VNIVERSITAT DE VALÈNCIA

La Genética en la conservación de especies vegetales: Estudio de la variabilidad genética intra e interespecífica en especies del género *Limonium* (Plumbaginaceae)

Memoria presentada por
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A mis padres, pues os debo lo que soy

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"Clasification makes organic diversity accesible to the other biological disciplines. Without it, most of them would be unable to give meaning to their findings."

Mayr & Ashlock

"Todas las plantas deberían de considerarse recursos fitogenéticos...Y quienes trabajamos con plantas en peligro, es bueno que lo hagamos con la conciencia de que nuestra actividad no es algo romántico o quijotesco, sino que con ella está directamente imbricado el futuro de la Humanidad".

César Gómez Campo

"Es muy importante que hagáis lo que de verdad os importe... sólo así podréis bendecir la vida cuando la muerte esté cerca".

Elisabeth Küber-Ross

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INTRODUCCIÓN Y OBJETIVOS

INTRODUCCIÓN

Es probable que los siglos XX y XXI se registren en el futuro como una de las mayores extinciones en masa desde finales del Cretácico, hace 65 millones de años, en que la mayoría de las especies que existían sobre la Tierra desaparecieron (Jablonski, 1986; Holsinger 1996). De hecho, se ha estimado que la tasa de extinción en este último siglo se ha incrementado hasta unas 5 veces sobre la existente en la mayoría de los 600 millones de años anteriores (Smith *et al.,* 1993). La causa, directa o indirecta, de este aumento de la pérdida de biodiversidad es la explosión en el crecimiento de la población humana global de 10 millones hace 10.000 años a 2,5 billones en 1950 y a 5.5 billones en 1993 (Avise 1996), con la consecuente destrucción de los espacios naturales y la fragmentación y degradación del hábitat. Así por ejemplo, para el caso particular de las plantas vasculares se ha calculado que casi el 25% de las 250.000 especies que existen en el mundo se extinguirán en los próximos 50 años (Schemske *et al.,* 1994). Las plantas ocupan un lugar clave en los ecosistemas terrestres, por lo que deberían también ocupar un lugar principal en las gestiones de conservación, más aún cuando la mayoría de especies raras o amenazadas son especies de plantas. Llegados a este punto cabría plantearse por qué es importante conservar la biodiversidad. Existen 2 argumentos principales a favor de la preservación de las especies (Norton, 1987). Una visión no antropocéntrica sería el problema ético que supone la desaparición de formas de vida que han evolucionado a lo largo de millones de años; una opinión más pragmática de la importancia de conservar la biodiversidad sería el valor potencial de todas las especies para el ser humano, tanto en la medicina, como en la agricultura, la industria... (Oldfield, 1984). Sin embargo, la sociedad occidental tiende a restringir cada vez más el número de especies que utiliza. Los países del norte y centro de Europa, al haber sufrido los efectos de las glaciaciaciones, han podido ofrecer al mundo muy pocos recursos fitogenéticos, pero trasladar esta mentalidad al Mediterráneo resulta absurdo, sobre todo si consideramos que en la Península Ibérica crece la mayor biodiversidad vegetal del continente europeo. Un ejemplo de ello lo representa el género *Limonium*; es cosmopolita, pero el mayor número de especies del mismo se concentran en el oeste de la región mediterránea (Erben, 1979). Este género presenta una serie de características que le hacen especialmente interesante para plantearse profundizar en su estudio, tanto desde el punto de vista evolutivo como de la conservación de sus especies. Los últimos avances de la Biología Molecular en la elaboración de herramientas conceptuales y empíricas para ahondar en la historia evolutiva presente y pasada de los distintos niveles de la biodiversidad han permitido resolver problemas antes inabordables tanto desde el punto de vista de la Filogenética como de la Genética de Poblaciones, ambas disciplinas clave en la Biología de la Conservación. Nosotros hemos aprovechado esta coyuntura para tratar de profundizar en distintos aspectos de la biología evolutiva del género *Limonium*, centrándonos particularmente en la sección Limonium, y para la posterior extrapolación de los resultados obtenidos a la conservación de sus especies.

LA SISTEMÁTICA MOLECULAR

La Sistemática Molecular estudia por medio del uso de marcadores moleculares tanto la variabilidad genética intraespecífica, tradicionalmente adscrita a la Genética de Poblaciones, como la diversidad interespecífica, tradicionalmente propia de la Sistemática Filogenética (Moritz & Hillis, 1996). Esta concepción integradora es fundamental para entender el sinergismo entre los estudios genéticos y de evolución molecular. La Sistemática Molecular usa los marcadores moleculares para inferir patrones microevolutivos (intraespecíficos) y macroevolutivos (interespecíficos). Estos patrones filogenéticos son usados en estudios de evolución para evaluar procesos, tasas, constricciones al cambio molecular a través del tiempo ((Kimura M, 1983); Gillespie, 1991; Li & Graur, 1991). A su vez, los resultados de estos estudios evolutivos proveen de más información para el uso de marcadores moleculares en los análisis filogenéticos y genético de poblacionales.

Perspectiva histórica

Esta conexión de la Sistemática y la Evolución Molecular está muy lejos de ser la visión tradicional. Sólo en los últimos años la Sistemática Molecular *per se* ha comenzado a considerarse una disciplina científica esencial, rica tanto empírica como conceptualmente (Avise, 1994).

La revolución que ha supuesto la integración de estas disciplinas no se inició hasta mediados de los 80. La causa de este retraso fue el hecho de que la Evolución Molecular, desde sus inicios a mediados de siglo, se ha visto dominada por una serie de controversias fundamentales sobre la naturaleza y significado evolutivo de la variación genética y su utilización para inferir relaciones evolutivas entre organismos. Estas controversias (ver a continuación), si bien han ayudado al desarrollo de conceptos y a entender mecanismos genéticos, retrasaron y desviaron la atención de la aplicación de los marcadores moleculares para elucidar la historia natural y la filogenética de los organismos.

El objetivo fundamental de la Filogenética es la identificación, descripción y clasificación de la diversidad. Las ideas de Linnaeus (1754) fueron esenciales para la formalización de la clasificación de los organismos, que hoy día continúa vigente. Cuando Darwin (1859) propuso la teoría de la evolución esa visión fijista del orden de las cosas cambió por completo y el principio metodológico de la clasificación natural pasó a ser estrictamente genealógico. Sin embargo, las clasificaciones continuaron basándose en las similitudes observadas a nivel morfológico y del desarrollo, sin haber una clara orientación filosófica en los aspectos de la evolución que debían ser reflejados en las clasificaciones. Sólo con el desarrollo teórico de la inferencia filogenética (Hennig, 1966) y con la disponibilidad de computadoras para la Taxonomía Numérica (Sokal & Sneath, 1963), la Sistemática tradicional (Simpson, 1961; Mayr, 1969) dio paso al enfrentamiento entre cladistas y fenetistas [revisado en Avise (1994) y Sneath (1995)]. A pesar de las críticas sufridas, la Taxonomía Numérica fue la que abrió el camino a la Sistemática Molecular. En cualquier

caso, ambas escuelas de clasificación han contribuido a la elaboración de las actuales aproximaciones para la reconstrucción filogenética (Swofford *et al.,* 1996, y ver también más adelante). Solapándose en gran medida con este debate, surgió la controversia asociada con el uso de marcadores moleculares en lugar de morfológicos para la reconstrucción filogenética (Patterson *et al.,* 1993). Recientemente parece que se ha entrado en una era más madura de la Filogenética, al reconocer que tanto los datos moleculares como morfológicos pueden ser igualmente informativos (p.e., Hillis, 1987; Sytsma, 1990; Donoghue & Sanderson, 1992; Wiens & Hillis, 1996). Sólo cuando la variación morfológica es limitada o la homología de los caracteres morfológicos está poco clara, pueden los caracteres moleculares ser la única vía de explorar la historia evolutiva de un grupo de organismos [p.e., en cetáceos marinos (Milinkovitch, 1995)]. De todas maneras, la delimitación de los taxones que van a ser estudiados con métodos moleculares se basa en muchos casos en caracteres morfológicos, por lo que estos no pueden ser nunca infravalorados (Donoghue & Sanderson, 1992).

Hasta mediados de los sesenta, la Genética de Poblaciones, por entonces una disciplina totalmente independiente, no entró en contacto de una manera clara con la Sistemática Molecular, y lo hizo a través de estudios de electroforesis en proteínas (alozimas) (Harris, 1966; Johnson *et al.,* 1966; Lewontin & Hubby, 1966). En un principio estos marcadores genéticos sirvieron para resolver problemas en relación con la teoría de la Genética de Poblaciones y proveyeron de información valiosa sobre la variación genética en poblaciones naturales, pero no se centraron en el contenido filogenético de la información molecular, en el estudio de los linajes génicos (Lewontin, 1985). Por el contrario, el descubrimiento de una mayor variabilidad genético-molecular de la predicha según la "escuela clásica" [Lewontin (1974) revisa la controversia entre esta escuela de pensamiento y la "escuela equilibradora"] estimuló el debate sobre el significado adaptativo de esta variación, que desembocó en una explicación alternativa a la misma, la teoría neoclásica o neutral de la evolución (Kimura, 1968; King & Jukes, 1969). La controversia neutralismo-seleccionismo continúa hoy día (Gillespie, 1991) y quizás, si hay una solución, ésta esté en un punto intermedio entre ambos polos (Powell, 1994).

El descubrimiento de otros marcadores genéticos asociados al DNA, especialmente los RFLPs (*Restriction Fragment Length Polymorphisms*) en sus distintas aplicaciones, y con ello el desarrollo de la Evolución Molecular (Nei, 1987), introdujeron una perspectiva histórica en la genética de poblaciones, en oposición a la perspectiva estática o de equilibrio previa (Felsenstein, 1982; Avise *et al.,* 1987). Las diferencias moleculares de los haplotipos o alelos se pueden relacionar jerárquicamente en las genealogías génicas o alélicas, como una filogenia (Avise, 1989). En los últimos años, el desarrollo de la teoría de la coalescencia [revisada por Hudson (1990) y Harvey *et al.* (1994)] y la filogeografía (Avise, 1989), por una parte, y en general el desarrollo de la estructura teórica de la genética de poblaciones molecular (Slatkin & Hudson, 1991; Felsenstein, 1992; Excoffier *et al.,* 1992; Templeton & Sing, 1993; Excoffier & Smouse 1994; por citar algunos ejemplos) han permitido la conexión definitiva entre la microevolución y la macroevolución, expandiendo la esfera de la

Sistemática a las genealogías intraespecíficas, la subestructura filogenética de la evolución (Fig. 1-1).

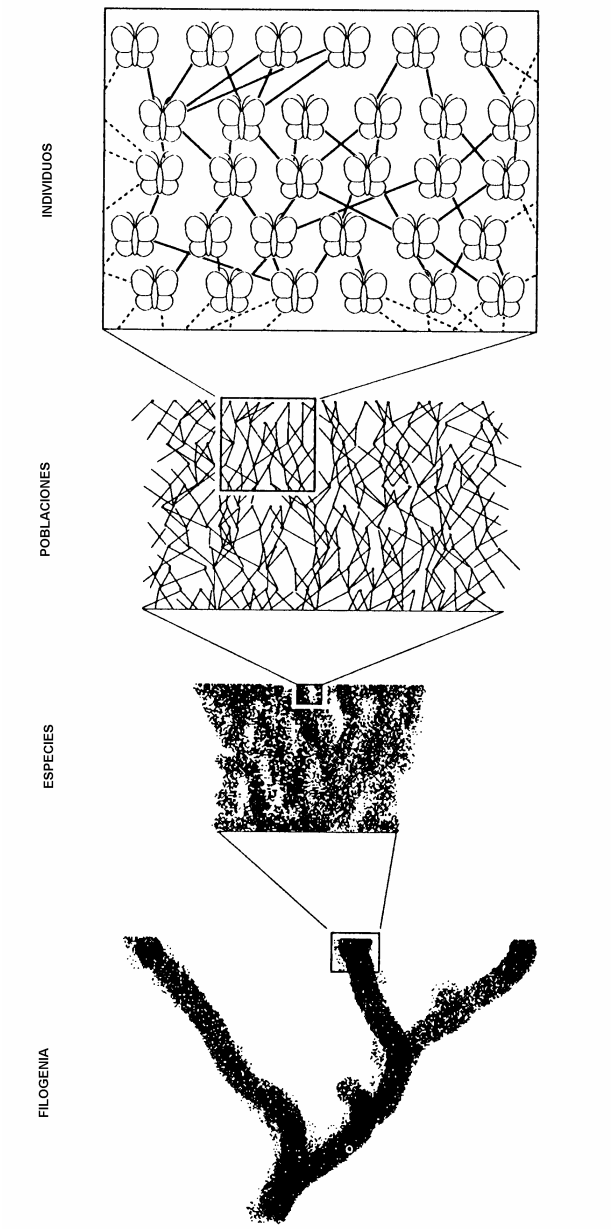


Fig. 1-1 Niveles de la Evolución: desde los individuos a los árboles filogenéticos (de Maddison y Maddison 1992)

HERRAMIENTAS DE LA SISTEMÁTICA MOLECULAR

La Sistemática Molecular necesita de herramientas para la inferencia de patrones y procesos micro y macroevolutivos. En todo estudio de este tipo la primera etapa consiste en la detección de la variabilidad genética usando alguno de los marcadores genético moleculares disponibles en el momento. Una vez extraída la información que estos marcadores pueden proporcionarnos es necesario analizarla adecuadamente. Generalmente, a la aparición de nuevos métodos empíricos le suele acompañar una evaluación exhaustiva de los métodos de

análisis de la diversidad génica que serán más apropiados para resolver problemas concretos de evolución y procesos históricos con ese nuevo marcador (Avise, 1994).

Marcadores genético moleculares

Los marcadores genéticos pueden diferir en su capacidad para integrar la genética de poblaciones y el análisis filogenético. Así, la secuenciación del DNA y el análisis de RFLPs, sí permiten una buena conexión entre ambas disciplinas al poder analizarse con estos métodos regiones del genoma con tasas de evolución diferentes, adecuándose a cada nivel. Por el contrario, con otros métodos, como la electroforesis isoenzimática o la hibridación DNA-DNA, no es posible la reconstrucción de genealogías génicas, por lo que es difícil que puedan beneficiarse de la interacción entre las dos disciplinas (Hillis *et al.,* 1996, cf. Sibley & Ahlquist, 1986). Asimismo, muchos de los métodos desarrollados recientemente para el análisis de la variación a nivel intraespecífico, como los de DNA *fingerprinting* basados en la PCR (Polymerase Chain Reaction) o los microsatélites, no permiten esta integración, pues la homología de los alelos detectados a niveles superiores es cuestionable (FitzSimmons *et al.*, 1995; Smith *et al.,* 1995).

La elección del marcador o marcadores moleculares apropiados para el sistema objeto de estudio dependerá de las características diferenciales de los mismos, de sus ventajas y desventajas, pero también de un compromiso basado muchas veces en necesidades opuestas (p.e., Karp & Edwards, 1997). Es importante destacar que la elección puede depender de lo que de antemano se sepa sobre ese grupo de estudio. Además, la mayoría de las veces no es posible trazar de antemano el esquema que se va a seguir, sino que a medida que se amplíe nuestro conocimiento del grupo y/o se desarrollen nuevas herramientas de la Sistemática Molecular, se irá a su vez viendo cuales de ellas son más adecuadas en los problemas biológicos que pretendamos abordar.

Más adelante se explican con más detalle aquellos marcadores moleculares que hemos utilizado para el análisis de la variabilidad genética intra e interespecífica en el género *Limonium*, que es el grupo de estudio en esta tesis.

Métodos de análisis de la diversidad genética

La nueva dimensión al estudio de patrones evolutivos aportada por la Sistemática Molecular ha supuesto no sólo su aplicación a nivel de la filogenia y la evolución de las especies, sino también al entendimiento de la distribución y la extensión de la variabilidad genética a nivel intra e interespecífico. La interpretación de la evolución molecular supone la utilización de aproximaciones alternativas dependiendo de la aplicación concreta y de la naturaleza de los propios datos. A continuación se pretende introducir las técnicas de análisis de la diversidad genética para la reconstrucción de genealogías génicas y los supuestos básicos de este tipo de análisis. También hemos considerado importante repasar los métodos para determinar la distribución de la diversidad génica a nivel intraespecífico y en especies con subdivisión poblacional.

Métodos de reconstrucción filogenética

Los métodos de inferencia filogenética tratan de reconstruir o estimar la historia evolutiva de un grupo de organismos basándose en información incompleta obtenida a partir del análisis de los datos proporcionados por las moléculas contemporáneas (Swofford *et al.,* 1996). La hipótesis resultante de esa inferencia se plasma en un árbol filogenético.

Dos supuestos básicos subyacen al uso de los datos en la reconstrucción de genealogías génicas. El primero es la independencia de los caracteres. Desde un punto de vista simplificado los datos moleculares son ciegos a la selección y, bajo la teoría neutral de la evolución (Kimura, 1983), los caracteres se comportarán como independientes. En realidad esta presunción es demasiado simple. El impacto de la selección en estudios sistemáticos dependerá de la proporción de marcadores afectados por ella, de la correlación entre loci, y de la robustez del método de análisis a desviaciones de la neutralidad. En general se supone que cuantos más loci examinados, menor será el efecto de la selección (Moritz y Hillis, 1996). Asimismo, los métodos de análisis suelen suponer no sólo esa independencia de los caracteres, sino también tasas iguales de cambio entre los caracteres o entre el estado de los mismos. Pero dentro de los genes existen efectos posicionales y patrones de sustitución no aleatorios, que violan los anteriores supuestos. Para resolver este problema se han desarrollado distintos métodos de ponderación de los caracteres, por ejemplo los que corrigen para cambios superpuestos (Nei, 1987; Tamura & Nei, 1993; Wakeley, 1994), o los que lo hacen para constricciones debidas a la formación de estructura secundaria en las moléculas de RNA (p.e., (Dixon & Hillis, 1993). Otros sirven para la ponderación del estado de los caracteres, como serían los métodos de corrección de la diferencia en la tasa de sustitución de transiciones y transversiones [p.e., la distancia de 2 parámetros de Kimura (Kimura M, 1980) o la parsimonia de transversión (Swofford *et al.,* 1996)], o en la probabilidad asimétrica de cambio en los sitios de restricción (Albert *et al.,* 1992). La suposición general de la homología de los caracteres es también crucial en el análisis filogenético. Por ejemplo, en el caso de las secuencias moleculares, por un lado se supone que las regiones comparadas a través de un grupo organismos son ortólogas (Fitch, 1970), por otro, el alineamiento de las mismas permite establecer las posiciones presuntamente homólogas entre taxones (Davidson D., 1985). La homología en los datos de algunos caracteres binarios, como los de las técnicas de DNA *fingerprinting*, se establece por la comparación de fragmentos, suponiéndose homólogos los de igual tamaño, de ahí la limitación en cuanto a la divergencia de las OTUs comparadas.

Otro problema de la inferencia filogenética es que el número de árboles filogenéticos que pueden potencialmente reflejar la verdadera historia evolutiva de un grupo de organismos alcanza cantidades astronómicas aún con un número de taxones relativamente bajo. Por el modo de seleccionar uno o más árboles de entre todo el conjunto de filogenias posibles, los métodos de inferencia filogenética se pueden dividir en dos grupos: a) los que lo hacen definiendo una serie de pasos (un algoritmo) que conduce a un solo árbol como resultado final; o b) los que definen un criterio para comparar filogenias alternativas y decidir cuál o cuáles son mejores.

Existen muchas revisiones más o menos recientes sobre el uso de los datos moleculares que nos hablan de estos métodos ((Felsenstein, 1988)1982, 1988; Nei, 1987; Swofford *et al.,* 1996; Avise, 1994).

En el primer grupo se incluyen todas las formas de análisis de cluster, que suponen ultrametricidad [p.e., UPGMA, Sokal & Sneath (1963)] y algunos métodos de árboles aditivos como neighbor-joining (Saitou & Nei, 1987). Brevemente, estos métodos parten de una matriz de distancias emparejadas, y a partir de ella construyen el árbol por adición sucesiva de OTUs según las reglas especificadas en el algoritmo, para a continuación calcular la longitud de las ramas que las conectan.

En el segundo grupo hay dos pasos. El primero es definir el criterio de optimización, y así tenemos: los métodos de máxima parsimonia (revisados en Swofford *et al.,* 1996) y los métodos basados en modelos de cambio evolutivo, entre estos últimos se incluyen el método de máxima verosimilitud (Felsenstein, 1981) y algunos métodos de distancias de árboles aditivos como (Fitch & Margoliash E., 1967) y otros relacionados (Felsenstein, 1982; (Rzhetsky, 1992). El segundo paso es el uso de algoritmos para encontrar el árbol o árboles óptimos según este criterio. Éstos pueden ser exactos (p.e., método de *branch-and-bound*) o heurísticos (p.e., método de *branch swapping*).

La elección de un método u otro para el análisis de los datos puede depender de varios factores, entre los que podemos destacar el tipo de marcador molecular y la naturaleza de los datos que nos proporcione, la "limpieza" de estos datos y las limitaciones computacionales (Swofford *et al.,* 1996).

Por último, siempre es deseable determinar los límites de confianza y la fiabilidad de las reconstrucciones filogenéticas obtenidas. La aproximación más comúnmente utilizada es el método de *bootstrapping* (Efron, 1982); (Felsenstein, 1985); (Hedges, 1992). Sin embargo, existen otros muchos tests estadísticos para ello, algunos de los cuales son aplicables en casos concretos. De ellos se tratará con más detalle a lo largo de la tesis.

Métodos de análisis de la estructura genético poblacional

El objetivo general de los estudios genético-poblacionales es cuantificar y caracterizar la variación genética dentro de una especie. La variación genética es el motor del cambio evolutivo, por lo que distintos niveles de variación en distintas poblaciones podrían suponer una evidencia de acontecimientos evolutivos pasados diferentes, así como del devenir futuro.

Los primeros análisis de los datos genético moleculares consisten en determinar el genotipo (o fenotipo, dependiendo del marcador utilizado) de cada individuo. A partir de los genotipos obtenidos se pueden establecer las relaciones entre ellos en forma de genealogías, utilizando los métodos de inferencia filogenéticos apuntados anteriormente. La filogenia resultante se puede relacionar con los patrones de distribución geográfica y elaborar hipótesis de la biogeografía histórica de las poblaciones, lo que en conjunto se conoce como *filogeografía intraespecífica* (Avise, 1987). La mayoría de métodos filogenéticos producen árboles dicotómicos (ver arriba). La reconstrucción de una red (*network*), con conexiones alternativas entre OTUs, parece ser mucho más realista bajo ciertas circunstancias. A nivel

intraespecífico, por ejemplo, es preferible la inferencia de las relaciones entre poblaciones en forma de árboles sólo cuando hay evidencias de independencia entre ellas; si no, se deben usar procedimientos alternativos, que no asuman una jerarquía (p.e., (Lessa, 1990); (Slatkin & Maddison, 1990); (Crandall & Templeton, 1996), para establecer las relaciones filobiogeográficas de las poblaciones (Moritz & Hillis, 1996).

En el siguiente nivel de análisis, el contaje de los genotipos lleva a obtener medidas de variación simples, como las frecuencias alélicas. En este sentido, y con el ya mencionado desarrollo de la teoría de la coalescencia, está adquiriendo importancia una trama teórica para tratar las genealogías génicas y las frecuencias alélicas y su distribución de una forma integrada. Esta nueva aproximación de las genealogías génicas ha probado ser muy útil para estudiar las relaciones genotipo-fenotipo ((Templeton *et al*, 1987), 1992), la selección natural ((Antonarakis S.E.C.D. *et al*, 1984); (Golding & Felsenstein, 1990); (Hartl & Sawyer, 1991)) y, lo que es más importante en nuestro caso, la estructura genético-poblacional tanto actual como histórica ((Avise JC *et al*, 1988); (Slatkin, 1989a); (Slatkin & Maddison, 1989b); Slatkin & Hudson, 1991; Excoffier *et al.,* 1992; (Neigel & Avise, 1993); Excoffier & Smouse, 1994).

A partir de la información genotípica se pueden determinar medidas de variación genética más complejas. Por ejemplo, la información de las distancias entre genotipos y sus frecuencias en las distintas poblaciones nos da una medida de diversidad génica entre y dentro de poblaciones [ver p.e., (Weir, 1990)), (González-Candelas F. & Palacios, 1997)]. La divergencia interpoblacional corregida se puede utilizar a su vez para, a través de los métodos mencionados anteriormente que parten de una matriz de distancias, representar las relaciones entre poblaciones en forma de dendrogramas. También es posible, a partir de estos datos, describir la estructuración genético-poblacional por medio de los estadísticos F de Wright ((Weir BS & Cockerham, 1984), o por derivados de estos como su extensión multialélica (Gst, Nei, 1987) o el análogo a nivel nucleotídico Nst (Lynch & Crease, 1990). El análisis de los estadísticos F en un contexto adecuado para el contraste de hipótesis se realiza en términos de un análisis de la varianza [ANOVA, Weir & Cockerham (1984), (Long, 1986)] que tiene en cuenta los distintos niveles de subdivisión poblacional (individuos, subpoblaciones y poblaciones). Excoffier *et al.* (1992) han introducido un método alternativo para partir la varianza genética en estos niveles jerárquicos: es el análisis molecular de la varianza (AMOVA). Este método utiliza información tanto de las distancias evolutivas entre genotipos como de las frecuencias de los mismos para obtener análogos de los estadísticos F (estadísticos Φ), cuya significación es contrastada usando métodos no paramétricos de permutaciones aleatorias.

Otro modo de estudiar la diferenciación genética entre poblaciones es a través de las medidas de similitud o distancia entre ellas, obtenidas de forma directa a través de las frecuencias génicas. Se han descrito muchas medidas de distancia que han sido posteriormente adaptadas para tener en cuenta los cambios evolutivos de las frecuencias génicas entre las poblaciones [p.e. (Mahalanobis P.C., 1936)), (Bhattacharyya, 1946)), (Rogers J.S., 1972)), o la distancia genética mínima y la estándar de Nei (1987)]. Una vez se tiene la

matriz de distancias genéticas entre poblaciones se pueden utilizar cualquiera de los métodos de mencionados anteriormente para representar las relaciones entre ellas en forma de dendrograma.

LA GENÉTICA EN LA CONSERVACIÓN

Una vez hemos visto la información que es posible extraer de los estudios genéticos, y más concretamente de la Sistemática Molecular, es posible entender mejor la importancia de la Genética en la Biología de la Conservación. Sin los últimos avances aportados por la incorporación de las herramientas moleculares, que permiten un acceso directo a las propiedades genéticas de los organismos naturales, y de las herramientas conceptuales para poder tratar esa nueva información, muchas de las cuestiones que son actualmente resueltas en la Biología de la Conservación ni siquiera sería posible plantearlas (Avise y Hamrick, 1996).

¿Cuál ha sido entonces la causa de que se haya dudado de la importancia de las consideraciones genéticas en los programas de conservación? (Lande, 1988; Simberloff 1988; (Caro & Laurenson, 1994)

Milligan *et al.* (1994) apuntan al hecho de que la variación de un locus marcador podría no proporcionar una medida real de la capacidad de una población para adaptarse a condiciones futuras o de la eficacia de los individuos con respecto a los marcadores. Mientras hay casos en que se ha demostrado una clara asociación entre los niveles de heterozigosidad *per se* y la eficacia biológica de una especie (Schaal & Levin, 1977); (Ledig, 1986); (Miller R.R. *et al*, 1989)) o su potencial adaptativo (Huenneke, 1991), otros apuntan a una relación contrapuesta entre esos factores (Fowler & Morris, 1977); (Mashbum S.J. *et al*, 1978); (Widen B. & Andersson S., 1993); (Leberg, 1992); (Pope T.R., 1995)6). Debemos, pues, tener claro que la falta de divergencia genética es más probable que sea un síntoma del riesgo de extinción que su causa (Caughley, 1994); (O'Brien, 1994)). Es decir, no hay duda de que la retención de variabilidad genética es esencial para asegurar el potencial evolutivo a largo plazo de cualquier especie, en peligro de extinción o no, pero estudiar los niveles de diversidad genética de un marcador molecular en la mayoría de las ocasiones es improbable que nos proporcione una información fiable sobre la viabilidad de las poblaciones, o que estos sean indicadores fiables de la diferenciación adaptativa de caracteres (normalmente cuantitativos) sujetos a distintas presiones de selección (Lewontin, 1984); Lynch, 1996), y ello es debido, precisamente, a la poco clara relación entre la eficacia o viabilidad ecológica y/o el potencial adaptativo con la variación genética de loci marcadores.

Por lo tanto, los objetivos de la Genética de la Conservación deben ir más allá de la mera documentación de los patrones de diversidad genética entre y dentro de poblaciones (cf. Templeton & Georgiadis 1996), y tratar de determinar los procesos responsables de esos patrones, las explicaciones causales subyacentes a los mismos.

Teniendo esto en cuenta, cabe destacar aquí la visión retrospectiva que Avise (1996) nos presenta de los papeles, tanto conceptuales como empíricos, que la Genética ha venido desempeñando en el campo de la Biología de la Conservación. Vamos a citar a continuación estos cinco papeles que se atribuyen a la Genética, y ejemplos de su utilidad real en materia de conservación (Avise y Hamrick, 1996 y referencias allí citadas).

Por un lado está la investigación de la extensión e importancia de la variabilidad genética dentro de las poblaciones. De esta forma se ha entrado a discutir en la gestión de la vida salvaje conceptos como el de la heterozigosidad, u otros como la depresión endogámica o el tamaño efectivo poblacional (*Ne*) ((Templeton & Read B., 1983); (Lande & Barrowclough, 1987); (Lacy, 1992); Ellstrand & Ellam, 1993).

Asimismo, usando marcadores genéticos es posible determinar patrones y relaciones de parentesco dentro de poblaciones y, a través de esta información genealógica, estimar procesos demográficos a corto y largo plazo como el sistema de cruzamiento, el *Ne*, o la migración (Milligan *et al.,* 1994), demostrándose claramente la interrelación entre la Genética y otras disciplinas como la Demografía o el estudio del comportamiento o la estructura social.

Dentro de esta línea, también destaca la utilidad de las aproximaciones genético-moleculares en la identificación de patrones de divergencia y estructuración poblacional, y las relaciones filogenéticas intraespecíficas. Esta información ha sido muy útil para guiar la gestión en programas de traslocación o aumento, y en el diseño de reservas [p.e., (Murphy & Noon, 1992)), (Pavlik *et al*, 1993); para revisiones ver (Schonewald-Cox CM *et al*, 1983)); Soulé & Simberloff (1986)]. También para revelar características, relevantes para la conservación, de la historia natural y demográfica de los organismos, puesto que la estructura genético poblacional es un reflejo de la historia filogeográfica y la ecología de las biotas ((Templeton & Georgiadis, 1996); (Bowen *et al*, 1995)). Sin querer entrar en la controversia sobre la importancia relativa de la Demografía y la Genética en estudios de conservación [para más información ver Lande (1988), Holsinger & Gottlieb (1991), Schemske *et al.* (1994)], cabe hacer mención de los últimos trabajos de Milligan *et al.* (1994) y Holsinger (1996), los cuales dejan ver claramente cuales son los límites de la Genética en la Biología de la Conservación y, sobre todo el primero, nos hace reflexionar en como la historia demográfica se refleja en la composición genética de las poblaciones y nos hace ver como sólo a través de los datos procedentes de los marcadores genéticos va a ser posible obtener esa información demográfica a largo plazo, tan relevante en los estudios de conservación (p.e., (Thomas C.D., 1990)), aunque en muchos casos habrá que esperar al desarrollo de nuevos métodos analíticos para ello.

También ha probado ser muy eficiente, en el ámbito de la Biología de la Conservación, la utilización de las herramientas de la Genética Molecular en la determinación de las relaciones filogenéticas entre especies u otros taxones, ya que ha permitido determinar críticamente el nivel de diferenciación evolutiva y filogenética de poblaciones o especies amenazadas, por ejemplo, en complejos de especies en peligro de extinción (como los felinos,

(O'Brien & and collaborators, 1996); o las "silverswords" hawaianas, (Rieseberg & Swensen, 1996).

Por último, y en relación con lo anterior, las técnicas genéticas han servido para identificar los puentes entre especies y para determinar fenómenos de hibridación e

introgresión, que son tan importantes en la conservación de especies amenazadas, para evitar, por ejemplo, la depresión exogámica resultante del flujo génico entre poblaciones de la misma especie o interespecífico [ver Ellstrand & Elland (1993), (Frankham, 1995), y ejemplos allí citados] o la puesta en marcha de programas de conservación en poblaciones que son en realidad producto de hibridaciones entre especies contemporáneas, como con la especie *Limonium neocastellonense* (Laguna *et al.*, 1994) o en el gorrión *Ammodramus maritimus*, (Avise & Nelson, 1989), o, por el contrario la puesta en marcha de los mismos en especies de origen híbrido pero que han evolucionado independientemente y se han diferenciado de sus progenitores ((Ryder, 1986); (Wayne & Jenks, 1991). Dentro de esta línea, los marcadores moleculares se han aplicado también en estudios forenses para identificar el origen de individuos que migran o la ilegalidad de material biológico comercializado, por proceder de especies protegidas.

En resumen, las herramientas de la Sistemática Molecular tienen una serie de aplicaciones en los programas de conservación a menudo interrelacionadas con otras disciplinas y otras veces claramente diferenciadas, algunas actualmente ya explotadas, y otras que van a necesitar un impulso en su desarrollo futuro.

No queremos concluir esta sección sin enfatizar este carácter multidisciplinar de la Biología de la Conservación. Investigadores de muy diversas ciencias como la Demografía, los estudios de Historia Natural, la Etología, la Ecología, la Biología de Poblaciones (que abarca la Genética de Poblaciones, la Ecología de Poblaciones y la Biología Evolutiva), la Genética Cuantitativa, la Filogenética..., están contribuyendo activamente a los esfuerzos conservacionistas. Los datos genéticos no son pues más que una de tantas consideraciones en las decisiones de conservación. A su vez, no sólo son importantes las consideraciones científicas, sino que también entran a formar parte de esta esfera de puntos de vista potencialmente contradictorios, principalmente por la limitación en la disponibilidad de tiempo y de recursos, las consideraciones políticas, sociales, y económicas [ver p.e., Schemske *et al.* (1994)].

EL GÉNERO *LIMONIUM*

El género *Limonium* Miller, perteneciente a la familia Plumbaginaceae, es cosmopolita y comprende aproximadamente 400 especies cuyos centros de diversificación se encuentran en las estepas asiáticas y en el oeste de la región mediterránea (Erben, 1993).

Sus especies suelen distribuirse a lo largo de las costas, en estepas, o en desiertos. Colonizan pues substratos salinos (marjales, malladas, lagunas interiores, litorales rocosos), yesosos o suelos muy áridos, conformando generalmente áreas pequeñas y aisladas. Durante

las últimas tres décadas estos ecosistemas tan frágiles han sido sometidos a una gran presión antrópica en el área Mediterránea, por el incremento de la actividad urbanística y la transformación agraria, lo que ha llevado a una alarmante reducción de estos hábitats, un aislamiento aún mayor de las poblaciones que los ocupan, y finalmente a la casi extinción de muchos endemismos propios de estas zonas, como es el caso de especies del género *Limonium* en el área iberolevantina tales como *L. dufourii* y *L. cavanillesii* (Laguna *et al.,* 1994).

Las especies de *Limonium* se conocen como lavándulas marinas o espliegos de mar. Son plantas lignificadas, anuales o perennes, con hojas simples, formando una roseta basal. Las inflorescencias son racemosas, pueden presentar ramas estériles que acompañan a las ramas fértiles, cuyas flores se disponen agrupadas en espiguillas (Fig. 1-2). Las relativamente pocas diferencias morfológicas a menudo existentes entre estas especies son una de las causas de la dificultad taxonómica del género. Generalmente son necesarias plantas con estructuras reproductoras para ser determinadas con seguridad.

Muchas especies de *Limonium* exhiben dimorfismo en la combinación polen-estigma asociado con autoincompatibilidad (Fig. 1-3). Estos caracteres están codificados por sendos loci dialélicos y estrechamente ligados (Baker, 1966).

A partir de este sistema se han derivado, por recombinación entre ambos genes, especies con autocompatibilidad monomórfica; en otros casos se ha desarrollado la agamospermia (reproducción asexual en la que el polen, en caso de intervenir, sólo lo hace para estimular la división del zigoto, pero no hay fecundación) asociada al monomorfismo autoincompatible y/o a la esterilidad del polen [Baker (1966) y ver también más adelante]. Los procesos de apomixis por agamospermia permiten el posterior establecimiento de taxones triploides. Esto ha llevado a pensar que la apomixis, junto con la hibridación, ha jugado un papel muy importante en la evolución del género *Limonium* dificultando todavía más su clasificación taxonómica (Baker, 1966; (Dolcher & Pignatti, 1971)). La poliploidía está también ampliamente extendida entre sus especies, con dotaciones cromosómicas que varían de 2n=12 a 2n=54. Todas las especies con números cromosómicos mayores de 18 (con la posible excepción de 2n=32, 36 y 54) parecen ser híbridos derivados de la combinación de los números cromosómicos básicos x=8 y x=9 (Erben, 1979).

En resumen, los principales mecanismos de la especiación propios de los vegetales ((Dobzhansky *et al*, 1980), que son el aislamiento poblacional geográfico, y el reproductivo, por los procesos de poliploidía e hibridación, con la posibilidad añadida del mantenimiento de los híbridos por apomixis, están presentes en el género *Limonium*, permitiendo la aparición y establecimiento de nuevas especies con relativa frecuencia. Estas características hacen de *Limonium* un género apasionante, pero complican en gran medida el estudio de las relaciones (filogenéticas) entre sus especies. La circunstancia añadida de que el aumento de la pérdida de biodiversidad en las costas iberolevantinas, causada por ese impacto humano sin precedentes, esté afectando en gran medida a especies de este género (Laguna *et al.,* 1994), fue el impulso definitivo para ahondar en su estudio.

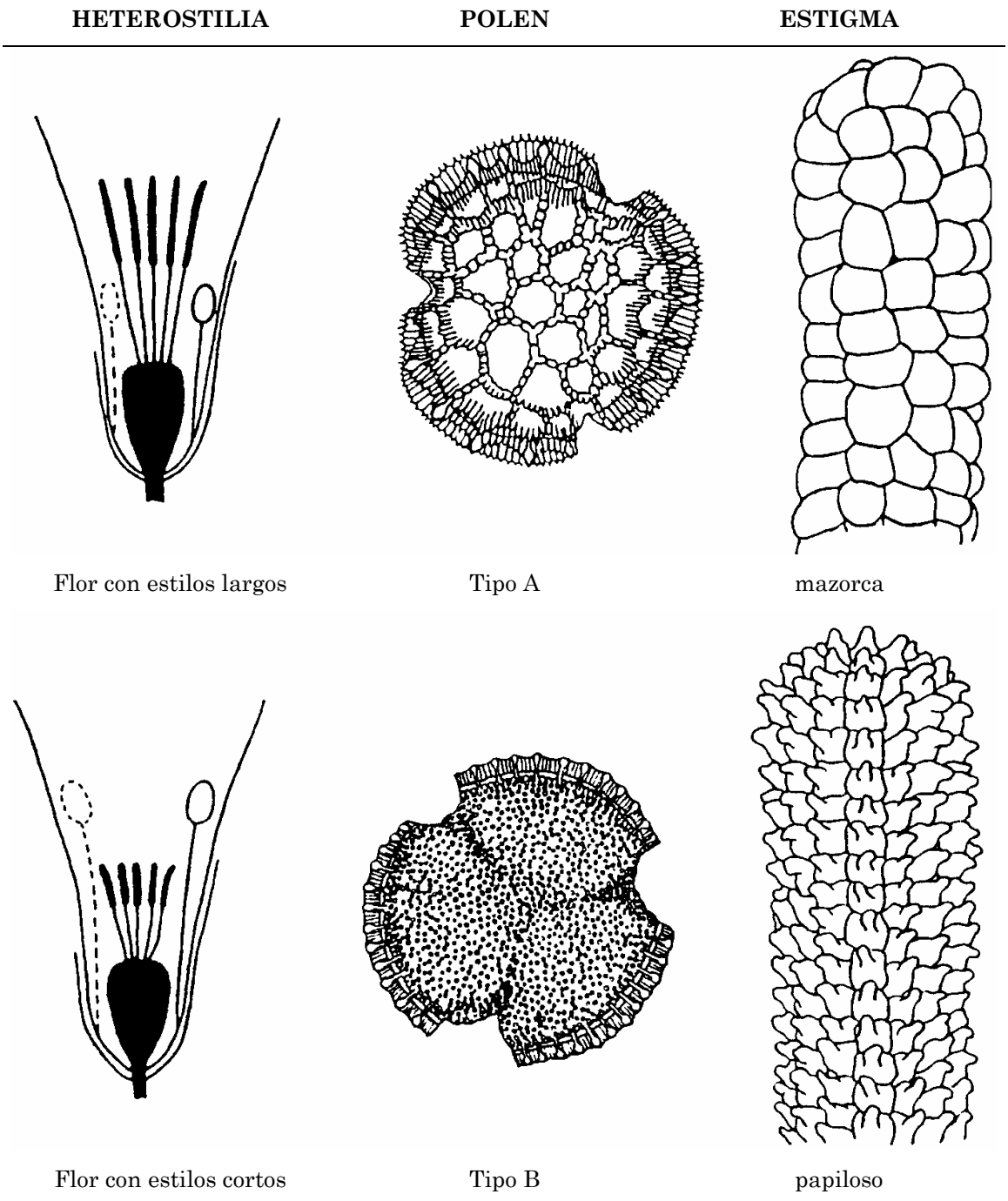


Fig. 1-3 Dimorfismo en la combinación polen-estigma del género *Limonium* y la heterostilia asociada a cada combinación cuando esta existe.

Hasta la fecha no se ha publicado ninguna clasificación taxonómica del género *Limonium* con tratamiento filogenético explícito. La primera revisión general sobre el mismo fue llevada a cabo hace un siglo y medio por Boissier (1848), el cual propuso un esquema jerárquico que dividía al género en secciones y algunas de éstas en subsecciones (Fig. 1-4), basándose en las características florales, tales como la forma del cáliz, la soldadura de los pétalos o los estambres, o el tipo de fruto. Esta clasificación, aunque ha sido reconocida como probablemente artificial (Baker, 1966), continúa aún teniéndose en cuenta en estudios de

diversa índole llevados a cabo en este siglo (Baker, 1966; (Erben, 1978), Ingrouille 1984). Bien es verdad que, por un lado, se han propuesto varios cambios a esta ordenación general en revisiones posteriores ((Benthan G. & Hooker J.D., 1873); (Pax F., 1897); (Pignatti, 1971), aunque éstos no han profundizado en la clasificación por debajo del nivel de sección, sino que tratan la clasificación a nivel más general; y, por otro lado, se han creado otras secciones para acomodar a especies descubiertas con posterioridad (p.e., Squizopetalum o Pterolimon).

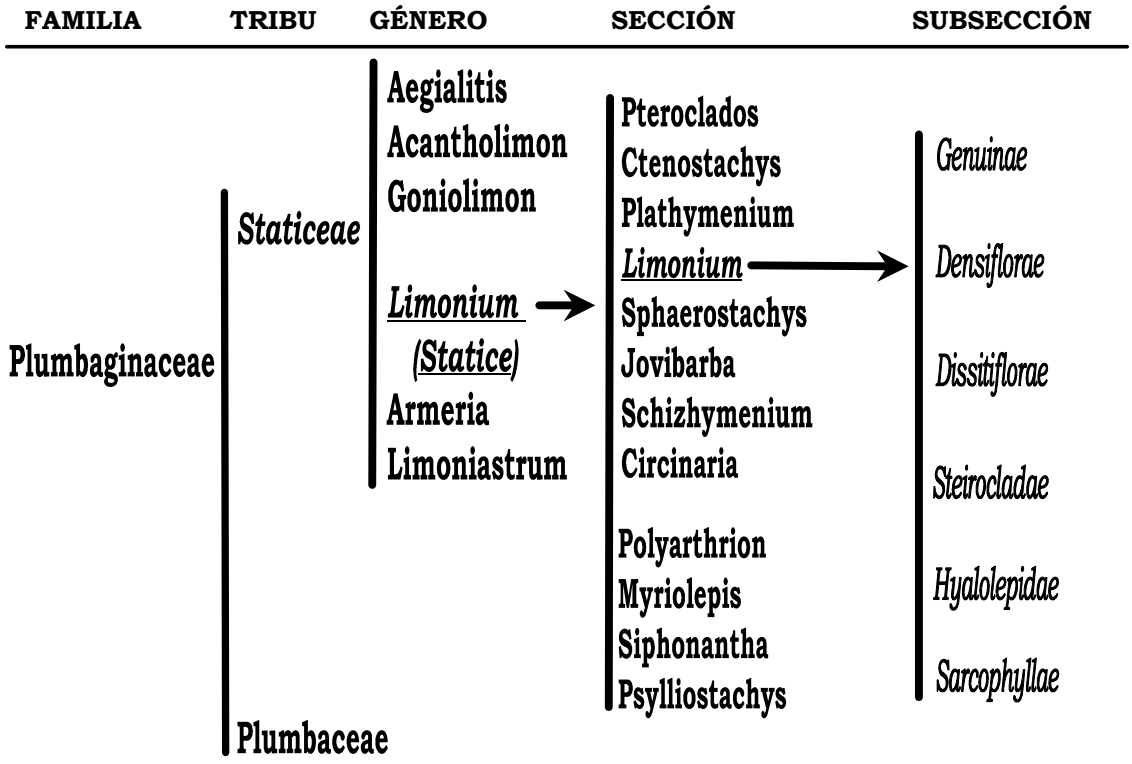


Fig. 1-4 Clasificación del género *Limonium* según Boissier (1848).

La sección Limonium

Una de las secciones más interesantes, pero poco estudiadas, del género es la sección Limonium. Boissier (1848) la dividió en seis subsecciones (ver Fig. 1-4), sin embargo, hay evidencias de que las especies adscritas a cada subsección algunas veces lo han sido innecesariamente, incluso es posible que tengan orígenes polifiléticos, ya que parece probable que en el género puedan hibridar especies poco cercanas morfológicamente (Baker, 1953).

La sección Limonium es la que tiene un mayor número de especies dentro del género *Limonium*. Sus especies también presentan todos los mecanismos reproductivos del género, incluso los más evolucionados (Fig. 1-5). Muchas especies del género *Limonium* son dimórficas (polen tipo A/estigmas patrón de mazorca o polen tipo B/estigmas patrón papiloso, ver Fig. 1-6), con fecundación cruzada obligada. Dentro del género *Limonium* sólo en la subsección Genuinae, perteneciente a la sección Limonium, hay especies que presentan también heterostilia (variación en la longitud de los estilos con respecto a los estambres)

(Baker, 1966). Este debe de ser el estado filogenético derivado en mayor grado, dentro del sistema de incompatibilidad heteromórfica (Fig. 1-5).

Este sistema ha sido roto en varias ocasiones a lo largo de la evolución (ver más adelante) y de dos formas diferentes. Algunas especies del género son monomórficas, con la combinación A/papilosa o B/mazorca (ésta última sólo presente en *L. echioides* de la sección Schizhymenium, Fig. 1-6). Estas combinaciones son justo opuestas a las de las especies dimórficas, y, por lo tanto, las especies que las presentan son capaces de autofecundarse. Esta autocompatibilidad monomórfica está presente en especies de las subsecciones Genuinae y Hialolepidae dentro de la sección Limonium. El otro mecanismo de ruptura de la incompatibilidad dimórfica ocurre únicamente dentro del género en algunas especies de las subsecciones Densiflorae, Dissitiflorae y Steirocladae, pertenecientes a la sección Limonium. Aquí, aunque las poblaciones sean monomórficas, la combinación polen-estigma es la de las especies dimórficas, o en algunos casos el polen no se produce. En todos estos casos se ha comprobado que hay una alta proporción de granos de polen malformados (Bokhari, 1971). Estas especies se ha demostrado que se reproducen por apomixis (Baker, 1953, 1954, 1966).

