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INCONGRUENCIA ENTRE SEÑAL MORFOLÓGICA Y MOLECULAR: UNA NUEVA PROPUESTA SISTEMÁTICA PARA EL COMPLEJO GRIMMIACEAE-PTYCHOMITRIACEAE (BRYOPHYTA)

Memoria para optar al grado de Doctor en Biología por la Universidad Autónoma de Madrid presentada por

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A los nadies

I. INTRODUCCIÓN

"La más necesaria de todas las ciencias
es la de olvidar el mal que una vez
se aprendió."
Aristóteles

I. Introducción

Los briófitos (antocerotas, hepáticas y musgos) se definen como plantas terrestres que comparten un ciclo de vida con alternancia de dos generaciones heteromórficas de las que, a diferencia de las plantas vasculares, la fase haploide (gametófito) es la generación con mayor diferenciación morfológica, realiza la fotosíntesis y es perenne, mientras que la diploide (esporófito) es fotosintética por un periodo de tiempo muy corto. El esporófito termina su desarrollo dependiendo de los nutrientes que le aporta el gametófito al que permanece unido. Representan el linaje más antiguo de las plantas terrestres y después de las angiospermas es el más diversificado. Estudios recientes apuntan a que quizá no representen un grupo natural (Garbary, Renzaglia, 1998; Hedderson *et al.*, 1998; Hedderson *et al.*, 1996; Lewis *et al.*, 1997; Mishler *et al.*, 1994), pero comparten características anatómicas y morfológicas y por este motivo se han estudiado conjuntamente. Los musgos (clase Bryopsida), con cerca de 12000 especies (Crosby et al., 1999), es el grupo más numeroso de los tres. A pesar de su pequeño tamaño presentan cierta complejidad estructural y diversidad de formas, aunque los caracteres macroscópicos son limitados y la mayoría de caracteres empleados en taxonomía se basan en variaciones de los distintos tejidos, formas y tamaños celulares. Con el objetivo implícito de reconstruir el árbol de la vida muchos autores han realizado distintas propuestas sistemáticas, que se han visto modificadas por la aportación de nuevos conocimientos en la biología de estos organismos. Desde el primer tratamiento completo de todas las familias de musgos (Brotherus, 1924; Brotherus, 1925) y, en consonancia con las propuestas de Fleischer (1904a; 1904b; 1908; 1923), los caracteres del peristoma se consideran de gran relevancia para definir unidades taxonómicas; visión que se ha mantenido durante este último siglo. El hecho de que los caracteres del peristoma estén relacionados con el proceso de la dispersión de esporas ha supuesto que algunos autores hayan cuestionado su validez para definir grupos naturales, al estar sometidos a una mayor presión

selectiva (Buck, 1991). Sin embargo, muchos caracteres del peristoma siguen siendo de gran utilidad en la sistemática de musgos (Edwards, 1984) Ver figura I.1.

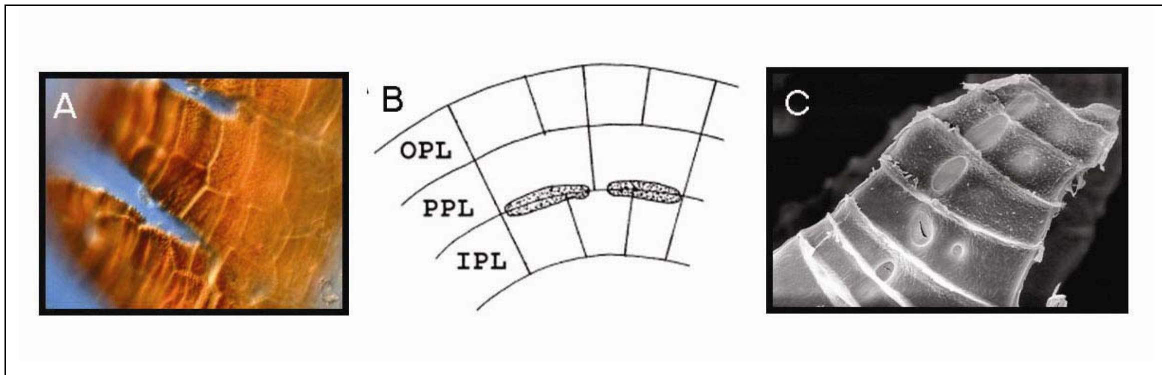

Fig. I.1. Representación esquemática de los dientes del peristoma haplolépido (clase Dicranidae). **A.** La cara interna de los dientes haplolépidos se origina a partir de dos células, por lo que hay tabiques longitudinales. **B.** Esquema de la formación de los dientes de un peristoma haplolépido. **C.** La cara externa se forma a partir de las paredes una única célula, por lo que no hay tabiques longitudinales. OPL (capa peristomial externa, del inglés "Outer Peristome Layer"), PPL (capa peristomial primaria, del inglés "Primary Peristome Layer"), IPL (capa peristomial interna, del inglés "Inner Peristome Layer"). A y C, J. Muñoz; B, tomada de Magombo (2003).

Según estudios recientes, los musgos con peristoma haplolépido parecen conformar un grupo monofilético (Beckert *et al.*, 2001; La Farge *et al.*, 2000; Magombo, 2003), que coincidiría con la subclase Dicranidae (Buck, Goffinet, 2000; Vitt *et al.*, 1998). Dilucidar las relaciones filogenéticas dentro de esa subclase es una tarea que, actualmente, es foco de atención de distintos laboratorios de briología. El orden Grimmiales, y dentro de él, las familias Grimmiaceae y Ptychomitriaceae, que representan su núcleo principal, es uno de los más ricos en número de especies. La Fig. I.2 muestra la posición sistemática del orden Grimmiales dentro del phylum Bryophyta atendiendo a propuestas filogenéticas recientes. Dentro de Grimmiales la interpretación de las relaciones de parentesco ha sido objeto de polémica. Buck & Goffinet (2000) incluyen a las Grimmiaceae, Ptychomitriaceae, Drummondiaceae y Scouleriaceae. Otros autores excluyen a las Drummondiaceae y Scouleriaceae e incluyen a las Seligeriaceae (Goffinet, Buck,

2004; Ochyra *et al.*, 2003; Tsubota *et al.*, 2003). Independientemente de cómo sean tratadas las Seligeraceae, Drummondiaceae y Scouleriaceae, siempre se han considerado como grupos más alejados del complejo de especies formado por Grimmiaceae y Ptychomitriaceae, mientras que la sistemática de estas dos familias ha resultado más controvertida y las relaciones filogenéticas de los géneros que las integran son cuestiones que permanecen abiertas. El objetivo del presente trabajo es presentar una propuesta de clasificación y de relaciones filogenéticas de las familias Grimmiaceae y Ptychomitriaceae, haciendo especial hincapié en el género *Grimmia* Hedw. en el sentido en el que lo han tratado Muñoz & Pando (2000) y Greven (2003).

1. Antecedentes del objeto de estudio

Las relaciones de parentesco de las familias Grimmiaceae y Ptychomitriaceae, así como los géneros que las componen, han sido sujeto de discusión desde la creación de la segunda (Schimper, 1860). Para algunos autores deberían considerarse como una sola (Allen, 2002; Allen, 2005; Brotherus, 1901-1909; Churchill, 1981; Deguchi, 1978; Deguchi, 1987; Dixon, Jameson, 1924; Gradstein *et al.*, 2001; Jones, 1933; Lawton, 1971; Noguchi, 1988; Tsubota *et al.*, 2003), mientras que para otros deberían ser consideradas como familias separadas (Buck, Goffinet, 2000; Gao, Crosby, 2003; Hedderson *et al.*, 2004; Ignatov, Afonina, 1992; Li, Crosby, 2001; Nyholm, 1956; Nyholm, 1960; Ochyra *et al.*, 2003; Scott *et al.*, 1976; Sharp *et al.*, 1994; Smith, 2004; Tsubota *et al.*, 2002). En un tratamiento más audaz, Limpricht (1885-1890) consideró acertado el separar a las Campylosteliaceae del núcleo de Ptychomitriaceae tal y como había sido propuesto por De Notaris (1869), algo que no ha sido aceptado por ningún autor posterior. Si ha habido debate en cuanto a si deberían tratarse como una, dos o tres familias, también ha habido propuestas muy diversas acerca de qué géneros deben incluirse en cada una. Sirva como ejemplo el tratamiento de Churchill (1981), que incluye a

Racomitrium en la subfamilia Ptychomitrioideae (= Ptychomitriaceae) por las características del peristoma.

Como base del presente trabajo hemos considerado la propuesta de Buck and Goffinet (2000), por ser la que cuando lo iniciamos parecía mejor documentada. Estos autores consideraban dos familias por separado, e incluían Campylosteliaceae dentro de Ptychomitriaceae. Según el criterio de estos autores, Ptychomitriaceae incluiría a las especies con filidios sin pelos hialinos y paredes celulares de la lámina rectas, mientras que las especies de Grimmiaceae tendrían filidios con pelos hialinos y paredes celulares de la lámina sinuosas, con la siguiente composición genérica:

Ptychomitriaceae: *Campylostelium* Bruch & Schimp., *Glyphomitrium* Brid., *Ptychomitriopsis* Dixon y *Ptychomitrium* Fürnr.

Grimmiaceae: *Aligrimmia* R.S. Williams, *Coscinodon* Spreng., *Coscinodontella* R.S. Williams, *Dryptodon* Brid., *Grimmia* Hedw., *Indusiella* Broth. & Müll. Hal., *Jaffueliobryum* Thér., *Leucoperichaetium* Magill, *Racomitrium* Brid. y *Schistidium* Bruch & Schimp.

La familia Grimmiaceae incluye tres de los géneros más complejos dentro de la clase Bryopsida: *Grimmia*, *Racomitrium* y *Schistidium*. La falta de una adecuada definición e interpretación de los caracteres que serían de mayor interés taxonómico es una limitación que se suma a esta complejidad. En los últimos tiempos este problema parece en curso de solución al estar los tres géneros en proceso de revisiones taxonómicas que rastrean nuevos caracteres y analizan críticamente los utilizados con anterioridad.

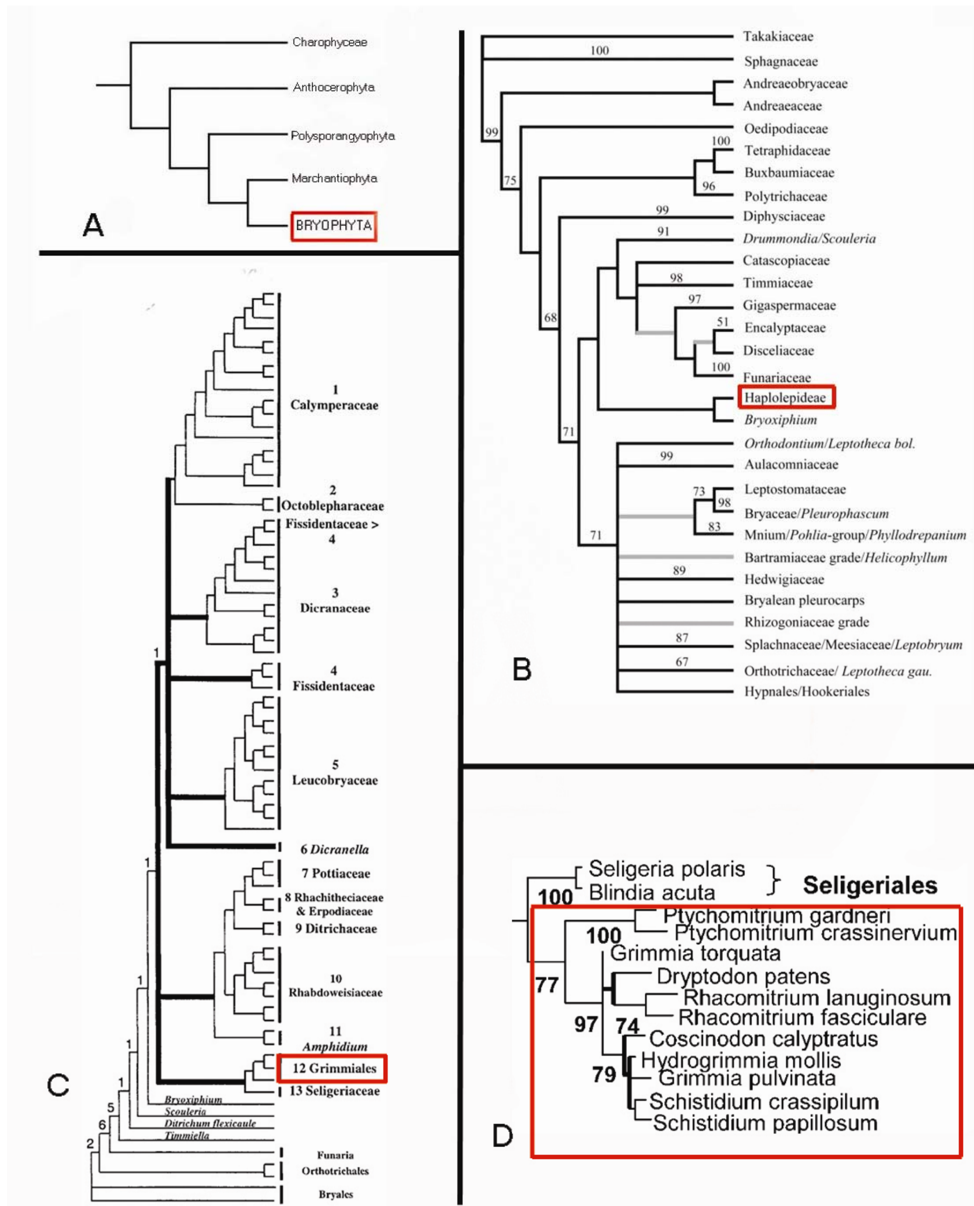

Fig. I.2. Estudios filogenéticos recientes que muestran la posición sistemática de las familias objeto de estudio atendiendo a distintos niveles jerárquicos de clasificación. **A.** Hipótesis de las relaciones filogenéticas de los briófitos (Crowe *et al.*, 1997; Hedderson *et al.*, 1998; Hedderson *et al.*, 1996; Nishiyama, Kato, 1999). **B.** Relaciones filogenéticas del phylum Bryophyta donde se indica la posición de los musgos haplolépidos (Goffinet et al., 2001). **C.** Relaciones filogenéticas de los musgos haplolépidos donde se indica la posición del orden Grimmiales (La Farge et al., 2000). **D.** Relaciones filogenéticas del orden Grimmiales donde se indica la posición de Ptychomitriaceae y Grimmiaceae (Hedderson et al., 2004).

Género *Grimmia*. Sin dejar de lado la sistemática de la familia, nuestro interés se centrará especialmente en las relaciones filogenéticas del género *Grimmia*. El

género consta de alrededor de 80 especies con distribución cosmopolita (Greven, 2003; Muñoz, Pando, 2000). De análisis cladísticos preliminares utilizando caracteres morfológicos se obtienen los siguientes grupos naturales infragenéricos: subgen. *Grimmia*; subgen. *Orthogrimmia*, que incluye las secciones *Donnianae* y *Montanae*; subgen. *Ovales*; y subgen. *Trichophyllae*, que incluye las secciones *Trichophyllae* y *Pulvinatae*.

En el subgénero *Grimmia* se incluyen especies con setas más o menos sigmoides, unidas asimétricamente a la cápsula, y cápsulas ventrudas. El subgénero *Trichophyllae* queda definido por la presencia de setas curvadas y cápsulas estriadas. En los subgéneros *Orthogrimmia* y *Ovales* se incluirían las especies de seta recta, y se diferenciarían porque *Orthogrimmia* tiene filidios aquillados y nervio diferenciado del resto de la lámina, mientras que en *Ovales* los filidios son cóncavos y el nervio está indiferenciado de la lámina. Algunos autores cuestionan la monofilia de *Grimmia* y consideran todos estos subgéneros como géneros independientes (Ochyra et al., 2003).

Independientemente de si consideramos a *Grimmia* como un género monofilético o no, la mayoría de los autores coinciden a grandes rasgos en la identificación de estos grupos (bien a nivel genérico o subgenérico). Además, todas las clasificaciones conceden mayor importancia al esporófito para definir unidades taxonómicas.

Sin embargo, diversos estudios han mostrado cómo la estructura de los esporófitos puede verse modificada ante determinados cambios ambientales, lo que demuestra que son caracteres más lábiles de lo que previamente se pensaba y que, en consecuencia, los grupos definidos por ellos podrían no ser naturales. Esta eventualidad fue apuntada por Mitten (1859), quien comprobó que en los musgos epífitos se producía una reducción de las distintas estructuras del esporófito, y confirmada por Stark (2001), quien observó que en veranos con menor cantidad de lluvia se reducía la longitud de la seta en *Grimmia orbicularis*, y Vanderpoorten et al. (2002), que encontraron una correlación entre hábitat y estructura del

esporófito dentro de las Amblystegiaceae. Nosotros hemos observado personalmente esta reducción en materiales de *Grimmia orbicularis* (expresión *moxleyi*) colectados en el desierto de Mojave a lo largo de una rambla en la que la cantidad de humedad variaba, con una variación correlacionada en la longitud de la seta y por tanto en la longitud en la que la cápsula sobresale por encima de los filidios periqueciales (Fig. I.3. A-F). Relacionado con la importancia relativa de las dos fases vitales en la sistemática de otra de las más importantes familias de musgos, las Pottiaceae, Zander (1993) propuso que la fase de gametófito era la que mostraba las relaciones filogenéticas en esta familia, lo que fue confirmado más tarde utilizando secuencias de ADN (Werner et al., 2002a; Werner et al., 2004; Werner et al., 2005).

A partir de los estudios de Muñoz (1998; 1999) surgió la hipótesis de que los diferentes subgéneros y secciones en los que se divide el género *Grimmia* según la clasificación infragenérica clásica (o genérica, según el rango utilizado para cada taxon por los diferentes autores), basada principalmente en caracteres del esporófito, no estaría reflejando grupos monofiléticos, sino el resultado de procesos de reducción y/o reticulación. En concreto, en el subgénero *Grimmia* se encuentran esporófitos similares a los abortivos observados en *Grimmia orbicularis* (Stark, 2001) y gametófitos característicos de cada uno del resto de subgenéros, lo que llevó a plantear la hipótesis de que en realidad ese grupo no es natural y que son híbridos derivados de táxones pertenecientes a subgéneros diferentes, de los que al menos uno de los progenitores pertenece, invariablemente, al subgénero *Trichophyllae*.

Otra hipótesis que surgió exclusivamente a partir del estudio de caracteres morfológicos es que la clasificación infragenérica debería estar basada en los caracteres del gametófito, ya que de esta forma disminuían las incoherencias observadas.

Ambas hipótesis estaban apoyadas por resultados preliminares obtenidos al realizar análisis cladísticos de los caracteres del gametófito y del esporófito por

separado. Los análisis basados en datos morfológicos no conseguían resolver con suficiente claridad las relaciones de parentesco debido al alto nivel de homoplasia, pero sirvieron para ilustrar posibles fuentes de conflicto entre caracteres con el fin de formular hipótesis de estudio en la sistemática del género.

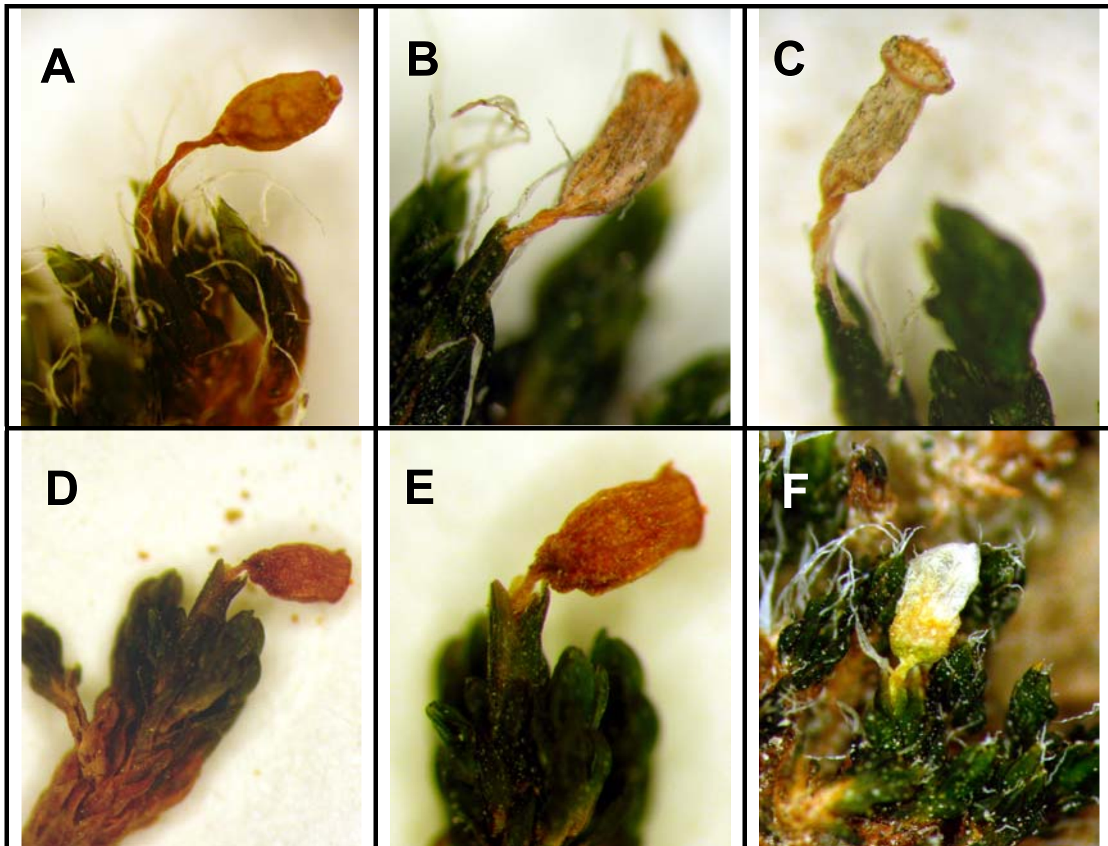

Fig. I.3. Esporófitos de la especie *Grimmia orbicularis* recolectados en el desierto de Mojave (Estados Unidos) en los que se observa la reducción del tamaño de la seta.

Todas estas observaciones basadas en datos morfológicos permitieron plantear hipótesis novedosas, pero no el avanzar más allá de ese punto, por lo que nos planteamos la necesidad de utilizar datos moleculares para contrastar las hipótesis propuestas de hibridación y poliploidización, así como la propuesta de nueva clasificación supraespecífica.

2. Antecedentes de los estudios moleculares

La sistemática molecular pretende reconstruir la historia de un determinado taxon a través de la información contenida en su ADN. Este puede ser abordado hoy día gracias al desarrollo de distintos métodos estadísticos y al desarrollo de las técnicas de secuenciación molecular. La amplificación por PCR permite obtener secuencias nucleotídicas de manera más o menos sencilla, lo que se ha reflejado en un aumento considerable en los estudios de sistemática en todos los grupos de organismos. En el ámbito de la briología este campo se desarrolló más tarde que en el resto de plantas terrestres por dos motivos principales: a) en sus comienzos se requerían grandes cantidades de material para conseguir ADN suficiente, lo que para algunos musgos de vida efímera y de pequeño tamaño suponía la pérdida completa del material de estudio, y b) las dificultades que presentan estos organismos para crecer en cultivo. Actualmente se han desarrollado protocolos de extracción de ADN que permiten obtener la cantidad suficiente a partir de pequeñas porciones de material vegetal de partida. Con el método de Werner et al. (2002b) se puede obtener ADN a partir de un único filidio. Una consecuencia inmediata es que el uso de estas técnicas se ha popularizado en muchos laboratorios de briología. A fecha de hoy existen numerosas filogenias disponibles para los grandes grupos de musgos, a nivel de orden y familia, aunque también es cierto que en otros grupos se carece de estudio alguno. Tal es el caso de las familias objeto de estudio en la presente tesis. Para ilustrar esta situación basta decir que al comenzar este trabajo apenas había disponibles en las bases de datos públicas GenBank y EMBL cuatro secuencias del intrón plastidial *trnL* [*Racomitrium canescens* (Hedw.) Brid., *R. microcarpum* (Hedw.) Brid., *Schistidium apocarpum* (Hedw.) Bruch & Schimp. y *Grimmia pulvinata* (Hedw.) Sm.] y ninguna de la región ITS completa.

En los últimos años han aumentado los trabajos en los que se han incluido especies de las familias Grimmiaceae y Ptychomitriaceae, aunque en todos ellos los objetivos eran resolver las relaciones a nivel de familia o superior. Estudios basados

en los genes *rps4* (Goffinet et al., 2001; Hedderson et al., 2004), *rbcL* (Tsubota et al., 2003) o ambos combinados con *trnL-F* (La Farge et al., 2000) han aclarado algunos aspectos que afectan a estas familias y que podemos resumir en que (1) Grimmiaceae y Ptychomitriaceae se resuelven como grupos hermanos, (2) Seligeriaceae se resuelve como grupo hermano de ambas familias, (3) *Glyphomitrium* no pertenece ni a Grimmiaceae ni a Ptychomitriaceae, sin que pueda proponerse su pertenencia a ninguna familia por el momento, y (4) el género *Grimmia* se resuelve polifilético.

Pese a la importancia de estos trabajos para entender las relaciones filogenéticas a niveles taxonómicos altos, sólo se había utilizado para inferirlas alrededor de 25 ejemplares para todo el conjunto de Grimmiaceae y Ptychomitriaceae, por lo que nos planteamos ampliar el estudio filogenético a niveles más bajos, género y especie, en dos familias que no habían sido estudiadas hasta entonces.

A medida que se va profundizando en estos estudios van surgiendo nuevas cuestiones en relación a la evolución molecular de las regiones de ADN empleadas, lo que invita a ser cautelosos a la hora de interpretar los resultados o realizar propuestas filogenéticas y taxonómicas prematuras. Recientes estudios sobre distintas regiones plastidiales en briófitos, como *psbT-H* (Quandt et al., 2003), *trnT-F* (Quandt, Stech, 2004) o el intrón *trnL* (Quandt, Stech, 2005) han mostrado como distintas mutaciones estructurales (inserciones, delecciones e inversiones) juegan un importante papel en la evolución molecular de estas regiones. Un tratamiento apropiado de estas mutaciones en nuestro alineamiento puede ayudar a disminuir la homoplasia de nuestros datos y hacerlos más fiables. En cuanto a la región ITS, Álvarez and Wendel (2003) describieron fenómenos como la falta de evolución concertada o la presencia de pseudogenes, que dificultaban la correcta identificación de secuencias homólogas. Por otro lado, detectar fuentes de incongruencia entre conjuntos de datos independientes, (distintos genes, por ejemplo) puede servirnos para detectar posibles episodios de reticulación entre genes o especies (Wendel, Doyle, 1998). Este hecho sugiere la necesidad de

realizar estudios filogenéticos basados en distintos compartimentos (cloroplasto, mitocondria, núcleo) y compararlos con distintas fuentes de datos independientes (morfológicos, histoquímicos, etc.) para inferir mejor la historia evolutiva de las especies que derive en una propuesta sistemática más sólida y estable.

Al comienzo de este estudio seleccionamos el intrón *trn*L (cpDNA) y la región ITS (nrDNA), que habían demostrado su eficacia en estudios similares, (Goffinet, Shaw, 2002; La Farge *et al.*, 2002; p.ej., Shaw, 2000; Vanderpoorten *et al.*, 2002). A medida que avanzábamos, y con el objetivo de aumentar el apoyo estadístico de los resultados y dar mayor solidez a nuestra propuestas, decidimos explorar nuevas regiones que habían sido utilizadas en angiospermas, pero que casi no se habían empleado en briófitos, como es el caso del espaciador plastidial *trnT-L* o el gen mitocondrial *nad5*, o que nunca se han empleado en musgos, como la región *trnK/matK*. La idea subyacente era que de la combinación de los datos de estas regiones con los morfológicos nos permitirían profundizar en la evolución de caracteres y táxones con el objetivo de comprender mejor la sistemática de este grupo de especies.

II. OBJETIVOS

"Justo cuando me supe
todas las respuestas de la vida
cambiaron las preguntas”.
Anónimo

II. Objetivos

A partir de las premisas anteriores nos planteamos como objetivos principales:

1) Proponer un marco de relaciones filogenéticas para el complejo de géneros formado por las familias Grimmiaceae y Ptychomitriaceae.

2) Estudiar las relaciones filogenéticas del género *Grimmia* (sensu Muñoz, Pando, 2000) y Greven (2003), así como valorar la monofilia tanto del propio género como de los distintos subgéneros reconocidos tradicionalmente.

3) Proponer una clasificación de *Grimmia* basada en datos morfológicos y de secuencias de ADN nuclear y cloroplástico.

Como una consecuencia de estos objetivos surgieron otros más específicos, que ha sido posible completar casi en su totalidad:

4) Valorar distintos marcadores moleculares que, o bien rara vez han sido empleados en briófitos, como es el caso del espaciador plastidial *trnT-L* o la región mitocondrial nad5, o bien no se han empleado nunca en musgos, como la región plastidial *trnK/matK*.

5) Estudiar la influencia de las mutaciones estructurales (inserciones, delecciones, inversiones) en el estudio de las relaciones filogenéticas.

6) Detectar posibles episodios de reticulación en *Grimmia* comparando filogenias obtenidas con secuencias nucleotídicas nucleares y plastidiales, y el conjunto con la información aportada por los datos morfológicos.

El Capítulo IV.1 estudia la posición sistemática de la especie *Grimmia pitardii* y sirve como estudio piloto para valorar la resolución que podemos obtener de los marcadores plastidiales *rps4* y *trnL-F* con vistas a elaborar filogenias a nivel de

familia y de género. De forma general responde a las preguntas planteadas en los objetivos 1 y 2, ya que aporta información sobre las relaciones del género *Campylostelium* y la posible necesidad de reconocer a la familia Campylosteliaceae, así como sobre la monofilia del subgénero *Grimmia*.

En el Capítulo IV.2 se abordan las relaciones filogenéticas a nivel de familia de Grimmiaceae, Ptychomitriaceae y Campylosteliaceae, y se tratan los procesos evolutivos que afectan a las secuencias obtenidas (procesos de inserción, delección e inversiones). En consecuencia responde a las preguntas planteadas en los objetivos 1 y 5.

Los capítulos IV.3 y IV.4 profundizan en el estudio filogenético del género *Grimmia*, con la intención de mostrar la evolución de caracteres y posibles procesos de reticulación. En concreto, el Capítulo IV.3, pretende responder a los objetivos 2 y 3, y de manera secundaria, al objetivo 4, al valorar la utilidad de la región plastidial *trnK/matK* para resolver relaciones filogenéticas. Por último, el Capítulo IV.4 responde a las preguntas planteadas en los objetivos 2 y 3, y de una manera más extensa, analiza posibles episodios de reticulación en Grimmia, por lo que responde al objetivo 6.

III. MATERIAL Y MÉTODOS

"La vida es todo aquello que pasa,
mientras uno está ocupado
haciendo otra cosa."
John Lennon

III. Material y métodos

Aunque en cada capítulo se tratará de manera detallada la metodología específica empleada, presentamos en este apartado, de manera resumida, la metodología común que no se ha incluido en el apartado correspondiente de los artículos enviados a las revistas.

Muestreo. Los sujetos de estudio de este proyecto son, mayoritariamente, las especies del género *Grimmia*. Desde 1993 el Dr. Muñoz ha reunido más de 3000 ejemplares de Grimmiaceae provenientes de todo el mundo, que en la actualidad están depositadas en el herbario del Real Jardín Botánico de Madrid (MA). Para completar el muestreo se solicitó material de los herbarios BCB, CHR, MO, MUB y S. La mayor parte de estos ejemplares se recolectaron hace menos de 10 años, por lo que son apropiados para estudios que utilizan secuencias de ADN. El muestreo incluye varios ejemplares para cada especie, haciendo hincapié en los táxones del subg. *Grimmia*.

Selección de regiones de ADN. La selección de las distintas regiones diana se hace fundamentalmente atendiendo al nivel taxonómico que deseamos investigar. El marcador molecular elegido deberá ser suficientemente variable para detectar cambios al nivel de estudio deseado. En un principio se secuenciaron las regiones *trnL-F* e ITS, y posteriormente añadimos las regiones plastidiales *trnS-rps4-trnT-L-F* y *trnK/matK* para poder completar los objetivos planteados. Se realizó un estudio piloto con el gen mitocondrial *nad5*, pero no mostró suficiente variabilidad como para proceder a su estudio en profundidad.

Obtención de secuencias. Los estudios moleculares basados en amplificación directa por PCR siguen un protocolo de trabajo en el laboratorio que se puede resumir en los siguientes pasos: 1) extracción de ADN, 2) amplificación de la región

concreta, 3) purificación de los productos amplificados y 4) secuenciación de los productos.

1) Para la extracción de ADN se suelen emplear distintos "kit" comerciales que optimizan la cantidad de ADN y la pureza del mismo, aunque son más caros que otros métodos y requieren gran cantidad de material. Teniendo en cuenta el tamaño de nuestros organismos probamos otros métodos, como el protocolo de extracción con NaOH (Werner et al., 2002c), que requería una menor cantidad de material vegetal de partida.

2) Para obtener la región concreta se prepara una mezcla de reactivos cuyas concentraciones pueden variar dependiendo de las características de cada secuencia que queremos amplificar. Generalmente, trabajamos con las siguientes concentraciones: En 50 µl de mezcla de reactivos añadimos 1.5 unidades de enzima "Taq polimerasa", 1 mM de bases nucleotídicas (dNTPs) a una concentración de 0.25 mM por base, tampón enzimático 1x, 1.5 mM de $MgCl_2$, 10 pmol para cada cebador y 1 µl de ADN total. Se probaron distintos cebadores universales para las distintas regiones. En aquellos casos en los que no obtuvimos amplificación procedimos a diseñar nuestros propios cebadores. Todos los iniciadores empleados en este trabajo se detallan en el capítulo correspondiente. Una vez preparada la mezcla de reactivos se procede a la amplificación de la secuencia diana por PCR, cuyo funcionamiento se basa en programar distintos ciclos de temperatura para desnaturalizar el ADN, permitir después la unión de los iniciadores y producir finalmente la extensión de la hebra correspondiente, tal y como se detalla en la Fig. III.1. Aunque los distintos ciclos empleados varían según las distintas regiones, presentamos un perfil standard de las distintas temperaturas empleadas. La desnaturalización se produce a una temperatura aproximada de 94º C durante 30'', la unión de los cebadores a una temperatura de 55º C durante 1' y la extensión a 72º C. Estos tres pasos se repiten un número de ciclos (25, más o menos). Sigue un paso final de extensión a 72º C y finalmente se detiene la reacción bajando la temperatura a 4º C.

3) Tras la amplificación, el siguiente paso consiste en obtener la secuencia que pretendemos estudiar libre de otro tipo de reactivos, como restos de cebadores, Taq polimerasa, etc. La purificación de esos productos se realiza por medio de columnas proporcionadas por distintas casas comerciales (p. ej., PCR Clean-up kit, MoBio Laboratories, California).

