuos de distintos genotipos.

3.- Las poblaciones de *Dryopteris oreades* albergan una diversidad isoenzimática considerable, probablemente reminiscente del mayor número y tamaño de sus poblaciones durante la última glaciación cuaternaria. A pesar de la distribución naturalmente fragmentada de esta especie, parece haber un flujo génico interpoblacional abundante que ralentiza la deriva genética intrapoblacional. Debido a esta conectividad, las poblaciones ibéricas de *D. oreades* están genéticamente poco diferenciadas entre sí.

4.- Por el contrario, *D. aemula* muestra signos evidentes de haber atravesado recientemente un cuello de botella genético. Los patrones isoenzimáticos obtenidos para las poblaciones ibéricas son monomórficos, y los datos obtenidos con microsatélites confirman que la diversidad genética de las poblaciones ibéricas de esta especie es realmente reducida. La mayor diversidad genética observada en la población de Azores sugiere que los archipiélagos macaronésicos funcionaron como un refugio glacial para *D. aemula*, a partir del cual se extendió por la Europa continental durante el último período interglacial. Si bien las poblaciones de *D. aemula* no parecen tener una estructura genética espacial, las poblaciones están muy diferenciadas genéticamente entre sí a consecuencia de un flujo génico reducido

entre las poblaciones. Es muy probable que la causa de este flujo génico reducido sea la remarcable predisposición a la autofecundación que posee esta especie.

5.- Los microsatélites desarrollados para *D. aemula* son transferibles a un buen número de táxones emparentados con esta especie, especialmente en alopoliploides e híbridos que incorporan al menos una copia del genoma de *D. aemula*.

6.- Cultivados sobre sustrato comercial, los gametófitos de las tres especies tienden a ser femeninos y liberan anteridiógenos inductores de la masculinidad en gametófitos asexuales. Esta inducción no parece ser específica, ya que los gametófitos de cada especie responden a los anteridiógenos de su propia especie y a los de las demás. La inespecificidad observada se puede interpretar como un mecanismo promotor de hibridación que funcionaría como un factor de selección contra el establecimiento del citotipo minoritario en poblaciones mixtas de especies de diferentes ploidías.

7.- En las tres especies, existe una relación evidente entre el tamaño del gametófito y su sexo. Los gametófitos más grandes tienden a ser femeninos o hermafroditas, mientras que los más pequeños son masculinos o se mantienen como asexuales. Sin embargo, la relación de los estímulos ambientales con el tamaño y el sexo es mucho menos directa, ya que en algunas condiciones limitantes los gametófitos son grandes y femeninos, mientras que otras condiciones hipotéticamente favorables los gametófitos tienden a ser más pequeños y masculinos. Estas observaciones reflejan que, si el efecto de los anteridiógenos está mediado por el tamaño del gametófito, ninguna de las condiciones de cultivo ensayadas es capaz de emular el efecto fisiológico de esta molécula.

8.- *Dryopteris corleyi* presenta algunas características que probablemente representan ventajas competitivas frente a sus parentales diploides. A pesar de tener tamaños similares, los gametófitos de *D. corleyi* cultivados en diferentes condiciones maduran sexualmente antes que los gametófitos de *D. aemula* y *D. oreades*. Esta maduración más temprana puede favorecer el emparejamiento clasificado entre gametófitos de *D. corleyi* y, por tanto, fecundaciones más tempranas que en los dos diploides. La formación más temprana de esporófitos de *D. corleyi* podría haber contrarrestado la exclusión del citotipo minoritario en poblaciones compartidas con sus parentales, favoreciendo así su establecimiento.

9.- Las especies estudiadas presentan diferentes sistemas reproductivos. Las isoenzimas y los cultivos de gametófitos indican que *D. oreades* se reproduce habitualmente por fecundación cruzada, mientras que los microsatélites sugieren que *D. aemula* se reproduce principalmente mediante autofecundación. El sistema reproductivo de *D. corleyi* continúa siendo una incógnita: en condiciones favorables, sus gametófitos desarrollan arquegonios y liberan anteridiógenos, lo que favorece la fecundación cruzada, pero lo mismo sucede en *D. aemula* a pesar de su notable tendencia a la autofecundación. Dilucidar el sistema reproductivo predominante en esta especie requiere, por tanto, estudios adicionales.

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LISTA DE MANUSCRITOS

Los siguientes capítulos de esta tesis doctoral han sido redactados en inglés con el objetivo de ser publicados en revistas científicas de ámbito internacional. Se detallan, por tanto, los datos de publicación de cada uno de ellos:

Capítulo 1. Ares Jiménez, Luis G. Quintanilla, S. Pajarón, E. Pangua. 2009. Genetic variation in the allotetraploid *Dryopteris corleyi* (Dryopteridaceae) and its diploid parental species in the Iberian Peninsula. *American Journal of Botany* 96: 1880-1886.

Capítulo 2. Ares Jiménez, Rolf Holderegger, Daniela Csencsics, Luis G. Quintanilla. Microsatellites reveal marked among-population genetic differentiation and strong inbreeding in the relict fern *Dryopteris aemula*. Manuscrito inédito.

Capítulo 3. Ares Jiménez, Luis G. Quintanilla, S. Pajarón, E. Pangua. 2008. Reproductive and competitive interactions among gametophytes of the allotetraploid fern *Dryopteris corleyi* and its two diploid parents. *Annals of Botany* 102: 353-359.

Capítulo 4. Ares Jiménez, Luis G. Quintanilla. Environmental sex determination in a diploid-polyploid complex of woodferns: the effects of nutrients and light availability on gametophyte development. Manuscrito inédito.

CAPÍTULO 1

Genetic variation in the allotetraploid *Dryopteris corleyi* (Dryopteridaceae) and its diploid parental species in the Iberian Peninsula

Ares Jiménez[1], Luis G. Quintanilla[1], Santiago Pajarón[2], Emilia Pangua[2]

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[1]Depargamento de Biología y Geología, Universidad Rey Juan Carlos, 28933 Móstoles

[2]Departamento de Biología Vegetal I, Universidad Complutense, 28040 Madrid

SUMMARY

Studies on genetic diversity help us to unveil the evolutionary processes of species and populations and can explain several traits of diploid–polyploid complexes such as their distributions, their breeding systems, and the origin of polyploids. We examined the allozyme variation of *Dryopteris aemula* and *D. oreades*, diploid ferns with highly fragmented habitats, and the allotetraploid *D. corleyi* to (1) analyze the putative relationship between both diploids and the tetraploid, (2) compare the levels of genetic variation among species and determine their causes, and (3) assess the breeding system of these taxa. The allozymic pattern of *D. corleyi* confirms that it derived from *D. aemula* and *D. oreades*. The lack of genetic diversity in *D. aemula*, a species of lowland habitats, may be due to genetic drift associated with the contraction of populations in the last glaciation. By contrast, the alpine *D. oreades* had moderate intrapopulation genetic variation, which may derive from the expansion of populations during the last glaciation. In the latter species, low interpopulational variation suggested effective gene flow (spore exchange), and genotype frequencies in Hardy–Weinberg equilibrium indicated cross-fertilization of gametophytes. Evolutionary history appears to be an essential element in the interpretation of genetic variation of highly fragmented populations.

INTRODUCTION

Genetic diversity maintains the evolutionary potential of taxa to adapt to changing environmental conditions (Ellstrand and Elam 1993, Lande and Shannon 1996), and the knowledge of how much genetic diversity there is and how it is distributed provides important insights into the evolutionary processes that species and populations undergo. Genetic diversity arises through mutation in local populations, is maintained and spread via gene flow among individuals, and decreases through inbreeding, genetic drift, and genetic bottlenecks (Usher 1997). Natural or anthropogenic habitat fragmentation during the evolutionary history of species is currently regarded as one of the main threats to genetic diversity (e.g. Peterson et al. 2008). Fragmentation leads to smaller patches of suitable habitat, thereby reducing population sizes and increasing distances among remaining populations, which restricts interpopulational gene flow (Young et al. 1996, Usher 1997). Under these conditions, characterized by the low probability of "genetic rescue" via gene flow, random genetic drift can lead to the fixation of different alleles at each population (Barrett and Kohn 1991, Robichaux et al. 1997). Consequently, drift not only causes genetic differentiation among populations, but also leads to a loss of genetic diversity both at the population and species levels.

Ferns have been the target of numerous studies on genetic diversity because of their particular life histories, potential for long-distance dispersal and frequently fragmented populations (e.g. Pajarón et al. 1999, Landergott et al. 2001, Schneller and Liebst 2007). The life history of these organisms typically consists of a haploid stage, the gametophyte, and a diploid stage, the sporophyte, which are independent of each other. Homosporous fern gametophytes are potentially hermaphroditic and can self-fertilize yielding completely homozygous sporophytes, thus having the possibility to found a population from a single spore. However, rather than selfing, many species tend to cross-fertilize even if their gametophytes can express both sexes (Ranker and Geiger 2008). Cross-fertilizations are facilitated by antheridiogens, gibberellin-related pheromones that are released by archegoniate gametophytes and influence presexual gametophytes by hindering their growth and inducing male sex expression (Yamane 1998, Tanurdzic and Banks 2004).

Hybridization and polyploidy have played a pre-eminent role in fern species diversification (Soltis and Soltis 1989). Allopolyploids, i.e. interspecific hybrids which have undergone a genome duplication, can normally produce balanced, viable gametes (Manton 1950, Comai 2000). As a result of nonsegregation of homeologous chromosomes during

meiosis, they are often reported to have "fixed heterozygosity" (e.g. Glover and Abbott 1995, Nelson and Elisens 1999). Genetic diversity of allopolyploid species can increase when multiple origins from different diploid parents take place, with each subsequent origin potentially adding new alleles to the previous pool of the allopolyploid (Werth et al. 1985).

With roughly 225 species described throughout the world (Hoshizaki and Wilson 1999), woodferns (genus *Dryopteris*) comprise one of the most diversified groups among homosporous ferns. Hybridization and allopolyploidy have been major factors in shaping evolutionary affinities within *Dryopteris*, with numerous diploid–polyploid complexes identified (e.g. Manton 1950, Darnaedi et al. 1990, Werth 1991). *Dryopteris corleyi* Fraser-Jenkins is an allotetraploid endemic to the northern Iberian Peninsula. Morphological and phytochemical characters (Fraser-Jenkins 1982, Fraser-Jenkins and Widén 1983) indicate that it derived from *D. aemula* (Aiton) Kuntze and *D. oreades* Fomin, two diploids with a highly fragmented distribution in the Iberian Peninsula. A partial phylogeny of the genus including *D. corleyi* and *D. aemula* supports the close relationship between these species (Geiger and Ranker 2005). Using allozyme electrophoresis, we addressed the following questions: (1) Did *Dryopteris corleyi* derive from *D. aemula* and *D. oreades*? (2) How much genetic variation is distributed within and among the populations, and what are their principal determinants? (3) What are the mating systems of these species?

MATERIALS AND METHODS

Studied species

Three species were sampled for this study: the tetraploid *Dryopteris corleyi* ($2n = 4x = 164$, Fraser-Jenkins and Gibby, 1986) and the sexual diploids *D. aemula* ($2n = 2x = 82$; Manton 1950) and *D. oreades* ($2n = 2x = 82$; Manton 1950). *Dryopteris corleyi*, endemic to a narrow coastal strip of the northern Iberian Peninsula, inhabits heathland banks and anthropogenic habitats such as eucalyptus and pine plantations from 50 to 650 m a.s.l. (Salvo and Arrabal 1986). *Dryopteris aemula* is present in western Europe, from Scotland to the Azores, Canary and Madeira archipelagos, and appears also in warm, oceanic enclaves in Turkey and Transcaucasia; in the Iberian Peninsula, it inhabits temperate deciduous forests within narrow, north-oriented valleys from sea level to 900 m a.s.l. (Salvo and Arrabal 1986).

These lowland forests have been heavily fragmented and transformed into cattle pastures in historical times (Izco 1994). *Dryopteris oreades* is distributed in the mountains of western, southern, and central Europe, and appears also in Turkey and the Caucasus mountains; in the Iberian Peninsula, it occurs in open noncalcareous rocky areas and screes that are snow-covered in winter, from 600 to 2400 m a.s.l. (Salvo and Arrabal 1986).

Plant material

Leaf fragments were sampled from 41 individuals from each of three wild populations of each species as listed in Table 1. For each individual, 1–2 pinnules were collected and stored in plastic bags for 4–6 d at 5°C until enzyme extraction. Pinnules were ground in polyvinylpyrrolidone-phosphate buffer (Soltis et al. 1983), and the resulting extracts were absorbed onto 4 × 6 rectangles of Whatman no. 3 chromatography paper and frozen at –80°C until electrophoresis.

Allozyme electrophoresis

Electrophoreses were conducted in 12.5% starch gels as per Soltis et al. (1983) and Haufler (1985). Ten enzymatic systems were studied, with nomenclature following Acquaah (1992): aspartate aminotransferase (AAT), hexokinase (HEX), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), 6-phosphogluconate dehydrogenase (6-PGD), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and shikimate dehydrogenase (SKDH). Genotypes were deduced from the electrophoretic phenotypes on the basis of the quaternary structure and subcellular locations reported for other plant species (Gottlieb 1982, Weeden and Wendel 1989). Presumed loci were numbered consecutively from anode to cathode; alleles were named alphabetically from anode to cathode.

Statistical analysis

The mean number of alleles per locus (A) and the percentage of polymorphic loci following the 0.99 criterion ($P_{0.99}$) were calculated for each population and averaged for each of the three species. For polymorphic loci, we determined the observed heterozygosity (H_o),

the expected heterozygosity under Hardy–Weinberg equilibrium (H_e), and Wright's (1943) fixation index $F = 1 - H_o/H_e$. The statistical significance of F was tested by χ^2 tests. We used the statistics H_I, H_S, H_T, F_{IS}, F_{IT} and F_{ST} to portray genetic diversity and population structure. Values of F_{ST} were averaged over loci to calculate G_{ST} as per Hamrick and Godt (1989). Additionally, as suggested by Culley et al. (2002), G_{ST} was also calculated following Nei's (1973) method. Nei's (1978) genetic distance D was calculated for all population pairs within species.

RESULTS

Band interpretation

The 10 enzymatic systems provided a total of 14 interpretable loci: *Aat*, *Hex*, *Idh*, *Lap*, *Mdh-1*, *Mdh-2*, *Mdh-3*, *Me*, *6-Pgd-1*, *6-Pgd-2*, *Pgi*, *Pgm-1*, *Pgm-2* and *Skdh* (Fig. 1). Some of these loci were not interpretable for some species or populations. Locus *Aat* could not be interpreted in populations O2 and O3 due to extremely faint bands in all individuals. *Mdh-2* could only be interpreted in *D. oreades* as presenting one expressed allele and one null allele; therefore, it did not permit a distinction between *aa* homozygotes and *an* (*n* = null allele) heterozygotes. Because no bands appeared in *Mdh-2* for *D. aemula* and *D. corleyi*, *Mdh-2* was considered to be uninterpretable for these species. *Me* could not be interpreted in *D. corleyi*; most individuals had unreliable, blurry bands. These problematic loci were included in calculations only if they provided valid data.

For loci *Hex*, *Idh*, *Lap*, *Mdh-1*, *6-Pgd-1*, *Pgm-1*, and *Sdh*, *D. corleyi* showed a fixed (i.e. nonsegregative among individuals) heterozygosity allozymic pattern, which could be readily interpretable as the sum of patterns of *D. aemula* and *D. oreades* (Fig. 1). For the remaining loci, *D. corleyi* was monomorphic for the same bands as *D. aemula*, and those bands were also present in some *D. oreades* individuals. All alleles present in the tetraploid were present in at least one of the two putative parents, or in other words, no orphan alleles were detected.

Genetic variation

In *D. aemula*, the 13 resolved loci were monomorphic, fixed for the same alleles in all individuals from all three populations (Fig. 1). Thus, the values of A and $P_{0.99}$ averaged over populations were 1.00 and 0.0, respectively (Table 2).

In *D. oreades*, seven of the 14 resolved loci showed polymorphism (Fig. 1). A and $P_{0.99}$ averaged over populations showed moderate values (Table 2). The values of G_{ST} calculated as per Nei (1973) and as per Hamrick and Godt (1989) (Table 3) were, respectively, 0.052 and 0.057. The genetic distances between population pairs were very low: $D_{O1\text{-}O2} = 0.005$, $D_{O1\text{-}O3} = 0.016$, and $D_{O2\text{-}O3} = 0.011$.

In *D. corleyi*, only one of the 12 loci resolved, *6-Pgd-1*, was polymorphic (Fig. 1). Loci showing fixed heterozygosity were regarded as monomorphic. The value of A overaged over populations was similar to that of *D. oreades*, but the value of $P_{0.99}$ was considerably low (Table 2). Some individuals were heterozygous for locus *6-Pgd-1*, with alleles present both in *D. aemula* and *D. oreades*, and the remaining individuals were homozygous for the allele fixed in *D. aemula* (Fig. 1). The observed frequencies of these heterozygotes in populations AO1, AO2 and AO3 were 0.00, 0.22 and 0.90, respectively. Given that *a–/b–* heterozygotes for *6-Pgd-1* might theoretically be *aa*/*ab*, *aa*/*bb* or *ab*/*bb*, and that these genotypes could not be reliably distinguished in gels, genotypic and allelic frequencies and statistics based on them could not be calculated.

