UNIVERSIDAD DE MURCIA

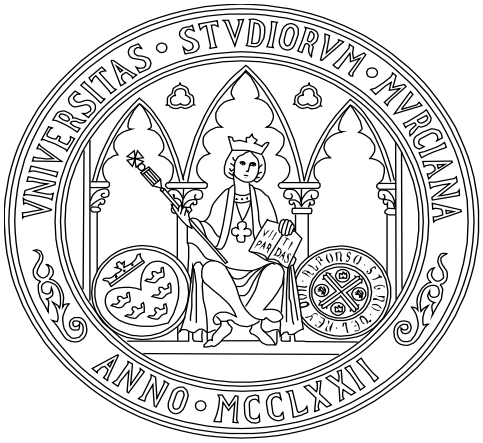
ESCUELA INTERNACIONAL DE DOCTORADO

The Moss Genus *Ceratodon*: Studies of Evolutionary Biology and Taxonomy in Southern Europe

El Género de Musgos *Ceratodon*: Estudios de Biología Evolutiva y Taxonomía en el Sur de Europa

Dña. Marta Nieto Lugilde

2019



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TESIS DOCTORAL

Marta Nieto Lugilde

2019

Directores

Olaf Franziskus Werner (Universidad de Murcia)

Rosa María Ros Espín (Universidad de Murcia)

Stuart Frazier McDaniel (University of Florida)

Tesis Doctoral que opta a mención de "Doctorado Internacional"

Expertos doctores que han informado sobre la tesis:

- Dr. Rafael Medina, University of Augustana. Rock Island, Illinois, EE.UU.
- Dr. Mahmoud Magdy Abdallah Awad, Ain Shams University, Cairo, Egipto.

FUENTES DE FINANCIACIÓN

La investigación llevada a cabo en el marco de esta Tesis Doctoral fue financiada por los proyectos CGL2011-22936/BOS del Ministerio de Ciencia e Innovación y CGL2014-52579-R del Ministerio de Economía y Competitividad, cofinanciados con fondos FEDER.

Asimismo, Marta Nieto Lugilde disfrutó de una beca-contrato del Programa de Formación de Personal Investigador (referencia BES-2012-056799) del Ministerio de Economía y Competitividad, desde diciembre de 2012 hasta noviembre de 2016, además de tres ayudas a la movilidad predoctoral para la realización de sendas estancias breves en centros de I+D: Gainesville, Florida, EE.UU. en 2014 (4 meses, referencia EEBB-I-14-08968); Gainesville, Florida, EE.UU. en 2015 (4 meses, referencia EEBB-I-15-10402); y České Budějovice, República Checa en 2016 (2 meses, referencia EEBB-I-16-11747).

A mis padres, y a mi familia

“De Norte a Sur”

AGRADECIMIENTOS

Este camino se termina, pero es el principio del resto. Durante estos casi siete años, he vivido y aprendido muchísimo, y espero que eso no cambie. Este es el momento de agradecer a todas aquellas personas que han contribuido en mi formación y que me han ayudado a llevar acabo esta tesis. Los aquí citados y los que no (por espacio) merecen mis más sinceros agradecimientos.

Existen dos tipos de familias: en la que naces y otra que es la que eliges. En mi caso tengo la suerte de tener tres, en la que nací, mis amigos y la gran familia que nos incorporó a la suya cuando mis padres se establecieron en el sur. No sé cómo agradecerles a mis padres, Mari Carmen y Juan Pedro, el amor por aprender cosas nuevas que nos inculcaron, el ímpetu por descubrir y el apoyo que siempre nos demostraron a los tres. A mis hermanos, Marcos y Diego, que son las mejores personas a las que puedo admirar, que a pesar de la distancia siempre han estado conmigo y me han dado libertad para que me formara yo misma. A mis sobrinas, Sara y Eva, con las que podría pasarme el día haciendo tartas, pintando tiestos o jugando a juegos de mesa, son tan diferentes como el azúcar y la sal, pero ambas son geniales y las quiero con locura. Que “el roce hace el cariño”, suena evidente… pero mantener el cariño a pesar de los cientos de kilómetros que separan a mi familia es pura magia y por ello quiero dedicarles especialmente esta tesis, a mi familia “De Norte a Sur”. A Encarni, Jose, Maribel, Nolo, Tino, Isa, Pepe, Fe, Iván, Ana, Juan Luis, Patri, Diego y Noemí, porque sois pura fuerza, dedicación, resiliencia, superación, alegría y cariño.

Me gustaría hacer especial mención a mis directores de tesis Rosa M. Ros, Olaf F. Werner y Stuart F. McDaniel. Rosa ha llegado a ser una amiga, y además gracias a ella he aprendido a ser meticulosa, la importancia de la taxonomía, el correcto estudio de los briófitos y el buen hacer científico. Olaf quizás pasa desapercibido por su carácter pero siempre tiene una opinión, una idea o unas palabras de apoyo en el momento adecuado, además no hay quien le gane en el laboratorio. Gracias a ambos por vuestra dedicación en mi formación durante todos estos años y ofrecerme la oportunidad de realizar mi tesis con vosotros. Por último conocí a Stuart, que desde el principio me

trató como si me conociera de toda la vida, ofreciéndome su amistad, simpatía y confianza. Además con sus extensos conocimientos sobre *Ceratodon* y biología en general, me enseñó a mirar más allá de mi "sistema" y a llevar mi investigación a ámbitos de interés general, "así es", muchas gracias.

Al llegar a la Universidad de Murcia, conocí a mucha gente que me ayudó en muchos aspectos y a los que estaré eternamente agradecida. Mis compañeros de laboratorio Mahmoud Magdy, Sergio Pisa, Inma Aledo, Agripina Ramírez, Elena de la Cruz, Samah Mohamed Rizk, Aurora Rodríguez y Juan Antonio Giménez, con los que compartí comidas, excursiones y muchas horas de aprendizaje. Por supuesto a la gente del laboratorio de palinología, el cual muchas veces parecía una prolongación del nuestro, Isa López, Elena Fierro (te echamos de menos, D.E.P.), Aline Freitas, Juan Ochando, Saúl Manzano, Santiago Fernández y Pepe Carrión. Mucho cariño les tengo a la gente de la cantina de Biología, Pascual, Alejandro, y al resto de personal, los cuales rompieron mi corazón cuando me anunciaron que no celebraríamos mi tesis juntos, gracias chicos por conseguir que desconectara al menos a la hora de comer. Trini, es el personaje más entrañable de toda la facultad de Biología, es atenta, alegre, servicial y muy eficaz. Gracias, Trini, por alegrarnos todas las mañanas, por preocuparte por todos y por avisarme cuando venían los festivos. También quiero agradecerles su trato a esas personas del Departamento de Biología Vegetal (Botánica) de la Universidad de Murcia que por circunstancias varias hemos tenido menos contacto, pero siempre me han respondido con una sonrisa y me han ayudado cuando lo he necesitado, Diego Rivera, Paco Alcaraz, Juan Guerra, Pilar Torrente, Mayte Gallego, José María Egea, María Jesús Cano, María Asunción Morte, Pedro Sánchez, Marta Alonso, Juan Antonio Jiménez, y Mari Ángeles Caravaca.

Quiero agradecer a Pepe Galián (coordinador del Programa de Doctorado en Biodiversidad y Gestión Ambiental) y a Rosa Mª Fernández Cabada (Vicerrectorado de Investigación y Transferencia), su inestimable ayuda durante los tediosos procesos burocráticos.

Quisiera destacar la labor de la UCC de la Universidad de Murcia, en especial la de Delfina Roca y la periodista María José Moreno con las que tuve la suerte de compartir algunos ratillos y que me enseñaron la importancia de comunicar la investigación que hacemos y acercarla a todo el mundo, muchas gracias.

Una de las mejores cosas que me ha pasado durante estos años fue la oportunidad de realizar estancias de investigación en el extranjero, donde crecí como investigadora y como persona. Y quiero agradecerles a todos aquellos que formaron parte de esas experiencias, porque hicieron que el estar lejos de casa no pesara tanto y alegraron los días con sonrisas, y buenos ratos. En Gainesville (Florida, EE.UU.): Gabriela Bloom y Catalina Pimiento, que me acogieron en su casa con todo el cariño del mundo; Sarah Carey, Barry Kaminsky, Leslie Kollar, Adam Payton y Lorena Endara que me ayudaron en el laboratorio y con los análisis, me integraron en las actividades del departamento y me acogieron como a una más del "McDaniel Lab"; No me olvido de Majo, Tania, Daniel, y el resto de mexicanos y argentinos que me enseñaron sus culturas y me aceptaron en su "familia multicultural"; Carmen Piñero, la murciana que conocí a miles de kilómetros, y que juntas disfrutamos de todos los rincones americanos que se pusieron a nuestro alcance. En České Budějovice (República Checa): Jan Kučera y Petr Koutecký, por aceptarme en su laboratorio y enseñarme todo lo que sé sobre citometría de flujo; Alžběta Manukjanová que me enseñó la ciudad y su cultura, me facilitó los papeleos en la Universidad y me proporcionó todo lo necesario para pasar varios días en el campo, gracias por ser tan amable y servicial conmigo.

Murcia llegó a ser mi casa y ha generado en mí esa necesidad de volver de vez en cuando, pero no solo por las "marineras", "salchichas secas" o "paparajotes", sino por esas personas que son parte fundamental de esta tesis. Porque mis pavas "murcianas", Cristina Pérez, Gemma Prieto, Ana Fernández, Elena Castro y Carmen Sica, han aguantado mis problemas y agobios, y los han aliviado con muchas risas y comida a borbotones, muchísimas gracias.

Uno no debe de olvidar de donde viene… pero es que además yo no quiero olvidarme de esa gente que me han apoyado y confiado en mí desde el principio y que siempre me reciben con un abrazo y una sonrisa. Del Departamento de Botánica de la Universidad de Granada especial cariño le tengo al equipo cervecero formado por Miguel Ballesteros, Antonio González, Paqui Alba, Julio Peñas y David Fernández, que han sido un pilar fundamental para terminar esta tesis, gracias por confiar en mí. Con los que viví mis primeros años de investigación y con los que formé, entre Papaveráceas y Pteridófitos, mi primera "familia científica": Víctor Suárez, Miguel Ángel Pérez, Ana

González, Laura Terrón y Samira Ben-Menni. Muchísimas gracias también a mis profesores de botánica Gabriel Blanca, Concha Morales y Joaquín Molero, que me descubrieron este apasionante mundo y me motivaron a estudiarlo.

Muchísimas gracias a mis amigos de Graná que llevan muchos años aguantando, que una ruta dure el doble porque me entretengo con todas las plantitas, que discutieron conmigo si la nueva especie debía llamarse *"amazonum"* o "*nevadensis"*, y que han vivido estos últimos meses con el mismo suspense que la final de la Champions: Jorge, Olmo, Andrés, Bilbo, Mabel, Miriam, Javi, Loles, Chitin, María, Luisi, Aurora, Brittany, Campillo y Carmencita. A Rafa Navajas y Cristina Aznarte, quisiera agradecerles su empatía y apoyo para que continúe investigando proponiéndome colaboraciones muy interesantes.

Por último pero no menos importante, quiero agradecerles su cariño y su acogida a esa tercera familia que tengo, mi familia de Graná: Matilde, José Manuel, María José, Juan, Mari Loli, Nacho, Mati (te fuiste muy pronto, D.E.P), Jerónimo, Anita, Nuria, Ángela, Pepe, Loli,… y todos los demás que componen una larga lista que hace que nos juntemos 45 personas en Navidad, más el equipo de futbol juvenil que ya podemos montar. Espero que sigamos juntándonos y disfrutando muchos años más.

¡Muchas gracias a todos!

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Resumen

La descripción y la cuantificación de la biodiversidad son dos de los grandes retos a los que se enfrentan los científicos. Bajo el escenario de cambio global en el que nos encontramos, ésta es una ardua tarea dado el riesgo de que muchas especies desaparezcan incluso antes de que sean descritas. La taxonomía tradicional se ha basado fundamentalmente en la morfología de las especies, aunque también a menudo se han tenido en cuenta datos sobre su ecología y fisiología. Con el desarrollo de las técnicas moleculares (principalmente de la PCR) se ha facilitado la obtención de marcadores genéticos que en muchas ocasiones clarifican las relaciones sistemáticas entre las especies, especialmente cuando éstas presentan un continuo en su variación morfológica o incluso son crípticas. Sin embargo, fenómenos como la introgresión o la segregación incompleta de los linajes complican la interpretación de los datos genéticos. La información morfológica y la molecular pueden apuntar hacia conclusiones diferentes, incluso se pueden observar inconsistencias entre diferentes marcadores moleculares. Todo ello pone en evidencia la necesidad de usar una metodología integrativa para arrojar luz sobre los mecanismos de especiación.

Los briófitos, que incluyen musgos, hepáticas y antocerotas, constituyen el segundo grupo de plantas terrestres más diverso después de las angiospermas. Su aparato vegetativo es pequeño y de anatomía sencilla. Una de sus características más destacables es la dominancia de la generación haploide en su ciclo de vida (el gametófito). Dado que el agua es necesaria para que se lleve a cabo la reproducción sexual, están frecuentemente asociados a ambientes húmedos. Pero a pesar de ello, los briófitos están presentes en todos los ambientes terrestres, desde las zonas polares hasta los trópicos, incluso en los desiertos.

En esta tesis se ha utilizado como objeto de estudio el género de musgos *Ceratodon* Brid., perteneciente a la familia Ditrichaceae, que fue revisado a nivel mundial por Burley and Pritchard (1990). En un admirable trabajo de síntesis, estos autores reconocieron tan solo cuatro especies, entre ellas, la cosmopolita *C. purpureus* (Hedw.) Brid. con una amplia variación morfológica, por lo que distinguieron tres subespecies, cada una con un patrón biogeográfico definido, al igual que el resto de los taxones. Sin embargo los datos genéticos obtenidos por McDaniel and Shaw (2005) a

nivel mundial no apoyaron estos resultados. *Ceratodon purpureus* es dioica y se reproduce abundantemente. Junto con *Physcomitrella patens* (Hedw.) Bruch & Schimp. y *Funaria hygrometrica* Hedw., es una de las especies de musgos empleadas como sistema modelo para estudios sobre fisiología, genética y evolución de plantas y además su genoma completo ha sido secuenciado. Por todo ello, el género *Ceratodon* es idóneo para estudiar uno de los mayores problemas de la taxonomía: si la variación morfológica de las especies en condiciones naturales es debida a la diferenciación genética y por tanto corresponden a unidades evolutivas distintas, o si es causada por la variación ambiental. La delimitación de especies en organismos con un elevado potencial de dispersión mediante esporas en ambientes muy variables y con una considerable variación morfológica sigue siendo un gran desafío.

Este trabajo de investigación se ha enfocado desde un punto de vista multidisciplinar con el objetivo general de estudiar la diversidad, la biología evolutiva y la taxonomía en el género *Ceratodon* en el sur de Europa. Se han combinado estudios de morfometría, análisis filogenético basado en secuencias de ADN y citometría de flujo para determinar el tamaño del genoma. Asimismo se han tenido en cuenta datos de biogeografía y ecología. Los objetivos específicos fueron los siguientes:

1. Revisar la taxonomía y nomenclatura del género *Ceratodon* en el sur de Europa.

2. Conocer la diversidad genética de las poblaciones de *Ceratodon* en las áreas montañosas del Mediterráneo.

3. Evaluar si las poblaciones del sur de Europa pertenecen a la misma especie o si por el contrario, la diversidad genética encontrada entre ellas define quizás especies diferentes y en este caso arrojar luz sobre el proceso de especiación.

4. Comprobar si los especímenes de *Ceratodon* del sur de Europa muestran diferencias en la proporción de sexos y en la cantidad de ADN en los núcleos.

5. Determinar la proporción de variación morfológica en *Ceratodon* que es debida al medio ambiente o si esta tiene una base genética.

6. Utilizando una metodología integrativa, obtener conclusiones taxonómicas sobre las muestras del sur de España que presentan una morfología diferente a la descrita para *C. purpureus*.

7. Reconstruir la historia evolutiva de las especies de *Ceratodon* presentes en el sur de Europa y sus interacciones vía hibridación.

8. Estimar los parámetros demográficos y evaluar los posibles escenarios evolutivos en las poblaciones del sur de Europa.

Esta tesis se estructura en cuatro capítulos íntimamente relacionados, presentados como artículos científicos.

Con el fin de conocer la morfología de las especies susceptibles de estar presentes en el área mediterránea, en el primer capítulo se llevó a cabo la revisión del tipo nomenclatural de *Ceratodon conicus* (Hampe ex Müll. Hal.) Lindb. y de los sinónimos propuestos por Burley and Pritchard (1990). Para ello se estudiaron los especímenes tipo designados por estos autores y se compararon con los datos de los protólogos. El lectótipo de *C. conicus*, basado en un ejemplar depositado en el herbario GOET, fue confirmado, pero el material de los isolectótipos de los herbarios FH, GOET, y MANCH no se correspondieron con el protólogo de la especie. Además, los tipos de los tres sinónimos de *C. conicus*: *C. cedricola* J.J Amann depositado en el herbario Z+ZT, *C. dimorphus* H. Philib. en el herbario BM, and *C. purpureus* var. *graefii* Limpr. en el BR, considerados como holótipos por Burley and Pritchard (1990), fueron aquí designados como lectótipos ya que no existe un ejemplar original inequívoco en ningún caso. Por último, *C. purpureus* var. *graefii* fue considerado por nosotros como sinónimo de *C. purpureus s.l.*, ya que sus características morfológicas coinciden con las descritas para esta especie y no con las de *C. conicus*.

Un periodo de alopatría se cree que es fundamental para que tenga lugar un aislamiento reproductivo. Sin embargo, una estricta alopatría puede ser difícil de lograr en especies cosmopolitas que se dispersan mediante esporas, como los musgos. En el segundo capítulo, con el fin de evaluar el papel de la alopatría y el cambio de ploidía en la divergencia de las poblaciones, se examinó la diversidad genética y el tamaño del genoma en las poblaciones del sur de Europa de *Ceratodon*. Para ello se muestrearon áreas montañosas y tierras bajas de la región Mediterránea, así como de Europa occidental y central. Se realizaron análisis filogenéticos y de coalescencia con las secuencias del ADN de cinco intrones nucleares y un locus cloroplastidial. También se estimó el tamaño del genoma mediante citometría de flujo y se determinó el sexo de las

plantas mediante un marcador genético ligado al sexo. Los análisis filogéneticos resolvieron dos clados bien diferenciados, que discriminaron dos grupos homogéneos: uno correspondiente a la especie cosmopolita *C. purpureus* y otro restringido a las montañas del sur de España. Las muestras de este grupo local también poseían un tamaño del genoma un 25% más grande que el de *C. purpureus*, y eran exclusivamente hembras. También se encontraron híbridos, y algunos de ellos tenían un tamaño de genoma equivalente a la suma del genoma de *C. purpureus* y el de los especímenes del sur de España, lo que hace pensar que se formaron por alopoliploidía. Todos estos datos sugieren que una especie nueva de *Ceratodon* surgió por un proceso de especiación peripátrica, lo que potencialmente implicó un cambio en el tamaño del genoma y una fuerte desviación en la proporción de sexos. También se observaron procesos de hibridación entre la nueva especie y *C. purpureus*.

Para evaluar el efecto de la variación ambiental en la taxonomía del género *Ceratodon*, se realizó un análisis biométrico basado en 22 caracteres morfológicos, tanto en plantas recolectadas en campo como en plantas cultivadas *in vitro* (crecidas a partir de las mismas plantas de campo). Estos datos se compararon con los del análisis filogenético basado en sus secuencias de ADN y los de tamaño del genoma obtenidos en el capítulo anterior. Se encontró que la expresión de varios rasgos gametofíticos cambió entre las muestras recolectadas en campo y las muestras cultivadas *in vitro*, lo que confirmó que la variabilidad ambiental complica las inferencias taxonómicas y sugirió que algunos caracteres deben usarse con precaución para la distinción entre especies. Sin embargo, coincidiendo con los datos genéticos y de citometría de flujo, se encontró una clara discontinuidad biométrica entre algunas plantas del sur de España y las de otras partes del mundo. Las muestras de origen híbrido fueron morfológicamente muy similares a las plantas de las montañas del sur de España. La aproximación integrativa basada en datos genéticos, del tamaño del genoma y morfométricos apoyó de manera inequívoca el reconocimiento de la nueva especie, que se describió formalmente con el nombre de *Ceratodon amazonum* Nieto-Lugilde, O. Werner, S.F. McDaniel & Ros. Los resultados también sugirieron que la previamente reconocida *C. conicus* es un recombinante entre *C. purpureus* y *C. amazonum*. Por ello se consideró como una notoespecie, para la cual se designó un epitipo porque el lectótipo es demostrablemente ambiguo.

La diferencia en la escala de tiempo entre la evolución que lleva al aislamiento reproductivo y el cambio en las condiciones ecológicas implica que las áreas actuales de distribución de las especies sean aproximaciones imperfectas de las que tenían en el momento de la especiación. Un desafío clave en la biología evolutiva es identificar fuentes alternativas de información que puedan proporcionar más datos sobre el proceso de especiación. Análisis estadísticos de la estructura genética actual y de la diversidad genética de las poblaciones de especies relacionadas pueden identificar procesos de vicarianza, la historia de la dispersión y los episodios de expansión y contracción de los tamaños de las poblaciones. En el cuarto capítulo de esta tesis se reconstruyó la historia demográfica y evolutiva de *C. purpureus*, su especie hermana, *C. amazonum*, y la recombinante *C. ×conicus*, algo que ha sido realizado en pocas especies de briófitos. Se generó un mayor número de datos genéticos que en capítulos anteriores aumentando tanto el número de muestras europeas analizadas como los marcadores genéticos empleados, que ascendieron a nueve. Se calcularon parámetros estadísticos de genética de poblaciones y se realizaron análisis filogenéticos; además, se estimaron los parámetros demográficos bajo un modelo de aislamiento en presencia de migración entre las especies y se infirieron posibles fluctuaciones históricas en el tamaño efectivo de las poblaciones. Finalmente, se realizó un análisis para determinar el número de eventos por los que se originaron los especímenes recombinantes, y se evaluaron los posibles escenarios evolutivos para las especies. Se encontró evidencia de flujo génico asimétrico entre *C. purpureus* y *C. amazonum*, lo que favoreció la introgresión de la especie cosmopolita en la especie aislada del sur de Europa. El tamaño efectivo de la población de *C. amazonum* resultó ser más pequeño que el de *C. purpureus*. Esto sugiere que si el evento de especiación que produjo estas dos especies hermanas involucró un cuello de botella poblacional, el tamaño efectivo de la población de *C. amazonum* se recuperó de manera relativamente rápida, potencialmente como consecuencia del flujo de genes recurrentes de *C. purpureus*. Finalmente, se encontraron evidencias claras de que *C. ×conicus* se formó por múltiples eventos de hibridación entre *C. purpureus* y *C. amazonum*, lo que apoya aún más el papel del flujo génico en el proceso de especiación en briófitos.

En conjunto esta tesis pone de manifiesto las carencias actuales en el conocimiento sobre la diversidad de briófitos y en los mecanismos que la generan, incluso en especies comunes y ampliamente distribuidas, y demuestra que las especies

silvestres cosmopolitas tienen el potencial de revelar las diversas causas genéticas de la especiación. Asimismo, confirma que los fenómenos de hibridación y poliploidía son mecanismos que también intervienen en la especiación de briófitos. Todavía hay aspectos importantes por tratar y descubrir, como la distribución real de *C. amazonum* y *C.* ×*conicus*, comparar los genomas de *C. amazonum* y *C. purpureus*, e incluso de los híbridos entre ellas, y conocer su arquitectura genética. Además, una línea de investigación muy interesante sería el estudio de los efectos epigenéticos a largo plazo en las poblaciones naturales para comprender sus consecuencias en la morfología o fisiología en función de las condiciones ambientales, para generar un conocimiento más profundo del origen de la variación morfológica y los mecanismos evolutivos.

General Introduction

THE STUDY OF BIODIVERSITY

The variety of life forms that we currently find on planet Earth is the result of long processes of evolution and diversification since its origin about 3 800 million years ago (mya) or possibly earlier (Schidlowski et al., 1979; Rosing, 1999; Hedges, 2002; Nutman et al., 2016). The biodiversity is a multi-dimensional concept, with features like genetic and phenotypic variation, diversity between species, and diversity with respect to functional roles in the ecosystems or network structures of whole communities (Page, 2010; Burch-Brown & Archer, 2017). Estimating the number of species inhabiting Earth is among the most fundamental questions in science (Erwin, 1991; May, 1992; Stork, 1993; Mora et al., 2011; Grosberg et al., 2012), and one of the individual measures commonly used to represent the biological diversity. The present number of formally described species is approximately 1.8 million (Roskov et al., 2019), however more species exist. Estimates of undescribed species range from 0.5-10 million to at least 1 to 6 billion (Mora et al., 2011; Costello et al., 2013; Larsen et al., 2017) and remain highly uncertain. The process of evolution and the increase in species number has not been gradual, but has been interrupted on multiple occasions by catastrophic extinction events, eliminating a high proportion of populations, which is a prelude to species extinction (Raup, 1986; McElwain & Punyasena, 2007; Ceballos et al., 2017). Extinction events are often viewed as disturbances to the evolutionary process. Although they are unpredictably destructive, extinction events may in the long term accelerate evolution by increasing evolvability (Lehman & Miikkulainen, 2015). Even though the disappearance of some populations or species may favor divergence, but very high levels of extinction may lead to the disappearance of species even before they have been described (Staab et al., 2015).

Species is the basic unit for studies in biodiversity, biogeography, ecology, evolutionary biology, as well as for conservation biology and therefore determining what constitutes a species is a crucial step. A universal species definition has not been found, and multiple attempts have been made (there are even more than 20 species concepts) based on morphology, reproductive isolation, ecological niche, phylogeny...

or combinations of them. Some scholars even argue that species are not individual real entities, but should be considered as man-made constructs (Hey, 2006; Mallet, 2013). Obviously the species concept used will influence the analyses, the interpretation of data and the conclusions of any study carried out in the fields mentioned above. Taxonomists are the scientists who describe, name, revise and synonymize taxa ultimately (Agapow, 2004). A key step is the reexamination and review by other subsequent authorities that support or deny the conclusions (Raczkowski & Wenzel, 2007). The traditional taxonomic classification is based on morphological similarities between species, an eminently descriptive approach, although sometimes ecological or physiological characteristics were noted (Wheeler, 2004). The origin of modern taxonomy is the publication of the first edition of *Systema Naturae* by Carolus Linnaeus in 1735, proposing a binomial naming of species and a standardized system of classification of living beings. But the morphological taxonomy is complicated by phenomena such as cryptic speciation (Shaw, 2001; Bickford et al., 2007; Struck et al., 2018), species with high morphological plasticity (de Kroons & Hutchings, 1995; reviewed by Price et al., 2003), or by sexual dimorphism (Punzalan & Hosken, 2010; Barrett & Hough, 2013; Berns, 2013; Charlesworth, 2018).

When the polymerase chain reaction (PCR) was developed, it became possible to sequence DNA markers with the help of universal primers in a routine manner (e.g.; Soltis et al., 1992; Hillis et al. 1996). Although molecular and especially DNA sequence data helped in many cases to clarify the systematic relationships between species and to delimit species boundaries (reviewed by Duminil & Michele, 2009; Huttunen & Ignatov, 2010; Stech et al., 2013) phenomena such as incomplete lineage sorting or hybridization may lead to severe difficulties in their application (Mallet, 2005; Vanderpoorten & Shaw, 2010; Naciri & Linder, 2015). Inconsistencies between morphological and molecular data on the one hand and between different molecular markers on the other hand may be due to the moment in which the speciation process occurred, due to the fact that distinct markers do not diverge in time at the same rate (de Queiroz, 2007; Heinrichs et al., 2009; reviewed by Vanderpoorten & Shaw, 2010).

There is not a single key methodology (e.g. morphology or DNA sequencing) for the determination of species. While technological advances are continuously integrated in systematic studies, established methods still contribute substantially. As a

consequence, integrative taxonomy emerged with the aim to use the richness of available methods from different fields that lead to more rigorous, accurate and complete scientific hypothesis on species diversity (Dayrat, 2005; Will et al., 2005; Renzaglia et al., 2007; Padial et al., 2010; Schlick-Steiner et al., 2010; Heethoff et al., 2011; Medina et al., 2012; Renner et al., 2013; Lee & Palci, 2015; Pante et al., 2015; Caparrós et al., 2016). Schlick-Steiner et al. (2010) recommended the use of at least three methodologies, among which morphology (phenotypic information) should be prioritized, along with genetic data (mainly nuclear but also plastidial markers) and finally another that provides complementary information. The incorporation of new techniques and bioinformatic tools to study diversity and its classification make the taxonomy a discipline under strong renewal (de Carvalho et al., 2007). There is even a wide debate in the taxonomic literature on the possibility of discarding the current biological codes based on Linnaean system, the International Codes of Nomenclature for algae, fungi, and plants (ICBN), animals (ICZN), bacteria (ICNB), and viruses (ICTV), and replace it by "PhyloCode", unranked classification and definition of taxa names strictly on the basis of phylogeny (Cantino & de Queiroz, 2000, 2014). Another proposition is to implement a system called "BioCode"; it governs the formation and choice of scientific names of taxa (using the binary names of Linnaean system) but not the circumscription, position, or rank of the taxa themselves (Greuter et al., 1998, 2011). But none of them, for the moment, is having too much impact and acceptance. From what has been said so far, we can deduce that species names give relatively little information, they do not tell us about the function or evolutionary history of species (Swenson, 2011). But having a name is an important step to be recognized and considered as entity (Delić et al., 2017). In this context an evolutionary biologist must collaborate or train together with a strict taxonomist (Raczkowski & Wenzel, 2007).

MAIN CHARACTERISTICS OF BRYOPHYTES

Land plants first appeared in the time from the Middle Cambrian-Early Ordovocian period, about 500 mya (Morris et al., 2018) to the Middle Ordovician period, about 470 mya (Strother et al., 1996; Wellman et al., 2003; Rubinstein et al., 2010; Lenton et al.,

2012). The bryophytes comprise about 15 000 known species (Hallingbäck & Hodgetts, 2000), including liverworts, hornworts, and mosses. There are discrepancies about this part of the tree of life when different phylogenetic methods are used, some data is consistent with monophylly of bryophytes (Goremykin & Hellwig, 2005; Ruhfel et al., 2014; Wickett et al., 2014; Morris et al., 2018; Puttick et al., 2018), with "Setaphyta" (liverworts and mosses) a sister clade of hornworts. However other analyses support the hypothesis that bryophytes are paraphyletic, with liverworts often placed sister to all other land plants, followed by mosses, and with hornworts sister to Tracheophyta (Qiu et al., 2006; Qiu, 2008; Ruhfel et al., 2014). From all other land plants, bryophytes are distinguishable in having a dominant gametophyte life cycle and unbranched sporophytes that develop from an embryo embedded within and nutritionally dependent on the gametophyte (Vanderpoorten & Goffinet, 2009). Even though a fraction of sperm cells are tolerant to environmental desiccation for extended periods, an aqueous medium is essential for the sperm to get from the antheridia to the eggs located in the archegonia (Rosenstiel & Eppley, 2009; Shortlidge et al., 2012). However these characteristics associated with water do not limit the expansion of mosses in terrestrial environments, and we can find them in deserts (Smith, 1982; Bowker et al., 2000; Zheng et al., 2011), artics or in tropical forests and from sea level to alpine peaks. (Richardson, 1981; Smith, 1982; Richards, 1984).

BRYOGEOGRAPHY

There are bryophyte species that show restricted to very specific distribution patterns and others are found in all continents with a very widespread distributions (van Zanten, 1978; van Zanten & Pocs, 1981; Schuster, 1983; Schofield, 1984, 1988, 1992; Vanderpoorten & Goffinet, 2009). Some examples are in Mediterranean climate: *Bartramia aprica* Müll. Hal., (Damayanti et al., 2012); Australia: *Archidium thalliferum* I.G. Stone (Stone, 1985); Fuerteventura: *Orthotrichum handiense* F. Lara, Garilleti & Mazimpaka (Patiño et al., 2013); or with worldwide distribution *Funaria hygrometrica* Hedw., (Ochyra et al., 2008) and *Bryum argenteum* Hedw. (Pisa et al., 2014). The bryophytes have low levels of endemicity compared to other plant groups. For example,

in the flora of the Canary Islands, endemic species reach 40% in angiosperms and only 1.5% in bryophytes (Patiño et al., 2014); and in peninsular Spain the percentage of endemicity of vascular plants arrives to 15.5% (Aedo et al., 2013), and in bryophytes (including even the territory of the Balearic Islands) it is only a scant 0.5% (Infante et al., 2017). The small size allows them to inhabit microhabitats and facilitate their survival on adverse environments (Anderson, 1963; Patiño et al., 2016). Moreover, bryophytes are totipotent: all cells have the ability to differentiate into a meristematic state and develop a new plant from any small fragment. This confers them great advantages in establishment, colonization, and maintenance in ecosystems; It has been shown that under the specific conditions of permafrost a successful regeneration of bryophytes after some centuries (even millennia) is possible (La Farge et al., 2013; Roads et al., 2014; Cannone et al., 2017).

Diaspores (spores, gemmae or gametophyte fragments) have an outstanding capability for dispersal including long distances, mainly by air currents (van Zanten, 1978; van Zanten & Pocs, 1981; Muñoz et al., 2004; Lönnell et al., 2012; Norros et al., 2014; Lönnell, 2014; Biersma et al., 2017), or through animals (Parsons et al., 2007; Lewis et al., 2014; Wilkinson et al., 2017; Chmielewski & Eppley, 2019). Around 60% of the moss species and 70% of liverworts are dioicous (Patiño & Vanderpoorten, 2018). Most of the reported sex ratios in dioicous bryophytes are female biased (Bisang & Hedenäs, 2005, 2013; Rydgren et al., 2010), including sex expressing and nonexpressing plants (Bisang & Hedenäs, 2013). In dioicous species the population sex ratios can be altered because one sex may be less stress tolerant than the other sex (Stark et al., 2005a, b; Benassi et al., 2011), affecting the probability of sexual reproduction (Casanova-Katny et al., 2016). An evolutionary adjustment of the size, shape and ornamentation of the spores, as well as the height and timing of the release, can significantly change the expected distribution of the dispersion of organisms with spores (Johansson et al., 2014; Zanatta et al., 2016). Usually monoicous species present sporophytes, given the high level of intragametophytic selfing (Anderson, 1963; Mishler, 2001; Eppley et al., 2007; Karlin et al., 2011). On the contrary, dioicous species more rarely produce sporophytes (Longton & Miles, 1982; Longton, 1992), given the difficulty of finding a male and a female relatively close (Vanderpoorten & Goffinet, 2009). The sporophyte frequency is correlated with sex ratio (Rydgren et al., 2010) and with microclimates (temperature: Casanova-Katny et al., 2016). Many

species do not reproduce by spores, and no differences were observed in effectiveness of diaspore dispersal between generative and vegetative propagation, even reaching several 100 km in both cases (Frahm, 2008). However other studies showed evidences of usually short distance dispersal for asexual propagules, due to their weight (Kimmerer, 1991, 1994; Löbel et al., 2006). Moreover, no correlation was found between the presence of asexual propagules and dioicy in mosses and liverworts (Laenen et al., 2016). The mating system does not cause changes on allele frequency in the absence of evolutionary forces, selfing bisexual or asexual bryophytes have no lower gametophytic variation than outcrossing unisexual ones. But this behavior changes in several ways when mating system is combined with (at least one) evolutionary forces: selection, gene flow, genetic drift, and mutation (Stenøien & Såstad, 2001).

Due to small size and ability to produce spores (and others dormant stages), which facilitate dispersal by air and migrating animals, Baas Becking (1934) proposed that "*in microorganisms, everything is everywhere, but the environment selects*". This tenet was tested for the cosmopolitan moss *Bryum argenteum* in which a genetic variation significantly correlated with the elevation was found in Spanish Sierra Nevada Mountains; the results indicated that genetic structure in cosmopolitan mosses may be due mainly to ecological specialization and not so much to the limitation of dispersion (Pisa et al., 2013; Magdy et al., 2016), supporting what Baas Becking said. On the other hand, in spite of the fact that some mosses have a high production of diaspores, not all of them have a wide distribution (Frahm, 2008). Even considering only seemingly suitables habitats, bryophytes present limitations to effective dispersal (Medina et al., 2011). Two alternative views can explain the main bryophyte disjunctions and they are frequently discussed and revised: vicariance, where the fragmentation of a previous continuous range takes place, and dispersal within and among species (Crum, 1972; Buck, 1990; Shaw et al., 2003; McDaniel & Shaw, 2003, 2005; Devos & Vanderpoorten, 2009; Heinrichs et al., 2009; Vanderpoorten et al., 2010; Shaw et al., 2014, 2015a; Biersma et al., 2017). Probably these distribution patterns are formed by events like short distance dispersal, long distance dispersal, extinction, recolonization and diversification (Heinrichs et al., 2009). In some species of Polytrichales with extreme bipolar disjuntion, major trans-equator dispersal events (very long-dispersal) have occured but are extremely rare and occur only sporadically on multi-million-year

timescales (Biersma et al., 2017). However, more typical long-distance dispersal events (around 1.5 to more than 100 km) may be the rule and not the exception in bryophyte metacommunities (Barbé et al., 2016; Lönnell & Hylander, 2018). But what we consider short or long distances of dispersal will depend on the ecosystem and the taxon (Barbé et al., 2016). To know the dispersal mechanisms of a species is important for understanding and predicting its distribution dynamics in space and time, being able to influence speciation processes (Johansson et al., 2014).

The Mediterranean area (Taberlet et al., 1998; Vogel et al., 1999; Mejías et al., 2007), Asia Minor (Ansell et al., 2011) and central Europe (Provan & Bennett, 2008) served as refugia for animals and plants along the period of multiple glacial cycles during the Quaternary (2.6 mya to present). Among the mountainous systems the Sierra Nevada Mountains in southern Spain are especially interesting (Fig. 1), because there populations of many species were moving up or down in the mountains according to the climatic conditions reigning during the glaciations (Gutiérrez Larena et al., 2002). This mountainous system began to rise during the Tertiary period from the collision of the continental plates of Africa and Eurasia (Channell & Medizza, 1981; Sanz de Galdeano & López Garrido, 2000). It is situated inside the Baetic range and presents the highest peak on Iberian Peninsula (Mulhacén: 3481 m above sea level), with other important peaks above 3000 m, and the southermost glacial cirques in Europe (Gómez-Ortiz et al., 2015). Sierra Nevada Mountains are exposed to Mediterranean climate in its pluviseasonal oceanic variant (Rivas Martínez et al., 2007), with a pronounced summer drought and precipitation as snow above 2000-2500 m almost always during the winter season (Blanca et al., 2009; Fernández Calzado et al., 2010). The peculiar situation and characteristic of these mountains allow them to have a relevant biological richness (Gil, 1976; Molero Mesa & Pérez Raya, 1987; Rams Sánchez, 2007; Rams et al., 2014; Lorite, 2016) and to host a large number of endemic vascular plants (Prieto Fernández, 1975; Blanca et al., 1998; Blanca & Molero, 1990; Blanca, 2002).

SPECIATION IN MOSSES

The bryophytes exhibit a comparatively lower extant diversity than tracheophytes (Laenen et al., 2014; reviewed by Patiño & Vanderpoorten, 2018), which might be due to a lack of opportunities for allopatric speciation by intense gene flow (Wilkinson, 2001; Finlay, 2002), or might result from lower net diversification due to elevated extinction or decreased speciation rates (Laenen et al., 2014). Alternatively, the species richness of bryophytes might be underestimated by the existence of cryptic species or taxonomic shortcomings (Konrat et al., 2010; Medina et al., 2012; Hedenäs et al., 2014; Caparrós et al., 2016; Renner et al., 2017).

Fig. 1. View of Sierra Nevada Mountains. Photo by Marta Nieto Lugilde

In bryophytes the limited number of good morphological diagnostic traits between species favors cryptic speciation, hiding the genetic structure (Shaw, 2001). Many taxa (especially dioicous species) rarely or never produce sporophytes (Longton & Schuster, 1983), making it difficult to identify taxa. Apparently some widely distributed bryophyte species actually correspond to complexes of multiple species with narrower distribution ranges (reviewed by Patiño & Vanderpoorten, 2018).

On island archipelagos the most frequent speciation mode in bryophytes is anagenesis (Patiño et al., 2014), in which changes accumulate over time within the same lineage (temporal continuum). This way founder events do not produce evolutionary radiation on an island and the number of species is not increased (Stuessy et al., 2006).

However, in angiosperms congeneric endemic species generally result from the diversification of a single common ancestor in a cladogenetic speciation (Patiño & Vanderpoorten, 2015).

Hybridization causes the so-called reticulated evolution and is due to genetic exchange in moments of sympatry both in animals and plants (Dowling & Secor, 1997; Natcheva & Cronberg, 2007). In many cases there are no clear limits (neither morphological nor reproductive) that allow to delimit the species without problems, being fundamental the study of genetic architecture of speciation (Heinrichs et al., 2009; Harrison & Larson, 2014).

Polyploidy is an important, well-established phenomenon in plants (Soltis et al., 2018), but an interesting recent debate about if, once established, new polyploid lineages enjoy or not increases in diversification rates has arisen (Wood et al., 2009; Mayrose et al., 2011; Arrigo & Barker, 2012; Soltis et al., 2014; Mayrose et al., 2015). Due to the lower incidence of genetic compatibility problems, most reported cases of polyploidy are autopolyploid (doubling of an individual taxon), but numerous cases of allopolyploid (doubling of a combination of the genomes of two taxa) speciation have occurred for example within the *Physcomitrium* (Brid.) Brid.-*Physcomitrella* Bruch & Schimp. complex (Beike et al., 2014), and there are other well-know examples in bryophyte genera like *Atrichum* P. Beauv. (Perley & Jesson, 2015), *Plagiomnium* T.J. Kop. (Wyatt et al., 1988, 1992; Jankowiak-Siuda et al., 2008) or the peatmoss *Sphagnum* L. (Karlin et al., 2009, 2010; Shaw et al., 2015 b).

THE GENUS *CERATODON* BRID.

Bridel (1826) created the genus *Ceratodon* when he separated *C. purpureus* (Hedw.) Brid. and *C. chloropus* (Brid.) Brid. from *Dicranum* Hedw. *Ceratodon* plants are characterized by the following description (Burley & Pritchard, 1990): acrocarpous, habit caespitose or crowded turf, scarcely branched, 5-60 mm high, reddish rhizoids at lower part of stems. Leaves ovate to linear-lanceolate, obtuse to acuminate; margin recurved, rarely plane, variably dentate to serrate near apex or entire; nerve sub-

percurrent to longly excurrent; mid-leaf cells quadrate to isodiametric, smooth and unistratose. Dioicous; occasionally with asexual filamentous propagules in axils of leaves. Seta yellow to red, 5-40 mm long; capsule ellipsoid to cylindrical; calyptra cuculate; annulus revoluble, 2-3 seriate; operculum conical to rostrate; peristome teeth 16, 250-600 µm long, bifid nearly to base, with 4-18 articulations (and 0-13 trabeculate), strongly papillose, usually with a membranous border. Spores papillose, sub-spherical, 10–21 µm in diameter.

The number of species of the moss genus *Ceratodon* is controversial (Burley & Pritchard, 1990; Ochyra, 1998; O'Shea, 2006; McIntosh, 2007), mainly due to their high polymorphism (Dixon & Jameson, 1896; Watson, 1968; Crum & Anderson, 1981), hindering the discrimination between them. Burley and Pritchard (1990), based on a worldwide morphometric study including an extensive taxonomic and nomenclatural synthesis, recognized four species: *C. antarcticus* Cardot, *C. conicus* (Hampe *ex* Müll. Hal.) Lindb., *C. heterophyllus* Kindb., and *C. purpureus* with three subspecies: subsp. *purpureus*, subsp. *convolutus* (Reichardt) Burley, and subsp. *stenocarpus* (Bruch & Schimp.) Dixon. But the grouping of subespecies on the basis of latitudinal variation in continuously varying morphological traits of Burley and Pritchard (1990), were not sustained by the genealogical results obtained by McDaniel and Shaw (2005); however they found limited population structure across the global distribution, suggesting that long-distance migration is common in the genus, at least within the Northern Hemisphere and Australasian regions.

The most abundant species with worldwide distribution is *C. purpureus* (Fig. 2; Crum, 1973), which grows in quite diverse habitats, usually in places where the competition between plants is limited due to stress or disturbance (Jules & Shaw, 1994; Eversman, 2001). It can colonize a wide range of substrata, such as bare ground, rock, wood, and sand (Crum, 1973). Moreover it is common from natural and well conserved to contaminated sites (Shaw et al., 1991), and it gets the name “fire moss” in North America for its habit of colonizing recently burned places (Duncan & Dalton, 1982; Foster, 1985; Clément & Touffet, 1990). This moss emits volatile cue substances (more by females) to guide microarthropods (springtails) and use them as “pollinators” (sperm transport vectors) during the sperm dispersion, increasing moss fertilization (Rosenstiel et al., 2012). In addition to this affinity for practically any habitat, its facility to produce

many sporophytes with reddish or purple capsules and hundreds of spores per sporophyte, make it a great pioneering species (Shaw et al., 1991; Beever et al., 1992; Shaw & Beer, 1999; Taylor et al., 2007).

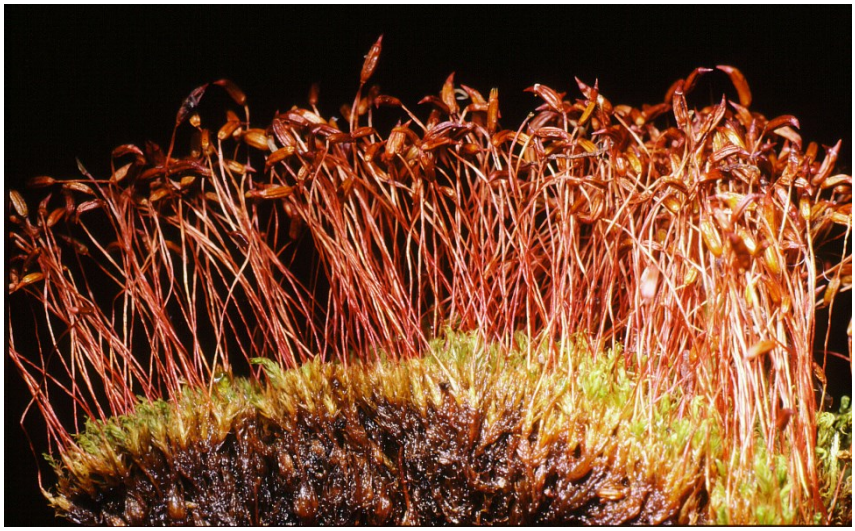

Fig. 2. The cosmopolitan moss *Ceratodon purpureus.* Photo by Olaf F. Werner.

With the discovery and development of homologous recombination technologies at a reasonably efficiency in the moss *Physcomitrella patens* (Hedw.) Brach & Schimps. (Schaefer & Zrÿd, 1997) and later in *C. purpureus* (Zeidler et al., 1999; Brücker et al., 2005), an efficient tool for the analysis of gene functions was provided. The use of bryophytes as model organisms in land plant studies is due to characteristics such as: free-living dominant haploid life stage, which allows the effects of recessive mutations to be observed directly; easily cultured through vegetative propagation under controlled conditions; excellent opportunity to observe developmental processes at the level of the single cell on protonemata stage; possession of relative small genome and tolerance to induced mutations (Cove et al., 1997). A major difference to other moss species used as model plants (*P. patens* and *Funaria hygrometrica*) is that *C. purpureus* is dioicous. Today the complete genome sequencing is done for *C. purpureus* (https://genome.jgi.doe.gov/portal/ThemosCpurpureus/ThemosCpurpureus.info.html).
An important advantage of the use of a common species like *C. purpureus* as a model plant is that it offers a substantial number of individuals, simplifying the logistics of the sampling, reducing the harmful impact on natural populations and facilitating the statistical validation of the results in studies of intraspecific variation (Steel et al., 2013).

