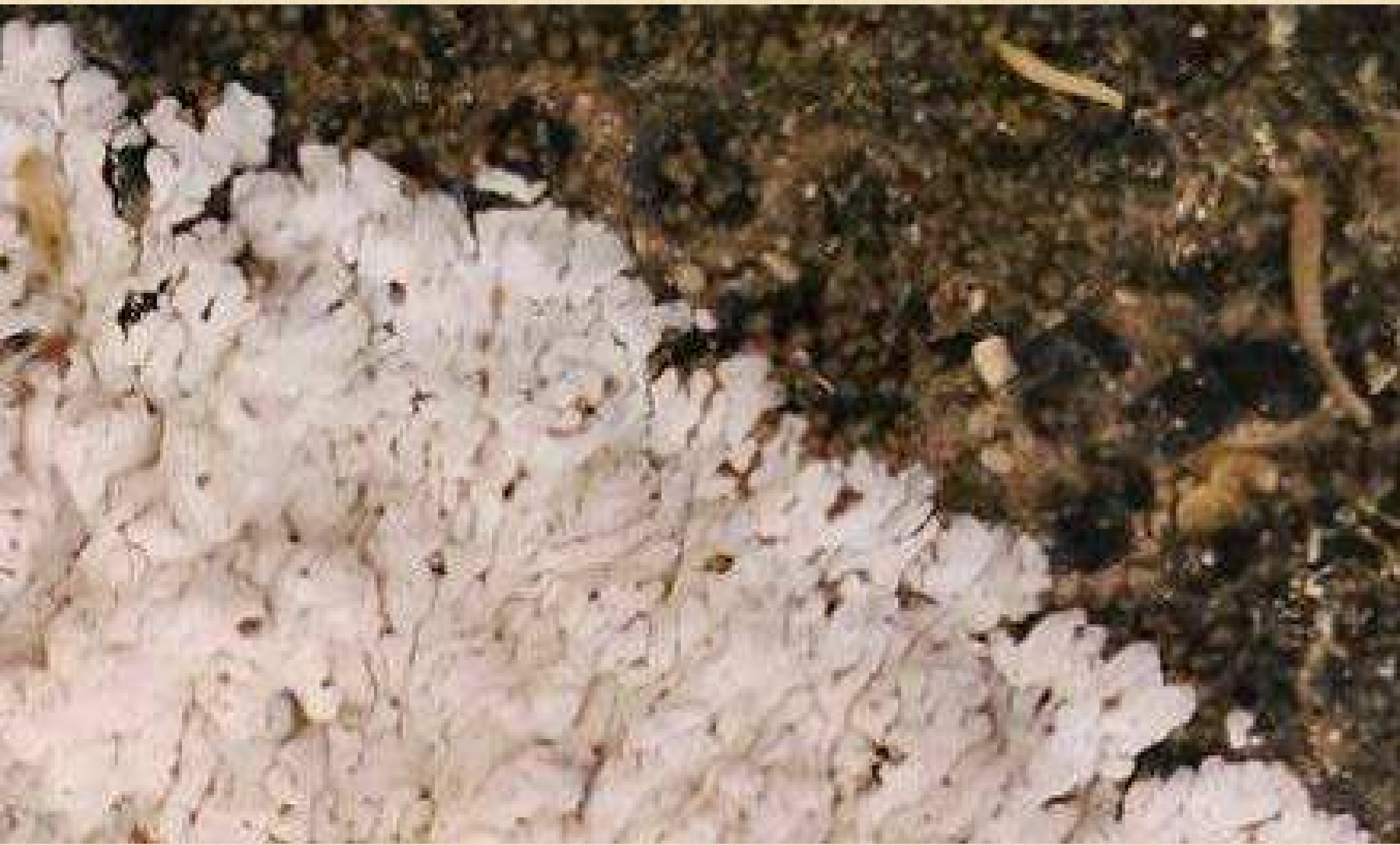
TESIS DOCTORAL

PATRONES DE SELECCIÓN DE MICROALGAS EN COMUNIDADES DE LÍQUENES TERRÍCOLAS EN BIOCOSTRAS

Salvador Chiva Natividad



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Facultat de Ciències Biològiques
Departamento de Botánica y Geología

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Tesis Doctoral

Patrones de selección de microalgas en comunidades de líquenes terrícolas en biocostras

José Salvador Chiva Natividad

Directora/Tutora: Eva Barreno Rodríguez
Directora: Patricia Moya Gay
Directora: Arantzazu Molins Piqueres

Programa de Doctorado en Biodiversidad y Biología Evolutiva
Valencia, enero 2020

Facultat de Ciències Biològiques
Departamento de Botánica y Geología

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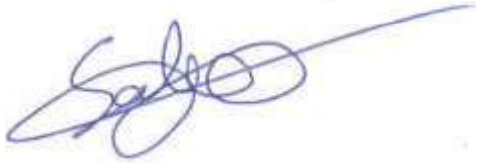
de Biodiversitat i Biologia Evolutiva

Tesis presentada por **José Salvador Chiva Natividad** para optar al grado de Doctor en Ciencias Biológicas por la Universitat de València, con el título:

Patrones de selección de microalgas en comunidades de líquenes terrícolas en biocostras

Firmado: José Salvador Chiva Natividad



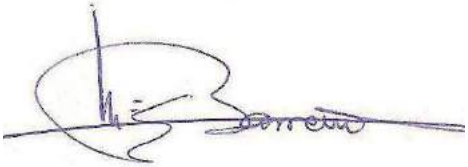
VNIVERSITAT DE VALÈNCIA

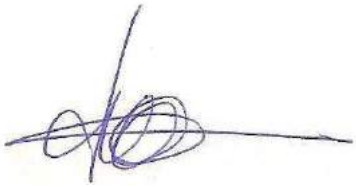
La Dra. **Eva Barreno Rodríguez**, Catedrática del Departamento de Botánica y Geología de la Facultad de Ciencias Biológicas de la Universitat de València; la Dra. **Patricia Moya Gay** y la Dra. **Arantzazu Molins Piqueres**.

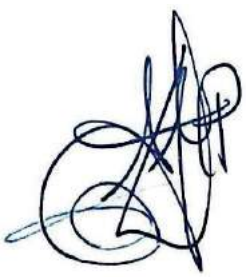
CERTIFICAN que el licenciado en Biología **José Salvador Chiva Natividad** ha realizado bajo su dirección el trabajo **Patrones de selección de microalgas en comunidades de líquenes terrícolas en biocostras**, y autorizan su presentación para optar al título de Doctor de la Universitat de València.

Y para que así conste, en cumplimiento de la legislación vigente, firmamos el presente certificado en Burjassot, en octubre de 2019.

Fdo.: Eva Barreno Rodríguez	Fdo.: Patricia Moya Gay	Fdo.: Arantzazu Molins Piqueres
Directora/ Tutora de la Tesis	Directora de la Tesis	Directora de la Tesis







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CERTIFICAN que las revistas donde se han publicado los artículos que se integran en la Tesis Doctoral de **José Salvador Chiva Natividad**, titulada: **Patrones de selección de microalgas en comunidades de líquenes terrícolas en biocostras**, se encuentran indexadas. A continuación, se especifica para cada revista el índice de impacto, el cuartil y la posición en el área que corresponde con el año de publicación.

Revista / Año		Índice de impacto	Cuartil	Posición en el área	
IJSEM	2015	2.493	Q3	65/123	Microbiology
Fottea	2018	1.727	Q2	100/228	Plant Sciences
Journal of Biogeography	2018	3.884	Q1	34/164	Ecology

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Directora de la Tesis

Fdo.: Arantzazu Molins Piqueres
Directora de la Tesis

Publicaciones científicas de la presente Tesis Doctoral

Como resultado de esta Tesis Doctoral se han publicado los siguientes artículos de investigación:

- Moya P, Škaloud P, **Chiva S**, García-Breijo FJ, Reig-Arminana J, Vancurova L & Barreno E (2015). Molecular phylogeny and ultrastructure of the lichen microalga A*sterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems. *International Journal of Systematic and Evolutionary Microbiology*, 65, 1838-1854.

- Moya P, **Chiva S**, Molins A, Jadrná I, Škaloud P, Peksa O, & Barreno E (2018). *Myrmecia israeliensis* as the primary symbiotic microalga in squamulose lichens growing in European and Canary Island terricolous communities. *Fottea*, 18, 72-85.

- **Chiva S**, Garrido-Benavent I, Moya P, Molins A & Barreno E (2019). How did terricolous fungi originate in the Mediterranean region? A case study with a gypsicolous lichenized species. *Journal of Biogeography*, 46, 515-525.

Manuscrito original para enviar:

- Moya P, Molins A, **Chiva S**, Bastida J & Barreno E. Symbiont interaction patterns in biocrust lichen communities located in semi-arid gypsum outcrops in the Central Iberian Peninsula. *Microbial ecology - Soil microbiology*.

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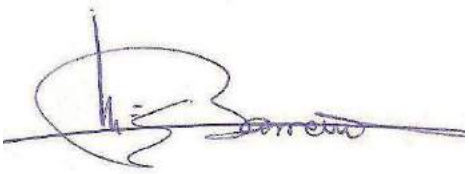
La Dra. **Eva Barreno Rodríguez**, Catedrática del Departamento de Botánica y Geología de la Facultad de Ciencias Biológicas de la Universitat de València; la Dra. **Patricia Moya Gay** y la Dra. **Arantzazu Molins Piqueres**.

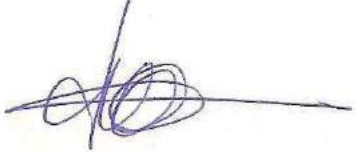
INFORMAN con el objetivo de cumplir con la legislación vigente en la Universitat de València, por lo que respecta a la presentación de Tesis Doctorales por compilación de artículos, que la Tesis Doctoral de **José Salvador Chiva Natividad**, titulada: **Patrones de selección de microalgas en comunidades de líquenes terrícolas en biocostras**, incluye tres artículos publicados en las revistas indicadas en el documento que corresponde. Destacamos que el doctorando es el primer autor de la publicación de la revista con mayor índice de impacto. En las otras dos publicaciones no es el primer autor, para la justificación de la autoría se redactó un informe donde las directoras indicaron las funciones del doctorando en cada uno de los artículos (a continuación). Dicho informe fue aceptado por la Comisión de Doctorado permitiendo la exposición de esta Tesis. Así mismo, es necesario indicar que el resto de firmantes de dichos artículos son Doctores, por lo cual, el trabajo no ha estado, ni estará, formando parte de otra Tesis doctoral.

Y para que así conste, en cumplimiento de la legislación vigente, firmamos el presente informe en Burjassot, en octubre de 2019.

Fdo.: Eva Barreno Rodríguez
Directora/ Tutora de la Tesis



Fdo.: Patricia Moya Gay
Directora de la Tesis



Fdo.: Arantzazu Molins Piqueres
Directora de la Tesis

Valencia, 21 de septiembre de 2018

Eva BARRENO RODRÍGUEZ, catedrática de Botánica de la Universitat de València, me dirijo a ustedes como directora del grupo de investigación del Instituto Cavanilles de Biodiversidad y Biología Evolutiva: "Biodiversidad Vegetal: Ecofisiología".

SOLICITO, de la comisión de doctorado, en mi nombre y en el de las otras dos directoras, el permiso para la presentación de la Tesis doctoral de José Salvador Chiva Natividad con título provisional: "Especificidad y variabilidad de los ficobiontes identificados en líquenes de comunidades gipsícolas" **por compendio de publicaciones.**

A continuación, se justifica por parte de la directora de la tesis Eva Barreno Rodríguez y las codirectoras Patricia Moya Gay y Arántzazu Molins Piqueres la posición del doctorando en la autoría de dos publicaciones incluidas en dicha tesis y su implicación en cada uno de ellos:

> **1**-Moya, P., Škaloud, P., **Chiva, S.**, García-Breijo, F. J., Reig-Armiñana, J., Vančurová, L., & Barreno, E. (2015). Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems. International journal of Systematic and evolutionary Microbiology, 65(6), 1838-1854.
>
> **2**- Moya, P., **Chiva, S.**, Molins, A., Jadrná, I., Škaloud, P., & Eva Barreno, O. P. (2018). *Myrmecia israeliensis* as the primary symbiotic microalga in squamulose lichens growing in European and Canary Island terricolous communities. FOTTEA, 18(1), 72-85.

En diciembre del 2012 Salvador Chiva presentó el trabajo de final de máster titulado "Especificidad y variabilidad de los ficobiontes identificados en líquenes de comunidades gipsícolas", debido a los novedosos resultados obtenidos se consideró interesante la realización de una tesis doctoral basada en dicho TFM, así pues, el estudiante se inscribió en el Programa de doctorado "Biodiversidad y biología evolutiva" en el curso 2014-2015.

1. La redacción del primer artículo mencionado y que suponía la descripción de una nueva especie del género *Asterochloris*, se realizó en colaboración con el mejor experto en este género que, debido a sus conocimientos en técnicas que se emplean en la publicación, se decidió colocar como segundo firmante (Pavel s). Para dicho artículo se utilizaron y ampliaron datos obtenidos por Salvador Chiva en su TFM.

A continuación, se especifican los métodos y análisis del estudio que llevó a cabo únicamente el estudiante de doctorado:

Taxa sampling/ Transmission electron microscopic/ Scanning electron microscopy /DNA isolation, amplification and sequencing/ Haplotype network.

Morphological and ultrastructural characterization/Phycobiont phylogenetic analyses/ Mycobiont phylogenetic analyses.

Sin embargo, fue la primera firmante y codirectora de esta tesis quien coordinó la redacción final y publicación del artículo (necesaria para la justificación de la financiación recibida), mientras el estudiante, que todavía estaba en su primer año de doctorado (2015) se encargaba de finalizar los muestreos, de las extracciones de ADN, del análisis de las secuencias y de la recopilación de datos para siguientes publicaciones. Dichos muestreos y procesados de datos, realizados exclusivamente por Salvador Chiva, han sido cruciales para la posterior redacción de varias publicaciones y de su tesis doctoral.

Por todo ello, se consideró adecuado la posición en tercer lugar del estudiante en este trabajo, aunque su implicación fue clave para la publicación del mismo.

2. De nuevo, mediante otra colaboración con el grupo de la Universidad de Praga, y utilizando los datos generados en el TFM de Salvador Chiva, se finalizó la redacción del segundo artículo. Dicho estudio, que fue de mayor complejidad, ha supuesto la confirmación de un género nuevo de microalga simbionte que es la que predomina en un amplio grupo de líquenes escuamulosos y con hábitat terrícola.

A continuación, se especifican los métodos y análisis del estudio que llevó a cabo únicamente Salvador Chiva:

Lichen material/Sample preparation/DNA extraction, amplification and sequencing/ Phycobiont and mycobiont phylogenetic analyses/ Microscopic investigations "in thallus"

Phycobiont and mycobiont phylogenetic analyses/ Morphological and ultrastructural characterization of *Myrmecia israeliensis* in the thallus

Debido a la mayor implicación del estudiante en la redacción de la publicación se encuentra en dicho artículo en segunda posición.

Además, el estudiante se ha formado en biogeografía y filogeografía para aplicar complejos análisis estadísticos al estudio poblacional de *Buellia zoharyi*, una interesante especie de liquen, de distribución circunmediterránea *s.o.* y canaria, el cual parte también de los datos generados en su TFM.

Actualmente, han aceptado en *Journol of Biogeography* el manuscrito titulado: "Phylogeography of the gypsicolous lichenized fungus *Buellia zoharyi* (Caliciaceae, Ascomycota) in the Mediterranean region. **Salvador Chiva**, Isaac Garrido-Benavent, Patricia Moya, Arantzazu Molins and Eva Barreno.

Dicha publicación se incluirá en la tesis doctoral, junto con un estudio en fase de redacción, en el que Salvador Chiva es primer firmante y que versa sobre la diversidad y coexistencia de ficobiontes de *B. zoharyi*. Con este artículo se cierra el compendio de artículos incluidos en esta tesis doctoral.

Por supuesto, ninguno de los artículos mencionados en este escrito han sido utilizados en la defensa de otras tesis y son un trabajo exclusivo de Salvador Chiva.

Por todo esto, consideramos adecuadamente justificada la implicación del estudiante en aquellos trabajos incluidos en su tesis doctoral en los que no figura como primer firmante.

A continuación, se detallan también los congresos en los que el estudiante ha presentado posters o comunicaciones orales relacionadas con su tesis doctoral:

Chiva, S., Moya, P., Molins, A. & Barreno E. Relaciones entre el micobionte y el ficobionte de las comunidades de la CBS. VIII Jornadas de Liquenología de la Sociedad Española de Liquenología. Sierra Nevada, Granada. Septiembre 2018. Tipo de comunicación: Oral

Chiva, S., Moya, P., Molins, A., Garrido-Benavent, I. & Barreno E. Hints that different geological events in the mediterranean had influence over the distribution of *Buellia zoharyi*. XXI Simpósio de Botánica Criptogámica Aranjuez, Madrid. Julio 2017. Tipo de comunicación: Oral.

Chiva, S., Moya, P., Molins, A., Jadrnà, I., Škaloud, P., Peksa, O. & Barreno, E. *Myrmecia israelensis* as the primary symbiotic microalga in terricolous squamulose lichens across Europe and Canary Islands. XXI Simpósio de Botánica Criptogámica Aranjuez, Madrid. Julio 2017. Tipo de comunicación: Póster

Chiva, S., Moya, P., Molins, A. & Barreno E. Análisis de la estructura poblacional de *Buellia zoharyi*: liquen con baja diversidad genética y distribución geográfica amplia y disyunta. VII Jornadas de Liquenología de la Sociedad Española de Liquenología. Ronda, Málaga. Septiembre 2016. Tipo de comunicación: Oral.

Moya, P., **Chiva, S.**, Molins, A., Reig-Armiñana, J., García-Breijo, F.J. & Barreno E. Improved propagation method, rapid molecular identification and ultrastructural characterization as a multidisciplinary approach for *Trebouxia* species delimitation. Meeting of the TREBOUXIA-WORKING group. Septiembre 2016. Tipo de comunicación: Oral.

Chiva, S., Moya, P., Molins, A., Reig-Armiñana, J., García-Breijo, F.J. & Barreno E. *Buellia zoharyi* populations show noticeable microalgal diversity throughout their entire range of distribution. The 8th IAL Symposium Lichens in Deep Time. Agosto 2016. Tipo de comunicación: Póster.

Moya, P., Molins, A., **Chiva, S.**, Reig-Armiñana, J., García-Breijo, F.J. & Barreno E. Complexity of lichen simbiosis: genomic diversity and coexistence of various green microalgae in the epigeous lichen Buellia zoharyi. EMBO. Exploring the genomic complexity and diversity of eukaryotes. Octubre 2015. Tipo de comunicación: Póster.

Chiva, S., Moya, P., Molins, A., Reig-Armiñana, J., García-Breijo, F.J. & Barreno E. Coexistence and prevalence of symbiotic microalgae in *Buellia zoharyi* lichen: are substrata and/or biogeographic barriers involved? XX Simpósio de Botánica Criptogámica Oporto, Portugal. Julio 2015. Tipo de comunicación: Oral.

Reciban un cordial saludo.

Eva Barreno Rodríguez Patricia Moya Gay Arantzazu Molins Piqueres

DIFUSIÓN DE RESULTADOS RELACIONADOS CON ESTA TESIS DOCTORAL

- **Chiva S**, Moya P, Molins A, Barreno E (2019) "Microalgal selectivity patterns in biocrusts lichen communities". *XXII Simposio de Botánica Criptogámica*. Lisboa, 24 – 26 julio. Tipo de participación: **oral**.
- **Chiva S**, Moya P, Molins A, Barreno E (2018) "Biological Soil Crusts: myco/phycobiont relationships in terricolous lichen communities". *The complexity of lichen symbiosis: novel interdisciplinary approaches from genomic to functional perspectives*. Valencia, 3 – 12 diciembre. Tipo de participación: **oral**.
- **Chiva S**, Moya P, Molins A, Barreno E (2018) "Relaciones entre el micobionte y el ficobionte de las comunidades de la CBS". *VIII Jornadas de Liquenología – Sociedad Española de Liquenología (SEL)*. Sierra Nevada, 8 septiembre. Tipo de participación: **oral**.
- **Chiva S**, Moya P, Molins A, Garrido-Benavent I, Barreno E (2017) "Hints that different geological events in the Mediterranean had influence over the distribution of *Buellia zoharyi*". *XXI Simposio de Botánica Criptogámica*. Aranjuez, 20 – 24 junio. Tipo de participación: **oral**.
- **Chiva S**, Moya P, Molins A, Jadrná I, Škaloud P, Peksa O, Barreno E (2017) "*Myrmecia israeliensis* as the primary symbiotic microalga in terricolous squamulose lichens across Europe and Canary Islands". *XXI Simposio de Botánica Criptogámica*. Aranjuez, 20 – 24 junio. Tipo de participación: **póster**.
- Moya P, **Chiva S**, Molins A, García-Breijo Fj, Reig-Armiñana J, Barreno E (2016) "Improved propagation method, rapid molecular identification and ultrastructural characterization as a multidisciplinary approach for *Trebouxia* species delimitation". *Trebouxia* workshop. Trieste, 26 - 28 septiembre. Tipo de participación: **oral**.
- **Chiva S**, Moya P, Molins A, Garrido-Benavent I, Barreno E (2016) "Análisis de la estructura poblacional de *Buellia zoharyi*: liquen con baja diversidad genética y distribución geográfica amplia y disyunta". *VII Jornadas de Liquenología – Sociedad Española de Liquenología (SEL*). Ronda, 6 – 10 septiembre. Tipo de participación: **oral**.
- **Chiva S**, Moya P, Molins A, Reig-Armiñana J, García-Breijo Fj, Barreno E (2016) "*Buellia zoharyi* populations show noticeable microalgal diversity throughout their entire range of distribution". *The 8th IAL (International Association for lichenology) Symposium*. Helsinki, 1 – 5 agosto. Tipo de participación: *póster*.
- Moya P, Molins A, **Chiva S**, García-Breijo Fj, Reig-Armiñana J, Barreno E (2015) "Complexity of lichen symbiosis: genomic diversity and coexistence of various green microalgae in the epigeous lichen *Buellia zoharyi*". *EMBO - Exploring the genomic complexity and diversity of eukaryotes*. Sant Feliu de Guíxols, 17-22 octubre. Tipo de participación: **póster**.
- **Chiva S**, Moya P, Molins A, Reig-Armiñana J, García-Breijo Fj, Barreno E (2015) "Coexistence and prevalence of symbiotic microalgae in *Buellia zoharyi* lichen: are substrata and/or biogeographic barriers involved?" *XX Simposio de Botánica Criptogámica*. Porto, 22 – 25 julio. Tipo de participación: **oral**.
- Moya P, **Chiva S**, Barreno E (2013) "Coexistence and switching of chlorobionts (symbiotic microalgae) between Lichens and Lichenicolous lichens". *EMBO - European Molecular Biology Organization*. Girona, 19 – 24 octubre. Tipo de participación: **póster**.

Esta Tesis Doctoral se ha financiado gracias a los siguientes proyectos:

- **Genoma de *Trebouxia* sp. TR9 como modelo de alga verde simbionte: caracterización, potencial metabólico y estructural. Implicaciones de la coexistencia con otros simbiontes en talos liquénicos y plantas soporte. PROMETEOII/2013/021**

  Entidad financiadora: Ayudas para el desarrollo de acciones científicas del programa de investigación de Excelencia Prometeo. Consellería de Educación, Dirección General de Política Científica, Generalitat Valenciana.

- **Nueva perspectiva interdisciplinar sobre la complejidad de las simbiosis liquénicas: estudio genómico y funcional de microalgas y bacterias. SYMBIOLICHEN. CGL2016-79158-P**

  Entidad financiadora: Ministerio Economía y Competitividad.

- **Lichen symbiosis as a complex mutualistic association and paradigm of resilience to adverse environments. Genomic, structural and functional diversity. PROMETEO/2017/039**

  Entidad financiadora: Ayudas para el desarrollo de acciones científicas del programa de investigación de Excelencia Prometeo. Consellería de Educación, Dirección General de Política Científica, Generalitat Valenciana.

AGRADECIMIENTOS

En primer lugar, quisiera agradecer a mi directora de tesis Eva Barreno el darme la oportunidad de aprender junto a ella (y su equipo) todo sobre los líquenes y este mundo de la ciencia. Aún recuerdo el día en que, después de recolectar líquenes en Monfragüe para hacer un trabajo de ecología, J. Antonio Gil-Delgado me acompañó a su despacho para que me aconsejara sobre este trabajo. Desde ese mismo momento tuve abiertas las puertas de su laboratorio. Gracias por tu confianza, Eva. Aunque una casualidad hizo que me presentara en tu despacho siendo un niño tímido con curiosidad por los líquenes, gracias a ti he podido desarrollarme profesionalmente. Tu gran entusiasmo por la investigación es contagiosa, y después de unos pocos días bajo tu lupa identificándolos, supe que los líquenes iban a ser importantes en mi vida. Gracias por contagiarme la pasión por ellos y confiar en mí. Muchas gracias Eva.

A mis otras directoras, Txa y Pata, no puedo estar menos agradecido, lo han sido todo para mí personal y profesionalmente durante estos años de trabajo en el equipo de Eva Barreno. Me han enseñado todo lo que sé sobre las técnicas de laboratorio y si me habéis podido transmitir una parte de vuestra calidad investigadora ya me doy por satisfecho. Hemos trabajado mucho para finalizar esta tesis y el agotamiento ha sido fuerte, pero también han sido fuertes las risotadas que hemos compartido. Muchas gracias por los consejos. Muchas gracias por todo, siempre voy a estar en deuda con vosotras.

Mi gratitud para con todos los compañeros que han formado parte del equipo, en especial a Cristina, Ernesto, Fernando, Guillermo, Isaac, Santi, Sergio y Raquel, por los buenos momento que hemos pasado.

Gracias a todas las personas que han colaborado de forma desinteresada en las recolecciones de líquenes para los distintos estudios de esta tesis.

También quiero agradecer a mis padres que siempre me han apoyado en todo lo que me he propuesto. Gracias por la paciencia que habéis tenido conmigo.

A mi hermano Víctor, no sabes lo orgulloso que estoy de ti. Eres uno de los motores de mi vida.

Patricia, tú sí que has tenido paciencia conmigo… Gracias por tu apoyo incondicional, sabes que te necesito.

Y por ultimo, dedicarle este trabajo a mi otro motor, Eloi. Hay niños que vienen con un pan debajo del brazo, y otros, como tú, que vienen con una tesis.

Salvador Chiva

Tabla de contenidos

RESUMEN

Las costras biológicas de los suelos (biocostras, CBS) están constituidas por una combinación de organismos, tanto fotoautótrofos como heterótrofos, que viven dentro o sobre la superficie de los suelos, los cuales, con sus interacciones y actividades, generan una capa conjunta con las partículas del sustrato. Los líquenes, debido a la complejidad de sus simbiosis son capaces de colonizar estos hábitats adversos para otros vegetales, gracias a sus adaptaciones ecofisiológicas. Las biocostras dominadas por líquenes son muy abundantes en los claros de los matorrales y pastizales que se desarrollan en los territorios yesíferos. Su diversidad, participación en los ciclos biogeoquímicos y potencial para formar grandes coberturas (a veces> 80%) sobre los suelos, es importante para la gestión y conservación de estos entornos tan frágiles y amenazados.

En la cuenca mediterránea hay amplias regiones cubiertas por distintos tipos de yesos que surgieron durante la crisis del Messiniense (5,96-5,33 m.a.). Estas áreas están colonizadas por comunidades de líquenes terrícolas, que se caracterizan principalmente por la abundancia de especies crustáceas, tales como *Diploschistes diacapsis*, *Acarospora placodiiformis*, *A. nodulosa, Buellia zoharyi*, *Diplotomma rivas-martinezii* y *Rhizocarpon malenconianum*, que conviven con líquenes escuamulosos de los taxones *Psora decipiens*, *P. saviczii*, *Clavascidium* spp. y *Placidium* spp., entre otros. Por otra parte, en aquellos enclaves que por sus características microambientales conservan un mayor grado de humedad en el suelo, son frecuentes los líquenes foliáceos y dimórficos del género *Cladonia*.

En este estudio, se han analizado las relaciones entre cada tipo de micobionte y las microalgas simbióticas de los líquenes de estas comunidades. Para ello, se realizaron análisis moleculares de los micobiontes con objeto de conocer su diversidad, construir filogenias, redes de haplotipos y tratar de elaborar posibles reconstrucciones biogeográficas. En el caso de los ficobiontes, se estudiaron marcadores genéticos nucleares (nrITS y actina) y cloroplásticos (LSU rADN). Además, se realizó la caracterización ultraestructural de las células mediante microscopía de transmisión y se diseñó un protocolo de aislamiento y propagación in vitro.

En estas comunidades se han detectado tres géneros diferentes de microalgas: *Trebouxia* en *Diploschistes diacapsis*, *Acarospora placodiiformis*, *A. nodulosa*, *Diplotomma rivas-martinezii* y *Rhizocarpon malenconianum*, *Asterochloris* en *Cladonia* spp. y *Myrmecia* en los líquenes escuamulosos. La coexistencia de distintas microalgas en un mismo talo y el *algal switching* se ha hallado, por lo general, en líquenes en los que las microalgas del género *Trebouxia* eran predominantes.

El objetivo principal de esta tesis ha sido analizar los patrones de asociación entre microalgas y hongos en estas comunidades liquénicas y el estudio se ha abordado desde distintas aproximaciones metodológicas. La variabilidad genética de los distintos componentes simbióticos puede ser un dato clave para poder entender mejor las relaciones que se establecen entre ellos. Esta variabilidad nucleotídica de los micobiontes parece estar relacionada con su dependencia por las características del sustrato, ya que las especies gipsófitas exclusivas presentan una menor variabilidad genética en comparación a las que tienen mayor tolerancia por otros suelos. La baja variabilidad de las especies gipsófitas podría ser consecuencia de los eventos geológicos/climáticos acontecidos en la cuenca mediterránea en el pasado. Otros factores que también podrían explicar los patrones de asociación entre los distintos simbiontes de la biocostras son los biotipos liquénicos, los tipos de sustratos e incluso las estructuras reproductoras y formas de dispersión. Aunque, en este último caso, la complejidad encontrada en dichas estructuras no permite establecer un claro patrón de asociación (selectividad y especificidad).

ABSTRACT

Biological soil crusts (biocrusts, BSCs) are made up of a combination of organisms, both photoautotrophic and heterotrophic, which live inside or on the surface of soils. By means of their interactions and activities, they generate a joint layer along with the particles of the substrate. Lichens, due to the complexity of their symbiosis, are able to colonize these adverse for-other-plant habitats, thanks to their ecophysiological adaptations. Biocrusts dominated by lichens are highly abundant in the clearings of scrublands and grasslands that develop in gypsiferous outcrops. Their diversity, participation in biogeochemical cycles and potential to form large coverages (sometimes > 80%) on soils are important for the management and conservation of these fragile and threatened ecosystems.

In the Mediterranean basin, there are large regions covered by different types of gypsum that emerged during the Messiniense crisis (5.96-5.33 Mya). These areas are colonized by communities of terricolous lichens, which are mainly characterized by the abundance of crustose species, such as *Diploschistes diacapsis*, *Acarospora placodiiformis*, *A. nodulosa*, *Buellia zoharyi*, *Diplotomma rivas-martinezii* and *Rhizocarpon malenconianum*, which co-occur with squamulose taxa such as *Psora decipiens*, *P. saviczii*, *Clavascidium* spp. and *Placidium* spp., among others. In those places which, due to their microenvironmental characteristics, conserve a greater degree of humidity in the soil, foliose and dimorphic lichens of the genus *Cladonia* are common.

In this study, the relationships between each type of mycobiont and the symbiotic microalgae of the lichens of these communities have been analysed. For this purpose, molecular analyses of the mycobionts were carried out in order to discover their diversity, build phylogenies, haplotype networks and possible biogeographic reconstructions. In the case of phycobionts, nuclear (nrITS and actin) and chloroplast (LSU rADN) genetic markers were studied. In addition, the ultrastructural characterization of the cells was performed by transmission microscopy, and an in vitro isolation and propagation protocol was designed.

Three different genera of microalgae have been detected in these communities: *Trebouxia* in *Diploschistes diacapsis*, *Acarospora placodiiformis*, *A. nodulosa*, *Diplotomma rivas-martinezii* and *Rhizocarpon malenconianum*, *Asterochloris* in *Cladonia* spp., and *Myrmecia* in squamulose lichens. The coexistence of different microalgae in the same thallus, and "algal switching" processes, have been found, generally, in lichens in which microalgae of the genus *Trebouxia* were predominant.

The main objective of this doctoral thesis has been to analyze the association patterns between microalgae and fungi in these lichen communities and the study has been addressed from different methodological approaches. The genetic variability of the different symbiotic components may be a key data to better understand the relationships established between them. This nucleotide variability of mycobionts seems to be related to their dependence on the characteristics of the substrate, since exclusive gypsophyte species have less genetic variability compared to those with greater tolerance for other soils. The low variability of gypsophyte species could be a consequence of the geological/climatic events that occurred in the Mediterranean basin in the past. Other factors that could also explain the patterns of association between the different symbionts of the biocrusts are lichen biotypes, kind of substrates and even reproductive structures and dispersal strategies. However, in the latter case, the complexity found in these strategies does not allow a clear association pattern (selectivity and specificity) to be established.

1 Introducción general

1 INTRODUCCIÓN GENERAL

1.1 Las costras biológicas de los suelos

Las costras biológicas de los suelos (biocostras o CBS), hacen referencia al conjunto de especies vegetales no vasculares (líquenes, musgos, hepáticas) y microorganismos unicelulares o de organización simple (cianobacterias libres, hongos, algas) que habitan en el suelo, y a la estrecha relación que mantienen con la capa más superficial del mismo (Figura I1) (Eldridge y Rosentreter 1999; Belnap et al. 2001, 2016; Concostrina-Zubiri et al. 2013).

Las biocostras se encuentran en todos los continentes, y cubren un área total de ~18 millones de km^2, aunque su área potencial puede llegar a ocupar casi el 40% de la superficie terrestre (Belnap 2006; Elbert et al. 2012; Rodríguez-Caballero et al. 2018).

Las condiciones climáticas del entorno afectan a la composición, apariencia y biomasa de los organismos presentes en las biocostras de cada hábitat (Belnap y Lange 2013). Dependiendo del grupo de organismos con mayor frecuencia y cobertura, las costras biológicas se pueden clasificar como costras dominadas por cianobacterias, por briófitos o por líquenes (Büdel et al. 2009; Weber et al. 2015). Además, las dominadas por líquenes se dividen en ciano- o cloroliquénicas según sean los componentes fotoautótrofos de los talos predominantes en la comunidad liquénica.

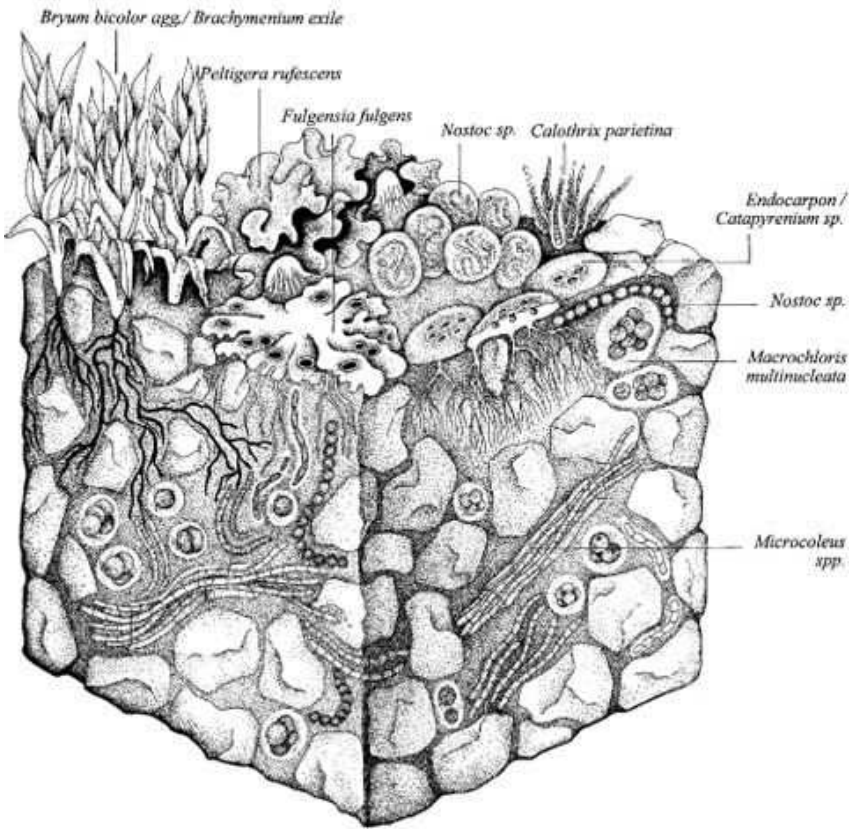


Figura I1. Esquema de la capa superficial del suelo con los colonizadores típicos de una costra biológica. Extraido de Belnap y Lange (2013).

Otro criterio de clasificación que se propone, sería la morfología de las especies dominantes de las biocostras, debido a los efectos que la morfología de los organismos participantes produce en las dinámicas ecosistémicas y a la difícil identificación de algunas de las especies. Dependiendo del modelo arquitectónico y del grado de complejidad estructural mayoritaria, se han establecido las siguientes categorías: cianobacterias, algas verde azuladas, hepáticas, musgos, líquenes foliáceos, líquenes crustáceos, líquenes gelatinosos, líquenes escuamulosos y líquenes fruticulosos (Eldridge y Rosentreter 1999; Belnap et al. 2001).

Las biocostras se desarrollan en muchos tipos de sustratos, ocupando superfícies calcáreas, silíceas, yesosas y volcánicas (Cameron y Blank 1965; Belnap y Gardner 1993; Bliss y Gold 1999; Rosentreter y Belnap 2001; Bowker y Belnap 2008; Moya et al. 2015, 2018; Chiva et al. 2019). Son particularmente frecuentes en suelos de baja productividad como zonas áridas, semiáridas, alpinas y polares (Maestre et al. 2002; Büdel y Veste 2008; Rippin et al. 2018). En estos hábitats pueden alcanzar hasta un 70% de cobertura (generalmente en los espacios no cubiertos por plantas vasculares perennes) debido a la disponibilidad de espacio y que favorece la entrada de radiación solar (Belnap 2006; Belnap y Lange 2013).

En los ecosistemas de zonas áridas, las biocostras son muy importantes ya que estabilizan y protegen el suelo desprovisto de vegetación vascular de la erosión producida por el agua de lluvia y de escorrentía, así como frente a la erosión del viento (Campbell et al. 1989; Lange et al. 1998; Beyschlag et al. 2008; Jiménez Aguilar et al. 2009; Belnap et al. 2016). Se ha demostrado que en las CBSs distintos grupos morfológicos de líquenes contribuyen a la estabilidad del suelo (Belnap y Harper 1995; Lange et al. 1998; Jiménez-Aguilar et al. 2009), y también influyen en la germinación de semillas y en el establecimiento de las plántulas (Li et al. 2005; Coe et al. 2012).

Los hongos y bacterias en las CBSs alcanzan una gran diversidad y afectan a la composición y estructura de los suelos (Maier et al. 2016, 2018). Además, las biocostras influyen en los ciclos del carbono y del nitrógeno. Recientes estudios sugieren que las biocostras y otros tipos de coberturas superficiales con microorganismos podrían contribuir con un 7% a la fijación global de CO_2 y un 50% a la fijación biológica de nitrógeno (Elbert et al. 2009, 2012; Delgado-Baquerizo et al. 2010; Reed et al. 2012). Las biocostras también intervienen en los procesos de intercambio de gases con la atmósfera (Porada et al. 2014; Lenhart et al. 2015; Weber et al. 2015; Meusel et al. 2018) y en el ciclo hidrológico, ya que pueden absorber y redistribuir el agua procedente de las lluvias desde una escala espacio-

temporal (Eldridge y Rosentreter 1999; Chamizo et al. 2013, 2016). Otra característica importante es que reducen la evaporación y la temperatura superficial modulando la reflectancia del suelo (Karnieli et al. 2001; Burgheimer et al. 2006; Kidron y Tal 2012).

Por estas razones, las costras biológicas de los suelos han conseguido despertar el interés tanto de la comunidad científica como de los gestores medioambientales. El estudio en profundidad de estos complejos permitirá la correcta implementación de estrategias para la conservación de estos hábitats con alto valor ecológico.

1.2 Los afloramientos yesíferos en la cuenca mediterránea

1.2.1 Origen y naturaleza del yeso

El yeso o evaporita ($CaSO_4$ –$2H_2O$), del griego γύψος (gypsos), es uno de los minerales más comunes de las rocas sedimentarias. Es un mineral blando y normalmente de color blanco o transparente, aunque con frecuencia suele presentar otra coloración debido a las impurezas, fundamentalmente de arcilla o de carbonato (Pérez-López et al. 2011).

El mineral de yeso se forma por precipitación química a partir de fluidos o masas de agua, como las de un lago o de un mar con un alto contenido en sales. El sedimento se forma por acumulación de cristales de yeso, provenientes de la precipitación del sulfato cálcico disuelto en el agua, por esta razón las capas de yeso tienen un origen químico (Gutiérrez et al. 2004; Pérez-López et al. 2011).

1.2.2 Tipos de yesos

Las evaporitas presentes en la cuenca mediterránea pueden ser de origen continental o marino:

Las evaporitas continentales se formaron sobre todo durante el Cenozoico y ocuparon extensiones relativamente importantes. Además, a lo largo del Terciario, tuvo lugar en muchas cuencas endorreicas la sedimentación evaporítica continental durante las fases orogénicas alpinas aumentando así la extensión de este tipo de evaporitas (Parrish et al. 1982).

Las evaporitas marinas más comunes son las del Triásico y las del Messiniense. Durante el Triásico se produjo una de las deposiciones de yesos más abundantes de la historia de la Tierra, debido principalmente a dos condiciones: un clima árido y una importante transgresión marina (Orti et al. 2017). Es decir, el nivel del mar

fue subiendo paulatinamente al mismo tiempo que iba cubriendo extensas zonas someras. La regresión marina posterior, que tuvo lugar en el Triásico Superior, provocó la evaporación y la precipitación química. Dando lugar a formaciones rocosas, de yesos y anhidritas entre otros sulfatos. Estas tienen enorme extensión mundial y abundan en el sector oriental de la Península Ibérica (Márquez-Aliaga 2017).

Los sedimentos evaporíticos marinos del mediterráneo, que se depositaron al final del Mioceno (Messiniense), son los más importantes conocidos en las épocas más recientes (Fortuin et al. 2000). Este gran volumen de evaporitas está asociado a lo que se ha llamado la crisis de salinidad del Mediterráneo (5,93- 5,33 millones de años), cuando sus aguas dejaron de tener comunicación con las del Océano Atlántico durante 600 mil años (Krijgsman et al. 2001; Duggen et al. 2003; Gutiérrez et al. 2004). El Mar Mediterráneo pasó a ser un mar cerrado, cuyas aguas al quedar aisladas se fueron saturando en sales por la evaporación. Produciéndose una importante precipitación de sales, conforme se iba evaporando el agua, hasta quedar casi toda la cuenca seca. Posteriormente, se produjo la gran inundación Zancleana (hace 5,33 millones de años) debida a la rotura del dique de separación entre el océano Atlántico y el antiguo mar Mediterráneo, configurando la nueva cuenca y restableciendo la conexión con el océano, esta vez por el estrecho de Gibraltar (Hsü et al.1977; Duggen et al. 2003).

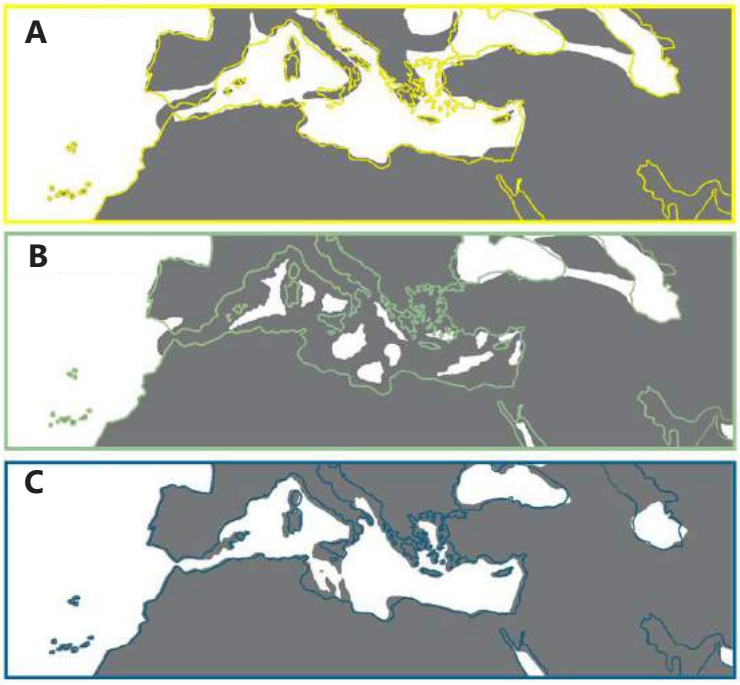


Figura I2. Historia paleogeológica simplificada de la cuenca mediterránea antes de la crisis del Messiniense (A, amarillo), durante la crisis (verde) y después de la crisis (azul oscuro). La distribución actual de las tierras emergidas está representada por la línea de color correspondiente. Basado en Chiva y coautores (2019).