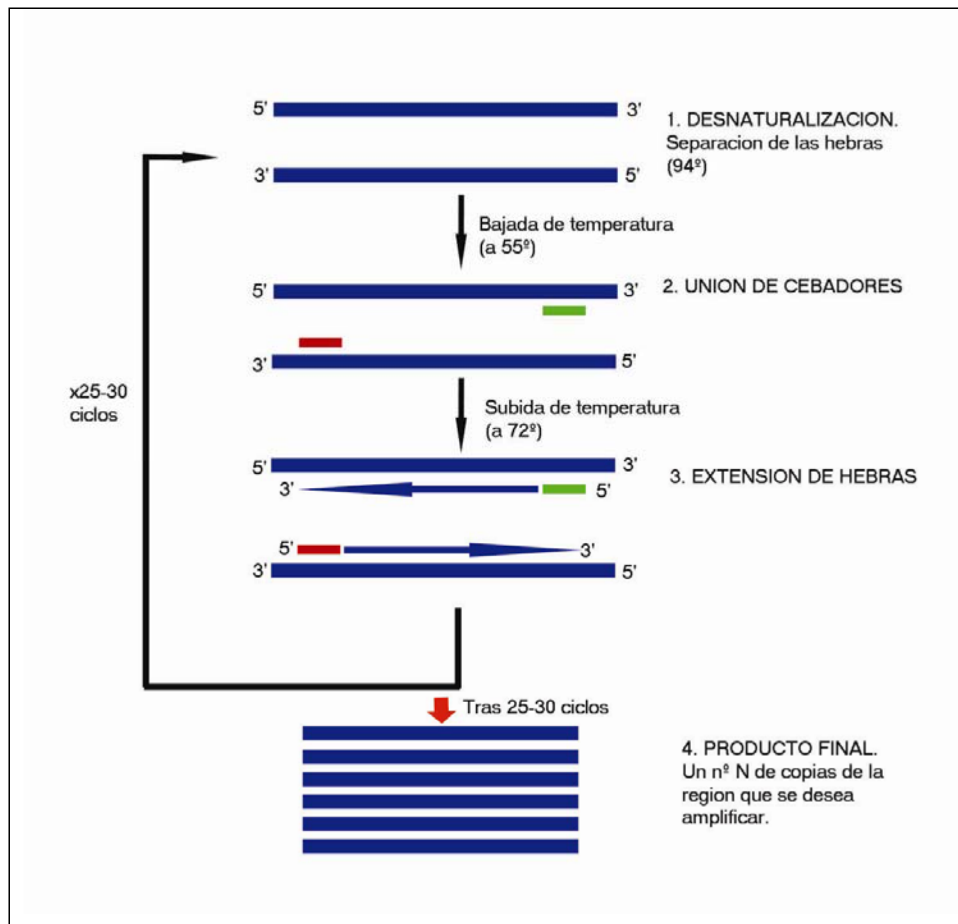

Fig. III.1. Representación esquemática de la reacción en cadena de la polimerasa (PCR). Los cebadores se indican en rectángulos rojos y verdes.

4) Por último, los productos se secuenciaron usando "Big Dye Terminator v 2.0" (Applied Biosystems, California).

Alineamiento de secuencias. Todas nuestras hipótesis filogenéticas dependen en última instancia de la calidad de nuestro alineamiento, por lo que es fundamental asegurarse de que las secuencias de ADN que comparamos son homólogas. Existen

muchos paquetes de software para alinear secuencias, tanto de manera manual (p.ej., Phyde v0.92: Müller et al., 2005), como automática (p.ej., ClustalX: Thompson et al., 1997).

Análisis filogenéticos. Toda hipótesis filogenética puede estar condicionada por el método de búsqueda de árboles. Para evitar errores debidos al método empleado hemos comparado las topologías obtenidas utilizando métodos de parsimonia, máxima verosimilitud e inferencia bayesiana. Se estimó el apoyo de los clados obtenidos mediante análisis de tipo "bootstrap" así como con índices de consistencia e índices de decaimiento o "decay values". En el caso de inferencia bayesiana obtuvimos el valor de probabilidad *a posteriori* como una medida adicional de apoyo estadístico. El programa winPAUP (Swofford, 2002) contiene la mayoría de las funciones para el análisis filogenético, y MrBayes 3.1 (Huelsenbeck, Ronquist, 2001) permite hacer los análisis bayesianos. Además, empleamos otros paquetes cuyas funciones los complementan, como Mesquite (Maddison, Maddison, 2006) y PRAP (Müller, 2004), entre otros.

IV. RESULTADOS

"La verdadera explicación
sencillamente no se puede explicar".
Julio Cortázar

IV.1. Chloroplast data reveal two conflicting hypotheses for the position of the *Campylostelium* and *Grimmia pitardii* (Bryophyta)

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ABSTRACT

Due to a variety of ambiguous morphological characters, the systematic placement of the Mediterranean-Central Asian *Grimmia pitardii*, lately considered a member of *Campylostelium* (Ptychomitriaceae), has been highly problematic. A molecular study based on the chloroplast gene *rps4* and the *trnL-F* region was performed to clarify its systematic affinities. According to the molecular analysis, *Grimmia pitardii* is resolved on a maximally supported clade together with the genus *Campylostelium,* sister to a similarly high supported clade comprising *Grimmia*, *Racomitrium, Coscinodon,* and *Schistidium*, and must be treated as *Campylostelium pitardii* (Corb.) E. Maier. At the same time, the systematic position of *Campylostelium* and *Ptychomitrium*, traditionally grouped in the family Ptychomitriaceae, was studied. Our results show two conflicting topologies: one groups *Ptychomitrium* and *Campylostelium,* whilst the second branches *Campylostelium* first, grouping *Ptychomitrium* with the Grimmiaceae.

Keywords: Bryophyta, *Campylostelium*, *Campylostelium pitardii,* chloroplast sequences, Grimmia, Grimmiaceae, Ptychomitriaceae, *Ptychomitrium,* trnL-F, trnS-rps4, systematics.

INTRODUCTION

Grimmia is one of the most complex and species-rich genera within Grimmiaceae. Without morphological synapomorphies to characterize it, and with an inordinate number of taxa described without critically evaluating existing ones, the genus challenges the use of only classical taxonomic methodologies for its study. Recent studies have reduced the number of accepted taxa to about 80 (Muñoz & Pando, 2000; Greven, 2003) and clarified many aspects of its taxonomy. However, many questions still remain to be solved around the classical *Grimmia* concept, as the treatment by Ochyra & al. (2003) lets suspect.

One example of the complexity within *Grimmia* is represented by the scarce and idiosyncratic *G. pitardii* Corb., originally described from Tunisia (Pitard & Corbière, 1909), and afterwards from Tajikistan (as *Usmania campylopoda* Laz.) and Irak (as *G. gibbosa* S. Agnew). It is indeed a rare species growing directly on the ground and not on rocks as it is the rule in *Grimmia*, distributed in southern Europe (Crete, Cyprus, France, Greece, Italy, and Spain), Canary Islands and Maghreb (Morocco and Tunisia), Turkey, and Central Asia (Tajikistan and Uzbekistan). Besides its habitat, this taxon deviates from any other *Grimmia* in habit, leaf morphology, costa anatomy, and peristome features.

Maier (1998) was the first noting its oddness in *Grimmia* (as defined by Limpricht, 1890), and compared *G. pitardii* with *G. plagiopodia* Hedw. (type of *Grimmia*), and *Campylostelium saxicola* (F. Weber & D. Mohr) Bruch & Schimp. and *C. strictum* Solms. On the basis of the plurilobed mitrate calyptra, costa anatomy, peristome teeth with basal membrane, and the outer peristome layer as thick as the inner peristome layer, she concluded that *Grimmia pitardii* was indeed a *Campylostelium*, and proposed the new combination *C. pitardii* (Corb.) E. Maier.

Neither Muñoz & Pando (2000) nor Greven (2003) adopted Maier's views, considering that although similar to *Campylostelium*, *G. pitardii* also shared important characters with members of *Grimmia* subg. *Grimmia* (e.gr., cygneous seta and ventricose capsule), leaving the question open to future studies.

The goal of this study is therefore to clarify the systematic position of *Grimmia pitardii* using a molecular approach based on the plastid *rps4* gene and *trnL-F* region (cpDNA). According to recent molecular studies as well as unpublished data from the authors we included representatives of the genera currently treated within Grimmiaceae and Ptychomitriaceae (Buck & Goffinet, 2000; La Farge & al., 2000; Tsubota & al., 2003; Hedderson & al., 2004).

A secondary aim was to know in what extension *Ptychomitrium* is related to *Campylostelium*, in order to present a phylogeny of Grimmiaceae/Ptychomitriaceae as currently considered.

MATERIAL AND METHODS

Plant Material: Vouchers are deposited in B, BCB, MA, MO, and MUB. EMBL accession numbers, voucher numbers of the herbaria as well as the origin of specimens are listed in Table IV.1.1.

DNA isolation amplifications and sequencing: Total DNA of gametophore tips from dried herbarium specimens or recent collections was isolated using the CTAB method described by Doyle & Doyle (1987), modified for bryophytes as described in Shaw (2000). PCR amplifications of the *rps4* gene, including the *trnS-rps4* spacer as well as the *trnL-F* region were performed in 50 µl–reactions containing 1.5 U *Taq* DNA polymerase, 1 mM dNTPs-Mix each 0.25 mM, 1 x buffer, 1.5 mM $MgCl_2$, 10 pmol of each amplification primer and 1µl of DNA. The *trnS-rps4* region was amplified using the primers trnS-R and rps4-5Fbryo described in Nadot & al. (1994), whereas the *trnL-F* region was amplified using the original Taberlet & al. (1991) primers, C and F. Amplification cycles were as follows: 2 min at 94ºC, 30 cycles with 2 min 94ºC, 1 minute 55ºC and 1 min 72ºC, and a final 7 min extension step at 72ºC. Amplified products were cleaned using spin filter columns (PCR Clean-up DNA Purification Kit, MoBIO Laboratories, California) following the manufacturers protocols. Cleaned products were directly sequenced using dye terminators (Big Dye Terminator v 2.0, Applied Biosystems, California).

Data analysis: Sequences were edited and manually aligned using PhyDE® (Müller & al., 2005) following alignment rules described in Kelchner (2000) and Quandt & Stech (2004, 2005). Following the approach in Quandt & al. (2003) and Quandt & Stech (2004, 2005), the data matrix was screened for inversions using secondary structure models calculated with RNAstructure (Matthews & al., 2004). Details on the inversions and structures are shown in Hernández-Maqueda & al. (in prep.). As discussed in Quandt & al. (2003) and Quandt & Stech (2004), presence or absence of detected inversions was not coded for the phylogenetic analyses. However, in order to gain information from substitutions within detected inversions they were reverse complemented and included in the analysis. Incomplete and ambiguous data were identified and excluded from subsequent analyses.

For phylogenetic inference, all characters were given equal weight, and gaps were treated as missing data. Parsimony analyses were conducted using *win*PAUP*4b10 (Swofford, 2002) and PRAP (Müller, 2004a). The latter program (available at http://www.botanik.uni-bonn.de/system/downloads/) generates command files for PAUP* that allow parsimony ratchet searches as designed by Nixon (1999) for analysis of large data sets. In the present study, 10 random addition cycles of 200 ratchet iterations each were used. Each iteration comprised two rounds of TBR branch swapping, one on a randomly re-weighted data set (25% of the positions), and the other on the original matrix saving one shortest tree. Since each random addition cycle rapidly converged to the same tree score, cycles were not extended to more than 200 iterations, nor were further cycles added. Shortest trees collected from the different tree islands were used to compute a strict consensus tree. Furthermore the data set was analysed employing a simple indel coding approach as advocated by Simmons & Ochoterena (2000) using the PAUP command file generated by Seqstate (Müller, 2004b) and the same options in effect.

Internal branch support was estimated by heuristic bootstrap searches with 1000 replicates and 10 addition sequence replicates per bootstrap replicate. Decay

values as further measurement of support for the individual clades were obtained using PRAP in combination with PAUP and the same options in effect as in the ratchet.

Maximum likelihood analyses were executed assuming a general time reversible model (GTR+G), and a rate variation among sites following a gamma distribution (four categories represented by mean). GTR+G was chosen as the model that best fits the data by Modeltest v3.6 (Posada & Crandall 1998) employing the interface MTgui (Nuin, 2005). The settings proposed by Modeltest v3.6 [BaseFreq=(0.3792 0.1178 0.1229), Nst=6, Rmatrix=(0.5689 2.3709 0.1546 0.1819 2.3709), Shape=0. 2102] were executed in PAUP. Maximum likelihood bootstrap searches were performed as faststep searches with 1000 replicates.

For further measurement of support, posterior probabilities were calculated using MrBayes v3.1 (Huelsenbeck & Ronquist, 2001). As in the maximum likelihood analysis, the GTR model of nucleotide substitution was employed, assuming site-specific rate categories following a gamma distribution. In addition an independent analysis with an appended indel matrix was performed employing the binary model for the indel partition. The a priori probabilities supplied were those specified in the default settings of the program. Posterior probability (PP) distributions of trees were created using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method and the following search strategies suggested by (Huelsenbeck & al., 2001, 2002). Two runs with four chains each were run simultaneously for 10^6 generations each run, with the temperature of the heated chains set to 0.2. Chains were sampled every 10 generations and the respective trees were written to a tree file. Calculation of the consensus tree and of the posterior probability of clades was done based upon the trees sampled after the burn-in (25 %). Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph (Müller & Müller, 2004).

RESULTS

The combined aligned data set (*rps4* and *trnL-F*) corrected for inversions is 1125 position long. 594 positons correspond to the *trnS-rps4* region and the remaining positions correspond to the *trnL-F* region. Primer sequences were trimmed from the sequences. Of 236 variable characters (118 each region) 169 were parsimony informative (84 from the *rps4* and 85 from the *trnL-F* region)

The MP ratchet analysis retained 18 most parsimonious trees (MPT, length=385, CI=0.735, RI=0.839, RC=0.617). With regard to the Ptychomitriaceae, two conflicting topologies were resolved by the combined analysis: one of them groups *Ptychomitrium* and *Campylostelium* and has been termed "Ptychomitriaceae monophyletic", PM, Fig. IV.1.1, left tree), whilst the other branches *Campylostelium* first, grouping *Ptychomitrium* with the Grimmiaceae ("*Campylostelium* first", CF, IV.1.1, right tree). Nine MPTs showed the Ptychomitriaceae monophyletic (PM; -ln 3548.23205) whereas in the other nine MPTs the clade comprising *Campylostelium* and *Grimmia pitardii* branched first followed by *Ptychomitrium* and than the *Grimmiaceae* (-ln 3549.36277). Figure IV.1.2 shows one of the 18 MPTs with decay values and bootstrap support (with and without indel coding) along the branches. As the hypothesis with Ptychomitriaceae being monophyletic had the better likelihood score and was in addition independently retrieved by the strict consensus of the simple indel coding approach as well as the maximum likelihood and Bayesian analyses of the combined data, one of the nine MPTs showing the Ptychomitriaceae monophyletic hypothesis was choosen for illustration (Fig. IV.1.2).

The maximum likelihood tree (-ln 3549.55239) with bootstrap support as well as posterior probabilities (with and without indel coding) is depicted in Fig. IV.1.3.

Separate analyses of the *trnS-rps4* and *trnL-F* matrices revealed a conflicting signal regarding the Ptychomitriaceae between both data sets (Fig. IV.1.1).

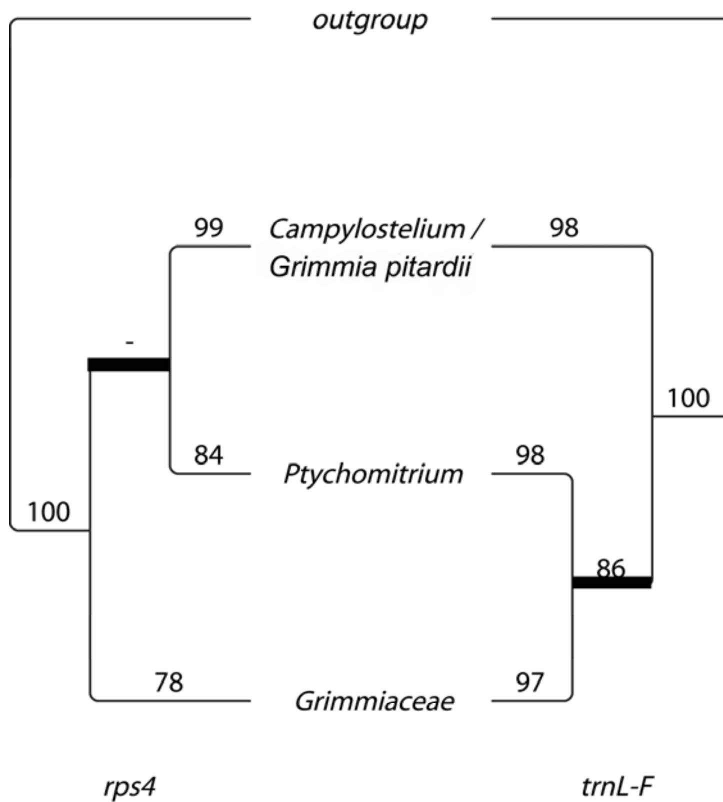

Fig. IV.1.1. Strict consensus topologies obtained from separate MP analyses of the *trnL-F* and *trnS-rps4* regions, with bootstrap support values along the branches

Whereas the *trnS-rps4* matrix favoured the PM-hypothesis (although without support), the *trnL-F* matrix resolved the *Campylostelium* first (CF) hypothesis with moderate support (BS 86). With almost equal amount of parsimony informative sites in each data partition, the observed conflicting signal might explain why neither the PM nor the CF hypothesis receives significant support in the combined analyses.

All analyses reveal *Grimmia pitardii* sister to *Campylostelium strictum* with maximal statistical support. Apart from *Campylostelium* (including *Grimmia pitardii*), both *Ptychomitrium* and the Grimmiaceae (including *Racomitrium, Coscinodon*, *Grimmia,* and *Schistidium*) form maximally supported groups in all analyses as well. Generally, *Campylostelium* (including *Grimmia pitardii*) is grouped with *Ptychomitrium*, although without significant support.

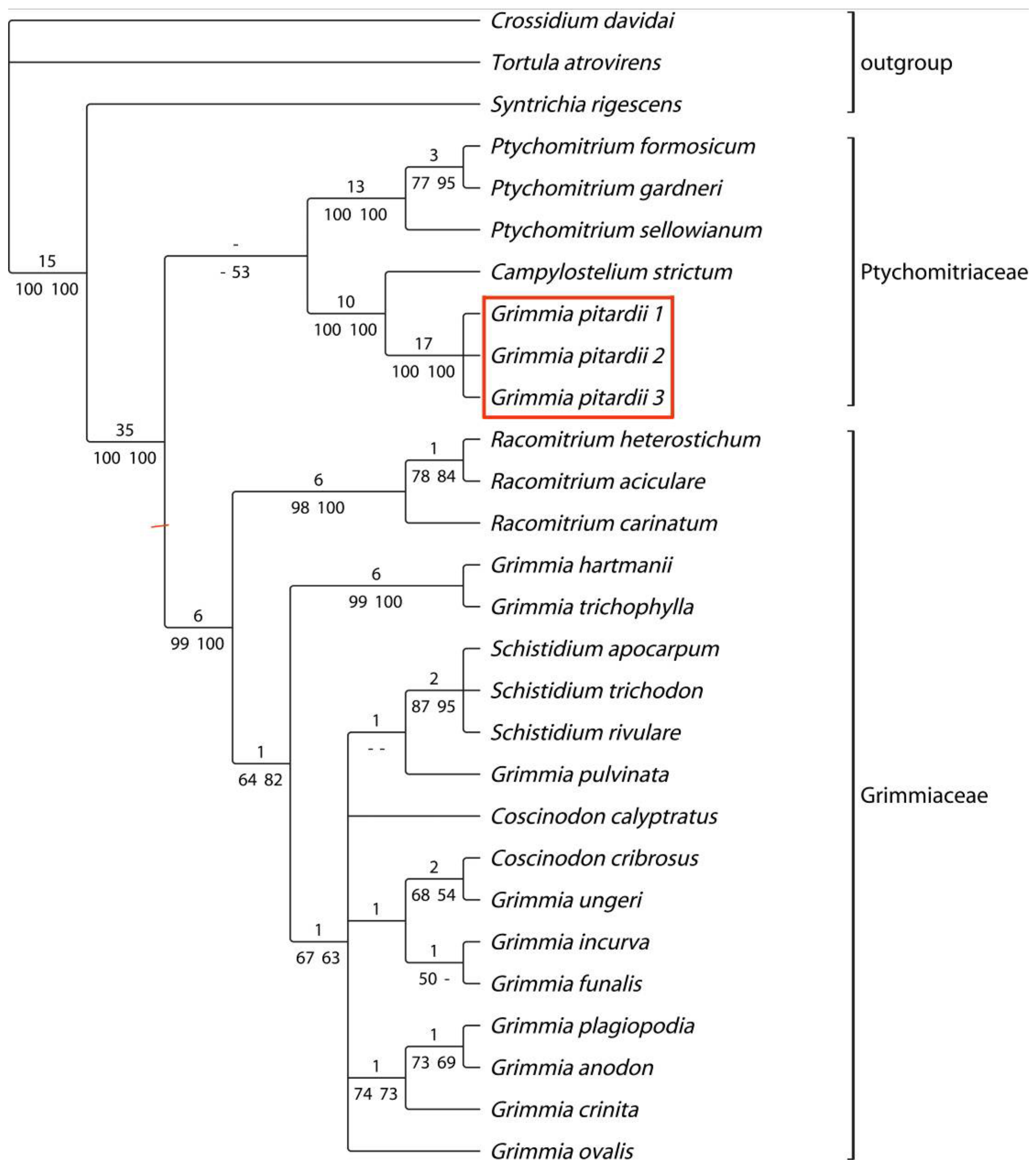

Fig. IV.1.2. One of 18 most parsimonious trees (length=385, CI=0.735, RI=0.839, RC=0.617) of the combined data. Decay indices are depicted above the branches; bootstrap support values are shown below the branches. The second value refers to bootstrap support obtained with the sic-indel matrix appended (sic = simple indel coding (Simmons and Ochoterena 2000)) as implemented in SeqState (Müller 2004b).

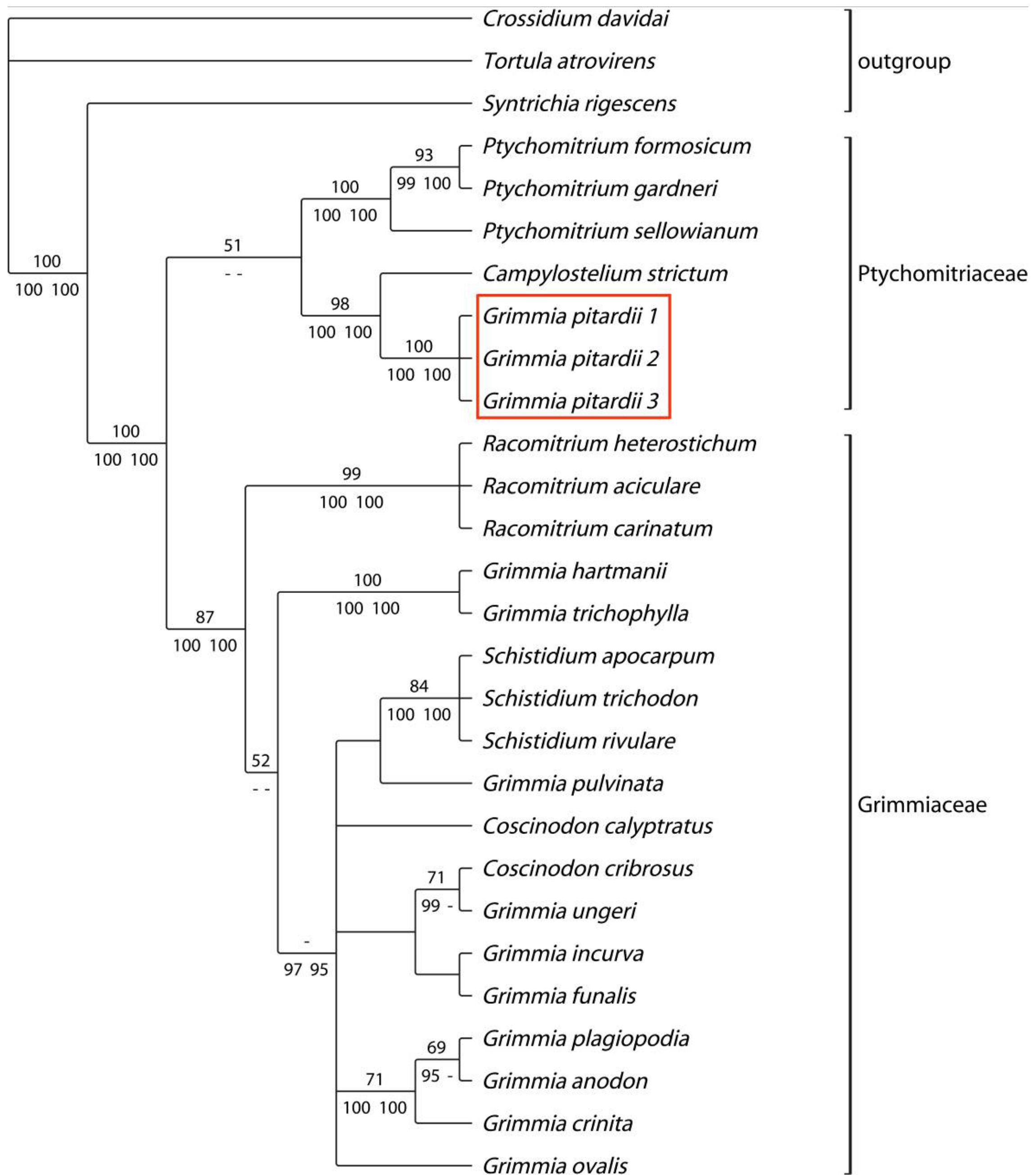

Fig. IV.1.3. Maximum likelihood (ML) tree of the combined data (-ln 3549.55239) with ML bootstrap support shown above the branches and posterior probabilities (with and without indel coding) below. The second value refers to bootstrap support obtained with the sic-indel matrix appended (sic = simple indel coding (Simmons and Ochoterena 2000)) as implemented in SeqState (Müller 2004b). Only significant posterior probabilities ≥ 95 are depicted.

Within the Grimmiaceae, *Racomitrium* is resolved monophyletic with maximal support and branching first, followed by a maximally supported clade consisting of *Grimmia hartmanii* Schimp. and *G. trichophylla* Grev*. Grimmia* is thus revealed paraphyletic with species of *Coscinodon* and the monophyletic *Schistidium* (MP: DC 2, BS 87/95; ML/Bayes: BS 93, PP 100/100) nested within.

DISCUSSION

Although *trnL-F* alone favors with morderate support the CF-hypothesis, *Ptychomitrium* and *Campylostelium* are resolved sister to the *Grimmiaceae* when *trnS-rps4* and *trnL-F* sequences are used in combination. This observation is in agreement with previous studies (Buck & Goffinet, 2000; Hedderson & al., 2004). However, the low support joining *Ptychomitrium* and *Campylostelium* does not allow conclusions concerning the phylogenetic relationships between these genera, especially as the phylogenetic signal provided by *trnL-F* matrix clearly supports the CF-hypothesis (Fig. IV.1.1). More sequence data of different regions from a denser sampling within Ptychomitriaceae might resolve this issue (Hernández-Maqueda & al., in prep.).

Based on our results, the *Grimmiaceae*, as defined by Buck & Goffinet (2000), form a monophyletic group with high to maximal statistical support. However, the genus *Grimmia* is resolved paraphyletic based on: a) the position of *Grimmia hartmanii* and *Grimmia trichophylla*, and b) the position of *Schistidium* and *Coscinodon* nested within *Grimmia*. *Grimmia hartmanii* and *Grimmia trichophylla* are considered by various authors (e.gr., Ochyra & al., 2003) as representatives of the genus *Dryptodon* Brid., although more information is required to confirm this taxonomic placement. To this end, an in-depth study using more DNA regions (ITS, *trnK/matK*, as well as the *rps4-trnT-trnL* spacers) from a large number of members of all the genera traditionally recognized within Grimmiaceae is currently under way

to resolve the phylogenetic relationships within the family (Hernández-Maqueda & al., in prep.).

Regarding *Grimmia pitardii,* our results clearly show its genetic proximity with *Campylostelium strictum*. Besides, the representatives of *Grimmia* subg. *Grimmia* in this study (*G. anodon, G. plagiopodia,* and *G. crinita*), which *Grimmia pitardii* belongs to (Loeske, 1930), appear in the Grimmiaceae clade far away from the *Campylostelium strictum*-*Grimmia pitardii* clade. Our results support the morphological evidences pointed out by Maier (1998, i.e., plurilobed mitrate calyptra, leaf costa with median guide cells larger than the ventral cells, peristome with basal membrane, and outer peristome layer as thick as the inner peristome layer), and we therefore consider the correct treatment of this taxon to be *Campylostelium pitardii,* as Maier (1998) proposed:

Campylostelium pitardii (Corb.) E. Maier, Candollea 53: 307. 1998. *Grimmia pitardii* Corb., Bull. Soc. Bot. France 56: LVI. 1909 (isotype, G not seen).

Usmania campylopoda Laz., Dopov. Akad. Nauk Ukrajins'k. RSR 11: 1040. 1970. *Grimmia campylopoda* (Laz.) K. Saito, J. Jap. Bot. 48(6): 163. 1973 (holotype, LWS not seen). Synonymized by Abramova & Abramov (1988).

Grimmia gibbosa S. Agnew, J. Bryol. 7: 339. 1973 (holotype in Herb. S. Agnew, isotypes in BUH, BM, not seen). Synonymized by Abramova & Abramov (1988).

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TABLE IV.1.1. List of investigated specimens, with GenBank accession numbers for the regions sequenced, including voucher numbers and the herbaria where the specimens are kept.

Especies	**Nº genbank** *rps4*	*trnL-F*	Localidad	Herbario/Nº de colección
Campylostelium strictum (Solms) Kindb.	*DQ399604*	*DQ399631*	Portugal.Marvao	BCB 43791
Coscinodon calyptratus (Drumm.) C.E.O. Jensen	*DQ399614*	*DQ399641*	USA. South Dakota	MO 5126877
Coscinodon cribrosus (Hedw.) Spruce	*DQ399615*	*DQ399642*	USA. Maine:	MO 4441357
Crossidium davidai Catches.	*DQ399626*	*DQ399627*	Spain. Canary Islands	MUB 5349
Grimmia anodon Bruch & Schimp.	*DQ399619*	*DQ399646*	USA.Nevada	MA 25617
Grimmia crinita Brid.	*DQ399620*	*DQ399647*	Spain.Huesca	MA 22641
Grimmia funalis (Schwágr.) Bruch & Schimp	*DQ399625*	*DQ399652*	Norway, Finmark	B 64173
Grimmia hartmanii Schimp.	*DQ399623*	*DQ399650*	Sweden. Värmlands Lan:	B 30709
Grimmia incurva Schwägr.	*DQ399622*	*DQ399649*	Sweden.Jamtlands Lan:	B 70022
Grimmia ovalis (Hedw.) Lindb.	*DQ399618*	*DQ399645*	USA.Nevada:	MO 5217105
Grimmia pitardii Corb. 1	*DQ399605*	*DQ399632*	Spain.Almeria	JM 6775
Grimmia pitardii Corb. 2	*DQ399606*	*DQ399633*	Spain.Almeria	MA 19751
Grimmia pitardii Corb. 3	*DQ399607*	*DQ399634*	Spain.Murcia	MUB 15032
Grimmia plagiopodia Hedw.	*DQ399616*	*DQ399643*	Sweden. Torne Lappmark:	B 70024
Grimmia pulvinata (Hedw.) Sm.	*DQ399617*	*DQ399644*	USA.California	MA 25026
Grimmia trichophylla Grev.	*DQ399624*	*DQ399651*	USA.California	MA 25700
Grimmia ungeri Jur.	*DQ399621*	*DQ399648*	USA.Nevada	MA 25618
Ptychomitrium formosicum Broth. & Yosuda	*DQ399601*	*DQ399628*	Taiwan.Taichung Co	MO 5219650
Ptychomitrium gardneri Lesq.	*DQ399602*	*DQ399629*	USA.Idaho	MO 5135689
Ptychomitrium sellowianum (Müll. Hal.) A. Jaeger	*DQ399603*	*DQ399630*	Paraguay.Paraguarí	MO 5215787
Racomitrium aciculare (Hedw.) Brid.	*DQ399609*	*DQ399636*	Spain.Cantabria	MA 22069
Racomitrium carinatum Cardot	*DQ399610*	*DQ399637*	South Korea.	MA 21356
Racomitrium heterostichum (Hedw.) Brid.	*DQ399608*	*DQ399635*	USA.California. Kyonggi-do	MO 5125302
Schistidium apocarpum (Hedw.) Bruch & Schimp .	*DQ399611*	*DQ399638*	Spain. León	MA 13294
Schistidium rivulare (Brid.) Podp.	*DQ399613*	*DQ399640*	Spain. Palencia	JM 6701
Schistidium trichodon (Brid.) Poelt	*DQ399612*	*DQ399639*	Austria.Totes Gebirge	MA 7455
Syntrichia rigescens (Broth. & Geh.) Ochyra	*AF481037*	*DQ400972*	Morocco, High Atlas	MUB 11378
Tortula atrovirens (Sm.) Lindb.	*AF480990*	*AY651833*	Spain, Sevilla	DQ552

IV.2. Phylogeny and classification of the Grimmiaceae/Ptychomitriaceae complex (Bryophyta) inferred from cpDNA

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ABSTRACT

Phylogenetic relationships within the Grimmiaceae/Ptychomitraceae were studied using a plastid tRNA cluster, including 4 tRNAs (*trnS, trnT*, *trnL, trnF*), a fast evolving gene (*rps4*), four spacers separating the coding regions, as well as one group I intron. Secondary structure analyses of the spacers as well as the *trnL* intron P8 domain identified several homoplastic inversions. Tracing the structural evolution of P8 we were able to identify lineage specific modifications that are mainly explained by inversions often in combination with large indel events. Phylogenetic analyses using maximum parsimony, maximum likelihood, and Bayesian methods indicate that *Jaffueliobryum* and *Indusiella* are closely related to *Ptychomitrium* and form the Ptychomitraceae s.str. As *Campylostelium* is neither resolved within Ptychomitriaceae s.str. nor Grimmiaceae s.str. we prefer to treat it in its own family, Campylosteliaceae De Not. The systematic position of *Glyphomitrium,* as also found by other authors, should be considered in a broader analysis of haplolepideous mosses as our analyses indicate that it is not part of Campylosteliaceae, Grimmiaceae or Pthycomitriaceae. Within Grimmiaceae s.str., *Racomitrium* is recognized as a monophyletic group sister to a clade including *Dryptodon, Grimmia*, and *Schistidium*. *Coscinodon* species appear disperse in *Grimmia* s.str. next to species sharing the same gametophyte morphology, and thus the genus is synonymyzed with *Grimmia*. Finally, *Schistidium* is resolved monophyletic with high statistical support, and seems to represent a rapidly evolving group of species. Our results are not fully congruent with recently published treatments splitting Grimmiaceae in a fairly high number of genera, neither with a comprehensive *Grimmia* including *Dryptodon* and *Grimmia* s. str.