El sistema genético subyacente al mecanismo de reproducción dimórfica autoincompatible (ver arriba) es lo suficientemente complejo como para que sea improbable que haya aparecido repetidamente en la naturaleza, por lo que puede proporcionar de una guía filogenética valiosa (Baker, 1966). Por otro lado, la ruptura de este mecanismo y el retorno a la autocompatibilidad con el monomorfismo en la combinación polen-estigma en el género *Limonium*, sí se corresponde con el retorno a la autocompatibilidad en otras Plumbaginaceas, pareciendo pues haberse producido de forma secundaria y repetidamente en géneros diferentes (p.e., *Armeria*). Incluso también se observa este fenómeno en otras familias de plantas como Limaceae o Primulaceae. Este hecho sugiere que, si prevalecen ciertas circunstancias ecológicas particulares que favorezcan la autocompatibilidad, el monomorfismo será seleccionado positivamente. Sin embargo, la aparición de la agamospermia como forma alternativa de producción de semillas por autofecundación ha sido sólo descubierta en el género *Limonium*, y más concretamente en especies pertenecientes a algunas subsecciones de la sección Limonium. Este hecho concede una gran importancia a esta sección, lo cual nos inclinó a elegirla como punto de partida para profundizar en el estudio de este género, más aún cuando, como ya se ha apuntado anteriormente, los estudios de conservación están de alguna manera subordinados a las clasificaciones sistemáticas y ,en nuestro caso, las especies en que era prioritario el estudio genético por estar gravemente amenazadas de extinción pertenecen ambas a esta sección. Estas especies son *L. dufourii* y *L. cavanillesii*.

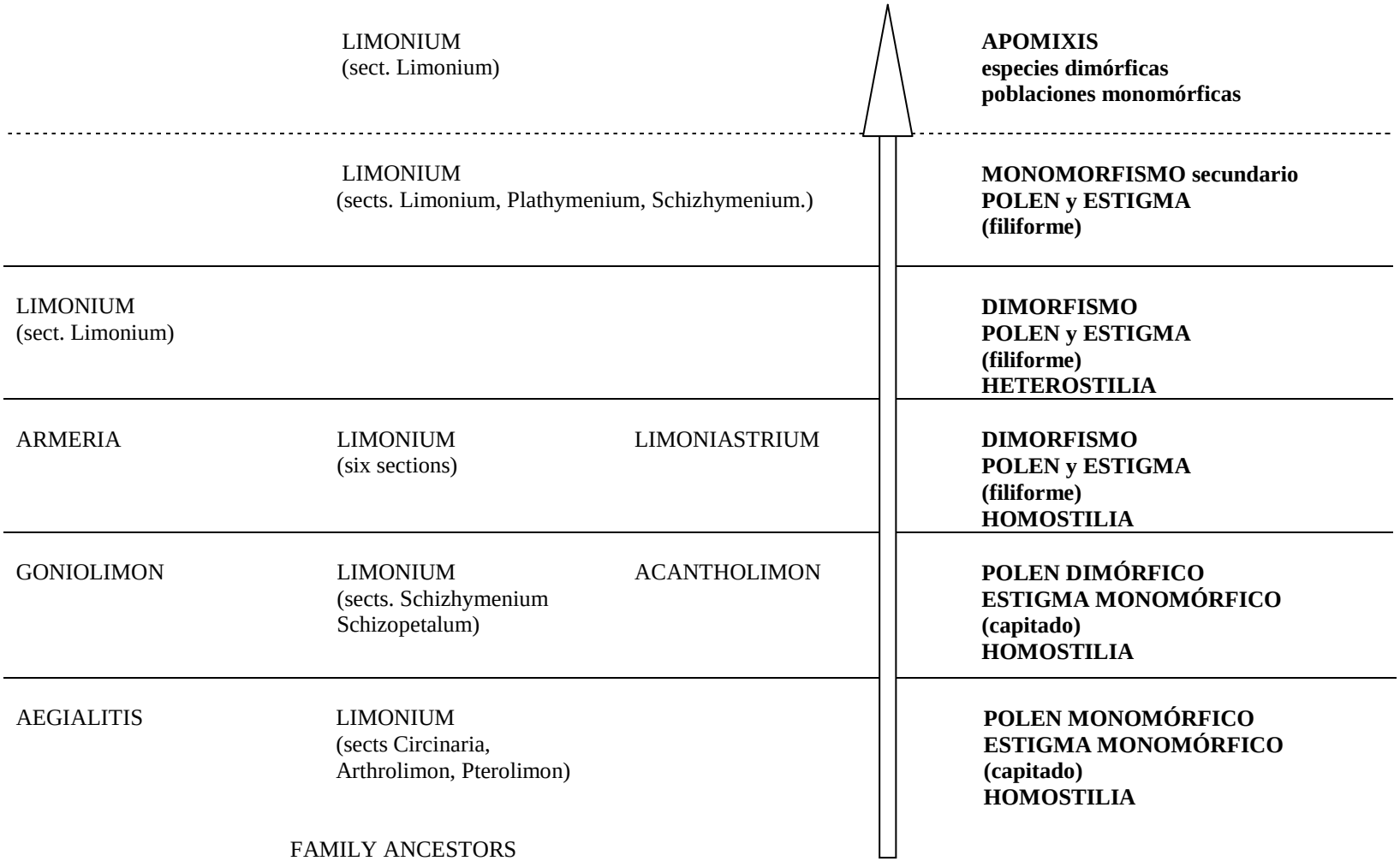


Fig. 1-5 Diagrama que muestra la secuencia evolutiva postulada para el desarrollo del sistema de incompatibilidad heteromórfica en la tribu Staticeae (modificado de Baker 1966).

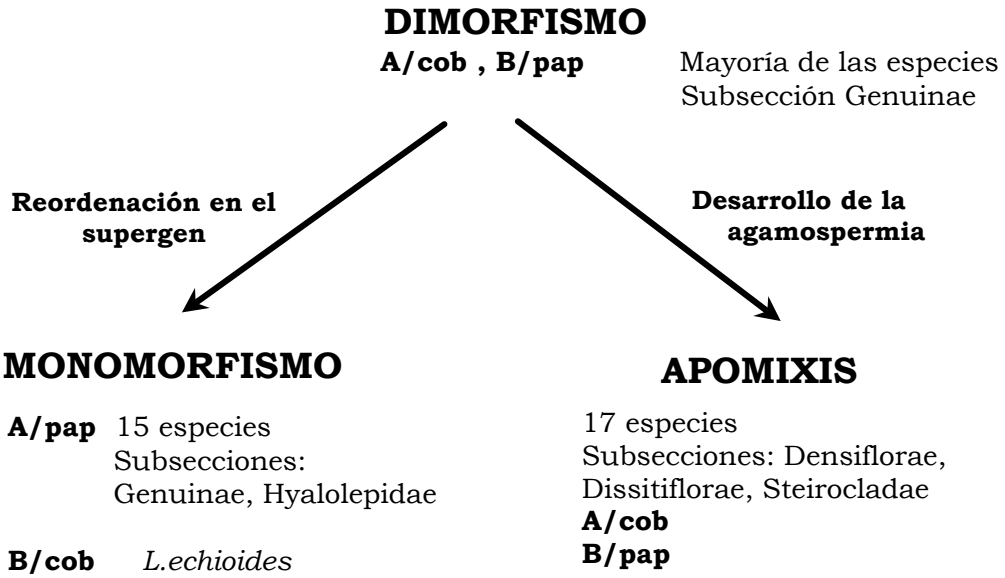


Fig. 1-6 Diagrama que muestra los dos caminos para la rotura del sistema de incompatibilidad heteromórfica en el género *Limonium* (tomado de Baker 1966).

Limonium dufourii (Girard) O. Kuntze

L. dufourii es el denominado comúnmente Limonio villoso o de Dufour o ensopeguera villosa o de Cullera. Es una planta perenne, con hojas dispuestas en una roseta basal, hemicriptófita y recubierta de tricomas en el tallo, las hojas y las brácteas (Fig. 1-7a). Presenta inflorescencias racemosas, las ramas superiores con flores de color violáceo (Fig. 1-7b), mientras que las inferiores son estériles. Es una especie con reproducción asexual por apomixis obligada ya que presenta un sistema de autoincompatibilidad monomórfico en la combinación polen/estigma (B/papiloso en todas las poblaciones) (Baker, 1966) y un número cromosómico triploide (2n=27) (J.A. Rosselló, comunicación personal).

La especie, antaño distribuida abundantemente a lo largo de las costas valenciano-castellonenses, sobre suelos de saladares arenosos o limosos o en acantilados litorales rocosos afectados por las salpicaduras marinas ((Crespo & Laguna, 1993)), hoy día se encuentra en retroceso, debido al incremento urbanístico de nuestras costas durante los últimos 20 años, con la consiguiente alteración o pérdida por completo de los hábitats donde esta especie habita (Laguna *et al.,* 1994). *L. dufourii* fue declarada protegida por el gobierno autonómico en 1985, y posteriormente catalogada como "en peligro crítico" atendiendo a las nuevas categorías de las listas rojas de la IUCN (1994).

Fig 1-7. Detalles de las inflorescencias de *Limonium dufourii*.

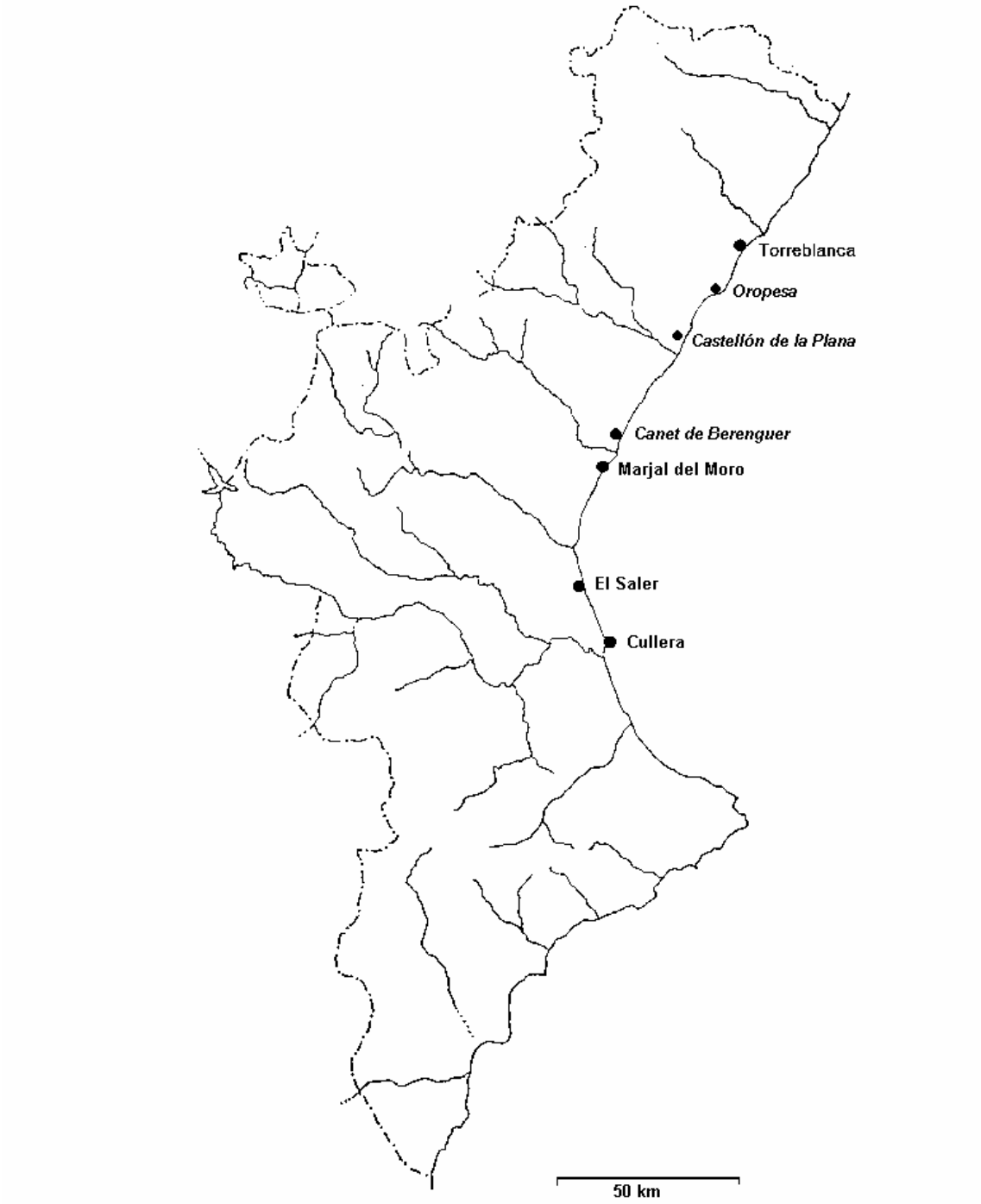


Fig 1-8. Mapa de la situación geográfica de las poblaciones de *Limonium dufourii*. En letra bastardilla se muestran las localidades históricas en las que la especie no es encuentra en la actualidad.

Muchas de las poblaciones originales de *L. dufourii* han desaparecido por completo (E. Laguna, comunicación personal). En la actualidad se conocen únicamente 6 poblaciones naturales de la especie (Fig. 1-8) ((Laguna & Escribá M.C., 1996). Cuatro de ellas están en inminente peligro de extinguirse por su extremadamente pequeño tamaño (concretamente las poblaciones de El Saler, Marjal del Moro I (Sagunto) y Cullera) y/o su ubicación en zonas con elevada afluencia turística y alteradas por el hombre (Cullera y Torreblanca). Las otras dos poblaciones, situadas en la Marjal del Moro, cuentan con unos miles de individuos Sin embargo, estas poblaciones fueron encontradas en época de sequía, durante el año 1995, por lo que la gran cantidad de individuos aparecidos puede considerarse un suceso puntual, resultado de la germinación de semillas tras la desecación de la marjal (E. Laguna, comunicación personal). La confirmación de este hecho está supeditada a estudios demográficos futuros.

Limonium cavanillesii Erben

L. cavanillesii es el limonio o la ensopeguera de Peñíscola o de Cavanilles. Es una especie anual-bianual, también rosulada y con inflorescencias racemosas, las inferiores estériles y las superiores con flores de color violeta (Fig. 1-9). Se la considera como una especie con reproducción apomíctica por presentar un número de cromosomas triploide (2n=27) en todos sus individuos, además de presentar una proporción elevada de granos de polen malformados (>95%) y una combinación polen-estigma autoincompatible (J.A. Rosselló, resultados no publicados).

L. cavanillesii fue descrita por primera vez por M. Erben a través de material de herbario de Sennen (1913). La especie es endémica de una parte muy restringida de nuestras costas, concretamente se distribuía a lo largo de terrenos marítimos desde Peñíscola a Benicarló (Castellón). Fue declarada bajo protección por el gobierno autonómico en 1986 (DOGV 336, Generalitat Valenciana). Sin embargo, después de no ser detectada durante casi dos décadas y las antiguas poblaciones haber sido destruídas por actividades humanas y por erosión marina (Aguilella, 1994), se pensó que en realidad se había extinguido. En 1994, la especie fue redescubierta en la Serra d'Irta, cerca de Peñíscola, como una población de 29 individuos distribuídos en un área muy pequeña alrededor del Torreón de Badún. Esta especie es una de las más amenazadas de extinción de este género pues presenta los mismos riesgos que el resto de sus congéneres (ver arriba) pero acrecentados debido al número tan pequeño de individuos encontrados en la naturaleza.

Fig 1-9 *Limonium cavanillesii*

MARCADORES MOLECULARES EN EL GÉNERO *LIMONIUM*

Para abordar distintos aspectos que nos resultaron interesantes sobre la biología evolutiva del género *Limonium*, nos planteamos el estudio de la diversidad génica de varias especies y a diferentes niveles evolutivos. Para ello se han utilizado distintas herramientas de la Sistemática Molecular. A continuación se detallan las principales características de los marcadores genético-moleculares finalmente utilizados, justificándose también las razones de su elección, que como ya se ha indicado anteriormente puede depender de lo que se vaya descubriendo a medida que se profundice en el estudio del grupo. En un principio el marcador elegido lo fue en función de la variabilidad encontrada en un estudio piloto usando como marcador el análisis de RFLPs en el DNA cloroplástico (cpDNA), y en base también a resultados previos obtenidos con isozimas (Rosellò, comunicación personal). En este último estudio se encontró una escasa variabilidad a nivel intraespecífico tras el análisis de varios sistemas isoenzimáticos; mientras que la variabilidad a nivel de cpDNA parecía la adecuada para determinar relaciones evolutivas a nivel interespecífico.

El genoma cloroplástico

En la Fig. 1-10 se muestran la estructura y orden génico algunas grandes líneas de evolución del genoma cloroplástico. El cpDNA de las plantas terrestres es un único cromosoma circular con un tamaño que varía entre 120 y 217 kb (Palmer, 1991). Su característica estructural más destacable es la presencia de una duplicación invertida (IR) que divide al cromosoma en una región de copia única pequeña (SSC) y una grande (LSC). Las dos copias de la IR son idénticas debido a la recombinación intramolecular que existe entre ellas. La mayor parte de la variación en tamaño del cpDNA es debida precisamente a variación en tamaño de la IR, sin ir acompañada de cambios en la secuencia. De hecho, solamente existen grandes cambios estructurales cuando la IR está alterada (geranio) o ha desaparecido (legumbres), mientras que hay sólo cuatro diferencias en el orden génico entre tabaco, arroz y la hepática *Marchantia*, que comprenden el rango completo de la diversidad de las plantas terrestres, siendo el orden génico en la inmensa mayoría de plantas vasculares aquél definido por primera vez en tabaco (Downie & Palmer, 1992). En estas tres últimas especies el cpDNA ha sido completamente secuenciado (Shinozaki *et al.,* 1986; Hiratsuka *et al.,* 1989; Ohyama *et al.,* 1986, respectivamente), así como en la dicotiledónea *Epifagus virginiana* (Wolfe *et al.,* 1992) y el alga *Euglena gracilis* (Hallick *et al.,* 1993). El genoma cloroplástico típico de las plantas terrestres está empaquetado densamente, con una media de 115 genes identificados en tres de los genomas secuenciados totalmente (Shimada & Sugiura, 1991). Estos genes codifican en su mayoría para proteínas que participan en la fotosíntesis o en la expresión génica (Palmer *et al.,* 1988). Muchos de estos genes están organizados en operones, y varios de estos son muy similares a los de eubacterias (Palmer, 1991), lo que demuestra, junto con otras líneas de evidencia, el origen endosimbiótico de los cloroplastos (revisado en Gray, 1992).

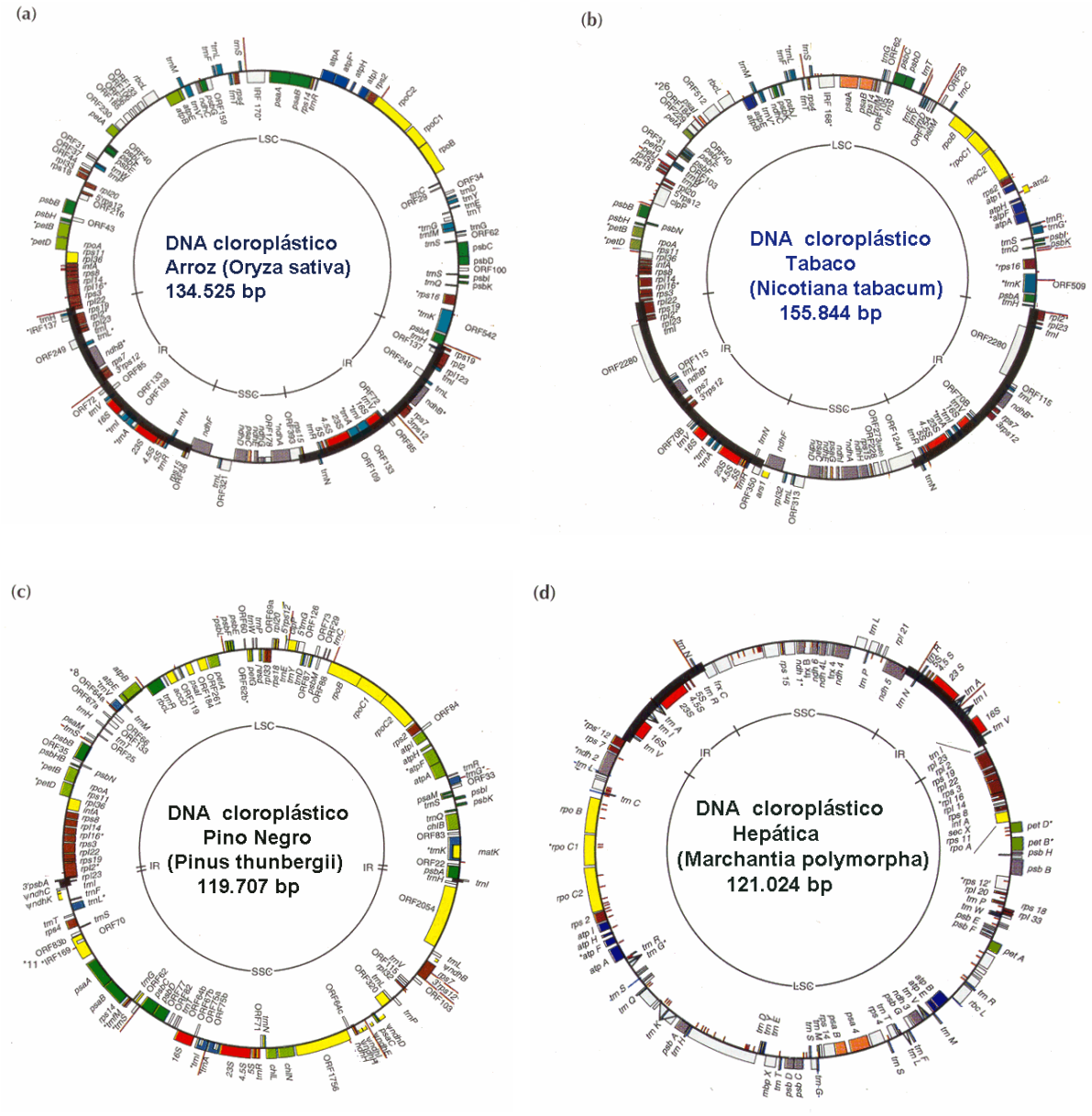


Fig 1-10. Estructura y orden génico de cuatro de las grandes líneas de evolución del genoma cloroplástico.

La comparación de las tasas de sustitución sinónimas de genes codificantes de proteínas de varios genomas eucarióticos ha revelado que el cpDNA de las angiospermas evoluciona lentamente, seguramente por presentar una tasa de mutación reducida (Clegg y Zurawski, 1992). No obstante, las tasas de evolución parecen variar no sólo entre genes (Shimada & Sugiura, 1991), sino también en el mismo gen en diferentes linajes de plantas (Wolfe *et al.,* 1987; Clegg *et al,* 1991; Palmer *et al.,* 1988).

Varios factores prácticos, incluyendo la abundancia del cpDNA en las células de las hojas, su facilidad de aislamiento, su tamaño relativamente pequeño, así como su modo de evolución simple y relativamente conservada, y su herencia clonal, generalmente uniparental

y en cualquier caso sin recombinación (Palmer, 1987), han hecho que esta molécula haya sido ampliamente utilizada en estudios con fines filogenéticos. Desde el nivel infraespecífico al de familia, la aproximación metodológica más frecuentemente empleada ha sido la comparación de fragmentos o de sitios de restricción (Palmer *et al.,* 1988). La mayoría de estos estudios se han centrado en determinar las relaciones entre especies congenéricas o de géneros cercanos, aunque también se ha detectado variación a nivel intraespecífico (revisado en Soltis *et al.,* 1992). La existencia de variación a este nivel con más frecuencia de lo esperado inicialmente previene de la necesidad de un muestreo adecuado para estimar relaciones filogenéticas a nivel específico o mayor. Al mismo tiempo advierte de la importancia de realizar análisis concomitantes con marcadores moleculares, sobretodo si hay sospechas de introgresión, ordenación filogenética (*phylogenetic sorting*, Avise 1986), o flujo génico entre poblaciones o hibridación entre especies, que son tan comunes en plantas, y que llevarían a discordancias entre las filogenias nucleares y organulares, por la ausencia de recombinación en estos últimos. Estos factores son los responsables de la discordancia entre árboles génicos y filogenias reales de los organismos objeto de estudio, que ha sido tratada en profundidad por muchos autores (p.e., Doyle, 1992; Rieseberg & Brunsfeld, 1992).

La metodología más frecuentemente empleada para el análisis de RFLPs consiste en la digestión de los cpDNAs de los individuos objeto de estudio con varios enzimas de restricción, generalmente que reconocen 4 o 6 nucleótidos. A continuación se separan los fragmentos en un gel de electroforesis y se localizan por hibridación con sondas generalmente no homólogas (heteroespecíficas) pero procedentes de clones del cpDNA de una especie cercana evolutivamente (Palmer, 1987). La construcción de una genoteca del cpDNA previamente al estudio de su divergencia evolutiva en una especie o grupo de especies suele ser preferible cuando no se dispone de información más o menos detallada de la estructura del mismo en ella/s. El empleo de sondas heteroespecíficas podría ser fallido o dar resultados erróneos si realmente la estructura del cpDNA es diferente a la del organismo del que proceden las sondas o la divergencia de estas sondas con el genoma del grupo de estudio es alta (Palmer *et al.,* 1988).

El hecho de que en el género *Limonium* los procesos de hibridación y poliploidía sean mecanismos activos de especiación (ver más arriba), nos inclinó a la utilización de un marcador nuclear alternativo al cpDNA, por las razones mencionadas anteriormente. El marcador elegido fue la región de los espaciadores transcritos internos (ITS) del DNA ribosómico nuclear.

El cistrón del DNA ribosómico nuclear. La región de los ITS.

En el genoma eucariótico nuclear, el DNA ribosómico (rDNA) está organizado en unidades repetidas en tándem separadas por espaciadores intergénicos (IGS), y a menudo distribuidas en diferentes cromosomas (Gerbi, 1985). En cada repetición de rDNA los genes que codifican para las subunidades grande y pequeña de los RNAs ribosomales (rRNA) están flanqueados por los extremos distales de los IGS, que son denominados espaciadores transcritos externos (ETS). Entre los tres genes del rDNA, a ambos lados del 5,8S, están los

espaciadores transcritos internos (ITS1 e ITS2) (Fig. 1-11). A esta zona se la conoce como la región de los ITS (Baldwin *et al.,* 1995).

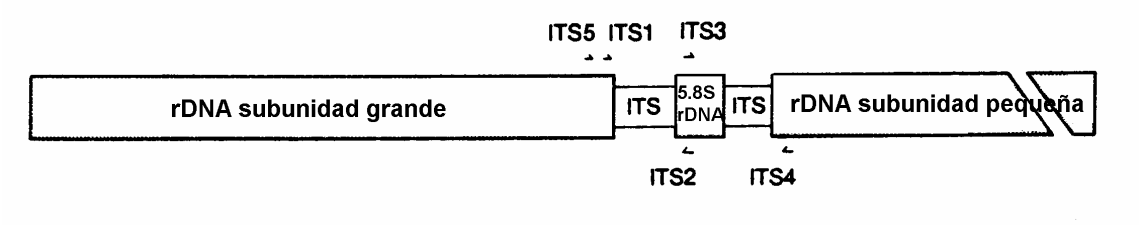


Fig 1-11 Cistrón del DNA ribosómico nuclear, en el que se muestra las zonas de unión de los cebadores ITS1-ITS5 (ver Capítulo 2 para una información más detallada).

El número de repeticiones cistrónicas por genoma se ha estimado que varía de varios cientos en mamíferos e insectos a varios miles en plantas, lo cual representa un uno por cien o más del genoma total (Long & Dawid, 1980). Sin embargo, la diversidad intragenómica del rDNA es generalmente baja; ello es debido a que los procesos de conducción molecular (entrecruzamiento desigual y evolución concertada) (Arnheim, 1983) actúan homogeneizando nuevas mutaciones entre loci ribosómicos.

Cada unidad de rDNA es transcrita a un precursor de rRNA (pre-RNA) que será subsecuentemente procesado por una serie de pasos ordenados, hasta convertirse en las moléculas de rRNAs maduras. Durante este procesado post-transcripcional del pre-RNA, los ITS son escindidos y eliminados, de ahí que se piense que estén relativamente libres de constricciones evolutivas, lo cual viene corroborado por su alta tasa de evolución en diferentes organismos (Savard *et al.,* 1993; Schlötterer *et al,* 1994). Se ha demostrado, sin embargo, que ciertas zonas de los ITS intervienen en la maduración de los rRNAs (Musters *et al.,* 1990; van der Sande *et al.,* 1992; van Nues *et al.,* 1994). Esta función atribuida a los ITS se traduce en constricciones en la estructura y la secuencia de los mismos. Así, existe un elemento estructural de 20-23 bp de longitud que está altamente conservado en la secuencia de ITS1 de 88 especies de angiospermas (Liu & Schardl, 1994), mientras que el resto de la secuencia de estas especies alineaba con dificultad. No obstante se han encontrado dominios conservados de estructura secundaria propios de organismos particulares. Por ejemplo, van Nues *et al.,* (1994) encontraron 5 dominios conservados en levadura. También se han descrito largas estructuras en horquilla, junto con complementariedad entre la región 3' del 18S rRNA y la 5' del ITS1 [p.e., en algas verdes (Aimi *et al.,* 1992) o en plantas superiores (Suh *et al.,* 1992; Venkateswarlu & Nazar, 1991)]. El ITS2 parece estar funcionalmente más constreñido que el ITS1, a juzgar por la mayor conservación de su secuencia entre las angiospermas (cf. Baldwin *et al.*, 1995) y su mayor sensibilidad a alteraciones estructurales (van Nues *et al.* (1994). Incluso Hershkovitz & Zimmer (1996) han llegado a proponer un modelo de estructura secundaria con las relaciones de apareamiento generalizadas en las angiospermas.

En resumen las características inherentes a los ITS les hace potencialmente ideales para estudios filogenéticos a niveles taxonómicos bajos (al nivel de especie o menor). Estas características serían, además de su alta tasa de evolución, su elevada repetición en el genoma nuclear, la rápida evolución concertada de su familia génica, su pequeño tamaño y la presencia de secuencias altamente conservadas que flanquean ambos espaciadores, lo que facilita su amplificación por PCR usando cebadores universales (White *et al.,* 1990). Su uso se ha extendido especialmente en especies vegetales, ya que en estos organismos urgía la necesidad de encontrar una secuencia, además nuclear, de evolución más rápida que el genoma cloroplástico para estimar relaciones filogenéticas a nivel específico, puesto que, al contrario de lo que ocurre en animales y hongos (Avise *et al.,* 1987), el mtDNA de vegetales no es un genoma apropiado para estos estudios por su baja tasa de evolución por sustituciones nucleotídicas y sus frecuentes reordenaciones (Palmer, 1992).

La única desventaja posible que se le puede atribuir a los ITS para su uso en análisis filogenéticos es la de cualquier otra familia génica: que las copias parálogas, o también llamadas *plerólogas* (Patterson 1988), no evolucionan como una única secuencia. Si la evolución concertada es más lenta que la especiación o si hay un sesgo en la misma sobre distintos tipos de ITS durante la deriva molecular, entonces existirá variación a nivel intra-individual, lo cual puede resultar en una filogenia entre especies potencialmente errónea si estos *parálogos divergentes* (Baldwin *et al.,* 1995) no son identificados (Sanderson & Doyle, 1992). Una aproximación por secuenciación directa de productos de PCR procedentes del DNA mezclado de varios individuos de una especie puede en muchas ocasiones detectar la existencia de varios tipos de ITS intragenómicos (Baldwin *et al.,* 1995). Si se detecta esta microheterogeneidad, será necesaria la posterior clonación y secuenciación de estos tipos individualmente, para extraer información útil de ellos (p.e., Ritland *et al.,* 1993, Buckler *et al.,* 1997).

Técnicas de DNA *fingerprinting* basadas en la PCR

Como ya se ha apuntado anteriormente, la secuenciación del DNA y los RFLPs pueden ser utilizados para estudios a distintos niveles evolutivos. Los resultados obtenidos con estos marcadores en el género *Limonium*, si bien dejan ver la importancia de un adecuado muestreo en la determinación de las relaciones entre especies cercanas evolutivamente, no proporcionarían variabilidad suficiente para estudios más exhaustivos a nivel intraespecífico. Una variedad de técnicas de reciente descubrimiento que están dando resultados muy satisfactorios en este sentido son las de DNA *fingerprinting* basadas en la PCR ((Schierwater *et al*, 1994), las cuales utilizan cebadores diseñados más o menos aleatoriamente para detectar productos de amplificación polimórficos, por lo que también se les ha denominado conjuntamente técnicas de PCR con cebadores aleatorios (Smith & Williams, 1994; Karp & Edwards, 1997) o técnicas MAAP (multiple arbitrary amplicon profiling) ((Caetano-Anollés G. & Gresshoff P.M., 1994).

Estas técnicas se caracterizan por requerir relativamente poca cantidad de DNA de partida, al estar basadas en la PCR. Además no necesitan de información previa específica de

secuencia, ni sondas clonadas o caracterizadas, y son relativamente simples y rápidas de manipular experimentalmente, principalmente por no incluir pasos de transferencia o hibridación. Entre ellas destacan los RAPDs (Random Amplified Polimorphic DNAs) (Williams *et al.,* 1990), que es la más frecuentemente utilizada; AP-PCR (Arbitrary primed PCR) (Welsh & MacClelland, 1990); y DAF (DNA amplification *fingerprinting*) ((Caetano-Anollés G. *et al*, 1991). Estos métodos se diferencian básicamente en la longitud de los cebadores que utilizan y las condiciones de la PCR. Una ventaja añadida a los mismos es que los fragmentos que amplifican nos proveen de un gran número de loci potencialmente polimórficos. De hecho, especialmente los RAPDs han probado ser muy útiles para revelar variación en especies con *a priori* escasa variabilidad genética, lo que ocurre precisamente en taxones en peligro de extinción y/o asexuales (Williams *et al.,* 1993), y especialmente cuando otras técnicas han fallado en revelar diferencias genéticas entre poblaciones (Dawson *et al.,* 1993; Karp & Edwards, 1997).

Todas estas características nos inclinaron a la elección de los RAPDs como método de análisis alternativo a los RFLPs o la secuenciación para estudiar la variabilidad genética intraespecífica en especies amenazadas del género *Limonium*.

Los RAPDs

Esta técnica consiste en la amplificación de fragmentos por PCR usando cebadores de secuencia aleatoria, entre 9 o 10 bases de longitud. Generalmente estos cebadores se consiguen a través de compañías comerciales, que aseguran la aleatoriedad y el buen diseño de los mismos, para que resulten en una amplificación eficiente. Los productos de los RAPDs son fragmentos de DNA de doble cadena derivados de la reacción de amplificación por PCR. Cada fragmento es una región del genoma que consiste en dos segmentos, flanqueados por zonas homólogas al único primer que se usa en la reacción, y situados en cadenas opuestas, pero lo suficientemente cercanas como para que la amplificación funcione. El siguiente paso es la separación de los productos de la PCR por medio de la electroforesis, normalmente usando geles con una concentración de agarosa media, relativamente grandes, para una mejor resolución de las bandas amplificadas, y teñidos con bromuro de etidio (EtBr); aunque también se han empleado en ocasiones geles de poliacrilamida con tinción de plata (Huff *et al.,* 1993).

Como ya se ha mencionado anteriormente el análisis de RAPDs posee una serie de ventajas sobre otras técnicas para el análisis genético de poblaciones (Hadrys *et al.,* 1992), y muchas de ellas lo hacen potencialmente ideal para estudios genéticos en especies raras o amenazadas (Gibbs *et al.,* 1994; Rosseto *et al.,* 1995; Nusser *et al.,* 1996). Sin embargo la técnica también tiene limitaciones.

En primer lugar, estas metodologías que usan cebadores aleatorios han sido cuestionadas en cuanto a su fiabilidad. La causa puede ser debida a que los fragmentos que finalmente resultan amplificados dependen de muchas variables como la concentración de DNA, la longitud, secuencia y concentración de los cebadores, los componentes químicos (especialmente el Mg^{++}), el tipo y la concentración de polimerasa Taq, las temperaturas y el

número de ciclos en los distintos pasos de la PCR, las condiciones de la electroforesis y el procedimiento de codificación de los datos (Penner *et al.,* 1993; (Schierwater & Ender, 1993); Wolff & Peters-van Rijn, 1993). Parece, sin embargo, que se pueden conseguir resultados reproducibles con una práctica de laboratorio cuidadosa y prestando mucha atención a todas estas variables, estableciendo protocolos estandarizados que las mantengan constantes, incluyendo muestras control, realizando estudios piloto de reproducibilidad, y empleando técnicas de codificación lo más objetivas posible ((Smith J.S.C. & Williams J.G.K., 1994). De esta manera los estudios que usan estos métodos de DNA *fingerprinting*, y en nuestro caso particular los RAPDs, pueden llegar a utilizarse sin más problema en este sentido que otros tipos de marcadores, solamente que el tiempo que se ha ahorrado en su aplicación tan directa a menudo se pierde en determinar su consistencia y en confirmar la reproducibilidad de los datos obtenidos.

Una limitación más importante de las técnicas que nos ocupan concierne a la naturaleza de los datos generados, estos marcadores son dominantes, no codominantes como los alozimas o los microsatélites (Williams *et al.,* 1993). Los caracteres que rinden son del tipo presencia (1) o ausencia (0), de forma que no es posible discernir entre el heterozigoto (10 ó 01) y el homozigoto dominante (11), con lo que no se tiene información de las frecuencias alélicas de forma directa, sólo se dispone de información fenotípica. En los últimos años varios autores han propuesto diferentes métodos para abordar el problema de la dominancia en el análisis genético poblacional de los datos de RAPDs (Clark & Lanigan, 1993; Lynch & Milligan, 1994; Stewart & Excoffier, 1996). En general, lo que estos autores reseñan es que los marcadores dominantes pueden usarse en el análisis de la genética de poblaciones, pero nunca pueden ser tan exactos como los marcadores codominantes, pues están sujetos a una serie de presunciones que disminuyen su precisión.

El método de AFLP

Recientemente se ha desarrollado la nueva metodología de AFLPs (Vos *et al.,* 1995), que está siendo extremadamente eficiente en revelar diversidad génica a nivel intraespecífico ya que puede cubrir grandes áreas del genoma en un único ensayo (Karp & Edwards, 1997).

Esta técnica está a caballo entre los RFLPs y los RAPDs, pudiendo anticiparse que ha heredado de cada uno de ellos muchas de sus características favorables para el análisis de la diversidad génica.

En la Fig. 1-12 se esquematizan los tres pasos sucesivos del método: restricción-ligación, amplificación preselectiva y amplificación selectiva. La primera reacción es una doble digestión del DNA genómico con enzimas de restricción, y simultáneamente se realiza la ligación de unos adaptadores. Estos adaptadores tienen una secuencia central y la específica del enzima de restricción. La reacción simultánea es posible gracias a que los adaptadores han sido diseñados de tal modo que su ligación al fragmento digerido no reconstituye el sitio de restricción ((Zabeau M. & Vos P., 1993). Las reacciones de PCR subsiguientes se llevan a cabo con cebadores que se unen a los adaptadores, amplificándose así los fragmentos de restricción generados en el paso anterior. El número de fragmentos

amplificados finalmente se reduce por medio de la adición de nucleótidos al extremo 3' de los cebadores, de forma que sólo se amplifican el subconjunto de fragmentos que comienza por esos nucleótidos selectivos y así pueden ser resueltos en un gel de electroforesis. Como sistema de electroforesis se utilizan normalmente geles de secuenciación. Para visualizar los fragmentos al menos uno de los cebadores está marcado, bien radioactivamente o con fluorescencia. Este último sistema de marcaje ha permitido la automatización de la técnica, de forma similar a la secuenciación. Para visualizar los fragmentos al menos uno de los cebadores está marcado, bien radioactivamente o con fluorescencia. Este último sistema de marcaje ha permitido la automatización de la técnica, de forma similar a la secuenciación.

La técnica de AFLP se asemeja, por tanto, a la de RAPD en ser una forma de DNA *fingerprinting* basada en la PCR que produce marcadores dominantes, presentando por los mismos problemas en el análisis de los datos para los parámetros genético-poblacionales. Sin embargo, el hecho de que los fragmentos sean generados por endonucleasas de restricción, de forma similar a los RFLPs, permite el uso de aproximaciones de tipo evolutivo (Nei & Li, 1979) al análisis de sus datos, con las ventajas que ello conlleva. Además, toda la evidencia apunta a que es una técnica mucho menos sensible a las condiciones de PCR y demás variables destacadas con anterioridad (Vos *et al.,* 1995; Janssen *et al.,* 1996), por las propias características del método. La flexibilidad de la técnica también es destacable. La complejidad de AFLPs puede ajustarse a los distintos tamaños de genoma y al sesgo en el contenido en G+C, a través del uso de diferentes enzimas de restricción y/o al cambio en las bases selectivas. Además la técnica está evolucionando hacia una completa automatización por el uso conjunto de cebadores marcados por fluorimetría y secuenciadores automáticos. La combinación de estas características ha permitido que potencialmente puedan detectarse simultáneamente gran número de marcadores, mucho más que otras técnicas. La caracterización de gran número de loci es una característica deseable no sólo en estudios genético poblacionales, sino también para la construcción de mapas de ligamiento físicos y génicos, especialmente si los marcadores son reproducibles entre laboratorios.

En resumen, el uso combinado de distintas herramientas de la Sistemática Molecular nos ha permitido estudiar la variabilidad genética intra e interespecífica en algunas especies del género *Limonium* pertenecientes principalmente a la sección Limonium para ahondar en los patrones y procesos subyacentes a su evolución. A su vez, el estudio de estos aspectos en la divergencia intraespecífcia de dos de sus especies, que se encontraban en inminente peligro de extinción, *L. dufourii* y *L. cavanillesii*, nos ha permitido intervenir en las decisiones de sus programas de conservación.

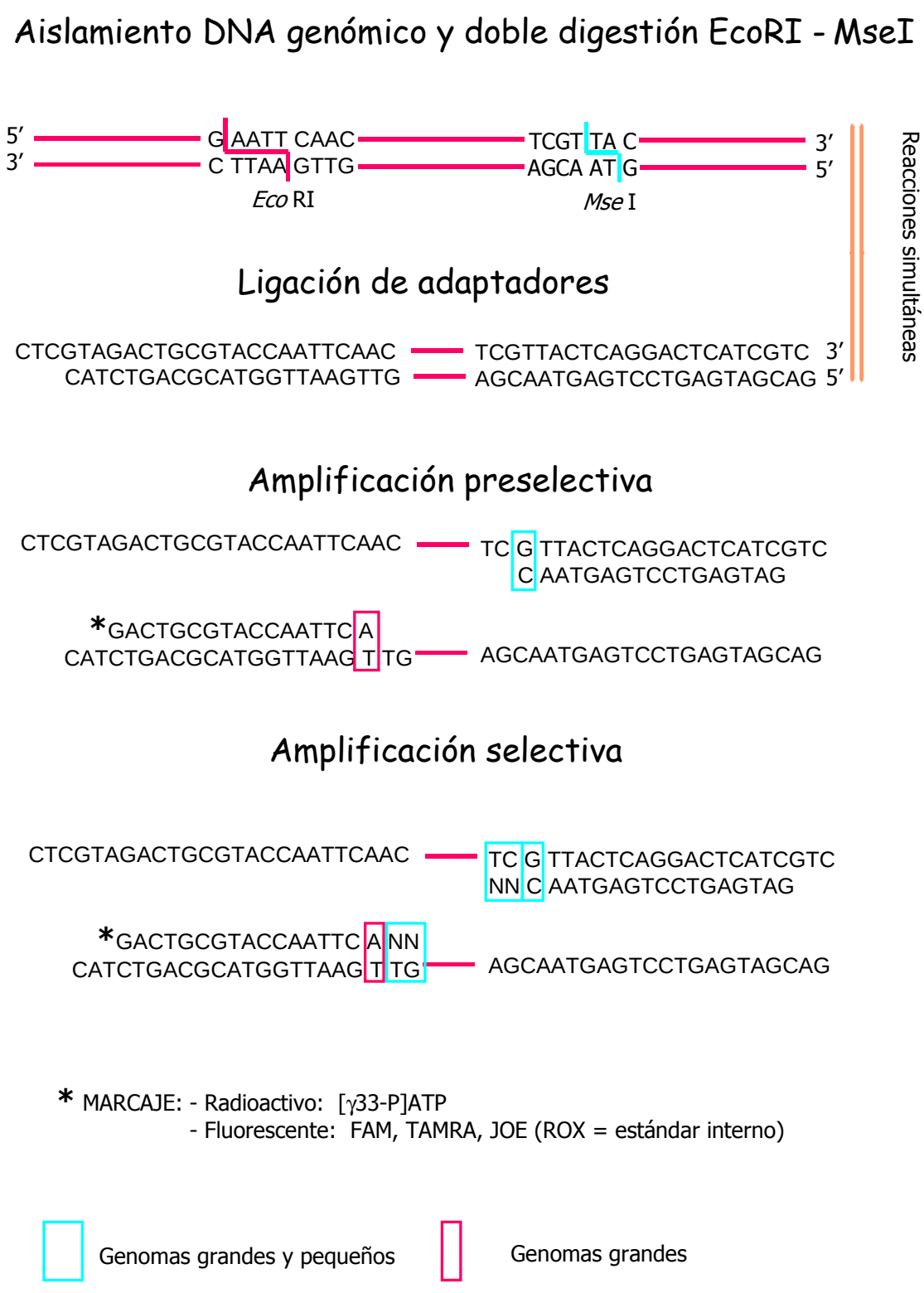


Fig 1-12 Esquematización de los distintos pasos de la técnica de AFLPs.

OBJETIVOS DE ESTA TESIS

El presente trabajo está enmarcado en una línea de investigación que abarca el estudio de la Genética Molecular y Evolutiva de diversos organismos. Más concretamente nuestro grupo de trabajo está centrado en el estudio de la variabilidad genética intra e interespecífica de especies vegetales, principalmente silvestres, con el uso de distintos marcadores moleculares, y su aplicación a la conservación *in situ* y/o *ex situ* de las mismas. El trabajo en el género *Limonium* se inició con el comienzo de esta tesis hace aproximadamente cinco años. En principio se pretendían clarificar distintos aspectos tanto microevolutivos como marcroevolutivos del género, empezando concretamente por la sección más prolífica del mismo, la sección Limonium. Todo ello bajo la propuesta inicial de determinar el *estatus* genético de algunas especies que estaban más amenazadas de extinción. Este objetivo básico pasó a plasmarse posteriormente en una serie de objetivos concretos y otros derivados que constituyen los objetivos de esta tesis y que pasamos a detallar a continuación.