Sex determination in dioicous mosses differs from vascular plants, because it takes place at the haploid gametophyte stage, where female and male plants carry different sex chromosomes called U and V, respectively (Bachtrog et al., 2011). The diploid sporophytes are heterozygous for the sex chromosomes. The sex specific region of the U-V chromosomes is non-recombining, but a part of the U-V chromosomes carries genes that are not sex related and can recombine during cross-over (Bachtrog et al., 2011). Because both chromosomes are subject to purifying selection during the haploid phase neither chromosome is expected to loose a high number of genes by degeneration, contrary to what is observed for example in mammals with their X-Y system (Bachtrog et al., 2011). As a consequence of this system in mosses both female and male sex chromosomes have a similar (although not always identical) size (Allen, 1945) and in *C. purpureus* they are even five times larger than average autosomes (McDaniel et al., 2007). Several studies addressed the ratio of female and male plants in *C. purpureus*. Shaw and Gaughan (1993) found a heterogeneous sex ratio between populations but male biases occur only in two of 11 populations, and 9/11 were female biased; moreover at time of germination a three females/two males ratio was observed with sexually dimorphic traits, probably associated with the differences of life history. However, Eppley et al. (2018) discovered that the ratio of sexes in artificial populations changed over time, but not in all populations. Those in which the females predominated retained their high female bias and produced a large number of sporophytes, but in the populations where the males predominated significantly, they moved towards a deviation for the females and most of the populations did not produce sporophytes. The sex ratios are influenced at spore stage (Norrell et al., 2014) but also during the gametophytic stage, probably caused by differential survival or growth of clones during both intra and interspecific competition (Eppley et al., 2018). The genetic differentiation in morphological and life history observations among populations showed sexually dimorphic traits with respect to size, physiology, maturation rates, and reproductive output between male and female gametophytes (Shaw & Gaughan, 1993; Shaw & Beer, 1999; McDaniel, 2005; Slate et al., 2017). Multiple complex genetic factors contribute to divergence among population of *C. purpureus*; some hybrid progeny from a cross between isolates of different populations that represent extremes of the morphological distribution of the species showed abnormal development phenotype and a segregation

pattern that it is consistent with Dobzhansky-Muller interactions (McDaniel et al., 2007, 2008).

AIMS OF THE THESIS

The main aim of this thesis was to study the diversity, evolutionary biology and taxonomy of the populations of the genus *Ceratodon* in southern Europe. The specific aims of the thesis were:

1. To review the taxonomy and nomenclature of the genus *Ceratodon* in southern Europe.

2. To know the genetic diversity of *Ceratodon* populations in Mediterranean mountain areas.

3. To evaluate if the *Ceratodon* populations of southern Europe belong to the same species or, on the contrary, if the genetic diversity found between them defines perhaps different species and in this case shed light on the speciation process.

4. To test if southern European *Ceratodon* specimens show differences in sex ratio and DNA amount in nuclei.

5. To determine in what proportion the morphological variation in *Ceratodon* is due to the environment or if it has a genetic basis.

6. Using an integrative methodology, to reach a taxonomic conclusion about samples from southern Spain that have different morphology from that described for *C. purpureus*.

7. To reconstruct the evolutionary history of *Ceratodon* species present in southern Europe and their interaction via hybridization.

8. To estimate demographic parameters and evaluate possible evolutionary scenarios in southern European populations of *Ceratodon*.

THESIS STRUCTURE

The bulk of this thesis is structured in four chapters. Each of them contains a study focused on different aspects of the evolutionary biology or the taxonomy of the moss genus *Ceratodon in* southern Europe. All the chapters are presented as scientific papers. These are followed by a general discussion and the conclusions of this thesis. The following is a brief summary of each chapter.

Chapter I. Taxonomical and nomenclatural notes on the moss *Ceratodon conicus* (Ditrichaceae, Bryophyta). In this work the revision of the nomenclatural type of *Ceratodon conicus* and the three synonyms proposed in the world study of the genus *Ceratodon* published by Burley and Pritchard (1990) was carried out (*C. cedricola*, *C. dimorphus*, and *C. purpureus* var. *graefii*) with the aim of knowing the morphology of the species susceptible of been present in the Mediterranean area (specific aim 1).

Chapter II. Peripatric speciation associated with genome expansion and female-biased sex ratios in the moss genus *Ceratodon.* The genetic and genome size diversity in European populations of the moss *Ceratodon purpureus s.l.* was examined to evaluate the role of allopatry and ploidy change in population divergence (specific aims 2, 3 and 4). Phylogenetic and coalescent analyses on sequences from five nuclear introns and a chloroplast *locus* were performed to reconstruct their phylogenetic history. The genome size using flow cytometry was also estimated, and the sex of the samples determined. These data suggested that a new species of *Ceratodon* arose via peripatric speciation, potentially involving a genome size change and a strong female-biased sex ratio. The new species hybridized in the past with *C. purpureus*.

Chapter III. Environmental variation obscures species diversity in southern European populations of the moss genus *Ceratodon.* Biometric analyses based on 22 morphological gametophytic characters on both field collected plants and cultivated plants of *Ceratodon* populations from Mediterranean mountain areas, other European mountain systems and lowlands were compared to a clustering based on DNA sequence and genome size data obtained on previous chapter, to evaluate the effect of

environmental variation on the taxonomy of the moss genus *Ceratodon* and reach a taxonomic conclusion (specific aims 5 and 6). It was confirmed that environmental variability complicates taxonomic inferences. However, integrative taxonomy based on genetic diversity, genome size and morphological data unambiguously supports the recognition of a new species, *Ceratodon amazonum* Nieto-Lugilde, O. Werner, S.F. McDaniel & Ros. The chapter includes the formal description of *C. amazonum*, a morphology based key for its distinction from *C. purpureus* and the nothospecies *C* ×*conicus,* which is considered to be of recombinant origin, and for wich an epitype is designated.

Chapter IV. Testing the evolutionary history of *Ceratodon amazonum*, *C. purpureus*, and the recombinant *C.* ×*conicus.* It is focused on testing the peripatric speciation and reconstructing the demographic and evolutionary history in the cosmopolitan moss *C. purpureus*, the sister species *C. amazonum*, and the recombinant *C.* ×*conicus*, based on nucleotide polymorphism data (specific aims 7 and 8). For these aims, populations genetic summary statistics were calculated, demographic parameters (divergence time, effective population sizes and migration rates) were estimated and evolutionary scenarios were simulated. Moreover, it was calculated if hybridization between parental taxa occurred in one or several events. *Ceratodon purpureus* almost always had higher levels of genetic diversity than *C. amazonum*. A recent speciation event was confirmed, the divergence time between both parental species was about 1.7 mya, and *C.* ×*conicus* was no originated by a unique and rare hybridization event.

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Chapter I. Taxonomical and nomenclatural notes on the moss *Ceratodon conicus* (Ditrichaceae, Bryophyta)

ABSTRACT

A revision of the nomenclatural and taxonomical data related to *Ceratodon conicus* (Hampe *ex* Müll. Hal.) Lindb. and its synonyms published by Burley and Pritchard (1990) was carried out. The lectotype designated from material filed in GOET was confirmed, but the material from duplicates of lectotype specimens filed in FH, GOET, and MANH was found not corresponding with the protologue of the species. In addition, the types of three synonyms of *Ceratodon conicus*, *C. cedricola* J.J Amann from Z+ZT, *C. dimorphus* H. Philib. from BM, and *C. purpureus* var. *graefii* Limpr. from BR, designated as holotypes by Burley and Pritchard, are here designated as lectotypes as no unequivocal original specimen exists in any case. Finally, *Ceratodon purpureus* var. *graefii* is better considered to be a synonym of *C. purpureus* (Hedw.) Brid. *sensu lato*, as its morphological characteristics match the description of this species and not that of *C. conicus*.

INTRODUCTION

Burley and Pritchard (1990) recognized four species in the genus *Ceratodon* Brid. in their worldwide taxonomical revision: *C. antarcticus* Cardot, *C. conicus* (Hampe *ex* Müll. Hal.) Lindb., *C. heterophyllus* Kindb., and *C. purpureus* (Hedw.) Brid. with three subspecies. Their study was based on an extensive morphometric study including a great taxonomic and nomenclatural synthesis.

Ceratodon conicus was described as *Trichostomum conicum* Hampe *ex* Müll. Hal. (Müller, 1849), based on material received *in litt*. from the German botanist Georg Ernst Ludwig Hampe (1795-1880). The protologue reads "*Germania septentrionalis, Flegesen circa Hameln prope Hohnsen in muris: Schlotheuber*". The species was first validly combined in the genus *Ceratodon* by Lindberg (1879). Interestingly, Carl Müller himself later (Müller, 1899) proposed the illegitimate homonymous combination *Ceratodon conicus*, based on *Barbula conica* Spreng. from South Africa, that according to Burley and Pritchard (1990) is a synonym of *C. purpureus* subsp. *stenocarpus* (Bruch & Schimp.) Dixon.

According to Burley and Pritchard (1990), the most important diagnostic features of *Ceratodon conicus* from the other species in the genus are: the ovate-lanceolate, slightly concave leaves, the entire, recurved to apex or just below margins, the costa excurrent in an arista of variable length, and the yellow to orange-reddish peristome teeth, 21-48 μm wide at base, usually with 3-5 trabeculae and 5-9 articulations, with narrow or absent border. The gametophytic characters were not found sufficiently stable by Burley and Pritchard (1990), which lead the authors to state that, in the absence of mature capsules, it is not possible to distinguish *C. conicus* from some morphs of *C. purpureus* with confidence.

Ceratodon conicus can be found with certainty in North America (Canada and U.S.A.), Europe (Austria, Germany, Norway, Switzerland, United Kingdom) and North Africa (Morocco); its habitat is terrestrial on bare earth, but it also occurs on soil-capped limestome walls, rock crevices and mountain ledges; it is probably is xerophytic and strictly calcicolous (Burley & Pritchard, 1990).

The acceptance of *C. conicus* at species level has been disputed, and some authors (Husnot, 1884; Dixon, 1896) have moved it to varietal rank given the scarce and unreliable diagnostic morphological characters of the gametophyte, and the usual absence of sporophytes. While the authors of major large European checklists (Hill et al., 2006; Ros et al., 2013) accepted the taxonomic view of Burley and Pritchard (1990), others have had taxonomic difficulties deciding about the certainty of its occurrence (Kučera & Váňa, 2003), or accepting the species at subspecific level as McIntosh (2007) in the Flora of North America.

Burley and Pritchard (1990) revised most of the approximately 70 names attributable to the genus *Ceratodon*, which were included in Index Muscorum (Wijk et al., 1959, 1969). They designated the lectotype and three isolectotypes for *Trichostomum conicum*, and put into its synonymy three additional names: *Ceratodon cedricola* Amann from Morocco, *C. dimorphus* Philib., and *C. purpureus* var. *graefii* Limpr., the latter two from Switzerland.

In the course of a taxonomical study of *Ceratodon* species, some nomenclatural and taxonomical inconsistencies in the treatment of Burley and Pritchard (1990) were found. Based on the study of the protologues and the types designated by these authors, in this paper intends to solve the problems detected and to know the morphology of the species susceptible of been present in the Mediterranean area.

MATERIALS AND METHODS

We revised the types of *Trichostomum conicum* designated by Burley and Pritchard (1990), which were the lectotype and isolectotypes from GOET at the Georg-August-Universität Göttingen and borrowed the isolectotype from FH. The label data of the isolectotype from MANCH was analyzed through photographs sent by the curator of the herbarium, together with other specimens identified under this name. We also borrowed a specimen from STU, mentioned by Meinunger and Schröder (2007) as a possible type. Additionally, we revised the types of the three synonyms proposed by Burley and

Pritchard (1990), namely *Ceratodon cedricola* from Z+ZT, *C. dimorphus* from BM, and *C. purpureus* var. *graefii* from BR.

RESULTS AND DISCUSSION

Type revision of *Ceratodon conicus*

Ceratodon conicus (Hampe *ex* Müll. Hal.) Lindb., *Musci Scand*. 27. 1879 (non *Ceratodon conicus* (Lindb.) Müll. Hal., *Hedwigia* 38: 98. 1899, *hom. illeg*.). **Basionym:** *Trichostomum conicum* Hampe *ex* Müll. Hal., *Syn. Musc. Frond*. 1: 575. 1849; *Ceratodon purpureus* var. *conicus* (Hampe *ex* Müll. Hal.) Husn., *Muscol. Gall*. 60. 1884; *Ceratodon purpureus* subsp. *conicus* (Hampe *ex* Müll. Hal.) Dixon, *Stud. Handb. Brit. Mosses* 68. 1896. **Lectotype** (designated by Burley & Pritchard, 1990): [Germany, Niedersachsen] Auf Mauern bei Flegesen … (unreadable) Hameln bei Hohnsen, Mai coll. *Schlotheuber* pastor eccl. 784, Hampe misit 15/2 48, C. Müller det. (GOET 011795!).

Burley and Pritchard (1990) designated the lectotype of *Ceratodon conicus* from GOET and also four isolectotypes from FH, GOET (2) and MANCH. Nevertheless, at present, only one specimen is kept at GOET, where duplicates of Hampe's Herbarium are preserved. All of them have a revision label handwritten by J. S. Burley in 1985, on which he writed: "Appears to be a good species *C. conicus* (Hampe) Lindb". This specimen is barcoded (GOET 011795) and considered an isotype by the herbarium keepers (Fig. I.1).

As no more specimens were found at GOET whose label matches exactly the protologue, and we have not found any other potential type specimen exhibiting a revision label of Burley, we consider the above described specimen as the lectotype. Nevertheless, there is a non-barcoded specimen in GOET with the label "*Trichostomum conicum* Hampe n.sp., Auf einer Gartenmauer in Hachmühlen unweit Hameln", lacking collector name and date. However, the locality data do not correspond with those of the lectotype, as it is in the neighboring zones of the locality of the lectotype (Flegessen) but at a certain distance. Although it has not a revision label of Burley, it could be the

GOET isolectotype mentioned by Burley and Pritchard (1990), since the locality data are the same as in the next two herbaria specimens, which were also considered isolectotypes by these authors.

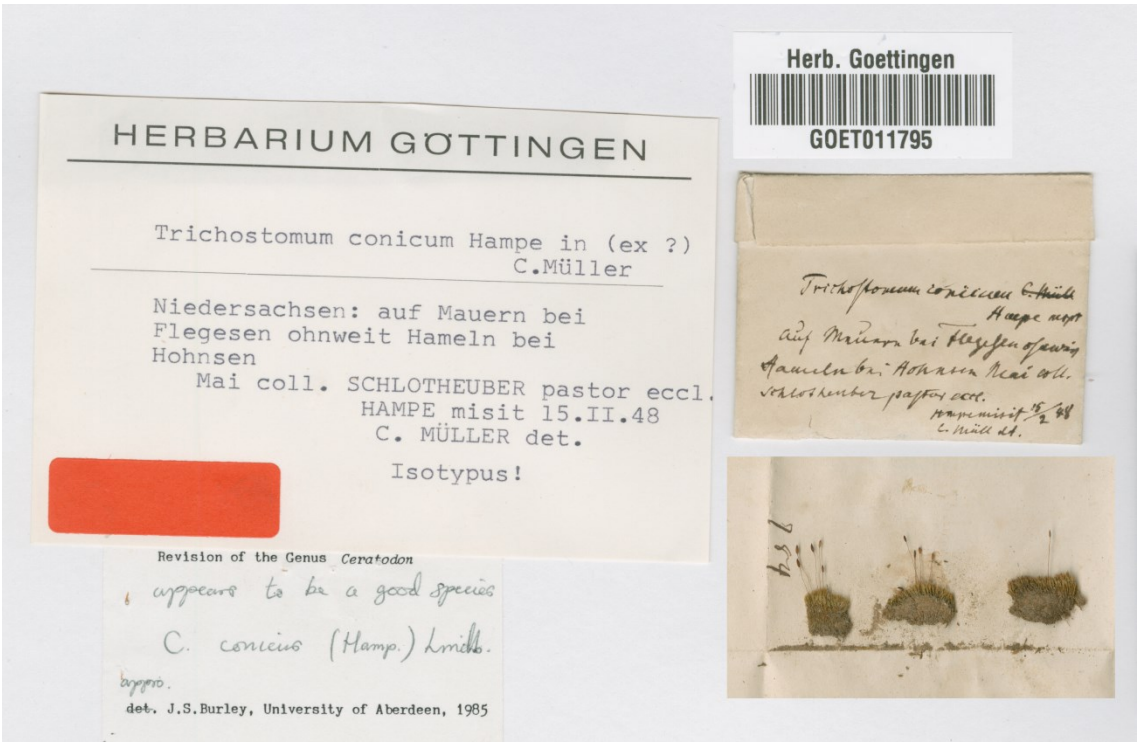

Fig. I.1. Leptotype of *Trichostomum conicum* Hampe *ex* Müll designated by Burley and Pritchard (1990). Photo by Marta Nieto Lugilde.

The MANCH specimen studied and annotated by Burley in 1985, and designated as isolectotype in Burley and Pritchard (1990) (labelled "Garden wall near Hachmühlen Hannover, Pastor *Schlotheuber*, May 17/47"), cannot be considered part of the type material. The reason is that it was collected from a different locality (the same as the above mentioned specimen from GOET). Besides, it was collected one year earlier than the lectotype specimen. There is another specimen kept at MANCH that was collected from one of the above mentioned neighboring localities by the same collector, but without collecting date "Ex herb. Hampe, *Ceratodon conicus*, Hannover, Legit *Schlotheuber*". Therefore, none of the specimens kept at MANCH can be considered part of the type material.

The specimen from the Hampe Herbarium at FH ("*Ceratodon purpureus minor*, *Trichostomum conicum* ..., Germania pr. Hachmühlen, FH00290580!"), although without collecting date, might also belong to above mentioned non-type Hachmühlen

collection, parts of which are filed at GOET and MANCH. Morphologically, the specimen seems to correspond to the concept of *C. conicus* of Burley and Pritchard (1990): ovate to lanceolate leaves, entire margin, excurrent costa in most of the leaves, cross-section of leaves without guide cells; two-three sheeting internal perichaetial leaves, widely ovate to orbicular, rounded to obtuse, apiculate or not, with narrow, poorly developed costa, excurrent in a short apiculus or not; capsule not strumose and erect, peristome teeth not bordered, 30-32 µm wide at the base each, with 3-5 trabeculae and 3-4 articulations; spores 10-12 µm in diameter.

Finally, the label information of the specimen kept at STU reads "Hannover, *Ceratodon purpureus* (L.) Brid. var. *conicus* (Hpe) … , loc. class. des *Trichostomum conicum* Kr. Hameln, Hachmühlen auf der Gartenmauer der Gastwirtschaft, VI. 1880, leg. *Schlotheuber*". This specimen was mentioned in The atlas of German mosses (Meinunger & Schröder, 2007), together with the lectotype as the only certain German occurrences of the species, considering that other collections from the country are sterile and therefore doubtful. Also in this case, the collecting site is different from that of the lectotype and the same as the above mentioned specimens from FH, GOET and MANCH; moreover label data given do not match the information on collecting date (June 1880). According to Wagenitz (1988), the Hannovarian ecclesiastic pastor and botanist Schlotheuber lived between 1789 and 1866. The label is hand-written by Fritz Koppe (Martin Nebel, curator of STU, pers. comm.). Probably, he or another person separated a part of the original specimen to a duplicate with a new label in which mistakes were inserted.

The type of *Ceratodon cedricola*

Ceratodon cedricola J.J. Amann, *Rev. Bryol*. 51: 57. 1924. Holotype (as considered by Burley & Pritchard, 1990): *Ceratodon* sp. nova, *cedricola* Amann, Maroc. Atlas moyen. Ras El Ma, sur tronc de Cèdre. 27.3.23, l. *P. Jaccard* (Z+ ZT!). **Lectotype, designated here**: based on the former specimen, instead of holotype (= *Ceratodon conicus* (Hampe *ex* Müll. Hal.) Lindb., *Musci Scand.* 27. 1879) (Fig. I.2).

Burley and Pritchard (1990) considered the specimen mentioned above to be the holotype of this name, kept at Z+ ZT, but due to the fact that Amann did not designate

any specimen as the nomenclatural type and no unequivocal specimen exists, in our opinion the specimen kept at Z+ ZT should be better designated as lectotype.

The study of the type allowed us to confirm that the morphological characteristics of the specimen correspond to those given by Burley and Pritchard (1990) for *C. conicus*: leaves ovate to lanceolate, with entire margin and excurrent costa, with 3-4 guide cells in cross-section; both internal perichaetial leaves wide ovate to orbicular, strongly sheathing, with rounded to obtuse, non apiculate apex, costa narrow, poorly developed and non excurrent; capsule slightly strumose, erect to inclinate; peristome teeth not bordered, 32 µm wide at the base, with 4-5 trabeculae and 3-5 articulations; spores inmature. Amann (1924), when describing the species, considered *C. cedricola* to be closely related to *C. corsicus* Bruch & Schimp. (= *C. purpureus* subsp. *stenocarpus*) and distinguishable from the last one by the dense, tight, felted and smaller sized tufts (4-5 mm), the characteristics of perichaetial leaves, the small and narrow capsule, and the smooth and not bordered peristome teeth. Nevertheless, most of these characters are shared also by *C. purpureus s.l.*

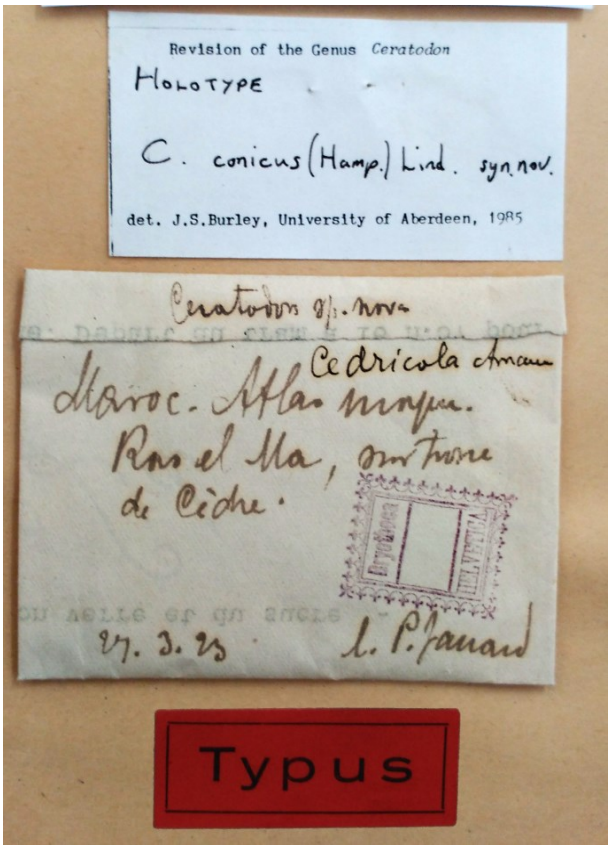

Fig. I.2. Lectotype of *Ceratodon cedricola* J.J. Amann designated as holotype by Burley and Pritchard (1990). Photo by Marta Nieto Lugilde.

The type of *Ceratodon dimorphus*

Ceratodon dimorphus H. Philib., *Rev. Bryol*. 15: 28. 1888; *Ceratodon purpureus* subsp. *dimorphus* (H. Philib.) Limpr., *Laubm. Deutsch*. 3: 683. 1901; *Ceratodon purpureus* var. *dimorphus* (H. Philib.) Mönk., *Allg. Bot. Z. Syst*. 15: 92. 1909; *Ceratodon purpureus* fo. *dimorphus* (H. Philib.) Mönk., *Laubm. Eur*. 158. 1927. Holotype (as considered by Burley & Pritchard, 1990): Husnot, *Musci Galliae* 814. *Ceratodon dimorphus* Phil. Sur le col du Simplon (Valais). Altitude 2000 m, juillet 1887, PHILIBERT (BM 000965220!). **Lectotype, designated here**: based on the former specimen, instead of holotype (= *Ceratodon conicus* (Hampe *ex* Müll. Hal.) Lindb., *Musci Scand*. 27. 1879) (Fig. I.3).

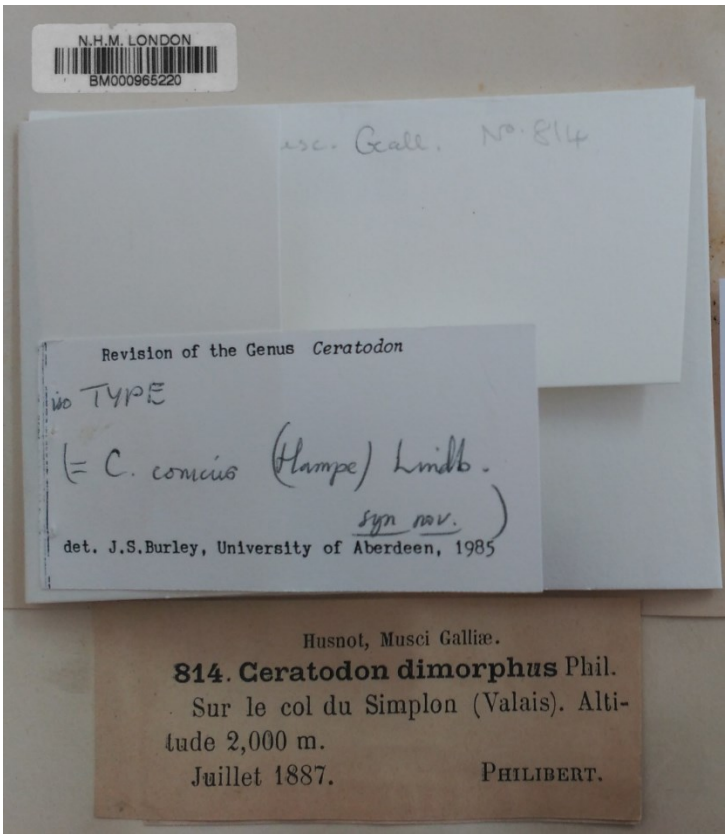

Fig. I.3. Lectolotype of *Ceratodon dimorphus* H. Philib designated as holotype by Burley and Pritchard (1990). Photo by Marta Nieto Lugilde.

Burley and Pritchard (1990) considered the BM specimen mentioned above as holotype, but as in *Ceratodon cedricola*, the author of the name did not designate any specimen as the nomenclatural type, and no unequivocal specimen exists. Therefore, it should be considered a lectotype, which is done here.

The specimen shows plane, ovate to linear-lanceolate leaves, with entire to dentate margins, costa excurrent in a short apiculus, rarely percurrent, with 2-3 guide cells in cross-section; sheathing and obtuse perichaetial leaves; capsule not strumose, slightly inclinate, peristome teeth narrowly bordered, 20-26 µm wide at the base, with 3-4 trabeculae and 4-6 articulations; spores 8-10 µm in diameter.

The morphological characteristics of the peristome of this specimen correspond to those given by Burley and Pritchard (1990) for *Ceratodon conicus*, but the leaves are more variable and are not typical for this species. It seems to present intermediate characteristics with *C. purpureus* subsp. *stenocarpus*, although we agree with Burley and Pritchard (1990) to identify it as *C. conicus*.

The type of *Ceratodon purpureus* var. *graefii*

Ceratodon purpureus var. *graefii* Schlieph. *ex* Limpr., *Laubm. Deutschl*. 1: 487. 1887; *Ceratodon graefii* Schlieph., *nom. nud. in synon*., *Laubm. Deutschl*. 1: 487. 1887; *Ceratodon purpureus* fo. *graefii* (Schlieph. *ex* Limpr.) Mönk., *Laubm. Eur*. 158. 1927. Holotype (as considered by Burley & Pritchard, 1990): *Ceratodon graefianus* Limpr. *e*x Schliephacke, Helvetia, Ober–Hasli ad terram, juni 1883 leg ... Dr. H. Graef com. Schliephacke (BR-BRYO 165426-41!). **Lectotype, designated here**: based on the former specimen, instead of holotype (= *Ceratodon purpureus* (Hedw.) Brid., *Bryol. Univ.* 1: 480. 1826) (Fig. I.4).

Burley and Pritchard (1990) selected the BR specimen mentioned above as holotype, but also in this case the author of the name did not designate any specimen as the nomenclatural type and no unequivocal specimen exists, and therefore, it should have been designated as lectotype. Also it seems that Burley and Pritchard were not aware that this name at the specific rank is invalid.

The morphological characteristics of the specimen are the followings: plane, ovate to linear-lanceolate leaves, with entire to crenulate margins, costa excurrent in an apiculus, cross section with 2 guide cells; internal perichaetial leaves sheathing with obtuse apex; capsule not strumose, erect or inclinate, slightly sulcate to smooth, peristome teeth strongly bordered, 46-50 µm wide at the base, with 4-5 trabeculae and 4-5 articulations; spores inmature. It is worth mentioning that some characteristics of

the capsule and the leaves do not fit the diagnostic characters given for *C. conicus* by Burley and Pritchard (1999), especially the basally wide, strongly bordered peristome teeth. Taking into consideration the characters considered in the revision of Burley and Pritchard (l.c.), this specimen falls within the variation described for *Ceratodon purpureus s.l.* and therefore should not be considered a synonym of *C. conicus*.

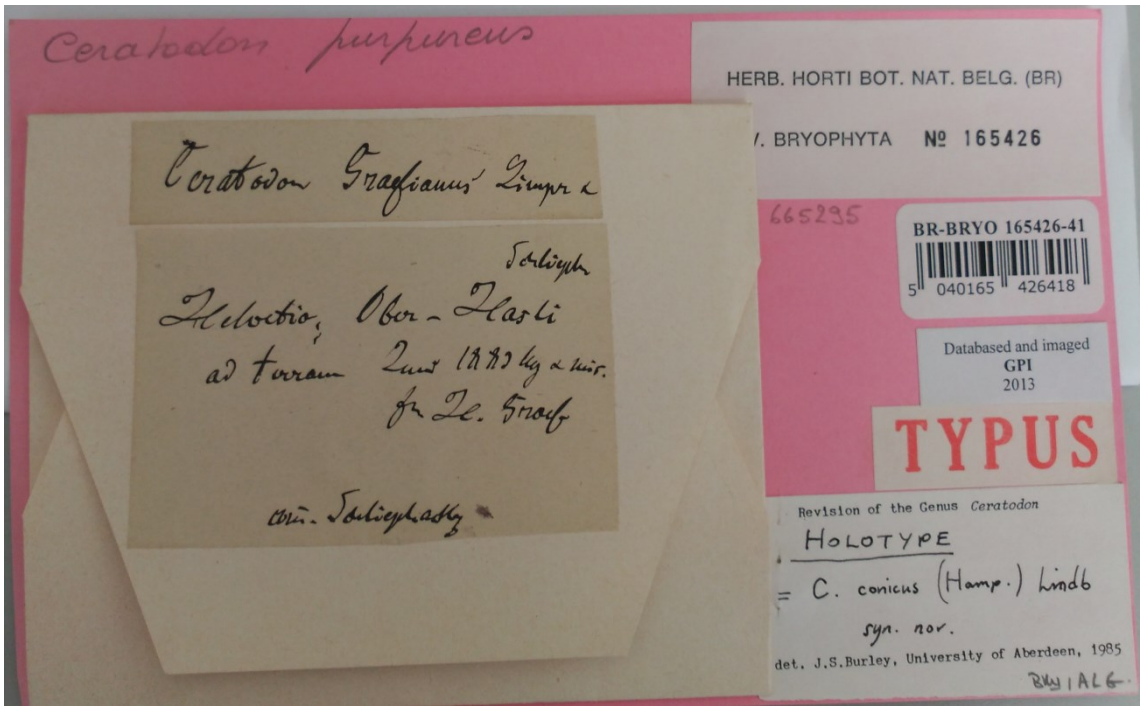

Fig. I.4. Lectotype of *Ceratodon purpureus* var. *graefii* Schlieph. *ex* Limpr. designated as holotype by Burley and Pritchard (1990). Photo by Marta Nieto Lugilde.

ACKNOWLEDGEMENTS

We express our gratitude to the curators of BM, BR, FH, GOET, MANCH, STU, and Z+ZT for the loan of specimens or for information about the specimens; to Marc Appelhans (Göttingen, Germany) for his help during the stay at the GOET herbarium of MNL; to Gabriela Blohm who kindly revised the English text on a previous version; to Jesús Muñoz for his advise on nomenclatural questions; to William R. Buck and Jan Kučera, reviewers of the manuscript, for their corrections and suggestions. This study was supported financially by the Spanish Ministry of Economy and Competitiveness (Projects CGL2011-22936/BOS and CGL2014-52579-R) and FEDER funds of the E.U.

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Chapter II. Peripatric speciation associated with genome expansion and female-biased sex ratios in the moss genus *Ceratodon*

ABSTRACT

A period of allopatry is widely believed to be essential for the evolution of reproductive isolation. However, strict allopatry may be difficult to achieve in some cosmopolitan, spore-dispersed groups, like mosses. We examined the genetic and genome size diversity in Mediterranean populations of the moss *Ceratodon purpureus s.l.* to evaluate the role of allopatry and ploidy change in population divergence. We sampled populations of the genus *Ceratodon* from mountainous areas and lowlands of the Mediterranean region, and from western and central Europe. We performed phylogenetic and coalescent analyses on sequences from five nuclear introns and a chloroplast locus to reconstruct their evolutionary history. We also estimated genome size using flow cytometry (employing propidium iodide) and determined the sex of samples using a sex-linked PCR marker. Two well-differentiated clades were resolved, discriminating two homogeneous groups: the widespread *C. purpureus* and a local group mostly restricted to the mountains in southern Spain. The latter also possessed a genome size 25% larger than the widespread *C. purpureus*, and the samples of this group consist entirely of females. We also found hybrids, and some of them had a genome size equivalent to the sum of the *C. purpureus* and Spanish genome, suggesting that they arose by allopolyploidy. These data suggest that a new species of *Ceratodon* arose via peripatric speciation, potentially involving a genome size change and a strong female-biased sex ratio. The new species has hybridized in the past with *C. purpureus*.

INTRODUCTION

The origin of new species represents a major unsolved problem in evolutionary biology (Rieseberg & Willis, 2007; Seehausen et al., 2014; Dev, 2015). Theory shows that the simplest mechanism for generating new species is through allopatric speciation, in which some portion of a species' range becomes geographically isolated, allowing natural selection or genetic drift to drive allele frequency changes that ultimately generate additional reproductive barriers (Mayr, 1963; Barraclough & Vogler, 2000; Coyne & Orr, 2004). This is because even modest levels of gene flow can homogenize allele frequencies between populations, retarding divergence (Wright, 1931). While local adaptation can drive peripatric or sympatric divergence in cases where the immigrant rate is less than the intensity of selection (Lenormand, 2002), most empirical studies cannot exclude the possibility that speciation was preceded by a period of allopatry (Nadachowska-Brzyska et al., 2013; Shaner et al., 2015). This presents a paradox in species-rich groups like mosses, where long-distance migration appears to be common, but speciation and diversification have occurred in spite of the fact that geographic barriers may not cause a long-term impediment to gene flow (Shaw et al., 2003; Piñeiro et al., 2012; Lewis et al., 2014a; Szövényi et al., 2014; Barbé et al., 2016).

One potential resolution to this paradox is sympatric speciation through polyploidy, which is frequent in flowering plants (Ramsey & Schemske, 1998; Mallet, 2005), and potentially in mosses (Crosby, 1980; Kuta & Przywara, 1997; Såstad, 2005; McDaniel et al., 2010; Rensing et al., 2013). Polyploidy generates a strong reproductive barrier in a single mutational event (Ramsey & Schemske, 1998; Madlung, 2013). However, the homogeneity in bryophyte genome sizes (Voglmayr, 2000) raises the possibility that the role played by polyploidy in moss speciation may be small in relation to other speciation mechanisms. The nature of the genomic, demographic, or ecological factors (beyond geographic isolation and polyploidy) that generate reproductive barriers between nascent species of mosses remain poorly characterized (McDaniel et al., 2010; Yousefi et al., 2017).

Within mosses, the genetic basis of reproductive barriers is best characterized among populations of *Ceratodon purpureus* (Hedw.) Brid. (Ditrichaceae) (McDaniel et al., 2007, 2008). Moreover, the developing genomic and laboratory tools make this species a promising model for further ecological genomic study (McDaniel et al., 2016). *Ceratodon purpureus* is abundant on every continent and grows on a wide variety of substrates (Crum, 1973). Molecular population genetic analyses indicated that gene flow among Northern Hemisphere and even Southern Hemisphere populations was frequent but that tropical populations were more genetically isolated (McDaniel & Shaw, 2005). These observations suggest that the current level of sampling may be insufficient to detect the full scope of population structure among populations in this taxon. Indeed, partial hybrid breakdown was clearly evident in crosses between a temperate and a tropical population, suggesting that reproductive barriers may be in the process of evolving between ecologically distinct regions of the distribution of *C. purpureus* (McDaniel et al., 2007, 2008). These barriers did not involve ploidy differences. However, the genome size of *C. purpureus* is well characterized in only a modest number of European samples (mean ± SD = 0.39 ± 0.0046 pg, n = 10; Voglmayr, 2000), leaving open the possibility that polyploidy contributes to reproductive isolation among isolates from other parts of its broad cosmopolitan distribution.

In a previous phylogeographic analysis (McDaniel & Shaw, 2005), the Mediterranean region contained several rare haplotypes that were distantly related to the common haplotypes found throughout the range of *C. purpureus*. In the present study, we tested for the existence of any relationship between the genetic diversity and DNA content found in the Mediterranean area in the moss genus *Ceratodon*. McDaniel and Shaw (2005) argued that frequent gene flow maintained the genetic homogeneity of the species, at least among the temperate Northern Hemisphere populations, but that the divergent populations were simply outside the main area of spore rain, and therefore had not yet been homogenized. Alternatively, these isolated populations could represent cryptic species, and reproductive isolation evolved in spite of this gene flow (McDaniel et al., 2007, 2008). To distinguish between these alternatives, we evaluated the patterns of polymorphism in five nuclear introns and a single chloroplast *locus* in plants sampled from mountainous areas of the Mediterranean region and other mountain regions and lowlands, mostly from southern Europe. We also estimated the genome size of these

isolates using flow cytometry. These data clearly show that a new species has evolved within the genus *Ceratodon*, accompanied by both large non-polyploid and allopolyploid changes in genome size and, potentially, by major changes in sexual system. These insights also highlight the complexity of peripatric speciation mechanisms in bryophytes.

MATERIALS AND METHODS

Plant material

For this study, we generated genetic data for a total of 93 samples, 71 (76.4%) from Mediterranean mountain areas (47 from the Spanish Sierra Nevada, 19 from the Spanish central mountain ranges, three from the Spanish southeastern mountains, and two from Sicilian Mount Etna). Of the remaining 22 samples, 11 (11.8%) were from other European mountainous systems (eight from the Alps and three from the Pyrenees) and 11 specimens (11.8%) were from lowlands (three from Czech Republic, two from Germany, two from Sweden, two from United Kingdom, and two from South Africa). Mainly between April and November 2011-2014 (for more detailed information, see Annex II.1), we collected 84 new samples for this study, all of which are deposited at MUB (Herbarium of the University of Murcia, Spain); nine samples were loaned from herbaria, including BOL (Bolus Herbarium, University of Cape Town, South Africa), CBFS (University of South Bohemia, Czech Republic), and S (Herbarium of the Swedish Museum of Natural History, Sweden); and two samples were donated from Laura Forrest (at Royal Botanic Garden Edinburgh, United Kingdom). We sequenced four specimens of *Cheilothela chloropus* (Brid.) Lindb. to use as an outgroup (voucher information and GenBank accession numbers are listed in Annex II.1).

DNA sequencing

To examine the genealogical relationships among the 93 isolates, we sequenced five nuclear exon-primed intron-spanning *loci*, including *rpL23A* and *TRc1b3.05* (McDaniel et al., 2013b; referenced by EST accessions AW086590 and AW098560), *hp23.9*, *PPR*, and *TBP* (McDaniel et al., 2013a, b), and a single chloroplast *locus* (*trn*L). We

amplified all *loci* from all individuals in 20 µL polymerase chain reaction (PCR) using DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The cycling conditions were 94°C for 2 min, then 10 cycles of 94°C for 15 s, an annealing temperature of 65°C that dropped one degree each cycle, and 72°C for 1 min, followed by 20 cycles of 94°C for 15 s, 56°C for 30 s, and 72°C for 1 min, and terminating with 72°C for 7 min (McDaniel et al., 2013a). To make the resulting PCR products ready for sequencing, we removed unincorporated primers and inactivated unincorporated nucleotides using PCR clean-up reaction with Exo I and FastAP Alkaline Phosphatase enzymes (Thermo Fisher Scientific). Both enzymes were heat inactivated by maintaining the mixture at 85°C for 15 min. Sequencing was accomplished on an ABI3730XL DNA Analyzer, Applied Biosystems (Macrogen Europe, Amsterdam, The Netherlands).

Cloning of DNA sequences

In samples where we observed double peaks in the chromatograms, we cloned all *loci*. PCR products were isolated from agarose gels, and cloned using the CloneJet PCR Cloning Kit (Thermo Fisher Scientific). Cloning efficiency and accuracy were checked using PCR reactions. Successful clones then were sequenced using an ABI3730XL DNA Analyzer (Macrogen).

Phylogenetic analyses

We aligned the DNA sequences using CLUSTALW (Larkin et al., 2007) as implemented in Bioedit (Hall, 1999) and manually resolved inconsistencies in the resulting alignment. DnaSP v5 (Librado & Rozas, 2009) was used to observe characteristics such as total length with and without gaps, number of constant positions and number of parsimony-informative variable positions about all *loci*. We coded gaps as informative with a simple indel coding strategy (Simmons & Ochoterena, 2000) implemented in SeqState (Müller, 2005). We performed phylogenetic analyses using MrBayes version 3.2 (Ronquist et al., 2012). The need for a priori model testing was removed using the substitution model space in the Bayesian MCMC analysis itself (Huelsenbeck et al., 2004) with the option nst = mixed. The sequence and indel data were treated as separate and unlinked partitions. The a priori probabilities supplied were those specified in the default settings of the program. Posterior probability distributions

of trees were generated using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method. To search for convergence in the phylogenetic analyses, we used two runs with different settings for some of the *loci*. For *hp23.9*, *TBP*, and *trn*L, four chains with 1×10^7 generations were run simultaneously, with the temperature of the single heated chain set to the default in MrBayes. Nevertheless, eight chains with 1×10^6 generations each were run, changing the temperature of the single heated chain set to 2 (*PPR*), 3 (*TRc1b3.05*), and 6 (*rpL23A*), because with the default temperature setting, convergence was not reached in initial runs. Chains were sampled every 1000 generations and the respective trees were written into a tree file. The first 25% of the total sampled trees of each run were discarded as burn-in. Consensus trees and posterior probabilities of clades were calculated by combining the two runs and using the trees sampled after the chains converged and had become stationary. The sump command of MrBayes was used to check whether an appropriate sample from the posterior was obtained. To do so, we first inspected visually the log likelihood plot, which should not show tendencies to decrease or increase over time; the different runs should show similar values. Then we checked that the effective sampling size (ESS) values for all parameters reached ≥ 500 and that the potential scale reduction factor (PSRF) for each parameter was ~ 1.00. The genealogies were rooted with sequences from *Cheilothela chloropus*. The final trees were edited with TreeGraph2 (Stöver & Müller, 2010). We performed phylogenetic analyses using the same setting as before, combining the new sequences generated here with other sequences for the *TBP locus* available on GenBank from Antarctica (1), Australia (1), and eastern North America (54), which were previously reported by McDaniel et al. (2013a).

Low resolution in phylogenetic reconstructions can sometimes be caused by incongruence or conflicts in the molecular datasets that lead to different, equally possible, solutions (Huson & Bryant, 2006; Draper et al., 2015). To evaluate this possibility, we reconstructed a phylogenetic network based on the neighbor-net method (Bryant & Moulton, 2004) using the program SplitsTree4 version 4.13.1 (Huson & Bryant, 2006) for the six concatenated *loci*. The calculations were based on uncorrected p-distances. To test the hypothesis of recombination in each graph, a pairwise homoplasy index (phi-test) was calculated, which is a robust and reliable statistic to detect recombination. This estimates the mean refined incompatibility score from nearby sites. Under the null hypothesis of no recombination, the genealogical

correlation of adjacent sites is invariant to permutations of the sites because all sites have the same history. The order of the sites is important when levels of recombination are finite, because distant sites will tend to have less genealogical correlation than adjacent sites (Bruen et al., 2006). The significance is then tested using a permutation test by default. In accordance with Bruen et al. (2006) for the phi-test of recombination, $P < 0.05$ indicates the presence of recombination signal.

Coalescent stochasticity analyses

Individual gene trees often differ from each other and from the species tree (Rosenberg, 2002; Mao et al., 2014). To check whether the differentiation we found between the Sierra Nevada (SN) and Worldwide (Ww) clades was a good fit to the multispecies coalescent model (MSCM), we employed an approach based on posterior predictive simulation implemented in the R statistical language package "P2C2M" (Gruenstaeudl et al., 2016). In this approach, a posterior distribution of gene genealogies estimated from empirical data is compared to a posterior predictive distribution of genealogies simulated under a model of interest. We first used *BEAST (Heled & Drummond, 2010) to infer genealogies and species trees, and the simulation of genealogies under the MSCM with ms program (Hudson, 2002) under a JC +I +G nucleotide substitution model selected as the most probable in all *loci* using jModelTest (Posada, 2008). Gaps were included as a character state in these analyses. The run was conducted assuming a strict clock for each *locus*. We selected "Yule Model" as the species tree prior, and employed "Piecewise linear and constant root" as the population size model. Finally, the default values for MCMC analysis were used. We compared the genealogies from the posterior distribution to the species trees and compared the genealogies from the posterior predictive distribution to the species tree using two descriptive summary statistics: *lcwt* (likelihood of the coalescent waiting times) and *ndc* (number of deep coalescences). When samples are drawn from data with a good fit to the MSCM, the summary statistics from each distribution should be approximately equal and the expected difference between the two is zero (Reid et al., 2014). Data that are a poor fit to the MSCM are indicated by a deviation from the expectation of a difference distribution that is centered on zero is encountered above a specified quantile level (Gruenstaeudl et al., 2016).

Genome size determination

We used flow cytometry (FCM) technology for 75 specimens to estimate nuclear DNA content. One shoot of each sample was chopped with a razor blade together with the internal standard *Carex acutiformis* Ehrh. 1C = 0.41 pg; Lipnerová et al., 2012) or *Bellis perennis* L. (1C = 1.56 pg; our own calibration against *Carex acutiformis*) in 1 mL of LB01 buffer (Doležel et al., 1989). The fluorochrome propidium iodide and RNase IIa (both at final concentration 50 µg/mL) were added immediately; the samples were stained for ≥ 10 min. The samples were analyzed using a Partec CyFlow SL flow cytometer equipped with a 532 nm (green) diode-pumped solid-state laser (100 mW output); the fluorescence intensity of 12 000 particles was recorded. When possible, we used in vitro cultivated fresh material, but for 47 samples that did not grow satisfactorily in vitro, we used dry material collected in the years 2009–2014. The fluorescence histograms were processed using FlowJo version 10.2 (https://www.flowjo.com/).

Sex determination

To determinate sex, one plant per sample was employed. We amplified the *rpS15A* sex-linked *locus* by PCR and digested the product with HindIII. An intron in the *rpS15A* amplicon contains a cut-site difference between the male and female products (Norrell et al., 2014) that is clearly observable in the banding patterns, which were visualized after electrophoresis in an agarose gel and scored by hand. We identified the sex of 82 samples, 88.17% of the total, which were from the Spanish Sierra Nevada (42), Spanish central mountain ranges (16), Spanish southeastern mountains (3), Sicilian Mount Etna (2), Alps (7), Pyrenees (3), South Africa (2), Germany (2), Czech Republic (3), and Sweden (2). For the remaining samples, we could not unambiguously interpret the pattern in the restriction-site fragment length polymorphism in the *rpS15A* amplicon. We express the results as a proportion of males and computed the 95% confidence interval (CI) for this estimate with the “dbinom” function in R (R Development Core Team, 2017).

Calculation of the binomial proportion confidence interval

If the total number of experiments and the number of positive outcomes of a success-failure experiment are known, it is possible to calculate the CIs for the probability of

success. As a consequence, the CI for the proportion of males (or females) in a population (success) based on the results of sex-determination of a given number of individuals can be calculated. We used the "Hmisc" package (Harrell, 2018) in R3.4.3 (R Development Core Team, 2017), with the options "Wilson" and "Exact" (= Clopper-Pearson) to calculate CIs (presented in parentheses below).

RESULTS

Phylogenetic analyses

The sequence alignments varied in total length between 207 (215 with coded gaps) and 848 (891 with coded gaps) positions, for *hp23.9* and *rpL23A*, respectively. The number of constant positions was between 186 and 715 for the above-mentioned *loci*, and the parsimony-informative variable positions differed between 5 and 95 for *trn*L and *rpL23A*, respectively (Table II.1). The *loci PPR*, *TBP*, *rpL23A*, and *TRc1b3.05* showed two well-differentiated clades with support of 0.87 (0.85–0.88) to 0.77 (0.75–0.79) posterior probability (pp), 0.96 (0.95–0.96) to 1.00 (1.00–1.00) pp, 1.00 (1.00–1.00) to 1.00 (1.00–1.00) pp, and 1.00 (1.00–1.00) to 1.00 (1.00–1.00) pp, respectively (Fig. II.1; see also the Annex II.2, Annex II.3, Annex II.4).