Keywords: Grimmiaceae, Ptychomitriaceae, Campylosteliaceae, *Schistidium*, *Racomitrium*, *Grimmia*, *Dryptodon*, *trnL*, inversions, group I intron, secondary structure, microstructural changes, inversions.

INTRODUCTION

Among arthodontous mosses the haplolepideous mosses have shown to represent a monophyletic lineage (e.g. La Farge et al., 2000; Beckert et al., 2001; Magombo, 2003; Werner et al., 2004) that traditionally has been recognized as the subclass Dicranidae (e.g. Vitt et al., 1998; Buck and Goffinet, 2000). In haplolepideous mosses the peristome consists only of an endostome that comprises a single row of teeth with externally undivided sides while the internal one is split in two asymmetric columns. One of the most speciose groups in the Dicranidae includes the families Grimmiaceae and Ptychomitriaceae, which form the core of the order Grimmiales. This order has been differently treated in the past, either with the Drummondiaceae and Scouleriaceae (Buck and Goffinet, 2000) included or without both, but Seligeriaceae included (Ochyra et al., 2003; Tsubota et al., 2003; Goffinet and Buck, 2004). Whatever the familial composition of the Grimmiales turns out to be in the near future (Hernández-Maqueda in prep.), the latter families are usually considered more distantly related (Goffinet and Buck, 2004), and thus not a source of much dispute. But the generic composition of the Grimmiaceae and Ptychomitriaceae as well as the relationship between both families have been discussed controversially in the past and are still unresolved (Hernández-Maqueda et al., 2007). Whereas, some authors have lumped both families into a single one (Brotherus, 1901-1909; Dixon and Jameson, 1924; Jones, 1933; Lawton, 1971; Deguchi, 1978; Churchill, 1981; Deguchi, 1987; Noguchi, 1988; Gradstein et al., 2001; Allen, 2002; Tsubota et al., 2003; Allen, 2005), while others treat them as independent families, either related (Nyholm, 1956; 1960; Scott et al., 1976; Ignatov and Afonina, 1992; Sharp et al., 1994; Buck and Goffinet, 2000; Li and Crosby, 2001; Gao and Crosby, 2003; Ochyra et al., 2003; Hedderson et al., 2004; Smith, 2004), or rather distant (Brotherus, 1924, 1925; Nyholm, 1979; Crum and Anderson, 1981).

The genera included in each family have varied considerably among authors. The most drastic change, with respect to the traditional view, was published by Churchill (1981) grouping *Racomitrium* within the subfam. Ptychomitrioideae solely based on peristome similarities. Table IV.2.1 summarizes the treatment of the Grimmiaceae/Ptychomitriaceae complex in several classification systems, however for a more detailed summary of Grimmiales sytematics we refer to Tsubota et al. (2003).

In recent years, several studies at ordinal level or above, using cpDNA sequences, have helped to delimit the circumscription of Grimmiaceae and Ptychomitriaceae when combined with morphological traits, which alone fail to provide uncontrovertible data at such scale (e.g., the inclusion of *Racomitrium* in subfam. Ptychomitrioideae based on peristome traits, cf. Churchill, 1981). Studies using the *rps4* gene (Goffinet et al., 2001; Hedderson et al., 2004), *rbcL* (Tsubota et al., 2003), or both combined with *trnL-F* (La Farge et al., 2000), rendered basically the same results, which can be summarized as: (1) Grimmiaceae and Ptychomitriaceae are sister groups, (2) closely related to Seligeriaceae; (3) *Glyphomitrium* does not pertain in Grimmiaceae or Ptychomitriaceae, a result also reached by Estébanez et al. (2002) using histochemichal data, and according to Tsubota et al. (2003), this genus should be included in the Dicranaceae or Rhabdoweisiaceae; (4) the systematic position of *Campylostelium* is controversial, as revealed by Tsubota et al. (2003) and corroborated by Hernández-Maqueda et al. (2007); (5) neither *Scouleria* nor *Drummondia* pertain in the Grimmiales, being in fact basal to the core of the Dicranidae (further confirmed by Cox et al., 2000); (6) finally, the genus *Grimmia* is polyphyletic, and *Dryptodon* should be recognized as an independent genus to render the former monophyletic.

Although, in a recent phylogenetic study, we were able to confidently resolve the phylogenetic position of the former *Grimmia pitardii* using *rps4* and *trnL-F* (Hernández-Maqueda et al., 2007), the obtained trees showed that the

phylogenetic relationships on generic level could not be confidently resolved using these markers only. Therefore, we explored more variable regions, namely the spacers between *rps4*, *trnT* and *trnL* as additional phylogenetic markers. Whereas, *rps4* and *trnL-F* have been widely used in phylogenetic reconstructions at all classification levels, both spacers mentioned above have never been used to resolve phylogenies within bryophytes (Quandt and Stech, 2004; Stech, 2004). However, recently the molecular evolution of *trnT-L* spacer as well as the adjacent *trnL-F* region has been addressed by Quandt and Stech (2004), suggesting its suitability for this purpose.

As already stated, the aims of the previous molecular phylogenetic studies were to resolve the systematic relationships at ordinal classification level and above, and therefore they do not present extensive discussion on generic relationships within the families. The objective of the present study is thus to elucidate the phylogenetic relationships within the Grimmiaceae and Ptychomitriaceae, as well as between these two families. More specifically, we try to answer: (1) Do the non-coding parts of the plastid *trnS-F* represent a useful marker at this classification level? (2) Which of the previously proposed familial schemes is supported by the DNA sequence data, if any? (3) Are the genera accepted for each family in such divergent treatments as Buck and Goffinet (2000) or Ochyra et al. (2003) -followed by Goffinet and Buck (2004)- monophyletic?

MATERIAL AND METHODS

Plant Material: Plant vouchers are deposited in BCB, MA, MO, MUB and S. GenBank accession numbers, herbarium number of the vouchers, as well as the geographical origin of the specimens are listed in Table IV.2.2.

DNA isolation amplifications and sequencing: Total DNA of gametophore tips from dried herbarium specimens or recent collections was isolated using the NaOH method following the protocol described by Werner et al. (2002), recommended for isolation of small quantities of dry material. PCRs of the total region were generally performed in three sets: a) the *rps4* gene, including the *trnS-rps4* spacer, b) the *rps4-trnL* region and c) the *trnL-F* region using the primers as indicated in Fig. IV.2.1. In some cases nested PCRs for the *rps4-trnL* region were performed with internal primers (compare Fig. IV.2.1). All amplifications were done in 50 µl–reactions containing 1.5 U *Taq* DNA polymerase, 1 mM dNTPs-Mix each 0.25 mM, 1 x buffer, 1.5 mM $MgCl_2$, 10 pmol of each amplification primer and 1µl of DNA. Primer sequences and references are listed in Table IV.2.3. Amplification cycles for all reactions were as follows: 2 min at 94ºC, 30 cycles with 2 min 94ºC, 1 minute 55ºC and 1 min 72ºC, and a final 7 min extension step at 72ºC. Amplified *trnS-rps4* and *trnL-F* products were directly cleaned using spin filter columns (PCR Clean-up DNA Purification Kit, MoBIO Laboratories, California) following the manufacturers protocols. For the *rps4-trnL* region three to four products were pooled and gel cleaned.

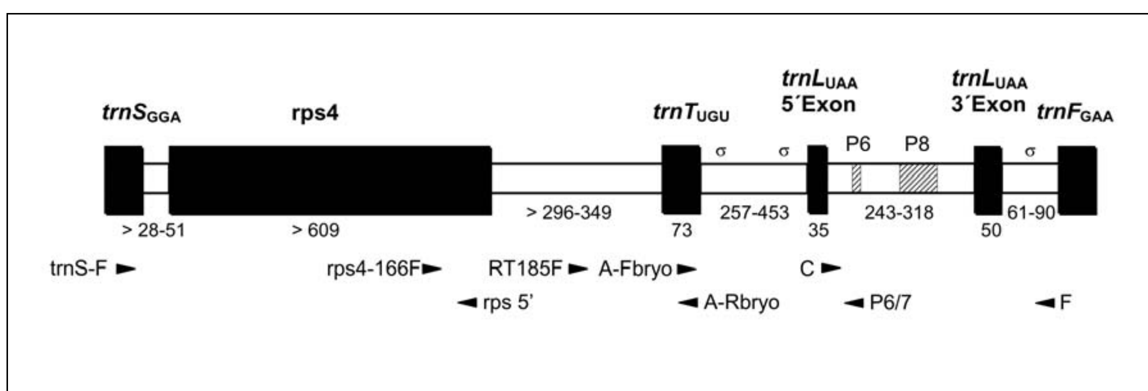

Fig. IV.2.1. Overview of the plastid *trnS-trnF* region. Black boxes indicate coding areas whereas the non-coding parts are represented by white boxes. Hattched boxes denote the location of the length variable P6 and P8 domains of the *trnL* intron. Locations of amplification and sequencing primers are specified below. Length variation of the region in the study group is shown below, putative promoter elements are indicate by σ (compare Quandt and Stech, 2003).

Cleaned products were directly sequenced using dye terminators (Big Dye Terminator v 2.0, Applied Biosystems, California). Unfortunately, the amplification of *Aligrimmia peruviana* R. S. Williams and *Indusiella bryanii* (R. S. Williams) S. P. Churchill extracts was unsuccessful, and *Ptychomitriopsis*, synonymyzed with *Ptychomitrium* by Churchill (1981), includes very rare species hardly ever collected, hence suitable material for DNA sequencing was unavailable.

Data analysis: Sequences were edited and manually aligned using PhyDE® (Müller et al., 2005) following alignment rules described in Kelchner (2000) and Quandt and Stech (2005). Following the approach in Quandt et al. (2003a) and Quandt and Stech (2004; 2005), the data matrix was screened for inversions using secondary structure models calculated with RNAstructure 4.2 (Mathews et al., 2004). Detected inversions were positionally separated in the alignment. As discussed in Quandt et al. (2003a) and Quandt and Stech (2004), presence or absence of detected inversions was not coded for the phylogenetic analyses. However, in order to gain information from substitutions within detected inversions, a second alignment file for the phylogenetic analyses was generated with the inversions included as reverse complemented. Alignments are available from www.treebase.com.

For phylogenetic inference, all characters were given equal weight, and gaps were treated as missing data. Parsimony analyses were conducted using *win*PAUP*4b10 (Swofford, 2002) and PRAP (Müller, 2004). The latter generates command files for PAUP* that allow parsimony ratchet searches as designed by (Nixon, 1999) for analysis of large data sets. In the present study, 10 random addition cycles of 200 ratchet iterations each were used. Each iteration comprised two rounds of TBR branch swapping, one on a randomly re-weighted data set (25% of the positions), and the other on the original matrix saving one shortest tree. Since each random addition cycle rapidly converged to the same tree score, cycles were not extended to more than 200 iterations, nor were further cycles added.

Shortest trees collected from the different tree islands were used to compute a strict consensus tree. Furthermore the data set was analysed employing a simple indel coding (sic) approach as advocated by Simmons and Ochoterena (2000) using the PAUP command file generated by SeqState (Müller, 2005) and the same options in effect.

Internal branch support was estimated by heuristic bootstrap searches with 1000 replicates and 10 addition sequence replicates per bootstrap replicate. Decay values as further measurement of support for the individual clades were obtained using PRAP in combination with PAUP and the same options in effect as in the ratchet.

Maximum likelihood analyses were executed assuming a general time reversible model (GTR+G+Γ), and a rate variation among sites following a gamma distribution (four categories represented by mean). GTR+G+Γ was chosen as the model that best fits the data according to the Akaike Information Criterion by Modeltest v3.6 (Posada and Crandall, 1998) employing the Windows® interface MTgui (Nuin, 2005). The proposed settings by Modeltest v3.6 were executed in PAUP 4.0b10. For the combined data set the following settings were used: BaseFreq=(0.4109 0.1016 0.1060), Nst=6, Rmatrix=(0.7745 2.3907 0.2275 0.8774 2.3907), Shape=1.2555, and Pinvar=0.4614.

For further measurement of support, posterior probabilities were calculated using MrBayes v3.1 (Huelsenbeck and Ronquist, 2001). The GTR model of nucleotide substitution was employed, assuming site-specific rate categories following a gamma distribution and a proportion of invariable sites. In addition, an independent analysis with an appended indel matrix was performed employing the binary model for the indel partition. The *a priori* probabilities supplied were those specified in the default settings of the program. Posterior probability (PP) distributions of trees were created using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method and the following search strategies suggested by

Huelsenbeck et al. (2001; 2002). Four runs with four chains each were run simultaneously for 10^6 generations each run, with the temperature of the heated chains set to 0.2. Chains were sampled every 10 generations and the respective trees were written to a tree file. Calculation of the consensus tree and of the posterior probability of clades was done based upon the trees sampled after the burn-in (we used a 25 % criterion as default). Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph (Müller and Müller, 2004).

RESULTS

Molecular evolution. The combined aligned data set (*trnS-rps4-trnT-trnL-trnF*) comprised 2359 positions, with five observed inversion that were positionaly separated in the original alignment. Three of the inversions were directly associated with structural changes of the P8 stem-loop region of the *trnL* intron as illustrated in Fig. IV.2.2, whereas the other two inversions are associated with hairpins located in the *trnT-L* (Fig. IV.2.3; Tab. IV.2.4) or the *trnL-F* spacer (not shown), respectively.

The inversion located in the *trnT-L* spacer (inversion 1, cf. Table IV.2.4 and Fig. IV.2. 3) affected the *Grimmia-Hydrogrimmia-Schistidium-Coscinodon* complex, and included two reverse complementary sequences spaning 12 nucleotides (Positions: 1280 to 1303). Inversion 2, involving the alignment positions 1903-1935 and 1955-2013, was confined to the *Racomitrium* clade (Fig. IV.2.2); inversion 3 (positions 1944-2015) was autapomorphic for *Grimmia ovalis* and affected almost the complete P8 stem-loop region (Fig. IV.2.2); inversion 4 (positions 2023-2044) distinguished the Grimmiaceae from the remainder but was shared with *Ptychomitrium sellowianum*, indicating the homoplastic nature of its occurence.

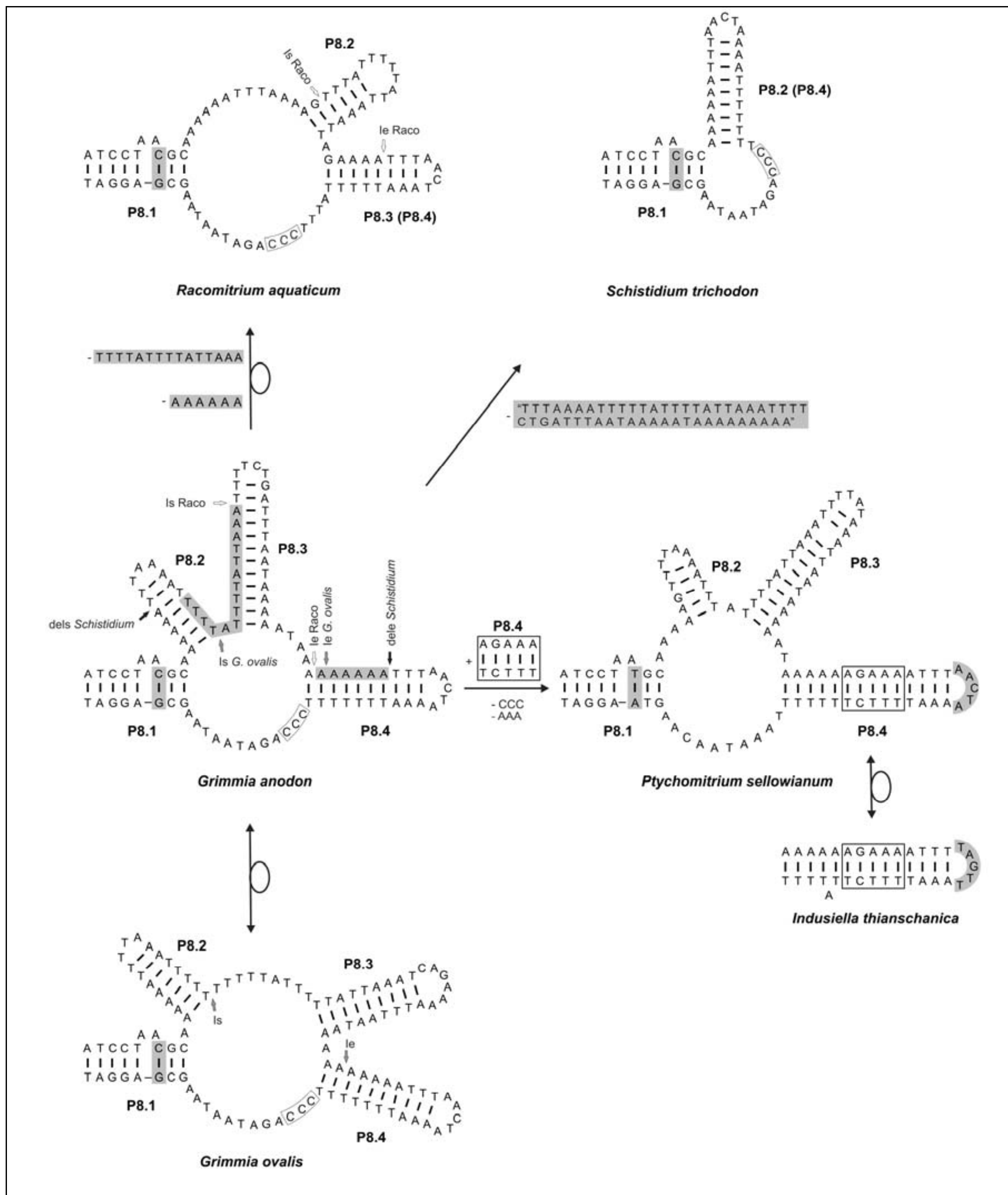

Fig. IV.2.2. Taxon or lineage specific P8 secondary structure models. All structures can be inferred by a few inversions, insertions and deletions events or combinations thereof from the common and, according to the phylogenetic analyses, ancestral type shared by the outgroups and the majority of ingroup taxa. Arrows with a circle on top indicate inversion events. Paired regions annotations in brackets indicate the homolog paired region in the common structure. Is = inversion start; Ie = inversion end; dels = deletion start; dele = deletion end; Raco = *Racomitrium*.

Finally, inversion 5 (positions 2210-2256) was located directly after the *trnL* 3'exon in the *trnL-F* spacer and differs from the previously recorded *trnL-F* inversion observed in pleurocarpous mosses (Quandt et al., 2003b; Quandt and Stech, 2004). Interestingly, the inversion of the hairpin formed by the putative sigma promotor elements in front of *trnF* (Quandt and Stech, 2004; Quandt et al., 2004) was not observed in the present data set. Except inversion 2, defining the *Racomitrium* species, all inversions were homoplastic and thus reduced tree resolution, which is in agreement with previous results (Quandt et al., 2003b; Quandt and Stech, 2004).

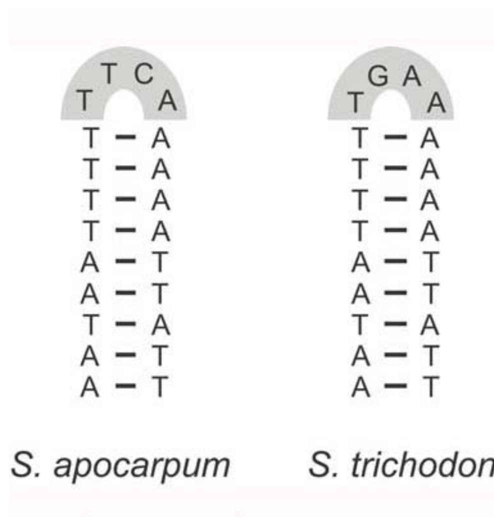

Fig. IV.2.3. Example of a hairpin associated inversion (inversion 1) as randomly found in *Schistidium* and *Grimmia* (compare Tab. 4 and Figs. IV.2.4-5).

Secondary structure calculations of the *trnL* intron P8 region revealed a simple multi-loop structure with, apart from the closing helix P8.1, three additional paired regions (P8.2 – P8.4) generally common for all taxa included in the study that is represented by the structure calculated for *Grimmia anodon* (Fig. IV.2.2). Compared to the Grimmiaceae the outgroups as well as *Glyphomitrium humillimum*, Ptychomitriaceae and Campylosteliaceae lack a CCC element in the multi-loop structure that is specific to the Grimmiaceae (Fig. IV.2.2). Apart from the autapomorphic inversion found in *Grimmia ovalis* that affected almost the entire P8, major deviations of the calculated structures are generally specific to inferred clades, such as for *Racomitrium*, *Schistidium* or Ptychomitriaceae and can be explained as derviates from the common structure as represented in *G. anodon*. For example, in the Ptychomitriaceae basically the same structure as in the Grimmiaceae and Campylosteliaceae is found, but P8.4 is extended by the insertion

of two paring repeats in the middle of the hairpin (Fig. IV.2.2) that according to the phylogenetic analyses (Fig. IV.2.4 and IV.2.5) were partly lost again in *Ptychomitrium formosicum* and *Jaffueliobryum wrightii*. *Indusiella* and *Jaffueliobryum* share the same P8 structure with the other Ptychomitriaceae. Here, the structure for *Ptychomitrium sellowianum* was choosen as it shares the inversion type B in the hairpin loop of P8.4 (inversion 4, Tab. IV.2.4) with the Grimmiaceae, whereas all other Ptychomitriaceae have the inversion type A (represented by *Indusiella thianschanica* below in Fig. IV.2.2; Tab. IV.2.4). The structure for *Schistidium* is characterised by the loss of the original P8.2 and P8.3. Similarly, the *Racomitrium* structure can be explained by a large deletion plus an inversion of large parts of the original P8.3 resulting in the loss of the original P8.2 and P8.3, and the increase of the multi-loop together with the formation of a new P8.2 (Fig. IV.2.2). However, in all structures P8.4 (P8.2 in *Schistidium* and P8.3 in *Racomitrium*) is consitently retained. In addition to the observed indels and inversions a compensating base pair change (CBC) in P8.1 was observed (Fig. IV.2.2).

Although all non-coding regions displayed considerable length variation, resulting in numerous indels that provided additional information, the spacers displayed a higher relative variability in terms of substitutions as well as indel events compared to the group I intron in *trnL* (Tab. IV.2.5). Interestingly, the relative amount of parsimony informative sites recorded for *trnL* was almost identical to the *rps4* values, indicating the fast evolving nature of the gene (Tab. IV.2.5).

Phylogenetics. Corrected for inversions the alignment comprised 2264 positions with 567 variable sites of which 371 have been parsimony informative, contributions of each region can be extracted from Tab. IV.2.5. After reverse complementing the inversions one parsimony informative site was lost. The simple

indel coding approach yielded annother 246 characters of which 152 were parsimony informative (61,79 %).

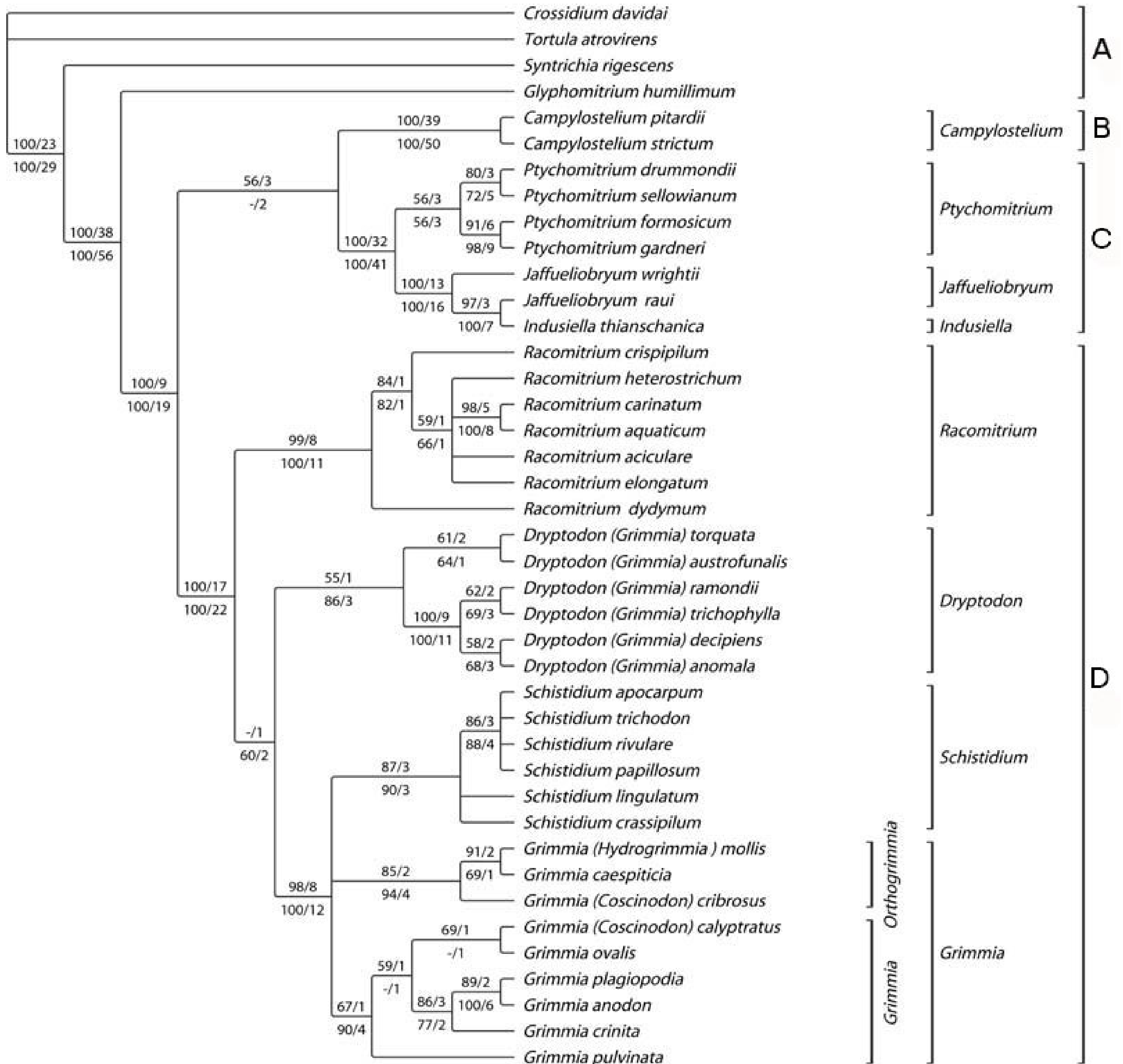

Fig. IV.2.4. Strict consensus tree of 79 most parsimonious trees (length = 1073, CI = 0.674, RI = 0.818, RC = 0.551). Bootstrap support (left) and decay values (right) without indel coding are shown above the branches, and with indel coding below the branches. Taxa indicated to the right follow the systematic arrangement proposed in this study. A. *outgroup*, B. *Campylosteliaceae*, C. *Ptychomitriaceae*, D. *Grimmiaceae*.

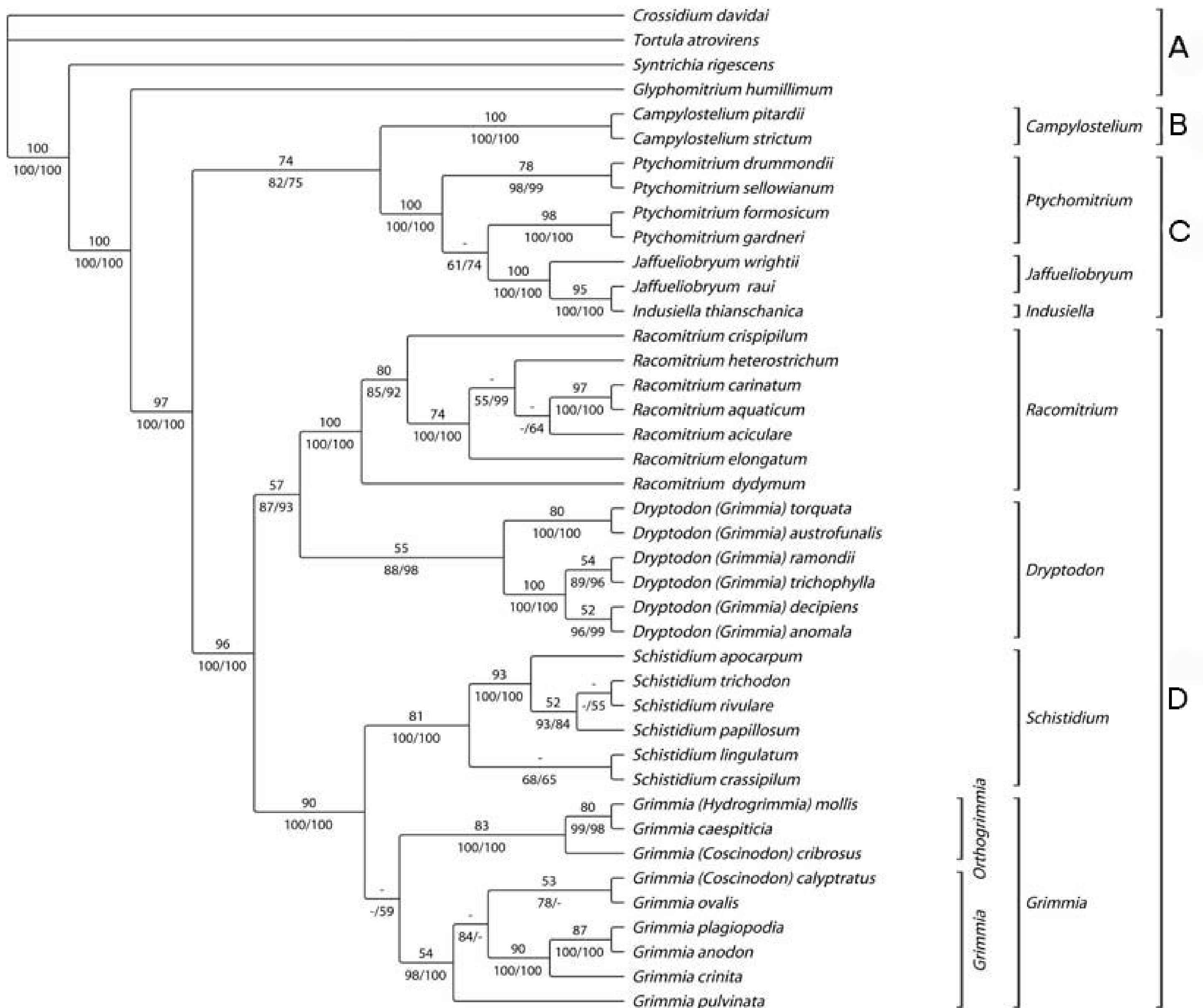

Fig. IV.2.5. The maximum likelihood tree (-ln = 8887.86914). Numbers above the branches indicate bootstrap support (>50%), while numbers below branches indicate Bayesian posterior probabilities (>50%) with (right) and without (left) indel coding. Taxa indicated to the right follow the systematic arrangement proposed in this study. A. *outgroup*, B. *Campylosteliaceae*, C. *Ptychomitriaceae*, D. *Grimmiaceae*.

The MP ratchet analysis retained 79 most parsimonious trees (MPT, length = 1073, CI = 0.674, RI = 0.818, RC = 0.551). Figure IV.2.4 depicts the strict

consensus tree, in which bootstrap support (left) and decay values (right) are shown above (without indel coding) and below (with indel coding) branches. The maximum likelihood tree (-ln 8887.86914) with bootstrap support indicated above the branches and posterior probabilities below (without/with indel coding) is depicted in Fig. IV.2.5. Coding of indels as characters according to Simmons and Ochoterena (2000) generally increased the statistical support for the clades especially at the tips of the tree as nicely illustrated by the example of *Racomitrium* (Fig. IV.2.6). Whereas the clade is largely unresolved in the MP analysis without indel coding, it is fully resolved and parts of the tree gain strong support with the sic-matrix appended.

Three clades are maximally supported in all analyses: the first one includes *Campylostelium* (Maximum Parsimony [MP]: 100/100 bootstrap support [bs], 39/50 decay value [dv]; Maximum Likelihood [ML]: 100 bootstrap support [bs]; Bayesian Inference [BI]: 100/100 posterior probability [pp]). It is defined by a sixteen nucleotide insertion located at the end of the *trnS* spacer (positions 48-63 in the aligned matrix) and another eleven nucleotides insertion in the *rps4*-*trnT* spacer (positions 1030-1040). The second includes *Ptychomitrium*, *Jaffueliobryum*, and *Indusiella* (MP: 100/100 bs, 32/41 dv; ML: 100 bs; BI: 100/100 pp). Finally, the third includes *Coscinodon*, *Grimmia, Racomitrium,* and *Schistidium* (MP: 100/100 bs, 17/22 dv; ML: 96 bs; BI: 100/100 pp).

Within Grimmiaceae (Figs. IV.2.4 and IV.2.5), *Racomitrium* is robustly resolved in a monophyletic clade (MP: 99/100 bs, 8/11 dv; ML: 100 bs; BI: 100/100 pp). The position of the *Dryptodon* clade depends on the analysis employed: with maximum parsimony it is resolved with the *Grimmia-Hydrogrimmia-Schistidium-Coscinodon* clade (Fig. IV.2.4), whilst with maximum likelihood or bayesian inference it branches with *Racomitrium* (Fig. IV.2.5).

The last clade is strongly supported (MP: 98/100 bs, 8/12 dv; ML: 90 bs; BI: 100/100 pp) in all the analyses. It includes as paraphyletic groups the remaining

species of *Grimmia* and *Coscinodon,* with *Hydrogrimmia* nested within as well as a strongly stupported monophyletic *Schistidium* clade (MP: 87/90 bs, 3/3 dv; ML: 81 bs; BI: 100/100 pp).

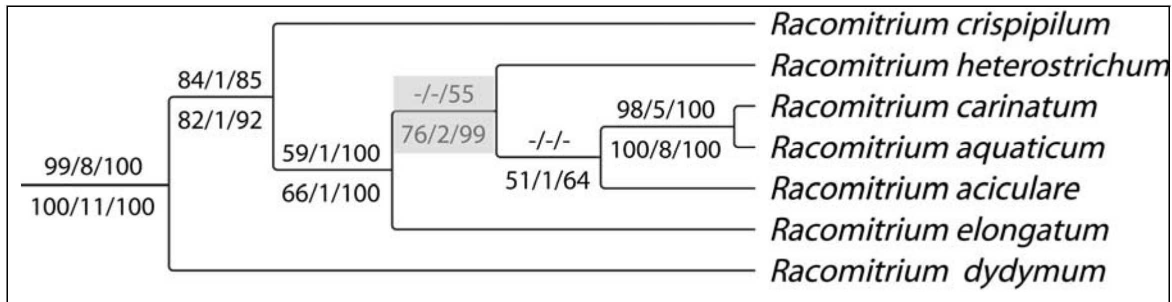

Fig. IV.2.6. Detailed summary of the *Racomitrium* clade showing the effect of indel coding on resolution and support values (BS/DV/PP). Support values above were inferred solely with the nucleotide matrix, whereas the values below are based on the nucleotide matrix with the indel matrix appended.