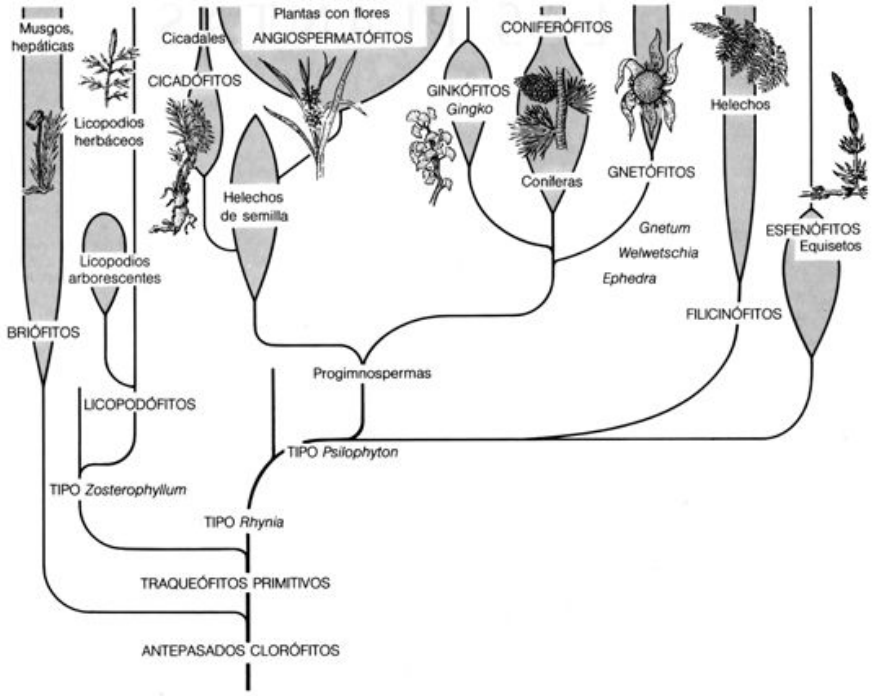
-Análisis de la variabilidad genética intra e interespecífica en algunas especies del género *Limonium*, centrándonos en el estudio de la sección Limonium. El principal objetivo era elucidar patrones filogenéticos micro y macroevolutivos de estas especies. Secundariamente se pretendía también comparar nuestros resultados con la clasificacion taxonómica (no filogenética) actualmente más aceptada de dicha sección. La forma más adecuada de abordar estos objetivos era contrastando la evolución de los genomas citoplasmático y nuclear. Para ello elegimos como marcadores moleculares los RFLPs en el cpDNA y la secuención de la región ITS del rDNA nuclear.

-Análisis de la diversidad genética de *L. cavanillesii*, una especie rara y críticamente amenazada de nuestra Comunidad. Este estudio fue propuesto para intervenir en la elaboración de los planes de recuperación de la especie, de la que sólo se conoce una única población natural. Se presentan los resultados obtenidos con dos técnicas de DNA *fingerprinting* basadas en la PCR: RAPDs y AFLPs.

-Análisis de la variabilidad genética intraespecífica y de la estructura genético-poblacional en la especie *L. dufourii*, para determinar su estatus genético. Los resultados obtenidos han ayudado también en la elaboración de los planes de conservación de este endemismo amenazado. Se usaron igualmente RAPDs y AFLPs como marcadores genéticos. También se comparan las ventajas y desventajas de la utilización de estos marcadores en los estudios de la diversidad genética intraespecífica y más concretamente en su aplicación a trabajos típicos de Genética de la Conservación en especies amenazadas.

2

Study of the evolutionary relationships among *Limonium* species (Plumbaginaceae) using nuclear and cytoplasmic molecular markers



RESUMEN

El género *Limonium* se caracteriza por presentar una especiación muy activa, debido a la frecuencia de procesos tales como la hibridación, la poliploidía y la apomixis. Estas características dificultan en gran medida su estudio taxonómico. Para intentar ahondar en la evolución de este género e iniciar así el estudio de su clasificación sistemática desde un punto de vista filogenético, comenzamos por estudiar la variabilidad genética intra e interespecífica en varias especies pertenecientes a la sección Limonium, la mayoría endémicas de la Comunidad Valenciana, con el interés añadido de que algunas de ellas están amenazadas de extinción, pues los ecosistemas que normalmente ocupan estas especies son muy susceptibles de alteración por parte del hombre. Como marcadores genéticos se utilizaron los RFLPs en el cpDNA y la secuenciación de la región ITS del rDNA nuclear. Para el análisis del cpDNA se emplearon 21 enzimas de restricción y 4 conjuntos de sondas no radioactivas, elaboradas a partir de 10 clones, procedentes en su mayoría de una genoteca del cpDNA de la especie *L. narbonense*. De los 779 fragmentos detectados, 490 eran variables y 339 informativos. Se encontraron en total 15 haplotipos de cpDNA en las 14 especies estudiadas, ya que *L. furfuraceum* resultó tener 2 haplotipos relativamente divergentes entre sí. Las relaciones filogenéticas obtenidas con las especies estudiadas resultaron básicamente coincidentes para los distintos métodos de análisis empleados. Sin embargo, la parsimonia ponderada parece ser el método más apropiado para el análisis de RFLPs, sobre todo si los pesos aplicados pueden corregir posibles violaciones a los supuestos subyacentes al análisis filogenético de los datos. Debido a la evolución reticular de las especies de este género, para poder llegar a extraer conclusiones acerca de la clasificación de las mismas era necesario contrastar los resultados obtenidos con aquellos procedentes de un marcador nuclear. Para ello se secuenció la región ITS del rDNA nuclear, que comprende las zonas ITS1, ITS2 y el 5,8 S. De los 776 caracteres resultantes del alineamiento de estas secuencias, 270 eran variables y 111 informativos. Basándonos en estudios previos en otras especies de angiospermas, se construyeron modelos de estructura secundaria para las zonas ITS1 e ITS2, que se utilizaron para ponderar diferencialmente las bases de los "stems" y los "loops". Se encontraron un total de 24 tipos diferentes de ITS en las 18 especies estudiadas: 6 en *L. delicatulum*, 2 en *L. interjectum* y uno en cada una de las restantes. En realidad dos tipos básicos de ITS, relativamente divergentes entre sí, pueden resumir la variabilidad intraespecífica observada tanto en *L. delicatulum* como en *L. interjectum*, lo que demuestra el origen común de estas especies, o incluso una relación de parentesco más directa. Por otro lado, los distintos métodos de inferencia filogenética utilizados rindieron topologías muy similares entre sí, con y sin utilizar la ponderación diferencial a que nos hemos referido anteriormente; y también tras considerar los "gaps" como datos adicionales. Sin embargo, en general, las relaciones entre las especies son controvertidas con respecto a las obtenidas con el genoma citoplásmico. Se discuten las posibles causas de las concordancias y discordancias en los resultados obtenidos con ambos marcadores, así como la necesidad de un muestreo intraespecífico más exhaustivo cuando se usan este tipo de marcadores en estudios sistemáticos.

ABSTRACT

The genus *Limonium* presents all principal mechanisms of rapid speciation in plants, as hybridization and polyploidy, as well as the possibility of reproduction through apomixis. These special features difficult its taxonomic study. As an initial approximation to the evolution in this genus and its systematic classification from a phylogenetic point of view, we have started the analysis of intra- and interspecific variability in several species that belong to section Limonium. Most of them are endemics to the western Mediterranean Basin, and some are also endangered species, due to the fragility of the ecosystems were these species are usually established. RFLP of cpDNA and ITS nuclear rDNA sequencing have been used as molecular markers for this study. For the cpDNA analysis, 21 restriction enzymes and 4 batches of non-radioactive probes, derived from 10 clones mainly from a *L. narbonense* cpDNA genomic library, were used. From the 779 resulting fragments, 490 were variable and 339 informative. A total of 15 cpDNA haplotypes resulted from the 14 species studied, as *L. furfuraceum* presented 2 relatively divergent haplotypes. Relationships encountered among the species studied were coincident among the different phylogenetic methods used. However, weighted parsimony seems to be the most appropriate method for the analysis of RFLP data, especially if the weights applied can correct for possible violations of the underlying assumptions of the analysis. Due to the reticular evolution of the genus *Limonium*, it seems obvious that in order to draw any conclusion on the actual classification of these species the results obtained should be compared with other sources of evidence from the nuclear DNA. ITS region sequence analysis was performed with this purpose. This region includes ITS1, ITS2 and 5.8S nuclear rDNA zones. The alignment contains 776 characters, of which 270 were variable and 111 informative. Based on previous studies on other angiosperms, secondary structure models for the ITS1 and ITS2 sequences were constructed and used for weighting differentially stem and loop positions. From the 18 species analysed, 24 ITS types were found: 6 from *L. delicatulum*, 2 from *L. interjectum*, and one from each of the rest. Two basic ITS types, relatively divergent between them, can account for the intraspecific variability detected in both *L. delicatulum* and *L. interjectum*, which demonstrates the possible common origin of these two species, or even a possible parentage relationship between them. The different phylogenetic inference methods used rendered very similar topologies, with or without the differential character weighting scheme pointed above, and also when gaps were considered as additional data. However, in general the relationships among the species studied are discordant with respect to those obtained with the cytoplasmic genome. Possible sources of discordances and concordances of the phylogenies obtained with both molecular markers are discussed, as well as the importance of intraspecific sampling when these types of markers are used on systematic studies.

MATERIAL AND METHODS

Plant samples

Twenty three *Limonium* populations representing 15 species from the section *Limonium* and three species classified under other sections of the genus were analyzed for cpDNA variation and/or sequence variation of the ITS region (Table 2-1). In this table it is also shown the number of individuals sampled per population, voucher specimen numbers, locality of origin of the populations, and chromosome numbers and mode of reproduction of each species. Our choice of species was mainly dictated by the availability of material. Most of them are endemic to the East Mediterranean coasts of Spain, and some are also endangered species, as *L. dufourii*, *L. cavanillesii, L. rigualii,* or *L. furfuraceum* (Gómez-Campo *et al.*, 1987). Nevertheless, at least one species from each of the currently recognized subsections of the section Limonium was selected, with the only exception of subsec. Sarcophyllae, for which no material was available. All the species chosen are polyploid, which can be accompanied by the development of a self-compatibility system or apomixis reproduction (Baker, 1966).

Total DNA isolation

Total cellular DNA was isolated using the CTAB method of Doyle (1991). One further chloroform-isoamylalcohol (24:1) extraction step was done when samples were still turbid after the first organic extraction.

Plant materials were collected as 1-3 g of fresh leaf tissue from the greenhouse or from the field and stored at -80ºC until DNA extraction. Usually leaves from two or more individuals from each population were pooled (Table 2-1). This approach has been suggested as a strategy to detect intraspecific or intraindividual variation both in cpDNA RFLPs and ITS sequence analyses ((Soltis *et al*, 1989); Baldwin *et al.,* 1995). To recover any useful information when polymorphisms were detected, posterior independent analysis of these polymorphisms is necessary, for which different approaches were followed depending on the molecular marker (see later).

RFLP analysis of the cpDNA genome

Variation among cpDNAs of *Limonium* was detected by digesting DNAs previously extracted with a total of twenty-one restriction endonuclease enzymes according to suppliers' instructions (Table 2-2). Restriction fragments were separated by electrophoresis on 0.7-1% agarose gels at aprox. 2.5 V/cm for 12 hours with TBE 0.5X buffer. Non-radioactive hybridization methods were used to detect cpDNA fragments. Transfer of DNA to nylon filters (Hybond-N, Amershan) by Southern blotting, and filter prehybridization, hybridization and detection methods followed manufacturer's instructions (Boehringer Mannheim, 1989) with some modifications. Probes used (see later) were labeled with digoxigenin-11-dUTP using the random priming method. Probe unions were allowed at a

level of 85% of homology by performing the hybridizations at 65ºC overnight and later washing the filters at 60ºC twice for 15 min in a prewarmed 0.1%SDS,

Table 2-1 *Limonium* species used for the study of cpDNA RFLP variation and ITS sequencing.

Species	Populations	Voucher number	EMBLaccesion no.	No.Individuals	Chromosome no. (2n)[a]	Reproduction mode
Sect. Limonium						
Subsections						
Genuinae						
L. narbonense	Almardà (Valencia)*	JAR-96132	AJ222838	2	36	Unknown
L. vulgare	Cantabria	JAR-96085	AJ222839	1	36	Unknown
Densiflorae						
L. dufourii	Cullera (Valencia)	JAR-96051	AJ222840	4	27	Apomixis
L. camposanum	Cala Pi (Mallorca)*	JAR-95111	AJ222841	1	27	Apomixis
L. gymnesicum	Sant Pere (Mallorca)*	JAR-94328	AJ222842	1	27	Apomixis
	Cala Blanca (Javea, Alicante)^	JAR-96127	AJ222843	1	Unknown	Apomixis
L. interjectum						
	El Llano (Javea, Alicante)^	JAR-96124	AJ222844	1		
	El Saler (Valencia)^	JAR-96027	AJ222845	1	26	Apomixis
L. girardianum						
Dissitiflorae						
L. delicatulum	CalaBlanca (Javea, Alicante)*	JAR-96018	AJ222846-51	8	25	Apomixis
L. cavanillesii	Torre Badún (Castellón)	JAR-96217	AJ222852	6	27	Apomixis
L. angustebracteatum	Pobla Farnals (Valencia)*	JAR-96127	AJ222853	1	26	Apomixis
L. rigualii	Cala Blanca (Javea, Alicante)	JAR-96126	AJ222854	2	27	Apomixis
	El Llano (Javea, Alicante)	JAR-96125		5		
Steirocladae						
L. virgatum	Cabo Salines (Mallorca)*	JAR-95025	AJ222855	1	27	Apomixis
	Cala Blanca (Javea, Alicante)^	JAR-96141		1		
	El Saler (Valencia)^	JAR-96028		1		
L. furfuraceum	Cabo Huertas (Alicante)	JAR-96219	AJ222856	20	18	Sexual
	Sta. Pola (Alicante)	JAR-96218		10		
L. tenuicaule	Artà (Mallorca)*	JAR-95112	AJ222857	1	18	Sexual
Hyalolepidae						
L. dichotomum	Aranjuez (Madrid)	JAR-96501	AJ222858	1	18	Sexual
Sect. Polyarthrion						

L.caesium	Villena (Valencia)*	JAR-94029	AJ222859	1	18	Sexual
Sect. Pteroclados						
L. sinuatum	C. Gata (Almería)^	JAR-96850	AJ222860	1	16	Sexual
Sect. Schizhymenium						
L. echioides	Cala Blanca (Javea, Alicante)^	JAR-96129	AJ222861	1	18	Sexual

* Samples from University of Valencia greenhouse facility.

^ Samples used on ITS sequencing study exclusively. The rest have been used on both studies except for the Sta Pola population sample of *L. furfuraceum* (see text for details).

a J: A. Rossellò personal communication.

0.5XSSC solution. Reutilization of the membranes was possible for at most twice, for optimal rehybridization and to avoid the presence of marks from previous hybridizations. Probe removal was performed by washing the membranes in distilled water for 1 min, followed by its incubation twice for 15 min at 60ºC in 0.4N NaOH, 0.1% SDS prewarmed solution, and finally rinsing them thoroughly in 2X SSC. Detection of hybridized probes in first hybridizations was performed by inmunochemioluminescence with CSPD® substrate (Boehringer Mannheim, B.M.) followed by autoradiography. Better results were obtained by employing colorimetric reagents (BCIP and NBT, B.M.) to detect the labels after rehybridizations.

Table 2-2. Restriction enzymes used in the RFLP cpDNA study.

Restriction enzyme	Type[a]	Freq.[b]	M.[c]
Asp700	6	H	B
AvaI	6	H	Ph
BamHI	6	H	B
BclI	6	H	B
BfrI	6	H	B
BglII	6	H	Ph
CfoI	4	H	B
ClaI	6	H	Ph
DraI	6	H	B
EcoRI	6	H	B
EcoRV	6	H	B
HaeIII	4	H	Ph
HindIII	6	H	B
MspI	4	H	B
NcoI	6	H	Ph
PstI	6	L	B
SacI	6	L	B
SalI	6	L	B
ScaI	6	H	B
XbaI	6	H	B
XhoI	6	L	B

[a]Enzyme type: 6 = six-cutter, 4 = four-cutter.
[b]Frequency with which the enzyme usually cut the cpDNA in studies based on other species Palmer, 1986): H = high frequency, L = low frequency.
[c]Manufacturer: B = Boehringer Manheim, Ph = Pharmacia.

A total of 10 previously cloned and mapped cpDNA restriction fragments obtained from a genomic library constructed from the species *L. narbonense* (see Fig. 2-1), and a clone from the cpDNA of *Nicotiana tabacum* (see later) were used as probes to detect homologous fragments among the different species (Fragment Ocurrence Analysis, FOA, Bremer, 1991).

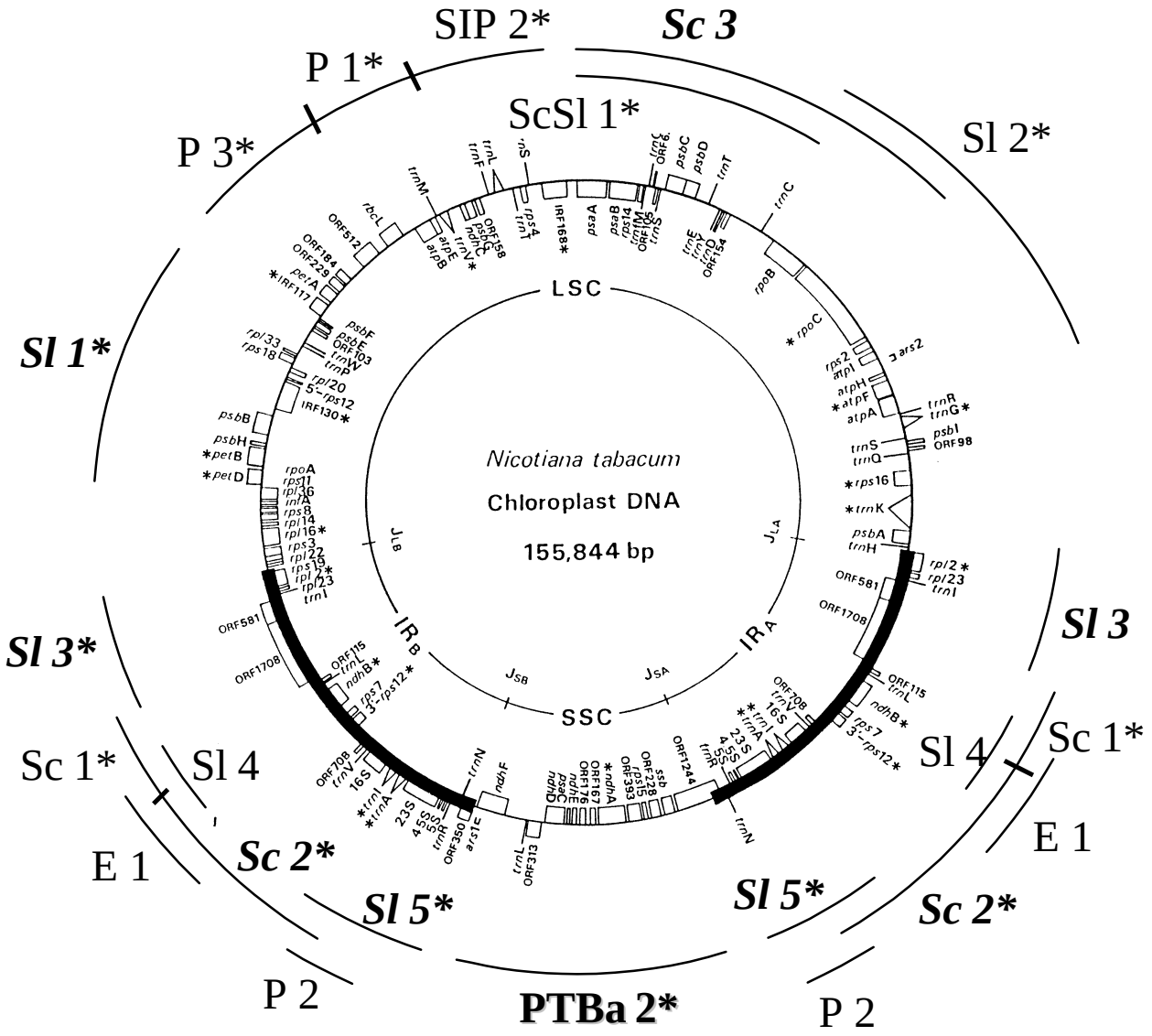


Fig. 2-1 cpDNA clones from *Limonium narbonense* superimposed on the chloroplast genetic map of tobacco. A genomic library from *L. narbonense* was obtained from enriched cpDNA extraction (Ko *et al.,* 1984) and posterior digestion and cloning of restriction fragments following standard protocols (Maniatis T *et al*, 1982)). cpDNA fragments of *L. narbonense* were detected and mapped by using cpDNA clones from *Nicotiana tabacum,* kindly provided by M. Sugiura ((Sugiura *et al*, 1986)), as probes. *L. narbonense* cpDNA clones are denoted by the restriction enzyme with which were obtained (Sl=SalI; Sc=SacI; E=EcoRV; P=PstI), followed by an arbitrary number. Clones showed in italics are in EMBL20 phage vector (B.M.), the rest are clones or subclones in PUCBM20 plasmid vector (B.M.). An asterisk indicates clones finally used in the study of the Limonium section. pTBa2 is a clone that covers the entire SSC region of the cpDNA from *N. tabacum* and was also used in this study (see text for details).

Clones were labeled separately but fragments, usually contiguous, were combined in four batches for filter hybridizations, comprising 127 kb in total, which represents a 84.6% of a presumable 150 kb cpDNA. Briefly, combinations of probes employed were as follows (see Fig. 2-1): group 1 consisted of fragments Sl1, P3, P1, and SlP2, while group 2 comprised contiguous fragments ScSl1 and Sl2 (these two batches cover approximately 62 kb of the large single copy region (LSC) of the cpDNA of *L. narbonense*); group 3 covers the IR region and consisted of the contiguous clones Sl3, Sc1, Sc2, and Sl5; finally, clone pTBA2 from *N. tabacum*, which covers the entire SSC region of its cpDNA (Sugiura *et al.,* 1986), was used as an additional heterologous probe in posterior hybridizations.

Fragment observation and reconstruction of the presence-absence character state data matrix was made directly on the autoradiograms from the different hybridizations. Care was taken not to score a mutation more than once by overlapping autoradiograms probed with adjacent probe/enzyme combinations. Any bands shared in both autoradiograms were scored only once.

In the FOA approach, homology of equal size fragments is assumed, which may be expected given the similarity of *Limonium* cpDNAs ($d<0.05$; Nei 1987, and see later). On the other hand, the difference between restriction site mutation analysis [called *site occurrence analysis* (SOA)] and FOA is that, for each restriction site gain or loss, there will be three characters in the FOA method for each one in the SOA method (3:1 ratio). This implies that, in the former approach, characters will be correlated. Consequently, some authors have rejected the use of this method in phylogenetic analysis (e.g. Swofford *et al.,* 1996) as the assumption of character independence underlies in all phylogenetic reconstruction methods (see Introduction). However, some further considerations are necessary. Bremer (1991) pointed out that if there are enough characters, the resulting phylogeny will not differ between the two methods, since the possible deviations from the 3:1 ratio in the character distribution are probably randomly distributed on the resulting tree. However, we believe that the difference in the probability of restriction site gains and losses would invalidate this argument, unless an appropriate phylogenetic treatment is applied. Different strategies have been followed when using both parsimony and distance methods to analyze the FOA data. These strategies take into account the asymmetric probability of gaining and losing a site, apart from correcting for the 3:1 ratio difference between FOA and SOA methods.

Parsimony analysis uses the presence-absence character state data matrix in a straightforward manner. Three different approaches to parsimony analysis have been followed in this study using PAUP 3.1 software package (Swofford, 1993). Wagner parsimony (Farris J.S., 1970)), in which all changes are equally weighted; Dollo parsimony ((LeQuesne W.J., 1974)), in which parallel restriction site gains are prohibited (one initial gain is allowed to occur, but a second gain is then weighted to infinity); and the weighted parsimony approach ((Sankoff K., 1975)), or "generalized parsimony" ((Swofford & Olsen, 1990)), in which different weights can be applied to gains and losses to reflect the different probabilities of these two evolutionary events.

The branch and bound search strategy was used for Wagner and Dollo parsimony analyses, ignoring invariant characters, and with ACCTRAN optimization. When more than one equally parsimonious tree was found, a 50% majority rule consensus tree was generated to summarize them. Successive approximations character weighting ((Farris J.S., 1969)), an *a posteriori* method that preferentially weights characters with low homoplasy, was performed when more than one MPT was obtained. It was implemented using the *reweight* option in PAUP, weighting each character proportional to the maximum (best fit) rescaled consistency index (RC) over the trees kept in memory each time, and using branch and bound searching with *simple sequence* addition. The process was repeated until identical tree topologies and branch lengths were found in two consecutive searches.

Character-state weighting uses the *step-matrix* option from PAUP. Implementation followed recommendations in Albert *et al.* (Albert *et al*, 1992) but with further considerations. These authors proposed a character-state weighting of gains over losses by a factor of 1.3, for low level analyses as species within a genus, which implies a probability of losing a site of 0.565 over 0.435 for gaining it. But we screened fragments instead of restriction sites, with the resulting 3:1 ratio character difference among these two types of markers. This means that a fragment gain can be derived from a gaining site with a probability of 2/3, but also from a losing site with a probability of 1/3, being these probabilities just the opposite for losing a fragment. Combining these two considerations, the final cost of fragment gains over fragment losses would be 21:19 (i.e. 1/[(0.565+2*0.435)/3]:1/[(0.435+2*0.565)/3]. Moreover, Albert *et al.* (1992) suggest trying other character weight factors in the range 1.0 and 2.5 for a comparative check. Consequently, we also tried the corresponding weights of these extreme values obtained by applying the previous argument, as alternative step-matrices for the FOA approach. These matrices weighted gains over losses equally for the first extreme value, which is equivalent to Wagner parsimony analysis, and 23:17 for the 2.5 factor. A heuristic search ignoring invariant characters and using ACCTRAN optimization was employed for the two step-matrix analyses. To avoid problems associated with tree islands ((Maddison, 1991)), a strategy suggested by Doyle & Doyle, (1993) was followed. It consisted of conducting searches using 100 *random addition sequence* option, followed by TBR branch swapping, retaining only a single tree from each run, and only the most parsimonious trees from all runs. Asymmetric weighting requires the definition of ancestral character states, and we chose the option *all missing-data* for it, and forced ingroup to monophyly ("enhanced Wagner" approach of Albert *et al.,* 1992).

Bootstrap analysis (Felsenstein, 1985) was used to assess the reliability of the phylogenetic hypothesis as implemented in PAUP. A minimum of 100 bootstrap replicates were performed for the three parsimony approaches with heuristic search options as above, except for weighted parsimony in which the stepwise addition was *closest* instead of *random*. In this last case, near-MPTs up to 10 steps longer were also examined as alternative phylogenetic hypotheses.

Phylogenetic information content of the entire data set was also estimated by the skewness coefficient (g_1, (Hillis, 1991)) using the *random trees* option of PAUP.

Pairwise distances among populations can be calculated from the character state data matrix using the nucleotide divergence estimate corrected for restriction fragment data according to the iteration method of Nei (1987, equations 5.53-5.55), for which we used the program described in González-Candelas *et al.* (1995). Divergence estimates from the three 4-cutters and the eighteen 6-cutters were averaged according to (Nei & Miller, 1990). An unrooted neighbor-joining (NJ) dendrogram (Saitou & Nei, 1987) was produced from this matrix using the NEIGHBOR program from the PHYLIP package (Felsenstein, 1993), that allows randomization of the input order of the species.

All trees were unrooted and rooted later for representation purposes using *L. caesium* as outgroup, except for Dollo parsimony, that requires an *a priori* specification of polarity although executed unrooted (Swofford & Olsen, 1990).

Sequence analysis of the ITS region

Amplification and sequencing strategies

The ITS region, that comprises the ITS1, ITS 2, and the 5.8S subunit of the rDNA cistron (Baldwin *et al.*, 1995), was amplified by PCR using universal eukaryote primers designed by White *et al.* (1990) (Table 2-3). General locations of all PCR and sequencing primers are given in Fig. 1-11. To avoid PCR artifacts, recommendations given by Wagner *et al.* (1994) and Baldwin *et al.* (1995) were followed. These include reducing the amount of DNA and the number of PCR cycles per reaction, pooling products from multiple PCRs, and sequencing both strands of the ITS region. PCR reactions were performed in 25 μL total volume containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2μM of each primer, approximately 3 ng of template DNA, 2.5 μL of 10X Taq buffer and 1 unit of Taq DNA polymerase. The profile for amplification reactions consisted of a start at 94ºC for 2 min, followed by 30 cycles at 94ºC for 1 min, 55ºC for 30 sec, and 72ºC for 45 sec; last cycle was followed by 9 min extension phase at 72ºC, then samples were hold at 6-4ºC. For this PCR reaction primers its5 and its4 were used, producing an amplification product of approximately 700 Kb, covering the entire ITS region.

Manual sequencing was performed for all species at least once. Previous PCR products were purified from primers and dNTPs using Ultrafree (Millipore) filters. Cycle-sequencing reactions of these purified products (200 ng, aprox.) were conducted using the AmpliCycle Sequencing kit (Perkin Elmer), following manufacturer instructions for the [γ^{33}P]-ATP end-labeling reaction procedure. This method essentially follows the chain-termination sequencing method of (Sanger F *et al*, 1977), but amplification reactions were performed in a thermal cycler (24 cycles of 1 min at 95ºC, 1 min at 52ºC and 30 sec at 72ºC). ^{33}P labeled primers its1 or its2, and its3 or its4 were used for the ITS1 and ITS2 regions, respectively. Fragments generated were separated in 6% polyacrylamide gels with 1X TBE buffer at 50W, followed by autoradiography at –70ºC for several days.

Table 2-3. Primer sequences used for PCR amplification and sequencing of ITS region, designed by White *et al.,* 1990.

Code	Seq.[a]	Sequence
Its1	F	5'-TCCGTAGGTGAACCTGCGG-3'
Its2	R	5'-GCTGCGTTCTTCATCGATGC-3'
Its3	F	5'-GCATCGATGAAGAACGCAGC-3'
Its4	R	5'-TCCTCCGCTTATTGATATGC-3'
Its5	F	5'-GGAAGTAAAAGTCGTAACAAGG-3'

[a]Sequencing direction: F = forward, R = reverse.

Automated sequencing was performed at least twice for each population using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc.) following manufacturer instructions with some modifications. Purification of PCR products (100ng, aprox.) from dNTPs and primers was performed by incubation at 37ºC for 15 min with exonuclease and shrimp alkaline phosphatase, followed by 15 min at 80ºC to inactivate the enzymes. Primers used were the same as in manual sequencing, without labeling. Centri-Sep (Princeton Separations, Inc) spin columns were used to purify extension products from dye terminators excess after cycle sequencing reactions. Electrophoresis was performed on 4% polyacrylamide at constant voltage (2500V) on ABI 377 or 373 automated DNA sequencers.

Some species show sequence uncertainties in certain nucleotide positions, which could be due to bad sequence resolution in those positions or to real polymorphisms. They were scored as ambiguities following the IUB code. Only DNA from the Cala Blanca population of *L. delicatulum* gave more general polymorphisms in both the ITS1 and ITS2 sequences, which were diagnosed, in the autoradiograms from manual sequences, by the presence of two or more nucleotide states at numerous sites of both complementary DNA strands (Ritland *et al.,* 1993; Baldwin *et al.,* 1995). To distinguish the different repeat-types, after isolation of DNA from four individuals separately, ITS PCR products were cloned using the pGEM-T Easy vector system (Promega Corp.) and at least two clones from each individual were sequenced (Bloch W., 1991). Cloning protocol is based on a modified typical blunt-end cloning procedure that modifies ITS fragments obtained via PCR by adding dATP to its ends, previously to be cloned in the vector (with 3' terminal thymidine on both ends) following standard procedures (Maniatis *et al.,* 1982). Recombinant plasmid DNAs were isolated following the modified mini alkaline-lysis/PEG precipitation procedure recommended by ABI (User bulletin Number 18). Cloned fragments were sequenced automatically using T7 and SP6 universal primers.

Sequence alignment

Manual or automatic sequences belonging to the same population or clone were assembled using Sequencher program (Gene Codes Corp., v. 3.0), and a consensus sequence was obtained for each of them, maintaining the ITS1 and ITS2 sequence data separate. The resulting consensus sequences were aligned using the PILEUP program from the UWGCG software package ((Edelman I. *et al*, 1995). Further adjustments of the alignment were done manually to increase similarity, using sequence editors: LINEUP (UWGCG) and GENEDOC (Nicholas & Nicholas, 1997). From the separate alignments of ITS1 and ITS2 regions, sequences were assembled into the complete ITS region, although most of them were not completely sequenced (Fig. 2-2). A consensus sequence of the ITS region of species from section Limonium was extracted from this alignment using LINEUP program. This *Limonium* consensus ITS sequence was tested against the Ribosomal DataBase Project, and showed the best alignment with *Arabidopsis thaliana* species.

Comparison of these two aligned sequences was useful to determine the boundaries of the coding and spacer regions from all *Limonium* aligned sequences.

Determination of secondary structure

Secondary structures were explored using the minimum free-energy [MFE algorithm, Zuker (1989)] program MFOLD in GCG. The ITS1 *Limonium* consensus sequence, excluding flanking coding regions 18S and 5.8S or including up to 20 bases of each of these coding regions, was folded at 37ºC. Structures within 2.9 kcal/mol of the optimal structures derived from both searches were recovered using the *Squiggles* option in PLOTFOLD (UWGCG). The general model of angiosperm ITS2 secondary structure proposed by Hershkovitz & Zimmer (1996) was employed to infer a consensus secondary structure model for the ITS2 region of *Limonium*. Based on the above alignment of the ITS2 *Limonium* consensus sequence with *A. thaliana*, general substructural features (c1 to c5) proposed by these authors were delimited. Then, the sequence was analyzed with MFOLD, forcing it to include these features by pairing 3'c1 with 5'c2, 3'c2 with 5'c3, and 3'c4 with 5'c5 (5, 13, and 4 bases at each end respectively); apart from forcing to pair the 3'-end of the 5.8S with the 5'-end of the 26S (19 bases each), an additional feature that seems to be common to eukaryotes (Hershkovitz & Lewis, 1996). Only simple, canonical base pairings, including G-U, were considered.

Compensatory mutations may be necessary to maintain the seemingly functional ITS secondary structures (see Introduction). Potential non-independence of characters due to these compensatory mutations should be considered on phylogenetic analysis. Positional downweighting of the non-independent positions provides a method to correct for this, but the extent of such correlation should be determined empirically (Baldwin *et al.,* 1995). Dixon and Hillis (1993) proposed a scheme of phylogenetic character-state weighting in which a relative weight for nucleotide substitutions occurring in double-stranded regions (stem characters) is calculated, after testing for deviations in the number of compensatory mutations that would be expected at random (i.e. no secondary structural constrains). This method was applied to our data set and the relative weighting scheme derived from it was used as alternative to equal character weights in all phylogenetic reconstruction methods employed in data analysis.

Data analysis

Different methods have been followed to analyze ITS sequence data. First, to evaluate the non-random structure of the combined data set the skewness coefficient of Hillis (1991) was used as above.

The Jukes and Cantor (JC) one parameter method (Jukes & Cantor C.R., 1969)) was chosen to calculate pairwise nucleotide divergence values for all sequences both, on the complete ITS region as well as on separate ITS1 and ITS2 data sets. DNADIST from the PHYLIP package was used for this purpose, as it is possible to take into account ambiguities in the nucleotide positions. To minimize information losses, gaps and missing data were deleted only on a pairwise basis.

Justification for combining ITS1 and ITS2 sets of characters in phylogenetic analyses is provided by several recent studies (e.g. Baldwin *et al.,* 1995). To test for the homogeneity of these sets we compared the correlation indices derived from the pairwise distance matrices by means of a t-test using program DIPLOMO (Weiller & Gibbs, 1993). Simultaneously we performed a Welch's approximate t-test of equality of distance means (Sokal & Rohlf, 1995). Significant correlation among ITS1 and ITS2 distance matrices was obtained ($t=0.456$, $df=275$, $P>0.5$; for the null hypothesis $r=1$), as well as, no significant differences among its distance means ($t'_s=0.338$, $df=275$, $P>0.5$; for the null hypothesis of equal means). Based on these results, the combined data matrix from the whole ITS alignment was used to perform the following phylogenetic data analyses, as the variation in the sequenced coding regions was minimal (eleven phylogenetically informative sites in total, Fig. 2-2). Based on selection guidelines set forth by (Kumar *et al*, 1994)(1993), we chose the JC method for phylogenetic inference, as divergence values were in general <0.05 for all ingroup species (Table 2-4). A NJ dendrogram was constructed, using the JC distance matrix obtained with and without considering relative character weights (categories). Bootstrap values for the different groups were calculated for 1000 replicates following PHYLIP manual recommendations.

Table 2-4. Average number of substitutions per 100 sites using Jukes & Cantor distance for the ITS region sequences of *Limonium* species in pairwise comparison, and when using the relative weighting scheme of Dixon & Hillis (1995) (see text for details).

	Ldel3	Ldel7	Ldel24	Ldel23	Lcavan	Lsinua	Lcampo	Lvirga	Ltenui	Ldel16	Ldel17	Lnarbo	Lvulga	Langus	Lcaesi	Lrigua	Ldicho	Ldufou	Lechio	Lfurfu	Lgirar	Lgymne	Linte1	Linte2
Ldel3	0.000																							
Ldel7	0.043	0.000																						
Ldel24	0.044	0.007	0.000																					
Ldel23	0.009	0.043	0.052	0.000																				
Lcavan	0.004	0.024	0.021	0.006	0.000																			
Lsinua	0.287	0.273	0.347	0.278	0.326	0.000																		
Lcampo	0.044	0.017	0.014	0.047	0.021	0.303	0.000																	
Lvirga	0.015	0.027	0.020	0.020	0.002	0.323	0.023	0.000																
Ltenui	0.038	0.011	0.007	0.041	0.015	0.300	0.007	0.017	0.000															
Ldel16	0.005	0.046	0.046	0.006	0.005	0.305	0.049	0.014	0.042	0.000														
Ldel17	0.044	0.012	0.011	0.045	0.027	0.267	0.012	0.025	0.014	0.046	0.000													
Lnarbo	0.105	0.111	0.132	0.101	0.098	0.292	0.105	0.106	0.101	0.108	0.108	0.000												
Lvulga	0.112	0.119	0.142	0.110	0.110	0.297	0.115	0.115	0.111	0.116	0.117	0.011	0.000											
Langus	0.027	0.011	0.005	0.032	0.011	0.344	0.006	0.010	0.004	0.032	0.006	0.098	0.107	0.000										
Lcaesi	0.057	0.071	0.079	0.058	0.053	0.331	0.064	0.051	0.063	0.061	0.064	0.115	0.121	0.049	0.000									
Lrigua	0.045	0.013	0.009	0.048	0.015	0.309	0.004	0.018	0.004	0.050	0.013	0.112	0.121	0.002	0.066	0.000								
Ldicho	0.041	0.014	0.010	0.042	0.015	0.318	0.011	0.019	0.006	0.043	0.016	0.118	0.126	0.006	0.067	0.007	0.000							
Ldufou	0.035	0.014	0.010	0.041	0.015	0.316	0.013	0.016	0.007	0.042	0.016	0.114	0.123	0.008	0.065	0.007	0.007	0.000						
Lechio	0.087	0.100	0.118	0.089	0.086	0.315	0.088	0.094	0.091	0.093	0.097	0.110	0.118	0.089	0.100	0.094	0.093	0.095	0.000					
Lfurfu	0.043	0.012	0.009	0.048	0.021	0.289	0.015	0.024	0.010	0.050	0.015	0.109	0.119	0.010	0.071	0.008	0.012	0.012	0.095	0.000				
Lgirar	0.005	0.015	0.011	0.010	0.000	0.280	0.016	0.007	0.010	0.007	0.015	0.087	0.096	0.002	0.040	0.011	0.014	0.012	0.074	0.015	0.000			
Lgymne	0.008	0.022	0.021	0.013	0.004	0.310	0.020	0.006	0.014	0.008	0.025	0.099	0.109	0.012	0.056	0.015	0.015	0.011	0.087	0.020	0.007	0.000		
Linte1	0.039	0.009	0.004	0.043	0.015	0.301	0.007	0.013	0.005	0.044	0.007	0.111	0.121	0.000	0.064	0.003	0.007	0.007	0.096	0.008	0.009	0.015	0.000	
Linte2	0.005	0.051	0.053	0.006	0.004	0.302	0.047	0.009	0.041	0.000	0.049	0.101	0.111	0.028	0.056	0.045	0.042	0.041	0.088	0.048	0.007	0.009	0.041	0.000

Felsenstein's (1981) maximum likelihood model was employed as an alternative phylogenetic reconstruction method, using FASTDNAML program ((Olsen *et al*, 1994)). Unlike DNAML from PHYLIP, FASTDNAML allows assigning categories as weights to particular nucleotide positions. Empirical transition/transversion (ts/tv) ratio calculated as an average over all sequences gave a value of 1.5. However, the best tree was searched for by using a range of ts/tv ratios from 0.5 to 4.0 as input for the program, and by using *global branch swapping* (N1=21) and *random addition of taxa*. To test whether alternatives to the ML topology were acceptable, the program evaluates the confidence limits of each branch length, and also gives its probability (Felsenstein, 1981).

Finally, PAUP 3.1 package was employed to conduct parsimony analyses with and without considering categories. Ambiguities were considered as *polymorphisms* or *partial uncertainties* character states, but the topologies obtained were exactly the same when using both possibilities, except for the length of the trees, as expected (Swofford, 1993). We will only comment the results obtained when they were considered as partial uncertainties. Heuristic search option was employed following the same strategy as in the previous section. 50% majority rule consensus trees were constructed from all most-parsimonious trees.

Phylogenetic reconstruction methods used until now did not considere insertion-deletion (indel) mutations. Because some length variation is present in the ITS alignment (Fig. 2-2), the effects of indels on evolutionary change of DNA sequences needed to be investigated. Gaps, which result from indels when sequences are aligned, were scored as additional presence/absence characters ((Brunsfeld *et al*, 1992); Swofford, 1993). Subsequently, by adding this additional set of characters from indel data to the sequence data matrix, parsimony analysis was performed as above.

Bootstrap analysis to assess statistical support for individual clades was not possible as the computer memory limits were reached before completion. An alternative method that compares the MPTs with near-MPTs, and constructs a consensus tree from these longer trees, was used instead ((Doyle & Doyle, 1993)).

All trees were unrooted and rooted later using *L. sinuatum* as outgroup based on the results from ITS pairwise divergence values.

Table 2-5 Number of shared bands between each pair of *Limonium* species. Bands generated with 4-cutters are shown in the upper hemimatrix, and those generated by 6-cutters in the lower hemimatrix. The main diagonal shows the number of bands (4-cutters/6-cutters) for each species.

	Lnarbo	***Lvulga***	***Ldufou***	***Lcampo***	***Lgymne***	***Ldelic***	***Langus***	***Lrigua***	***Lvirga***	***LfurfuB***	***LfurfuA***	***Ltenui***	***Ldicho***	***Lcaesi***	***Lcavan***
Lnarbo	115/368	114	78	79	79	78	83	81	77	78	80	78	80	88	83
Lvulga	358	118/367	79	80	80	79	84	82	78	79	81	79	81	90	84
Ldufou	256	258	119/365	116	116	116	107	109	113	117	105	118	115	91	106
Lcampo	258	260	358	120/366	119	117	107	108	113	116	105	117	114	90	105
Lgymne	259	261	358	365	120/366	117	107	108	112	116	105	117	114	90	105
L.delic	257	259	353	359	360	120/365	108	109	112	117	105	118	115	89	106
Langus	259	261	328	330	331	332	118/357	109	103	107	105	108	106	86	107
Lrigua	258	260	340	344	345	343	337	121/365	105	108	117	109	107	88	118
Lvirga	258	260	345	352	353	350	328	338	116/362	113	103	114	111	90	102
LfurfuB	260	262	352	359	360	356	330	341	350	120/372	104	119	116	90	105
LfurfuA	257	259	332	336	337	335	331	354	332	349	118/365	105	103	87	114
Ltenui	257	259	354	360	361	358	333	344	354	358	338	120/366	117	91	106
Ldicho	256	258	345	351	352	350	330	340	347	351	335	355	120/365	94	108
Lcaesi	264	266	271	271	272	273	272	270	267	271	267	271	267	124/365	91
Lcavan	261	263	327	331	332	331	336	350	328	331	347	334	330	274	121/361

RESULTS

Chloroplast DNA variation

CpDNA polymorphism was observed only on pooled DNA from the Cabo de las Huertas population of *L. furfuraceum*, which was diagnosed by two distinct RFLP patterns, of different intensity, with various probe-enzyme combinations. Independent analysis of these polymorphisms was performed. DNAs from 11 individuals of this population, and a pooled DNA sample of approximately 10 individuals from the Santa Pola population, were isolated separately and characterized for those probe-enzyme combinations that showed polymorphism. Six individuals presented cpDNA haplotype A of *L. furfuraceum*, while the rest have haplotype B, except from the Santa Pola population, which usually show pattern A for all probe-enzyme combinations, but one probe-enzyme combination rendered a different pattern, thus this population was excluded from analysis. As pointed in the introduction, independent analysis of these polymorphisms is necessary. Consequently, a total of 15 different cpDNA haplotypes, characteristic of the 14 *Limonium* species investigated, were subjected to further analysis.

A total of 779 different restriction fragments were scored using 21 restriction enzymes, of which 490 fragments were variable and 339 were phylogenetically informative (Appendix I). The number of bands shared by each pair of haplotypes is summarized in Table 2-5. The cpDNA data were strongly left-skewed, with g_1 of approximately –2.0 ($P<<0.001$), indicating a high phylogenetic information content of the entire data set ((Hillis & Huelsenbeck, 1992)).

Wagner parsimony analysis identified 3 equally most parsimonious trees (MPTs) with lengths of 654, consistency indices (ci) of 0.765 (with autapomorphies), and retention indices (ri) of 0.796. Fig. 2-3 shows the 50% majority rule consensus tree that summarizes these MPTs; bootstrap values obtained for individual clades are also shown. The basal nodes of the trees are highly resolved. *L. caesium*, chosen as outgroup species as it is classified under another section of the genus, is in fact situated basal to the rest of the species. It shares on average 269.64 bands with them (Table 2-5). However, *L. narbonense* and *L. vulgare*, from subsection Genuinae, also form a basal monophyletic group, sister to the rest of the species of the section. Besides, the mean number of bands that these two species share with the rest is 259.08, around 100 fewer bands than those shared between each pair of the other ingroup species. *L. angustebracteatum* is the most external taxon to the clade formed by these remaining ingroup species, which form two well-resolved monophyletic groups. The first one is formed by (*L. rigualii*, (*L. furfuraceum* A, and *L. cavanillesii*)), and the second comprises the rest of the species. Variation among the three topologies, and therefore lower bootstrap values, is located at these more internal nodes of the trees, involving *L. furfuraceum* B*, L. dufourii* and *L. delicatulum* species, which differ in their position with respect to *L. camposanum* and *L. gymnesicum*. However, all these later species form a monophyletic

group, always situated internal to *L. tenuicaule*, *L. virgatum* and *L. dichotomum*. Successive approximation character weighting stabilized after three cycles, and identified one shortest tree with ci=0.932 and ri=0.940. This tree has the same topology as one of the shortest Wagner trees, which situates *L. delicatulum* as a sister taxon to *L. camposanum* and *L. gymnesicum*, followed by *L. dufourii*, and then *L. furfuraceum* B. Contrarily, Dollo parsimony analysis gave a unique tree, with length 740, ci=0.676 and ri=0.905 (Fig. 2-4), that differed from all the above trees. It places *L. furfuraceum* B external to the monophyletic group formed by two clades that include (1) *L. dichotomum*, *L. virgatum* and *L. tenuicaule*, and (2) *L. delicatulum*, *L. dufourii*, *L. camposanum* and *L. gymnesicum*, respectively. On the other side, *L. furfuraceum* A form a monophyletic group with *L. rigualii* instead of *L. cavanillesii*, which is basal to them. In general, bootstrap values were also low for these groups. The two other different character-state transformational weights render identical MPT topologies, which were also coincident with one of the three Wagner MPTs. Fig. 2-5 illustrates the tree obtained with the weighting scheme 21:19 and its bootstrap values, which are also low for the most internal clades. Besides, the topologies of near-MPTs, include the three different topologies resulting as MPTs in Wagner parsimony analysis.