Actualmente, en zonas emergidas alrededor del Mediterráneo, encontramos numerosos yacimientos yesíferos del Messiniense, causados por los acontecimientos anteriormente citados.

1.3 Los líquenes en las costras biológicas de yesos

Diversos estudios han puesto de manifiesto que los suelos de los yesares albergan gran diversidad de líquenes, muchos de ellos especializados (Llimona 1974; Crespo y Barreno 1975; Martínez-Sanchez et al. 1994; Llimona y Navarro-Rosinés 1999). Las biocostras que se desarrollan en los yesos, contribuyen a incrementar de manera significativa la biodiversidad en muchos tipos de ecosistemas y pueden ser especialmente importantes en regiones donde la diversidad de plantas vasculares es baja (Maestre et al. 2011; Belnap et al. 2016). Por ejemplo, en todo el Desierto del Colorado (USA) los suelos de yesos tienen menos de cinco especies de plantas vasculares perennes, mientras que albergan hasta 28 especies de líquenes y musgos (Bowker y Belnap 2008). En estas situaciones, la presencia de biocostras bien desarrolladas puede convertir los *coldspots* de biodiversidad basados en el cálculo de las plantas vasculares en *hotspots*, cuando se incluyen las especies liquénicas (Belnap et al. 2016). Debido a su importancia, muchos estudios proponen el uso de las biocostras como un sistema modelo para estudiar las interrelaciones entre biodiversidad y funciones del ecosistema (Bowker et al. 2013, 2014).

1.3.1 Flora liquénica de las costras biológicas de yesos

La composición de las comunidades liquénicas de las biocostras de los yesos está condicionada por pequeñas variaciones del medio (dureza del sustrato, orientación, vegetación vascular próxima, disponibilidad de nutrientes, etc.) (Martínez-Sánchez et al. 1994; Ochoa-Hueso et al. 2011). De acuerdo con Rundel (1978) y Gutiérrez-Carretero y Casares-Porcel (1994, 2011) la diversidad de especies varía según la mayor o menor riqueza en contenido de yeso del sustrato, así como por el grado de continentalidad y/o la proximidad a las costas.

Los líquenes que colonizan las biocostras de los yesos son predominantemente especies basófilas, con un porcentaje pequeño (20 %) de gipsófitos (exclusivos y preferentes) (Gutiérrez-Carretero y Casares-Porcel 2011). Se consideran gipsófitos exclusivos las especies de líquenes que únicamente se desarrollan sobre sustrato de yeso, por ejemplo, *Acarospora clauzadeana*, *Acarospora placodiiformis*, *Buellia almeriensis*, *Xalocoa ocellata* var. *almeriensis*, *Gyalolechia poeltii*, *Lecidea circinarioides* y *Diplotomma rivas–martinezii* (Casares-Porcel et al. 1994, 1996; Gutiérrez-Carretero y Casares-Porcel 1994, 2011). Los gipsófitos preferentes son líquenes que presentan su óptimo sobre los yesos, pero también se pueden desarrollar sobre otros tipos de sustratos (p. ej. *Acarospora nodulosa*, *Buellia zoharyi*, *Diploschistes diacapsis*, *Lecidea gypsicola, Lepraria isidiata, Psora saviczii, etc.) (Casares-Porcel et al. 1994,*

1996; Gutiérrez-Carretero y Casares-Porcel 1994, 2011).

Especies con amplia distribución como *Cladonia foliacea*, *Gyalolechia fulgida*, *Lecanora crenulata*, *L. dispersa*, *Candelariella aurella*, etc., constituyen en torno al 70% de las costras biológicas de yesos. Estos líquenes, aparentemente indiferentes al yeso, que en su mayoría son comunes en suelos calcáreos (basófilos) presentan adaptaciones que les permiten sobrevivir en estos ambientes áridos (ver apartado 1.3.2; Llimona 1981; Gutiérrez-Carretero y Casares-Porcel 1994, 2011).

El biotipo más generalizado de los líquenes gipsófitos es el crustáceo con más del 50% de las especies (p. ej. *D. diacapsis*, *B. zoharyi*, *A. nodulosa* o *A. placodiiformis*). Otro biotipo importante es el escuamuloso con representantes de varios géneros tales como *Psora*, *Toninia* o *Clavascidium* (Prieto et al. 2012; Moya et al. 2018). En los microambientes donde es posible que se produzca un aporte extra de agua debido a la formación del rocío o a la ausencia de evaporación pueden aparecer talos fruticulosos, foliáceos o leprariodes (p. ej. *Cladonia pocillum*, *C. foliacea*, *Seirophora lacunosa* o *Lepraria isidiata*) (Gutiérrez-Carretero y Casares-Porcel 1994; Maestre 2003), y en los suelos muy duros o sobre cristales de yeso se instalan biotipos crustáceos con talos muy poco desarrollados debido a la inestabilidad del sustrato, como *B. almeriensis*, *A. clauzadeana* o *D. rivas–martinezii* (Barreno et al. 1975; Llimona y Werner 1975; Barreno 1991; Hafellner y Casares-Porcel 1992; Gutiérrez-Carretero y Casares-Porcel 1994, 2011).

En los ambientes de suelos yesíferos, la vegetación vascular tiene baja cobertura y en ellos son frecuentes los vientos a nivel de superficie (Concostrina-Zubiri et al. 2013). Por esta razón, en estos ambientes no son raros los líquenes vagrantes, escasamente adheridos al sustrato que se desprenden y son transportados por el viento. Facilitando de esta manera su fragmentación y dispersión (p. ej. *S. lacunosa*, *C. foliacea*, *D. diacapsis*) (Büdel y Scheidegger 2008; Gutiérrez-Carretero y Casares-Porcel 2011).

Los líquenes de las costras biológicas de los yesos también pueden presentar numerosos hongos parásitos y/o parasimbiontes (Hafellner y Casares-Porcel2003). Son los llamados "hongos liquenícolas", especies que establecen un amplio abanico de relaciones con sus hospedadores, que van desde situaciones próximas al comensalismo hasta el parasitismo (Ametrano et al. 2019). Todos ellos contribuyen a aumentar la biodiversidad de los yesares (Navarro-Rosinés et al. 1995; Calatayud y Atienza 2000; Calatayud y Etayo 2001; Calatayud y Triebel 2003; Hafellner y Casares-Porcel2003; Etayo 2008; Van den Boom y Etayo 2014).

1.3.2 Ecofisiología y adaptaciones de los líquenes a los yesos

Los ambientes gipsícolas pueden considerarse ambientes extremos debido a las características del sustrato, a las altas temperaturas superficiales y a la escasa humedad existente (Rivas-Martínez y Costa 1970; Crespo y Barreno 1975). Los líquenes han desarrollado una serie de adaptaciones morfológicas y fisiológicas que les permite sobrevivir en estos hábitats adversos donde otros organismos no pueden sobrevivir (Llimona 1981; Nash 2008).

Los briófitos y plantas vasculares de estos ambientes áridos suelen tener su ciclo de vida sincronizado con los periodos de lluvia (Oliver et al. 2011). Sin embargo, en estos ambientes, el hecho de que la estructura y composición de las comunidades liquénicas permanezcan durante todo el año, es un indicador de que las precipitaciones estacionales no les afectan de forma apreciable, sino que es la integración de la variabilidad interanual la responsable de las mismas (Galun 1963; Nash 2008).

El rocío, la niebla o la humedad relativa elevada constituyen los principales aportes hídricos para los organismos de las biocostras (Kappen 1988; del Prado y Sancho 2007). Así mismo, los frecuentes descensos de las temperaturas nocturnas o la influencia de brisas costeras que son fenómenos frecuentes en los territorios áridos, incrementan el contenido hídrico de los sustratos (Lange et al. 2006; Raggio et al. 2014). La mayoría de los organismos de las costras biológicas son poiquilohídricos, carecen de mecanismos para regular el contenido hídrico (Frahm et al. 2010; Belnap et al. 2016). Como propuso Lange (2001), los líquenes están fisiológicamente inactivos cuando los talos presentan déficit hídrico, pero al rehidratarse son capaces de activarse muy rápidamente sin sufrir daños irreversibles. Algunas de las adaptaciones de los líquenes a las condiciones extremas de sequía ya fueron descritas por Galun (1963) a partir de observaciones en el desierto del Negev, y son plenamente aplicables a los líquenes de los afloramientos de yesos (Lange 1969; Llimona 1974, 1981; Lange et al. 2006; del Prado y Sancho 2007; Wu et al. 2013).

En los ambientes yesíferos, los organismos presentan ciertas adaptaciones para reducir la radiación ultravioleta y la evaporación (Karsten y Holzinger 2014). Adaptaciones que, junto con la poiquilohidría, les permite resistir temperaturas muy superiores a sus óptimos (Souza–Egipsy y Sancho 2001; Sancho et al. 2014). La coloración clara de los talos en ambientes de alta exposición solar reduce la incidencia de radiaciones solares, por esta razón el color predominante de los líquenes de yesos es el blanco en seco, aunque en los momentos de hidratación las

superficies de los talos muestran tonalidades intensamente coloreadas (Gutiérrez-Carretero y Casares-Porcel 2011). Por ejemplo, el liquen *Psora decipiens* se cubre de una densa pruina blanca en las biocostras, mientras que en climas menos secos esta pruina permanece ausente (Barreno y Rico 1984). En talos de *Seirophora lacunosa* se ha observado diferente coloración cuando se desarrolla sobre suelos de yeso (gris) o arcillosos (pardo–naranja) (Llimona et al. 1998).

Aunque la dominancia de los biotipos crustáceos y escuamulosos parece estar favorecida por la retención de humedad del sustrato (debido a su íntima relación con la superficie del suelo), todavía se desconocen qué características químicas del sustrato hacen que el yeso favorezca la presencia de ciertos líquenes. El sulfato podría tener un papel importante en la especialización que presentan algunas especies por el yeso como sustrato, ya que algunas especies gipsícolas también habitan el borde de manantiales ricos en azufre (Belnap y Lange 2013). Por otra parte, el hecho de que muchos de los líquenes que viven sobre yesos también se encuentren sobre suelos calcáreos hace suponer que su presencia, obedece a una apetencia genérica por los sustratos básicos entre los que se incluyen los yesos (Gutiérrez-Carretero y Casares-Porcel 2011).

1.4 Las simbiosis liquénicas

Como hemos mencionado anteriormente, los líquenes son un componente importante en muchas de las biocostras. Para poder entender su papel es necesario conocer la singularidad de estas especies simbióticas.

Los líquenes, también llamados hongos liquenizados, son sistemas simbióticos complejos que se individualizan a partir de asociaciones simbióticas cíclicas (Khakhina et al. 1993; Chapman y Margulis 1998). Los líquenes cumplen con los criterios de individualidad en el mundo de los organismos eucarióticos, ya que son compuestos, complejos y multidimensionales a nivel biológico, morfológico, de desarrollo, fisiológico y molecular (Margulis 1993). Las simbiosis liquénicas están constituidas por, al menos, un hongo heterótrofo (micobionte) y uno o varios socios fotosintéticos (fotobiontes) que pueden ser microalgas verdes, (ficobiontes) y / o cianobacterias (cianobiontes), dando como resultado una entidad única: talo liquénico u holobionte (fenotipo simbiogenético) (Nash 2008; Hawksworth y Lücking 2017).

El desarrollo de nuevas técnicas de microscopía y biología molecular en los últimos años ha permitido reconocer la coexistencia de diversas especies de algas

en un mismo talo liquénico (Casano et al. 2011; del Campo et al. 2013; Catalá et al. 2016; Molins et al. 2018b; Škaloud et al. 2018). Además, durante los últimos años, nuevas aproximaciones a la composición de los líquenes han revelado la presencia de comunidades de bacterias no fotosintéticas en los talos liquénicos, consideradas como acompañantes multifuncionales de los holobiontes (Grube et al. 2009). La presencia de bacterias en líquenes había sido descrita muchos años atrás (Iskina 1938; Panosyan y Nikogosyan 1966; Henkel y Plotnikova 1973; González et al. 2005; Cardinale et al. 2006; Selbmann et al. 2010), pero la alta cantidad y diversidad de bacterias asociadas a talos liquénicos, se ha empezado a revelar gracias a técnicas moleculares y de localización *in situ* (Grube y Berg 2009; Hodkinson y Lutzoni 2009; Bates et al. 2012; Muggia et al. 2013a; Fernández-Brime et al. 2019). Y más recientemente, mediante avanzadas técnicas de aislamiento y cultivo que se han adaptado a las bacterias liquénicas (Biosca et al. 2016). Por otro lado, diferentes técnicas "ómicas" también han puesto de relevancia el papel que juegan varios grupos de bacterias en el aprovisionamiento de nutrientes y protección contra factores de estrés bióticos y abióticos en la asociación liquénica (Hodkinson & Lutzoni 2009; Grube et al. 2015).

Asimismo, se han encontrado hongos no simbióticos asociados a los talos liquénicos (hongos liquenícolas) (Grube y Berg 2009; Muggia et al. 2016; Aschenbrenner et al. 2014; Grube et al. 2015; Cernava et al. 2016) y otros endoliquénicos (Suryanarayanan et al. 2017; Huang et al. 2019). También, se han descubierto levaduras de basidiomicetos embebidas en el córtex de ciertos líquenes, cuya abundancia parece estar relacionada con variaciones en el fenotipo liquénico y la síntesis de ciertos metabolitos secundarios (Spribille et al. 2016; Tuovinen et al. 2019).

Con todos estos nuevos "participantes", el paradigma clásico del complejo sistema simbiótico basado en una visión micocéntrica está cambiando a un concepto más amplio. El holobionte queda definido como el organismo huésped que provee de soporte estructural al resto de elementos simbiontes (Ahmadjian 1993; Hawksworth y Honegger 1994). Por ello, se consideran los talos liquénicos como ecosistemas diminutos (microecosistemas, micobiomas) en los que pueden interactuar numerosos diferentes socios simbióticos (Honegger 1992, 1998; Margulis y Barreno 2003; Grube et al. 2014; Aschenbrenner et al. 2016; Cernava et al. 2017). Esta unión tiene como resultado la integración morfológica y metabólica del micobionte con los fotobiontes y los otros microorganismos simbiontes, lo que posibilita la aparición de innovaciones evolutivas simbioespecíficas (Honegger 1992, Margulis y Barreno 2003; Barreno 2013, 2019).

Las relaciones entre los biontes proporcionan no solo intercambio de nutrientes, sino interacciones diversas entre los genomas (Guerrero et al. 2013) que son importantes para conferir nuevas capacidades y afrontar condiciones adversas sin desnaturalizarse (resiliencia). Los líquenes podrían ser considerados como verdaderos supervivientes de la paleohistoria terrestre, poniendo de manifiesto el gran éxito de estas asociaciones para la adaptación de seres poiquilohídricos y poiquilotermos a ambientes atmosféricos estresantes (Barreno 2004, Schoch et al. 2009). Estos organismos poiquilotermos sometidos a ciclos repetidos de desecación/rehidratación, pueden sobrevivir en ambientes extremos, a menudo muy secos, como los desiertos o los hábitats árticos y antárticos e incluso el espacio exterior (Sancho 2015; Determeyer-Wiedmann et al. 2019). Por tanto, las simbiosis liquénicas son una estrategia exitosa para enfrentarse a las dificultades inherentes de adaptarse a cambios en las condiciones ambientales aéreas, debido a que presentan tolerancia a múltiples tipos de estrés abiótico (Honegger 2001; Margulis y Barreno 2003).

1.4.1 Microalgas simbióticas en líquenes de las biocostras

Las costras biológicas de los suelos están formadas, entre otros, por cianobacterias y microalgas de vida libre que constituyen el *pool* fotosintético de ficobiontes y cianobiontes disponibles para, en su caso, asociarse con los líquenes terrícolas. Entre los ficobiontes de las biocostras, las trebouxiofíceas son el grupo más importante de microalgas liquénicas.

Las trebouxiofíceas comprenden algas unicelulares (desde cocoides hasta elipsoidales) y colonias (simples, filamentosas o en láminas); que se desarrollan en entornos terrestres y acuáticos. Algunos representantes de esta clase han perdido la capacidad fotosintética, y han desarrollado su estrategia vital como parásitos heterótrofos (Lemieux et al. 2014; Darienko et al. 2015).

La clase Trebouxiophyceae (Chlorophyta) ha sido objeto de múltiples revisiones taxonómicas, se han reconocido hasta 16 linajes distintos, aunque está siendo difícil, en muchos casos, discernir las posibles relaciones filogenéticas que hay entre ellos (Neustupa et al. 2011, 2013a, b; Marin 2012; Gaysina et al. 2013; Fučíková et al. 2014; Lemieux et al. 2014; Sun et al. 2016; Zahradníková et al. 2017). Recientemente, Martínez-Alberola y coautores (2019) han reconstruido la filogenia de las Chlorophyta basándose en siete genes mitocondriales (Figura I3). Sus resultados sugieren que este filum está compuesto por cuatro clases (Chlorophyceae, Prasinophyceae,

Trebouxiophyceae y Ulvophyceae). Además, separa las trebouxiofíceas en dos subclados (I y II); dentro del subclado II, se encuentra la familia Trebouxiaceae (Trebouxiales). Este linaje es el grupo más conocido, extendido y rico en especies de las microalgas asociadas a líquenes. En concreto, los géneros *Trebouxia* Puymaly (1924), *Asterochloris* Tschermak-Woess (1980) y *Myrmecia* Printz (1921) son los más comunes, en los líquenes de las costras biológicas de yesos.

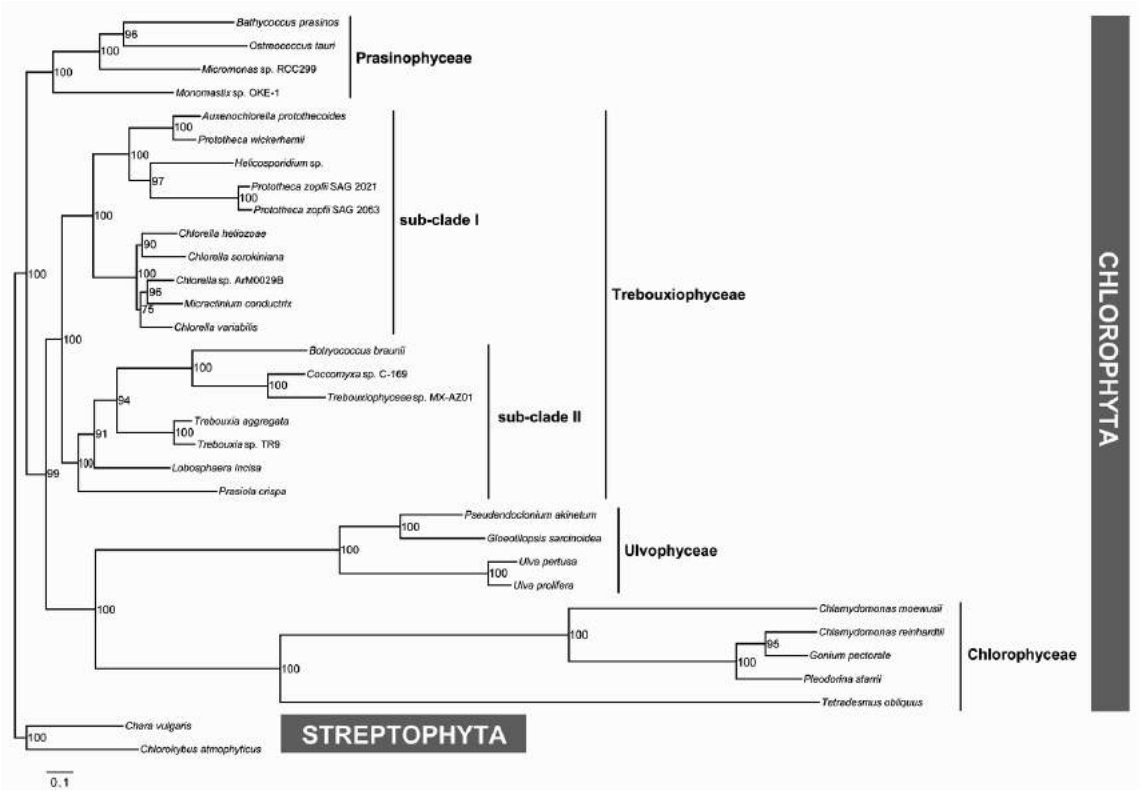


Figura I3. Reconstrucción filogenética de los linajes del filum Chlorophyta basado en el análisis de las secuencias de siete genes mitocondriales en 32 especies de este filum. Extraído de Martínez-Alberola y coautores (2019).

TREBOUXIA: Las especies del género *Trebouxia* son los simbiontes más comunes dentro de las principales familias de hongos liquenizados, con una representación particularmente alta en las familias Parmeliaceae y Lecanoraceae, aunque este dato probablemente sea debido a un efecto de sesgo en los muestreos, debido a que estas microalgas fueron las primeras en ser identificadas como simbiontes en líquenes (Archibald 1975).

Actualmente, han sido descritas 28 especies de *Trebouxia* (Ver Anexo I), aunque según Muggia y coautores (2018) este dato es claramente una subestimación de la diversidad real en el rango específico. Inicialmente, los taxones del género *Trebouxia* se distinguieron de los de *Asterochloris*, un género próximo, únicamente

por la morfología de los cloroplastos y de los pirenoides (Tschermak-Woess 1980a, b; Friedl 1989). La posterior introducción de diversas técnicas filogenéticas supuso la reorganización de ambos géneros mediante múltiples revisiones taxonómicas (p. ej. Rambold et al. 1998; Helms et al. 2001; Škaloud y Peksa 2008), hasta que Škaloud y Peksa (2010) los separaron formalmente.

Tradicionalmente, las especies del género *Trebouxia* han sido identificadas mediante el uso de técnicas microscópicas (principalmente con microscopía óptica). Friedl, en 1989, describió 8 tipos diferentes de microalgas del género *Trebouxia* según la morfología del pirenoide con el uso de la microscopía electrónica. Posteriormente, Škaloud y Peksa (2010) clarificaron los géneros *Trebouxia* y *Asterochloris*, y se pudieron establecer cinco tipos de pirenoide para *Trebouxia* ('gigantea', 'impressa', 'arboricola', 'gelatinosa' y 'corticola') y tres para *Asterochloris* ('irregularis', 'erici' y 'magna'). Además, basándose en filogenias de la región nrITS, Helms y coautores (2001, 2003) y Beck (2002) establecieron cuatro clados principales dentro del género *Trebouxia* ('A' de *T. arboricola*, 'G' de *T. galapagensis*, 'I' de *T. impressa* y 'S' de *T. simplex*); para la asignación del nombre de cada clado se usó la inicial de una de las especies en representación del grupo. En estudios posteriores, se encontró una fuerte congruencia entre los datos ultraestructurales descritos por Friedl (1989) y los moleculares de las microalgas localizadas dentro del talo (del Campo et al. 2010; Muggia et al. 2010, 2014; Casano et al. 2011; Leavitt et al. 2015; Català et al. 2016; Moya et al. 2017; Molins et al. 2018a).

En una reciente revisión de la clase Trebouxiophyceae, Muggia y coautores (2018) sugieren como criterio de delimitación de especies en *Trebouxia*, además de las filogenias inferidas a partir de los datos de nrITS (*barcode*) o de multilocus, la caracterización morfológica y ultraestructural de los taxones en simbiosis en los talos y en cultivos de algas axénicos.

Son numerosos los trabajos que se han realizado sobre microalgas del género *Trebouxia* que simbiotizan en líquenes terrícolas (p. ej. Beck 2002; Voytsekhovich & Beck 2016; Moya et al. 2015, 2018; Molins et al. 2018a, b; Škaloud et al. 2018). Estos líquenes podrían formar parte de algún tipo de biocostra ya que en ocasiones participan en ellas. En cambio, en estos trabajos se han estudiado otros aspectos y no se ha analizado la influencia del sustrato en sus simbiontes. En esta tesis, se relaciona el sustrato con las microalgas *Trebouxia* presentes en los líquenes de las biocostras.

ASTEROCHLORIS: Las microalgas del género *Asterochloris* se asocian con especies de unos diez géneros de líquenes, principalmente con los de las familias Cladoniaceae (~500 especies) y Stereocaulaceae (~250 especies).

Hasta el momento han sido descritas 16 especies de *Asterochloris* (Ver Anexo I). La delimitación molecular de especies en este género es reciente. Las primeras filogenias fueron inferidas a partir de fragmentos de la subunidad nuclear pequeña del rDNA (nrSSU) y la subunidad nuclear grande (nrLSU) (Friedl y Zeltner 1994; Friedl 1995; Friedl y Rokitta 1997). El carácter monofilético del género *Asterochloris* fue respaldado con un análisis multilocus (nrITS y 18S) basado en secuencias obtenidas de microalgas en simbiosis y de cultivos axénicos (Piercey- Normore y DePriest 2001; Friedl y Büdel 2008). En 2010, Škaloud y Peksa propusieron la delimitación formal del género *Asterochloris* basándose en el análisis filogenético del nrITS, en un intrón que se encuentra en el gen de la actina tipo I y en la estructura secundaria del nrITS (Škaloud y Peksa 2010). En este trabajo, los autores destacaron la diversidad genética oculta en este género y reubicaron las especies del género *Trebouxia* y *Asterochloris*. Recientemente, en Škaloud y coautores (2015) se describieron seis nuevas especies de *Asterochloris* y se propuso la morfología del cloroplasto como el mejor marcador morfológico.

La mayoría de estudios relacionados con microalgas del género *Asterochloris* se refieren a las que son simbióticas de líquenes del género *Cladonia* (p. ej. Yahr et al. 2004, 2006; Beiggi & Piercey-Normore 2007; Škaloud et al. 2015; Steinová et al. 2019). Algunos de estos líquenes, se encuentran en microhábitats sombríos de las biocostras, aquellos en los que la disponibilidad hídrica es mayor. Sería interesante averiguar si en las especies de *Cladonia* de las biocostras el microalga predominante es también *Asterochloris* y qué especies de este género simbiotizan con líquenes de ambientes tan extremos como estos en distintas áreas.

MYRMECIA: Diferentes especies del género *Myrmecia* son el microalga predominante en varios géneros de líquenes de talos escuamulosos, como *Psora*, *Heteroplacidium*, *Placidium* o *Clavascidium* (Thüs et al. 2011; Williams et al. 2017; Moya et al. 2018). También, se ha detectado *M. biatorellae* en el liquen crustáceo endolítico *Sarcogyne privigna* (Tschermak-Woess 1978; Perez-Ortega et al. 2012).

En este género se han descrito hasta el momento nueve especies (Ver Anexo I). La escasez de estudios sobre *Myrmecia* podría deberse a la falta de trabajos sobre los ficobiontes de líquenes escuamulosos y a que, hasta 2018, se carecía de secuencias del nrITS (*barcode*) en el GenBank de las especies de *Myrmecia* para su potencial uso en análisis filogenéticos (Moya et al. 2018).

En el estudio realizado por Moya y coautores (2018) (incluido en esta tesis, R 3.2) se sugiere que la combinación de diferentes técnicas (moleculares, de aislamiento y microscópicas) y la correcta selección de los cebadores moleculares *barcode* (nrITS) son clave en los estudios de delimitación de microalgas liquénicas en el género *Myrmecia*.

En las biocostras son abundantes los líquenes de las familias Psoraceae y Verrucariaceae, todos ellos presentan como socios simbiontes especies del género *Myrmecia* (Thüs et al. 2011; Williams et al. 2017; Moya et al. 2018). Determinar las causas por las que estos líquenes seleccionan las microalgas de este género en vez de otras (en principio) más abundantes en las biocostras, es uno de los objetivos de esta tesis.

1.4.2 Selectividad y especificidad de las microalgas simbióticas

En la formación de un talo liquénico, los simbiontes implicados y los mecanismos de interacción, son un tema crucial y ampliamente estudiado en liquenología.

Tradicionalmente, las estrategias de dispersión de los hongos liquenizantes se han dividido según la presencia de uno (micobionte) o de ambos simbiontes (micobiontes+fotobiontes) en las diásporas. Cuando el desplazamiento tiene lugar conjuntamente con ambos socios simbióticos, la dispersión se produce a través de pequeños fragmentos del talo o por estructuras reproductivas especiales (p. ej. soredios o isidios) (Heinken 1999; Büdel y Scheidegger 2008). Si se colonizan nuevos hábitats mediante la dispersión exclusiva del micobionte, los propágulos son esporas generadas sexualmente (ascosporas) o asexualmente (conidiosporas). En estos últimos casos, las especies se enfrentan al desafío de encontrar microalgas adecuadas para restablecer la simbiosis (Bowler y Rundel 1975; Hestmark 1991, 1992; Nash 2008). Además, hay que valorar como una potencial fuente de microalgas, los propágulos simbióticos dispersados por otras especies de líquenes (Beck et al. 1998; Fedrowitz et al. 2011, Belinchón et al. 2015).

La captación de uno o varios linajes de ficobiontes por parte de un protoliquen está mediada por distintos factores relacionados con los patrones de asociación que permitan esta unión. Diversos autores (Rambold et al. 1998; Yahr et al. 2004, 2006) han propuesto la utilización de los términos "especificidad" para designar el posible rango taxonómico de los socios admisibles; y "selectividad", para expresar la frecuencia de asociación entre los socios compatibles. Actualmente, ambos conceptos

tienen un carácter multidireccional ya que pueden atribuirse del microalga hacia el micobionte y viceversa (Muggia et al 2018).

Las condiciones ambientales del medio en que se desarrollan los simbiontes son clave en la disponibilidad (presencia o ausencia) de un determinado simbionte para el desarrollo de los talos liquénicos (Peksa y Škaloud 2011; Muggia et al. 2018, Steinová et al. 2019). Con estas premisas, la disponibilidad de determinadas microalgas dependerá de sus requerimientos ambientales y límites de tolerancia ecofisiológicos, que afectan directamente a la distribución de las distintas especies de líquenes (Yahr et al. 2006; Casano et al. 2011; Peksa y Škaloud 2011). Peksa y coautores (2015) sugieren que las comunidades de líquenes que prosperan en unas condiciones ambientales específicas se asocian con una o varias microalgas localmente adaptadas, y estas microalgas son distintas en una comunidad con ecología diferente. En ambientes con condiciones climáticas adversas, las comunidades liquénicas (*lichen guilds*) usan el mismo *pool* de microalgas como socios simbiontes (*photobiont-mediated lichen guild*) (Rikkinen et al. 2002; Peksa y Škaloud 2011; Perez-Ortega et al. 2012; Dal Grande et al. 2014b, 2017; Park et al. 2015; Engelen et al. 2016; Singh et al. 2017).

La variedad de patrones de especificidad y selectividad que se pueden encontrar en los líquenes explicaría la diversidad detectada en los holobiontes. Y es que, el grado de especificidad entre los fotobiontes y el micobionte depende del taxón liquénico (Muggia et al. 2018).

También, se ha hipotetizado que las asociaciones que se producen en los talos liquénicos podrían facilitar la coevolución de ambos socios y favorecer la diversificación concertada (Rambold et al. 1998; Wornik y Grube 2010; del Campo et al. 2013). Los mecanismos de reproducción asexual que tienen algunos líquenes pueden conducir a una alta tasa de coevolución entre ambos simbiontes, pero dicha especialización puede disminuir la diversidad genética de ambos socios (Wornik y Grube 2010; Otálora et al. 2013). En cambio, cuando el micobionte se dispersa sin su pareja fotosimbiótica (esporas) aumentan las oportunidades de evolución adaptativa al tener que unirse a nuevos fotobiontes (Wornik y Grube 2010; Otálora et al. 2013).

Hasta el momento, pocos estudios han evaluado la influencia de las estrategias reproductivas de los líquenes con la especificidad de las microalgas asociadas (Wornik y Grube 2010; Otálora et al. 2010; Fedrowitz et al. 2011; Cao et al. 2015). Recientemente, Steinová y coautores (2019) analizaron en líquenes del género *Cladonia* las estrategias de reproducción y dispersión y observaron que las especies

con reproducción sexual solían adoptar una estrategia generalista al asociarse con numerosos linajes de *Asterochloris*. En cambio, las especies de *Cladonia* que se reproducen asexualmente se asociaron exclusivamente con *A. glomerata* o *A. irregularis*, aunque se detectaron otras especies de *Asterochloris* disponibles en esas mismas localidades. Según Fedrowitz y coautores (2012), el uso estricto de pocas especies de fotobiontes en líquenes de amplia distribución geográfica podría señalar la existencia de alta especificidad entre sus simbiontes. Resultados similares se habían observado en otras especies de *Cladonia* asociadas con *Asterochloris* spp. (Yahr et al. 2004, 2006) y en géneros como *Nephroma* y *Degelia* que se simbiotizan con *Nostoc* (Fedrowitz et al. 2011; Otálora et al. 2013).

En 2001, Piercey-Normore y DePriest utilizan el término *algal switching* para designar el proceso por el que ciertos líquenes pueden tener la capacidad de cambiar la especie predominante de microalga simbiótica. Esta hipótesis se estableció para el caso de comunidades liquénicas en donde es común que varias especies de líquenes se asocien con las mismas microalgas predominantes (Piercey-Normore y DePriest 2001, Romeike et al. 2002, Blaha et al. 2006, Nelsen y Gargas 2009, Wornik y Grube 2010). Ohmura y coautores (2019) encontraron los mismos haplotipos de *Trebouxia corticola* en talos de *Parmotrema tinctorum* y en el sustrato sobre donde estos crecen. Estos autores sugieren que la diversidad de fotosimbiontes dentro de las poblaciones de un liquen con reproducción asexual se puede generar mediante el cambio de microalga predominante (*algal switching*) entre la microalga original del talo y los socios de algas compatibles del sustrato circundante. Además, establecieron las siguientes observaciones que también apoyarían el fenómeno del *algal switching:* 1) alta diversidad genética de microalgas en poblaciones con pocos individuos; 2) coexistencia de microalgas en un único talo; 3) no correspondencia en los análisis genéticos entre el micobionte y el microalga predominante; y 4) clara selectividad de las microalgas simbióticas en diversos líquenes (Ohmura et al. 2019).

Dentro de una comunidad liquénica se establecen distintos tipos de asociaciones entre los simbiontes y los holobiontes; el rango de especificidad de cada uno de los participantes de la simbiosis genera un complejo mosaico de relaciones entre ellos. En las comunidades de líquenes con entornos pobres en nutrientes, como en las costras biológicas de yesos, la correcta selección de microalgas es determinante para el establecimiento de los talos.

1.4.3 Coexistencia

La coexistencia de microalgas en un mismo talo liquénico es un fenómeno que pese a su constatación en diferentes especies (Casano et al. 2011; Dal Grande et al. 2018; Molins et al. 2018b; Škaloud et al. 2018) sigue generando controversia en el mundo de la liquenología (Paul et al. 2018). Esta controversia surge por que tradicionalmente, se asociaba la existencia de una única población homogénea de microalga a un micobionte en un único talo liquénico. Este paradigma ha ido evolucionando hasta concluir actualmente que múltiples especies de microalgas pueden coexistir dentro de un único talo liquénico. De hecho, la coexistencia de múltiples microalgas en un mismo talo es un fenómeno más común de lo que se pensaba anteriormente en la simbiosis liquénica (Muggia el al. 2013b; Dal Grande et al. 2014a; Moya et al. 2017; Ohmura et al. 2019).

La mayoría de trabajos sobre diversidad de microalgas se ha realizado utilizando exclusivamente la metodología Sanger (Sanger et al. 1977). El uso de este método ha sido recientemente discutido; ya que, en algunos talos según la composición de la diversidad de microalgas presentes, los resultados de la amplificación podrían o no ser coherentes y/o producirse una amplificación al azar (U'Ren et al. 2014; Onuț-Brännström et al. 2018; Paul et al. 2018). Los trabajos anteriormente citados coinciden en que el uso de la secuenciación mediante Sanger presenta resultados coherentes para determinados análisis, pero en el caso de especies con coexistencia de microalgas y una predominante, este método podría sesgar hacia esa microalga como único producto de amplificación, de modo que se estaría obviando la presencia del resto de microalgas.

Dicha coexistencia, en las trebouxiofíceas, ha sido mayoritariamente estudiada entre especies de los géneros *Trebouxia*, pero también ocurre entre otros géneros de microalgas simbióticas de esta clase. Por ejemplo, en el hongo liquenizado *Schizoxylon albescens* se han identificado múltiples taxones de microalgas *Coccomyxa* (Muggia et al. 2011), igualmente en *Micarea peliocarpa* con varias microalgas del género *Elliptochloris* (Voytsekhovich et al. 2011).

La coexistencia no es exclusiva entre microalgas de un mismo género, es un proceso en el que pueden interactuar microalgas de distintos géneros en un mismo talo. En algunas especies de *Micarea* se detectó coexistencia entre microalgas del género *Elliptochloris* y *Pseudococcomyxa* (Voytsekhovich et al. 2011). En Moya y coautores (2017) que aplicaron la metodología de pirosecuenciación por 454 para analizar la diversidad de microalgas en un único talo del liquen *Ramalina farinacea*, se pudieron

detectar hasta 31 OTUs representativos de diferentes géneros de microalgas, 26 de ellos se correspondian con especies de *Trebouxia*.

La existencia de más de una especie de microalga trebouxiofícea dentro de un único talo de *R. farinacea* se constató por primera vez mediante la combinación de técnicas moleculares y ultraestructurales (del Campo et al. 2010; Garcia-Breijo et al. 2010; Casano et al. 2011). Posteriormente, en numerosos trabajos se detectó la coexistencia de distintas especies de *Trebouxia* en un mismo talo en otras especies de líquenes (p. ej. Mansournia et al. 2012; Muggia et al. 2013b, 2014; Dal Grande et al. 2014a, 2019; Park et al. 2014; Voytsekhovich y Beck 2016; Singh et al. 2017; Molins et al. 2018b).

La coexistencia es dependiente del momento en que las microalgas son adquiridas por el micobionte. Esta adición de simbiontes fotosintéticos se puede producir al principio de la asociación simbiótica (protoliquen), o cuando el talo está totalmente formado, si es que presenta selectividad baja hacia su microalga y/o si necesita una fuente adicional de carbono (Dal Grande et al. 2014a; Voytsekhovich y Beck 2016). En Moya y coautores (2017) se observó que en las partes basales de los líquenes fruticulosos (*R. farinacea*) hay mayor diversidad de microalgas que en los ápices de las lacinias del talo, apoyando la idea de que en ese liquen la mayor adquisición de microalgas tiene lugar en las fases iniciales del desarrollo.

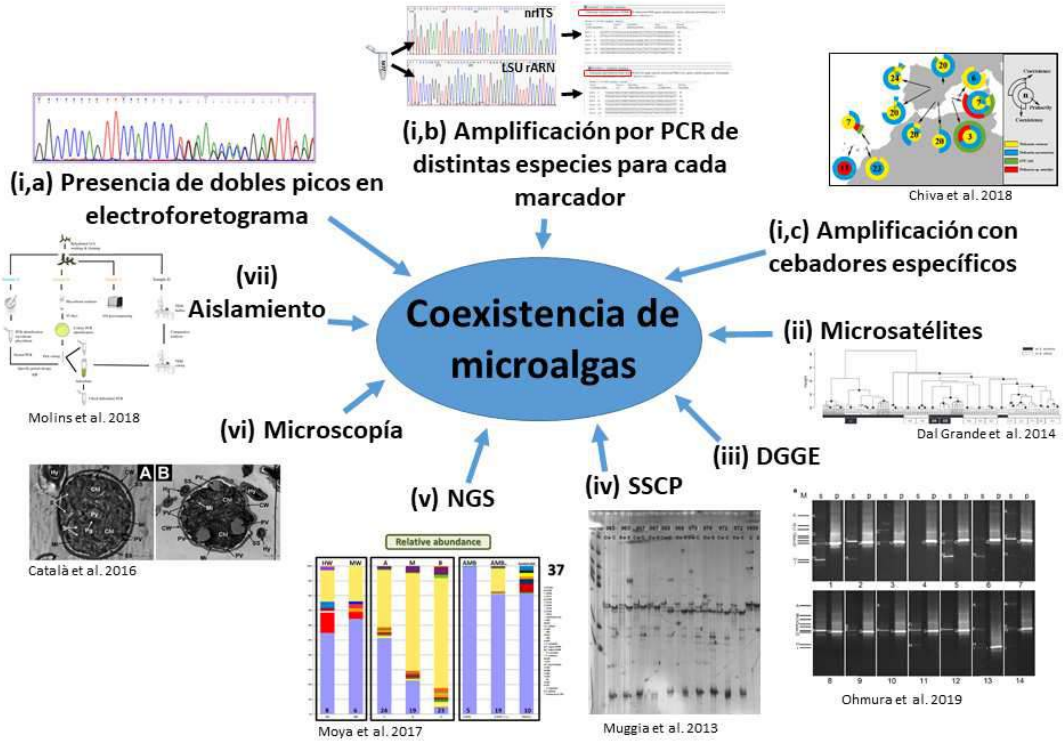


Figura I4. Esquema con las diferentes metodologías que permiten la detección de coexistencia de distintas microalgas en un mismo talo liquénico.

En general la coexistencia se ha puesto de manifiesto mediante el uso de distintas metodologías (Figura I4):

1. secuenciación Sanger,
    - presencia de dobles picos en electroforetograma (R 3.3),
    - incongruencia por la detección de distintas algas con diferentes marcadores moleculares (R 3.3),
    - detección de microalgas no predominantes mediante cebadores específicos (Chiva et al. 2016);
2. análisis de microsatélites (Dal Grande et al. 2014a);
3. análisis de la diversidad de microalgas con DGGE: electroforesis en gel con gradiente de desnaturalización (Ohmura et al. 2019);
4. análisis de la diversidad de microalgas con SSCP (*Single Stranded Conformational Polymorphism*): polimorfismo de conformación de cadena sencilla (Muggia el al. 2013b);
5. secuenciación masiva o "NGS" (Moya et al. 2017; Molins et al. 2018b; R 3.3);
6. observación directa de distintas microalgas en simbiosis en un talo con técnicas microscópicas (del Campo et al. 2010; Molins et al. 2018a) y
7. aislamiento de cultivos axénicos de microalgas (Gasulla et al. 2010; Muggia et al. 2017; Molins et al. 2018b).

1.4.4 Transferencia/adquisición de microalgas entre líquenes

Entre los componentes de las comunidades liquénicas se puede producir el proceso de transferencia (o "robo") de microalgas entre los distintos líquenes de una misma comunidad. Normalmente esta captura de microalgas es realizado por líquenes que son permanente o transitoriamente parásitos de otros líquenes. El clásico ejemplo de este proceso fue descrito por Friedl (1987), donde el liquen *Diploschistes muscorum* adquiere el microalga *Asterochloris irregularis* durante su fase parásita sobre el liquen hospedador (*Cladonia* spp.). Posteriormente, cuando *D. muscorum* tiene capacidad de desarrollarse independientemente del hospedador cambia de compañero fotosintético *algal switching*, y lo sustituye por *Trebouxia showmanii*. Otros estudios realizados sobre comunidades liquénicas sugieren la transferencia de microalgas entre diferentes especies, géneros y familias de hongos liquenizados (Ahmadjian, 1987; Beck et al. 1998; Rambold et al. 1998).

Otra comunidad de líquenes terrícolas en la que se ha analizado este proceso es la compuesta por *Gyalolechia bracteata*, *G. fulgens*, *Toninia sedifolia*, *Squamarina cartilaginea*, *Psora decipiens* y *Lecidea lurida*, dichas especies también forman parte de CBSs (Tabla I1; Ott et al. 1995; de Vera, 2000). Schaper y Ott (2003) reliquenizaron cultivos axénicos del micobionte de *G. bracteacta* (parásita) con microalgas simbióticas aisladas de la misma comunidad y demostraron la presencia de sustancias liquénicas exclusivas de *Gyalolechia* en el interior del talo de sus hospedadores (*Toninia sedifolia*, *Squamarina cartilaginea*, *Psora decipiens* y *Lecidea lurida*). La presencia de estos metabolitos secundarios permitió demostrar que había penetración de hifas de *Gyalolechia* el interior que, posiblemente, eran capaces de captar microalgas del hospedador.

Liquen hospedador	**Liquen parásito (obligado o transitorio)**
Circinaria hofffmanniana	*Caloplaca inconnexa*
Diploschistes diacapsis	*Acarospora nodulosa*
	Acarospora placodiiformis
	Rhizocarpon malenconianum
Psora decipiens	*Gyalolechia* spp.
Squamarina cartilaginea	*Gyalolechia* spp.
Toninia sedifolia	*Gyalolechia* spp.