DISCUSSION

As illustrated by Figs. IV.2.2 and IV.2.3 as well as Tab. IV.2.4 applying rapidly evolving non-coding molecular markers for phylogenetic reconstructions is not as straight forward as using rather slow evolving genes displaying low degrees of microstructural change. Length mutations and especially hairpin associated inversions considerably complicate the homology assessment and might mess up the phylogenetic structure of the data set leading to low resolution and unsupported and in the worst case to erroneous trees (c.f. Kelchner, 2000; Quandt et al., 2003a). However, using alignment approaches based on repeat recognition (possibly guided by secondary structures) and applying mechanisms of molecular evolution as advocated by Kelchner (2000), Borsch et al. (2003), and Quandt and Stech (2005) as well as Quandt et al. (2003b) in alignment construction enables the utilisation of more complex evolving regions such as spacers and introns. Though more difficult to treat, the addition of both spacers (*rps4-trnT, trnT-trnL*) improved the tree resolution in comparison to a previous study by the same

authors (Hernández-Maqueda et al., 2007), especially within the *Grimmia-Hydrogrimmia-Schistidium-Coscinodon* complex. Although we increased the number of taxa in the present study of the Grimmiaceae/Ptychomitriaceae complex, the use of the spacers between *rps4* and *trnL* in combination with *trnS-rps4* and *trnL-F* rendered a better structured and supported topology. Especially, the additional information gained from indels increased the number of parsimony informative sites considerably and overall resulted in higher support values as nicely illustrated in Fig. IV.2.4. In contrast to the observed inversions that are highly homoplastic in the present study indels seem to provide a high quality signal that is similar to substitutions (CI indels = 0.656; CI substitutions = 0.674; CI inversions = 0.455).

Our results corroborate previous findings that *Glyphomitrium* is not a member of the complex and suggest a different systematic arrangement of the genera in the Grimmiaceae/Ptychomitriaceae complex different to any previously proposed. In addition our results indicate the need of accepting Campylosteliaceae as an independent family, although its systematic affinities are not yet confidently resolved due reported incongruences when comparing different DNA regions, analysis techniques, and morphological traits around *Campylostelium* (Hernández-Maqueda et al., 2007).

***Glyphomitrium*.** The exclusion of *Glyphomitrium* from either Grimmiaceae or Ptychomitriaceae is corroborated by our results, although we are not able to yet answer its phylogenetic relationships. Its familial placement has varied widely (Table IV.2.1), mostly due to its small size and paucity of distinct morphological characters that allow disentangling its phylogenetic relationships. Based on morphology, Churchill (1981) was the first in removing it from the Grimmiaceae/Ptychomitriaceae complex, although he did not formally propose any alternative placement. His views were corroborated using *rbcL* sequence data by Tsubota et al. (2003), who proposed a close relationship with *Arctoa* Bruch & Schimp. in the Dicranales not refuted yet.

Campylosteliaceae De Not. In a previous study using *rps4* and *trnL-F*, Hernández-Maqueda et al. (2007) found a conflicting signal regarding the systematic position of *Campylostelium*. Using *trnL-F Campylostelium* retained a sister group relationship to the Grimmiaceae, whereas based on *rps4* data it clustered with the Ptychomitriaceae. The addition of the *rps4-trnT* and *trnT-trnL* spacers now joined *Campylostelium* sister to the Ptychomitriaceae, but with low support (MP: 56/- bs, 3/2 dv; ML 74 bs; BI: 82/75 pp). Under these circumstances, it seems more appropriate to consider *Campylostelium* in its own family. The family Campylosteliaceae was described by De Notaris (1869) to include only *Campylostelium* which, according to this author, would differ from Ptychomitriaceae and Grimmiaceae in the shining leaves gradually tapering in a subulate apex. This familial arrangement has been only followed by Limpricht (1885-1890), who also included *Brachydontium* Fürnr., which according to recent studies (Goffinet and Buck, 2004; Hedderson et al., 2004) is not related to *Campylostelium* beyond superficial morphological similarities.

Ptychomitriaceae. According to our results (Figs. IV.2.4 and IV.2.5), this family should change its composition rather dramatically. Not only *Campylostelium* and *Glyphomitrium* are excluded from it, but *Jaffueliobryum* and *Indusiella* (includes *Coscinodontella*), formerly considered in the Grimmiaceae s. str. are robustly nested within (Table IV.2.1).

Although striking, this proposal is supported by two molecular synapomorphies, the presence of a deletion spanning > seven nucleotides in the *rps4-trnT* spacer (positions 860-868), a > seven nucleotide deletion within the hairpin loop P6 of the *trnL* intron (positions 1827-1835) as well as a insertion of a helical element in P8.4 (Fig. IV.2.2). Moreover, there are at least two morphological synapomorphies: (1) the costa with well-differentiated cell layers as seen in cross-section (except *Jaffueliobryum*, which costa is rather reduced and variable, and never has guide-cells sandwiched between two stereid bands), and (2) the cryptoicous sexual

condition, first demonstrated for *Ptychomitrium* by Deguchi (1977), and later found in *Aligrimmia* and *Indusiella* (Murray, 1984) and *Jaffueliobryum* (Churchill, 1987; Spence, 2006), but unknown in Grimmiaceae s. str., *Glyphomitriuim* and *Campylostelium*.

Although solidly resolved in the Ptychomitriaceae, we have to admit that the placement of *Jaffueliobryum* is a little bit odd in the family. First, morphologically it deviates in having a rather boring costa, and two of its three species have merely mitrate, although large, calyptrae, similar if not identical to the calyptrae found in species of the Grimmiaceae. However, in the Grimmiaceae the calyptrae never have the characteristic lobation at the base, which makes them similar to a Hawaian skirt in the Ptychomitriaceae. Secondly, the two species studied, morphologically very similar, resulted segregated in our analyses (Figs. IV.2.3 and IV.2.4), with *J. raui* branching with the morphologically very different *Indusiella thianschanica*. The independence of both genera is firmly fastened on morphological grounds: *Jaffueliobryum* species have broadly ovate leaves ended in a hair-point, and rather indistinct costae, while *Indusiella* species have lanceolate, muticous leaves, and a costa with strongly differentiated cell layers. The phylogenetic relationships of these genera (and *Aligrimmia*) were already raised by Murray (1984) and Churchill (1987). The incongruence we found could derive of incomplete sampling: our original design did not include *J. arsenei* (Thér.) Thér., and all attempts to sequence *Aligrimmia peruviana* and *Indusiella bryanii*, which would help to resolve the relationships of this small group of species were in vain.

Grimmiaceae. The clade joining the Grimmiaceae s. str. genera is maximally supported in all analyses (Figs. IV.2.4 and IV.2.5). Morphologically, the family is characterized by leaves with sinuose cell walls and costae of Kawai's (1968) type A, B, or C (in *Glyphomitrium*, Campylosteliaceae, and Ptychomitriaceae they are of type D or E), and outer peristome layer thicker than the inner layer (equally thickened in *Glyphomitrium*, Campylosteliaceae, and Ptychomitriaceae). Within the

family, either MP, ML, or Bayesian methods clearly show that *Racomitrium* and *Schistidium* are well supported monophyletic genera, while *Coscinodon* and *Grimmia* are non-monophyletic taxa. The circumscription of the genera in the family are subject to controversy after the rather revolutionary system proposed by Ochyra et al. (2003), who presented a very detailed account of the history of the taxa they accept at generic rank.

Whatever the taxonomic rank is considered to be, *Racomitrium* is a morphologically well-characterized taxon that in this study appears maximally supported in all analyses. In addition the genus is well defined by several molecular peculiarities such as an synapomorphic inversion of a large P8 fraction in combination with two considerable deletions or a ten nucleotide deletion in the *rps4-trnT* spacer (positions 1027-1047). In addition, *Racomitrium* species share several morphological synapomorphies, like the cladocarpous habit, the sinuose and porose cell walls of the vaginula, and the strongly sinuose-nodulose basal leaf cells. Recently, it was split in four genera (Ochyra et al., 2003; followed by Goffinet and Buck, 2004, cf. Table IV.2.1), a proposal that appears to be well supported on morphological grounds. *Racomitrium* has been included most often in the Grimmiaceae, although several authors (e.g., Jones, 1933; Churchill, 1981) have considered it more closely related to *Ptychomitrium* as both share some peristome characteristics, like the divided teeth and the presence of a basal membrane. Our results, and the fact that both of these characters are also present in *Grimmia* s. lat. firmly anchor it within Grimmiaceae, though.

Dryptodon has been treated usually as an intermediate genus between *Grimmia* and *Racomitrium* (Crundwell, 1971; Deguchi, 1978; Smith, 1978), sharing with the first the leaf areolation, seta posture and capsule morphology, and with the latter the general habit and the structure of the peristome, deeply divided in two prongs and with a basal membrane. Some authors did not consider it at any rank, but as synonym to *Grimmia* (Nyholm, 1998; Muñoz and Pando, 2000; Greven, 2003;

Ignatov and Ignatova, 2003; Hill et al., 2006). After Ochyra et al. (2003), the genus has gained acceptance and included the species formerly treated as *Grimmia* subg. *Rhabdogrimmia* (Goffinet and Buck, 2004; Hedderson et al., 2004). According to Ochyra et al. (2003: 118-121), *Dryptodon* is characterized by the variously curved setae, symmetric and mostly ribbed capsules, recurved leaf margins, and leaf costa protruding in dorsal side, although this definition is not without problems. Our results corroborate the paraphyletic nature of *Grimmia* (Hedderson et al., 2004; Streiff, 2006), which supports the recognition of *Dryptodon* as an independent genus, but considerably more restricted in the number of species included as well as in the characters which define it. The present study is however focused on the familial relationships, and not in resolving the phylogeny of *Grimmia* s. lat. (i.e., *Grimmia, Dryptodon, Guembelia, Hydrogrimmia, Orthogrimmia*, and *Streptocolea*, in the sense of Ochyra et al., 2003) that will be treated exclusively and in depth in a forthcoming paper by the same authors.

The results in the present study are in agreement with the view of a genus intermediate between *Grimmia* and *Racomitrium*. When the data are analyzed under MP (Fig. IV.2.4), *Dryptodon* branches with *Grimmia-Hydrogrimmia-Schistidium-Coscinodon* complex, although poorly supported. In contrast, when maximum likelihood or Bayesian methods are used (Fig. IV.2.5), it is resolved next to *Racomitrium*. Apart from shared substitutions *Racomitrium* and *Dryptodon* are linked by a thirteen base insertion in the *trnT-trnL* spacer (positions 1314-1326). As a molecular synapomorphy, all *Dryptodon* share a sixteen base insertion at the end of the *trnS* spacer (positions 29-44 in the matrix). In contrast, morphologically the genus is difficult to define beyond the presence of vegetative reproduction by specialized gemmae (Streiff, 2006). Interestingly these are also present in a peculiar *Racomitrium* species (*R. vulcanicola* Frisvoll & Deguchi).

The clade including the *Grimmia-Hydrogrimmia-Schistidium-Coscinodon* complex is strongly supported in all the analyses (Figs. IV.2.4 and IV.2.5). It

includes very similar taxa in terms of sequence variation, and the branch lengths are also similar when analyzed under likelihood methods, which could be the result of rapid radiation processes. Morphologically, they differ in sporophytic traits, but have very similar gametophytes, therefore they have been treated as closely related taxa. Even relatively recent treatments have considered them as members of an encompassing *Grimmia* (Lawton, 1971; Crum and Anderson, 1981; Noguchi, 1988; Sharp et al., 1994), although latter works have split them in at least three genera: *Grimmia, Schistidium*, and *Coscinodon*, and included *Hydrogrimmia* in *Grimmia*. As noted above, Ochyra et al. (2003) proposed a radical division of *Grimmia* and offered an outstanding summary on the historical systematic arrangements involving the taxa around this genus. Subsequent authors either embraced this proposal (Goffinet and Buck, 2004) or rejected it (Allen, 2005), and it is here tested for the first time using molecular data. From Figs. IV.2.4 and IV.2.5, two obvious conclusions arise: *Schistidium* must be considered as an independent genus, while *Grimmia*, *Hydrogrimmia*, and *Coscinodon* must be combined in one for which the prioritary name is the former.

Schistidium represents a monophyletic lineage strongly supported by the molecular data (Figs. IV.2.4 and IV.2.5). The main DNA sequence synapomorphy involves a fifty one base deletion in the P8 region of the *trnL* intron (Fig. IV.2.2), which support the morphological synapomorphies that separate this genus from *Grimmia*, like the reddish-brick color of the plants, the perichaetial leaves larger than the vegetative ones and of different shape, and -specially- the systylious capsules (columella attached to the operculum and falling with it at capsule dehiscence).

Hydrogrimmia has been considered an independent genus including only *H. mollis* on the basis of soft, unistratose, rounded-obtuse and muticous leaves, and straight setae (Abramova, 1969; Churchill, 1981; Ignatov and Ignatova, 2003; Ochyra et al., 2003). However, although its gametophyte is distinctive, caused by

the habitat it grows (cold running water), its sporophyte is virtually identical to that of *Grimmia* subg. *Orthogrimmia* (genus *Orthogrimmia* sensu Ochyra et al., 2003), which led other authors to include it in *Grimmia* s. str. (Nyholm, 1998; Muñoz and Pando, 2000; Ignatova and Muñoz, 2004; Norris and Shevock, 2004; Hastings and Greven, 2006). cpDNA sequences strongly support the latter view firmly rooting this taxon within *Grimmia* (Figs. IV.2.3 and IV.2.4).

Coscinodon species have gametophytes identical to species in *Grimmia*, and both genera can only be distinguished by sporophytic traits. Confusions of sterile plants involve thus more often members of different genera: i.e., *Coscinodon cribrosus* is confused with *Grimmia caespiticia*, and *Coscinodon calyptratus* with *Grimmia pulvinata*. Our results suggest that *Coscinodon* has to be merged with *Grimmia*, and also that gametophytic traits are more important than sporophytic to resolve the relationships within *Grimmia*.

Grimmia is a large and difficult genus even after chopping *Dryptodon* and *Schistidium* from it. Inclusion of *Hydrogrimmia* does not add complexity to it, but inclusion of *Coscinodon* increases the variability of sporophyte traits in the encompasing genus considerably. If *Grimmia* should be split in several further genera, as advocated by Ochyra et al. (2003), or maintained as a genus of broader scope, cannot be resolved in the present study: the DNA regions employed were not informative enough at this scale. To clarify the phylogeny of *Grimmia* as proposed in the present study is beyond the scope of a paper like this focused on the familial relationships. A molecular phylogenetic study including more species and more plastid (*trnK-matK*) and nuclear (ITS) genes is now under way.

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Table IV.2.1. Several systematic treatments of the Grimmiaceae/Ptychomitriaceae complex. The systematic arrangement suggested by our data is presented as This Study, with families arranged in alphabetical order. Under *incertae sedis* we include *Leucoperichaetium*, a very rare taxon not treated in this study, and *Glyphomitrium*, for which our results are not concluding. Goffinet and Buck (2004) treatment follows the systematic arrangement proposed by Ochyra et al. (2003) on a worldwide basis.

Limpricht (1885-1889)	Brotherus (1901-1902)	Brotherus (1924-1925)	Churchill (1981)	Buck & Goffinet (2000)	Goffinet & Buck (2004)	This Study
Campylosteliaceae	Grimmiaceae	Grimmiales	Grimmiaceae	Grimmiales	Grimmiales	Grimmiales
Campylostelium	Orthotrichaceae	Grimmiaceae	Grimmioideae	Grimmiaceae	Grimmiaceae	Campylosteliaceae
Brachydontium	*Aulacomitrium*	Scoulerioideae	"Guembelia"	*Aligrimmia*	*Aligrimmia*	*Campylostelium*
Grimmiaceae	(= *Glyphomitrium*)	*Scouleria*	"Rhabdogrimmia"	*Coscinodon*	*Bucklandiella*	Grimmiaceae
Cinclidonteae	Ptychomitrieae	Grimmioideae	*Grimmia*	*Coscinodontella*	*Codriophorus*	*Dryptodon*
Cinclidotus	*Glyphomitrium*	*Coscinodon*	*Schistidium*	*Dryptodon*	*Coscinodon*	*Grimmia*
Grimmieae	*Ptychomitrium*	*Indusiella*	*Hydrogrimmia*	*Grimmia*	*Coscinodontella*	*Racomitrium*
Schistidium	*Euglyphomitrium*	*Aligrimmia*	Coscinodontoideae	*Indusiella*	*Dryptodon*	*Schistidium*
Coscinodon	(= *Glyphomitrium*)	*Grimmia*	*Coscinodon*	*Jaffueliobryum*	*Grimmia*	Ptychomitriaceae
Grimmia	*Campylostelium*	*Schistidium*	*Jaffueliobryum*	*Leucoperchaetium*	*Guembelia*	*Aligrimmia*
Dryptodon	Scoulerieae	*Racomitrium*	*Indusiella*	*Racomitrium*	*Hydrogrimmia*	*Indusiella*
Racomitrium	*Scouleria*	Isobryales	*Aligrimmia*	*Schistidium*	*Indusiella*	*Jaffueliobryum*
Ptychomitrieae	Grimmieae	Ptychomitriaceae	Ptychomitrioideae	Ptychomitriaceae	*Jaffueliobryum*	*Ptychomitrium*
Brachysteleum	*Coscinodon*	*Campylostelium*	*Racomitrium*	*Campylostelium*	*Leucoperchaetium*	*Incertae sedis*
(Ptychomitrium)	*Indusiella*	*Ptychomitrium*	*Campylostelium*	*Glyphomitrium*	*Niphotrichum*	*Glyphomitrium*
(Glyphomitrium)	*Grimmia*	*Glyphomitrium*	*Ptychomitrium*	*Ptychomitriopsis*	*Orthogrimmia*	*Leucoperichaetium*
	Grimmia		*Incertae sedis*	*Ptychomitrium*	*Racomitrium*	
	Schistidium		*Glyphomitrium*		*Schistidium*	
	Racomitrium				*Streptocolea*	
					Ptychomitriaceae	
					Campylostelium	
					Ptychomitriopsis	
					Ptychomitrium	

Table IV.2.2. List of the species included in the analysis with the voucher's reference and GenBank accession number for each particular molecular region, as well as the geographic origin of the specimens

Species	Voucher herbarium reference	GENBANK accesion nº			Geographical origin
		rps4	***rps4-trnL***	***trnL-F***	
Campylostelium pitardii Corb.	MA 19752	DQ399605	FORTHCOMING	DQ399632	Spain. Almería
Campylostelium strictum (Solms) Kindb.	MA 4527	DQ399604	FORTHCOMING	DQ399631	Portugal. Marvao
Crossidium davidai Catches.	MUB 5349	DQ399626	FORTHCOMING	DQ399627	Spain. Canary Islands
Dryptodon (Grimmia) anomala Hampe	MA 24709	FORTHCOMING	FORTHCOMING	FORTHCOMING	Russia Altay Republic
Dryptodon (Grimmia) austrofunalis Müll. Hal.	MO 5211690	FORTHCOMING	FORTHCOMING	FORTHCOMING	Bolivia. La Paz
Dryptodon (Grimmia) decipiens (Schultz.) Lindb.	MA JM7131	FORTHCOMING	FORTHCOMING	FORTHCOMING	Spain. Toledo
Dryptodon (Grimmia) ramondii (Lam. & DC.) Margad.	MO 5142675	FORTHCOMING	FORTHCOMING	FORTHCOMING	U.S.A.: Alaska
Dryptodon (Grimmia) torquata Drumm.	MA 25588	FORTHCOMING	FORTHCOMING	FORTHCOMING	U.S.A.: California
Dryptodon (Grimmia) trichophylla Grev.	MA 25700	DQ399624	FORTHCOMING	DQ399651	U.S.A.: California
Grimmia (Coscinodon) calyptratus (Drumm.) C.E.O. Jensen	MO 5126877	DQ399614	FORTHCOMING	DQ399641	U.S.A.: South Dakota
Grimmia (Coscinodon) cribrosus Spruce	MO 4441357	DQ399615	FORTHCOMING	DQ399642	U.S.A.: Maine
Glyphomitrium humillimum (Mitt.) Cardot	MA 32763	FORTHCOMING	FORTHCOMING	FORTHCOMING	Japon. Kyoto
Grimmia anodon Bruch & Schimp.	MA 25617	DQ399619	FORTHCOMING	DQ399646	U.S.A.: Nevada
Grimmia crinita Brid.	MA 22641	DQ399620	FORTHCOMING	DQ399647	Spain. Huesca
Grimmia (Hydrogrimmia) mollis Bruch & Schimp.	S B6791	FORTHCOMING	FORTHCOMING	FORTHCOMING	Austria. Tirol
Grimmia ovalis (Hedw.) Lindb.	MO 5217105	DQ399618	FORTHCOMING	DQ399645	U.S.A.: Nevada
Grimmia plagiopodia Hedw.	S B70024	DQ399616	FORTHCOMING	DQ399643	Sweden.Torne Lappmark
Grimmia pulvinata (Hedw.) Sm.	MA 25026	DQ399617	FORTHCOMING	DQ399644	U.S.A.: California
Grimmia caespiticia (Brid.) Jur.	MA 19713	FORTHCOMING	FORTHCOMING	FORTHCOMING	Spain. Ávila
Indusiella thianschanica Broth. & Müll. Hal.	MO 4435504	FORTHCOMING	FORTHCOMING	FORTHCOMING	China. Qinghai
Jaffueliobryum raui (Austin) Thér.	MO 4420291	FORTHCOMING	FORTHCOMING	FORTHCOMING	U.S.A.: New Mexico
Jaffueliobryum wrighti (Sull.) Thér.	MO 3684962	FORTHCOMING	FORTHCOMING	FORTHCOMING	U.S.A.: Nebraska
Ptychomitrium drummondii (Wilson) Sull.	MO 5123797	FORTHCOMING	FORTHCOMING	FORTHCOMING	U.S.A.: Arkansas
Ptychomitrium formosicum Broth. & Yosuda	MO 5219650	DQ399601	FORTHCOMING	DQ399628	Taiwan. Taichung Co
Ptychomitrium gardneri Lesq.	MO 5135689	DQ399602	FORTHCOMING	DQ399629	U.S.A.: Idaho
Ptychomitrium sellowianum (Müll. Hal.) A. Jaeger	MO 5215787	DQ399603	FORTHCOMING	DQ399630	Paraguay. Paraguarí
Racomitrium aciculare (Hedw.) Brid.	MA 22609	DQ399609	FORTHCOMING	DQ399636	Spain. Cantabria
Racomitrium aquaticum (Schrad.) Brid.	MA 22070	FORTHCOMING	FORTHCOMING	FORTHCOMING	Spain. Santander
Racomitrium carinatum Cardot	MA 21356	DQ399610	FORTHCOMING	DQ399637	South Korea. Kyonggi-do
Racomitrium crispipilum (Taylor) A. Jaeger	MA 14328	FORTHCOMING	FORTHCOMING	FORTHCOMING	Colombia. Usme
Racomitrium didymum (Mont.) Jaeger	MA 25251	FORTHCOMING	FORTHCOMING	FORTHCOMING	Chile. Región de los Lagos
Racomitrium elongatum Frisvoll	MA 13319	FORTHCOMING	FORTHCOMING	FORTHCOMING	Spain. Palencia
Racomitrium heterostichum (Hedw.) Brid.	MO 5125302	DQ399608	FORTHCOMING	DQ399635	U.S.A.: California
Schistidium apocarpum (Hedw.) Bruch & Schimp.	MA 13294	DQ399611	FORTHCOMING	DQ399638	Spain. León
Schistidium crassipilum H.H. Blom	MA 14862	FORTHCOMING	FORTHCOMING	FORTHCOMING	Spain. Granada
Schistidium lingulatum Blom	MA 26281	FORTHCOMING	FORTHCOMING	FORTHCOMING	U.S.A.: Washington
Schistidium papillosum Culm.	MA 26557	FORTHCOMING	FORTHCOMING	FORTHCOMING	Spain. Lérida
Schistidium rivulare (Brid.) Podp.	MA 20932	DQ399613	FORTHCOMING	DQ399640	Spain. Palencia
*Schistidium trichodon (*Brid.) Poelt	MA 7455	DQ399612	FORTHCOMING	DQ399639	Austria. Totes Gebirge
Syntrichia rigescens (Broth. & Geh.) Ochyra	MUB 11378	AF481037	FORTHCOMING	DQ400972	Morocco. High Atlas
Tortula atrovirens (Sm.) Lindb.	MUB 11352	AF480990	FORTHCOMING	AY651833	Spain. Sevilla

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Table IV.2.4. Alignment and distribution of the inversions 1 and 4 detected in the data set. The alignment position for each inversion is indicated. In both cases the reverse complement of each particular block derivates in the subsequent particular block.

species	*trnT-L* spacer alignment position : 1280-1303	*trnL-F* spacer alignment position : 2023-2044
Glyphomitrium humillimum	------------------------	TTTTAGTTAAA-----------
Crossidium davidai	------------------------	----------------------
Tortula atrovirens	------------------------	----------------------
Syntrichia rigescens	------------------------	----------------------
Jaffueliobryum wrightii	------------------------	TTTTAGTTAAA-----------
Jaffueliobryum raui	------------------------	TTTTAGTTAAA-----------
Indusiella thianschanica	------------------------	TTTTAGTTAAA-----------
Ptychomitrium formosicum	------------------------	TTTTAGTTAAA-----------
Ptychomitrium gardneri	------------------------	TTTTAGTTAAA-----------
Ptychomitrium drummondii	------------------------	TTTTAGTTAAA-----------
Ptychomitrium sellowianum	------------------------	-----------TTTAACTAAAA
Campylostelium pitardii	------------------------	TTTTAGTTAAA-----------
Campylostelium strictum	------------------------	TTTTAGTTAAA-----------
Racomitrium heterostrichum	------------------------	-----------TTTAACTAAA-
Racomitrium carinatum	------------------------	-----------TTTAACTAAA-
Racomitrium aciculare	------------------------	-----------TTTAACTAAA-
Racomitrium elongatum	------------------------	-----------TTTAACTAAA-
Racomitrium crispipilum	------------------------	-----------TTTAACTAAA-
Racomitrium dydymum	------------------------	-----------TTTAACTAAA-
Racomitrium aquaticum	------------------------	-----------TTTAACTAAA-
Dryptodon austrofunalis	------------------------	-----------TTTAACTAAA-
Dryptodon torquata	------------------------	-----------TTTAACTAAA-
Dryptodon decipiens	------------------------	-----------TTTAACTAAA-
Dryptodon ramondii	------------------------	-----------TTTAACTAAA-
Dryptodon trichophylla	------------------------	-----------TTTAACTAAA-
Dryptodon anomala	------------------------	-----------TTTAACTAAA-
Schistidium apocarpum	------------TTTTTTCAAAAA	-----------TTTAACTAAAA
Schistidium trichodon	TTTTTGAAAAAA------------	-----------TTTAACTAAAA
Schistidium rivulare	------------TTTTTTCAAAAA	-----------TTTAACTAAAA
Schistidium lingulatum	------------TTTTTTCAAAAA	-----------TTTAACTAAAA
Schistidium crassipilum	------------TTTTTTCAAAAA	-----------TTTAACTAAAA
Schistidium papillosum	------------TTTTTTCAAAAA	-----------TTTAACTAAA-
Coscinodon cribrosus	TTTTTGAAAAAA------------	-----------TTTAACTAAAA
Coscinodon calyptratus	TTTTTGAAAAAA------------	-----------TTTAACTAAAA
Grimmia mollis	------------------------	-----------TTTAACTAAAA
Grimmia plagiopodia	------------TTTTTTCAAAAA	-----------TTTAACTAAAA
Grimmia pulvinata	------------TTGTTTCAAAAA	-----------TTTAACTAAAA
Grimmia ovalis	------------TTTTTTCAAAAA	-----------TTTAACTAAAA
Grimmia anodon	TTTTTGAAAAAA------------	-----------TTTAACTAAAA
Grimmia caespiticia	TTTTTTTAAAAA------------	-----------TTTAACTAAAA
Grimmia crinita	------------TTTTTTCAAAAA	-----------TTTAATTAAAA
	typ A typB inversion 1	typ A typ B inversion 4

Table IV.2.5. Summary of sequence length, divergence and proportional contribution of the different regions to the data matrix as well as ti/tv ratios number and distribution of indels and inversions. The uncorrected values refer to the original alignment, whereas the corrected values are based on the matrix with the inversions included as reverse complement.

					uncorrected		**corrected**		**uncorrected**		**corrected**		**uncorrected**	**corrected**	**uncorrected**	**corrected**		
Character Set	**No. Characters**	**length range**	**mean**	**S.D.**	**divergence [%]**	**S.E.**	**divergence [%]**	**S.E.**	**ti/tv**	**S.E.**	**ti/tv**	**S.E.**	**variable [%]**	**variable [%]**	**informative [%]**	**informative [%]**	**No. indels**	**No. inversions**
trnS-rps4 spacer*	75	28-51	37.5	7.05	11.833	2.925	11.833	3.103	1.004	0.519	1.004	0.532	28	28	14.667	14.667	8	0
rps4	609	609	609	0	4.082	0.407	4.082	0.387	3.926	0.838	3.926	0.936	23.153	23.153	15.435	15.435	0	0
rps4-trnT spacer	455	296-349	323.9	11.38	9.731	0.894	9.731	0.883	1.423	0.313	1.423	0.339	31.429	31.429	21.099	21.099	81	0
trnT	73	73	73	0	0.535	0.225	0.535	0.226	-	-	-	-	8.219	8.219	2.74	2.74	0	0
trnT-trnL spacer	445	257-445	291.3	26.79	6.722	0.713	6.723	0.706	1.06	0.272	1.063	0.243	27.133	27.865	17.287	17.753	75	1
trnL 5'exon	35	35	35	0	0.408	0.228	0.408	0.232	0.051	-	0.051	-	8.571	8.571	0	0	0	0
trnL-intron	371	243-318	290.6	20.16	5.042	0.684	5.069	0.646	2.408	0.67	2.288	0.618	20.814	24.259	12.896	15.364	64	3
trnL 3'exon	50	50	50	0	0.278	0.277	0.278	0.28	0	-	0	-	2	2	2	2	0	0
trnL-trnF spacer	111	62-90	65.6	4.68	13.615	2.288	13.675	2.184	1.991	0.791	1.689	0.723	30.081	30.631	24.39	26.126	18	1
*trnF**	40	40	40	0	1.17	0.657	1.17	0.66	0.216	-	0.216	-	10	10	5	5	0	0
	∑ 2264																**∑ 246**	**∑ 5**

IV.3. Phylogenetic analysis and morphological evolution of the *Grimmia-Coscinodon-Hydrogrimmia-Dryptodon-Schistidium* complex (Grimmiaceae, Bryopsida) inferred from chloroplast DNA and morphological data

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ABSTRACT

Recent studies based on molecular data have revealed *Grimmia* as a paraphyletic taxon, with *Dryptodon* resolved as an independent genus, and *Coscinodon* and *Hydrogrimmia* nested with *Grimmia* species having a similar gametophytic morphology, and termed thereafter as *Grimmia* s.l. A previous study on 114 taxa of Grimmiaceae using *rps4* and *trnL-F* regions confirmed the isolated position of *Dryptodon*, but could not fully resolve the relationships within the *Grimmia s.l.-Schistidium* clade. With the aim of disentangling such relationships, a phylogenetic analysis of the species in this clade was done employing two plastid regions, *trnK/matK* and a tRNA cluster (*trnS-F*) that includs the gene *rps4,* the *trnL* intron, as well as three spacers (*rps4-trnT, trnT-L*, *and trnL-F*). The phylogenetic reconstructions combined with morphological data aimed to test: 1) the phylogenetic relationships within this taxonomic complex; 2) the monophyly of the genera accepted by Ochyra et al. (2003), formerly included in a broadly understood *Grimmia*; 3) the distribution of the gametophytic and sporophytic traits in the chloroplast tree in order to elucidate which characters better reflect the phylogeny of the group.

The results show that the phylogenetic relationships in the group are very complex at the least, especially considering the sporophytic traits. The only diagnostic character to separate *Dryptodon* from *Grimmia* s.l. is the presence of gemmae in the former, shared with the rare *Racomitrium vulcanicola*. *Schistidium* is resolved sister to the redefined *Grimmia* s.l. Considering that the support for *Grimmia* s.l. is only low to moderate and that no morphological synapomorphies have been found the taxonomic value of this clade is questionable and opens the possibility of its division into smaller clearly monophyletic entities with uncertain relationship. These would broadly correspond with the infrageneric groups traditionally considered in *Grimmia* once *Dryptodon* and *Schistidium* are excluded (*Grimmia* s.str., *Guembelia*, *Hydrogrimmia*, *Orthogrimmia*, and *Streptocolea*),

although there are several incongruences that need to be eliminated to make the morphological and the molecular evidences fully compatible. The current subdivision of *Grimmia* rests on sporophytic traits, and it is not reflected in the molecular or morphological trees. In addition, there are several species pairs with identical gametophytes but sporophytes characteristic of different subgenera. The above groups are only monophyletic if the species traditionally included in subg. *Grimmia* (= *Grimmia* s.str.) are transferred to the taxon where they should be place based on gametophyte morphology. We conclude that the gametophytic traits reflect more accurately the evolutionary history in the genus, although they are affected by different levels of homoplasy.

Key words: Grimmiaceae, phylogeny, *Grimmia, Dryptodon, Schistidium*, *Guembelia*, *Hydrogrimmia*, *Orthogrimmia*, *Streptocolea, trnK/matK, trnS-rps4-trnT-trnL-trnF*.

1. INTRODUCTION

The Grimmiaceae is a monophyletic family (Tsubota et al., 2003; Hedderson et al., 2004; Hernández-Maqueda et al., 2007a, 2007b) sister to Ptychomitriaceae (La Farge et al., 2000; Goffinet et al., 2001; Hernández-Maqueda et al., 2007b). Recently, *Jaffueliobryum* Thér. and *Indusiella* Broth. & Müll. Hal., formerly part of the Grimmiaceae, have been shown to pertain to the Ptychomitriaceae, along with *Ptychomitrium* Fürnr., while *Campylostelium* Bruch & Schimp. either should be included in the Ptychomitriaceae or in its own family, the Campylosteliaceae (Hernández-Maqueda et al., 2007b). The re-circumscribed family Grimmiaceae includes *Racomitrium* Brid., a large monophyletic lineage that on morphological grounds has been split in four genera, i.e., *Bucklandiella* Roiv., *Codriophorus* P. Beauv., *Nipotrichum* (Bednarek-Ochyra) Bednarek-Ochyra and Ochyra, and *Racomitrium* (Ochyra et al., 2003; Bednarek-Ochyra, 2006), although this proposal is not universally accepted (i.e., Allen, 2005; Hill et al., 2006), nor has it been tested using molecular methods. This lineage is sister to the complex including the also speciose *Schistidium* Bruch & Schimp., *Dryptodon* Brid., and *Grimmia* Hedw. s.l., the latter including *Grimmia, Coscinodon* Spreng., and *Hydrogrimmia* Loeske (Hernández-Maqueda et al., 2007b). The first is a well defined genus both on morphological and molecular grounds. *Dryptodon* is monophyletic, but its species composition is still debatable, particularly because the characters used by Ochyra et al. (2003) to define the genus are highly homoplastic. Finally, *Grimmia* s.l., as currently understood, is paraphyletic (Hedderson et al., 2004; Streiff, 2006; Hernández-Maqueda et al., 2007b), and would include *Coscinodon* and *Hydrogrimmia* (Hernández-Maqueda et al., 2007b).