Table II.1. Characteristics of the *loci* used for molecular evolutionary analyses. The genomic location "nuclear-putative autosomal" is based on unpublished data.

Locus	**Genomic location**	**Sequence length (with gaps)**	**Invariant sites**	**Parsimony-informative sites**
hp23.9	Nuclear-autosomal	207 (215)	186	15
PPR	Nuclear-U/V	331 (334)	309	8
rpL23A	Nuclear-putative autosomal	848 (891)	715	95
TBP	Nuclear-autosomal	365 (365)	337	11
TRc1b3.05	Nuclear-putative autosomal	402 (417)	362	28
*trn*L	Chloroplast	320 (320)	311	5

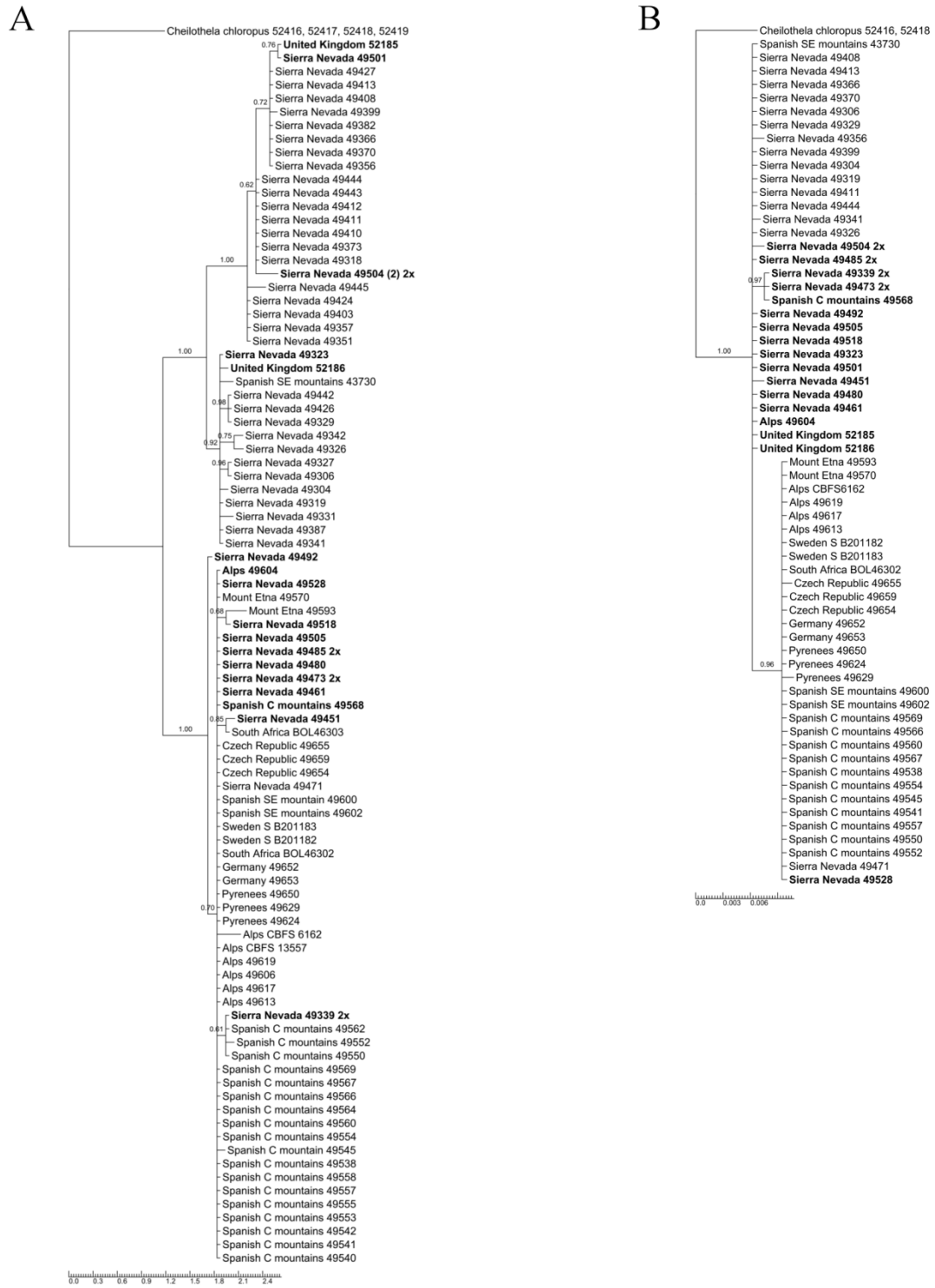

Fig. II.1. Phylogenetic trees inferred from two of the studied *loci*: (A) nuclear *TRc1b3.05* and (B) chloroplast *trn*L. For each tip in the trees, geographical origin and herbarium number are given (numbers without letters are from MUB); "2x" indicates diploid samples; the number of sequences obtained by cloning is in parentheses if there was more than one; and bold letters indicate recombinant samples.

In the case of *rpL23A*, sequences of *Cheilothela chloropus* were not obtained for use as outgroup, but again two clades were resolved. The *hp23.9 locus* had a support for one clade of 1.00 (1.00–1.00) pp but the other clade had a value of 0.55 (0.45–0.61) pp (Annex II.5). In all the five nuclear *loci* studied, one of the clades was formed always by 34 Sierra Nevada samples and one of the Spanish southeastern mountains (hereafter "SN group"). The second clade consistently included 42 specimens coming from the rest of the sampled areas, including one from the Sierra Nevada and two from the Spanish southeastern mountains (hereafter "Ww group"). For one marker (*TBP*) we added sequences available at GenBank, including samples from Antarctica, Australia, and North America. The resulting tree topology shows that our samples give a reasonable good representation of the Ww group and that none of these additional sequences is closely related to the SN samples (Annex II.6). The remaining 17 sequenced samples were strongly resolved in either the SN clade or the Ww clade, depending on the studied *locus* (they did not present intermediate sequences between both clades; Annex II.7); we considered these samples recombinants. The term "hybrid" applied to bryophytes should strictly be used only for the sporophytic hybrids (2n) (Anderson, 1980); for their gametophytic progeny (n) showing combination of parental alleles after meiosis, "recombinants" should be used (Shaw, 1994, 1998) in order not to confuse these with hybrids observed among vascular plants. The recombinants were derived mainly from the Sierra Nevada, but also from the Spanish central mountain ranges, the Alps, and the lowlands of the United Kingdom (Fig. II.2). The chloroplast *locus* showed one well-supported clade 0.96 (0.95–0.98) pp, and all remaining samples with deeper coalescent events (Fig. II.1). All the samples considered as recombinants based on the nuclear markers were closely related and sister to the rest of the SN samples, with the only exception of one specimen from the Sierra Nevada (MUB 49528), which is a recombinant and belongs to the Ww chloroplast clade.

The apparently uncertain position of some individuals is clarified by the result of the Neighbor-Net network (Fig. II.3). Moreover, for the phi-test when the six *loci* were studied together, a highly significant *P* value (0.0) was obtained, confirming the presence of recombination signal. Graphically, two extreme groups can be observed, the SN group and the Ww group, with some individuals in intermediate positions.

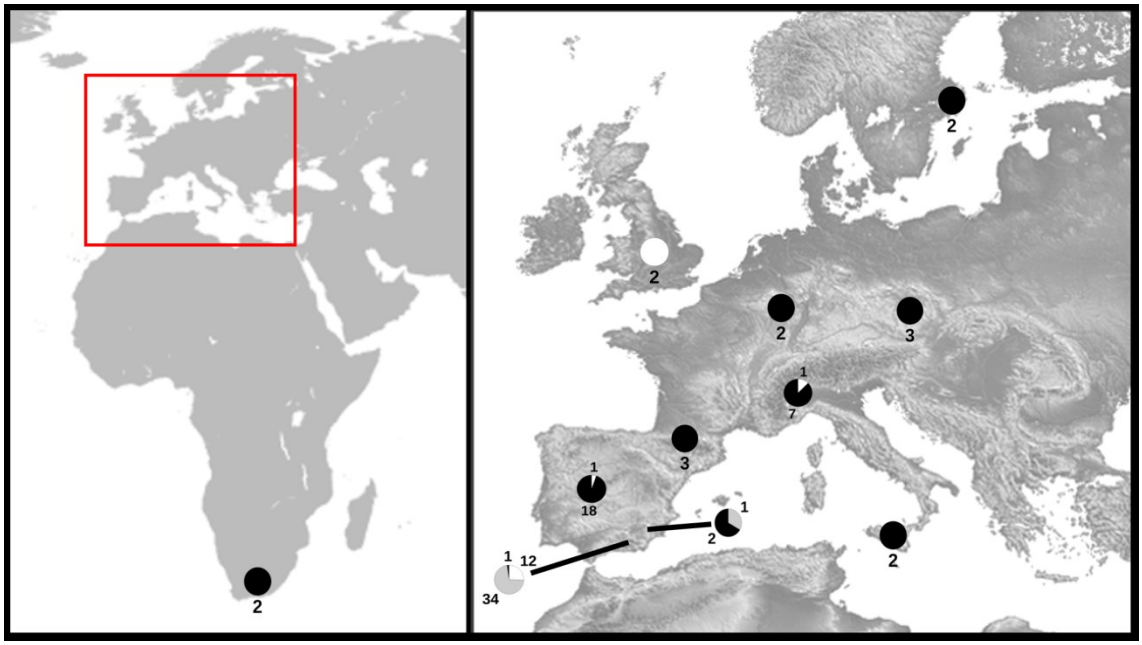

Fig. II.2. Geographic location of *Ceratodon* samples included in this study. Pie charts indicate proportion of samples of each genomic group by area (black: Ww group; gray: SN group; white: recombinant samples), with number of samples for each.

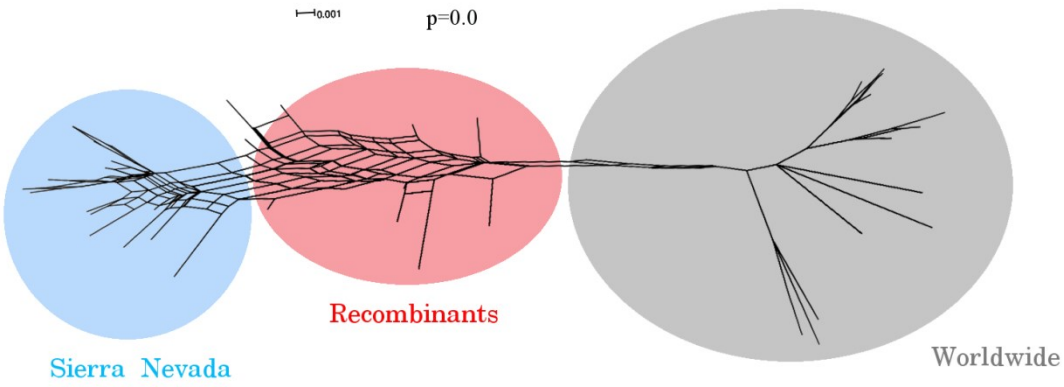

Fig. II.3. Neighbor-Net network to test signals of reticulate evolution between the samples. The main groups are highlight by colored circles matched to their names. The *P* value from the phi-test of recombination is indicated.

Cloning DNA sequences

Cloning of *loci* confirmed that diploid specimens (see below) present two different copies of the same *loci* in most cases. The *loci TRc1b3.05*, *PPR*, and *rpL23A* presented predominantly a single copy, although some individuals presented the two copies in other *loci* (Annex II.7). Some haploid individuals presented two different copies of a

locus. This may be due to the possibility of gene redundancy, which can result from unequal crossing over, retroposition, or chromosomal (or genome) duplication (Magadum et al., 2013).

Coalescent stochasticity analyses

Although our data suggested the existence of recombinants between the two groups, incomplete lineage sorting and hybridization may result in similar molecular signals. Nevertheless, the two summary statistics *ndc* and *lcwt*, employed for the comparison of the genealogies from the posterior distribution to the species trees and from the posterior predictive distribution to the species trees, show significant ($P < 0.05$) differences between our data with respect to MSCM (Table II.2), indicating that incomplete lineage sorting (coalescent model) alone cannot explain the different tree topologies.

Table II.2. Results of P2C2M analysis in which *lcwt* and *ndc* descriptive summary statistics are shown for each DNA *locus* analyzed. All *loci* under study are of nuclear origin, except *trn*L. Asterisks indicate poor model fit at a probability level of 0.05; n.a. = not applicable.

Locus	***lcwt***	***ndc***
hp23.9	510.16 ± 117.86*	-57.46 ± 29.93*
PPR	530.67 ± 121.31*	-59.88 ± 29.87*
rpL23A	462.75 ± 110.74*	-53.04 ± 29.16*
TBP	525.94 ± 120.55*	-59.56 ± 29.77*
TRc1b3.05	516.43 ± 121.96*	-58.31 ± 29.96*
*trn*L	n.a.	-65.33 ± 29.26*
Sum of all genes	n.a.	-353.59 ± 105.62*

Flow cytometry analyses

We obtained three clearly differentiated groups of cytotypes for both fresh and dry material (Table II.3 and Fig. II.4). Measurements from dry material gave higher values (by 18% on average) than those from fresh material, and therefore a conversion factor (1/1.18 = 0.85) was applied to the former. When fresh and dry materials were

Table II.3. Nuclear DNA content as measured by flow cytometry. Cytotypes considered, number of samples used in the analyses (*n*), mean value of DNA, standard deviation, and range of values obtained for each cytotype are given (asterisk indicates conversion factor of 0.85 applied to dry material when fresh and dry material are combined).

	Cytotype	*n*	Mean (pg)	Standard deviation	Minimum (pg)	Maximum (pg)
Fresh material	a	5	0.36	< 0.01	0.36	0.37
	b	20	0.46	0.01	0.45	0.48
	c	3	0.81	0.01	0.81	0.82
Dry material	a*	25	0.44	0.01	0.41	0.45
	b*	21	0.54	0.01	0.52	0.57
	c*	1	0.97	--	--	--
Fresh + dry material (*)	a + a*	30	0.37	0.01	0.35	0.38
	b + b*	41	0.46	0.01	0.44	0.48
	c+c*	4	0.82	0.01	0.81	0.82
		75				

considered together, the first cytotype had a mean value of 1C = 0.37 pg, and the second one showed 25.4% more DNA content (1C = 0.46 pg). The third cytotype had 1C = 0.82 pg mean value of DNA content. All specimens of the Ww group belonged to the smallest cytotype, while all those of the SN group were categorized in the second cytotype, and the recombinant specimens were found in both the second and the third cytotype (Annex II.7).

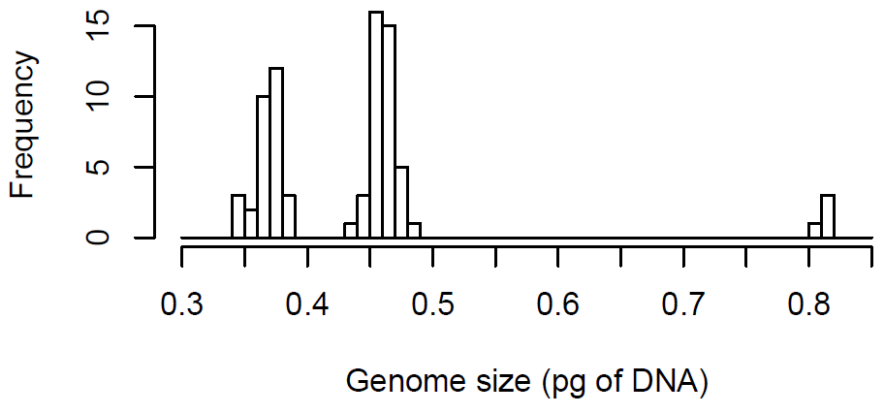

Fig. II.4. Histogram of genome sizes of representative samples of *Ceratodon* generated by flow cytometry. A conversion factor of 0.85 was applied to the data obtained from dry material.

Sex determination

All samples from the SN group (29) and all the recombinant samples (15) were females, while the Ww group (38) consisted mainly of females and two males (one from the Sierra Nevada and the other from the Alps; see Annex II.1). The high proportion of females in the Ww samples may be due to a collection bias, given that we preferentially chose moss cushions with sporophytes, because sporophyte morphology is one of the few characters that enabled us to make a clear distinction in the field between *Ceratodon* and other morphologically similar genera (e.g., in the Bryales Limpr. and Pottiales M. Fleisch.). The presence of sporophytes, however, indicates that males must have been present. Male buds (perigonia) are deciduous and may not be produced each season, which means that sterile plants may have been males. In the Sierra Nevada, we never observed sporophytes (the identity of all samples was verified in the laboratory by examining microscopic gametophytic characters). Similarly, Rams et al. (2014) reported finding no sporophytes in deep sampling carried out in the Sierra Nevada from early spring to autumn from 2002 to 2004, and García-Zamora et al. (1998) found only one fructified specimen identified as *C. purpureus* in a survey of a zone close to the Sierra Nevada in 1990–1991. Moreover, none of the *Ceratodon* samples from southeastern Spain in the MUB and GDA/GDAC (University of Granada, Spain) herbaria showed sporophytes. If we exclude a possible bias in the case of the Sierra Nevada samples, we can conclude, based on the binominal distribution, that the 95% CI for the probability of encountering males in the SN-type populations lies in the range of $p = 0.00$ for the lower limit and $p = 0.12$ for the upper limit for both tested methods ("Wilson" and "Clopper-Pearson"), which means that males might even be completely absent.

DISCUSSION

In most major models of speciation, a period of allopatry is essential to the evolution of reproductive isolation (Coyne & Orr, 2004). However, in many cosmopolitan species, including many mosses and ferns, the entire habitable range of the species is within the range of the dispersal distance of its spores (Muñoz et al., 2004; Frahm, 2007; Pisa et al., 2013), making strict allopatry unlikely. Therefore, it is reasonable to propose that

speciation mechanisms that either occur in sympatry or accommodate some gene flow contribute to generating the extant diversity in such groups. The two best-studied sympatric speciation mechanisms in plants are polyploidy and the evolution of self-fertilization (Barringer, 2007). Here we show that the evolution of a new species, closely related to the cosmopolitan *Ceratodon purpureus*, was associated with a 25% increase in genome size and a significant decrease in frequency of males (Nieto-Lugilde et al., 2018). Surprisingly, although we have found neither males nor evidence of recent sexual reproduction (i.e., sporophytes) in the new species, the genetic diversity among members of this species is relatively high. Despite the long period of isolation suggested by the sequence divergence between *C. purpureus* and the new species, we have found evidence of interspecific hybridization, suggesting that the new species apparently has retained the capacity for sexual reproduction. In a separate paper, we discuss the taxonomic implications of this discovery (Nieto-Lugilde et al., 2018). Here we use genealogical and genome size data to make inferences regarding the genetic architecture of speciation, and the demographic parameters that permit such divergence.

Taxonomists have struggled with species delimitation in the genus *Ceratodon* since the description of the genus. Burley and Pritchard (1990) found references for nearly 50 specific or subspecific taxa within *Ceratodon*, but—based on an extensive survey of herbarium specimens—recognized only four species: *C. antarcticus* Cardot., *C. conicus* (Hampe) Lindb., *C. heterophyllus* Kindb., and *C. purpureus*, including three infraspecific taxa (subsp. *convolutus* (Reichardt) Burley, subsp. *purpureus*, and subsp. *stenocarpus* (Bruch & Schimp.) Dixon). Previous molecular population genetic analyses indicated that disjunct populations of *C. purpureus* were sometimes very closely related, clearly showing that long-distance dispersal, even among continents, was frequent enough to erase any signal of strong population structure (McDaniel & Shaw, 2005). However, these data did not provide strong genealogical support either for or against the existence of distinct species other than *C. purpureus*. Subsequent classical genetic analyses showed that geographically and ecologically distant populations were partially reproductively isolated from one another (McDaniel et al., 2007, 2008), but these appeared to be somewhat porous reproductive barriers, and it was unclear that the populations represented different species.

McDaniel and Shaw (2005) did find some isolates of *C. purpureus* that were genetically distant from the more common haplotypes found in northern temperate regions. Here we found strong evidence that haplotypes that are distantly related to the typical *C. purpureus* haplotypes are locally abundant in the Sierra Nevada of southern Spain. We also found populations containing SN haplotypes and recombinants, together with some rare samples with exclusively the typical *C. purpureus* haplotypes. To evaluate the possibility that the segregation of these divergent haplotypes in the SN populations represents the retention of ancestral variation in the species (i.e., coalescent stochasticity causing incomplete lineage sorting), we generated coalescent simulations using *BEAST and P2C2M. These analyses showed that the divergence between these two haplotypic classes was too great to be explained by coalescent stochasticity. The fact that this polymorphism is found in all the nuclear *loci* that we sampled, and that it is geographically concentrated to the Sierra Nevada region, suggests that balancing selection is also an unlikely explanation. Collectively, these data suggest that the SN haplotypes constitute a rare species, sister to and partially reproductively isolated from the cosmopolitan *C. purpureus*.

The default mode for the evolution of reproductive isolation is allopatric speciation. The sympatric occurrence of typical *C. purpureus* haplotypes and SN haplotypes, even at modest frequencies, contradicts the suggestion by McDaniel and Shaw (2005) that the Mediterranean populations were isolated from the rest of the species as a result of decreased spore rain in peripheral populations separated by prevailing global wind patterns. If we assume that the current dispersal capabilities of *C. purpureus* represent the ancestral condition, this suggests that geography may not have been the primary isolating mechanism between the nascent species. Morphological analysis of plants of both species grown in a common garden (as well as putative recombinants between them; Nieto-Lugilde et al., 2018) indicate that members of the Ww group can be distinguished morphologically from the SN group on the basis of multivariate biometrical evaluation of microscopic features of the caulidia and phyllidia (stem length, presence or absence of apical comal tuft, leaf size and shape, leaf costa width at base of lamina, and leaf costa excurrence). Nevertheless, we were unable to distinguish between the SN group plants and recombinants in field samples, which suggests that the environment influences the variance in taxonomically important

characters. It is certainly possible that an extrinsic factor, like a habitat preference, isolated the two species.

It is also possible that an intrinsic factor isolated the two species. Remarkably, however, we detected only females in the SN species, which suggests that male lethality could contribute to isolating the two species. Sex in dioecious bryophytes like *C. purpureus* is determined at meiosis, by the segregation of a UV chromosome pair, meaning that ~ 50% of the spores produced in a population should be males. Some meiotic sex ratio variation has been observed in this species in natural populations (overall mean of proportion of males was 0.41 [95% CI: 0.17–0.72]; Norrell et al., 2014) and artificial crosses (male-biased sex ratio = 60%; McDaniel et al., 2008). Even given our sample size (n = 29, with no males), we can conclude that the percentage of males in the SN populations is much lower (95% CI included 0-12%; additional samples not included in this study lower the 95% CI to a range of 0-6.7%). We do not know whether the decrease in males coincided with the speciation event, or occurred subsequent to the evolution of reproductive isolation. The evolution of apomixis or obligate selfing from historically outcrossing lineages is a well-documented route to the evolution of new species in plants (Stebbins, 1974; Barrett, 2010; Wright et al., 2013), and parthenogenetic lineages associated with the loss of males are frequent in some animal lineages (insects: Hagimori et al., 2006; Montelongo & Gómez-Zurita, 2015; vertebrates: Neaves & Baumann, 2011; Gutekunst et al., 2018). However, we know of no other cases where the loss of males has been associated with speciation in bryophytes.

The presence of recombinants containing both typical *C. purpureus* alleles and alleles from the SN species indicated that rare interspecies hybridization has occurred between individuals of the two species. Most of the recombinants possessed the SN chloroplast type, based on the *trn*L sequence data, which suggests that this species was more often the maternal parent (consistent with the rarity of males). We found one instance of a recombinant plant that had a typical *C. purpureus trn*L sequence, but we cannot determine whether this was a rare case of a hybridization involving an SN male (i.e., a cross in the opposite direction) or whether this resulted from a backcross of a male recombinant to a typical *C. purpureus* female. Intrinsic genetic incompatibilities are often manifest as Dobzhansky-Muller interactions, which result in asymmetric

introgression patterns at the causative *loci* (McDaniel et al., 2008) due to the death of incompatible multilocus genotypes. Although we sampled only six *loci* across the genome, the recombinants tended to have the SN alleles at the *TBP* and *rpL23A loci*. We are currently examining the frequency of polymorphism across the genome of the SN and recombinant genotypes to distinguish among forms of extrinsic and intrinsic isolation between the SN and typical *C. purpureus* populations.

The flow cytometric data also showed that members of the SN species had a genome ~ 25% larger than typical members of *C. purpureus*. It is possible that the speciation involved a whole-genome duplication event followed by rapid genome reduction, the duplication of large chromosomes (Inoue et al., 2015; Panchy et al., 2016), or the accumulation of transposable elements (TEs), which contribute to the extraordinary variation in genome size within even closely related species in angiosperms (Vitte & Bennetzen, 2006). Although the current data represent the most comprehensive sampling of variation in genome size in *Ceratodon*, we still lack cytological data to determine whether variation in nuclear DNA content is due to an increase in the size of chromosomes or to the increase in the number of chromosomes. The variance in genome size is almost equal between the two groups, which suggests that the SN species is fixed for whatever *loci* underlie the genome size change. Additionally, recombinants between the two groups have the genome size of SN species, not an intermediate value, suggesting that the increase in genome size may come from a single genomic change, rather than many small changes across genome. One hypothesis is that these plants have gained DNA on the sex chromosome, which comprises nearly one-third of the genome (Heitz, 1932; Jachimsky, 1935; McDaniel et al., 2007). Sex chromosomes in other organisms are known to accumulate genomic material rapidly, sometimes in large translocations, and potentially generating pronounced evolutionary and ecological consequences (Tennessen et al., 2018). We are now attempting to generate artificial crosses to evaluate the genetic basis of the genome size difference.

We also found a third rare cytotype with a genome size approximately twice that of either SN plants or typical *C. purpureus* plants. These isolates all had mixed haplotypes (i.e., gene sequences from both the SN and typical *C. purpureus* clades) and a genome size very close to the sum of the SN group and Ww group (~ 1.2% smaller

than the sum of the group means), suggesting that they arose from an allopolyploid event. Without more sequence or cytological data, we cannot formally eliminate the possibility that the larger cytotype arose from autopolyploidy followed by hybridization, although this would require the gain of ~ 10% or loss (~ 12%) of the genomic DNA. Additionally, allopolyploidy is a widely observed mechanism to restore the fertility of F_1 hybrids between partially reproductively isolated species with karyotypic differences and exhibit meiotic abnormalities (De Storme & Mason, 2014).

Finally, the new SN species apparently maintains levels of genetic diversity nearly equivalent to typical populations of its sister species *C. purpureus* without obviously undergoing sexual reproduction. Sexual reproduction in mosses occurs when males and females grow in close proximity, and sperm cells disperse, typically in humid conditions, from male to female plants, producing sporophytes, a very common observation in most populations of *C. purpureus*. Given the complete absence of sporophytes in observed Sierra Nevada samples, reproduction seems to be predominantly by fragmentation of the gametophores. Moss gametophores can persist for many years, even in relatively stressful conditions, and easily spread clonally by fragmentation. In some cases, such fragments may be dispersed a considerable distance (Frahm, 2007; Lewis et al., 2014b). It is clear that spatially heterogeneous selection (Vrijenhoek, 1978) or frequency-dependent selection (Weeks and Hoffmann, 2008) can maintain high genetic diversity in clonal organisms. Antarctic populations of *C. purpureus*, which similarly lack any sexual reproduction, were also quite variable, although less polymorphic than was observed in the closely related nearby populations from Australia (Clarke et al., 2009). Also similar to the Antarctic studies, we found polymorphic nuclear ITS sequences between samples collected a few meters apart (M. Nieto-Lugilde, O. Werner, S. F. McDaniel, R. M. Ros, unpublished data), indicating that these localities were colonized several times independently. However, unlike the Antarctic case, the SN isolates are genetically distinct from any known spore source. It is possible that sexual reproduction in the SN species generated the current variation under a past climate regime, or in undetected localities, although it is clearly far rarer than in *C. purpureus*. Further analyses of the evolutionary history of the SN population are likely to produce a better understanding of the phenomena that generate new species in cosmopolitan taxa.

ACKNOWLEDGMENTS

The authors thank the curators of herbaria BOL, GDA/GDAC, and S for providing plant material; L. Forrest of Royal Botanic Garden Edinburgh, United Kingdom, for donating samples; the management group of the National Park of Sierra Nevada Natural Space for allowing us to collect samples in the National and Natural Parks; and two anonymous reviewers for their valuable comments, which improved the manuscript. This study was supported financially by the Spanish "Ministerio de Ciencia e Innovación" (project CGL2011-22936/BOS), by "Ministerio de Economía y Competitividad" (project CGL2014-52579-R), co-financed by ERDF of European Union, and by a U.S. National Science Foundation grant (DEB 1541005) to S.F.M. M.N.-L. also thanks the Spanish "Ministerio de Economía y Competitividad" for a "Formación de Personal Investigador" fellowship (FPI program 2012; reference BES-2012-056799).

DATA ACCESIBILITY

The aligned sequences and trees are available at TreeBase:
http://purl.org/phylo/treebase/phylows/study/TB2:S22472.

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ANNEXES

Annex II.1. Voucher information for the studied specimens. For each sequenced sample the next information is given: herbarium code (herbaria acronyms follow Index Herbariorum); geographical origin, collection date (year-month-day), gender if known (F for female, M for male), presence of sporophyte if appropriate, indicated by an asterisk (*), GenBank accession numbers for the six *loci* studied, given in the next order: *hp23.9*, *PPR*, *rpL23A*, *TBP*, *TRc1b3.05* and *trnL*; sequences obtained by cloning are indicated by their GenBank accession number given in parentheses.

Ingroup

Mediterranean mountain areas

MUB 43730: Spanish southeastern mountains, 2011-11-13, F, KP825628, KP826017, KP826181, KP826402, KP826531, KY229001. **MUB 49304:** Sierra Nevada Mountains, 2012-07-20, F, KP825703, KP826091, KP826265, KP826473, KP826601, MG050779. **MUB 49306:** Sierra Nevada Mountains, 2012-07-20, F, KP825701, KP826089, KP826263, KP826471, KP826599, KY229023. **MUB 49318:** Sierra Nevada Mountains, 2012-07-20, KP825698, KP826086, KP826260, KP826468, KP826596, –. **MUB 49319:** Sierra Nevada Mountains, 2012-07-20, KP825697, KP826085, KP826259, KP826467, KP826595, MG050780. **MUB 49323:** Sierra Nevada Mountains, 2012-07-20, F, KP825696, KP826084, KP826258, KP826466, KP826594, KY229040. **MUB 49326:** Sierra Nevada Mountains, 2012-07-20, F, KP825693, KP826081, KP826255, KP826463, KP826591, MG050781. **MUB 49327:** Sierra Nevada Mountains, 2012-07-20, F, KP825692, KP826080, KP826254, KP826462, KP826590, –. **MUB 49329:** Sierra Nevada Mountains, 2012-07-20, F, KP825690, KP826078, KP826252, KP826460, KP826588, KY229024. **MUB 49331:** Sierra Nevada Mountains, 2012-07-20, F, KP825688, KP826076, KP826250, KP826459, KP826586, –. **MUB 49339:** Sierra Nevada Mountains, 2012-07-20, F, (MG050789, MG050790, MG050791, MG050792, MG050793, MG050794, MG050795, MG050796, MG050797, MG050798, MG050799), (KP826073, MG050748, MG050749, MG050750, MG050751, MG050752), KP826248, (KP826456, MG050761, MG050762, MG050763, MG050764, MG050765), KP826583, KY229035. **MUB 49341:** Sierra Nevada Mountains, 2012-07-20, F, KP825683, KP826071, KP826246, KP826454, KP826581, MG050782. **MUB 49342:** Sierra Nevada Mountains, 2012-07-20, F, KP825682, KP826070, KP826245, KP826453, KP826580, –. **MUB 49351:** Sierra Nevada Mountains, 2012-07-20, F, KP825681, KP826069, KP826244, KP826452, KP826579, –. **MUB 49353:** Sierra Nevada Mountains, 2012-07-20, F, KP825679, KP826067, KP826242, KP826450, –, –. **MUB 49356:** Sierra Nevada Mountains, 2012-07-20, KP825677, KP826065, KP826239, KP826448, KP826577, KY229030. **MUB 49357:** Sierra Nevada Mountains, 2012-07-20, F, KP825676, KP826064, KP826241, KP826447, KP826576, –. **MUB 49366:** Sierra Nevada Mountains, 2012-07-21, F, KP825670, KP826058, KP826238, KP826442, KP826570, KY229011. **MUB 49370:** Sierra Nevada Mountains, 2012-07-21, KP825674, KP826062, KP826234, KP826446, KP826574, KY229015. **MUB 49373:** Sierra Nevada Mountains, 2012-07-21, F, KP825671, KP826059, KP826233, KP826443, KP826571, –. **MUB 49382:** Sierra Nevada Mountains, 2012-07-21, F, KP825669, KP826057, KP826180, KP826441, KP826569, –. **MUB 49387:** Sierra Nevada Mountains, 2012-07-21, F, KP825666, KP826054, KP826230, KP826438, KP826565, –. **MUB 49399:** Sierra Nevada Mountains, 2012-07-21, F, KP825663, KP826051, KP826224, KP826435, KP826563, KY229033. **MUB 49403:** Sierra Nevada Mountains, 2012-07-21, F, KP825660, KP826048, KP826182, KP826432, KP826560, –. **MUB 49408:** Sierra Nevada Mountains, 2012-07-21, F, KP825657, KP826045, KP826222, –, KP826557, KY229005. **MUB 49410:** Sierra Nevada Mountains, 2012-07-21, F, KP825655, KP826043, KP826220, KP826428, KP826555, –. **MUB 49411:** Sierra Nevada Mountains, 2012-07-21, F, KP825654, KP826042, KP826219, KP826427, KP826554, MG050783. **MUB 49412:** Sierra Nevada Mountains, 2012-07-21, F, KP825653, KP826041, KP826218, KP826426, KP826553, –. **MUB 49413:** Sierra Nevada Mountains, 2012-07-21, F, KP825652, KP826040, KP826217, KP826425, KP826552, KY229008. **MUB 49424:** Sierra Nevada Mountains, 2012-07-21, F, KP825651, KP826039, KP826216, KP826424, KP826551, –. **MUB 49426:** Sierra Nevada Mountains, 2012-07-21, F, KP825649, KP826037, KP826214, KP826422, KP826549, –. **MUB 49427:** Sierra Nevada Mountains, 2012-07-21, F, KP825648, KP826036, KP826213, KP826421, KP826548, –. **MUB 49442:** Sierra Nevada Mountains,

2012-07-21, F, KP825643, KP826031, KP826208, KP826417, KP826544, –. **MUB 49443:** Sierra Nevada Mountains, 2012-07-21, F, KP825642, KP826030, KP826207, KP826416, KP826543, –. **MUB 49444:** Sierra Nevada Mountains, 2012-07-21, F, KP825641, KP826029, KP826206, KP826415, KP826542, MG050784. **MUB 49445:** Sierra Nevada Mountains, 2012-07-21, KP825640, KP826028, KP826209, KP826414, KP826541, –. **MUB 49451:** Sierra Nevada Mountains, 2013-07-12, F, (KP825639, MG050800, MG050801, MG050802, MG050803, MG050804, MG050805, MG050806, MG050807, MG050808), (KP826027, MG050753), (KP826204, MG050869, MG050870), KP826413, KP826540, KY229045. **MUB 49461:** Sierra Nevada Mountains, 2013-07-12, F, KP825638, KP826026, KP826203, KP826412, KP826539, KY229052. **MUB 49471:** Sierra Nevada Mountains, 2013-07-12, M, KP825706, KP826094, KP826201, KP826476, KP826604, KY229043. **MUB 49473:** Sierra Nevada Mountains, 2013-07-12, F, (KP825637, MG050809, MG050810, MG050811, MG050812, MG050813, MG050814, MG050815, MG050816, MG050817, MG050818, MG050819), KP826025, (MG050871, MG050872, MG050873, MG050874, MG050875, MG050876), (MG050766, MG050767, MG050768, MG050769, MG050770), KP826538, KY229041. **MUB 49480:** Sierra Nevada Mountains, 2013-07-12, F, (KP825636, MG050820, MG050821, MG050822, MG050823, MG050824, MG050825, MG050826), KP826024, KP826199, KP826410, KP826537, KY229046. **MUB 49485:** Sierra Nevada Mountains, 2013-07-12, F, (KP825635, MG050827, MG050828, MG050829, MG050830, MG050831, MG050832, MG050833), (KP826023, MG050754, MG050755, MG050756, MG050757, MG050758), (MG050877, MG050878, MG050879, MG050880, MG050881, MG050882), (KP826409, MG050771, MG050772, MG050773, MG050774, MG050775, MG050776), KP826536, KY229032. **MUB 49492:** Sierra Nevada Mountains, 2013-07-13, F, (MG050834, MG050835, MG050836, MG050837, MG050838, MG050839, MG050840), KP826022, KP826198, KP826408, –, KY229037. **MUB 49501:** Sierra Nevada Mountains, 2013-07-13, F, KP825633, –, KP826197, KP826407, KP826535, KY229042. **MUB 49504:** Sierra Nevada Mountains, 2013-07-13, F, (KP825632, MG050841, MG050842, MG050843, MG050844, MG050845), KP826021, KP826196, KP826406, (MG050867, MG050868), KY229047. **MUB 49505:** Sierra Nevada Mountains, 2013-07-13, F, KP825631, KP826020, KP826195, KP826405, KP826534, KY229031. **MUB 49518:** Sierra Nevada Mountains, 2013-07-13, F, (KP825630, MG050846, MG050847, MG050848, MG050849, MG050850, MG050851, MG050852, MG050853, MG050854), (KP826019, MG050759), KP826194, KP826404, KP826533, KY229038. **MUB 49528:** Sierra Nevada Mountains, 2013-07-13, F, (KP825629, MG050855, MG050856, MG050857, MG050858, MG050859, MG050860), (KP826018, MG050760), KP826193, (MG050777, MG050778), KP826532, KY229027. **MUB 49538:** Spanish central mountain ranges, 2011-10-27, F, KP825762, KP826150, KP826192, KP826528, KP826659, KY229021. **MUB 49540:** Spanish central mountain ranges, 2011-10-27, F, KP825760, KP826148, KP826191, KP826526, KP826657, –. **MUB 49541:** Spanish central mountain ranges, 2011-10-27, F, KP825759, KP826147, KP826190, KP826525, KP826656, MG050785. **MUB 49542:** Spanish central mountain ranges, 2011-10-27, F, KP825758, KP826146, KP826188, KP826524, KP826655, –. **MUB 49545:** Spanish central mountain ranges, 2011-10-27, KP825755, KP826143, KP826186, KP826521, KP826652, KY229029. **MUB 49550:** Spanish central mountain ranges, 2011-10-27, F*, KP825750, KP826138, KP826179, KP826516, KP826647, MG050786. **MUB 49552:** Spanish central mountain ranges, 2011-10-27, F*, KP825748, KP826136, KP826177, KP826514, KP826645, MG050787. **MUB 49553:** Spanish central mountain ranges, 2011-10-27, F*, KP825747, KP826135, KP826176, KP826513, KP826644, –. **MUB 49554:** Spanish central mountain ranges, 2011-10-27, F*, KP825746, KP826134, KP826175, KP826512, KP826643, KY229017. **MUB 49555:** Spanish central mountain ranges, 2011-10-27, KP825745, KP826133, KP826174, KP826511, KP826642, –. **MUB 49557:** Spanish central mountain ranges, 2011-10-27, F, KP825743, KP826131, KP826173, KP826509, KP826640, MG050788. **MUB 49558:** Spanish central mountain ranges, 2011-10-28, F, KP825742, KP826130, KP826172, KP826508, KP826639, –. **MUB 49560:** Spanish central mountain ranges, 2011-10-28, F*, KP825740, KP826128, KP826170, KP826506, KP826637, KY229013. **MUB 49562:** Spanish central mountain ranges, 2011-10-28, KP825738, KP826126, –, KP826504, KP826635, –. **MUB 49564:** Spanish central mountain ranges, 2011-10-28, F*, KP825736, KP826124, KP826168, KP826502, KP826633, –. **MUB 49566:** Spanish central mountain ranges, 2011-10-28, F, KP825734, KP826122, KP826167, KP826500, KP826631, KY229044. **MUB 49567:** Spanish central mountain ranges, 2011-10-28, F*, KP825733, KP826121, KP826166, KP826499, KP826630, KY229003. **MUB 49568:** Spanish central mountain ranges, 2011-10-29, F, KP825732, –, KP826165, –, KP826629, KY229048. **MUB 49569:** Spanish central mountain ranges, 2011-10-29, F*, KP825731, KP826119, KP826164, KP826497, KP826628, KY229009. **MUB 49570:** Sicilian Mount Etna, 2013-09-07, F, KP825714, KP826107, –, KP826478, KP826606, KY229016. **MUB 49593:** Sicilian Mount Etna, 2013-09-08, F, KP825715, KP826106, KP826163, KP826479, KP826607, KY229034. **MUB 49600:** Spanish southeastern mountains, 2013-11-15, F*, KP825722, KP826104, KP826159, KP826486, KP826613, KY229022.

MUB 49602: Spanish southeastern mountains, 2013-11-15, F, KP825723, KP826105, KP826160, KP826487, KP826614, KY229050.

Other mountainous systems

CBFS 6159: Alps, 1997-08-14, KP825712, KP826100, –, KX503294, –, –. **CBFS 6162:** Alps, 1997-08-14, F, KP825711, KP826099, KP826154, KP826483, KP826611, KY229028. **CBFS 13557:** Alps, 2009-07-23, F, KP825708, KP826096, KP826151, –, KP826608. **MUB 49604:** Alps, 2012-09-14, F*, KP825627, KP826016, KP826162, KP826401, KP826530, KY229053. **MUB 49606:** Alps, 2012-09-14, F*, KP825727, KP826115, KP826161, KP826493, KP826624, --. **MUB 49613:** Alps, 2012-09-16, F*, KP825726, KP826114, –, KP826492, KP826623, KY229051. **MUB 49617:** Alps, 2012-09-16, F*, KP825725, KP826113, –, KP826491, KP826622, KY229002. **MUB 49619:** Alps, 2012-09-17, M, KP825724, KP826112, –, KP826490, KP826621, KY229000. **MUB 49624:** Pyrenees, 2012-08-31, F*, KP825730, KP826118, –, KP826496, KP826627, KY229007. **MUB 49629:** Pyrenees, 2012-08-31, F*, KP825729, KP826117, KP826158, KP826495, KP826626, KY229055. **MUB 49650:** Pyrenees, 2012-09-02, F*, KP825728, KP826116, KP826157, KP826494, KP826625, KY229004.

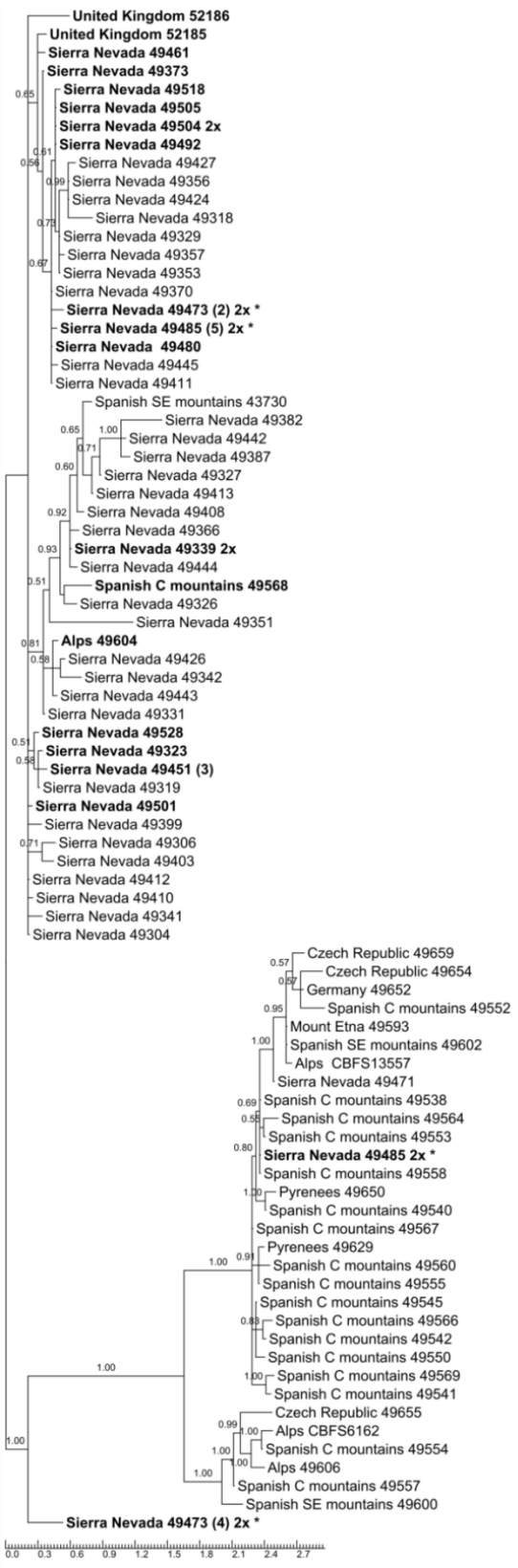
Lowlands

BOL 46302: South Africa, 2002-10-25, F*, KP825717, KP826109, –, KX503295, KP826618, KY229010. **BOL 46303:** South Africa, 2002-11-16, F*, KP825716, KP826108, –, –, KP826617, --. **MUB 49652:** Germany, 2011-08-11, F*, KP825718, KP826110, KP826156, KP826488, KP826619, KY229039. **MUB 49653:** Germany, 2011-02-09, F*, KP825719, KP826111, –, KP826489, KP826620, KY229020. **MUB 49654:** Czech Republic, 2014-04-11, F*, KX503276, –, KX503286, KX503291, KX503306, KY229012. **MUB 49655:** Czech Republic, 2014-04-11, F*, KX503275, –, KX503288, KX503290, KX503305, KY228999. **MUB 49659:** Czech Republic, 2014-04-12, F*, KX503274, –, KX503287, KX503289, KX503304, KY229006. **MUB 52185:** United Kingdom, 2014-12-10, KX503277, KX503282, KX503284, KX503292, KX503307, KY229049. **MUB 52186:** United Kingdom, 2014-04-03, (MG050861, MG050862, MG050863, MG050864, MG050865, MG050866), KX503283, KX503285, KX503293, KX503308, KY229054. **S B201182:** Sweden, 1985-07-16, F*, KP825721, KP826103, –, KX503296, KP826616, KY229018. **S B201183:** Sweden, 1985-07-21, F*, KP825720, KP826102, –, KP826485, KP826615, KY229014.

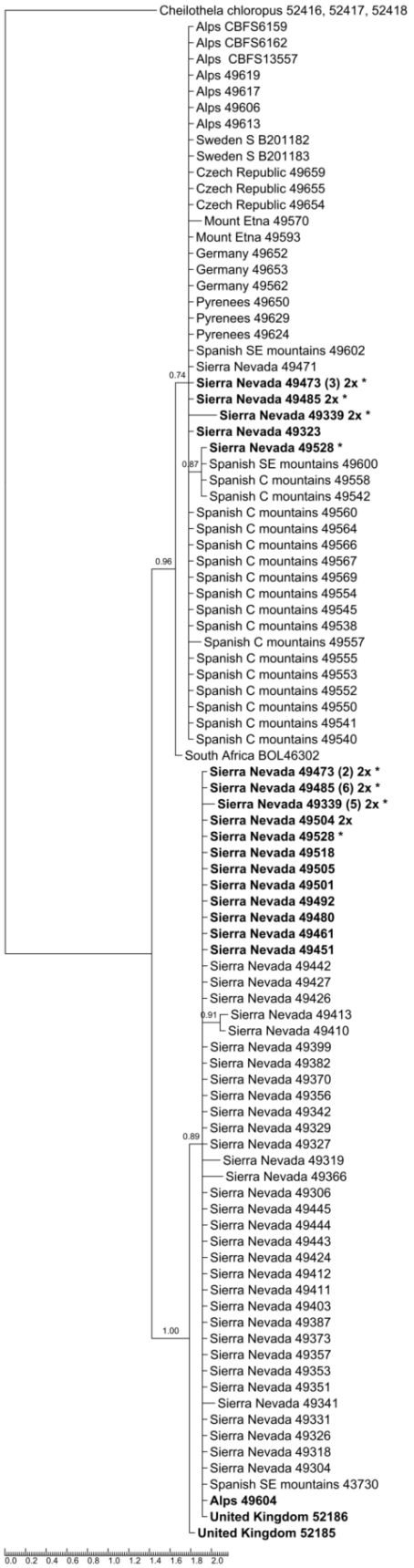
Outgroup: Cheilothela chloropus

MUB 52416: Sierra Nevada Mountains, 2011-04-28, KX503273, KX503281, –, KX503299, KX503303, KY229025. **MUB 52417:** Sierra Nevada Mountains, 2011-04-28, –, KX503280, –, KX503298, KX503302, –. **MUB 52418:** Sierra Nevada Mountains, 2011-04-28, –, KX503279, –, KX503297, KX503301, KY229026. **MUB 52419:** Sierra Nevada Mountains, 2011-04-28, –, KX503278, –, –, KX503300, –.