Tabla I1. Tabla de especies de líquenes hospedadores y parásitos de las biocostras de yesos de la Península Ibérica. Basado de Gutiérrez-Carretero y Casares-Porcel 2011.

Los casos de transferencia de microalgas no son exclusivos de líquenes terrícolas. Se han descrito entre especies de líquenes umbilicados (L*asallia pustulata* y *Umbilicaria spodochroa*) con microalgas del género *Trebouxia* (Hestmark et al. 2016). En el hongo liquenizado *Lecanographa amylacea* se ha analizado la posible adquisición de microalgas (*Trentepohlia* o *Trebouxia*) a partir de otros líquenes de la misma comunidad (Ertz, et al. 2018).

En las comunidades liquénicas de las CBSs de yesos son frecuentes las relaciones parasitarias (o transitoriamente parasitarias) entre las distintas especies de líquenes (Tabla I1). Un caso bien conocido es el de los gipsófitos *Acarospora nodulosa* y *A. placodiiformis*. Ambas especies se desarrollan en sus estadios iniciales como parásito transitorio sobre los talos de *Diploschistes diacapsis*. En cambio, el liquen *Rhizocarpon malenconianum*, se desarrolla sobre *D. diacapsis* parasitándolo de

forma permanente (parásito obligado) (Llimona y Werner, 1975). Igual que en los casos donde el liquen parásito es una especie de *Gyalolechia*, la estrategia de estos consiste en que las primeras hifas que surgen de la germinación de sus esporas, adquieren las microalgas contenidas en los talos de *D. diacapsis* (Rundel 1978; Gutiérrez-Carretero y Casares-Porcel 2011).

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2 Objetivos generales y específicos

2 0BJETIVOS GENERALES Y ESPECÍFICOS

En los últimos años el estudio de las costras biológicas de los suelos se ha incrementado de forma notable y son numerosos los trabajos dedicados al estudio de los organismos que la conforman, de su taxonomía, ecofisiología, distribución y contribución en la modelización de la dinámica de los flujos de agua y nutrientes. Los estudios referentes a los líquenes de las biocostras se han realizado desde perspectivas focalizadas en los micobiontes y, por esta razón, son escasos los que contribuyen con sus resultados al conocimiento de las microalgas simbiontes (ficobiontes) de las comunidades liquénicas.

El objetivo general de la presente memoria doctoral es el estudio de los distintos patrones de asociación y los factores que pueden influir en la interacción de los simbiontes de líquenes en hábitats de biocostras. Con este propósito se han estudiado las microalgas y los micobiontes presentes en estos líquenes desde múltiples enfoques: ultraestructurales, filogenéticos, secuenciación masiva...

Los objetivos específicos que se plantean para alcanzar el objetivo general son los siguientes (ver códigos en Resultados):

1.-. Analizar la selectividad y especificidad de las microalgas simbióticas presentes en los líquenes de las biocostras (R 3.1, R 3.2 y R 3.3).

2.- Determinar si la variabilidad genética del micobionte de los talos influye en la selección de las microalgas (R 3.1, R 3.2, R 3.3 y R 3.4).

3.- Relacionar los distintos eventos climáticos/geológicos de la cuenca mediterránea en la generación de variabilidad genética en los micobiontes (R 3.4).

4.- Analizar si el biotipo (forma etológica) de los distintos talos liquénicos influye en el proceso de selección de microalgas simbiontes (R 3.1, R 3.2 y R 3.3).

5.- Precisar si el tipo de sustrato interviene en el proceso de selección de los ficobiontes (R 3.1, R 3.2 y R 3.3).

6.- Determinar la influencia de los distintos tipos de estructuras reproductoras y la dispersión de los líquenes en la selección de microalgas simbiontes (R 3.1, R 3.2 y R 3.3).

7.- Estudiar de los procesos de coexistencia, *algal switching* y transferencia/ captación de microalgas en los líquenes analizados (R 3.1, R 3.2 y R 3.3).

3 Resultados

Esta tesis está basda en cuatro capítulos, mendcionadas en el texto como "R 3.1, R 3.2, R 3.3 y R 3.4":

R 3.1

Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems.

R 3.2

Myrmecia israeliensis as the primary symbiotic microalga in squamulose lichens growing in European and Canary Island terricolous communities.

R 3.3

Symbiont interaction patterns in biocrusts lichen community located in semi-arid gypsum outcrops at the Central Iberian Peninsula.

R 3.4

How did terricolous fungi originate in the Mediterranean region? A case study with a gypsicolous lichenized species.

3.1 Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems (R 3.1)

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Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems

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The microalgae of the genus *Asterochloris* are the preferential phycobionts in *Cladonia*, *Lepraria* and *Stereocaulon* lichens. Recent studies have highlighted the hidden diversity of the genus, even though phycobionts hosting species of the genus *Cladonia* in Mediterranean and Canarian ecosystems have been poorly explored. Phylogenetic analyses were made by concatenation of the sequences obtained with a plastid – LSU rDNA – and two nuclear – internal transcribed spacer (ITS) rDNA and actin – molecular markers of the phycobionts living in several populations of the *Cladonia convoluta-Cladonia foliacea* complex, *Cladonia rangiformis* and *Cladonia cervicornis* s. str. widely distributed in these areas in a great variety of substrata and habitats. A new strongly supported clade was obtained in relation to the previously published *Asterochloris* phylogenies. Minimum genetic variation was detected between our haplotypes and other sequences available in the GenBank database. The correct identification of the fungal partners was corroborated by the ITS rDNA barcode. In this study we provide a detailed characterization comprising chloroplast morphology, and ultrastructural and phylogenetic analyses of a novel phycobiont species, here described as *Asterochloris mediterranea* sp. nov. Barreno, Chiva, Moya et Škaloud. A cryopreserved holotype specimen has been deposited in the Culture Collection of Algae of Charles University in Prague, Czech Republic (CAUP) as CAUP H 1015. We suggest the use of a combination of several nuclear and plastid molecular markers, as well as ultrastructural (transmission electron and confocal microscopy) techniques, both in culture and in the symbiotic state, to improve novel species delimitation of phycobionts in lichens.

INTRODUCTION

Lichens exemplify the details of complex individuality since they are the outcome of cyclical obligate associations involving at least two very different organisms, a heterotrophic fungus (mycobiont) and a photoautotrophic (photobiont) cyanobacterium (cyanobiont) or/and a unicellular green alga (phycobiont, chlorobiont) (Barreno, 2013). Lichenization allows the partners to thrive in habitats that would otherwise be unavailable to either one on its own, and they are frequently successful in outperforming vascular plants, and even bryophytes, in terms of biodiversity as well as biomass. Lichens also host diverse and heretofore little explored communities of non-phototrophic lichenic bacteria

Abbreviations: BI, Bayesian inference; CBC, compensatory base change; CM, confocal microscopy; ITS, internal transcribed spacer; LM, light microscopy; ML, maximum-likelihood; SEM, scanning electron microscopy; TEM, transmission electron microscopy; wMP, weighted maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the ITS1-5.8S rRNA gene-ITS2-26S rRNA gene region of *Asterochloris mediterranea* are KP257366 to KP257398, those for the LSU rDNA gene are KP257300 to KP257332, and those for the actin gene are KP257333 to KP257365. The GenBank/EMBL/DDBJ accession numbers for the ITS1-5.8S rRNA gene-ITS2 region of the *Cladonia convoluta-Cladonia foliacea* complex, *Cladonia rangiformis and Cladonia cervicornis* s. str. are KP257399 to KP257424.

Two supplementary tables and one supplementary figure are available with the online Supplementary Material.

(Aschenbrenner *et al.*, 2014). Several patterns for mycobiont–phycobiont interactions have been described, but the underlying mechanisms may differ considerably depending on the lichen species. Most of the studies on population structure have reported the presence of a single primary phycobiont species per thallus (Yahr *et al.*, 2004; Muggia *et al.*, 2008; Nelsen & Gargas, 2008) or multiple phycobiont genotypes in a single thallus (Ohmura *et al.*, 2006; Dal Grande *et al.*, 2014; Muggia *et al.*, 2014; Nyati *et al.*, 2014). Additional complexity has been reported (Casano *et al.*, 2011; del Campo *et al.*, 2013; Molins *et al.*, 2013) inside a single lichen thallus by the intrathalline coexistence of different algal species and/or genera.

Because of the obligate and intimate relationship between the photobionts and the mycobiont, it has been hypothesized that lichen symbioses undergoes long-term coevolution or concerted diversification (Ahmadjian, 1987; Rambold *et al.*, 1998; del Campo *et al.*, 2013). On the other hand, no overall co-speciation was evidenced between algal and fungal partners in the worldwide-distributed genus *Cladonia* (Piercey-Normore & DePriest, 2001). This genus represents one of the largest genera of lichen-forming fungi with more than 400 described species (Ahti, 2000). Species of the genus *Cladonia* are often major contributors to overall biomass in diverse habitats and ecosystems (Lechowicz & Adams, 1974; Munger *et al.*, 2008). Moreover, *Cladonia* thalli are among the most complex of lichens, and the interpretation of phenotypic variation of their thalli has been controversial (Stenroos & DePriest, 1998; Stenroos *et al.*, 2002a, b; Grube & Hawksworth, 2007). Several recent molecular studies have revealed a lack of correlation between morphological and molecular data, and many traditionally well-delimited species seem to be problematic (Myllys *et al.*, 2003; Kotelko & Piercey-Normore, 2010; Piercey-Normore *et al.*, 2010; Pino-Bodas *et al.*, 2012a, b, c; Steinová *et al.*, 2013).

Cladonia represents a genus known for its prevailing specificity to *Asterochloris* algae (Piercey-Normore & DePriest, 2001; Nelsen & Gargas, 2006, 2008). This genus of microalga has been studied recently from the lichen fungi in the Cladoniineae (genera *Cladonia*, *Lepraria*, *Stereocaulon*) and in *Diploschistes muscorum*, a common parasite of species of the genus *Cladonia* (Škaloud & Peksa, 2010; Škaloud *et al.*, 2015). Phylogenetic analysis based on the concatenated set of internal transcribed spacer (ITS) rDNA and actin type I intron sequences revealed 20 well-resolved clades among *Asterochloris* phycobionts and particular clades were found to be associated with taxonomically different, but ecologically similar, lichens. Additional large hidden diversity in *Asterochloris* lineages was revealed in species of the genus *Cladonia* from India (Řídká *et al.*, 2014).

Piercey-Normore & DePriest (2001) showed that there are very few algal genotypes shared among variously related taxa of the family Cladoniaceae, implying that selectivity is not equal between lichen-forming fungi and algae. Contrasting findings suggested that different patterns of selectivity and specificity may occur in different lichen taxa (Piercey-Normore, 2004; Yahr *et al.* 2004; Nelsen & Gargas, 2008, 2009; Fernández-Mendoza *et al.*, 2011; Magain & Sérusiaux, 2014). Subsequent analyses (Peksa & Škaloud, 2011) revealed that these *Asterochloris* phycobionts could exhibit clear preferences for environmental factors. These algal preferences may limit the ecological niches available to lichens and lead to the existence of specific lichen guilds. Likewise, recent physiological studies evidenced that dehydration rate and time of desiccation affect recovery in *Asterochloris erici* cultures; in addition proteomic and transcript analyses suggest that desiccation tolerance seems to be achieved by constitutive mechanisms in this alga (Gasulla *et al.*, 2009, 2013).

Similar to other characteristics, the secondary structure of rRNA has also been used in evolutionary comparisons (Coleman *et al.*, 1998; Lott *et al.*, 1998; Hausner & Wang, 2005). Patterns in the secondary structure of the ITS rRNA transcripts in *Asterochloris* and in other microalgae have been used as an additional attribute to delimit species boundaries (Beiggi & Piercey-Normore, 2007; Škaloud & Peksa, 2010; Škaloudová & Škaloud, 2013). However, it was demonstrated that differences in the ITS rRNA secondary structures are often not diagnostic at the species level in green algae (Caisová *et al.*, 2011; Škaloud & Rindi, 2013).

This work was focused on four species of the genus *Cladonia*: *Cladonia foliacea* (Huds.) Willd., which is the most common and widely distributed species in the Mediterranean area, sharing its habitat with *Cladonia convoluta* (Lam.) Anders; *Cladonia rangiformis* Hoffm. and *Cladonia cervicornis* s. str. (Ach.) Flot. (Pino-Bodas *et al.*, 2013a). All of these taxa grow preferentially in scrublands and forest clearings under prevailing dry bioclimates (Burgaz & Ahti, 1992, 2009). Several studies (Burgaz & Ahti, 1992, 2009; Litterski & Ahti, 2004) have reported the controversy concerning how to morphologically, chemically and phylogenetically identify the differences between *C. convoluta* and *C. foliacea*; up to the present, it has been impossible to separate them into monophyletic groups, and the term *C. convoluta-C. foliacea* complex has been established (Pino-Bodas *et al.*, 2010). The majority of molecular studies of species of the genus *Cladonia* have focused on mycobiont analyses, but the phycobiont has been mostly ignored and is poorly known.

In this study, we describe a novel phycobiont species, *Asterochloris mediterranea* sp. nov., discovered during our investigations into the phycobionts of *Cladonia* and other terricolous genera in the Mediterranean and Canary Islands ecosystems. Molecular and ultrastructural approaches led us to identify, and characterize in detail, this novel species both from thalli and isolated monoclonal cultures. Moreover, a key to the species of the genus *Asterochloris* has been drawn up.

METHODS

Taxa sampling. Specimens of *C. foliacea* (Huds.) Willd. and *C. convoluta* (Lam.) Anders (*C. convoluta-C. foliacea* complex), *C. rangiformis* Hoffm.

and *C. cervicornis* s. str. (Ach.) Flot. were collected from different locations in the Iberian Peninsula and the Canary Islands (Fig. 1; Table S1 available in the online Supplementary Material). Samples were dried out in the shaded open air for 1 day and then stored at −20 °C until needed.

Isolation and cultivation of phycobionts. The algal symbionts were isolated by the thallus fragmentation method (Ahmadjian, 1993; Peksa & Škaloud, 2008) as follows: small fragments of lichen thalli were plated onto 2 % agar slants of Bold's Basal Medium (BBM) as modified by Bischoff & Bold (1963). The Petri dishes with thalli fragments were incubated at 18 °C, under an illumination of 20–30 mmol m^{-2} s^{-1} and a 16 : 8 h light/dark cycle. If fungal contamination occurred during the cultivation, the contaminants were carefully removed or the thalli fragments were transferred to new plates. After 2–3 weeks, groups of dividing algal cells were observed associated with some of the fragments. To obtain unialgal cultures, small populations of phycobionts were transferred onto the fresh BBM agar slants and incubated accordingly.

Microscopic investigations. Light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques were performed for morphological analysis of 'in thallus' lichen phycobionts. Pieces of rehydrated *C. convoluta-C. foliacea* complex thalli from Fuentidueña de Tajo (Madrid) C6 and Bujaraloz (Zaragoza) C16 were used to examine the *Asterochloris*-type phycobionts inside the thalli. To analyse the morphology of chloroplasts in isolated phycobionts, confocal microscopy (CM) was used. TEM and SEM examinations were made at the SCSIE Service of the University of Valencia.

Light microscopy (LM). For LM analyses, 15–20 μM sections were cut from frozen samples with a sliding microtome (Leica SM 2000R). The sections were observed with an Olympus BX40 microscope equipped with an Olympus SC100-10.6 camera.

Transmission electron microscopy (TEM). For TEM, the cells were fixed in 2 % Karnovsky fixative for 6 h at 4 °C, and washed three times for 15 min with 0.01 M PBS, pH 7.4, and post-fixed with 2 % OsO_4 in 0.01 M PBS, pH 7.4, for 2 h at room temperature. Thereafter, they were washed three times in 0.01 M PBS, pH 7.4, for 15 min and then dehydrated at room temperature in a graded series of ethanol, starting at 50 % and increasing to 70 %, 95 % and 100 % for no less than 20–30 min for each step (Casano *et al.*, 2011; Molins *et al.*, 2013). The fixed and dehydrated samples were embedded in Spurr's resin according to the manufacturer's instructions (Spurr, 1969). Sections (90 nm) were cut with a diamond knife (DiATOME Ultra 45°) using an ultramicrotome (LKB Bromma Nova Ultratome), mounted on copper grids of 100 mesh and post-stained using a SynapTek Grid Staining kit (Electron Microscopy Sciences; http://www.ems-diasum.com/microscopy/technical/datasheet/71175.aspx). The sections were observed with a JEOL JEM-1010 (80 kV) electron microscope, equipped with a MegaView III digital camera and AnalySIS image acquisition software.

Scanning electron microscopy (SEM). SEM was undertaken in order to observe the surface ultrasculpture of the squamules. Fractured

Fig. 1. Distribution of the *Cladonia* samples collected for this study in the Iberian Peninsula and the Canary Islands. Sites 1 to 13 and the various types of substrate are indicated by symbols: quartzites, siliceous (white square □), limestones, calcareous (black square ■), sandstones, siliceous (black triangle ▶), Miocene gypsum (black circle ●) and volcanic (white discontinuous circle ◌). Details of the sampling sites are given in Table S1.

thalli were attached to the holder, coated with palladium/gold and viewed with a Hitachi S-4800 field emission scanning electron microscope.

Confocal microscopy (CM). A Leica TCS SP2 laser scanning confocal microscope equipped with an argon-krypton laser was used. We applied a 488 nm excitation line and an AOBS filter-free system collecting emitted light between 498 and 700 nm. The autofluorescence of chlorophyll was exploited for visualization of the chloroplast structure. A series of optical sections through chloroplasts was captured and used for three-dimentional reconstruction of their morphology. The chloroplast reconstructions were produced by the ImageJ 1.34p program (Abràmoff *et al.*, 2004), using the 'Volume viewer' plugin.

DNA isolation, amplification and sequencing. Individuals from localities 1 to 13 (Fig. 1) were analysed after washing performed following the protocol of Muggia *et al.* (2013). Samples were given an alphanumeric code from C1 to C33 as shown in Table 1. Total genomic DNA was isolated and purified using a Dneasy Plant Minikit (Qiagen) following the manufacturer's instructions.

Three algal loci were amplified from genomic DNA from each lichen thallus. As chloroplast genome marker, we studied a region of the LSU rRNA gene by using the algal-specific primers 23SU1 and 23SU2 (del Campo *et al.*, 2010a). We also amplified two nuclear loci encoding the nrITS RNA, using the algal-specific primer nr-SSU-1780 (Piercey-Normore & DePriest, 2001) and the universal primer ITS4 (White *et al.*, 1990), and actin type 1, using the algal-specific primer pair ActinF2 Astero (Škaloud & Peksa, 2010) and a-nu-act1-0818-3′ (Nelsen & Gargas, 2006). The primers used to amplify the nuclear ITS rRNA from the mycobiont were ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990).

Table 1. List of taxa included in this study, code (C1 to C33) used in phylogenetic analysis, and the GenBank accession numbers for the newly determined ITS rDNA, LSU rDNA and actin sequences, locality and type of substrate (see also Fig. 1 and Table S1)

Taxon	Code	GenBank accession number				Locality & type of substrate
		Phycobiont			Mycobiont	
		ITS rDNA	LSU rDNA	Actin	ITS rDNA	
C. conv-fol complex	C1	KP257366	KP257300	KP257333	KP257399	2 ●
C. conv-fol complex	C2	KP257367	KP257301	KP257334	KP257400	2 ●
C. conv-fol complex	C3	KP257368	KP257302	KP257335	KP257425	2 ●
C. conv-fol complex	C4	KP257369	KP257303	KP257336	KP257427	2 ●
C. conv-fol complex	C5	KP257370	KP257304	KP257337	KP257426	2 ●
C. conv-fol complex	C6	KP257371	KP257305	KP257338	KP257428	2 ●
C. conv-fol complex	C7	KP257372	KP257306	KP257339	KP257401	2 ●
C. conv-fol complex	C8	KP257373	KP257307	KP257340	KP257402	2 ●
C. conv-fol complex	C9	KP257374	KP257308	KP257341	KP257403	2 ●
C. conv-fol complex	C10	KP257375	KP257309	KP257342	KP257404	2 ●
C. conv-fol complex	C11	KP257376	KP257310	KP257343	KP257405	2 ●
C. conv-fol complex	C12	KP257377	KP257311	KP257344	KP257406	2 ●
C. conv-fol complex	C13	KP257378	KP257312	KP257345	KP257407	2 ●
C. conv-fol complex	C14	KP257379	KP257313	KP257346	KP257408	3 ●
C. conv-fol complex	C15	KP257380	KP257314	KP257347	KP257409	3 ●
C. conv-fol complex	C16	KP257381	KP257315	KP257348	KP257410	6 ●
C. conv-fol complex	C17	KP257382	KP257316	KP257349	KP257411	6 ●
C. conv-fol complex	C18	KP257383	KP257317	KP257350	KP257412	6 ●
C. conv-fol complex	C19	KP257384	KP257318	KP257351	KP257413	11 ●
C. conv-fol complex	C20	KP257385	KP257319	KP257352	KP257414	1 □
C. conv-fol complex	C21	KP257386	KP257320	KP257353	KP257415	1 □
C. conv-fol complex	C22	KP257387	KP257321	KP257354	KP257416	1 □
C. conv-fol complex	C23	KP257388	KP257322	KP257355	KP257417	4 ■
C. conv-fol complex	C24	KP257389	KP257323	KP257356	KP257418	5 ■
C. conv-fol complex	C25	KP257390	KP257324	KP257357	KP257419	7 ▶
C. conv-fol complex	C26	KP257391	KP257325	KP257358	KP257420	8 ▶
C. conv-fol complex	C27	KP257392	KP257326	KP257359	KP257421	10 ■
C. rangiformis	C28	KP257393	KP257327	KP257360	KP257429	12 ◌
C. rangiformis	C29	KP257394	KP257328	KP257361	KP257430	13 ◌
C. rangiformis	C30	KP257395	KP257329	KP257362	KP257431	1 □
C. cervicornis s. str.	C31	KP257396	KP257330	KP257363	KP257422	9 ▶
C. cervicornis s. str.	C32	KP257397	KP257331	KP257364	KP257423	1 □
C. cervicornis s. str.	C33	KP257398	KP257332	KP257365	KP257424	1 □

C. convoluta-C. foliacea complex are indicated as *C. conv-fol* complex.

PCRs were performed in 50 µl using EmeraldAmp GT PCR Master Mix (Takara). The only user-supplied reagents that need to be added are template DNA, specific primers and water, allowing for improved reproducibility while minimizing the potential for contamination. Negative controls, without a DNA template, were included in every round of PCR amplification to ensure against false-positive results caused by contaminants in the reagents. The PCR programme for amplifications comprised an initial denaturation at 94°, 2 min, and 30 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min, followed by a final elongation at 72 °C for 5 min. Amplifications were carried out on a 96-well SensoQuest labcycler (Progen Scientific). The PCR products were separated on 2 % agarose gels and purified using Real Clean Spin (Durviz). The amplified PCR products were sequenced with an ABI 3100 Genetic analyser using the ABI BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Sequence analyses. A multiple alignment of the newly determined fungal ITS rDNA (accession nos KP257399 to KP257424), algal ITS rDNA (KP257366 to KP257398), actin (KP257333 to KP257365) and LSU rDNA (KP257300 to KP257332) sequences and selected fungal ITS rDNA sequences described by Pino-Bodas *et al.* (2010) was built using MAFFT, version 6, applying the Q-INS-i strategy (Katoh *et al.*, 2002). The alignment of actin sequences was improved by eliminating the ambiguously aligned regions using the program Gblocks v. 0.91b (Castresana, 2000). The three loci were concatenated, yielding an alignment of 2036 characters. The final matrix contained 62 ITS rDNA, 62 actin and 34 LSU rDNA sequences. For each locus, the most appropriate substitution model was estimated using the Akaike information criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander, 2004). This AIC-based model selection procedure selected the GTR + Γ model for the mycobiont ITS rDNA, and the three following models for the phycobiont datasets: (1) GTR + I + Γ for ITS rDNA, (2) GTR + Γ for the actin gene, and (3) HKY + I for LSU rDNA.

The phylogenetic trees were inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist *et al.*, 2012), carried out on partitioned datasets using the different substitution models selected by PAUP/MrModeltest 1.0b. All parameters were unlinked among partitions. Two parallel Markov chain Monte Carlo (MCMC) runs were carried out for ten million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0.0025. Finally, the burn-in values were determined using the 'sump' command.

Bootstrap analyses were performed by maximum-likelihood (ML) and weighted maximum-parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl, 2006) and PAUP*, version 4.0b10 (Swofford, 2002), respectively. ML analysis consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100 000). The analysis was performed on partitioned datasets using the different substitution models. The wMP bootstrapping (1000 pseudo-replicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data. Character weights were assigned using the rescaled consistency index on a scale of 0 to 1000. New weights were based on the mean fit values for each character over all trees in the memory.

Haplotype network. To show the genetic diversity within the newly characterized lineages, we reconstructed the haplotype networks on the basis of MP analyses of all available sequences, including those selected from the GenBank database (Table 2), using the Haplotype Viewer (G. Ewing, available at www.cibiv.at/~greg/haploviewer).

Phycobiont ITS2 secondary structure. The coding regions, required for the basal stems in ITS2 secondary structures, were delimited by comparison between our sequences and known sequences of *Asterochloris* (Beiggi & Piercey-Normore, 2007). The stem–loop structures were folded using the ITS2DATABASE (Koetschan *et al.*, 2010). If more than one fold was produced, the final fold was based on comparisons with those previously published for *Asterochloris* (Beiggi & Piercey-Normore, 2007), maximizing the hydrogen bonding forming solid stems, and the largest negative Δg value (free energy).

RESULTS

Asterochloris mediterranea sp. nov. Barreno, Chiva, Moya et Škaloud (Figs 2, 3 and 4)

Description. Mature vegetative cells spherical in shape, but oval, pyriform and kidney shapes are not uncommon, 8.2–8.5 to 12–16.3 µm in diameter. Single central chloroplast

Table 2. GenBank fungal ITS rDNA sequences described by Pino-Bodas *et al.* (2010) included in the phylogenetic analysis and algal ITS rDNA (Pino-Bodas *et al.*, direct submission; Piercey-Normore & DePriest, 2001) included in the haplotype network analysis

Mycobiont	Collection	Fungal ITS	Algal ITS	Locality	Substrate
C. convoluta	MACB 90565	FM205886.1b	FM205732	Granada, Spain	Limestone ■
C. convoluta	MACB 90565	FM205886.1c	FM205730	Granada, Spain	Limestone ■
C. convoluta	MACB 91687	FM211899	FM205729	Guadalajara, Spain	Sandstone ▶
C. foliacea	MACB 95599	FM205914	FM205728	Trás-os-Montes, Portugal	Granite ▷
C. foliacea	MACB 90533	FM205897	FM205727	Guadalajara, Spain	Granite ▷
C. foliacea	MACB 90574	FM205895	FM205725	Tarragona, Spain	Granite ▷
C. foliacea	MACB 91639	FM205899	FM205724	Ávila, Spain	Quartzite □
C. convoluta	MACB 90440	FM205901	FM205723	Barcelona, Spain	Limestone ■
C. convoluta	MACB 90499	FM205900	FM205722	Navarra, Spain	Limestone ■
C. convoluta	MACB 90565	FM205886.1	FM205721	Granada, Spain	Limestone ■
C. foliacea	MACB 90503	FM205898	FM205720	Alto Alentejo, Portugal	Quartzite □
C. rangiformis	Frost-Olsen 5501	–	AF345435	Yugoslavia	–
Cladonia fimbriata	Gustavsson & Thollesson s.n.	–	AF345434	Gothenburg, Sweden	–

with margins extended into finger-like, divided lobes. Central pyrenoid, spherical or irregularly elongated, of *irregularis*-type. One nucleus with nucleolus. Cell wall thin, 0.3–0.5 μm wide, with flat local thickening and irregular secretory spaces. Asexual reproduction by 64–128 spherical aplanospores.

Type locality. Phycobiont of *C. convoluta-C. foliacea* complex, collected on gypsum soils in Fuentidueña de Tajo (Madrid, Spain), 40° 07′ 87″ N 03° 09′ 12″ W, altitude 571 m, upper mesomediterranean low dry (leg. Barreno, Chiva, Molins & Salvá 24 February 2012). The lichen specimen was deposited in herbarium PRC (no. 2939). Samples of the same population are in MA-Lich (no. 18201), VAL_Lich (no. 30278) and MAF-Lich (no. 19479).

Holotype. Cryopreserved cells of strain C6, deposited at the Culture Collection of Algae of the Charles University in Prague (CAUP), as item TYPE – H 1015.

Reference strains. CAUP H 1015 and E. Barreno's Lab in the Universitat de València (no. 131).

Molecular signatures. Hemi-CBCs in helix III (**C**-G : **U**-G; unique) and (**G**-G : **C**-G) of the ITS2 as compared with the ITS2 rRNA secondary transcripts of *Asterochloris phycobiontica*.

Etymology. *Asterochloris mediterranea* (me.di.ter.ra′ne.a.).

Etymology: L. fem. adj. *mediterranea* midland, inland and, in late Latin, used to refer to the Mediterranean Sea or bioclimatic types (Rivas-Martínez & Rivas-Sáenz, 2009).

The specific epithet refers to the wide but not exclusive Mediterranean distribution and the relative abundance of several *Cladonia* mycobionts, which are up to now the most frequent hosts of this phycobiont.

Distribution. So far only known in the Iberian Peninsula, former Yugoslavia, Sweden and the Canary Islands.

Ecology. At the time of writing it has been found in symbiosis with *C. convoluta-C. foliacea* complex, *C. rangiformis*, *Cladonia fimbriata* and *C. cervicornis* s. str. lichen thalli growing in a wide variety of habitats, from lowlands to Mediterranean mountains, and on soils derived from very different types of rocks (gypsum, limestones, quartzites, granites, sandstones, volcanic).

Morphological and ultrastructural characterization

The squamules analysed in this study showed a strongly fissured and cracked surface (Fig. 2a, b), similar to the features previously noticed by Osyczka & Rola (2013), who found a full range of surface roughness under SEM in different species of the genus *Cladonia* growing in non-arid or dry habitats.

In this study, LM, SEM and TEM were used to characterize the structure and ultrastructure of the cells of *Asterochloris mediterranea* sp. nov. found in *C. convoluta-C. foliacea* complex, *C. rangiformis* and *C. cervicornis* s. str. (Figs 2 and 3). The observations were made on samples C6 and C16, and no differences were found between them. The phycobionts were usually located in close contact with the hyphae in the phycobiont layer; the interactions between photobiont cells and fungal hyphae were the 'simple' type (Honegger, 1986), without invaginations or haustoria (Fig. 2a, b, c). Mature vegetative cells were mostly spherical, but oval, pyriform and kidney shapes were also common, 8.2–8.5 to 12–16.3 μm in diameter. The cell wall exhibited a variable thickness, ranging from 0.3 to 0.5 μm, with flat local thickening and irregular secretory spaces giving a characteristic appearance to the young and mature vegetative cells. Both in thallus and culture, the cells showed a large, axial and lobed chloroplast with margins extended into finger-like lobes in the periphery characteristic of the genus *Asterochloris* (Fig. 2c, d). The thylakoid membranes were grouped in stacks shaped by 4–5 membranes, similar to the grana in vascular plants (Fig. 3b, c, e, f). A large central portion of the chloroplast was taken up by the pyrenoid (Figs 2e, f and 3a, c, d). Usually, there was only one pyrenoid per cell, although in some cases two could appear (Fig. 3d). This pyrenoid might be spherical or irregularly elongated (Fig. 3a, c), with a small number of thin, arched thylakoid tubules invaginating within the matrix. Numerous pyrenoglobuli (80–100 nm in diameter) were connected with these tubules (Fig. 3b). This ultrastructure fits into the *irregularis*-type (Friedl, 1989). Spherical non-electron-dense vesicles appeared throughout the cytoplasm and were especially numerous at the periphery (Figs 2e, f and 3a). Also, some small mitochondria were observed (Fig. 3e). Most of the cells presented numerous ribosomes in the cytoplasm (Fig. 3e, f) and showed a clear and apparent nucleus with nucleolus (Figs 2f and 3d). The plasmatic membrane showed invaginations in many areas connected with secretory vesicles (Fig. 3e). Secretory spaces were irregular in thickness and myelin-like bodies could be seen in some cases (Fig. 3e). Two types of myelin-like bodies were detected, the plasmalemmasomes in the secretory spaces (Fig. 3e) outlined by evagination of the plasmalemma as well as by fusion of vesicles, and lomasomes (Fig. S1) associated with the rough endoplasmic reticulum (Marchant & Robards, 1968; Robards, 1968). Aplanospores by 64–128 in the sporocysts were spherical (Fig. 2d).

Chloroplast morphology of isolated lichen phycobionts

Once unialgal cultures were obtained as previously described, confocal reconstructions showed that the majority of the cell volume was occupied by the chloroplast (Fig. 4). In young cells, the chloroplast was central, axial, with simple lobes spreading towards the cell periphery (Fig. 4a). Mature vegetative cells could possess a similarly formed, shallowly lobed plastid with simple lobes. However, the lobes were

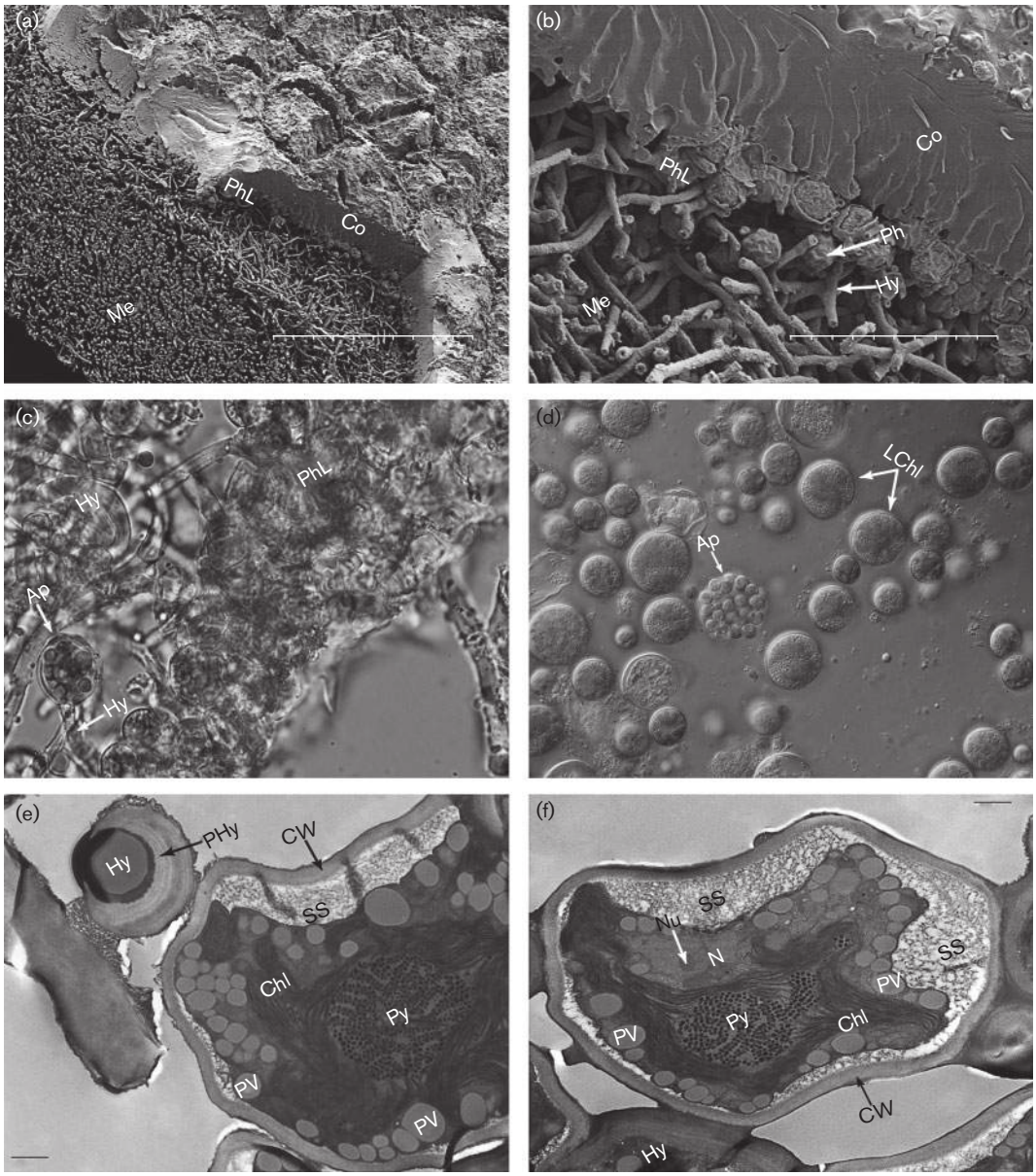

Fig. 2. Ultrasculpture architecture of *C. convoluta*-*C. foliacea* complex thalli C16 by SEM. (a) Transversal section of the primary thallus with upper cortex (Co), phycobiont layer (PhL) and medulla (Me); bar, 200 μm. (b) Detail of the phycobiont layer (PhL) showing the interaction between phycobiont (Ph) and hypha (Hy) of the type 'simple' (Honegger, 1986); bar, 40 μm. (c) Cross section of the thallus by LM (sample C16, fresh). Phycobiont layer (PhL) showing asexual reproduction by aplanospore (Ap). (d) Isolated *Asterochloris*-type phycobiont showing aplanospore (Ap) and characteristic 'lobed' *Asterochloris* chloroplast (LChl). Cross section of C16 thallus by TEM (e) and (f) *Asterochloris mediterranea* sp. nov. phycobiont inside thallus. Bars, 1 μm. PHy, Peripherial hypha; CW, cell wall; SS, secretory space; Chl, chloroplast; Py, pyrenoid; PV, peripheral vesicles; N, nucleus; Nu, nucleolus.

often branched at their ends, so the chloroplast margin was extended into finger-like, divided lobes (Fig. 4b, c). Rarely, the chloroplast assumed a parietal position, having short, simple lobes. In the late ontogenetic stages, specifically prior to zoo- or aplanosporogenesis, the chloroplast transformed into the parietal type with smooth, never lobed, margins. After a short time, it began to divide into numerous parts in preparation for asexual reproduction (Fig. 4d).

Phycobiont phylogenetic analysis

At the time of writing, apart from sequences obtained in this study, a total of 19 nrITS phycobiont sequences from *C.*

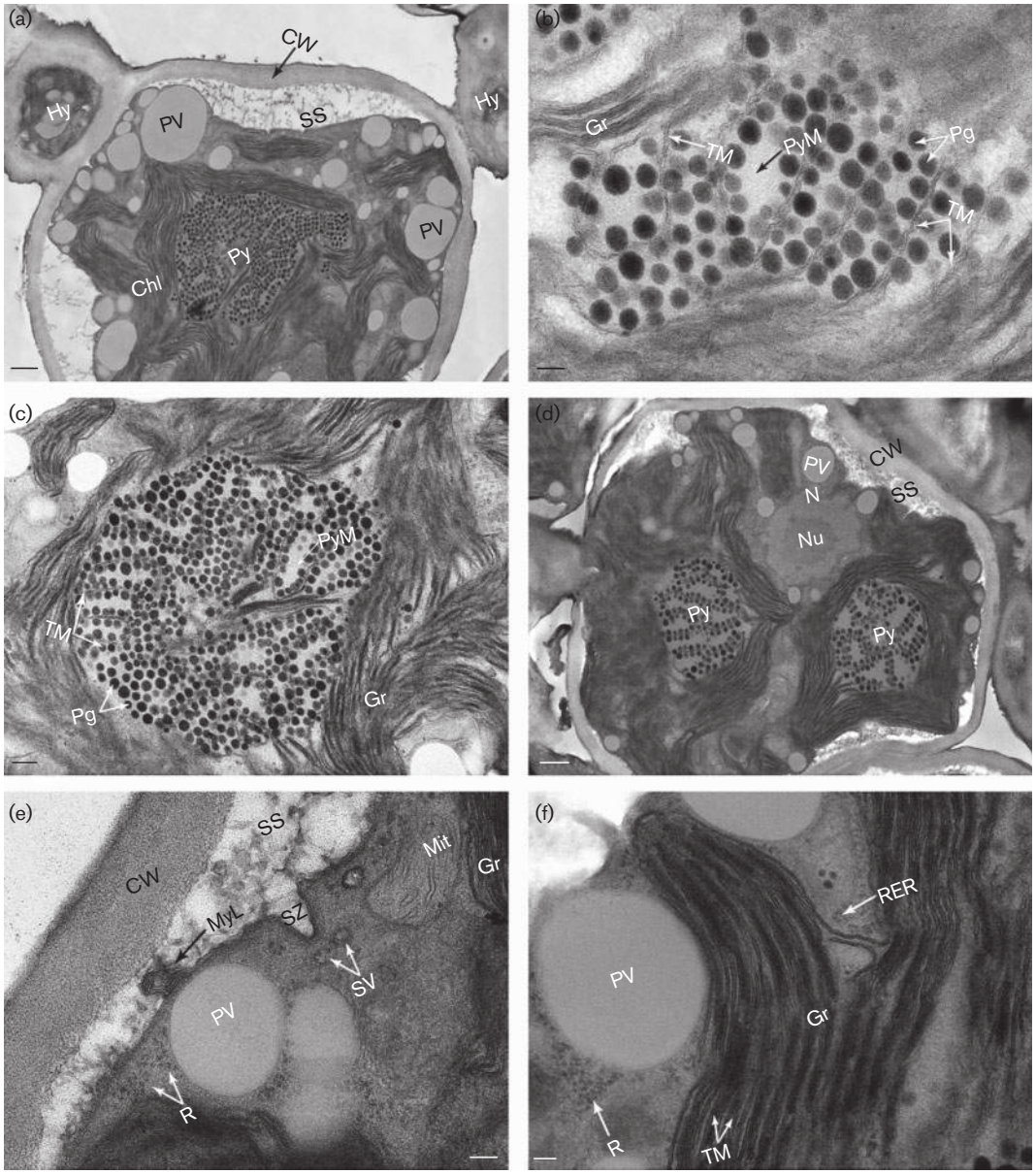

Fig. 3. *C. convoluta*-*C. foliacea* complex. Cross section of C16 thalli by TEM. (a) *Asterochloris mediterranea* sp. nov. phycobiont inside thallus; bar 1 μm. (b) and (c) Detail of a pyrenoid (Py), pyrenoglobuli (Pg) associated with the pyrenoid matrix (PyM), grana (Gr) and thylakoid membrane (TM); bars 0.2 μm and 0.5 μm, respectively. (d) Phycobiont showing chloroplasts in duplication phase with two pyrenoids (Py), peripheral vesicles (PV), nucleus (N), nucleolus (Nu), cell wall (CW), secretory space (SS); bar 1 μm. (e) Detail showing secretory complex: myelin-like bodies (MyL), peripheral vesicles (PV), mitochondria (Mit), ribosome (R), secretory space (SS), secretory vesicle (SV), secretion zone (SZ) and rough endoplasmic reticulum (RER); bar 0.2 μm. (f) Detail of chloroplast showing peripheral vesicles (PV), ribosome (R), thylakoid membrane (TM), rough endoplasmic reticulum (RER) and grana (Gr). Bar 0.2 μm

convoluta, *C. foliacea*, *C. rangiformis* and *C. cervicornis* subsp. *verticillata* were available in GenBank (Piercey-Normore & DePriest, 2001; Beiggi & Piercey-Normore, 2007; Bačkor *et al.*, 2010; Pino-Bodas *et al.*, 2010). Only 11 sequences from the *C. convoluta*-*C. foliacea* complex, submitted by Pino-Bodas (Table 2), fitted with those of *Asterochloris mediterranea*. All of them incorporated information about mycobiont nrITS and substrate. In addition, two sequences (AF345434 and AF345435) from *C. fimbriata* and *C. rangiformis* produced by Piercey-Normore & DePriest (2001) also fitted with those of *A. mediterranea*, but without any specific information concerning the habitat, location or nrITS sequence of the mycobiont. We included these sequences (Table 2) in the haplotype parsimony networks analysis, but they were excluded from the phycobiont phylogeny analysis due to the lack of actin and LSU rDNA information.