Index Muscorum, the monumental compilation of names of mosses, recognized almost 800 taxa in the genus *Grimmia* s.l. (van der Wijk et al., 1962, 1969). Such vastness, and the traditional stress on the importance of sporophytic characters to

recognize the taxa in the genus –although most species can be identified with confidence using exclusively the gametophyte–, led to its consideration as an almost intractable genus. Several recent treatments have reduced the final number to around 80 accepted species (Maier and Geissler, 1995; Muñoz, 1998; Muñoz and Pando, 2000; Maier, 2002; Greven, 2003; Hastings and Greven, 2006), although none of them have dealt with its infrageneric classification in detail.

As reviewed by Ochyra et al. (2003) and Streiff (2005), over 90 infrageneric taxa have been proposed in *Grimmia* (including *Dryptodon*), most of them between the publication of Bruch and Schimper (1845) and Brotherus (1924). Usually, subgenera definition essentialy rest on sporophytic traits, like seta orientation (straight, curved, or sigmoid) and capsule form (ovoid and symmetric vs. ventricose and asymmetric), while gametophytic traits like leaf margins stance (plane, recurved, or incurved) or cross-sectional shape of the leaves (keeled vs. concave) are used for sectional and lower taxa delimitation. Table IV.3.1 shows some of the proposed classification systems, of which the most widely used was formulated by Hagen (1909) and popularized by Brotherus (1924). Recently, Ochyra et al. (2003) raised Hagen's seven subgenera to generic rank, on the idea that they represent monophyletic groups. However, while evidence has been accumulated to support *Dryptodon* (= *Rhabdogrimmia* sensu Hagen, 1909) as a monophyletic independent lineage, the rest of Hagen's infrageneric divisions, as traditionally interpreted, are not monophyletic (Hedderson et al., 2004; Streiff, 2005; Streiff, 2006; Hernández-Maqueda et al., 2007a, 2007b). The incongruences in this system are evident from a macroscopic analysis of gametophytic and sporophytic traits. The most obvious problem is that species pairs exist that are gametophytically indistinguishables but possess sporophytes so diverse that are classified in separate subgenera (or genera, depending on the author). For example, *G. tergestina* and *G. poecilostoma* are identical gametophytically, but the first has straight setae and symmetric capsules and is included in subg. *Guembelia*

(≡ subg. *Litoneuron* ≡ subg. *Ovales*), while the latter has sigmoid setae and ventricose capsules and consequently is included in subg. *Grimmia*. Another stricking example involves the pair *G. ovalis*-*G. olneyi*; the first is the nomenclatural type of subg. *Guembelia*, characterized by straight setae and symmetric capsules, while the latter, which cannot be separated from *G. ovalis* on gametophytic grounds, is included in *Dryptodon* by its curved setae (Ochyra et al., 2003). The most striking species-pair incongruences invariably involve one taxon from the subg. *Grimmia* as currently circumscribed and a species of every other subgenera: they are identical gametophytically, but very disparate sporophytically. Hagen's system was created to accommodate the European taxa known to date, but from the above it is evident that in order to include all the variability observed in the genus it needs some refinements.

The objective of this study is to elucidate the phylogenetic relationships within a broadly understood *Grimmia* (Muñoz and Pando, 2000; Greven, 2003), as well as the systematic position of *Coscinodon* and *Hydrogrimmia*. In particular, we aim 1) to clearly define the species boundaries of *Dryptodon* and *Grimmia;* 2) to resolve the phylogenetic relationships within the *Grimmia* s.l. clade (including *Coscinodon*, *Hydrogrimmia*, and *Schistidium*) of Hernández-Maqueda et al. (2007b); 3) to evaluate the monophyly of the different infrageneric taxa recently resuscitated to generic status (*Grimmia* s.str., *Guembelia*, *Hydrogrimmia*, *Orthogrimmia*, and *Streptocolea*); 4) To test the distribution of the gametophytic and sporophytic traits in the chloroplast tree in order to elucidate which of the two phases better reflect the phylogeny of the complex.

For the first aim we selected the *rps4-trnL* region as target, and for subsequent aims we concatenated the *trnS-rps4-trnT-trnL-trnF*, and *trnK/matK* cpDNA regions. The use of the *trnK/matK* region for phylogenetic reconstructions is tested for the first time in bryophytes, and should be considered as an extra outcome of this study.

2. MATERIALS AND METHODS

2.1. Taxon and DNA sampling

The study was performed using 5 data sets. For our first aim, to correctly delimitate *Dryptodon* from *Grimmia* s.l., we selected 114 exemplars: 56 *Grimmia* and *Dryptodon* species, 8 *Schistidium*, and 12 outgroup taxa belonging to *Campylostelium*, *Indusiella*, *Jaffueliobryum*, *Ptychomitrium*, and *Racomitrium*. About 50% of the *trnL* and *rps4* sequences were taken from Streiff (2005), whereas the remaining sequences were generated for this study. For the second and third aims, we analyzed separately the region *trnS-rps4-trnT-L-F*, and the region *trnK/matK*, which allow us to search for incongruences between both partitions. Fifty nine terminals for the first region, and forty four for the second were successfully sequenced. Furthermore, a third matrix was analyzed for both regions combined for a total of forty four taxa. For the forth aim, ancestral state reconstruction for morphological traits was done using an independent matrix with forty one terminals including molecular and morphological data combined (total evidence approach). Vouchers are deposited in BCB, CHR, MA, MO, MUB, and S. Taxa authorship, Genbank accession numbers, and voucher information for newly sequenced taxa are collated in Table IV.3.2.

2.2. DNA isolation amplifications and sequencing

Total DNA of gametophore tips from dried herbarium specimens or recent collections was isolated using the NaOH method following the protocol described by Werner et al. (2002b), recommended for isolation of small quantities of dry material. PCR amplifications of the *rps4* gene, including the *trnS-rps4* spacer as well as the *rps4-trn-L*, *trnL-F*, and *trnK/matK* regions were performed in 50 µl–reactions containing 1.5 U *Taq* DNA polymerase, 1 mM dNTPs-Mix each 0.25 mM, 1 x buffer, 1.5 mM $MgCl_2$, 10 pmol of each amplification primer and 1µl of DNA. The

region amplified, primers used and its sequence, and reference authors are indicated in Table IV.3.3. Amplification cycles for the *rps4*, *rps4-trnL* and *trnL-F* were as follows: 2 min at 94ºC, 30 cycles with 2 min 94ºC, 1 minute 55ºC and 1 min 72ºC, and a final 7 min extension step at 72ºC. For the *trnK/matK* region: 3 min at 96º, 3 min at 50º and 3 min at 72º, 39 cycles with 30 seconds at 94º, 1 minute and 30 seconds at 48º and 3 minutes at 72º, followed with a 20 minutes extension step at 72º. Amplified products were cleaned using spin filter columns (PCR Clean-up DNA Purification Kit, MoBIO Laboratories, California) following the manufacturer's protocol. Cleaned products were directly sequenced using dye terminators (Big Dye Terminator v 2.0, Applied Biosystems, California) on automated sequencers, type ABI 3730XL (Applied Bioscience Inc.) at the sequencing service Centro de Sequenciación - Facultad de Ciencias Biológicas, Universidad Complutense de Madrid (Spain).

2.3. Data analysis

Sequences were edited and manually aligned using PhyDE® (Müller et al., 2005) following alignment rules described in Kelchner (2000) and Quandt and Stech (2004; 2005). Following the approach in Quandt et al. (2003) and Quandt and Stech (2004; 2005), the data matrix was screened for inversions using secondary structure models calculated with RNAstructure (Mathews et al., 2004). Detected inversions were positionally separated in the alignment. As discussed in Quandt et al. (2003) and Quandt and Stech (2004), presence or absence of detected inversions was not coded for the phylogenetic analyses. However, in order to gain information from substitutions within detected inversions they were reverse complemented and included in the analysis similarly to Hernández-Maqueda et al. (2007a; 2007b). For details on the complex intron evolution and the obserevd inversions we refer to Hernández-Maqueda et al. (2007b). Incomplete and

ambiguous data were identified and excluded from subsequent analyses. Alignments are available from www.treebase.com.

For phylogenetic inference, all characters were given equal weight, and gaps were treated as missing data. Parsimony analyses were conducted using winPAUP*4b10 (Swofford, 2002) and PRAP (Müller, 2004). The latter program generates command files for PAUP* that allow parsimony ratchet searches as designed by Nixon (1999) for analysis of large data sets. In the present study, 10 random addition cycles of 200 ratchet iterations each were used. Each iteration comprised two rounds of TBR branch swapping, one on a randomly re-weighted data set (25% of the positions), and the other on the original matrix saving one shortest tree. Since each random addition cycle rapidly converged to the same tree score, cycles were not extended to more than 200 iterations, nor were further cycles added. Shortest trees collected from the different tree islands were used to compute a strict consensus tree. Furthermore the data set was analysed employing a simple indel coding approach as advocated by Simmons and Ochoterena (2000) using the PAUP command file generated by Seqstate (Müller, 2005) and modified later by Müller (2006), with the same options in effect.

Internal branch support was estimated by heuristic bootstrap searches with 1000 replicates and 10 addition sequence replicates per bootstrap replicate. Decay values as further measurement of support for the individual clades were obtained employing a rachet search (command file generated by PRAP) in PAUP and the same options in effect as in the ratchet.

For further measurement of support, posterior probabilities were calculated using MrBayes v3.1 (Huelsenbeck and Ronquist, 2001) with a GTR model of nucleotide substitution and assuming site-specific rate categories following a gamma distribution and a proportion of invariable sites (GTR+Γ+I). In addition an independent analysis with an appended indel matrix was performed employing the binary model for the indel partition. The a priori probabilities supplied were those

specified in the default settings of the program. Posterior probability (PP, x100 in the figures) distributions of trees were created using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method and the following search strategies suggested by Huelsenbeck et al. (2001; 2002). Two runs with four chains each were run simultaneously for 10^6 generations each run, with the temperature of the heated chains set to 0.2. Chains were sampled every 10 generations and the respective trees were written to a tree file. Calculation of the consensus tree and of the posterior probability of clades was done based upon the trees sampled after the burn-in (25 %).

Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph (Müller and Müller, 2004). In those cases where we obtain congruent topologies employing different methods (MP or BI), the statistical values were represented in one consensus tree. Bootstrap frequencies > 75% and posterior probabilities > 95 are considered indicative of robustness.

The significance of differences between alternative tree topologies were evaluated by Shimodaira-Hasewaga tests under maximum parsimony (Shimodaira and Hasegawa, 1999) as implemented in winPAUP*4b10.

2.4. Combined analysis

To asses the homogeneity of the signals obtained from both regions, *rps4-trnT-L-F* and *trnK/matK*, we conducted an ILD test (homogeneity test) as implemented in winPAUP4.0b using 1000 replicates for each test.

2.5. Morphological analysis

Twenty five characters (Table IV.3.4) considered taxonomically important (Deguchi, 1978; Muñoz, 1998; Greven, 2003; Streiff, 2005) were included and analyzed for *Grimmia* s.l., *Dryptodon*, and *Schistidium*; the two latter genera were

included to facilitate the reconstruction of ancentral states. Characters reconstruction was done using parsimony with Mesquite 4.0 (Maddison and Maddison, 2006). Quantitative characters difficult to code as binary were not included.

3. RESULTS

The statistical information for the different analyses are presented in Table IV.3.5.

3.1. Analysis of rps4 and trnL-F sequences

From previous molecular studies (Hedderson et al., 2004; Streiff, 2006; Hernández-Maqueda et al., 2007b), *Dryptodon* and *Grimmia* seem to represent different lineages, but neither of the above studies clearly defined their species composition. Using 114 terminals, including some 70% of the species included in both genera, phylogenetic reconstructions based on the combined *rps4* gene and the *trnL-F* region using maximum parsimony (MP) resulted in a mostly unresolved tree (Fig. IV.3.1). However the separation of *Dryptodon* form *Schistidium* and *Grimmia* s.l. was evident and moderatly supported (77-79 bs) with *Dryptodon* species grouping togther with *Racomitrium* (77 bs). Adding cpDNA regions strongly increases the support for *Dryptodon*, as shown in Fig. IV.3.2 (Maximum Parsimony [MP]: 88 bs, 3 decay value [dv]; Bayesian Inference [BI]: 82 posterior probability [pp]). The clade (Fig. IV.3.1) including *Schistidium* and *Grimmia* s.l. is resolved with 79 bs. Inside *Grimmia* s.l. some groups can be recognized, e.g., *Grimmia* s.str. (91 bs), or *Orthogrimmia* (82 bs). With the exception of *Grimmia tergestina*, no significant infraspecific variation were detected (less than 1%).

3.2. Analysis of the rps4-trnT-L-F region

The consensus topology is ilustrated in Fig IV.3.2. The inclusion of the two non-coding spacers between *rps4* and *trnL* (*rps4-trnL* region) resulted in a more structured topology and stronger support.

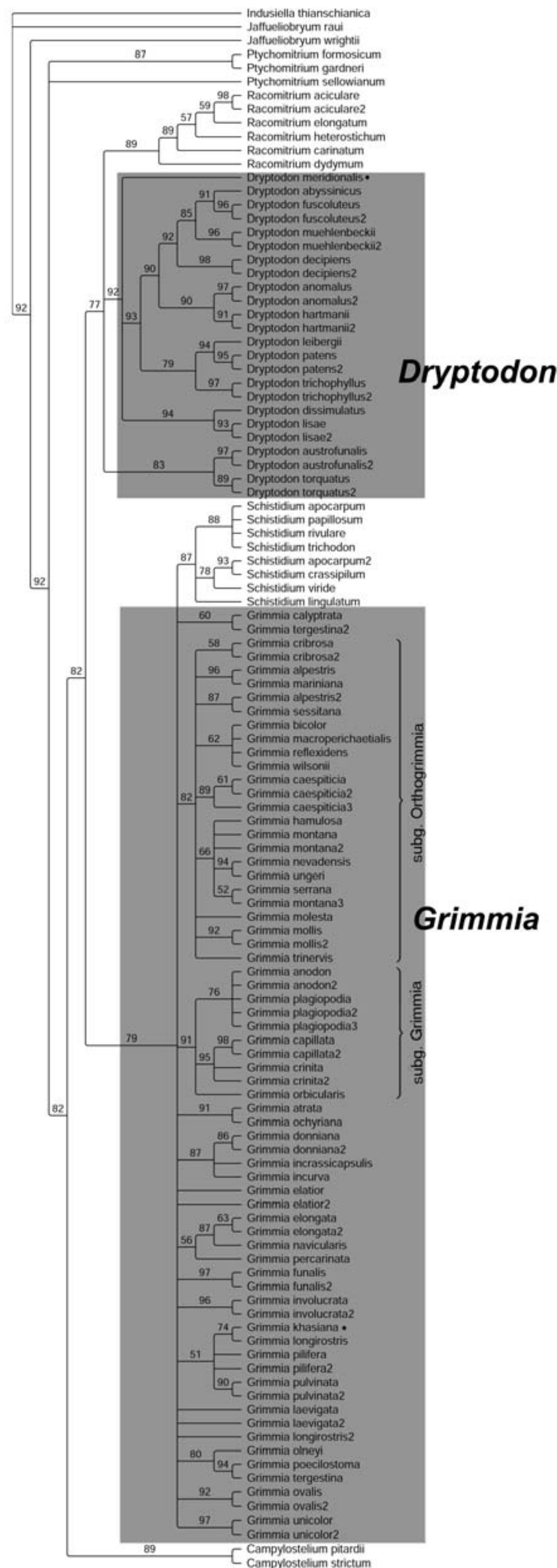


Fig. IV.3.1. Strict consensus tree of 1693 most parsimonious trees based on a combined analysis of *rps4* and *trnL-F*. Numbers above the branches indicate bootstrap values >50. * indicates species not accepted as independent in this study; *D. meridionalis* and *G. khasiana* are taxonomic synonyms of *Dryptodon trichophyllus* and *Grimmia longirostris*, respectively.

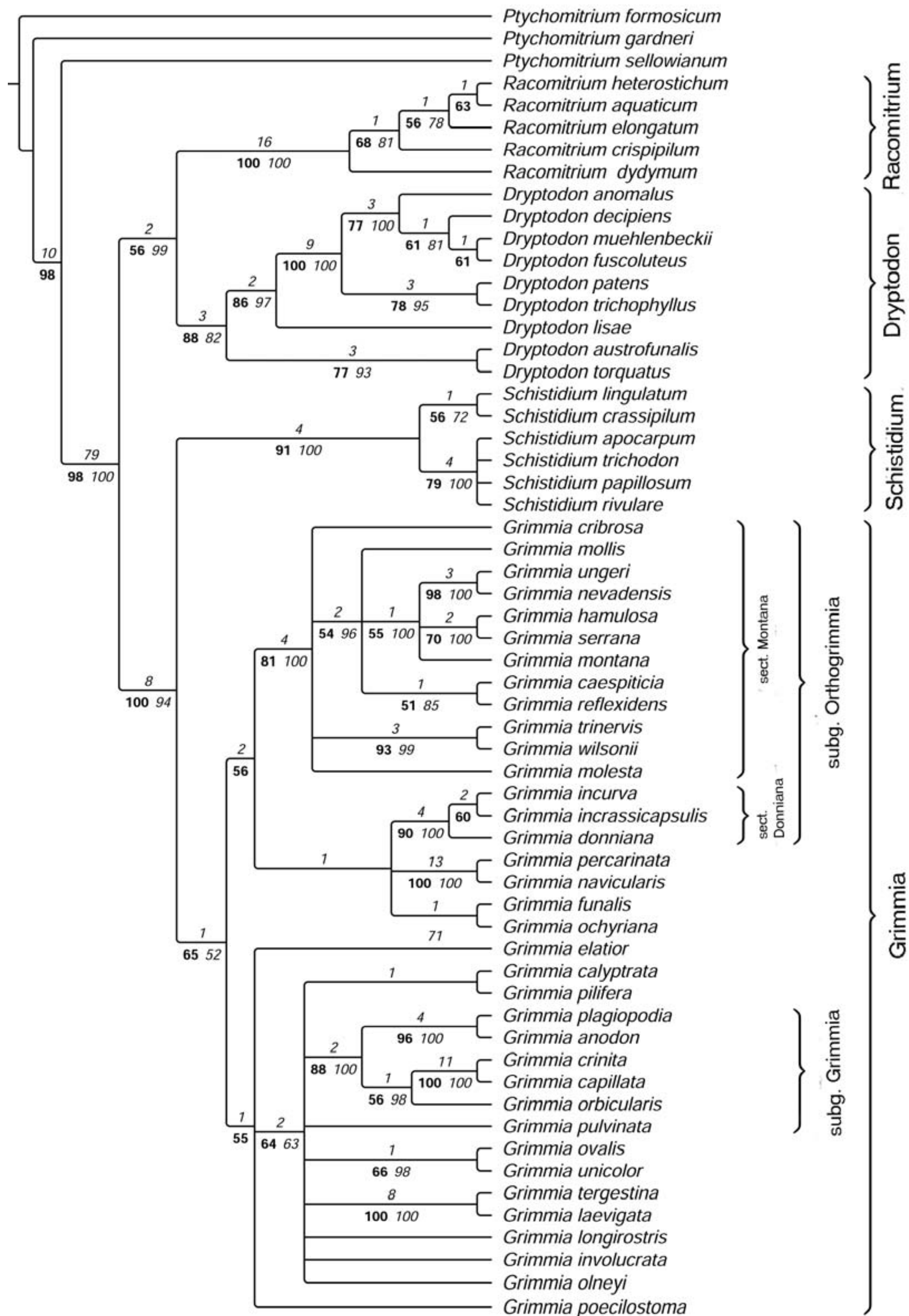


Fig. IV.3.2. Strict consensus tree of 386 most parsimonious trees employing sequence data from the plastid *trnS-rps4-trnT-L-F* cluster. Decay values (DV) are indicated in bold above the branches, whereas below the branches bootstrap support (BS) is indicated in bold and posterior probabilities (PP, ×100) obtained with Bayesian inference in italics. Taxa to the right are those recognized in the present study.

The final alignment had 2246 base pairs, of which 117 were variable but parsimony uniformative, and 258 were parsimony informative. Codification of indels as recommended in Müller (2006) increased the statistical support significantly. Of particular importance was the *P8* region of the *trnL* intron (Quandt and Stech, 2005). Its secondary structure models show highly conserved structures for the complete *P8* for *Grimmia* (compare Hernández-Maqueda et al., 2007b). Inside the Grimmiaceae two major clades are evident a) the weakly supported *Racomitrium*/*Dryptodon* clade and b) the maximally supported *Schistidium*/*Grimmia* s.l. clade. Whithin the shallow *Racomitrium*/*Dryptodon* clade *Racomitrium* is maximally supported (MP: 100 bs, 16 dv; BI: 100 pp) and a more elusive *Dryptodon* moderatly (MP: 88 bs, 3 dv; BI: 82 pp).

A monophyletic *Schistidium* (MP: 91 bs, 4 dv; BI: 100 pp) is resolved sister to *Grimmia* s.l., but without support (MP: 65 bs, 1 dv; BI: 52 pp). Within *Grimmia* s.l. the phylogenetic relationships are not fully resolved although some groups stand, like sect. *Montanae* (MP: 81 bs, 4 dv; BI: 100 pp), sect. *Donnianae* (MP: 90 bs, 4 dv; BI: 100 pp), or the pair *G. percarinata* and *G. navicularis* (MP: 100 bs, 13 dv; BI: 100 pp). Finally, *Grimmia* s.str. (including *G. plagiopodia*, type of the genus), of which the circumscription is amended in this study is also highly supported (MP: 88 bs, 2 dv; BI: 100 pp).

3.3. Analysis of the trnK/matK region (Fig. IV.3.3)

Forty eight taxa were analyzed; 40 of the ingroup and 8 outgroups. The final alignment comprised 2220 base pairs, of which 224 were variable but parsimony uniformative, and 286 were parsimony informative. No significant structural mutations were detected in the group II intron nor in the *matK* coding frame. The recovered tree basically depicts the same structure as found with the previous regions, but has considerably lower statistical suport values.

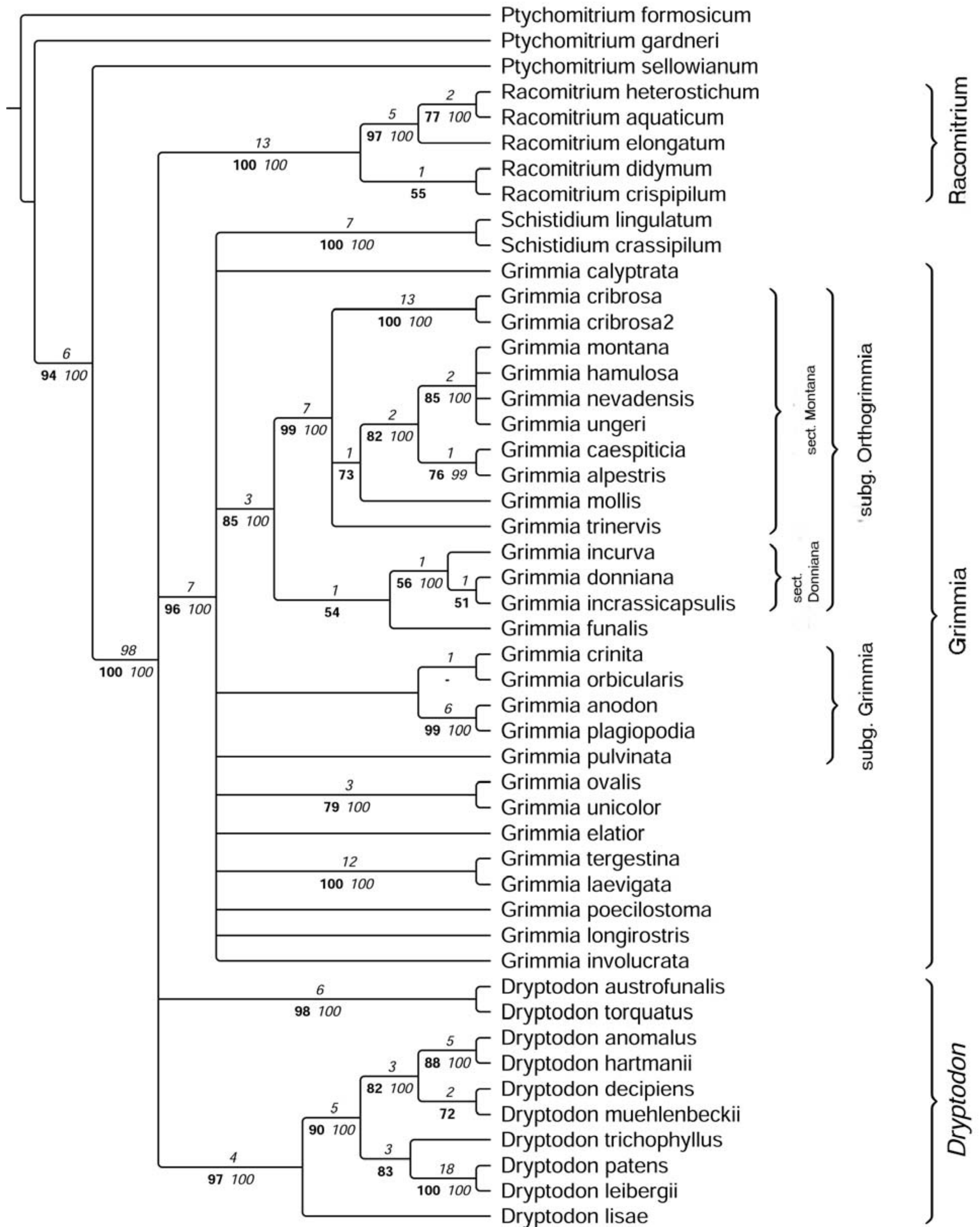


Fig. IV.3.3. Strict consensus tree of 811 most parsimonious trees based on the plastid *trnK/matK* region. Decay values (DV) are indicated in bold above the branches, whereas below the branches bootstrap support (BS) is indicated in bold and posterior probabilities (PP, ×100) obtained with Bayesian inference in italics. Taxa to the right are those recognized in the present study.

3.4. Analysis of rps4-trnT-L-F and trnK/matK combined

The final alignment had 4468 base pairs, of which 426 were variable but parsimony uniformative, and 502 were parsimony informative. The ILD test indicated that the combination of both data partitions in one matrix did not result in significantly incongruent trees (*P value* = 0.95). Figure IV.3.4 shows that the consensus topology obtained with the *rps4-trnT-L-F* region is better resolved than the corresponding *trnK/matK* consensus tree. The consensus tree for the forty four terminals using the combined data matrix is illustrated in Fig. IV.3.5. Compared to the separate analyses above, the resolution of the tree is highly increased for all clades (Fig. IV.3.5). Strikingly, *Schistidium* clearly retains a sister group position relative to *Grimmia* s.l., which now appears moderately supported (MP: 76 bs, 4 dv; BI: 89 pp).

3.5. Reconstruction of ancestral character states

The used of morpholgy and cpDNA combined (total evidence approach) using maximum parsimony produced the consensus topology in Fig. IV.3.6 and statistical values collated in Table IV.3.5.

Dryptodon is defined by three synapomorphic characters (Fig. IV.3.6, clade A): curved setae (character 8), ribbed capsule (19) and presence of gemmae (21). Characters 8 and 19 are also present in clade D (Fig. IV.3.6), and some terminals in *Grimmia* (*G. elatior* and *G. funalis*). Curiously, *Dryptodon austrofunalis* and *D. torquatus* share with the rare *Racomitrium vulcanicola* Frisvoll & Deguchi the presence of gemmae in stalks (22).

Schistidium is defined by five synapomorphic characters (Fig. IV.3.6): immersed (character 7) and systilius (9) capsules, perichaetial leaves much larger than the vegetative leaves (17), calyptra small (18), and annulus undifferentiated (24). The latter is diagnostic, in *Grimmia* s.l., for sect. *Montanae* (Fig. IV.3.6, clade B).

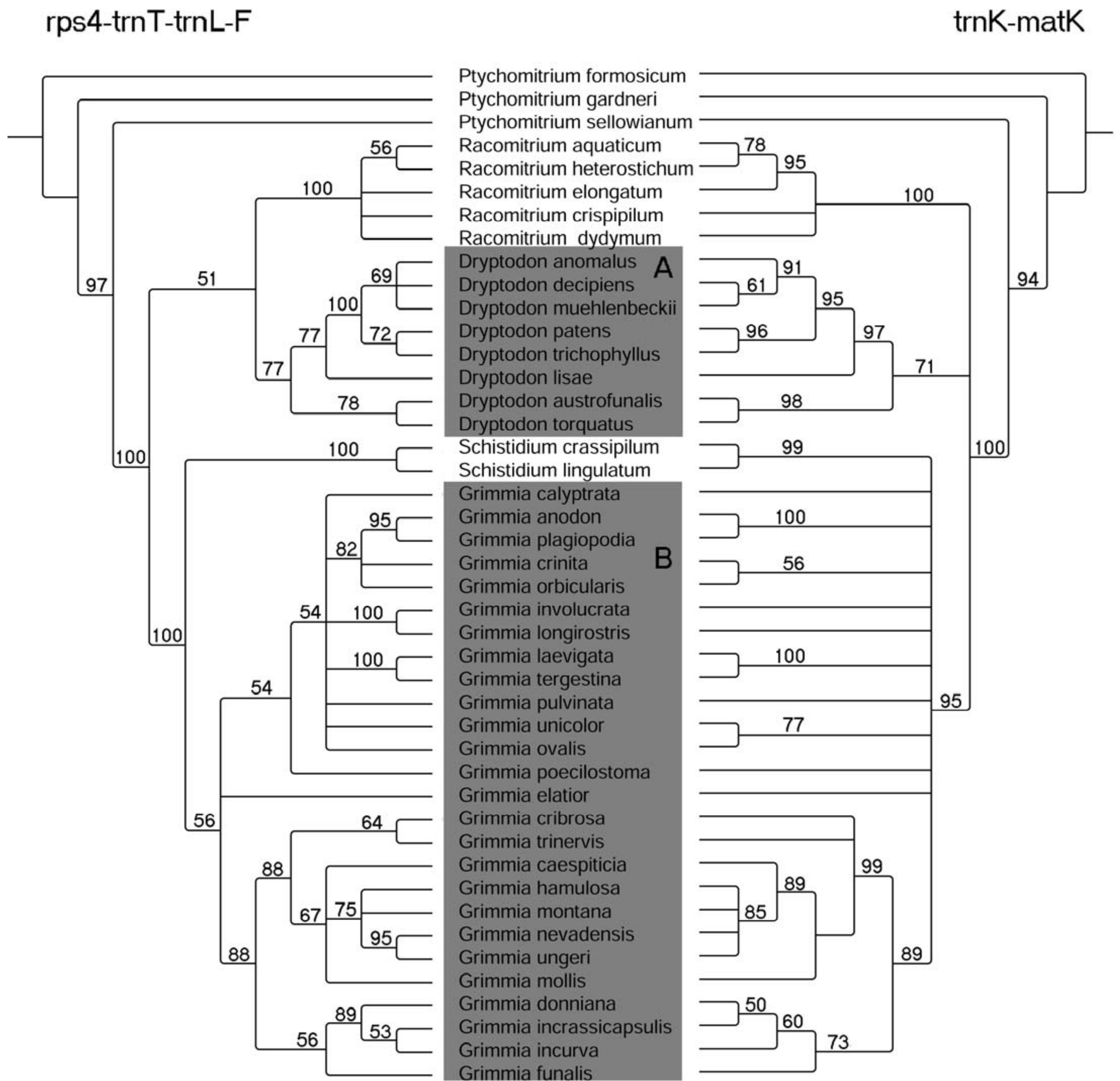


Fig. 4. Strict consensus topologies obtained under the assumption of maximum parsimony. The left tree represents the *trnS-F* region and the right tree the *trnK/matK* region. Boxes A and B represent *Dryptodon* and *Grimmia*, respectively.