Hardy–Weinberg equilibrium

In *D. oreades*, most polymorphic loci were in Hardy–Weinberg equilibrium in the three populations (Table 4). The only exceptions were *6-Pgd-2*, which had a significant excess of heterozygotes in population O1, and *6-Pgd-1* and *Idh*, which had a significant deficit of heterozygotes in populations O2 and O3, respectively. Hardy–Weinberg equilibrium could not be tested in *D. aemula* or *D. corleyi*, as a result of the absence of genetic variation in *D. aemula* and the impossibility of assigning genotypes for the *6-Pgd-1* locus of *D. corleyi*, as described earlier.

DISCUSSION

Origin of D. corleyi

Our data strongly support the hypothesis that *D. aemula* and *D. oreades* gave rise to *D. corleyi* via allopolyploidization. First, the allozyme banding pattern of *D. corleyi* showed fixed heterozygosity. Second, this pattern was readily interpretable as the additive pattern of *D. aemula* and *D. oreades.* And third, all alleles present in *D. corleyi* were present in at least one of the two putative parents. This absence of orphan alleles in *D. corleyi* can be due to the lack of evolutionary time for genetic divergence between the allotetraploid and its diploid parents to occur, thus also hinting at a recent origin of this species. This conclusion agrees with several traits of *D. corleyi*, including narrow distribution range, occupation of recent habitats (Mayor and Fernández 1988), low genetic divergence from *D. aemula* at the chloroplast DNA level (Geiger and Ranker 2005), and highly variable percentage of spore abortion (Quintanilla and Escudero 2006). We must remark, however, that the sampling range of our study, restricted to Iberian populations only, may be insufficient to provide evidence of local formation of *D. corleyi* in its current distribution area. Assuming a recent origin of this species, a wider sampling including several non-Iberian populations of *D. aemula* and *D. oreades* might reveal additional alleles which could confirm an Iberian origin of *D. corleyi*.

We found two allozymic phenotypes in locus *6-Pgd-1* of *D. corleyi*: *bb*/*bb* and *a*–/*b*– (Fig. 1). Given that in all *D. aemula* individuals sampled the allele *b* was fixed, the most likely interpretation is that *a*–/*b*– individuals of *D. corleyi* actually represent genotypes *aa*/*bb* and *ab*/*bb*. Under this assumption, *D. oreades* would have contributed alleles *a* and *b* in at least two separate origins of *D. corleyi.* However, the supposition of multiple origins for *D. corleyi* based only on the polymorphism in the *6-Pgd-1* locus seems unwarranted because other explanations, such as postpolyploidization mutations resulting in different allozymic phenotypes or repeated origin in a hybrid swarm from the same diploid parents, are possible (Vogel et al. 1999). Additionally, two routes of polyploid formation involving unreduced gametes, rather than diploid hybridization and subsequent polyploidization, could explain our results under the supposition of a single hybridization event (e.g. Gastony 1986, Ramsey and Schemske 1998). The first route would consist of the direct union of two abnormally

produced diploid gametes, one *bb* (from *D. aemula*) and one *ab* (from *D. oreades*). The second one would involve a triploid bridge, where an unreduced *ab* gamete from a heterozygous diploid would combine with a normal *b* gamete; the resulting *ab/b* triploid would be mostly sterile but could eventually produce a functional unreduced triploid spore, whose gametes could combine with a new *b* gamete. In both cases, the resulting initial *ab/bb* sporophyte would produce *a/b* and *b/b* gametophytes and gametes, which could then generate *aa/bb*, *ab/bb*, and/or *bb/bb* sporophytes.

Genetic diversity

We found very distinct genetic diversities in the diploid species studied, with extremely low levels in *D. aemula* and moderate levels in *D. oreades.* The disparate evolutionary histories of these species can explain these results. Concretely, their different environmental requirements could have determined their response to glacial–interglacial cycles. During the last glacial stage (the Würm glaciation), lowland species, such as *D. aemula*, would have withdrawn to warmer southern refugia in the Iberian Peninsula or Macaronesia, whereas the populations of alpine species, such as *D. oreades*, would have expanded across the Iberian Peninsula. At the end of the glacial stage, the generalized rise of temperatures in the Iberian Peninsula would have facilitated the spread of lowland taxa from warm refugia and the retreat of alpine taxa to cooler altitudes and latitudes, as explained by the contraction–expansion model suggested by Hewitt (1996).

The outcome of these events for lowland taxa would be a general genetic impoverishment due to population fragmentation and extinction during the ice spread, genetic drift in isolated refugial populations, and founder effects during the subsequent Holocene expansion (Hewitt 1996). Our results for *D. aemula* agree with those found in other Macaronesian relict ferns such as *Trichomanes speciosum*, *Culcita macrocarpa*, and *Woodwardia radicans* (Rumsey et al. 1999, Quintanilla et al. 2007). These species have similar ecological requirements and are often found sharing habitats with *D. aemula* in the northern Iberian Peninsula, and also have a very low genetic diversity at the allozyme level in fragmented populations close to their distribution limits. Some northern Iberian coastal spots could have acted as warm refugia for temperate Atlantic taxa during glacial maxima, as suggested by the presence of pollen of trees such as *Quercus*, *Alnus*, and *Corylus* usually found sharing habitats with *D. aemula*, *C. macrocarpa*, and *W. radicans* in Würmian sediments in the northern Iberian coast (Mary et al.

1975 and 1777, Ramil-Rego et al. 1998). Preliminary results obtained with microsatellite markers confirm that genetic diversity in Iberian populations of *D. aemula* is very low (Jiménez et al., Capítulo 2).

On the other hand, alpine species would have sustained high levels of genetic diversity during the last glaciation due to population expansion, arrival of new alleles from northern latitudes and abundant gene flow among populations. After the retreat of ice, remaining, shrunken populations would then begin to experience a loss of genetic diversity and an increase of genetic divergence among them because of fragmentation-driven genetic drift (Hewitt 1996, Knowles and Richards 2005). The presently high fragmentation in the southern distribution limit of *D. oreades* in the Iberian Peninsula could be expected to determine low within-population genetic diversity and high genetic distances among populations, as observed in other plants with fragmented distributions (e.g. Landergott et al. 2001, Lönn and Prentice 2002, Jump and Peñuelas 2006). However, according to our results, this is not the case. Genetic diversity values averaged for the studied populations (A = 1.62, $P_{0.99}$ = 44.9; Table 2) do not depart greatly from those reported for other fern species (A = 1.6, $P_{0.99}$ = 38.4, Ranker and Geiger 2008) and are only slightly lower than those of seed plants (A = 1.95, $P_{0.99}$ = 50.5, Hamrick and Godt 1989). G_{ST} and D values were low, suggesting abundant interpopulation gene flow and low genetic differentiation among populations, in accordance with the expectations for long-lived plant species with potential for long-range gene movement (Hamrick and Godt 1989). It appears, therefore, that long-distance spore dispersal can be instrumental in delaying the drift-mediated erosion of genetic diversity in *D. oreades*. Wind-driven homosporous fern spores can travel distances of up to thousands of kilometers (Muñoz et al. 2004) and have been previously reported to play an important role in shaping the genetic diversity of several species (e.g. Wolf et al. 1991, Sciarretta et al. 2005, Schneller and Liebst 2007). Even though the great majority of released spores fall near the source plant (Raynor et al. 1976), very low levels of gene flow via long-distance dispersal can offset the loss of variation due to genetic drift and reduce genetic distances among populations (Ellstrand and Elam 1993). Consequently, occasional arrival of wind-dispersed spores from other populations can maintain the levels of genetic variation observed in *D. oreades*. This result is in accordance with those for other alpine ferns that may have undergone similar evolutionary events, such as *Cryptogramma crispa* in the Iberian Peninsula (Pajarón et al. 1999) and *Athyrium distentifolium* in North America (Woodhead et al. 2005).

The allotetraploid *D. corleyi* presented little genetic variation, with only one polymorphic locus, *6-Pgd-1*. Although the average number of alleles per locus is similar to the average value reported for seed plants and other ferns (Hamrick and Godt 1989, Ranker and Geiger 2008), this comparison is debatable as *A* is intrinsically inflated for allopolyploids due to fixed heterozygosity. Given the efficient wind spore dispersal in ferns and the short distances separating the three *D. corleyi* populations studied (AO1-AO2: 5 km; AO1-AO3: 7 km; AO2-AO3: 4 km), one might expect comparable proportions of *a-/bb* and *bb/bb* genotypes at the *6-Pgd-1* locus in the three populations. However, the *a-/bb* genotype is absent in population AO1 and represents only 22% of genotypes in population AO2, whereas in AO3 it is present in 90% of the sampled individuals. This uneven distribution may obey a recent appearance of the *a-/bb* genotype or to populations having been founded by individuals with different genotypes.

Breeding systems

For *D. oreades*, the fact that most polymorphic loci were in Hardy–Weinberg equilibrium in all three populations, with *F* values close to zero (Table 4), strongly supports the hypothesis that this woodfern is mostly an outbreeder, as also suggested by the presence of an antheridiogen system operating in this species (Jiménez et al. 2008). This result is in accordance with the observations for most diploid fern species (Masuyama and Watano 1990). The lack of genetic variation in *D. aemula* and the impossibility to score *6-Pgd-1* genotypes in *D. corleyi* prevented deducing the breeding system of these species. The presence of an antheridiogen system in these species should favor outcrossing in the studied populations (Jiménez et al. 2008).

Conclusions

Dryopteris corleyi originated by allopolyploidization from the diploids *D. aemula* and *D. oreades.* The absence of genetic divergence from its parental species indicates a recent origin of the polyploid, although a local Iberian origin cannot be confirmed. The lack of genetic variation in *D. aemula* seems to correspond to the influence of Pleistocene glaciations. On the other hand, *D. oreades* harbors a moderate genetic diversity within its populations, probably as a consequence of windborne spore dispersal delaying the genetic drift which should

accompany the natural fragmentation of its habitat. The genotypic frequencies of *D. oreades* are in Hardy–Weinberg equilibrium, thus confirming that this species frequently outcrosses in nature. As shown by this work, the occurrence of population contraction–expansion cycles can explain the amount and distribution of genetic diversity in current fern populations. Further studies on seed plants comparing lowland and alpine species with fragmented distributions may also arrive at the conclusion that particular evolutionary histories can be, rather than the fragmentation-mediated processes, the main factor shaping intra- and interpopulational genetic diversity.

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TABLES AND FIGURES

Table 1. Populations sampled to study allozyme variation of the three *Dryopteris* species in the Iberian Peninsula.

Population	Locality	Latitude, Longitude	Altitude (m a.s.l.)
D. aemula			
A1	A Coruña, Fragas do Eume.	43°14′24″ N, 8°30′19" W	50
A2	Asturias, Santianes del Agua	43°25′42″ N, 5°20′40" W	50
A3	Gipuzkoa, Mutriku	43°18′40″ N, 2°24′51" W	90
D. oreades			
O1	Madrid, Puerto de Navafría	40°59′60″ N, 3°49′36" W	2060
O2	León, Puerto de Ancares	42°50′59″ N, 6°49′40" W	1790
O3	Burgos, Lagunas altas de Neila	42°30′40″ N, 3°30′59″ W	1930
D. corleyi			
AO1	Asturias, between Purón and La Borbolla	43°23′22″ N, 4°41′90″ W	50
AO2	Asturias, Pendueles	43°23′57″ N, 4°38′85″ W	75
AO3	Asturias, between Buelna and Santiuste	43°23′27″ N, 4°35′49″ W	65

Table 2. Genetic variation statistics for the three *Dryopteris* species in the Iberian Peninsula. For each population, 41 individuals were sampled. *A*, mean number of alleles per locus; $P_{0.99}$, percentage of polymorphic loci following the 0.99 criterion; SD, standard deviation.

Species	Population	*A*	$P_{0.99}$
D. aemula	A1	1.00	0.0
	A2	1.00	0.0
	A3	1.00	0.0
Mean (SD)		1.00 (0.00)	0.0 (0.0)
D. oreades	O1	1.69	50.0
	O2	1.33	38.5
	O3	1.83	46.2
Mean (SD)		1.62 (0.26)	44.9 (5.9)
D. corleyi	AO1	1.58	0.0
	AO2	1.67	8.3
	AO3	1.67	8.3
Mean (SD)		1.64 (0.05)	5.6 (4.8)

Table 3. *H* and *F* statistics for three *Dryopteris oreades* populations from the Iberian Peninsula. H_I, H_S and F_{IS} are averaged over populations. The mean value of F_{ST} equals G_{ST} calculated as per Hamrick and Godt (1989). Loci *Hex*, *Mdh-1*, *Mdh-3, Me, Pgm-1* and *Pgm-2* were monomorphic in all populations and thus $H_I = H_S = H_T = 0$. Loci *Aat* and *Mdh-2* were excluded from calculations (see Results).

Locus	H_I	H_S	H_T	F_{IS}	F_{ST}	F_{IT}
Idh	0.130	0.185	0.226	0.298	0.181	0.425
Lap	0.423	0.469	0.484	0.099	0.030	0.126
6-Pgd-1	0.390	0.438	0.439	0.108	0.003	0.111
6-Pgd-2	0.260	0.211	0.227	–0.234	0.072	–0.144
Pgi	0.016	0.016	0.016	–0.025	0.016	–0.008
Skdh	0.049	0.061	0.063	0.201	0.037	0.231
Mean	0.106	0.115	0.122	0.075	0.057	0.126

Table 4. Observed (H_o) and expected (H_e) heterozygosities and fixation indexes (*F*) for three *Dryopteris oreades* populations from the Iberian Peninsula. *, significant deviation from Hardy-Weinberg equilibrium; n.r., not resolved; —, monomorphic population.

	Population O1			Population O2			Population O3		
Locus	H_o	H_e	*F*	H_o	H_e	*F*	H_o	H_e	*F*
Aat	0.390	0.405	0.035	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Idh	0.000	0.000	—	0.122	0.115	–0.065	0.268	0.442	0.393*
Lap	0.415	0.487	0.148	0.366	0.356	–0.028	0.488	0.565	0.137
6-Pgd-1	0.439	0.414	–0.060	0.317	0.457	0.306*	0.415	0.442	0.061
6-Pgd-2	0.512	0.381	–0.344*	0.112	0.115	–0.065	0.146	0.137	–0.067
Pgi	0.049	0.048	–0.025	0.000	0.000	—	0.000	0.000	—
Sdh	0.024	0.024	–0.012	0.000	0.000	—	0.122	0.159	0.234

Figure 1. Zymograms of the 10 enzymatic systems studied and genotype interpretation in the three *Dryopteris* species. Thin horizontal lines represent single allele dosage; thick horizontal lines represent double allele dosage; vertical lines represent unresolved loci; -, unresolved allele; ***n*,** null allele. Anode toward top of the figure.

	D. aemula	*D. corlevi*	*D. oreades*
Aat	*cc*	*cc/cc*	*aa ac bc cc*
Hex	*bb*	*aa/bb*	*aa*
Idh	*bb*	*aa/bb*	*aa ac cc*
Lap	*bb*	*bb/dd*	ac *bc bd cc cd ce dd de ee*
1			
2			
3			
Mdh-1	*aa*	*aa/bb*	*bb bb*
Mdh-2	*nn*	*nn/nn*	*a- nn*
Mdh-3	*aa*	*aa/aa*	*aa aa*
Me	*bb*	--/--	*aa*
1			
2			
6-Pgd-1	*bb*	*aa/bb bb/bb*	*ab aa ab bb aa ab bb*
6-Pgd-2	*cc*	*cc/cc cc/cc*	*ac bc bc bc cc cc cc*
Pgi	*bb*	*bb/bb*	*ab bb*
1			
2			
Pgm-1	*bb*	*aa/bb*	*aa*
Pgm-2	*aa*	*aa/bb*	*bb*
Sdh	*bb*	*bb/cc*	*ac cc cd dd*

CAPÍTULO 2

Microsatellites reveal marked among-population genetic differentiation and strong inbreeding in the relict fern *Dryopteris aemula*

Ares Jiménez[1], Rolf Holderegger[2], Daniela Csencsics[2], and Luis G. Quintanilla[1]

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[1]Departamento de Biología y Geología, Universidad Rey Juan Carlos, 28933 Móstoles, Spain

[2]WSL Swiss Federal Research Institute, 8909 Birmensdorf, Switzerland

SUMMARY

We used microsatellite markers to explore the population genetics of the diploid fern *Dryopteris aemula*. A previous study detected no allozyme diversity in Iberian populations of this species. We developed eight microsatellite loci for *D. aemula* and tested their cross-amplification with other ferns. Five polymorphic loci were used to characterize the amount and distribution of genetic diversity in *D. aemula* populations from the Iberian Peninsula and the Azores, to assess the breeding system of natural populations, and to identify the spatial genetic structure within a population of this species. Most developed microsatellite markers were transferable to taxa close to *D. aemula*. We observed low genetic variation in *D. aemula* (H_T = 0.447) and high genetic differentiation of populations (F_{ST} = 0.520). All polymorphic loci surveyed strongly departed from Hardy-Weinberg equilibrium. No spatial genetic structure was found in *D. aemula*. The higher genetic diversity observed in the Azorean population studied suggested a possible refugium in this region from where mainland Europe had been recolonized after the Pleistocene glaciations. The deviations from Hardy-Weinberg equilibrium reflect high inbreeding probably due to intragametophytic selfing. The absence of spatial genetic tructure indicates effective spore dispersal over short distances.