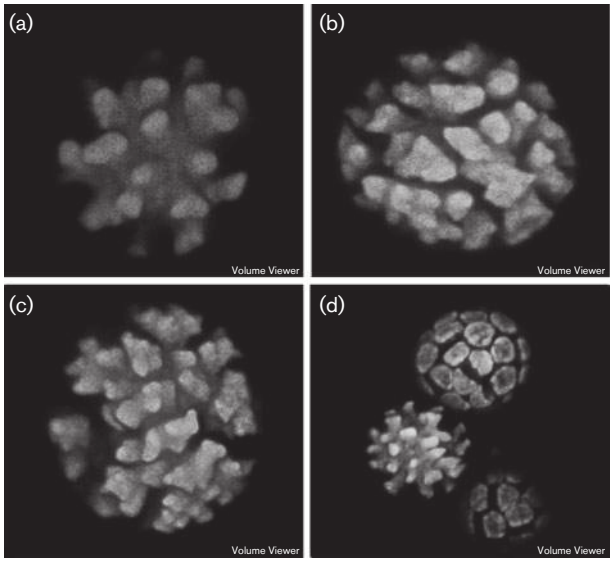

Fig. 4. Confocal microscopy of chloroplast structures in isolated lichen phycobionts (*A. mediterranea* sp. nov.). (a) A young cell with simple lobes, (b) a mature vegetative cell showing shallowly lobed plastid with simple lobes, (c) a mature vegetative cell with finger-like divided lobes, (d) two globular aplanosporangia with a large number of aplanospores.

The concatenated Bayesian analysis of the new phycobiont ITS rDNA, actin and LSU rDNA dataset revealed the existence of more than 20 well-resolved lineages within the genus *Asterochloris* (Fig. 5). The relationships among the lineages correspond well with the phylogeny presented by Škaloud & Peksa (2010) and Škaloud *et al.* (2015), including the presence of three moderately to well-supported major clades, A, B and C. The 13 previously described species (*A. erici*, *A. excentrica*, *A. glomerata*, *A. irregularis*, *A. italiana*, *A. magna*, *A. phycobiontic*a, *A. echinata*, *A. friedlii*, *A. gaertneri*, *A. leprarii*, *A. lobophora* and *A. woessiae*) formed well-recognized, distinct lineages. All phycobionts investigated in this study formed a distinct, statistically well-supported lineage, inferred within clade C, in relation to the lineage represented by a single *Lepraria* phycobiont Nelsen 2585.

Genetic relationships of sequences of *A. mediterranea* from samples C1 to C33 (Table 1) and the sequences selected in Table 2 were analysed by statistical parsimony networks of the ITS rDNA showing minimum differences. All the haplotypes were included in one linked network pointing out a single taxon. Six haplotypes of *A. mediterranea* were found in the *C. convoluta-C. foliacea* complex, and two more haplotypes in the three *Cladonia* taxa studied here (Fig. 6a). Haplotype 7 (AF345434) and Haplotype 9 (AF345435) were inferred from GenBank. In lichens growing at localities with Miocene gypsum bedrock, only four haplotypes were detected (Fig. 6b), whereas another four haplotypes appeared randomly in the remaining types of substrates.

ITS2 secondary structure

A common overall organization of the ITS2 secondary structure could be identified in *A. mediterranea* (Fig. 7). The ITS2 secondary structure possessed conserved motifs among green algae (Mai & Coleman, 1997), i.e. four-fingered hand (helix I-IV), a pyrimidine-pyrimidine mismatch in helix II, and a conserved sequence of UGGU on the 5′ side of helix III (Fig. 7). The ITS2 secondary structures were compared first among the *A. mediterranea* genotypes found in this study to check the occurrence of compensatory base changes (CBCs: nucleotide changes at both sides of paired bases) and hemi-CBCs (changes at only one side of a nucleotide pair, but still preserving pairing) according to Coleman (2003). One insertion, one single base change and one hemi-CBC at positions 18, 80 and 88 were identified; these changes were not previously identified by Škaloud & Peksa (2010).

The ITS2 secondary structure of *A. mediterranea* was also compared with the previously published structures of *Asterochloris* lineages 1–16 (Škaloud & Peksa, 2010). In total, two single base changes at positions 35 and 37, and one hemi-CBC at position 91, were newly identified.

Mycobiont phylogenetic analysis

To validate the correct lichen identifications, 33 samples were analysed, all of them with the corresponding algal sequences (Table 1). We also included 11 mycobiont sequences selected from those described by Pino-Bodas *et al.* (2010) in which the phycobiont partner sequences coincided with those of *A. mediterranea* (Table 2).

The aligned fungal ITS was 481 bp long, including ITS1, 5.8S rDNA and partial ITS2, with 91 variable characters of which 64 were parsimony-informative. We resolved 20 fungal ITS genotypes: 14 in the *C. convoluta-C. foliacea* complex, three in *C. rangiformis* and two in *C cervicornis* s. str. (Fig. 8). BLAST searches produced significant matches with other fungal accessions of species of the genus *Cladonia* described by Pino-Bodas *et al.* (2010, 2013a) and Stenroos *et al.* (2002b). Phylogenetic analysis including mycobiont ITS sequences from Pino-Bodas *et al.* (2010) selected in this study, showed three well-supported clades corresponding with the *C. convoluta-C. foliacea* complex, *C. rangiformis* and *C. cervicornis* s. str. (Fig. 8) as we previously determined.

DISCUSSION

The present study contributes to the understanding of the symbiont microalgae of *Cladonia* lichens providing new insights into the hidden phycobiont diversity found in the genus *Asterochloris* (Tschermak-Woess, 1980; Bačkor *et al.*, 2010; Škaloud & Peksa, 2010; Peksa & Škaloud, 2011; Škaloud *et al.*, 2015). Both morphological and molecular analyses pointed out the presence of a previously unknown taxon described here as *Asterochloris mediterranea* sp. nov.

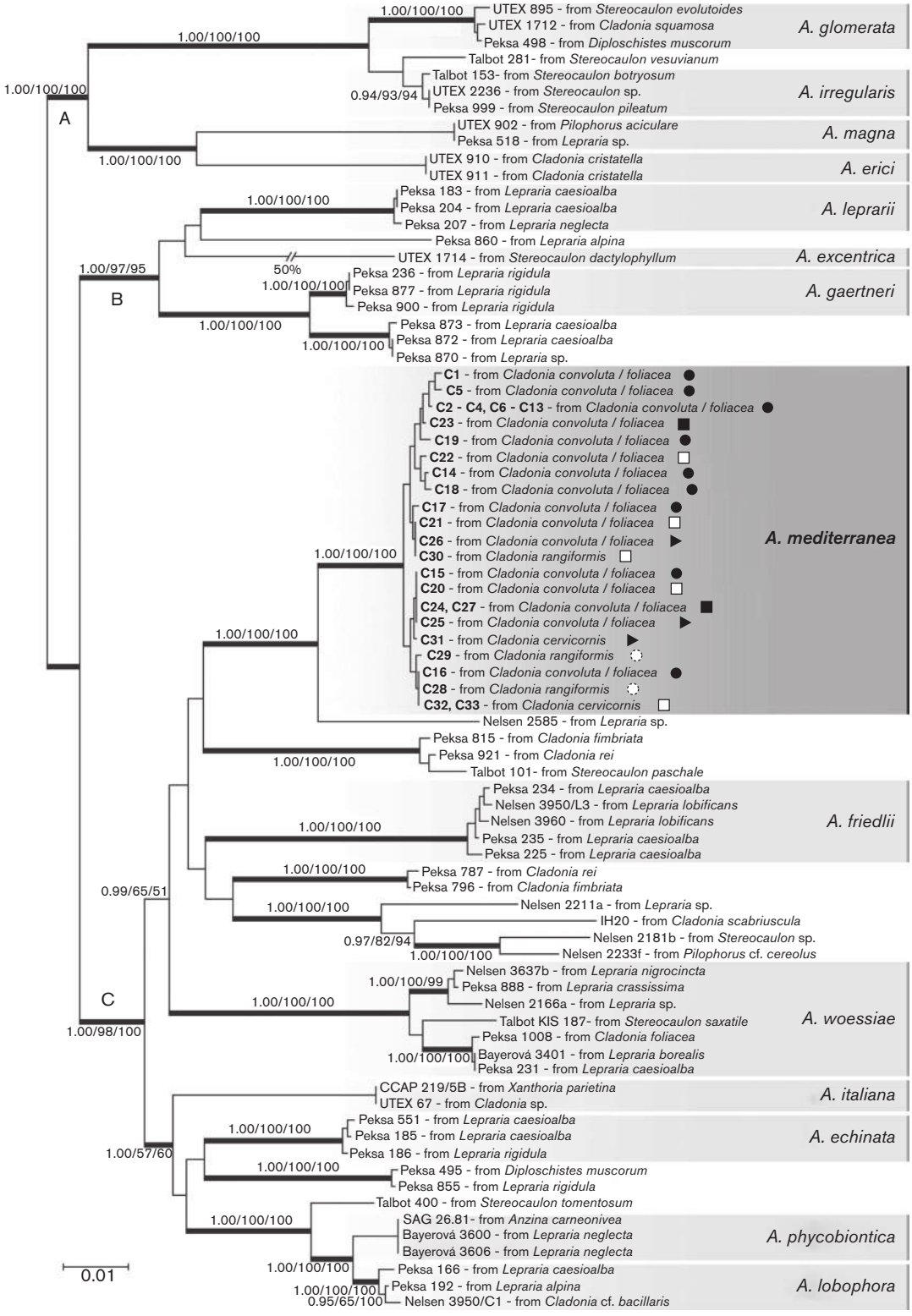

Fig. 5. Unrooted BI analysis based on the combined ITS rDNA + LSU rDNA + actin dataset using the GTR + I + Γ model for ITS rDNA, HKY + I model for LSU rDNA and GTR + Γ model for the actin gene. Values at nodes indicate statistical support estimated by three methods – MrBayes posterior node probability (left), ML bootstrap (middle) and MP bootstrap (right). Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly obtained sequences are given in bold type with the type of substrate indicated by symbols: quartzites, siliceous (white square □), limestones, calcareous (black square ■), sandstones, siliceous (black triangle ►), Miocene gypsum (black circle ●) and volcanic (white discontinuous circle

◌). Authentic strains of the genus *Asterochloris* are marked in grey and the newly defined *A. mediterranea* is given in bold type. Strain affiliation to three major clades (A–C) is indicated. Bar, estimated number of substitutions per site.

Barreno, Chiva, Moya et Škaloud linked to the thalli of several members of the genus *Cladonia* which are widely but not exclusively distributed in Mediterranean and Canarian ecosystems.

This genus was described by Tschermak-Woess (1980) who separated it from the genus *Trebouxia* on the basis of chloroplast morphology, and later this was supported by more recent confocal and molecular analyses (del Campo *et al.* 2010a; Peksa & Škaloud 2008; Škaloud & Peksa, 2008a, b, 2010; Škaloud *et al.*, 2015). In this work, additional features based on TEM observations and on symbiotic state are proposed for the characterization of species of the genus *Asterochloris*. The plastid molecular marker LSU rDNA (23S) recommended by del Campo *et al.* (2010a) was incorporated to build the algal phylogeny, together with the traditionally used nuclear ITS rDNA and actin intron markers (Škaloud & Peksa, 2010). The correct

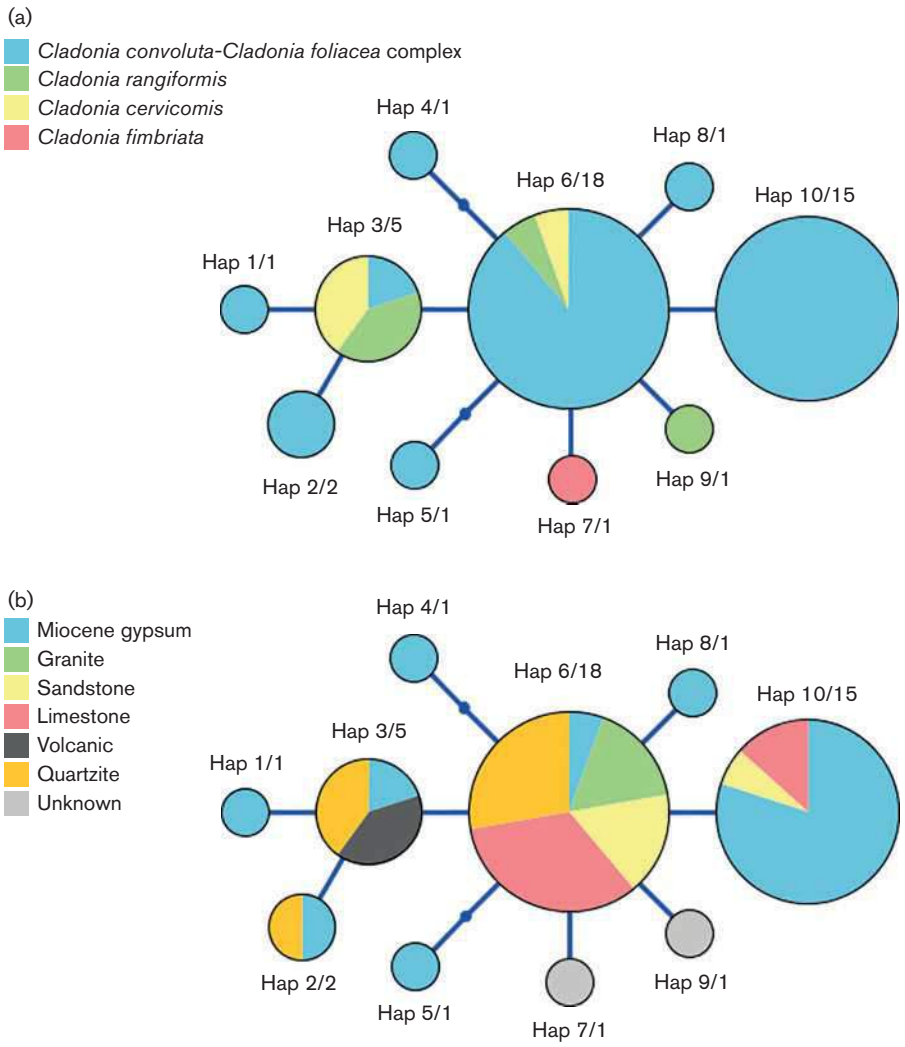

Fig. 6. Statistical parsimony networks obtained for the ITS rDNA *A. mediterranea* haplotypes found in this study including all available sequences selected from the GenBank database. Size of the circles is proportional to the number of samples sharing that haplotype. Number of samples found sharing that haplotype is given after the haplotype number. Colours in the upper network (a) denote taxa: *C. convoluta-C. foliacea* complex blue, *C. rangiformis* green, *C. fimbriata* pink and *C. cervicornis* s. str pale yellow. Colours in the lower network (b) denote type of substrate: Miocene gypsum blue, granite green, sandstone pale yellow, limestone pink, volcanic dark grey, quartzite orange, unknown grey.

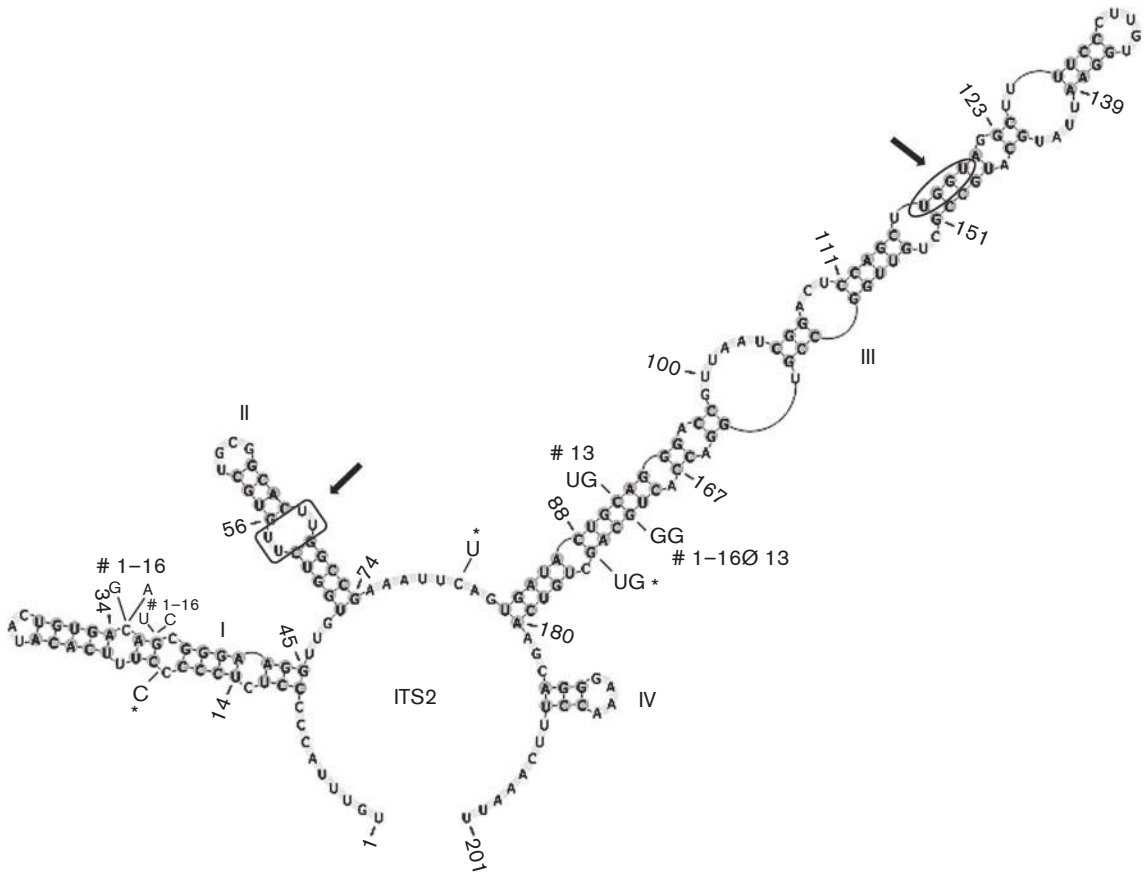

Fig. 7. Predicted secondary structure of the ITS2 transcript of *A. mediterranea*. Base changes between *A. mediterranea* genotypes found in this study are indicated with an asterisk; one single base change, one insertion and one hemi-compensatory base change (CBC). New base changes between *A. mediterranea* and 16 *Asterochloris* lineages defined by Škaloud & Peksa (2010) are also indicated; two single base changes and one hemi-CBC. The numbers next to the number sign (#1–16) specify the *Asterochloris* clades in which the base changes occurred. The highly conserved U-U in helix II and UGGU motif in helix III (both arrows) are highlighted (Schultz *et al.*, 2005).

identification of the fungal partners was corroborated by the nrITS barcode.

Cellular characteristics in species of the genus *Asterochloris* have been mostly described from *in vitro* cultures by CM and/or LM techniques. Algae undergo a variety of structural, physiological, and biochemical modifications as a result of lichenization (Galun, 1988; Friedl & Büdel, 2008); therefore, it seems likewise necessary to know the symbiotic state. TEM ultrastructural observations in symbiosis indicated that in mature cells one large central pyrenoid was always present; sometimes two appeared when the chloroplast was in the duplication phase (Fig. 3d). The pyrenoid ultrastructure fits with the *irregularis*-type (Fig. 3c) (Friedl, 1989) observed in most species of the genus *Asterochloris* delimited by Škaloud & Peksa (2010) (i.e. *A. excentrica*, *A. glomerata*, *A. irregularis*, *A. italiana* and *A. pyriformis*).

Škaloud & Peksa (2008a) proposed that chloroplast morphology could be considered as an important morphological marker for delimitation of species of the genus *Asterochloris* under culture conditions. The shallowly lobed plastid with either simple or finger-like lobes, observed in *A. mediterranea*, also occurred in *A. excentrica*, *A. irregularis* and *A. friedlii* (Table S2) (Škaloud & Peksa, 2008b). Nevertheless, this study has illustrated that CM and TEM techniques should be complementary in the characterization of the chloroplast morphologies (Figs 2e, f and 4) due to the differences in thylakoid arrangements (Fig. 3b, f), as proposed by del Campo *et al.* (2010b) and Casano *et al.* (2011) for the taxa *Trebouxia* TR1 and *Trebouxia* TR9. The frequent presence of evident nuclei with nucleoli and high amounts of ribosomes could be related to intense metabolic activity of the cells (Boisvert *et al.*, 2007). In addition, the different myelin-like bodies (plasmalemmasomes, lomasomes) in secretory spaces and endoplasmic reticulum highlight the strong cell activity which is required to remove the excess of membranes (Marchant & Robards, 1968; Robards, 1968). The variable shape of mature cells (spherical to pyriform or kidney-shaped) together with the irregular secretory spaces and the flat and

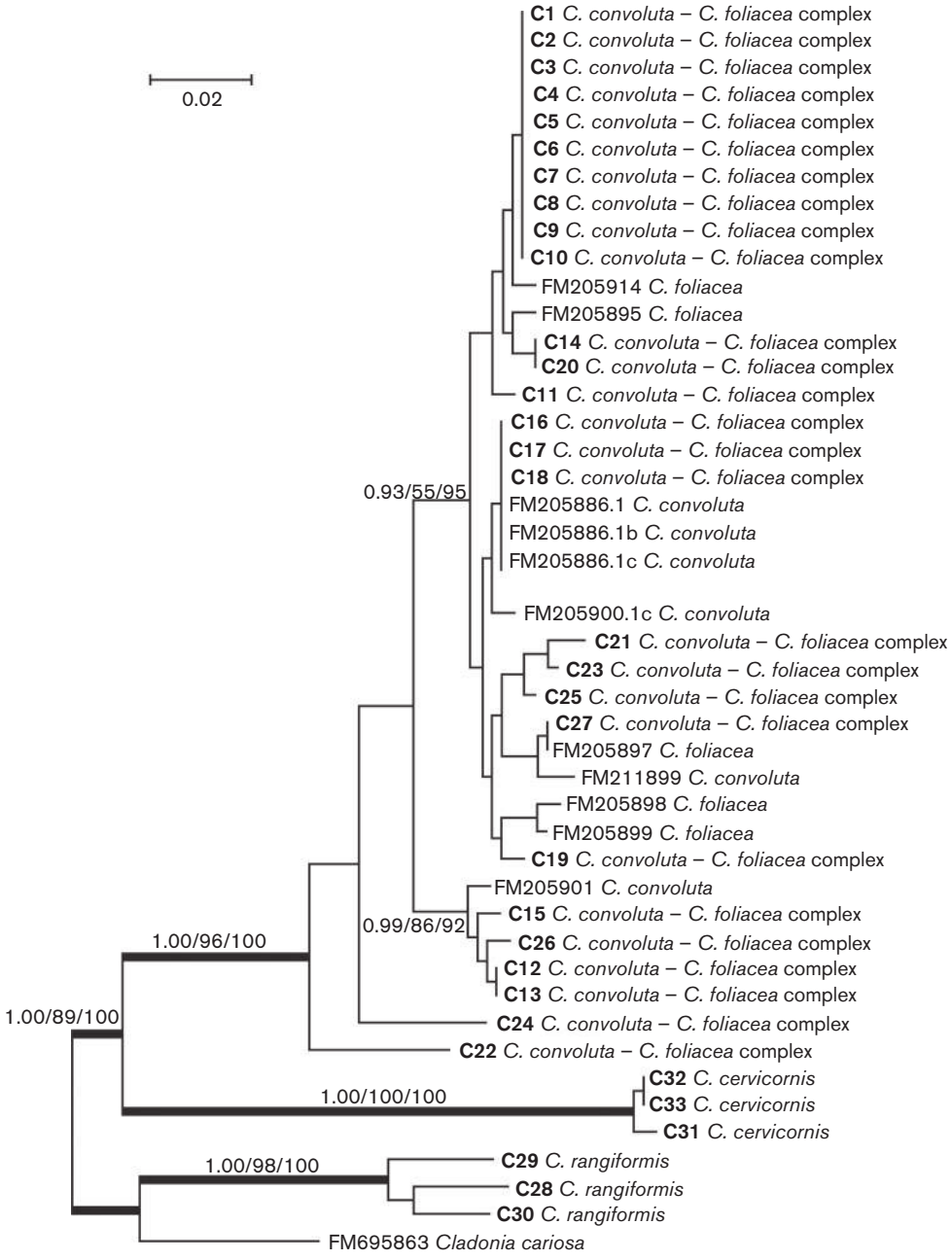

Fig. 8. The BI analysis of mycobiont ITS rDNA using the GTR + Γ model. Values at nodes indicate statistical support estimated by three methods – MrBayes posterior node probability (left), ML bootstrap (middle) and MP bootstrap (right). Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly obtained sequences are given in bold type. Accession numbers of sequences selected from Pino-Bodas *et al.* (2010) accompany each species name. Bar, estimated number of substitutions per site.

delimited thickening of the cell wall could be useful features to help distinguish species of the genus *Asterochloris* from those of the genus *Trebouxia* (data not published).

Species delimitation methods based on single-locus data rely on the assumption that a single gene genealogy is sufficient to illustrate species phylogeny. In *Asterochloris* algae, evolutionary inferences based on two molecular markers (ITS rDNA and actin sequences) revealed extensive diversity of this algal genus (Škaloud & Peksa, 2010; Škaloud *et al.*, 2015). In this work, evolutionary inferences based on multiple loci, both nuclear (nrITS and actin) and chloroplast-encoded (LSU rRNA), helped us to reinforce the *Asterochloris*

phylogeny suggested by Škaloud & Peksa (2010) and revealed a new well-supported clade (100 % MrBayes/ML/MP) here described as *Asterochloris mediterranea* sp. nov.

Parsimony networks using our samples C1 to C33 and the 13 GenBank sequences showed minimum intraspecific genetic variation among all the haplotypes detected. In addition, we were not able to find any relationship between the haplotype distributions, neither in the types of substrata nor the *Cladonia* taxa studied here, although Peksa & Škaloud (2011) demonstrated clear ecological preferences among the majority *of Asterochlor*is lineages.

Putative models of secondary structures have been characterized for the ITS regions of rRNA (Coleman *et al.*, 1998; Joseph *et al.*, 1999; Lalev & Nazar1998, 1999; Mai & Coleman, 1997). The maintenance of these structures is important for the proper functioning of the rRNA (Coleman, 2003) and, therefore, ITS rRNA transcripts in *Asterochloris* have been used as an additional attribute to delimit species boundaries (Beiggi & Piercey-Normore, 2007; Škaloud & Peksa, 2010; Škaloudová & Škaloud, 2013). However, it has recently been suggested that differences in the ITS rRNA secondary structures are often not diagnostic at the species level in green algae (Caisová *et al.*, 2011; Škaloud & Rindi, 2013). Therefore, we would rather consider the presence of nucleotide substitutions in stem regions of the ITS2 rRNA transcript as an attribute of elapsed evolutionary time, indicating that sufficient time has passed to produce a speciation event. Comparing *A. mediterranea* ITS rRNA secondary structure with the *Asterochloris* lineages described by Škaloud & Peksa (2010), low genetic variation was found in ITS rDNA gene regions, which correlates with the absence of CBCs.

In the genus *Cladonia*, identification of the fungal partners is often problematic and BLAST searches have showed high failure rates (Kelly *et al.*, 2011), thus the DNA barcoding for fungi (nrITS sequences) proposed by Schoch *et al.* (2012) was used. Even using the barcoding molecular marker, some lichen taxa still remain problematic (Pino-Bodas *et al.*, 2013a). Several studies have shown that the nrITS region provides a poor resolution for certain species in the genus *Cladonia* (Fontaine *et al.*, 2010; Kotelko & Piercey-Normore, 2010; Pino-Bodas *et al.*, 2010, 2013b; Steinová *et al.*, 2013). Specifically, *C. convoluta* (Lam.) Anders and *C. foliacea* (Huds.) Willd. the currently available data hindered the delimitation of two monophyletic groups (Pino-Bodas *et al.*, 2010). Only three species of the genus *Cladonia* were separated as independent monophyletic groups, with *C. convoluta* and *C. foliacea* joined together in the same clade, confirming the results found by Pino-Bodas *et al.* (2010). Although, the case of *C. cervicornis* complex was clearly solved by Pino-Bodas *et al.* (2013b).

In summary, the genus *Asterochloris* is the preferential phycobiont in *Cladonia*, *Lepraria* and *Stereocaulon* lichens, and the diversity of this algal genus needs to be deeply explored in different mycobionts, areas and habitats. The combination of several nuclear and plastid molecular markers as well as ultrastructural (TEM and CM) techniques both in culture and in the symbiotic state should be utilized. *Asterochloris mediterranea* might exemplify this assertion.

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SUPPORTING INFORMATION

Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems

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Fig. S1. Detail of myelin-like bodies type lomasomes (L) in the cytoplasm. The lomasomes appeared near the peripheral vesicles (PV) arose from endoplasmic reticulum while those marked with arrow heads arose from the thylakoid membranes (TM). In both cases the accumulation of inside concentric membranes were observed. Abbreviations; Chloroplast (Chl), Mitochondria (M).

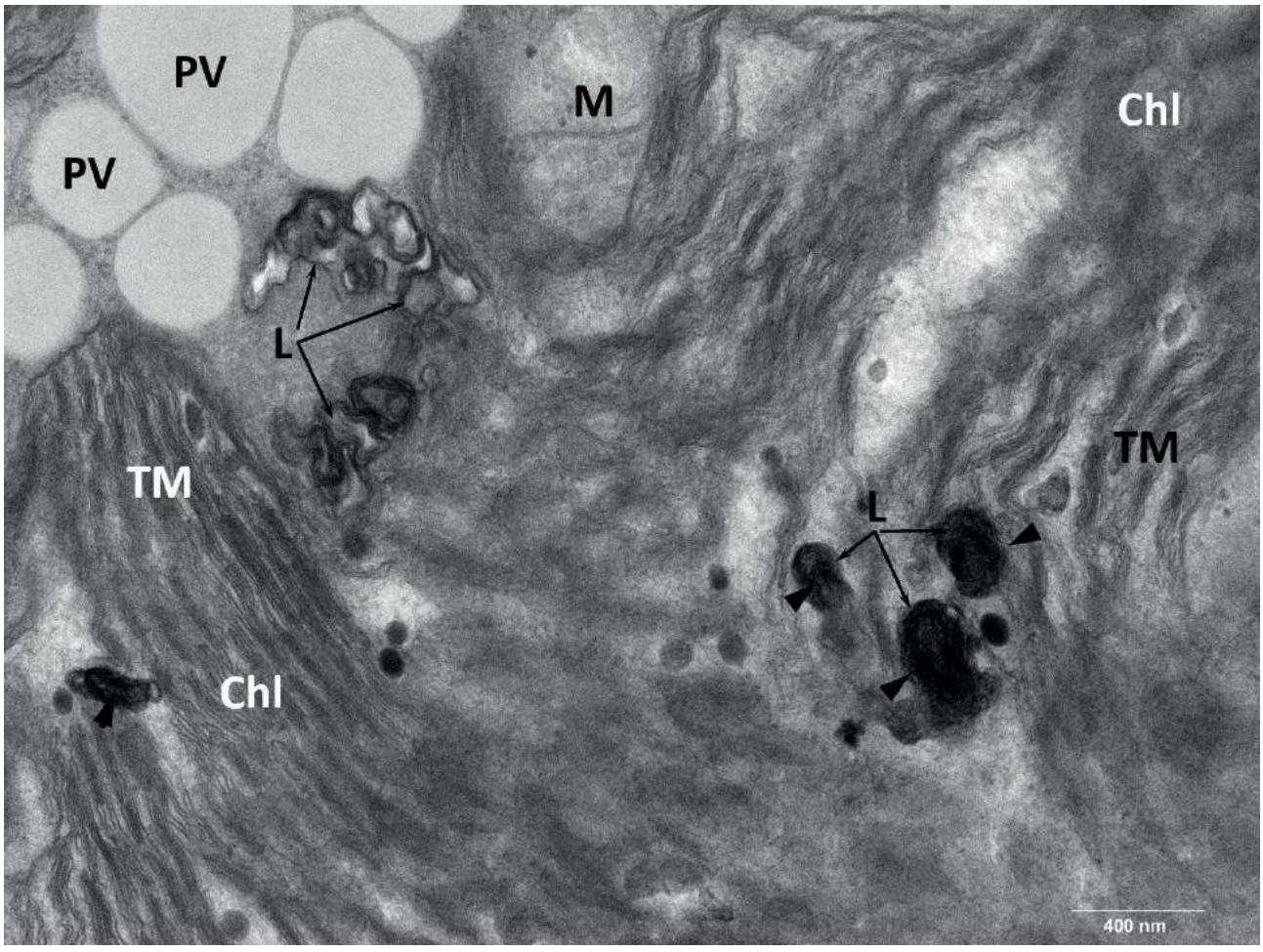

Table S1. Location for collections of *Cladonia* spp. samples used in this study, n= number of samples collected.

Locality /geographic coordinates /altitude /bioclimatic belt /collection data	**Type of substrate**	**n**
1: Spain, Villareal de San Carlos (Cáceres) / N39º50'45'' W6º01'41'' / 402 m / low mesomediterranean low subhumid / (leg. García 20/02/2014)	Quartzites, Siliceous	6
2: Spain, Fuentidueña de Tajo (Madrid) / N40º07'87'' W03º09'12'' / 571 m / upper mesomediterranean low dry / (leg. Barreno, Chiva, Molins & Salvá 24/02/2012)	Miocene gypsum	13
3: Spain, Sorbas (Almería) / N37º08'77'' W02º08'75'' / 415 m / upper thermomediterranean low semiarid / (leg. Barreno, Chiva, Moya & Salvá 9/01/2014)	Miocene gypsum	2
4: Spain, Puebla de Valverde (Teruel) / N40º11'51'' W0º55'01'' / 1130 m / low supramediterranean upper dry / (leg. Barreno 13/05/2013)	Limestones, Calcareous	1
5: Spain, Rubielos de Mora, Mijares river (Teruel) / N40º09'06'' W0º42'34'' / 925 m / low supramediterranean upper dry / (leg. Barreno 13/05/2013)	Limestones, Calcareous	1
6: Spain, Bujaraloz (Zaragoza) / N41º49'01'' W0º25'28'' / 351m / upper mesomediterranean low dry / (leg. Barreno, Chiva, Moya & Salvá 14/11/2014)	Miocene gypsum	3
7: Spain, Mora de Rubielos (Teruel) / N40º14'34'' W0º44'26'' / 1039 m / low supramediterranean upper dry / (leg. Barreno 13/05/2013)	Sandstone, Siliceous	1
8: Spain, Rubielos de Mora (Teruel) / N40º13'38'' W0º42'30'' / 932 m / low supramediterranean upper dry / (leg. Barreno 13/05/2013)	Sandstone, Siliceous	1
9: Spain, Chóvar (Castellón) / N39º51'14'' W0º19'13'' / 415 m / upper thermomediterranean low dry / (leg. Barreno 13/05/2013)	Sandstone, Siliceous	1
10: Spain, Algar de Palancia (Valencia) / N39º46'12'' W0º23'18'' / 208 m / low thermomediterranean low dry / (leg. Moya & Salvá 8/05/2013)	Limestones, Calcareous	1
11: Spain, Villena (Alicante) / N38º39'26'' W0º56'13'' / 518 m / upper mesomediterranean low dry (leg. Barreno, Chiva, Moya & Salvá 14/11/2014)	Miocene gypsum	1
12: Spain, Arico (Tenerife) / N28º24'43'' W16º25'28'' / 560 m / low thermomediterranean low semiarid / (leg. Barreno & Molins 21/11/2013)	Volcanic	1
13: Spain, Haría (Lanzarote) N29º07'31'' W13º31'25'' / 270 m / low thermomediterranean upper dry / (leg. Barreno & Molins 21/11/2013)	Volcanic	1

Key to species of the genus *Asterochloris*

1. Cells mainly of pyriform and oval shape.....2
   Cells predominantly spherical.....3
2. Asexual reproduction by 16-32 aplanospores.....*A. irregularis*
   Asexual reproduction by 32-128 aplanospores.....*A. glomerata*
3. Vegetative cells up to 16 mm in diameter.....4
   Vegetative cells larger, up to 21-29 mm in diameter.....5
4. Only 16 aplanospores produced.....*A. erici*
   Asexual reproduction by 32-128 aplanospores.....*A. mediterranea*
5. Pyrenoids absent .....*A. magna*
   Chloroplasts contain usually 1, rarely 2 pyrenoids.....6
   Chloroplasts can contain 3 or even more pyrenoids.....7
6. Only deeply and shallowly lobed chloroplasts are formed.....*A. excentrica*
   Chloroplasts of crenulate and parietal types are often formed.....8
7. Chloroplasts are usually shallowly lobed; a deeply lobed type is not formed. .....*A. lobophora*
   Deeply lobed chloroplasts are formed, as well.....*A. friedlii*
8. Cells contain echinate chloroplasts.....9
   Echinate chloroplasts are never formed.....10
9. Cells contain only echinate and crenulate chloroplasts, with simple lobes .....*A. echinata*
   Chloroplasts of deeply and shallowly lobed types are often forme.....*A. gaertneri*
10. Only a shallowly chloroplast type is formed.....*A. italiana*
    Other chloroplast types are formed, as well.....11
11. Cells usually contain parietal lobed chloroplasts.....*A. phycobiontica*
    A parietal chloroplast type is formed very rarely.....12
12. Cells up to 25 mm in diameter; chloroplasts usually deeply lobed, sometimes a flat lobe type is formed, as well.....A. woessiae
    Cells up to 28 mm in diameter; chloroplasts usually shallowly lobed and crenulate, a flat lobe chloroplast type is never formed .....A. leprarii

Table S2. Morphological charateristics of *Asterochloris* strains defined by Škaloud et al. (2015) including *A. mediterranea* and a key to identify the species of the genus *Asterochloris* in culture.

Species	Predominant cell shape	Cell size (mm)	Plastid types							Lobe extensions					Max. number of pyrenoids	Aplanospores			
			Shallowly lobed	Deeply lobed	Crenulate	Parietal	Echinate	Flat lobed	Globular	Elongated	Simple	Flat	Finger-like	Not formed		16	32	64	128
A. glomerata	oval, pyriform	5.3 - 22.9	yes	yes	-	-	-	yes	-	yes	-	yes	yes	-	3 or more	-	yes	yes	yes
A. irregularis	oval, pyriform	6.3 - 26.3	yes	yes	-	yes	-	yes	-	yes	-	-	yes	-	3 or more	yes	yes	-	-
A. magna	spherical	3.3 - 22.9	-	-	-	yes	-	-	yes	-	-	-	yes	yes	0	-	-	yes	yes
A. erici	spherical	4.7 - 15.8	yes	-	yes	-	-	-	-	yes	yes	-	yes	-	1	yes	-	-	-
A. excentrica	spherical	5.0 - 22.8	yes	yes	-	-	-	-	-	yes	-	yes	yes	-	2	-	-	yes	-
A. leprarii	spherical	0.5 - 28.3	yes	yes	yes	yes	-	-	-	yes	yes	yes	yes	-	3 or more	-	-	yes	yes
A. gaertneri	spherical	5.5 - 29.4	yes	yes	yes	-	yes	-	-	yes	yes	yes	yes	-	3 or more	-	yes	yes	yes
A. woessiae	spherical	5.0 - 25.4	yes	yes	yes	yes	-	yes	-	yes	yes	yes	yes	-	3 or more	-	-	-	yes
A. friedlii	spherical	4.4 - 21.0	yes	yes	yes	yes	-	-	-	yes	yes	yes	yes	-	1	-	-	yes	yes
A. italiana	spherical	4.8 - 22.5	yes	-	-	-	-	-	-	yes	-	-	yes	-	3 or more	-	yes	yes	-
A. echinata	spherical	4.9 - 21.0	-	-	yes	-	yes	-	-	-	yes	-	-	-	3 or more	-	-	yes	yes
A. phycobiontica	spherical	5.0 - 24.3	yes	-	yes	yes	-	-	-	yes	yes	-	yes	-	3 or more	-	-	yes	yes
A. lobophora	spherical	5.9 - 25.4	yes	-	yes	yes	-	-	-	yes	yes	yes	yes	-	1	-	-	yes	yes
A.mediterranea	spherical	8.2 - 16.3	yes	-	-	yes	-	-	-	-	yes	-	yes	-	2	-	-	yes	yes

3.2 *Myrmecia israeliensis* as the primary symbiotic microalga in squamulose lichens growing in European and Canary Island terricolous communities (R 3.2)

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Myrmecia israeliensis as the primary symbiotic microalga in squamulose lichens growing in European and Canary Island terricolous communities

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Abstract: *Myrmecia israeliensis* has been traditionally considered as a green coccoid free–living microalga. This microalga was previously suggested as the primary phycobiont in the lichens *Placidium* spp., *Heteroplacidium* spp., and *Psora decipiens*. However, due to the absence of ITS rDNA sequences (barcode information) published along with these investigations, the symbiotic nature of *M. israeliensis* might be confirmed by using the DNA barcoding and different microscopic examinations both in the symbiotic state and in culture. The aim of this study was to settle the presence of *M. israeliensis* as the primary microalga in squamulose lichens growing in terricolous communities (*Psora* spp., *Placidium* spp. and *Claviscidium* spp.) in 32 localities within European and Canary Island ecosystems by using both molecular and ultrastructural techniques. The lichen–forming fungi were identified using ITS rDNA as a barcode, and in the case of *P. decipiens* specimens, the mycobiont analyses showed an unexpected variability. Phycobiont phylogenetic analyses were made using both chloroplast (LSU rDNA) and nuclear (ITS rDNA) molecular markers. Our results proved that *M. israeliensis* is the primary symbiotic microalga in all the chosen and analyzed lichens. In addition, fluorescence microscopy, transmission electron microscopy and scanning electron techniques were used to characterize *M. israeliensis*. Finally, the presence of this microalga in lichen thalli was verified using different microscopic observations. A combination of different techniques, both molecular and microscopic, allowed for the accurate identification of this symbiotic microalga, beforehand mainly known as free living. Here, we suggest the combination of these techniques to prevent incorrect identification in microalgal lichen studies.

Key words: *Clavascidium* spp., ITS rDNA (barcoding), LSU rDNA, *Myrmecia israeliensis*, phycobiont, *Placidium* spp., *Psora* spp., ultrastructure

Introduction

The dynamics and ecology of biological soils crusts (BSC) in arid and semiarid regions of the world have been well documented over the last decade (Belnap 2003; Maestre et al. 2011; Pointing & Belnap 2012; Weber et al. 2016). Recently, the European research initiative ''Soil Crust International'' (SCIN) focused on the relevance of the biodiversity of BSC and the functional aspects in their specific environment (Büdel et al. 2014; Belnap & Büdel 2016).

Many lichen species are adapted to dry environments and are components of BSC in semiarid regions, and they play an important role in the functioning of these ecosystems (Belnap & Lange 2001; Rosentreter 2007; Bowker et al. 2011; Belnap & Büdel 2016). In particular, terricolous squamulose lichens, such as *Psora decipiens* (Hedwig) Hoffm. and *P. saviczii* (Tomin) Follmann et A. Crespo, form a compact and stable zone in the upper millimeters of the substratum (Belnap & Lange 2001), and the occurrence of these pioneer lichens is dependent on their phycobiont availability. The microalgae pool is crucial to settle these communities, and points out the relevance of studying the algal diversity; however, phycobiont identification in several *Psora* spp. has been controversial.

The primary phycobiont of *P. decipiens* and *P. globifera* (Ach.) A. Massal has been identified as *Myrmecia biatorellae* (Tschermak–Woess et Pessl) Petersen (Geitler 1963; Galun et al. 1971; Tschermak–Woess 1988), although Schaper & Ott (2003) claimed to have found a species of *Asterochloris* in *P. decipiens.* Ruprecht et al. (2014) associated *P. decipiens* from Spanish and North and Central European localities with several

species mostly belonging to the genera *Asterochloris* and *Trebouxia*. Recently, Williams et al. (2017) suggested the green algal genus *Myrmecia* as the primary phycobiont in *P. decipiens*, but their analyses were based only on 26S rDNA and *rbc*L molecular markers and no ITS rDNA information was provided.

M. biatorellae and *M. israeliensis* (Chantanachat et Bold) T. Friedl have also been found associated with a lineage in the lichen family Verrucariaceae (Thüs et al. 2011). Representatives of this family, such as *Placidium* spp. and *Heteroplacidium* spp., are also associated with BSC and share with *Psora* spp. their terricolous squamulose appearance worldwide (Tschermak–Woess 1988; Friedl & Büdel 2008).

The diversity, ecology, and distribution of the genus *Myrmecia* as a lichen phycobiont have been overlooked in the past, and some interesting questions about this genus are still unresolved. The aim of this study was to settle the presence of *M. israeliensis* as the primary phycobiont in different terricolous squamulose lichen species (*Psora* spp., *Placidium* spp. and *Claviscidium* spp.) growing on xerothermic soil crusts by using both molecular (DNA barcoding) and ultrastructural techniques, in 32 localities within European and Canary Island ecosystems.

Material and Methods

Lichen material. *Psora decipiens* (n= 31), *Psora saviczii* (n= 7), *Placidium pilosellum* (Breuss) Breuss 1 (n= 4), *Placidium* sp. 1 (n= 2), *Placidium* sp. 2 (n= 4), *Clavascidium* sp. 2 (n= 3) and *Clavascidium* sp. 3 (n= 1) were collected from 32 locations within Europe and the Canary Islands (Table 1 and Supplementary Table 1). Samples were dried and stored at –20 °C until processing.