The NJ tree derived from the pairwise genetic distance matrix of restriction fragment data (Table 2-6) renders a tree that identified the same groups as in the weighted parsimony analyses (Fig. 2-5). Divergence values ranged from 0.0003-0.0229. The extreme values of this range are due, on the lower side, to *L. camposanum* and *L. gymnesicum*, two highly related species from Mallorca island included in subsection Denssiflorae, which always form a monophyletic group in all phylogenetic approximations tried. The upper value corresponds to the typical level of divergence of the species from subsection Genuinae, *L. narbonense* and *L. vulgare*, with respect to the other species (mean 0.0219), including *L. caesium*. However, this outgroup species has levels of pairwise divergence with all the other ingroup species ranging from 0.0176-0.0196. Levels of divergence among these other ingroup species ranged from 0.0010 to 0.0066. Note that intraspecific pairwise divergence between *L. furfuraceum* A and B haplotypes are in the middle of this range.

Based on these cpDNA RFLP results, the current classification of *L. narbonense* and *L. vulgare* within section Limonium may be questioned, as divergence levels with other species of the same section were similar to those obtained with the outgroup species. Consequently, we decided to include as possible outgroups for the analysis of the nuclear ITS region two species that are classified under other sections of the genus, *L. sinuatum* and *L. echioides* (Table 2-1). Other species included in the ITS study, from which cpDNA was not studied, correspond to a parallel survey on the presumed hybrid species *L. interjectum*, whose hypothetical ancestors could be *L. virgatum* and *L. girardianum* based on morphological characters ((Pau C., 1898); (Soler & Rosselló, 1997)).

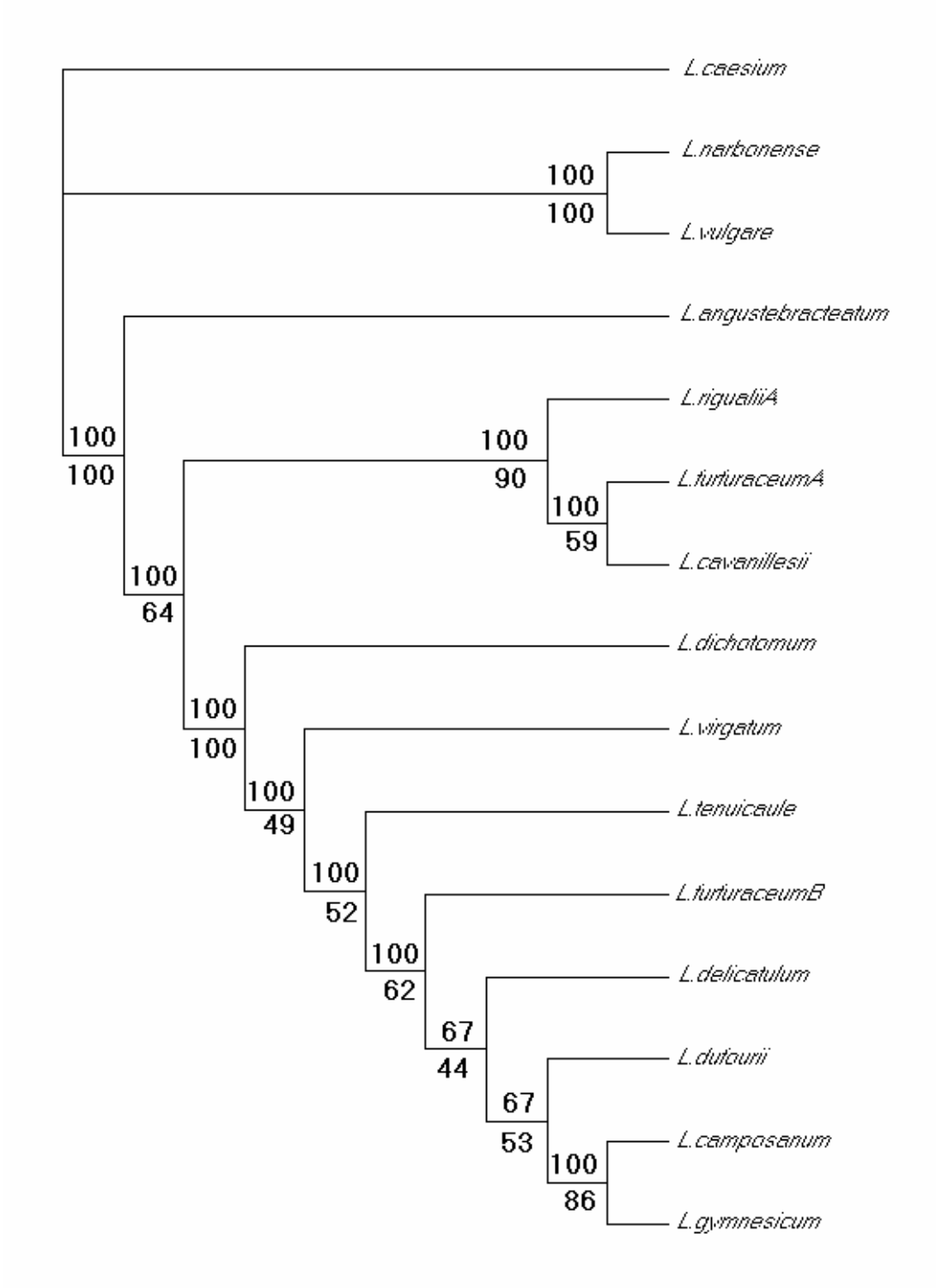


Fig. 2-3 50% majority rule consensus tree from *Limonium* cpDNA analysis using Wagner parsimony method. Bootstrap values are those below the branches of the tree.

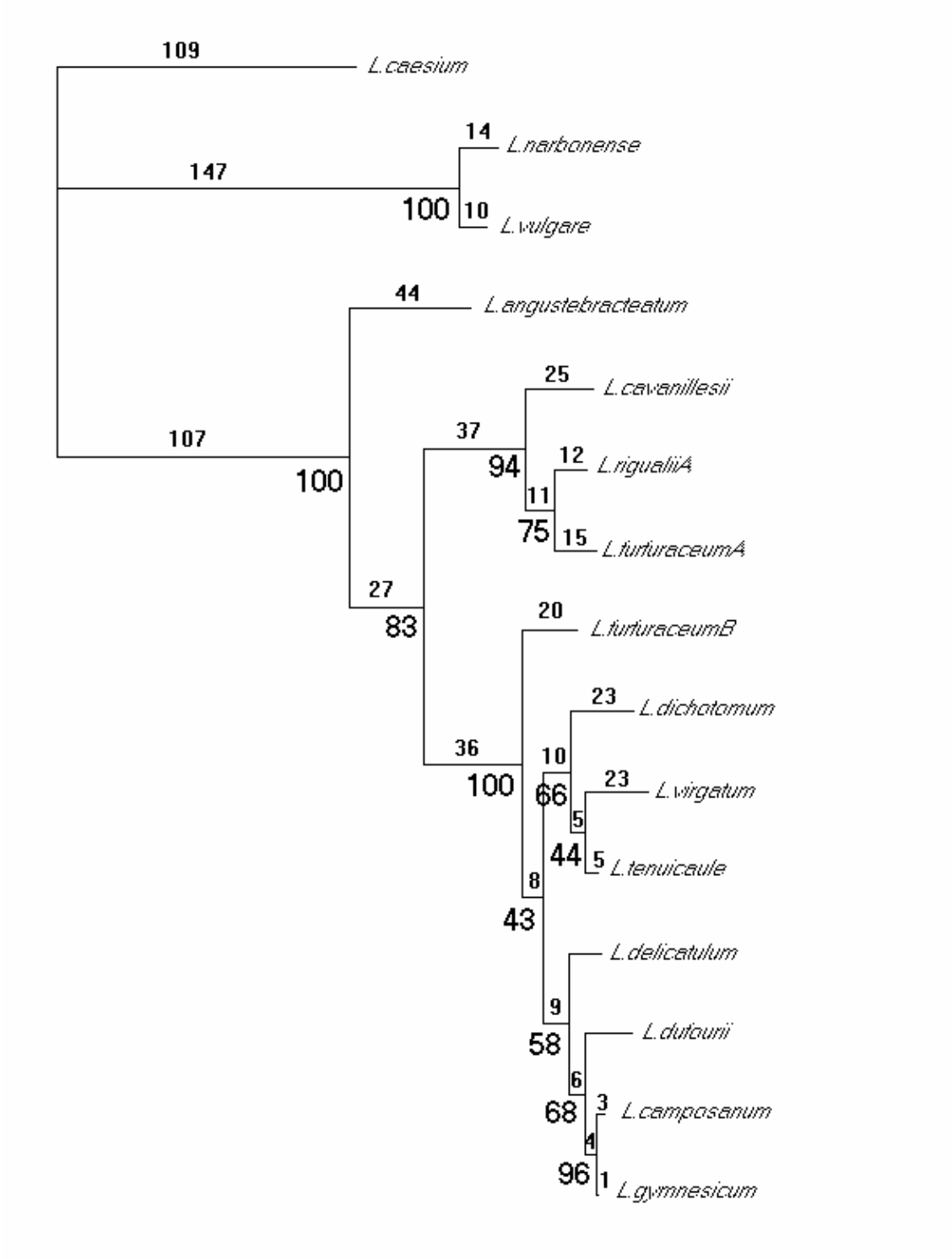


Fig. 2-4. Maximum parsimony tree derived from Dollo parsimony analysis of cpDNA restriction fragment data from *Limonium* species. Numbers along the branches represent mutational steps and those below are bootstrap values for each clade.

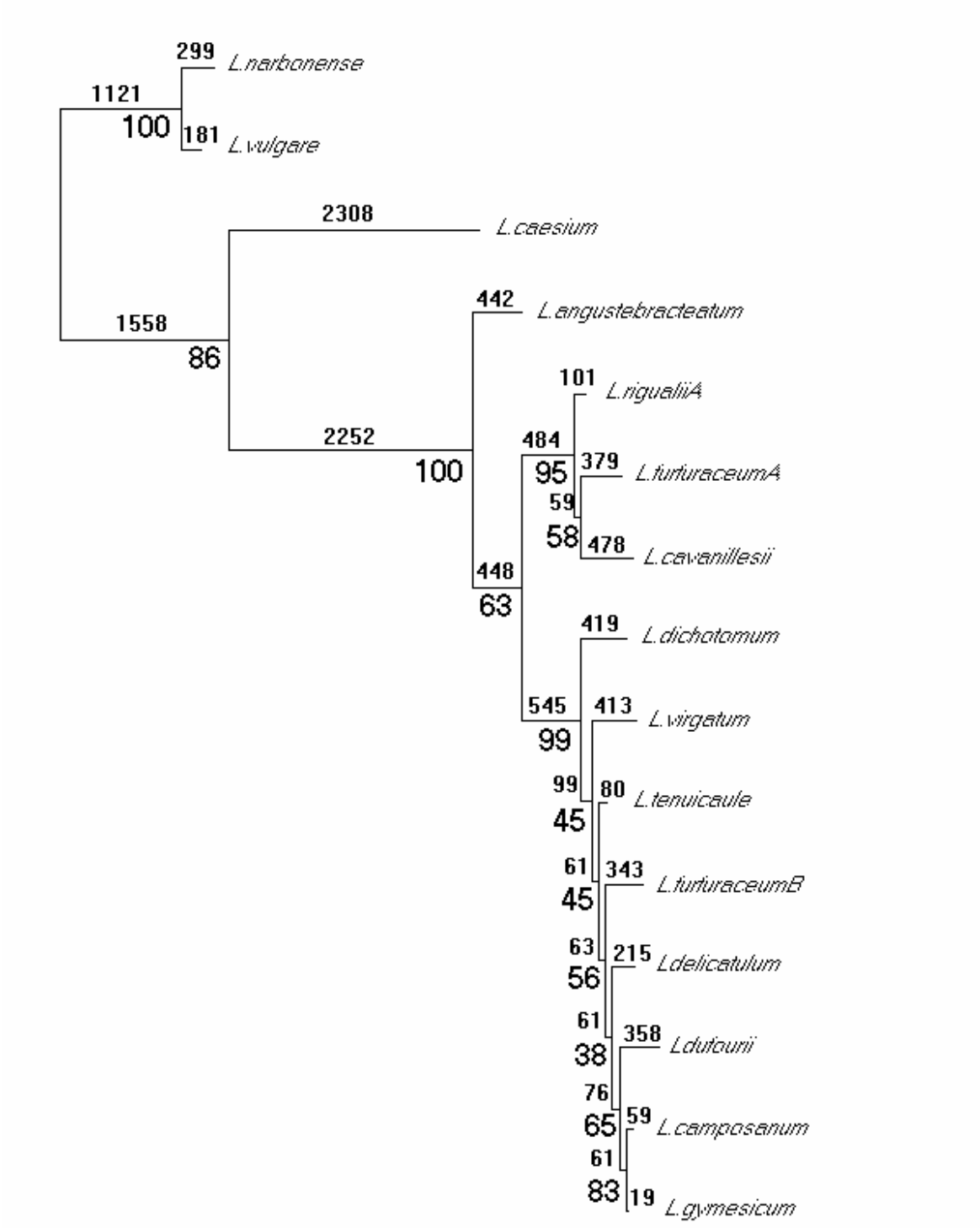


Fig. 2-5 Maximum parsimony tree derived from 'enhanced Wagner' parsimony analysis using the stepmatrix 21:19. Numbers along the branches represent mutational steps and those below are bootstrap values for each clade. This topology is identical to that of the NJ tree.

Table 2-6 Distance matrix relating cpDNAs from the different OTUs obtained using Nei & Miller (1990) procedure to 4- and 6-cutter restriction enzyme fragments in *Limonium* species.

	Lnarbo	**Lvulga**	**Ldufou**	**Lcampo**	**Lgymne**	**Ldelica**	**Langus**	**Lrigua**	**Lvirga**	**L.furfuB**	**L.furfuA**	**Lteniui**	**Ldicho**	**Lcaesi**	**Lcavan**
Lnarb	0.0000														
Lvulg	0.0015	0.0000													
Ldufo	0.0229	0.0225	0.0000												
Lcamp	0.0225	0.0221	0.0014	0.0000											
Lgym	0.0223	0.0219	0.0014	0.0002	0.0000										
Ldelic	0.0228	0.0224	0.0020	0.0012	0.0011	0.0000									
Langu	0.0209	0.0205	0.0059	0.0058	0.0056	0.0053	0.0000								
Lrigua	0.0222	0.0217	0.0047	0.0044	0.0043	0.0043	0.0045	0.000							
Lvirga	0.0223	0.0219	0.0030	0.0022	0.0022	0.0025	0.0061	0.005	0.000						
Lfurfu	0.0227	0.0222	0.0024	0.0018	0.0016	0.0020	0.0062	0.005	0.002	0.0000					
Lfurfu	0.0223	0.0219	0.0062	0.0057	0.0056	0.0058	0.0057	0.001	0.006	0.0044	0.0000				
Ltenui	0.0229	0.0224	0.0016	0.0011	0.0010	0.0012	0.0052	0.004	0.001	0.0015	0.0054	0.000			
Ldich	0.0227	0.0222	0.0032	0.0026	0.0025	0.0026	0.0059	0.005	0.003	0.0027	0.0061	0.001	0.0000		
Lcaesi	0.0201	0.0195	0.0183	0.0186	0.0185	0.0184	0.0184	0.019	0.018	0.0190	0.0197	0.018	0.0186	0.000	
Lcava	0.0210	0.0206	0.0067	0.0064	0.0062	0.0062	0.0047	0.002	0.006	0.0067	0.0028	0.005	0.0060	0.017	0.0000

ITS region variation

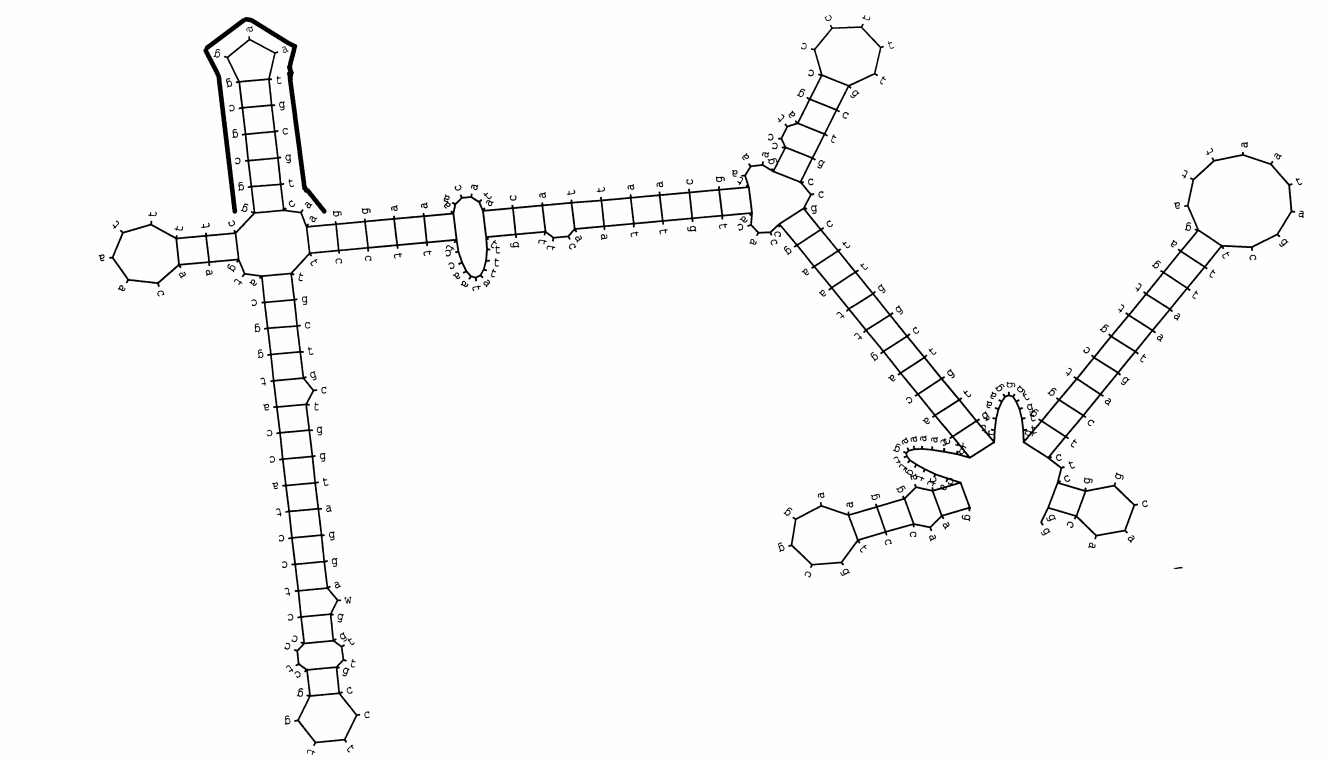
Aligned sequences of ITS1 and ITS2, including part of the 5.8rRNA gene and flanking coding regions were obtained for all species classified under section Limonium and the three outgroup species with 776 characters in total (Fig. 2-2). All sequences have been submitted to EMBL and the corresponding accession numbers are shown in Table 2-1. The length of the ITS1 and ITS2 regions varied from 200 to 221 bp and from 232 to 249 bp, respectively. Most of the length variation encountered is due to small insertion/deletion (indel) events of only one or two base pairs. Only outgroup species have larger indels, especially *L. sinuatum*. This species also presents a zone at the end of the ITS2 region that was not possible to align unambiguously.

Among the 22 populations from the 18 *Limonium* species studied, 24 different ITS sequence types were identified. Six derived from 8 clones of *L. delicatulum* Cala Blanca population, which gave six different ITS types, two per individual studied, except one individual that has the same sequence as individual 0 (see later). A unique ITS type was extracted from the other 21 populations surveyed. A consensus sequence was then constructed from those sequences that belong to more than one population of the same species, each representative of that species respectively, as no differences were observed among their populations. The only exception was the species *L. interjectum* that presented two ITS types, one from each population studied. Note, therefore, that ambiguities scored were present in all sequences gathered as a consensus for that particular species or population finally analyzed.

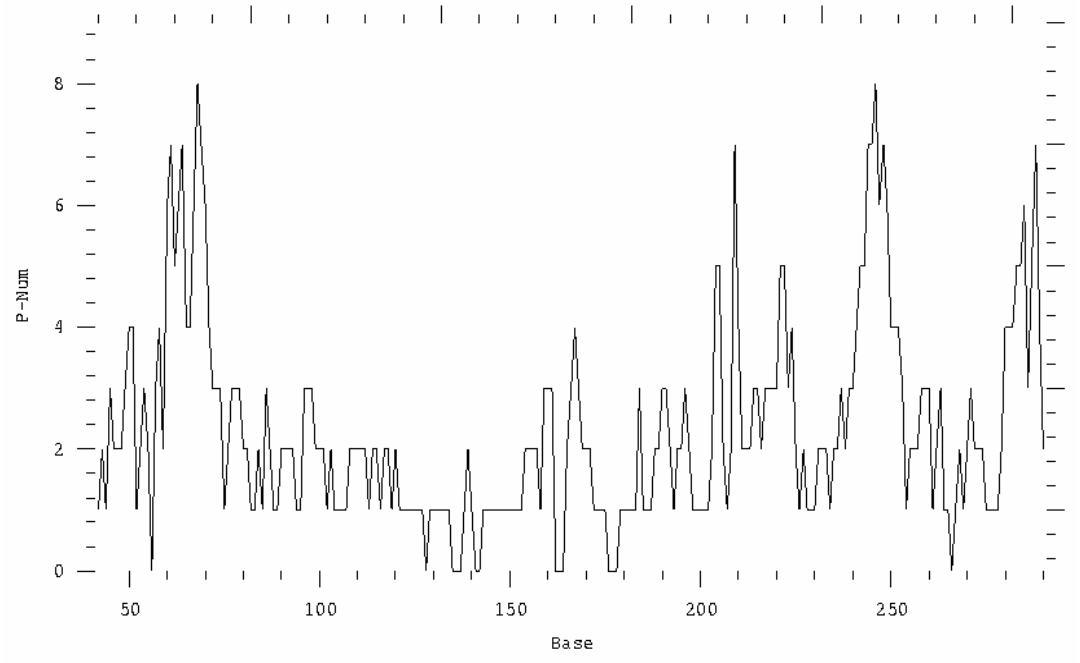
ITS secondary structure and character weighting

The consensus secondary-structural model for the ITS1 and ITS2 regions from the corresponding *Limonium* consensus sequences is shown in Fig. 2-6a and 2-6b respectively.

The optimal minimum free-energy (MFE) secondary-structure of the ITS1 region from *Limonium* derived from the search that includes the flanking coding regions (see Material and Methods) was finally chosen as the most plausible. The graphic output from p-numplot option in PLOTFOLD was useful to take this decision (Fig. 2-6a). The secondary-structure ITS1 model contains a series of hairpin structures present in at least one of the structures derived from our folding analysis. The p-num graphic shows that the majority of nucleotides involved in these hairpin structures complement with at most 3 different nucleotides, which gives further support to this MFE structure. For instance, the hairpin found among the 3'-end of the ITS1 and the 5'-end of the 5.8S was present in all MFE structures. This feature is common to other organisms (Yeh L.C.C. & Lee J.C., 1991); (Bakker *et al*, 1995). Besides, the second MFE structure derived from the search when coding regions were excluded was identical to our selection. Liu and Schardl (1994) described a highly conserved sequence among flowering plant species that could have a key function in rRNA transcripts. This sequence is present in all *Limonium* species (bases 187 to 207, Fig 2-2), except for one base in *L. sinuatum* (position 188) and one in *L. narbonense* and *L. vulgare* (position 207). The nucleotides involved in this



a



b

Fig. 2-6a (a) Hypothetical (MFE) secondary structure of ITS1 obtained when flanking coding regions were included. The conserved sequence of Liu and Schardl (1994) is highlighted. (b) The graphic shows the *P-Num Plot* derived from this search.

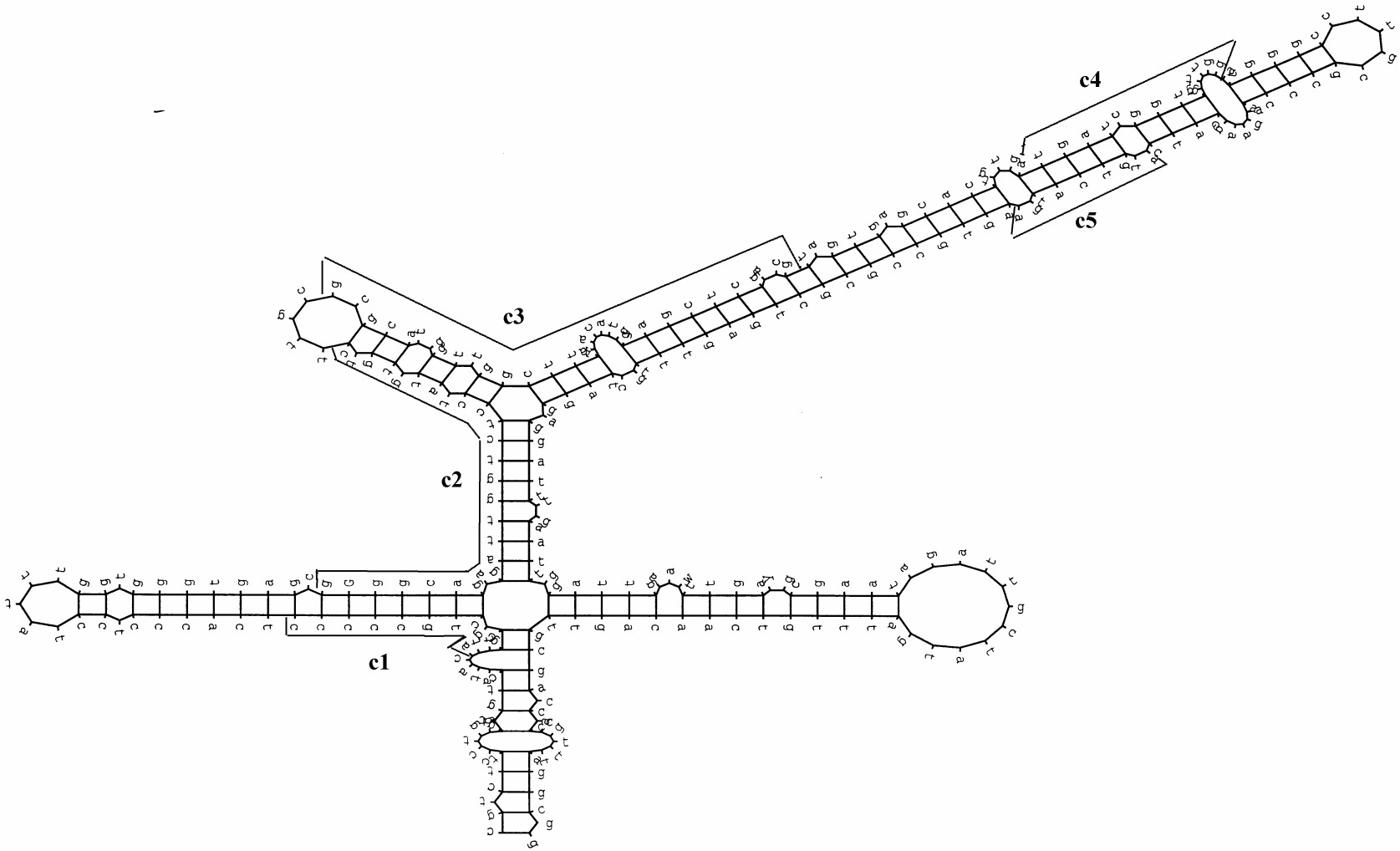


Fig. 2-6b Hypothetical (MFE) secondary structure of ITS2 obtained when flanking coding regions were included. Conserved features identified by Hershkovitz & Zimmer (1996) for other angiosperms are shown for the *Limonium* ITS2 consensus sequence as c1 to c5.

region form a hairpin that is present in all ITS1 MFE structures obtained (e.g. Fig 2-6a). This result could reinforce the idea of a specific functional role for this sequence.

Fig. 2-6b also shows the c1 to c5 ITS2 conserved regions identified by Hershkovitz & Zimmer (1996) for other angiosperms. The MFE ITS2 secondary-structure recovered from our search is very similar to that proposed by these authors as common for angiosperms, but c6 substructural feature was not encountered. Instead, a 5'-end hairpin structure was present in all ITS2 MFE structures recovered with and without constraining options.

To address the question of independence of stem characters (those at base-pairing regions) we performed the χ^2-test proposed by Dixon & Hillis (1993). First from final ITS1 and ITS2 secondary-structure models, the observed number of single and double compensatory and non-compensatory mutations were calculated, excluding outgroup species, and considering ambiguities both as uncertainties and polymorphisms separately. Expected values were generated from the probabilities of random substitutions as proposed by these authors. It was not possible to apply the test for double changes, as the minimum expected value was less than 5 (Table 2-7). Results are only shown when ambiguities were considered as uncertainties, but there were significantly more compensatory mutations than expected by chance among single changes for both cases (χ^2 values ranged from 14.9-54.0, df=1, $P<0.001$). Therefore, as stem bases are not evolving independently, we proceeded to calculate the appropriate relative weight for these characters assuming linear scaling as suggested by the authors. The values obtained in each case ranged from 0.79 to 0.83. Consequently, a relative weighting scheme of 0.8:1.0, for stem vs. loop characters, was chosen for further analysis, as alternative to equal character state weights.

ITS sequence divergence

Table 2-4 shows the average number of substitutions per 100 sites using JC distance for the ITS region sequences in pairwise comparisons, and when using the relative weighting scheme described above. ITS sequence divergence values range from 0 to 0.35. The highest value corresponds to the typical divergence between *L. sinuatum* and all the other species (mean ≈ 0.30). Levels of divergence between *L. narbonense* and *L. vulgare* sequences with respect to the remaining species are around 0.11. Pairwise comparison of *L. echioides* with all the other ingroup species was slightly lower (≈0.09). Besides, when comparing *L. caesium* with these other ingroup species, divergence values were still lower, ranging from 0.08 to 0.04. Excluding *L. narbonense* and *L. vulgare*, ITS divergence values for ingroup species range from 0.00 to 0.05. Note that null divergences are found among *L. interjectum2* and *L. delicatulum16, L. interjectum1* and *L. angustebracteatum*, and *L. girardianum* and *L. cavanillesii*. However, percentages of identity between these species are 99.8, 94.2, and 92.2, respectively. Except for the former pair, which only differs in two gaps in the ITS2 sequence, the other species differ in positions that are ambiguous. This accounts for the difference among divergence values of the species of each pair with respect to other species, which will logically influence their final location in the trees. It is also remarkable that intraspecific ITS pairwise divergence values have identical range limits (0.00 to 0.05), both for *L. delicatulum*

clones (Ldel) *and L. interjectum* populations. Two major types of sequences are observed in these species: type A involving Ldel7, Ldel24, Ldel17 and Linte1, with pairwise sequence variation ranging from 0.004-0.008; and type B involving L. del3, Ldel23, Ldel16 and Linte2. Their range of pairwise variation is 0.00 to 0.008. Note that clones Ldel7-Ldel3, Ldel17-Ldel16 and Ldel23-Ldel24 belong to the same sampled individual (0, 1, and 2), respectively.

Table 2-7 Substitutions observed in *Limonium* (a) ITS1, and (b) ITS2, sequence data on the basis of their models of secondary structure proposed in Figs. 2-6a and 2-6b, respectively. Ambiguities are considered as uncertainties.

(a)ITS1

Type of substitution (no. of ways[a])	No. Expected[b]	No. Observed
Sigle:		
Base pairing to base pairing (4)	2.88	9
Base paring to non-base pairing (28)	20.15	14
Double		
Base pairing to base pairing (11)	0.51	2
Base paring to non-base pairing (32)	1.49	0

(b)ITS2

Type of substitution (no. of ways[a])	No. Expected[b]	No. Observed
Sigle:		
Base pairing to base pairing (4)	5.25	21
Base paring to non-base pairing (28)	36.8	21
Double		
Base pairing to base pairing (11)	0.26	0
Base paring to non-base pairing (32)	0.74	1

[a] Number of ways (of 120 possible) in which one can choose two pairs of nucleotides that have this character.
[b] Based on the frequency of complementary pairs expected at random (Fig. 3 from Dixon and Hillis).

Phylogenetic analyses

Out of the 776 aligned positions from the whole ITS region, 270 sites were variable, of which 111 were phylogenetically informative. The skewness tests suggested non-random structure in this data set, $g_1 = -1.15$, which is well beyond the $P<<0.01$ level of significance (Hillis & Huelsenbeck, 1992).

Fig. 2-7 shows the NJ dendrogram derived from the JC distance matrix of Table 2-4. The NJ tree obtained when equal weights for stem and loop positions were considered has the same topology. Bootstrap values are high at the basal nodes of the tree, involving outgroup species, and the monophyletic group of (*L. narbonense* and *L. vulgare*). The other ingroup species form a monophyletic group supported by a 90% bootstrap value. Within this group there are two well-supported groups. One is formed by ITS type B sequences described above, with 99% bootstrap value. The other, supported by a 98% bootstrap value, places *L.*

angustebracteatum as basal to two other groups, one including those individuals with ITS type A and *L. furfuraceum*, the other formed by ((*L. rigualii*, *L. camposanum*), ((*L. dufourii*, *L. dichotomum*), *L. tenuicaule*)). However, bootstrap values for these later groups are in general lower than 50%, as those for the rest of these ingroup species.

Maximum-likelihood analysis of *Limonium* ITS sequences rendered exactly the same topologies for the different ts/tv ratios tested (from 0.5 to 4.0) and with and without considering categories. However, the tree with the highest ML value (-2675.7) was that obtained for a ts/tv ratio = 1.0, and without using categories, which is shown in Fig. 2-8. Basal branches are all significantly positive and do not differ from that described in the NJ tree. A group formed by all the other ingroup species is also well-supported. *L. girardianum* is located basal to all these ingroup sequences, as it was in the NJ tree. Although a large number of other inner branches have confidence limits that include the zero value, there are also some groups with significantly positive branches. One group is formed by ITS type B sequences. The other group includes those species that form a monophyletic group with a 98% bootstrap value in the NJ tree. Within this last group there are three well-supported clusters: (*L. del7, L.del24*); (((*L. dichotomum*, *L. dufourii*), *Ltenuicaule*), *L. rigualii*); and (((*L. del*17, *L. angustebracteatum*), *L. camposanum*), *L. interjectum*1).

Wagner parsimony analysis from the ITS data matrix identified large numbers of equally most parsimonious trees (>11,000 before memory limits were reached), with lengths of 330, and ci and ri values of 0.833 and 0.971, respectively. Identical strict consensus topologies were obtained with and without considering categories. Fig. 2-9 represents the 50% majority rule consensus tree when equal character weights were used. Despite the large number of MPTs, the consensus tree retained considerable resolution, especially at basal nodes, being the relationships coincident with those obtained with previous phylogenetic analyses. Polytomies at inner branches are responsible for the large number of equally parsimonious trees encountered. However, the relationships among these ingroup species have features in common with those commented above using other phylogenetic inference methods, especially with the ML tree.

Although ITS sequences have provided enough phylogenetic information to resolve some stable relationships among these ingroup, very closely related species, in order to check whether additional information could be gleaned by considering indel mutations, parsimony analysis was performed with the additional data matrix of 38 presence/absence of gap characters. The skewness index from the whole data set was -1.12 ($P<<0.01$). A total of 177 MPT topologies were obtained when using equal character weights, with 384 steps, ci=0.812, and ri=0.771. When relative character weighting was implemented as above, but giving gap characters a relative weight of 1.0, as loop bases, the number of trees was reduced to 21. The consensus topologies derived from these MPTs were very similar in both analyses. The only difference was a higher resolution in the clade that includes *L. dufourii* and *L. dichotomum*, by placing these two species basal to this clade when categories were considered. However,

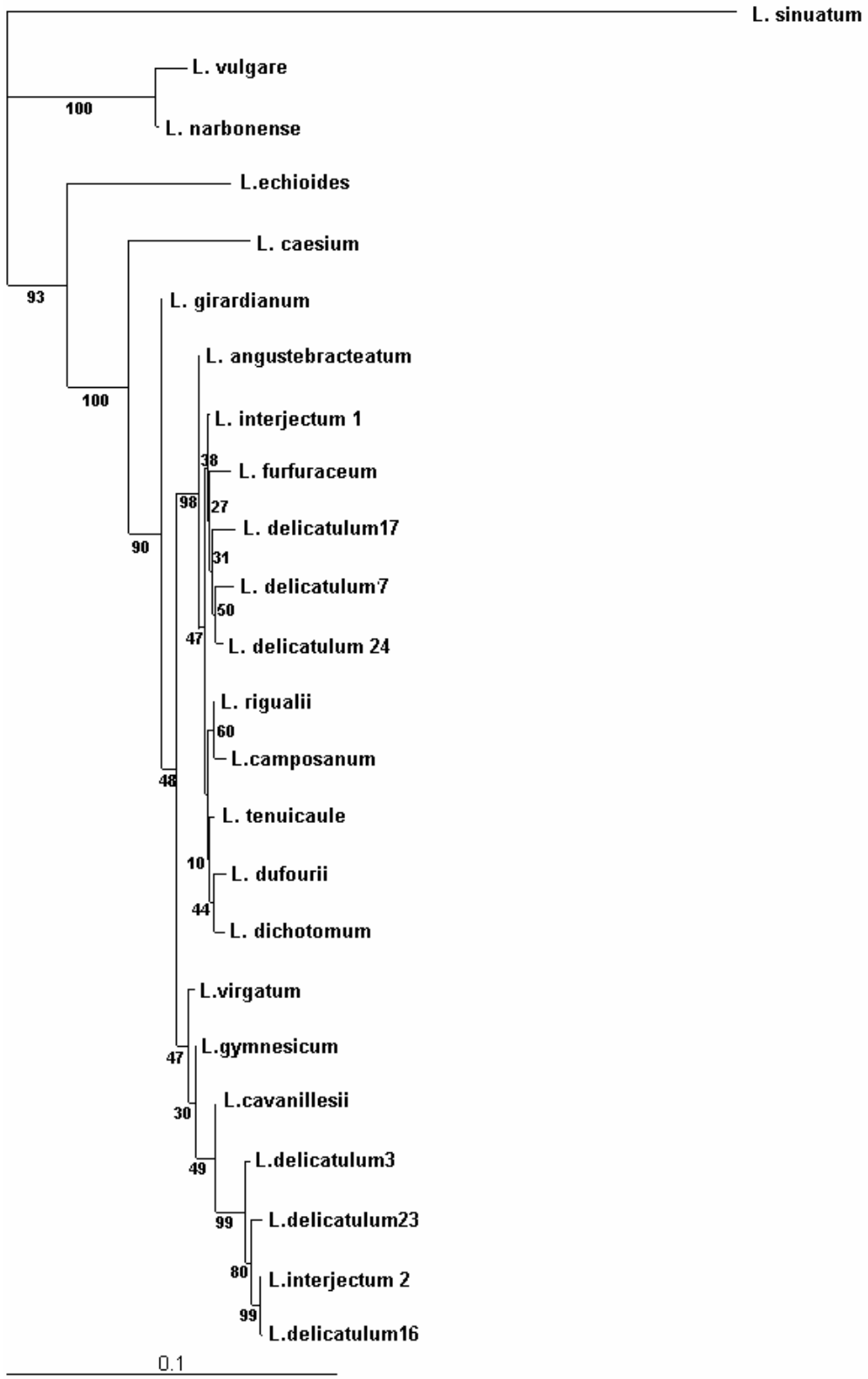


Fig. 2-7 Neighbor-joining dendrogram obtained from ITS sequences using JC distance matrix. Bootstrap values for each group are shown along the branches

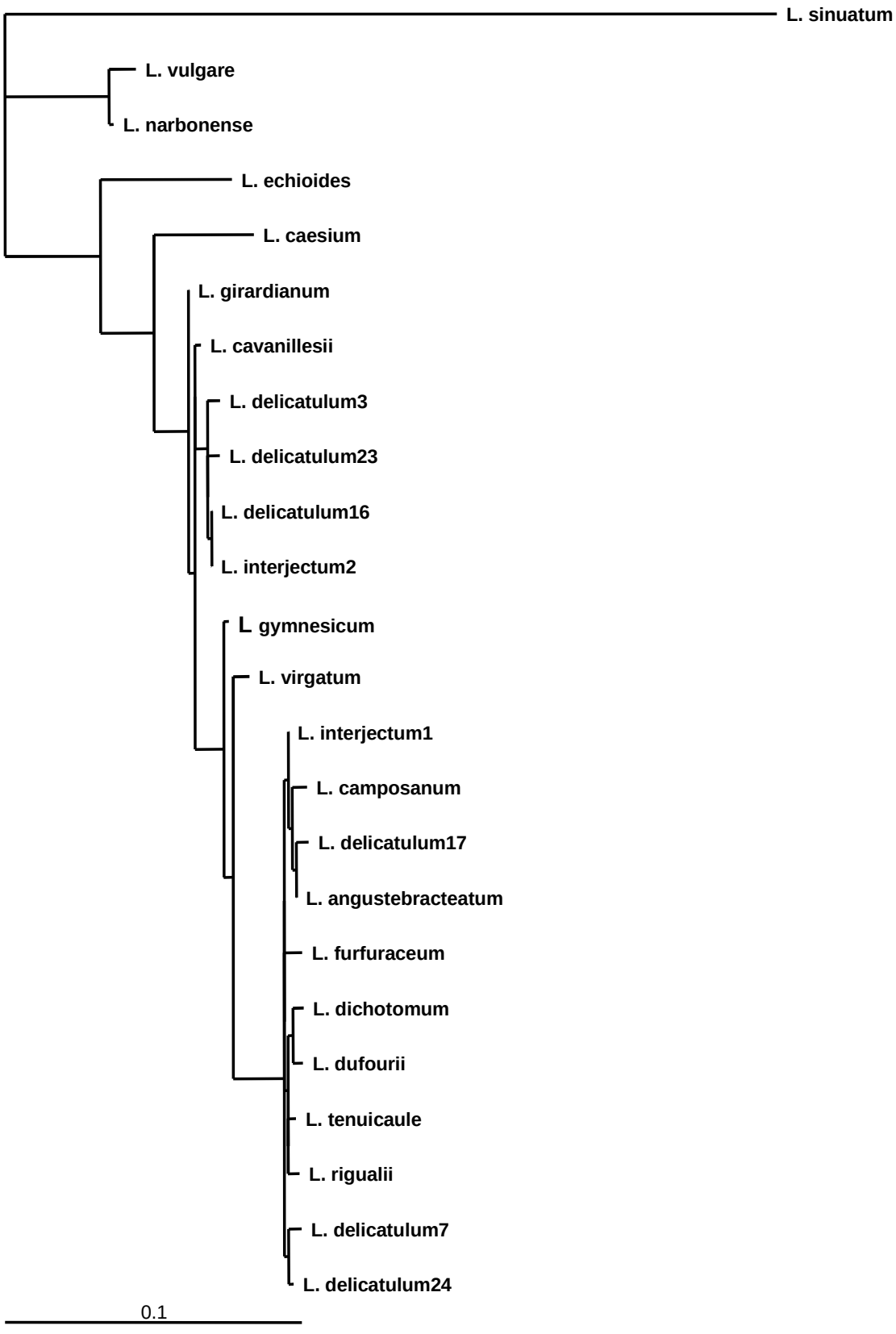


Fig. **2-8** Maximum likelihood tree derived from ITS sequence analysis with a ts/tv range from 0.5 to 4.0. The maximum likelihood value was obtained for the tree with ts/tv ratio = 1.0 and applying equal character weights to stem and loop positions.

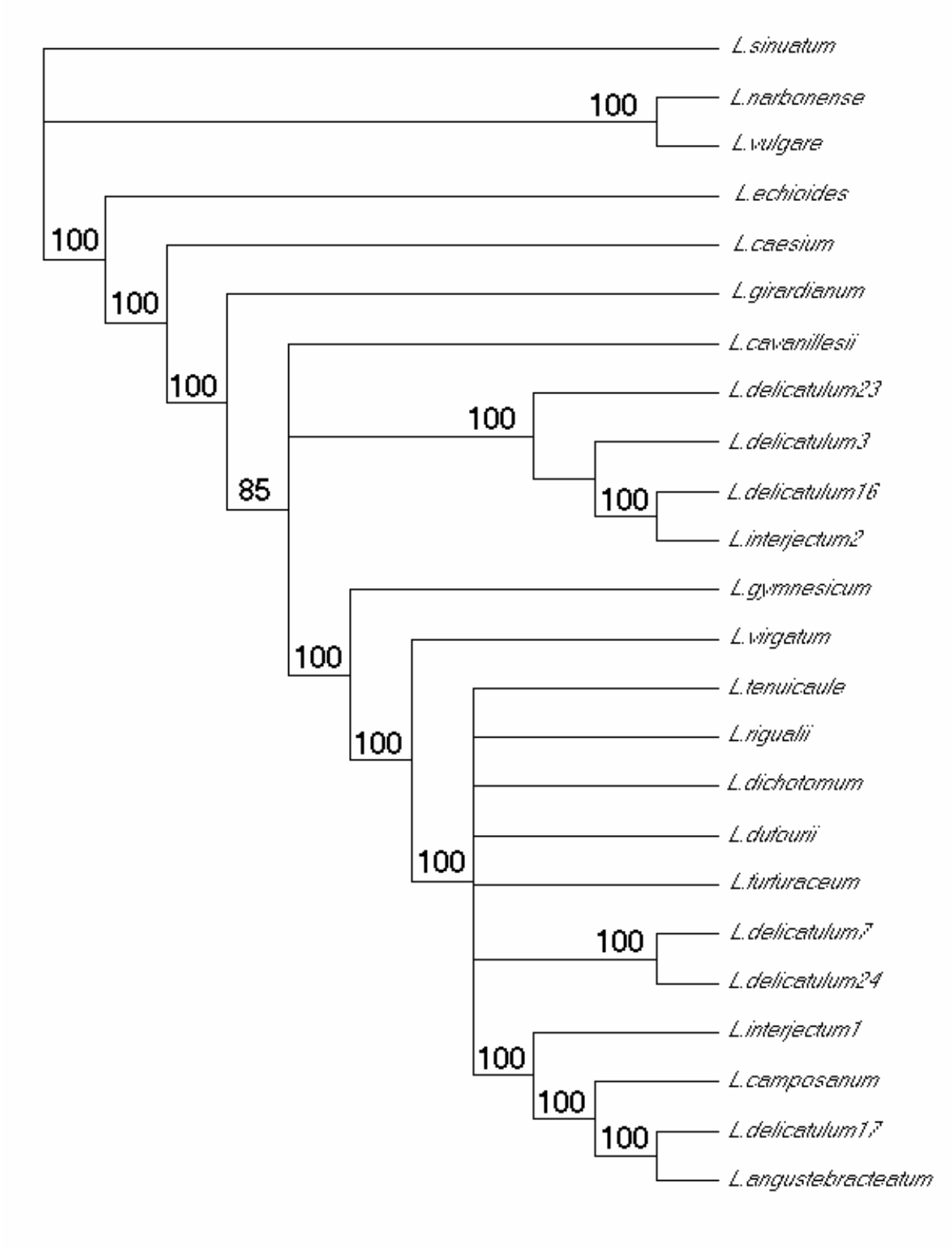


Fig. 2-9 50% majority rule consensus tree derived from Wagner parsimony analysis of *Limonium* ITS sequences considering equal character weights.