Annex II.2. Phylogenetic tree inferred from the nuclear *rpL23A locus*. For each tip in the trees geographical origin and number of herbarium are given (numbers without letters are from MUB); 2x is used to highlight diploid samples; number of equal sequences obtaine by cloning is indicated between parentheses if there was more than one; asterisk (*) is used for indicating samples with more than one copy for the *locus*; bold letters indicate recombinant samples.

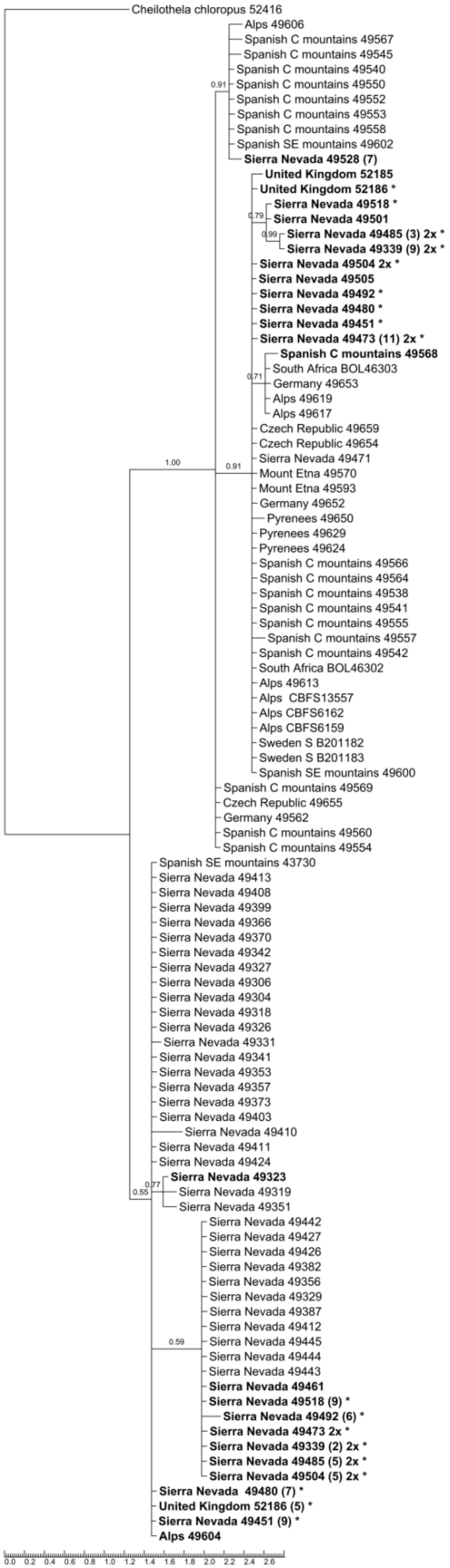


Annex II.3. Phylogenetic tree inferred from the nuclear *TBP locus*. Information about the data given for each tip in the tree as in Annex II.2.

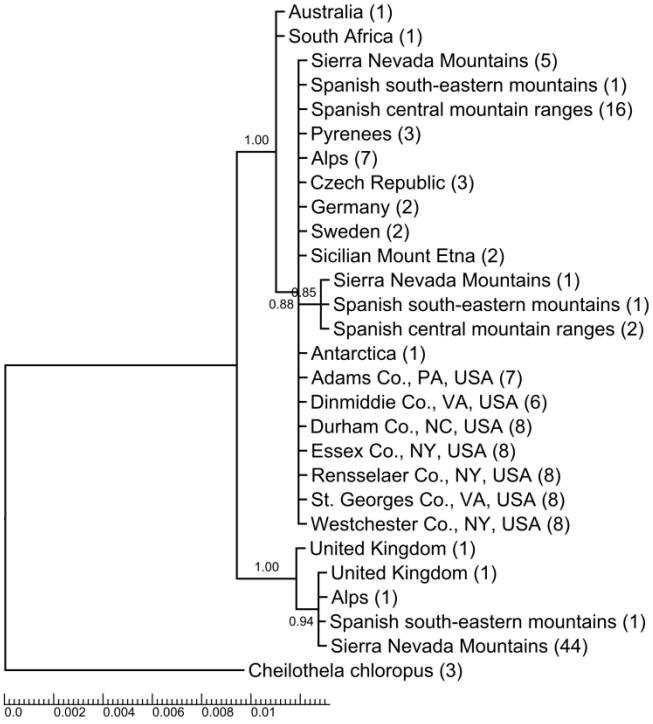


Annex II.4. Phylogenetic tree inferred from the nuclear *PPR locus*. Information about the data given for each tip in the tree as in Annex II.2.

Annex II.5. Phylogenetic tree inferred from the nuclear *hp23.9 locus*. Information about the data given for each tip in the tree as in Annex II.2.



Annex II.6. Phylogenetic tree inferred from the nuclear *TBP locus* adding to the samples used in this work other Ceratodon samples from Antarctica, Australia, and North America (GenBank accession numbers: KC436690 to KC436698, KC436701 to KC436706 and KC436710 to KC436750); To simplify the representation, we collapsed terminals of each group of individuals by area (total number of samples is given in parentheses).



Annex II.7. List of samples employed, indicating for each DNA *locus* analyzed, to which clade obtained in the phylogenetic analysis they belong (blue: SN clade, grey: Ww clade), the state of material used in cytometry analysis, and the amount of DNA (in case of dry material corrected by a factor of 0.85).

Specimen	*hp23.9*	*PPR*	*rpL23A*	*TBP*	*TRc1b3.05*	*Trn*L	State of material used	Amount of DNA (pg)
BOL 46302	Ww	Ww		Ww	Ww	Ww	--	--
BOL 46303	Ww	Ww			Ww		--	--
CBFS 13557	Ww	Ww	Ww		Ww		dry	0,36
CBFS 6159	Ww	Ww		Ww			--	--
CBFS 6162	Ww	Ww	Ww	Ww	Ww	Ww	--	--
MUB 43730	SN	SN	SN	SN	SN	SN	dry	0,44
MUB 49304	SN	SN	SN	SN	SN	SN	dry	0,47
MUB 49306	SN	SN	SN	SN	SN	SN	--	--
MUB 49318	SN	SN	SN	SN	SN		fresh	0,47
MUB 49319	SN	SN	SN	SN	SN	SN	fresh	0,45
MUB 49323	SN	SN	SN	Ww	SN	SN	fresh	0,46
MUB 49326	SN	SN	SN	SN	SN	SN	dry	0,46
MUB 49327	SN	SN	SN	SN	SN		--	--
MUB 49329	SN	SN	SN	SN	SN	SN	dry	0,45
MUB 49331	SN	SN	SN	SN	SN		fresh	0,46
MUB 49339	Ww/SN	Ww/SN	SN	Ww/SN	Ww	SN	fresh	0,82
MUB 49341	SN	SN	SN	SN	SN	SN	fresh	0,45
MUB 49342	SN	SN	SN	SN	SN		fresh	0,46
MUB 49351	SN	SN	SN	SN	SN		fresh	0,47
MUB 49353	SN	SN	SN	SN			dry	0,45
MUB 49356	SN	SN	SN	SN	SN	SN	fresh	0,47
MUB 49357	SN	SN	SN	SN	SN		dry	0,48
MUB 49366	SN	SN	SN	SN	SN	SN	fresh	0,46
MUB 49370	SN	SN	SN	SN	SN	SN	dry	0,46
MUB 49373	SN	SN	SN	SN	SN		dry	0,47
MUB 49382	SN	SN	SN	SN	SN		dry	0,48
MUB 49387	SN	SN	SN	SN	SN		dry	0,47
MUB 49399	SN	SN	SN	SN	SN	SN	fresh	0,47
MUB 49403	SN	SN	SN	SN	SN		fresh	0,47
MUB 49408	SN	SN	SN		SN	SN	dry	0,45
MUB 49410	SN	SN	SN	SN	SN		dry	0,45
MUB 49411	SN	SN	SN	SN	SN	SN	fresh	0,47
MUB 49412	SN	SN	SN	SN	SN		dry	0,48
MUB 49413	SN	SN	SN	SN	SN	SN	dry	0,45
MUB 49424	SN	SN	SN	SN	SN		dry	0,46
MUB 49426	SN	SN	SN	SN	SN		dry	0,44
MUB 49427	SN	SN	SN	SN	SN		fresh	0,46
MUB 49442	SN	SN	SN	SN	SN		dry	0,45
MUB 49443	SN	SN	SN	SN	SN		dry	0,46
MUB 49444	SN	SN	SN	SN	SN	SN	fresh	0,47
MUB 49445	SN	SN	SN	SN	SN		dry	0,47
MUB 49451	Ww/SN	Ww/SN	SN	SN	Ww	SN	fresh	0,46
MUB 49461	SN	SN	SN	SN	Ww	SN	dry	0,47
MUB 49471	Ww	Ww	Ww	Ww	Ww	Ww	fresh	0,37
MUB 49473	Ww/SN	Ww	Ww/SN	Ww/SN	Ww	SN	fresh	0,81
MUB 49480	Ww/SN	Ww	SN	SN	Ww	SN	dry	0,47
MUB 49485	Ww/SN	Ww/SN	Ww/SN	Ww/SN	Ww	SN	dry	0,82
MUB 49492	Ww/SN	Ww	SN	SN	Ww	SN	fresh	0,46
MUB 49501	Ww		SN	SN	SN	SN	--	--
MUB 49504	Ww/SN	Ww	SN	SN	SN	SN	fresh	0,82
MUB 49505	Ww	Ww	SN	SN	Ww	SN	--	--
MUB 49518	Ww/SN	Ww/SN	SN	SN	Ww	SN	fresh	0,45

Specimen	*hp23.9*	*PPR*	*rpL23A*	*TBP*	*TRc1b3.05*	*Trn*L	State of material used	Amount of DNA (pg)
MUB 49528	Ww	Ww/SN	SN	Ww/SN	Ww	Ww	fresh	0,46
MUB 49538	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,35
MUB 49540	Ww	Ww	Ww	Ww	Ww		dry	0,37
MUB 49541	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,37
MUB 49542	Ww	Ww	Ww	Ww	Ww		--	--
MUB 49545	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,35
MUB 49550	Ww	Ww	Ww	Ww	Ww	Ww	fresh	0,36
MUB 49552	Ww	Ww	Ww	Ww	Ww	Ww	fresh	0,36
MUB 49553	Ww	Ww	Ww	Ww	Ww		dry	0,38
MUB 49554	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,35
MUB 49555	Ww	Ww	Ww	Ww	Ww		dry	0,37
MUB 49557	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,37
MUB 49558	Ww	Ww	Ww	Ww	Ww		dry	0,38
MUB 49560	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,36
MUB 49562	Ww	Ww		Ww	Ww		dry	0,36
MUB 49564	Ww	Ww	Ww	Ww	Ww		dry	0,37
MUB 49566	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,36
MUB 49567	Ww	Ww	Ww	Ww	Ww	Ww	--	--
MUB 49568	Ww		SN		Ww	SN	--	--
MUB 49569	Ww	Ww	Ww	Ww	Ww	Ww	--	--
MUB 49570	Ww	Ww		Ww	Ww	Ww	fresh	0,37
MUB 49593	Ww	Ww	Ww	Ww	Ww	Ww	fresh	0,36
MUB 49600	Ww	Ww	Ww	Ww	Ww	Ww	--	--
MUB 49602	Ww	Ww	Ww	Ww	Ww	Ww	--	--
MUB 49604	SN	Ww	SN	SN	Ww	SN	fresh	0,48
MUB 49606	Ww	Ww	Ww	Ww	Ww		dry	0,37
MUB 49613	Ww	Ww		Ww	Ww	Ww	dry	0,38
MUB 49617	Ww	Ww		Ww	Ww	Ww	dry	0,38
MUB 49619	Ww	Ww		Ww	Ww	Ww	dry	0,37
MUB 49624	Ww	Ww		Ww	Ww	Ww	dry	0,37
MUB 49629	Ww	Ww	Ww	Ww	Ww	Ww	--	--
MUB 49650	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,38
MUB 49652	Ww	Ww	Ww	Ww	Ww	Ww	dry	0,37
MUB 49653	Ww	Ww		Ww	Ww	Ww	dry	0,37
MUB 49654	Ww		Ww	Ww	Ww	Ww	dry	0,37
MUB 49655	Ww		Ww	Ww	Ww	Ww	dry	0,38
MUB 49659	Ww		Ww	Ww	Ww	Ww	dry	0,38
MUB 52185	Ww	Ww	SN	SN	SN	SN	--	--
MUB 52186	Ww/SN	Ww	SN	SN	SN	SN	fresh	0,47
S B201182	Ww	Ww		Ww	Ww	Ww	--	--
S B201183	Ww	Ww		Ww	Ww	Ww	--	--

Chapter III. Environmental variation obscures species diversity in southern European populations of the moss genus *Ceratodon*

ABSTRACT

A major problem in taxonomy is to determine if morphological variation in field-collected specimens is caused by genetic differentiation, and therefore corresponds to evolutionary distinct units, or is caused by environmental variation acting on a single interbreeding population. To evaluate the effect of environmental variation on the taxonomy of the moss genus *Ceratodon,* we compared biometric analyses based on 22 morphological characters on both field-collected plants and cultivated plants to a clustering based on DNA sequence and genome size data published previously. We sampled *Ceratodon* species from mountainous areas of the Mediterranean region, and other mountain regions and lowlands, mostly from southern Europe. We found that the expression of several gametophytic traits changed between field and laboratory conditions, confirming that environmental variability complicates taxonomic inferences, and suggesting that some characters should be used with caution in distinguishing among species. However, consistent with the genetic and flow cytometry data, we found a clear biometric discontinuity between some plants collected from southern Spain, and those from other parts of the world. Samples considered of hybrid origin, based on genetic data, were morphologically indistinguishable from plants from the southern Spanish mountains. Integrative taxonomy based on genetic, genome size and morphological data unambiguously support the recognition of a new species, *Ceratodon amazonum.* These data also suggest that the previously recognized *C. conicus* is a recombinant between *C. purpureus* and *C. amazonum* and is considered here to be a nothospecies, for which an epitype is here designated because the lectotype is demonstrably ambiguous.

INTRODUCTION

Phenotypic variation within species may be caused by genotypic differences (Såstad et al., 1999; Savolainen et al., 2013) or by differential gene expression induced by environmental variability (Price et al., 2003; Pigliucci et al., 2006). Assessing the adaptive significance of morphological trait variation is a fundamental issue in evolutionary ecology (Såstad et al., 1999; Yousefi et al., 2017) and in systematics (Shaw & Bartow, 1992; Såstad, 1999), avoiding the overestimation of biodiversity. On the other hand, relying on morphological methods alone may miss cryptic species and therefore underestimate species diversity (Bickford et al., 2007). Cases of cryptic speciation have been widely documented in bryophytes (Shaw, 2001; McDaniel & Shaw, 2003; Hedenäs & Eldenäs, 2007; Kreier et al., 2010; Heinrichs et al., 2010, 2011; Ramaiya et al., 2010; Hutsemékers et al., 2012; Medina et al., 2012; Caparrós et al., 2016; Patiño et al., 2017), in which substantial genetic divergence within morphologically complex groups was observed. Genetic adaptation to habitats with relatively minor differences in ecological conditions was demonstrated in mosses as well (Såstad et al., 1999). Controlled experiments are the only way to determine functional correspondence between morphological, genotypic and environmental variability (Såstad, 1999; Såstad et al., 1999; Yousefi et al., 2017).

The delimitation of species in organisms with high dispersal potential by spores across variable environments and with considerable morphological variation remains a great challenge, such that any single methodology may not be sufficient to discriminate between species (Medina et al., 2012, 2013; Renner et al., 2013; Hedenäs et al., 2014; Draper et al., 2015; Caparrós et al., 2016; Gama et al., 2017; Patiño et al., 2017). In such cases, an integrative taxonomic approach (combining morphology, phylogeny, biogeography, ecological niche or genome size studies) is necessary.

The genus *Ceratodon* Brid. is distinguishable from other members of the family Ditrichaceae by molecular data (Cox et al., 2010; Stech et al., 2012; Fedosov et al., 2016) and morphological characters (Magill, 1981; Allen, 1994; Chien et al., 1999; Frey et al., 2006; McIntosh, 2007). Nevertheless, species within the genus are highly polymorphic (Dixon & Jameson, 1896; Watson, 1968; Crum & Anderson, 1981). The

most abundant species, *C. purpureus* (Hedw.) Brid. occurs on every continent (Crum, 1973). Wijk et al. (1959, 1969) listed 22 *Ceratodon* species, and within *C. purpureus* alone two subspecies and 31 varieties. In a global survey of the genus, Burley and Pritchard (1990) recognized four species. *Ceratodon antarcticus* Cardot grows on bare soil, rock crevices and ledges in the Antarctic region. *Ceratodon conicus* (Hampe) Lindb. is found on calcareous substrates of arid habitats in North America, Europe and Africa. *Ceratodon heterophyllus* Kindb. is known from arctic bare soils in North America, Europe and Asia. Finally, *C. purpureus* is frequent on a wide range of substrata (acid-calcareous), bare disturbed ground, soil covered rock ledges, sand dunes or on rotten wood. This latter species is common from natural and well conserved to contaminated sites (Shaw et al., 1991), and is a frequent colonizer of recently burned places (Duncan & Dalton, 1982; Foster, 1985; Clément & Touffet, 1990). Burley and Pritchard (1990) recognized three infraspecific taxa for *C. purpureus*: subsp. *purpureus* and subsp. *convolutus* (Reichardt) Burley, both found in temperate areas, and subsp. *stenocarpus* (Bruch & Schimp.) Dixon, found in tropical regions. *Ceratodon purpureus* subsp. *purpureus* and *C. purpureus* subsp. *stenocarpus* are fairly well differentiated based on morphological characteristics, largely due to sporophyte features, and they would be recognized with the rank of species if it were not for the existence of plants with intermediate characteristics between both of them, which they called *C. purpureus* subsp. *convolutus*.

The taxonomic treatment of Burley and Pritchard (1990) has been challenged by numerous authors based on the heterogeneous distribution of morphological variation within the range of the taxa and on the apparent gradation in all the diagnostic characters. For example, Ochyra (1998) considered both *C. heterophyllus* and *C. antarcticus* to be synonyms of *C. purpureus*; O'Shea (2006) questioned the value of *C. purpureus* subsp. *purpureus* and *C. purpureus* subsp. *convolutus*; and McIntosh (2007) considered *C. conicus* a subspecies within the *C. purpureus* complex based on the apparent gradation and reduction of all the characters that Burley and Pritchard (1990) used in their treatment. Phylogenetic analyses of DNA sequences generally have not strongly supported recognizing these taxa as distinct. For example, several authors found no evidence that the Antarctic specimens formed distinct genetic clusters relative to Australasian and Subantarctic isolates, in spite of finding high levels of genetic variability (Clarke et al., 2009). Additionally, McDaniel and Shaw (2005) performed

population genetic analyses of DNA sequence data from three unlinked *loci* to examine biogeographical patterns in a global sampling of *Ceratodon*. They found limited population structure across the global distribution, suggesting that long-distance migration is common, at least within the Northern Hemisphere and Australasian regions, although migration among equatorial populations was not frequent.

In parallel to the morphometric study presented here, we carried out a genetic and genome size study, including identifying the sex of not fructified individuals (Nieto-Lugilde et al., 2018a). In that paper we describe a phylogenetic analyses based on five nuclear introns and a single chloroplast *locus* of *Ceratodon* specimens from Mediterranean mountainous areas, other European mountainous systems and western and central European and South African lowlands. We could clearly distinguish two groups of specimens, corresponding to the cosmopolitan *C. purpureus* and a newly discovered species, the latter only known at present from the Sierra Nevada and the southeastern mountains of Spain. Coalescent simulations showed that the divergence between *C. purpureus* and the newly discovered species was too great to be explained by coalescent stochasticity. Several isolates had sequences from both species, which we interpreted as recombinants produced by meiosis from a hybrid sporophyte. We also examined the genome sizes of these taxa using flow cytometry. We discovered that specimens of *C. purpureus* had the smallest genome, while the new species possessed a genome 25% larger than *C. purpureus*, and the recombinants had the genome size either of the new species or the sum of the genome sizes of *C. purpureus* and the new species, the latter group potentially arising by allopolyploidy. Curiously, we found no male individuals in samples of the new species, either scanning for antheridia using a dissection microscope or using the PCR-based approach proposed by Norrell et al. (2014).

Here we use a common garden experiment to test the role of environmental variance in shaping the phenotype in the field of these distinct genetic entities. This is generally significant because we have a relatively poor understanding of the potential for the environment to shape our taxonomic concepts of bryophyte species (Vanderpoorten et al., 2002). In the present study we carried out a morphometric study, both from field collected and *in vitro* cultivated plants (laboratory isolates grown from field plants), on a subset of the plants used previously in the phylogenetic and genome

size study (Nieto-Lugilde et al., 2018a). We had three main goals: (i) to determine what the relevance of environmental variance in morphological variation in the genus *Ceratodon* is (i.e. to determine which proportion of the morphological variation has a genetic basis, and which proportion is due to environment); (ii) to search for relevant characters to discriminate molecularly defined species in the genetically-induced morphological variation, and (iii) to make a taxonomical proposal according to the results obtained.

MATERIALS AND METHODS

Plant material

We performed a morphological analysis of 64 samples of the genus *Ceratodon* (Voucher information is listed in Annex III.1). We collected 43 specimens (67.2% of the total) from Mediterranean mountain areas (28 from Spanish Sierra Nevada; ten from the Spanish Sistema Central, three from the Spanish southeastern mountains [Eastern Sierra del Segura and Sierra de Alcaraz], and two from Sicilian Mount Etna). Of the remaining 21 samples, nine (14.1%) were from other European mountainous systems (six from the Alps and three from the Pyrenees) and 12 specimens (18.7%) were from lowlands (three from Czech Republic, three from Germany [including the type of *Ceratodon conicus*], two from Sweden, two from United Kingdom, and two from South Africa). Most of the studied samples (55) were collected by the authors and are deposited at MUB (Herbarium of the University of Murcia, Spain), and the rest (nine) were loaned from several herbaria: BOL (Bolus Herbarium, University of Cape Town, South Africa), CBFS (University of South Bohemia, Czech Republic), GOET (University of Göttingen, Germany), S (Herbarium of the Swedish Museum of Natural History, Sweden), and two samples were donated by Laura Forrest (at Royal Botanic Garden Edinburgh, United Kingdom).

In vitro cultures

We generated *in vitro* cultures for 32 out of the 64 specimens. Apart from the type specimen that could not be used for axenic culture, 31 field specimens did not grow

successfully *in vitro* (Annex III.1). As most of the samples did not produce sporophytes (only 11 presented mature capsules), the cultures were prepared from gametophytic material for all the specimens. First, several gametophore fragments from field collected plants were put on polystyrene Ø 55 x 14 mm petri dishes with moistened quartz sand containing a nutrient solution (Murashige and Skoog basal salt solution 0,4x) and kept in a growth chamber with 22 ± 3ºC and 16/8h of light to darkness supplied by cool-white fluorescent tubes (Lumilux, Osram Germany) at a photon fluency rate of 33.5-55.0 µmol $m^{-2}s^{-1}$. To reduce the risk of death of the samples by contamination of fungi and algae when the fragments began to develop protonemata, they were sterilized with 0.10% sodium dichloroisocyanurate ($C_3Cl_2N_3NaO_3$) for 10s. We then washed the samples with sterile distilled water and dried them on absorbent paper to remove any residual of sodium dichloroisocyanurate, and put them on polystyrene Ø 55 x 14 mm petri dishes containing Gelzan^{TM} with MS (half-strength Murashige and Skoog basal medium) with cellophane overlays, and maintained in the growth chamber with the same conditions as above. When the protonemata developed rhizoids, caulidia and several phyllidia (80-278 days), they were used for biometric study as it was done with the field collected plants. During the period in growth chamber, plates were changed from position in the chamber, two times per week, to reduce chamber effect (Measures et al., 1973; Porter et al., 2015).

Biometric study

We selected 22 descriptive morphological characters, following the morphological results of Burley and Pritchard (1990) and our own observations (Table III.1). Of these, nine were qualitative and 13 quantitative characters. Terms used in the present work are based on "*Glossarium polyglottum bryologiae*, a multilingual glossary for bryology" (Magill, 1990). No sporophytic characters were considered for the biometric study because of the absence of capsules in the field-collected specimens and because the plants obtained *in vitro* never developed sporophytes. When they were available, sporophyte traits were only studied to confirm the identity of the specimens. We also estimated the pH of the substrate on which the moss was growing by placing a few drops of hydrochloric acid (HCl) in soil present in each sample. HCl reacts with carbonated minerals (calcite, $CaCO_3$, is the most commonly encountered) such that

Table III.1. Descriptive morphological characters included in the biometric study and HCl soil reaction of the substrate of the *Ceratodon* Brid. samples, with indication of the number of items studied, abbreviations used, type (QL = qualitatitive, QT = quantitative) and status characters.

	Abbreviation	Character	Type and status character
Substrate	SR	HCl soil reaction	QL: Acidic (0); Basic (1)
Caulidium (2 caulidia per collection)	PP	Phyllidium posture in the caulidium when moist	QL: Straight (1); Curved (2)
	CT	Comal tuft in the caulidium apex	QL: Absent (0); Present (1)
	CL	Caulidium length	QT (mm)
Phyllidium (10 phyllidia: 5 from each caulidium)	LL	Lamina length	QT (µm)
	LLW	Lamina length from apex to widest part	QT (µm)
	LW	Lamina width at widest part	QT (µm)
	AT	Teeth at apical part of lamina	QL: Absent (0); Dentate (1); Serrate (2)
	PM	Phyllidium margins	QL: Plane at midle part of lamina (0); Recurved at midle part of lamina (1); Recurved at midle and apical part of lamina (2)
	PA	Phyllidium apex	QL: Acute (1); Obtuse (2)
	EN	Excurrent nerve	QL: Absent (0); Present (1)
	ENL	Excurrent nerve length	QT (µm)
	NW	Nerve width at base of lamina	QT (µm)
	NC	Nerve color	QL: Reddish (1); Greenish (2)
	CS	Middle laminal cells shape	QL: Rounded (0); Quadrate (1)
	LL/LLW	Lamina length/lamina length from apex to widest part ratio	QT
	LL/LW	Lamina length/lamina width in widest part ratio	QT
	LL/NW	Lamina length/nerve width at base of lamina ratio	QT
	ENL/LL	Excurrent nerve length/lamina length ratio	QT
	NW/LW	Nerve width at base of lamina/lamina width at widest part ratio	QT
Middle laminal cells (12 cells: 3 from each 2 phyllidia coming from 2 caulidia)	CW	Cells width	QT (µm)
	CWT	Cells wall thickness	QL: ≤ 2µm (1); > 2µm (2)
Phyllidium cross sections (6 sections: 3 from each phyllidium coming from 2 caulidia)	NGC	Number of guide cells	QT (number of cells)

carbon dioxide bubbles (effervescence) are produced if the soil presented basic characteristics, and no effervescence if the soil was acidic.

In each collection, the HCl soil reaction (SR) was tested and two gametophore shoots were taken, in which the next characters were observed: phyllidium posture in the caulidium when moist (PP), presence/absence of a comal tuft in the caulidium apex (CT) and caulidium length (CL). Five phyllidia of the middle part of each caulidium were dissected, in which the following characters were studied (Fig. III.1): lamina length (LL), lamina length from apex to widest part (LLW), lamina width at widest part (LW), presence /absence of teeth at apical part of lamina and apical phyllidium margins if present (AT), phyllidium margins as seen in section (PM), phyllidium apex (PA), presence/absence of excurrent nerve (EN), excurrent nerve length (ENL), nerve width at base of lamina (NW), nerve color (NC), middle laminal cells shape (CS), lamina length /lamina length from apex to widest part ratio (LL/LLW), lamina length/lamina width in widest part ratio (LL/LW), lamina length/nerve width at base of lamina ratio (LL/NW), excurrent nerve length /lamina length ratio (ENL/LL), and nerve width at base of lamina /lamina width at widest part ratio (NW/LW). Two phyllidia from each of the two caulidia were selected, in each of them three measures were taken of the middle laminal cells width (CW) and their wall thickness (CWT). After selection of one phyllidium coming from each caulidium, at least three cross sections were made at the basal part near the middle of each phyllidium for observation of the number of guide cells (NGC). We measured the morphological parameters using a light microscope (Olympus BH2) with a micrometer inserted in an ocular and in a stereomicroscope (Leica A8APO) with a video camera connected (Leica camera: DFC295) in order to transfer the images to a computer. The Leica Application Suite, Version 4.1.0 was used for image analysis.

Statistical analyses

All characters were entered in a LibreOffice spreadsheet (Calc program, The Document Foundation) and then imported in R (R Core Team, 2016). For the evaluation of morphological characters and multivariate analyses, the specimens were assigned to one of three groups based on the phylogenetic analyses of the specimens carried out by Nieto-Lugilde et al. (2018a): 1) Sierra Nevada (SN) group: formed by 14 Sierra Nevada

samples and one of the Spanish southeastern mountains, included in a highly supported clade by all the six *loci* studied (SN clade); 2) Worldwide (Ww) group, that consistently included 31 specimens mostly coming from the rest of the sampled areas, but also including a low number from SN and Spanish southeastern mountains, also in a highly supported clade by all the six *loci* studied (Ww clade); and 3) Recombinant group: formed by 17 samples mainly coming from Sierra Nevada, but also from Spanish Sistema Central, Alps and United Kingdom; that were strongly resolved in either the SN clade or the Ww clade, depending on the studied *locus*. Each group was subdivided into two subgroups: field-collected plants and cultivated plants. All samples from SN group and Ww group for which the genome size could be determined previously were haploid (13 and 20 respectively); the recombinant samples contained four putative allopolyploids and nine haploids (Annex III.1). The type of *Ceratodon conicus* was studied morphologically and considered a recombinant based on its morphological characteristics, because neither the genotype nor the genome size could be determined.

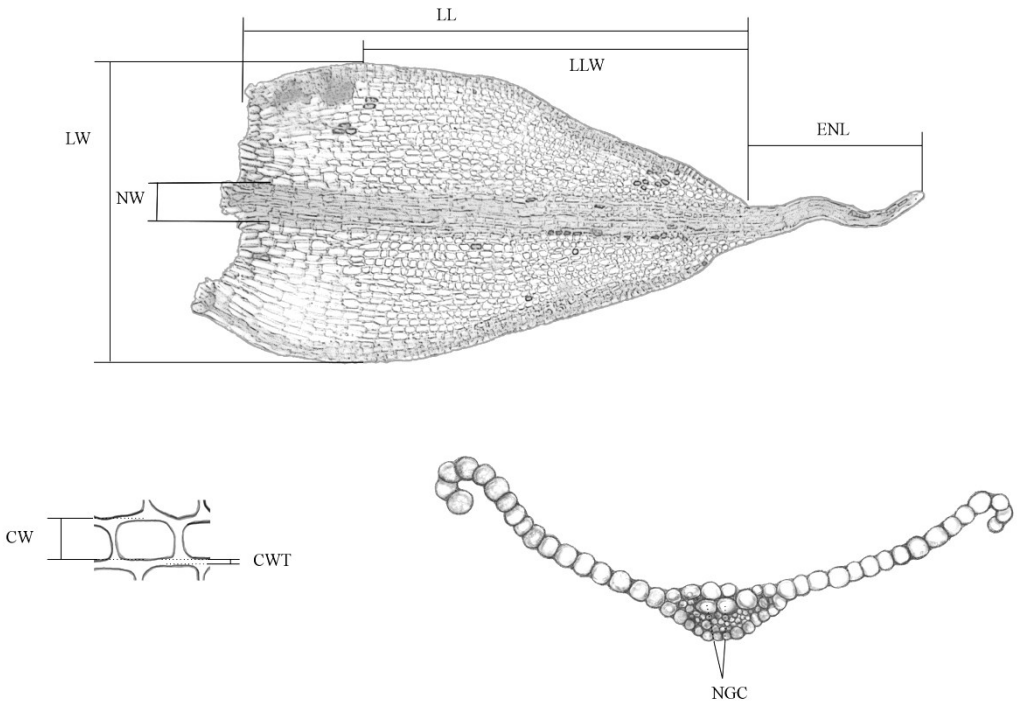


Fig. III.1. Diagrammatic definition of the quantitative morphological characters included in the biometric study of *Ceratodon*. LL: lamina length, LLW: lamina length from apex to widest part, ENL: excurrent nerve length, LW: lamina width, NW: nerve width at base of lamina, CW: middle laminal cells width, CWT: middle laminal cells wall thickness, and NGC: number of guide cells in phyllidium cross section.

We used Wilcoxon test (non-parametric test; Wilcoxon, 1945) to examine the significance of the differentiation between different subgroups, and we adjusted the *p*-value to test the significance level using the Benjamini-Hochberg correction (Benjamini & Hochberg, 1995). We plotted the reaction norms for each quantitative morphological character for the cultivated plants and the corresponding field-collected plants for each genetic group. The set of R functions for the morphometric analysis was based on Koutecký (2015). The transformations performed to improve their distribution were: Log_{10} (CL), Log_{10} (LL/LLW), Log_{10} (ENL + 1) and Log_{10} (ENL/LL × 100 + 1). The characters AT and PM were split in two binary characters: AT d (dentate), AT s (serrate), PM p (plane) and PM r2 (recurved at middle and apical part of lamina). We performed two-way ANOVAs on each quantitative morphological character to study the effect of environmental conditions and the genotype, and the interaction term between them. The environment where the plants were grown was employed as variation factor with two levels (field collected plants and cultivated plants) and the genetic groups obtained in Nieto-Lugilde & al. (2018a) were used as variation factor with three levels (SN group, Ww group and Recombinant group). Finally we performed three multivariate morphometric analyses: Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA) and Classificatory Discriminant Analysis (CDA). PCA converts a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components (PC). The essential part of LDA is a dimensionality reduction, which allows replacing the original variables-classifiers by the linear discriminants (LD), a smaller number of derivate classifiers. LDA finds the line that best separates the classes, on the contrary to PCA that is not optimal for classification. In addition we performed two types of significance tests of individual characters, first the marginal effects (i.e., when a character is alone in the model) and second the unique contributions of the characters (i.e., the addition of each character into the model with all other characters). The CDA quantifies the proportion of specimens correctly assigned to a species by the best combination of morphological characters. We performed a LDA using the real morphological character as variables in the discriminant analysis; moreover we tested the use of the PC as variables in LDA (PCA-LDA). To avoid using highly correlated characters ($r > |0.95|$) in the discriminant analysis (Koutecký, 2015) we calculated a Spearman's matrix of the correlation coefficients of the characters. Only the characters LL and LLW showed high correlation

between them (r = 0.989). For this reason, LL was omitted for discriminant analysis. The PCs with eigenvalues > 1 were selected as optimal number of variables (Kaiser, 1960), moreover the amount of explained variance was 70-80%. The most useful characters for separating the three groups of samples both field-collected plants and cultivated plants were determined by a higher absolute value of contribution of characters to individual axes (both LD1 and LD2). These values were standardized by within group variance and reflected better the relative importance of the characters (Lepš & Šmilauer, 2003).

RESULTS

Environmental variance in morphological variation

The statistical results of qualitative and quantitative characters included in the biometric study are shown in Table III.2, and the results of analyses of the variance (two-way ANOVAs) of the quantitative morphological characters are given in Table III.3.

Most of the analyzed traits (92%) showed significant (α = 0.05) effects of environment (except LL/NW) and genotype (except NW/LW) on ANOVAs. Only Log_{10} (ENL + 1) and Log_{10} (ENL/LL × 100 + 1) did not show significant effects due to the interaction between genotype and the environment (G × E). The p-value for some characters are greater for the genotype factor than for the environment. The environment factor explains more than 50% of the observed phenotypic variation for Log_{10} (CL), LW, Log_{10} (ENL + 1), NW. The genetic factor explains more than 50% of the variation in LL, LLW, LL/LW, LL/NW, Log_{10} (ENL/LL × 100 + 1) and NGC. Finally the interaction between the two factors explains more than 50% of the variation in Log_{10} (LL/LLW) and NW/LW, but in these cases the environment factor is more important than the genetic factor. Furthermore, the character CW does not show a clear explanation by only one factor (E = G x E = 40.24%). In general, the morphological variation within the genus *Ceratodon* is more influenced by environmental variation in seven characters and by genetic variation in six characters. Reaction norm plots for each

Table III.2. Statistical results of qualitative and quantitative characters of *Ceratodon* Brid. specimens included in the biometric study. Character abbreviations follow those given in Table III.1. Specimens were grouped according to the three genetic groups obtained from phylogenetic analyses (Nieto-Lugilde et al., 2018a) and each of them was subdivided in two subgroups: the field collected plants and the *in vitro* cultivated plants (SN Field: Sierra Nevada group samples from field, Ww Field: Worldwide group samples from field, Recombinant Field: Recombinant group samples from field, SN Cultivated: Sierra Nevada group samples from *in vitro* cultures, Ww Cultivated: Worldwide group samples from *in vitro* cultures, and Recombinant Cultivated: Recombinant group samples from *in vitro* cultures). Number (n) of specimens examined for each group is given. Descriptive statistics (mean ± SD [range]) for quantitative characters are presented. All measurements are given in μm, except for CL in mm. Values with statistically significant difference (α = 0.05) are written in bold.

Abbreviation of characters studied	SN Field (*n* = 15)	Ww Field (*n* = 31)	Recombinant Field (*n* = 17)	SN Cultivated (*n* = 9)	Ww Cultivated (*n* = 14)	Recombinant Cultivated (*n* = 9)
SR	**50.00% Acidic**	96.60 % Acidic	82.30% Acidic			
PP	93.75% Straight	**67.20% Straight**	97.00% Straight	100.00% Curved	100.00% Curved	100.00% Curved
CT	59.30% Absent	**96.90% Absent**	50.00% Absent	100% Absent	100% Absent	100% Absent
CL	4.46 ± 2.30 [2.05-9.45]	**10.65 ± 5.54 [2.81-29.26]**	5.17 ± 2.24 [2.26-10.01]	3.65 ± 1.93 [1.30-7.50]	2.53 ± 1.13 [0.60-4.93]	2.20 ± 0.50 [1.52-2.94]
LL	**594.10 ± 162.30 [350.00-1150.00]**	**1142± 357.48 [362.70-2586.00]**	**717.20 ± 192.16 [354.60-1350.00]**	512.90 ± 129.09 [266.00-806.00]	**811.10 ± 295.84 [338.50-1656.00]**	558.90 ± 130.26 [322.40-846.30]
LLW	**462.30 ± 151.81 [241.80-950.00]**	**946.30 ± 334.26 [241.80-2182.00]**	**569.30 ± 182.68 [241.80-1200.00]**	411.20 ± 127.04 [161.20-750.00]	**637.30 ± 289.15 [241.80-1515.00]**	441.90 ± 125.66 [225.70-725.40]
LW	378.00 ± 80.71 [200.00-556.10]	387.80 ± 72.76 [230.00-612.60]	384.00 ± 102.39 [193.40-710.00]	235.60 ± 40.78 [129.00-346.60]	**297.50 ± 64.28 [177.30-483.60]**	226.90 ± 46.96 [137.00-346.60]
AT	98.10% Absent: 1.90% Dentate	**91.90% Absent: 8.10% Dentate**	98.80% Absent: 1.20% Dentate	56.00% Absent: 44.00% Dentate	49.30% Absent: 50.00% Dentate: 0.70% Serrate	66.70% Absent: 33.30% Dentate
PM	**89.40% (2): 6.90% (0): 3.70% (1)**	97.80% (2): 2.20% (1)	97.60% (2): 1.20% (0): 1.20% (1)	44.00% (2): 29.00% (0): 27.00% (1)	38.00% (0): 31.30% (1): 30.70% (2)	36.60% (2): 32.20% (1): 31.20% (0)
PA	90.00% Acute	**98.80% Acute**	94.10% Acute	98.00% Acute	97.30% Acute	100.00% Acute

Table III.2. Continued.

Abbreviation of characters studied	SN Field (*n* = 15)	Ww Field (*n* = 31)	Recombinant Field (*n* = 17)	SN Cultivated (*n* = 9)	Ww Cultivated (*n* = 14)	Recombinant Cultivated (*n* = 9)
EN	**75.00% Present**	**54.40% Present**	**94.10% Present**	59.00% Absent	**76.00% Absent**	58.90% Present
ENL	**128.60 ± 69.45 [40.00-322.40]**	**107.40 ± 58.60 [24.18-350.00]**	**159.20 ± 91.37 [40.00-600.00]**	58.47 ± 35.22 [16.12-200.00]	61.12 ± 30.61 [16.12-137.00]	70.72 ± 45.46 [16.12-255.70]
NW	64.22 ± 17.43 [25.00-114.00]	**79.13 ± 17.36 [38.00-140.00]**	67.48 ± 19.19 [25.00-140.00]	48.38 ± 13.13 [20.00-72.00]	**57.84 ± 17.16 [26.00-108.00]**	50.02 ± 12.24 [25.00-90.00]
NC	74.4% Greenish	66.9% Greenish	**87.6% Greenish**	84.0% Greenish	92.7% Greenish	74.4% Greenish
CS	**92.5% Quadrate**	**62.2% Quadrate**	**99.4% Quadrate**	97.0% Quadrate	90.0% Quadrate	100.0% Quadrate
LL/LLW	**1.30 ± 0.14 [1.07--1.80]**	**1.23 ± 0.14 [1.03--2.20]**	**1.29 ± 0.16 [1.00--2.00]**	1.27 ± 0.15 [0.92--2.00]	1.33 ± 0.19 [1.05--2.12]	1.29 ± 0.14 [1.06--1.66]
LL/LW	**1.58 ± 0.32 [0.85--2.78]**	**2.96 ± 0.83 [1.06--5.83]**	**1.91 ± 0.42 [1.07--3.18]**	**2.18 ± 0.44 [1.25--3.18]**	**2.78 ± 1.06 [1.33--6.52]**	**2.52 ± 0.65 [1.39--4.16]**
LL/NW	**9.56 ± 2.30 [5.40--18.00]**	**14.51 ± 3.96 [5.79--34.26]**	**10.99 ± 2.65 [5.33--21.25]**	11.06 ± 2.83 [6.16--21.49]	**14.33 ± 4.06 [7.31--32.83]**	11.65 ± 3.26 [6.24--22.00]
ENL/LL	0.22 ± 0.12 [0.05-0.74]	**0.09 ± 0.05 [0.02-0.29]**	0.22 ± 0.11 [0.05-0.60]	0.11 ± 0.06 [0.03-0.29]	**0.06 ± 0.03 [0.02-0.14]**	0.13 ± 0.09 [0.03-0.47]
NW/LW	0.17 ± 0.04 [0.08-0.30]	**0.21 ± 0.04 [0.10-0.30]**	0.18 ± 0.04 [0.10-0.28]	0.21 ± 0.05 [0.10-0.30]	0.20 ± 0.06 [0.09-0.36]	0.23 ± 0.06 [0.10-0.35]
CW	8.17 ± 2.01 [4.00-15.00]	7.75 ± 1.70 [3.00-12.50]	8.44 ± 1.96 [4.00-16.00]	8.26 ± 1.44 [6.00-12.00]	**10.22 ± 3.00 [4.00-18.00]**	8.96 ± 2.64 [4.00-16.94]
CWT	77.22% ≤ 2	77.30% ≤ 2	75.56% ≤ 2	96.60% ≤ 2	95.83% ≤ 2	100.00% ≤ 2
NGC	**0.98 ± 1.02 [0.00-3.00]**	**3.28 ± 1.32 [0.00-6.00]**	**1.37 ± 1.35 [0.00-5.00]**	0.00 ± 0.00 [0.00-0.00]	**1.45 ± 1.68 [0.00-4.00]**	0.00 ± 0.00 [0.00-0.00]

quantitative morphological trait (except NGC) were shown in Annex III.2. All morphological characters presented environmental variance for all or at least some of the genotypes. The magnitude of environmental variance was quantified by the slope of the line which varies enormously between genotypes. It is usually observed that the characters vary in the same sense although each genotype varies with a different magnitude, the exceptions were LL/LLW, LL/LW, LL/NW, NW/LW, and CW. Wilcoxon´s test also confirmed clear differences between field-collected plants and cultivated plants in most of the studied characters (Table III.4); in the samples from SN (Sierra Nevada) and Ww (Worldwide) groups 81.8% of the characters studied were different, but the recombinants presented a little more variation between different environments (86.4%). We could not detect a significant environmental response in samples from SN group in the characters CL, NC, CS and CW ($p > 0.05$), and LLW and PA characters were marginally significant ($\alpha = 0.01$-0.05).

Table III.3. Analyses of the variance (ANOVAs, two way factorial design) of the quantitative morphological characters. The environment of growing (E) was employed as variation factor with two levels: field collected plants and cultivated plants under laboratory conditions; the genetic species (G; obtained in Nieto-Lugilde et al. 2018a) was used as variation factor with three levels: SN = Sierra Nevada group samples, Ww = Worldwide group samples, and Recombinant group samples. The residuals tell us about the variation within each level. Character abbreviations follow those given in Table III.1.; the transformations performed to improve their distribution were: Log_{10} (CL), Log_{10} (LL/LLW), Log_{10} (ENL + 1) and Log_{10} (ENL/LL × 100 + 1). Df = Degree of freedom; Values with statistically significant difference ($\alpha = 0.05$) are written in bold.

Characters studied	Variation factors	Df	Sum of Squares	Mean Squares	F value	*P* value (> F)	Variance explained
CL	E	1	5.856	5.856	155.68	**< 2e^{-16}**	66.95%
	G	2	0.985	0.492	13.09	**7.07e^{-06}**	11.26%
	G x E	2	1.906	0.953	25.34	**6.27e^{-10}**	21.79%
	Residuals	122	4.590	0.038			
LL	E	1	13098465	13098465	216.3	**< 2e^{-16}**	25.89%
	G	2	33017908	16508954	272.6	**< 2e^{-16}**	65.27%
	G x E	2	4469967	2234984	36.9	**6.93e^{-16}**	8.84%
	Residuals	634	38400805	60569			
LLW	E	1	10536668	10536668	193.90	**< 2e^{-16}**	26.40%
	G	2	24834829	12417414	228.51	**< 2e^{-16}**	62.22%
	G x E	2	4544316	2272158	41.81	**< 2e^{-16}**	11.38%
	Residuals	634	34451281	54340			
LW	E	1	3078582	3078582	567.966	**< 2e^{-16}**	88.19%
	G	2	345789	172894	31.897	**6.32e^{-14}**	9.91%
	G x E	2	66654	33327	6.149	**0.00227**	1.90%
	Residuals	634	3436510	5420			

ENL	E	1	7.324	7.324	136.387	**< 2e^{-16}**	89.73%
	G	2	0.665	0.332	6.190	**0.00229**	8.15%
	G x E	2	0.173	0.086	1.608	0.20185	2.12%
	Residuals	336	18.043	0.054			
NW	E	1	78322	78322	271.023	**< 2e^{-16}**	74.05%
	G	2	23613	11807	40.855	**< 2e^{-16}**	22.32%
	G x E	2	3841	1920	6.645	**0.00139**	3.63%
	Residuals	634	183219	289			
LL/LLW	E	1	0.0476	0.04756	21.968	**3. 40e^{-06}**	34.07%
	G	2	0.0203	0.01016	4.693	**0.00948**	14.53%
	G x E	2	0.0718	0.03592	16.590	**9.49e^{-08}**	51.40%
	Residuals	634	1.3726	0.00217			
LL/LW	E	1	4.6	4.63	8.787	**0.00315**	2.45%
	G	2	148.4	74.20	140.897	**< 2e^{-16}**	79.15%
	G x E	2	34.5	17.27	32.792	**2.81e^{-14}**	18.40%
	Residuals	634	333.9	0.53			
LL/NW	E	1	1	0.60	0.047	0.8283	0.030%
	G	2	3104	1552.2	127.384	**< 2e^{-16}**	94.55%
	G x E	2	178	89.20	7.318	**0.000721**	5.42%
	Residuals	634	7725	12.2			
ENL/LL	E	1	2.122	2.1223	48.071	**2.11e^{-11}**	24.73%
	G	2	6.235	3.1176	70.614	**< 2e^{-16}**	72.66%
	G x E	2	0.224	0.1121	2.539	0.0805	2.61%
	Residuals	336	14.834	0.0441			
NW/LW	E	1	0.0543	0.05428	25.737	**5.15e^{-07}**	29.88%
	G	2	0.0127	0.00634	3.007	0.0502	6.99%
	G x E	2	0.1147	0.05737	27.202	**4.63e^{-12}**	63.13%
	Residuals	634	1.3372	0.00211			
CW	E	1	231	230.78	50.27	**3.05e^{-12}**	40.24%
	G	2	112	55.90	12.18	**6.22e^{-06}**	19.52%
	G x E	2	231	115.56	25.17	**2.59e^{-11}**	40.24%
	Residuals	762	3498	4.59			
NGC	E	1	254.2	254.20	188.72	**< 2e^{-16}**	42.01%
	G	2	318.7	159.33	118.28	**< 2e^{-16}**	52.67%
	G x E	2	32.2	16.09	11.95	**9.49e^{-06}**	5.32%
	Residuals	357	480.9	1.35			

In the samples from the Ww group, the CT, PA, LL/LW and NW/LW characters were not different. In the recombinant samples the CS, LL/LLW and LL/NW characters were not different, and again the PA character was marginally significant (α = 0.01-0.05).