Sample preparation. Lichen squamules were examined under a stereo–microscope to remove surface contamination (e.g. sand, mosses, epiphytic algae, fragments of other lichen species, or infection by lichenicolous fungi). The squamules were sterilized by sequential immersion in 96% ethanol (10 s), 0.5% NaOCl (2 min) and 70% ethanol (2 min) (Arnold et al. 2009). Two to five squamules from each location were randomly selected and pooled together.

DNA extraction, amplification and sequencing. Total genomic DNA of the Iberian Peninsula and Canary Islands samples (IB_CI) as well as from free–living microalgae inside saccharoid gypsum crystals from Madrid Province (collected from rock faces in the same location as the lichen samples), was isolated and purified using the DNeasy TM Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The standard CTAB protocol (Doyle & Doyle 1987) was performed in the remaining samples from North and Central Europe (N_CE).

The mycobiont and primary phycobiont (both from thalli and/or from isolated culture) were identified by Sanger sequencing. Fungal ITS rDNA was amplified using the primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). Two algal loci were amplified; a region of the chloroplast LSU rDNA gene using the algal–specific primers 23SU1 and 23SU2 (Del Campo et al. 2010) and the ITS rDNA. The ITS rDNA was amplified using the two specific primer pairs. The first combination consisted of two specific primers designed based on the *M. israeliensis* sequence, MI_F (5'– GCC CGT TGT TGC CCT TCA–3', located in the ITS1 region) + MI_R (5'– CAG TAT GTC ACA ACA GGC CA– 3', located in the ITS2 region). The second combination included the newly designed primer specific to green algae zeleny_F2 (5'–TTC TTA GTT GGT GGG TTG CC–3', located at the end of 18S rDNA) + the universal primer ITS4 (White et al. 1990), respectively. In the IB_CI samples, PCR reactions and Sanger sequencing were performed as described in Molins et al. (2017). In the N_CE samples, PCRs were performed in 20 µl using MyTaq™ DNA polymerase (Bioline, London, UK) containing: 4 µm of buffer, 0,3 µm zeleny_F2/ITS4 primers, 0,2 µm MyTaq polymerase and 1 µl of template DNA.Sterile Milli–Q water was used to bring to volume. The PCR program for amplification comprised of an initial denaturation at 94 °C for 4 min, and 35 cycles at 94 °C for 60 s, 56 °C for 60 s and 72 °C for 90 s, followed by a final elongation at 72 °C for 10 min. Amplifications were carried out on a 96–well labcyclers SensoQuest (Progen Scientific Ltd., South Yorkshire, UK) or Mastercycler gradient (Eppendrorf). The PCR products were visualized on 0.8% agarose gels and purified using MagJET Magnetic Bead–Based Nucleic Acid Purification (ThermoFischer Scientific, Massachusetts, USA). All the Sanger sequencing experiments were performed at Macrogen Inc. (Seoul, Korea).

Sequence analyses. **Phycobiont phylogenetic analysis.** A multiple alignment was prepared including: i) the newly determined algal ITS rDNA (KY981643 to KY981701) and LSU rDNA (KY981702 to KY981749) sequences from the lichen thalli, the cultures and the gypsum crystals, ii) a selection of *Trebouxia*, *Myrmecia* and *Asterochloris* species available from the Culture Collection of Algae at Göttingen University (SAG), from the Culture Collection of Algae at the University of Texas (UTEX) and from Culture Collection of Algae at the University of Prague (CAUP) downloaded from the GenBank, and iii) selected Chlorophyta ITS rDNA sequences obtained by Ruprecht et al. (2014). The alignment was carried out using MAFFT v 7.0 (Katoh et al. 2002; Katoh & Standley 2013) with default parameters, visualized and manually adjusted. GBlocks 0.91b (Castresana 2000) was used to remove ambiguously aligned regions and large gaps by means of a less stringent option allowing smaller final blocks and gap positions within the final blocks. Alignment was 1237 bp in length for the ITS rDNA+LSU rDNA region. The best–fit substitution model for this alignment (GTR+I+G) was chosen using jModelTest v 2.0 (Darriba et al. 2012) and applying the Akaike Information Criterion (Akaike 1974, 2011). Maximum Likelihood (ML) analysis was implemented in RAxML v 8.1.11 (Stamatakis 2014) using the GTRCAT substitution model. Bootstrap support was calculated based on 1,000 replications (Stamatakis et al. 2008). Bayesian phylogenetic analyses were carried out in MrBAYES v 3.2 (Ronquist et al. 2012). Settings included two parallel runs with six chains over 20 million generations starting with a random tree, and sampling after every 200th step. We discarded the first 25% of data as burn–in. MAFFT, jModelTest, ML and Bayesian analyses were implemented at the CIPRES Science Gateway v 3.3 webportal (Miller et al. 2010). Phylogenetic trees were visualized in FigTree v 1.4.1 (Rambaut 2014).

Mycobiont phylogenetic analysis. Two multiple alignments were prepared. The first one included the newly determined

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Table 1. Location for collections of *Psora* spp., *Claviscidium* spp. and *Placidium* spp. samples used in this study.

Locality/geographic coordinates/altitude/bioclimatic belt/collection data	**Type of substrate**	**Sample code**
Spain, Asturias, Puerto Somiedo, Puerto/ 43º02'23"N, 06º14'29"W / 1250/ Upper supratemp submediterranean low hyperhumid / leg. Vázquez & Fernández 04/10/2014	Limestone, calcareous	AST
Spain, León, Rabanal de Luna, Ermita de la Virgen de Pruneda/ 42º56'17"N, 05º58'25"W / 1150/ Upper supratemp submediterranean upper humid / leg. Vázquez 27/09/2014	Limestone, calcareous	LEN
Spain, Madrid, Fuentidueña de Tajo/ 40º07'41"N, 03º09'14"W / 571/ Upper mesomediterranean low dry / leg. Barreno, Chiva, Molins & Salvà 24/02/2012	Miocene gypsum	MAD_FT
Spain, Madrid, Titulcia/ 40º07'32"N, 03º33'15"W / 521/ Upper mesomediterranean low dry / leg. Barreno, Chiva, Molins & Salvà 24/02/2012	Miocene gypsum	MAD_ TIL
Spain, Zaragoza, Pina de Ebro/ 41º29'29"N, 0º15'30"W / 351/ Upper mesomediterranean low dry / leg. Barreno, Chiva, Moya & Salvà 14/11/2014	Miocene gypsum	ZGZ
Spain, Almería, Sorbas/ 37º08'44"N, 02º08'43"W / 415/ Upper thermomediterranean low semiarid / leg. Barreno, Chiva, Moya & Salvà 09/01/2014	Miocene gypsum	ALM
Spain, Alicante, Villena/ 38º39'26"N, 0º56'13"W/ 518/ Upper mesomediterranean low dry / leg. Barreno, Chiva, Moya & Salvà 11/01/2014	Miocene gypsum	ALC
Spain, Cádiz, El Gastor, Ventas Nuevas/ 36º50'08"N, 05º20'55"W / 461/ Low subhumid / leg. Chiva 07/09/2016	Triassic gypsum	CAD
Spain, Málaga: Ronda, P. Nat. de la Sierra de las Nieves, Cañada de las Ánimas/ 36º42'13"N, 05º01'09"W / 1459/ Upper supramediterranean low humid / leg. Chiva 09/09/2016	Limestones, calcareous	MLG
Spain, Cataluña, Lleida, Ponts Guisona/ 41º52'45"N, 01º13'15"E / 382/ Low supramediterranean low subhumid / leg. Salvà 15/12/2012	Oligocene gypsum	CAT
Spain, Lanzarote, Orzola/ 29º13'20"N, 13º27'10"W / 9/ Inframediterranean arid / leg. Barreno & Molins 24/11/2013	Volcanic	LNZ
Spain, Valencia, Algar de Palancia/ 39º46'12"N, 0º23'18"W / 208/ Upper thermomediterranean low dry / leg. Barreno & Salvà 8/05/2013	Limestones, calcareous	VLC
Czech Republic, Nové Dobrkovice, Český Krumlov/ 48º49'09"N, 14º17'32"E / 521/ Upper supratemperate low humid/ leg. Jadrná 21/09/2015	Metamorphic limestone	NOV
Czech Republic, Beroun, Merhout's rock/ 49º57'26"N, 14º05'50"E / 250-260/ Upper supratemperate low subhumid / leg. Peksa & Jadrná 11/08/2014	Diabase calcareous	BER
Czech Republic, Sbrsko - NPR Karlštejn/ 49º55'56"N, 14º08'11"E / 230-250/ Upper supratemperate low subhumid / leg. Jadrná 29/10/2015	Devonian Limestones, calcareous	SBR
Turkey, Akseki/ 38º38'42"N, 34º50'05"E / 1228/ Upper supramediterranean upper dry/ leg. Jadrná 14/05/2015	Limestones, calcareous	TUK
Cyprus, Dipotamos Reservoir/ 34º51'09"N, 33º21'05"E / 195/ Upper semiarid low dry/ leg. Jadrná 30/03/2016	Miocene gypsum	DIP
Cyprus, Avkas gorge/ 34º55'26"N, 32º20'32"E / 85/ Low thermomediterranean low dry/ leg. Jadrná 30/03/2016	Limestones, calcareous	AVK
Cyprus, Akamas peninsula, Neo Chorio/ 35º03'02"N, 32º21'16"E / 12/ Low thermomediterranean low dry/ leg. Jadrná 30/03/2016	Miocene gypsum	AKA
Cyprus, Akrotiri/ 34º36'03"N, 32º58'15"E / 1/ Upper inframediterranean low dry/ leg. Jadrná 30/03/2016	Limestones, calcareous	AKR

Table 1 Cont.

Cyprus, Akrotiri 2/ 34º36'01"N, 32º58'21"E / 1/ Upper inframediterranean low dry / leg. Jadrná 30/03/2016	Limestones, calcareous	AKR_2
Croatia, Velebit Mts, Starigrad Paklenica/ 44º17'34"N, 15º27'24"E / 30/ Low mesomediterranean low subhumid/ leg. Malíček 28/06/2016	Limestone, calcareous	STA
Germany, ruine Homburg/ 50º01'38"N, 09º47'58"E / 300/ Low supratemperate upper subhumid/ leg. Peksa, Jadrná 7/11/2014	Triassic, calcareous	HOM
Slovakia, Tematín, Lúka, Považský Inovec/ 48º40'03"N, 17º54'57"E / 350/ Low supratemperate upper subhumid / leg. Peksa 13/4/2015	Wetterstein dolomite, calcareous	TEM
Slovakia, Lúka 1, Považský Inovec/ 48º39'43"N, 17º53'37"E / 221/ Low supratemperate upper subhumid / leg. Jadrná 11/10/2016	Wetterstein dolomite, calcareous	LUK_1
Slovakia, Lúka 2, Považský Inovec/ 48º40'03"N, 17º54'54"E / 337/ Low supratemperate upper subhumid / leg. Jadrná 11/10/2016	Wetterstein dolomite, calcareous	LUK_2
Slovakia, Za Šípem, Stankovany/ 49º09'54"N, 19º10'23"E / 1092/ Loew orotemperate upper humid/ leg. Jadrná 11/10/2016	Limestones, calcareous	ZAS
Slovakia, Turnianský hradný vrch/ 48º36'35"N, 20º52'34"E / 319/ Low supratemperate low humid/ leg. Jadrná 11/01/2016	Limestones, calcareous	TUR
Slovakia, Lančár/ 48º35'58"N, 17º38'49"E / 247/ Low supratemperate upper subhumid/ leg. Jadrná 11/01/2016	Dolomite, calcareous	LAN
Slovakia, Pustá Ves/ 48º38'28"N, 17º36'48"E / 295/ Low supratemperate upper subhumid / leg. Jadrná 11/01/2016	Wetterstein dolomite, calcareous	PUS
Slovakia, Velký Plešivec/ 48º42'05"N, 17º44'12"E / 465/ Low supratemperate low humid/ leg. Jadrná 11/01/2016	Dolomite, calcareous	VEL
Slovakia, Dolný Lopašov/ 48º35'18"N, 17º38'02"E / 250/ Low supratemperate upper subhumid/ leg. Jadrná 11/01/2016	Limestones, calcareous	DOL

fungal ITS rDNA sequences from *Psora* spp. lichen thalli (KY981596 to KY9816339), and a selection of *Psora* spp. sequences downloaded from the GenBank. We included *Protoblastenia rupestris* (KT695366) as the outgroup. The second was built using newly determined fungal ITS rDNA sequences from *Placidium* spp. and *Clavascidium* spp. lichen thalli (KY981582 to KY981595), and selected sequences downloaded from the GenBank. We included *Placidiopsis cinerascens* (GQ344607) as the outgroup. The alignments and phylogenetic analyses were carried out as previously described for the phycobiont. The best–fit substitution model for this alignment (GTR+I+G) was chosen using jModelTest v 2.0 (Darriba et al. 2012) and applying the Akaike Information Criterion (Akaike 1974, 2011).

Microscopic investigations "in thallus". Fluorescent Microscopy (FM), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) techniques were performed for morphological analysis of symbiotic lichen microalgae. To analyse the morphology of chloroplasts in *Myrmecia israeliensis* inside the thalli, FM was used in the sample *Psora decipiens* HOM_PD_1. The chloroplast morphology was analysed by an Olympus CX21 camera with an LED Fluorescent Illuminator. Phycobionts were characterized in the samples *Psora decipiens* MAD_FT and *Psora savizcii* MAD_FT by SEM and TEM. The ultrasculpture (Osyczka & Rola 2013) of the squamules was visualized by SEM. Fractured thalli were attached to the holder, coated with palladium/gold and viewed with a Hitachi (S4800). For TEM, the cells were fixed and dehydrated as described in Molins et al. (2017). Samples were embedded in Spurr's resin according to the manufacturer's instructions. Sections (90 nm) were cut with a diamond knife (DIATOME Ultra 45°) using an ultramicrotome (Reichert Ultracut E), mounted on oval hole copper grids coated with formvar and post–stained with 2% (w/v) aqueous uranyl acetate and 2% lead citrate, using the "SynapTek Grid Staining Kit" (http://www.ems–diasum.com/microscopy/technical/datasheet/71175.aspx). The sections were observed with a JEOL JEM–1010 (80 kV) electron microscope, equipped with a MegaView III digital camera and "AnalySIS" image acquisition software. SEM and TEM examinations were made at the SCSIE Service of the University of Valencia.

Isolation and cultivation of phycobionts. Phycobionts from selected squamules from the Fuentidueña de Tajo population (Supplementary Table 1) were isolated using the micromethod described by Gasulla et al. (2010). Samples were homogenized with a mortar and pestle in an isotonic buffer (0.3 M sorbitol, 50 mM HEPES, pH 7.5) and filtered through muslin. Isolation was carried out by a gradient centrifugation method using Percoll®. The algal suspension was diluted with sterile water, and 10 µl was spread using the streak method on sterile 1.5% agar Bold's Basal Media Petri dishes (BBM) (Bold 1949; Bischoff & Bold 1963). The isolated algae were maintained under 15 $\mu mol.m^{-2}.s^{-1}$ (PPFD) for a 12 h photoperiod at 21 °C. Phycobionts from samples encoded as NOV, LUK1, TEM, HOM, BER, SBR and VEL (Supplementary Table 1) were isolated by the thallus fragmentation method (Ahmadjian 1993; Peksa & Škaloud 2008) as described in Moya et al. (2015). After six weeks, groups of dividing algal cells were observed associated with some of the fragments. To obtain unialgal cultures, small populations of phycobionts were transferred onto the fresh BBM agar slants and incubated accordingly.

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Microscopic investigations of phycobionts "in culture". Light microscopy (LM) was performed on selected unialgal cultures obtained from *Psora decipiens* HOM_PD_3. For LM analyses, cultures were observed with an Olympus BX51 microscope equipped with a Canon EOS 1100D digital camera. To compare the ultrastructure obtained in symbiosis, TEM examinations were also performed on selected unialgal cultures on the 21st day of cultivation (Peksa & Škaloud 2008) from *M. israeliensis* UTEX 1181. TEM analyses were performed as previously described for the thallus.

Results

Phycobiont phylogenetic analysis

A total of 57 new sequences for *Psora* spp., *Placidium* spp. and *Clavascidium* spp. phycobionts were obtained by Sanger sequencing from every thallus and/or isolated phycobionts cultures, and also from free–living microalgae living into gypsum crystals. All phycobionts investigated in this study (including the sequence obtained from gypsum crystals) formed a statistically well–supported clade, including a sequence of *Myrmecia israelensis* from UTEX 1181 (authentic strain) and four sequences generated by Ruprecht et al. (2014) labeled as Chlorophyta spp. in the GenBank (Fig 1).

Mycobiont phylogenetic analysis

Psora decipiens and *P. saviczii* fungal phylogeny was constructed from 38 sequences and showed that the samples included in this study fall into six well–supported clades (Fig. 2). The fungal ITS were resolved into six clades: *P. saviczii*, *P. decipiens* s. str, Clade I, Clade II, Clade III and Clade IV. IB_CI samples were randomly distributed in the six clades, but only *P. decipiens* s. str. and Clade III appear to occur in the N_CE samples included in this study.

Placidium spp. and *Clavascidium* spp. fungal phylogeny was constructed, including 14 newly obtained sequences. The 14 sequences included in this study fell into five well–supported clades (Fig. 3). In the case of *Placidium* spp. we resolved three fungal clades (*Placidium pilosellum* 1, *Placidium* sp. 1, *Placidium* sp. 2), and two for *Clavascidium* spp. (*Clavascidium* sp. 2 and *Clavascidium* sp. 3) which appear to occur only in Iberian localities.

Morphological and ultrastructural characterization of *Myrmecia israeliensis* in the thallus

FM examinations of squamules from the *Psora decipiens* s. str. sample (HOM_PD_1) revealed the interaction between *M. israeliensis* and the fungal hyphae (Fig. 4), also the phycobionts showed a chloroplast characteristic of the *Myrmecia* genus Tschermak-Woess & Plessl (1948). Cells showing chloroplast morphologies related to *Trebouxia* or *Asterochloris* were not detected.

To further investigate the appearance of the phycobiont layer, the SEM was employed. The phycobionts cells were located in close contact with the hyphae (Fig. 5A, B, C). The cell wall exhibited a thickness ranging from 0.08 ±0.001 to 0.18±0.003 µm. In the thallus, the cells showed a bipartite cup–shaped parietal chloroplast without a pyrenoid, which is characteristic of the *Myrmecia* genus (Fig. 5D). The thylakoid membranes were grouped in loose stacks of three to seven (Fig. 5G, H). Numerous pyrenoglobuli were distributed along the thylakoids (Fig. 5D, F, G, H). Spherical non electron–dense vesicles appeared throughout the cytoplasm and were especially numerous at the periphery (Fig. 5D, F, H). Secretory spaces were irregular in distribution and thickness.

Morphological and ultrastructural characterization of *Myrmecia israeliensis* in culture

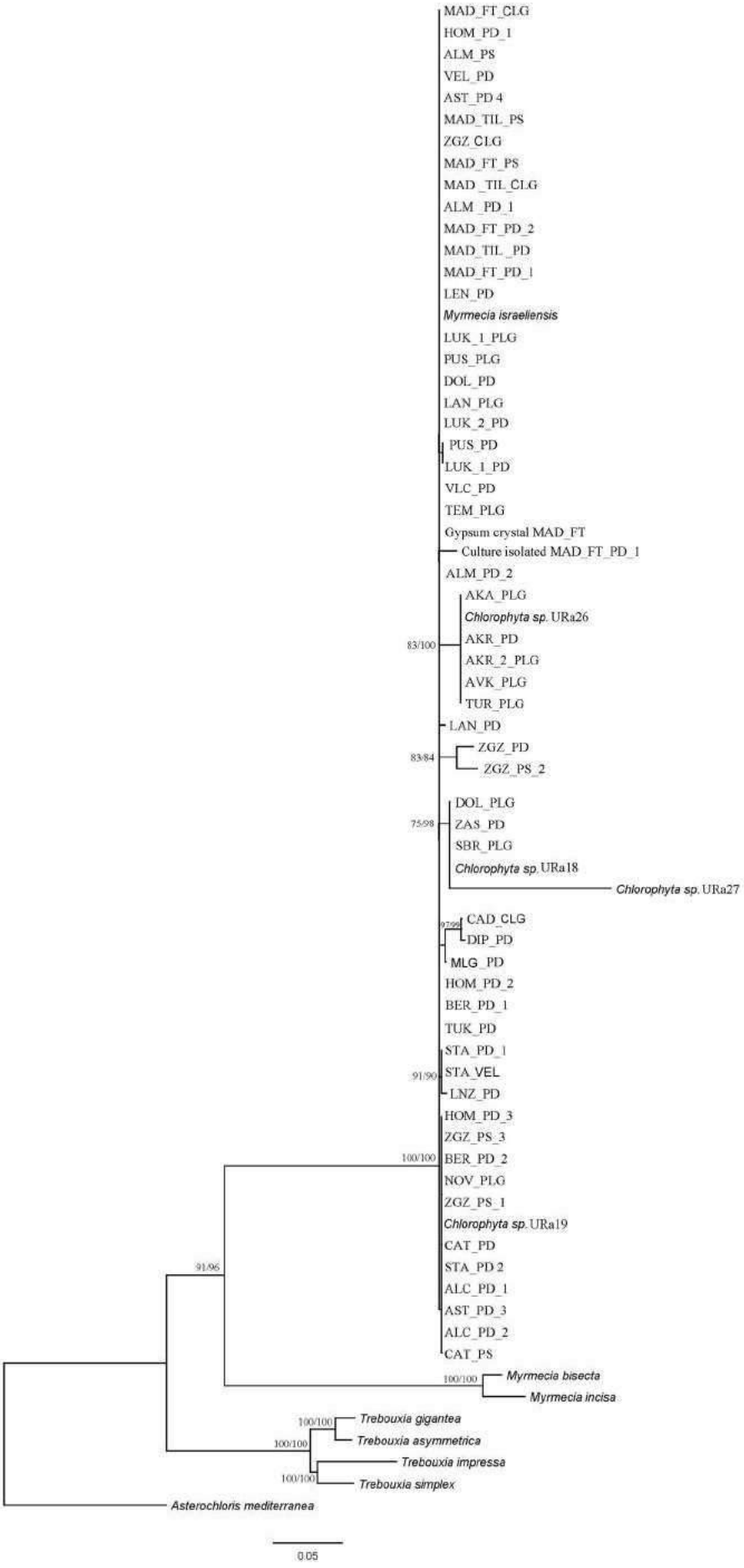
Mature vegetative cells were mostly spherical as seen using LM (Fig. 6A, C). Also in culture, both with TEM and LM, the cells showed the characteristic bipartite cup–shaped parietal chloroplast without a pyrenoid (Fig. 6).

Discussion

The class Trebouxiophyceae is generally known to comprise the majority of eukaryotic phycobionts (i.e. lichenized symbiotic microalgae). The genera *Trebouxia* Puymaly (1924), *Asterochloris* Tschermak–Woess (1980), *Coccomyxa* Schmidle (1901), *Symbiochloris* Škaloud et al. (2016), and *Myrmecia* Printz (1921) are among the most common primary symbiotic microalgae distributed. The coccoid green alga *Friedmannia israeliensis* isolated as free–living from Negev desert gypsum soils was described by Chantanachat & Bold (1962). Friedl (1995) demonstrated the monophyletic origin of *Myrmecia astigmatica*, *Myrmecia biatorellae* and *F. israeliensis*, and proposed synonymizing the genus *Friedmannia* with *Myrmecia*. Therefore, a new taxonomic combination, *Myrmecia israeliensis*, was proposed. These three green algae, forming a sister group with *Trebouxia* spp., also showed the characteristic chloroplast architecture described for *Myrmecia* spp.: cup–shaped, usually a lobed parietal chloroplast without a pyrenoid. However, the diversity, ecology, and distribution of *Myrmecia* genus as a lichen phycobiont have been overlooked in the past, and some interesting questions about this genus are still unresolved.

Studies on lichen microalgae have been performed mainly by Sanger sequencing (Molins et al. 2013; Voytsekhovich & Beck 2015). However, using this procedure some results could be controversial: only the primary phycobiont could usually be detected and primer biases might limit the detection of specific taxa (U'Ren et al. 2014). A combination of different techniques (molecular, isolation and microscopic) as well as the accurate selection of the molecular primers are key parameters in microalgal lichen studies and prevent any incorrect identification.

The present study contributes to the understanding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ALM _PD_1
MAD_FT_PD_2
MAD_TIL _PD
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LEN_PD
Myrmecia israeliensis
LUK_1_PLG
PUS_PLG
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VLC_PD
TEM_PLG
Gypsum crystal MAD_FT
Culture isolated MAD_FT_PD_1
ALM_PD_2
AKA_PLG
Chlorophyta sp. URa26
AKR_PD
AKR_2_PLG
AVK_PLG
TUR_PLG
LAN_PD
ZGZ_PD
ZGZ_PS_2
DOL_PLG
ZAS_PD
SBR_PLG
Chlorophyta sp. URa18
Chlorophyta sp. URa27
CAD_CLG
DIP_PD
MLG_PD
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BER_PD_1
TUK_PD
STA_PD_1
STA_VEL
LNZ_PD
HOM_PD_3
ZGZ_PS_3
BER_PD_2
NOV_PLG
ZGZ_PS_1
Chlorophyta sp. URa19
CAT_PD
STA_PD 2
ALC_PD_1
AST_PD_3
ALC_PD_2
CAT_PS
Myrmecia bisecta
Myrmecia incisa
Trebouxia gigantea
Trebouxia asymmetrica
Trebouxia impressa
Trebouxia simplex
Asterochloris mediterranea
83/100
83/84
75/98
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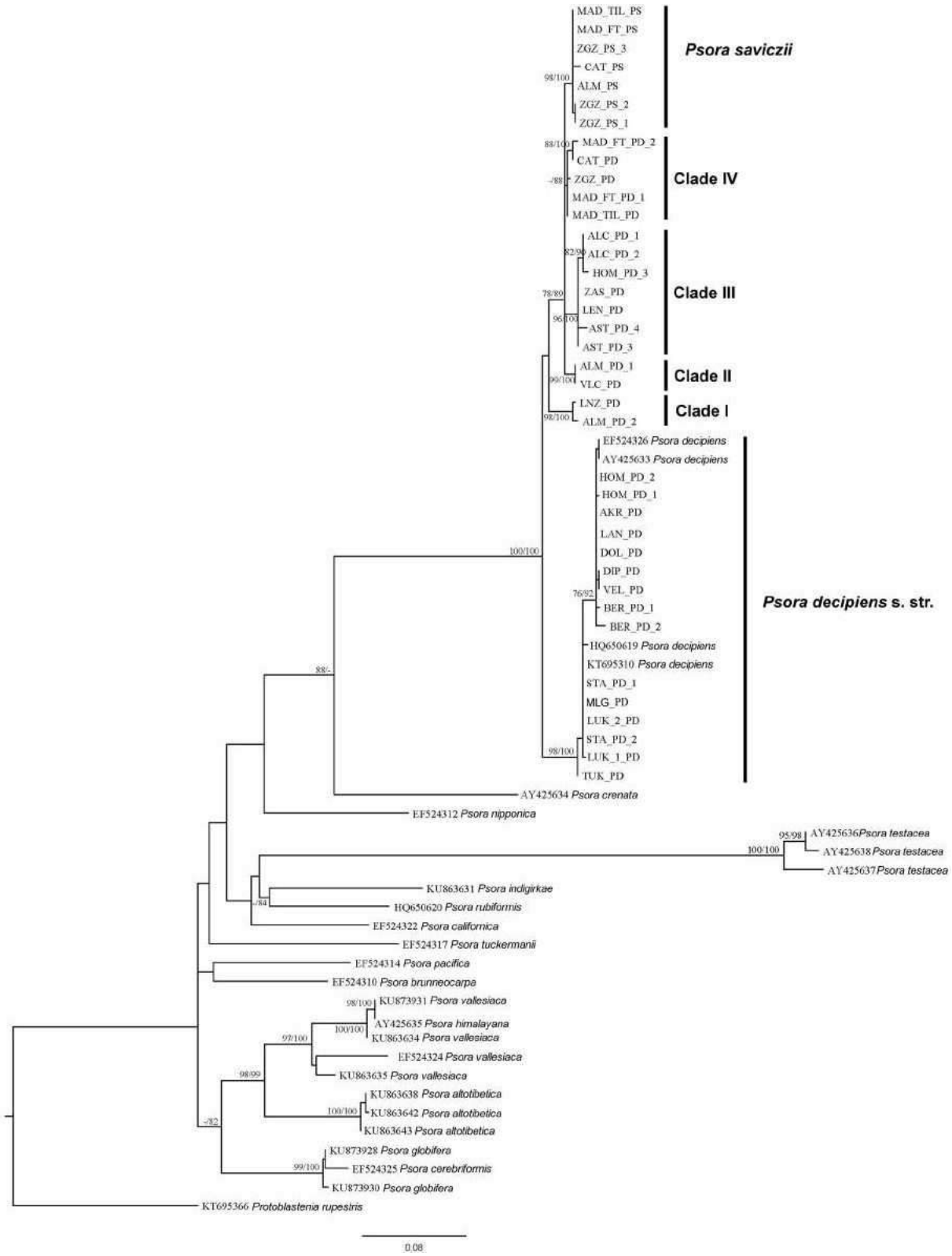

Fig. 2. Phylogenetic analysis of the ITS rDNA mycobiont from *Psora* spp. Values at nodes indicate statistical support estimated by two methods: MrBayes posterior node probability and maximum–likelihood bootstrap. Branches with a statistical support ≥ 75% in both analyses are indicated in the tree. Newly obtained sequences are grouped in clades named as*: Psora saviczii*, *Psora decipiens* s. str., Clade I, Clade II, Clade III and Clade IV. Accession numbers from *Psora* spp. and *Protoblastenia rupestris* sequences retrieved from the GenBank accompany each species name. Scale bar shows the estimated number of substitutions per site.

Fig. 1. *Myrmecia israeliensis* diversity detected by Sanger sequencing. A rooted and combined ITS rDNA and LSU rDNA gene tree representing 69 sequences is displayed, including selected sequences retrieved from the GenBank. Values at branches refer to Bayesian posterior probabilities ≥ to 0.75 and ML bootstrap values ≥ 75%, respectively. Scale bar shows the estimated number of substitutions per site.

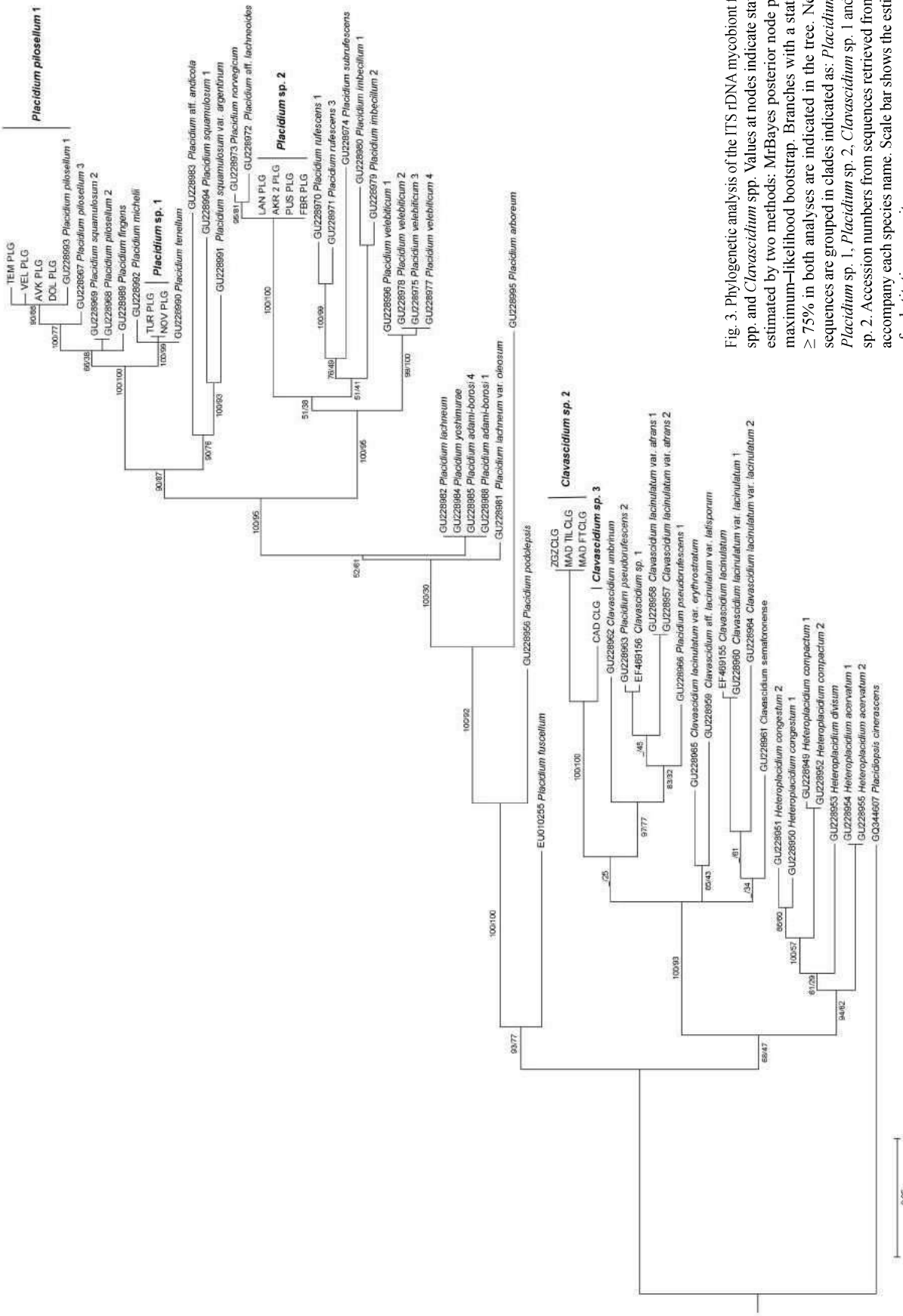

Fig. 3. Phylogenetic analysis of the ITS rDNA mycobiont from *Placidium* spp. and *Clavascidium* spp. Values at nodes indicate statistical support estimated by two methods: MrBayes posterior node probability and maximum–likelihood bootstrap. Branches with a statistical support ≥ 75% in both analyses are indicated in the tree. Newly obtained sequences are grouped in clades indicated as: *Placidium pilosellum* 1, *Placidium* sp. 1, *Placidium* sp. 2, *Clavascidium* sp. 1 and *Clavascidium* sp. 2. Accession numbers from sequences retrieved from the GenBank accompany each species name. Scale bar shows the estimated number of substitutions per site.

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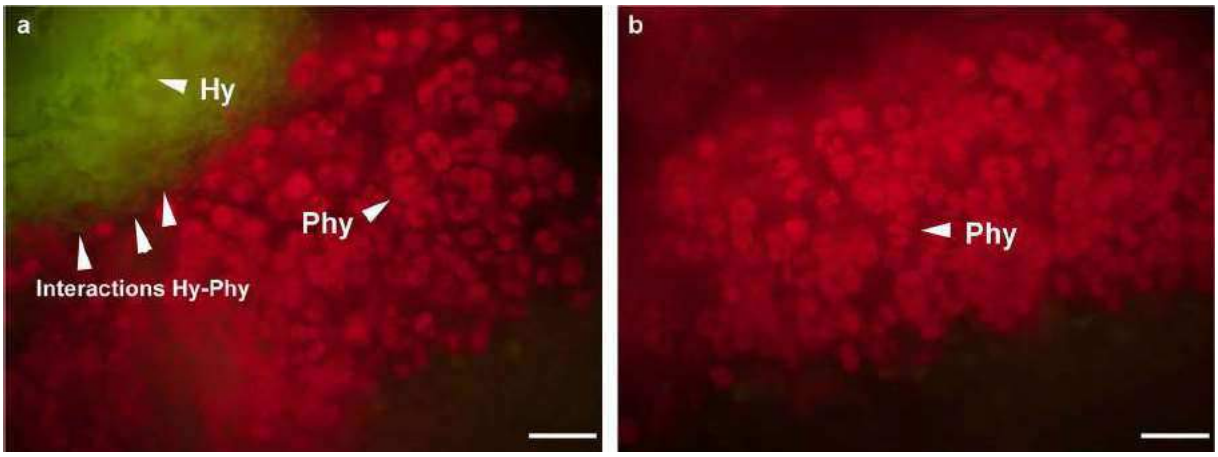


Fig. 4. Localization of *Myrmecia israeliensis* in squamules of *Psora decipiens* s. str. by FM. Abbreviations; Phy (Phycobionts) and Hy (Hyphae). Scale bar 20 μm.

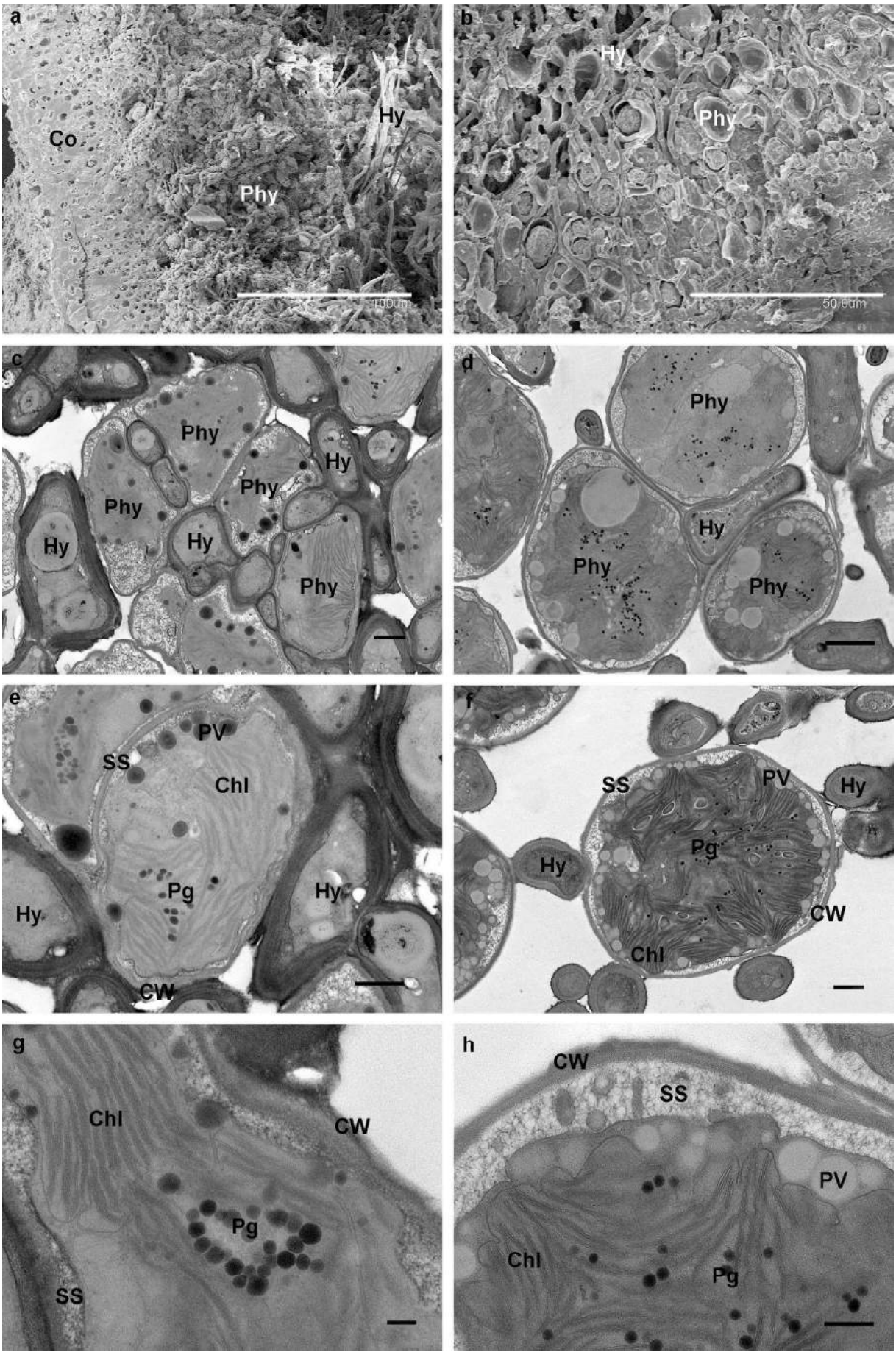
FM examinations of squamules from the *Psora decipiens* s. str. sample (HOM_PD_1) revealed the interaction between *M. israeliensis* and the fungal hyphae (Fig. 4), also the phycobionts showed a chloroplast characteristic of the *Myrmecia* genus Tschermak–Woess & Plessl 1948. Cells showing chloroplast morphologies related to *Trebouxia* or *Asterochloris* were not detected.

of the primary symbiont microalgae associated with *Psora* spp., *Placidium* spp. and *Clavascidium* spp. Both morphological and molecular analyses pointed out the undeniable presence of *Myrmecia israeliensis* linked to several terricolous squamulose lichen specimens distributed in European and Canary Island ecosystems. The nuclear ITS rDNA (clearly amplified with primers designed for this study) was added to build the algal phylogeny, together with the plastid molecular marker LSU rDNA (Del Campo et al. 2010). In this work, isolation and microscopic observations on algae in both the symbiotic and the cultivated state, were crucial to corroborate the presence of *M. israeliensis* as the primary symbiotic microalga in these lichens.

All the phycobionts investigated in this study formed a statistically well–supported clade, including a sequence of the authentic strain of *Myrmecia israeliensis* UTEX 1181 (corroborating the determination of our sequences as *M. israeliensis*) and four *Myrmecia* sequences generated by Ruprecht et al. (2014), who labeled them as Chlorophyta spp. These sequences were obtained by sequencing *Psora* lichens collected in the Ruine Homburg locality, along with numerous phycobionts determined as *Trebouxia* spp. and *Asterochloris* spp. However, our investigations, including the analysis of *Psora* lichens re–sampled from the Ruine Homburg locality, clearly show that neither *Trebouxia* nor *Asterochloris* were detected. Such bias can be explained by the fact that Ruprecht et al. (2014) used highly specific primers designed in this study to amplify the *Trebouxia* and *Asterochloris* lichen phycobionts. However, these primers do not match target sites for several green algal lineages, including the genus *Myrmecia*. Consequently, the primary microalga *Myrmecia* remained undetected. Indeed, electropherograms showing double peaks and/or polymorphic phycobiont sequences have been frequently reported in lichens, but these samples were usually removed from the analysis (Muggia et al. 2014; Leavitt et al. 2015; Voytsekhovich & Beck 2015). In this work, we included novel and clear barcode information (ITS rDNA) which provides the basic information for a precise delimitation of the microalgae identities.

M. israeliensis was also detected by PCR as free–living in Miocene gypsum crystals from Spain. Other lichens sharing the same habitats (BSC) with the species studied here, showed other genera as primary microalgae such as *Trebouxia* in: *Buellia zoharyi* (Chiva et al 2015; Moya et al 2016; Muggia et al 2016), *Acarospora* spp., *Diplotomma rivas–martinezii* and *Rhizocarpon malenconianum* (Chiva 2012) or *Asterochloris* in *Cladonia* spp. (Moya et al. 2015). These results raise questions about the levels of specificity and the strategies followed by lichenized fungi to associate with a certain alga available in the substrate pool (Muggia et al. 2013; Meessen & Ott, 2013; Williams et al 2017).