INTRODUCTION

Microsatellites are tandem repeats of two to six nucleotides found in most nuclear genomes (Tautz 1989, Chambers and MacAvoy 2000). Since their discovery over three decades ago and with the rise of PCR technology, microsatellite markers have progressively become the current molecular marker of choice for population genetics studies. Their popularity is based on their ubiquity across most organisms, their single-locus co-dominant inheritance, and their high variability owing to high mutation rates (Tautz 1989, Sunnucks 2000, Selkoe and Toonen 2006). Microsatellites are thus a valuable tool for detecting population genetic structure, evaluating genetic diversity, testing parentage relationships and interpreting recent population history (Zhang and Hewitt 2003). The widespread use of microsatellites is, however, largely confined to vertebrate animals and seed plants, whereas they remain largely unexploited in organisms such as lichens, algae, lycophytes and ferns.

Homosporous ferns have aroused the interest of researchers due to their ability for long-distance dispersal and their exceptional life cycle. Their haploid spores germinate into potentially bisexual gametophytes, which are able to self-fertilize via intragametophytic selfing and produce completely homozygous sporophytes, a trait which enables these plants to found new populations from a single spore. Although these populations harbour little genetic diversity during their first generations, wind dispersal of spores over long distances may lead to abundant gene flow among populations. Fern populations are therefore viewed as exhibiting low genetic differentiation, with most variation being intrapopulational (Soltis and Soltis 1989), in agreement with the expectations for long-lived plant species with potential for long-range gene movement (Hamrick and Godt 1989). Most genetic studies on ferns have used allozyme electrophoresis (Ranker and Geiger 2008). However, the low allelic diversity usually found at allozyme loci limits their applicability in population genetic studies as compared to microsatellites (Estoup et al. 1998, Degen et al. 1999), especially in the case of recently founded or bottlenecked populations (e.g. Quintanilla et al. 2007). Only recently, a few studies applied microsatellites to populations of ferns (Pryor et al. 2001, Vitalis et al. 2002, Woodhead et al. 2005, Kang et al. 2008) and demonstrated that these markers are useful for the characterization of genetic diversity and structure of populations of ferns.

Dryopteris aemula is a diploid homosporous fern. It is considered to be a relict from the tropical flora that covered the Mediterranean area during the Tertiary period (Pichi-Sermolli 1979). The actual distribution range is scattered in oceanic refugial areas along the coasts of Western Europe, some islands of the Macaronesian archipelagos, Northeastern Turkey and Southwestern Transcaucasia. It is listed as a vulnerable species in Spain because of the rarity

of the species' habitat, i.e. deciduous, temperate oceanic forests at low altitude close to the sea (Bañares et al. 2004). The production and response to antheridiogen, a pheromone released by female or hermaphrodite gametophytes promoting maleness in close asexual gametophytes, suggests that *D. aemula* may be mainly outbreeding (Jiménez et al. 2008). A previous survey found no genetic variation at 13 allozyme loci in three populations spanning the distribution area of *D. aemula* in the Iberian Peninsula (Jiménez et al. 2009). Genetic bottlenecks and founder effects as the consequence of cycles of local extinction and recolonization from Macaronesian refugia during Pleistocene glaciations are a possible cause of the lack of allozyme diversity in the Iberian populations of this species. The use of more variable genetic markers is necessary to ascertain whether the lack of allozyme diversity in *D. aemula* is reflected in other genomic regions than the allozyme coding genes. Here, we report on eight newly developed nuclear microsatellite markers isolated from *D. aemula*, also checking for cross-amplification in other fern taxa including hybrids and polyploids incorporating the "aemula" parental genome. Using five of these polymorphic microsatellite loci, we tested the following hypotheses. (1) Overall genetic variation is low, as indicated by allozymes, and higher in Azores than in the Iberian Peninsula, because this archipelago acted as a refugium during glaciations. (2) Among-population genetic differentiation is low on account of long-distance gene flow via spore dispersal. (3) Genotype frequencies within populations are in Hardy-Weinberg equilibrium, as a consequence of outcrossing due to the action of antheridiogens. (4) Genetic variation within populations is not spatially structured because of effective spore dispersal and outcrossing.

MATERIALS AND METHODS

Sampling design

Four *Dryopteris aemula* (Aiton) Kuntze populations were sampled, three of them in the northern Iberian Peninsula (SAN, MUT, and EUM), and a fourth in São Miguel Island, Azores (AZO, Table 1). Individuals were randomly sampled in all populations except in EUM, which was selected to additionally study the spatial genetic structure within a population. Here, all individuals present along a 30 × 6 m transect were sampled, and the position of each individual mapped. One or two pinnules per individual were collected and directly dried in silica-gel until DNA extraction.

Microsatellite development and genotyping

DNA was extracted with the DNeasy 96 Plant Kit (Qiagen). Genomic DNA of one *D. aemula* individual sampled from SAN was used by Ecogenics (Zurich, Switzerland) to make an enriched library from size selected genomic DNA ligated into TspADshort/TspADlong-linker (Armour et al. 1994) and enriched by magnetic bead selection with biotin-labelled $(GT)_{13}$, $(CT)_{13}$, $(AAG)_{10}$ and $(TAC)_{10}$ oligonucleotide repeats (Gautschi et al. 2000a,b). Out of 378 recombinant colonies screened, 59 gave positive signals after hybridization. Plasmids from these 59 positive clones were sequenced and primers were designed for 18 microsatellite inserts with PRIMER3 (Rozen and Skaletsky 2000). Eight consistently amplifying microsatellite loci (Dae04, Dae05, Dae06, Dae07, Dae09-1, Dae09-2, Dae11 and Dae15; Table 2) were tested for cross-amplification in 13 *Dryopteris* species and hybrids of various ploidy levels (*D. oligodonta*, *D. oreades*, *D. affinis* subsp. *affinis*, *D. carthusiana*, *D. corleyi*, *D. crispifolia*, *D. dilatata*, *D. filix-mas*, *D. guanchica*, *D. ×arecesiae*, *D. ×asturiensis, D. ×fraser-jenkinsii*, *D. ×madalenae*), three other Dryopteridaceae species (*Polystichum lonchitis*, *P. setiferum* and *P. aculeatum*), and two species of Woodsiaceae (*Gymnocarpium dryopteris* and *Athyrium filix-femina*). Five of these loci (Dae06, Dae07, Dae09-2, Dae11 and Dae15) were finally used to genotype all *D. aemula* individuals sampled. As primer pair Dae09 seemed to amplify two loci, with two distinct amplification products consistently appearing 7-39 bp apart, the presence of an AG microsatellite in Dae09-2 was proven by sequencing of PCR products cut from 2 % agarose gels using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Forward primers of each primer pair were labeled with a fluorescent dye (6FAM, NED, PET or VIC; Applied Biosystems). Multiplexed PCR amplifications were performed with 20 to 40 ng of genomic DNA using the Multiplex PCR Kit (Qiagen) in final reaction volumes of 10 µl. PCR profiles consisted on 15 min of initial denaturation at 95 ºC, followed by 30 cycles of 94 ºC for 30 s, 56-62 ºC (depending on primer pair; Table 2) for 90 s and 72 ºC for 60 s, and a final elongation step at 72 ºC for 30 min. PCR products were separated on an ABI 3130 Avant automated sequencer (Applied Biosystems), and fragment sizes determined with GENEMAPPER 3.7 (Applied Biosystems) using the internal size standards GeneScan™ 400HD ROX™ for primer pair Dae15 and GeneScan™ 500 LIZ® (Applied Biosystems) for all other primer pairs.

Statistical analyses

Levels of genetic diversity were assessed by the average number of alleles per locus (A) and, for comparison with allozyme studies, overall total heterozygosity (H_T). Genetic differentiation among populations was assessed with F_{ST} (Weir and Cockerham 1984). Population structure was also studied by estimating R_{ST}, an analogue of F_{ST} developed for microsatellite loci mutating under the stepwise mutation model (Slatkin 1995). The number of reproductively successful migrants per generation (Nm) was estimated using the private allele method (Barton and Slatkin 1986). The observed heterozygosity (H_o), the expected heterozygosity (H_e) under Hardy-Weinberg equilibrium, and Wright's (1943) fixation index $F = 1 - H_o / H_e$ were calculated for each locus in each population. Hardy-Weinberg equilibrium was tested using Chi-squared tests. Linkage disequilibrium was tested among all pairs of loci and its significance determined after applying sequential Bonferroni correction (Rice 1989). All statistics except H_T and R_{ST} were estimated with GENEPOP 4.0 (Rousset 2008). H_T was calculated according to Nei (1973), and R_{ST} was estimated with FSTAT 2.9.3.2 (Goudet 2002). The spatial autocorrelation of genotypes in population EUM was explored using Moran's I in SGS (1000 permutations; Degen et al. 2001) taking into consideration the four loci polymorphic in this population and ten distance classes of 3 m width between 0 m to 30 m.

RESULTS

Microsatellite development and cross-taxa amplification

Out of the 18 primer pairs tested in *D. aemula*, seven failed to amplify the target microsatellites and four amplified several fragments, whereas seven amplified products consistently amenable to interpretation. One of these primer pairs (Dae04) amplified monomorphic products, whereas the other six (Dae05, Dae06, Dae07, Dae09, Dae11, and Dae15) amplified polymorphic products (Table 2). Primer pair Dae09 amplified two microsatellite loci (Dae09-1 and Dae09-2), which showed fragments varying in size according to an AG-microsatellite.

Transferability to other *Dryopteris* species and hybrids, especially those including an "aemula" parental genome, was feasible for most microsatellites isolated from *D. aemula* (Table S1). Transferability to *Polystichum* species was limited and there was no cross-amplification to *Gymnocarpium* and *Athyrium*. Locus Dae04, which was monomorphic in *D. aemula*, showed polymorphisms in other species.

Overall genetic variation

Total genetic variation considering all loci was $H_T = 0.447$. Overall, the Azorean population (AZO) showed a higher microsatellite variation than the three Iberian populations (EUM, MUT and SAN; Table S2). The mean number of alleles per locus (*A*) in AZO, EUM, MUT and SAN was 5.0, 2.6, 2.2 and 2.0, respectively, with an average of 3.0. In these populations, the number of private alleles was 13, one, three and two, respectively.

Among-population genetic structure

Genetic differentiation was high ($F_{ST} = 0.520$), and the value of R_{ST} (0.409) was lower than that of F_{ST}, although still high. In consequence, the estimated number of migrants per generation was low ($Nm = 0.25$).

Hardy-Weinberg and linkage equilibria

All loci significantly departed from Hardy-Weinberg equilibrium in all populations, exhibiting a remarkable deficit of heterozygotes as shown by the positive *F* fixation indexes, which ranged from 0.650 to 1.000 (Table 3) and had a value of 0.855 averaged over all loci and populations. Loci pairs Dae09-2/Dae11 and Dae11/Dae15 were in linkage disequilibrium ($P < 0.05$ after sequential Bonferroni correction) in some, but not all populations: loci Dae02-2 and Dae11 were linked in SAN, and loci Dae11 and Dae15 in SAN and MUT.

Within-population genetic structure

In population EUM, all values of mean Moran's *I* per distance class fell between the confidence interval of the correlogram, indicating no significant spatial autocorrelation at any distance class (Fig. 1). Hence, there was no spatial genetic structure within this population.

DISCUSSION

Microsatellite development and cross-taxa amplification

Only seven out of 18 originally designed primer pairs for *D. aemula* consistently amplified interpretable products, a result comparable to the attrition rates reported by Squirrell et al. (2003) for primer optimization steps. Primer pair Dae09 amplified two putatively independent loci, Dae09-1 and Dae09-2, and it appeared that Dae09-2 was a duplicated locus. This locus duplication was probably a consequence of repeated polyploidization and gene silencing

cycles in the evolution of genus *Dryopteris*, as reported formerly for many ferns (Nakazato et al. 2008).

The microsatellite loci developed for *D. aemula* were largely transferable to other species and hybrids of the genus and, to some extent, to other Dryopteridaceae. Product amplifications were more successful in taxa which incorporated at least one "aemula" parental genome, indicating that the flanking regions of these microsatellites were largely conserved. The microsatellites presented herein have thus the potential to be succesfully used in other taxa phylogenetically close to *D. aemula*.

Overall genetic variation

In agreement with our first hypothesis, total genetic variation was low (H_T = 0.447). Even if the value of H_T is similar to those reported for ferns in allozyme studies (Gitzendanner and Soltis 2000), microsatellites are likely to show much higher values due to their high allelic varialibity (Hedrick 1999, Woodhead et al. 2005). In the Iberian populations, the maximum number of microsatellite alleles per locus was four (Table S2), which indicates an overall low genetic diversity as compared to other microsatellite-based studies on species with restricted and scattered distribution ranges (Salgueiro et al. 2003, Mitrovski et al. 2007, Kang et al. 2008). Population AZO showed a maximum of seven microsatellite alleles per locus and was polymorphic for all five microsatellite loci studied, whereas populations SAN, MUT, and EUM were polymorphic for only a portion of them. In addition, the number and proportion of private alleles in population AZO was also larger than in the Iberian populations, and a large number of the alleles present in the Iberian populations were shared with population AZO (Table S2). These results generally reinforce the hypothesis of a recent strong genetic bottleneck in the Iberian populations of *D. aemula* due to a recent founder effect as suggested by the absence of allozyme diversity of *D. aemula* in the Iberian Peninsula (Jiménez et al., 2009). Rather than the persistence of *D. aemula* in Iberian refugia during Pleistocene glaciations (Hewitt 1996, Quintanilla et al. 2007), the higher genetic diversity of population AZO could suggest that the Macaronesian archipelagos acted as a glacial refugium from which *D. aemula* spread and recolonised mainland Europe. This hypothesis is not only congruent with the higher genetic diversity of population AZO and with the fact that a high proportion of the alleles are shared among Azorean and Iberian populations, but also with the finding that the identity of the most frequent allele differed among Iberian populations for loci Dae09-2, Dae11 and Dae15, as expected under a scenario of repeated but independent colonization events (Schneller and Holderegger 1996).

Among-population genetic structure

In marked disagreement with our second hypothesis, among-population genetic differentiation was very high, as shown by the values of both F_{ST} and R_{ST} (0.520 and 0.409, respectively). Populational differentiation is intimately linked to the frequency and magnitude of gene flow among populations (Loveless and Hamrick 1984) which, in ferns, takes place in the form of spore dispersal. Given the remarkable potential of homosporous ferns for long-distance dispersal and colonization, as illustrated by the fact that they are an important component of oceanic island floras (Muñoz et al. 2004, Ranker and Geiger 2008) and by empirical studies showing low population differentiation in other fern species (Sciarretta et al. 2005, Schneller and Liebst 2007), the marked differentiation among the four *D. aemula* populations studied seems surprising. Theory predicts that, in the long run, a migration rate of 1.0 is generally sufficient to offset drift-mediated population differentiation (Spieth 1974), and empirical studies suggest that even higher values of *Nm* are necessary to counteract drift (Lacy 1987, Mills and Allendorf 1996), but the effective number of migrants estimated for *D. aemula* was much lower with $Nm = 0.25$.

High population differentiation is well in agreement with the above presented colonization scenario of *D. aemula* on the Iberian Peninsula. However, other studies reporting high genetic population differentiation in ferns attributed it to the action of inbreeding. Substantial genetic differentiation among homosporous fern populations has, for instance, been observed in *Alsophila spinulosa* (Su et al. 2004), which has functionally bisexual, self-fertilizing gametophytes, as well as in the polyploid taxa *Asplenium septentrionale*, *A. ruta-muraria* and *Polypodium vulgare* (Schneller and Holderegger 1996), which are expected to self-fertilize due to polyploidy. Therefore, inbreeding might have contributed to high genetic population differentiation in *D. aemula*.

Hardy-Weinberg and linkage equilibria

Our third hypothesis, that populations of *D. aemula* were in Hardy-Weinberg equilibrium, was clearly rejected. All loci departed from Hardy-Weinberg equilibrium in all populations, and inbreeding coefficients were high, exhibiting heterozygote deficits. Several factors such as allelic dropout or null alleles can bias results obtained with microsatellites towards the inference of homozygous genotypes (Selkoe and Toonen 2006). However, the substantial deviation from Hardy-Weinberg equilibrium found in *D. aemula* congruent across loci and populations rather indicates that our results most likely point to inbreeding. In addition, the

linkage disequilibrium detected may also be a consequence of successive generations of inbreeding (Loveless and Hamrick 1984), which lead to the fixation of different multilocus genotypes within populations.

Averaged over loci and populations, the inbreeding coefficient detected in *D. aemula* (F = 0.855) is very high even for ferns (Ranker and Geiger 2008) and approaches those found in *Botrychium* species, which have subterranean gametophytes that obligately self-fertilize via intragametophytic selfing (McCauley et al. 1985, Hauk and Haufler 1999). Though *D. aemula* gametophtyes cultured under rich growth conditions produce male and female gametangia asynchronously (Jiménez et al. 2008), thus promoting outcrossing, culture experiments carried out under a wide range of environmental conditions showed that *D. aemula* gametophytes may spend a long phase in bisexual stage, opening a time window for self-fertilization (Jiménez et al., Capítulo 4).

The marked tendency to inbreeding observed in natural populations of *D. aemula* contrasts with most diploid ferns, which generally display high outbreeding rates (Ranker and Geiger 2008), and also with the substantial tendency to outcrossing in other diploid *Dryopteris* species such as *D. oreades* (Jiménez et al. 2008). High levels of inbreeding are, however, typical for polyploids and colonizing or invasive ferns (Lott et al. 2003, Flinn 2006, Ranker and Geiger 2008). Intragametophytic selfing greatly facilitates long-distance colonization, as it enables a single spore to found populations. In the absence of subsequent immigration of spores, such populations founded by single spores are genetically uniform and fully homozygous (Schneller and Holderegger 1996, Vogel et al. 1999). Thus, the high inbreeding coefficients observed in *D. aemula* populations might result from rather recent (post-glacial) colonization events, again congruent with the above mentioned colonisation of the Iberian Peninsula.