We have highlighted differences between field-collected plants and cultivated plants in the box-plots (Figs. III.2-III.3), in which the data obtained for 12 of the 13 quantitative characters studied were shown (with the exception of CW). Most quantitative plant characters presented low mean values for cultivated plants in comparison with field collected plants, as observed in LL, LLW, LW, ENL, NW, ENL/LL or NGC (Table III.2).

Relevant characters to discriminate molecularly defined species

Studying the three genetic groups of field-collected plants separately, approximately one-third (36.4%) of the means of the measured morphological characters differed between the SN group samples and Recombinant group samples from Ww group samples (PP, CT, CL, AT, PA, NW, ENL/LL and NW/LW; Tables III.2-III.4). Slightly higher proportion of the characters (40.9%) separated the three genetic groups (LL, LLW, EN, ENL, CS, LL/LLW, LL/LW, LL/NW and NGC; Tables III.2-III.4). In these characters, the differences between the mean values in the Ww group and the other two groups were always larger (2-5.5 times) than the difference between the SN group and the Recombinant group. Two characters were homogeneous among the three groups (LW and CWT; Table III.4). The SN group samples were indifferent to the type of soil (50% acid), while the Ww group and Recombinant group samples showed a preference for acid soils, 96.6 and 82.3% respectively (Table III.2).

In the PCA, 46.08% of the total variance was explained with the first two PC (32.35% PC1 and 13.73% PC2), and 14 PC explained 96.51% of the total variance. PC1 separated the Ww samples from the SN and recombinant samples (Fig. III.4A), while the other PCs did not clearly separate any group. Six PCs had an eigenvalue higher than one (73.77% of the variance); the contributions of morphological characters are shown in Annex III.3. We performed a LDA using the morphological variables (Fig. III.4B). The LD1 (74.21%) separated two clear groups: Ww group with positive score values and SN group together with Recombinant group with negative score values. The LD1 was influenced primarily by LL/LW and LLW, and the next most important characters were NGC, and Log_{10} (ENL/LL × 100 +1) (Annex III.4). The LD2 (25.79%) slightly separated SN group and Recombinant group, and the Ww group samples show value ranges as broad as the other two groups together; the LL/LW loaded heavily also on LD2, together with PM p, PM r2 and LL/NW (Annex III.4).

Table III.4. *P*-values of the morphological characters included in the biometric study and HCl soil reaction of the substrate of the *Ceratodon* Brid. samples (Wilcoxon test) between genetic groups from field and *in vitro* cultivated plants. Character abbreviations follow those given in Table III.1. Significant *p*-values < 0.01 are written in bold after being corrected with Benjamini-Hochberg test; * means 0.01 < *p*-values < 0.05. Groups obtained from phylogenetic analyses (Nieto-Lugilde et al., 2018a): 1 = Sierra Nevada group samples from field, 2 = Sierra Nevada group samples from *in vitro* cultures, 3 = Worldwide group samples from field, 4 = Worldwide group samples from *in vitro* cultures, 5 = recombinant samples from field, and 6 = recombinant samples from *in vitro* cultures.

	Groups compared	SR	PP	CT	CL	LL	LLW	LW	AT	PM	PA	EN	ENL
Field vs. Cultures	1-2		**0.0000**	**0.0012**	0.2251	**0.0001**	**0.0161***	**0.0000**	**0.0000**	**0.0000**	**0.0137***	**0.0000**	**0.0000**
	3-4		**0.0000**	0.3384	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.2696	**0.0000**	**0.0000**
	5-6		**0.0000**	**0.0003**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0193***	**0.0000**	**0.0000**
Field	1-3	**0.0002**	**0.0043**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.1208	**0.0068**	**0.0000**	**0.0068**	**0.0000**	**0.0081**
	1-5	**0.2583***	0.5338	0.4525	0.1453	**0.0000**	**0.0000**	0.8093	0.6061	**0.0021**	0.6061	**0.0000**	**0.0000**
	3-5	0.2464	**0.0008**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.1563	**0.0016**	0.8930	**0.0016**	**0.0000**	**0.0000**
Cultures	2-4		--	--	0.0670	**0.0000**	**0.0000**	**0.0000**	0.2828	0.0392	0.7389	**0.0044**	0.5516
	2-6		--	--	0.0217	0.0156	0.0749	0.0703	0.1336	0.4191	0.1810	0.0141	0.0086
	4-6		--	--	0.2918	**0.0000**	**0.0000**	**0.0000**	**0.0083***	0.2429	0.1200	**0.0000**	0.5345

	Groups compared	NW	NC	CS	LL/LLW	LL/LW	LL/NW	ENL/LL	NW/LW	CW	CWT	NGC
Field vs. Cultures	1-2	**0.0000**	0.0687	0.1313	**0.0069**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.7660	**0.0000**	**0.0000**
	3-4	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.4499	**0.0002**	**0.0001**	0.0662	**0.0000**	**0.0000**	**0.0000**
	5-6	**0.0000**	**0.0070**	0.4718	0.6254	**0.0000**	0.1695	**0.0000**	**0.0000**	**0.0017**	**0.0000**	**0.0000**
Field	1-3	**0.0000**	0.0933	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0004**	0.2688	**0.0000**
	1-5	0.2102	**0.0021**	**0.0013**	**0.0206***	**0.0000**	**0.0000**	0.8891	0.1416	0.0435	0.8959	**0.0158***
	3-5	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.2612	0.3307	**0.0000**
Cultures	2-4	**0.0000**	0.0310	0.6159	0.0379	**0.0000**	**0.0000**	**0.0000**	0.2459	**0.0000**	1.0000	**0.0000**
	2-6	0.6298	0.1048	0.0998	0.3039	**0.0005**	0.2070	0.4080	0.0066	0.0621	0.0567	--
	4-6	**0.0005**	**0.0001**	0.1795	0.2774	**0.0000**	**0.0000**	**0.0000**	**0.0011**	**0.0001**	0.0560	**0.0000**

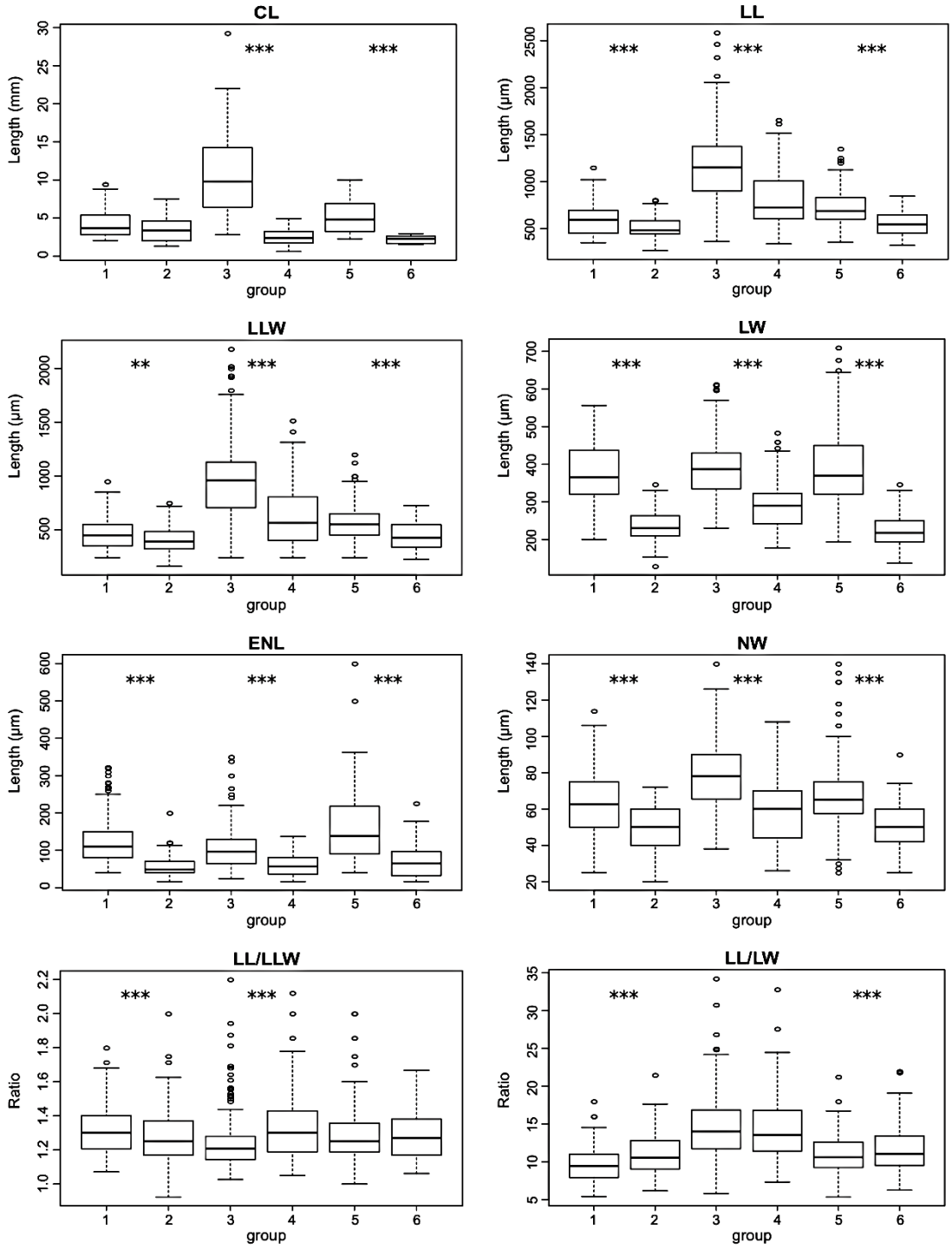


Fig. III.2. Box-plots of some of the principal morphological characters studied: caulidium length (CL), lamina length (LL), length of lamina from apex to widest part (LLW), lamina width at widest part (LW), excurrent nerve length (ENL), nerve width at base of lamina (NW), lamina length/length of lamina from apex to widest part ratio (LL/LLW) and lamina length/lamina width in widest part ratio (LL/LW). Number of groups as in Table III.4. Significant p-value < 0.01 and 0.01 < p-value < 0.05 after being corrected with Benjamini-Hochberg test are indicated with *** and **, respectively.

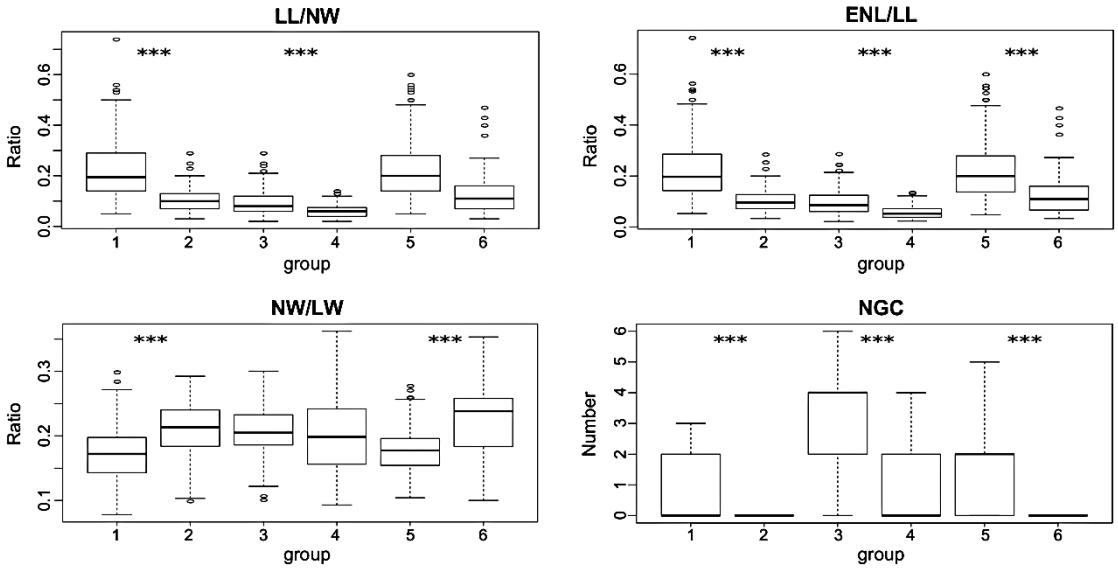


Fig. III.3. Box-plots of some of the principal morphological characters studied: lamina length/nerve width at base of lamina ratio (LL/NW), excurrent nerve length/lamina length ratio (ENL/LL), nerve width at base of lamina/lamina width at widest part ratio (NW/LW) and number of guide cells (NGC). Number of groups as in Table III.4. Significant *p*-value < 0.01 after being corrected with Benjamini-Hochberg test is indicated with asterisks.

The first significance test (marginal effects) highlighted all characters with $p < 0.05$, except LW and CWT. On the other hand, the second significance test (unique contributions) showed only LL/NW and Log_{10} (ENL/LL × 100 + 1) with $p < 0.05$. The CDA showed the correctness of classification. For the Ww group it was 90.32%, while for the SN group and Recombinant group it was 63.33% and 63.88% respectively.

Two caulidia of the lectotype of *Ceratodon conicus* were included in multivariate analyses. In the PCA analysis they are together with the Recombinant group and SN group, but in LDA and PCA-LDA analysis they are grouped with the recombinants and separated from the SN group (Fig. III.4A, B). One sample from Austria without sporophytes (CBFS 6162, included in this study) showed some morphological characteristics typical of *C. heterophyllus*, such as broadly ovate, concave, almost cucullate phyllidia, and nerves ending shortly below the obtuse apex. Nevertheless, despite these morphological characteristics, the Austrian sample belongs to the Ww group based on genetic data. Our PCA results slightly discriminate this sample from most of the Ww group samples, but LDA results indicated that this sample could not be separated by morphological gametophytic characters from *C. purpureus* (Fig. III.4A, B). Two samples with sporophytes from South Africa studied here (BOL

46302 and BOL 46303) presented the morphological diagnostic characters of *C. purpureus* subsp. s*tenocarpus*: erect to sub-erect, smooth to slightly sulcate when dry, ± lacking struma capsules, and peristome teeth usually narrowly bordered, with 0-5 trabeculae (*C. purpureus* subsp. *purpureus*, in contrast, presents inclined to horizontal, deeply sulcate when dry, strumose capsules, and peristome teeth with broad and prominent border, and with (5)7-9(13) trabeculae). Based on genetic data, these two African samples also belonged to the Ww group. According with that, our morphological analyses did not differentiate them from the rest of samples from Ww group.

Comparing each genotype from cultivated plants, we observed that nine out of 22 morphological characters separate the SN group samples and Recombinant group samples from Ww group samples (LL, LLW, LW, EN, NW, CW, NGC, LL/NW and ENL/LL). Only one character (LL/LW) differed among the three genetic groups in culture, and nine characters did not differ among groups (PP, CT, CL, PM, PA, ENL, CS, CWT and LL/LLW). The other three characters (AT, NC and NW/LW) separate the Ww group samples and Recombinant group samples. The groups were barely intermingled with each other in the PCA, but no clear-cut discontinuity was detected among them (Fig. III.4C). Six PCs had an eigenvalue higher than 1 (77.06% of the variance), the contribution of morphological characters is shown in Annex III.3. In LDA analyses we obtained results similar to field-collected plants, but LD1 discriminated 62.09% and LD2 37.91% (Fig. III.4D). Looking at the contribution of characters (Annex III.4), it was observed that LW, Log_{10} (ENL/LL × 100 + 1), together with LL/NW were the characters with the highest influence on LD1, while on LD2 were Log_{10} (CL), NW/LW, NC and Log_{10} (ENL/LL × 100 + 1). The first significance test (marginal effects) showed less amount of significant characters than in field-collected plants; Log_{10} (CL), LLW, LW, Log_{10} (ENL + 1), NW, LL/LW, LL/NW, NW/LW, Log_{10} (ENL/LL × 100 + 1), CW and NGC presented $p < 0.05$. The second significance test (unique contributions) showed only CL and CW with $p < 0.05$. The CDA showed here less capacity to discriminate groups than for field-collected plants. For the Ww group it was 89.29%, while for SN group and Recombinant group it was 62.50% and 58.82% respectively.

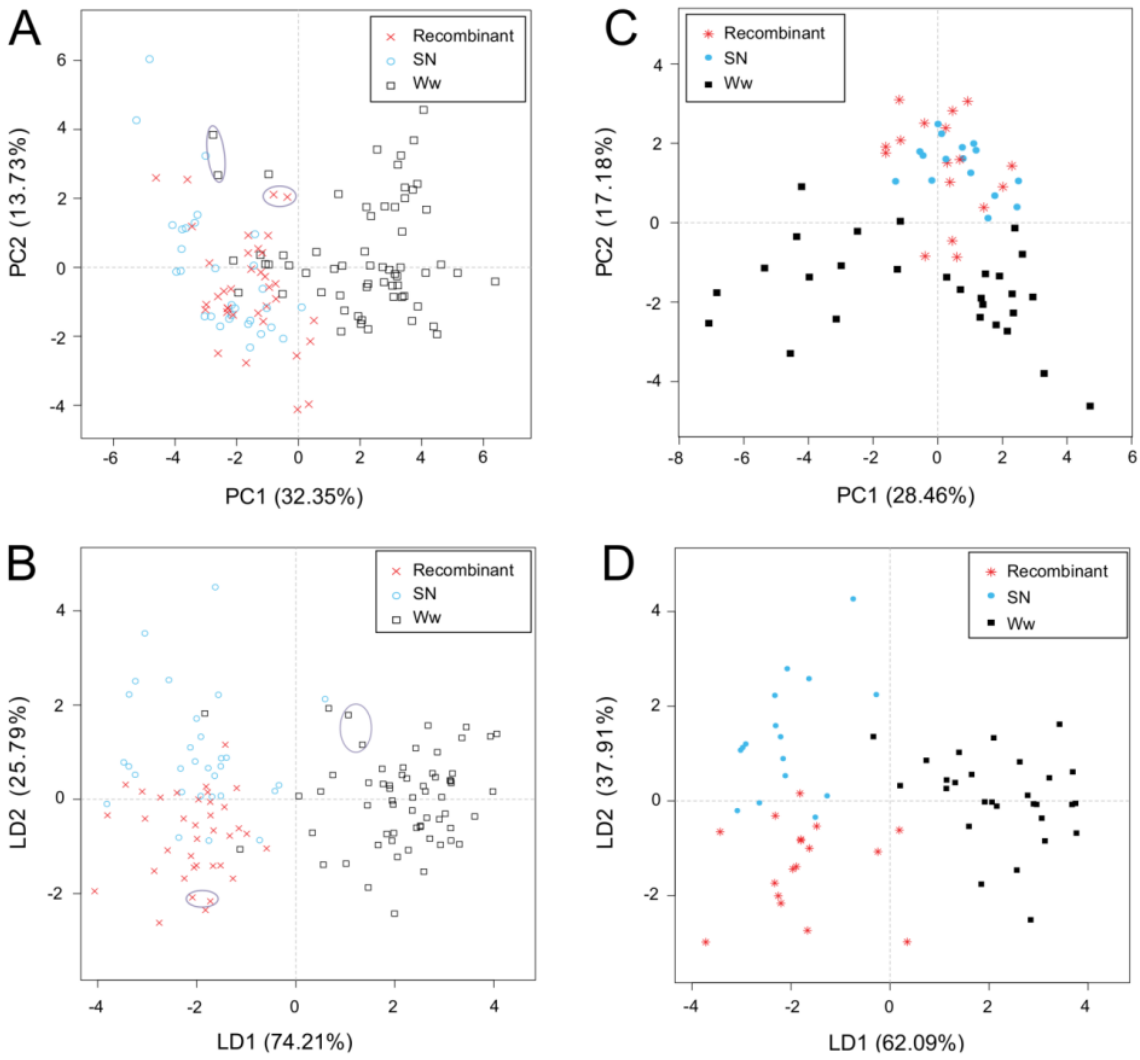


Fig. III.4. Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) plots for *Ceratodon* samples showing separation of morphotypes. Grouping samples according to Nieto-Lugilde et al. (2018a): SN = Sierra Nevada group samples, Ww = Worldwide group samples, and Recombinant = Recombinant group samples. The grouped samples from Ww group correspond with *C. heterophyllus* sample and the two grouped recombinants are the type of *C. conicus*. A) PCA plot analyzing only field collected samples; B) LDA plot analyzing only field collected samples; C) PCA plot analyzing only *in vitro* cultivated samples; D) LDA plot analyzing only *in vitro* cultivated samples.

In addition, for field-collected plants as well as cultivated plants we performed a LDA analysis employing the six most important PC as variables, PCA-LDA and a CDA (data not shown). The results were very similar but with less capacity of discrimination between conflicting groups (Recombinant group and SN group).

We also conducted the same analyses using genome size data obtained by flow cytometry as a clustering criterion. Although only four allopolyploid individuals were

found, we obtained biometric data of all, from field-collected plants and cultivated plants. The morphological characters CW and NGC showed significant *p*-values between 0.01-0.05 after being corrected with Benjamini-Hochberg test, and LL, LLW, LW, ENL, NW presented significant *p*-value < 0.01 from field-collected plants (Table III.5). From cultivated plants only ENL, ENL/LL and NC showed significant *p*-values. Although the Wilcoxon test showed these differences when we performed the LDA, the allopolyploid recombinants were mixed with the haploid recombinants, hindering their signaling in the graphs (data not shown). We obtained four LD from field-collected plants and, only LD3 (15.03%) showed the allopolyploid recombinants in an intermediate zone between the group of SN (haploid) and the haploid recombinants, but without any clear separation. The CDA showed 37.50% of the correctness of classification for the allopolyploid recombinants, the SN group and haploid recombinants were 56.66% and 57.69% respectively, and the Ww group was 88.70%. On other hand, the LDA from cultivated plants showed three LD, but any of them showed clear separation between allopolyploids and haploids (both SN group or Recombinant group). Finally the CDA highlighted similar results for allopolyploid recombinants, SN group and Ww group (37.50%, 56.25% and 85.71%, respectively), but haploid recombinants presented minor correctness of classification (22.22%).

DISCUSSION

A central challenge in taxonomy is to identify morphological variants that distinguish separate species but remain homogeneous among groups of interbreeding populations. This is particularly difficult in widespread species with distributions that span multiple environmental gradients. Our most important finding is that a higher proportion of morphological characters in *Ceratodon* varies stronger due to environmental factors than to genetic factors compared to vice versa. This was observed for the three cytotypes and for the genetic groups found previously (SN group, Ww group and Recombinant group). Furthermore, the genetic factor has a very important role, too. We found that in spite of the great variability in phyllidia and sporophytes within the Ww

Table III.5. Statistical results of qualitative and quantitative morphological characters of recombinants samples included in the biometric study. Character abbreviations follow those given in Table III.1. The field-collected plants and *in vitro* cultivated plants were subdivided according to the cytotypes obtained from flow cytometry analyses (Nieto-Lugilde et al., 2018a). Number (*n*) of specimens examined for each group is given. Descriptive statistics (mean ± SD [range]) for quantitative characters are presented. All measurements are given in µm, except for CL in mm. Significant *p*-values < 0.01 are written in bold after being corrected with Benjamini-Hochberg test; * means 0.01 < *p*-value < 0.05.

	Field collected plants		**Cultivated plants**	
Characters studied	**Haploids (*n* = 9)**	**Diploids (*n* = 4)**	**Haploids (*n* = 3)**	**Diploids (*n* = 4)**
SR	77.77% Acidic	100.00% Acidic	--	--
PP	94.44% Straight	100.00% Straight	100.00% Curved	100.00% Curved
CT	61.11% Present	62.50% Present	100.00% Absent	100.00% Absent
CL	5.58 ± 2.43 [2.94-10.01]	4.16 ± 1.70 [2.26-6.92]	2.00 ± 0.42 [1.52-2.54]	2.36 ± 0.55 [1.60-2.94]
LL	**781.50 ± 210.22 [400.00-1350.00]**	**581.00 ± 121.69 [354.60-830.00]**	514.50 ± 127.19 [322.40-800.00]	569.40 ± 135.96 [370.80-846.30]
LLW	**621.30 ± 210.90 [270.00-1200.00]**	**457.50 ± 112.41 [241.80-700.00]**	395.40 ± 111.32 [241.80-700.00]	444.70 ± 123.19 [225.70-685.10]
LW	**401.90 ± 94.00 [230.00-710.00]**	**321.90 ± 86.36 [193.40-510.00]**	215.50 ± 34.07 [137.00-270.00] *****	247.00 ± 55.07 [153.10-346.60] *****
AT	100% Absent	100% Absent	70.00% Absent: 30.00% Dentate	55.00% Absent: 45.00% Dentate
PM	**98.88% (2): 1.11% (0)**	**95.00% (0): 2.50% (1): 2.50% (2)**	46.67% (2): 40.00% (0): 13.33% (1)	40.00% (0): 32.50% (2): 27.50% (1)
PA	94.44% Acute	95.00% Acute	100.00% Acute	100.00% Acute
EN	92.22% Present	95.00% Present	50.00% Present	75.00% Present
ENL	**185.50 ± 106.16 [40.00-600.00]**	**121.10 ± 65.13 [40.00-310.00]**	**107.48 ± 57.72 [16.12-225.68]**	**48.09 ± 26.64 [24.18-128.96]**
NW	**69.30 ± 16.05 [25.00-135.00]**	**55.99 ± 15.70 [30.00-85.00]**	46.27 ± 13.70 [25.00-74.00]	52.85 ± 11.83 [30.00-90.00]
NC	86.66% Greenish	80.00% Greenish	**53.33% Reddish**	**87.50% Greenish**
CS	98.88% Quadrate	100% Quadrate	100% Quadrate	100% Quadrate
LL/LLW	1.29 ± 0.18 [1.00-2.00]	1.29 ± 0.12 [1.10-1.57]	1.32 ± 0.14 [1.09-1.67]	1.30 ± 0.13 [1.07-1.66]
LL/LW	1.96 ± 0.40 [1.25-2.93]	1.88 ± 0.49 [1.34-3.18]	2.41 ± 0.58 [1.55-4.00]	2.36 ± 0.60 [1.39-3.86]
LL/NW	11.51 ± 2.76 [5.33-21.25]	10.77 ± 2.15 [6.26-15.52]	11.98 ± 4.32 [6.33-22.00]	11.05 ± 2.54 [6.24-15.58]
ENL/LL	0.24 ± 0.13 [0.05-0.60]	0.20 ± 0.09 [0.05-0.54]	**0.22 ± 0.13 [0.03-0.46]**	**0.09 ± 0.04 [0.04-0.17]**
NW/LW	0.17 ± 0.03 [0.10-0.28]	0.17 ± 0.03 [0.12-0.26]	0.22 ± 0.06 [0.10-0.35]	0.22 ± 0.05 [0.11-0.32]
CW	**7.88 ± 1.94 [4.00-12.50] ***	**8.84 ± 1.67 [5.00-12.50] ***	9.54 ± 2.37 [6.00-15.00]	9.62 ± 2.69 [6.00-16.00]
CWT	74.07% ≤ 2	70.83% ≤ 2	100.00% ≤ 2	100.00% ≤ 2
NGC	**1.519 ± 1.37 [0.00-5.00] ***	**0.50 ± 0.88 [0.00-2.00] ***	0.00 ± 0.00 [0.00-0.00]	0.00 ± 0.00 [0.00-0.00]

group of *C. purpureus*, as well as the demonstrable importance of environmentally induced variation in some characters, other morphological traits allowed us to clearly distinguish the Ww group from the SN group and Recombinant group. Similarly, in agreement with Burley and Pritchard (1990), we could not separate *C. heterophyllus* (CBFS 6162 sample) from members of the Ww group of *C. purpureus* by morphological analysis of gametophytic characters, nor could we morphologically differentiate (with gametophytic characters) the African samples (BOL 46302 and BOL 46303) from the Ww genetic group (although these samples presented sporophytes typical of *C. purpureus* subsp. *stenocarpus*). Together these data highlight the complexity of the morphological variation within the Ww group of *C. purpureus*.

The fact that some morphological characters were influenced strongly by environmental factors are is shown in the SN and Ww groups by the gametophytic characters PP, CT, CL, AT, PM, PA, ENL, CS, LL/LLW and NW/LW in field-collected plants, which were clearly different, but these differences disappeared when comparison was made in culture plants. This is not surprising because the field-collected plants studied here grew in locations with different climates (mainly from Mediterranean mountains, but also from Alpine mountains and Atlantic lowlands). Nevertheless, these data suggest that such characters should be used with caution when delimiting species based on morphology.

However, some characters differing between species appear to be regulated by genetic factors independent of the environment, such as NW, LL/LW and ENL/LL because they vary the same way both in field and in cultures. An additional factor that cannot be discounted is that variation observed under controlled growth conditions would not be expressed in the field, due to G × E. Our data show a high proportion of characters clearly influenced by this interaction. Some studies performed a reciprocal transplant experiment (Såstad, 1999; Såstad et al., 1999; Hassel et al., 2005; Yousefi et al., 2017) to advise on the adaptive capacity of morphological characters. Ecological gradients have been studied extensively in the moss genus *Sphagnum* L. species from the same natural area, where variations in pH or water level are easily observable (Såstad, 1999; Såstad et al., 1999). In the case of *Ceratodon*, these key ecological gradients have not yet been detected, but the genus seems to have a widespread ecological distribution. We should caution that relying exclusively on cultivated plants

could lead to an under-estimation of the range of variation that could be displayed along an environmental gradient (Såstad, 1999). Whereas characters that do not differ much between field-collected plants and cultivated plants may be useful for identification, characters that are expressed in the specific natural habitats may be even more indicative (shown by the fact that the field collected plants were more frequently placed in the correct genotype group by the CDA). Such diagnostic characters may not be expressed under axenic culture (Anderson et al., 1992).

These data show that plants of the Ww group can be distinguished morphologically from the SN group and Recombinant group samples using careful, multivariate biometrical study of caulidia and phyllidia, although the differences are quantitative rather than qualitative: longer caulidia without a comal tuft, phyllidia straight or curved when moist, with margins sometimes dentate, acute apex, wider nerve at base of lamina, excurrent nerve length/lamina length ratio smaller, and nerve width/lamina width ratio bigger are characteristic of *C. purpureus*. On the contrary, plants of the SN group and Recombinant group samples often present shorter caulidia, sometimes with a comal tuft, phyllidia straight when moist, with margins mostly entire, apex generally acute but sometimes obtuse, narrower nerve at base of lamina, excurrent nerve length/lamina length ratio bigger, and nerve width/lamina width ratio smaller. The Ww and SN groups are also clearly distinguishable by flow cytometry (Nieto-Lugilde et al., 2018a), as the Ww group had a DNA mean content of 0.37 pg versus 0.46 pg in the SN group.

Based on our morphological results in field-collected samples it was not possible to distinguish clearly between the SN group and Recombinant group. The recombinants did manifest a higher percentage of phyllidia showing excurrent nerves, and the average length of the awn was greater than in the pure SN genotype, although these differences were based on statistical tests, the distributions between the species are broadly overlapping. Depending upon the genetic architecture of the group differences, recombinants may be morphologically intermediate between the two parental species or virtually indistinguishable from one of the parental species. The few available studies reveal that the viable recombinant progeny tend to resemble one of the parental species (bibliographic review in bryophytes by Natcheva & Cronberg, 2004), probably because incompatibility between the genomes makes complete mixtures unviable (Cronberg &

Natcheva, 2002; Natcheva & Cronberg, 2007, McDaniel et al., 2007, 2008). We also observed that the Recombinant group had two different cytotypes (Nieto-Lugilde et al., 2018a), one the size of the SN species (0.46 pg) and another the sum of the genome sizes of the SN species and *C. purpureus* (0.82 pg). We observed no clear morphological differences between haploid recombinants and allopolyploids, although we cannot rule out that it this is due to the low number (four) of putative allopolyploids studied. However, no observable morphological differences are rarely recorded between haploids and diploids of bryophyte taxa in nature (Anderson, 1980; Chopra, 1998; Ricca et al., 2008).

It is possible that the lack of males in our sample of SN individuals could elevate the morphological and genetic differences between the Ww and SN groups. Since *C. purpureus* is strongly sexually dimorphic (Shaw & Beer, 1999; McDaniel, 2005; Slate et al., 2017), the trait distributions for the SN group could be narrower and shifted toward the female value. While different sex ratios in the samples from the SN and Ww groups may play a role, we do not believe that sexual dimorphism can explain the strong differences that we found between these groups for two main reasons. First, we could not morphologically distinguish between the SN group, in which we detected no males nor any signs of sexual reproduction, and recombinants, which has sporophytes and genetic signatures of recombination, indicating that males must be present. Thus, the recombinant males are likely to be similar to the SN females. Additionally, in the Ww group the male and female trait distributions are statistically different but broadly overlapping (Shaw & Beer, 1999; McDaniel, 2005). Slate et al. (2017) observed higher levels of variation among females within and among populations than among males. Therefore, we conclude that the morphological gaps observed between species in our data are greater than those that may exist between males and females within the same species.

These morphological analyses, combined with DNA sequence and genome size data, indicate that two clearly separated lineages exist in the studied samples of *Ceratodon*. Moreover, we find evidence for hybridization signals between them. One lineage was only found in southern Spanish mountains, mainly in Sierra Nevada. The other one has a worldwide distribution. The SN genotype is very abundant locally, but seems to decline rapidly in frequency northwards in the Iberian Peninsula, as it has not

been found in Spanish Sistema Central or even in sites with similar Mediterranean climatic conditions, like the Sicilian Mount Etna, which is situated at almost the same latitude and has a very similar altitude as Sierra Nevada. We think that the potential distribution of the SN genotype in southern Spain is broader than presently known, as many other recorded samples from neighboring areas have been previously reported as *C. purpureus* or *C. conicus* (Martínez Sánchez et al., 1991; García-Zamora et al., 1998; Cano et al., 2010; Rams et al., 2014), but show the morphology of SN and Recombinant group samples, as observed after revision of MUB and GDA/GDAC (Herbarium of the University of Granada, Spain) herbaria samples. Outside southern Spanish mountains, only Ww genotype and recombinants were found. Therefore, if the SN genotype is present outside southeastern Spain, it is probably rare and seems to be less frequent than recombinants, at least in central Europe where the type of *C. conicus* comes from.

Our morphological multivariate analyses situated the lectotype of *C. conicus* together with the recombinant samples including two samples identified as *C. conicus* from United Kingdom lowlands (MUB 52185 and MUB 52186), where *C. conicus* has been found several times but is not common (Smith, 2004; Porley, 2013; Martin, 2014). We must highlight that *C. conicus* has never been found again in the type locality (Flegessen, Lower Saxony, Germany) (Nieto-Lugilde & al., 2018b), despite of the efforts of multiple many bryologists (Meinunger & Schröder, 2007). Moreover, we identified a sample from the Alps (MUB 49604) that was a recombinant, based on sequence data, with a genome size equivalent to the SN pure genotype (Nieto-Lugilde & al., 2018a), and presented sporophytic and gametophytic characters similar to the lectotype of *Ceratodon conicus* (GOET 011795). The samples identified as *C. conicus* from Europe that we have sequenced are also clearly morphologically similar to the recombinants. The broad distribution of recombinants across Europe suggests that they originate not simply through regional introgression where the two species are in contact. The genotype of the type of *C. conicus* is unknown. Therefore, there will always be a doubt related to its genetic composition. *Ceratodon conicus* is scarce outside of Spain and we suspect that we have not fully examined the variability of the species. Nevertheless, although a higher number of samples with morphology similar to *C. conicus* from outside southeastern Spain would have been desirable, the basic outline of our results seems to be reliable.

The most parsimonious conclusion from these data is that there are three entities: the pure SN genotype represents a one species, the Ww genotype corresponds to another species, and a swarm of recombinants (hybrids) exists. There is no doubt that the Ww genotype is *C. purpureus*. Whether the type of *C. conicus* is a recombinant or a pure SN type is not known at this moment, but the genetic, biogeographic, and reproductive data strongly favor the recombinant status. Therefore, we formally propose that *C. conicus* is a nothospecies, and the parents are the cosmopolitan *C. purpureus* and the SN species. The latter represents a new species of the genus *Ceratodon*, here described, for which we propose the name *Ceratodon amazonum*. We consider that recombinants and the new genotype must not be considered the same species, as the *ICN* (Turland et al., 2018) allows describing nothoespecies based on their morphology. There are some cases of moss species described as hybrids (Williams, 1966; Anderson & Lemmon, 1972; Guerra et al., 1994; Ros et al., 1994; Werner et al., 2014). In our case, the nothospecies (*C. conicus*) and one parent (*C. purpureus*) were first described, and later we have discovered the other parent. Therefore, we consider that the temporal sequence in the description of species or nothospecies should not be taken as an argument for not given giving a name already described to a hybrid. We cannot exclude the possibility that some of the samples identified as pure SN based on our genetic data are indeed recombinants that lack the Ww-type allele at the marker genes we employed in our genetic analysis. In contrast to vascular plants, where the F1 generation of hybrids is long-lived, bryophyte F1 sporophytes are quite ephemeral, meaning that the persistent recombinant gametophytes produced from F1 meiosis will not be heterozygous across the genome but rather will show different patterns of interspecies haplotypes. However, it is well known from other studies that the assignment of individuals to pure species or hybrid lines is not always perfect, especially in homoploid hybrids where backcrosses are possible (Nielsen et al., 2006; Gramlich et al., 2016).

In Sierra Nevada metamorphic and carbonate rocks predominate and soils are very diverse, some of them having acidic and other basic characteristics (Molero Mesa et al., 1992). Most of our Sierra Nevada samples were observed to grow on acid substrates, although some samples were found on basic soils. Burley and Pritchard (1990) and many floras (e.g. Smith, 2004; Frey et al., 2006) described *C. conicus* as strictly calcicolous. Perhaps for this reason previous authors identified samples collected in Sierra Nevada as xeric forms of *C. purpureus* and discarded the presence of

C. conicus in this area (Höhnel, 1895; Rams et al., 2014; Brugués & Ruiz, 2015). Unfortunately, Burley and Pritchard (1990) did not describe the methodology employed to elucidate the type of substrate on which the 18 representative specimens they studied grew (from Canada, England, Norway, Scotland and United States). It cannot be discarded that the substrate of these specimens and maybe others (probably excluded because they do not present sporophytes) would have given an acid reaction according to the method applied by us. The recombinant samples found outside Sierra Nevada grew mainly on basic substrate, except MUB49568 from Spanish Sistema Central. Our representatives of *C. conicus* are limited as we have previously stated, but our results indicate that both recombinants and *C. amazonum* sp. nov. can be found on basic and acid substrates. The type of substrate does not seem to be a limiting factor in the distribution of *Ceratodon* species here studied.

TAXONOMIC CONCLUSIONS

Species description

Ceratodon amazonum Nieto-Lugilde, O. Werner, S.F. McDaniel & Ros, **sp. nov.**

Holotype: SPAIN, Granada province, Sierra Nevada, ascent to El Dornajo by the road A4025, km 3, 37°07'46.7"N, 03°25'34.9"W, bare soil between spiny pillow bushes, 1850 m a.s.l. (sample 6/16), *R.M. Ros & O. Werner s.n.*, 21/07/2012 (MUB 49413; isotype: FLAS B66910).

See Fig. III.5 for images of the holotype and other specimens, and Fig. III.6 for images of habitats and turfs of the species in Sierra Nevada.

Specific diagnosis

Plants in compact turfs. Caulidia 2.05-6.76(9.45) mm high. Phyllidia sometimes forming a comal tuft, phyllidia of the middle part of caulidia plane or concave, ovate or longly ovate, rarely lanceolate, (0.35)0.43-0.76(1.15) x (0.20)0.30-0.46(0.56); nerves (25.0)46.8-86.7(114.0) µm wide at base of lamina, generally excurrent in a smooth awn,

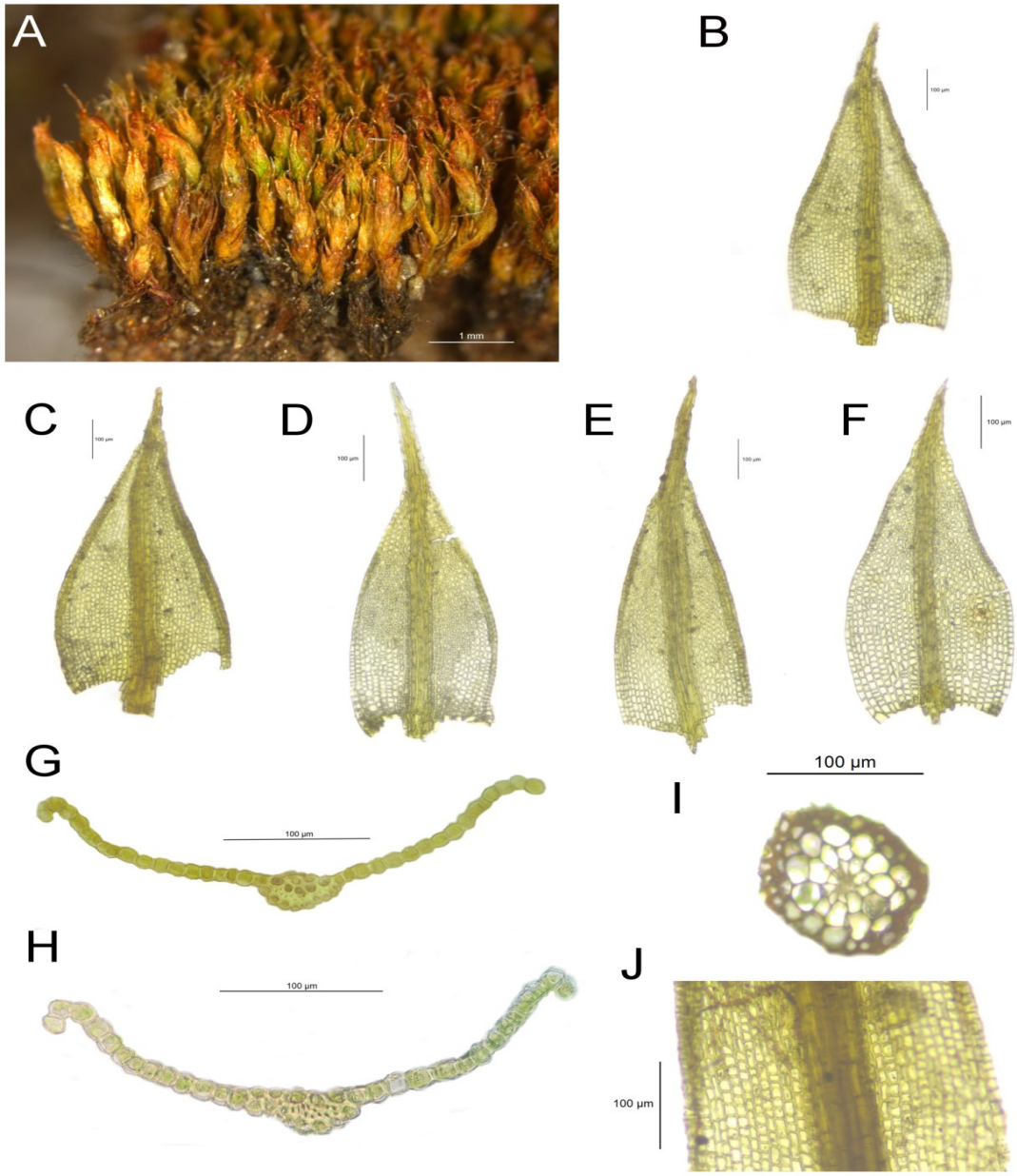


Fig. III.5. *Ceratodon amazonum*. A, habit; B-F, phyllidia of the middle part of caulidium; G-H, middle phyllidia cross-sections; I, caulidium cross-section; J, middle laminal cells. --A, D, E, F and J, MUB 49413 (holotype); B and C, MUB 49427; G, MUB 49306; H and I, MUB 43730. Photos by Marta Nieto Lugilde.

(40.0)60.0-198.0(322.0) µm long, excurrent nerve length/lamina length ratio between (0.05)0.10-0.34 (0.74), rarely percurrent, lamina length/nerve width ratio (5.4)7.0-12.0(18.0); middle phyllidia cross-section crescent-shaped, with 0-3 guide cells. Propagules absent. Gametangia and sporophyte unknown. It differs from *C. purpureus* by having shorter caulidia, comal tuft often present, phyllidia of the middle part of

caulidia mostly ovate or longly ovate, nerve narrower at base of lamina, and generally excurrent in a relatively long awn.

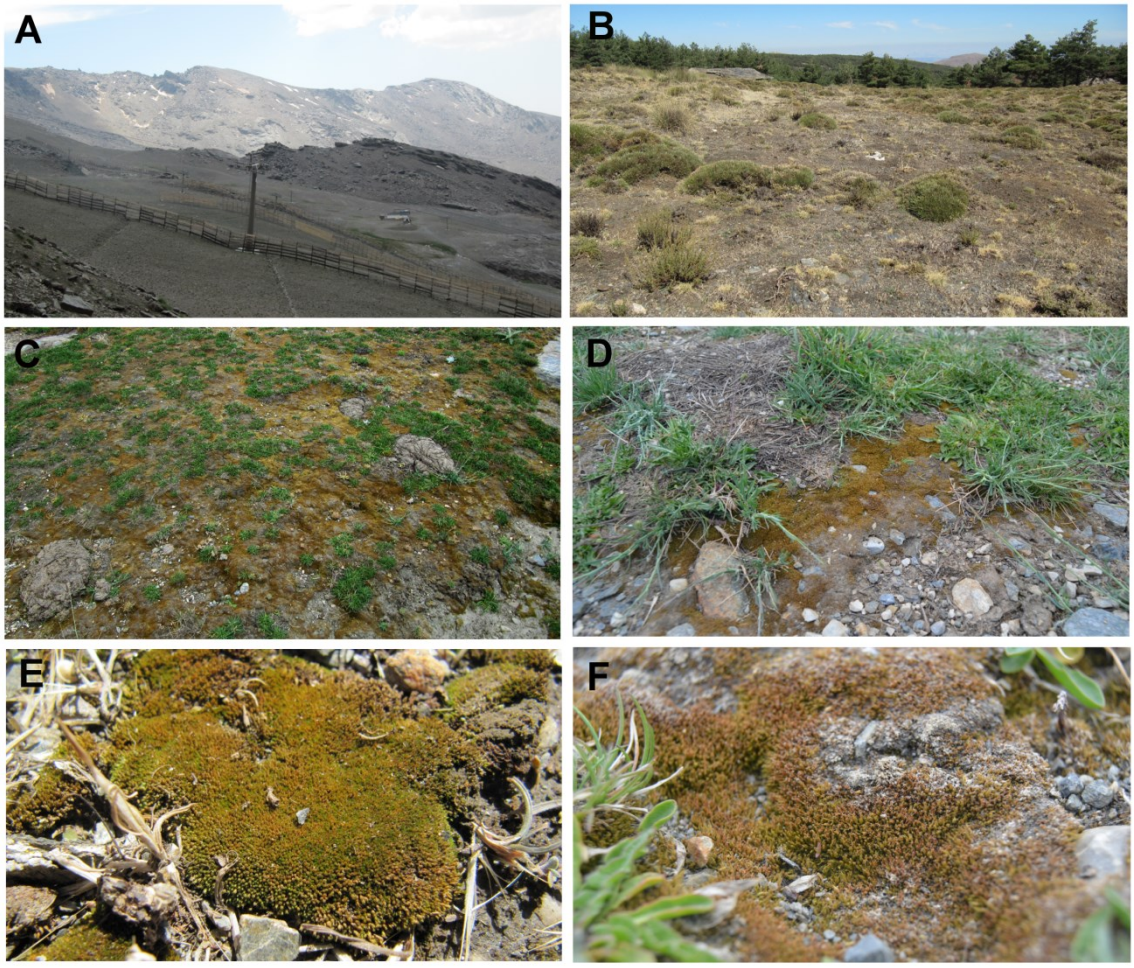


Fig. III.6. Habitats and turfs of *Ceratodon amazonum* in Sierra Nevada. A, Ski resort "Borreguiles", at 2 690 m a.s.l.; B, El Dornajo, at 1 850 m a.s.l. C-D, turfs on bare soil; E-F, details of turfs. Photos A, B, C and E by Rosa M. Ros Espín; D and F by Marta Nieto Lugilde.