Mycobiont identification was confirmed by the DNA barcoding proposed by Schoch et al. (2012) in all the specimens included in this study. Timdal (1984, 1986) studied the anatomy and chemistry of *Psora decipiens* and *P. saviczii*, detecting several taxonomic problems at the specific level. In the European *P. decipiens* specimens he delimited three chemical strains: strain I (no lichen substances present) was the most frequent in Scandinavia and Central Europe; strain II (norstictic acid) was the most frequent in the Mediterranean region, and strain III (hyposalazinic acid and hypostictic acid) appeared in Austria, Hungary and Spain. Recently, Williams et al. (2017) included a mycobiont phylogeny (26S rDNA and *rbc*L molecular markers) of *P. decipiens* specimens from Germany, Sweden, Spain and Austria. They also found a surprising mycobiont variability (at least four clades), but no ITS rDNA information was provided. In this work, high genetic variability was also detected (five well–supported clades), but we were not able to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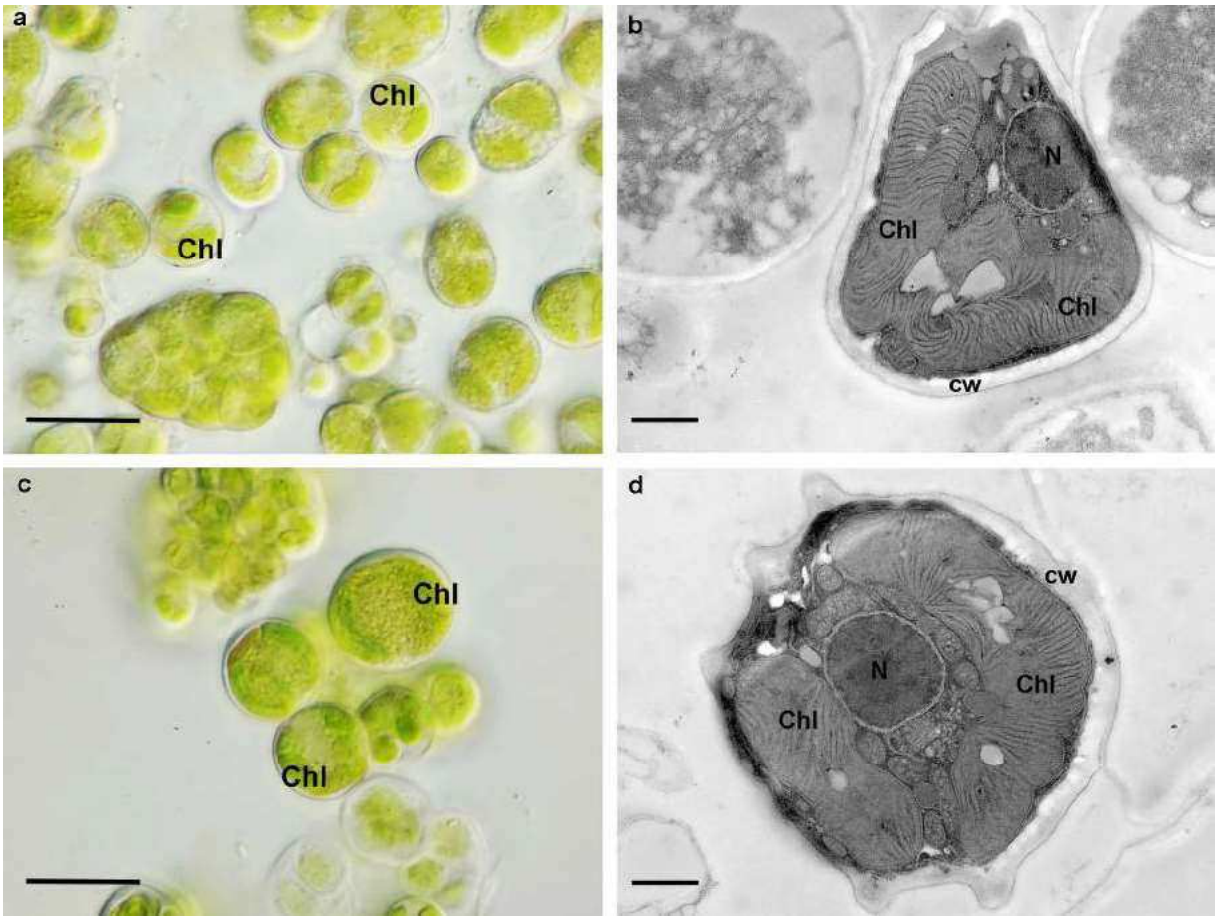

Fig. 6. *Myrmecia israeliensis* cells in isolated state by LM and TEM. Scale bar 800 nm. Abbreviations; Chl (Chloroplast), N (Nucleus), CW (Cell Wall).

Fig. 5. Cross section of *Psora decipiens* and *P. saviczii* thalli, and *Myrmecia israeliensis* microalgae in symbiosis. A–C–E MAD_FT_PD. B–D–F MAD_FT_PS. G–H *M. israeliensis* in detail associated with these thalli. Scale bars 200 nm (G), 400 nm (H), 800 nm (E), 1 µm (C, F), 2 µm (D), 50 µm (B) and 100 µm (A). Abbreviations; Phy (Phycobionts), Hy (Hyphae), Co (cortex), CW (Cell wall), SS (Secretory space), Chl (Chloroplast), Pg (Pyrenoglobuli), PV (Peripheral vesicles).

link our ITS rDNA sequences of *P. decipiens* with the sequences published by Williams et al. (2017) due to different analyzed loci. The advisable inclusion of chemical analyses and further research is required to understand the genetic diversity and the biogeographical distribution of *P. decipiens*.

Prieto et al. (2012) resolved the relationships within the *Placidium* group (*Placidium* spp., *Clavascidium* spp. and *Heteroplacidium* spp.). In this work, evolutionary inference based on ITS rDNA reinforces the *Placidium* phylogeny suggested by Prieto et al. (2012), and revealed four new well–supported clades, here described as *Placidium* sp. 1*, Placidium* sp. 2*, Clavascidium* sp. 2 and *Clavascidium* sp. 3.

Besides molecular techniques, *M. israeliensis* occurrence was validated in these lichen taxa through the examination of lichenized as well as isolated algae by microscopy including Transmission Electronic Microscopy (TEM). Recent literature (Casano et al. 2011; Molins et al. 2013; Catalá et al. 2015; Moya et al. 2015; Molins et al. 2017) proved that TEM observations should be considered as key methodology for the ultrastructural characterization of phycobiont species inside lichen thalli. The maintenance of the ultrastructural traits of the *Myrmecia* genus allowed us to identify and corroborate the presence of *M. israeliensis* as the primary microalga. Several authors pointed out some ultrastructural modifications when comparing isolated phycobionts *vs* lichenized states (e. g. cell wall thickness and the amount of mitochondria and ribosomes) (Galun 1988; Friedl & Büdel 2008; Melkonian & Berns 1983). Despite this, ultrastructural characteristic traits of cells from the *Myrmecia* genus remained recognizable enough in culture to allow for the correlation of both states (symbiotic and isolated).

In summary, our results proved *M. israeliensis* to be the primary symbiotic microalga in all the lichens analyzed here, and the presence of this microalga was verified using different molecular and microscopic observations. The combination of different techniques, molecular, isolation and microscopic, allowed for the accurate identification of this symbiotic microalga,

previously mainly known as free living.

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Supplementary material

the following supplementary material is available for this article:

Table S1. GenBank accession number for specimens and culture included in this study.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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SUPPORTING INFORMATION

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Table S1. GenBank accession number for specimens and culture included in this study.

Lichen species	**Code**	**LSU rDNA**	**nrITS DNA phycobiont**	**nrITS DNA mycobiont**
Placidium sp. 1	NOV_PLG	KY981733	KY981685 (culture)	KY981586
Psora decipiens s. str.	BER_PD_1	KY981709 (culture)	KY981651 (culture)	KY981596
Psora decipiens s. str	BER_PD_2	KY981710 (culture)	KY981652 (culture)	KY981597
Placidium sp. 2	SBR_PLG	KY981736 (culture)	KY981670 (culture)	KY981589
Psora decipiens s. str	TUK_PD	KY981737	KY981695	KY981598
Psora decipiens s. str	DIP_PD	-	KY981701	KY981599
Placidium pilosellum 1	AVK_PLG	-	KY981681	KY981582
Placidium sp.	AKA_PLG	-	KY981686	-
Psora decipiens s. str	AKR_PD	-	KY981687	KY981600
Placidium sp. 2	AKR_2_PLG	-	KY981688	KY981588
Psora decipiens s. str	STA_PD_1	KY981740	KY981671	KY981601
Psora decipiens s. str	STA_PD_2	KY981741	KY981672	KY981602
Psora decipiens s. str	HOM_PD_1	KY981715 (culture)	KY981657 (culture)	KY981603
Psora decipiens s. str	HOM_PD_2	KY981716 (culture)	KY981697 (culture)	KY981604
Psora decipiens clade III	HOM_PD_3	KY981717 (culture)	KY981696 (culture)	KY981605
Psora decipiens clade IV	MAD_TIL_PD	KY981728	KY981664	KY981606
Psora saviczii	MAD_TIL_PS	KY981730	KY981666	KY981607
Clavascidium sp.2	MAD_TIL_CLG	KY981729	KY981665	KY981593
Psora decipiens clade IV	MAD_FT_PD_1	KY981724	KY981660	KY981608
Psora decipiens clade IV	MAD_FT_PD_2	KY981725	KY981661	KY981609
Psora saviczii	MAD_FT_PS	KY981727	KY981663	KY981610
Clavascidium sp.2	MAD_FT_CLG	KY981726	KY981662	KY981594
Psora decipiens clade III	ALC_PD_1	KY981702	KY981643	KY981611
Psora decipiens clade III	ALC_PD_2	KY981703	KY981644	KY981612
Psora decipiens clade II	ALM_PD_1	KY981704	KY981646	KY981613
Psora decipiens clade I	ALM_PD_2	KY981705	KY981647	KY981614
Psora saviczii	ALM_PS	KY981706	KY981648	KY981615
Psora decipiens clade III	LEN_PD	KY981719	KY981658	KY981616
Psora decipiens clade III	AST_PD_3	KY981707	KY981649	KY981617
Psora decipiens clade III	AST_PD_4	KY981708	KY981650	KY981618
Psora decipiens clade IV	ZGZ_PD	KY981745	KY981675	KY981619
Psora saviczii	ZGZ_PS_1	KY981747	KY981677	KY981620
Psora saviczii	ZGZ_PS_2	KY981748	KY981678	KY981621
Psora saviczii	ZGZ_PS_3	KY981749	KY981645	KY981622

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Table S1 Cont.

Clavascidium sp.2	ZGZ_CLG	KY981746	KY981676	KY981595
Psora decipiens s. str	MLG_PD	KY981731	KY981667	KY981623
Clavascidium sp.3	CAD_CLG	KY981732	KY981668	KY981592
Psora decipiens clade IV	CAT_PD	KY981711	KY981653	KY981624
Psora saviczii	CAT_PS	KY981712	KY981654	KY981625
Psora decipiens clade I	LNZ_PD	KY981720	KY981659	KY981626
Psora decipiens clade II	VLC_PD	KY981743	KY981674	KY981627
Placidium pilosellum 1	TEM_PLG	-	KY981679 (culture)	KY981584
Psora decipiens s. str	LUK_1_PD	KY981721 (culture)	KY981699 (culture)	KY981628
Placidium sp.	LUK_1_PLG	KY981722	KY981700	-
Psora decipiens s. str	LUK_2_PD	KY981723	KY981698	KY981629
Psora decipiens clade III	ZAS_PD	KY981744	KY981693	KY981630
Placidium sp. 1	TUR_PLG	KY981738	KY981694	KY981587
Psora decipiens s. str	LAN_PD	-	KY981689	KY981631
Placidium sp. 2	LAN_PLG	KY981718	KY981692	KY981590
Psora sp.	PUS_PD	KY981734	KY981683	-
Placidium sp. 2	PUS_PLG	KY981735	KY981684	KY981591
Psora decipiens s. str	VEL_PD	KY981739 (culture)	KY981682 (culture)	KY981632
Placidium pilosellum 1	VEL_PLG	KY981742	KY981680	KY981585
Placidium pilosellum 1	DOL_PLG	KY981714	KY981691	KY981583
Psora decipiens s. str	DOL_PD	KY981713	KY981690	KY981633
	Gypsum crystal_MAD_FT	-	KY981656	-
	Culture isolated MAD_FT_PD_1	-	KY981655	-

	LSUrDNA	**nrITS DNA**
Myrmecia israeliensis UTEX 1181	KM462861	KY981669
Chlorophyta sp. URa18	-	KF907689
Chlorophyta sp. URa19	-	KF907687
Chlorophyta sp. URa26	-	KF907699
Chlorophyta sp. URa27	-	KF907700
Myrmecia bisecta SAG 2043	GQ168957	-
Myrmecia incisa SAG 2466	KM821265	KM020046
Trebouxia gigantea UTEX 2231	JQ921008	AJ249577
Trebouxia asymmetrica SAG 48.88	EU725860	AJ249565
Trebouxia impressa UTEX 893	JQ921009	AF345890
Trebouxia simplex SAG 101.80	FJ804756	FJ626735
Asterochloris mediterranea CAUP 1015	KP257332	KP257398

3.3 Symbiont interaction patterns in biocrust lichen communities located in semi-arid gypsum outcrops in the Central Iberian Peninsula (R 3.3)

Symbiont interaction patterns in biocrust lichen communities located in semi-arid gypsum outcrops in the Central Iberian Peninsula

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Abstract

This study aims to be a contribution to a comprehensive understanding of the relationships among the crustose lichens which grow in gypsum outcrops in the Central Iberian Peninsula, in order to obtain an overview of the symbiotic patterns and the factors shaping these relationships in the whole community. This work is complementary to previous studies focused on microalgae diversity in the foliose and dimorphic *Cladonia* spp. and the squamulose *Psora decipiens*, *P. saviczii*, *Clavascidium* spp. and *Placidium* spp. developed on the same biocrusts. In addition, due to the peculiar life style of the selected crustose species we implemented several analyses focused on phycobiont coexistence, diversity and switching. This crustose portion was composed of: *Acarospora nodulosa* and *Acarospora placodiiformis*, transient parasites of *Diploschistes diacapsis*, which in mature stages are able to develop independent thalli. *Rhizocarpon malenconianum*, an obligate lichenicolous lichen on *D. diacapsis* as the host, and the saxicolous *Diplotomma rivas-martinezii*, which always occurred in contact with *D. diacapsis*. In the whole community, three different microalgae genera were detected: *Trebouxia* in *D. diacapsis*, *A. placodiiformis*, *A. nodulosa*, *D. rivas-martinezii* and *R. malenconianum*; *Asterochloris* in *Cladonia* spp. and *Myrmecia* in the squamulose guild. Due to the peculiarity of biocrust gypsum substrates, several analyses of the chemical soil properties were included. Lichen morphology, reproductive and dispersal strategies and mycobiont diversity were investigated as factors driving the symbiotic association patterns in the whole community.

Introduction

Biological soil crusts (BSC; biocrusts) are complex communities of multiple organisms, both autotrophic and heterotrophic, which live on top of the soil surface creating a consistent layer and binding soil particles due to their architecture and activity [1]. Many studies have emphasized the role of BSCs in defining an ecosystem's structure and function through their interactions with top soil layers and other soil organisms, and due to their participation in carbon and nitrogen fixation as well as hydrological [2] and nutrient cycling [3-5]. Biocrusts are present in a wide variety of ecosystems; however, their abundance is generally greater in arid environments [6] with sparse vascular vegetation cover, where they are considered pioneers in colonizing soil surfaces [7]. The presence, abundance and frequency of BSCs respond differently to diverse environmental factors, particularly to the climate and soil types [3, 8].

Biocrusts dominated by lichens are particularly notable in gypsum ecosystems [9, 10]. These lichen species are mainly basiphilous (frequently associated with alkaline substrates), and around 20% of them are exclusive gypsophytes; well-adapted and occurring exclusively on gypsum soils such as *Acarospora placodiiformis*, *Diplotomma rivas–martinezii* and *Rhizocarpon malenconianum*, or preferential gypsophytes; species with a great preference for gypsum soils, but also being found outside these substrates, such as in *Acarospora nodulosa*, *Buellia zoharyi*, *Diploschistes diacapsis* or *Psora saviczii* [11]. Biocrust lichens have a high conservation value due to their potential to form extended covers, and their contribution to the diversity of the ecosystems where they develop [12]. Different areas throughout the Central Iberian Peninsula evidenced biocrust gypsum outcrops deposited in the late Miocene period [13]. These peculiar BSCs are colonized by a predominant group of crustose species (*D. diacapsis*, *A. placodiiformis*, *A. nodulosa* and *B. zoharyi*), occasionally accompanied by *D. rivas-martinezii* [14] and *R. malenconianum* [15]. There is also a relevant squamulose community composed of *Psora decipiens* [16], *P. saviczii* [17, 18], *Clavascidium* spp. and *Placidium* spp. [19], and foliose *Cladonia* spp. covering micro-areas with an extra water supply due to condensation, i.e. under shrubs [20]. Crustose and squamulose morphotypes seem to be favored in gypsum biocrusts, probably due to their capacity to retain the humidity [21]; however, the geochemical characteristics of the gypsum likely to influence the presence of specific lichen species are still unknown [11].

The crustose fraction of these communities shows a peculiar life style [11]: *A. nodulosa* and *A. placodiiformis* are parasites of *D. diacapsis* during their first growth stages (Fig. 1A parasitic state), while in mature stages they are able to develop independent thalli (Fig. 1B). *R. malenconianum* is an obligate lichenicolous lichen on the *D. diacapsis* host [15]. Additionally, the epilithic species *D. rivas-martinezii*, occurring on efflorescent rocks at soil level, is always found in contact with *D. diacapsis* (Fig. 1). The way that phycobionts are shared in species growing as a parasite, or in close proximity to one another, has been traditionally documented with the case of the lichenicolous lichen *Diploschistes muscorum* growing on *Cladonia* spp. [22], as well as the mycobiont of *Gyalolechia bracteata* as a parasite of *Thalloidima sedifolium* [23]. In general, parasitic lichen communities are good candidates to study photobiont switching and coexistence, and specifically the microalgal diversity in parts of these crustose parasitic lichens have been mostly ignored, and little information is available concerning the phycobionts in these lichens. Photobiont switching was reported for lichen communities where algal morphospecies and genotypes were shared among different fungal genera and families [24]. The coexistence of multiple *Trebouxia* species within a single lichen thallus was studied in depth in the species *Ramalina farinacea* from the Mediterranean region [25-29]. Subsequently, this co-occurrence has been reported in other lichens from diverse geographic origins and growth forms [26, 30-37]. In fact, it now appears that multiple phycobiont species within a single lichen thallus is a relatively common phenomenon in lichen symbioses.

In previous studies [38, 39], we reported the microalgae diversity of the foliose *Cladonia* spp. and squamulose fraction (*Psora* spp., *Clavascidium* spp. and *Placidium* spp.) growing in Central Iberian Peninsula Miocene gypsum biocrusts. Moya et al. [38] provided a detailed characterization of a novel phycobiont species (*Asterochloris mediterranea*) detected in the thalli of *Cladonia* spp., and Moya et al. [39] proved that *Myrmecia israeliensis* is the primary symbiotic microalga in squamulose lichens. In the present study, we deal with the crustose fraction of these lichen guilds [40, 41] to analyze the symbiont interaction patterns in the gypsum lichen communities. To achieve these objectives, the microalgae nrITS was studied using different techniques: Sanger sequencing, 454-pyrosequencing and ultrastructural characterization. In order to analyze the fungal identification and diversity, barcode nrITS mycobiont analyses were performed to construct phylogenies, haplotype networks and diversity indices. Finally, to characterize the gypsum soils, different chemical and spectrometric techniques were applied in samples collected from each location.

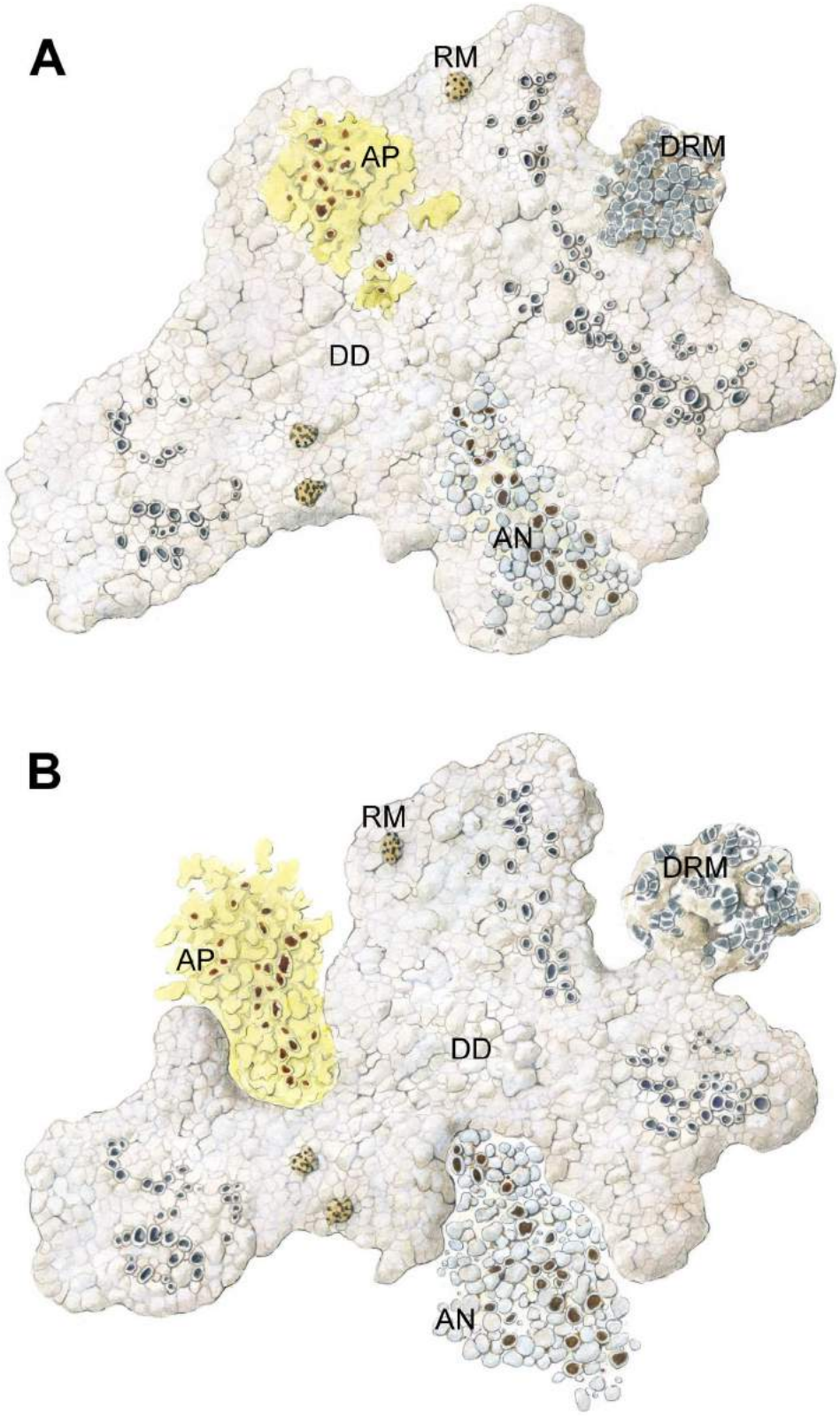

Fig. 1 Depiction of the crustose lichen communities growing in gypsum biocrusts composed of *Diploschistes diacapsis* (DD), *Acarospora nodulosa* (AN), *Acarospora placodiiformis* (AP), *Rhizocarpon malenconianum* (RM) and *Diplotomma rivas-martinezii* (DRM). During their first growth stages (Fig. 1A parasitic state), *A. nodulosa* and *A. placodiiformis* are parasites of *D. diacapsis*, while in mature stages (Fig. 1B) they develop independent thalli. *R. malenconianum* is an obligate lichenicolous lichen on the *D. diacapsis* host, and the epilithic species *D. rivas-martinezii*, occurring on efflorescent rocks at soil level is always found in contact with *D. diacapsis*

Materials and Methods

Sampling

Two representative locations in the Upper Mesomediterranean low dry area of Madrid (Spain) with gypsum BSCs were selected: Fuentidueña de Tajo (FU) (40º07'41"N, 03º09'14"W / 571 m a.s.l.) and Titulcia (TI) (40º07'32"N, 03º33'15"W / 521 m a.s.l.). At least 40 independent (Fig. 1B) specimens of *Diploschistes diacapsis* (Ach.) Lumbsch (DD), *Acarospora nodulosa* (Dufour) Hue (AN) and *Acarospora placodiiformis* H. Magn. (AP) were collected from both locations, we included the lichenicolous lichen *Rhizocarpon malenconianum* (Llimona & Werner) Hafellner & Mayrhofer (RM; n=7) (only detected in Fuentidueña) and the epilithic *Diplotomma rivas-martinezii* Barreno & A. Crespo (DRM; n=7) (Fig. 1B) when they appeared. The samples were dried and stored at −20 °C until processing. Each lichen specimen used in this study was encoded as lichen code species_number of sample_location code.

Geological background of sampled rocks

The considered substrates are gypsiferous rocks from Miocene outcrops of lacustrine deposits from the Madrid basin. The sampled substrates in FU, are units of macroscrystalline massive grey – green gypsums with intercalated beds of green gypsiferous clays from the Lower – Mid Miocene [42]. The sampled substrates of TI are a micrograined laminated unit of secondary gypsums and lutites from the Mid Miocene [43]. The evaporite minerals (gypsum and other salts) in the lacustrine deposits from the Lower – Mid Miocene in the Madrid Basin, come from the recycling of Mesozoic and Paleogene evaporite formations related to the uplift of the Sierra de Altomira and the Iberian mountain range on the eastern side of the Madrid Basin [44].

Basic Soil Chemical properties

The total carbon (TC; g 100 g^{-1}), total nitrogen (TN; g 100 g^{-1}) and total organic carbon (TOC; g 100 g^{-1}) content of the BSCs (5 replicates per location) were determined using a CN element analyser (Leco TruSpec CN628; LECO Corp., St Joseph, MI, USA). 0.05-0.07g of air-dried soil was weighed into a tin capsule and combusted at 120 ºC; $CaCO_3$ was determined by reaction with a 2N HCl solution and then air-dried for 8 hours. Inorganic carbon (IC) was calculated as a 10.3%

share of the $CaCO_3$ content. TOC was quantified by subtracting the IC from the TC content. The solid organic matter (SOM) was calculated from the TOC by multiplication with the factor 1.724 (van Bemmelen factor). These analyses were carried out at the Laboratorio de Ionómica (CEBAS-CSIC).

Determination of macro- and micro elements in gypsum samples

Macro and micro element contents were determined according to ISO 11.885 [45] by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) using Thermo ICAP 6500 Duo equipment (Thermo Fisher Scientific, Waltham, MA, USA). In the microwave furnace equipment, for each sample, 200 mg were added to a 25 ml tube with a mixture of 4 ml of HNO_3 (68% purity) and 1 ml H_2O_2(33% purity) for their subsequent digestion. 300 ml high-purity de-ionized water, 30 ml H_2O_2 (33% purity) and 2 ml H_2SO_4(98% purity) were also added in the Teflon reactor. The microwave heating digestion program consisted of 3 steps: starting at 20 ºC and 40 bar; increasing 10 bar/minute for 30 minutes up to 220 ºC; and remaining at 220 ºC for 20 minutes. After cooling, the mineralized samples were transferred to double gauge tubes (10 mL for the micro elements and 25 mL for the macro elements) and the volume was made up with high-purity de-ionized water. A multimineral standard solution containing 31 minerals, supplied by SCP Science (Quebec, Canada), was used to prepare calibration standards in high-purity de-ionized water. For ICP-OES analyses, two control samples containing high-purity de-ionized water and a multimineral standard were used. Each mineral determination was performed at specific wavelengths ranging from 167.1 to 670.8 nm. The concentration of macro and micro elements was calculated according to the formula "mg Kg^{-1} or µg Kg^{-1}= (C x D) / W"; where C was the mineral concentration, D was the dilution factor and W was the sample weight. These analyses were carried out at the Laboratorio de Ionómica (CEBAS-CSIC).

X Ray powder diffraction mineralogical analysis

X-ray powder diffraction (XRD) was performed to investigate the mineralogical compositions of three samples from FU (FU1-FU2-FU3), and three from TI (TI1-TI2-TI3). The FU1 and TI1 samples were collected from the soil immediately below the corresponding biocrust (0-0.5 cm), the FU2 and TI2 samples spanned a depth of about 2-5 cm, and the FU3 and TI3 samples were obtained as a mixture of the corresponding FU1+FU2 and TI1+TI2 samples. Dried samples were ground in a Fritz Pulverisette P9 to

powder, to pass through a 230 ASTM sieve. Random powders were obtained using the Niskanen [46] method, and oriented aggregates of fractions used for identification of sheet silicates according to the Pansu & Gautheyrou [47] method. The collection of XRD data was performed using a Bruker D8 instrument with the Diffrac Plus System, Cu-Kα radiation, beam voltage and a current of 40 kV and 20 mA, respectively; Ni filter, step size: 0.03°2θ, step time: 96 s. The Eva® program working with the ICDD data base was used for data evaluation following the Warshaw & Roy [48] method for identification of sheet silicates. Semi-quantitative estimates of the mineral phases were carried out according to Davis & Smith [49]. XRD analysis was done at the X Ray Powder laboratory of the SCSIE Service of the University of Valencia.

Sample handling, DNA extraction, amplification and Sanger sequencing

Lichen thalli were examined under a stereomicroscope to remove soil particles and were sterilized by sequential immersion in ethanol and NaOCl [50]. Fragments from different parts of each thallus were randomly excised and pooled together. The mycobiont and the primary phycobiont were identified by Sanger sequencing.

Total genomic DNA from all the samples was isolated and purified using the DNeasy TM Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Phycobiont locus encoding the nrITS (internal transcribed spacer) was amplified using the primer pair nr-SSU-1780 [24] and ITS4 [51]. Fungal nrITS was amplified using the primer pair ITS1F [52] and ITS4 [51]. PCR reactions were performed as described in Molins et al. [37, 53].

454-pyrosequencing analyses

One additional thalli of DD, with AN and RM in their parasitic state from Fuentidueña were pyrosequenced following the protocol described in Moya et al. [29] and Molins et al. [53]. The number of cycles of PCR I and PCR II were determined by the average Ct (cycle threshold) of the RT-PCR I (DD=20, AN=17, and RM=15) and RT-PCR II (DD=6, AN=7, and RM=7). Algal nrITS sequences were determined using a GS Junior 454 system (Roche 454 Life Sciences, Branford, CT, USA) following the Roche Amplicon Lib-L protocol at the Genomics Core Facility at the University of Valencia (Spain). Reads were processed as described in Moya et al. [29] and clustered based on S 99 -L 0.9 criteria. The

consensus sequences of the OTUs (operational taxonomic units) were identified using the BLAST tool in the GenBank data base [54], and were encoded as lichen code species_number of OTU_number of sequences found for this OTU.

Phycobiont phylogenetic analysis

For the nrITS, a multiple alignment was prepared including: the phycobiont obtained by Sanger sequencing (S1), the consensus sequence OTUs obtained by 454-pyrosequencing analysis (S1), selected sequences described by Leavitt et al. [55], Moya et al. [29] and Molins et al. [37], and a selection of *Trebouxia* species available from the Culture Collection of Algae at Göttingen University (SAG), from the Culture Collection of Algae at the University of Texas (UTEX) and *Trebouxia* sp. TR9 (FJ418565). We included *Asterochloris erici* (AF345439) as an outgroup. The alignment was carried out with MAFFT v 7.0 [56, 57] using default parameters. The best-fit substitution model for this region (GTR+G) was chosen using jModelTest v 2.0 [58], and by applying the Akaike Information Criterion [59]. Maximum likelihood (ML) analysis was implemented in RAxML v 8.1.11 [60] using the GTRGAMMA substitution model. Bootstrap support was calculated based on 1,000 replications [61]. Bayesian inferences (BI) were carried out in MrBAYES v 3.2 [62]. Settings included two parallel runs with six chains over 20 million generations starting with a random tree, and sampling after every 200th step. We discarded the first 25% of data as burn-in. MAFFT, jModelTest, ML and BI analyses were implemented at the CIPRES Science Gateway v 3.3 webportal [63]. Phylogenetic trees were visualized in FigTree v 1.4.1 [64].

Microscopic examinations

The ultrastructure of the phycobionts was characterized by transmission electron microscopy (TEM) from at least one specimen of each species. These five specimens were sampled in the parasitic state from the same DD thallus (Fig. 1A), and their primary phycobionts were identified by Sanger sequencing. For TEM, the cells were fixed and dehydrated as described in Molins et al. [37, 53]. Samples were embedded in Spurr's resin according to the manufacturer's instructions. Sections (90 nm) were cut and mounted as described in Moya et al. [29]. The sections were observed with a JEOL JEM-1010 (80 kV) electron microscope, equipped with a MegaView III digital camera and 'AnalySIS' image acquisition software. TEM examinations were done at the SCSIE Service of the University of Valencia.

Mycobiont phylogenetic analysis, nucleotide diversity and genealogical relationships of haplotypes

For each lichen species, a multiple alignment was prepared including the newly determined fungal nrITS sequences (S2) and a selection of sequences downloaded from the GenBank. *Thelotrema* spp. (HQ650717 and AJ508684) were selected as outgroup DD, *Pycnora sorophora* (KX132977) for AN/AP, *Fuscidea intercincta* (AF483605) for RM and *Physconia grisea* (AF542506) for DRM. The alignments and phylogenetic analyses were carried out as previously described for the phycobiont (GTR+I+G for DD, AN, AP and RM and GTR+G for DRM).

The nucleotide diversity (π) was calculated using DnaSP 5.10 [65]. This software ignores positions with gaps, and consequently pools haplotypes that only differ by insertions and deletions (indels). Therefore, indel positions in the nrITS alignments were recoded as 5th characters [66].

In order to infer the genealogical relationships among haplotypes of the nrITS, a statistical parsimony haplotype network was constructed with POPART v 1.7 [67]. We first generated haplotype alignments with the FABOX v 1.41 online toolbox [68], and then constructed networks using TCS [69] under the 95% parsimony probability criterion [70], with gaps treated as a 5th character state.

Results

Comparative trends in soil chemical properties between both locations

The elemental contents of gypsum soils analyzed showed higher values for TOC and SOM in TI, meanwhile TN and TC were more uniform between both locations. The carbonate content was similar between FU and TI, with only slight differences (Table 1).

	TN g/100g	TC g/100g	TOC g/100g	SOM g/100g	CaCO3 g/100g
FU	0.09(0.04)	1.19(0.57)	0.64(0.38)	1.10(0.67)	4.61(3.52)
TI	0.13(0.09)	1.74(1.40)	1.31(1.35)	2.26(2.33)	3.59(1.36)

Table 1 Summary of geochemical values of elemental contents of gypsum soils analyzed in Fuentidueña de Tajo (FU) and Titulcia (TI): Total nitrogen (TN), total carbon (TC), total organic carbon (TOC) and solid organic matter (SOM)

The micro and macro element contents in soil samples collected from FU and TI are shown in S3. The pattern of micro and macro-elements found in both locations was very similar: the most abundant micro elements were Fe-Sr-Si and Ti, their values being 4484.78-2889.06-209.26 and 115.85 in FU, and 1801.72-955.82-195.46 and 85.54 in TI, the remaining elements had values ranging from <0.01 to 33.65. Fe and Sr (the most abundant elements in both locations) showed higher values in FU compared to TI.

Mineralogical analysis of soil samples from FU and TI was also performed by X Ray powder diffraction. The patterns of the studied samples (FU1 to FU3 and TI1 to TI3) are shown in S4 and S5, including the quantitative estimates of the identified phases. The mineral assemblages of the studied substrates consist mainly of gypsum, calcite and quartz, with minor contents of sheet silicates (illitte, kaolinite and chlorite) and feldspars (orthoclase and anorthite), with the presence of calcium oxalates, whewellite (calcium oxalate monohydrate), and weddellite (calcium oxalate dihydrate), concentrated in the outer rim of the substrates. Both locations showed a similar mineralogical assemblage.

Analysis of phycobiont coexistence

To test phycobiont coexistence, nrITS was analyzed and only samples without double peaks in the electropherogram were included in the study, the remaining samples considered as coexistence were removed. From a total of 55 specimens analyzed in DD, 33 showed coexistence and only 18 showed unique sequences. The ratio coexistence vs unique in the remaining lichen species is shown in Fig. 2. All species selected showed coexistence ranging from 33% in RM, to 72% in the case of AN and DRM.

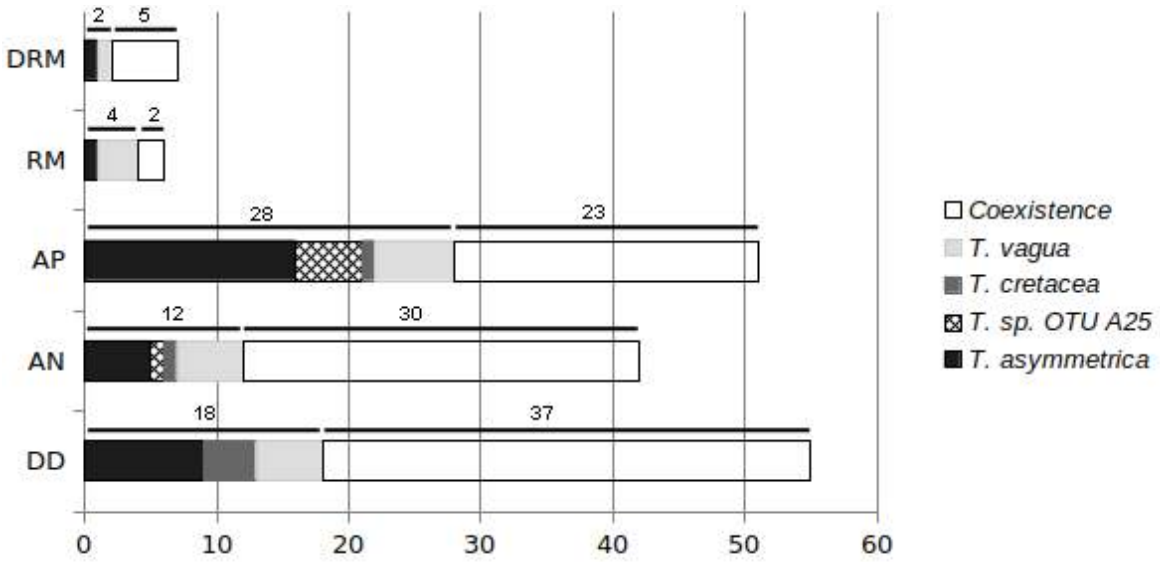

Fig. 2 Coexistence (white squares) and *Trebouxia* diversity detected by Sanger sequencing in the crustose lichen species composed of *Diploschistes diacapsis* (DD), *Acarospora nodulosa* (AN), *Acarospora placodiiformis* (AP), R*hizocarpon malenconianum* (RM) and *Diplotomma rivas-martinezii* (DRM). *Trebouxia* species detected are indicate as light grey squares for *T. vagua*, dark grey squares for *T. cretacea*, frame squares for *Trebouxia* sp. OTU A25 and black squares for *T. asymmetrica*. The total number of specimens showing unique sequence vs coexistence are indicated.

Phycobiont diversity detected by Sanger sequencing (nrITS) and 454-pyrosequencing. Taxonomic assignment by phylogeny

Trebouxia phycobionts detected by Sanger sequencing were grouped into four well-supported clades (S6): 32 of them from DD/AN/AP/RM/DRM linked with *Trebouxia asymmetrica* (100/97), 20 detected in the same 5 lichen species matched with *Trebouxia vagua* (62/86), 6 detected in DD/AN/AP with *Trebouxia cretacea* (-/97) and 6 from AN/AP with *Trebouxia* sp. OTU A25 [55] (-/79). *Trebouxia asymmetrica*, *Trebouxia vagua* and *Trebouxia* sp. OTU A25 appeared in both locations (FU and TI), *Trebouxia cretacea* was only detected in FU (S6).

	DD	AN	RM
Total			
T. asymmetrica	OTU1_4111	OTU1_3027	OTU1_4972
T. sp. OTU A25	OTU2_2008	OTU4_3	OTU2_370
T. cretacea	OTU3_83		OTU3_181
T. vagua	OTU4_16	OTU3_6	OTU4_11
Myrmecia israeliensis		OTU2_1247	
Bracteacoccus		OTU5_3	
Vulcanochloris		OTU6_2	
T. sp. OTU I53		OTU7_2	

Table 2 Taxonomic identification and summary of number of sequences obtained by pyrosequencing of *Trebouxia* spp., *Myrmecia* and other minor chlorophyte for each particular OTU in the three analyzed thalli, *Diploschistes diacapsis* (DD), *Acarospora nodulosa* (AN) and *Rhizocarpon malenconianum* (RM). The consensus sequences of the OTUs were encoded: number of OTU_number of sequences found for this OTU

Three specimens (DD, AN and RM) in the parasitic state from the same DD from FU were selected for the pyrosequencing analysis. 454-pyrosequencing of nrITS amplicons produced 6.218 sequence reads for DD, 4.290 for AN and 5.534 for RM. Raw read datasets obtained from these thalli were individually trimmed, and singletons and unreliable reads were filtered and removed. Clustering these nrITS

amplicons with a 99% similarity cut-off, 4 *Trebouxia* OTUs for DD and RM were detected: *Trebouxia asymmetrica*, *Trebouxia vagua*, *Trebouxia cretacea* and *Trebouxia* sp. OTU A25 (Table 2 and S6). In the case of AN, only 3 *Trebouxia* OTUs were determined: *Trebouxia asymmetrica*, *Trebouxia vagua* and *Trebouxia* sp. OTU A25, also *Myrmecia* and other minor chlorophyta were recognized (Table 2 and S6).

Trebouxia photobiont switching and ultrastructural characterization of microalgae

Considering only *Trebouxia* microalgae detected by both Sanger and 454-pyrosequencing, we detected a total of four *Trebouxia* species in DD considered to be the donor lichen: *Trebouxia asymmetrica*, *Trebouxia vagua, Trebouxia cretacea* and *Trebouxia* sp. OTU A25. The same four *Trebouxia* species were found in the parasitic lichen species: AN, AP and RM, meanwhile the epilithic DRM only showed *Trebouxia asymmetrica* and *Trebouxia vagua* (Fig. 3).

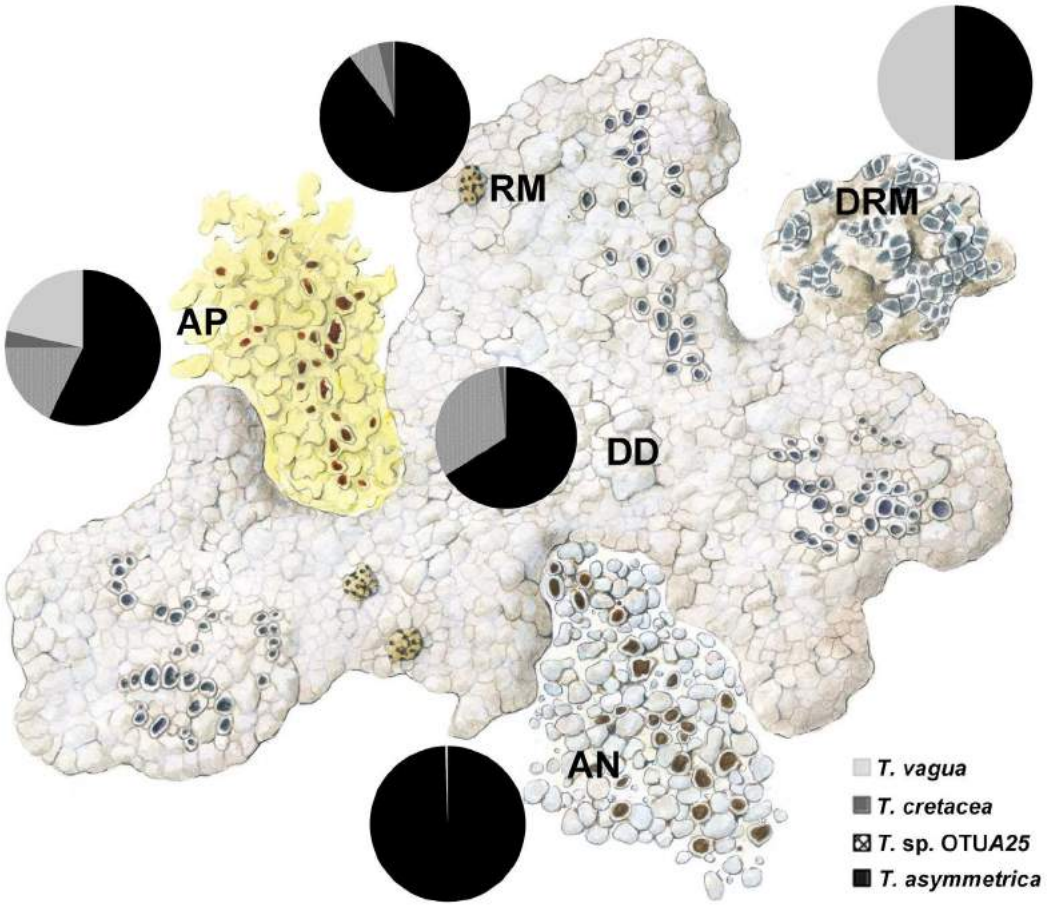

Fig. 3 *Trebouxia* diversity detected by Sanger and 454-pyrosequencing in the crustose lichen species composed of *Diploschistes diacapsis* (DD), *Acarospora nodulosa* (AN), *Acarospora placodiiformis* (AP), *Rhizocarpon malenconianum* (RM) and *Diplotomma rivas-martinezii* (DRM). *Trebouxia* species detected are indicated as black, dark grey, grey and light grey

To try to relate the molecular and ultrastructural information [53], the phycobiont nrITS from the five specimens selected for TEM was analyzed by Sanger sequencing. DD, AN, RM and DRM showed coexistence, and only AP showed a unique sequence which matched with *T. cretacea* (included in the phylogeny: S6). TEM analyses of phycobionts based on the ultrastructure of pyrenoids (Py) and plastids distinguished two mainly *Trebouxia* morphotypes (Fig. 4). Morphological characteristics of each morphotype in detail can be seen in S7 and S8, and more cells of each type in S9. One morphotype found in DD, AP and RM cells (Fig. 4A) showed a single central Py related to the gigantea-type described by Friedl [71], with pyrenoglobuli (Pg) uniformly distributed within the Py matrix, and a dense thylakoid membrane disposition. The second morphotype found in DD, AN and DRM showed the same Py type, but with a lax: thylakoid membrane disposition (Fig. 4B). Only in DD did we find both morphotypes and a few cells together, showing a Py related to the gelatinosa-type (Fig. 4C).