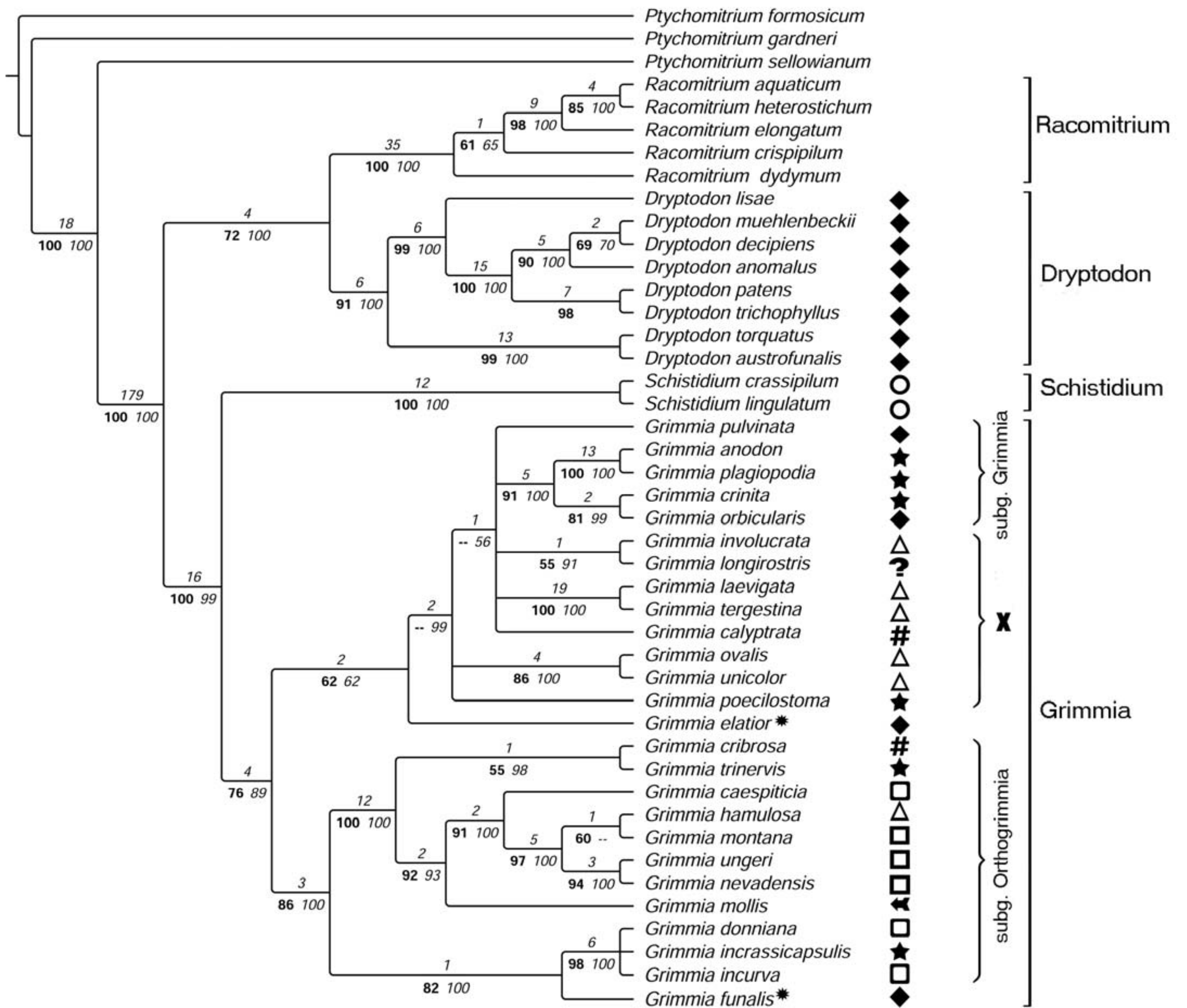
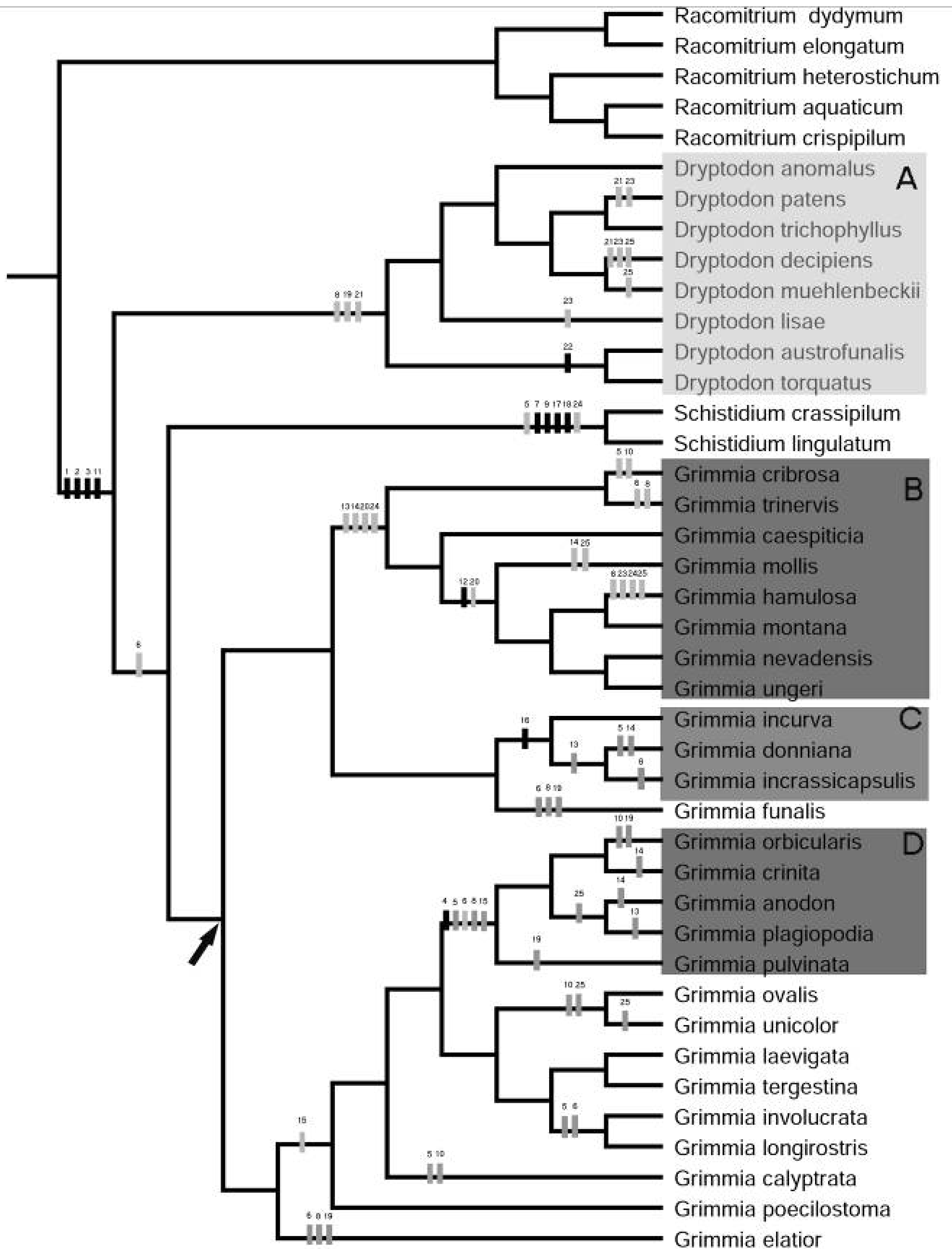


Fig. IV.3.5. Strict consensus tree of 37 most parsimonious trees based on the combined analysis of the *trnS-rps4-trnT-L-F*, and *trnK/matK* region (cpDNA). Decay values (DV) are above the branches, whereas below branches are bootstrap support (BS) in bold and posterior probabilities (PP, ×100) obtained with Bayesian inference in italics. The symbols represent different groups traditionally recognized for this species (rank unespecificied): ◆ = *Rhabdogrimmia*; **O** = *Schistidium*; **#** = *Coscinodon*; ★ = *Grimmia*; Δ = *Guembelia*; **?** = *Ovatae*; □ = *Orthogrimmia*; ◀ = *Hydrogrimmia*. Names to the right are taxa recognized in the present study, ✖ includes *Ovatae* and *Guembelia*.


Racomitrium dydymum
Racomitrium elongatum
Racomitrium heterostichum
Racomitrium aquaticum
Racomitrium crispipilum
Dryptodon anomalus
A
Dryptodon patens
Dryptodon trichophyllus
Dryptodon decipiens
Dryptodon muehlenbeckii
Dryptodon lisae
Dryptodon austrofunalis
Dryptodon torquatus
Schistidium crassipilum
Schistidium lingulatum
Grimmia cribrosa
B
Grimmia trinervis
Grimmia caespiticia
Grimmia mollis
Grimmia hamulosa
Grimmia montana
Grimmia nevadensis
Grimmia ungeri
Grimmia incurva
C
Grimmia donniana
Grimmia incrassicapsulis
Grimmia funalis
Grimmia orbicularis
D
Grimmia crinita
Grimmia anodon
Grimmia plagiopodia
Grimmia pulvinata
Grimmia ovalis
Grimmia unicolor
Grimmia laevigata
Grimmia tergestina
Grimmia involucrata
Grimmia longirostris
Grimmia calyptrata
Grimmia poecilostoma
Grimmia elatior

The reconstruction of ancentral states using maximum parsimony failed to identify synapomorphies defining *Grimmia* s.l. However, groups inside it are rather well characterized morphologically. The species around *G. montana* (sect. *Montanae*, Fig. IV.3.6, clade B) have plane margins (character 13), lamina regularly 2-4-stratose (14), and a simple and persistent annulus (24); only in this group appear species with plicate leaves (20). Also solidly joined are the species around *G. donniana* (sect. *Donnianae*, Fig. IV.3.6, clade C) by having basal marginal cells of the leaves with all the walls very thin and similar (16).

Grimmia s.str. (including *G. plagiopodia*, type of the genus) is defined by three synapomorphies (Fig. IV.3.6, clade D): leaves of epilose morphs are strongly modified and boat-shaped (character 4), a character not reported previously in the literature, monoicous sexual condition (5), and rather obtuse leaf apex (6). Of a total of 25 characters studied, 12 resulted non-homoplasious (Fig. IV.3.6, black rectangles), 4 for *Racomitrium*, 4 for *Schistidium*, 1 for *Dryptodon*, while only 3 characters resulted non-homoplaisous for *Grimmia*. The character setae straight vs. curved has been widely used to define groups in *Grimmia* s.l., and is a good choice to illustrate the high levels of homoplasy found in the studied species (Fig. IV.3.7).

4. DISCUSSION

The major result of this study is that the supraspecific taxa defined by cpDNA show a general correlation with gametophytically defined groups (Fig. IV.3.6).

Fig. IV.3.6. Reconstruction of ancentral states for 25 morphological characters under the maximum parsimony criterion. *Racomitrium* was chosen as outgroup. Boxes A, B, C, and D represent groups supported by morphological synapomorphies; the arrow indicates the origin of *Grimmia* as treated in the present study and the absence of synapomorphies to support it. Boxes over the branches represent non-homoplasious synapomorphies (black), and homoplasious synapomorphies (gray).

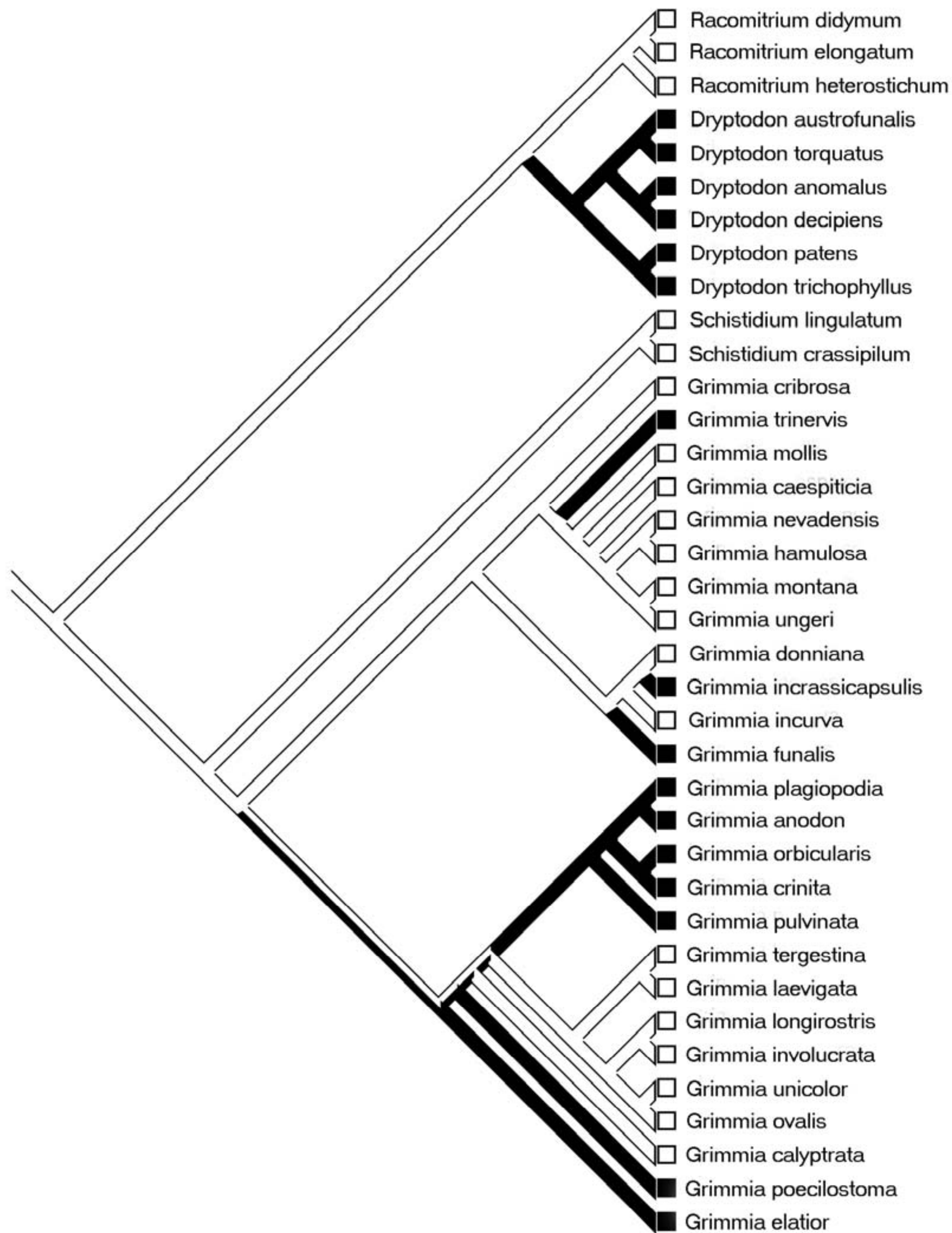


Fig. IV.3.7. Reconstruction of ancentral states for the character setae straight-curved under the assumption of maximum parsimony. Black branches show taxa with curved seta.

This is in open contradiction with the traditional and current taxonomy of *Grimmia* s.l. and *Dryptodon*, where the sporophyte was the main, sometimes the only, source of characters to define supraspecific taxa.

4.1. Delimitation of Dryptodon

Although all the analyses clearly separated *Dryptodon* from *Grimmia* s.l., the use of *rps4* and *trnL-F* to decide which species belong in either genera resulted in poorly structured topologies, as already found by Streiff (2006); also our results corroborate that the only diagnostic character to separate morphologically both genera are the presence of gemmae in *Dryptodon*.

On molecular grounds, *Dryptodon* occupies an intermediate position between *Racomitrium* and *Grimmia*. In a previous study (Hernández-Maqueda et al., 2007b) we found that its relationships depended on the tree-building algorithm used: maximum parsimony linked it with the *Grimmia-Hydrogrimmia-Schistidium-Coscinodon* clade, whilst with maximum likelihood or bayesian inference it branched with *Racomitrium*. In the present study the addition of the *trnK/matK* sequences resolved *Dryptodon* more closely related to *Racomitrium*, in contrast with the traditional view in which all taxa in *Dryptodon* would pertain to *Grimmia* and would be only distantly related to *Racomitrium* (except *D. patens*, which according to some authors, e.g., Smith, (1978), would share characters of both genera). Although the genus has gained acceptance after Ochyra et al. (2003; but see Allen, 2005), the morphological circumscription of the genus is not clear and should be amended, specially because the characters employed by Ochyra et al (2003) to define *Dryptodon* –curved setae, ribbed capsules, and partially recurved margins– have proved to be homoplastic (Figs. IV.3.6 and IV.3.7). Although *Dryptodon* species are morphologically rather characteristic and share several synapomorphies as the racomitrioid habit, the lanceolate leaves unaltered in epilose morphs, with margins partially recurved, and the sporophyte with curved setae and ribbed capsules, only the presence of gemmae –specialized structures for asexual reproduction produced either on the leaf lamina or axils– unambiguously separate it from *Grimmia* s.l. The presence of gemmae also links *Dryptodon* and *Racomitrium*; *Dryptodon austrofunalis*, *D. torquatus*, and *Racomitrium vulcanicola* (Frisvoll, 1988)

share the production of gemmae on branched stalks formed on the dorsal surface of the costa basis (character 22, Fig. IV.3.6), and in the latter species the stalks can also arise from stem epidermic cells at the leaves axils, like in *D. lisae* and *D. muehlenbeckii*.

Despite the strength of phylogenetic signal and the high resolution of the reconstructed relationships (Figs. IV.3.1-3, IV.3.5), a morphological delimitation for *Dryptodon* is problematic due to two species, *Grimmia funalis* and *G. elatior*, that unequivocally pertain to it morphologically, appear however solidly anchored in *Grimmia* s.l. according to the molecular phylogenies. Forcing *G. elatior* and *G. funalis* into *Dryptodon* significantly increased tree length (Shimodaira-Hasewaga test: Δ -ln L = 145.605, $P << 0.001$), confirming that the high support values in the cpDNA trees reflect true relationships. The lack of morphological correspondence with the molecular phylogeny in these two species is puzzling, and we cannot offer a solid explanation at the moment. Further research at population level could help resolving these intriguing results, and what phenomenon (e.g., lateral gene transfer, hybrydization, neoteny, adaptative convergence, etc.) could be responsible for this inconsistence.

When the cpDNA regions are analyzed individually, the clade joining *Dryptodon* species is internally more structured than the clade grouping *Grimmia*. This was previously noted by Streiff (2006), who hypothesized that *Dryptodon* (= "Rhabdogrimmia" in Streiff, 2006) could have evolved earlier and contains more phylogenetic information. As an additional explanation we suggest that the presence of two mechanisms of reproduction, sexual and asexual by the presence of gemmae, could facilitate mutational events that would increase variability at molecular level. However, it must be noted that the addition of cpDNA regions (Fig. IV.3.5) resulted in similarly structured patterns, which would indicate that Streiff's (2006) observation could be the consequence of using individual genes, and would refer more to particular gene evolution rates than taxa evolutionary phenomena.

Besides, in the initial sampling there are more than two times as many *Grimmia* a.l. taxa in the sampling than *Dryptodon*. This taxon sampling difference was leveled out with increasing markers, hence the branching differences might be just a taxon sampling artefact. However, a specific study of the gene rate effect could help understanding the evolution of this particular group of species.

4.2. The position of Coscinodon, Hydrogrimmia, and Schistidium

Most of the species in those genera were originally described as *Grimmia*, and their status as independent genera has been long debated. Of them, *Schistidium* forms a monophyletic clade strongly supported according to all the analyses (Figs. IV.3.1-6), and it is well-defined morphologically by the costa cells undifferentiated in cross-section, the minute calyptrae, systilius capsules, and the lack of an differentiated annulus at the capsule mouth.

The genus *Coscinodon* was created to include the species with a large, campanulate and plicate calyptra covering most of the urn, and leaves usually plicate longitudinally. Gametophytically it is rather heterogeneous, with a set of taxa with strongly plicate leaves and one taxon with smooth leaves. Sporophytically it also shows high diversity, including taxa with long or short, straight setae, and also a taxon with coiled setae (Hastings, 1996, 1999). To complicate the scenario, variation of both life phases is not correlated. According to our results (Figs. IV.3.1-6), *Coscinodon* is polyphyletic, and their species show the closest relationships with the species of *Orthogrimmia* or *Guembelia* (whatever their taxonomic rank) with which they share a high level of gametophytic similarity. *Grimmia cribrosa* and *G. trinervis* (*Coscinodon cribrosus* and *C. trinervis*, respectively) are nested within *Orthogrimmia*, sharing the strongly plicate leaf lamina of *G. caespiticia* or, to a lesser degree, *G. alpestris* and *G. reflexidens*. On the other hand, *Grimmia calyptrata* (= *Coscinodon calyptratus*) falls within *Guembelia*, which includes the taxa more similar to it on gametophytic grounds. From our results it is clear that

the genus does not deserve taxonomic recognition, but its taxa should be treated as members of *Orthogrimmia* and *Guembelia*.

The genus *Hydrogrimmia* was described to individualize *Grimmia mollis* on the basis of its soft, broadly ovate to rounded leaves. Considered sometimes as an independent genus (e.g., Ochyra et al., 2003), its morphological divergence from other *Grimmia* species is surely due to its ecological requirements: irrigated rocks in or near mountain streams at high elevations or latitudes. Based on cpDNA, *Grimmia mollis* falls in the *Orthogrimmia* core next to the species with erect setae and lacking differentiated annulus (sect. *Montana*), and hence *Hydrogrimmia* cannot be maintained at any taxonomic rank but considered part of this taxon.

4.3. Monophyly of Grimmia s.str. (= subg. *Grimmia*)*, Guembelia, Orthogrimmia, and Streptocolea*

From the previous points it follows that *Grimmia* s.l. in the sense of this paper includes *Coscinodon*, *Hydrogrimmia* and the species of *Grimmia* (sensu Muñoz and Pando, 2000; or Greven, 2003) that are not part of *Dryptodon* (as amended in this paper) or *Schistidium*, clearly independent genera. Unfortunately, *Grimmia* s.l. as genus is difficult to define morphologically. Some subgroups within are easily discernible, but homoplasy in both gametophytic and sporophytic characters makes difficult to unequivocally characterize them. It can be argued that the generated trees are based on cpDNA, and consequently do not reflect the evolutionary history of the genus correctly, as hybridisation events and later introgression might have been occur. To test if the phylogenetic signal from the cpDNA is congruent with the other genomes we are currently expanding the study to include nuclear markers, mainly ITS. Streiff (2006) found this region to be highly variable in *Grimmia* (including *Dryptodon*), and could not use it in her studies. However, our ITS preliminary data set shows highly conserved parts in the alignment that contains phylogenetic information, providing a new source of characters to study deeper

relationships within *Grimmia* s.l. The taxonomic rank of the recognized groups inside *Grimmia* s.l. is not fully resolved by the present data set, and thus the question remains open pending further studies. However, we can derive a number of conclusions from the cpDNA and morphology analyses, that we present below in detail, treating conservatively the groups at subgeneric rank.

The traditional supraspecific groups defined by sporophytic traits in which *Grimmia* s.l. has been segregated are only relativately reflected in the molecular tree. There are however several species, that might serve as a key for a generic understanding of *Grimmia* in its broadest sense, with sporophytic traits conflicting with both gametophytic traits and cpDNA signal. Namely, the species with sigmoid to coiled setae and ventricose capsules, which have been so far grouped as subg. *Grimmia* (genus *Grimmia* according to Ochyra et al., 2003), fall scattered across the cpDNA cladograms and group with the species that are close in terms of gametophyte morphology. On the one hand we have found that *Grimmia plagiopodia* (type of the genus), *G. anodon* and *G. crinita* share with *G. capillata* (with short, straight setae, and symmetrical, plicate capsules), *G. orbicularis* and *G. pulvinata* (both with curved setae and symmetrical, ribbed capsules) the ovate-oblong leaves adopting a very peculiar boat shape when lacking hair-points, a character not noticed previously (Figs. IV.3.2-5). On the other hand, *Grimmia incrassicapsulis* and *G. trinervis*, that should be included in subg. *Grimmia* according to their sporophyte morphology, fall in all the cpDNA analyses within sect. *Donnianae* and sect. *Montanae*, respectively, next to the species to which they are identical gametophytically; *G. poecilostoma* is ambiguosly resolved sister to *Guembelia*; and *G. pitardii* pertains in fact to *Campylostelium* (Maier, 1998; Hernández-Maqueda et al., 2007a).

Grimmia subg. *Grimmia* is thus polyphyletic (Figs. IV.3.2-3, IV.3.5-6), and it must be re-defined to include the species with ovate-oblong, broadly acute to

obtuse leaves boat-shaped when epilose. Sporophytically, its species have variously flexuous to curved to coiled setae, and capsules from symmetrical to variously asymmetrical at the base. We interpret this variation as a reduction series from long, curved setae and ribbed, symmetrical capsules to short, sigmoid or coiled setae and ventricose capsules. This reduction series can be observed in natural populations of plants growing in extremely harsh and dry environments, as *G. orbicularis* ("moxleyi" expression, from the Mojave desert in U.S.A., or "persica" expression, from Iraq). Gametophytically, *Grimmia crinita* and *G. capillata* are basically small expressions of *G. orbicularis*, sharing with it leaf morphology and anatomy, calyptrae shape, cladautoicous sexual condition, habitat, and geographic distribution. They are here interpreted as two independent endpoints of a reduction series in sporophyte size and structure derived from a *G. orbicularis*-related ancestor (amplification of the *trnK/matK* was unsucessful for *G. capillata*, which is thus not included in the total evidence tree in Fig. IV.3.6). Finally, the position of *G. pulvinata* is ambiguous, although in the total evidence tree it is resolved sister to this group. Such reduction series have not been recognized so far in Grimmiaceae, but they are a common pattern in Pottiaceae, where the *Astomum-Hymenostomum-Weissia*, or *Desmatodon-Phascum-Pottia-Tortula* complex are striking examples (Zander, 1993; Werner et al., 2002a; Werner et al., 2004; Werner et al., 2005; Zander, 2006). Similarly, the highly distinctive entomophilous syndrome of the Splachnaceae sporophyte or the origin of cleistocarpy in this family are now recognized to have independent origins (Goffinet and Shaw, 2002; Goffinet et al., 2004).

Grimmia subg. *Guembelia* is not defined in our study by any synapomorphy (Fig. IV.3.5), although the close relationships of the pairs *G. ovalis*-*G. unicolor* and *G. laevigata*-*G. tergestina* are supported either by cpDNA and morphology (Figs. IV.3.2-3, IV.3.5-6). Morphologically, subg. *Guembelia* has been characterized by

the leaves with plane margins and costa undifferentiated from the lamina, cucullate calyptrae, and straight seta. In our results, species with these characteristics are somewhat related to taxa with costa differentiated from the lamina, recurved leaf margins, and mitrate calyptra (*G. calyptrata*, *G. longirostris*, and *G. pilifera*), which in our opinion would be better considered as a separate taxon (subg. *Ovatae* Loeske). However, the lack of support leaves the question open to future studies.

Grimmia subg. *Streptocolea* would include only *G. atrata* according to Ochyra et al. (2003). It is morphologically similar to *G. ochyriana*, which according to *rps4* and *trnL-F* sequences is its closest relative in the data set. Unfortunately, we were unsucessful in getting sequences from other partitions, which hinder any further conclusion regarding their relationships.

Grimmia subg. *Orthogrimmia*, morphologically defined by the keeled leaves with plane margins (except very rare expressions of *G. reflexidens*), and straight setae, is resolved monophyletic and related to the previous groups according to all the cpDNA analyses (Figs. IV.3.2-3, IV.3.5-6), which precludes its segregation at generic rank. Muñoz (1998) considered that two sections, *Donnianae* and *Montanae*, could be recognized within *Orthogrimmia*; sect. *Donnianae* includes species with marginal basal leaf cells with uniformly thin walls, mitrate calyptrae, and *elongata*-type annulus (compound and revoluble, cells quadrate), while sect. *Montanae* includes species with marginal basal leaf cells with transverse walls thicker than the longitudinal walls, cucullate calyptrae, and *Schistidium*-type annulus (simple and persistent, cells undifferenciated). The molecular data also clearly separate both sections (Fig. IV.3.5). Additionally, sect. *Montanae* is the only taxon including species which lack stomata in the base of the urn. This non-homoplasious character grouping *G. mollis, G. hamulosa, G. montana, G. nevadensis* and *G. ungeri* is also found in *G. alpestris*. The latter is joined with the

former species by *rps4-trnL-F* (Fig. IV.3.1) and *trnK/matK* (Fig. IV.3.3) partitions, but lack of amplification of the *trnT* region precluded its inclusion in the total evidence analysis.

4.4. The position of Grimmia funalis and *Grimmia elatior*

The cpDNA relationships of *G. funalis* and *G. elatior* are puzzling at the very least (Fig. IV.3.5), and we cannot offer any simple explanation for our results at present. Neither of the two share any particular morphological resemblance with the species with which they are grouped; on the contrary, they pertain in *Dryptodon* based on morphology, both gametophytically and sporophytically. These results would question the validity of the sporophytic and gametophytic traits currently used to define groups in *Grimmia*-*Dryptodon*. However, of a total of 53 species of *Grimmia* sampled (~ 70% of the accepted species), only these two taxa show cpDNA relationships highly deviating from gametophyte morphology based relationships. Further research at population level could help to disentangle the reasons for such relationships.

4.5. Use of the trnK/matK region for phylogenetic reconstructions and multiple gene analyses

Our results show that the *trnK/matK* region provides a higher number of parsimony informative sites than the *rps4-trnT-L-F* region, and would be useful to infer phylogenetic relationships at genus and species level in bryophytes. However, the lack of structural changes in the region (insertions/deletions, inversions, etc) most likely explains the poor resolution of the trees obtained, as compared with the *rps4-trnT-L-F* region.

In order to increase the resolution of the phylogenetic trees, we explored the concatenation of DNA regions as proposed by Rokas (2003), Wortley et al. (2005), or Quandt and Stech (2004; 2005) for bryophytes. This multiple gene analysis

using a selected group of species improved the resolution of the phylogenetic trees, increased the statistical support in most cases, and help separating *Schistidium* from *Grimmia* (Fig. IV.3.5).

5. CONCLUSIONS

The major result of this study is that the supraspecific taxa defined by cpDNA show a general correlation with gametophytically defined groups (Fig. IV.3.6), but not with the sporophytic traits that have been traditionally used for *Grimmia* systematics. This is in agreement what the situation in Pottiaceae and Splachnaceae, where a switch of paradigm, from sporophyte- to gametophyte-based taxonomy was confirmed by studies using molecular data (Goffinet and Shaw, 2002; Werner et al., 2002a; Goffinet et al., 2004; Werner et al., 2004; Werner et al., 2005).

The high homoplasy found on sporophytic characters (e.g., setae stance, Fig. IV.3.7) could be the explanation for the instability of the different classifications mainly based on sporophytic traits (illustrated by symbols in Fig. IV.3.5). Although the gametophytic traits also show high levels of homoplasy, this could be due to the influence of environmental conditions, as discussed by Vanderpoorten et al. (2002). *Grimmia* is a highly complex genus with only three non-homoplasious characters, two of them related to the gametophyte: leaves muticous in otherwise pilose species boat-shaped as opposed to leaved unmodified in epilose morphs, and basal leaf cells with transverse walls uniformly thin as opposed to transverse walls thicker than longitudinal walls. As both the gametophytic and sporophytic traits seem to be more labile than previously thought, an exhaustive search for new morphological characters seems mandatory in order to unequivocally define the groups outlined by cpDNA and morphology.

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Table IV.3.1. Several systematic treatments of the *Grimmia-Coscinodon-Hydrogrimmia-Dryptodon-Schistidium* complex. The systematic arrangement suggested by our data is presented as “Present Study”.

Schimper (1856)	Limpricht (1885-1890)	Hagen (1909)Brotherus (1924)	Loeske (1930)	Ochyra et al. (2003)	Present study
Genus *Grimmia*	Genus *Grimmia*	Genus *Grimmia*	Genus *Grimmia*	Genus *Grimmia*	Genus *Grimmia*
subg. *Gasterogrimmia*	subg. *Gasterogrimmia*	subg. *Gastrogrimmia*	subg. *Gastrogrimmia*	Genus *Dryptodon*	subg. *Grimmia*
subg. *Grimmia*	subg. *Grimmia*	subg. *Rhabdogrimmia*	subg. *Litoneuron*	Genus *Guembelia*	subg. *Guembelia*
subg. *Orthogrimmia*	subg. *Rhabdogrimmia*	sect. *Trychophyllae*	subg. *Alpestres*	Genus *Orthogrimmia*	subg. *Ovatae*
subg. *Guembelia*	subg. *Guembelia*	sect. *Torquatae*	subg. *Alpinae*	Genus *Streptocolea*	subg. *Orthogrimmia*
subg. *Schistidium*	Genus *Dryptodon*	subg. *Litoneuron*	subg. *Pulvinatae*	Genus *Hydrogrimmia*	sect. *Montanae*
Genus *Coscinodon*	Genus *Schistidium*	subg. *Guembelia*	subg. *Torquatae*	Genus *Schistidium*	sect. *Donnianae*
	Genus *Coscinodon*	sect. *Montanae*	subg. *Rhabdogrimmia*	Genus *Coscinodon*	Genus *Schistidium*
		sect. *Ovales*	Genus *Drypdodon*		Genus *Dryptodon*
		sect. *Funales*	Genus *Schistidium*		
		subg. *Streptocolea*	Genus *Coscinodon*		
		subg. *Hydrogrimmia*			
		subg. *Schistidium*			
		Genus *Coscinodon*			

Table IV.3.2. List of investigated specimens, with GenBank accession numbers for the regions sequenced, including voucher numbers and the herbaria where the specimens are kept. The new sequences obtained for this study are in italics.

species	Voucher or reference	*rps4* Genbank Accesion nº	*trnT-L* Genbank Accesion nº	*trnL-trnF* Genbank Accesion nº	*trnK-matK* Genbank Accesion nº
Campylostelium pitardii (Corb.) E. Maier	Hernández-Maqueda et al. (2007a)	DQ399605	Forthcoming	DQ399632	--
Campylostelium strictum (Solms.) Kindb.	Hernández-Maqueda et al. (2007a)	DQ399604	Forthcoming	DQ399631	--
Dryptodon abyssinicus (Müll. Hal.) A. Jaeger (*)	MA 21868	Forthcoming	--	Forthcoming	--
Dryptodon anomalus (Hampe) Loeske	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon anomalus2	Streiff (2005, as *Grimmia anomala*)	AJ845210	--	AJ847860	--
Dryptodon austrofunalis (Müll. Hal.) Ochyra & Zarnowiec	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon austrofunalis2	Streiff (2005, as *Grimmia austrofunalis*)	AJ845211	--	AJ847861	--
Dryptodon decipiens (Schultz.) Lindb.	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon decipiens2	Streiff (2005, as *Grimmia decipiens*)	AJ845215	--	AJ847865	--
Dryptodon dissimulatus (E. Maier) Ochyra & Zarnowiec	Streiff (2005, as *Grimmia dissimulata*)	AJ845216	--	AJ847866	--
Dryptodon fuscoluteus (Hook.) Ochyra & Zarnowiec	MA 21398	Forthcoming	Forthcoming	Forthcoming	--
Dryptodon fuscoluteus2	Streiff (2005, as *Grimmia fuscolutea*)	AJ845221	--	AJ847871	--
Dryptodon hartmanii (Schimp.) Limpr.	Hernández-Maqueda et al. (2007a)	DQ399623	--	DQ399650	Forthcoming
Dryptodon hartmanii2	Streiff (2005, as *Grimmia hartmanii*)	AJ845222	--	AJ847872	--
Dryptodon leibergii (Paris) Ochyra & Zarnowiec	MA 25022	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon lisae (De Not.) Loeske	S B13712	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon lisae2	Streiff (2005)	AJ845226	--	AJ847876	--
Dryptodon meridionalis (Müll. Hal.) Ochyra & Zarnowiec	Streiff (2005, as *Grimmia meridionalis*)	AJ845228	--	AJ847878	--
Dryptodon muehlenbeckii (Schimp.) Loeske	MA 22709	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon muehlenbeckii2	Streiff (2005, as *Grimmia muehlenbeckii*)	AJ845230	--	AJ847880	--
Dryptodon patens (Hedw.) Brid..	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon patens2	Streiff (2005, as *Grimmia ramondii*)	AJ845214	--	AJ847864	--

Dryptodon torquatus (Drumm.) Brid.	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Dryptodon torquatus2	Streiff (2005, as *Grimmia torquata*)	AJ845239	--	AJ847889	--
Dryptodon trichophyllus (Grev.) Brid.	Hernández-Maqueda et al. (2007a)	DQ399624	Forthcoming	DQ399651	Forthcoming
Dryptodon trichophyllus2	Streiff (2005, as *Grimmia trichophylla*)	AJ845240	--	AJ847890	--
Grimmia alpestris (F. Weber & D. Mohr) Schleich.	MA 21346	Forthcoming	--	Forthcoming	Forthcoming
Grimmia alpestris2	Streiff (2005)	AJ845237	--	AJ847887	--
Grimmia anodon Bruch & Schimp.	Hernández-Maqueda et al. (2007a)	DQ399619	Forthcoming	DQ399646	Forthcoming
Grimmia anodon2	Streiff (2005)	AJ845209	--	AJ847859	--
Grimmia atrata Miel. ex Hornsch.	S B70026	Forthcoming	--	Forthcoming	--
Grimmia bicolor Herzog	MO 4461458	Forthcoming	--	Forthcoming	--
Grimmia caespiticia (Brid.) Jur.	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia caespiticia2	Streiff (2005)	AJ845212	--	AJ847862	--
Grimmia caespiticia3	MA 24716	Forthcoming	--	Forthcoming	--
Grimmia calyptrata Drumm.	Hernández-Maqueda et al. (2007a)	DQ399614	Forthcoming	DQ399641	Forthcoming
Grimmia capillata De Not.	MA 24719	Forthcoming	Forthcoming	Forthcoming	--
Grimmia capillata2	MA 18789	Forthcoming	--	Forthcoming	--
Grimmia cribrosa (Hedw.) Spruce	Hernández-Maqueda et al. (2007a)	DQ399615	Forthcoming	DQ399642	Forthcoming
Grimmia cribrosa2	Streiff (2005)	AJ845205	--	AJ847855	Forthcoming
Grimmia crinita Brid.	Hernández-Maqueda et al. (2007a)	DQ399620	Forthcoming	DQ399647	Forthcoming
Grimmia crinita2	Streiff (2005)	AJ845213	--	AJ847863	--
Grimmia donniana Sm.	MA 15356	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia donniana2	Streiff (2005)	AJ845217	--	AJ847867	--
Grimmia elatior Bruch ex Bals.-Criv. & De Not.	S B51986	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia elatior2	Streiff (2005)	AJ845218	--	AJ847868	--
Grimmia elongata Kaulf.	S B53421	Forthcoming	--	Forthcoming	--
Grimmia elongata2	Streiff (2005)	AJ845219	--	AJ847869	--
Grimmia funalis (Schwägr.) Bruch & Schimp.	Hernández-Maqueda et al. (2007a)	DQ399625	Forthcoming	DQ399652	Forthcoming
Grimmia funalis2	Streiff (2005)	AJ845220	--	AJ847870	--
Grimmia hamulosa Lesq.	MA 25701	Forthcoming	Forthcoming	Forthcoming	Forthcoming