Description

Plants in compact turfs, usually yellow-green or yellowish. **Caulidia** 2.05-6.76(-9.45) mm, often branched, 110-160 µm diameter in cross-section, with central strand 15-25 µm wide, central cylinder and cortex with 1(2) layers of cells sometimes differentiated as sclerodermis. **Rhizoids** orange, smooth or slightly papillose. **Phyllidia** straight to slightly twisted when dry, sometimes forming a comal tuft, erecto-patent to spreading when wet, phyllidia of the middle part of caulidia plane or concave, ovate or longly

ovate, rarely lanceolate, (0.35)0.43-0.76(1.15) x (0.20)0.30-0.46(0.56) mm; margins recurved to near apex, rarely plane, usually entire; apices mostly acute, rarely obtuse; **nerves** mostly greenish, rarely reddish, (25.0)46.8-86.7(114.0) µm wide at base of lamina, generally excurrent in a smooth awn, (40.0)60.0-198.0(322.0) µm long, excurrent nerve length/lamina length ratio between (0.05)0.10-0.34(0.74), rarely percurrent, lamina length/nerve width ratio (5.4)7.0-12.0(18.0), superficial cells elongate ventrally, rectangular dorsally; middle phyllidia cross-section crescent-shaped, with 0-3 guide cells, stereid dorsal band well developed, ventral stereid band absent, and ventral epidermal cells well developed, often so big as the guide cells; **middle laminal cells** quadrate, rarely isodiametric, smooth, (4.0)6.2-10.2(15.0) µm wide, cell walls rarely thicker than 2 µm. Propagules absent. Gametangia and sporophyte unknown.

Distribution

Southern Spain: Sierra Nevada Mountains (Andalusia) and mountains in NW of Murcia Region.

Habitat

Acid or basic soils, very frequently without plant cover in open areas, sometimes in more developed soils and somehow protected under herbaceous plants or small shrubs, at 1290-2870 m a.s.l. altitude.

Etymology

The specific epithet refers to the Amazons, name given in Greek mythology to a tribe formed and governed entirely by women warriors, given the absence of males in the present known populations of the species.

Paratypes

SPAIN, Murcia province, mountains in the NW, El Sabinar, 38°13'41.2" N, 02°02'46.6" W, 1290 m a.s.l., *R.M. Ros & O. Werner s.n.*, 13/11/2011 (MUB 43730). Granada province, Sierra Nevada Mountains, ski resort "Borreguiles", 37°04'21.3"N, 03°23'06.5"W, 2690 m a.s.l., *R.M. Ros & O. Werner s.n.*, 20/07/2012 (MUB 49306). *Ibidem*, down to Barranco de San Juan, 37°05'12.4"N, 03°22'44.4"W, 2540 m a.s.l., *R.*

M. Ros & O. Werner s.n., 20/07/2012 (MUB 49327 and MUB 49329). *Ibidem*, Hoya de la Mora, 37°05'36.7"N, 03°23'11.3"W, 2510 m a.s.l., *R.M. Ros & O. Werner s.n.*, 20/07/2012 (MUB 49342). *Ibidem*, "Albergue militar de montaña General Oñate", 37°06'47.9"N, 03°25'10.0"W, 2205 m a.s.l., *R.M. Ros & O. Werner s.n.*, 20/07/2012 (MUB 49356). *Ibidem*, "Centro de visitantes El Dornajo", 37°07'57.9"N, 03°26'06.2"W, 1667 m a.s.l., *R.M. Ros & O. Werner s.n.*, 21/07/2012 (MUB 49366 and MUB 49370). *Ibidem*, road GR-3200 near to "Casas Rurales Telecabina Las Catifas", 37°08'24.8"N, 03°25'34.4"W, 1539 m a.s.l., *R.M. Ros & O. Werner s.n.*, 21/07/2012 (MUB 49382). *Ibidem*, road GR-3200, 1 km after the junction with A4025 going from "Centro de visitantes El Dornajo", 37°08'44.8"N, 03°25'30.2"W, 1431 m a.s.l., *R.M. Ros & O. Werner s.n.*, 21/07/2012 (MUB 49399). *Ibidem*, ascent to El Dornajo by the road A4025, km 3, 37°07'46.7"N, 03°25'34.9"W, 1850 m a.s.l., *R.M. Ros & O. Werner s.n.*, 21/07/2012 (MUB 49408). *Ibidem*, ascent to El Dornajo by the road A4025, km 5, 37°07'09.1"N, 03°26'23.4"W, 2020 m a.s.l., *R.M. Ros & O. Werner s.n.*, 21/07/2012 (MUB 49426 and MUB 49427). *Ibidem*, ascent to El Dornajo by the road A4025, km 7, 37°07'28.8"N, 03°25'52.2"W, 2093 m a.s.l., *R.M. Ros & O. Werner s.n.*, 21/07/2012 (MUB 49442).

Key to species

In order to facilitate the distinction of *C. amazonum* from *C. purpureus* a key is presented based on morphological characteristics of field plants studied in this work. As in many individuals it is impossible to assign them to either *C. amazonum* or the nothospecies *Ceratodon* ×*conicus* based on morphological data, they are keyed together. Nevertheless, based on results of the statistical analyses of the biometric study presented here, the most easily observable differences that allow identifying samples at the extremes of the variability are indicated for each of them.

1. Caulidia length (2.8)5.1-16.2(29.0) mm, comal tuft usually absent; phyllidia of the middle part of caulidia ovate, ovate-lanceolate, lanceolate or triangular-lanceolate, (0.4)0.8-1.5(2.6) mm long; lamina length/lamina width in widest part ratio (1.06)2.13-3.79 (5.83); nerve (38.0)61.8-96.5(140.0) µm wide at base of lamina, percurrent or excurrent in a smooth awn 24-350 µm long; excurrent nerve length/lamina length ratio (0.02)0.04-0.14 (0.30); lamina length/nerve width ratio (5.8)10.5-18.5(34.0)..……..……...……..……***Ceratodon purpureus***

1. Caulidia length (2.0)2.5-7.1(10.0) mm, comal tuft usually present; phyllidia of the middle part of caulidia ovate or longly ovate, rarely lanceolate, 0.4-0.9(1.4) mm long; lamina length/lamina width in widest part ratio (0.85)1.26-2.33(3.18); nerves (25.0)46.8-86.7(140.0) µm wide at base of lamina, rarely percurrent, usually excurrent in a smooth awn 40-600 µm long; excurrent nerve length/lamina length ratio between (0.05)0.11-0.34(0.74); lamina length/nerve width ratio (5.3)7.0-13.6(21.25).....……**2**

2. Phyllidia of the middle part of caulidia ovate or longly ovate, rarely lanceolate; lamina length/lamina width in widest part ratio (0.85)1.26-1.90 (2.78); nerve (25.0)46.8-86.7(114.0) µm wide at base of lamina, generally excurrent in a smooth awn, 40-322 µm long; lamina length/nerve width ratio (5.4)7.0-12.0(18.0)…….....……...…...***Ceratodon amazonum***

2. Phyllidia of the middle part of caulidia ovate, longly ovate or ovate- lanceolate; lamina length/lamina width in widest part ratio (1.07)1.49-2.33 (3.18); nerve (25.0)48.3-86.7(140.0) µm wide at base of lamina, generally excurrent in a smooth awn, 40-600 µm long; lamina length/nerve width ratio (5.3)8.3-13.6(21.25) …….……...……..…….…....***Ceratodon ×conicus***

Typification of the name *Ceratodon ×conicus*

Ceratodon ×conicus (Hampe *ex* Müll. Hal.) Lindb. (*Ceratodon amazonum* × *Ceratodon purpureus*) in *Musci Scand.* 27. 1879 (non *Ceratodon conicus* (Lindb.) Müll. Hal. in *Hedwigia* 38: 98. 1899, *hom. illeg.*). **Basionym:** *Trichostomum conicum* Hampe ex Müll. Hal. in *Syn. Musc. Frond.* 1: 575. 1849 ≡ *Ceratodon purpureus* var. *conicus* (Hampe ex Müll. Hal.) Husn. in *Muscol. Gall*. 60. 1884 ≡ *Ceratodon purpureus* subsp. *conicus* (Hampe ex Müll. Hal.) Dixon in *Stud. Handb. Brit. Mosses* 68. 1896. **Lectotype** (designated by Burley & Pritchard, 1990): [Germany, Niedersachsen] Auf Mauern bei Flegesen … (unreadable) Hameln bei Hohnsen, Mai coll. *Schlotheuber* pastor eccl. 784, Hampe misit 15/2 48, C. Müller det. (GOET barcode 011795!). **Epitype here designated**: ITALY, Südtirol, Stilfser Joch, 46°31'43.7"N, 10°27'09.7"E, on accumulated earth at the base of an artificial wall in anthropized area, frequently cover by snow, 2763 m a.s.l., R.M. Ros & O. Werner s.n., 14/09/2012 (MUB 49604!).

More data on the taxonomy and nomenclature of this species are given in Nieto-Lugilde et al. (2018b).

It was considered necessary to select an epitype of *Ceratodon* ×*conicus* to serve as an interpretative type because the lectotype is demonstrably ambiguous with regards to its genotype and amount of nuclear DNA and cannot be critically identified for purposes of the precise application of the name to the nothospecies (Art. 9.9 of *ICN*, Turland et al., 2018). The MUB 49604 sample was designated as epitype as it presents similar morphology and diagnostic characteristics (gametophytic and sporophytic) to those showed by the lectotype of *C.* ×*conicus*, it was sequenced showing a recombinant genotype and its DNA amount was measured, being 0.48 pg.

ACKNOWLEDGEMENTS

The authors thank the curators of herbaria BOL, CBFS, GDA/GDAC, GOET, and S for providing plant material; Laura Forrest from Royal Botanic Garden Edinburgh, United Kingdom, for donating samples; Petr Koutecký for his recommendations and help with statistical analyses; Vicente Mazimpaka for his help with the botanical latin; Juana María González-Mancebo for her assistance in the biometric study; the management group of the National Park of Sierra Nevada Natural Space for allowing us to collect samples in the National and Natural Parks; and two anonymous reviewers and Alain Vanderpoorten (associate editor in charge of the manuscript) for their valuable comments, which helped to improve a first draft of the manuscript. This study was supported financially by the Spanish “Ministerio de Ciencia e Innovación” (Project CGL2011-22936/BOS), by “Ministerio de Economía y Competitividad” (Project CGL2014-52579-R) and co-financed by ERDF of the European Union. M N-L also thanks the Spanish “Ministerio de Economía y Competitividad” for a "Formación de Personal Investigador” fellowship (FPI program 2012; reference BES-2012-056799).

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ANNEXES

Annex III.1. Voucher information of the specimens studied. For each sample, information is given as follows: herbarium code; geographic origin; MC letters in the case that in vitro cultivated plants were included in the biometric study; genomic group based on phylogenetic study of Nieto-Lugilde et al., (2018a); genome size determined by flow cytometry technology if known (H for haploid sample, A for allopolyploid sample) from Nieto-Lugilde et al., (2018a); sex if known (F = female, M = male) from Nieto-Lugilde et al., (2018a); presence of sporophyte if appropriate (indicated by an asterisk). For the type specimen, also collection data are given.

Mediterranean mountain areas

MUB 43730: Spanish south eastern mountains (Eastern Sierra del Segura), SN group, H, F. **MUB 49306:** Spanish Sierra Nevada (hereafter Sierra Nevada), SN group, F. **MUB 49323:** Sierra Nevada, MC, Recombinant group, H, F. **MUB 49327:** Sierra Nevada, SN group, F. **MUB 49329:** Sierra Nevada, MC, SN group, H, F. **MUB 49339:** Sierra Nevada, MC, Recombinant group, A, F. **MUB 49342:** Sierra Nevada, MC, SN group, H, F. **MUB 49356:** Sierra Nevada, SN group, H. **MUB 49366:** Sierra Nevada, MC, SN group, H, F. **MUB 49370:** Sierra Nevada, SN group, H. **MUB 49382:** Sierra Nevada, SN group, H, F. **MUB 49399:** Sierra Nevada, MC, SN group, H, F. **MUB 49408:** Sierra Nevada, MC, SN group, H, F. **MUB 49413:** Sierra Nevada, MC, SN group, H, F. **MUB 49426:** Sierra Nevada, MC, SN group, H, F. **MUB 49427:** Sierra Nevada, MC, SN group, H, F. **MUB 49442:** Sierra Nevada, MC, SN group, H, F. **MUB 49451:** Sierra Nevada, Recombinant group, H, F. **MUB 49461:** Sierra Nevada, MC, Recombinant group, H, F. **MUB 49471:** Sierra Nevada, MC, Ww group, H, M. **MUB 49473:** Sierra Nevada, MC, Recombinant group, A, F. **MUB 49480:** Sierra Nevada, Recombinant group, H, F. **MUB 49485:** Sierra Nevada, MC, Recombinant group, A, F. **MUB 49492:** Sierra Nevada, MC, Recombinant group, H, F. **MUB 49501:** Sierra Nevada, MC, Recombinant group, F. **MUB 49504:** Sierra Nevada, MC, Recombinant group, A, F. **MUB 49505:** Sierra Nevada, Recombinant group, F. **MUB 49518:** Sierra Nevada, Recombinant group, H, F. **MUB 49528:** Sierra Nevada, Recombinant group, H, F. **MUB 49538:** Spanish Sistema Central, MC, Ww group, H, F. **MUB 49545:** Spanish Sistema Central, Ww group, H. **MUB 49554:** Spanish Sistema Central, Ww group, H, F. **MUB 49560:** Spanish Sistema Central, MC, Ww group, H, F*. **MUB 49562:** Spanish Sistema Central, Ww group, H. **MUB 49564:** Spanish Sistema Central, Ww group, H. **MUB 49566:** Spanish Sistema Central, MC, Ww group, H, F. **MUB 49567:** Spanish Sistema Central, Ww group, F. **MUB 49568:** Spanish Sistema Central, MC, Recombinant group, F. **MUB 49569:** Spanish Sistema Central, Ww group, F. **MUB 49570:** Sicilian Mount Etna, Ww group, H, F. **MUB 49593:** Sicilian Mount Etna, Ww group, H, F. **MUB 49600:** Spanish south eastern mountains (Sierra de Alcaraz), MC, Ww group, F*. **MUB 49602:** Spanish south eastern mountains (Sierra de Alcaraz), MC, Ww group, F.

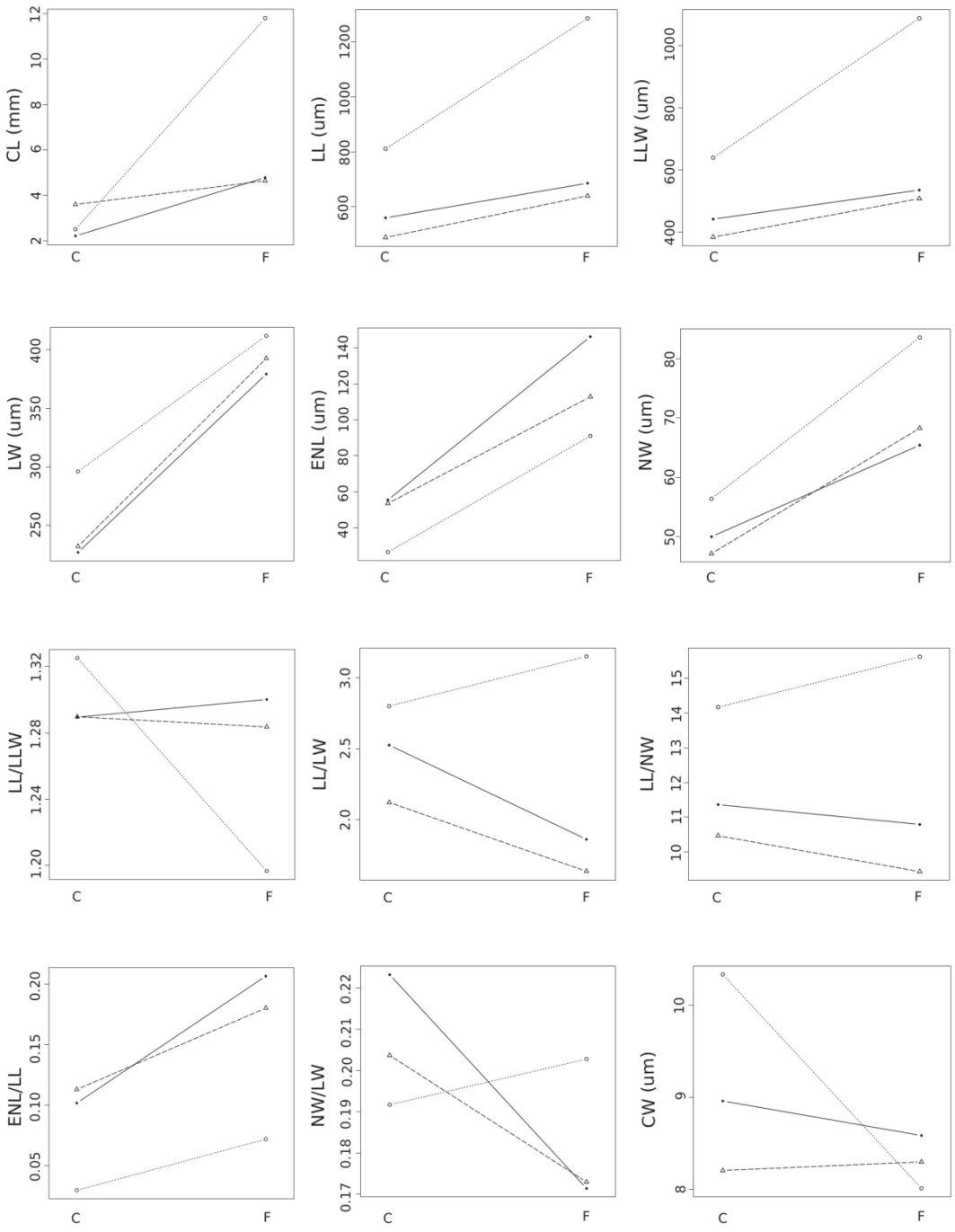
Other European mountainous systems

CBFS 6159: Alps, Ww group. **CBFS 6162:** Alps, Ww group, F. **MUB 49604:** Alps, Recombinant group, H, F*. **MUB 49613:** Alps, MC, Ww group, H, F*. **MUB 49617:** Alps, Ww group, H, F*. **MUB 49619:** Alps, Ww group, H, M. **MUB 49624:** Pyrenees, MC, Ww group, H, F*. **MUB 49629:** Pyrenees, MC, Ww group, F. **MUB 49650:** Pyrenees, MC, Ww group, H, F*.

Lowlands

BOL 46302: South Africa, Ww group, F*. **BOL 46303:** South Africa, Ww group, F*. **GOET 011795:** Germany, *, *Trichostomum conicum* Hampe *ex* Müll. Hal. lectotype, *Schlotheuber* s.n., 15/2/1848. **MUB 49652:** Germany, MC, Ww group, H, F*.**MUB 49653:** Germany, Ww group, H, F*. **MUB 49654:** Czech Republic, MC, Ww group, H, F. **MUB 49655:** Czech Republic, MC, Ww group, H, F. **MUB 49659:** Czech Republic, MC, Ww group, H, F. **MUB 52185:** United Kingdom, Recombinant group. **MUB 52186:** United Kingdom, Recombinant group, H. **S B201182:** Sweden, Ww group, F. **S B201183:** Sweden, Ww group, F.

Annex III.2. Reaction norm plots for morphological characters: caulidium length (CL), lamina length (LL), length of lamina from apex to widest part (LLW), lamina width at widest part (LW), excurrent nerve length (ENL), nerve width at base of lamina (NW), lamina length/length of lamina from apex to widest part ratio (LL/LLW), lamina length/lamina width in widest part ratio (LL/LW), lamina length/nerve width at base of lamina ratio (LL/NW), excurrent nerve length/lamina length ratio (ENL/LL), nerve width at base of lamina/lamina width at widest part ratio (NW/LW), and cells width (CW). Continuous line: Recombinants; dashed line: SN group; and dotted line: Ww group. The environmental growth conditions are indicated from *in vitro* cultures (C) and from field (F).



Annex III.3. Contribution of morphological characters in the six most informative Principal Components (PC) in Principal Component Analysis (PCA) for field collected plants and in vitro cultivated plants. Variables with the highest contributions are written in bold. Character abbreviations follow those given in Table III.1; the characters AT and PM were split in two binary characters: AT d (dentate), AT s (serrate), PM p (plane) and PM r2 (recurved at middle and apical part of lamina); the transformations performed to improve their distribution were: Log_{10} (CL), Log_{10} (LL/LLW), Log_{10} (ENL + 1) and Log_{10} (ENL/LL × 100 + 1).

	Field collected plants						Cultivated plants					
Characters studied	PC1	PC2	PC3	PC4	PC5	PC6	PC1	PC2	PC3	PC4	PC5	PC6
PP	0.5017	0.1484	0.3861	**-0.3525**	**-0.3764**	-0.0546						
CT	-0.3789	-0.3701	-0.0946	0.0332	-0.1705	**0.5699**						
CL	0.6394	0.1261	-0.0567	-0.1791	0.0599	**0.4496**	-0.4752	0.3257	0.0565	0.1121	0.2984	**-0.4486**
LL	**0.9498**	-0.0709	-0.0569	-0.1608	-0.0148	0.1383	**-0.8583**	-0.4764	0.0129	0.0828	0.0042	0.1093
LLW	**0.9535**	-0.0860	-0.0309	-0.1620	-0.0485	0.1558	**-0.9155**	-0.3565	-0.0526	0.0656	0.0236	0.0730
LW	0.2849	**-0.6789**	**-0.4285**	-0.2121	-0.1266	0.1704	-0.1857	**-0.6739**	**0.4854**	0.3155	0.2402	0.0262
AT d	0.3063	**0.4721**	-0.0715	0.0107	**-0.4957**	0.0938	-0.1171	-0.2609	0.2541	0.0822	**-0.6791**	**-0.4333**
AT s							-0.2428	-0.2257	**0.5146**	-0.0075	0.1600	-0.1603
PM p	-0.4014	0.4190	-0.2538	**-0.6457**	0.1816	0.0266	0.5711	-0.1941	-0.0925	**0.5025**	-0.2503	0.2160
PM r2	0.3217	-0.3523	0.2999	**0.6618**	-0.2578	-0.1754	-0.3104	0.2300	**0.4944**	**-0.5385**	-0.0841	-0.1176
PA	-0.3245	0.2508	0.3473	**-0.3732**	-0.1089	-0.1560	0.3234	-0.2887	0.3824	-0.3363	-0.0968	**0.4738**
ENL	-0.2365	**-0.7269**	**0.4176**	**-0.3133**	0.0913	0.0074	-0.4159	**0.6593**	0.4603	0.1530	-0.1222	0.1383
NW	0.6760	**-0.5845**	-0.1382	-0.1153	0.1104	-0.0565	-0.6708	-0.1342	0.2352	**0.5666**	0.1342	0.2245
NC	-0.2276	-0.1469	**0.4548**	-0.3411	**-0.3833**	-0.1633	0.3027	-0.1064	0.3534	**0.4804**	**-0.3199**	-0.2817
CS	-0.5086	0.1305	0.0963	0.1443	**-0.5255**	-0.0051	0.0792	-0.0311	**-0.6195**	**0.4766**	-0.0585	-0.1087
LL/LLW	-0.5988	0.2461	-0.1251	-0.0435	0.2308	-0.1919	0.6462	-0.3540	0.2302	-0.0915	-0.1744	0.2676
LL/LW	**0.8961**	0.2584	0.1742	-0.0397	0.0364	0.0782	**-0.8606**	-0.1237	-0.3450	-0.1053	-0.1482	0.1771
LL/NW	**0.7783**	0.3690	-0.0012	-0.1223	-0.1588	0.2323	-0.5635	-0.5961	-0.2447	-0.3439	-0.0901	-0.0622
NW/LW	0.6301	-0.0284	0.3747	0.1393	0.3296	-0.2805	-0.5295	0.5624	-0.2002	0.2994	-0.1204	0.3221
ENL/LL	-0.4953	**-0.6313**	0.4046	-0.2327	0.0252	-0.0039	-0.1955	**0.7579**	0.4680	0.1296	-0.1499	0.1824
CW	-0.2714	-0.2865	**-0.5134**	-0.1756	-0.2611	-0.2534	0.5738	-0.6352	0.1169	0.1345	0.1060	0.0519
CWT	-0.0352	0.2534	**0.5020**	-0.0182	0.3219	**0.5000**	-0.4494	-0.0834	-0.2666	-0.2109	**-0.4715**	0.0752
NGC	**0.8054**	-0.2646	-0.1169	-0.0891	0.0268	-0.0644	**-0.7037**	-0.3733	0.2506	0.0672	-0.0404	-0.0090

Annex III.4. Correlation of morphological characters studied in each derivate classifier (CCA1 and CCA2) in Linear Discriminant Analysis (LDA) for field collected plants and *in vitro* cultivated plants. Variables with the highest contributions are written in bold. Character abbreviations follow those given in Table III.1; the characters AT and PM were split in two binary characters: AT d (dentate), AT s (serrate), PM p (plane) and PM r2 (recurved at middle and apical part of lamina); the transformations performed to improve their distribution were: Log_{10} (CL), Log_{10} (LL/LLW), Log_{10} (ENL+1) and Log_{10} (ENL/LL*100+1).

	Field collected plans		Cultivated plants	
Characters studied	CCA1	CCA2	CCA1	CCA2
PP	0.1443	-0.0670		
CT	-0.2660	-0.0489		
CL	0.3749	-0.2203	-0.0953	**0.3327**
LLW	**0.4366**	-0.2816	0.2724	-0.0922
LW	0.0342	0.0151	**0.3452**	0.0414
AT d	0.1129	0.0129	0.0989	0.1054
AT s			0.0678	0.0081
PM p	-0.1188	**0.4427**	0.0571	0.0290
PM r2	0.0828	**-0.4496**	-0.1031	0.0552
PA	-0.1297	-0.0820	0.0395	0.1040
ENL	-0.2303	-0.2744	-0.2682	-0.1702
NW	0.2125	-0.0985	0.1511	-0.1117
NC	-0.1258	-0.2128	0.0590	**0.2112**
CS	-0.3048	-0.1390	-0.0043	-0.1498
LL/LLW	-0.1450	0.1448	0.0636	0.0028
LL/LW	**0.4753**	**-0.3926**	0.1455	-0.1808
LL/NW	0.3710	**-0.3557**	**0.3434**	-0.0554
NW/LW	0.2723	-0.2119	-0.1559	**-0.2342**
ENL/LL	**-0.4097**	-0.2577	**-0.3557**	**-0.2099**
CW	-0.1081	0.2625	0.2217	-0.0949
CWT	-0.0359	-0.1174	0.0347	0.1665
NGC	**0.4115**	-0.2240	0.2874	0.0342

Chapter IV. Testing the evolutionary history of *Ceratodon amazonum*, *C. purpureus*, and the recombinant *C.* ×*conicus*

ABSTRACT

The difference in time scale between the evolution or reproductive isolation and changing ecological circumstances means that current species ranges are likely to be imperfect proxies for distributions at the time of speciation. A key challenge, therefore, is to identify alternative sources of information that can provide insights into the speciation process in particular groups. To test the peripatric speciation and reconstructing the demographic and evolutionary history in the cosmopolitan moss *C. purpureus*, the sister species *C. amazonum*, and the recombinant *C. ×conicus*, nine nuclear loci were sequenced. Population genetic summary statistics and phylogenetic analyses were calculated. Moreover demographic parameters (divergence time, effective population sizes and migration rates) were estimated and evolutionary scenarios were simulated. Finally, it was calculated if hybridization between parental taxa occurred in one or several events. *Ceratodon purpureus* almost always had higher levels of genetic diversity than *C. amazonum*, and asymmetric gene flow was found between them. The effective population size of *C. amazonum* was smaller than *C. purpureus*, but estimations were broadly overlapping. Finally, we found clear evidence that *C. ×conicus* was formed by multiple hybridization events between *C. purpureus* and *C. amazonum*, further supporting the role of gene flow in the speciation process in bryophytes.

INTRODUCTION

The evolution of reproductive isolation involves the cessation of gene flow between nascent species (Endler, 1977; Barton & Bengtsson, 1986; Futuyma, 1998). Studies of speciation have frequently used geography as a proxy for gene-flow – allopatric speciation implies a complete lack of gene flow, while peripatric, parapatric, and sympatric speciation imply increasing levels of genetic exchange over the course of the speciation process (Coyne & Orr, 2004). However, it is increasingly apparent that the evolutionary time scale over which speciation occurs is typically longer than the ecological time scale of range expansion or contraction in response to climate change; the difference in time scale between the evolution or reproductive isolation and changing ecological circumstances means that current species ranges are likely to be imperfect proxies for distributions at the time of speciation (Parmesan, 2006). A key challenge, therefore, is to identify alternative sources of information that can provide insights into the speciation process in particular groups.

In principle, genetic data could provide insights into the demography integrated over many generations, potentially providing more direct insights into the interaction between external factors, such as climate and community shifts, and features of the organisms in question. Statistical analysis of present genetic structure and diversity of populations of related species can identify historical vicariance, dispersal history and episodes of expansion and contraction of population size from species using a handful of DNA markers (Feng et al., 2014; Merceron et al., 2017). For speciation an event long ago that involved equal population sizes, we expect that the daughter species should show similar patterns of population genetic variation; however, for peripatric speciation events involving unequal population sizes or population bottlenecks, we expect asymmetric patterns of population genetic variation; gene flow homogenizes allele frequencies among descendant species, and can obscure some of these patterns (Templeton, 1980; Charlesworth et al., 1997). However, recent migration leaves a signiture that is detectable by methods that examine the genealogical relationships among alleles (Rosenberg & Nordborg, 2002). Inferences drawn from an Approximate

Bayesian Computation (ABC) framework may help to understand the demographic history of species (Csilléry et al., 2010; Roux et al., 2011; Leroy et al., 2014).

The use of ABC approaches is particularly valuable in species like bryophytes, where numerous moss species exhibit limited genetic differentiation among widely disjunct populations (Shaw et al., 2003; McDaniel & Shaw, 2005; Désamoré et al., 2016; Lewis et al., 2017). Under these circumstances geographic barriers are particularly poor proxies for species barriers; however, evaluating the demographic history of speciation requires a quantity of genetic data which has been generated for relatively few bryophyte species (Yousefi et al., 2017). One such species is *Ceratodon purpureus* (Hedw.) Brid., a cosmopolitan species that grows on a wide variety of substrates (Crum, 1973). It frequently produces sporophytes and abundant spores that may maintain their viability for up to 16 years (Malta, 1922). A global view of the molecular population genetics of *C. purpureus* was provided by McDaniel and Shaw (2005), confirming that this species can disperse long distances (Muñoz et al., 2004). Moreover this species is considered a promising model for further ecological and genomic studies (McDaniel et al., 2016).

The taxonomy of the genus *Ceratodon* Brid. is complex, largely because many of the historically recognized species within the genus are scarcely distinguishable, morphologically or genetically, from the widespread and polymorphic *C. purpureus* (Burley & Pritchard, 1990; McDaniel & Shaw, 2005). Recently a new species, *C. amazonum* Nieto-Lugilde, O. Werner, S.F. McDaniel & Ros, was described, which is distinct from the cosmopolitan species *C. purpureus* based on a 25% increase in genome size as well as by genetic markers and morphology and a strong female-biased sex ratio (Nieto-Lugilde et al., 2018a, b). These authors observed that *C. amazonum* is locally abundant in Sierra Nevada Mountains in southern Spain and almost totally displaced *C. purpureus* in this region. Based on analyses of these data, Nieto-Lugilde et al. (2018a, b) suggested that *C. amazonum* may have arisen via peripatric speciation. Interestingly, *C. amazonum* was never was found with sporophytes, so sexual reproduction and dispersal by spores currently may be limited or absent. Population genetic theory predicts that small and isolated populations progressively lose genetic diversity as a consequence of genetic drift (Hedrick, 2001), making them less able to adapt to environmental change and diminishing their long-term reproduction and

survival. However, Nieto-Lugilde et al. (2018a, b) also reported evidence that a hybridization event between *C. amazonum* and *C. purpureus* gave rise to the previously recognized species *C.* ×*conicus* (Hampe) Lindb. some of which were diploid, presumably of allopolyploid origin. Populations with high level of hybridization may have a greater chance at long term survival, but not remain distinct species (Wang et al., 1999; Keller & Waller, 2002; Hufbauer et al., 2015; Huisman et al., 2016). Thus, the zone of sympatry between *C. purpureus*; and *C. amazonum* allows to inquire about the demography of speciation in cosmopolitan taxa.

The main goal of the present work was to reconstruct the demographic and evolutionary history of the cosmopolitan moss *C. purpureus,* the sister species *C. amazonum,* and the recombinant *C.* ×*conicus,* based on coalescent analyses of nucleotide polymorphism data. Specifically we asked: i) Did the speciation event between *C. purpureus* and *C. amazonum* involve a population bottleneck, consistent with peripatric speciation? or ii) Did speciation proceed in the presence of ancestral or ongoing gene flow, consistent with parapatric speciation?, and iii) Did the recombinant *C.* ×*conicus* form once or multiple times? We found evidence for asymmetric gene flow between between *C. purpureus* and *C. amazonum*, favoring introgression from the common, cosmopolitan species to the rare southern European species. We estimated the effective population size of *C. amazonum* to be smaller than *C. purpureus*, although the distribution of probable values was broadly overlapping between the two species. These observations suggest that if the event that produced these two daughter species involved a population bottleneck, the effective population size of *C. amazonum* has recovered relatively quickly, potentially as a consequence recurrent of gene flow *C. purpureus*. Finally, we find clear evidence that *C.* ×*conicus* was formed by multiple hybridization events between *C. purpureus* and *C. amazonum,* further supporting the role of gene flow in the speciation process in bryophytes.

MATERIALS AND METHODS

Plant material

A total of 190 specimens of the genus *Ceratodon* were used for this study. Detailed information is listed in the Annex IV.1. We generated genetic data for 56 specimens of *C. amazonum*, 26 of *C.* ×*conicus*, and 51 of *C. purpureus* (of these 34, 17 and 37 respectively were used before, Nieto-Lugilde et al., 2018a, b). We collected 45 of these samples, all of which were deposited at MUB (Herbarium of the University of Murcia, Spain). Nine samples were loaned from herbaria, including BOL (Bolus Herbarium, University of Cape Town, South Africa), CBFS (University of South Bohemia, Czech Republic), and S (Herbarium of the Swedish Museum of Natural History, Sweden), and two samples were donated by Laura Forrest (at Royal Botanic Garden Edinburgh, United Kingdom). Moreover the Genbank accessions for 57 specimens from Antarctica, Australia and North America, previously published by McDaniel et al. (2013b) were downloaded to increase the sampling area of *C. purpureus*. We sequenced four specimens of *Cheilothela chloropus* (Brid.) Lindb. from Spanish Sierra Nevada Mountains, to use as an outgroup (GenBank accession numbers are listed in Annex IV.1). *Ceratodon amazonum* specimens were from Spanish Sierra Nevada Mountains and one from Spanish southeastern mountains. *Ceratodon* ×*conicus* specimens were from Alps, Czech Republic, Sierra Nevada Mountains, Spanish central mountain ranges and United Kingdom. *Ceratodon purpureus* specimens were from Alps, Antarctica, Australia, Czech Republic, Germany, North America, Pyrenees, Sicilian Mount Etna, South Africa, Spanish central mountain ranges, Spanish Sierra Nevada Mountains, Spanish southeastern mountains and Sweden.

DNA sequencing

We sequenced the nuclear ribosomal Internal Transcribed Spacer 2 (ITS2) and eight nuclear exon-primed intron-spanning *loci*, including *hp23.9*, *PPR*, *TBP* (McDaniel et al., 2013a, b; Nieto-Lugilde et al., 2018a), *rpL23A*, *TRc1b3.05* (McDaniel et al., 2013a; Nieto-Lugilde et al., 2018a), *hp23.3*, *KIAA0187* and *rpS18A* (McDaniel et al., 2013a, b). Genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB)

extraction (Doyle & Dickson, 1987; Doyle & Doyle, 1987; Cullings, 1992). We amplified all *loci* from all individuals in 20 μL using Thermo Scientific DreamTaq DNA Polymerase (Thermo Fisher Scientific Inc.), following the PCR conditions employed before by McDaniel et al. (2013b) and Nieto-Lugilde et al. (2018a). In samples where we observed double peaks in the chromatograms, we cloned the *loci* by isolating the PCR products from agarose gels and cloning using the CloneJet PCR Cloning Kit (Thermo Fisher Scientific). We checked cloning efficiency and accuracy using PCR. Unincorporated primers and unincorporated nucleotides were removed using Exo-AP Clean-up reaction. The resulting cleaned PCR products were sequenced on an ABI3730XL DNA Analyzer, Applied Biosystems (Macrogen Europe, The Netherlands, Amsterdam).

Phylogenetic analyses

We followed the methodology employed before by Nieto-Lugilde et al. (2018a). In general, we aligned the DNA sequences, we coded gaps as informative, and we performed phylogenetic analyses using MrBayes v.3.2 (Ronquist et al., 2012). To search for convergence in these phylogenetic analyses we used two runs with different setting for some of the *loci*; for *hp23.9*, *TBP*, *PPR*, *TRc1b3.05* and *rpL23A* we employed the same condition as used in Nieto-Lugilde et al. (2018a); And four chains with 1×10^7 generations were run simultaneously for *hp23.3*, *ITS2*, *KIAA0187* and *rpS18A*, with the temperature of the single heated chain set to the default in MrBayes. The genealogies were rooted with sequences from *Cheilothela chloropus*, when it was possible. The final trees were edited with TreeGraph2 (Stöver & Müller, 2010).

Analysis of divergence and polymorphism

The population genetic summary statistics were calculated for each *locus* using the software DnaSP v5 (Librado & Rozas, 2009). All multiple alignment columns with missing data or gaps were ignored. For this reason, we make estimates using only the complete sequences. All markers included in this study were clustered by genealogies, as *C. amazonum* or *C. purpureus*. We calculated the number of segregating (polymorphic) sites, estimates of proportion of segregating sites (θw, Watterson, 1975), the nucleotide diversity (π, Nei, 1987; Nei & Miller, 1990), Fu's Fs (Fu, 1997), the mutation frequency spectrum statistic Tajima's D (Tajima, 1989a) and Fu & Li's F* (Fu

& Li, 1993) for both species separately in each *locus*. We also tested for evidence of historical recombination (Rm, Hudson & Kaplan, 1985) at each *locus* separately, observing possible recombination events in each species. Moreover the average number of nucleotide substitutions per site (Dxy, Nei, 1987) between *C. amazonum* and *C. purpureus* was calculated.

Analysis of parameters of the isolation–migration model

In order to estimate the parameters of the isolation–migration model between species, we generated molecular demographic parameters, such as historical (N_A) and current effective population sizes for *C. amazonum* and *C. purpureus* (N_{ca} and N_{cp}), rates of gene flow between species (m_{Ca-Cp} and m_{Cp-Ca}) and divergence time (T_{split}), using the program IMa2 (Hey & Nielsen, 2004, 2007; Hey, 2010a, b). This software implements a Markov Chain Monte Carlo (MCMC) search strategy to identify maximum likelihood estimates (MLEs) of demographic parameter values given in the sampled data. The "Isolation with Migration" (IM) model implemented in IMa2 involves several simplifying assumptions. These include no recombination within each *locus*, free recombination among all *loci*, no population structure within each species, no genetic contribution from unsampled populations or species and selective neutrality. The samples were grouped by species excluding specimens of *C. ×conicus*, based on morphological and phylogenetic previous studies (Nieto-Lugilde et al., 2018a, b). We divided the sequences into segments that did not violate the four-gamete test (historical recombination), based on the output from DnaSP. The *locus rpL23A* showed a minimum of ten recombination events, for this reason the *locus* was excluded for the testing. Based on the Akaike information criterion, the Hasegawa-Kishino-Yano mutation model (Hasegawa et al., 1985) was used for all *loci*. Each *locus* was assigned an inheritance scalar, 1.0 for autosome *loci* and 0.5 for sex-linked *loci* (*PPR* and *rpS18A*). We calculated values for the prior distributions according to program documentations (q = 30, t = 12 and m = 0.33), and conducted an initial run of the program to establish parameter maxima where the MLEs reached zero probability, and an appropriate burn-in period. We then conducted three additional runs of more than one million steps (run of 1 119 119 steps for data shown), each with a burn-in of 30 000 steps, a random different seed number, the lowest effective sample size (ESS) among the parameters was at least 100, and checked convergence among the three runs.

Calculation of ancestral effective population size is in the most of cases really complex due to presence of migration between species (Edwards & Beerli, 2000; Tseng et al., 2014). To test the size of ancestral effective population without migration we conducted three additional runs. We assumed that *C. purpureus* produces one sporophyte generation per year, and used rates of 1×10^{-6} neutral mutations per site per generation (μ); however, because we have no bryophyte-based calibration for this molecular clock, we provided the dates only as a rough estimate. All parameters were scaled by μ as follow: $m = M/\mu$, $t = T \times \mu$, $\theta = 2N\mu$, where M represents migration rate per generation per gene copy, T represents divergence time estimated in years, and N represents the effective population size.

Population size fluctuations through time

The Extended Bayesian Skyline Plot (EBSP), a coalescent-based graphical method, was carried out in BEAST v.2.3.2 (Drummond & Rambaut, 2007; Drummond et al., 2012; Bouckaert et al., 2014) for both species independently to infer potential historical fluctuations in effective population size. EBSP analysis was run using recommendations of Joseph Heled (Extended Bayesian Skyline Plot tutorial for BEAST 2), empirical HKY substitution model, specific mode of inheritance for each *locus* and strict clock models. Per species mutation rate (μ) 1×10^{-6} was used. All analyses were run three times to check for convergence with 5×10^{7} generations, and sampling every 5×10^{3} generations. The first 10 % of the genealogies were discarded as burn-in. Convergence, stationarity, effective sample size each parameter of interest and the appropiate burn-in were evaluated using the software TRACER v. 1.6 (Rambaut et al., 2014). We considered a generation time of one year to rescale the uncorrected population size, because it is the time that *C. purpureus* employs in developing the sporophytic generation in its life cycle that reach an age of 2-5 years (During, 1979).

Test of diversification scenarios

To investigate in more detail the divergence history of *C. amazonum* and *C. purpureus*, we used ABC approaches to make inferences with complex models based on molecular data. We carried out a statistical evaluation of a total of 14 models of speciation (Fig. IV.1). All of these models included the subdivision of an ancestral panmictic population into two daughter species (*C. amazonum* and *C. purpureus*) at time T_{split}. The scenarios

were divided into two types: A) the species had independent effective population size that remained constant (homogeneus) over time and, B) heterogeneus effective population size over time. For each one four general models were tested. Three of the scenarios assumed substantial gene flow since T_{split}: isolated with migration (constant migration over time, IM), ancient migration (AM), and secondary contacts (SC).

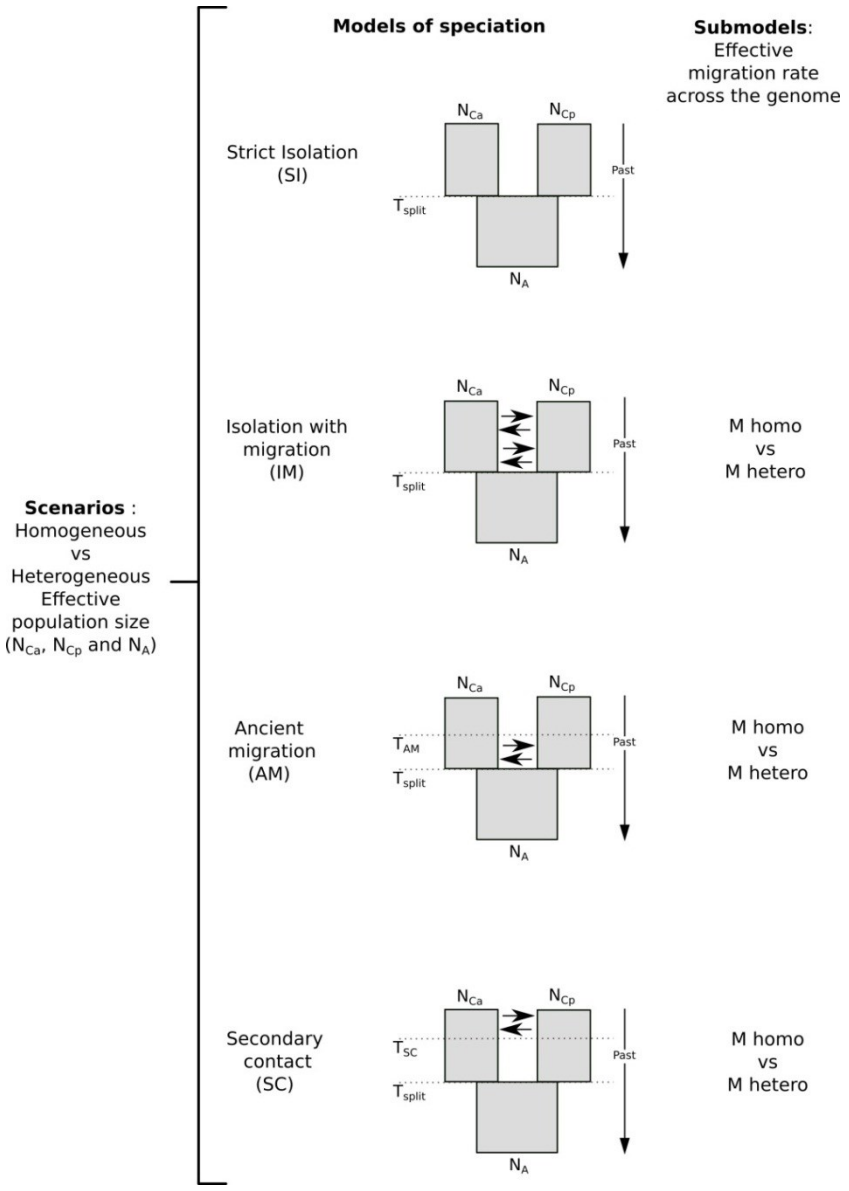


Fig. IV.1. Alternative scenarios and models of speciation for *Ceratodon amazonum* and *C. purpureus*. All models assume an ancestral population of effective population size (N_A) split at divergence time (T_{split}) into two populations of effective population size (N_{ca} and N_{cp}). Two scenarios including homogeneity and heterogeneity of effective population size, each one with four models are compared: SI (strict isolation, no migration), IM (isolation with migration over time), AM (ancient migration assuming that populations started diverging in the presence of gene flow, T_{AM}), and SC (secondary contact in which populations diverged in the absence of gene flow followed by a single period of secondary contact, T_{SC}). For each model including migration (asymmetric migration between both species in all cases) two different submodels are depicted assuming either homogeneity ("homo") or heterogeneity ("hetero") of effective migration rate across the genome.