Within-population genetic structure

Genetic structure within populations is defined as the nonrandom spatial distribution of alleles or genotypes (Loveless and Hamrick 1984). In agreement with our fourth hypothesis, no significant spatial structure of genetic variation was observed in population EUM. However, this result conflicts with the results for highly inbred populations, which often present genetically structured populations (Loveless and Hamrick 1984, Soltis et al. 1989). It has been observed that the spore dispersal ability absolutely determines intrapopulational genetic structure in ferns (Cousens 1988, Soltis et al. 1989, Pryor et al. 2001). It seems that the habitat homogeneity of *D. aemula* populations, where microsites suitable for spore

germination and gametophyte development are abundant and randomly distributed in space, as well as the effective mixing of selfed genotypes by spore dispersal, largely determine the lack of genetic structure in *D. aemula*. Therefore, as observed in *Cheilanthes gracillima*, which exhibited genetically structured populations in spite of high outcrossing rates (Soltis et al. 1989), ecological factors may be more important than the breeding system in determining the genetic structure of *D. aemula* populations. Regardless, we can not completely rule out the possibility that the absence of spatial genetic structure in population EUM is partly consequence of using microsatellites, as very high mutation rates can reduce the estimates of spatial structure of populations (Epperson 2005).

Conclusions

Microsatellites revealed low levels of genetic diversity in *D. aemula*, as often observed in bottlenecked populations. Iberian populations harboured a lower number of alleles across loci than a Macaronesian population studied, probably as a consequence of post-glacial long-distance founder effects. Restricted gene flow among populations within the scattered distribution of *D. aemula* on the Iberian Peninsula maintained a strong genetic differentiation among populations, an effect reinforced by high inbreeding in this fern. Substantial heterozygote deficits showed that, despite its diploidy, *D. aemula* is in fact mostly inbreeding. Homogeneity of its habitats as well as effective spore dispersal over smaller distances caused, in spite of inbreeding, a lack of spatial genetic structure within a population of *D. aemula*.

The microsatellite loci reported here can potentially be used to design conservation strategies for the endangered *D. aemula*, to evaluate genetic diversity in other *Dryopteris* species or to clarify parental identities among diploid and polyploid members of this genus. Given the advantages of using microsatellites, we believe that this marker type will help in understanding the importance of evolutionary factors shaping population genetic variation and structure in ferns.

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TABLES AND FIGURES

Table 1. Populations studied with five microsatellite loci in *Dryopteris aemula*.

Acronym	Locality	N latitude	W longitude	Altitude (m a.s.l.)	Numbers of individuals
SAN	Spain, Asturias, Santianes del Agua	43º 25'	5º 20'	50	49
MUT	Spain, Gipuzkoa, Mutriku	43º 18'	2º 24'	90	50
EUM	Spain, A Coruña, Fragas do Eume	43º 23’	8º 00’	350	74
AZO	Portugal, Azores, São Miguel Island	37º 48’	25º 14’	950	56

Table 2. Characterization of eight microsatellites from *Dryopteris aemula*. GenBank accession number, repeat motif, size range in base pairs, annealing temperature (T_a), forward (F) and reverse primer (R) sequences are given for each locus.

Locus	Genbank accession number	Repeat motif	Size range (bp)	Primer sequences (5'- 3')	T_a (ºC)
Dae04	FJ455846	$(AAG)_{16}$	111	F: GCCTCCTTGAAGGTCTACCC	56
				R: CCCCCGGAAAAGAGTGTATG	
Dae05	FJ554839	$(CTT)_8$ (CTA) (CCT) $(CTT)_3$	102-108	F: ATGGTCACCTTCGACCTTTG	59
				R: TAACCGACCATGAGTCCTTG	
Dae06	FJ455847	$(TC)_{21}$	107-117	F: TGGCAAAAATAGAGAGAGGCAAC	59
				R: ATCAGGCGGCCAGTAGGAG	
Dae07	FJ455848	$(TG)_{22}$	77-101	F: GTACTACGCGCGCCACAAG	59
				R: CGTGAGCTTCTGTGAAGAGC	
Dae09-1	FJ455849	$(AG)_{13}$	95-97	F: CTTTGCAGGCGGCATAGC	56
				R: AGTTGACAAGAACTACTCGGATGC	
Dae09-2	FJ455849	$(AG)_{26}$	102-134	F: CTTTGCAGGCGGCATAGC	56
				R: AGTTGACAAGAACTACTCGGATGC	
Dae11	FJ455850	$(AG)_{21}$	151-157	F: TCAACTCCTTGTAAGCCCAAG	56
				R: CCGTGGACGGTAATAAACTACTC	
Dae15	FJ455851	$(AG)_{27}$	106-140	F: TATGTTGGTCTTGCGTGTGC	62
				R: AAACCATCAGTAGCCTCGACA	

Table 3. Observed (H_o) and expected (H_e) heterozygosity and fixation index (F) for four *Dryopteris aemula* populations from the Iberian Peninsula (SAN, MUT, EUM) and Azores (AZO). Asterisks indicate significant deviation from Hardy-Weinberg equilibrium; — : monomorphic within population.

Population	Locus	H_o	H_e	F
SAN	Dae06	0	0	—
	Dae07	0	0	—
	Dae09-2	0.122	0.595	0.795***
	Dae11	0.061	0.414	0.853***
	Dae15	0.102	0.484	0.789***
MUT	Dae06	0	0	—
	Dae07	0	0.04	1.000***
	Dae09-2	0	0	—
	Dae11	0.02	0.134	0.851***
	Dae15	0.02	0.170	0.882***
EUM	Dae06	0.027	0.153	0.824***
	Dae07	0.014	0.040	0.650***
	Dae09-2	0	0	—
	Dae11	0	0.027	1.000***
	Dae15	0.054	0.581	0.907***
AZO	Dae06	0.071	0.385	0.816***
	Dae07	0.089	0.604	0.853***
	Dae09-2	0.054	0.444	0.878***
	Dae11	0.089	0.400	0.778***
	Dae15	0.018	0.336	0.946***

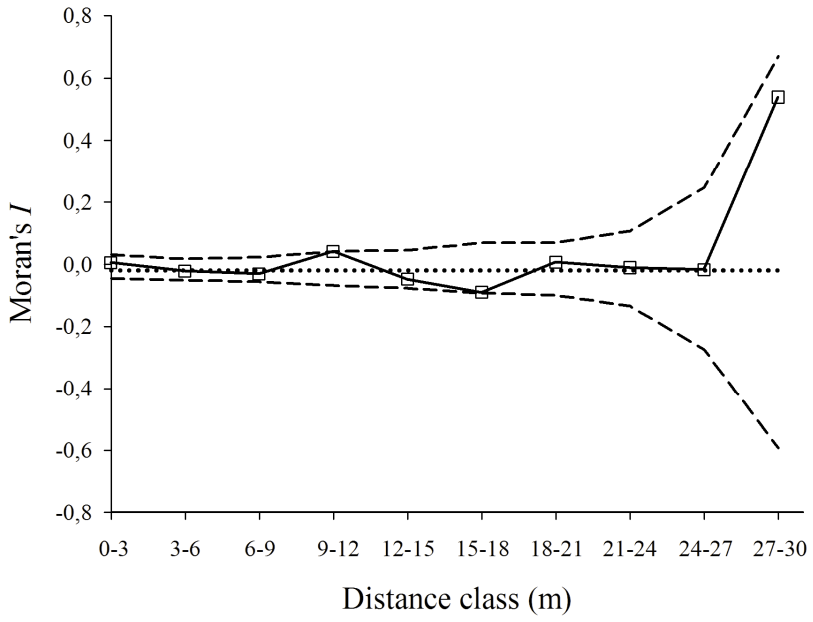
Table S1. Cross-amplification and fragment size of *Dryopteris aemula* microsatellites in 18 other fern taxa and hybrids. The number of individuals genotyped (n) and an indication of ambiguous, multiple or weak amplification products (±) and no amplification (–) are also given. *Dryopteris* genome constitution abbreviations: A: aemula; C: caucasica; E: expansa; G: oligodonta; H: hypothetical "pure affinis" ancestor; I_a: intermedia subsp. azorica; I_i: intermedia subsp. intermedia; I_m: intermedia subsp. maderensis; O: oreades; S: hypothetical "semicristata" ancestor.

Taxon	*Dryopteris* genome	n	Dae04	Dae05	Dae06	Dae07	Dae09-1	Dae09-2	Dae11	Dae15
D. oligodonta	GG	2	111	103	–	125	–	109	–	–
						231		125		
						241		141		
						267				
D. oreades	OO	2	111	102	–	135	±	±	201	–
				120						
D. affinis subsp. *affinis*	OH	1	–	102	–	135	±	±	185	–
				105						
D. carthusiana	I_iI_iSS	1	±	–	–	94	±	±	±	–
D. corleyi	AAOO	3	111	102	109	99	95	109	151	116
						135			191	118
D. crispifolia	AAI_aI_a	1	111	102	113	77	95	131	153	114
D. dilatata	EEI_mI_m	1	±	–	–	111	±	±	–	–
D. filix-mas	CCOO	1	–	99	–	135	±	±	–	–
				120						
D. guanchica	AAI_mI_m	2	111	±	101	77	95	–	169	138
						91				
D. ×arecesiae	AAO	1	111	102	111	99	95	109	151	116
						135				
D. ×asturiensis	AOOH	2	111	102	109	99	95	109	151	116
					111	135			185	
D. ×fraser-jenkinsii	COOH	1	111	±	–	77	–	127	179	144
						93			185	
						135				
D. ×madalenae	AI_aI_a	1	111	102	113	77	95	131	153	114
						95				
Polystichum lonchitis		1	111	–	–	185	–	±	–	–
P. setiferum		1	111	–	–	–	–	±	–	–
			117							
P. aculeatum		1	–	–	–	171	–	±	–	–
Athyrium filix-femina		1	–	–	–	–	–	–	–	–
Gymnocarpium dryopteris		1	–	–	–	–	–	–	–	–

Table S2. Allele frequencies of five microsatellite loci in four *Dryopteris aemula* populations from the Iberian Peninsula and Azores. Give meaning of SAN, MUT, EUM and AZO.

		Population						Population			
Locus	Allele	SAN	MUT	EUM	AZO	Locus	Allele	SAN	MUT	EUM	AZO
Dae06	a	—	—	0.053	0.080	Dae11	a	0.255	0.930	0.986	0.746
	b	1.000	1.000	0.920	0.045		b	0.724	—	0.014	0.228
	c	—	—	0.027	0.777		c	0.02	0.040	—	0.026
	d	—	—	—	0.080		d	—	0.030	—	—
	e	—	—	—	0.018						
						Dae15	a	—	—	—	0.035
Dae07	a	—	—	0.007	0.228		b	—	—	0.033	—
	b	—	—	—	0.193		c	—	0.020	—	—
	c	—	—	—	0.026		d	—	—	0.593	0.816
	d	1.000	0.980	0.980	0.553		e	0.602	0.050	0.140	0.018
	e	—	0.020	0.013	—		f	0.398	0.020	0.233	0.009
							g	—	0.910	—	—
Dae09-2	a	—	—	—	0.035		h	—	—	—	0.035
	b	0.214	1.000	1.000	0.719		i	—	—	—	0.035
	c	0.224	—	—	—		j	—	—	—	0.053
	d	0.561	—	—	—						
	e	—	—	—	0.079						
	f	—	—	—	0.140						
	g	—	—	—	0.009						
	h	—	—	—	0.018						

Figure 1. Moran's *I* correlogram (10 distance classes) for five microsatellite loci in population EUM of *Dryopteris aemula*. Values were not significant in any distance class. Given are the observed values (solid line with squares), the 95% confidence intervals (dashed lines), and the reference mean (dotted line).



CAPÍTULO 3

Reproductive and competitive interactions among gametophytes of the allotetraploid fern *Dryopteris corleyi* and its two diploid darents

Ares Jiménez[1], Luis G. Quintanilla[1], Santiago Pajarón[2], Emilia Pangua[2]

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[1]Departamento de Biología y Geología, Universidad Rey Juan Carlos, 28933 Móstoles, Spain

[2]Departamento de Biología Vegetal I, Universidad Complutense, 28040 Madrid, Spain.

SUMMARY

Several models predict that the establishment of polyploids within diploid populations is enhanced by nonrandom mating (i.e. selfing and assortative mating) of cytotypes and by a higher relative fitness of polyploids. This report assesses the role that antheridiogens (i.e. maleness-inducing pheromones) and intercytotype differences in growth rate have on polyploid performance. Three buckler-fern species were studied: the allotetraploid *Dryopteris corleyi* and its diploid parents, *D. aemula* and *D. oreades*. In one experiment, gametophytes of these species were cultured under rich growth conditions to compare the timing of gametangia production. The substrata on which these gametophytes had grown were used as antheridiogen sources in a second experiment. The three species were combined as source and target of antheridiogen (i.e. nine species pairs). Timing of antheridia production and gametophyte size were determined after those antheridiogen treatments. Under rich growth conditions the allotetraploid produced archegonia earlier than those of diploid parents. Female gametophytes of the three species produced antheridiogens that inhibited growth and favoured maleness both within and among species. Gametophyte size was similar in the three species but antheridia formed earlier in the allotetraploid. Unisexuality, promoted by nonspecific antheridiogens, enhances random mating both within and among species. The resulting hybridization can favour the reproductive exclusion of the allopolyploid in sites where it is outnumbered by diploids. However, the earlier production of gametangia in the allotetraploid favours assortative mating and may thus counterbalance reproductive exclusion.

INTRODUCTION

Polyploidy is a major evolutionary force in numerous eukaryotic and prokaryotic lineages (Masterson 1994, Otto and Whitton 2000, Tobiason and Seifert 2006). Polyploid organisms arise in low numbers within diploid parental populations. The minority cytotype exclusion model (Levin 1975) predicts that, in populations where individuals of different cytotypes mate randomly, newly arisen polyploids (i.e. neopolyploids) will be excluded if they can not counterbalance their frequency-dependent mating disadvantage, as a consequence of ineffective matings between minority diploid and majority haploid gametes. Several additional evolutionary models have analysed the effects of factors that can enhance polyploid establishment success, such as nonrandom mating among individuals of different ploidy level, higher relative fitness of polyploids, niche differentiation, recurrent polyploid formation, migration or stochastic events (Felber 1991, Rodríguez 1996, Rausch and Morgan 2005).

Two different types of nonrandom mating may facilitate the establishment of polyploids: assortative mating based on ploidy-related phenotypic traits (Husband and Sabara 2004) and selfing (Levin 1975, Rodríguez 1996, Rausch and Morgan 2005). Some recent studies on *Chamerion angustifolium* show that ploidy-related assortative mating can enhance the performance of autotetraploids (Husband and Sabara 2004, Kennedy et al. 2006), but more studies are necessary to demonstrate the importance of this factor on a broader range of taxa, including allopolyploids. Polyploids are expected to have increased levels of self-fertilization for several reasons (Barringer 2007). One of these reasons is that selfing would prevent neopolyploids from hybridizing with diploid individuals, circumventing the fitness disadvantage of being a minority cytotype. This prediction is supported by several empirical studies in angiosperms (Husband and Schemske 1997, Barringer 2007, but see Mable 2004) and homosporous ferns (Masuyama and Watano 1990).

Ferns are the vascular plant group with the highest proportion of polyploids (Grant 1981, but see also Vida 1976, and Soltis and Soltis 1989). Homosporous fern gametophytes are potentially hermaphroditic and may thus self-fertilize, producing completely homozygous sporophytes. However, there is evidence that homosporous ferns actually show environmental sex determination, i.e. the development of female, male or both sex organs largely depends on environmental conditions (Bull 1981, Korpelainen 1998). Mainly based on a high cost of female reproduction, Haig and Westoby (1988) predicted female gender expression under favourable growth conditions and male expression under poor growth conditions for homosporous fern gametophytes. In many species, gender is also environmentally determined by antheridiogens, i.e. maleness inducing pheromones. Antheridiogens are gibberellin-like

molecules released by archegoniate (female or hermaphrodite) gametophytes (Yamane 1998). These compounds inhibit vegetative growth and induce protandry (i.e. antheridium-first gender expression) on asexual gametophytes (Döpp 1950, Fernández et al. 1999, Tanurdzic and Banks 2004). Antheridiogens thus promote outcrossing among gametophytes. Antheridiogens induce maleness not only between conspecific gametophytes, but also between gametophytes of different fern species (Näf 1956, Chiou and Farrar 1997). In several allopolyploid complexes it has even been found that the gametophytes of polyploid taxa respond to the antheridiogens released by parental species and vice versa (Haufler and Ranker 1985, Pangua et al. 2003). Thus, antheridiogens may promote hybridization and favour reproductive exclusion.

The competitive interactions between allopolyploid species and their diploid relatives are poorly understood (Levin 2002). Allopolyploidization (interspecific hybridization either preceded or followed by chromosome doubling) can affect fitness-related traits through two mechanisms. First, chromosome doubling has biophysical effects such as increased nucleus and cell sizes, which can in turn affect whole-plant morphology (Stebbins 1971). Second, hybridization involves important genetic and epigenetic modifications (Wendel 2000), such as increased heterozygosity, which may increase vigour in some fitness-related traits (e.g. Soltis and Rieseberg 1986). The homosporous fern genus *Dryopteris* shows both types of effects as the spores of allotetraploid species are bigger (biophysical effect) and germinate faster (hybrid vigour) than those of diploid parental species (Quintanilla and Escudero 2006).