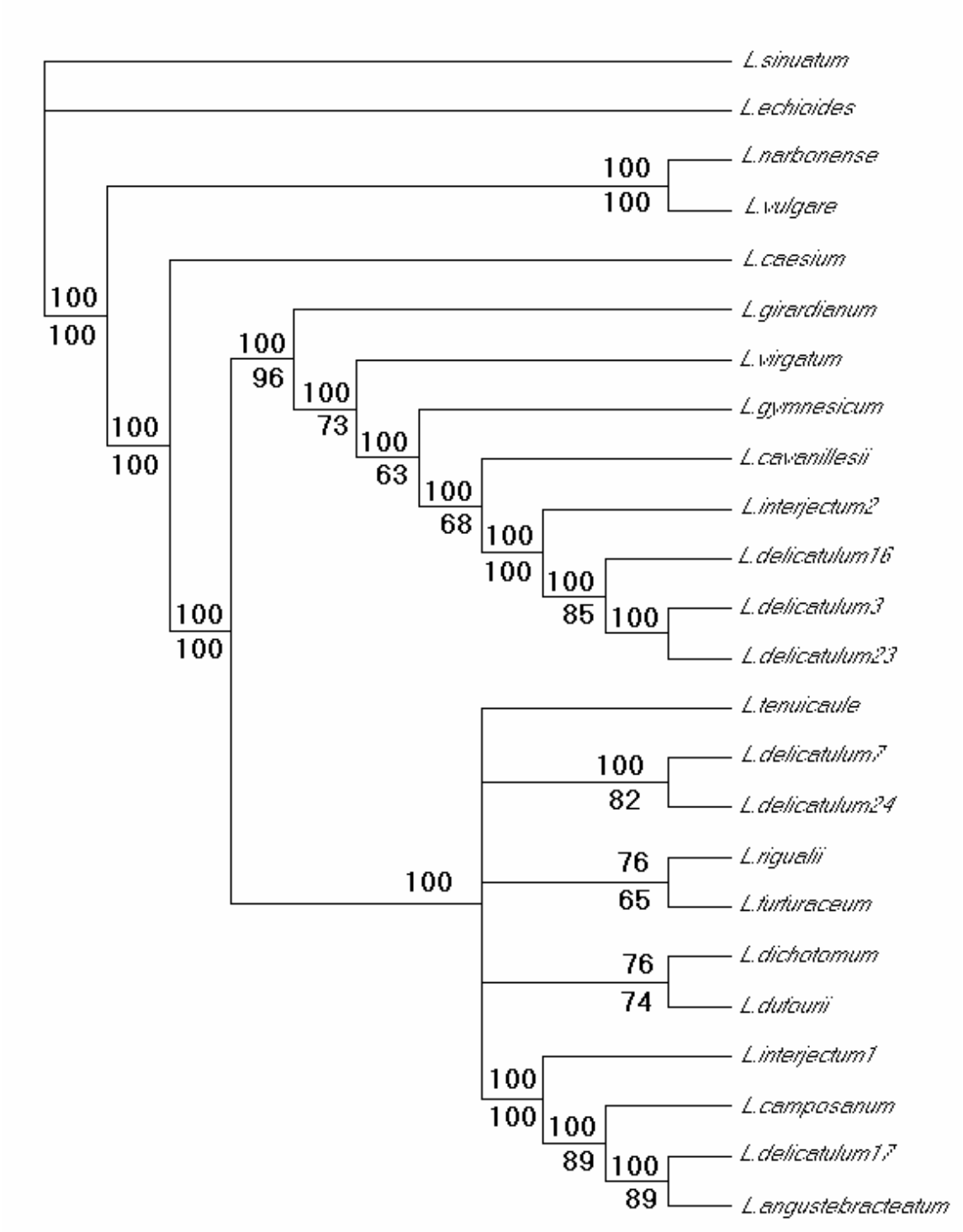


Fig. 2-10 50% majority rule consensus tree from parsimony analysis of ITS sequences when gaps were considered as additional data. Consensus values under the branches are those obtained from the 50% majority rule consensus tree derived from near-MPT topologies when gaps were taken into account.

when near-MPTs (13 steps longer, 9302 trees in total) were studied, the consensus tree derived from them has the same topology as the analysis without using categories. Fig. 2-10 represents the majority-rule consensus tree obtained in this later case, as well as the consensus values derived from the study of near-MPTs when categories were used. The tree is highly resolved not only at basal nodes, but also at more internal branches. *L. echioides* and *L. sinuatum* are located as outgroups to the rest of the species. *L. narbonense* and *L. vulgare* are basal, and still external to *L. caesium*, which continues as a sister taxon to the monophyletic group constituted by the rest of the ingroup species. When gaps were considered as additional data, the monophyletic ingroup subdivided into two major clades which were present in all MPTs (100% consensus value). One includes ITS type B sequences, and basal to them are situated *L. cavanillesii*, *L. gymnesicum*, *L. virgatum* and *L. girardianum*, in this order, very similar to that encountered in the NJ tree. The rest of the species, which show basically the same relationships as in previous parsimony and ML phylogenetic analyses, form the other clade. Most polytomies from these previous analyses are now well resolved. In fact, consensus values are also 100% for nearly all the monophyletic groups formed; likewise, near-MPTs give high support to them.

DISCUSSION

Intraspecific variation

Our results illustrate the importance of analyzing intraspecific variability on both cpDNA RFLP and ITS sequence studies. In some cases, conspecific samples rendered identical genotypes. In others, intraspecific variability has been detected through pooled DNA samples from different individuals of the same population. It was necessary to perform a posterior independence analysis of these different genotypes in order to extract information from them.

L. furfuraceum, a sexual species with a very conspicuous morphology and endemic to the Alicante province (Spain), presents two different cpDNA haplotypes, but no variability was found in its rDNA. This pattern of variation can be explained in several ways ((Rieseberg & Brunsfeld, 1992)). The most plausible explanation is that those individuals with cpDNA haplotype B obtained their cytoplasm by introgression from an unidentified *Limonium* species (as haplotype A was also present in the other population of the species analyzed), but introgression would have not been suspected based only on nuclear rDNA. Another possible explanation would be that the species was formed by crosses between two species with different cpDNAs, being it possible that both ancestors are the maternal parents; but the offspring would have the same rDNA (perhaps the sum of both parents, but see later). It is also possible that this pattern of variation could actually result from phylogenetic sorting from a polymorphic ancestor (in terms of cpDNA), although this is a less likely hypothesis (see later too). Likewise, *L. delicatulum* represents another case in which intraspecific variability has been detected, but with an opposite rDNA-cpDNA pattern of variability. This

is an asexual species, with apomictic reproduction mode, endemic to the Southeast of the Iberian Peninsula. (Mertens L., 1993) summarizes the possible explanations for this pattern of association in asexual polyploids formed through hybridization. Several rDNA genotypes associated with a single cpDNA haplotype could be explained if a single species with a distinct cpDNA haplotype was the maternal parent in all cases of hybridization, being the different ITS types those from both hybridizing species, which remain as different rDNA intraindividual arrays in the genome (Bobola M.S. *et al*, 1992)). On the other side, the polymorphisms within each of these ITS types can be explained as a remnant of the polymorphisms in the ancestral species, but they could also be due to the accumulation of mutations. In other *Limonium* species some ambiguities in rDNA sequences have been detected, that could actually represent intraspecific polymorphisms, or uncertainties, or both. It could be worth to investigate the levels of intraspecific variability in these species, especially in those whose sequences have more ambiguities, such as *L. angustebracteatum*, *L. cavanillesii*, *L. girardianum*, or *L. gymnesicum* (with 20-30 ambiguities, Fig. 2-2). It should be done through cloning of individual sequences from several individuals, as in *L. delicatulum*.

The hybrid species *L. interjectum* represents a special case of variation. Its rDNA sequencing demonstrated the presence of two different ITS types in each of the two populations of the species, which are very closely related to types A and B found in each individual of *L. delicatulum*. Therefore, *L. delicatulum* and *L. interjectum* could have a common origin, or even a more direct parentage relationship. A more exhaustive study of the intraspecific variation would reveal whether the two ITS types are present on both populations. Moreover, based on these results it does not seem plausible that *L. virgatum* and *L. girardianum* could have been the parental species of *L. interjectum* as previously hypothesized. This observation is confirmed by an additional analysis of cpDNA RFLPs with some probe-enzymes combinations in pooled DNA samples from two populations of each of these species (data not shown). *L. interjectum* did not present any of the cpDNA haplotypes of its potential ancestors; however, the cpDNA patterns observed were not discernable from those of the group *L. delicatulum, L. dufourii, L. camposanum* and *L. gymnesicum* (see for instance Fig. 2-5). Further studies are necessary to draw any conclusion about the origin of this species.

Summarizing, intraspecific rDNA or cpDNA types of *L. delicatulum, L. interjectum* and *L. furfuraceum*, were paraphyletic (Figs. 2-5 and 2-10). They show levels of divergence similar to those detected between other *Limonium* species, which reinforce the introgression or hybridization hypothesis to explain the origins of these DNAs. Besides, when multigene families are studied, the special mechanisms involved in their evolution (unequal crossing over, gene conversion, etc.) may override the classical factors (selection, mutation and genetic drift) as agents regulating genetic variation, resulting in unexpected variation patterns based on the biology of the species (Ritland *et al.,* 1993). These factors tend to homogenize the genome, and can hide the source of one parental rDNA, which could be the case of *L.*

furfuraceum and *L. interjectum*. Although it has been demonstrated that these mechanisms are active in asexual species (Hillis *et al*, 1991); (Crease T.J. & Lynch M., 1991), they can fail to act across repeat units contributed by the different parental species of a hybrid if the hybridization event was recent or rDNA types are at different loci in the parental taxa and interlocus gene conversion is inoperative in the hybrid (Baldwin *et al.,* 1995). This could be the case of *L. delicatulum*.

Data presented here indicate the need for adequate sampling strategies in molecular phylogenetic studies, especially when there is evidence from hybridization or introgression and when repetitive gene families are studied. Intraspecific variability is ubiquitous, but systematists seldom deal with polymorphism explicitly; for instance, by excluding morphological characters that show polymorphism, or, in molecular studies, by sampling a single individual per species. Unfortunately, analysis of many population samples in phylogenetic studies is often deterred because it is expensive and time consuming. Pooled DNA samples from different individuals of the same population has been the method used to detect intraspecific variability in this study of the genus *Limonium*. This method might be advantageous in preliminary studies to determine the levels of variability in a group of species and/or with a new molecular marker; and also when levels of intraspecific variability have been shown not be very high. Contrarily, in phylogenetic studies in which the levels of intraspecific variability have been reported to be high, this method is not recommended as it implies additional isolation of the DNA types encountered, and posterior full independent analysis of them. Perhaps a way to circumvent this problem and to deal with intraspecific variability in phylogenetic studies would be the analysis of additional population samples from the species studied, but only with diagnostic mutations (Rieseberg & Brunsfeld, 1992). Another possibility would be the use of rapid assays of DNA sequence variation through non-sequencing methods such as denaturing gel electrophoresis ((Lessa, 1992)).

Interspecific variability using cpDNA RFLP data

The present variability analysis in cpDNA has revealed a relatively stable phylogenetic structure, coincident when using different parsimony methods (Figs. 2-3 to 2-5). At low taxonomic levels, the asymmetry in the way restriction sites evolve is almost nonexistent (Holsinger & Jansen, 1993), and the choice of parsimony method is only expected to affect the results if there is homoplasy ((Olmstead *et al*, 1990)). Differences are found only in parts of the trees where none of the alternatives are strongly supported, as shown by bootstrap analysis. When there is disagreement in the support given to some taxa by a parsimony method and its bootstrap values (e.g. *Lfurfuraceum* B in Dollo's MPT, or *L. dichotomum*, *L. virgatum* and *L. tenuicaule* in Wagner consensus tree), it is suggestive of large homoplasy in the corresponding tree ((Olmstead & Palmer, 1994)). However, ci and ri values for these trees are relatively high (see Results).

Although Wagner and Dollo parsimony analyses have been considered biologically unrealistic (Albert *et al.,* 1992; Felsenstein, 1992), they were conducted to compare their results with those obtained with a more realistic range of weights. Our results agree with

Albert *et al.* (1992) predictions about how the different parsimony methods should behave. For instance, a Dollo parsimony topology (Fig. 2-4) will never be obtained with the 'enhanced Wagner' approach, but only with weighted parsimony implemented with outgroup = ancestor and an unreasonable high weight (which implies evolutionary assumptions at which data should be rejected). Contrarily, 'enhanced Wagner' parsimony can help to discriminate among equally parsimonious Wagner topologies, as it is the case here (Figs. 2-3 and 2-5). Besides, the relationships obtained are coincident with those from the NJ tree, which is based on a distance that also corrects for the use of fragments instead of sites as characters. In conclusion, weighted parsimony is currently considered the best choice, among all parsimony methods, for RFLP analysis (Albert *et al.,* 1992; Felsenstein, 1992; (Holsinger & Jansen, 1993); Huelsenbeck & Hillis, 1993). Although which weights to apply remains a controversial issue (Swofford *et al.,* 1996), those that correct for possible violations of the assumptions of the phylogenetic inference methods should be considered. However, the use of other methods for inferring phylogenetic relationships is advisable, because they can help to interpret the results obtained.

Presumable uniparental (maternal) inheritance of cpDNA in *Limonium* (Clegg, 1987); (Harris & Ingram, 1991)) and absence of intermolecular recombination, disallow reticulation in cpDNA phylogenies, as opposed to morphological, plant-mtDNA or nuclear DNA phylogenies. However, caution is needed in the interpretation of results where lineage sorting, hybrid origin or introgression may have resulted in the transfer of cpDNA from one lineage to another (Palmer *et al.,* 1983; Soltis *et al.,* 1992; Doyle, 1992). In the section Limonium polyploidy and apomixis are common and diploid species scarce, numerous hybrids occur naturally, and as a consequence, reticulate evolution seems to be the rule rather than the exception. Therefore, before drawing any conclusion on the classification of these species, an *a posteriori* search of inconsistencies with other types of data, preferentially from nuclear DNA, as hybridization is less permeable to this biparentally inherited DNA ((McDade, 1992)), was necessary.

Interspecific variability with ITS sequence data

ITS1 and ITS2 sizes in members of the genus *Limonium* studied here, are similar to those reported for other flowering plants, as with the entire ITS region length (~700bp). ITS1 is longer than ITS2, which seems to be common in angiosperms (Baldwin *et al.,* 1995). ITS2 pairwise divergence values are, in general, lower than those of ITS1. For instance, among ingroup species (excluding *L. narbonense* and *L. vulgare*), ITS2 divergence values range from 0-0.09, while ITS1 range from 0-0.44. However, their average pairwise distance values were not significantly different (see materials and methods). Some authors have already pointed out that this may not be an adequate measure of relative evolutionary rate ((Muse & Weir, 1992)). In any case, combining data from ITS1 and ITS2 seems to be justified, as the possible sources of the small number of discrepancies from its separate analyses (data not shown) could be due, mainly, to sampling error, for the low number of informative characters (49 in ITS1, 53 in ITS2).

Within section Limonium, the ITS region has evolved primarily by point mutations, which agrees with other studies on closely related plants ((Soltis & Kuzoff, 1995)). The conservation of ITS sequences is presumably due to their role in the production of mature DNA. As pointed before, it has been demonstrated that the functionality of ITS regions depends on evolutionarily conserved secondary structural motifs. Inference of non-independence at directly opposing sites in these secondary structures can be determined empirically (Dixon & Hillis, 1993). However, in our case the use of differential character weights for stem vs. loop positions has not lead to differences in the analysis of ITS sequences from *Limonium*. It has been demonstrated that rDNA processing mechanisms could be labile enough to allow readjustments of intrastrand RNA pairing, which could imply mutations at non-paired positions (cryptic non-independence) ((Olsthoorn R.C.L. *et al*, 1994); (Baldwin *et al*, 1996). These would have important implications in phylogenetic analysis. But this could also mean that selection for compensatory mutations may be weaker for these spacers than for nrDNA coding regions, alleviating the concern about non-independence of characters.

None of the methods currently available for phylogenetic analysis is ideal, and it is always recommended the use of different approaches for data analysis (e.g. (Holsinger & Jansen, 1993)). The performance of methods for phylogenetic inference can be viewed as a problem of how well the model of evolution assumed by the estimation method fits the actual processes of evolution ((Huelsenbeck & Hillis, 1993). It is reasonable to expect that well-supported groups identified by the different techniques are likely to reflect the evolutionary history of the taxa involved (Holsinger & Jansen, 1993). In our case, the relationships obtained among ITS *Limonium* sequences when using NJ, ML and parsimony approaches are, in general, coincident for those monophyletic groups that are highly supported in all these trees (Figs. 2-7 to 2-9). Besides, (Kishino & Hasegawa, 1989) likelihood test between the trees obtained showed no significant differences among them, meaning that none of the trees was worse or better than the others. The same result was obtained when one of the 21 MPTs derived from the parsimony analysis using gaps (Fig. 2-10) was included in this analysis. However, the use of character states within indel regions has shown that they may be phylogenetically informative, as they have given support to otherwise weakly supported lineages, especially for more closely related species. Similarly, these characters have shown to be less homoplastic than other types of substitutions in other ITS studies in angiosperms ((Baum *et al*, 1994); (Baldwin & Robichaux, 1995); (Buckler IV & Holtsford, 1996)).

Comparison of ITS and cpDNA phylogenetic analysis

In the absence of a comprehensive phylogenetic analysis of the section Limonium based on other independent characters, our results can only be discussed in relation to the current classification of the group, which is clearly not based on phylogenetic principles. This is an important limitation (e.g. (Donoghue & Cantino C.R., 1988); (Doyle *et al*, 1990)), but

more so in case of striking disagreements ((Doyle & Doyle, 1993)), which is generally the case here.

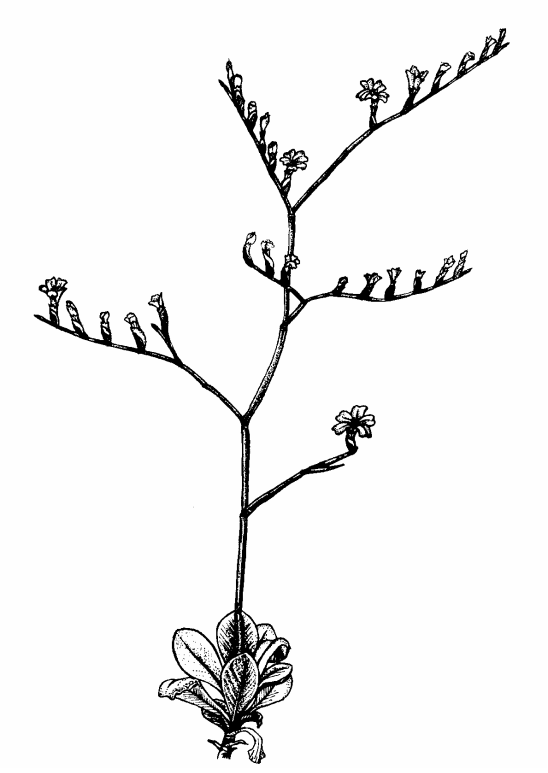
For comparative purposes, we will take into account the topologies from Fig. 2-5 and Fig. 2-10, which seem to be the most plausible hypothesis for the phylogenetic relationships among the *Limonium* species studied, based on cpDNA and rDNA molecules, respectively. Although these trees show obvious discordances, there are, however, areas of coincidence. *L. narbonense* and *L. vulgare*, from subsection Genuinae, form a clade basal to the rest of the ingroup species in all trees obtained. These species present also levels of divergence with the rest of the ingroup species similar to those of species that belong to other sections of the genus for both molecular markers. These results cast doubts on the actual placement of these two species within section Limonium. The analysis of other species from section Genuinae will allow us to determine whether this subsection belongs to section Limonium or whether only the classification of *L. narbonense* and *L. vulgare* in that subsection has to be questioned. Another area of concordance was that the remaining ingroup species, which belong to other subsections, always form a monophyletic group, denoted section Limonium for further discussion. Even so there was in general a lack of resolution within this clade with both types of data, which could mainly be due to the reticular evolution among these closely related species. This is confirmed by the significant discordance between ITS and cpDNA phylogenies at this level. cpDNA RFLP data reveal two major monophyletic groups within section Limonium. Only *L. angustebracteatum* is excluded as a sister group to these two clades formed by: (1) *L. rigualii, L. cavanillesii,* and *L. furfuraceum* cpDNA type A; and (2) the rest of the species and *L. furfuraceum* haplotype B. However, these two groups are not present in the ITS trees, where two different major lineages are observed. One includes *L. girardianum, L. virgatum, L. gymnesicum,* and *L. cavanillesii*, as sister species to ITS type B of *L. delicatulum* and *L. interjectum*; and the other is formed by *L. tenuicaule, L. furfuraceum, L. rigualii, L. dichotomum, L. dufourii, L. camposanum, L. angustebracteatum,* and ITS type A of *L. delicatulum* and *L. interjectum*.

As pointed above, the use of multiple data sets in Systematics is advisable, but it has raised to the controversy regarding how to integrate this information in our search to find the true organismal phylogeny ((Bull *et al*, 1993); (de Queiroz *et al*, 1995); (Huelsenbeck *et al*, 1996). Topological differences among data sets can be artifactual due to sampling error or the use of an inappropriate evolutionary model. In such case, data can be combined with appropriate treatment ((Lutzoni F.M., 1997)). When topological differences among data sets reflect different phylogenetic histories due to reticulation, lineage sorting, recombination,..., these data cannot be combined. In our case, sampling error can be discarded, as the phylogenetic signal from both, cpDNA and ITS data sets, is quite high. Different models agree in the phylogenies inferred, so the use of an inappropriate model of evolution can not be the cause of disagreement. Although other factors may contribute to the phylogenetic discordance in section Limonium (e.g. long branch attraction, convergence, unequal rates of evolution, etc.), different evolutionary histories of organellar and nuclear DNAs due to

reticulate evolution of these closely related species is the most likely explanation for the discrepancies between these phylogenies. Hence, our results confirm previous concerns in the use of organellar variation for retrieving a phylogeny, particularly at lower taxonomic levels in groups noted for introgressive hybridization (Harris & Ingram, 1991; (Doyle, 1992); (Rieseberg & Soltis, 1991); Rieseberg & Brunsfeld, 1992; (Soltis & Kuzoff, 1995); Baldwin *et al.,* 1995). Consequently, it is likely that the cpDNA tree is not reflecting the species tree but we can neither be certain that the ITS tree does reflect it. Other phylogenetic sources of evidence obtained by using other types of data, from both nuclear and chloroplast genomes, and by increasing the number of species to study (see later), should help to understand the discrepancies in the relationships among the species from section Limonium, which have emerged from our study of this genus.

3

Analysis of the genetic variation in the rare and endangered *Limonium cavanillesii*



RESUMEN

Limonium cavanillesii es una especie en extremo peligro de extinción, endémica de la provincia de Castellón, en la costa este del Mediterráneo español. Se pensó que estaba extinguida, pero recientemente se descubrió una nueva población natural de pequeño tamaño, lo que aceleró la adopción de medidas para su conservación por parte de las agencias oficiales. Como parte del esfuerzo común para su conservación, hemos analizado la variabilidad genética en esta población por medio del uso de RAPDs y AFLPs como marcadores moleculares. El análisis de 29 individuos con 11 cebadores tipo RAPD produjo 131 bandas monomórficas. A nuestro juicio este es el nivel más bajo de variabilidad detectada con el uso de marcadores tipo RAPD. Como consecuencia de este resultado decidimos llevar a cabo un nuevo análisis de diversidad en la especie usando la recientemente descubierta técnica de AFLP. El uso de 3 tipos diferentes de cebadores de AFLP proporcionó 231 bandas marcadoras, 13 de las cuales eran polimórficas entre los 29 individuos analizados, permitiendo su clasificación en 11 fenotipos diferentes, pero altamente relacionados. El análisis de estos datos sugiere que la población estuvo probablemente en equilibrio en el pasado. Sin embargo, la baja variabilidad genética encontrada en *L. cavanillesii* podría explicarse por su sistema de reproducción apomíctico y por haber sufrido un cuello de botella reciente, después del cual no ha habido oportunidad para que la mutación reestablezca los niveles de variabilidad esperables. Los AFLPs parecen representar una clase de marcadores más variables que los RAPDs, al menos en este caso. Se discuten también las ventajas de los AFLPs como marcadores moleculares para el análisis de la variabilidad intraespecífica con respecto a otras técnicas de DNA *fingerprinting* basadas en la PCR.

ABSTRACT

Limonium cavanillesii is an extremely endangered plant species endemic to the East Mediterranean region of Spain. Taken as extinct for several years , the recent discovery of a small population has prompted the adoption of measures for its conservation by official agencies. As part of this effort, we have analyzed genetic variation in this population by means of random amplified polymorphic DNA (RAPDs) and AFLP markers. The analysis of 29 individuals with 11 different RAPD primers produced 131 monomorphic bands. To our knowledge, this is the lowest level of genetic variation detected in plants using RAPD markers. As a consequence of this result we decided to perform an additional analysis of diversity in the species using the recently developed AFLP technique, to see whether some variation could be found. The use of three different AFLP primers provided 231 marker bands, 13 of which were polymorphic among the 29 individuals assayed, thus allowing their classification into 11 distinct, but very closely related phenotypes. The analysis of these data suggests that the population was probably in equilibrium. The low genetic variability encountered in *L. cavanillesii* could be explained both by the apomictic reproductive system of this species and by the passage through a severe bottleneck in recent times, after which there has been no chance for mutation to restore detectable genetic variation. AFLPs seem to represent a significantly more variable class of markers than RAPDs, at least in this case. The advantages of AFLPs as molecular markers for analysis of intraspecific variability over other PCR-based DNA fingerprinting methods is discussed.

MATERIALS

Plant samples

A total of 29 wild plants were used in this study. These comprise all the known individuals of *L. cavanillesii* and thus represent the species as a whole.

Plant materials were collected from the field and kept on ice until storage at -80ºC. Two to three small leaves, ranging from 26 to 594 mg depending on the availability of material, were sufficient to perform both RAPD and AFLP analyses.

Template DNA isolation and quantification

Frozen tissue was homogenized using liquid nitrogen. DNA was extracted using a CTAB protocol developed by Doyle (1991), with the only modification that one more chloroform-isoamylalcohol (24:1) extraction step was done when samples were still turbid after the first extraction step.

DNA contents were estimated by direct comparison with standard DNA concentrations in 0.8% agarose gels stained with ethidium bromide (0.5 µg/ mL). These DNAs were directly used for AFLP assays. For the RAPD method, after quantification samples were diluted to a final approximate DNA concentration of 1 ng/µL.

METHODS

RAPD method

DNA amplification and fragment visualization

To generate RAPD profiles we used 20 ten base pair primers (OPA-1-OPA-20) from the Operon Technologies Primer Kit A in PCR amplifications. Amplification reactions were carried out in 20 µL total volume containing 1X Taq buffer (Pharmacia), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 15 ng of primer (Operon), 1.0 unit of *Taq* DNA polymerase (Pharmacia), 5 µL of DNA previously diluted, and deionized and distilled water. Each reaction mix was overlaid with mineral oil (Sigma) when using Robocycler (Stratagene) (see below). Negative controls in which water was added instead of DNA were included in each run in order to verify the absence of contamination.

DNA amplification was initially performed for each primer in a Stratagene Robocycler Gradient 96. When the pattern obtained with this thermal cycler was not reproducible, a PE-2400 thermal cycler was used instead, and only if the results were reproducible in this machine the RAPD markers obtained with this particular primer were considered. The program used was the same for both thermal cyclers; an initial melting step at 94ºC (5 min), followed by 45 cycles each at 94ºC (1 min), 39ºC (2 min) and 72ºC (2 min). A final extension step at 72ºC (7 min) was performed after the 45 cycles.

Amplification products were separated on 1.4% agarose gels stained with ethidium bromide to a final concentration of 0.5 µg/mL. Gels were run in 0.5X TBE buffer during

approximately four hours at 7.5 V/cm and then visualized under ultraviolet light. Monochrome photographic negatives (Agfapan, APX100) were taken of the gels using a Polaroid camera.

Table 3-1 Summary of data obtained in RAPD analysis with eleven primers for *Limonium cavanillesii*. The number of fragments for each primer represents the number of reproducible, scored bands in at least two independent assays. All analyses were made using Robocyler as the thermal cycler, except for primers OPA-08 and OPA-11 for which PE-2400 was used (see text for more details).

Primer	Nucleotide sequence	Thermal cycler used	No. of fragments	Band size range (bp)
OPA-04	AAT CGG GCT G	Robocycler	21	550-1800
OPA-07	GAA ACG GGT G	Robocycler	18	300-2500
OPA-09	GGG TAA CGC C	Robocycler	15	700-3500
OPA-10	GTG ATC GCAG	Robocycler	13	550-2400
OPA-15	TTC CGA ACC C	Robocycler	14	600 - 2400
OPA-16	AGC CAG CGA A	Robocycler	7	900-2900
OPA-18	AGG TGA CCG T	Robocycler	13	300-2500
OPA-19	CAA ACG TCG G	Robocycler	10	700-2700
OPA-20	GTT GCT ATC C	Robocycler	5	1700-1300
OPA-08	GTG ACG TAG G	PE 2400	9	600-1700
OPA-11	CAA TCG CCG T	PE 2400	6	800-1300
Total			131	300-3500

Initially we surveyed all primers for a sample of three randomly chosen individuals, in order to evaluate their suitability with the Stratagene Robocycler. Thirteen primers gave a clear profile in this pilot study, nine of which gave reproducible marker patterns (Table 3-1). The other four were tested for reproducibility in the PE 2400 and only two of them gave reproducible banding patterns (Table 3-1). Hence, a total of eleven primers were used in the final study.

Fragments included in the final analysis were tested for reproducibility. Whenever a new RAPD band appeared in the final study and it was not present in the pilot study, two

replicate PCR reactions, one with the same DNA dilution and one with a new DNA dilution, were performed.

AFLP method

AFLP reactions, data collection and scoring procedure

AFLP assays were performed with the Perkin-Elmer/Applied Biosystems (PE/ABI) AFLP™ plant mapping kit for small genomes according to the manufacturer instructions. It is based on the method of Vos *et al.* (1995) but it uses non-radioactive, fluorescent dyes to label the primers, thus enabling the multiplexing of up to three different reactions from one individual in a single gel lane. The kit uses MseI-C in the pre-selective amplification (PSA) and Dye-EcoRI-AN/TN and MseI-CNN primers in the selective amplification (SA), where N represents any of the four nucleotides. Following Janssen *et al.* (1996), a preliminary study was done to determine which primer combination would be more appropriate. Primer selection (Table 3-2) was based only on the number of fragments amplified, and there was no bias in favor of those primers that gave more polymorphism, which is important in order to make comparisons among different techniques or with other species (Clark & Lanigan, 1993).

Modifications to the original protocol were minimal. The number of cycles in the final step of the SA was increased for the green and yellow fluorescent dyes (Table 3-2). The SA reactions were multiplexed in order to load the 3 primer combinations from one individual in a single lane in the gel. Multiplexing was carried out by adding an uneven quantity of each SA, depending on the dye. Electrophoresis was performed on 4% polyacrylamide gels at constant voltage (3000 V) for 3 hours at 51ºC on an automated DNA sequencer (Model 377, PE/ABI) equipped with GeneScan Analysis software (version 2.1, PE/ABI).

Table 3-2. AFLP primers used, color dye, number of cycles on the selective amplification reaction (SA), number of µl used on the multiplexing reaction (MR), and number of monomorphic and polymorphic bands respectively.

AFLP Primers	Color Dye	Cycles SA	µL MR	Total Bands	Polymorphic Bands
MseI-CTG EcoRI-AC	FAM (blue)	23	5	75	6
MseI-CAA EcoRI-AG	JOE (green)	25	10	84	3
MseI-CTG EcoRI-AT	TAMRA (yellow)	27	20	72	4

Reproducibility studies

A multifactorial experiment was carried out to determine the influence of DNA concentration and possible random differences in the three steps of each AFLP reaction on the final AFLP profiles. DNA quantities of 50, 100, and 350 ng were tested, and no differences were observed in the profiles obtained

among the 14 reactions performed for each DNA concentration. Consequently, the amount of DNA used per AFLP reaction was 5.5 μL of the original extraction, which implies variations in DNA concentrations in the range mentioned above.

Further reproducibility tests were performed by repeating the complete AFLP procedure, including running the samples in different gels, with 5 individuals. Besides, samples from other 12 individuals were run twice in different gels.

Data Analysis

A matrix with all the different AFLP phenotypes (patterns) was assembled with programs GeneScan and Genotyper (version 1.1, PE/ABI), with further visual inspection of the electrophoretograms to correct for any misinterpretations from both programs. The resulting presence/absence data matrix was analyzed using the package RAPDistance (Armstrong *et al.*, 1996). Estimates of pairwise distances were made using the Euclidean distance proposed by Excoffier *et al.* (1992), and Dice similarity coefficient (S, equivalent to that of Nei & Li, 1979) proposed by Lynch (1990) for DNA fingerprinting data. The similarity coefficient was transformed to a distance (D) according to $D = \sqrt{1 - S}$ (Armstrong *et al.*, 1996). The resulting distance matrices were compared using DIPLOMO (Weiller & Gibbs, 1993) to test the significance of their correlation by means of a t-test (Sokal & Rohlf, 1995 pp. 575-583). These two distances were chosen as representatives of several other possible distance measures for binary data that either take into account the sharing of the absence of a character (Excoffier) or do not (Dice) (Armstrong *et al.*, 1996, see also Chapter 4).

We have employed several approaches to ascertain the relationships among the different AFLP patterns using both distance measures. First, the neighbor-joining procedure (Saitou & Nei, 1987) was used to construct an unrooted dendrogram. In order to make a statistical assessment of the resulting trees two methods were used: the Permutation Test Probability (PTP) analysis (Faith & Cranston, 1991), and a correlation test to compare the original distance matrices with the patristic distance matrices of the trees (Armstrong *et al.*, 1996), because conventional approaches for estimating the reliability of inferred trees, such as bootstrapping or jackknifing, are better suited for interspecific studies and may lack statistical power at the intraspecific level (Templeton *et al.*, 1992; Templeton & Georgiadis, 1996). Second, principal coordinates analysis (PCO) was performed using NTSYS (Rohlf, 1993) to provide a visual representation of the connections between phenotypes. The multidimensional scaling was completed with the superposition of a minimum spanning tree (MST, Dunn & Everitt, 1982), obtained with NTSYS, on the corresponding plot. Finally, program MINSPNET was used to obtain a minimum spanning network (Excoffier & Smouse, 1994). The network represents all possible connections among patterns with the minimum number of mutational steps (presence/absence of bands) that separate them. This kind of representation is very useful for intraspecific studies because it allows to visualize the

connections among the patterns integrating the information from gene genealogies simultaneously with the frequencies of those patterns (Excoffier & Smouse, 1994; Templeton *et al.*, 1995)

The analysis of nucleon diversity within populations by means of the distribution of pairwise differences among molecular variants (Tajima, 1983) has been used to infer historical demographic patterns (Slatkin & Hudson, 1991; Rogers & Harpending, 1992; Marjoram & Donnelly, 1994). Although the most frequent application has been to nucleotide sequences, it can also be applied to other molecular markers (Lavery *et al.*, 1996; Nybom, 1993). Given the lack of recombination in the nuclear genome of apomictic species, which is the case for *L. cavanillesii*, and the large number of potential markers that can be studied with DNA fingerprinting techniques, it is possible to consider the whole genome of that species as a single, non-recombining gene with infinite alleles. We have used this approach for the present study as it may shed some light on the historical processes that have occurred in *L. cavanillesii* population.

RESULTS

RAPD results

Figure 3-1 shows examples of RAPD profiles obtained with some primers. The negative controls, in which DNA was omitted, were always free of DNA fragments. Table 3-1 summarizes the data on the number of fragments detected per primer. The total number of fragments scored was 131, ranging in size from 300 bp (OPA-07 and OPA-18) to 3500 bp (OPA-09), with an average of 11.9 fragments per primer.

Primers OPA-04, OPA-08, OPA-09, OPA-10, OPA-15, and OPA-19 showed the same banding pattern for all the individuals of *L. cavanillesii*. On the contrary, primers OPA-07, OPA-11, OPA-16, OPA-18, and OPA-20 gave some bands that were different between individuals but that were not reproducible in a second test. Problems with band reproducibility, even using standardized conditions and reagents, seem to be the norm rather than the exception in RAPD studies. They may be due merely to a random difference among PCR reactions or to the sensitivity of the technique to small differences in template DNA concentration between samples, which is a quite common drawback in DNA fingerprinting methods (Vos *et al.,* 1995). Once all the possible differences were tested and non-reproducible bands were discarded from the results, we concluded that no RAPD variation was found in *L. cavanillesii*. To our knowledge, this is the most extreme case of low genetic variability detected in a natural plant population by means of the RAPD technique. The ability of the RAPD technique to identify variation within populations when it exists has been well demonstrated (Huff *et al.*, 1993; Rossetto *et al.*, 1995; Gibbs *et al.*, 1994; M'Ribu & Hilu, 1994). We also have evidence for the ability of RAPD analysis to detect within species

variability in the genus *Limonium*, as a concomitant RAPD survey has been performed on six populations of the apomictic species *L. dufourii* (see Chapter 4).

Therefore, the absence of RAPD variation in *L. cavanillesii* is a reflection of its isogenic state and it is in agreement with the reproductive mechanism of this species, apomictic and without possibility of sexual reproduction due to its uneven number of chromosomes. In this situation, mutation is the only force that can cause genetic differentiation between parents and offspring. This indicates that the recently rediscovered, unique population of the species must have gone through a recent bottleneck, and there has been no time for mutation to restore detectable genetic variation. As a consequence of this lack of variability we decided to perform an additional analysis of diversity in the species using the recently developed AFLP technique, to see whether some variation could be found.

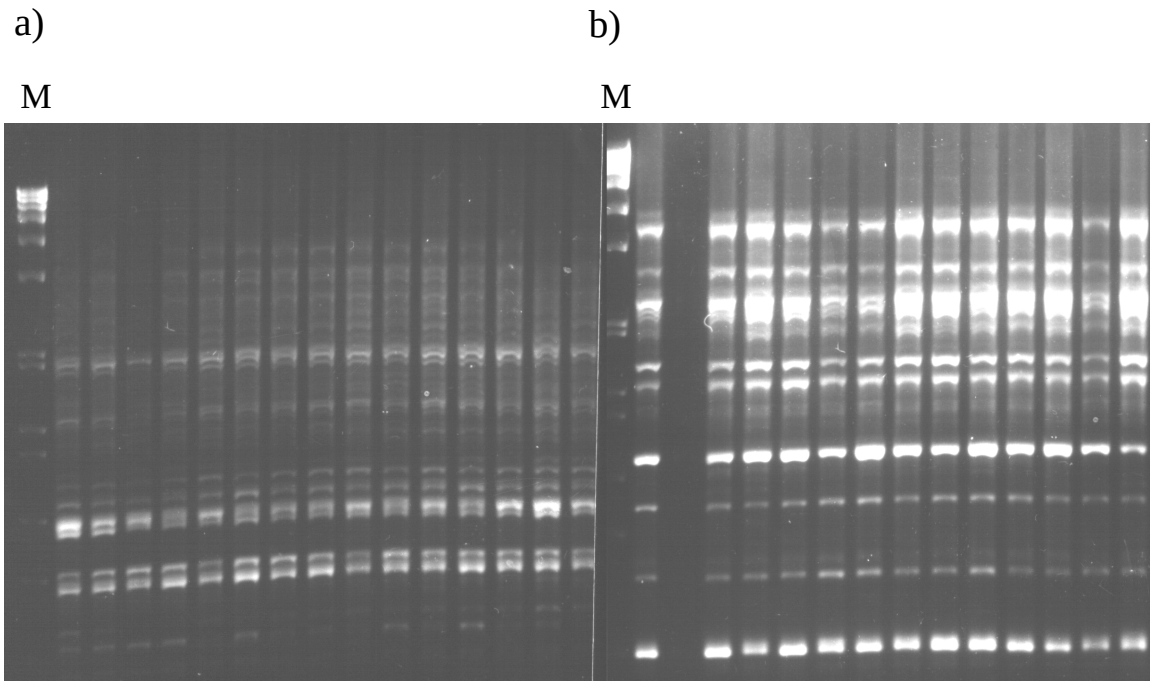


Fig. 3-1 Representative RAPD results with primers OPA-04 (a) and OPA-07 (b) showing lack of genetic variation in *L. cavanillesii* individuals. Lanes labeled M represent molecular weight markers.

AFLP results

A clear difference between AFLP and RAPD methods was already detected in the pilot study. No variation among the AFLP profiles was found when significantly different amounts of DNA were used, as opposed to the different profiles obtained with RAPDs with smaller differences in the amount of DNA. Furthermore, the remaining reproducibility tests carried out in the pilot study also gave identical results for each individual tested. Hence, the reliability of the AFLP technique at its different steps was confirmed in this study.

Fig. 3-2 shows an example of an AFLP polyacrilamide gel using fluorescent-dye primers. These AFLP primers finally used for analysis are shown in Table 3-2. A total of 231 fragments were generated, ranging from 75 to 500 bp, with an average of 222.6 fragments

per individual and 77 bands per primer combination. These 231 markers were able to distinguish 11 different AFLP phenotypes in *L. cavanillesii* (Table 3-3), but the variability observed was relatively low, which is in agreement with the previous RAPD study where no polymorphic markers were obtained. Only 13 (5.6%) AFLP markers were polymorphic. Using this proportion of polymorphic fragments, the number of variable bands expected in the RAPD study (with 131 markers screened) would have been 7.34. This implies that both techniques differ significantly ($P<0.001$) in their levels of polymorphic loci. Therefore, it is not simply the larger number of markers detected with the AFLP technique what marks the difference with the result obtained with RAPDs.

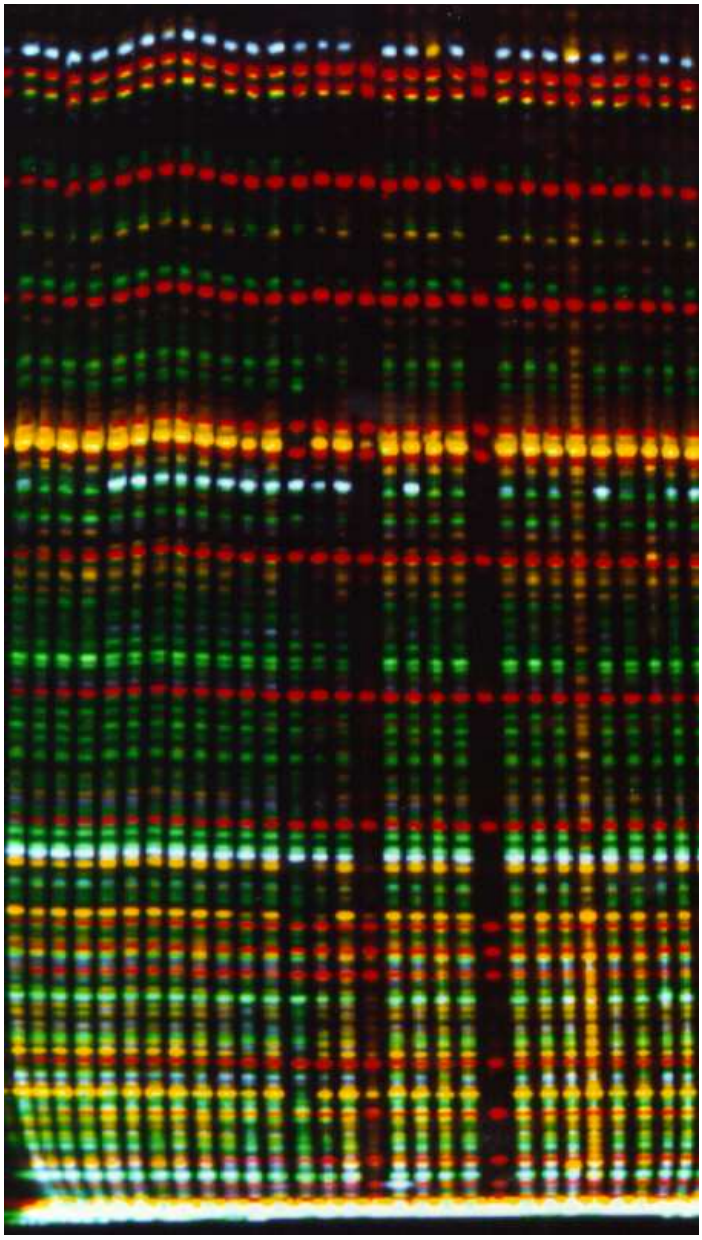


Fig. 3-2 AFLP gel from multiplexing reactions of the three fluorescent-dye primers used for analyses (BLUE, GREEN AND YELLOW bands). RED bands are those from the standard molecular weight marker.

Table 3-3. Presence and absence data matrix for the 13 polymorphic AFLP markers of the 11 phenotypes, and absolute frequency of each phenotype in the only remaining *L. cavanillesii* population.

Phenotype	***Polymorphic bands***													***Individuals***
1	0	0	0	0	0	1	0	1	0	0	1	1	0	3
2	0	0	0	0	1	1	1	1	0	0	1	0	0	1
3	0	0	0	0	0	1	0	1	0	0	1	1	1	1
4	0	0	0	0	0	1	0	1	1	0	1	1	0	3
5	0	0	0	1	1	0	1	1	0	0	0	0	0	11
6	0	0	0	1	1	0	1	1	0	0	1	0	0	5
7	0	0	1	1	1	1	1	1	0	0	0	0	0	1
8	0	0	1	1	1	0	1	0	0	0	0	0	0	1
9	1	0	0	0	1	1	1	1	0	0	1	0	0	1
10	0	0	0	0	0	1	0	1	0	0	1	0	0	1
11	0	1	0	0	0	1	0	1	0	1	1	1	1	1

All the analyses performed using Excoffier and Dice distance matrices (Table 3-4) gave very similar results, as expected from the highly significant correlation found between them (r=0.988; t_s=0.087, P>0.90, for the null hypothesis r=1). The relationships among AFLP patterns obtained with the neighbor-joining procedure are represented in Fig. 3-3.

Table 3-4. Distance matrices using Excoffier (upper hemimatrix) and Dice (lower hemimatrix) coefficients among *L. cavanillesii* phenotypes obtained with AFLP markers.

Phenotype											
1	-	3	1	1	6	5	6	8	4	1	3
2	0.082	-	4	4	3	2	3	5	1	2	6
3	0.047	0.095	-	2	7	6	7	9	5	2	2
4	0.047	0.095	0.067	-	7	6	7	9	5	2	4
5	0.116	0.082	0.125	0.125	-	1	2	2	4	5	9
6	0.106	0.067	0.116	0.116	0.047	-	3	3	3	4	8
7	0.116	0.082	0.125	0.125	0.067	0.082	-	2	4	5	9
8	0.134	0.106	0.142	0.142	0.067	0.082	0.067	-	6	7	11
9	0.095	0.047	0.106	0.106	0.095	0.082	0.094	0.116	-	3	7
10	0.048	0.067	0.067	0.067	0.106	0.095	0.106	0.126	0.082	-	4
11	0.082	0.116	0.067	0.094	0.142	0.134	0.142	0.157	0.125	0.095	-

There is no difference between the topologies obtained with both distance measures and only the one obtained from Excoffier distances is shown. Hence, it can be concluded that, at least in this case, considering the sharing of the absence of matches only influences the branch-lengths but not the topology of the tree.

Principal coordinates analyses based on Excoffier and Dice distance matrices have a low resolution power, as only the first dimension is significant for both distances (Table 3-5). Excoffier distance explains about 20% more of the total variation with the first dimension in the PCO analysis than Dice distance, despite the high correlation between them. The topologies obtained are very similar, and basically there is a spreading of the different patterns along the axis, showing no particular structure.