Mycobiont barcoding identification, nucleotide diversity and haplotype networks

Mycobiont barcoding of each specimen included in the study was analyzed to validate the correct lichen identification. Independent multiple alignments were performed, including the new mycobiont sequences generated in this study and selected sequences from previously described phylogenies [72-75] (S10, S11, S12 and S13). The dataset of DD from both locations linked in a clade (100/100) with three sequences deposited in the GenBank from Fernandez-Brime et al. [72] (S10). AN and AP sequences from both locations also matched into a well-supported clade (100/100 and 98/97), including previous sequences from these two lichen species [73] (S11), and RM from FU and TI appeared as a new well-supported clade (100/100) in the previously published phylogeny from this genus [74] (S12). The new DRM sequences from FU matched into a well-supported clade (100/96), including the DRM sequences downloaded from Molina et al. [75] (S13). Nucleotide diversity π ranged between 0 in RM and DRM and 0.00505 in DD for the mycobiont (Table 3).

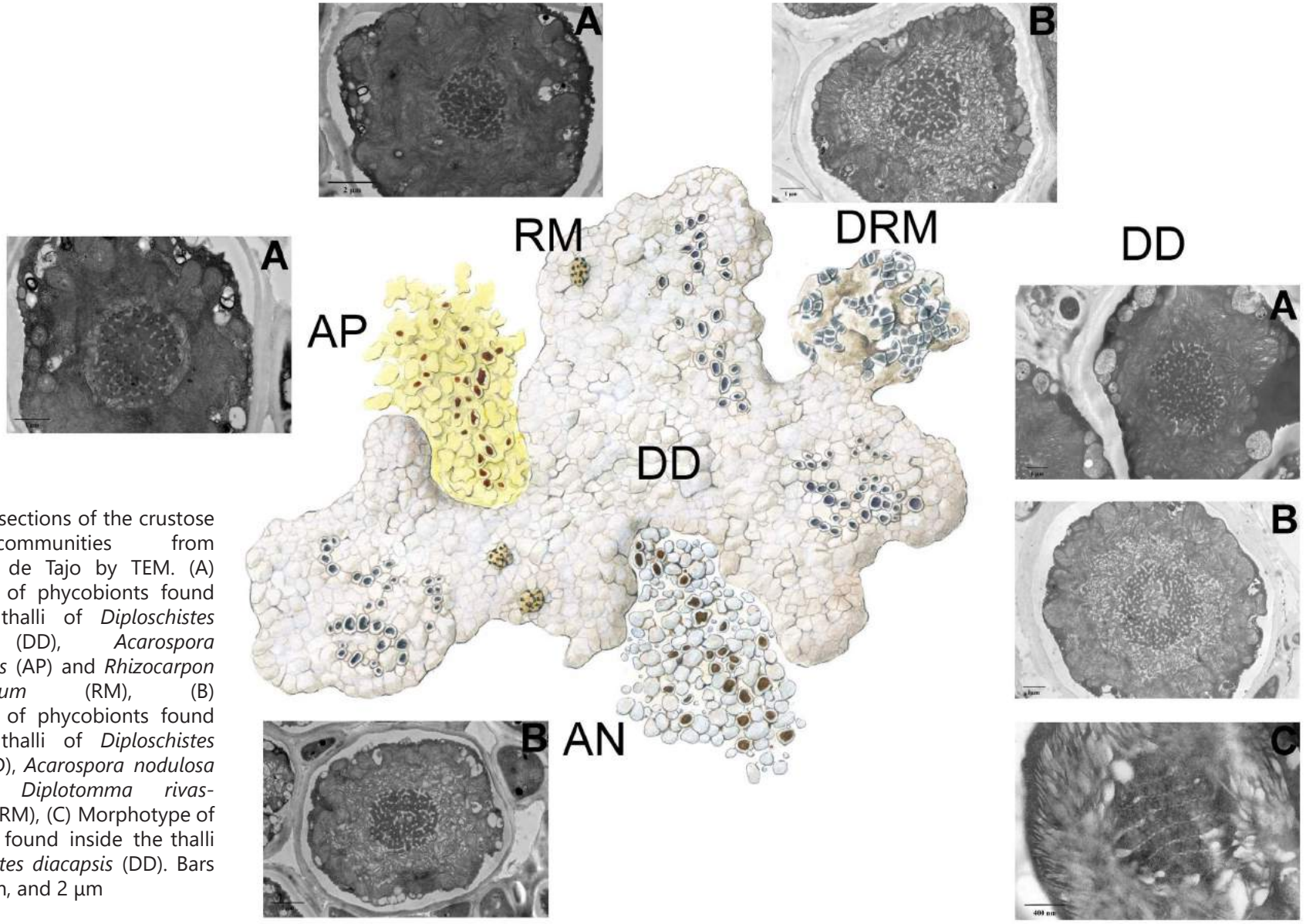

Fig. 4 Cross sections of the crustose lichen communities from Fuentidueña de Tajo by TEM. (A) Morphotype of phycobionts found inside the thalli of *Diploschistes diacapsis* (DD), *Acarospora placodiiformis* (AP) and *Rhizocarpon malenconianum* (RM), (B) Morphotype of phycobionts found inside the thalli of *Diploschistes diacapsis* (DD), *Acarospora nodulosa* (AN) and *Diplotomma rivas-martinezii* (DRM), (C) Morphotype of phycobionts found inside the thalli of *Diploschistes diacapsis* (DD). Bars 400 nm, 1 µm, and 2 µm

RM and DRM were excluded from the analyses because no haplotype differences were detected. Genetic relationships of mycobiont sequences of DD, AN and AP were analyzed by statistical parsimony networks of the nrITS (S14). Specimens of all the lichen species studied growing in FU or TI with Miocene gypsum bedrock appeared randomly distributed.

	π	SD
DD	0,00505	0,00051
AN	0,00182	0,0003
AP	0,00046	0,00011
RM	0	0
DRM	0	0

Table 3 Mycobiont nucleotide diversity (π) of D*iploschistes diacapsis* (DD), *Acarospora nodulosa* (AN), *Acarospora placodiiformis* (AP), *Rhizocarpon malenconianum* (RM) and *Diplotomma rivas-martinezii* (DRM) with standard deviation (SD)

Discussion

The present study contributes to complete our understanding of the crustose lichen species growing in gypsum outcrops in the Central Iberian Peninsula, and allows us to obtain an overview of the symbiotic patterns and the factors that determine these relationships in the whole community. This study is complementary with Moya et al. [38, 39], where microalgae diversity in these biocrusts were analysed in the foliose and dimorphic *Cladonia* spp. and in the squamulose *Psora decipiens*, *P. saviczii*, *Clavascidium* spp. and *Placidium* spp. In addition, due to the peculiar life style of the selected crustose species we can implement some interesting analysis focused on phycobiont coexistence, diversity and switching.

Pedological analysis of BSC from Polar Regions [76, 77] showed no significant correlation with these chemical soil parameters and species composition, moreover a statistically significant influence of precipitation on species composition was determined. In Polar Regions, the soil organic matter content ranged from the level of mineral soils in the BSCs (calculated soil organic matter content: <3%) to the level of peaty soils (>50% soil organic matter). Meanwhile the gyspum biocrusts included in this study showed similar values to the mineral soils. Schulz et al. [78] reported the total phosphorous content as the main driver for the BSC microalgal species composition collected from coastal dunes of the Baltic Sea,

BSC microalgal species composition collected from coastal dunes of the Baltic Sea, but FU and TI biocrusts showed extremely low values of phosphorous in the micro and macro element analyses.

However, at smaller local, intra-site, or microscales, gypsum biocrust could be tightly coupled to a relatively narrow range of the soils physical and chemical properties, often in idiosyncratic ways. Gypsum, calcite, sheet silicates and feldspars are usual constituents of the gypsiferous rocks from FU and TI. High content of oxalic acid (more than 66% dry weight) has been reported in lichen thalli [79], which may act as an effective "solvent" of clay materials and iron-oxides and react with mineral constituents of the substrate to form weddellite (COD) or whewellite (COM) [80]. The presence of COM and/or COD detected in our samples could be associated with organic rims, and they have been previously reported as products of biomineralization [81]. There is evidence of their presence on the surface of foliose lichens [82], on lichen biocrusts and in a wide variety of rock substrates [83]. Macro and micro element patterns showed a significant presence of Fe and Sr in both localities, these elements could be influencing both the microalgae *pool* and the presence of specific lichen species [84-92]. Similarly, Fe, Mg, and Ca have been shown to be positively associated with lichen cover, while Mn, and sometimes Zn, have been negatively associated with lichen diversity [92]. Other elements such as Cd, Li, Cu, Mo, and Sr are known to negatively affect either the abundance or diversity of algae [93]. It was also suggested that the substrate determines the sets of cyanobionts available for sharing among mycobionts of terrestrial and epiphytic lichen guilds [40]. In the present study, the presence of *A. mediterranea*, *M. israeliensis* and the *Trebouxia* guild found could not be related with the substrate type, due to its presence in gypsum, calcareous, siliceous and also volcanic areas. However, due to possible implications in phycobiont availability we consider that the analysis of gypsum soil chemical properties should not be avoided when lichen microalgae diversity is investigated.

For decades, studies dealing with phycobiont diversity in lichen thalli were primarily based on Sanger sequencing. In recent years, metabarcoding analyses have uncovered a huge species diversity associated with lichen symbioses [94-97] because HTS techniques allow for the detection of a vast number of genotypes that would otherwise remain undetected using conventional PCR amplifications [98].

In fact, the discovery of this phycobiont multiplicity within a single lichen thallus [29, 32, 53] and the exclusive use of Sanger sequencing in the majority of studies, pointed out to the lichenologist community to consider if terms such as specificity and selectivity related to symbiotic association patterns introduced years ago, should be revised under this new perspective. In Paul et al. [99], the potential of Sanger sequencing and HTS metabarcoding to infer photobiont diversity were compared in *Lasallia hispanica* and *L. pustulata*. In this work, they assumed that in the majority of studies focused on microalgae diversity, researchers only detected a single species of photobiont based on unambiguous Sanger sequence electrophoretograms. Nevertheless, several authors specify that electrophoretograms showing double peaks, or polymorphic sequences, were removed from the analyses [31, 35, 55]. These removed sequences constituted the without considered percentage of coexistence detected.

In the present study, all the species selected showed coexistence (double peaks in the electrophoretograms) ranging from 33% in *Rhizocarpon malenconianum* to 72% in the case of *Acarospora nodulosa* and *Diplotomma rivas-martinezii* (Fig. 2). Moreover, Chiva et al. [100] detected in the crustose *Buellia zoharyi* growing in 11 locations including FU and TI, three *Trebouxia* spp. (*T. cretacea*, *T. asymmetrica* and *Trebouxia* sp. OTU A25) and the coexistence of these *Trebouxia* species was detected in 75% of the specimens analysed. High coexistence percentage (up to 100%) was also detected by Sanger sequencing in vagrant and semi vagrant *Circinaria* spp. growing in very continental areas in the Iberian Peninsula [37]. Also, in the Canarian lichen *Parmotrema pseudotinctorum* coexistence of *Asterochloris* [101] and *Trebouxia crespoana* [102] was detected. Moya et al [28] and Català et al. [29] validated phycobiont multiplicity inside *Ramalina farinacea* and *R. fraxinea*, respectively. Muggia et al. [32, 103] also showed this promiscuity in saxicolous *Protoparmeliopsis muralis* and *Tephromela atra*.

The ability of Sanger sequencing to address a wide variety of questions in lichen ecology and evolution is unquestionable, but it is important to point out that phycobiont coexistence could vary depending on the lichen species, and could even be the general rule in some lichen species. We hypothesized that coexistence could be related or favored with thallus architecture, development and/or life style, and these factors should be considered when studies about patterns of fungal-algal associations are carried out.

As mentioned, microalgae diversity was analysed in the foliose and dimorphic *Cladonia* spp. and in the squamulose *Psora decipiens*, *P. saviczii*, *Clavascidium* spp. and *Placidium* spp. [38, 39]. In terms of local scale specificity, all these species could be considered specialists, in contrast, the crustose portion included in this study are associated with four different *Trebouxia* species with little or no apparent selectivity (generalists). As mentioned in Muggia et al. [104], 'specificity' was initially defined as the possible taxonomic range of acceptable partners, in contrast to 'selectivity', which indicated the frequency of association between compatible partners [105-108]. Currently, 'selectivity' and 'specificity' may also be regarded as multidirectional in terms of characterizing relationships among lichen symbionts.

Reproductive and dispersal strategies are the key factors shaping photobiont diversity. Recently Steinová et al. [108], showed that asexually reproducing *Cladonia* are highly specific to their phycobionts, associating with only two closely related *Asterochloris* species. In contrast, sexually reproducing lichens associated with seven unrelated *Asterochloris* lineages, thus being photobiont generalists. The reproductive mode had the largest explanatory power, explaining 44% of total photobiont variability. Crustose lichen species included in this study, both preferential or exclusive gypsophyte, showed a mixture of dispersal and reproductive strategies; thallus fragmentation, sexual and asexual spores which require re-synthesis of symbiosis *de novo*, and co-propagation of symbiotic patterns. Thus establishing that the implication of these factors in the symbiotic patterns could be risky in these species.

Some parasitic lichens acquire their compatible photobiont by theft from the host lichen, others associate with a different algal partner. Parasitic relationships in lichens were traditionally documented with two specific cases: a) the case of *Cladonia* spp. and its lichenicolous lichen *Diploschistes muscorum*. Ascospores of this crustose, parasitic lichen germinate on thallus squamules or podetia of diverse *Cladonia* spp. and associate with their microalgae, *Asterochloris irregularis*, which is later replaced by *Trebouxia showmanii* [71], if available; and b) ascospores of *Gyalolechia bracteata* which parasitizes the thallus surface of *Thalloidima sedifolium* in order to take over the host phycobiont which they can then use in the development of a new thallus of *G. bracteata* [23].

Parasitic lichen species such as *A. nodulosa*, *A. placodiiformis* and *R. malenconianum* are suitable systems to analyze photobiont switching, as different algal genotypes/species are shared among different fungal genera [24] and set a challenge for concepts such as selectivity or specificity. The present study reveals a *Trebouxia* mediated guild [40] shared between these lichen species; *D. diacapsis* considered as the photobiont donor could harbor four all of these *Trebouxia* species which seem to be randomly transferred to *A. nodulosa*, *A. placodiiformis* and *R. malenconianum*. In *Diplotomma rivas-martinezii* we only detected two *Trebouxia*, this decrease in phycobiont diversity could be influenced by its different life style with respect to *D. diacapsis* (epilithic not parasite), or by the low number of specimens analyzed. Photobiont switching seems to be a ubiquitous phenomenon in lichens, and appears to play a vital role in lichen adaptation to environment [53, 103, 109-113]. Associating with a wide range of symbionts may help species survive in harsh environmental conditions [114-116] such as gypsum biocrusts.

In many organisms, the genetic diversity of populations has been related with the capacity to adapt to different environments and face possible long-term changes [117-119]. Combining the data obtained in this study with those obtained in Moya et al. [38, 39] and Chiva et al. [120], we tried to analyze the relationship between genetic diversity and substrate preference from nine lichen species showing varied distribution and different soil requirements (S15). Cosmopolite species, such as *C. foliacea* or *P. decipiens*, showed high nucleotide variability in comparison with other lichens with preferential gypsophyte characteristics, such as *A. nodulosa*, *D. diacapsis*, *P. saviczii* and *B. zoharyi* (S15). These species present their optimum coverage and frequency on gypsum soils, although they may colonize other substrates in restricted areas. *A. placodiiformis* and *D. rivas-martinezii* are exclusive gypsophytes; they only colonize gypsiferous substrates [75, 121] and showed less genetic diversity (S15). These data underline a possible relationship between nucleotide variability and substrate preference, since lichen species with less preference for gypsum showed greater genetic variability and vice versa. Although studies on the genetic diversity of mycobiont/phycobionts are relatively frequent [122, 123], studies of these relationships in gypsum lichens are scarce. Domaschke et al. [122] revealed a decrease in genetic diversity in

isolated populations of *Cetraria aculeata* from Antarctica, probably due to a founding effect during long-distance colonization. Recently, Beck et al. [123] also analyzed Antarctic populations of Placopsis spp. and determined that phycobiont haplotypes showed different environmental preferences, possibly related to their ability to adapt to changing and/or extreme environmental conditions. These data could be extrapolated to other isolated populations, such as those that occur in gypsum biocrusts. In general, the loss of genetic diversity tends to reduce the potential for adaptation of populations and increases the risk of extinction. The risk of extinction, therefore, seems to increase when species are under greater environmental stress [124, 125], such as hyperspecificity by a single type of substrate such as gypsum soils.

Lichen morphology and development have usually been studied under a descriptive or ecophysiological point of view [126-129]. However, Souza-Egipsy et al. [130, 131] analyzed the ultrastructural and biogeochemical features of different substrates (gypsum, volcanic and sedimentary rock) to explore the relationships between lichen symbiosis and the lichen-soil interface, and revealed that thallus morphology is a key character to condition contact with the surface. Our results evidence a clear correlation between symbiotic patterns and life architecture, and we suggest the morphology and/or growth type could be influencing the selection of each mycobiont for a particular microalgal genus. Therefore, three microalgae genera are available in the same area, but a multidirectional selection is performed by the mycobiont/phycobionts and probably influenced by each characteristic lichen growth type.

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Supporting information

Symbiont interaction patterns in biocrust lichen communities located in semi-arid gypsum outcrops in the Central Iberian Peninsula

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S1) Phycobiont nrITS GenBank accession number for specimens in this study

Sample Code	Locality	Accesion Number
DRM 4 FU	Fuentidueña de Tajo	MN517147
DRM 6 FU	Fuentidueña de Tajo	MN517148
RM 1 FU	Fuentidueña de Tajo	MN517149
RM 2 FU	Fuentidueña de Tajo	MN517150
RM 3 FU	Fuentidueña de Tajo	MN517151
RM 1 TI	Titulcia	MN517152
DD 5 FU	Fuentidueña de Tajo	MN517153
DD 8 FU	Fuentidueña de Tajo	MN517154
DD 10 FU	Fuentidueña de Tajo	MN517155
DD 11 FU	Fuentidueña de Tajo	MN517156
DD 15 FU	Fuentidueña de Tajo	MN517157
DD 22 FU	Fuentidueña de Tajo	MN517158
DD 23 FU	Fuentidueña de Tajo	MN517159
DD 25 FU	Fuentidueña de Tajo	MN517160
DD 26 FU	Fuentidueña de Tajo	MN517161
DD 2 TI	Titulcia	MN517162
DD 4 TI	Titulcia	MN517163
DD 5 TI	Titulcia	MN517164
DD 9 TI	Titulcia	MN517165
DD 10 TI	Titulcia	MN517166
DD 14 TI	Titulcia	MN517167
DD 17 TI	Titulcia	MN517168
DD 18 TI	Titulcia	MN517169
DD 20 TI	Titulcia	MN517170
AN 8 FU	Fuentidueña de Tajo	MN517171
AN 10 FU	Fuentidueña de Tajo	MN517172
AN 14 FU	Fuentidueña de Tajo	MN517173
AN 15 FU	Fuentidueña de Tajo	MN517174
AN 20 FU	Fuentidueña de Tajo	MN517175
AN 23 FU	Fuentidueña de Tajo	MN517176
AN 1 TI	Titulcia	MN517177
AN 2 TI	Titulcia	MN517178
AN 4 TI	Titulcia	MN517179
AN 14 TI	Titulcia	MN517180
AN 16 TI	Titulcia	MN517181
AN 17 TI	Titulcia	MN517182

Sample Code	Locality	Accesion Number
AP 6 FU	Fuentidueña de Tajo	MN517183
AP 11 FU	Fuentidueña de Tajo	MN517184
AP 12 FU	Fuentidueña de Tajo	MN517185
AP 14 FU	Fuentidueña de Tajo	MN517186
AP 15 FU	Fuentidueña de Tajo	MN517187
AP 20 FU	Fuentidueña de Tajo	MN517188
AP 21 FU	Fuentidueña de Tajo	MN517189
AP 22 FU	Fuentidueña de Tajo	MN517190
AP 23 FU	Fuentidueña de Tajo	MN517191
AP 24 FU	Fuentidueña de Tajo	MN517192
AP FU TEM	Fuentidueña de Tajo	MN517193
AP 1 TI	Titulcia	MN517194
AP 2 TI	Titulcia	MN517195
AP 4 TI	Titulcia	MN517196
AP 6 TI	Titulcia	MN517197
AP 7 TI	Titulcia	MN517198
AP 10 TI	Titulcia	MN517199
AP 11 TI	Titulcia	MN517200
AP 12 TI	Titulcia	MN517201
AP 14 TI	Titulcia	MN517202
AP 15 TI	Titulcia	MN517203
AP 16 TI	Titulcia	MN517204
AP 17 TI	Titulcia	MN517205
AP 18 TI	Titulcia	MN517206
AP 20 TI	Titulcia	MN517207
AP 22 TI	Titulcia	MN517208
AP 23 TI	Titulcia	MN517209
AP 24 TI	Titulcia	MN517210

S2) Mycobiont nrITS GenBank accession number for specimens in this study

Sample Code	Locality	Accesion Number
RM 1 FU	Fuentidueña de Tajo	MN510996
RM 2 FU	Fuentidueña de Tajo	MN510998
RM 3 FU	Fuentidueña de Tajo	MN510999
RM 4 FU	Fuentidueña de Tajo	MN511000
RM 454 FU	Fuentidueña de Tajo	MN510997
RM FU TEM	Fuentidueña de Tajo	MN511000
RM 1 TI	Titulcia	MN511002
DRM 1 FU	Fuentidueña de Tajo	MN509005
DRM 2 FU	Fuentidueña de Tajo	MN509006
DRM 3 FU	Fuentidueña de Tajo	MN509007
DRM 4 FU	Fuentidueña de Tajo	MN509008
DRM 5 FU	Fuentidueña de Tajo	MN509009
DRM 6 FU	Fuentidueña de Tajo	MN509010
DRM FU TEM	Fuentidueña de Tajo	MN509011
DD 1 FU	Fuentidueña de Tajo	MN511089
DD 2 FU	Fuentidueña de Tajo	MN511090
DD 3 FU	Fuentidueña de Tajo	MN511091
DD 4 FU	Fuentidueña de Tajo	MN511092
DD 5 FU	Fuentidueña de Tajo	MN511093
DD 6 FU	Fuentidueña de Tajo	MN511094
DD 7 FU	Fuentidueña de Tajo	MN511095
DD 8 FU	Fuentidueña de Tajo	MN511096
DD 9 FU	Fuentidueña de Tajo	MN511097
DD 10 FU	Fuentidueña de Tajo	MN511098

Sample Code	Locality	Accesion Number
AN 1 FU	Fuentidueña de Tajo	MN511023
AN 2 FU	Fuentidueña de Tajo	MN511024
AN 3 FU	Fuentidueña de Tajo	MN511025
AN 4 FU	Fuentidueña de Tajo	MN511026
AN 5 FU	Fuentidueña de Tajo	MN511027
AN 6 FU	Fuentidueña de Tajo	MN511028
AN 7 FU	Fuentidueña de Tajo	MN511029
AN 8 FU	Fuentidueña de Tajo	MN511030
AN 9 FU	Fuentidueña de Tajo	MN511031
AN 10 FU	Fuentidueña de Tajo	MN511032
AN 11 FU	Fuentidueña de Tajo	MN511033
AN 12 FU	Fuentidueña de Tajo	MN511034
AN 13 FU	Fuentidueña de Tajo	MN511035
AN 14 FU	Fuentidueña de Tajo	MN511036
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AN 16 FU	Fuentidueña de Tajo	MN511038
AN 17 FU	Fuentidueña de Tajo	MN511039
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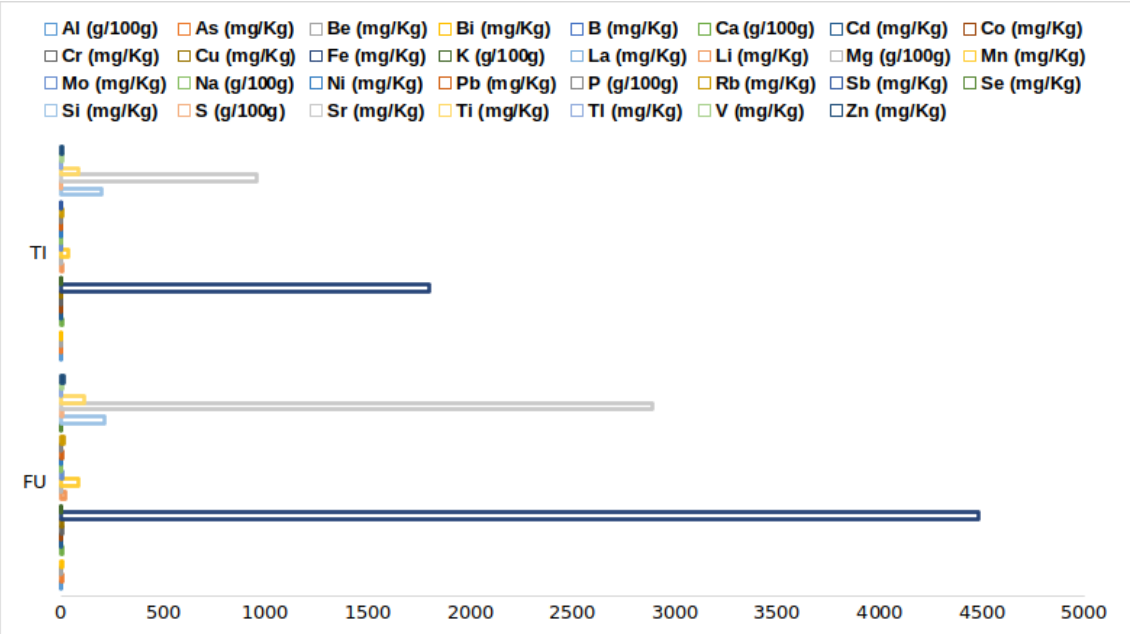
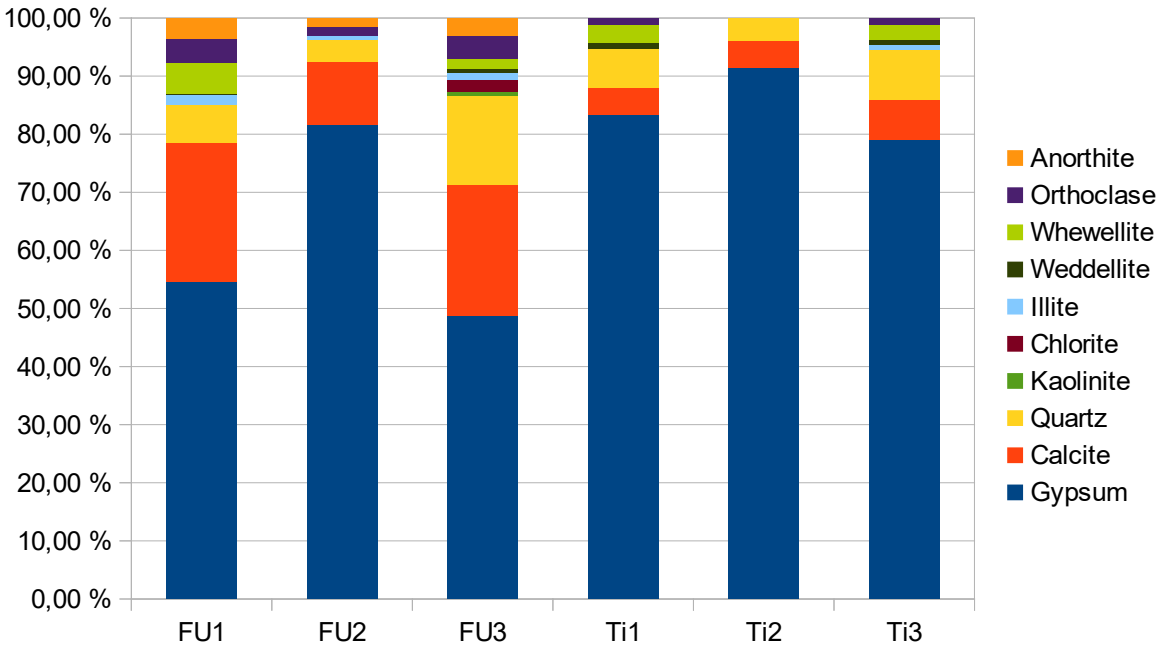
AN 19 FU	Fuentidueña de Tajo	MN511041
AN 20 FU	Fuentidueña de Tajo	MN511042
AN 21 FU	Fuentidueña de Tajo	MN511043
AN 22 FU	Fuentidueña de Tajo	MN511044
AN 23 FU	Fuentidueña de Tajo	MN511045
AN 24 FU	Fuentidueña de Tajo	MN511046

S3) Concentration (g/100g) and (mg/Kg) of micro and macro elements detected in soil samples collected in Fuentidueña de Tajo (FU) and Titulcia (TI) by ICP OES



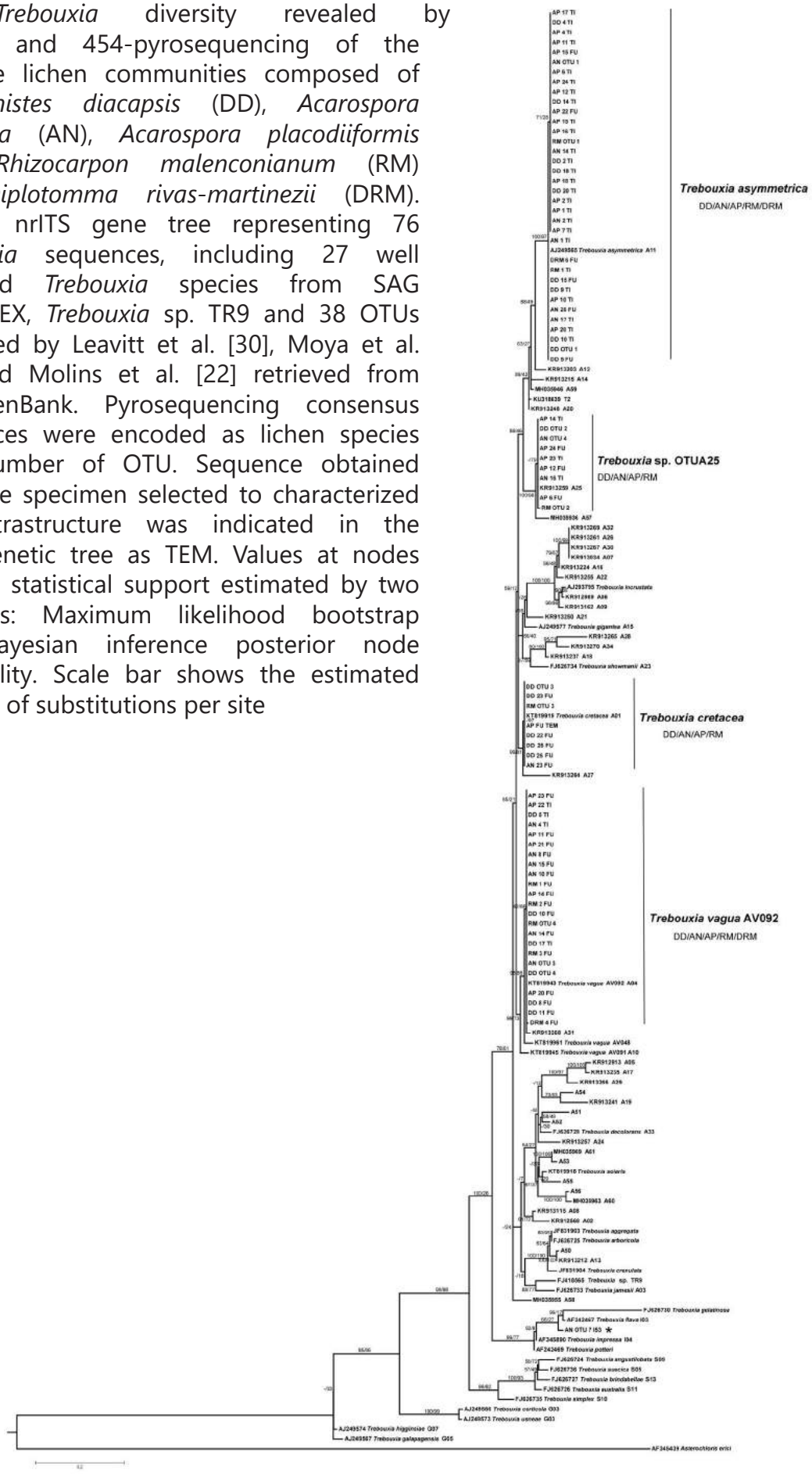
S4) Mineralogical compositions distribution of soil samples from Fuentidueña de Tajo (FU) and Titulcia (TI) performed by X Ray powder diffraction



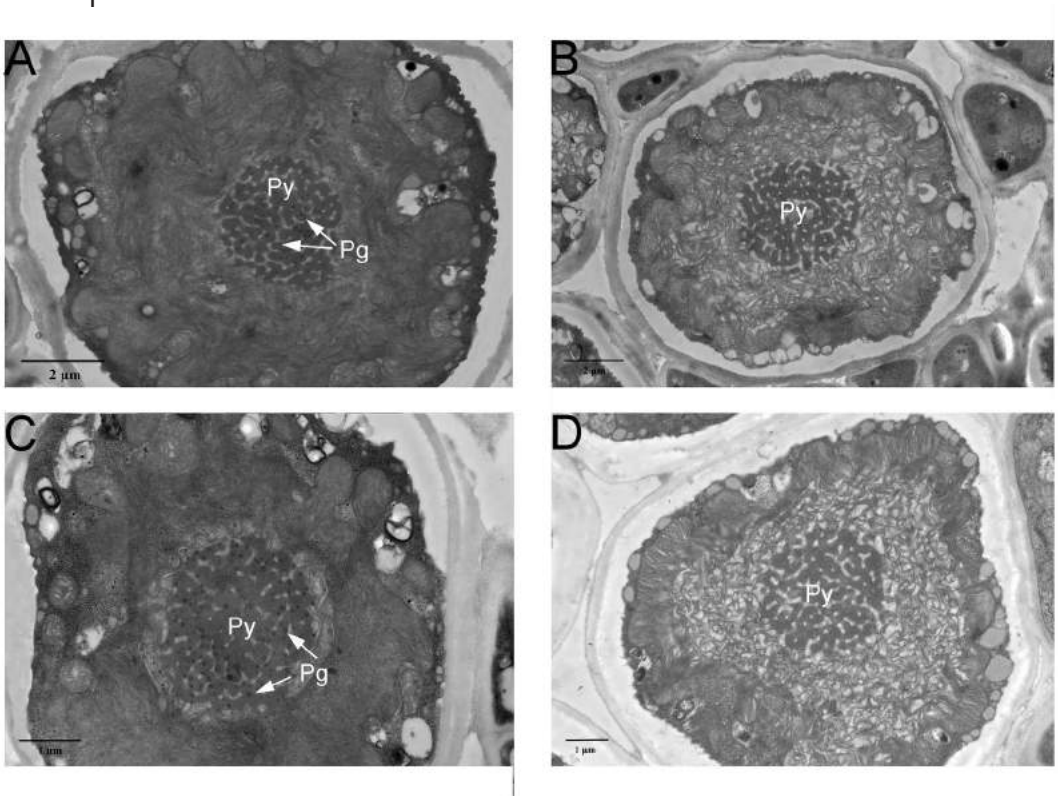
S5) The semi-quantitative mineralogical compositions of soil samples from Fuentidueña de Tajo (FU) and Titulcia (TI) performed by X Ray powder diffraction. ICDD file: Number file of ICDD data base. I/Ic: Reference intensity of compound. SQ: Quantitative estimation (%). RE: Relative error

Sample				FU1	FU2	FU3	TI1	TI2	TI3	
ICDD file	Compound name	Formula	I/Ic	SQ	SQ	SQ	SQ	SQ	SQ	RE
00-036-0432	Gypsum	$CaSO_4{\cdot}2H_2O$	1,7	54,6	81,5	70,7	83,4	91,6	78,9	0,1
00-005-0586	Calcite, syn	$CaCO_3$	2,0	23,9	10,8	17,3	4,6	4,7	7,0	0,1
00-033-1161	Quartz, syn	SiO_2	3,6	6,6	3,9	6,2	6,6	3,8	8,6	0,1
01-083-0971	Kaolinite	$Al_2(Si_2O_5)(OH)_4$	1,0	0,0	0,0	0,0	0,0	0,0	0,0	0,5
01-078-1997	Chlorite	$(Mg4.715Al.694Fe.269Fe.109Cr.128Ni.011)(Si3.056Al.944)O_{10}(OH)_8$	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,5
00-002-0056	Illite	$KAl_2Si_3Al_4O_{10}\ (OH)_2$	2,0	1,6	0,6	0,6	0,0	0,0	0,8	0,5
01-075-1314	Weddellite (COD)	CaC_2O_4 (H2O) 2.375	1,4	0,3	0,0	0,1	1,2	0,0	0,9	0,5
01-075-1313	Whewellite (COM)	CaC_2O_4 (H_2O)	1,1	5,3	0,0	0,7	3,0	0,0	2,6	0,5
01-075-1592	Orthoclase	$KAlSi_3O_8$	0,8	4,2	1,6	2,0	1,2	0,0	1,1	0,5
01-078-2330	Anorthite	Na.25Ca.71 ($Al_2Si_2O_8$)	0,5	3,5	1,5	2,3	0,0	0,0	0,0	0,5

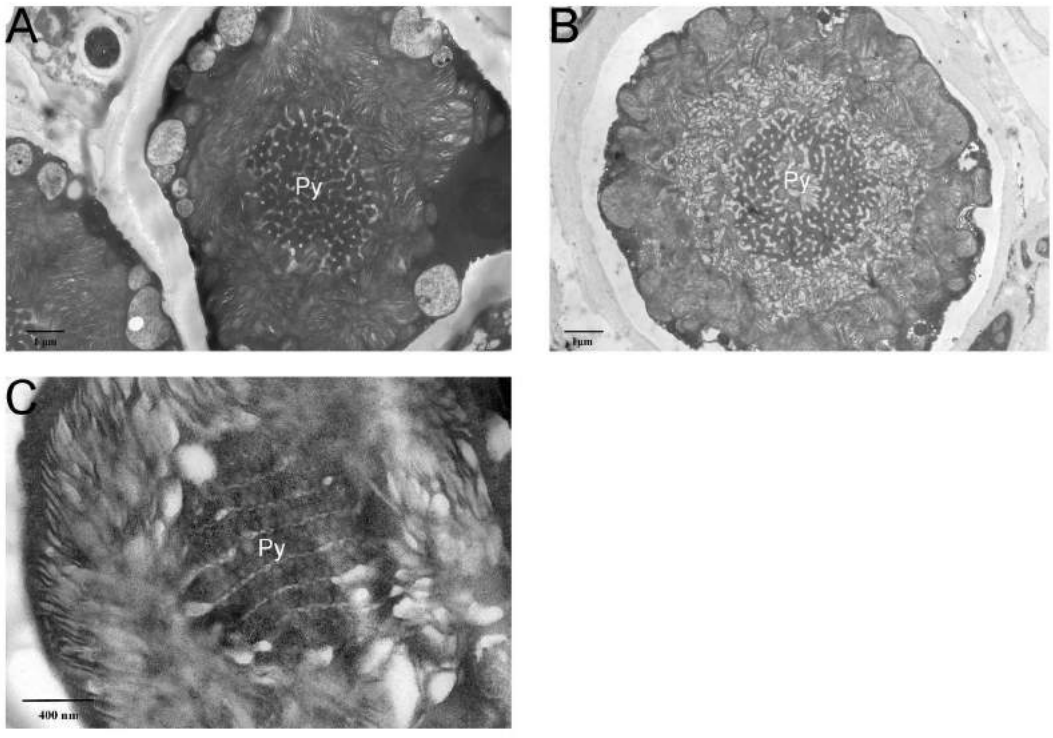
S6) *Trebouxia* diversity revealed by Sanger and 454-pyrosequencing of the crustose lichen communities composed of *Diploschistes diacapsis* (DD), *Acarospora nodulosa* (AN), *Acarospora placodiiformis* (AP), *Rhizocarpon malenconianum* (RM) and *Diplotomma rivas-martinezii* (DRM). Rooted nrITS gene tree representing 76 *Trebouxia* sequences, including 27 well accepted *Trebouxia* species from SAG and UTEX, *Trebouxia* sp. TR9 and 38 OTUs described by Leavitt et al. [30], Moya et al. [14] and Molins et al. [22] retrieved from the GenBank. Pyrosequencing consensus sequences were encoded as lichen species code_number of OTU. Sequence obtained from the specimen selected to characterized the ultrastructure was indicated in the phylogenetic tree as TEM. Values at nodes indicate statistical support estimated by two methods: Maximum likelihood bootstrap and Bayesian inference posterior node probability. Scale bar shows the estimated number of substitutions per site



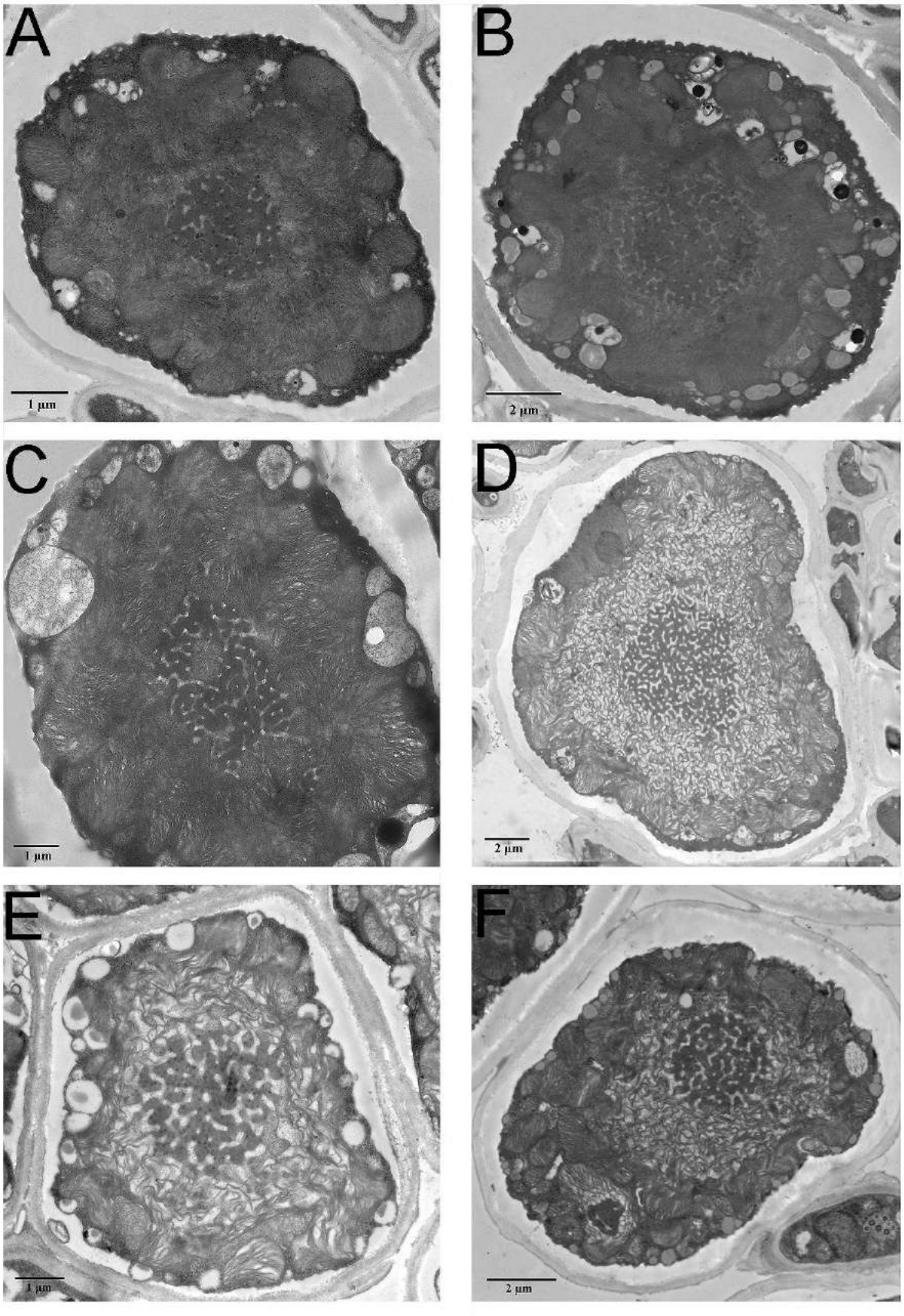
S7) Detail of pyrenoid. A) and C) morphotype A in *Rhizocarpon malenconianum* and *Acarospora placodiiformis*, B) and D) morphotype B in *Acarospora nodulosa* and *Diplotomma rivas-martinezii*. Abbreviations: Py, Pyrenoid; Pg, Pyrenoglobuli. Bars 1 µm and 2 µm