In this study two culture experiments were used to elucidate the reproductive and competitive interactions between the gametophytes of the allotetraploid *Dryopteris corleyi* Fraser-Jenkins and its diploid parental species, *D. aemula* (Aiton) O. Kuntze and *D. oreades* Fomin. These interactions may affect mating system and fitness, and therefore influence polyploid establishment. The following hypotheses were tested: (1) gametophytes of *D. corleyi* and of its two diploid parental species become female under rich growth conditions, as predicted by the Haig and Westoby (1988) model of sex expression; (2) female gametophytes of all three taxa release antheridiogens that reduce the growth rate and induce maleness among neighbouring gametophytes, both within and among species; (3) *D. corleyi* gametophytes show higher rates of vegetative and reproductive growth than the two diploid parental species.

MATERIALS AND METHODS

Study species

The three following buckler-ferns were studied: the allotetraploid *Dryopteris corleyi* ($2n = 4x = 164$; Fraser-Jenkins and Gibby 1986) and its diploid parental species, *D. aemula* ($2n = 2x = 82$; Manton 1950) and *D. oreades* ($2n = 2x = 82$; Manton 1950). *Dryopteris corleyi* originated from the hybrid *D. ×pseudoabbreviata* Jermy (*D. aemula* × *D. oreades*), as based on morphological and phytochemical traits (Fraser-Jenkins 1982, Fraser-Jenkins and Widén 1983) and allozyme markers (Jiménez et al. 2009). It is endemic to a narrow coastal strip of Northern Spain, where it inhabits eucalyptus and pine plantations and heathlands from 50 to 650 m a.s.l. (Salvo and Arrabal 1986). *Dryopteris aemula* lives in shady riparian forests from sea level to 900 m a.s.l., whereas *D. oreades* occurs in open, non-calcareous rocky areas and scree slopes from 600 to 2400 m a.s.l. (Salvo and Arrabal 1986). In some coastal mountains, the three species live in close proximity, especially *D. aemula* and *D. corleyi* which form the sterile hybrid *D. ×arecesiae* F.J. Pérez Carro & T.E. Díaz González (Pérez Carro and Díaz González 1990). Several traits suggest a recent origin of *D. corleyi*: low genetic divergence from *D. aemula* at the chloroplast DNA level (Geiger and Ranker 2005), presence of all allozymes of *D. corleyi* in its two parental species (Jiménez et al. 2009), high percentage of spore abortion (Quintanilla and Escudero 2006), narrow distribution range, and occupation of disturbed, potentially recent habitats (Mayor and Fernández 1988).

Plant material

Spores of the three study species were collected in Northern Spain from one population per species: *D. corleyi* in Asturias province, Pendueles; *D. aemula* in A Coruña province, Castro river; and *D. oreades* in León province, Laguna Ferreira (see Quintanilla and Escudero 2006 for further information on sampled populations and spore harvesting). Spores were stored in glass vials at 5 ºC, conditions appropriate for maintaining viability in these species (Quintanilla et al. 2002).

Experiment 1: Gender under rich growth conditions

Spores of each species were sown at high density in 5.5 cm-diameter Petri dishes containing sterilized mineral agar (Dyer 1979) to which Nystatin (100 units mL^{-1}) was added to minimize fungal contamination. Dishes were sealed with Parafilm and placed into a growth chamber with 16 ºC-light /10 ºC-dark temperatures and 13h photoperiod (daylight fluorescent tubes, photon irradiance 30–45 µmol m^{-2} s^{-1} within the 400–700 nm region). Temperatures

and photoperiod were chosen on the basis of late summer and early spring conditions in the studied populations (Quintanilla and Escudero 2006). Both seasons seem the most favourable for germination: in the former, spores are released, and the latter would represent the end of a hypothetical winter dormancy.

Eight weeks after sowing, 200 presexual gametophytes per species were transplanted to transparent plastic trays completely divided into 25 square cells with 2-cm sides (eight trays per species, 600 gametophytes in total). Into each cell, one gametophyte was transplanted on 3 mL of moist, autoclaved nutrient-rich commercial substratum (Compo Sana Semilleros, COMPO, Barcelona, Spain). Trays were sealed with Parafilm and placed in a growth chamber under the same conditions described above. Trays were periodically randomized to prevent possible effects of environmental heterogeneity within the chamber. For each gametophyte, gender (female, hermaphrodite, or male) was determined every three weeks using a dissection microscope until week 29. After gender determinations, each gametophyte was watered with 3-5 droplets of distilled water. Temporal differences among species required to start producing gametangia were analyzed with a Kruskal-Wallis test followed by Nemenyi tests (Zar 1999). Statistical analyses were conducted with SPSS (2003).

Experiment 2: Gender and size under antheridiogen effect

A second experiment was performed to study the intra- and interspecific effects of antheridiogen pheromone. The substrata of Experiment 1, pooled per species, were used as pheromone sources, as all gametophytes of the three species were female at the end of the experiment and had potentially released antheridiogens. To increase the quantity of exudates in the substrata, gametophytes were rinsed with 1 mL distilled water prior to removal. Each of the species-specific substrata was mixed with one third (volume) of fresh substratum to compensate for nutrient uptake by the removed gametophytes. The resulting substrata were autoclaved and stored at 5 ºC.

Dense gametophyte cultures were obtained as in Experiment 1. The substrata with the exudates of the three species were again distributed into trays divided into 25 square cells (2-cm sides, 3 mL substratum per cell). Eight-week old presexual gametophytes were transplanted into these trays, one gametophyte per cell, making up the nine possible antheridiogen source-target species combinations (i.e. *D. aemula – D. aemula*, *D. aemula – D. corleyi*, etc.). One hundred gametophytes were transplanted per source-target combination (four trays per combination, 900 gametophytes in total). Additionally, 50 asexual gametophytes per species were transplanted into cells with fresh antheridiogen-free

substratum. Trays were sealed with Parafilm, placed into the growth chamber under the same conditions as given above, and periodically randomized. For each antheridiogen-target gametophyte, gender was determined every two weeks until week 18. Percentages of male gametophytes were calculated on the basis of the four trays ($N = 4$) per antheridiogen source-target combination. In addition, gametophyte size was measured 12 and 18 weeks after spore sowing. At each date, 25 gametophytes grown without antheridiogens and 25 grown under antheridiogen influence were randomly sampled per species. For this variable, gametophytes of the three antheridiogen-source treatments were pooled per antheridiogen-target species. Images of sampled gametophytes were obtained with a high resolution scanner (94.5 pixel mm^{-1}), and their area was determined with ImageTool 3.0 (http://ddsdx.uthscsa.edu/dig/itdesc.html).

The effects of antheridiogen-source and antheridiogen-target species (both fixed) on arcsine-transformed percentage of male gametophytes were explored with a two-factor ANOVA. A posteriori pairwise comparisons were done with Tukey tests. Differences among target species in time required to initiate antheridia were analyzed with a Kruskal-Wallis test followed by Dunn tests for unbalanced data (Zar 1999). Gametophyte sizes were analyzed by ANOVA and Tukey tests with three fixed factors: antheridiogen (present or absent), antheridiogen-target species (*D. aemula*, *D. corleyi* and *D. oreades*) and time (12 and 18 weeks). All statistical analyses were done with SPSS (2003).

RESULTS

Experiment 1

All gametophytes of the three species became female during the 29 weeks of observation (Fig. 1). Time required to initiate archegonia differed significantly among species ($H = 184.56$, df = 2, $P < 0.001$, Kruskal-Wallis test). The allotetraploid *D. corleyi* became female earlier (species means ± SE, $N = 200$: 13.7 ± 0.1 week) than the two diploid species, *D. aemula* (15.9 ± 0.1 week) and *D. oreades* (15.9 ± 0.2 week). The two diploid species were not significantly different according to Nemenyi tests ($P < 0.05$).

Experiment 2

Most gametophytes became male in all the nine antheridiogen source-target species combinations. There were significant differences in the percentages of males among antheridiogen-source species and among antheridiogen-target species (Table 1). The source ×

target species interaction was not significant. Antheridiogen of *D. oreades* induced maleness in more gametophytes than *D. corleyi* antheridiogen, whereas maleness induction of *D. aemula* antheridiogens was not significantly different from those of *D. oreades* and *D. corleyi* (Fig. 2). Percentage of males in antheridiogen-target gametophytes decreased in the order *D. corleyi* > *D. oreades* > *D. aemula* (Fig. 2).

Time required to initiate antheridia differed among species ($H = 59.84$, df = 2, $P < 0.001$, Kruskal-Wallis test). *Dryopteris corleyi* became male significantly earlier (13.2 ± 0.1 weeks, $N = 300$) than *D. oreades* (13.7 ± 0.1 weeks, $N = 290$), which in turn became male earlier than *D. aemula* (14.6 ± 0.1 weeks, $N = 249$) ($P < 0.05$, Dunn tests).

In ANOVA of gametophyte sizes, all factors and interactions were significant except for target species and the target species × time interaction (Table 1). The antheridiogen × time × target species interaction means that differences of size among target species are not independent of the other two factors. At 12 weeks, there were no significant differences in gametophyte size between antheridiogen absent/present or between species (Fig. 3). At 18 weeks, gametophytes grown with antheridiogens were significantly smaller than those grown without antheridiogens. In the antheridiogen absent treatment, gametophyte size decreased in the order: *D. oreades* > *D. corleyi* > *D. aemula* (Fig. 3).

DISCUSSION

Archegonia-first strategy in rich growth conditions

The hypothesis that archegonia develop before antheridia under rich growth conditions (Haig and Westoby 1988) was supported by results for all three *Dryopteris* species studied in the present work. A similar archegonia-first strategy under good growth conditions has been observed in other diploid (e.g. *D. ludoviciana*, Cousens and Horner 1970) and allotetraploid (e.g. *D. filix-mas*, Barker and Willmot 1985, Korpelainen 1994) species of *Dryopteris* as well as in other fern groups (e.g. *Phegopteris decursive-pinnata*, Masuyama 1979; *Lygodium microphyllum*, Lott et al. 2003). Conversely, poor growth conditions (low nutrient availability and low light incidence) partially favor maleness in the three study species (Capítulo 4). According to theory, the relationship between plant size (growth rate) and gender expression may be adaptive because of the different costs of male (sperm production) and female (production of egg cells and young sporophyte development) functions. In brief, female function demands a higher energetic investment (Ghiselin 1969) and a longer time commitment (Paquin and Aarssen 2004).

Antheridiogen effects within and among species

In agreement with our second hypothesis, we found that female gametophytes of the three studied species produced antheridiogens which reduced growth rate and gametophyte size and induced maleness in asexual gametophytes within and among species. Maleness induction was more intense with *D. oreades* as antheridiogen-source species and with *D. corleyi* as antheridiogen-target species (Fig. 2). However, one should note that maleness induction was very high (> 80%) for all species irrespective of whether they acted as antheridiogen source or target.

Several models predict that polyploids have higher selfing rates than their diploid progenitors (reviewed by Barringer 2007). Our results do not support this prediction. The gametophytes of the three studied *Dryopteris* species had an initial unisexual phase, i.e. female under rich growth conditions and male under the effect of antheridiogens. Similarly, Cousens (1975) found high proportions of unisexual gametophytes in eight *Dryopteris* species, including some allopolyploids. The balance between intragametophytic selfing and intergametophytic mating can be expected to depend on the time periods of the different sexual phases, on whether or not they overlap, and on the length of the period of overlap (Cruden and Lloyd 1995). Unisexuality, promoted by antheridiogens, favours cross-fertilization in the studied species, or at least our results suggest that they have similar balances of intra- and intergametophytic mating. In contrast, experimental evidence in homosporous ferns indicates that polyploidy is associated with increased levels of selfing (Masuyama and Watano 1990, Suter et al. 2000, Pangua et al. 2003). In the genus *Dryopteris*, Flinn (2006) found higher intragametophytic selfing in the allotetraploid *D. carthusiana* than in one of its parental species, *D. intermedia.* However, a number of studies on angiosperms contradict this relationship between polyploidy and inbreeding (Husband and Schemske 1997, Galloway et al. 2003, Yeung et al. 2005). The most recent review of this topic did support increased selfing in allopolyploids but not in autopolyploids (Husband et al. 2008). The presumed recent origin of *D. corleyi* (see Material and Methods: Study species) may explain the proposed deviation from the breeding behaviour expected for allopolyploids. As suggested by Cook and Soltis (1999), if both diploid parental species were outcrossers, then the derived allotetraploid would inherit the traits responsible for cross-fertilization. For self-fertilization to evolve, substantial time would be required for selection to favour it (Cook and Soltis 1999). Genotype frequencies of *D. oreades* at allozyme loci show Hardy-Weinberg equilibrium, in accordance with random mating (Jiménez et al. 2009). In *D. aemula* and *D. corleyi*, however,

determining the mating system could not be accomplished owing to a lack of allozyme variation.

The nonspecific antheridiogen system that we found in the three study species could also favour the coexistence among females and males of different species, which might promote random mating among species. Antheridiogens would favour hybridization in multispecies gametophyte communities (Haufler and Ranker 1985, Schneller et al. 1990) and may thus select against the establishment of minority cytotypes (Levin 1975).

Intercytotype differences in growth rate

The third hypothesis, that gametophytes of the allotetraploid *D. corleyi* will have faster vegetative and reproductive growth than those of diploid parental species, was only partially supported by our results. In Experiment 2, the three species showed similar vegetative growth rates. The only exception was the antheridiogen treatment at week 18, in which the size of *D. corleyi* was intermediate between its diploid parental species (Fig. 3). However, in Experiment 1, archegonia appeared earlier in the allotetraploid than in the diploids. Similarly, in Experiment 2, the allotetraploid produced antheridia earlier than diploids. It can thus be concluded that reproductive –but not vegetative– growth rate is faster in *D. corleyi*. It has been shown that spore germination of some allotetraploid homosporous ferns is faster than that of their diploid parental species (e.g. *Dryopteris*, Whittier 1970; *Polypodium*, Kott and Peterson 1974). This pattern was also found in the three studied species (Quintanilla and Escudero 2006). However, it seems that the initial advantage of *D. corleyi* at germination is not sustained in subsequent vegetative growth of the gametophyte.

A shorter time to sexual maturity of the gametophytes of polyploid species has been observed in other ferns. For example, the allotetraploid *Polypodium virginianum* attains sexual maturity faster than one of its diploid parental species (Kott and Peterson 1974), and the autotetraploid *Phegopteris decursive-pinnata* produces sporophytes earlier than the diploid cytotype (Masuyama 1979). Early gametangia production found in *D. corleyi* in the present study may favour ploidy-related assortative mating. Two traits can promote germination of the studied species in the same time and place. First, spore dispersal of the three studied *Dryopteris* species occurs synchronously in the late summer and thus favours simultaneous germination (Quintanilla and Escudero 2006). Second, microsites suitable for the growth of gametophytes are rare and lead to a spatial clustering of different species (Cousens et al. 1985). However, following germination, the gametophytes of *D. corleyi* will produce archegonia and release antheridiogens earlier than its two parental species.

Furthermore, antheridia formation in response to antheridiogens will also be faster in *D. corleyi*. This time advantage can promote intraspecific mating in *D. corleyi*. Given that early sexual maturity reduces hybridization, it may allow *D. corleyi* gametophytes to circumvent minority cytotype exclusion (Levin 1975). This assortative fertilization we propose is analogous to the assortative pollination due to flowering asynchrony that has been observed in some autopolyploid flowering plants (Maceira et al. 1993, Husband and Sabara 2004).

Conclusions

The gametophytes of *D. corleyi* and its two diploid parental species show two traits with opposite effects for the establishment of polyploids. First, unisexuality, enhanced by nonspecific antheridiogens, favours random mating both within and among species. The resulting hybridization may hinder the persistence of *D. corleyi* in sites where it is outnumbered by diploid parental species. Second, earlier gametangia production by *D. corleyi* promotes intraspecific (assortative) mating and may therefore counterbalance minority cytotype exclusion. Future studies should test if these gametophyte traits observed under culture conditions also occur in nature. Additionally, analysing the distribution and abundance of different cytotypes in polyploid–diploid hybrid zones (Petit et al. 1999) could answer the questions of the importance of hybridization and competition among cytotypes, thus complementing experimental approaches (Baack 2004).

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TABLES AND FIGURES

Table 1. ANOVAs to explore the effects of antheridiogens on arcsine-transformed percentage of male gametophytes and gametophyte size of *Dryopteris* spp.

Variable	**Effect**	d.f.	m.s.	***F***	
Male gametophytes (%)	Source species	2	138.220	3.800	*
	Target species	2	1699.869	46.728	***
	Source × target species	4	55.019	1.512	n.s.
	Error	27	36.378		
Gametophyte size	Antheridiogen (present or absent)	1	4327.707	234.371	***
	Target species	2	43.199	2.340	n.s.
	Time	1	5126.119	277.609	***
	Antheridiogen × target species	2	63.428	3.435	*
	Antheridiogen × time	1	3971.059	215.056	***
	Target species × time	2	46.756	2.532	n.s.
	Antheridiogen × time × target species	2	68.493	3.709	*
	Error	288	18.465		

The analysis of the percentage of male gametophytes included the two fixed factors antheridiogen-source species and antheridiogen-target species (*D. aemula*, *D. oreades* and *D. corleyi*). The analysis of gametophyte size included the three fixed factors antheridiogen absent or present (data from the three source species were pooled), antheridiogen-target species (*D. aemula*, *D. oreades* and *D. corleyi*), and time (12 or 18 weeks). d.f., degrees of freedom; m.s., mean square; n.s., non significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 1. Means (± SE) of the percentages of female gametophytes for three *Dryopteris* species during a 29 week period. Each mean female percentage is for 8 trays ($N = 8$) of 25 gametophytes. No gametophyte became male or hermaphrodite.