Grimmia incrassicapsulis B.G. Bell	CHR 503516	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia incurva Schwägr.	Hernández-Maqueda et al. (2007a)	DQ399622	Forthcoming	DQ399649	Forthcoming
Grimmia involucrata Cardot	MA 27659	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia involucrata2	MA 27658	Forthcoming	Forthcoming	Forthcoming	--
Grimmia khasiana Mitt.	Streiff (2005)	AJ845224	--	AJ847874	--
Grimmia laevigata (Brid.) Brid.	MA 25401	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia laevigata2	Streiff (2005)	AJ845225	--	AJ847875	--
Grimmia longirostris Hook.	MA 21394	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia longirostris2	Streiff (2005)	AJ845227	--	AJ847877	--
Grimmia macroperichaetialis Greven	MO 5137774	Forthcoming	--	Forthcoming	--
Grimmia mariniana Sayre	MA 25490	Forthcoming	--	Forthcoming	--
Grimmia molesta J. Muñoz	MA 26046	Forthcoming	Forthcoming	Forthcoming	--
Grimmia mollis Bruch & Schimp.	S- B6791	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia mollis2	Streiff (2005, as *Hydrogrimmia mollis*)	AJ845206	--	AJ847856	--
Grimmia montana Bruch & Schimp.	MA 13305	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia montana2	Streiff (2005)	AJ845229	--	AJ847879	--
Grimmia navicularis Herzog	MO 4430352	Forthcoming	Forthcoming	Forthcoming	--
Grimmia nevadensis Greven	CAS	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia ochyriana J. Muñoz	MA 21454	Forthcoming	Forthcoming	Forthcoming	--
Grimmia olneyi Sull.	MA 19068	Forthcoming	Forthcoming	Forthcoming	--
Grimmia orbicularis Bruch	MO 5217118	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia ovalis (Hedw.) Lindb.	Hernández-Maqueda et al. (2007a)	DQ399618	Forthcoming	DQ399645	Forthcoming
Grimmia ovalis2	Streiff (2005)	AJ845232	--	AJ847882	--
Grimmia percarinata (Dixon & Sakurai) Nog. ex Deguchi	MA 26664	Forthcoming	Forthcoming	Forthcoming	--
Grimmia pilifera P. Beauv.	MA 24934	Forthcoming	Forthcoming	Forthcoming	--
Grimmia pilifera2	Streiff (2005)	AJ845233	--	AJ847883	--
Grimmia plagiopodia Hedw.	Hernández-Maqueda et al. (2007a)	DQ399616	Forthcoming	DQ399643	Forthcoming
Grimmia plagiopodia2	Streiff (2005)	AJ845234	--	AJ847884	--
Grimmia plagiopodia3	MA 21686	Forthcoming	--	Forthcoming	--

Grimmia poecilostoma Cardot & Sebille	MA 24655	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia pulvinata (Hedw.) Sm.	Hernández-Maqueda et al. (2007a)	DQ399617	Forthcoming	DQ399644	Forthcoming
Grimmia pulvinata2	Streiff (2005)	AJ845235	--	AJ847885	--
Grimmia reflexidens Müll. Hal.	MO 5233641	Forthcoming	Forthcoming	Forthcoming	--
Grimmia serrana J. Muñoz, Shevock & D.R. Toren	MA 25708	Forthcoming	Forthcoming	Forthcoming	--
Grimmia sessitana De Not.	Streiff (2005)	AJ845236	--	AJ847886	--
Grimmia tergestina Bruch & Schimp.	MA 25013	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia tergestina2	Streiff (2005)	AJ845238	--	AJ847888	--
Grimmia tergestina3	MA14653	Forthcoming	--	Forthcoming	--
Grimmia trinervis R.S. Williams	MUB 13530	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia ungeri Jur.	Hernández-Maqueda et al. (2007a)	DQ399621	Forthcoming	DQ399648	Forthcoming
Grimmia unicolor Hook.	S B51960	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Grimmia unicolor2	Streiff (2005)	AJ845241	--	AJ847891	--
Grimmia wilsonii Greven	MO 5125736	Forthcoming	Forthcoming	Forthcoming	--
Indusiella thianschianica Broth. & Müll. Hal.	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	--
Jaffueliobryum raui (Austin) Thèr.	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	--
Jaffueliobryum wrighti (Sull.) Thèr.	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	--
Ptychomitrium formosicum Broth. & Yosuda	Hernández-Maqueda et al. (2007a)	DQ399601	Forthcoming	DQ399628	Forthcoming
Ptychomitrium gardneri Lesq.	Hernández-Maqueda et al. (2007a)	DQ399602	Forthcoming	DQ399629	Forthcoming
Ptychomitrium sellowianum (Müll.Hal.) A. Jaeger	Hernández-Maqueda et al. (2007a)	DQ399603	Forthcoming	DQ399630	Forthcoming
Racomitrium aciculare (Hedw.) Brid.	Hernández-Maqueda et al. (2007a)	DQ399609	Forthcoming	DQ399636	--
Racomitrium aciculare2	Streiff (2005)	AJ845207	--	AJ847857	--
Racomitrium aquaticum	MA22070	--	--	--	Forthcoming
Racomitrium carinatum Cardot	Hernández-Maqueda et al. (2007a)	DQ399610	Forthcoming	DQ399637	--
Racomitrium dydymum (Mont.) Jaeger	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Racomitrium elongatum Frisvoll	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Racomitrium heterostichum (Hedw.) Brid.	Hernández-Maqueda et al. (2007a)	DQ399608	Forthcoming	DQ399635	Forthcoming
Schistidium apocarpum (Hedw.) Bruch & Schimp.	Hernández-Maqueda et al. (2007a)	DQ399611	Forthcoming	DQ399638	--
Schistidium apocarpum2	Streiff (2005)	AJ845208	--	AJ847858	--

Schistidium crassipilum H.H. Blom	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Schistidium lingulatum Blom	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Schistidium papillosum Culm.	Hernández-Maqueda et al. (2007b)	Forthcoming	Forthcoming	Forthcoming	--
Schistidium rivulare (Brid.) Podp.	Hernández-Maqueda et al. (2007a)	DQ399613	Forthcoming	DQ399640	--
Schistidium trichodon (Brid.) Poelt	Hernández-Maqueda et al. (2007a)	DQ399612	Forthcoming	DQ399639	--
Schistidium viride H.H. Blom	MA 22105	Forthcoming	--	Forthcoming	--

Table IV.3.3. Primer sequences used in this study.

Region amplified	primer	Sequence 5'—3'	Reference
trnS-rps4	trnS-F	TAC CGA GGG TTC GAA TC	Souza-Chies et al., 1997
trnS-rps4	rps 5'	ATG TCC CGT TAT CGA GGA CCT	Nadot et al., 1994
trnL-F	C	CGA AAT CGG TAG ACG CTA CG	Taberlet et al., 1991
trnL-F	F	ATT TGA ACT GGT GAC ACG AG	Taberlet et al., 1991
rps4-trnL spacer	rps4-166F	CCA TAA TGA AAA CGT AAT TTT TG	Hernández-Maqueda et al., 2007a
rps4-trnL spacer	P6/7	CAT YGA GTC TCT GCA CCT	Quandt et al., 2004
rps4-trnL spacer*	RT185F	TCA AAA ACA TCA TAA CAT AAG AGA	Hernández-Maqueda et al., 2007a
rps4-trnT spacer*	A-Rbryo	AGA GCA CCG CAC TTG TAA TG	Hernández-Maqueda et al., 2007a
trnT-L spacer*	A-Fbryo	CAT TAC AAG TGC GGT GCT CT	This study (modification of Taberlet et al., 1991 primer A)
trnK/matK	trnKFbryo1	GGG TTG CTA ACT CAA TGG TAG AG	This study
trnK/matK	trnKRbryo4	TGG GTT GCC CGG GGC TCG AAC	This study
matK	trnKF426Grim	AAA TYA CCA GTG TGC TGA CT	This study
matK	matK1024F	TTC GTC GAC GTA TMC AAG ACA CTT C	This study
matK	psbARbryo	CGC TTT CGC GTC TTT CTA AAG	This study

Table IV.3.4. Characters included in the morphological data set coded as nominal.

Characters included in the morphological data set
1. Growth form cladocarpous (0) or acrocarpous (1)
2. Peristome with basal membrane (0), or without basal membrane (1)
3. Peristome teeth deeply divided (0), or undivided to shortly divided (1)
4. Leaves shape, in specimens lacking hairpoints of otherwise pilose species, unmodified (0), or boat-shaped, weakly to strongly cucullate, differing in shape from normally pilose leaves (1)
5. Sexual condition dioicous (0), or monoicous (1)
6. Leaf apex acute to obtuse (0), acute (1), or acuminate (2)
7. Capsules inmersed (0), or exerted (1)
8. Setae curved to sigmoid (0), or straight (1)
9. Capsules systilious, opercula and collumella falling together at capsules's dehiscence (0), or not systilious, collumella remaining inside the urn at dehiscence (1)
10. Calyptrae mitrate (0), cucullate (1), or campanulate (2)
11. Basal yuxtacostall cells strongly sinouse (0), or not to scarcely sinuose (1)
12. Stomata in the base of urns present (0), or absent (1)
13. Margen recurved at least in one side (0), or plane or incurved (1)
14. Laminal unistratose, only locally bistratose and then usually to the margins (0), or regularly 2-4-stratose (1)
15. Costa dorsally differentiated from the lamina (0), or undifferentiated from the lamina (1)
16. Proximal marginal cells of leaves with all walls similar, thin (0), or with transverse walls thicker than the longitudinal walls (1)
17. Perichaetial leaves much larger than the vegetative leaves (0), or similar to the vegetative leaves.
18. Calyptrae covering the urn (0), or covering only part of the urn (1)
19. Capsule ribbed (0), or smoth (1)
20. Leaves strongly plicate (0), or nor to weakly plicate (1)
21. Gemmae lacking (0), or present (1)
22. Gemmae grouped on branched stalks (0), or sessile on leaves (1)
23. Ventral layer of costa of 2 cells wide (0), or more than 2 cells wide (1)
24. Annulus simple and persistent (0), or compound and revoluble (1)
25. Hairpoints entire to weakly denticulate (0), strongly denticulate to dentate (1), or hairpoints absent (2)

Table IV.3.5. Statistical values obained for different combinations of regions. Length = length of the most parsimonious tree; Pi = number of parsimony informatives characters; CI = Consistency Index; RI = Rescaled index; RC = Rescaled consistency.

region	nº of taxa	nº of characters	nº of trees	lenght	Pi	CI	RI	RC
rps4-trnL-F	114	1286	1693	677	202	0.59	0.80	0.47
rps4-trnT-L-F	59	2246	386	820	258	0.68	0.77	0.53
trnK-matK	48	2220	811	860	286	0.66	0.76	0.50
rps4, *trnT-L-F*, *trnK-matK*	44	4486	37	1527	502	0.69	0.77	0.53
cpDNA + morphology	41	4511	9	1532	358	0.65	0.73	0.48

IV.4. Testing reticulation and adaptative convergence in the Grimmiaceae.

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ABSTRACT

Phylogenetic relationships based on plastid DNA sequences have been recently explored for the genus *Grimmia*, revealing a complex evolutionary history and many incongruencies with respect to traditional views. Based on empirical observations it was postulated that episodies of allopollyploydy and various hybrydization events triggered speciation in the genus *Grimmia*. Comparisons of genes from different genomes could therefore help to detect putative reticulations that can not be detected employing a single genome. For this purpose phyloegentic inferences based on the complete ITS region of nuclear ribosomal DNA were contrasted against plastid (*trnS-trnF*, *trnK/matK*) ones. The ITS region proved to be highly variable in *Grimmia*, with various lineage-specific indels interspersed among a considerable number of conserved regions that contained important phylogenetic information. The sectional placement of most of species is in congruence with previous results based on plastid DNA. However, some species seemingly combine the nuclear sequences of one section with chloroplast sequences of another. With the exception of *G. pulvinata*, the species of *Grimmia* subg. *Grimmia* are nested within *Grimmia* in plastid phylogenies, but sister to remaining *Grimmia* groups and closer to *Dryptodon* using nuclear DNA sequences. These observations could be explained by past reticulation events. However, according to the Shimodaira-Hasegawa (SH) test an alternative hypothesis with *Grimmia* subg. *Grimmia* being nested within *Grimmia* could not be rejected with the available data, and hence further research is needed to check this incongruence. On the other hand, an alternative topology with *G. tergestina* close to *G. laevigata* as revealed by plastid data was clearly rejected by a SH test, which confirms the observation that *G. tergestina* has the nuclear sequence of section *Orthogrimmia* and a chloroplast sequence of section *Guembelia*. We hypothesized that the best explanation for the origin of this species would be past reticulation events.

Keywords: Bryophyta, Grimmiaceae, *Dryptodon*, *Schistidium*, *Grimmia*, ITS, nuclear DNA sequences, reticulation events, adaptative convergence

1. INTRODUCTION

Current discussions regarding the systematics of *Grimmia* face the same problems as in many other groups: splitting versus lumping. Whereas Muñoz & Pando (2000) or Greven (2003) view *Grimmia* as one large genus, others treat the different subgenera in which the genus has been divided as independent genera (Ochyra et al., 2003; Goffinet y Buck, 2004). Recent molecular studies focused on the Grimmiaceae (Streiff, 2006; Hernández-Maqueda et al., 2007b, 2007c) partially resolved questions about the generic relationships in the family. For example, *Dryptodon* and *Schistidium* are now considered as independent genera, *Coscinodon* and *Hydrogrimmia* demonstrate to be better considered part of *Grimmia* (Hernández-Maqueda et al., 2007c), and *Grimmia pitardii* was transferred to the Campylosteliaceae (Hernández-Maqueda et al., 2007b). Despite the segregation of *Dryptodon* and *Schistidium*, two main issues remain: a) the ancestral character state reconstructions did not identify synapomorphies for the redefined *Grimmia*; and b) the species composition of the subgenera accepted in *Grimmia* are clearly in conflict with traditional views. The most stricking examples are *G. funalis* and *G. elatior*. Both are gametophytically and sporophytically similar to *Dryptodon*, but plastid DNA phylogenies place them nested within *Grimmia*. The adaptative convergence could be an explanation for such placement, but morphological similarities indicate that *Grimmia* could have experienced some episodes of hybridization (Muñoz, pers. observ.). Interespecific hybridization has long been recognized as an important phenomenon in plant evolution (Rieseberg, 1995; Burke y Arnold, 2001). Natcheva & Cronberg (2004) present an updated overview on bryophyte hybridization, which is rarely considered as an important evolutionary phenomenon in mosses; most often, bryologists consider that the effects of interspecific hybridization in bryophytes are confined to the sporophytic phase (e.g., Philibert, 1873). Incongruence between chloroplast and nuclear DNA sequences could be employed to detect putative reticulation events and thus

potential hybrids. Unfortunately, not much bryological literature deals with the topic, except for the work on *Sphagnum* (Shaw y Goffinet, 2000).

The present study is therefore aiming to explore the congruence between a nuclear region with the plastid phylogenies already published. For this purpose the internal transcribed spacer (ITS) region (18S-5.8s-26S) of the nuclear ribosomal DNA (nrDNA) was sequenced and compared to previous results (Hernández-Maqueda et al., 2007a; Hernández-Maqueda et al., 2007c). The ITS region has been widely used at generic and infrageneric levels in other plant groups (Baldwin et al., 1995), and Quandt and Stech (2003) reviewed its use in bryophytes. In particular, the ITS region has been employed to resolve phylogenetic relationships in *Sphagnum* (Shaw, 2000b), *Amblystegium* (Vanderpoorten et al., 2001), *Campylopus* (Stech, 2004), *Trichostomum* and related genera (Werner et al., 2005b), or *Dydymodon* (Werner et al., 2005a). From these studies it becomes evident that sequence variation is largely lineage dependent as, for example, the ITS region exhibits as much variation across certain Hypnalean families as it is observed among populations of single species of the genus *Mielichhoferia* (Shaw, 2000a). In addition, the lack of complete concerted evolution, putative presence of pseudogenes and paralogy are phenomena that can increase the homoplasy in phylogenetic relationships performed with ITS (Álvarez y Wendel, 2003). Testing alternative hypotheses by constraining phylogenetic relationships that reflect different taxonomic treatments could be a useful tool to detect the accuracy of the phylogenetic relationships obtained.

The present work tries to answer the following questions: a) how useful is the ITS region to study the phylogeny of *Grimmia*?, b) how congruent are the phylogenetic inferences based on the nuclear ITS region compared to plastid DNA?, and if not, how can we explain this incongruences?, and c) can we propose a solid classification of *Grimmia* considering all available data (morphology, plastid DNA and nuclear DNA)?

2. MATERIAL AND METHODS

2.1. Taxon and DNA sampling

Fourty eight taxa including species of *Grimmia*, *Schistidium* and *Dryptodon* were included in the analysis. Four *Racomitrium* species were selected as outgroup. Vouchers are deposited in BCB, CHR, MA, MO, MUB, and S. GenBank accession numbers, voucher numbers of the herbaria and origin of the specimens are listed in Table IV.4.1. A second reduced data set (44 terminals) with sequences from both plastid and nuclear DNA was used in a single matrix to evaluate the degree of congruence between both genomes. Plastid sequences derive from previous studies by the same group (Hernández-Maqueda et al., 2007a; Hernández-Maqueda et al., 2007b, 2007c).

2.2. DNA isolation amplifications and sequencing

Protocols followed for DNA isolation, amplification, purification and sequencing are described in detail in Hernández-Maqueda et al. (2007b). Amplification products for ITS region were generated using the following program: 2 min at 96 ºC, 30 cycles with 2 min 96 ºC, 1 minute 49 ºC, and 1 min 72 ºC, with the annealing temperature being increased 1 ºC per cycle that was stabilized once it reached 60 ºC. The final extension step at 72 ºC was set to 7 min. As amplification and sequencing primers we used 18S (5'- GGA GAA GTC GTA ACA AGG TTT CCG) designed by Spagnuolo et al. (1999) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC - 3') as reverse primer (White *et al.*, 1990).

2.3. Data analysis

Sequences were edited and manually aligned using PhyDE® (Müller et al., 2005). Direct sequences were thoroughly screened to detect putative superimposed nucleotide additivity patterns (SNAP) following Whittall et al. (2000). In addition,

each sequence was analyzed in order to detect putative pseudogenes. For this purpose obtained sequences were screened for pecularities in nucleotide composition (GC content) as well as substitution rates as recommended by Álvarez and Wendel (2003) and Bailey et al. (2003).

For phylogenetic inference, all characters were given equal weight, and gaps were treated as missing data. Parsimony analyses were conducted using *win*PAUP*4b10 (Swofford, 2002) and PRAP (Müller, 2004). The latter program generates command files for PAUP* that allow parsimony ratchet searches as designed by Nixon (1999) for analysis of large data sets. In the present study, 10 random addition cycles of 200 ratchet iterations each were used. Each iteration comprised two rounds of TBR branch swapping, one on a randomly re-weighted data set (25% of the positions), and the other on the original matrix saving one shortest tree. Since each random addition cycle rapidly converged to the same tree score, cycles were not extended to more than 200 iterations, nor were further cycles added. Shortest trees collected from the different tree islands were used to compute a strict consensus tree. Furthermore the data set was analysed employing a simple indel coding approach as advocated by Simmons and Ochoterena (2000) using the PAUP command file generated by Seqstate (Müller, 2005) and modified later by Müller (2006) with the same options in effect.

Internal branch support was estimated by heuristic bootstrap searches with 1000 replicates and 10 addition sequence replicates per bootstrap replicate. Decay values as further measurement of support for the individual clades were obtained using PRAP in combination with PAUP and the same options in effect as in the ratchet.

Maximum likelihood analyses were executed assuming a general time reversible model (GTR+Γ+I), and a rate variation among sites following a gamma distribution (four categories represented by mean). GTR+Γ+I was chosen as the model that best fits the data according to the Akaike Information Criterion by Modeltest v3.6

(Posada y Crandall, 1998) employing the Windows® interface MTgui (Nuin, 2005). The proposed settings by Modeltest v3.6 were executed in PAUP 4.0b10. For the ITS region the following settings were used: BaseFreq=(0.2240 0.2805 0.2711), Nst=6, Rmatrix=(0.1.1491 2.5223 0.6444 0.5111 2.7457), Shape=1.6661, and Pinvar=0.3445.

For further measurement of support, posterior probabilities were calculated using MrBayes v3.1 (Huelsenbeck y Ronquist, 2001), the GTR model of nucleotide substitution was employed, assuming site-specific rate categories following a gamma distribution and a proportion of invariable sites. In addition, an independent analysis with an appended indel matrix was performed employing the binary model for the indel partition. The *a priori* probabilities supplied were those specified in the default settings of the program. Posterior probability (PP) distributions of trees were created using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method and the following search strategies (Huelsenbeck et al., 2001; Huelsenbeck et al., 2002): two runs with four chains each were run simultaneously for 10^6 generations each run, with the temperature of the heated chains set to 0.2. Chains were sampled every 10 generations and the respective trees were written to a tree file. Calculation of the consensus tree and of the posterior probability of clades was done based upon the trees sampled after the burn-in (25%) Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph (Müller y Müller, 2004). In cases with congruent topologies employing different methods (MP or BI), the statistical values were represented in one consensus tree.

2.4. Statistical test for accuracy of the phylogenetic observations

<u>Incongruence length difference test (ILD)</u>. The degree of congruence between the ITS versus the plastid partition was evaluated based on the obtained ITS data

in combination with the *rps4-trnT-trnL-trnF* and *trnK/matK* sequence matrix of Hernández-Maqueda et al. (2007b; 2007c). We use the test proposed by Farris et al. (1995) based on the incongruence length difference (I_{MF}) of Mickevich and Farris (1981) as implemented in PAUP* ("partition homogeneity test"). The metric is computed for a number of random partitions of the combined data set. When 95% or more of those random partitions show an I_{MF} smaller than the original, we reject the null hypothesis and conclude that the data sets are significantly heterogeneous.

Shimodaira-Hasegawa Test (SH). We use Shimodaira and Hasegawa (1999) nonparametric test to compare alternative phylogenetic hypotheses statistically, using the GTR+Γ+I model with the settings proposed by Modeltest. Only taxa that showed conflicting positions with previous studies were explored: *Grimmia* subg. *Grimmia*, *G. funalis*, *G. elatior*, and *G. tergestina*. The analysis were run in *win*PAUP*b4.0 using 1000 bootstrap replicates and full parameter optimization of the model.

3. RESULTS

3.1. ITS Sequences

The ITS sequences of the species studied are highly variable. Length variations range from 515 nt in the species *Racomitrium elongatum* to 670 nt in *Grimmia elongata*. The greatest distance was found between *Schistidium crassipilum* and *Racomitrium elongatum*, with pairwise distances values up to 0.117. Variations within taxa range from 0.023 between species of sect. *Montanae* to 0.061 between species of the genus *Dryptodon*. For 6 species (*Grimmia caespiticia*, *G. funalis*, *G. involucrata*, *G. montana*, *G. orbicularis*, and *G. pulvinata*), multiple populations were sequenced. The highest intraspecific distance was observed in *G. orbicularis* (0.027), whereas no variation was observed in *G. involucrata* and *G. pulvinata*. Many structural repeat units were detected in the matrix, ranging in size from 1-2

nt to 86 nt in *Grimmia elongata* (position 457-543 of the final alignment). These long structural mutations also occurred in other species, which made the alignment occasionally difficult. In addition, some lineage-specific indels often associated with highly variable regions (hot spots) alternated with conserved regions, which caused that assessing homology was in some cases a problematic task. As a consequence, some regions, mainly located in the ITS1 spacer, had to be excluded from the analysis. After exclusion, the final matrix included 1511 characters. Positions 1-840 corresponding to the ITS1 spacer, 841-1019 to the 5.8s gene and 1020-1511 to the ITS2 spacer.

3.2. Polimorphysms in ITS

After a careful analysis of the different pherograms we could not identify polymorphys due to SNAP processes. Double peaks detected for a particular position in some pherograms were unambiguosly solved after analyzing the reverse primer (i.e., *G. involucrata* and *G. reflexidens*). Moreover, taxa with double peaks were re-sequenced to corroborate our observations. In every case a cleaner sequence resulted in the absence of these ambiguous positions. We conclude that the superimposed peaks observed are better explained by technical causes instead of presence of polymorphisms among the ITS copies of an individual.

GC-contents range from 59.6 % in the genus *Racomitrium* to 55.8 % in *Grimmia* sect. *Montanae.* Relative ratio tests revealed higher variation values for the spacers ITS1 and 2 compared to the 5.8S gene.

3.3. Phylogenetic analysis

The final alignment consists of 1511 base pairs, of which 156 were variable but parsimony uniformative, and 246 were parsimony informative.

The simple indel coding approach yielded another 254 parsimony informative characters.

The MP ratchet analysis retained 12 most parsimonious trees (MPT, length = 1639, CI = 0.636, RI = 0.769, RC = 0.489). Figure IV.4.1 shows the strict consensus tree with decay values and bootstrap support either with indel coding above branches and without below branches. The maximum likelihood tree (-ln 6979.03094) with bootstrap support indicated above the branches and posterior probabilities below is depicted in Fig. IV.4.2. Although an indel coding approach could not be employed in the likelihood analyses, it has been included in the Bayesian inference. Posterior probabilities with indel coding is indicated as second value below branches.

Coding of indels as characters according to Simmons and Ochoterena (2000) generally increased the statistical support for the clades especially at the tips of the tree, both in the MP and the bayesian analyses. Whereas some clades are largely unresolved in the MP analysis without indel coding, the support of some parts of the tree increases with the sic-matrix appended.

Using *Racomitrium* as outgroup, *Dryptodon*, *Schistidium* and *Grimmia* are grouped with strong support (Maximum Parsimony [MP]: 100/100 bootstrap support [bs], 13/11 decay value [dv]; Maximum Likelihood [ML]: 100 bootstrap support [bs]; Bayesian Inference [BI]: 1.0/1.0 posterior probability [pp]). *Dryptodon* is unresolved (MP: --/-- bs, 1/-- dv; ML: -- bs; BI: --pp). *Grimmia* subg. *Grimmia* (including *G. orbicularis, G. crinita, G. capillata, G. anodon*, and *G. plagiopodia*) form a monophyletic clade sister to remaining taxa (MP: 100/100 bs, 13/10 dv; ML: 100 bs; BI: 1.0/1.0 pp). *Schistidium* and the remaining species of G*rimmia* form a strongly supported clade (MP: 96/93 bs, 8/7 dv; ML: 100/95 bs; BI: 0.96/0.98 pp) in which *Schistidium* was maximally supported (MP: 100/100 bs, 31/22 dv; ML: 100 bs; BI: 1.0/1.0 pp) whereas *Grimmia* was moderately resolved (MP: 73/64 bs, 5/4 dv; ML: 85 bs; BI: 0.97/0.98 pp). Within the genus *Grimmia*,

subg. *Guembelia* obtains moderate support (MP: 88/75 bs, 4/3 dv; ML: 88 bs; BI: 1.0/1.0 pp). Finally, the two sections recognized within subg. *Orthogrimmia* are clearly resolved with high support (sect. *Montanae*: MP: 100/100 bs, 18/14 dv; ML: 100 bs; BI: 1.0/1.0 pp; sect. *Donnianae*: MP: 100/93 bs, 8/5 dv; ML: 100 bs; BI: 1.0/1.0 pp). Sister to sect. *Donnianae*, *Grimmia funalis* is resolved high support values (MP: 91/83 bs, 5/3 dv; ML: 91 bs; BI: 1.0/1.0 pp).

3.4. Accuracy of the phylogenetic observations

ILD test. The trees obtained with the ITS region provided slightly different topologies compared to trees obtained with plastid DNA (Hernández-Maqueda et al., 2007b, 2007c). The most significant conflicts were found in the position of the clade including *Grimmia orbicularis, G. crinita, G. capillata, G. anodon* and *G. plagiopodia* (*Grimmia* subg. *Grimmia*). The chloroplast sequences group them within genus *Grimmia*, but the nuclear sequences placed them in a intermediate position between *Dryptodon* and *Grimmia*. The position of *Grimmia tergestina* is also conflictive. It combines ITS sequences of *Orthogrimmia*-type and plastid sequences of *Guembelia*-type (Fig. IV.4.3). The ILD test showed a significant heterogeneity between plastid and nuclear data sets ($P < 0.001$). If the problematic species (i.e. *Grimmia* subg. *Grimmia* and *Grimmia tergestina*) were excluded, the *P* value increases ($P = 0.05$), indicating homogeneity between the two data sets.

Shimodaira-Hasegawa test. The results of the SH test evaluating alternative hypotheses for the placement of *Grimmia* subg*. Grimmia, G. funalis, G. elatior*, and *G. tergestina* are summarized in Table IV.4.2. Of the 5 alternative hypotheses tested, two of them, namely, a) the systematic position of *Grimmia funalis* and *G. elatior* nested within *Dryptodon*, and b) the placement of *G. tergestina* as a close relative of *G. laevigata*, were rejected with $P < 0.001$.

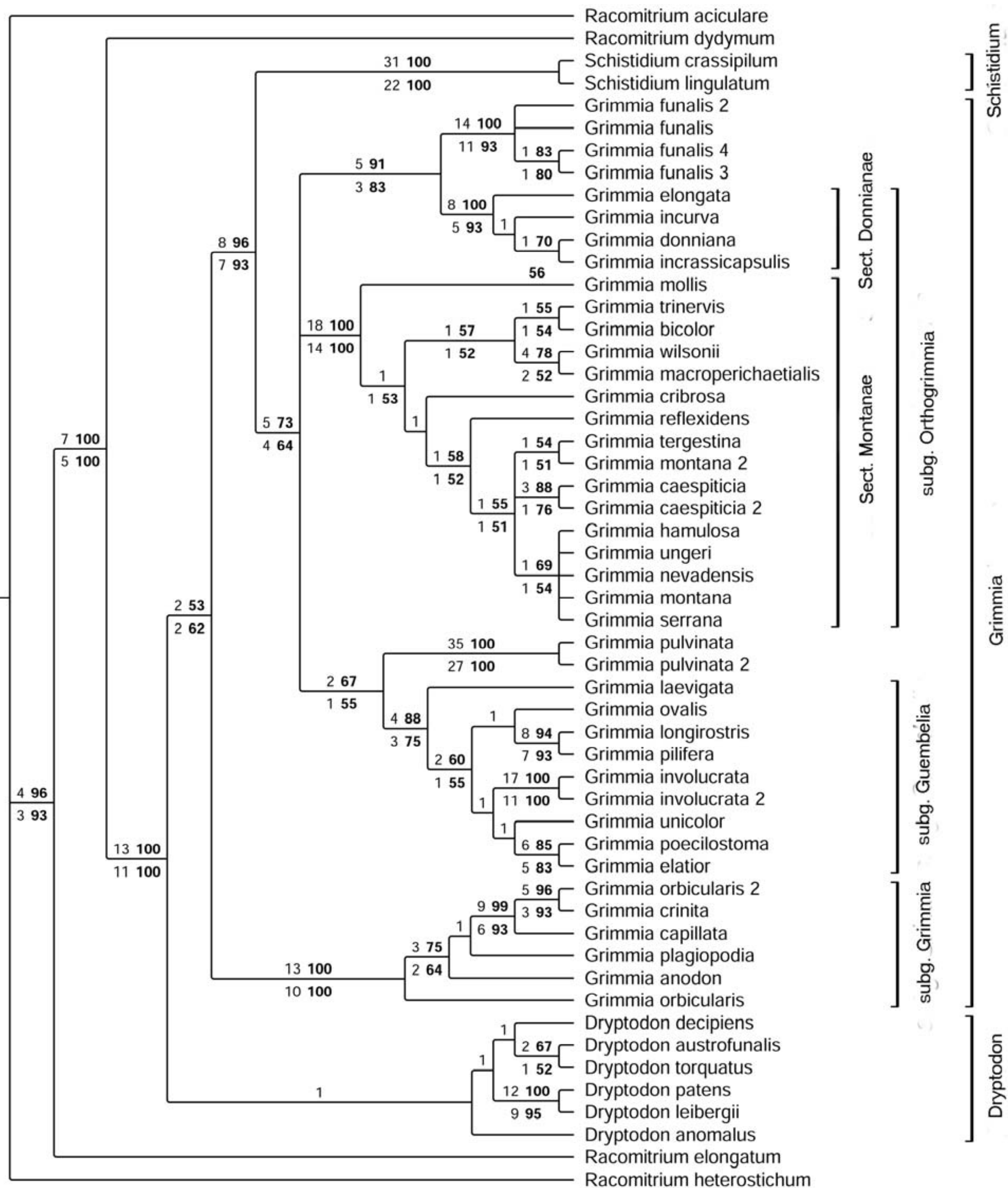

Fig. IV.4.1. One of 12 most parsimonious trees (length = 1639, CI = 0.636, RI = 0.769, RC = 0.489) found based on the analysis of the ITS region. Decay (left) and bootstrap support (right) values are shown above (with indel coding) and below (without indel coding) the branches.