The other model assumed strict isolation (SI), without migration. Each scenario was tested assuming homo- and heterogeneus migration over the genome but asymmetric between them. Following Roux et al. (2016) homogeneous effective population size scenarios are assumed, which means that most of the genetic variation in the genome is unaffected by selection at linked sites. For this reason a single value of effective population size shared by all *loci* across the genome was used, but effective population size differed among populations. On the contrary, heterogeneous effective population size scenarios account for local genomic effects of directional selection (background selection, selective sweeps) by considering a variable effective population size among *loci*. Submodels with homogeneous migration rate assume that all *loci* share the same probability to receive alleles from the sister species. Alternatively, the heterogeneous migration rates submodels account for the existence of local barriers to gene flow, of variable strengths, and of variable levels of genetic linkage to the sampled *loci* (more data see Roux et al., 2016). We simulated ten million multilocus data sets with msnsam, a modified version of Hudson's ms program allowing variability of sample size across *loci* (Hudson, 2002; Ross-Ibarra et al., 2008). Each data set was composed of 21 fragments of *loci* fitting the length and sampling size of the 21 fragments of observed *loci* (employed for IMa2). Simulations were performed with random priors drawn from a modified version of priorgen (Ros-Ibarra et al., 2008; Leroy et al., 2017). We used the following summary statistics for all simulations: nucleotide diversity (π_1 and π_2, Tajima, 1983) and Tajima's D (D_1 and D_2, Tajima, 1989b, c) for each population, and the F_{ST} between populations. The average and standard deviation across the *loci* of these statistics were calculated with the program MScalc (Ross-Ibarra et al., 2008, 2009; Roux et al., 2011; Leroy et al., 2017). Large uniform prior distributions were used for all parameters common to all scenarios, based on previous information given for IMa2 analysis. Prior θ_{Ca}/θ_{ref} and θ_{Cp}/θ_{ref} distributions were uniform at intervals 0-10, with $\theta_{ref}= 2N_{ref}\mu$, N_{ref} is the number of effective individuals of the reference population, arbitrarily set at 100 000, and μ is the mutation rate of 1×10^{-6}/bp/generation, as we commented before. The prior distribution for $\theta_{anc}/\theta_{ref}$ was uniform over the 0-25 interval. The $T_{split}/(2N_{ref})$ ratio was sampled from the interval 0-20 generations, conditioning the parameters T_{AM} and T_{SC} to be uniformly chosen within the $0\text{-}T_{split}$ interval. For speciation scenarios assuming homogeneous migration of *loci*, all *loci* moving in a given direction had the same effective migration rate, which was independent of that for

migration in the opposite direction. M_{ca} (= $2N_{Ca}m_{Cp\text{-}Ca}$) and M_{cp} (= $2N_{Cp}m_{Ca\text{-}Cp}$) were sampled from uniform distributions (0-0.5), where $m_{Cp\text{-}Ca}$ and $m_{Ca\text{-}Cp}$ are the proportions of migrants from *C. purpureus* in *C. amazonum* and migrants from *C. amazonum* in *C. purpureus*, respectively. There are two important genomic features known to bias demographic inferences: genomic heterogeneities in effective migration rates and effective population sizes. For scenarios assuming genomic heterogeneity in effective migration rates, *locus*-specific effective migration rates were randomly sampled from a beta distribution shaped by parameters "shape1" and "shape2" (0-20, and 0-200 respectively). We statistically evaluated alternative speciation models by a hierarchical procedure (Fagundes et al., 2007; Oliveira et al., 2015). First, for each model we evaluated the posterior probabilities of the two migration alternative "Homo" and "Hetero" versions. Second, we separately compared the best version between the two effective population size alternative "Homo" and "Hetero" versions. Finally the best supported versions of the four scenarios were evaluated. Posterior probabilities for each scenario were estimated with a neural network under the ABC framework, in the R package "abc" (Csilléry et al., 2012). The 10 000 replicate simulations closest to the observed values of the summary statistics were selected for the two first comparison and for the last one we used 200 000 replicate simulations. Finally, we checked simulated models fit to the observed data, plotting the histogram of the null distribution under different models on which we superimpose the observed value.

Analysis of recombinant specimens origin

The hypothesis of a monophyletic origin of *C.* ×*conicus* was explicitly tested by contrasting the likelihood of two competing topologies, one that resulted from the unconstrained analyses, and one produced under the constraint that all recombinant specimens are included within the same clade, following the approach described in Bergsten et al. (2013). Model marginal likelihoods were estimated with the stepping-stone method (Xie et al., 2011) as implemented in MrBayes 3.2 (Ronquist et al., 2012) with the following settings: ngen = 25 000 000; diagnfreg = 250 000. A log diference above five is considered very strong evidence (Kass & Raftery, 1995).

RESULTS

Cloning DNA sequences

Cloning of *loci* confirmed that some specimens presented two different copies of the same *loci* in most cases. Only from few samples we obtained cloned sequences of *KIAA0187*. The *ITS2* together with *TRc1b3.05*, *PPR*, and *rpL23A* presented predominantly a single copy, although some individuals presented the two copies in other *loci*. These results are similar to those reported before (Nieto-Lugilde et al., 2018a). Finally we employed for this study a total of 1632 accessions of genus *Ceratodon* (Annex IV.1).

Phylogenetic analyses

For *locus ITS2* two clades were resolved 1.00-0.92 pp (Annex IV.2), but sequences of *Cheilothela chloropus* were not obtained for using as outgroup. In the case of *hp23.3* the sequences of *C. chloropus* were identical with one clade of *Ceratodon*, for this reason it was not useful to root the tree, but the other clade was well differentiated 1.00 pp (Annex IV.3). The *loci rpS18A* (Annex IV.4) and *KIAA0187* (Annex IV.5) showed one well supported clade 0.99 pp and 1.00 pp respectively, and all remaining samples with deeper coalescent events. The remaining *loci* showed similar results according with those obtained in Nieto-Lugilde et al. (2018a) (data not shown). One of these clades corresponded with the socalled SN clade in Nieto-Lugilde et al. (2018a), formed always mainly by Sierra Nevada Mountains samples but also by Spanish southeastern mountains. The second clade consistently included specimens coming from the rest of the sampled areas, including Sierra Nevada and Spanish southeastern mountains (named Ww clade in Nieto-Lugilde et al., 2018a). Some samples were resolved in either the SN clade or the Ww clade, depending on the studied *locus*, they were considered as recombinants.

Genetic diversity

The *TBP* and *hp23.3* alignments did not present indels. No indels larger than one and four bases were evident for *PPR* and *ITS2* alignments respectively. The *rpL23A*

alignments showed six bases indel for *C. amazonum* while some sequences of *C. purpureus* had an insertion of seven bases. Samples of *C. amazonum* had an indel (GTGT) for the *hp23.9* alignment; and for *KIAA0187* alignment, the samples of *C. purpureus* presented an indel of ten bases. And finally the *rpS18A* and *TRc1B3.05* alignment showed indels of 30 and eight bases, respectively, but they were not useful to discriminate between species. Several standard summary statistics (Table IV.1) were calculated to understand the population genetic processes shaping variation on the two species. The sex-linked *loci* (*rpS18A* and *PPR*) exhibited lower values of segregating sites compared with most of the autosomal *loci*, but two autosomal *loci* (*hp23.9* and *TBP*) had similar low values. *Ceratodon purpureus*, with values ranging from nine (*TRc1B3.05*) to 60 (*rpL23A*), was higher than *C. amazonum*, with values ranging from one (*PPR*) to 30 (*rpL23A*). The exception was the *TRc1B3.05 locus* in which the value of segregating sites was larger in *C. amazonum* (22) than in *C. purpureus* (nine). The levels of Watterson estimation (θ_w) in *C. purpureus* were higher than that in *C. amazonum*. The mean value was 0.0071 for *C. amazonum*, 0.0007 (*PPR*) to 0.0121 (*TR C1B3.05*), and 0.0100 for *C. purpureus*. The nucleotide diversity in most of the *loci* studied had higher values in *C. purpureus* than *C. amazonum* (except *ITS2*, *KIAA0187*, *TBP* and *TRc1B3.05 loci*). The Tajima's D values suggested that the *loci* have experienced different histories. They were negative in *C. amazonum* for six of nine *loci* (mean value = - 0.56) indicating a recent population expansion or chromosome-wide selective sweep, but only one of them was significantly different, having a positive value (*TBP*). In *C. purpureus*, seven *loci* were negative (mean value = - 0.95) and four of them were significantly different (*ITS2*, *KIAA0187*, *TBP* and *TRc1b3.05*). Positive Tajima's D potentially indicated decrease in population size and/or balancing selection but any of the positive *loci* were significantly different. For Fu's Fs neutrality statistic, *C. amazonum* showed four *loci* (*ITS2*, *KIAA0187*, *rpL23A* and *TBP*) with statistical significant values (only TBP with a positive value) and *C. purpureus* three (*hp23.9*, *KIAA0187* and *TBP*) and another time the same *locus* with positive value. The rest of the *loci* do not deviate from neutrality. Fu's Fs values were significantly negative at the 2% level, which corresponds to a 5% rejection rate (Fu, 1997). Again, most of the *loci* did not deviate from the neutrality for the Fu & Li's F* values, presenting only two significantly negative values for *C. amazonum* (*rpS18A* and *TBP*) and three for *C. purpureus* (*hp23.9*, *TBP* and *TRc1b3.05*). The *loci hp23.3*, *PPR*, *rpS18A* and *TBP* were

Table IV.1. Summary statistics for the studied loci for *Ceratodon amazonum* (Ca) and *C. purpureus* (Cp). Θ_w: Watterson estimation, π: nucleotide diversity, Fu's Fs, mutation frequency spectrum statistic Tajima's D and Fu & Li's F*(for the last two parameters, *P*-value < 0.05 and < 0,02 respectively are shown in bold), Rm: historical recombination events, Dxy: average number of nucleotide substitutions per site between species. The genomic location "nuclear–putative autosomal" for *rpL23A* and *TRc1B3.05* is based on unpublished data.

			Sample size		Segregating sites		θ_w		π		Fu's Fs		Tajima's D		Fu & Li's F*		Rm		Dxy
Locus	**Length**	**Genomic location**	**Ca**	**Cp**	**Ca**	**Cp**	**Ca**	**Cp**	**Ca**	**Cp**	**Ca**	**Cp**	**Ca**	**Cp**	**Ca**	**Cp**	**Ca**	**Cp**	
hp23.3	283	Nuclear - Autosomal	44	87	12	14	0.010	0.010	0.007	0.009	-0,031	-0,330	-0.976	-0.391	-1.694	-0.667	0	0	0.057
hp23.9	215	Nuclear - Autosomal	56	87	6	14	0.006	0.012	0.006	0.007	-0.306	**-0,554**	-0.267	-1.426	-0.570	**-2.793**	1	2	0.056
ITS2	492	Nuclear – Autosomal	42	35	19	26	0.009	0.014	0.009	0.006	**-1,261**	-0,339	-0.624	**-1.997**	-1.530	-2.353	4	1	0.043
KIAA0187	489	Nuclear - Autosomal	51	64	10	19	0.005	0.009	0.003	0.003	**-0,710**	**-0,134**	-0.967	**-2.067**	-1.708	-2.235	3	0	0.024
PPR	334	Nuclear - U/V	47	70	1	12	0.001	0.008	0.001	0.010	0,103	-0,219	0.699	0.933	0.684	0.167	0	0	0.081
rpL23A	892	Nuclear-Putative Autosomal	15	21	30	60	0.011	0.020	0.011	0.019	**-0,079**	-0,005	0.231	-0.217	-0.782	-0.413	2	1	0.042
rpS18A	365	Nuclear - U/V	51	62	9	11	0.006	0.007	0.003	0.009	-0,042	-0,087	-1.262	1.096	**-3.091**	1.022	0	0	0.024
TBP	365	Nuclear-Autosomal	51	94	6	10	0.004	0.006	0.001	0.001	**0,077**	**0,053**	**-1.948**	**-2.217**	**-2.473**	**-4.160**	0	0	0.051
TRc1b3.05	447	Nuclear-Putative Autosomal	52	34	22	9	0.012	0.005	0.0124	0.001	-0,572	0,049	0.079	**-2.296**	-0.783	**-3.975**	1	0	0.051

the only ones that did no present evidences of recombination in both species. The average proportion of nucleotide differences between species ranged from 0.0238 (*rpS18A*) to 0.0808 (*PPR*).

Past population history

The estimations scaled of current and ancestral effective population sizes, migration rates, and divergence times calculated with the program IMa2 for *Ceratodon amazonum* and *C. purpureus* are shown in Table IV.2 and Fig. IV.2.

Table IV.2. Parameter estimates in IMa2 analyses. HiPt: the bin with the highest probability in the estimation; HPD95Lo: the lower bound of the estimated 95% highest posterior density (HPD) interval; HPD95Hi: the upper bound of the estimated 95% HPD interval; N: effective population size (individuals) for *Ceratodon amazonum* (Ca), *C. purpureus* (Cp) and ancestral population (A); m: population migration rate for both directions (from *C. amazonum* to *C. purpureus* and vice versa); T: divergence time in years. †: unstable along different runs. The mutation rate per generation (μ) employed was 1×10^{-6}.

	Value	N_{Ca}	N_{Cp}	N_A	m_{Ca-Cp}	m_{Cp-Ca}	T_{split}
Analysis with migration	HiPt	277 500	427 500	277 500 †	1.3×10^{-8}	4.2×10^{-8}	1 686 000
	HPD95Lo	157 500	247 500	22 500 †	0.0	0.0	930 000
	HPD95Hi	472 500	622 500	1 450 000 †	10.1×10^{-8}	16.4×10^{-8}	2 358 000
Analysis without migration	HiPt	292 500	427 500	1 102 500	–	–	1 074 000
	HPD95Lo	157 500	247 500	427 500	–	–	426 000
	HPD95Hi	472 500	637 500	2 137 500	–	–	1 638 000

The results showed that the current effective population size for *C. amazonum* (N_{Ca}) and *C. purpureus* (N_{Cp}) was approximately 277 500 individuals (HPD95= 157 500-472 500) and 427 500 (HPD95= 247 500-622 500; Fig. IV.2A, B) respectively. The ancestral effective population size (N_A) was unstable, flat, converged and reached zero along different runs. But when we tested the same runs without migration between the species, it was stable and more than double of N_{Cp} (N_A = 1 102 500 individuals, HPD95= 427 500-2 137 500; Fig. IV.2B), whereas the current effective population sizes were approximately equal. The maximum likelihood estimates (MLE) of migration rates from *C. purpureus* to *C. amazonum* ($m_{Cp-Ca} = 4.2 \times 10^{-8}$, HPD95= 0.000-$16.4 \times 10^{-8}$) was larger than that from *C. amazonum* to *C. purpureus* ($m_{Ca-Cp} = 1.3 \times 10^{-8}$, HPD95= 0.000-$10.1 \times 10^{-8}$; Fig. IV.2C). Neither of the two converged but both reached zero. Also we estimated the divergence time between species found around 1.68 (HPD95= 0.93-2.35) million years ago (mya; Fig. IV.2D). Reconstruction of the population size

history by means of EBSP suggested an expansion episode for *C. amazonum* c. 6 600 years before present (Fig. IV.3). The mean number of population size changes throughout its history was estimated as 1.3 (95% HPD 1-2). However our data failed to find convergence when we studying *C. purpureus*.

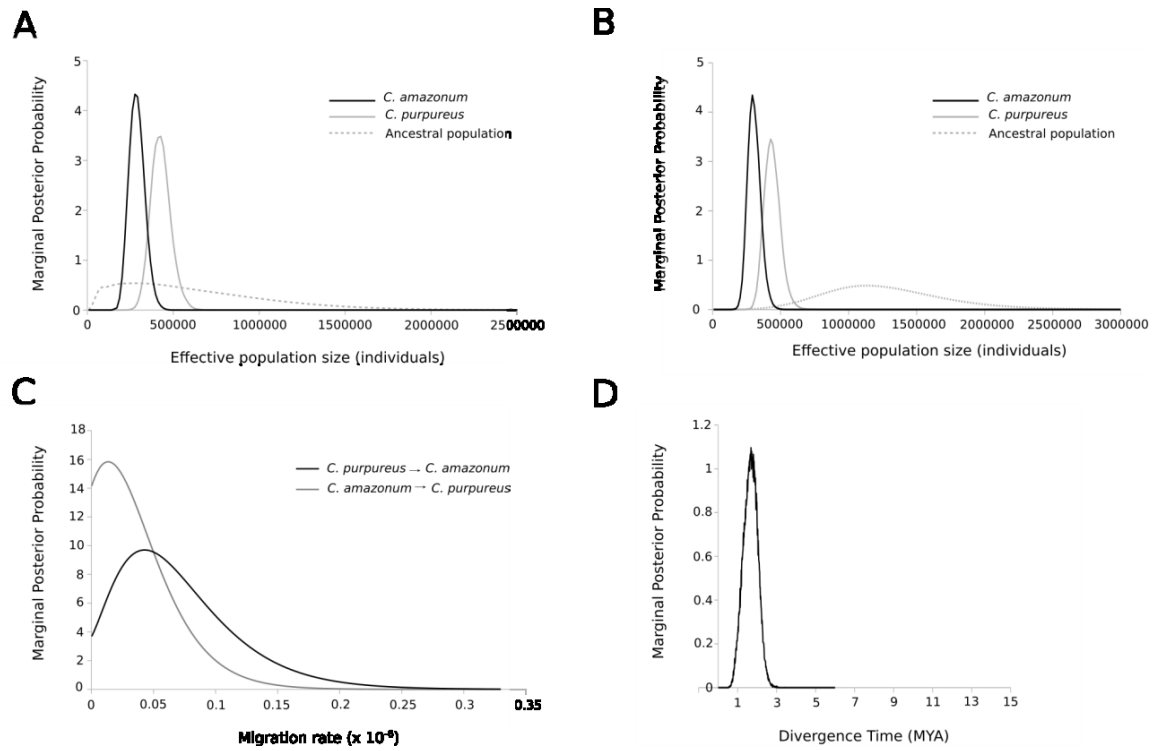


Fig. IV.2. Marginal posterior probability distribution of the parameters estimated using the Isolation with Migration model between *Ceratodon amazonum* and *C. purpureus*. A) Effective population size of species and the ancestral population considering migration; B) Effective population size of species and the ancestral population without migration between them; C) Migration rate between species; D) Divergence time in millions of years since the split of species.

Diversification scenarios

Analysis of IMa2 suggested the existence of gene flow during the formation of the two current species. However, migration could have occurred at different stages of speciation. To reveal the divergence history, 14 models were proposed and simulated. First we compared the submodels of mutation rate between them across the three models with migration (IM, AM and SC). For AM and SC comparison the submodels homogeneous mutation rate across the genome were selected (PP = 0.59-0.77) but in the case of IM was heterogeneous mutation rate (PP = 0.57-0.76; Annex IV.6), both in the case of homogeneous and heterogeneous effective population size scenarios. When we compared the best models selected in the step before between the two scenarios

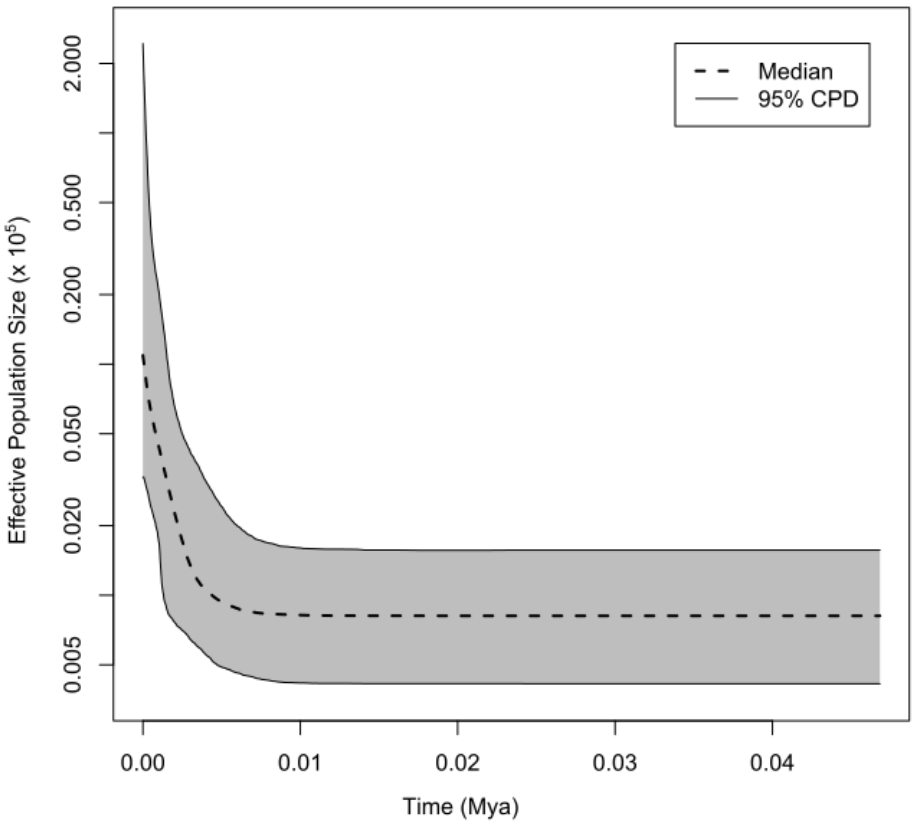


Fig. IV.3. Extended Bayesian Skyline Plots of *Ceratodon amazonum*. The dotted line represents the median of the effective population size parameter and the grey zone the 95% Central Posterior Density (CPD) interval.

(homogeneous vs. heterogeneous effective population size) in all cases were the homogeneous effective population size scenarios selected (PP = 0.62-0.94). Finally all general models (SI, IM, AM and SC) were compared using the best models of step before, the selected model was SC (PP = 0.8304). During the step of fit, we did not get a good fit; the simulated data did not approximate our observed data. For this reason we should not consider these results as reliable.

Origin of recombinant specimens

The Bayesian analyses consistently favored the unconstrained tree topology over the constrained topologies for all *loci* (Table IV.3). *Ceratodon* ×*conicus* was found to be polyphyletic and not monophyletic. Indicating that the recombinants between *C. amazonum* and *C. purpureus* currently found have evolved from several independent hybridization events and not from a single hybridization event.

Table IV.3. Estimates of the marginal model likelihoods (ln units) for the studied *loci*. Topologies with and without constrained recombinant samples are compared. The statistically better model for each *locus* is highlighted in bold. A log diference above five is considered very strong evidence.

Locus	Unconstrained model	Constrained model
hp23.3	**-1392.04**	-1462.20
hp23.9	**-1699.35**	-1780.42
ITS2	**-3773.92**	-3957.17
KIAA0187	**-1665.25**	-1689.34
PPR	**-1437.27**	-1461.54
rpL23A	**-5289.10**	-5695.93
rpS18A	**-2076.01**	-2343.98
TBP	**-1360.31**	-1400.03
TRc1b3.05	**-1586.14**	-1727.56

DISCUSSION

A major problem in evolutionary biology is to identify the processes that lead to the generation of reproductive isolation and the cessation of gene flow between new species. The geographic distribution of daughter species has long provided critical information regarding the patterns of gene flow that occurred over the course of a speciation event. However, geography may be a poor proxy for gene flow in species capable of frequent long distance dispersal, like free-spawning marine animals, ferns, and bryophytes. In this study we used genetic data to reconstruct the demographic and evolutionary history of the cosmopolitan moss *C. purpureus,* its geographically restricted sister species *C. amazonum,* and the nothospecies *C.* ×*conicus*. Previous data suggested that *C. amazonum* arose from a peripatric speciation event with *C. purpureus*, and *C.* ×*conicus* subsequently formed from hybridization between *C. amazonum* and *C. purpureus*. Using coalescent analyses we show that the speciation event between *C. purpureus* and *C. amazonum* occurred approximately 1.7 mya, but introgression occurred between the two species, more often from *C. purpureus* into *C. amazonum*. We also find that *C.* ×*conicus* was formed by multiple such hybridization events. These results highlight the complex role that gene flow can play during the speciation process.

We had initially predicted that the nucleotide diversity in *C. amazonum* would be much lower than that in *C purpureus*. Given the difference in the sizes of their geographic distributions, we hypothesized that *C. amazonum* diverged peripatrically from a geographically widespread common ancestor (Nieto-Lugilde et al., 2018a), a process would presumably involve a population bottleneck. Additionally, *C. amazonum* was never observed reproducing sexually. Indeed, our results show that the more abundant species (*C. purpureus*) almost always had higher levels of diversity than the less common species (Neutral theory: Kimura, 1983), probably due to a combination of demographic factors, including faster generation times, high rates of gene flow and large local population size (and effective population sizes).

Surprisingly, current effective population size of *C. amazonum* is relatively close to that of *C. purpureus*, despite the differences in census size. And neither species showed evidence of a population expansion. Demographic changes (bottleneck, population split) affect all *loci* whereas selection is expected to be *locus*-specific and is therefore distinguishable if multiple *loci* are analyzed. Our current results do not support a genome-wide historical event (for any of both species), which influenced all regions in the same way regardless of genomic position. Similar conclusion were obtained previously in *C. purpureus*, estimated by the distribution of theta value ($\theta = Ne\ \mu$) with mean = 0.014 (0.0-0.14). This degree of variation illustrates the among-*locus* heterogeneity in evolutionary history within this species (McDaniel et al., 2013a). The *loci* that are more diverged reflect *locus*-specific rather than genome-wide evolutionary processes (McDaniel & Shaw, 2005; McDaniel et al., 2013a). Overall nucleotide diversity was low in both species (*C. amazonum* and *C. purpureus*) compared to estimations in seed plants (reviewed in Wright & Gaut, 2004), which might be a general trend in bryophytes (Szövényi et al., 2007).

It is possible that low levels of introgression from *C. purpureus* into *C. amazonum* maintain similar levels of nucleotide diversity in the two species. We found clear evidence of ongoing asymmetric gene flow between the two species, with the bias favoring introgression into *C. amazonum*. In addition, the fact that the recombinant *C. ×conicus* was formed by multiple events is also consistent with ongoing interspecific gene-flow. This indicates that reproductive isolation between the two species is not complete. In addition, recombinant diploid gametophytes were reported in *Ceratodon*

(Nieto-Lugilde et al., 2018a) and their sequences do not differ from those of haploid recombinants, this indicates that both haploid and diploid hybridization events are very recent. Our data revealed that *C. purpureus* was more introgressed than *C. amazonum*, moreover multiple independent hybridization events have occurred. The fact that the hybridization between both lineages is not rare, probably indicates that the genomic imbalances derived from this hybridization are solved by both haploid and diploid recombinant individuals.

The lack of obvious population structure in either *C. purpureus* or *C. amazonum* suggests that both species have the capacity for some long-distance dispersal making it unlikely that the speciation event separating them involved a long period of allopatry. Accordingly with the worldwide sampling (McDaniel & Shaw, 2005), in our sampling little or no genetic structure has been found associated with geography in *C. purpureus* (even considering the samples from North America), probably due to a really efficient spore dispersal. For *C. amazonum*, no genetic population structure was observed, despite the fact that sporophytes were never observed (Nieto-Lugilde et al., 2018a). Thus, the fact that in Sierra Nevada Mountains *C. purpureus* is displaced by *C. amazonum* remains mysterious. It could be explained by density-dependent processes, such as gene surfing, high-density blocking, and competitive exclusion ("founder takes all" principle: Waters et al., 2013) or other not necessarily density-dependent and not mutually exclusive processes (dispersal ability or social behavior, niche specialization, genetic incompatibility or assortative mating: Buckley et al., 2013). It is possible that *C. amazonum* is locally adapted to the region, but *C. purpureus* occupies many sites that are superficially similar to habitats in the Sierra Nevada.

Molecular clocks are widely used to date phylogenetic events (Bromham & Penny, 2003; Kumar, 2005; Weir & Schluter, 2008), and there are evidences that substitution rates of mosses are considerably lower than in vascular plants (Stenøien, 2008). Previously, a mutation rate of 1×10^{-8} neutral mutations per site per generation was employed in *C. purpureus* (McDaniel et al., 2013b). However greater mutation rates can be considered for *Ceratodon* species, based on the results in the Moss and Liverwort Tree of Life with five species and calculated by three different methods, which indicate that the median age of the stem of *Ceratodon* genus is $12.6\text{-}19.0 \times 10^{6}$ years (Laenen et al., 2014). We estimated a divergence time near to 1.7 mya, however,

if a lower mutation rate (around to 1×10^{-7} mutations per site per generation) is considered, the split time will be of 17 mya and is in this hypothetical case near to the origin of the genus. However, mutation rate variation has been associated with dramatic changes in genome size and complexity (Sloan et al., 2012), much like that in *C. amazonum*, meaning that mutation processes may not be consistent between the two species or over time.

Results from IMa2 program show that probably the effective population size of the ancestor was near the double of the current *C. purpureus* effective population size. Usually effective population sizes are lower than ecologically observed population sizes, but not always (Braude & Templeton, 2009; Nunney, 2016). The structure of the population through breeding individuals (sex ratio or gene flow), as well as variation in "family size" (Wang et al., 2016; Bobay & Ochman, 2018) could vary over time, explaining this decrease. Probably a sterile condition associated with a significant decrease in the presence of males in *C. amazonum* (Nieto-Lugilde et al., 2018a) may depress the effective population size of this species. However, *C. purpureus* is very common now, reproduces frequently, and has a close to even sex ratio. Several of these features would have had to be quite different for this species to have had an effective population size twice as big in the past.

One possible explanation for the decreased genetic diversity relative to the estimated ancestral population size is recurrent selection reducing nucleotide diversity in our sampled *loci*. The deviations from neutrality can be due to selective and/or demographic events, confounding the interpretation of tests. The three tests to study the deviation of neutrality selected here were different by definition, so Tajima's D and Fu and Li's F* use information on the frequency of mutations and are based on the differences between estimators of the population mutation rate and differed in their results, and Fu's Fs is based on the haplotype distribution. The power of these statistics can vary depending on sample size, number of segregating sites, recombination rates and demographic events (Ramos-Onsins & Rozas, 2002; Ramírez-Soriano et al., 2008). The most powerful test is Fu's Fs but it is strongly affected by recombination. Therefore, because of the risk to over- or underestimate Fu's Fs due to unknown facts, we present the other two tests. The combined results suggest that the *loci* have experienced different histories, and indicate that the species has expanded recently

across the whole investigated areas. This was also supported by the results of EBSP at least for *C. amazonum*.

Similarly, the ABC analyses are difficult to apply to such circumstances, in spite of their flexiblity and power (Bertorelle et al., 2010). Models are always approximations of reality, and they should be as simple as possible, but not more so, as Einstein said in one of the great quotes in science (Saracci, 2006; Csilléry et al., 2010). We considered different testable scenarios that would cope to the extant distribution of genetic variation. It is difficult and practically impossible to find a simulation model that fits the data. For this reason, it is a common practice in ABC to use one or more summary statistics of the data rather than the data itself (Sadegh & Vrugt, 2014). We tried six to 22 summary statistics used successfully in other works, but they were not sufficient to provide as much information for the model parameters as the original data set itself. The effect of competing models, the number of simulations, the choice of summary statistics, or the acceptance threshold should be evaluated and tested in each study (Bertorelle et al., 2010). We also considered important to incorporate the heterogeneity in migration rates among *loci*, and to account for the semi-permeability of the barrier to gene flow between recently diverged species (Roux et al., 2013, 2016). In this way we can incorporate the effect of background selection and genetic hitchhiking in regions of low recombination in demographic inferences (Sousa & Hey, 2013; Roux et al., 2016; Fraïsse et al., 2018). Despite our efforts to simulate data, these were not successful, perhaps due to greater selective pressure or maybe due to more complex scenarios that we did not take into account.

Taken as a whole, the demographic history that we have inferred from DNA sequencing data indicates that *C. amazonum* experienced a recent population expansion, whereas it was not conclusive for *C. purpureus*. These results indicate that more complex phenomena are affecting the populations of *C. purpureus* and possibly are the culprits of the failure to simulate the data. Despite these limitations, demographic insights from this study illustrate the value of a long term evolutionary perspective, as well as an understanding of ongoing population processes. These mosses form a complex scenario that are of particular interest for such a study given their obscure morphologies species diversity, and their contrasting distribution around the Earth, from a restricted (*C. amazonum*) to a worldwide distribution (*C. purpureus*). Disentangling

the effects of selective and demographic processes poses a serious challenge, due to the fact that both may result in similar patterns of nucleotide variability (Csilléry et al., 2010).

ACKNOWLEDGEMENTS

The authors thank the curators of herbaria BOL, CBFS, and S for providing plant material; Laura Forrest from Royal Botanic Garden Edinburgh, United Kingdom, for donating samples; the management group of the National Park of Sierra Nevada Natural Space for allowing us to collect samples in the National and Natural Parks. This study was supported financially by the Spanish "Ministerio de Ciencia e Innovación" (Project CGL2011-22936/BOS), by "Ministerio de Economía y Competitividad" (Project CGL2014-52579-R) and co-financed by ERDF of the European Union. M N-L also thanks the Spanish "Ministerio de Economía y Competitividad" for a "Formación de Personal Investigador" fellowship (FPI program 2012; reference BES-2012-056799).

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ANNEXES

Annex IV.1. Voucher information for the studied specimens. For each sequenced sample the next information is given: herbarium code (or Genbank identification code); geographical origin, Genbank accession numbers for the nine *loci* studied, given in the next order: *hp23.3*, *hp23.9*, *ITS2*, *KIAA0187*, *PPR*, *rpL23A*, rpS18A, *TBP* and *TRc1b3.05*. Sequences obtained by cloning (Nieto-Lugilde et al., 2018a) are indicated by their GenBank accession number given in parentheses.

Ceratodon amazonum

MUB 43730: Spanish south-eastern mountains, KP825494, KP825628, KP825765, KP825883, KP826017, KP826181, KP826270, KP826402, KP826531. **MUB 49302:** Sierra Nevada Mountains, KP825570, KP825705, KP825825, KP825958, KP826093, KP826267, KP826346, KP826475, KP826603. **MUB 49303:** Sierra Nevada Mountains, KP825569, KP825704, KP825817, KP825957, KP826092, KP826266, KP826345, KP826474, KP826602. **MUB 49304:** Sierra Nevada Mountains, KP825568, KP825703, KP825824, KP825956, KP826091, KP826265, KP826344, KP826473, KP826601. **MUB 49305:** Sierra Nevada Mountains, KP825567, KP825702, KP825823, KP825955, KP826090, KP826264, KP826343, KP826472, KP826600. **MUB 49306:** Sierra Nevada Mountains, KP825566, KP825701, KP825822, KP825954, KP826089, KP826263, KP826342, KP826471, KP826599. **MUB 49317:** Sierra Nevada Mountains, KP825564, KP825699, KP825819, KP825952, KP826087, KP826261, KP826340, KP826469, KP826597. **MUB 49318:** Sierra Nevada Mountains, KP825563, KP825698, KP825818, KP825951, KP826086, KP826260, KP826339, KP826468, KP826596. **MUB 49319:** Sierra Nevada Mountains, KP825562, KP825697, KP825816, KP825950, KP826085, KP826259, KP826338, KP826467, KP826595. **MUB 49321:** Sierra Nevada Mountains, KP825560, KP825695, KP825814, KP825948, KP826083, KP826257, KP826336, KP826465, KP826593. **MUB 49326:** Sierra Nevada Mountains, KP825558, KP825693, KP825812, KP825946, KP826081, KP826255, KP826334, KP826463, KP826591. **MUB 49327:** Sierra Nevada Mountains, KP825557, KP825692, KP825821, KP825945, KP826080, KP826254, KP826333, KP826462, KP826590. **MUB 49328:** Sierra Nevada Mountains, KP825556, KP825691, KP825811, KP825944, KP826079, KP826253, KP826332, KP826461, KP826589. **MUB 49329:** Sierra Nevada Mountains, –, KP825690, KP825810, KP825943, KP826078, KP826252, KP826331, KP826460, KP826588. **MUB 49330:** Sierra Nevada Mountains, KP825555, KP825689, KP825809, KP825942, KP826077, KP826251, KP826330, –, KP826587. **MUB 49331:** Sierra Nevada Mountains, KP825554, KP825688, KP825808, KP825941, KP826076, KP826250, KP826329, KP826459, KP826586. **MUB 49341:** Sierra Nevada Mountains, KP825549, KP825683, KP825806, KP825937, KP826071, KP826246, KP826324, KP826454, KP826581. **MUB 49342:** Sierra Nevada Mountains, KP825548, KP825682, KP825805, KP825936, KP826070, KP826245, KP826323, KP826453, KP826580. **MUB 49351:** Sierra Nevada Mountains, KP825547, KP825681, KP825804, KP825935, KP826069, KP826244, KP826322, KP826452, KP826579. **MUB 49352:** Sierra Nevada Mountains, KP825546, KP825680, KP825803, KP825934, KP826068, KP826243, KP826321, KP826451, –. **MUB 49353:** Sierra Nevada Mountains, KP825545, KP825679, KP825802, KP825933, KP826067, KP826242, KP826320, KP826450, –. **MUB 49355:** Sierra Nevada Mountains, KP825544, KP825678, –, KP825932, KP826066, KP826240, KP826319, KP826449, KP826578. **MUB 49356:** Sierra Nevada Mountains, KP825543, KP825677, KP825801, KP825931, KP826065, KP826239, KP826318, KP826448, KP826577. **MUB 49357:** Sierra Nevada Mountains, KP825542, KP825676, KP825800, KP825930, KP826064, KP826241, KP826317, KP826447, KP826576. **MUB 49366:** Sierra Nevada Mountains, KP825537, KP825670, KP825797, KP825924, KP826058, KP826238, KP826311, KP826442, KP826570. **MUB 49370:** Sierra Nevada Mountains, KP825540, KP825674, –, KP825928, KP826062, KP826234, KP826315, KP826446, KP826574. **MUB 49373:** Sierra Nevada Mountains, KP825538, KP825671, KP825798, KP825925, KP826059, KP826233, KP826312, KP826443, KP826571. **MUB 49375:** Sierra Nevada Mountains, KP825539, KP825672, KP825799, KP825926, KP826060, KP826236, KP826313, KP826444, KP826572. **MUB 49377:** Sierra Nevada Mountains, –, KP825673, –, KP825927, KP826061, KP826237, KP826314, KP826445, KP826573. **MUB 49382:** Sierra Nevada Mountains, KP825536, KP825669, KP825796, KP825923, KP826057, KP826180, KP826310, KP826441, KP826569. **MUB 49384:** Sierra

Nevada Mountains, KP825535, KP825668, KP825795, KP825922, KP826056, KP826232, KP826309, KP826440, KP826568. **MUB 49387:** Sierra Nevada Mountains, KP825533, KP825666, –, KP825920, KP826054, KP826230, KP826307, KP826438, KP826565. **MUB 49389:** Sierra Nevada Mountains, KP825532, KP825665, KP825793, KP825919, KP826053, KP826228, KP826306, KP826437, KP826564. **MUB 49390:** Sierra Nevada Mountains, KP825531, KP825664, –, KP825918, KP826052, KP826229, KP826305, KP826436, KP826566. **MUB 49399:** Sierra Nevada Mountains, KP825530, KP825663, KP825792, KP825917, KP826051, KP826224, KP826300, KP826435, KP826563. **MUB 49400:** Sierra Nevada Mountains, KP825529, KP825662, KP825791, KP825916, KP826050, KP826223, KP826299, KP826434, KP826562. **MUB 49403:** Sierra Nevada Mountains, KP825527, KP825660, KP825790, KP825914, KP826048, KP826182, KP826303, KP826432, KP826560. **MUB 49405:** Sierra Nevada Mountains, KP825525, KP825658, –, KP825912, KP826046, KP826225, KP826301, KP826430, KP826558. **MUB 49408:** Sierra Nevada Mountains, KP825524, KP825657, –, KP825911, KP826045, KP826222, KP826298, –, KP826557. **MUB 49409:** Sierra Nevada Mountains, KP825523, KP825656, KP825789, KP825910, KP826044, KP826221, KP826297, KP826429, KP826556. **MUB 49410:** Sierra Nevada Mountains, KP825522, KP825655, KP825788, KP825909, KP826043, KP826220, KP826296, KP826428, KP826555. **MUB 49411:** Sierra Nevada Mountains, KP825521, KP825654, KP825787, KP825908, KP826042, KP826219, KP826295, KP826427, KP826554. **MUB 49412:** Sierra Nevada Mountains, KP825520, KP825653, KP825786, KP825907, KP826041, KP826218, KP826294, KP826426, KP826553. **MUB 49413:** Sierra Nevada Mountains, KP825519, KP825652, KP825785, KP825906, KP826040, KP826217, KP826293, KP826425, KP826552. **MUB 49424:** Sierra Nevada Mountains, KP825518, KP825651, KP825784, KP825905, KP826039, KP826216, KP826292, KP826424, KP826551. **MUB 49425:** Sierra Nevada Mountains, KP825517, KP825650, KP825783, KP825904, KP826038, KP826215, KP826291, KP826423, KP826550. **MUB 49426:** Sierra Nevada Mountains, KP825516, KP825649, KP825782, KP825903, KP826037, KP826214, KP826290, KP826422, KP826549. **MUB 49427:** Sierra Nevada Mountains, KP825515, KP825648, KP825781, KP825902, KP826036, KP826213, KP826289, KP826421, KP826548. **MUB 49428:** Sierra Nevada Mountains, KP825514, KP825647, –, KP825901, KP826035, KP826212, KP826288, –, KP826547. **MUB 49429:** Sierra Nevada Mountains, KP825513, KP825646, KP825780, KP825900, KP826034, –, KP826287, KP826420, –. **MUB 49439:** Sierra Nevada Mountains, KP825512, KP825645, KP825779, KP825899, KP826033, KP826211, KP826286, KP826419, KP826546. **MUB 49440:** Sierra Nevada Mountains, KP825511, KP825644, –, KP825898, KP826032, KP826210, KP826285, KP826418, KP826545. **MUB 49442:** Sierra Nevada Mountains, KP825510, KP825643, KP825778, KP825897, KP826031, KP826208, KP826284, KP826417, KP826544. **MUB 49443:** Sierra Nevada Mountains, KP825509, KP825642, KP825777, KP825896, KP826030, KP826207, KP826283, KP826416, KP826543. **MUB 49444:** Sierra Nevada Mountains, KP825508, KP825641, KP825776, KP825895, KP826029, KP826206, KP826282, KP826415, KP826542. **MUB 49445:** Sierra Nevada Mountains, KP825507, KP825640, KP825775, KP825894, KP826028, KP826209, KP826281, KP826414, KP826541.

Ceratodon* ×*conicus

CBFS3416: Czech Republic, KP825495, –, KP825764, KP825969, KP826015, –, KP826268, KP826400, KP826529. **CBFS13223:** Czech Republic, KP825579, KP825710, KP825763, KP825965, KP826098, –, KP826353, KP826482, KP826610. **MUB 49307:** Sierra Nevada Mountains, (KP825565, MH790737), (MH790742, MH790743, MH790744, MH790745, MH790746, MH790747, MH790748, MH790749), (MH790800, MH790801, MH790802, MH790803, MH790804), –, (KP826088, MH790821), KP826262, (MH790841, MH790842), (KP826470, MH790850), KP826598. **MUB 49323:** Sierra Nevada Mountains, KP825561, KP825696, KP825815, KP825949, KP826084, KP826258, KP826337, KP826466, KP826594. **MUB 49337:** Sierra Nevada Mountains, (KP825553, MH790738), (MH790750, MH790751, MH790752, MH790753, MH790754, MH790755, MH790756, MH790757, MH790758, MH790759), (MH790805, MH790806, MH790807, MH790808, MH790809), –, (KP826075, MH790822), KP826249, (KP826328, MH790847), (KP826458, MH790851), KP826585. **MUB 49338:** Sierra Nevada Mountains, (KP825552, MH790739), (MH790760, MH790761, MH790762, MH790763, MH790764, MH790765, MH790766, MH790767, MH790768), KP825829, –, (MH790823, MH790824), –, KP826327, (KP826457, MH790852), KP826584. **MUB 49339:** Sierra Nevada Mountains, KP825551, (MG050789, MG050790, MG050791, MG050792, MG050793, MG050794, MG050795, MG050796, MG050797, MG050798, MG050799), KP825820, KP825938, (KP826073, MG050748, MG050749, MG050750, MG050751, MG050752), KP826248, (KP826326, MH790844), (KP826456, MG050761, MG050762, MG050763, MG050764, MG050765), KP826583. **MUB 49340:** Sierra Nevada Mountains, (KP825550, MH790740), (MH790769, MH790770, MH790771, MH790772), KP825830, –, (MH790825, MH790826), KP826247, (KP826325, MH790848), (KP826455, MH790853), KP826582. **MUB 49385:**

Sierra Nevada Mountains, KP825534, KP825667, KP825794, KP825921, KP826055, KP826231, KP826308, KP826439, KP826567. **MUB 49404:** Sierra Nevada Mountains, KP825526, (MH790778, MH790779, MH790780), (MH790810, MH790811, MH790812, MH790813, MH790814, MH790815), (KP825913, MH790820), (MH790828, MH790829, MH790830, MH790831, MH790832, MH790833), KP826226, (KP826302, MH790849), (KP826431, MH790855), KP826559. **MUB 49407:** Sierra Nevada Mountains, (KP825528, MH790741), (MH790773, MH790774, MH790775, MH790776, MH790777), KP825827, –, (KP826049, MH790827), –, (KP826304, MH790843), (KP826433, MH790854), KP826561. **MUB 49451:** Sierra Nevada Mountains, KP825506, (KP825639, MG050800, MG050801, MG050802, MG050803, MG050804, MG050805, MG050806, MG050807, MG050808), (KP825774, MH790781, MH790782, MH790783, MH790784), KP825893, (KP826027, MG050753), (KP826204, MG050869, MG050870), KP826280, KP826413, KP826540. **MUB 49461:** Sierra Nevada Mountains, KP825505, KP825638, (KP825773, MH790785, MH790786, MH790787, MH790788), KP825892, KP826026, KP826203, KP826279, KP826412, KP826539. **MUB 49473:** Sierra Nevada Mountains, KP825504, (KP825637, MG050809, MG050810, MG050811, MG050812, MG050813, MG050814, MG050815, MG050816, MG050817, MG050818, MG050819), KP825826, KP825891, KP826025, (MG050871, MG050872, MG050873, MG050874, MG050875, MG050876), (KP826278, MH790845), (MG050766, MG050767, MG050768, MG050769, MG050770), KP826538. **MUB 49480:** Sierra Nevada Mountains, (KP825503, MH790735), (KP825636, MG050820, MG050821, MG050822, MG050823, MG050824, MG050825, MG050826), KP825772, KP825890, KP826024, KP826199, KP826277, KP826410, KP826537. **MUB 49485:** Sierra Nevada Mountains, (KP825502, MH790736), (KP825635, MG050827, MG050828, MG050829, MG050830, MG050831, MG050832, MG050833), MH790797, KP825889, (KP826023, MG050754, MG050755, MG050756, MG050757, MG050758), (MG050877, MG050878, MG050879, MG050880, MG050881, MG050882), (KP826276, MH790846), (KP826409, MG050771, MG050772, MG050773, MG050774, MG050775, MG050776), KP826536. **MUB 49492:** Sierra Nevada Mountains, KP825501, (MG050834, MG050835, MG050836, MG050837, MG050838, MG050839, MG050840), (KP825771, MH790789, MH790790, MH790791, MH790792), KP825888, KP826022, KP826198, KP826275, KP826408, –. **MUB 49501:** Sierra Nevada Mountains, KP825500, KP825633, KP825770, KP825887, –, KP826197, KP826274, KP826407, KP826535. **MUB 49504:** Sierra Nevada Mountains, KP825499, (KP825632, MG050841, MG050842, MG050843, MG050844, MG050845), KP825769, –, KP826021, KP826196, (MH790836, MH790837), KP826406, (MG050867, MG050868). **MUB 49505:** Sierra Nevada Mountains, KP825498, KP825631, KP825768, KP825886, KP826020, KP826195, KP826273, KP826405, KP826534. **MUB 49518:** Sierra Nevada Mountains, KP825497, (KP825630, MG050846, MG050847, MG050848, MG050849, MG050850, MG050851, MG050852, MG050853, MG050854), (KP825767, MH790793, MH790794, MH790795, MH790796), KP825885, (KP826019, MG050759), KP826194, KP826272, KP826404, KP826533. **MUB 49528:** Sierra Nevada Mountains, KP825496, (KP825629, MG050855, MG050856, MG050857, MG050858, MG050859, MG050860), KP825828, KP825884, (KP826018, MG050760), KP826193, (MH790838, MH790839), (MG050777, MG050778), KP826532. **MUB 49568:** Spanish central mountain ranges, –, KP825732, KP825851, KP825984, –, KP826165, KP826369, –, KP826629. **MUB 49604:** Alps, KP825493, KP825627, KP825766, KP825882, KP826016, KP826162, KP826269, KP826401, KP826530. **MUB 52185:** United Kingdom, MH790732, KX503277, –, MH790818, KX503282, KX503284, MH790840, KX503292, KX503307. **MUB 52186:** United Kingdom, (MH790733, MH790734), (MG050861, MG050862, MG050863, MG050864, MG050865, MG050866), (MH790798, MH790799), MH790819, KX503283, KX503285, –, KX503293, KX503308.