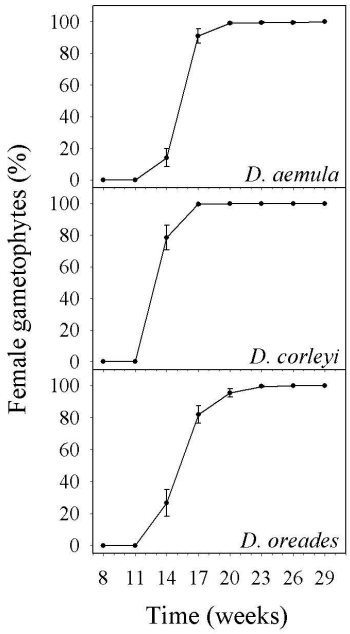


Figure 2. Means (+ SE) of the percentages of male gametophytes with different antheridiogen-source and antheridiogen-target *Dryopteris* species. Percentages were calculated on the basis of the four trays ($N = 4$) per antheridiogen source-target combination. Within each graph, different letters indicate significantly different means ($P < 0.05$, Tukey tests).

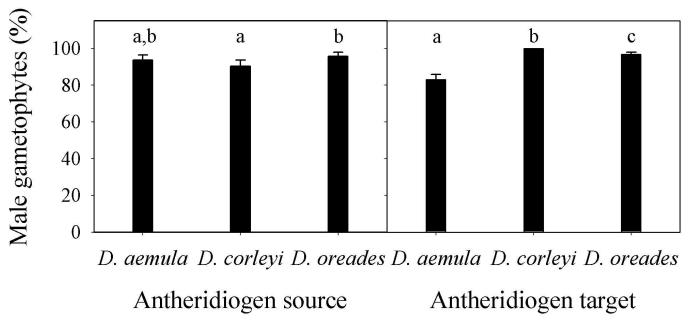
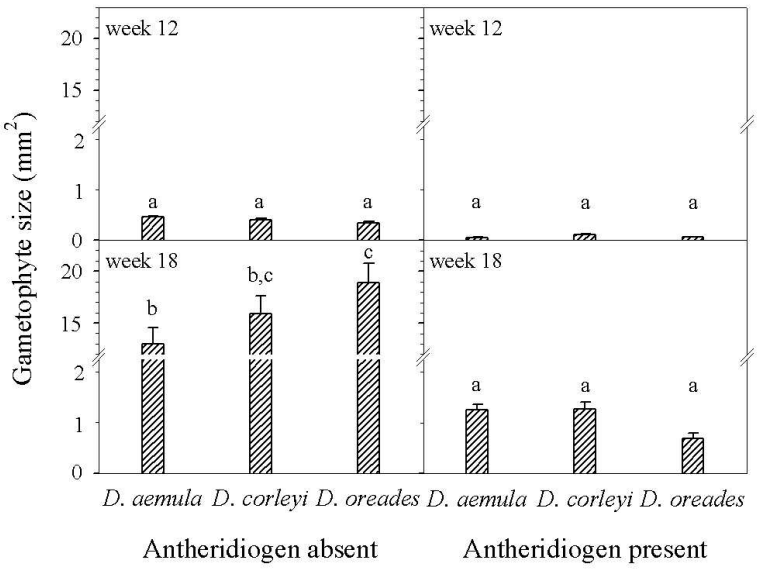


Figure 3. Means (+ SE) of gametophyte sizes of *Dryopteris* spp. in the presence or absence of antheridiogens at weeks 12 and 18. Different letters among graphs indicate significantly different means ($P < 0.05$, Tukey tests).



CAPÍTULO 4

Environmental sex determination in a diploid-polyploid complex of woodferns: the effects of nutrients and light availability on gametophyte development

A. Jiménez[1] and L. G. Quintanilla[1]

Manuscrito inédito.

[1]Departamento de Biología y Geología, Universidad Rey Juan Carlos, 28933 Móstoles, Spain.

SUMMARY

Homosporous ferns are the only group of vascular plants in which sex is mainly determined by the environment, but the effects of abiotic environmental factors such as nutrient and light availability on gametophyte growth and sex expression have received little attention. Here, we cultured gametophytes of three *Dryopteris* species which compose a diploid-polyploid complex with different breeding systems to study the effects of varying nutrient and light conditions on vegetative growth and sex expression. In agreement with the size advantage hypothesis, most females and hermaphrodites were bigger than males and asexuals, although there was no clear association between nutrient or light level and size. Gametophyte size was correlated with number or archegonia, but not with number of antheridia. Hermaphrodites were more frequent in inbreeding than in outcrossing species, although percentages varied among experimental levels, suggesting that both breeding systems and varying environmental conditions may determine different sexual ontogenies. Finally, the polyploid species showed an earlier maturation of female sex organs under some conditions unfavorable to vegetative growth, which may represent a competitive advantage respective to its diploid parental species.

INTRODUCTION

Sexual reproduction has been a key evolutionary milestone for the diversification of life, as it enables organisms to share and spread new genetic mutations. Despite the predominance and conservation of this trait across taxa, the mechanisms which ultimately determine reproduction as a male and/or female parent are remarkably diverse (Tanurdzic and Banks 2004, Haag and Doty 2005). All these mechanisms fall into two major groups: genotypic sex determination (GSD) and environmental sex determination (ESD). While GSD, such as our own XX/XY chromosome system, is generalized in animals and plants, many species are subject to ESD, where gender is decided at some point after fertilization following the extrinsic signals which come from the milieu (Korpelainen 1990). Light availability, concentration of nutrients in the medium, photoperiod, temperature, water availability, trauma, parasite effects and pH have been identified as major environmental determinants of sex (Freeman et al. 1980, Bierzychudek and Eckhart 1988, Korpelainen 1998).

Abundant empirical evidence shows that, in plants with labile sex expression, female-biased ratios predominate under more favorable or less stressful conditions, thus suggesting that favourable and unfavourable growth conditions promote, respectively, femaleness and maleness (e.g. Freeman et al. 1980, Bierzychudek and Eckhart 1988, Schlessman 1988). The core explanation for this recurrent observation is the size advantage hypothesis (SAH), which was advanced by Ghiselin (1969) and explains sex change on the basis of different fitnesses for each gender at each size or time. Following this hypothesis, organisms will mature sexually assuming the function which benefits more at a given size or age. However, gender will shift when individual size reaches a threshold in which the other sex increases its reproductive advantages more than the sex first expressed (Warner 1975, Charnov and Skúladóttir 2000, Munday et al. 2006). The translation of this hypothesis to plants involves that, given that female reproduction (ovule, seed and fruit production) is more costly than male reproduction (pollen production) nutrient- and effort-wise, it is more advantageous for small individuals to reproduce as males, and for large individuals to reproduce as females (Schlessman 1988).

Some traits such as the breeding system and ploidy level have an undeniable importance upon many aspects of plant reproduction. The breeding system of potentially bisexual organisms largely depends on their sexual ontogeny. Asynchronous maturation of the male and female sex organs, i.e. dichogamy, is regarded as an adaptation to promote cross-fertilizations and outbreeding, whereas the simultaneous maturation of both male and female sexes facilitates self-fertilizations and inbreeding (Bertin and Newman 1993). On the other

hand, polyploidy can also influence the reproductive biology of species, as genome doubling leads to increased nucleus and cell sizes, which in turn affects whole-individual morphology including reproductive structures (Stebbins 1971). Additionally, allopolyploids, which rise through interspecific hybridization in addition to genome doubling, can potentially show heterosis (hybrid vigor) because of genetic and epigenetic interactions between parental genomes, which may also influence the reproductive system (Pikaard 2001). Polyploids generally grow faster than related diploids in their earlier life stages, and therefore they reach sexual maturity earlier, and are also able to develop in more limiting environmental conditions (Levin 2002). Despite the relevance of breeding system and ploidy in plant reproduction, the influence of these factors on the patterns of sexual maturation under an ESD perspective has been barely explored.

Among plants, homosporous ferns are the only group where ESD is the rule, rather than the exception (Korpelainen 1998). Their diploid sporophytes produce a single kind of haploid spore which develops into a potentially bisexual gametophyte. The gender of gametophytes is labile, and the development of sex organs (gametangia), either male (antheridia) or female (archegonia), depends on environmental stimuli (Haig and Westoby 1988, Korpelainen 1998). Paradoxically, althought sexual lability makes homosporous fern gametophytes a suitable model to study ESD, few study cases focusing on the effects of particular environmental factors other than antheridiogens have been published. Antheridiogens, which are present in many homosporous fern species studied up to date (Yamane 1998, Schneller 2008), are gibberellin-related molecules which are released by archegoniate gametophytes and have a double effect on young, asexual gametophytes: they reduce vegetative growth and induce the formation of antheridia (Döpp 1950, Tanurdzic and Banks 2004, Jiménez et al. 2008). The uniqueness of this kind of sex-determining signalling among plants drew most of the early attention devoted to the reproductive biology of ferns (e.g. Döpp 1950, Näf 1956, Voeller 1964), whereas the influence of other environmental factors such as light and nutrient availability was practically ignored until recently (e.g. Guillon and Raquin 2002, Guillon and Fievet 2003, Huang et al. 2004, DeSoto et al. 2008). The few observations of the influence of non-antheridiogen factors on gametophyte sex determination seem to agree with the SAH: relatively large gametophytes tend to develop as females or hermaphrodites, whereas relatively small gametophytes tend to develop as males or remain asexual (Huang et al. 2004, DeSoto et al. 2008). Archegoniate gametophytes do not invest resources in seeds or fruits, but they do transfer nutrients to young sporophytes until they become functionally independent, which is more resource- and time-consuming than sperm formation (Haig and Westoby

1988). Additionally, it has been found that bigger sizes not only favor femaleness, but also that bigger females bear more archegonia than smaller ones whereas the number of antheridia is not correlated with male gametophyte size (DeSoto et al. 2008). This observation is in accordance with the theoretical expectations of Warner (1975) that female fitness, but not male fitness, is correlated with size in species where male size does not act as a selection factor for sexual reproduction.

The complex integrated by three homosporous fern species, the allopolyploid *Dryopteris corleyi* Fraser-Jenkins and its diploid parents *D. aemula* (Aiton) Kuntze and *D. oreades* Fomin, provides a suitable model to study the relationship between ESD, breeding system and ploidy level. As the effects of environmental resources limitation on gametophyte growth and sex determination are unknown for these three species, we pursued to test the following hypotheses: (i) under good growth conditions, gametophytes will tend to be female; (ii) gametophyte size will be positively correlated with the number of archegonia but not with the number of antheridia; (iii) the proportion of bisexual gametophytes will be greater in inbreeding species than in outbreeding species; and (iv) greater allopolyploid vigor will entail faster vegetative and reproductive growth.

MATERIAL AND METHODS

Study species

Dryopteris corleyi is an allotetraploid ($2n = 4x = 164$; Fraser-Jenkins and Gibby 1986) endemic to northern Spain, and occupies man-modified habitats, such as the understorey of eucalypt and pine plantations from 50 to 650 m a.s.l. (Salvo and Arrabal 1986). It derived from the diploids *D. aemula* ($2n = 2x = 82$; Manton 1950) and *D. oreades* ($2n = 2x = 82$; Manton 1950), as suggested by morphological and phytochemical traits (Fraser-Jenkins, 1982; Fraser-Jenkins and Widén, 1983) and by allozyme markers (Jiménez et al. 2009). *Dryopteris aemula* appears in scattered oceanic enclaves across Europe and Macaronesian archipelagos, where it inhabits temperate, north-oriented, deciduous riparian woodlands along narrow valleys from sea level to 900 m a.s.l. *Dryopteris oreades* can be found in the mountains of western, southern, central and eastern Europe. It inhabits open, non-calcareous rocky areas and screes which are covered by snow in winter, from 600 to 2400 m a.s.l.

The three species have an antheridiogen system, with asexual gametophytes developing into females when grown on antheridiogen-free substratum and into males when grown on antheridiogen-soaked substratum (Jiménez et al. 2008). Results obtained with genetic

molecular markers indicate that *D. oreades* is mainly outbreeding (Jiménez et al. 2009), whereas *D. aemula* has a strong tendency to self-fertilize (Jiménez et al., Capítulo 2). Low allozyme diversity in *D. corleyi* populations prevented assessing its breeding system (Jiménez et al. 2009), but the presence of heterozygous individuals in an allozymic locus suggest that this species is, at least partially, an outcrosser.

Plant material

Spores of the three study species were collected from one population per species in northern Spain: *D. corleyi* in Pendueles, Asturias province; *D. aemula* in Castro River, A Coruña province; and *D. oreades* in Laguna Ferreira, León province (see Quintanilla and Escudero, 2006 for further information on sampled populations and spore harvesting methods). Spores were kept in glass vials at 5 ºC, as described in Quintanilla et al. (2002), until the beginning of culture experiments.

Experimental treatments

We carried out two independent culture experiments: nutrient or light manipulation. Spores of the three study species were sown on agar containing the mineral medium of Dyer (1979) in 5.5-cm-diameter Petri dishes subsequently sealed with Parafilm (American National Can, Chicago, IL) to prevent drying. The dishes were incubated in a phytotron (13h photoperiod,16 ºC-light /10 ºC-dark temperatures) under a range of nutrient and light regimes. For the nutrient experiment, spores were sown on three dilution levels of Dyer mineral agar: undiluted, diluted 100 times, and diluted 10000 times, levels which have been reported to affect sex expression in *Woodwardia radicans* (DeSoto et al. 2008). In this experiment, the photosynthetically active radiation (PAR) was fixed at 60 µmol m^{-2} s^{-1}. For the light experiment, spores were sown on undiluted Dyer medium, with a series of light filters which allowed PARs of 40, 20, 10, 5 and 2.5 µmol m^{-2} s^{-1}. These light levels are similar to those reported to influence sex expression on *Equisetum* gametophytes (Guillon and Fievet 2003). Nine weeks after sowing, the resulting gametophytes, still asexual at that time, were transplanted to transparent plastic trays divided into 25 square cells of 4 cm^2 each (Bibby Sterilin; Barloworld Scientific, Stone, Staffordshire, UK), each cell containing 3 mL of culture medium. To exclude the effects of competition and pheromones (antheridiogens), only one gametophyte was transplanted to each cell, maintaining its corresponding light and nutrient levels. Eight gametophytes of each species (24 gametophytes total) were grown within each tray to avoid possible tray effects within species; the central cell of each 25-cell

tray was left empty. The trays were sealed with Parafilm and their location periodically randomized to minimize possible effects of environmental heterogeneity within the phytotron.

Gametophytes were grown until 24, 29 and 34 weeks after spore sowing, times at which one third of the gametophytes of each species were randomly sampled for each level and experiment. Total sample sizes were 1080 gametophytes for the nutrient experiment (3 species × 3 levels × 3 times × 40 gametophytes) and 1800 gametophytes for the light experiment (3 species × 5 levels × 3 times × 40 gametophytes). Sampled gametophytes were stained with acetocarmine-chloral hydrate (Edwards and Miller 1972) and mounted on microscope cover glasses to allow the examination of both sides under the light microscope. For each gametophyte, we determined gender (asexual, male, female or hermaphrodite), number of antheridia and number of archegonia using a light microscope. We also measured the area of each gametophyte by scanning gametophytes with a high resolution scanner and using the software ImageJ (Abramoff et al. 2004).

Statistical analyses

The effects of nutrient and light availability on gametophyte sex expression and size were separately assessed by fitting generalized linear models (GLMs) with the GENMOD prodecure of SAS 9.0 (SAS Institute 2002). These models were chosen because of unbalanced sample sizes and variables departing from a normal distribution. A multinomial distribution with cumulative logit link function was set for gender, and a normal distribution with identity link function was used for size. Models included the effects of species, treatment level and harvest time; all of these were considered as fixed effects. Gender was included as a fixed effect in the models for size.

The relationship between gametophyte size and number of gametangia (archegonia, antheridia, or archegonia + antheridia) for each gender was analyzed with Pearson correlation coefficient with SPSS 17.0 (SPSS 2008).

RESULTS

Nutrient experiment

Gender was significantly affected by the factors species, time and nutrient level, as well as by all their interactions except by the triple interaction time × nutrient level × species (Table 1). Level 1/100 showed higher female percentages than the other nutrient levels in the three species and times (Fig. 1). Overall, *D. corleyi* had more females than both diploids. The

percentage of hermaphrodites was higher in *D. aemula* than in *D. oreades* and *D. corleyi* in most nutrients levels in all times, although percentages varied widely among nutrient levels within each species (Fig. 1).

Size was significantly affected by time, gender and nutrient level, as well as by all the interactions between species, gender, and nutrient level (Table 1). Gametophytes cultured in level 1/100 were bigger than those cultured in levels 1 and 1/10000 in the three species and times (Fig. 2). Females and hermaphrodites were consistently larger than asexuals and males. Differences in size between females and hermaphrodites and between males and asexuals were not consistent and depended on time and nutrient level. Gametophytes of *D. corleyi* had similar sizes to those of both diploid species in all times and nutrient levels (Fig. 2).

Across nutrient levels, there were some significant relationships between gametophyte size and number of gametangia in the species studied (Table 2). For females, the number of archegonia increased with gametophyte size for all species. For males, the number of antheridia increased with size only for *D. corleyi*, whereas both variables were not correlated for *D. aemula* and *D. oreades*. For bisexuals, the number of antheridia did not increase with size for any species, whereas the number of archegonia and total gametangia increased with size in *D. aemula* and *D. corleyi*, but not in *D. oreades*. In general, *D. corleyi* gametophytes of all sexes had fewer gametangia than gametophytes of both diploid species (Table 2).