Table 3-5. Result of the percentage of variation explained by the 2 first dimensions in the principal coordinates analysis of AFLP phenotypes in *L. cavanillesii* when using Excoffier and Dice distance matrices.

	Excoffier		**Dice**		**Expected**
Dimension	Eigenvalue	Percent	Eigenvalue	Percent	
1st	12.94	52.72	0.16	32.41	27.45
2nd	4.02	16.37	0.08	15.28	18.36

The superposition of the MST obtained with NTSYS on the PCO analysis (Fig. 3-4) shows that the previous relationships are preserved in the multidimensional mapping. Whereas in the plot derived from Excoffier distance pattern 8 is connected to pattern 5, in that derived from Dice distance it is connected to pattern 7. This difference arises because Excoffier distance takes into account an absent band shared by patterns 5 and 8 (Table 3-3), which is not considered in Dice distance. In the neighbor-joining tree, these patterns are included in the same cluster with pattern 7. The minimum spanning network (Fig. 3-5) shows that alternative connections among OTUs 5, 7, and 8 are equally likely, thus explaining the previous results. Consequently, it seems that alternative ways of representing the relationships among the variants, not by strictly bifurcating trees, may be more adequate at the within species level. These results are especially relevant when dealing with larger sample sizes and/or intraspecific studies of population structure (see Chapter 4).

The absolute frequency of each pattern is shown in Table 3-3. The most abundant patterns are 5 and 6, separated by only one mutational step (Fig. 3-5). The remaining patterns appear with a lower frequency, and only one or two mutational steps separate them from each other or from the most common patterns. Hence, all individuals are very closely

related, which confirms the results of the cluster analysis, as branch-lengths in the NJ trees are very small, and it is also reflected in the absence of any particular structure in the PCO analysis.

The pairwise difference distribution (Fig. 3-6) does not match with the population expansion model of Rogers & Harpending (1992), and Harpending (1994) raggedness index (r = 0.0238) is not significantly different from the values for an equilibrium population (Prob(r<0.0238) = 0.0487 for 10000 random simulations of equilibrium populations with parameters as in the observed distribution). Also, because of its bimodality, the distribution resembles that found in simulated stable populations (Rogers & Harpending, 1992).

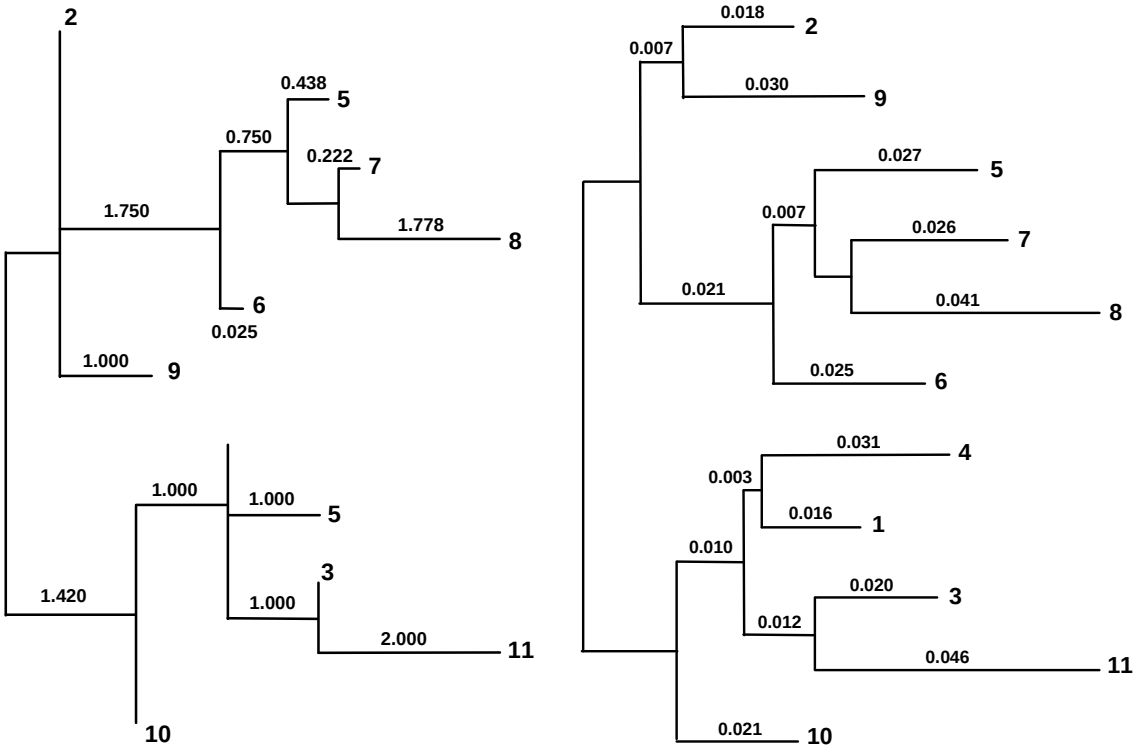


Fig. 3-3. Unrooted neighbor-joining dendrograms based on pairwise differences among the AFLP patterns from *L. cavanillesii* obtained using (a) Excoffier distance, and (b) Dice distance.

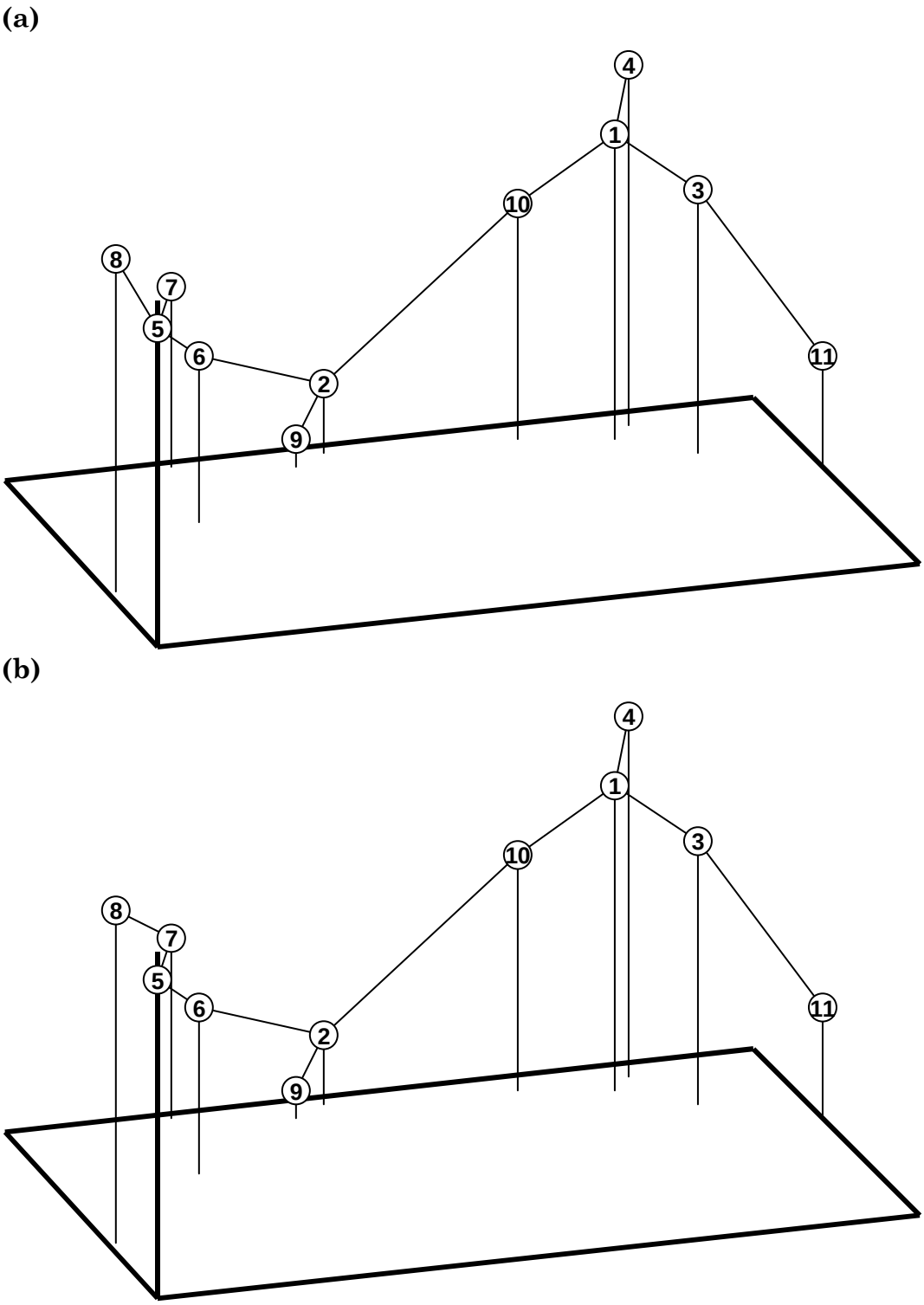


Fig. 3-4. Principal coordinates analysis with superimposed minimum spanning network for AFLP phenotypes from *L. cavanillesii* (a) Excoffier distance, and (b) Dice distance.

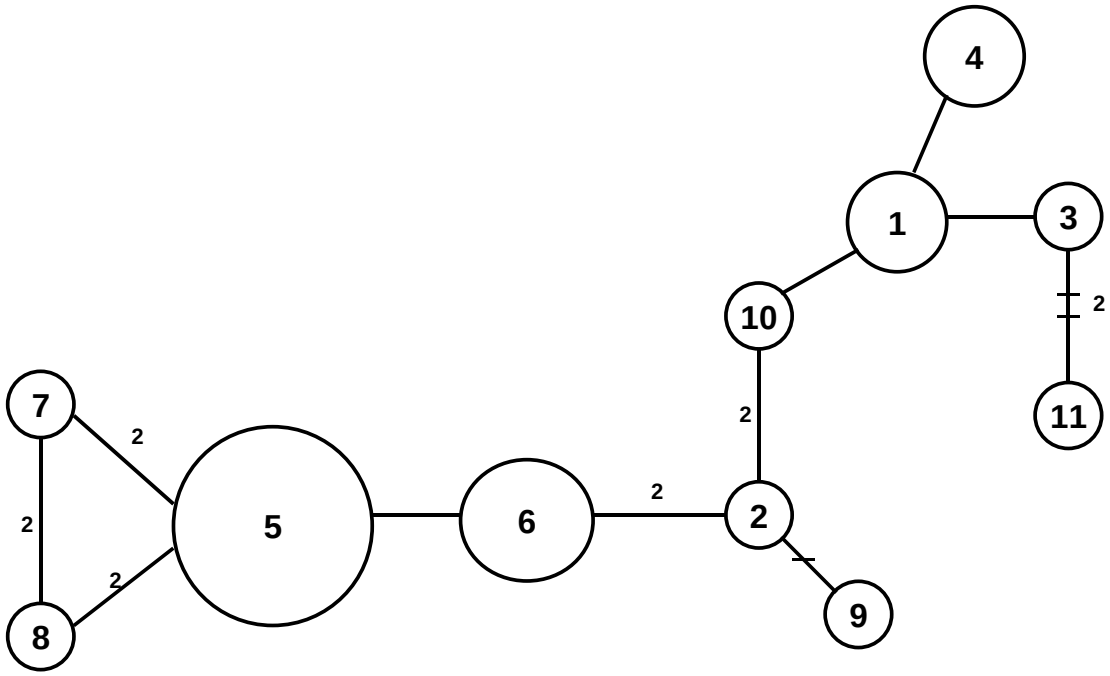


Fig. 3-5. Minimum spanning network showing the relationships among AFLP phenotypes from *L. cavanillesii*. Numbers on branches indicate mutational steps between patterns when larger than one. Bars over branches represent singleton mutations.

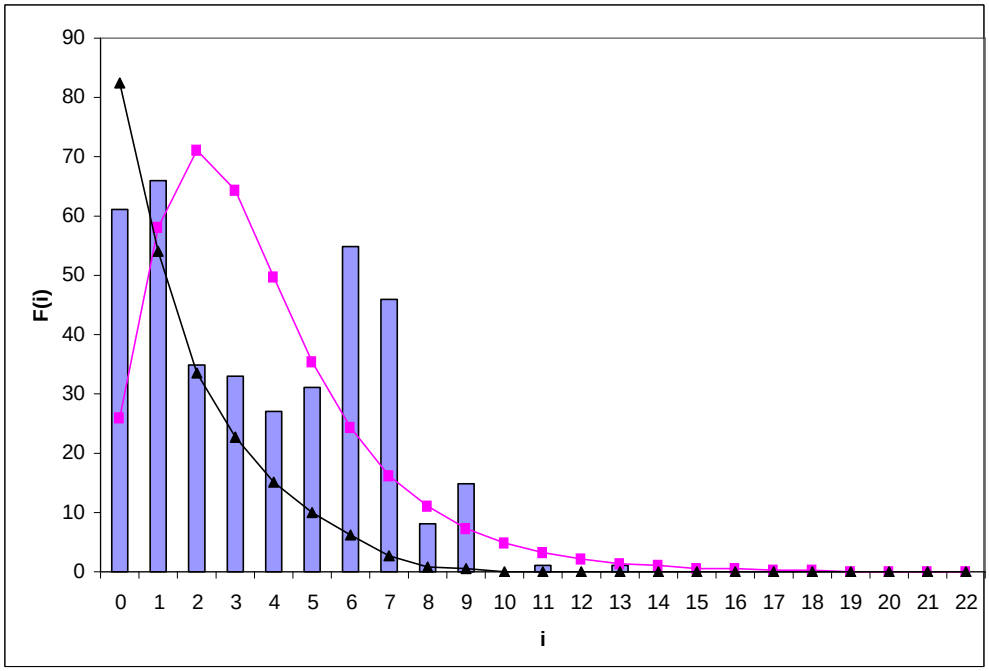


Fig. 3-6. Pairwise difference distribution for comparisons among individuals from *Limonium cavanillesii* population. Lines represent the expected distributions of pairwise differences under constant population size (triangles) and under the population expansion model (squares).

DISCUSSION

The low levels of genetic variability found in *L. cavanillesii* could be explained by the joint effects of the apomictic reproductive system of this species and the passage through a severe bottleneck in recent times, after which there has been no chance for mutation to restore genetic variation. However, the mismatch distribution shows a genetic equilibrium of the population. This apparent discrepancy could be explained if the genetic signal is reflecting the demographic pattern of a population in equilibrium in the past, that masks the effects of later events (Lavery *et al.*, 1996).

The need of polymorphic markers for planning recovery strategies in *L. cavanillesii* is important for its *ex situ* conservation in germplasm collections and the adequate establishment of new natural populations (see later). In both cases, samples taken from nature should represent the variability found in the species. The ability to recognize individual plants is also desirable to monitor the success of each phenotype on the new populations. AFLPs provide a suitable class of markers for this goal. Contrarily, RAPDs do not seem to present enough variability for this particular species. Comparative analysis of variability levels detected with AFLPs and RAPDs from the same *L. cavanillesii* individuals indicates that these techniques show significant differences, which cannot be explained simply by the larger number of markers derived from the AFLP procedure. Our lack of knowledge of the genome organization and mapping location of these markers in this species prevents us from drawing further conclusions with regard to the explanation for this observation. However, as more studies employing both techniques appear, it will be possible to determine whether this is a particular or general phenomenon.

In any case, the advantages attributed to AFLP over other PCR-based DNA fingerprinting methods have been confirmed in the present study. Its handling could seem to be cumbersome by the use of automated sequencers and additional software, but, under our experience, this initial appreciation is far from reality. Automation is a desirable feature in every molecular technique, to avoid the subjectivity of manual data collection (e.g. Rossetto *et al.*, 1995; Janssen *et al.*, 1996). Although, admittedly, AFLP data collection is still semiautomatic, the technique is evolving towards full automation, which is always an advantage over other, less objective, ways of collecting data. Besides, our pilot study has demonstrated the reproducibility of these markers, which makes of AFLP a very time-efficient method, as it is not necessary to establish the reproducibility of every fragment considered. Reproducibility and reliability are at the very heart the most common critics to RAPDs and other DNA fingerprinting techniques, especially for the impossibility to interchange results among laboratories.

Summarizing, when PCR-based DNA fingerprinting methods are chosen as the most appropriate technique to face a particular problem, as for instance in the analysis of diversity in endangered species or species for which there are indications of very low genetic

variability, AFLP fingerprinting has remarkable characteristics that made it advantageous over other methods.

Prospects for the future

Now that the biology of *Limonium cavanillesii*, and the causes leading to its decline are better understood, and the genetic variability of the only wild population of the species has been studied, decisions for future *in situ* and *ex situ* conservation of the species can be made.

The adoption of conservation measures for apomictic species can be controversial. Questions about their status as proper species arise very often, especially when they have a hybrid origin as it is the case for this species. Besides, the taxonomy of endemic apomictics can be quite confounded, as clearly distinct ecotypes may have been classified as different species on the basis of morphological characters, but the use of molecular markers can lead to very different conclusions (van Heusden *et al.*, 1991; Kraft & Nybom, 1995; Kraft *et al.*, 1996). This situation is frequent in the genus *Limonium*, in which reticulate evolution has lead to a quite confounded and complicated taxonomy as has been pointed out in Chapter 2. In fact, the presumptive monophyly of the *duriusculum* complex, in which *L. cavanillesii* is included, is currently being studied (J.A. Rosselló, personal communication). Until this revision is completed, a conservative policy should preserve as much of the current diversity as possible, because this policy can always be revised, whereas the opposite one cannot, and especially when dealing with species in so meager conditions as *L. cavanillesii*. In the meantime, the study of AFLP makers on *L. cavanillesii* opens the possibility of performing population genetic studies on closely related species, which may help to resolve the taxonomic complexity of the *duriusculum* complex.

A separate issue for obligate apomictic species is how to decide what to preserve: populations, clones, or individuals. Given the nearly genetic homogeneity of the only extant population of *L. cavanillesii*, it seems obvious that the whole population should be preserved. Due to its low genetic variability, *L. cavanillesii* is likely to be very sensitive to environmental changes. Conservation of the unique wild population of the species must be an urgent measure, via preservation and protection of the area where it is currently established. Some authors have stressed the potential value of small reserves to provide a wider choice of sites for protection and emphasize that they can play an important role in plant conservation (Lesica & Allendorf, 1991; Reznicek, 1987). Besides, re-establishment of the species in suitable and ecologically secure areas, creating new self-sustaining populations, would also be desirable.

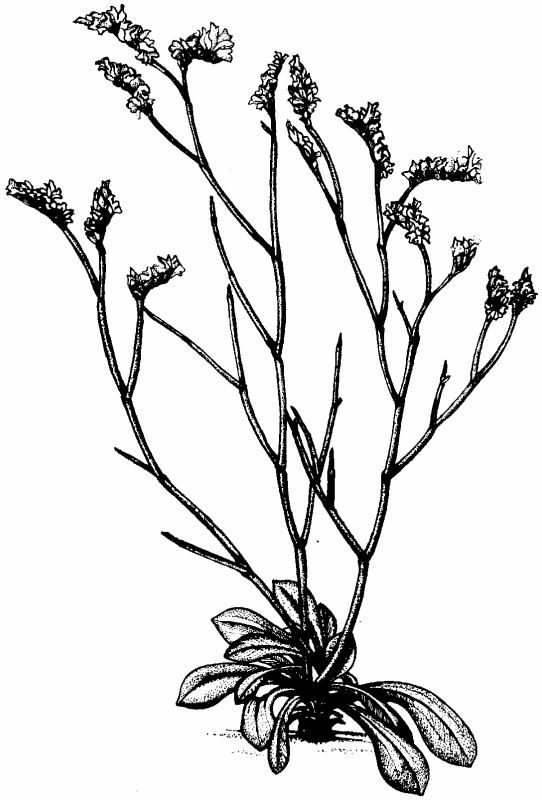
Ex-situ conservation is another measure for the preservation of the species in the future that should be considered. Conservation of seeds in germplasm banks has not been possible in some species of the genus (M.D. Lledó, personal communication), but good seed germination results have been obtained with material from *L. dufourii*, after one year of dormancy (J.A. Rosselló, personal communication). Although costly and time consuming, conservation b y micropropagation of some individuals might be desirable. At the same time,

a study of the germination capacity of the seeds, after being maintained in germplasm banks during one or more years, may be important for the possibility of replacing micropropagation by this cheaper technique in the future.

We believe that the critical situation of *L. cavanillesii* deserves attention and that it should be catalogued as critically endangered according to the IUCN categories of threat.

4

Analysis of genetic variation in the endangered species *Limonium dufourii*



RESUMEN

Limonium dufourii es una especie triploide, con reproducción apomíctica, endémica de las costas del este mediterráneo español, donde está presente en sólo 6 poblaciones con unos pocos individuos en la mayoría de ellas. Inicialmente se estudió la variabilidad genética y la estructura poblacional usando como marcadores los RAPDs. Los doce primers utilizados para los 165 individuos analizados rindieron un total de 124 bandas reproducibles, de las cuales 33 resultaron polimórficas. Las bandas polimórficas permitieron definir 44 fenotipos diferentes. Se han utilizado varios métodos de evaluación estadística para el análisis de la variabilidad genética a nivel intra- e interpoblacional. Las relaciones entre los patrones llevaron a identificar 4 clusters principales. Dos de ellos se corresponden con las poblaciones de Cullera y Torreblanca, mientras que los otros dos (grupos A y B), incluyen patrones de individuos que coexisten en las poblaciones de la Marjal del Moro y El Saler. La mayor parte de la variabilidad es debida a diferencias entre las poblaciones según el análisis molecular de la varianza. Por último, el análisis de homogeneidad de varianzas mostró que existían diferencias sustanciales en la cantidad de variación genética presente en las 6 poblaciones.

Debido a las serías dudas que se han planteado sobre la reproducibilidad de los marcadores tipo RAPD, decidimos comparar los resultados obtenidos con otro método desarrollado recientemente, que ha resultado ser mucho más reproducible: la técnica de los AFLPs. Los 3 primers de AFLPs utilizados produjeron un total de 51 marcadores polimórficos, que permitieron distinguir 65 fenotipos de AFLPs.
La diversidad genética media encontrada con esta técnica fue algo menor que con RAPDs. Además, el índice de clasificación indicó una cierta falta de correspondencia entre las clasificaciones obtenidas con ambos marcadores. Sin embargo, a pesar de estos resultados, el análisis de los datos de AFLPs proporcionó la misma subdivisión en 4 grupos que con RAPDs, y además, los individuos concretos que intervenían en esos clusters también eran coincidentes. Asimismo, los análisis AMOVA y HOMOVA rindieron resultados muy similares a los RAPDs respecto a la estructuración poblacional y niveles comparativos de variabilidad intrapoblacional. Se muestran también los resultados del análisis de los datos de AFLPs y RAPDs considerados en conjunto. Por último, la similitud de los AFLPs con los marcadores tipo RFLP ha permitido aplicar con un alto grado de fiabilidad una aproximación evolutiva al cálculo de diversidad genética. Esta es una de las principales ventajas de los AFLPs sobre otras técnicas de DNA *fingerprinting* basadas en la PCR.

Los resultados obtenidos han servido para comprender la historia evolutiva y demográfica de *L. dufourii*, consideradas como requisitos indispensables para establecer medidas de conservación eficaces en cualquier especie.

ABSTRACT

Limonium dufourii (Plumbaginaceae) is a triploid species, with apomictic reproduction, endemic to the East Mediterranean coast of Spain, where it is present in only 6 populations with a few individuals in most of them. Initially, genetic variation and population structure was studied using RAPDs as markers. Twelve different RAPD primers provided 124 reliable bands, of which 33 were polymorphic among the 165 individuals analyzed. The polymorphic bands were able to define 44 different patterns. Several methods for statistical evaluation have been used for intra- and interpopulation analysis of genetic variability. Relationships among patterns have led to the identification of 4 main clusters. Two corresponded to Cullera and Torreblanca populations, respectively; while the other two (groups A and B) include patterns from individuals coexisting in Marjal del Moro and El Saler populations. Most of the variation found in this species is due to differences among populations as shown by the analysis of molecular variance. The analysis of homogeneity of variance shows that substantial differences in the amount of genetic variability present in the 6 populations exist.

Due to the general concern about the lack of reproducibility ofRAPD markers, we decided to compare the results obtained with a newly developed method, which has been reported as highly reproducible: the AFLP technique. The use of three different AFLP primers rendered 51 polymorphic markers that distinguished 65 AFLP phenotypes. The average diversity found with this technique was slightly lower than with RAPDs. Besides, the index of classification indicates a certain lack of correspondence between the classifications obtained with both markers. However, in spite of these results, the analyses performed with AFLP data revealed the same principal groups obtained with RAPDs and the particular individuals involved in those clusters are also coincident. Besides, AMOVA and HOMOVA analysis rendered similar results to RAPDs respect to population structure and comparative levels of variation. A combined analysis of AFLP and RAPD data was also performed.

The similarity of AFLP to RFLP markers makes it possible to apply an evolutionary approach to calculate genetic divergence with good confidence. This is one of the main advantages of the AFLP technique over other DNA fingerprinting methods.

The results obtained have been used to understand the evolutionary and demographic history of *L. dufourii*, which is a requisite in order to establish efficient conservation measures for any species.

MATERIALS

Plant sampling.

The number of individuals sampled from each of the six remaining populations of *L. dufourii* is shown in Table 4-1. For the three populations in which the census was lower than 100 individuals, all individuals were sampled for analysis. From the other populations, a representative sample size was collected. Two or three small leaves ranging from 20 to 800 mg of tissue, depending on the availability of material, were taken from each individual and kept refrigerated until storage at -80ºC.

Table 4-1. Location, number of individuals analyzed, and population sizes (approximate for populations larger than 100 individuals) of the six *Limonium dufourii* populations used in this study.

Population	Individuals analyzed	Population size
Cullera	34	34
Torreblanca	40	200
El Saler	7	7
Marjal del Moro-1	25	25
Marjal del Moro-2	29	3000
Marjal del Moro-3	30	2000

DNA isolation and quantification

DNA was extracted using the CTAB protocol developed by Doyle (1991), as for *L. cavanillesii*. Then, DNA contents were estimated following also the same procedure as in the previous chapter.

METHODS

RAPD method

DNA amplification and fragment visualization

Initial RAPD profiles were generated using the 20 OPAs and amplification reactions were carried out as for *L. cavanillesii*, including always negative controls. All DNA amplifications were done in a Perkin Elmer 2400 thermal cycler.

Fragment visualization followed the same procedure as in the previous chapter. To help scoring the bands, each 30-well gel consisted of alternating individuals from each of the six populations of *L. dufourii.*

Pilot study: Primers used and reproducibility of banding patterns

Usually some primers give more reliable results than others depending on the species. Therefore, a pilot study was carried out to evaluate the suitability of the available primers with *L. dufourii*.

Initially, each primer was used on a subset of four randomly chosen individuals from different populations. Based on the clarity of the patterns obtained (i.e. bright staining, sharp, and not too many

amplification products per lane), 14 primers were selected to test the reproducibility of their profiles. Each reaction was repeated three times using the same sample subset. Only those primers that gave reproducible banding patterns between reactions were chosen for the analysis of the whole sample set of the species (Table 4-2).

Table 4-2 Primers used, their sequences, base pair range scored, and number of polymorphic and monomorphic markers observed for each primer

			Bands	
Primer	Sequence	bp range	monomorphic	polymorphic
OPA-01	CAGGCCCTTC	600-2500	7	1
OPA-07	GAAACGGGTG	200-1500	10	5
OPA-08	GTGACGTAGG	250-2500	8	7
OPA-09	GGGTAACGCC	1000-2000	8	0
OPA-10	GTGATCGCAG	300-1250	6	5
OPA-12	TCGGCGATAG	500-2500	5	5
OPA-14	TCTGTGCTGG	600-1250	4	0
OPA-15	TTCCGAACCC	350-1250	12	0
OPA-16	AGCCAGCGAA	300-1300	4	0
OPA-18	AGGTGACCGT	350-1250	10	3
OPA-19	CAAACGTCGG	800-2500	6	4
OPA-20	GTTGCGATCC	800-2500	10	3

Furthermore, all the fragments included in the final analysis were tested for reproducibility. Whenever a new RAPD band that was not present in the pilot study appeared, two to four replicate PCR reactions were performed with that sample. The new band was included in the analyses only if it was present in all the reactions.

It is important to emphasize that primer selection was not biased in favor of those that revealed the most polymorphism. There is also no *a priori* reason to assume that there was a bias in scoring polymorphic versus monomorphic bands [Clark and Lanigan (1993) discuss how these biases can influence measures of genotypic diversity].

By heeding all the precautions mentioned above, most of the assumptions usually made in the analysis of dominant markers (Lynch & Milligan, 1994; Stewart & Excoffier, 1996) could be validated, allowing us the use of RAPDs, but at the cost of a decreased precision compared to codominant markers.

Data Analysis

The presence or absence of homologous bands included in the final analysis was determined for all individuals and a matrix of the different RAPD phenotypes was assembled. Fifteen different metric distances for all pairwise combinations of RAPD patterns were computed using RAPDistance (Armstrong *et al.*, 1996). These distance matrices were compared with the Euclidean metric (Excoffier *et al.*, 1992) using DIPLOMO (Weiller & Gibbs, 1993) to test their correlation by means of a t-test (Sokal & Rohlf, 1995). This distance was chosen for further analyses due to its adequacy for AMOVA (see below) and

because it uses both presence and absence of matches, which may be considered as a safety margin when dealing with rare organisms (Rossetto *et al.*, 1995).

Several approaches to assess relationships among RAPD phenotypes were used. First, the Euclidean distance matrix was used to construct a dendrogram using the neighbor-joining method (Saitou & Nei, 1987) as implemented in NEIGHBOR from the PHYLIP package (Felsenstein, 1993). As pointed in the previous chapter, conventional approaches for the estimation of the reliability of inferred trees, such as bootstrapping or jackknife, were originally developed for interspecific studies. These techniques sometimes lack statistical power at the intraspecific level because they focus on differences among taxa rather than between individuals, which are expected to differ minimally (Templeton *et al.*, 1992; Templeton & Georgiadis, 1996). Techniques that make an overall assessment of the tree rather than generating confidence limits for individual branches are better in this situation. We have used two different methods for this goal. First, we have compared the distance matrix with the patristic distances in the resulting neighbor-joining tree using DIPLOMO by means of their correlation coefficient (Armstrong *et al.*, 1996). Second, we have performed a Permutation Test Probability (PTP) analysis to test whether the resulting tree reflects an actual tree-like signal in the data or merely an artifact of the algorithm (Faith & Cranston, 1991).

Even though parsimony cannot be applied directly to RAPD data except in haploid organisms (Clark & Lanigan, 1993), we have also used the original RAPD phenotype data matrix to perform maximum parsimony analysis using PAUP (Swofford, 1993).

The above mentioned techniques are the classical approaches to determine the relationships among different operational taxonomic units (OTUs), but a theoretical framework for integrating the information from gene genealogies and frequency distributions of the variants at the intraspecific level is arising (Excoffier & Smouse, 1994; Templeton *et al.*, 1995; see also Chapter 3). Moreover, the finding of a large number of most parsimonious trees (see Results) suggested that an alternative way of representing the relatedness between the patterns obtained, not simply by means of strictly bifurcating trees, would be more adequate. We have therefore used estimation of a minimum spanning tree (MST, Dunn & Everitt, 1982) as the third approach for inferring the intraspecific phylogeny of *L. dufourii* using NTSYS (Rohlf, 1993). Alternative MSTs were obtained with MINSPNET, using the modified Prim procedure proposed by Excoffier and Smouse (1994). NTSYS was also used to study the relationships among RAPD phenotypes using principal coordinates (PCO) analysis. The multidimensional scaling was completed with the superposition of the MST on the corresponding plot. By using these last approaches we expected to be able to extract additional information from the plot obtained, such as the multidimensional relationships among the populations (Whitty *et al.*, 1994; Travis *et al.*, 1996), and to formulate a hypothesis on the intraspecific phylogeography of the species (Avise, 1994), which cannot be inferred from a simple dendrogram representation.

The distance matrices between RAPD patterns for 15 different metrics available in RAPDistance were used to calculate pairwise genetic distances between populations as:

$d'_{XY} = d_{XY} - (d_X + d_Y/2)$,

where d_{XY} represents the uncorrected distance between populations X and Y, and d_X, d_Y correspond to intrapopulation diversity measures. The resulting population distance matrices were compared to test their correlation with the Euclidean metric as in the previous section. The results of this correlation test allowed us to reduce the number of distance matrices to be employed in subsequent analyses, using only the Euclidean distance. An unrooted tree using the neighbor-joining method mentioned above was constructed

for *L. dufourii* populations. To test whether genetic and linear geographical distances between populations were correlated, the corresponding matrices were compared by means of the correlation test as described above.

For studying the genetic structure of *L. dufourii* populations we have used the extension of the analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) recently developed by Stewart and Excoffier (1996) to accommodate RAPD profile data by taking into account the dominant nature of these markers. The technique can be applied to diploid populations with assumed levels of self-fertilization. However, as *L. dufourii* is a triploid species, some further correction for dominance was necessary. In an apomictic species, departures from equilibrium result both from deviations in the founding populations and because of other evolutionary forces (selection, drift, mutation, etc.) which cannot be counteracted by random mating. Because Hardy-Weinberg cannot be checked with dominant markers, we either assumed random mating ($S = 0$) or complete self-fertilization ($S = 1$), as the two extremes for the range of possible values of the selfing rate, S. For the former case, the equations for the conditional expectations of the squared distance between individuals (7a) through (8b) in Stewart and Excoffier (1996), were transformed to be applied to a triploid species in Hardy-Weinberg equilibrium (Appendix 1). The two-step strategy proposed by the authors was applied to calculate the F-statistic analog (Φ_{ST}) and its significance (Excoffier *et al.*, 1992). For the case of $S = 1$, transformations were not necessary, because this condition is equivalent to a purely phenotypic comparison without correction for dominance (Huff *et al.*, 1993), except for a proportion factor. Under conditions of complete selfing, any individual showing a band is assumed to be homozygous for that allele. When compared to another individual showing the same band, there would be no differences among them, and when compared to an individual lacking the band, there would be 9 differences (3 x 3 chromosomes in each individual being compared). Finally, we used the nonparametric test for homogeneity of molecular variance (HOMOVA), based on Bartlett's statistic (Bartlett, 1937), to test for within population genetic diversity. Both AMOVA and HOMOVA analyses were performed using WINAMOVA (available from L. Excoffier).

Finally, inferences about the historical population genetic and demographic processes that have affected L. *dufourii* populations can be drawn from the analysis of within population diversity by calculating the pairwise mismatch distribution among RAPD patterns from each population (see also Chapter 3).

AFLP method

AFLP procedure and reproducibility study

The same protocol as in the previous chapter was followed for the AFLP method, data collection and scoring procedure, with the only modification that a modified "core mix" for the PSA and SA PCR reactions was employed. It consisted of 1.5mM $MgCl_2$, 0.2mM of each dNTP, 1X PCR Buffer II (PE), and 0.2U AmpliTaq (PE). Primers selected were the same as for *L. cavanillesii*, based on its preliminary study (Table 4-3).

Table 4-3. AFLP primers used, their fluorescent dye label, number of cycles on the selective amplification reaction (SA), amount used on the multiplexing reaction (MR), and number of monomorphic and polymorphic

bands obtained in this study.

AFLP Primers	Color Dye	Cycles on SA	µl on MR	Total no. Bands	Polymorphic Bands
MseI-CTG EcoRI-AC	FAM (blue)	23	5	75	22
MseI-CAA EcoRI-AG	JOE (green)	25	10	84	16
MseI-CTG EcoRI-AT	TAMRA (yellow)	27	20	73	13

The multifactorial experiment carried out in this last species was also repeated in *L. dufourii* to see if the use of our own "core mix" had any influence on the reproducibility of the reactions. In this case only intensity differences among reactions were observed, but they did not correlate with the quantity of DNA used. The immediate consequence of this difference was a discrepancy in the peaks finally called by program Genotyper. To diminish these automatic misinterpretations in the final matrix, AFLP reactions under a certain empirical intensity threshold were repeated. Fortunately, poor amplifications occurred consistently only with a few individuals, which were excluded from analysis. This accounts for the different samples used between this study and the previous RAPD survey.

Data analysis

AFLP phenotypes from the original matrix were extracted using the RAPDistance package (Armstrong *et al.*, 1996). The resulting data matrix was used to calculate two kinds of pairwise genetic distances among these phenotypes. First, based on the results of RAPD data, pairwise comparisons among all AFLP patterns were assessed using only two of the phenetic metrics employed there, which are those most commonly used on PCR-based DNA fingerprinting methods (Lynch, 1990; Huff *et al.*, 1993; Travis *et al.*, 1996): the Euclidean distance (Excoffier *et al.*, 1992) and Dice similarity coefficient (see Chapter 3). For the second kind of genetic distance, Nei and Li's method (Nei, Li, 1979) for estimating nucleotide divergence (or number of substitutions per site; equations 5.53 and 5.55 (Nei, 1987) between two phenotypes was employed. Therefore, we considered that AFLP data could be treated analogously to restriction-fragment presence/absence data (RFLP). However, apart from assumptions usually made for the analysis of DNA fingerprinting markers (see above), which are applied for both kinds of genetic distances, two further assumptions are necessary for estimating genetic divergence (d) from AFLP data. First, a single nucleotide substitution in the restriction-site or the selective nucleotides (that together conform the actual restriction site) is sufficient to prevent the amplification of a particular fragment. Second, Nei and Li's approach gives a fairly accurate estimate of d when $d<0.05$ (Nei, 1987), as F (the expected proportion of polymorphic fragments) decreases when d increases because multiple hits are treated as a single substitution. Hence, this approximation will be valid only for closely related OTUs.

The phenetic distance matrices were analyzed to ascertain the relationships among AFLP patterns and the relationships and degree of differentiation among *L. dufourii* populations following the same approaches as for RAPD markers. Likewise, the nucleotide divergence matrix was employed to construct a NJ dendrogram from AFLP patterns, and to perform AMOVA and HOMOVA analyses. On the other side,

using this evolutionary distance measure it is possible to calculate the nucleotide differentiation statistic proposed by Lynch and Crease (1990) as Nst = $D_{between}/(D_{between}+D_{within})$; being D the average of all inter- or intrapopulation divergence values.

Besides, gene diversity for each locus and Wright's Fst values (Weir BS, Cockerham, 1984) as measures of the percentage of the total variance due to interpopulation differences, and Ewens-Watterson's test of selective neutrality (Watterson G., 1978) on the frequency distribution of AFLP patterns in each population, were computed using program ARLEQUIN (available from L. Excoffier). Finally, computation of the distribution of the number of pairwise differences (mismatch distribution) between all pairs of molecular variants was assessed with the purpose of comparing historical demographic patterns for each population deduced from these markers with those obtained with RAPD data. In this case, a Monte-Carlo simulation was performed to obtain a null distribution for (Harpending, 1994) raggedness parameter *r* under the constant population size model for the mismatch distribution.

RESULTS

RAPD results

RAPD profiles and their geographic distribution.

Figure 4-1 shows examples of RAPD profiles generated with some primers. Negative controls, in which DNA was omitted, were always free of amplification products. Individuals that shared profiles with a polymorphic primer also shared their profiles with almost all the other polymorphic primers (e.g. compare patterns for primers 7 and 8 in Fig. 4-1). These results are characteristic for apomictic species, in which each individual is expected to be identical to its progenitor [i.e. they are 'ramets' from the same 'genet' according to Harper (1985)], unless a mutation event has occurred. Thus, if two individuals are descendant from the same progenitor, they are expected to present the same RAPD profile with all primers (barring mutation). This is corroborated by the fact that no band is exclusive to a single individual.

Primers used, their sequences, size range scored, and numbers of polymorphic and monomorphic markers observed for each primer are presented in Table 4-2. RAPD products in the middle molecular weight range usually produced much more reliable results, as some authors have already pointed out (Penner *et al.*, 1993; Stewart & Porter, 1995). The 12 primers used in the final study of 165 individuals from *L. dufourii* gave a total of 124 markers, 91 of which (74%) were monomorphic. A total of 33 (26%) polymorphic markers allowed distinguishing 44 different RAPD phenotypes (Appendix 2), whose frequency distribution in the 6 populations is shown in Appendix 3. Torreblanca and Cullera populations do not have any pattern in common with other populations, whereas El Saler and Marjal del Moro populations share some phenotypes. A total of 6 (13.6%) RAPD phenotypes are shared by more than one population, while the remaining 38 (86.4%) are confined to single populations. Hence, these results suggest a high degree of population differentiation, as expected for an apomictic species.

Pattern divergence and intraspecific phylogeny

Among the available measures to calculate distances among RAPD patterns we have used the Euclidean metric proposed by Excoffier *et al.* (1992), because the correlation among this and the remaining distance matrices was not significantly different from 1 and for the reasons mentioned in Material and Methods.

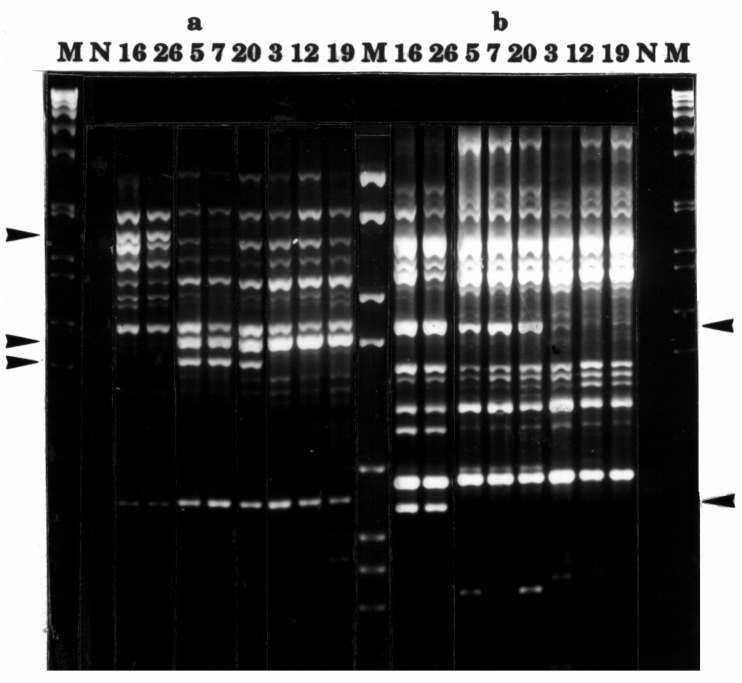


Fig. 4-1 Example of RAPD profiles in *L. dufourii* obtained with two different primers (a) OPA-8, (b) OPA-7. M and N lanes correspond to molecular size markers and to negative controls for each primer, respectively. Arrows indicate polymorphic bands. Note that individuals that share profiles with primer 8, also share their profiles with primer 7.

The unrooted dendrogram obtained using the neighbor-joining method (Fig. 4-2) shows the relationships among the 44 patterns based on RAPD analysis. The tree revealed clear differentiation of two major groups. One group includes patterns restricted to Marjal del Moro populations, denoted group A for further discussion. The remaining patterns are grouped in three subclusters, where phenotypes from Cullera and Torreblanca populations form two well separated groups, and the remaining patterns form another group, except for some phenotypes (23, 28, 31, 32) that are not clearly included in any of these subclusters. The last subcluster and the four separate patterns are denoted as group B. A majority-rule consensus tree derived from the 100 most parsimonious trees revealed similar relationships, including the two main clusters and the subclusters present in the neighbor-joining tree. Other minor groups with lower consensus values were also present in the neighbor-joining tree. The consensus values for groups coincident in both analyses are shown in the nodes of the tree depicted in Fig. 4-2.

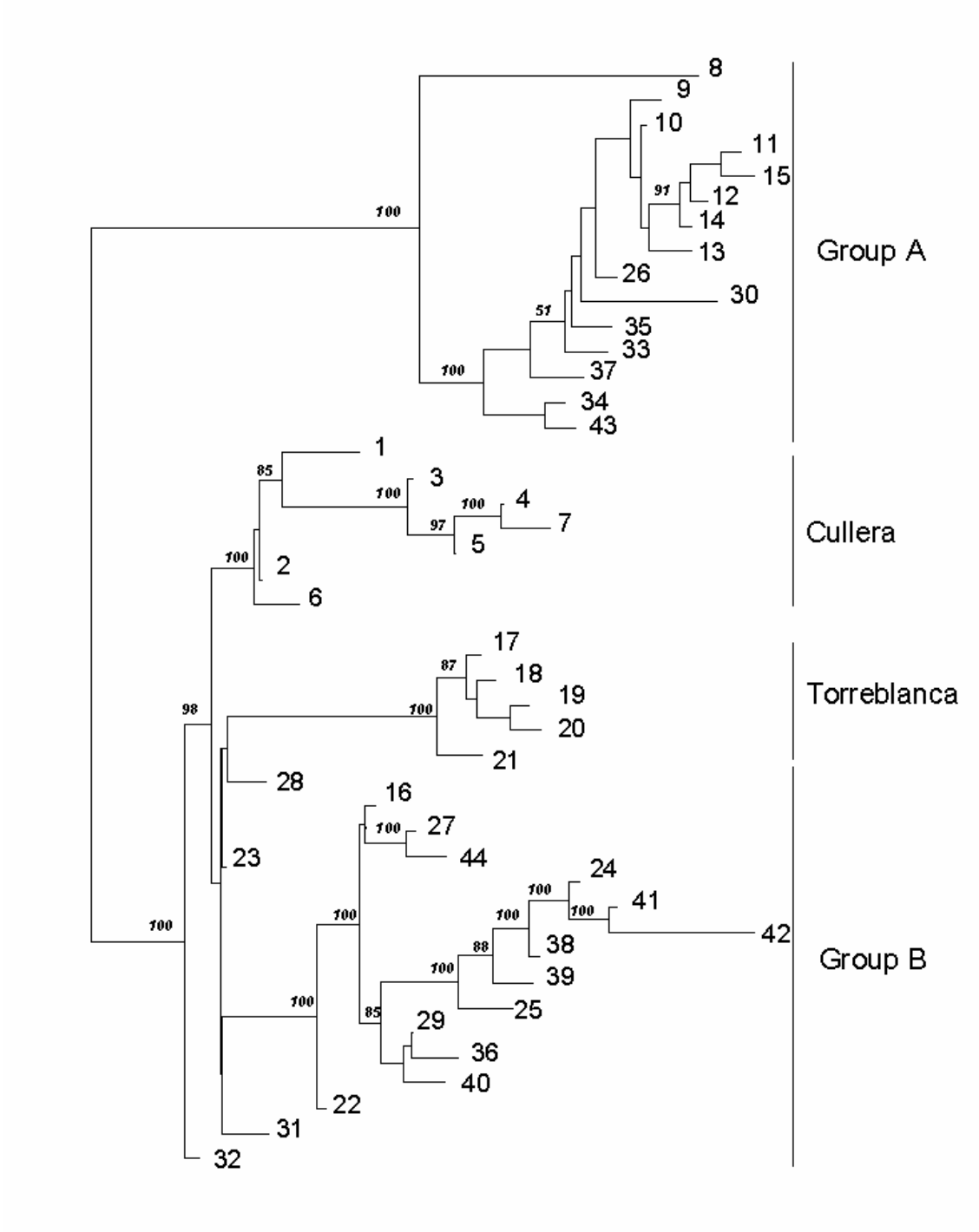


Fig. 4-2 Neighbor-joining tree showing the relationships among the 44 different RAPD patterns obtained in *L. dufourii*. The Euclidean distance matrix was used. Numbers at nodes represent the values of the majority-rule consensus tree obtained by parsimony analysis of the raw presence/absence matrix.

The two methods used for testing the tree-like signal in the data gave consistent results. The comparison between the genetic and the patristic distance matrices gave a significantly positive correlation value ($r = 0.976$, $P<0.001$ for the nule hypothesis of $r=0$). The PTP test gave a Z-value of 44.61, implying that the probability of this tree to occur simply by chance is practically null.