Ceratodon purpureus

ADM1: North America, KC436450, KC436508, –, KC436546, KC436899, –, KC437031, KC436690, –. **ADM2:** North America, KC436451, KC436509, –, –, KC436900, –, KC437032, KC436691, –. **ADM3:** North America, KC436452, KC436510, –, KC436547, KC436901, –, KC437033, KC436692, –. **ADM4:** North America, KC436453, –, –, KC436548, –, –, –, KC436693, –. **ADM5:** North America, KC436454, KC436511, –, KC436549, KC436902, –, KC437034, KC436694, –. **ADM6:** North America, KC436455, –, –, KC436550, –, –, –, –, –. **ADM7:** North America, KC436456, KC436512, –, KC436551, KC436903, –, KC437035, KC436695, –. **ADM8:** North America, KC436457, KC436513, –, KC436552, KC436904, –, –, KC436696, –. **ANT:** Antarctica, KC436458, –, –, KC436553, –, –, KC437036, KC436697, –. **AUST:** Australia, KC436459, –, –, KC436554, –, –, KC437037, KC436698, –. **BOL 46302:** South Africa, KP825574, KP825717, –, KP825970, KP826109, –, –, KX503295, KP826618. **BOL 46303:** South Africa, KP825573, KP825716, –, –, KP826108, –, –, –, KP826617. **CBFS 13557:** Alps, KP825577, KP825708, KP825835, KP825963, KP826096, KP826151, KP826351, KP826480, KP826608. **CBFS 14587:** Czech Republic, KP825582, KP825713, KP825838, KP825968, KP826101, KP826155, –, KP826484, KP826612. **CBFS 14724:** Czech Republic, KP825578, KP825709, –,

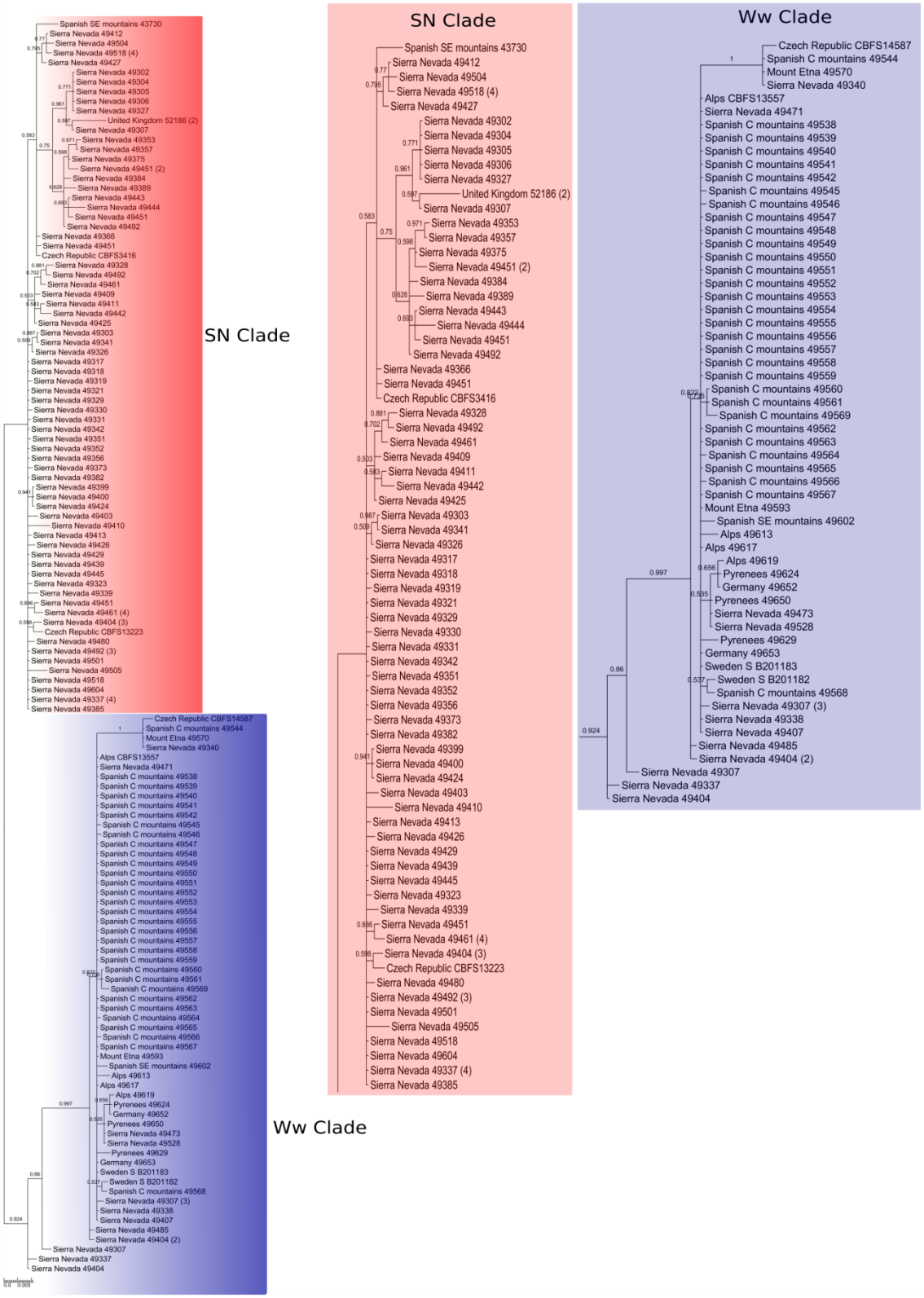
KP825964, KP826097, KP826152, KP826352, KP826481, KP826609. **DIN1:** North America, KC436460, KC436514, –, KC436555, KC436905, –, KC437038, KC436701, –. **DIN2:** North America, KC436461, KC436515, –, KC436556, KC436906, –, KC437039, KC436702, –. **DIN3:** North America, KC436462, –, –, KC436557, –, –, –, KC436703, –. **DIN4:** North America, KC436463, –, –, –, –, –, –, –, –. **DIN6:** North America, KC436464, –, –, KC436558, KC436907, –, KC437040, KC436704, –. **DIN7:** North America, KC436465, –, –, KC436559, KC436908, –, KC437041, KC436705, –. **DIN8:** North America, KC436466, –, –, KC436560, –, –, KC437042, KC436706, –. **DUR1:** North America, KC436468, KC436517, –, KC436563, KC436909, –, KC437045, KC436710, –. **DUR2:** North America, KC436469, KC436518, –, KC436564, –, –, KC437046, KC436711, –. **DUR3:** North America, KC436470, –, –, KC436565, –, –, –, KC436712, –. **DUR4:** North America, KC436471, –, –, KC436566, –, –, –, KC436713, –. **DUR5:** North America, KC436472, KC436519, –, KC436567, –, –, KC437047, KC436714, –. **DUR6:** North America, KC436473, KC436520, –, KC436568, KC436910, –, KC437048, KC436715, –. **DUR7:** North America, KC436474, KC436521, –, KC436569, KC436911, –, KC437049, KC436716, –. **DUR8:** North America, KC436475, KC436522, –, KC436570, KC436912, –, KC437050, KC436717, –. **ESX1:** North America, KC436476, –, –, KC436571, KC436913, –, KC437051, KC436718, –. **ESX2:** North America, KC436477, KC436523, –, KC436572, KC436914, –, KC437052, KC436719, –. **ESX3:** North America, KC436478, –, –, KC436573, KC436915, –, KC437053, KC436720, –. **ESX4:** North America, KC436479, KC436524, –, KC436574, KC436916, –, KC437054, KC436721, –. **ESX5:** North America, KC436480, –, –, KC436575, KC436917, –, KC437055, KC436722, –. **ESX6:** North America, KC436481, KC436525, –, KC436576, KC436918, –, KC437056, KC436723, –. **ESX7:** North America, KC436482, –, –, –, –, –, –, KC436724, –. **ESX8:** North America, KC436483, KC436526, –, KC436577, KC436919, –, KC437057, KC436725, –. **MUB 49471:** Sierra Nevada Mountains, KP825571, KP825706, KP825831, KP825959, KP826094, KP826201, KP826347, KP826476, KP826604. **MUB 49538:** Spanish central mountain ranges, KP825626, KP825762, KP825881, KP826014, KP826150, KP826192, KP826399, KP826528, KP826659. **MUB 49539:** Spanish central mountain ranges, KP825625, KP825761, KP825880, KP826013, KP826149, KP826205, KP826398, KP826527, KP826658. **MUB 49540:** Spanish central mountain ranges, KP825624, KP825760, KP825879, KP826012, KP826148, KP826191, KP826397, KP826526, KP826657. **MUB 49541:** Spanish central mountain ranges, KP825623, KP825759, KP825878, KP826011, KP826147, KP826190, KP826396, KP826525, KP826656. **MUB 49542:** Spanish central mountain ranges, KP825622, KP825758, KP825877, KP826010, KP826146, KP826188, KP826395, KP826524, KP826655. **MUB 49544:** Spanish central mountain ranges, KP825620, KP825756, KP825875, KP826008, KP826144, KP826189, KP826393, KP826522, KP826653. **MUB 49545:** Spanish central mountain ranges, KP825619, KP825755, KP825874, KP826007, KP826143, KP826186, KP826392, KP826521, KP826652. **MUB 49546:** Spanish central mountain ranges, KP825618, KP825754, KP825873, KP826006, KP826142, KP826185, KP826391, KP826520, KP826651. **MUB 49547:** Spanish central mountain ranges, KP825617, KP825753, KP825872, KP826005, KP826141, KP826184, KP826390, KP826519, KP826650. **MUB 49548:** Spanish central mountain ranges, KP825616, KP825752, KP825871, KP826004, KP826140, KP826183, KP826389, KP826518, KP826649. **MUB 49549:** Spanish central mountain ranges, KP825615, KP825751, KP825870, KP826003, KP826139, –, KP826388, KP826517, KP826648. **MUB 49550:** Spanish central mountain ranges, KP825614, KP825750, KP825869, KP826002, KP826138, KP826179, KP826387, KP826516, KP826647. **MUB 49551:** Spanish central mountain ranges, KP825613, KP825749, KP825868, KP826001, KP826137, KP826178, KP826386, KP826515, KP826646. **MUB 49552:** Spanish central mountain ranges, KP825612, KP825748, KP825867, KP826000, KP826136, KP826177, KP826385, KP826514, KP826645. **MUB 49553:** Spanish central mountain ranges, KP825611, KP825747, KP825866, KP825999, KP826135, KP826176, KP826384, KP826513, KP826644. **MUB 49554:** Spanish central mountain ranges, KP825610, KP825746, KP825865, KP825998, KP826134, KP826175, KP826383, KP826512, KP826643. **MUB 49555:** Spanish central mountain ranges, KP825609, KP825745, KP825864, KP825997, KP826133, KP826174, KP826382, KP826511, KP826642. **MUB 49556:** Spanish central mountain ranges, KP825608, KP825744, KP825863, KP825996, KP826132, –, KP826381, KP826510, KP826641. **MUB 49557:** Spanish central mountain ranges, KP825607, KP825743, KP825862, KP825995, KP826131, KP826173, KP826380, KP826509, KP826640. **MUB 49558:** Spanish central mountain ranges, KP825606, KP825742, KP825861, KP825994, KP826130, KP826172, KP826379, KP826508, KP826639. **MUB 49559:** Spanish central mountain ranges, KP825605, KP825741, KP825860, KP825993, KP826129, KP826171, KP826378, KP826507, KP826638. **MUB 49560:** Spanish central mountain ranges, KP825604, KP825740, KP825859, KP825992, KP826128, KP826170, KP826377, KP826506, KP826637. **MUB 49561:** Spanish central mountain ranges, KP825603, KP825739, KP825858, KP825991, KP826127, KP826169, KP826376, KP826505, KP826636. **MUB 49562:** Spanish central mountain ranges, KP825602, KP825738, KP825857,

KP825990, KP826126, –, KP826375, KP826504, KP826635. **MUB 49563:** Spanish central mountain ranges, KP825601, KP825737, KP825856, KP825989, KP826125, –, KP826374, KP826503, KP826634. **MUB 49564:** Spanish central mountain ranges, KP825600, KP825736, KP825855, KP825988, KP826124, KP826168, KP826373, KP826502, KP826633. **MUB 49565:** Spanish central mountain ranges, KP825599, KP825735, KP825854, KP825987, KP826123, –, KP826372, KP826501, KP826632. **MUB 49566:** Spanish central mountain ranges, KP825598, KP825734, KP825853, KP825986, KP826122, KP826167, KP826371, KP826500, KP826631. **MUB 49567:** Spanish central mountain ranges, KP825597, KP825733, KP825852, KP825985, KP826121, KP826166, KP826370, KP826499, KP826630. **MUB 49569:** Spanish central mountain ranges, KP825596, KP825731, KP825850, KP825983, KP826119, KP826164, KP826368, KP826497, KP826628. **MUB 49570:** Sicilian Mount Etna, KP825576, KP825714, KP825833, KP825962, KP826107, –, KP826350, KP826478, KP826606. **MUB 49593:** Sicilian Mount Etna, KP825575, KP825715, KP825834, KP825961, KP826106, KP826163, KP826349, KP826479, KP826607. **MUB 49600:** Spanish south-eastern mountains, KP825587, KP825722, –, KP825974, KP826104, KP826159, KP826357, KP826486, KP826613. **MUB 49602:** Spanish south-eastern mountains, KP825588, KP825723, KP825847, KP825975, KP826105, KP826160, KP826358, KP826487, KP826614. **MUB 49606:** Alps, KP825592, KP825727, –, KP825979, KP826115, KP826161, KP826364, KP826493, KP826624. **MUB 49613:** Alps, KP825591, KP825726, KP825846, KP825978, KP826114, –, KP826363, KP826492, KP826623. **MUB 49617:** Alps, KP825590, KP825725, KP825845, KP825977, KP826113, –, KP826362, KP826491, KP826622. **MUB 49619:** Alps, KP825589, KP825724, KP825844, KP825976, KP826112, –, KP826361, KP826490, KP826621. **MUB 49624:** Pyrenees, KP825595, KP825730, KP825841, KP825982, KP826118, –, KP826367, KP826496, KP826627. **MUB 49629:** Pyrenees, KP825594, KP825729, KP825840, KP825981, KP826117, KP826158, KP826366, KP826495, KP826626. **MUB 49650:** Pyrenees, KP825593, KP825728, KP825839, KP825980, KP826116, KP826157, KP826365, KP826494, KP826625. **MUB 49652:** Germany, KP825583, KP825718, KP825842, KP825971, KP826110, KP826156, KP826359, KP826488, KP826619. **MUB 49653:** Germany, KP825584, KP825719, KP825843, KP825972, KP826111, –, KP826360, KP826489, KP826620. **REN1:** North America, KC436485, KC436527, –, KC436578, KC436920, –, –, KC436727, –. **REN2:** North America, KC436486, –, –, KC436579, KC436921, –, KC437058, KC436728, –. **REN3:** North America, KC436487, KC436528, –, KC436580, KC436922, –, KC437059, KC436729, –. **REN4:** North America, KC436488, KC436529, –, KC436581, KC436923, –, KC437060, KC436730, –. **REN5:** North America, KC436489, –, –, –, –, –, –, KC436731, –. **REN6:** North America, KC436490, KC436530, –, KC436582, KC436924, –, KC437061, KC436732, –. **REN7:** North America, KC436491, KC436531, –, KC436583, –, –, KC437062, KC436733, –. **REN8:** North America, KC436492, KC436532, –, KC436584, KC436926, –, KC437063, KC436734, –. **S B201182:** Sweden, KP825586, KP825721, KP825849, –, KP826103, –, KP826356, KX503296, KP826616. **S B201183:** Sweden, KP825585, KP825720, KP825848, KP825973, KP826102, –, KP826355, KP826485, KP826615. **STG1:** North America, KC436493, KC436535, –, KC436586, KC436927, –, KC437064, KC436735, –. **STG2:** North America, KC436494, KC436536, –, KC436587, KC436928, –, KC437065, KC436736, –. **STG3:** North America, KC436495, –, –, KC436588, –, –, –, KC436737, –. **STG4:** North America, KC436496, KC436537, –, KC436589, KC436929, –, KC437066, KC436738, –. **STG5:** North America, KC436497, KC436538, –, KC436590, KC436930, –, KC437067, KC436739, –. **STG6:** North America, KC436498, –, –, KC436591, KC436931, –, KC437068, KC436740, –. **STG7:** North America, KC436499, KC436539, –, KC436592, KC436932, –, KC437069, KC436741, –. **STG8:** North America, KC436500, KC436540, –, KC436593, –, –, KC437070, KC436742, –. **WST1:** North America, KC436501, –, –, KC436594, KC436933, –, KC437071, KC436743, –. **WST2:** North America, KC436502, KC436541, –, KC436595, KC436934, –, KC437072, KC436744, –. **WST3:** North America, –, KC436542, –, KC436596, KC436935, –, KC437073, KC436745, –. **WST4:** North America, KC436504, KC436543, –, KC436597, KC436936, –, KC437074, KC436746, –. **WST5:** North America, KC436505, –, –, KC436598, –, –, –, KC436747, –. **WST6:** North America, –, KC436544, –, KC436599, KC436937, –, KC437075, KC436748, –. **WST7:** North America, KC436506, KC436545, –, KC436600, KC436938, –, KC437076, KC436749, –. **WST8:** North America, KC436507, –, –, KC436601, –, –, KC437077, KC436750, –.

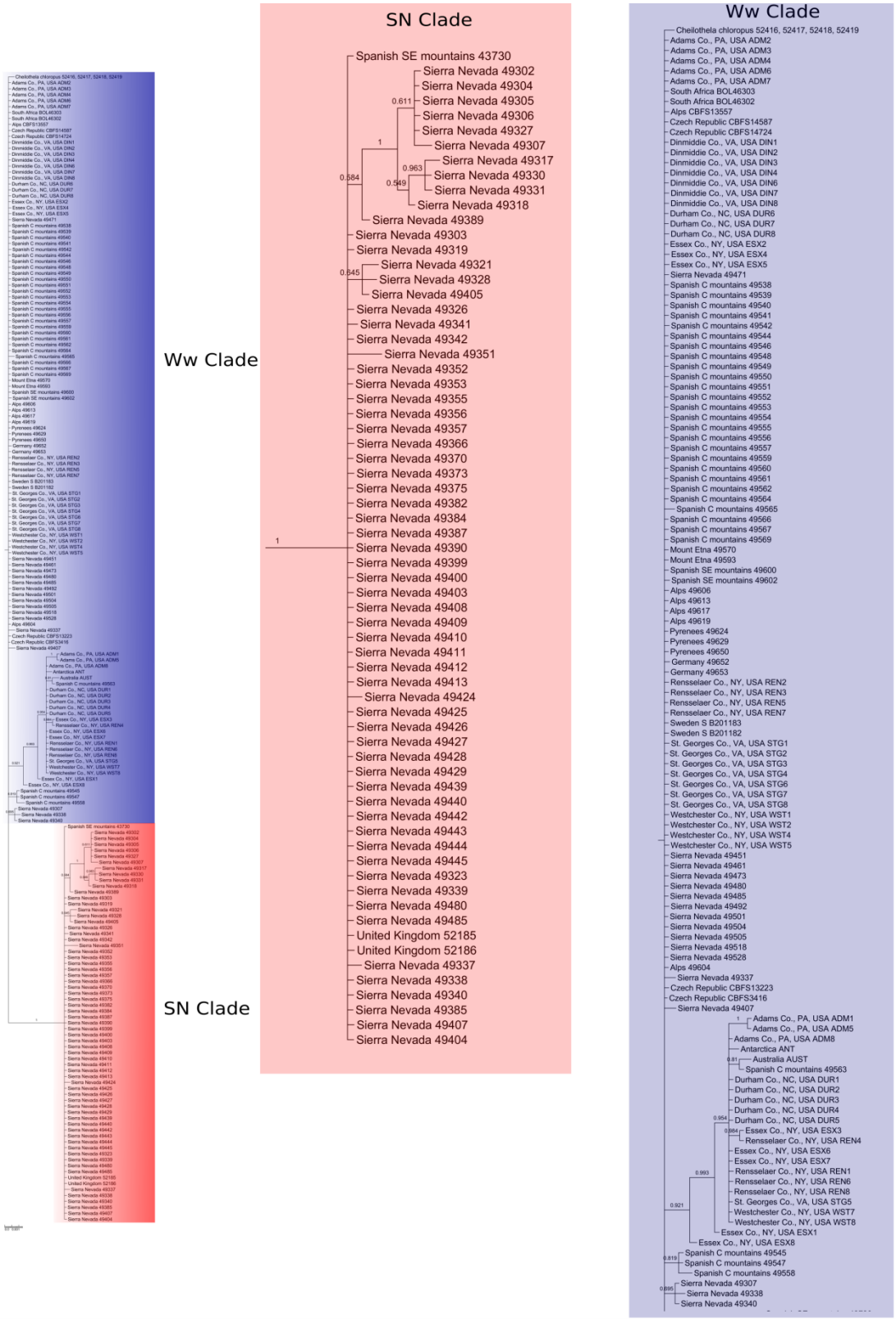
***Cheilothela chloropus* (Outgroup)**

MUB52416: Sierra Nevada Mountains, MH790731, KX503273, –, –, KX503281, –, MH790834, KX503299, KX503303. **MUB52417:** Sierra Nevada Mountains, MH790730, –, –, MH790817, KX503280, –, MH790835, KX503298, KX503302. **MUB52418:** Sierra Nevada Mountains, MH790729, –, –, –, KX503279, –, –, KX503297, KX503301. **MUB52419:** Sierra Nevada Mountains, MH790728, –, –, MH790816, KX503278, –, –, –, KX503300.

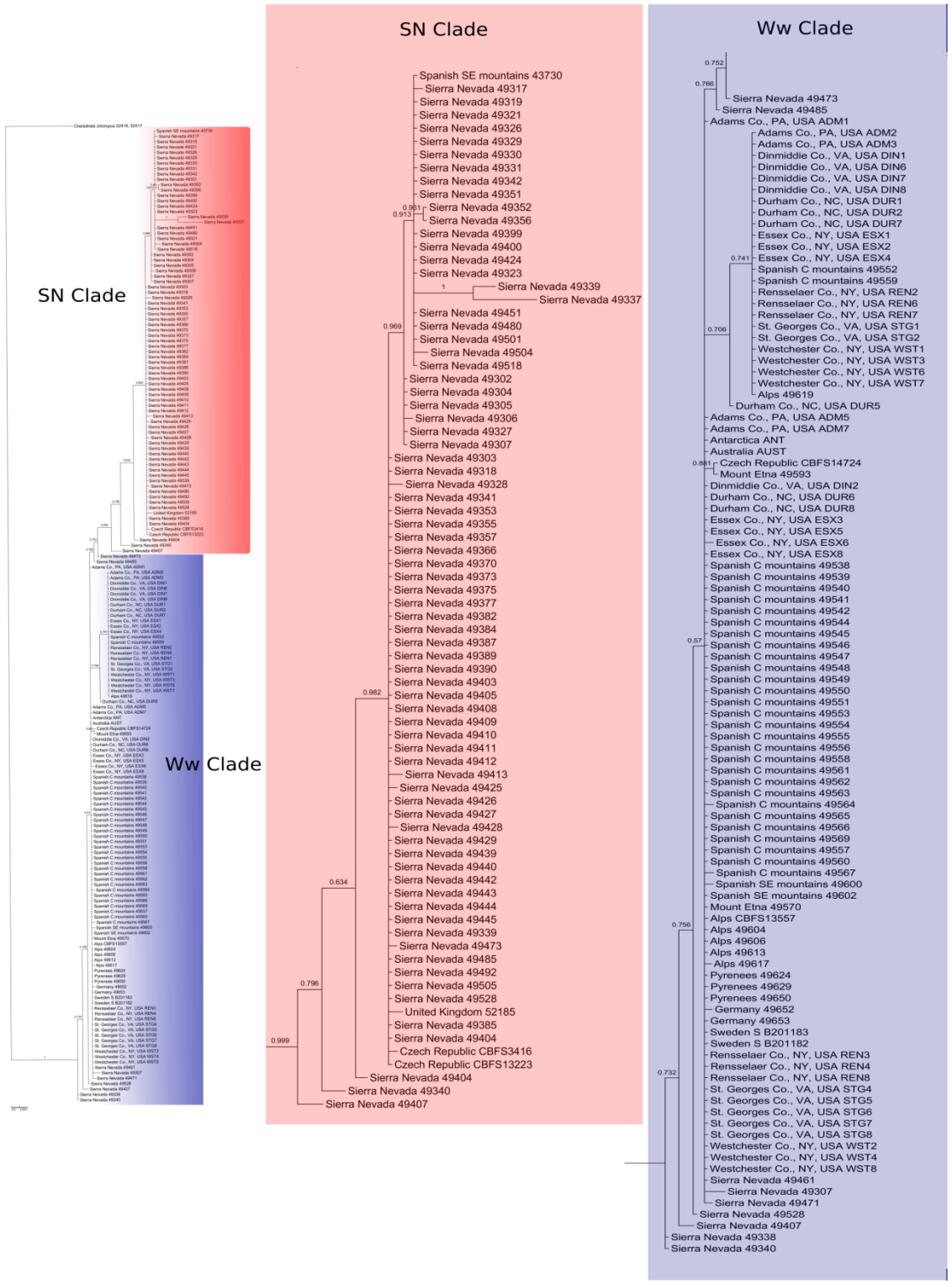
Annex IV.2. Bayesian phylogenetic tree inferred from the nuclear *ITS2 locus*. For each tip in the trees geographical origin and number of herbarium are given (numbers without letters are from MUB); number of equal sequences obtained by cloning is indicated between parentheses if there was more than one.



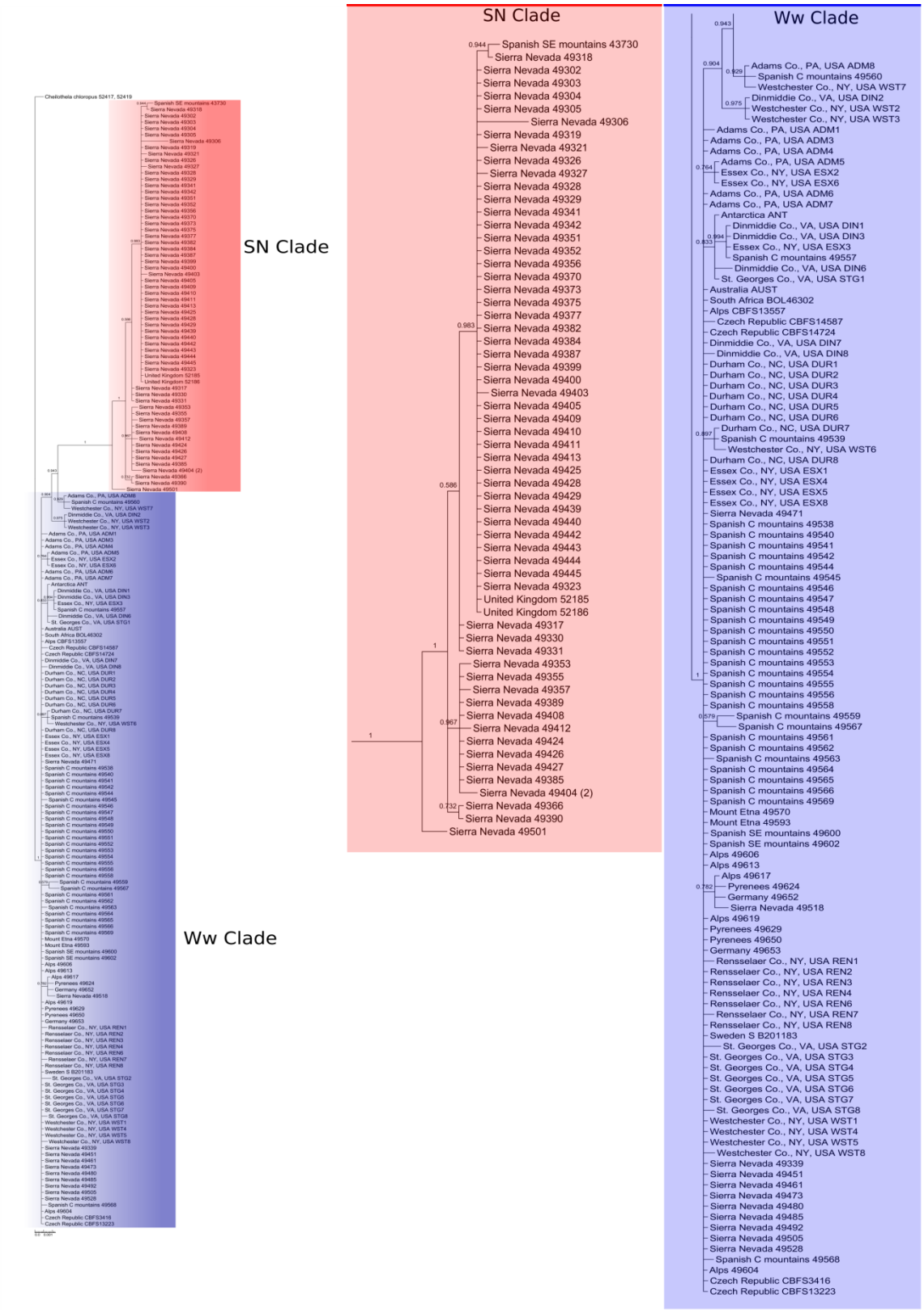
Annex IV.3. Bayesian phylogenetic tree inferred from the nuclear *hp23.3 locus*. Data for each tip as in Annex IV.2.



Annex IV.4. Bayesian phylogenetic tree inferred from the nuclear *rpS18A locus*. Data for each tip as in Annex IV.2.



Annex IV.5. Bayesian phylogenetic tree inferred from the nuclear *KIAA0187 locus*. Data for each tip as in Annex IV.2.



Annex IV.6. Results from ABC analyses assuming different scenarios of diversification and models of speciation for *Ceratodon amazonum* and *C. purpureus* as shown in Fig. IV.1. Values represent posterior probabilities of comparisons within each scenario, model or submodel. Preferred model at each comparison is highlighted in bold. A) Comparisons between the same scenario and model but with different migration patterns (submodels: homogeneous or heterogeneus across the genome; tolerance of 0.0005). B) Comparisons for different effective population sizes (homogeneous vs heterogeneous along the time of species; tolerance of 0.0005) among the best submodels selected for each before comparison. C) Comparison among the best models selected for each before comparison (tolerance of 0.005). Homo N: homogeneous effective population size; Hetero N: heterogeneous effective population size; IM: isolation with migration over time; AM: ancient migration assuming that populations started diverging in the presence of gene flow, T_{AM}; SC: Secondary contact in which populations diverged in the absence of gene flow followed by a single period of secondary contact, T_{SC}; SI: strict isolation, no migration; M homo: homogeneity of effective migration rate across the genome; M hetero: heterogeneity of effective migration rate across the genome.

	Scenarios	Models	Submodels	PP
A)	Homo N	IM	M homo	0.2353
			M hetero	**0.7647**
		AM	M homo	**0.6455**
			M hetero	0.3545
		SC	M homo	**0.7086**
			M hetero	0.2914
	Hetero N	IM	M homo	0.4228
			M hetero	**0.5772**
		AM	M homo	**0.5932**
			M hetero	0.4068
		SC	M homo	**0.7714**
			M hetero	0.2286
B)	Homo N	SI	--	**0.7657**
	Hetero N			0.2343
	Homo N	IM	M hetero	**0.9459**
	Hetero N		M hetero	0.0541
	Homo N	AM	M homo	**0.6995**
	Hetero N		M homo	0.3005
	Homo N	SC	M homo	**0.6235**
	Hetero N		M homo	0.3765
C)	Homo N	SI	--	0.0545
	Homo N	IM	M hetero	0.0591
	Homo N	AM	M homo	0.0560
	Homo N	SC	M homo	**0.8304**

General Discussion

DELIMITATION OF SPECIES IN BRYOPHYTES

One of the great current challenges in biology is to understand the diversity that surrounds us (Erwin, 1991; May, 1992; Stork, 1993; Mora et al., 2011; Grosberg et al., 2012), not only the number of species but also the evolutionary processes that generate this diversity (Rieseberg & Willis, 2007; Seehausen et al., 2014; Dev, 2015). Within this diversity, some species or species complexes possess elevated levels of morphological variation and a wide distribution. In these cases it is often difficult to determine whether the sub-units should be recognized as distinct species, because sometimes single species contain a morphological continuum with very different extremes (van Zanten & Pócs, 1981). Although the taxonomic treatment in these cases is often complicated, they can be very interesting models to study speciation mechanisms "in action". The results of the work carried out in this thesis highlight the gaps in knowledge about bryophytes diversity (and the mechanisms that generate it), even in common and abundant species, and demonstrate that wild cosmopolitan species have the potential to illustrate the several genetic causes of speciation despite of a certain level of gene flow between them. The broad morphological variation, together with the mistaken perception of the supposedly limited dispersion of the bryophytes, led to the past the overestimation of the diversity of the genus *Ceratodon*, reaching 22 species, and even within *C. purpureus* two subspecies and 31 varieties (Wijk et al., 1959, 1969) were described. But later the diversity of the genus *Ceratodon* was also underestimated when *C. purpureus* was considered a species with a high morphological plasticity, and probably the frequent demonstration of long-distance dispersal of certain species of bryophytes (van Zanten & Pócs, 1981; Muñoz et al., 2004; Parsons et al., 2007; Lönnell et al., 2012; Lewis et al., 2014; Norros et al., 2014; Biersma et al., 2017), and specifically *C. purpureus* (McDaniel & Shaw, 2005) may have contributed to this underestimation as well.

In this thesis has been shown that despite of the capacity for long-distance dispersal in the genus *Ceratodon*, the existence of *C. amazonum*, the determination of *C.* ×*conicus* as recombinant between *C. purpureus* and *C. amazonum*, and the presence of polyploid individuals, confirm that the number of species of *Ceratodon* may be

greater than was previously considered. Numerous authors have discussed the number of bryophyte species, due to the demonstration of the existence of cryptic species (Medina et al., 2012; Vigalondo et al., 2016, 2019; Patiño et al., 2017), in which the interpretation of morphological characters are complex and a broad concept of species is traditionally employed. The use of an integrative taxonomy is already widely accepted (Dayrat, 2005; Padial et al., 2010; Schlick-Steiner et al., 2010; Heethoff et al., 2011; Pante et al., 2015; Zamora et al., 2015; Renner et al., 2017) and can often solve the problems generated by each methodology individually, and provide data, not only to discriminate species, but also to understand the evolutionary processes that take place in different taxonomic groups. Employing at least three techniques seems adequate to reach a consensus on the discrimination between species in complex cases and also be able to infer the mechanisms of speciation.

In the present thesis, the delimitation of *Ceratodon* species has been made based on the results obtained with three different techniques (morphometry, phylogenetic analysis based on DNA sequences, and flow cytometry). If we had not used flow cytometry, we would have overlooked polyploidy an important mechanism of speciation in this genus. Polyploid recombinants have a similar morphology, both to *C. amazonum* and to haploid recombinants and their DNA sequences belong to the clade of *C. purpureus* or to *C. amazonum* depending on the *locus* studied and the specific individual. In *Ceratodon* it is possible to check the sex of plants by a simple test using restriction fragment length polymorphism of PCR amplified DNA fragments. If we had not checked the sex of the plants with this technique, in spite of not showing any external sign of sexual reproduction, we would not have detected the important bias of female individuals versus males in *C. amazonum*. Although three or more methodologies have been used in some animals (mites: Heethoff et al., 2011; bats: Taylor et al., 2018) and even in bryophytes (hornworts: Villarreal et al., 2017), the most studies on these plants use two of these methods: morphology (sometimes using statistical analysis) and DNA sequencing (employing two to six molecular markers, both nuclear and plastidial). Both seem sufficient, in most cases, to delimit species (Werner et al., 2003a, b, 2004, 2007, 2009, 2013, 2014, 2015; Ros & Werner, 2007; Köckinger et al., 2010, 2018; Medina et al., 2012, 2013; Draper et al., 2015; Caparrós et al., 2016; Renner et al., 2017; Mamontov & Vilnet, 2017; Schäfer-Verwimp et al., 2017; Sim-Sim et al., 2017; Kučera et al., 2018; Vigalondo et al., 2019).

However, we must prioritize the use of a greater number of genetic markers to obtain more solid results on the delimitation of species (Rokas & Carroll, 2005), as well as other methodologies that enrich evolutionary perspectives (Schlick-Steiner et al., 2010). It is important to note that in molecular plant systematics the use of several chloroplast markers is studied (e.g. *rps4*, *rbcL*, *trnL-F*…), but the only widely used nuclear marker is the *ITS* region (Liu et al., 2010; Stech & Quandt, 2010). But chloroplast markers are genetically linked and not independent, because chloroplast DNA has reduced recombination and usually chloroplasts are uniparentally inherited (reviewed in Birky, 2001; Odahara et al., 2015). On the contrary, nuclear markers recombine freely if they are located on different chromosomes and even if they are on the same chromosome recombination occurs during meiosis with certain frequency. As a consequence, different nuclear markers provide truly independent information while for chloroplast markers this is not the case. One of the major obstacles that impede a wider use of nuclear markers is the lack of universal primers to amplify specific nuclear regions. McDaniel et al. (2013) published a list of potential nuclear markers for bryophytes based on *Physcomitrella patens* and *Ceratodon purpureus* genome data. In this thesis we used a total of nine nuclear markers. This proved to be essential to establish the recombinant status of many samples because almost all of the recombinants share the chloroplast haplotypes of *C. amazonum*.

MECHANISMS OF SPECIATION IN BRYOPHYTES

The increase in the amount of DNA in the nuclei, polyploidization and the phenomena of hybridization and introgression are mechanisms that can give rise to groups of taxa with complex relationships with each other (Wyatt et al., 1988; Derda & Wyatt, 2000; Shaw et al., 2005; Ricca et al., 2008). We now know that polyploidy events occur in almost all vascular plant lineages of most of the current species (Wood et al., 2009; Alix et al., 2017; Soltis et al., 2018). These processes can generate taxonomic confusion and numerous misinterpretations during identification species process (Ennos et al., 2005). In some bryophytes such as *Anthoceros* L., few studies have documented phenomena of hybridization and polyploidy (Villarreal et al., 2014). However, in mosses and liverworts these issues have been treated more frequently. For example, in the moss

Racomitrium canescens (Hedw.) Brid., investigators have found no evidence of hybridization and the "morphological" species (despite the extensive morphological variability) correspond to the clades resulting in the phylogenetic analyses (Stech et al., 2013). However, it is common to find in the literature on bryophytes numerous examples with complex relationships. The family Funariaceae presents interfertility among its species, forming intricate networks such as the *Physcomitrella-Physcomitrium* complex, in which hybrid taxa such as *Physcomitrium collenchymatum* Gier and *P. eurystomum* Sendtn. have been formed from the same parents, *P. sphaericum* (C. Ludw.) Fürnr. and *P. pyriforme* (Hedw.) Hampe (McDaniel et al., 2010), with significant variations in the size of their genome (Beike et al., 2014). Moreover the genera *Entosthodon* Schwägr., *Physcomitrella* and *Physcomitrium* were determinated as polyphyletic entities with potential hybridizations (Liu et al., 2012; Medina et al., 2018), even using only sequences of organellar *loci* (reflecting the maternal history). Medina et al. (2018) found rapid diversification of the *Entosthodon-Physcomitrium* complex., potentially facilitated by a whole genome duplication event. In the liverwort complex *Aneura pinguis* (L.) Dumort, in which different morphologically indistinguishable but genetically different species are accepted, three clades with an intimate relationship with *A. mirabilis* (Malmb.) Wickett & Goffinet. were distinguished, demonstrating that the cryptic species of *A. pinguis* do not derive directly from a common ancestor (Myszczyński et al., 2017).

In *Ceratodon purpureus*, despite being a widely studied species, evidence for species beyond the widespread *C. purpureus* was lacking. Studying the intricate species complexes demonstrates our ability to delimit species and our vision of evolution and ecology. The greater size of the genome of *C. amazonum*, as well as the existence of polyploid individuals, provides valuable sources of genetic diversity, even in the future we could identify functionally important variants for ecological speciation. The distribution patterns of the species provide valuable data to understand their ecology and their possible current relationship with other species. But it is not known if cryptic speciation occurs between sympatric populations, or if it is allopatric, with a subsequent dispersal that leads to secondary sympatric distributions (Shaw, 2001). Probably the origin of many cryptic species is due to restricted mating and different ecological preferences, rather than geographical disjunction (Shaw, 2001). Signs of local adaptation (ecotypes) have been observed in bryophytes in different circumstances

(Jules & Shaw, 1994; Såstad et al., 1999; Gunnarsson et al., 2007; Szövényi et al., 2009; Hutsemekers et al., 2010; Pisa et al., 2013; Magdy et al., 2016). The pH and magnesium showed an adaptive role in the populations of *Sphagnum warnstorfii* Russow (Mikulášková et al., 2015, 2017), thus supporting some ecological isolation (due to strong environmental filters), without excluding the existence of a large-scale metapopulation. Even the mechanisms and processes of the origin of the diversity of cryptic species or species with a wide morphological variation are little known, especially in relation to environmental factors (Yu et al., 2013).

The morphological variation of the bryophyte species can be truly misleading due to a high morphological plasticity (Buryová & Shaw, 2005; Hassel et al., 2005) or by the adaptive convergence of distant taxa (Feldberg et al., 2010; Huttunen et al., 2012). Different environments directly induce changes in the morphology and physiology of a genotype, but if these changes (plasticity) are adaptive or not, has been widely discussed (Price et al., 2003; Pigliucci et al., 2006). The degree of plasticity is specific to each individual trait and environmental conditions (Pigliucci et al., 2006). The morphological characters can be determined at genetic level in different ways (different genes, different number of genes, epistasia...) in the different taxonomic groups; therefore, the way in which the attributes are expressed under the same environmental conditions can vary enormously (Huttunen et al., 2012). In addition, epigenetic mechanisms driven by environmental signals can promote long-term phenotypic changes without affecting the nucleotide sequences (reviewed in Balao et al., 2018).

The data presented here show that a greater percentage of morphological characters in *Ceratodon* vary more strongly due to environmental factors than to genetic factors, and only a few of them seem to be regulated more strongly by genetic factors, but we must not forget that there are also characters that are expressed differently according to the specific genotype-environment interaction and that can be very useful in the identification of species in field. Phenotypic plasticity influences environmental tolerance. Therefore, the greater the plasticity, the greater the range of environmental variation in which the species can inhabit will be (Ackerly et al., 2000). In addition, it allows the appearance of a new phenotype induced by the environment, and after new selective pressures on the expression of said phenotype appearance, it can end up

"fixing" it, that is, assimilated genetically (Pigliucci et al., 2006). Local adaptation may be impeded by weak selection or gene flow between populations, even if sufficient genetic variation is present (Antonovics, 1976). The complexity of the genetic structure that underlies phenotypic evolution is one of the classic problems of evolutionary biology (Orr & Coyne, 1992). Genetic analyzes and experiments with transplants are essential to discriminate between phenotypic plasticity and local adaptation (Nahum et al., 2008), and could explain a great variety of ecological evolutionary processes (Pigliucci et al., 2006). Studies of rapid climate change scenarios suggest that phenotypic plasticity may eclipse the importance of genetic diversity in species persistence, at least in the short term (Vitasse et al., 2010).

Establishing divergence time and understanding the processes of speciation are intimately connected and are central issues in the study of the evolutionary history of organisms (Soltis & Soltis, 2009), but their study is really complicated. The difficulty lies in the variations in the size of the ancestral population or in the genealogical variation between the different *loci*, indicating the mode of speciation according to the presence or absence of gene flow (Edwards & Beerli, 2000; Wu, 2001; Tseng et al., 2014). The divergence between species will be more complex if there is gene flow between them (Abbott et al., 2013; Leaché et al., 2014), and it is fundamental to consider the heterogeneity along the genome in the rates of introgression (Roux et al., 2013). Evidence of genetic exchange between *C. purpureus* and *C. amazonum* is clear when observing the incongruities between the phylogenetic trees made independently for each *locus*, which also allows the detection of recombinant individuals. But also, in the case studied, these have originated from repeated hybridization events, giving different genetic mosaics among the recombinants. All this, together with the determination of the demographic parameters, reveals a recent episode of speciation possibly formed from an isolated peripheral population (peripatric speciation), with gene flow, a female biased sex-ratio, and an increase in the size of the genome.

ONGOING PROJECTS AND FUTURE PERSPECTIVES

Although the delimitation of *Ceratodon* species has been made integrating different methodologies, there are still important aspects to be treated and discovered, such as the biogeography of *C. amazonum* and *C. ×conicus*. If the determining factor in speciation were microclimatic, the distribution of *C. amazonum* could spread through North Africa or other areas of the Mediterranean, since morphologies similar to *C. amazonum* / *C. ×conicus* have been observed in Morocco (for example, the type specimen of *C. cedricola*). However, certain genetic incompatibilities have been found at the extremes of the morphological variation of *C. purpureus* (McDaniel et al., 2007, 2008), which together with the genetic porosity (*C. ×conicus*) observed in the crosses between *C. amazonum* and *C. purpureus*, and the presence of polyploid individuals, highlight the need to study the genomes of these species and understand the genetic architecture that allows the formation of new species in *Ceratodon*. We are already on the way to explore a greater number of genes through massive sequencing techniques (GBS), both of *C. amazonum* and of *C. ×conicus*, which will be compared with the worldwide data of *C. purpureus*. In addition, studying the epigenetic effects of natural populations in the long term to understand their morphological or physiological responses to environmental fluctuations would generate a deeper knowledge of the origin of morphological variation and evolutionary mechanisms.

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Conclusions

The main conclusions of this thesis are the followings:

1. The lectotype of *Ceratodon conicus* designated by Burley and Pritchard (1990) is confirmed, but not the isolectotypes, as they cannot be considered part of the type material.

2. The types of the three synonyms of *C. conicus* proposed by Burley and Pritchard (1990), namely *C. cedricola*, *C. dimorphus*, and *C. purpureus* var. *graefii*, designated as holotypes, are here considered as lectotypes, because in any case the author of the names designated any specimen as the nomenclatural type and no unequivocal specimen exists.

3. The conspecifity of *C. cedricola* and *C. dimorphus* with *C. conicus* is confirmed, but not that of *C. purpureus* var. *graefii* which is identified as *C. purpureus s.l.*, as its morphological characteristics match the description of this species and not that of *C. conicus*.

4. A high genetic diversity is observed in Mediterranean populations of *Ceratodon* delimiting two phylogenetic entities, which show also different genome size. One corresponds to the widespread *C. purpureus* and the other to a local group to our present knowledge restricted to the mountains in southern Spain. Also recombinants are detected.

5. The local phylogenetic entity is considered to be a new species, which is described as *C. amazonum*, and differs from the cosmopolitan *C. purpureus* by a 25% increase in genome size, significant differences in several DNA sequence markers, and a big decrease in frequency of males. Given the complete absence of sporophytes in *C. amazonum*, the predominant reproduction way seems to be by fragmentation of the gametophores.

6. Recombinant specimens are haploid and polyploid. *Ceratodon* ×*conicus* belongs to this group. Therefore it is considered a nothospecies, with cosmopolitan *C. purpureus* as male parental line and *C. amazonum* as female parental line.

7. The number of morphological characters in *Ceratodon* that vary stronger due to environmental factors than to genetic factors is higher than the number of morphological characters that vary stronger due to genetic factors.

8. *Ceratodon purpureus* can be distinguished morphologically from *C. amazonum* and *C.* ×*conicus*. But it is not possible to distinguish between the last two in field samples. Neither there are morphological differences between haploid and polyploid *C.* ×*conicus.*

9. The most relevant characters to discriminate *C. amazonum* and *C.* ×*conicus*. from *C. purpureus* are the followings: caulidia length, presence/ausence of comal tuft, phyllidia shape and length, lamina length/lamina width ratio, nerve wide, percurrent/excurreny awn, awn length, excurrent nerve length/lamina length ratio, and lamina length/nerve width ratio. While *C. amazonum* and *C.* ×*conicus* can be weakly differentiated by shape of phyllidia, lamina length/lamina width ratio, nerve wide, awn length, and lamina length/nerve width ratio.

10. The complexity of the morphological variation within *C. purpureus* is highlighted. Neither *C. heterophyllus* nor *C. purpureus* subsp. *stenocarpus* can be differentiated by morphological analysis of gametophytic characters.

11. *Ceratodon amazonum* is until now only known from southern Spain, mainly in Sierra Nevada Mountains, where it is very abundant. The opposite occurs with *C. purpureus*, being prevalent in other geographical areas but almost absent in Sierra Nevada. *Ceratodon* ×*conicus* has a broad distribution across Europe from Sierra Nevada Mountains to United Kingdom and central Europe, but it is very scarce. The type of substrate does not seem to be a limiting factor in the distribution of *Ceratodon* species here studied.

12. A peripatric speciation event in the genus *Ceratodon* seems to have occurred around 1.7 mya, giving rise to *C. amazonum* and *C. purpureus*. Both have high levels of genetic diversity although *C. purpureus* produces abundant spores, whereas *C. amazonum* does not. *Ceratodon* ×*conicus* was originated from multiple, independent and recent hybridization events.