Light experiment

Gender was significantly affected by time and light level, as well as by all the interactions between species, time and light level except the triple interaction time × light level × species (Table 1). The only clear pattern of sex expression was a tendency towards more females in the lower light levels, especially in *D. oreades* and *D. corleyi* (Fig. 3). In all light levels and sampling dates, the percentage of hermaphrodites was generally similar in the three species, but *D. aemula* generally had fewer females (Fig. 3). Regardless, the observed differences were not significant (Table 1).

Size was significantly affected by species, time, gender and light level, as well as by all the interactions between species, gender, and light level except the triple interaction species × gender × light level. Gametophytes tended to be larger in higher light levels in all times and species (Fig. 4). Females and hermaphrodites were systematically larger than asexuals and males. Differences in size between females and hermaphrodites were not consistent and depended on time and light level, whereas male gametophytes generally had a similar or larger size than asexuals. On average, gametophytes of *D. oreades* were larger than those of

D. aemula and *D. corleyi* at the three sampling dates, although these differences depended on the light level (Fig. 4).

In this experiment, all relationships between gametophyte size and number of gametangia were significant (Table 2). Number of archegonia in females, of antheridia in males, and of archegonia, antheridia and total gametangia in hermaphrodites consistently increased with gametophyte size. In general, *D. corleyi* gametophytes of all sexes had fewer gametangia than gametophytes of both diploid species (Table 2).

DISCUSSION

The SAH in Dryopteris: do good growth conditions favor femaleness?

Gametophyte gender of the three species studied was correlated with size in both experiments (Table 1). In agreement with the SAH, and as observed in other empirical studies (Huang et al. 2004, Quintanilla et al. 2007, DeSoto et al. 2008), females and hermaphrodites were consistently larger than males and asexuals for all species, treatments and sampling times (Fig. 2, 4). As pointed out by Haig and Westoby (1988), big sizes enable gametophytes to reproduce as females because they will be able to nourish the young sporophyte. Conversely, small gametophytes have little reproductive resources and will gain more fitness if they reproduce as males. The similar sizes of females and hermaphrodites in our experiments suggest that hermaphrodites mostly developed from protogynous gametophytes, as hermaphrodites developing from males would be smaller than females (DeSoto et al. 2008). The most common explanation for this transition from female to hermaphrodite is that it serves as a reproductive assurance since self-fertilizing is better than not reproducing (Haig and Westoby 1988, Jarne and Charlesworth 1993). This mechanism of reproductive assurance gives predominantly outcrossing species the possibility to found populations from single spores via intragametophytic selfing (Soltis et al. 1988).

Even though there was a pattern in the relationship between gametophyte size and gender across species and experimental levels, the relationship of different experimental levels with size and gender was irregular. In general, high and low nutrient concentrations in the culture medium favored the formation of antheridia whereas intermediate nutrient concentrations favored the formation of archegonia, although the degree of response was species-dependent (Fig. 1). This observation departs from the expectations, as the number of male gametophytes should increase towards lower nutrient concentrations (Korpelainen 1994, DeSoto et al. 2008). Similarly, the results obtained in the light experiment also contradict the expectations

of decreasing femaleness and increasing maleness towards less luminous conditions (Guillon and Fievet 2003), as the ratio female/male gametophytes generally increased towards lower light levels in the three species (Fig. 3).

Even in very small concentrations, antheridiogens notably stunt vegetative growth of young, asexual gametophytes (Näf 1955, Stevens and Werth 1999), and induce them to produce antheridia even if gametophytes of the same species are mostly protogynous under rich growth conditions (e.g. Quintanilla et al. 2007, Jiménez et al. 2008). In our experiments, an almost darkness and lack of mineral nutrients were not able to produce significantly more males than less restrictive conditions, in contrast with the clear effects that antheridiogens have on gametophytes. There has been a debate for years between two conflicting opinions on the operating mechanism of antheridiogens. The first one says that antheridiogens especifically induce antheridia formation, and gametophytes decrease their growth rate as a consequence of resources being redirected to antheridia production (Näf 1956). The second one says that antheridiogens rather have an allelopathic effect which reduces growth and thus stimulates antheridia formation in other younger gametophytes (Korpelainen 1994). Even if our results can be considered as an indirect proof of the specific masculinizing effect of antheridiogens, there is the possibility that even the lowest nutrient and light levels of our experiments can not accurately emulate the growth-restrictive physiological effect of antheridiogens. Further laboratory studies including antheridiogens and wider ranges of environmental conditions seem necessary to dilucidate whether the effect of antheridiogens is especially concerned with sex determination or if it can be simulated by specific abiotic conditions.

Different fitness gains: do bigger gametophytes bear more gametangia?

Warner (1975) theoretically predicted that, if there is no size-based sexual selection for reproduction which allows males to capitalize on a bigger size, there will be a positive relationship between female fitness and size, but no correlation between size and male fitness. This pattern has been previously observed in *Woodwardia radicans*, where gametophyte size and number of archegonia, but not gametophyte size and number of antheridia, were correlated (DeSoto et al. 2008). In the nutrients experiment, the three study species were mainly in agreement with this prediction as the number of archegonia produced by females (a proxy of female fitness in homosporous ferns) increased with size, whereas the number of antheridia produced by males (a proxy of male fitness) was not significantly correlated with size (Table 2). The only exception were *D. corleyi* males, where number of antheridia was

significantly correlated with size. On the other hand, the results of the light experiment were not in accordance with the predictions, as the number of both archegonia and antheridia increased with size in the three species (Table 2). However, regardless of significance, Pearson's correlation statistic *r* still showed higher values for female-archegonia than for male-antheridia correlations in all cases, which points to a general agreement with our second hypothesis.

The influence of breeding system: do inbreeders tend to hermaphroditism?

In potentially bisexual organisms, the separate maturation of both sexes would favor cross-fertilization and outbreeding, whereas simultaneous maturation would favor self-fertilization and inbreeding (Bertin and Newman 1993). Consequently, simultaneous hermaphroditism should prevail in gametophytes of inbreeding species such as *D. aemula* to make self-fertilization more likely, whereas unisexuality should prevail in outbreeding species such as *D. oreades* and, possibly, *D. corleyi*, to promote cross-fertilization. This prediction was met in our work. In the nutrients experiment, the percentage of bisexual gametophytes was greater in *D. aemula* than in *D. oreades* and *D. corleyi* in most nutrient levels and sampling dates (Fig. 1). However, in the light experiment, all species showed similar percentages of bisexuals in most light levels and sampling dates. Althoug not statistically significant, the proportion of females was lower in the *D. aemula* than in the outcrosser *D. oreades* in most light levels and sampling times. In fern gametophytes, protoginy promotes outcrossing because of antheridiogen effects (Flinn 2006, Jiménez et al. 2008), which can explain this lower proportion of females in *D. aemula* in the light experiment.

None of the study species showed a uniform sexual ontogeny across experimental levels, which shifted from unisexuality to bisexuality or vice versa depending on nutrient and light conditions (Fig. 1, 3). The comparison of gametophyte sex of each species among experimental levels indicated that the degree to which different conditions affect sex expression may be species-dependent. For example, *Dryopteris aemula* gametophytes were mainly female in nutrient levels 1/100 and 1/10000, but predominantly male in nutrient level 1 and throughout all light levels (Fig. 1, 3). On the other hand, *Dryopteris oreades* gametophytes were predominantly female or hermaphrodite in nutrient levels 1 and 1/100 and all levels 40 to 10, but mostly male in nutrient level 1/10000. This result suggests that sex expression in *Dryopteris* is influenced by the dominant breeding system of each species, but that this influence depends, for its part, on the environmental conditions.

The influence of ploidy: do polyploids have reproductive advantages?

The bigger nucleus and cell sizes and the coexistence of two different genomes within the same organism can trigger different vegetative and sexual growth patterns in allopolyploids in comparison with their diploid parents (Stebbins 1971, Soltis and Rieseberg 1986, Wendel 2000). Quicker polyploid spore germination and gametophyte development has been previously documented in the fern genus *Polypodium* (Kott and Peterson 1974). A recent study also demonstrated ploidy-related differences between *D. corleyi* and its diploid parental species: although no differences in vegetative growth were detected between ploidies, *D. corleyi* produced both male and female gametangia earlier than *D. aemula* and *D. oreades* (Jiménez et al. 2008). In the nutrient and light experiments, *D. corleyi* gametophytes did not have a greater vegetative growth than both diploids for any experimental level, sex or sampling time (Fig. 2, 4). In angiosperms, tetraploid seedlings are bigger and grow faster than diploids, but this greater vigor does not carry over to the adult stage (Levin 2002). The same phenomenon seems to take place in *Dryopteris corleyi*, whose spores germinate earlier than those of *D. aemula* and *D. oreades* (Quintanilla and Escudero 2006). Young *D. corleyi* gametophytes were bigger than their haploid counterparts in all nutrients and light levels, but size differences disappeared after some weeks of culture (personal obervation). Therefore, monitoring gametophyte growth at a finer temporal scale than that used in the present work could reveal the possible advantages of *D. corleyi* over *D. aemula* and *D. oreades* during early gametophyte development.

In both experiments, *D. corleyi* did not show an earlier sexual maturation than its diploid parents for any experimental level or date, except for nutrient level 1 (Fig. 1, 3). In the latter level, which was clearly unfavorable for gametophyte development, a high proportion of *D. aemula* gametophytes remained asexual or became male and most *D. oreades* gametophytes remained asexual, whereas most *D. corleyi* gametophytes became female or hermaphrodite (Fig. 1). Earlier sexual maturation may be advantageous for the allopolyploid, which will outcompete nearby diploids by producing sporophytes first (Jiménez et al. 2008). This results is well in agreement with the generally acknowledged ability that tetraploids have to stablish in habitats unoccupied by related diploids (Levin 2002).

The allotetraploid *D. corleyi* consistently produced fewer archegonia, antheridia and total gametangia than its two diploid parents in both experiments (Table 2). These differences can be explained by the larger sizes of polyploid cells in comparison to diploids (Stebbins 1971). Fern gametangia are integrated by a low number of cells (Raghavan 1989), and gametangia of *D. corleyi* are consequently bigger than those of *D. aemula* and *D. orea*des (personal

observation). If sexually mature gametophytes of different ploidies have similar sizes (Fig. 2, 4), then polyploids should have fewer gametangia than diploids per area unit because their bigger gametangia will need more space to develop.

Conclusions

In general, the three *Dryopteris* species studied here support the size advantage hypothesis. We observed a clear relationship between gametophyte size and sex, with big gametophytes being mostly female or hermaphrodite and small gametophytes being mostly male or asexual. In most cases, the investment in female reproduction increased with size, whereas investment in male reproduction was independent of size. Additionally, this pioneer aproach to study the influence of the breeding system and ploidy level on the sexual ontogeny of species with labile sex determination showed that both factors can actually explain some interspecific differences in sex expression. Mostly inbreeding or outcrossing evolutionary histories dictate synchronous or asynchronous maturation of both sexes, respectively, but varying environmental conditions in this work modulated different sexual ontogenies. On the other hand, earlier maturation of female sex organs in *D. corleyi* under some poor growth conditions highlight the ability of this species to outcompete its diploid parents at the gametophyte stage.

The overwhelming majority of previous gametophyte culture experiments have been, and still are, conducted on nutrient-enriched agar under artifical light (Ranker and Houston 2002). Bearing in mind the uneven size and sex responses of gametophytes to decreasing nutrient and light conditions, we strongly suggest being cautious when drawing biological conclusions from culture experiments. Further works studying the effects that environmental factors have on the sexuality of ferns will undoubtedly help us to understand the sexual behaviour of these plants both in the laboratory and in their natural habitats.

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TABLES AND FIGURES

Table 1. Results of the GLMs for the effects of several levels of nutrient and light availability on sex and size of three *Dryopteris* species. Values in bold are significant.

Experiment	Variable	Effect in model	d.f.	χ^2	*P*
Nutrient	Gender	Species	2	139.66	**< 0.0001**
		Nutrient	2	24.99	**< 0.0001**
		Time	2	55.54	**< 0.0001**
		Species × nutrient	4	110.14	**< 0.0001**
		Species × time	4	25.57	**< 0.0001**
		Nutrient × time	4	34.88	**< 0.0001**
		Species × nutrient × time	8	2.84	0.9442
	Size	Species	2	3.51	0.1728
		Nutrient	2	102.48	**< 0.0001**
		Time	2	308.88	**< 0.0001**
		Gender	3	95.76	**< 0.0001**
		Species × nutrient	4	71.60	**< 0.0001**
		Species × gender	6	23.62	**0.0006**
		Nutrient × gender	6	35.18	**< 0.0001**
		Species × nutrient × gender	12	41.25	**< 0.0001**
Light	Gender	Species	2	2.72	0.2571
		Light	4	53.61	**< 0.0001**
		Time	2	176.25	**< 0.0001**
		Species × light	8	42.82	**< 0.0001**
		Species × time	4	18.84	**0.0008**
		Light × time	8	25.57	**0.0012**
		Species × light × time	16	19.54	0.2415
	Size	Species	2	126.35	**< 0.0001**
		Light	4	169.82	**< 0.0001**
		Time	2	240.78	**< 0.0001**
		Gender	3	632.11	**< 0.0001**
		Species × light	8	22.45	**0.0041**
		Species × gender	6	57.09	**< 0.0001**
		Light × gender	12	134.41	**< 0.0001**
		Species × light × gender	24	34.59	0.0748

Table 2. Number of gametangia per gametophyte (mean ± standard error) and Pearson correlation between number of gametangia and gametophyte size (*r*) for three *Dryopteris* species in nutrients and light availability experiments. Values in bold indicate significant correlations. Numbers in brackets represent sample sizes. Data of different sampling times and experimental levels were grouped for each experiment and species.

Gender	Nutrients			Light		
Gametangia	*D. aemula*	*D. oreades*	*D. corleyi*	*D. aemula*	*D. oreades*	*D. corleyi*
Female						
Archegonia	30.2 ± 2.4	20.9 ± 2.9	15.4 ± 1.3	20.2 ± 3.4	21.7 ± 3.9	7.8 ± (1.5)
	r = **0.575**	*r* = **0.530**	*r* = **0.581**	*r* = **0.911**	*r* = **0.777**	*r* = **0.798**
	(162)	(112)	(217)	(59)	(128)	(129)
Male						
Antheridia	21.7 ± 3.0	15.5 ± 3.1	18.5 ± 3.7	31.1 ± 3.6	25.3 ± (3.8)	24.2 ± (3.5)
	r = 0.141	*r* = 0.212	*r* = **0.438**	*r* = **0.529**	*r* = **0.248**	*r* = **0.550**
	(56)	(63)	(36)	(152)	(102)	(160)
Bisexual						
Archegonia	22.5 ± 2.4	8.0 ± 1.9	7.5 ± 1.9	15.5 ± 1.7	34.5 ± (3.6)	12.3 ± (2.3)
	r = **0.695**	*r* = 0.244	*r* = **0.653**	*r* = **0.775**	*r* = **0.831**	*r* = **0.845**
Antheridia	7.9 ± 1.2	17.5 ± 4.6	7.8 ± 1.7	22.1 ± 3.1	64.0 ± (8.5)	27.2 ± (3.6)
	r = -0.062	*r* = 0.074	*r* = 0.093	*r* = 0.263	*r* = **0.364**	*r* = **0.304**
Archegonia + antheridia	30.4 ± 2.3	25.4 ± 4.6	15.3 ± 2.5	37.6 ± 3.5	98.5 ± 10.2	39.5 ± (4.5)
	r = **0.670**	*r* = 0.177	*r* = **0.550**	*r* = **0.619**	*r* = **0.597**	*r* = **0.666**
	(94)	(19)	(32)	(127)	(138)	(102)

Figure 1. Percentages of gametophytes of each sex in three *Dryopteris* species cultured under varying nutrient conditions 24, 29 and 34 weeks after spore sowing. White bars, asexual; grey plain bars, male; black bars, female; grey striped bars, bisexual.