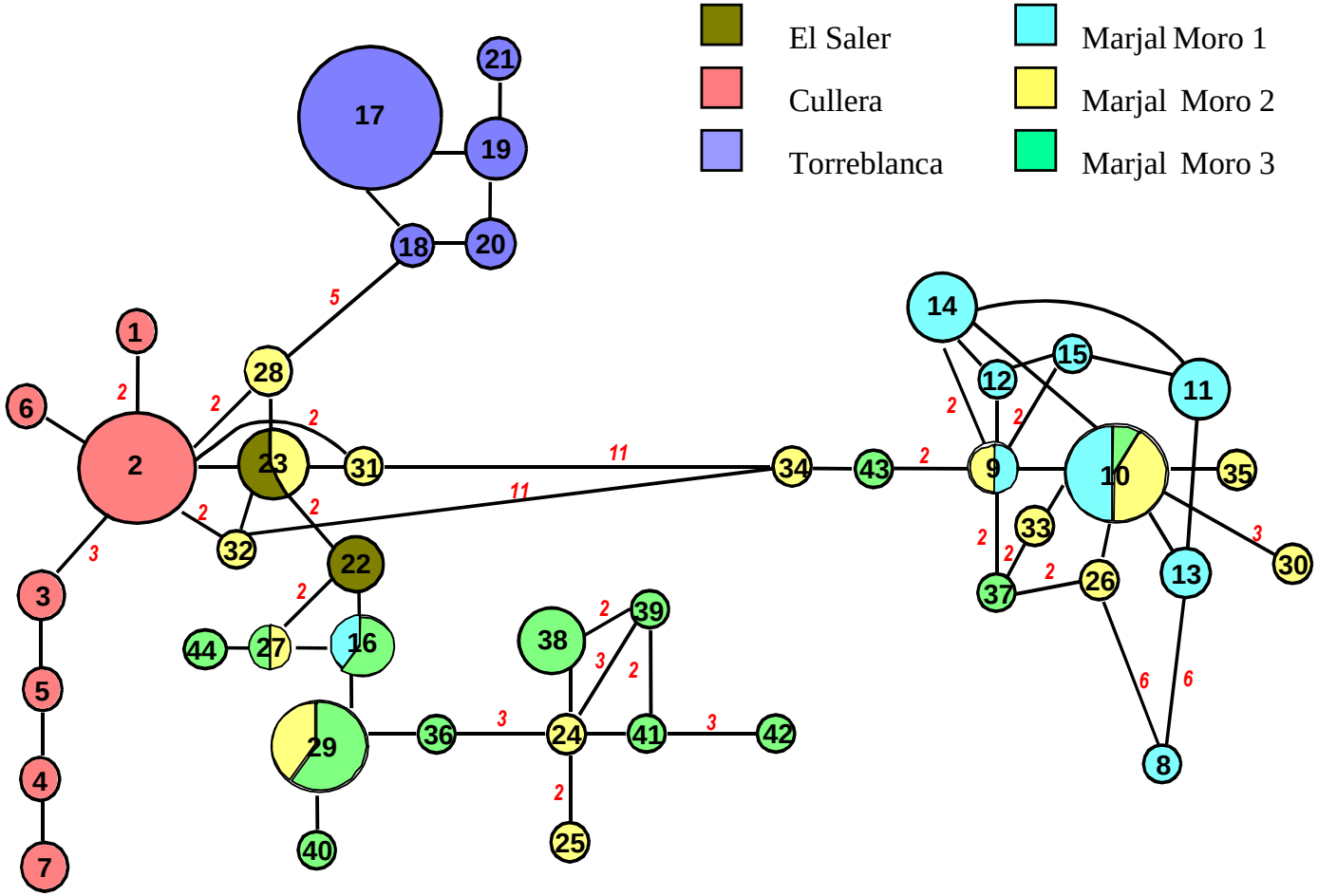


Fig. 4-3 Minimum spanning network obtained with MINSPNET showing the relationships among 44 RAPD patterns found in *L. dufourii*. The area of each circle is proportional to the frequency of individuals showing the corresponding pattern. Numbers above the segments connecting circles represent the number of differences among them when these are larger than 1.

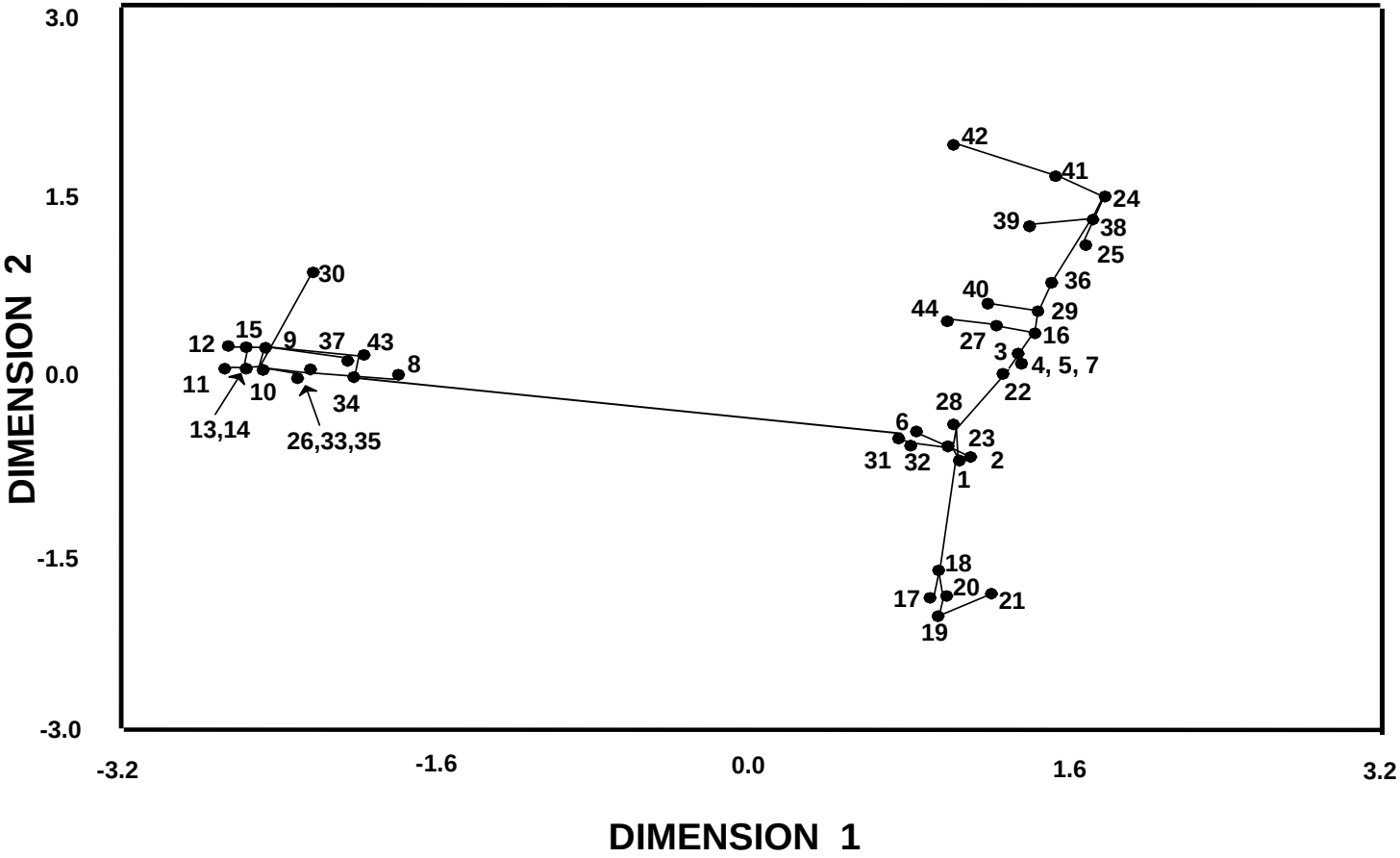


Fig. 4-4 Principal coordinates plot with the minimum-spanning tree superimposed for the two first dimensions in the PCO analysis of the 44 RAPD patterns obtained in *L. dufourii*.

Population frequency information and relationships among the patterns can be represented simultaneously on a minimum spanning network (Fig. 4-3). In general, the plot supports the clusters suggested by the phenetic method, but it shows that alternative relationships among patterns can be derived from this network, especially within groups. Consequently, a simple bifurcating tree is an inadequate representation of the evolutionary relationships at the intraspecific level.

The superposition of the MST on the bidimensional plot of the principal coordinates analysis derived from the Euclidean distance matrix between the 44 RAPD phenotypes (Fig. 4-4) confirms that the previous relationships are preserved in the multidimensional mapping. The first dimension of the PCO explains 51.5% of the total variation by separating group A of patterns found in the three Marjal del Moro populations from the rest. Note that in the minimum spanning network (Fig. 4-3) at least 11 mutational steps separate these groups. The second dimension explains an additional 14.2%, essentially spreading along its axis the remaining patterns and separating Torreblanca from the other populations. The third dimension (not shown) explains almost 10% of the total variation by enhancing the differences among Cullera patterns and the rest.

It is remarkable that only one pattern is common to all 3 Marjal del Moro populations and only 5 patterns are found in two populations. This low incidence of pattern sharing among Marjal del Moro populations is a preliminary indication of genetic structuring among them.

Divergence at the population level

Interpopulation distances obtained using the Euclidean metric (Table 4-4) and the remaining metrics available in RAPDistance showed a significant positive correlation, with the only exception of Russell and Rao metric (data not shown). This measure overestimates the distance between OTUs because it only considers mismatches in the denominator. It showed low correlation with the other distance measures, and we did not consider it adequate

Table 4-4 Interpopulation distances obtained using the Euclidean metric (lower hemimatrix) and linear geographic distances (upper hemimatrix) among *Limonium dufourii* populations.

	Cullera	**Torreblanca**	**El Saler**	**Marjal Moro-1**	**Marjal del Moro-2**	**Marjal Moro-3**
Cullera	-	63.072	12.530	28.000	28.003	28.001
Torreblanca	6.124	-	52.086	35.128	35.125	35.127
El Saler	1.495	5.364	-	18.026	18.029	18.027
Marjal del Moro-1	13.027	15.189	12.235	-	0.003	0.001
Marjal del Moro-2	4.136	7.207	3.085	3.515	-	0.002
Marjal del Moro-3	4.291	8.173	2.311	11.956	3.209	-

for RAPD data. The Euclidean distance previously used for comparing RAPD patterns was therefore used in comparisons at the population level.

The neighbor-joining tree (Fig. 4-5) constructed from the Euclidean distances among populations did not show concordance between the geographic location of the populations and the topology of the dendrogram. This result agrees with the correlation test performed, which did not reveal a significant correlation between genetic and the actual geographic distances between populations (Table 4-4, r = 0.151, P > 0.05 for the null hypothesis r=0).

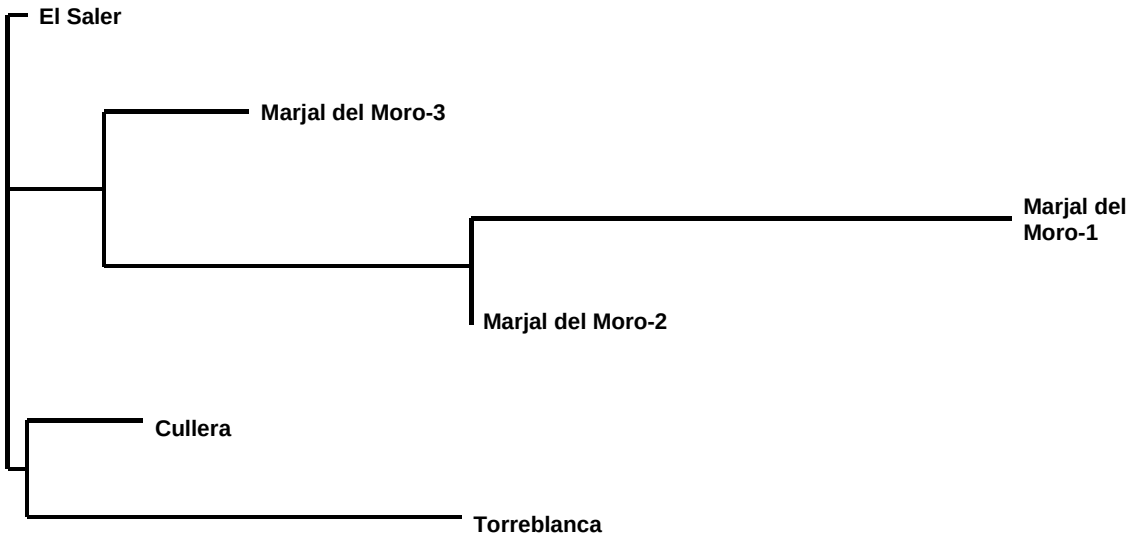


Fig. 4-5 Neighbor-joining trees for the six *L. dufourii* populations analyzed in this study using Excoffier distance.

Population genetic structure

Partitioning of RAPD variance within and among populations was performed using the AMOVA procedure with the modifications proposed for RAPD data by Stewart and Excoffier (1996). The 3 Marjal del Moro populations showed significant heterogeneity (data not shown) and were therefore treated as separate populations in subsequent analyses. The phenotypic distance matrix was used in the AMOVA analysis of the six *L. dufourii* populations according to Huff *et al.* (1993). This analysis is equivalent to the complete selfing case (S = 1). AMOVA analyses with distances among individuals corrected for the dominant nature of RAPDs (genotypic analyses) followed the two-step procedure from Stewart and Excoffier (1996) and were based on equations derived in Appendix 1 for the case of random mating (S = 0). The corrected distance matrix used in step 1 of the genotypic analysis is based on the frequency across all populations of each marker. Because this analysis showed significant population structure, the second step was performed based on a new corrected distance matrix that takes into account the frequency of each marker in each population. A summary of the phenotypic and two genotypic AMOVA analyses is shown in Table 4-5.

Table 4-5 Summary of the AMOVA and HOMOVA analyses. Population statistics are shown according to different assumptions on the data. DP: Analysis based on the matrix of phenotypic distances between individuals. DG1 and DG2: Analyses based on the matrices of genotypic distances among individuals when total or intrapopulation frequencies of each marker were considered (see text for further details). The first 6 rows refer to within population variance estimates for the six *L. dufourii* populations analyzed. Φ_{ST} represents the F_{ST} statistic analog from the AMOVA. Bartlett's test for the HOMOVA within populations is shown in the bottom row. Nonparametric tests of significance for variance components and homogeneity are based on 1000 random permutations.

Population statistics	DP	DG1	DG2
$\sigma^2_{Cullera}$	0.023	0.281	0.111
$\sigma^2_{Torreblanca}$	0.006	0.207	0.031
$\sigma^2_{El\ Saler}$	0.082	1.656	1.257
$\sigma^2_{Marjal\ del\ Moro\text{-}1}$	0.085	1.044	2.099
$\sigma^2_{Marjal\ del\ Moro\text{-}2}$	0.162	1.147	3.186
$\sigma^2_{Marjal\ del\ Moro\text{-}3}$	0.089	0.908	1.690
σ^2_{among}	3.586^{***}	16.690^{***}	37.116^{***}
σ^2_{within}	1.878^{***}	19.213^{***}	34.861^{***}
Φ_{ST}	0.656^{***}	0.465^{***}	0.516^{***}
Bartlett's test	73.074^{***}	25.561^{***}	159.886^{***}

***: $P < 0.001$

AMOVA analyses show significant population differentiation in *L. dufourii*, with independence of the selfing rate (phenotypic and genotypic analyses). Φ_{ST} values range between 0.515 and 0.656 for the possible values of *S*. The proportion of variation attributable to population differences is very high, as a consequence of the reproductive system, the low seed dispersal rate, and the demographic history of these populations. In addition, all pairwise comparisons of population variance were significant (data not shown).

Bartlett's tests for homogeneity of variance indicate significant levels of variability among populations (Table 4-5). Intrapopulation variances for phenotypic and genotypic AMOVAs are also shown in Table 4-5. The lowest level of genetic variation was observed in the Torreblanca population, followed by the Cullera, El Saler, and Marjal del Moro populations, respectively. Among the latter, Marjal del Moro-2 showed the highest diversity.

Figure 4-6 shows the observed distribution of pairwise differences for individuals from *L. dufourii* populations (plots a-e), and for individuals from the 3 Marjal del Moro populations pooled together (plots f

and g). El Saler population has not been considered due to its small sample size. The expected distribution of pairwise differences under a constant population size (Watterson, 1975) is also shown. Except for Torreblanca, all populations exhibit nonequilibrium distributions. However, there is also a clear difference between the Cullera and Marjal del Moro populations. The Cullera distribution shows a larger than expected number of comparisons with zero differences, while in the three Marjal del Moro populations there is a larger number of cases with large number of differences than expected. This is obviously due to the presence of patterns belonging to groups A and B in each Marjal del Moro population, thus generating a bimodal distribution in each of them as well as, and perhaps even more clearly, in the plot of the pooled populations (Fig. 4-6, plot f).

These signatures in the distribution of pairwise differences are indicative of different demographic histories in *L. dufourii* populations. The Cullera graph matches the distribution expected after a recent and sustained decline in population size (Rogers & Harpending, 1992, Fig. 4-6). This is a likely explanation in this case, since a reduction in the size of this population due to expanding urban development is well documented (Crespo & Laguna, 1993). The pattern observed in the Marjal del Moro populations requires a different kind of explanation. It is evident that the bimodality in these plots is due to the existence of two well-defined groups of patterns. Slatkin and Hudson (1991) argue that this situation is likely to arise in natural populations where the first split in a gene genealogy gives rise to two equally represented groups of variants. This is a plausible explanation given the hybrid nature of this species, but other alternatives should not be discarded. For instance, there is some evidence against this explanation in plot (g) from Fig. 4-6. This graph shows the observed and expected distributions of pairwise differences for all Marjal del Moro individuals when groups A and B are considered separately. If the above mentioned explanation was true, it would be expected that both plots overlap. The average number of pairwise differences between individuals with patterns from group A is 2.0 and for those from group B is 3.4, with variances 3.21 and 6.10, respectively. In consequence, it seems evident that group A is younger than group B, assuming that mutation rate and population size have been similar for both groups.

AFLP results

AFLP phenotypes

After pooling of the data from the three AFLP primer combinations used for analysis (Table 4-3), 252 DNA fragments were scored, ranging from 75 to 500 bp, with an average of 219.38 fragments per individual and 84.66 bands per primer combination.

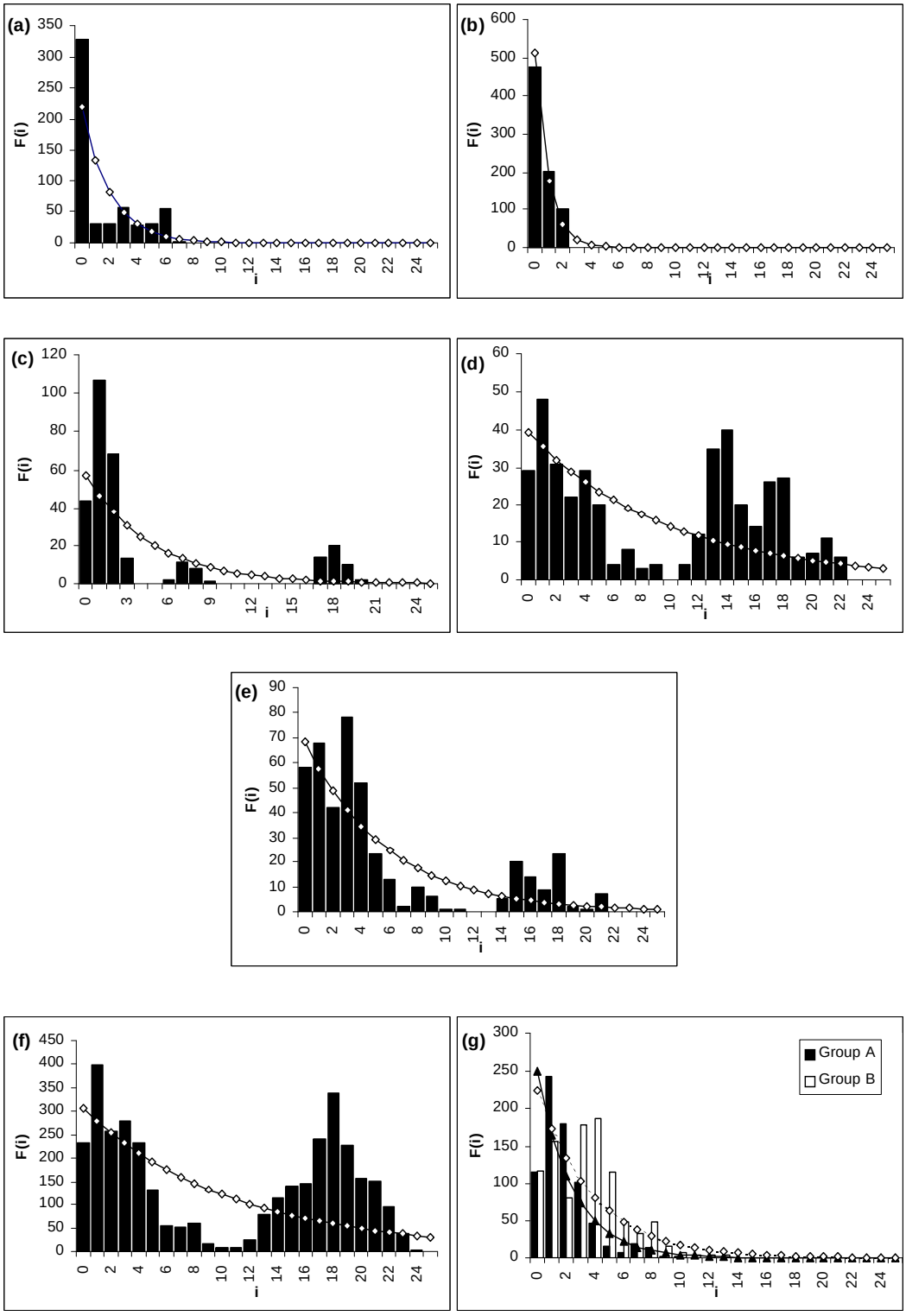


Fig. 4-6 Pairwise difference distribution for comparisons among individuals from *L. dufourii* populations. (a) Cullera; (b) Torreblanca; (c) Marjal del Moro-1; (d) Marjal del Moro-2; (e) Marjal del Moro-3; (f) individuals from the 3 Marjal del Moro populations pooled into one single population; (g) same as (f) when groups A and B are considered separately. The continuous line represents the expected distribution of pairwise differences under constant population size (Watterson, 1975).

There were 51 (20.24%) polymorphic markers that allowed distinguishing 65 AFLP phenotypes in the 6 populations of *L. dufourii* (Appendix 4), whose frequency distribution in the 6 populations is shown in Appendix 5. Only two patterns were present in more than one population, the rest were present in only one. It is also noteworthy the large number of individuals with a unique pattern in the three Marjal del Moro populations, in contrast with Cullera and Torreblanca. This is a preliminary indication of a higher diversity in these populations.

Intraspecific phylogeny and genetic population structure using phenetic distances

Both the non-Euclidean (Dice) and the Euclidean phenetic distance matrices were used for analysis. The results obtained with the two metrics were nearly interchangeable, as predicted by the highly significant correlation between them ($r=0.988$, $df=2078$, $P>0.29$ for the null hypothesis of $r=1$). Consequently only the results from the Euclidean measure are reported here.

Two main clusters can be distinguished from the NJ dendrogram for the 65 AFLP patterns (Fig. 4-7a). Cluster I includes only individuals from Marjal del Moro populations (denoted group A for later discussion). The other, cluster II, is subdivided into two subclusters, one including individuals from Marjal del Moro and El Saler (denoted group B), the other exclusively with patterns from Torreblanca. Patterns that belong to Cullera and pattern 1, that is present in three populations, do not form a monophyletic group, but are dispersed among other clusters. Phenotypic relationships within clusters are slightly different among Excoffier and Dice trees, but the above mentioned four cluster and subclusters include exactly the same patterns. The PTP test gave Z-values of 33.32 and 47.62 for Euclidean and Dice distances, respectively, indicating an extremely low probability ($P<0.001$) for the trees to have arisen by chance alone.

PCO analysis (Fig. 4-8) confirms the main relationships among phenotypes found in the cluster analysis. The first dimension explains 50% of the total variation, whereas only a 7.3% was expected under a broken stick model, and it results in the separation of clusters I and II. The second and third dimensions represent a very small fraction of the total variance (8% and 5.5%, respectively, as compared to 5.8% and 5.0% expected under a broken stick model) by spreading the patterns from clusters I and II along their axes. However, in this case patterns from cluster II form 3 separate groups that include, respectively, individuals from Torreblanca, from El Saler and Marjal del Moro, and those patterns, mainly from Cullera, that were dispersed among clusters in the NJ tree. These results are in agreement with the observed relationships among patterns obtained in the MSN (Fig. 4-9). Alternative relationships also represented in this network could be the reason for the small intracluster differences observed between the neighbor-joining trees from the Euclidean and Dice distances.

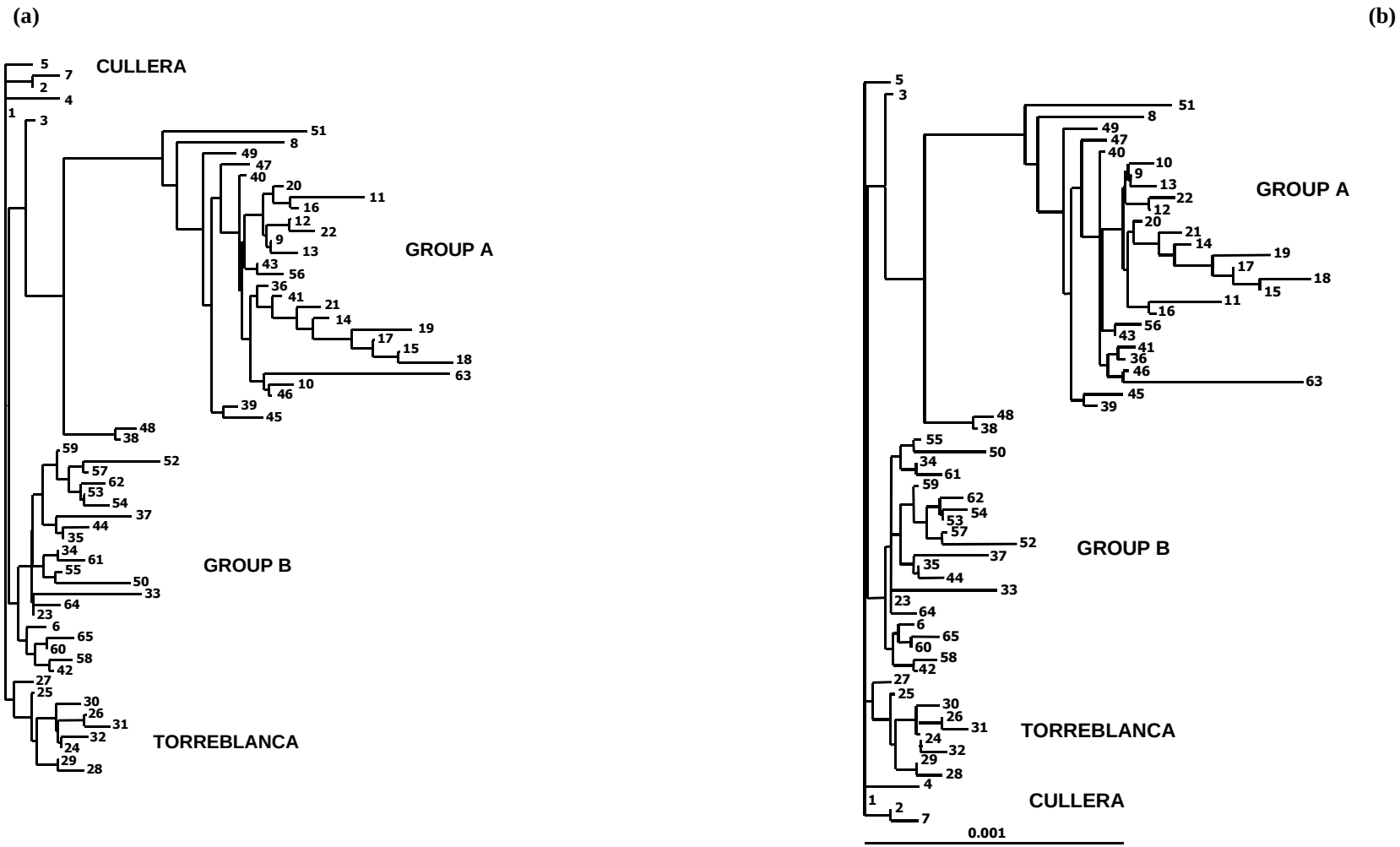
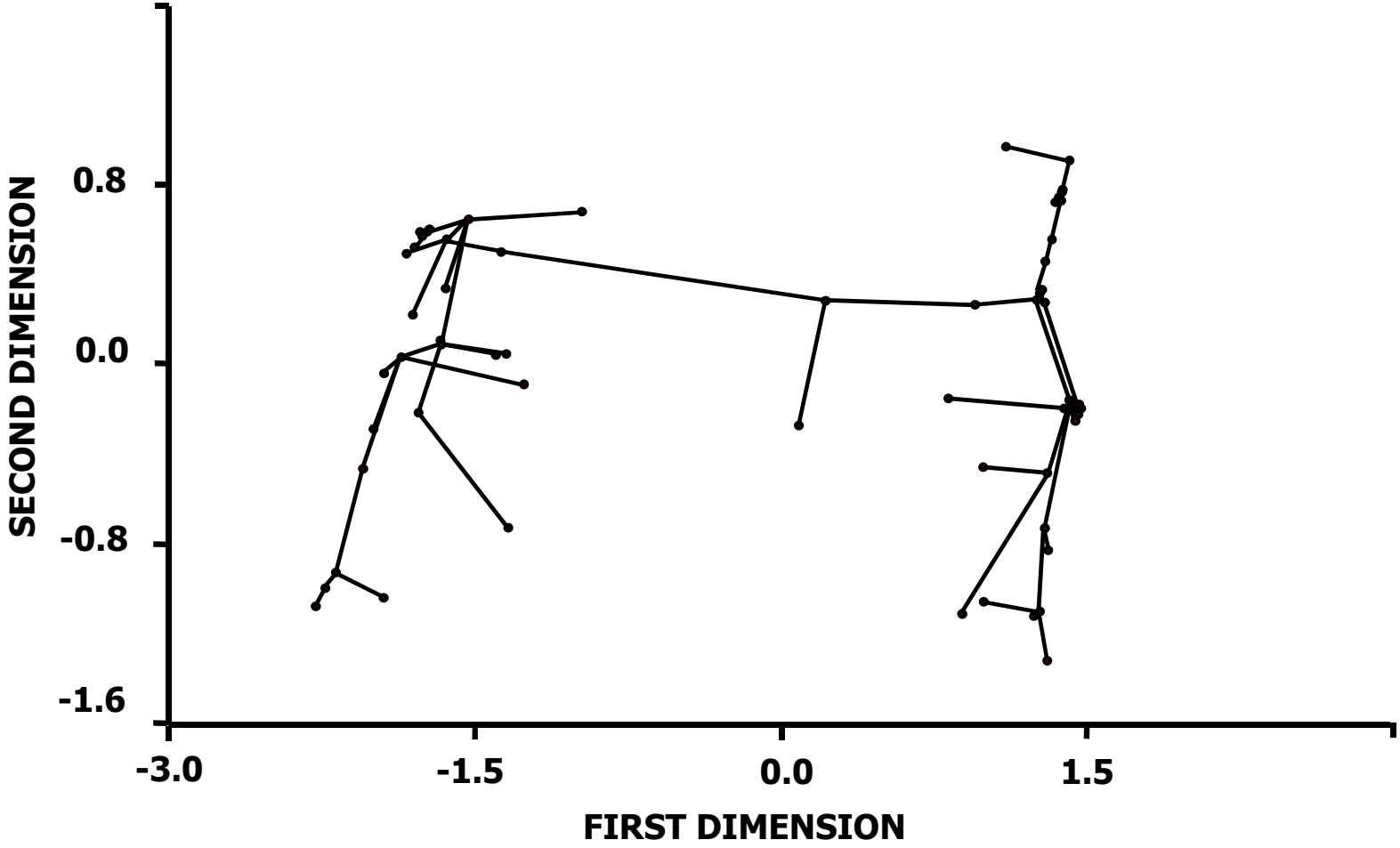


Fig. 4-7 Neighbor-joining tree derived from (a) Excoffier and (b) nucleotide divergence distance matrices among the AFLP patterns from *L. dufourii*.

Fig. 4-8 Principal coordinates plot with the minimum spanning tree superimposed for the two first dimensions in the PCO analysis for the AFLP patterns from *L. dufourii*.



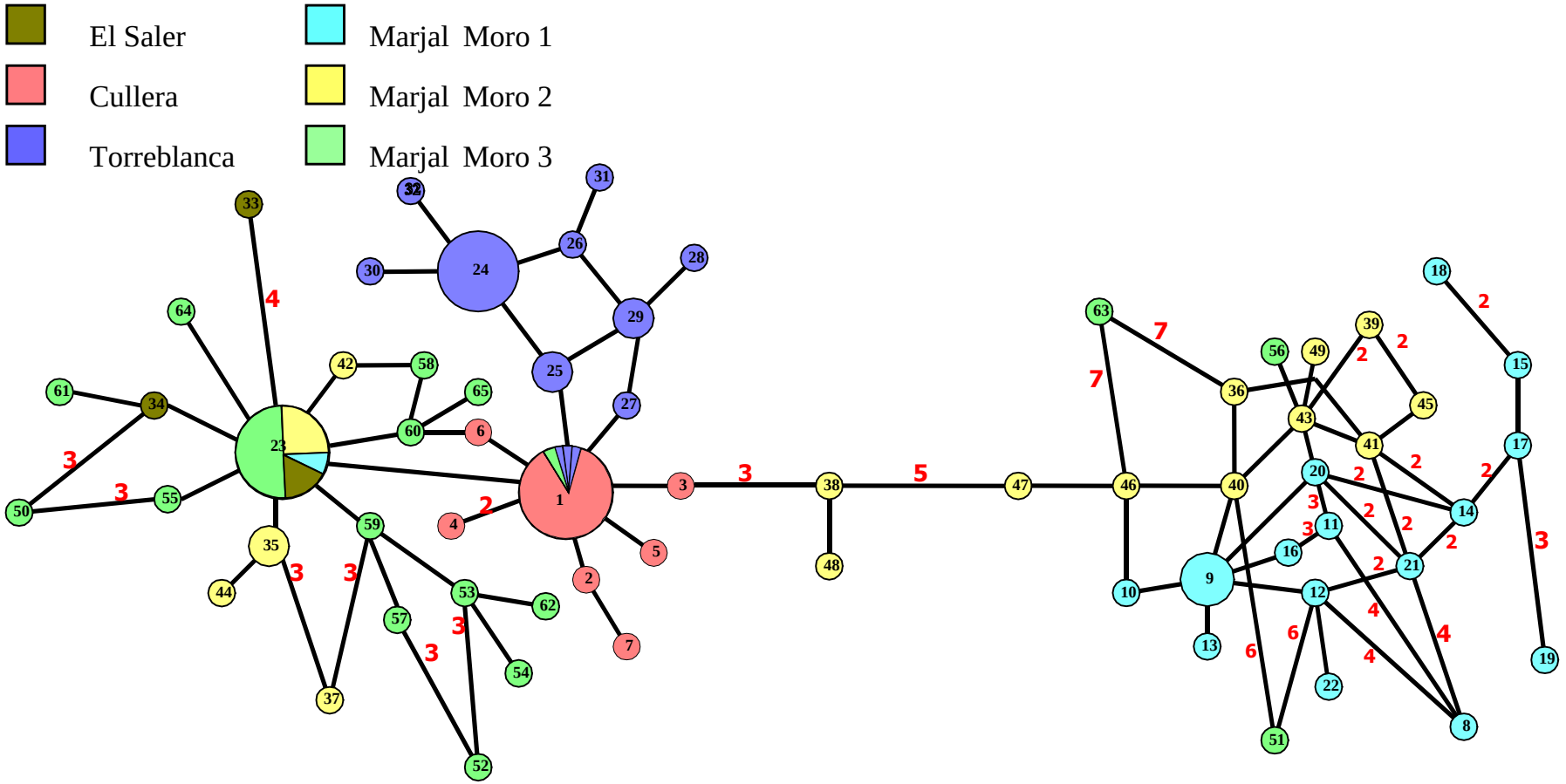


Fig. 4-9 Minimum spanning network obtained with MINSPNET showing the relationships among the 65 AFLP patterns found in *L. dufourii*. The area of each circle is an approximation to the frequency of that pattern in the species. The numbers next to the segments connecting nodes represent the number of differences among them when they are larger than 1.

The NJ tree obtained from the Euclidean interpopulation distance matrix is shown in Fig. 4-10. Marjal del Moro populations form a monophyletic group, and Cullera and Torreblanca are also placed in the same cluster. Furthermore a non-significantly different from zero correlation between this distance matrix and the linear geographic distance between populations was obtained (r=-0.069, df=13, P>0.5). This is an indication that isolation by distance is not the process accounting for the distribution of genetic variation among populations.

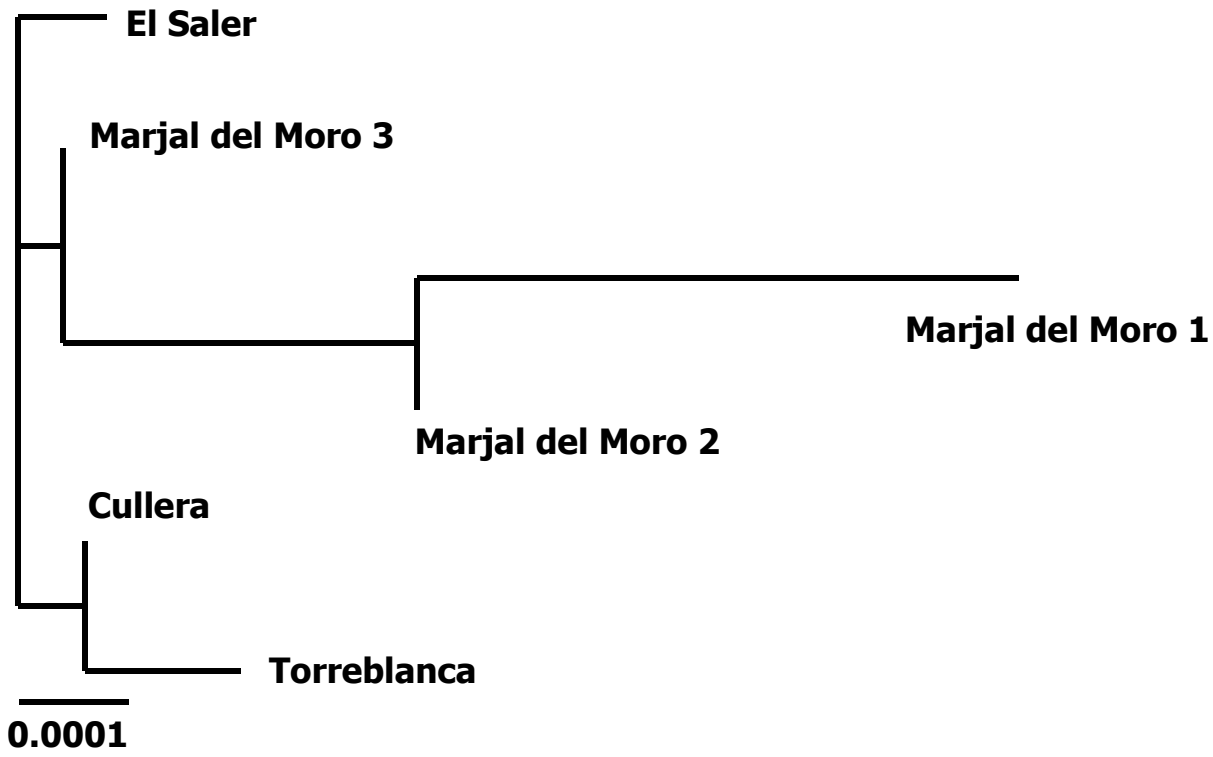


Fig. 4-10 Neighbor-joining tree from Excoffier and nucleotide divergence interpopulation distance matrices. The bar scales represents nucleotide substitutions per site

To determine the partitioning of the genetic variation within and among *L. dufourii* populations, the AMOVA procedure described for RAPD data analysis was also performed. Both phenotypic and genotypic AMOVA analyses result in a significant population genetic structure (Table 4-6), with Φst values of 0.538 and 0.531 respectively, which means that *L. dufourii* populations are genetically differentiated, regardless of the actual selfing rate in the species. All pairwise Φst values between populations were significant except for Marjal del Moro-3 and El Saler populations, possibly because they share the most frequent pattern (Appendix 5). When only the frequency of each pattern was considered, the resulting Fst value for this pair of populations was lower (0.243), but still significant (P<0.001).

Table 4-6 Results from the genotypic and phenotypic AMOVA analyses using the Euclidean distance (PE and GE, respectively), and for the analysis using the nucleotide divergence matrix (d) are summarized in the three first columns. The first six rows are the within population variance estimates for the six *L. dufourii* populations. These can be compared with the gene diversity estimate from the last column. Next rows are the Φst values from each AMOVA analyses, which can be compared with the Fst and Nst statistics bellow. Results from the HOMOVA analysis are shown in the bottom row. Non-parametric tests of significance for variance components and homogeneity are based on 1000 random permutations.

Population statistics	***PE***	**GE**	**d**	**Gene diversity**
$\sigma^2_{\text{Cullera}}$	0.304	9.486	0.000032	0.440
$\sigma^2_{\text{Marjal del Moro-1}}$	2.507	39.058	0.000258	0.920
$\sigma^2_{\text{Marjal del Moro-2}}$	3.185	46.885	0.000331	0.932
$\sigma^2_{\text{Marjal del Moro-3}}$	2.220	51.207	0.000231	0.821
$\sigma^2_{\text{Torreblanca}}$	0.607	7.310	0.000061	0.667
$\sigma^2_{\text{El Saler}}$	1.095	25.198	0.000117	0.662
σ^2_{among}	1.852^{***}	31.995^{***}	0.000194^{***}	
σ^2_{within}	1.593^{***}	28.205^{***}	0.000166^{***}	
Φ_{ST}	0.538^{***}	0.531^{***}	0.539^{***}	
Fst	0.243^{***}			
Nst	0.552^{***}			
Bartlett's test	51.594^{***}	47.735^{***}	51.950^{***}	

***: $P < 0.001$

The intrapopulation variances derived from the molecular analyses of variance and the average gene diversities are shown in Table 4-6. HOMOVA analyses showed significant differences in the amount of intrapopulation variation among populations (Table 4-6) as a whole, and in all but two pairwise comparisons. Although the different analyses do not agree completely, it seems evident that the Marjal del Moro populations show the highest levels of variation, El Saler is next, despite its low population size, and the highest genetic uniformity is found in Torreblanca and Cullera populations.

Distributions of pairwise differences among AFLP patterns of *L. dufourii* populations are shown in Fig. 4-11. The expected distributions under a constant population size (Watterson, 1975) and under the population expansion model (Rogers & Harpending, 1992) are also represented. Table 4-7 shows the relevant parameters and goodness of fit statistics for both models. Most populations do not show departures in the pairwise distributions from the expectation under a constant population size model according to the values of Harpending's raggedness index (Harpending, 1994). Only the Marjal del Moro-3 population departs marginally from the constant population size expectation using a Monte-Carlo approach for testing the null hypothesis, whereas all Marjal del Moro populations show significant differences from the expectation under Rogers and Harpending's population expansion model.

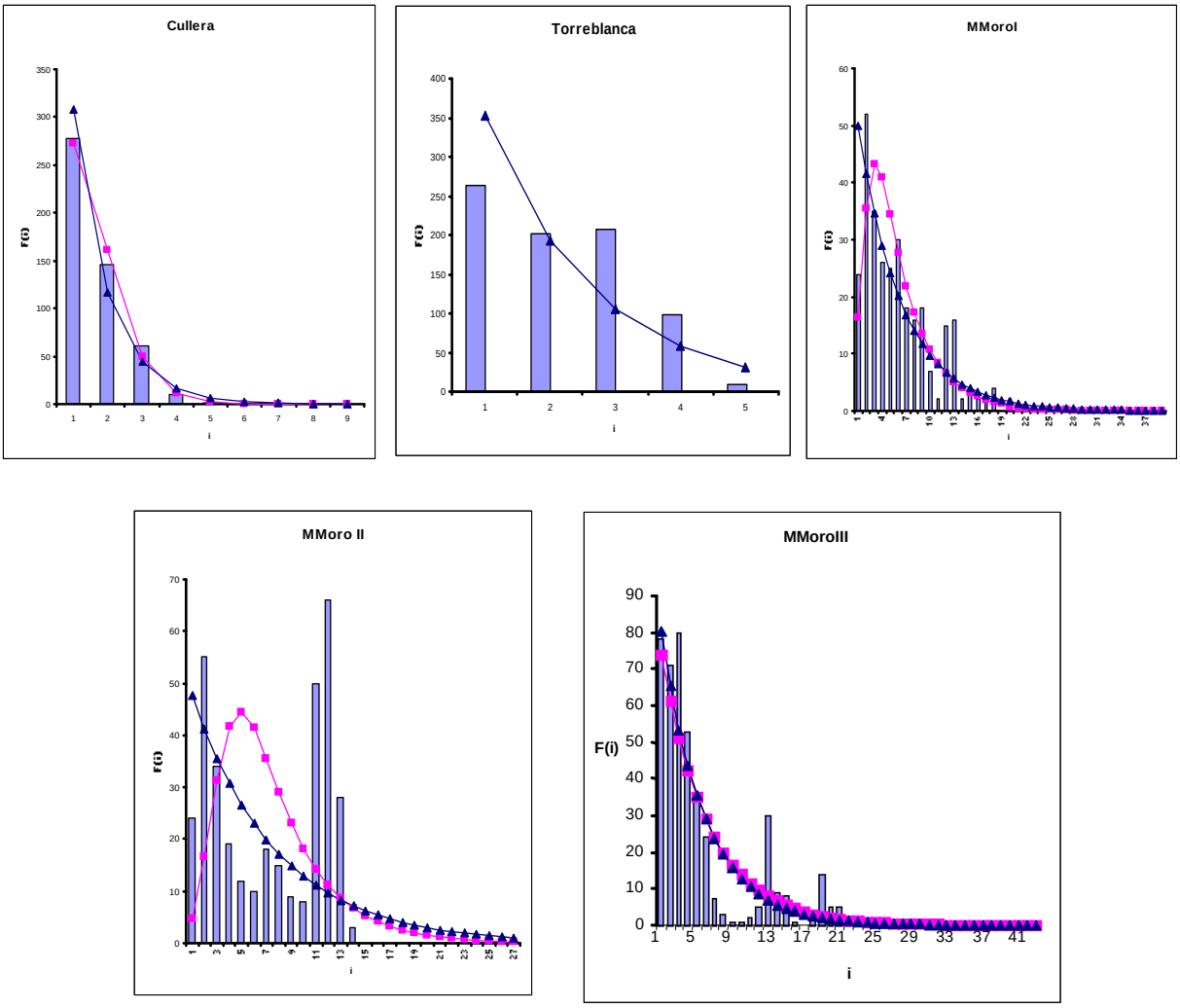


Fig. 4-11 Pairwise difference distribution for comparisons among individuals from *L. dufourii* populations. (a) Cullera; (b) Torreblanca; (c) Marjal del Moro-1; (d) Marjal del Moro-2; (e) Marjal del Moro-3. The continuous lines represent the expected distributions of pairwise differences under constant population size (triangles) and population expansion (squares).