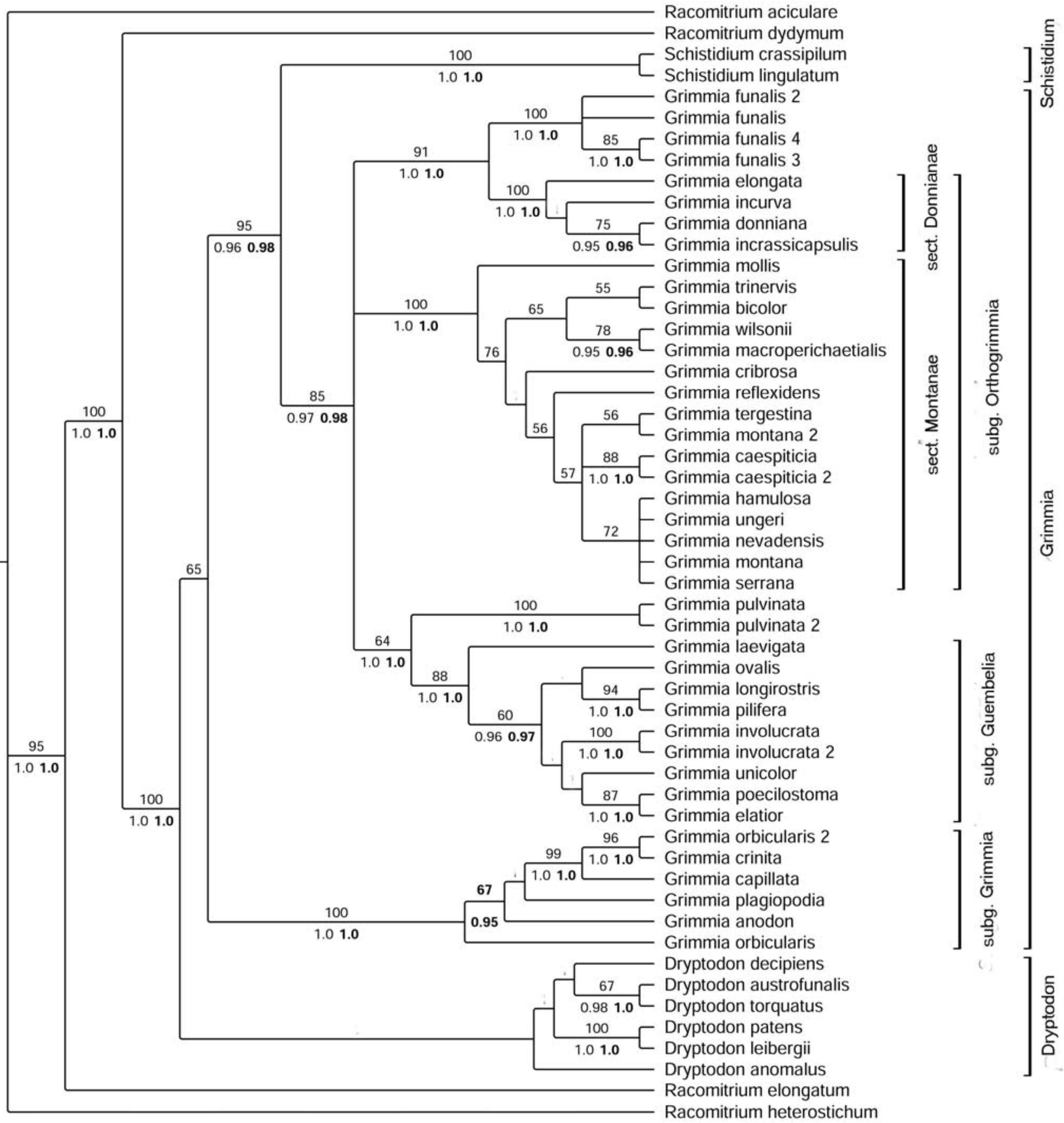

Fig. IV.4.2. Maximum likelihood tree (-ln 6979.03094) with ML bootstrap support values indicated above the branches and posterior probabilities (with and without indel coding), below. Only significant posterior probabilities ≥ 95 are depicted.

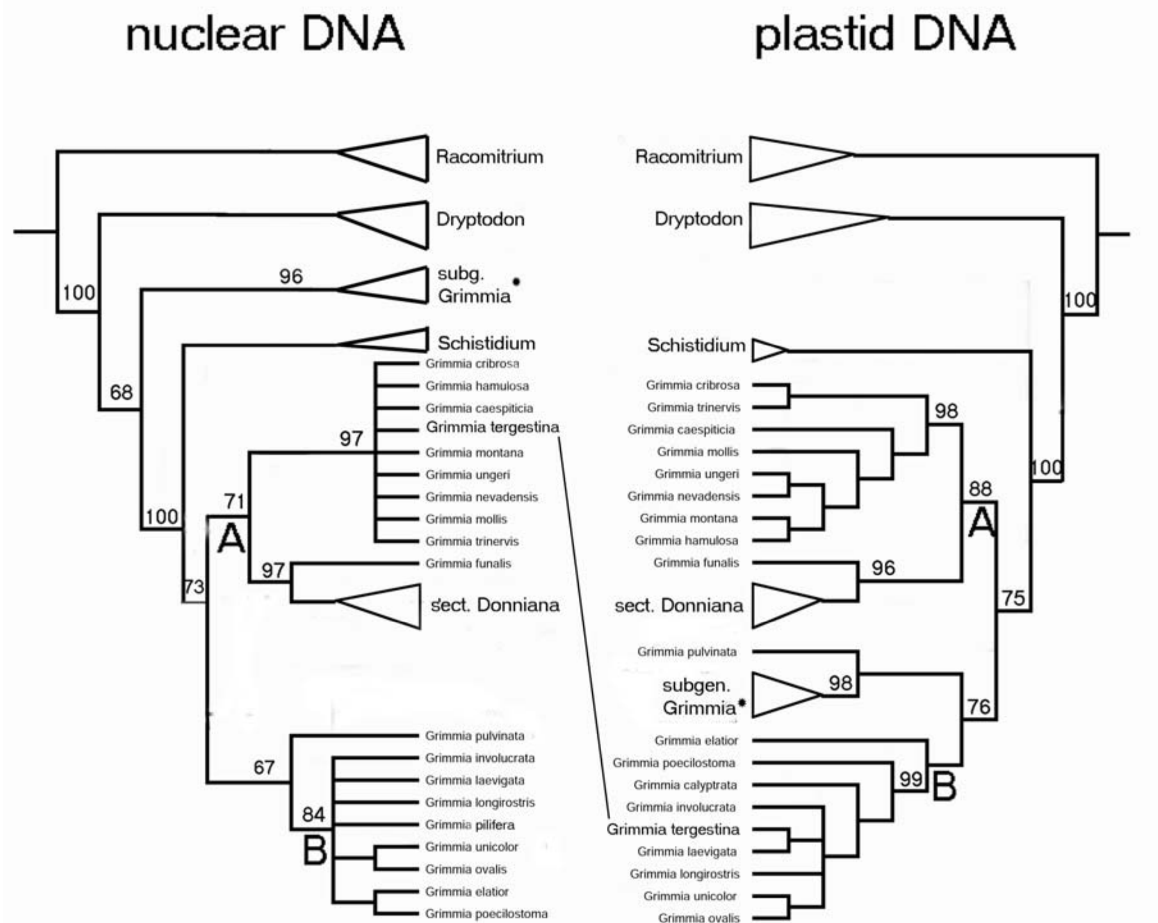

Fig. IV.4.3. Strict consensus topologies obtained from separate MP analyses of the ITS region and the combind chloroplast regions (*rps4-trnT-trnL-trnF* & *trnK/matK*), with bootstrap support values above the branches. A and B indicates the subgenera *Orthogrimmia* and *Guembelia*, respectively.

4. DISCUSSION

4.1. Molecular variability of the ITS region

The variability of the ITS region proved to be high and lineage-dependent in the *Grimmia-Dryptodon-Schistidium* complex. When *Dryptodon* and *Racomitrium* are included in the data set, a high number of gaps must be introduced in the matrix for a correct alignment. However, a large proportion of rather conserved regions were easily alignable and contained important phylogenetic information (Figs.

IV.4.1 and IV.4.2). The data reveals high inter- but very low intragroup variation. The most extreme case is the sequence variation within sect. *Montanae*, ranging from 0 to 0.023 and reflected in a large politomy (Fig. IV.4.3, in Figs. IV.4.1 and IV.4.2 those branches are poorly supported). For several conflicting taxa, namely, *G. caespiticia*, *G. funalis*, *G. involucrata*, *G. montana*, *G. orbicularis*, and *G. pulvinata*, more than one sequence was obtained, but the infraspecific variability detected does not have serious effects on the results. Two species, *G. pulvinata* and *G. orbicularis*, have polyploid populations (n = 13, 14, 26, 26+m, cf. Fritsch, 1991), and are therefore prime candidates for intraspecific ITS variability, although pairwise distances within them are not noteworthy. All sequenced populations of *G. pulvinata* cluster in the same clade, but this is not the case for *G. orbicularis*, for which presence of potential pseudogenes was studied as a source of variability. We rejected this hypotheses based on the higher substitution rates found in ITS1 and ITS2 compared to 5.8s and the lack of significant differences in GC-contents. According to Bailey et al. (2003), functional copies of the ITS region maintain the functional parts (5.8S gene) that are very conserved compared to the spacers ITS1 and ITS2. In contrast, the non-functional copies will show similar substitution rates across the complete region.

4.2. Phylogeny of Grimmia

Phylogenetic relationships within Grimmiaceae and specially within *Grimmia* and related genera have been recently discussed employing plastid DNA and morphology (Streiff, 2006; Hernández-Maqueda et al., 2007b, 2007c). According to these results, *Dryptodon* and *Schistidium* are independent genera, whereas *Coscinodon* and *Hydrogrimmia* pertain in *Grimmia*, which would include four subgenera: *Grimmia*, *Guembelia*, *Orthogrimmia*, and *Ovatae*. Results using plastid

data reflected conflicts with traditional views, pointing to complex evolutionary processes that can not be understood in the light of plastid DNA alone.

The current ITS data basically recognized the same groups as plastid DNA, and the placement of most of the species is also similar, but there are also differences. First, although the ITS matrix does not resolve *Dryptodon* as a monophyletic clade, it is well separated from *Grimmia*. This might be attributed to high rates of sequence divergence in the conserved blocks, which would provide considerable homoplasy at the deeper level. Besides, the region is quite short and more characters might be required to resolve *Dryptodon* monophyletic as reported for plastid data. Second, moderate support (Figs. IV.4.1 and IV.4.2) was obtained for *Guembelia*, which was only weakly supported with plastid DNA. Finally, the most dramatic changes with regard to plastid DNA were found in the position of *Grimmia* subg. *Grimmia* and the placement of *G. tergestina*, treated in more detail in points 4.3 and 4.5 below, respectively.

As the general aspects of the phylogenetic relationships of *Grimmia* have been discussed in detail elsewhere (Hernández-Maqueda et al., 2007c), in the following we will focus on the taxa which relationships inferred from ITS data are in conflict with traditional views.

4.3. Alternative hypotheses for the placement of Grimmia subg. Grimmia

Species in *Grimmia* subg. *Grimmia* are characterized by the leaves boat-shaped when muticous. According to recent molecular data (Hernández-Maqueda et al., 2007b, 2007c), *Grimmia* subg. *Grimmia* would include *Grimmia orbicularis, G. crinita, G. capillata, G. plagiopodia, G. anodon*, and *Grimmia pulvinata*. In all the cpDNA analyses, accessions of *G. pulvinata* were resolved sister to remaining species of the subgenus, and were included in this subgenus based on morphological affinities. Excluding *G. pulvinata*, remaining species were resolved in

different positions depending on the data set employed: nested within the genus *Grimmia* based on cpDNA, but sister to *Grimmia+Schistidium* employing ITS data (Fig. IV.4.3). The observed incongruence could reflect past reticulation events; polyploids are common in these species (Fritsch, 1991), which made them prime candidates to assume a hybrid origin. However, when we tested the placement of *Grimmia* subg. *Grimmia* within *Grimmia* as plastid DNA suggested, we found that the hypothesis cannot be rejected. This might be attributed to the low number of substitutions supporting the basal position of *Grimmia* in the ITS data, given that the region is rather short. Besides, a significant amount of information was obtained using an indel coding approach that has not been considered in the SH tests. Despite the fact that we can not demonstrate the hybrid origin of these species with the data on hand, we think that further investigations on nuclear DNA would corroborate this hypothesis.

4.4. Alternative hypotheses for the placement of G. funalis and G. elatior

Although these two species are equally resolved with plastid and nuclear DNA, we tested alternative hypotheses for the placement of both species as molecular data are incongruent with morphology and thus, traditional views. *Grimmia funalis* and *G. elatior* are morphologically indistinguishables from *Dryptodon* members, but resolved within the genus *Grimmia*, close to species morphologically very distant, based on molecular data. The four accessions of *G. funalis* sequenced show some puntual mutational differences, which makes this species interesting for population studies. As an alternative placement of *G. elatior* and *G. funalis*, we tested their inclusion in *Dryptodon*, but this hypothesis was rejected according to the SH test ($P < 0.001$, Table IV.4.2). In the light of the available data, their systematic position only can be explained by adaptive convergence.

4.5. Alternative hypotheses for the placement of G. tergestina

Grimmia tergestina shares nuclear sequences from subgenus *Orthogrimmia* and chloroplast sequences of subgenus *Guembelia*. Several molecular synapomorphies clearly support the relationships with each data set, while an origin from species in other sections is unlikely, both on molecular and morphological grounds.

Additionally, *G. tergestina* not only is of hybrid origin, but we hypothesize that it also acts as a parental species. It naturally hybridize with other *Grimmia* species and produces seemingly viable sporophytes, although it is unknown if they produce viable spores. One of such hybrids was described as *G. orbicularis* × *G. tergestina*, an invalid name later validated as *G. philibertii* Giacom. (Philibert, 1873; J. Muñoz, pers. obser., NY; Giacomini, 1950). Additionally, putative hybridization phenomena in *Grimmia* usually mention *G. tergestina* (Culmann, 1926; Loeske, 1930; Greven, 1995). *Grimmia tergestina* strongly resembles *G. americana*, *G. involucrata*, *G. poecilostoma* or *G. ovalis* (subg. *Guembelia*), and in fact it cannot be separated from the former three when sterile. *Grimmia americana* only differs in its gonioautoicous sexual condition, whereas *G. involucrata* (gonioautocous) and *G. poecilostoma* (dioicous) have curved setae and ventricose capsules. We hypothesize that these species have been originated by hybridization with *Grimmia* subg. *Grimmia* members (*G. orbicularis*?) based on the similarity of their sporophytes to some hybrid sporophytes (e.g., *G. philibertii*). The lack of intermediate characters is not a definitive reason to discard a hybrid hypothesis, as hybrids are not necessarily intermediate (Rieseberg, 1995). In bryophytes, gametophytic progeny results from recombination during meiosis and recombinants may inherit different parts of the parental genomes, express various combinations of parental traits, or may be more similar to one or the other parent. We believe that incongruence detected between plastid and nuclear DNA points to past reticulation events affecting the evolution of this species.

5. CONCLUSIONS

The ITS region has proved to be a useful molecular marker to infer phylogenetic relationships within *Grimmia*. Large structural mutations as well as lineage-specific indels affected the correct homology, especially between different genera (*Dryptodon*, *Racomitrium*, and *Grimmia*), but conserved regions show relevant phylogenetic signal. Detailed analyses and comparisons of nuclear and plastid DNA revealed that the evolutionary history of the genus *Grimmia* is much more complex than previously thought. The incongruence detected between phylogenetic relationships derived from plastid and nuclear DNA could be explained by reticulate evolution. However, in some cases reticulation could not be fully accepted due to either the rather low number of informative characters or the high levels of homoplasy in the ITS region. Alternative hypotheses placing *Grimmia* subg. *Grimmia* within *Grimmia* or nested within *Dryptodon* are found to be equally likely. In the case of *G. tergestina*, the reticulate evolution seems to be the better explanation for the detected incongruence. The relationships are supported by numerous base substitutions, and placements into other sections are very unlikely. If *G. tergestina* is an isolated example within the evolution of the genus or not is something that should be tested in further investigations. We feel that reticulation events should be taken into consideration in future research on the systematics of *Grimmia*, and bryophytes in general.

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Table IV.4.1. List of investigated specimens, with GenBank accession numbers for the regions sequenced, including voucher numbers of the herbaria where the specimens are kept and origin of each specimen.

Species	Voucher or reference	Origin	*ITS* Genbank Accesion nº
Dryptodon anomalus (Hampe) Loeske	MA-24709	Russia. Altay Republic	Forthcoming
Dryptodon austrofunalis (Müll. Hal.) Ochyra & Zarnowiec	MO-5211690	Bolivia. La Paz	Forthcoming
Dryptodon decipiens (Schultz.) Loeske	MA *JM7131*	Spain. Toledo	Forthcoming
Dryptodon leibergii (Paris) Ochyra & Zarnowiec	MA-25022	U.S.A. California	Forthcoming
Dryptodon patens (Hedw.) Brid.	MO-5142675	U.S.A. Alaska	Forthcoming
Dryptodon torquatus (Drumm.) Brid.	MA-25588	U.S.A. California	Forthcoming
Grimmia anodon Bruch & Schimp.	MA-25617	U.S.A. Nevada	Forthcoming
Grimmia bicolor Herzog	MO-4461458	Bolivia. La Paz	Forthcoming
Grimmia caespiticia (Brid.) Jur.	MA-24716	Spain. Avila	Forthcoming
Grimmia caespiticia2	MA-19713	Spain. Avila	Forthcoming
Grimmia capillata De Not.	MA-24719	Kazajistan. Mangyshlak	Forthcoming
Grimmia cribrosa (Hedw.) Spruce	MO-4441357	USA, Maine	Forthcoming
Grimmia crinita Brid.	MA-22641	Spain. Huesca	Forthcoming
Grimmia donniana Sm.	MA-15356	Italy. Val Venosta	Forthcoming
Grimmia elatior Bruch ex Bals.-Criv. & De Not.	S-B51986	Norway. Trams	Forthcoming
Grimmia elongata Kaulf.	S-B53421	Sweden. Torne Lappmark	Forthcoming
Grimmia funalis (Schwägr.) Bruch & Schimp.	MA-21988	Spain. Huesca	Forthcoming
Grimmia funalis2	MA-22007	Spain. Cantabria	Forthcoming
Grimmia funalis3	S-B64173	Norway, Finmark	Forthcoming
Grimmia funalis4	MA-21468	Russian. Chukotka	Forthcoming
Grimmia hamulosa Lesq.	MA-25701	U.S.A. California	Forthcoming
Grimmia incrassicapsulis B.G. Bell	CHR-503516	New Zealand. Otago	Forthcoming
Grimmia incurva Schwägr.	S-B70022	Sweden. Jamtlands Lam	Forthcoming
Grimmia involucrata Cardot	MA-27659	Mexico. Hidalgo	Forthcoming
Grimmia involucrata2	MA-27658	Mexico. Hidalgo	Forthcoming
Grimmia laevigata (Brid.) Brid.	MA-25401	Spain. Zamora	Forthcoming
Grimmia longirostris Hook.	MA-21394	Siberia. Yakutskaya	Forthcoming
Grimmia macroperichaetialis Greven	MO-5137774		Forthcoming
Grimmia mollis Bruch & Schimp.	S-B6791	Austria. Tirol	Forthcoming
Grimmia montana Bruch & Schimp.	MA-13305	Spain. Asturias	Forthcoming
Grimmia montana2	MA-14721	U.S.A. California	Forthcoming
Grimmia nevadensis Greven	CAS-C50Grev		Forthcoming
Grimmia orbicularis Bruch	MA-25043	U.S.A. California	Forthcoming
Grimmia orbicularis2	MO-5217118	U.S.A. Nevada	Forthcoming
Grimmia ovalis (Hedw.) Lindb.	MO-5217105	U.S.A. Nevada	Forthcoming
Grimmia pilifera P. Beauv.	MA-24934	Russia. Khabarovsk Kray	Forthcoming
Grimmia plagiopodia Hedw.	S-B70024	Sweden. Torne Lappmark	Forthcoming
Grimmia poecilostoma Cardot & Sebille	MA-24655	Siberia. Yakutskaya	Forthcoming
Grimmia pulvinata (Hedw.) Sm.	MA-25045	U.S.A. California	Forthcoming
Grimmia pulvinata2	MA-25026	U.S.A. California	Forthcoming
Grimmia reflexidens Müll. Hal.	MO-5233641	U.S.A. Colorado	Forthcoming
Grimmia serrana J. Muñoz, Shevoch & D.R. Toren	MA-25708	U.S.A. California	Forthcoming
Grimmia trinervis R.S. Williams	MO *Price 1547*	Bolivia. Cochabamba	Forthcoming
Grimmia ungeri Jur.	MA-25618	U.S.A. Nevada	Forthcoming
Grimmia unicolor Hook.	S-B51960	Sweden. Västra Götaland	Forthcoming
Grimmia wilsonii Greven	MO-5125736		Forthcoming
Racomitrium aciculare (Hedw.) Brid.	MA-22069	Spain. Cantabria	Forthcoming

Racomitrium dydymum (Mont.) Jaeger	MA-25251	Chile. Región de los Lagos	Forthcoming
Racomitrium elongatum Frisvoll.	MA-13319	Spain. Palencia	Forthcoming
Racomitrium heterostichum (Hedw.) Brid.	MO-5125302	U.S.A. California	Forthcoming
Schistidium crassipilum H.H. Blom	MA-14862	Spain. Granada	Forthcoming
Schistidium lingulatum Blom	MA-26281	USA: Washington	Forthcoming

Table IV.4.2. Results of Shimodaira-Hasegawa (SH) test for comparison of alternative phylogenetic hypotheses- A p value < 0.05 rejects the hypotheses.

Hypotheses	Shimodaira-Hasegawa test		
	-lnL	**diff-lnL**	**P values**
ML tree	6979.03094	--	--
Grimmia funalis and *Grimmia elatior* included in a monophyletic *Dryptodon*	7053.78692	74.75598	0.001*
Grimmia subgen. *Grimmia* nested within *Grimmia*	6987.17943	8.14849	0.508
Grimmia subgen. *Grimmia* nested within *Dryptodon*	6988.25067	9.219743	0.487
Schistidium basal to *Grimmia*	6997.40764	18.37670	0.270
Grimmia tergestina as sister to *G. laevigata*	7070.28780	91.25686	0.001*

V. DISCUSIÓN GENERAL

"Si no te equivocas de vez en cuando,
quiere decir que no estás aprovechando
todas tus oportunidades".
Woody Allen

V. Discusión general

Como principal aportación del presente trabajo podríamos decir que en el complejo Campylosteliaceae-Grimmiaceae-Ptychomitriaceae existe una incongruencia generalizada entre la señal filogenética aportada por los datos moleculares y la taxonomía basada en datos morfológicos tanto a nivel de familia, de género y de especie. Esto se ve reflejado de formas diferentes en cada uno de los estudios realizados.

El empleo de los marcadores plastidiales *rps4* y *trnL-F* resuelven inequívocamente a *Grimmia pitardii* junto a *Campylostelium strictum* y cuestiona los caracteres morfológicos empleados para situarla dentro de *Grimmia* (seta sigmoidal y cápsula ventricosa). A nivel de familia este estudio sirve como acercamiento preliminar de las relaciones filogenéticas del complejo formado por Grimmiaceae-Ptychomitriaceae. Lo más destacado es la posición ambigua de *Campylostelium* entre *Ptychomitrium* y Grimmiaceae, aunque no se puede aventurar nada más debido a la limitación de los datos.

Esa falta de resolución se resuelve, en parte, añadiendo a nuestro conjunto de datos el espaciador *trnT-L* (Capítulo IV.2). En esta región detectamos distintas mutaciones estructurales de gran tamaño e inversiones asociadas a ”hairpins” que complicaron el alineamiento y que, de no detectarse, hubiesen disminuído la resolución y podido contribuir a inferir filogenias erróneas. Para su alineamiento empleamos modelos basados en estructuras secundarias y aplicamos las reglas de alineamiento que para tal efecto están descritas en (Borsch *et al.*, 2003; Kelchner, 2000; Quandt, Stech, 2005).

Mediante el estudio combinado de la región *rps4-trnT-trnL-trnF* se obtuvieron propuestas filogenéticas para las familias tratadas que entran en conflicto con los tratamientos taxonómicos tradicionales. La familia Ptychomitriaceae cambia

drásticamente su composición. Nuestros datos corroboran que *Glyphomitrium* no pertenece ni a Grimmiaceae ni a Ptychomitriaceae, y que *Jaffueliobryum* e *Indusiella* se resuelven junto a *Ptychomitrium*. Estos resultados, aunque sorprendentes, se ven apoyados por distintas sinapomorfías morfológicas como la estructura del filidio en corte transversal y la condición sexual criptoica presente en todos los géneros que la componen. *Campylostelium*, por su parte, se resuelve como grupo hermano de las Ptychomitriaceae, por lo que preferimos tratarlo en la familia independiente Campylosteliaceae De Not.

La familia Grimmiaceae se resuelven como un grupo natural (Capítulos IV.1 y IV.2) definido por las hojas con paredes celulares sinuosas, nervios de tipo A, B o C (Kawai, 1968) y la capa externa del peristoma más gruesa que la interna (en Ptychomitriaceae, Campylosteliaceae y *Glyphomitrium* son del mismo grosor). Los géneros que componen esta familia según los datos moleculares serían *Racomitrium, Schistidium, Dryptodon* y *Grimmia* s.l. (incluidos *Coscinodon* e *Hydrogrimmia*). *Racomitrium* se resuelve como un grupo natural fuertemente apoyado por distintas sinapomorfías morfológicas como el hábito cladocárpico y las células basales de la hoja nodulosas. Además se han detectando ciertas sinapomorfías moleculares de tipo estructural (inserciones y delecciones) que apoyan estos resultados.

Las relaciones filogenéticas de *Schistidium, Dryptodon* y *Grimmia* se han discutido desde varios puntos de vista en los Capítulos IV.2, IV.3 y IV.4.

Según los datos moleculares, *Grimmia* se resuelve como un genero parafilético y *Dryptodon* debe reconocerse como un género independiente (Capítulo IV.2). Aunque las especies incluidas en *Dryptodon* presentan algunas sinapomorfías, la presencia de yemas para la reproducción asexual es el único carácter morfológico para diferenciarlo de *Grimmia*. *Coscinodon* e *Hydrogrimmia* se resuelven dentro de *Grimmia*, por lo que se consideran parte del mismo.

Schistidium representa un grupo natural apoyado tanto por datos moleculares (Capítulos IV.1, IV.2, IV.3 y IV.4) como morfológicos (Capítulos IV.2 y IV.3), aunque sólo con ITS (Capítulo IV.4) y con todos los genes plastidiales empleados en conjunto (Capítulo IV.3) se resuelve como grupo hermano separado de *Grimmia*.

Respecto a la sistemática de *Grimmia*, existen numerosas fuentes de incongruencia. Por un lado se observan conflictos entre la señal morfológica y molecular: el empleo de la región plastidial *trnK-matK* combinado con la región *rps4-trnT-trnL-trnF* y con datos morfológicos (Capítulo IV.3) muestran una correlación general con los grupos definidos morfológicamente, pero no con los caracteres del esporófito empleados tradicionalmente en la sistemática de *Grimmia*. La homoplasia detectada en los caracteres del esporófito (Capítulo IV.3, Fig. 7) pudiera ser la explicación para la inestabilidad de las diferentes clasificaciones propuestas en *Grimmia* (ilustrado por símbolos en el Capítulo IV.3, Fig. 5).

Sin embargo, los caracteres del gametófito muestran también cierto grado de homoplasia. *Grimmia funalis* y *G. elatior* son especies que tanto gametofítica como esporofíticamente se asemejan a las especies de *Dryptodon*, pero que se resuelven inequívocamente dentro de *Grimmia* tanto con datos plastidiales (Capítulos IV.1 y IV.3) como con datos nucleares (Capítulo IV.4). La convergencia adaptativa parece ser la única explicación plausible para explicar la posición sistemática de estas especies.

Para añadir complejidad a la sistemática de *Grimmia*, el empleo de datos moleculares procedentes del núcleo (Capítulo IV.4) muestra episodios de reticulación en al menos una especie -*Grimmia tergestina*- del complejo. Esta especie comparte ADN nuclear del subg. *Orthogrimmia* y ADN cloroplástico del subg. *Guembelia*. En varias especies de *Grimmia* subg. *Grimmia* se observa un fenómeno parecido aunque la limitación de los datos en este caso no nos permite asegurar episodios de reticulación. Futuras investigaciones en *Grimmia* deberían

tener muy en cuenta la reticulación como proceso evolutivo generador de especies en el género.

El problema de base que se refleja en el presente trabajo es un problema común en la sistemática de briófitos. La incongruencia entre la señal filogenética derivada del análisis de secuencias de ADN y de conceptos taxonómicos basados en morfología. Aunque en nuestro caso se aprecian ciertas sinapomorfías para apoyar algunos clados (Capítulo IV.3, Fig. 6), lo común en la sistemática de briófitos es su falta. Por ello todo este tipo de estudios, incluida la presente memoria, apuntan a la imperiosa necesidad de estudiar en profundidad la ontogenia de los caracteres, o explorar nuevos caracteres de tipo anatómico o ultraestructural que puedan reflejar mejor la historia evolutiva de los briófitos.

VI. RESUMEN Y CONCLUSIONES

"La ciencia se suicida
cuando adopta un credo".
Thomas H. Huxley

VI. Resumen y conclusiones

El objetivo de esta tesis es analizar las relaciones filogenéticas de los géneros incluidos actualmente en las familias Grimmiaceae y Ptychomitriaceae. Para este fin seleccionamos 124 ejemplares de 90 especies, de las que setenta pertenecen al género *Grimmia*, un 80% de las especies en él reconocidas. Se secuenciaron ~6000 pares de bases correspondientes a los genes *rps4*, *matK*, *5.8s*, los exones *trnS, trnT, trnL, trnF*, los espaciadores *rps4-trnT, trnT-L, trnL-F, ITS1, ITS2* y el intrón *trnL*. Unos 4300 pares de bases pertenecen al genoma plastidial y los 1700 restantes pertenecen al nucear. Se analizaron un total de 416 secuencias, de las que 344 han sido obtenidas durante el desarrollo de esta tesis: 87 de *rps4*, 88 de *trnL-F*, 68 de *trnT-L*, 48 de *trnK/matK* y 53 de ITS. Se diseñaron nuevos cebadores específicos para estas familias correspondientes a las regiones *rps4-trnL* (rps4-166F, RP185F, ARbryo y AFbryo) y *trnK/matK* (trnK-Fbryo1, trnKRbryo4, trnKF426Grim, matK1024F, psbARbryo). La región *trnK/matK* ha sido utilizada por primera vez en estudios filogenéticos en briófitos.

El análisis de las secuencias ha mostrado cómo las mutaciones estructurales juegan un papel importante en las reconstrucciones filogenéticas tanto del complejo Campylosteliaceae-Grimmiaceae-Ptychomitriaceae como del género *Grimmia*. Las inserciones o delecciones apoyan los clados obtenidos, por lo que contienen importante información filogenética. Sin embargo, las inversiones son altamente homoplásicas y de no detectarse pueden interferir negativamente en los resultados, tanto estructuralmente como en términos de resolución de los clados obtenidos. Del análisis de todas las secuencias y todos los datos morfológicos disponibles y tras los pertinentes análisis filogenéticos, podemos concluir que:

1. La familia Ptychomitriaceae no es un grupo natural.

2. El género *Glyphomitrium* no es un miembro ni de Grimmiaceae ni de Ptychomitriaceae, aunque su posición sistemática permanece sin resolver.

3. Existe un conflicto respecto a la posición sistemática de *Campylostelium*. Las regiones plastidiales empleadas dan resultados incongruentes, y su posición permanece sin resolver entre las familias Grimmiaceae y Ptychomitriaceae. En esta tesis se propone considerar a este género en su propia familia, Campylosteliaceae, tal y como fue propuesto por De Notaris en 1869 y Limpricht en 1889.

4. La proximidad genética de *Grimmia pitardii* a *Campylostelium strictum,* sumado a los caracteres morfológicas compartidos sugieren que el correcto tratamiento de este taxon debería ser *Campylostelium pitardii*.

5. *Jaffueliobryum* e *Indusiella* se resuelven junto a *Ptychomitrium* si empleamos marcadores plastidiales (*rps4-trnT-L-F)*. Distintos caracteres morfológicos compartidos como la condición sexual criptoica, las caliptras mitradas plurilobuladas y los nervios con una capa central de estereidas, apoyan el tratamiento de estos tres géneros dentro de la familia Ptychomitriaceae.

6. La familia Grimmiaceae es monofilética según datos del genoma plastidial e incluye a los géneros *Racomitrium, Schistidium, Dryptodon* y *Grimmia*.

7. *Racomitrium* es un grupo monofilético, claramente apoyado en todos los análisis filogenéticos y con varias sinapomorfías morfológicas como el hábito cladocárpico, las paredes celulares de la vagínula sinuosas y porosas, y las células basales de los filidios fuertemente sinuosas.

8. *Schistidium* representa un grupo natural con un gran apoyo estadístico y sinapomorfías como las cápsulas inmersas y sistilias, el característico color negro-rojizo de sus hojas y las hojas periqueciales más grandes que las vegetativas.

9. Según datos plastidiales y morfológicos, *Grimmia* incluiría a *Coscinodon* e *Hydrogrimmia*.

10. Para que *Grimmia* -sensu Muñoz & Pando (*Monogr. Syst. Bot. Missouri Bot. Gard.* 83: 1-133. 2000) y Greven (*Grimmias of the world*. 2003)- represente un grupo natural, *Dryptodon* debe reconocerse como género independiente.

11. Los datos moleculares y morfológicos indican que *Grimmia*, incluso una vez excluído *Dryptodon*, representa un grupo de especies muy complejo, por el momento no definido por sinapomorfías, y con una composición de especies diferente a la aceptada en las floras y tratamientos al uso.

12. En *Grimmia*, tanto los caracteres del esporófito como del gametófito son altamente homoplásicos. Sin embargo, tras estudiar la distribución de caracteres en los árboles filogenéticos vemos cómo ciertos caracteres del gametófito retienen algun tipo de información y parecen ajustarse mejor a la historia evolutiva de las especies.

13. El empleo de datos nucleares (ITS) en combinación con los plastidiales (*rps4-trnT-L-F*, *trnK/matK*) muestra incongruencias en la posición de ciertas especies (*Grimmia* subg. *Grimmia* y *G. tergestina*), lo que añade complejidad a la historia evolutiva del género. La posición de *Grimmia* subg. *Grimmia* puede explicarse por la falta de resolución en el marcador nuclear, pero la incongruencia entre

marcadores nucleares y cloroplásticos en *G. tergestina* se explica mejor por posibles episodios de reticulación que afectaron a la evolución de la especie.

14. *Grimmia elatio*r y *G. funalis* se resuelven tanto con ITS como con datos plastidiales dentro de *Grimmia*. Este hecho contrasta con la visión tradicional que las sitúa como especies de *Dryptodon* conforme a su morfología. A la espera de nuevos datos, la convergencia adaptativa parece ser la única explicación plausible para entender la evolución de estas dos especies.

15. El conjunto de resultados obtenidos nos lleva a proponer la siguiente clasificación:

Orden Grimmiales
- Familia Campylosteliaceae
  - Género *Campylostelium*
- Familia Ptychomitriaceae:
  - Género *Jaffueliobryum*
  - Género *Indusiella*
  - Género *Ptychomitrium*
- Familia Grimmiaceae
  - Género *Grimmia*
    - Subgénero *Grimmia*
    - Subgénero *Guembelia*
    - Subgénero *Ovatae*
    - Subgénero *Orthogrimmia*
      - Sección *Montanae*
      - Sección *Donnianae*
  - Género *Schistidium*

Género *Dryptodon*

Incertae sedis

Género *Glyphomitrium*

Género *Leucoperichaetium*

16. Mediante el uso combinado de datos moleculares y morfológicos se han resuelto muchas cuestiones problemáticas en el estudio taxonómico del complejo Campylosteliaceae-Grimmiaceae-Ptychomitriaceae, aunque también se ha puesto de manifiesto la complejidad de la evolución de estas especies. Las incongruencias detectadas parecen indicar que la historia evolutiva en el grupo es muy compleja, y que las relaciones filogenéticas posiblemente no puedan reflejarse en diagramas jerárquicos como los comúnmente empleados en estudios filogenéticos. Por este motivo creemos que prestar atención a posibles procesos de reticulación supondría una fuente alternativa de información para entender la evolución de este grupo y, en general, de los briófitos.

VII. REFERENCIAS BIBLIOGRÁFICAS

"De cerca,
nadie es normal."
Caetano Veloso

VII. Referencias bibliográficas

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