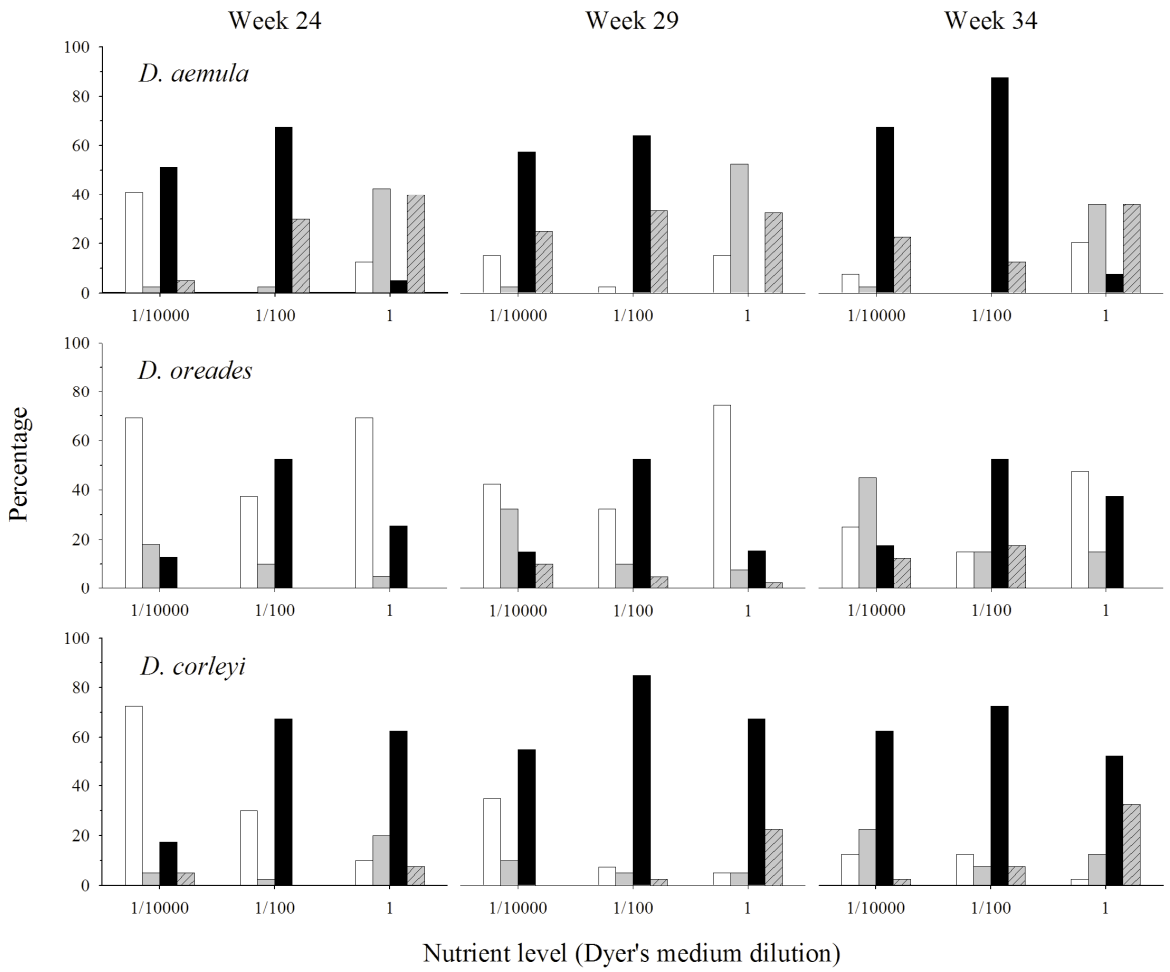


Figure 2. Gametophyte size (mean ± SE) in three *Dryopteris* species cultured under varying nutrient levels 24, 29 and 34 weeks after spore sowing. Black circles, asexual; white circles, male; black triangles, female; white triangles, hermaphrodite; squares, average over all sexes.

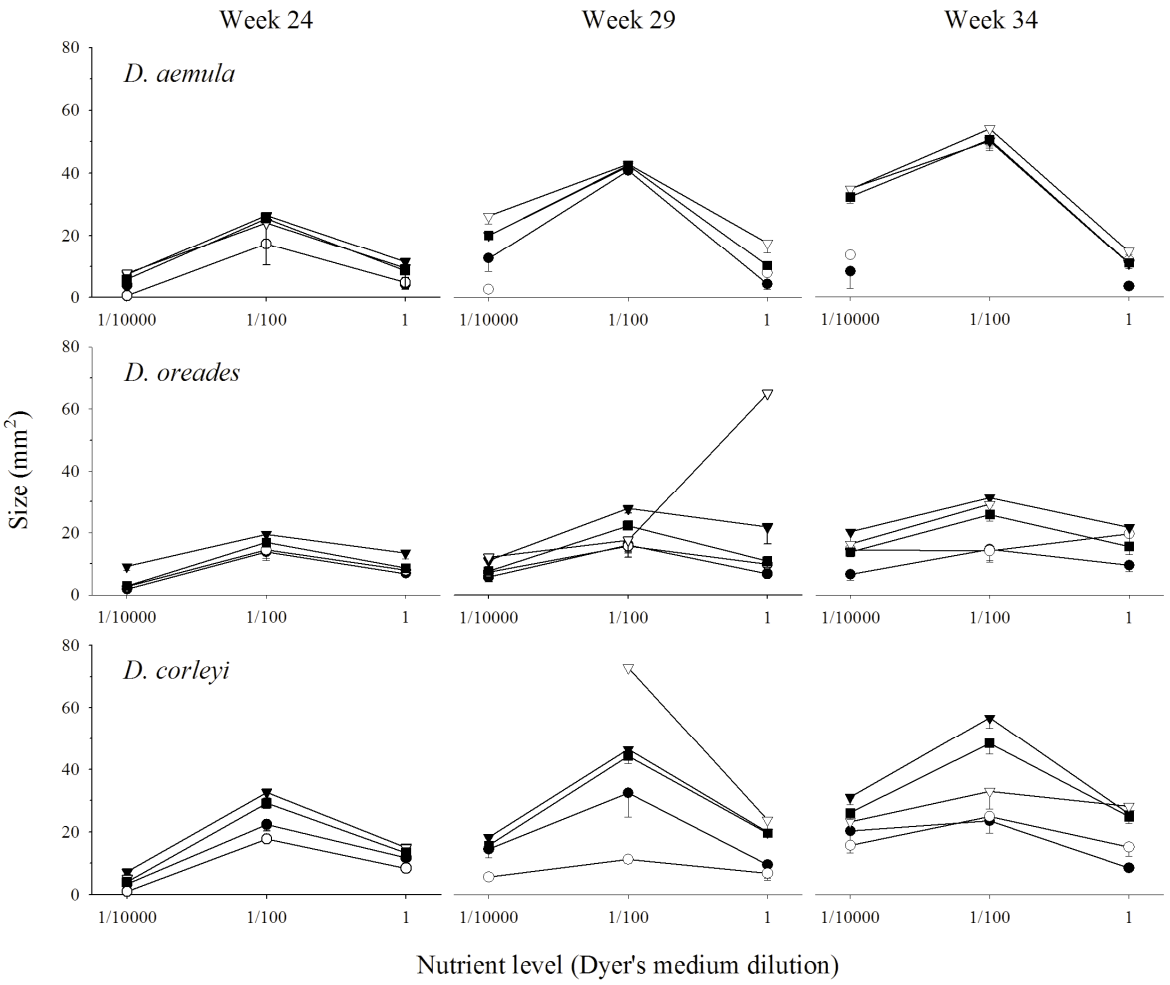


Figure 3. Percentages of gametophytes of each sex in three *Dryopteris* species cultured under varying nutrient conditions 24, 29 and 34 weeks after spore sowing. White bars, asexual; grey plain bars, male; black bars, female; grey striped bars, bisexual.

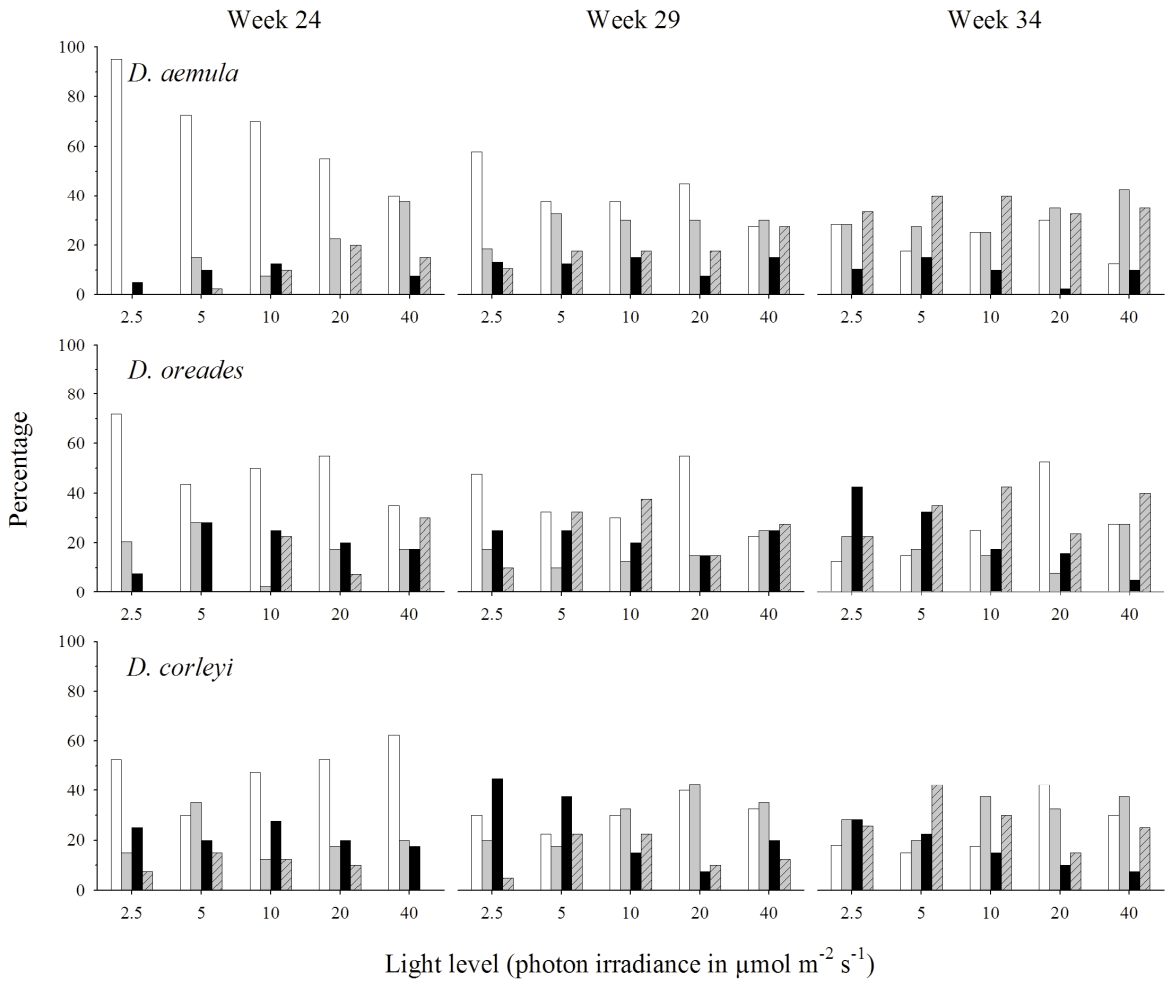
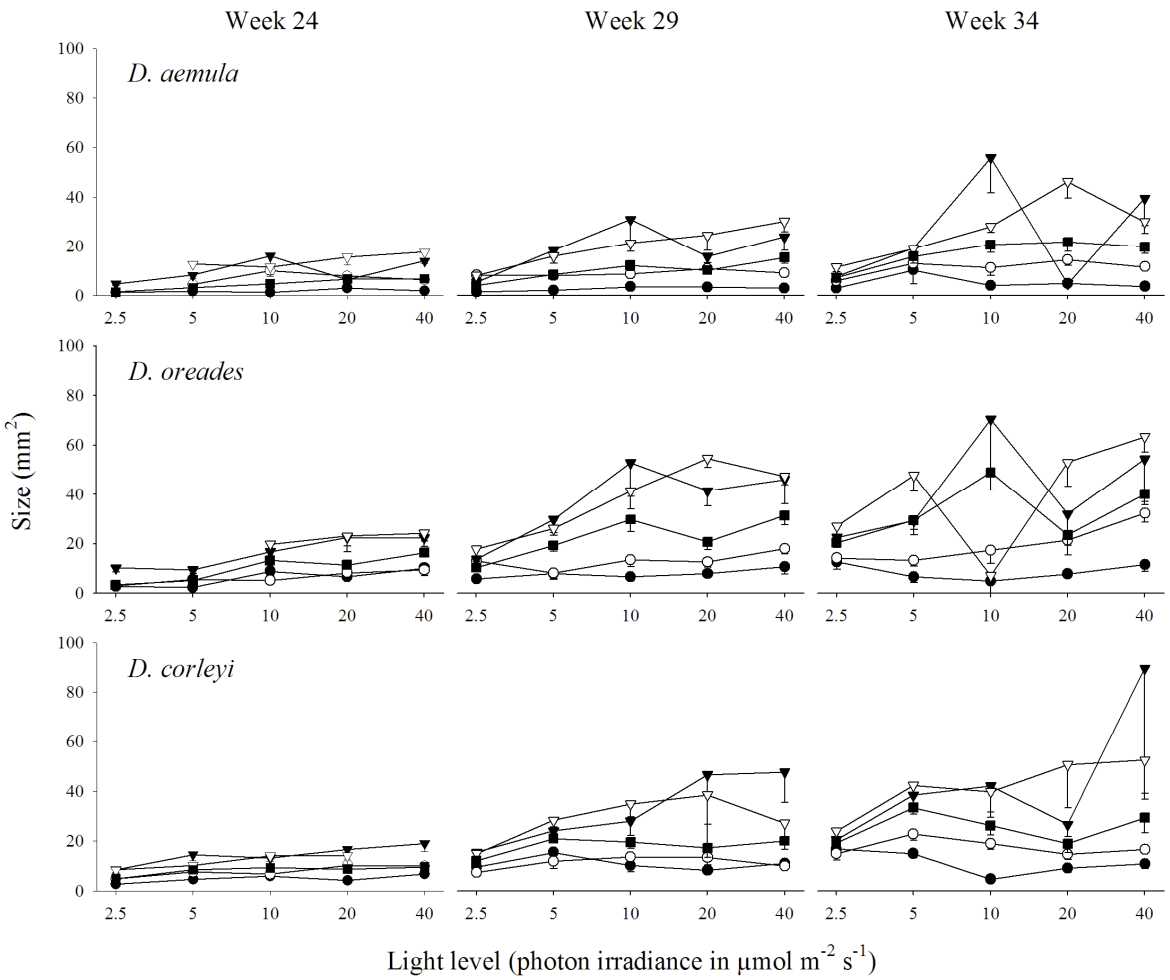


Figure 4. Gametophyte size (mean ± SE) in three *Dryopteris* species cultured under varying light levels 24, 29 and 34 weeks after spore sowing. Black circles, asexual; white circles, male; black triangles, female; white triangles, hermaphrodite; squares, average over all sexes.



CONCLUSIONS

1.- *Dryopteris corleyi* recently derived from *D. aemula* and *D. oreades*, as indicated by an allozymic pattern which is a combination of those of both parental species and by the absence in *D. corleyi* of "orphan alleles" not shared *D. aemula* and *D. oreades*. The existence of two different allozymic genotypes in *D. corleyi* suggests at least two different origins of this species from independent polyploidization events.

2.- *Dryopteris corleyi* has a low genetic diversity, with only one variable allozyme locus. The uneven distribution of different genotypes among the sampled populations suggests that either one of the genotypes had a recent origin and still has not had time enough to spread across populations or that these populations were founded by individuals with different genotypes.

3.- *Dryopteris oreades* populations harbour a considerable amount of allozyme diversity, which is probably a remnant of the greater number and size of *D. oreades* populations during the last Pleistocene glaciation. Despite the naturally fragmented distribution of this species, there seems to be a dynamic interpopulational gene flow which slows down genetic drift. Due to this connectivity, genetic differentiation among Iberian populations of *D. oreades* is low.

4.- Conversely, *D. aemula* shows clear signs of having suffered a recent genetic bottleneck. Allozymic patterns obtained for Iberian populations are monomorphic, and the data obtained with microsatellites confirm that genetic diversity in Iberian populations of this species is remarkably low. The greater genetic diversity observed in the Azorean population suggests that Macaronesian archipelagos acted as a glacial refugium for *D. aemula* from which it spread to continental Europe during the last interglacial period. Although genetic diversity in *D. aemula* populations does not seem to be spatially structured, interpopulational differentiation is high as a consequence of little gene flow among populations. It seems likely that the reason of this reduced gene flow is the marked tendency that this species has to inbreeding.

5.- The microsatellites developed for *D. aemula* are transferable to a large number of taxa related with this species, especially allopolyploids and hybrids incorporating at least one copy of the "aemula" genome.

6.- When cultured on good growth conditions, gametophytes of the three species tend to be female and release antheridiogens which induce maleness in asexual gametophytes. This

induction seems to be unspecific, as gametophytes of each species respond to its own antheridiogens and to those released by the other species. The observed unspecificity can be interpreted as a hybridization-promoting mechanism which selects against te establishment of the minority citotype in populations of mixed ploidies.

7.- In the three species, there is a clear relationship between gametophyte size and sex. Bigger gametophytes tend to be female or bisexual, whereas smaller ones tend to be male or asexual. However, the relationship between environmental conditions and sex is not so straightforward, as in some limiting conditions gametophytes are big and female, whereas in other theoretically favorable conditions gametophytes tend to be smaller and male. These observations reflect that, if the effect of antheridiogens is mediated by gametophyte size, none of the culture conditions used can accurately simulate the physiological effect of these substances.

8.- *Dryopteris corleyi* presents some traits which probably represent competitive advantages over its diploid parental species. Despite having similar sizes, *D. corleyi* gametophytes cultured under different conditions develop sex organs earlier than gametophytes *of D. aemula* and *D. oreades*. This earlier maturation can favour assortative mating in *D. corleyi* and, consequently, earlier fertilizations. The earlier sporophyte formation in *D. corleyi* could have counterbalanced the minority cytotype exclusion in mixed populations, thus favouring this species' stablishment.

9.- The species studied have different breeding systems. Allozymes and gametophyte culture experiments indicate that *D. oreades* is mostly an outcrosser, whereas microsatellites suggest that *D. aemula* is predominantly an inbreeder. The breeding system of *D. corleyi* remains unknown: in favourable conditions, its gametophytes develop archegonia and release antheridiogens, which favour cross-fertilizations, but this production of antheridiogens also takes place in *D. aemula* despite its tendency to inbreeding. Dilucidating the predominant breeding system of *D. corleyi* requires thus further studies.