Table 4-7 Summary of analyses of the distribution of pairwise differences among individuals from each *L. dufourii* population. The basic parameters shown are the number of individuals sampled (n), the number of segregating sites (S), the expected pairwise differences (θ), and Harpending's raggedness index (r). The

range shown for each population in column "constant" represents the 95% confidence interval for r obtained after 10000 simulations of a population with the same parameter values as the corresponding *L. dufourii* population. Under column·"expansion", chi-square value for the goodness of fit between the observed and expected value of mismatch counts under the population expansion model of Rogers and Harpending (1992) is shown.

Population	N	S	θ	r	Constant	Expansion
Cullera	32	7	0.147	0.111	0.054 - 1.0	4.119
Torreblanca	40	7	0.000	0.039	0.036 - 0.812	-
El Saler	7	5	1.368	0.283	0.052 - 1.0	3.742
Marjal del Moro 1	25	26	3.643	0.021	0.016 - 0.261	66.217***
Marjal del Moro 2	27	15	3.571	0.048	0.014 - 0.196	625.912***
Marjal del Moro 3	30	31	4.878	0.016	0.017 - 0.267	187.582***

*** P < 0.001

Evolutionary divergence of AFLP patterns: intraspecific phylogeny and population genetic structure.

The nucleotide diversity estimated according to Nei and Li (1979) between pairs of AFLP patterns ranged from 0.0001 to 0.0024. The mean nucleotide diversity across all patterns in the whole sample is 0.0009. The unrooted NJ dendrogram derived from the nucleotide divergence data matrix is shown in Fig. 4-7b. No major differences are observed with the tree derived from the Euclidean distance (Fig. 4-7a). The main clusters and subclusters are coincident. Only relationships within cluster I are slightly different. Similarly, the relationships among populations in the NJ tree obtained from the Excoffier interpopulation distance matrix are identical to those obtained from this genetic approximation (Fig. 4-10).

The AMOVA analysis performed with the nucleotide divergence matrix gave a Φst value of 0.5392. Pairwise genetic differentiation between populations was not significant for Marjal del Moro-3 and El Saler as in the previous section. The alternative measure of population genetic structure when using this evolutionary approach (Nst) gave a value of 0.52, which is very similar to the one obtained with the AMOVA approach. Barlett's heteroscedasticity index was highly significant (P<0.001), and no pairwise comparisons except for those between El Saler and Cullera and Marjal del Moro-3 showed significant intrapopulation variance differences. Results both from AMOVA and HOMOVA analyses using nucleotide divergence are also summarized in Table 4-6.

DISCUSSION

Comparative analysis of AFLP and RAPD markers

AFLP analysis resulted in 20.24% polymorphic fragments (65 from the 252 analyzed), whereas the previous survey with RAPDs, using nearly the same DNA samples, gave a proportion of 26.6% polymorphic bands (44 and 125, respectively). The average diversity per marker locus for AFLPs (0.0247 ± 0.0051) is significantly lower than for RAPDs (0.0763 ± 0.0128). This result is contrary to the study performed on *Limonium cavanillesii* (Chapter 3), for which no variation was detected with RAPDs and

some was present when the same individuals where assayed with AFLPs. Likewise, (Sharma et al. 1996) performed a comparative study with *Lens* and found that both, the average fraction of polymorphic loci and the mean heterogeneity per locus, were usually lower for RAPDs than for AFLPs in several populations of the genus. This implies that comparative results of variability levels among these techniques cannot be generalized, even for closely related species.

The intraspecific phylogeny obtained from the AFLP study revealed that the two principal groups resulting from the NJ tree of the RAPD survey (Fig. 4-2) were also present in this case (Fig. 4-7). It is remarkable that the particular individuals involved in those clusters are also coincident.

We have investigated up to what point RAPDs and AFLPs provide concordant classifications of the same individuals. We have used the index of classification, I_C, (Estoup *et al.*, 1995) by considering the degree of monophyly of the individuals sharing the same RAPD pattern when compared to their corresponding AFLP pattern, i.e., we were interested in learning how well the classification obtained with one kind of molecular marker is maintained when a different marker is used on the same individuals. The index of classification is defined as:

$$I_C = \frac{d_T - d_G}{d_T - d_M}$$

where d_T, d_G, and d_M are average distances between two OTUs taken from the total sample, from the group under study and from a monophyletic group of the same size than the group being considered, respectively. In this case the distance between two OTUs is defined as the number of OTUs deriving from the most external node linking the two OTUs. This index takes values between 1 (perfect correspondence between two classifications) and -0.5 (when a group includes only OTUs which are paraphyletic to all others).

There were 44 different groups in the RAPD study that could be compared with the corresponding AFLP patterns. The average value for *Ic* was 0.893 when all the groups were considered. However, only in 16 of those 44 groups there is more than one individual, and those are the groups for which the comparison is meaningful. The average value of I_C for them is 0.685. Although we lack a statistical test for the significance of this value, it seems clear that the correspondence between the classification of the same *L. dufourii* individuals with RAPDs and AFLPs is higher than the expected under pure chance, but it is far from being a perfect one.

A combined analysis of AFLP and RAPD data has been performed from the 159 *L. dufourii* individuals coincident on both studies (data not shown). The level of resolution obtained with the 84 polymorphic markers derived from this combined study was higher than in the separate analyses, as reflected in the 95 different phenotypes obtained. In the NJ tree obtained using the Euclidean distance (Fig. 4-12) 4 clusters are observed, that include the same individuals of the four principal groups derived from both previous studies (group A from Marjal del Moro, group B from Marjal del Moro and El Saler, Cullera, and Torreblanca), but they are better resolved here. It is remarkable that all individuals noted above as dispersed in the RAPD tree, form here a monophyletic subcluster within group B. Also individuals from Cullera that were dispersed in the AFLP tree, are grouped in a monophyletic cluster, as occurred in the RAPD study. Individuals that occupy an intermediate position between group A and cluster II in the AFLP MSN are the same in the combined tree, and coincide also with intermediate patterns in the RAPD MSN.

The application of a range of statistical techniques for the analysis of the intraspecific relationships among the patterns obtained allows a better understanding of the biological phenomenon under study. This has been particularly clear in this study. A mere cluster analysis is an inadequate description of the

relationships among the different patterns and populations, especially because it seems to be general that phenotypes that act as connectors among main clusters in the PCO and MSN do not form monophyletic groups in the NJ tree. For instance, RAPD patterns 23, 28, 31, and 32 are not clearly included in any of the clusters in the neighbor-joining dendrogram (Fig. 4-2), but the minimum spanning network (Fig. 4-3) places all them together in a central place, where they act as connectors among patterns belonging to the four main groups: Cullera, Torreblanca and groups A and B. Likewise, in the AFLP study, those patterns that were not clearly grouped in the NJ tree (1-7, Fig. 4-7) form a compact group in the PCO and the MSN (Figs. 4-8 and 4-9). From this result, it can be argued that a simple bifurcating tree could be a too strict representation of the evolutionary relationships at the within species level. Usually, alternative connections among patterns exist, which explain the differences observed at the within-cluster relationships depending on the method or distance used (see also Chapter 3). Besides, from multifurcating representations it is even possible to hypothesize which patterns have acted as ancestors. Patterns that are not fixed to any population (e.g. RAPD patterns 2, 10, or 23; or AFLP patterns 1, 23 or 9; Figs. 4-3 and 4-8, respectively) usually act as the hub of star-like clusters, meaning that they are connected to several other group-specific phenotypes. This suggests that recurrent evolution from these phenotypes has taken place (Templeton *et al.*, 1992; Excoffier & Smouse, 1994). On the other hand, principal coordinates analysis allows a quantification of the diversity responsible for the appearance of the aforementioned groups. In general, both in AFLP and RAPD analysis, the highest percentage of variation is explained by the separation of the patterns belonging to group A from the rest. Usually, the second and third dimensions explain the remaining variability by the separation of the patterns from cluster II in the other subgroups.

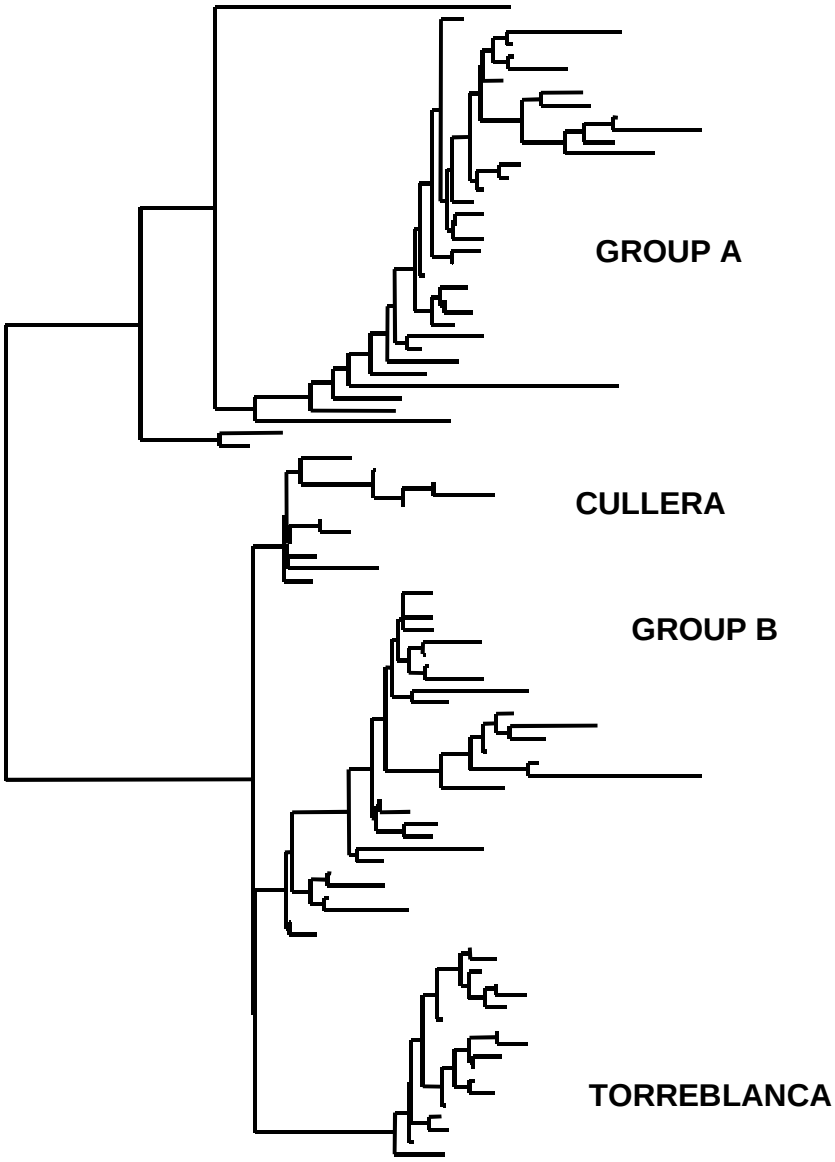


Fig. 4-12 Neighbor-joining tree derived from the Euclidean distance matrix of the combined AFLP and RAPD data matrix.

These four main groups encountered in *L. dufourii* (Cullera, Torreblanca, group A and group B), do not match with the observed population subdivision except for Cullera and Torreblanca, the two most extreme populations. This situation is unexpected for several reasons. First, the Cullera and El Saler populations are geographically much closer to each other than the El Saler population is to the 3 Marjal del Moro populations, but all the individuals from El Saler are included in group B patterns. This is unlikely to have been originated recently by gene flow given the low seed dispersal capacity of this species. Furthermore, the presence of a large urban area, Valencia, between El Saler and Marjal del Moro, would further contribute to reduce gene flow among these populations, at least in the last 2000 years. Second, phenotypes from Marjal del Moro populations are dispersed in two separate groups, A and B, hence resulting in large intrapopulation divergence. These patterns do not conform to any general structure. Furthermore, there is no correlation among geographic and genetic distances among populations (Table 4-

3). Given that there are no known forces currently acting on these populations that can explain these results, it seems plausible that historical processes in these populations have to be invoked.

The observed genetic diversity pattern can be explained if the 3 Marjal del Moro populations were united in the past and group B patterns arose first. These individuals dispersed, colonizing suitable habitats north- and southwards. The currently remaining border populations, Torreblanca and Cullera, present patterns related to those of group B. A second group of patterns appeared in the Marjal del Moro population, either derived from the existing one or from another hybridization event. In any case, this happened after the start of divergence of group B, which explains the younger age of group A and their absence from other populations. Reduction in population size led to the subdivision of the Marjal del Moro population into 3 subpopulations, and next genetic drift occurred, resulting in the observed structuring of genetic diversity. This would explain the presence of individuals with patterns from both groups A and B in the 3 current populations. Reduction of the El Saler populations has led to its almost complete extinction, while in Cullera, only a few individuals remain. The pairwise difference distribution shows that all these populations were in equilibrium in the past. The current reduction of suitable habitats for the species has caused the observed decline in the survival populations, as well as the extinction of some others (see Introduction).

However, there are still questions that cannot be answered by using fingerprinting methods such as RAPDs and AFLPs. In our study of *L. dufourii* populations there is one important and yet unanswered question: How did the different patterns assigned to groups A and B arise and have been maintained in the Marjal del Moro populations? Did they result from one single hybridization event and later differentiation or are at least two separate hybridizations necessary to explain the observed patterns? We think that only with more detailed information at the nucleotide level from these variants and other *Limonium* species will it be possible to provide answers for this questions.

Advantages of AFLP over other PCR-based DNA fingerprinting techniques

Since their development early this decade (Welsh & McClelland, 1990; Williams *et al.*, 1990), RAPDs have been used in many different applications in evolutionary biology, ranging from genomic mapping (Al-Janabi *et al.*, 1993; Rieseberg *et al.*, 1993; Sobral & Honeycutt, 1993), to the characterization of genetic resources in plants (Kresovich *et al.*, 1992; Orozco-Castillo *et al.*, 1994), or the identification of species, subspecies, hybrids, clones, or genotypes (Benito *et al.*, 1993; Cenis *et al.*, 1993; Transue *et al.*, 1994), among others. However, their utilization in population genetic studies has been hampered by the severe restrictions posed by some of the assumptions underlying the methods proposed for their analysis (Clark & Lanigan, 1993; Lynch & Milligan, 1994). Nevertheless, some of these difficulties can be overcome by the use of new methods (Stewart & Excoffier, 1996; cf. Martínez-Torres *et al.*, 1997). In spite of all the remaining drawbacks and considerations, the existence of other restrictions often encountered in the survey of natural populations still makes the use of RAPDs advisable when there is only a small amount of biological material for analysis, closely related individuals are being compared, little (or none at all) sequence information is known of the particular species, and general evaluations of variability at, for

instance, the population level are sought. All these conditions apply also for AFLP markers. However, this technique has been reported as highly reproducible (Vos *et al.*, 1995; Janssen *et al.*, 1996). Our pilot experiment on AFLP confirms the low sensitivity of the method to changes in DNA concentrations, contrarily to the other fingerprinting methods (Ellsworth *et al.*, 1993;(Caetano-Anollés G. et al. 1992); Micheli *et al.*, 1994); see also Introduction). This advantage of AFLP overcomes some limits of PCR-based DNA fingerprinting markers, especially with regards to its potential use among laboratories.

As already pointed out in Chapter 3, the flexibility of the AFLP technique (through the use of different enzymes and/or selective nucleotides) makes it really superior to other methods because of the large potential number of markers that can be detected simultaneously, especially after its evolution towards full automation. This feature becomes an advantage over other DNA fingerprinting methods in studies where the characterization of a large number of markers is desirable, such as in population genetics or genome mapping.

Finally, attempts to estimate evolutionary divergence relatedness on DNA fingerprinting data have been performed before (Clark & Lanigan, 1993). However, the similarity between AFLPs and RFLP markers makes it possible to apply Nei and Li's approach with good confidence, because the required assumptions are better satisfied here (Nei, 1987). To our knowledge this is the first time that an evolutionary approach has been used for AFLP data analysis at either intra- or interspecific levels. Even though we did not obtain appreciable differences on the relationships derived from this evolutionary genetic distance and the other phenetic distances, by using an evolutionary approach we can be more confident about the inferred phylogenetic relationships. Another advantage of this approach is that an estimate of nucleotide diversity provides a measure that makes the comparison among different species feasible. Infraspecific molecular studies in plant species in which nucleotide divergence estimates have been used are strongly biased towards RFLPs (Böhle U.-R. et al. 1994); (Soltis et al. 1992) reported intraspecific nucleotide divergence values in a revision of several cpDNA studies ranging from 0.000 to 0.003. Milligan (1991) reported levels of intraspecific cpDNA divergence commonly ranging from 0.0003 to 0.0015. Typical values for congeneric species range from 0.0004 (for very closely related species) to 0.0214 (for species of the same section of a genus), or even 0.0805 (for species of different sections) (Soltis *et al.*, 1992; Milligan, 1991; (Wang, Szmidt, 1993). Besides, our phylogenetic study using also RFLPs on cpDNA as markers with some *Limonium* species of the same section as *L. dufourii* (Chapter 2) shows nucleotide diversity values ranging from 0.0048 to 0.0347. Hence, nucleotide levels of diversity in *L. dufourii* (0.000-0.0024) indicate that the divergence values among individuals from groups A and B are within the intraspecific levels expected for other species of the same genus.

The conservation of *Limonium dufourii*

The original aim of this work was to provide some insight into the population biology from the genetic analysis of *Limonium dufourii* to possibly guide conservation measures. There has been much debate about the uses and virtues of Population Genetics in conservation [see for instance Avise and Hamrick (1996) and references therein]. The discussion is especially pertinent in the case of non-sexually reproducing organisms. These organisms will eventually be composed of a series of more or less divergent clones, whose fate will be determined either by random forces or by the action of natural selection, but without any possibility of increasing their survival potential by means of establishing new genetic combinations through recombination and segregation. Hence, conservation of genes can only be achieved through conservation of whole genotypes, and this is usually unaffordable. The logic in the reasoning is that

preservation of genes and their combinations will keep adaptability potential for future generations, hence decreasing the risk of extinction. However, this vision has been widely criticized (Lande, 1988; Milligan *et al.*, 1994; Lynch, 1996) and we agree that it is possibly not the best way of using Population Genetics in conservation. The identification of those populations that hold the most genetic variability and their subsequent preservation could be a solution under economic shortage (but see later).

Results from the present study can be used in devising conservation measures for this species. *L. dufourii* is a highly endangered species in which only two very close and localized populations (Marjal del Moro2 and 3) seem to be thriving, whereas the rest are at the verge of extinction. Information on the population genetic differentiation and distribution of variability is important for directing preservation measures, which may include the reintroduction of species in suitable areas. Given the high level of population subdivision encountered among *L. dufourii* populations, transplantation of individuals or seeds from one population to another should probably be avoided, except in the case of El Saler population, which is a clear candidate for reintroduction, because of its extremely small population size. The close phylogenetic proximity to Marjal del Moro populations would make it advisable to reintroduce seeds from group B Marjal del Moro individuals. However, this rule of thumb of avoiding artificial gene flow with high population structure is based on the assumption that genotypes are adapted to local environments. Ewens-Watterson tests of selective neutrality on the frequency distribution of RAPD patterns (data not shown) failed to reject the null hypothesis in 5 of the 6 populations studied, thus indicating that the current RAPD patterns frequency distribution does not seem to be due to adaptation to local conditions. It remains to be evaluated whether this is the case for other loci, which are or not selectively neutral as it is assumed for these kind of markers, in order to sustain this rule. There is a risk in using neutral genetic markers for making inferences about adaptive processes, unless selection is still acting and/or there is a very close linkage between the selected locus and the neutral marker. In any case, if asexual taxa do represent general-purpose genotypes (Bierzychudek, 1989) then variation in neutral markers is not an indication of any adaptive process but these markers become very useful for the study of the evolutionary history of the corresponding populations and also for the monitoring of different conservation measures.

Not all *L. dufourii* populations currently hold the same amount of variability (Tables 4-5 and 4-6). When this is an issue for choosing what natural populations to preserve, the analyses performed in this case are especially relevant. Populations can be ordered in terms of “phylogenetic variability”, as both frequencies and ancestrality relationships are used in evaluating within population diversity by means of the analysis of molecular variance. These results should not be taken as an absolute guidance for preserving some populations and disregarding others. Instead, they provide one more piece of information for taking a decision, but other considerations should be taken into account. For instance, the Cullera and Torreblanca populations are well differentiated from the rest and, although they do not rate as high in the variability scale as the Marjal del Moro populations, they should also be preserved due to their distinctiveness. So, Cullera is the only population whose individuals live on rocky soil, rather than in marshes as the rest. What the analysis is really worth for is in helping to decide, in this case, on which Marjal del Moro population should conservation measures be implemented first, or, alternatively, what sampling strategy should be used to setup an *ex situ* germplasm collection to complement other *in situ* preservation measures.

We believe that *L. dufourii* should be catalogued as endangered according to the IUCN categories of threat.

5

DISCUSIÓN GENERAL Y PERSPECTIVAS

DISCUSIÓN GENERAL Y PERSPECTIVAS

Los bajos niveles de divergencia nucleotídica encontrados entre la mayoría de las especies de la sección Limonium, tanto en el genoma cloroplástico como en la región ITS del rDNA nuclear, son consistentes con un origen reciente para este grupo de especies. Quizás en este tipo de géneros en los que la proliferación de (micro)especies es tan común, al estar presentes los principales mecanismos de especiación de los vegetales (Dobzhansky *et al.,* 1979) empezar por la indagación de los niveles de variabilidad, tanto a nivel intra como interespecífico y las relaciones evolutivas entre especies altamente relacionadas ha sido una buena aproximación. Para poder llegar a determinar con mayor exactitud las relaciones entre las especies parentales originales y las formas apomícticas derivadas del complejo agámico formado por estas especies de la sección Limonium, deberá intensificarse el muestreo, tanto en especies sexuales como asexuales. En este sentido los marcadores de DNA citoplasmático y nuclear, usados en conjunto, han demostrado ser unas herramientas muy buenas no sólo en el caso del grupo que nos ocupa (Palacios *et al.*, en preparación A), sino en otras muchas especies en las que la hibridación y la poliploidía son procesos comunes (Mertens, 1993 y ejemplos allí citados). En especies sexuales el DNA nuclear, al tener herencia biparental, permite la identificación de los ancestros de los híbridos, mientras que el DNA citoplasmático, al heredarse uniparentalmente, puede utilizarse para inferir la dirección de la hibridación y para identificar linajes clónicos. Por otro lado, los estudios que se realicen en un futuro para investigar las relaciones evolutivas del género deberían incluir especies monogenómicas (2n=16, 2n=18). En cualquier caso, una vez estudiados más profundamente los procesos de tipo microevolutivo, la elucidación de aspectos macroevolutivos será más factible. Podemos por lo tanto concluir que la resolución de la Sistemática del género *Limonium* estará supeditada a la obtención de datos micro y macroevolutivos en la cantidad y calidad suficientes como para su comprensión desde el punto de vista filogenético.

Con respecto a los estudios genéticos llevados a cabo con marcadores moleculares para determinar los niveles de variabilidad intraespecífica y la estructura genético-poblacional de especies con *a priori* escasa variabilidad genética, raras o amenazadas, y para su aplicación en la conservación de las mismas, podemos afirmar que los marcadores tipo DNA *fingerprinting* basados en la PCR poseen una serie de características que les convierten en potencialmente ideales para ello. Entre estos marcadores los AFLPs parecen ser los más efectivos (Palacios, en revisión; Palacios, en preparación A) al ser una técnica automatizada y más reproducible. Pero el método de los RAPDs no es en absoluto desdeñable: estos marcadores han resultado ser igualmente fiables siempre que se tomen las precauciones adecuadas (Palacios & González-Candelas, 1997), más aún cuando en condiciones de escasez económica podría ser el único método practicable. Sin embargo, por el carácter dominante de estos marcadores, el tratamiento analítico que requieren es a menudo diferente al de los marcadores codominantes (González-Candelas & Palacios, 1997). En este sentido sería de gran interés contrastar los resultados obtenidos con los de otros marcadores pero

codominantes, como los alozimas o los microsatélites (SSRs). De esta manera se podría determinar hasta que punto la disminución en la precisión asociada a la dominancia es relevante en las conclusiones finalmente extraídas de los análisis genéticos con este tipo de marcadores, lo cual podría contrarrestar las ya mencionadas importantes ventajas que poseen este tipo de marcadores.

Concretando, el estudio con este tipo de marcadores moleculares en las especies más amenazadas de extinción de nuestra Comunidad, *L. dufourii* y *L. cavanillesii*, han resultado ser esenciales para poder diseñar planes efectivos de recuperación y gestión de estas especies (Palacios & González-Candelas, 1997 A y B; Palacios *et al.*, en revisión; Palacios *et al.*, en preparación B). Por supuesto otro tipo de estudios sobre la autoecología y demografía de las poblaciones son aproximaciones alternativas que han demostrado ser igualmente efectivos en la conservación de este tipo de especies (Schemske *et al.,* 1994), aunque nosotros abogamos más por una complementariedad entre las distintas disciplinas. En este sentido, el desarrollo de métodos analíticos y empíricos en el campo de la Sistemática Molecular está cumpliendo con las predicciones hechas por Milligan *et al.* (1994) sobre el interés de estas herramientas en la Biología de la Conservación, porque a partir de ellas no sólo hemos podido determinar las frecuencias y patrones de diversidad, sino que también se ha podido conseguir información de la historia demográfica de las poblaciones y de su filobiogeografía, precisamente por que los patrones de variación de estos marcadores están influidos por esos factores que se ha reconocido son tan importantes en Biología de la Conservación. Por otro lado, es importante reseñar que la variabilidad estudiada se ha analizado en loci predominantemente neutrales, que podrían no estar correlacionados con la capacidad de supervivencia de las poblaciones, por lo que las decisiones tomadas en la gestión de las poblaciones existentes, o la reintroducción de posibles poblaciones y su seguimiento, no pueden basarse en la adaptabilidad de los genotipos observados. Estudios futuros deberían incluir el análisis de la variación genética cuantitativa, ya que ésta permite un acceso más directo a las propiedades evolutivas de los caracteres morfológicos y del comportamiento, los cuales son ecológicamente muy importantes por estar a menudo directamente relacionados con la supervivencia y/o la eficacia de una población (Lynch, 1996), y, por lo tanto, nos darán una perspectiva más completa del *status* genético de la misma, para de esta forma poder adoptar decisiones más informadas sobre la conservación de la especie. Sin embargo, hay que tener en cuenta que este tipo de estudios requiere una mayor cantidad de tiempo y mayores tamaños muestrales, por lo que, en el caso de especies en las que es urgente tomar decisiones para su gestión, han de ser relegados a un segundo plano, para consideraciones a más largo plazo. Un intento de integrar ambos tipos de técnicas para estudios en conservación ya ha sido planteado por Storfer (1996).

Dada la considerable disminución en el número de especies que se está produciendo en la actualidad, cualquier esfuerzo que se haga para cambiar el destino de aunque sólo sea una de ellas, no será en vano. Sin embargo, sólo hasta que no se produzca una reversión de la principal causa de esta crisis de extinción, que es la destrucción del hábitat por el ser

humano, los esfuerzos conservacionistas no pasarán de meramente contrarrestar esta cadena de destrucción.

6

CONCLUSIONS/CONCLUSIONES

CONCLUSIONS

The results obtained through the study of intra and interspecific variability in the different *Limonium* species by using empirical and conceptual tools from the Population Genetics and Phylogenetics, has allowed us to draw several conclusions, not only from micro and macroevolutionary patterns and processes of these species, but also for the management of the populations of the most endangered species in the Comunidad Valenciana, *Limonium cavanillesii* and *L. dufourii*.

- It has been demonstrated the need of an adequate sampling strategy to detect intraspecific variability in molecular phylogenetic studies, especially when there is evidence of hybridization and/or introgression, and cytoplasmic DNA or repetitive gene families are used as markers.

- The different rDNA and cpDNA types detected in *L. delicatulum, L. interjectum* and *L. furfuraceum* are paraphyletic, with divergence levels similar to those of other species from section Limonium. This result reinforces the introgression and/or hybridization hypotheses for the origin of these species.

- In all phylogenies obtained *L. narbonense* and *L. vulgare*, from subsection Genuinae, form a sister clade (monophyletic group) to the rest of the species from section Limonium, with levels of divergence similar or even higher than those of species from other sections of the genus. Consequently, the actual placement of *L. narbonense* and *L. vulgare* within section Limonium is questioned.

- The remaining species from section Limonium always form a monophyletic group in all the topologies resulting from phylogenetic analyses. The low levels of divergence encountered at this level imply a recent origin of these species. In general, there is a lack of resolution within this clade that could be due to the reticular evolution of these highly related species. This fact is confirmed by the lack of concordance between cpDNA and ITS phylogenies.

- It has been demonstrated the usefulness of comparing the results obtained from cytoplasmic and nuclear markers. Likewise, the use of different methods of phylogenetic analysis has demonstrated to be very helpful to interpret the results obtained and to extract more reliable conclusions about the evolution of these genomes.

- Besides, in those species in which a more exhaustive analysis of intraspecific variability has been performed, the use of appropriate analytical methods to determine the intraspecific relationships and the population structure has been essential to extract as much information as possible from the molecular data, in spite of the restrictions imposed by

dominant markers. It is noteworthy that dicotomic trees are very strict representations of the evolutionary relationships among the phenotypes or populations studied. Other type of representations, such as MSN (minimum spanning networks), have demonstrated to be more adequate, as it is possible to extract more information from the same data.

- The low levels of variability encountered in the species *L. cavanillesii* could be due to its obligate apomictic reproductive system. However, only one population with very low numbers has been discovered in nature, which implies that it has passed through a severe bottleneck, after which there has been no chance for mutation to restore the levels of genetic variability expected in a population that could have been in equilibrium in the past, as pointed by the analysis of nucleon diversity.

- Conservation of this unique *L. cavanillesii* population must be an urgent measure, as its low genetic variability makes it very sensible to environmental changes. The best strategy might be the protection of the area where it is currently established, declared as a small reserve, and the re-establishment of new self-sustaining populations in suitable habitats. Micropropagation of a representative sample of the variability encountered would be the most appropriate *ex situ* conservation measure. Finally, we believe that *L. cavanillesii* should be catalogued as critically endangered according to the IUCN categories of threat.

- The study of intraspecific variability in the species *L. dufourii* has demonstrated that there are two divergent phenotype groups (A and B) within Marjal del Moro populations. Moreover, phenotypes that belong to other populations present a higher similarity to those from group B, which points to a common origin of these phenotypes and its posterior dispersion, colonizing suitable habitats north and southwards. But divergence levels among phenotypes from groups A and B are within those expected for intraspecific studies.

- The high level of population structuring within *L. dufourii* makes it advisable to prevent transplantation of individuals or seeds from one population to another, with the possible exception of El Saler population, with only 7 individuals, in which reintroduction of new variability from group B individuals of Marjal del Moro populations is recommendable. *In situ* measures of conservation for the different populations should be complemented by *ex situ* measures, which must take into account the diversity and population subdivision encountered in the species. *L. dufourii* should be catalogued as endangered according to the IUCN categories of threat.

- Comparative results of detectable variability levels with different molecular markers, as AFLPs and RAPDs, can not be generalized even for evolutively close species. However, when PCR based DNA fingerprinting methods are the best choice in a particular study, AFLP method represents the most advantageous technique, if the necessary equipment and the economic resources are available.

CONCLUSIONES

Los resultados obtenidos a través del estudio de la variabilidad genética intra e interespecífica en distintas especies del género *Limonium* por medio del uso de algunas de las herramientas conceptuales y empíricas de la Genética de Poblaciones y la Filogenética, nos han permitido extraer una serie de conclusiones, no sólo sobre los patrones y procesos micro y macroevolutivos de estas especies, sino también acerca de las medidas a adoptar para la gestión de las poblaciones de dos de las especies vegetales más amenazadas de la Comunidad Valenciana, *Limonium cavanillesii* y *L. dufourii.*

- Se ha demostrado la necesidad de una adecuada estrategia de muestreo, que contemple la posibilidad de detectar variabilidad a nivel intraespecifico, en estudios filogenéticos moleculares, especialmente cuando existe evidencia de hibridación y/o introgresión, y se usen marcadores tanto de DNA citoplasmático como de familias génicas repetitivas.

- Los diferentes tipos de rDNA y cpDNA intraespecíficos detectados en *L. delicatulum, L. interjectum* y *L. furfuraceum* han resultado ser parafiléticos, con niveles de divergencia entre ellos similares a los detectados entre otras especies de la sección Limonium. Este resultado refuerza las hipótesis que postulan sucesos de hibridación o introgresión para explicar el origen de estos taxones.

- Las especies *L. narbonense y L. vulgare*, pertenecientes a la subsección Genuinae, forman un clado (grupo monofilético) hermano al resto de especies de la sección Limonium en todas las filogenias obtenidas, presentando niveles de divergencia similares o incluso mayores a los de especies clasificadas bajo otras secciones del género. Como consecuencia de estos resultados cuestionamos el actual emplazamiento de *L. narbonense* y *L. vulgare* dentro de la sección Limonium.

- El resto de especies de la sección Limonium siempre forman un grupo monofilético en todas las topologías resultantes del análisis filogenético. Los bajos niveles de divergencia encontrados a este nivel apuntan hacia un origen reciente de estas especies. En general se ha encontrado una falta de resolución dentro de este clado, que puede ser debida a la evolución reticular entre estas especies tan altamente relacionadas. Este hecho viene confirmado por la falta de concordancia entre las filogenias del cpDNA y de los ITS.

- Ha quedado demostrada también la utilidad del contraste de los resultados filogenéticos con marcadores citoplasmáticos y nucleares. Del mismo modo, el empleo de distintos métodos de análisis filogenético ha demostrado ser de gran ayuda para la interpretación de los resultados y poder así llegar a conclusiones más fiables en cuanto a la evolución de estos genomas.

- Asimismo, en las especies en que se ha hecho un análisis exhaustivo de la variabilidad genética intraespecífica, el uso de métodos analíticos adecuados para determinar las relaciones intraespecíficas y la estructuración poblacional ha sido esencial para poder extraer la mayor información posible presente en los datos moleculares, a pesar de las restricciones impuestas por haber empleado marcadores dominantes. En este sentido cabe destacar que los árboles dicotómicos son representaciones demasiado estrictas de las relaciones evolutivas tanto entre los fenotipos observados como entre las poblaciones objeto de estudio. Otro tipo de representaciones como las MSN (redes de expansión mínima) han demostrado ser mucho más adecuadas, ya que a partir de ellas se puede extraer más información partiendo de los mismos datos.

- Los bajos niveles de variabilidad genética encontrados en la especie *L. cavanillesii* podrían deberse a su sistema de reproducción apomíctico obligado. Sin embargo, el hecho de haberse encontrado una única población en la naturaleza con muy pocos individuos apunta a que la misma ha pasado por un cuello de botella muy severo en tiempos recientes, después del cual la mutación no ha podido restaurar los niveles de variabilidad genética esperables en una población que, según indica el análisis de la diversidad de los variantes encontrados, parecía estar en equilibrio en el pasado.

- La conservación de la única población natural remanente de *L. cavanillesii* debe ser priorizada, ya que su escasa diversidad genética y escaso número de individuos la hace muy sensible a los cambios ambientales. La mejor estrategia sería la preservación y protección del área donde está ubicada a través de la creación de una microrreserva. Además, sería apropiado el reestablecimiento de nuevas poblaciones de la especie en áreas adecuadas para asegurar su supervivencia futura. Por otro lado, la micropropagación de una muestra representativa de los variantes encontrados sería la medida de conservación *ex situ* más inmediata en este caso. Por último, proponemos que *L. cavanillesii* sea catalogada como en peligro crítico según las categorías de la IUCN.

- El estudio de la variabilidad genética intraespecífica en *L. dufourii* ha demostrado la presencia de dos grupos (A y B) de fenotipos divergentes dentro de las poblaciones de la Marjal del Moro, así como la mayor similitud de los fenotipos presentes en el resto de poblaciones con los fenotipos del grupo B, lo que es indicativo de un origen común de los mismos, y su dispersión posterior al norte y sur de nuestras costas. Pero cabe reseñar también que los niveles de divergencia entre los fenotipos de los grupos A y B están dentro de lo esperable a nivel intraespecífico.

- Los elevados niveles de subestructuración poblacional encontrados en *L. dufourii* nos indican que el transplante de individuos o semillas de una población a otra debería evitarse. Sólo en el caso de la población de El Saler, reducida a 7 individuos en la actualidad, es recomendable la reintroducción de nuevos variantes procedentes del grupo B de las poblaciones de la Marjal del Moro. Las medidas de conservación *in situ* de las distintas poblaciones de la especie podrían complementarse con otras de conservación *ex situ* que deben tener en cuenta los niveles de diversidad y estructuración de las distintas poblaciones de la especie. *L. dufourii* debería ser catalogada como en peligro según las categorías de la IUCN.

- Los resultados comparativos de los niveles de variabilidad detectados por el uso de distintos marcadores, como en nuestro caso AFLPs y RAPDs, no pueden generalizarse ni siquiera en el caso de especies cercanas evolutivamente. Sin embargo, cuando estos métodos tipo DNA *fingerprinting* basados en la PCR sean elegidos como los más apropiados en un estudio genético determinado, la técnica de AFLPs presenta una serie de características que la hacen más ventajosa sobre el resto de métodos de este tipo, siempre y cuando se disponga de los medios materiales y económicos para su utilización.

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APPENDIXES

Appendix 1

We have followed Stewart & Excoffier (1996) approach for deriving the corrected distance estimates for RAPD markers when triploid individuals are being compared. We assume that only two alleles (presence = +; absence = -) are present in the *s*-th site with frequencies p_A and q_A in population A, respectively. For any individual at each site, there are four possible genotypes (+/+/+, +/+/-, +/-/-, and -/-/-), and we further assume that the 3 genotypes that present at least one + allele are not distinguishable, and, in consequence, any individual can show only two phenotypes (+ or -) for that site. There are 4 different possible pairwise comparisons among individuals (+/+, +/-, -/+, and -/-), each involving nine (3 x 3) chromosomal comparisons. Assuming Hardy-Weinberg equilibrium for genotype frequencies in populations A and B, the conditional expectations for the inter-individual distances are:

$$E\left(\Delta^2_{sjk}\middle|j=[+],k=[+]\right)=\frac{1}{\left(p_A^3+3p_A^2+3p_A\right)\left(p_B^3+3p_B^2+3p_B\right)}$$

$$E\left(\Delta^2_{sjk}\middle|j=[+],k=[-]\right)=\frac{9p_Aq_B^3\left(p_A^2+2p_Aq_A+q_A^2\right)}{q_B^3\left(p_A^3+3p_A^2q_A+3p_Aq_A^2\right)}=\frac{9}{1+q_A}$$

$$E\left(\Delta^2_{sjk}\middle|j=[-],k=[+]\right)=\frac{9p_Bq_A^3\left(p_B^2+2p_Bq_B+q_B^2\right)}{q_A^3\left(p_B^3+3p_B^2q_B+3p_Bq_B^2\right)}=\frac{9}{1+q_B}$$

$$E\left(\Delta^2_{sjk}\middle|j=[-],k=[-]\right)=0$$

The corresponding values for intra-individual comparisons are

$$E\left(\Delta^2_{sjj}\middle|j=[+]\right)=\frac{36q_A^2\left(p_A^2+q_A^2\right)}{p_A^4+p_A^2q_A^2+q_A^4}$$

$$E\left(\Delta^2_{sjj}\middle|j=[-]\right)=0$$

By using these expectations, and summing up over *m* RAPD sites, we can obtain the expected number of RAPD differences among two individuals

$$E\left(\Delta^2_{jk}\right)=\sum_{s=1}^{m}E\left(\Delta^2_{sjk}\right)$$

The sum of squared deviations needed for the AMOVA can be obtained for all possible pairs of populations by means of

$$SSD(T)=\frac{1}{9N}\sum_{j=1}^{3N}\sum_{k=1}^{3N}E\left(\Delta^2_{jk}\right)$$

Appendix 2

RAPD patterns from 6 populations of *Limonium dufourii* as defined by 33 polymorphic bands (1 = presence, 0 = absence, of the corresponding band).

Pattern																																	
1	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0	0	1	0
2	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0
3	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0
4	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	0	0	0	1	0
5	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0	0	1	0
6	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1
7	1	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	0	0	0	1	0
8	0	0	0	1	0	1	1	1	1	0	1	1	0	0	0	1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	0	1
9	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
10	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
11	0	0	0	1	1	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
12	0	0	0	0	1	1	0	1	1	1	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
13	0	0	0	1	0	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
14	0	0	0	0	1	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
15	0	0	0	1	1	1	0	1	1	1	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
16	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
17	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0
18	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0
19	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0
20	0	0	0	0	0	1	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0
21	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	0	1	0	0	1	0
22	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
23	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0
24	1	1	1	0	0	1	0	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
25	0	1	1	0	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
26	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1
27	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	0	1	0	1	0
28	0	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0
29	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
30	1	1	1	0	0	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
31	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1	0	0	0	0	1	0
32	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0
33	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
34	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
35	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	1	0	1
36	0	0	0	0	0	1	0	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
37	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1
38	1	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
39	0	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	1
40	0	0	0	0	0	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	0
41	1	1	1	0	0	1	0	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	1	1
42	1	1	1	0	0	1	0	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	1	0	1	1	1	1	0	1	1	0	1
43	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1
44	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	0	1	0	1	0

Appendix 3

Frequency distribution of RAPD phenotypes (see Appendix 2) in the 6 populations of *Limonium dufourii*.

Phenotype	Cullera	Torreblanca	El Saler	Marjal del Moro-1	Marjal del Moro-2	Marjal del Moro-3
1	1	0	0	0	0	0
2	26	0	0	0	0	0
3	2	0	0	0	0	0
4	1	0	0	0	0	0
5	1	0	0	0	0	0
6	1	0	0	0	0	0
7	2	0	0	0	0	0
8	0	0	0	1	0	0
9	0	0	0	1	2	0
10	0	0	0	7	6	1
11	0	0	0	4	0	0
12	0	0	0	1	0	0
13	0	0	0	2	0	0
14	0	0	0	6	0	0
15	0	0	0	1	0	0
16	0	0	0	2	0	4
17	0	31	0	0	0	0
18	0	1	0	0	0	0
19	0	5	0	0	0	0
20	0	2	0	0	0	0
21	0	1	0	0	0	0
22	0	0	3	0	0	0
23	0	0	4	0	3	0
24	0	0	0	0	1	0
25	0	0	0	0	1	0
26	0	0	0	0	1	0
27	0	0	0	0	1	1
28	0	0	0	0	2	0
29	0	0	0	0	4	9
30	0	0	0	0	1	0
31	0	0	0	0	3	0
32	0	0	0	0	1	0
33	0	0	0	0	1	0
34	0	0	0	0	1	0
35	0	0	0	0	1	0
36	0	0	0	0	0	1
37	0	0	0	0	0	1
38	0	0	0	0	0	6
39	0	0	0	0	0	1
40	0	0	0	0	0	2
41	0	0	0	0	0	1
42	0	0	0	0	0	1
43	0	0	0	0	0	1
44	0	0	0	0	0	1

Appendix 4

AFLP patterns from 6 populations of *Limonium dufourii* as defined by 51 polymorphic bands (1 = presence, 0 = absence, of the corresponding band).

Pattern																																																			
1	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
2	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
3	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
4	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0
5	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0
6	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
7	1	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
8	1	0	1	1	0	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
9	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
10	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	1
11	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	1	0	0	1	1
12	1	0	1	1	0	1	1	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
13	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
14	1	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	1	0	1	1
15	1	1	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	1	1
16	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
17	1	1	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	1	1
18	1	1	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	0	0	1	1	0	0	1	0	1	1
19	1	1	1	1	0	1	0	1	0	0	1	1	0	1	0	1	1	0	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	0	1	1
20	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
21	1	0	1	1	0	1	0	1	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
22	1	0	1	1	0	1	1	0	0	0	1	1	0	1	0	1	1	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
23	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
24	1	0	1	1	0	0	1	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
25	1	0	1	1	0	0	1	0	0	0	1	0	0	1	1	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
26	1	0	1	1	0	0	1	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
27	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
28	1	0	1	1	1	0	1	0	0	0	1	0	0	1	1	1	0	1	1	1	0	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
29	1	0	1	1	0	0	1	0	0	0	1	0	0	1	1	1	0	1	1	1	0	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
30	1	0	1	1	0	1	1	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
31	1	0	1	1	0	0	1	0	0	0	1	0	0	0	1	1	0	1	1	1	1	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0

32	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
33	1	0	1	1	1	0	1	0	0	0	1	0	1	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
34	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
35	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
36	1	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
37	1	0	1	1	0	0	0	1	0	0	1	0	0	1	0	1	1	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
38	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	1
39	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	1	1	1	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
40	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
41	1	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
42	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
43	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
44	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
45	1	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1	1	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1
46	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	1
47	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	1	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	1
48	1	0	1	1	0	0	1	1	0	0	1	0	0	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	1
49	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	1	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1
50	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
51	1	0	1	0	0	1	1	0	0	0	1	1	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
52	1	1	1	1	0	0	1	1	0	1	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
53	1	1	1	1	0	0	1	1	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
54	1	1	1	1	0	0	1	1	0	0	1	0	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
55	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
56	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1
57	1	0	1	1	0	0	1	1	0	1	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
58	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
59	1	0	1	1	0	0	1	1	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
60	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
61	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
62	1	1	1	1	0	1	1	1	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
63	1	0	1	1	1	0	0	0	0	0	1	1	0	1	0	1	1	1	0	0	1	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	1	1
64	1	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
65	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0

Appendix 5

AFLP phenotypes (see Appendix 4) in the six *Limonium dufourii* populations.

Phenotypes	Cullera	Torreblanca	El Saler	Marjal del Moro-1	Marjal del Moro-2	Marjal del Moro-3
1	24	3	0	0	0	1
2	2	0	0	0	0	0
3	1	0	0	0	0	0
4	1	0	0	0	0	0
5	2	0	0	0	0	0
6	1	0	0	0	0	0
7	1	0	0	0	0	0
8	0	0	0	1	0	0
9	0	0	0	7	0	0
10	0	0	0	1	0	0
11	0	0	0	1	0	0
12	0	0	0	2	0	0
13	0	0	0	1	0	0
14	0	0	0	1	0	0
15	0	0	0	1	0	0
16	0	0	0	1	0	0
17	0	0	0	1	0	0
18	0	0	0	1	0	0
19	0	0	0	1	0	0
20	0	0	0	2	0	0
21	0	0	0	1	0	0
22	0	0	0	1	0	0
23	0	0	4	2	6	13
24	0	23	0	0	0	0
25	0	3	0	0	0	0
26	0	2	0	0	0	0
27	0	2	0	0	0	0
28	0	1	0	0	0	0
29	0	3	0	0	0	0
30	0	1	0	0	0	0
31	0	1	0	0	0	0
32	0	1	0	0	0	0
33	0	0	2	0	0	0
34	0	0	1	0	0	0
35	0	0	0	0	4	0
36	0	0	0	0	1	0
37	0	0	0	0	1	0
38	0	0	0	0	1	0
39	0	0	0	0	2	0
40	0	0	0	0	1	0
41	0	0	0	0	1	0
42	0	0	0	0	2	0
43	0	0	0	0	2	0
44	0	0	0	0	1	0
45	0	0	0	0	1	0
46	0	0	0	0	1	0
47	0	0	0	0	1	0
48	0	0	0	0	1	0
49	0	0	0	0	1	0
50	0	0	0	0	0	1
51	0	0	0	0	0	1
52	0	0	0	0	0	1

53	0	0	0	0	0	1
54	0	0	0	0	0	1
55	0	0	0	0	0	1
56	0	0	0	0	0	1
57	0	0	0	0	0	1
58	0	0	0	0	0	1
59	0	0	0	0	0	1
60	0	0	0	0	0	1
61	0	0	0	0	0	1
62	0	0	0	0	0	1
63	0	0	0	0	0	1
64	0	0	0	0	0	1
65	0	0	0	0	0	1