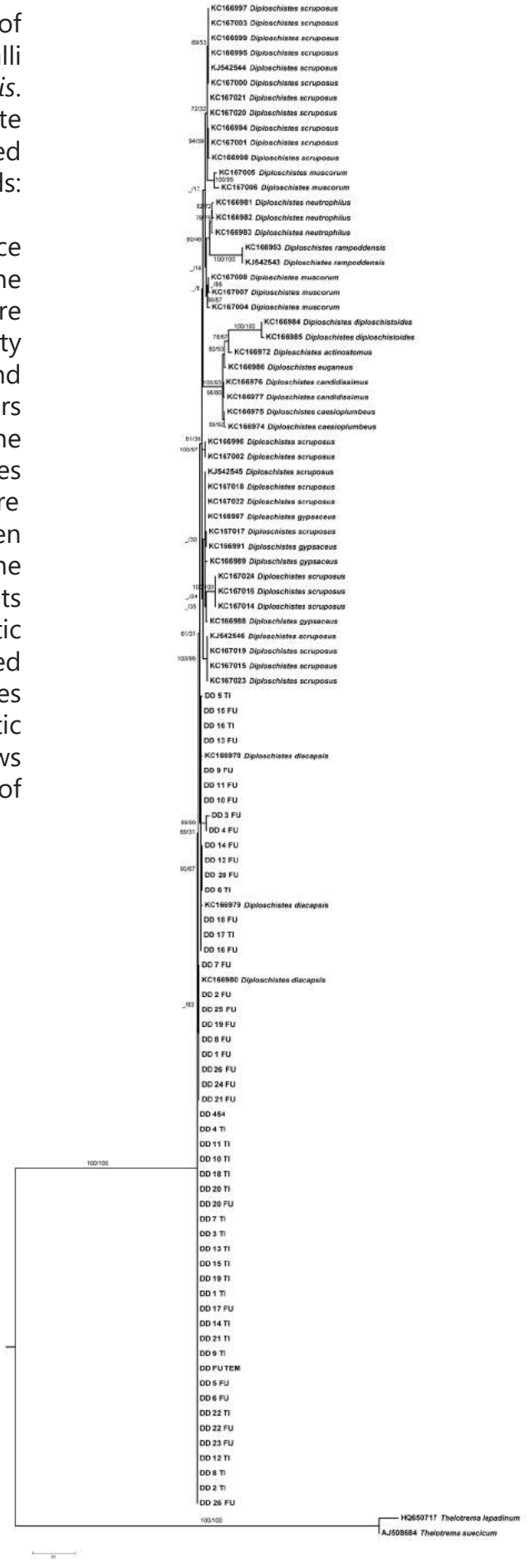


S8) Detail of pyrenoid from A) morphotype A, B) morphotype B and C) morphotype C found in D*iploschistes diacapsis*. Abbreviation: Py, Pyrenoid. Bars 400 nm and 1 µm



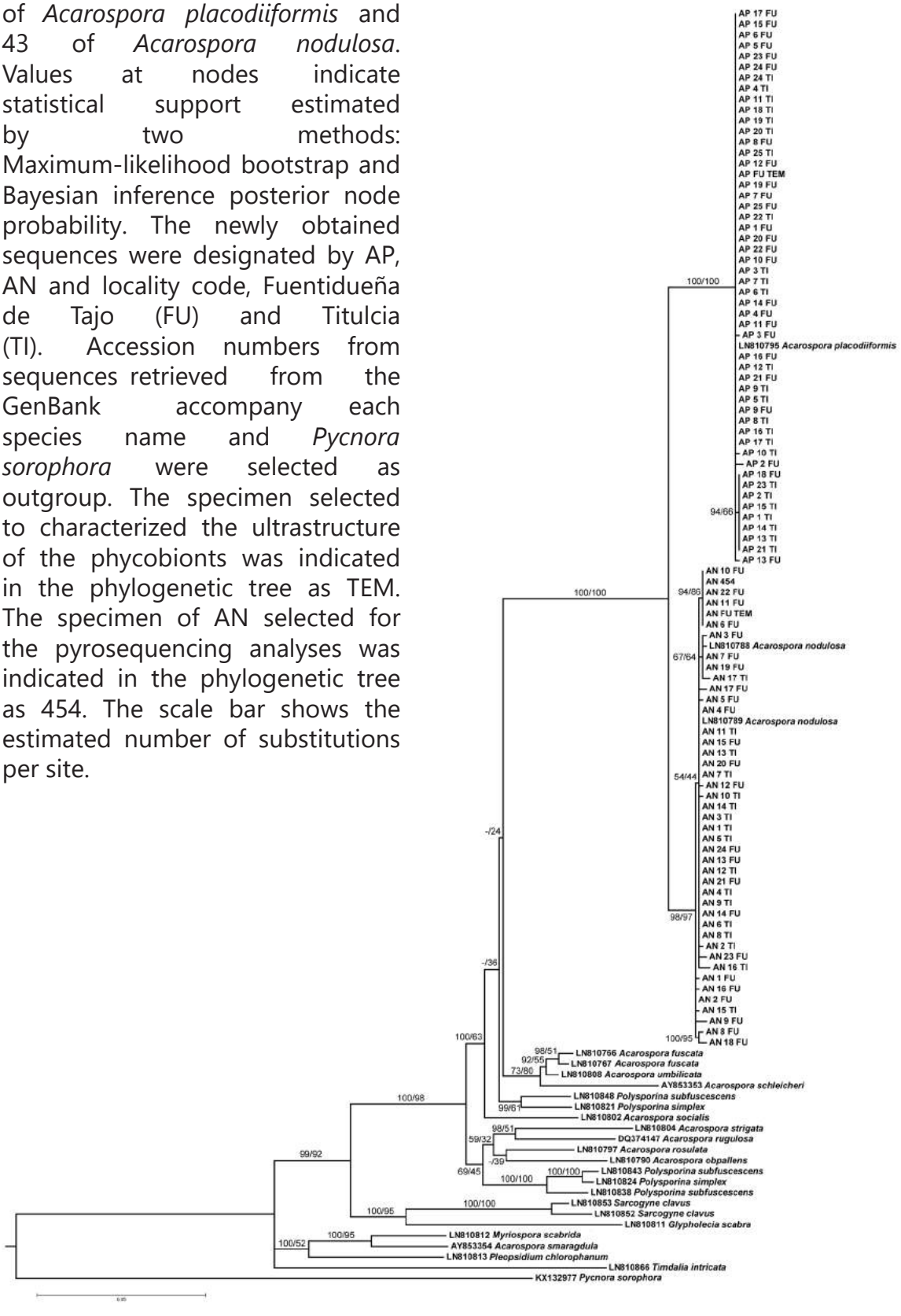
S9) Cross sections of morphotype A (A, B and C) and morphotype B (D, E and F). Bars 1 µm and 2 µm



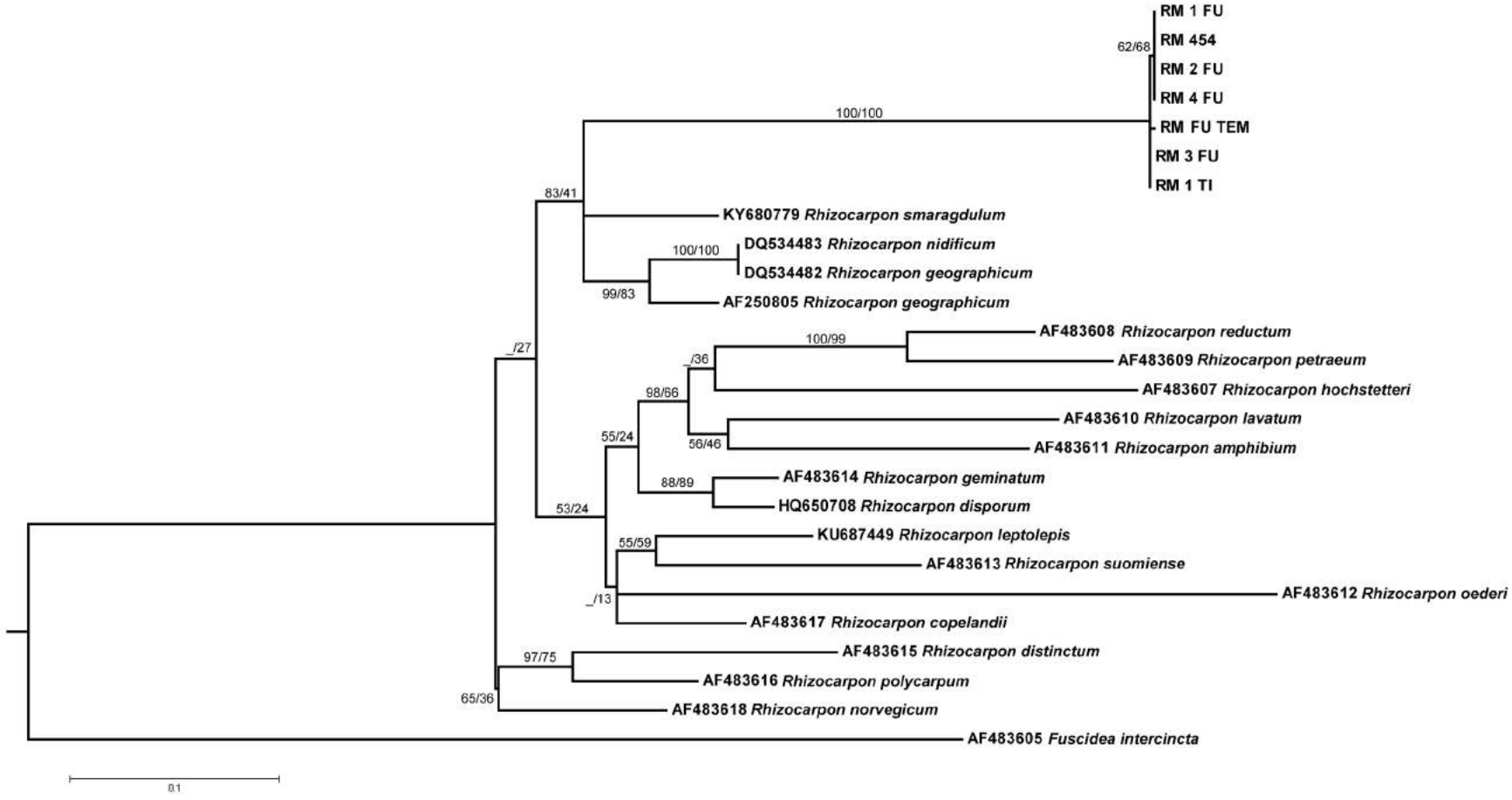


S10) Phylogenetic analyses of nrITS mycobiont from 51 thalli of *Diploschistes diacapsis*. Values at nodes indicate statistical support estimated by two methods: Maximum-likelihood bootstrap and Bayesian inference posterior node probability. The newly obtained sequences were designated by DD and locality code, Fuentidueña de Tajo (FU) and Titulcia (TI). Accession numbers from sequences retrieved from the GenBank accompany each species name and *Thelotrema* spp. were selected as outgroup. The specimen selected to characterized the ultrastructure of the phycobionts was indicated in the phylogenetic tree as TEM. The specimen selected for the pyrosequencing analyses was indicated in the phylogenetic tree as 454. The scale bar shows the estimated number of substitutions per site.

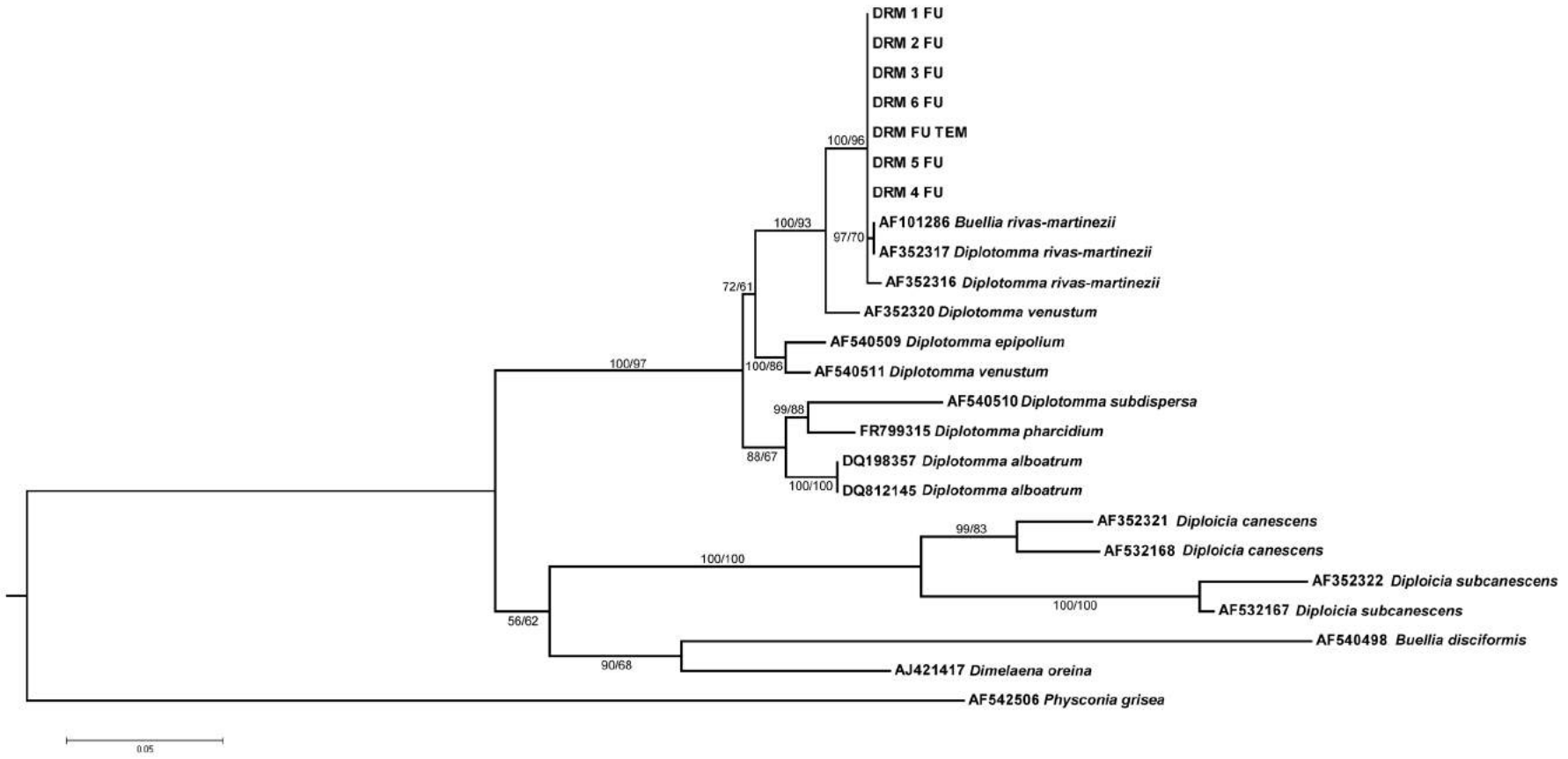
S11) Phylogenetic analyses of nrITS mycobiont from 51 thalli of *Acarospora placodiiformis* and 43 of *Acarospora nodulosa*. Values at nodes indicate statistical support estimated by two methods: Maximum-likelihood bootstrap and Bayesian inference posterior node probability. The newly obtained sequences were designated by AP, AN and locality code, Fuentidueña de Tajo (FU) and Titulcia (TI). Accession numbers from sequences retrieved from the GenBank accompany each species name and *Pycnora sorophora* were selected as outgroup. The specimen selected to characterized the ultrastructure of the phycobionts was indicated in the phylogenetic tree as TEM. The specimen of AN selected for the pyrosequencing analyses was indicated in the phylogenetic tree as 454. The scale bar shows the estimated number of substitutions per site.



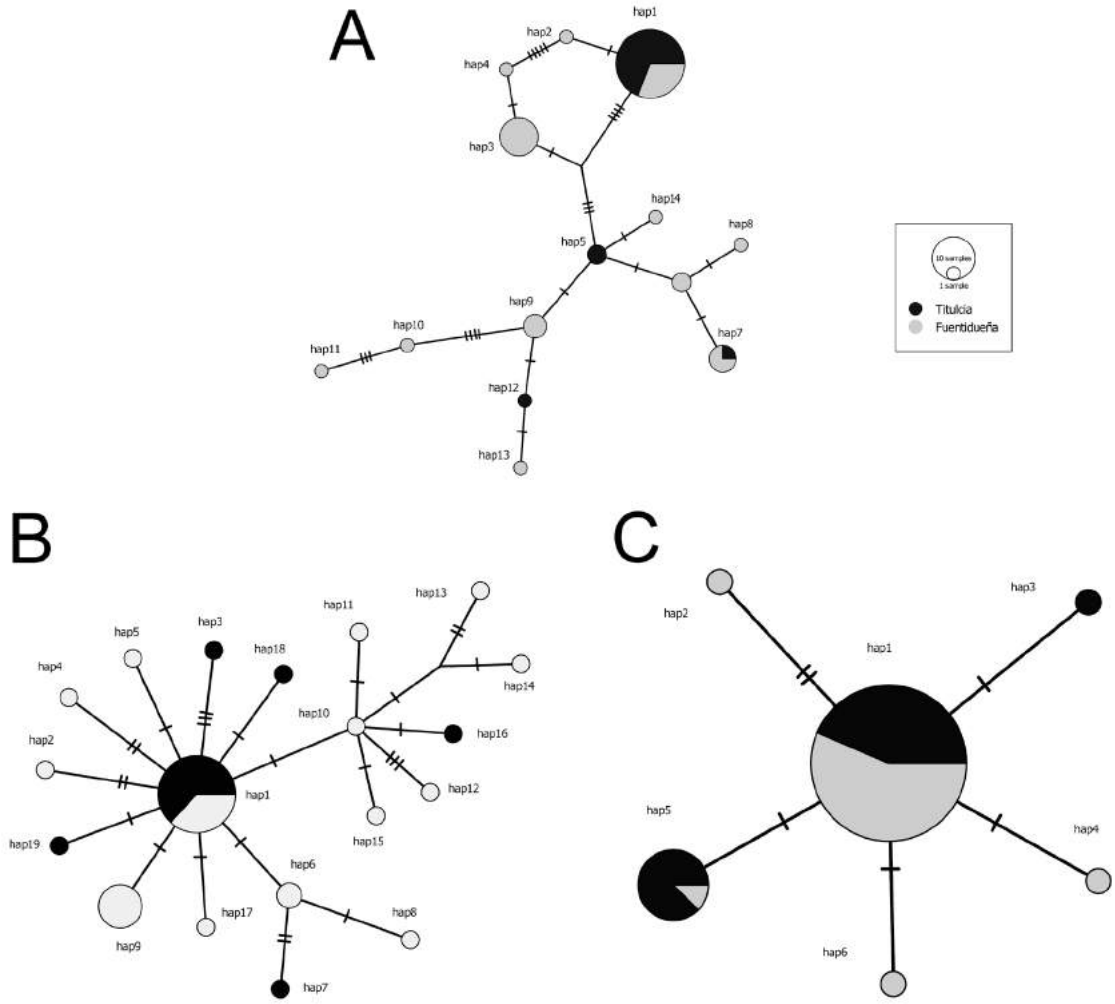
S12) Phylogenetic analyses of nrITS mycobiont from seven thalli of *Rhizocarpon malenconianum*. Values at nodes indicate statistical support estimated by two methods: Maximum-likelihood bootstrap and Bayesian inference posterior node probability. The newly obtained sequences were designated by RM and locality code, Fuentidueña de Tajo (FU) and Titulcia (TI). Accession numbers from *Rhizocarpon* spp. and *Fuscidea intercincta* sequences retrieved from the GenBank accompany each species name. The specimen selected to characterized the ultrastructure of the phycobionts was indicated in the phylogenetic tree as TEM. The specimen selected for the pyrosequencing analyses was indicated in the phylogenetic tree as 454. The scale bar shows the estimated number of substitutions per site.



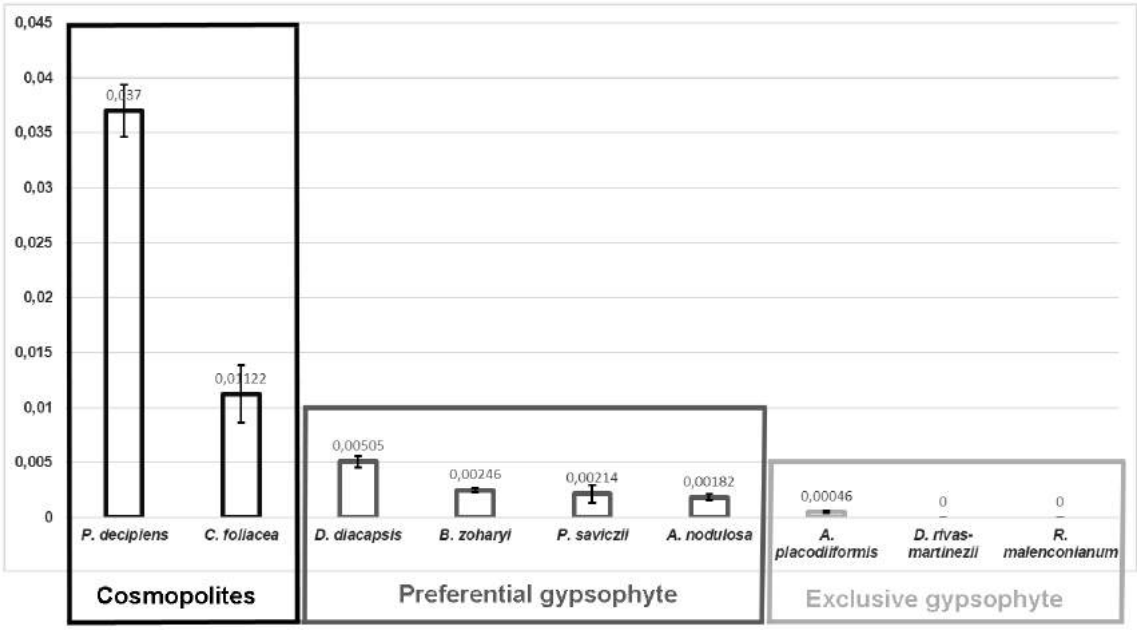
S13) Phylogenetic analyses of nrITS mycobiont from seven thalli of *Diplotomma rivas-martinezii*. Values at nodes indicate statistical support estimated by two methods: Maximum-likelihood bootstrap and Bayesian inference posterior node probability. The newly obtained sequences were designated by DRM and locality code, Fuentidueña de Tajo (FU). Accession numbers from *Diplotomma* spp., *Diploicia* spp., *Dimelaena* spp. and *Physconia grisea* sequences retrieved from the GenBank accompany each species name. The specimen selected to characterized the ultrastructure of the phycobionts was indicated in the phylogenetic tree as TEM. The scale bar shows the estimated number of substitutions per site.



S14) Statistical parsimony networks obtained for the nrITS of A) *Diploschistes diacapsis*, B) *Acarospora nodulosa* and C) *Acarospora placodiiformis*. The size of the circles is proportional to the frequency of each haplotype in the total sample. Each line in the network represents one mutational step. Grey circles represent haplotypes from Fuentidueña de Tajo and black circles from Titulcia.



S15) Bar charts of mycobiont nucleotide diversity (π) with standard deviation (SD) of cosmopolite lichen species underlined in black: *Psora decipiens* and *Cladonia foliacea*, preferential gypsophyte in drak grey: *Diploschistes diacapsis*, *Buellia zoharyi*, *Psora saviczii* and *Acarospora nodulosa* and exclusive gypsophyte in light grey: *Acarospora placodiiformis*, *Diplotomma rivas-martinezii* and *Rhizocarpon malenconianum*



3.4 How did terricolous fungi originate in the mediterranean region? A case study with a gypsicolous lichenized species (R 3.4)

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How did terricolous fungi originate in the Mediterranean region? A case study with a gypsicolous lichenized species

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Abstract

Aim: The historical causes responsible for the wide distribution of terricolous, crustose lichenized fungi across the Mediterranean Basin and the Canary Islands have never been explored. Here, we used the terricolous, circum-Mediterranean/Macaronesian species *Buellia zoharyi* (*Caliciaceae*, Ascomycota) to infer the time frame, and the climatic, geological and ecological factors influencing the origin and current spatial distribution of this species.

Location: Mediterranean Basin and Canary Islands.

Methods: Data from two nuclear markers (nrITS and *tef*1) obtained from 226 specimens of 23 populations covering the entire distribution range of *B. zoharyi* were used to calculate genetic diversity indices and haplotype networks and to investigate population size changes and structure. Three secondary calibrations were used to estimate the timing of the divergence of *B. zoharyi* from its hypothesized sister species, *B. elegans*, and the diversification of *B. zoharyi*.

Results: We found low nucleotide diversity and two geographically differentiated haplogroups, with a contact zone in the Iberian Peninsula. The three dating approaches established wide temporal windows for the divergence of *B. zoharyi* from *B. elegans* (Eocene-Pliocene) and its diversification (Miocene-Pleistocene). These intervals overlap with the origin and diversification ages found in other lichen-forming fungi and vascular plants inhabiting the Mediterranean region.

Main conclusions: In the context of lichen biogeography, our results support ecological specialization as well as geological and climatic events as drivers of the evolutionary history of *B. zoharyi* in the Mediterranean. In particular, the combined effects of the Messinian salinity crisis and the subsequent Zanclean Flood on the availability of gypsum soils in the Mediterranean Basin, as well as the Quaternary climatic oscillations, seem to have collectively shaped the amount and distribution of *B. zoharyi* population genetic diversity.

KEYWORDS
biogeography, *Buellia zoharyi*, ecological speciation, gypsum soils, Messinian salinity crisis, Zanclean Flood

1 | INTRODUCTION

There is a large gap in the knowledge of the phylogeographical patterns shown by cryptogams, in contrast to the considerable number of works on vascular plant phylogeography existing today (e.g. Feliner, 2014; Sork, Gugger, Chen, & Werth, 2016). One of the more striking examples is that of lichen-forming fungi, a group of symbiotic organisms which includes members of the phyla Ascomycota and Basidiomycota, with at least 19,400 currently accepted species so far (Lücking, Hodkinson, & Leavitt, 2017), for which comparatively few detailed phylogeographical studies are available (Leavitt & Lumbsch, 2016; Werth, 2011, and references therein).

Since the early 2000s, lichen-forming fungi phylogeography has focused on interpreting, within either a phylogenetic or population genetics framework, the distribution of single species showing remarkable disjunctions in one or both hemispheres (e.g. Crespo et al., 2002; Fernández-Mendoza & Printzen, 2013; Leavitt, Westberg, et al., 2018; Printzen, Ekman, & Tønsberg, 2003; Spribille, Klug, & Mayrhofer, 2011). Other works have described genetic diversity patterns at a more regional scale (e.g. Bendiksby, Mazzoni, Jørgensen, Halvorsen, & Holien, 2014; Sork & Werth, 2014; Widmer et al., 2012). In contrast, relatively few studies have explored genetic diversity and population structure in lichenized fungi occurring in the Mediterranean, a region that includes the Mediterranean Basin and Macaronesia and is considered a major hotspot of biodiversity (Myers, Mittermeier, Mittermeier, Da Fonseca, & Kent, 2000; Quézel, 1978; Rivas-Martínez et al., 2011). For instance, Núñez-Zapata, Cubas, Hawksworth, and Crespo (2015) and Alors et al. (2017) analysed specimens of two epiphytic, foliose species of lichenized ascomycetes (*Parmelina tiliacea* (Hoffm.) Hale, and *Parmelina carporrhizans* (Taylor) Hale) and showed contrasting patterns of population differentiation. Thus, populations of the asexually reproducing *P. tiliacea* were highly differentiated and more genetically diverse in Macaronesia; the opposite was true for the sexually reproducing *P. carporrhizans*, a scenario coherent with the hypothesis of the Macaronesian Islands as dead ends for colonization routes (Alors et al., 2017). The study of the terricolous fruticose *Cetraria aculeata* (Schreb.) Fr. provided quantitative evidence of a high genetic connectivity among populations in the Canary Islands, the Iberian Peninsula and the eastern Mediterranean Basin (Fernández-Mendoza & Printzen, 2013). All the latter works have focused on epiphytic or terricolous lichenized fungi forming macrolichens. However, nothing is known about the genetic diversity, population structure and the potential historical and ecological drivers of the evolutionary history of terricolous species with a crustose growth form in the Mediterranean Basin and the Canary Islands.

Here, we focus on *Buellia zoharyi* Galun (Figure 1a), which belongs in the *Buellia epigaea* group and whose most closely related species seems to be *Buellia elegans* Poelt (Poelt & Sulzer, 1974; Trinkaus & Mayrhofer, 2000). *Buellia zoharyi* is a circum-Mediterranean/Macaronesian species forming crustose placodioid lichens that usually occurs in biological soil crusts (BSCs) in semi-arid areas of the Mediterranean region (Gutiérrez-Carretero & Casares-Porcel, 2011). Specifically, this species predominantly grows on Miocene gypsum soils (Barreno, 1994; Crespo & Barreno, 1975; Trinkaus & Mayrhofer, 2000), and more rarely on other types of gypsum, volcanic and limestone soils (Etayo, 2011; Giralt & van den Boom, 2011; Roux & Poumarat, 2015). Gypsiferous soils usually host a diverse assemblage of plant species, many of which are endemic and/or threatened (Escudero, Palacio, Maestre, & Luzuriaga, 2015; Meyer, 1986; Mota, Sola, Jiménez-Sánchez, Pérez-García, & Merlo, 2004). Although gypsophytes have lately received some attention from a phylogeographical perspective (Gómez-Fernández, Alcocer, & Matesanz, 2016; Martínez-Nieto, Segarra-Moragues, Merlo, Martínez-Hernández, & Mota, 2013; Salmerón-Sánchez, Martínez-Ortega, Mota, & Peñas, 2017), there are no data regarding the evolutionary history of gypsicolous lichenized fungi in spite of their undoubted role in structuring cryptogamic communities in these ecosystems (Escudero et al., 2015; Maestre et al., 2011; Rosentreter, Eldridge, Westberg, Williams, & Grube, 2016).

To untangle the historical processes that may have driven the evolutionary history of *B. zoharyi* in space and time in the Mediterranean region, here we estimate: (a) the genetic diversity of specimens collected all over the entire range of the species distribution; (b) the population genetic structure and evidence of past changes in population size and (c) the time frame in which this species diverged from its alleged sister species and diversified. Our findings will provide a potential avenue to better understand the factors controlling the origin and spatial distribution of crustose lichen populations occurring in Mediterranean gypsiferous soils under semi-arid conditions.

2 | MATERIALS AND METHODS

2.1 | Sampling, pre-treatment of the samples and DNA extraction

In this study, 226 thalli collected from 23 geographical populations, covering the entire distribution range of the species, were analysed (Figure 1b; Table S1 in Appendix S1). We included fresh (n = 172) and herbarium samples (n = 54), the oldest one dating back to 1957. Fresh specimens were air-dried for 1 day and then stored at −20°C. Thalli were cleaned with a sterile blade under a stereomicroscope to remove soil particles, and then were superficially sterilized following Arnold et al. (2009). A piece of each thallus was randomly excised and pulverized using a Mixer Mill type MM 301 Retsch® tissue lyser (Retsch, Germany). Total genomic DNA was isolated and purified using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.2 | PCR amplification and sequencing

Six loci were initially selected to screen the genetic diversity of the target species: the nuclear ribosomal internal transcribed spacer (nrITS) and the large subunit (nuLSU), the mitochondrial small subunit (mtSSU) and the genes encoding the translation elongation factor

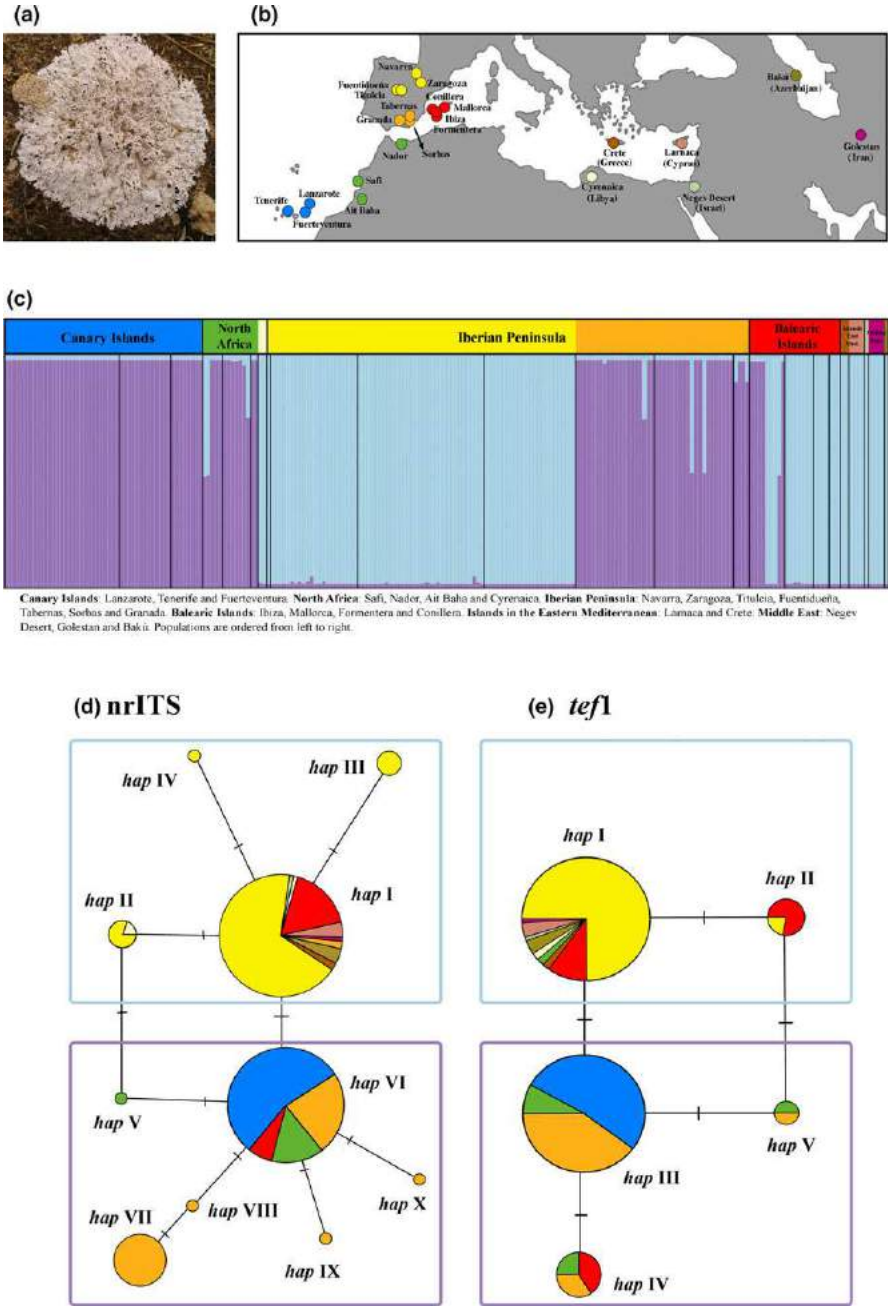

FIGURE 1 Sampling localities, haplotype networks and population structure of *Buellia zoharyi*. (a) Specimen from Mallorca (Balearic Islands). Photo: Arantzazu Molins. (b) Distribution map of the sampling localities; different colour labels are given to localities according to their geographical proximity: Canary Islands = blue, Morocco = green, southern Iberian Peninsula = orange, northern Iberian Peninsula = yellow, Balearic Islands = red, Cyrenaica (Libya) = beige, Crete (Greece) = brown, Larnaca (Cyprus) = pink, Negev Desert (Israel) = soft green, Bakú (Azerbaijan) = dark green and Golestan (Iran) = fuchsia. (c) Population genetic admixture inferred with STRUCTURE under $K = 2$. (d) and (e) nrITS and *tef*1 statistical parsimony haplotype networks, with different haplotypes coded with roman numerals (I–X); different colours are given to different sampling localities (see Figure 1b), and circle sizes are proportional to the number of samples sharing that haplotype; the two haplogroups (Blue and Purple) highlighted in networks largely correspond to the two main genetic clusters obtained in the STRUCTURE analysis [Colour figure can be viewed at wileyonlinelibrary.com]

1-α (*tef*1), the β-tubulin protein (β-*tub*) and the largest subunit of RNA polymerase II (*rpb*2). Semi-nested PCRs were performed for herbarium samples using a set of universal primers for the first PCR, and newly designed primers for the second PCR. Data on primers used for amplifying these loci are reported in Table S2 (Appendix S1). PCR reactions were performed following Moya et al. (2018). The amplified PCR products were sequenced with an ABI 3730XL sequencer, using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Samples with double peaks in any electropherogram were excluded from the analyses ($n = 3$; samples IB2, IR1 and LI1). Due to the lack of substantial nucleotide diversity in the nuLSU, mtSSU, β-tub and *rpb*2 datasets, only nrITS and *tef*1 were further considered. Potential recombination events within these two datasets were screened using seven methods (RDP, GENECONV, MAXCHI, BOOTSCAN, CHIMAERA, SISCAN and 3SEQ) implemented in the Recombination Detection Program 4.46 (RDP4, Martin, Murrell, Golden, Khoosal, & Muhire, 2015).

2.3 | Phylogenetic, polymorphism and neutrality analyses

We first constructed a phylogenetic tree to ascertain the ascription of all studied collections to the target species *B. zoharyi*, and to identify its phylogenetically closest *Buellia* species (i.e. sister species) according to data available in public databases. Settings of the phylogenetic analyses using the nrITS fungal barcode are detailed in Appendix S2 in Supporting Information. Second, genetic diversity indices and neutrality tests were calculated using DNASP 5.10

(Librado & Rozas, 2009). This software ignores positions with gaps, and consequently pools haplotypes that only differ by insertions and deletions (indels). Therefore, indel positions in the nrITS and *tef*1 alignments were recoded as 5th characters (Shi & Zhu, 2007). Specifically, we calculated the number of segregating sites (*s*), the number of haplotypes (*h*), the haplotype diversity (*Hd*), the average number of nucleotide differences (*k*) and the nucleotide diversity (π). Departures from neutrality which could indicate past changes in population size were tested with Tajima's *D* and Fu's *Fs* statistics using the number of segregating sites. The significance of these tests was assessed based on 10^4 coalescent simulations.

2.4 | Inference of population structure and genealogical relationships of haplotypes

The Bayesian clustering algorithm implemented in STRUCTURE 2.3.4 (Falush, Stephens, & Pritchard, 2003) was used to infer the population genetic structure in *B. zoharyi*. PGDSPIDER 2.0.7.2 (Lischer & Excoffier, 2012) was employed to transform the nrITS and *tef*1 datasets into allelic numbers to avoid bias due to genetic relatedness in multi-locus analyses. Then, 10 replicate runs consisting of 50,000 burn-in generations, followed by 500,000 iterations, with *K* ranging from 1 to 10 were performed. The analysis used a model allowing admixture, no prior population information, a uniform alpha prior, whereas allele frequencies were kept independent among gene pools in order to avoid overestimating the number of gene pools (Falush et al., 2003). The online platform CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015) was used to delimit the optimum number of clusters (best *K*) according to Evanno, Regnaut, and Goudet (2005), and the POPHELPER R package (Francis, 2017) was employed to graphically represent the admixture results.

In order to infer the genealogical relationships among haplotypes of the two variable genetic markers (nrITS and *tef*1), a statistical parsimony haplotype network was constructed with POPART 1.7 (Leigh & Bryant, 2015). We first generated haplotype alignments with the FABOX 1.41 online toolbox (Villesen, 2007), and then constructed networks using TCS (Clement, Snell, Walker, Posada, & Crandall, 2002) under the 95% parsimony probability criterion (Templeton, Crandall, & Sing, 1992), with gaps treated as a 5th character state.

2.5 | Molecular dating

A temporal framework for the origin and diversification of *B. zoharyi* was estimated using three secondary calibrations representing different dating strategies in BEAST 1.7 (Drummond, Suchard, Xie, & Rambaut, 2012). These types of calibrations, which are based on the results of previous molecular dating studies, are expected to provide inaccurate dating results. Even so, they constitute a commonly applied approach in divergence time analyses for organismal groups that lack fossil data, such as most lichen-forming fungi (Schenk, 2016). In the present study, a phylochronogram was firstly inferred using a five-locus dataset (mtSSU, *mcm7*, nrITS, nuLSU and β-*tub*; Table S1 in Appendix S3) constructed based on the phylogeny of *Caliciaceae* presented in Prieto and Wedin (2017). Individual alignments were done in MAFFT 7.308 (Katoh & Standley, 2013) as implemented in GENEIOUS® 9.0.2 using the FFT-NS-i x1000 algorithm and the scoring matrix 200 PAM/K2, and ambiguously aligned regions were delimited manually and removed. GBLOCKS 0.91b (Castresana, 2000) was further used as an automatic procedure to deal with gappy regions in the nuLSU dataset, allowing for smaller final blocks and half gap positions. The subsequent BEAST analysis used seven unlinked partitions and substitution models as inferred with PARTITIONFINDER 1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012), an RAXML-based starting tree, a Yule tree prior, and an uncorrelated lognormal relaxed clock model for each marker, imposing an age interval of 132–199 Myr on the ingroup node following 'analysis A9' in Prieto and Wedin (2017).

We also performed two additional date estimations using two different nrITS substitution rates. Since these have never been inferred from *Caliciaceae*, first we used a rate inferred for the parmelioid lichen-forming fungal genus *Melanohalea* (3.41×10^{-9} substitutions per site per year, Leavitt, Esslinger, Divakar, & Lumbsch, 2012); and second a rate for the non-lichenized fungal order Erysiphales (2.52×10^{-9} substitutions per site per year, Takamatsu & Matsuda, 2004). These alternative rates were imposed on an nrITS dataset including all *B. zoharyi* lineages together with selected sequences of *Buellia* species included in GenBank. Chronograms were then calculated in BEAST using a Yule tree prior and an uncorrelated lognormal relaxed clock model. Two independent MCMC runs ranging from 1.5×10^8 (multi-locus analysis) to 2.5×10^7 steps (single-locus analyses), always saving 10,000 trees, were carried out. We used TRACER 1.5, TREEANNOTATOR 1.8.1 and FIGTREE 1.4 (all available at: http://tree.bio.ed.ac.uk/) to check for convergence, to annotate the mean heights of the post-burn-in tree samples, and to construct 50% majority rule consensus trees respectively.

3 | RESULTS

3.1 | Phylogeny, genetic diversity, population structure and haplotype networks

The identity of all newly-produced nrITS sequences (n = 223) as *B. zoharyi* was confirmed by BLAST searches, as well as by phylogenetic inference. The new sequences were clustered within a supported monophyletic clade including a GenBank sample labelled as *B. zoharyi* with the accession number AJ421418 (Figure S1; Tables S3 & S4 in Appendix S1). *Buellia elegans* was revealed to be the sister species of *B. zoharyi*, as suggested by Trinkaus and Mayrhofer (2000). Neither locus showed statistically significant recombination events. Polymorphism statistics and neutrality tests for both loci are summarized in Table 1. In general, genetic diversity indices for the nrITS and *tef*1 loci showed low values, and although the negative values of either Tajima's *D* or Fu's *Fs* statistics could point to population expansion, the signal was not significant in any case.

Multi-locus inference of admixture populations carried out in STRUCTURE divided *B. zoharyi* into two geographically well-delimited

TABLE 1 Polymorphism statistics and neutrality test results for each genetic marker (nrITS and *tef*1) considering all individuals, or only individuals belonging to a particular haplogroup (see Figure 1c–e for graphical information). The calculated statistics were the number of segregating sites (*s*), number of haplotypes (*h*), haplotype diversity (*Hd*), average number of nucleotide differences (*k*) and nucleotide diversity (π). ns: not significant

	Polymorphism statistics					Neutrality tests	
Dataset	*s*	*h*	*Hd*	*k*	π	Tajima's *D*	Fu's *Fs*
nrITS all individuals	7	10	0.627	0.97305	0.00246	−0.59248 (ns)	−2.990 (ns)
nrITS (haplogroup Blue)	5	6	0.331	0.62773	0.00159	−0.98526 (ns)	−1.727 (ns)
nrITS (haplogroup Purple)	3	4	0.170	0.25520	0.00064	−0.96991 (ns)	−1.924 (ns)
*tef*1 all individuals	3	5	0.603	0.71482	0.00188	−0.67094 (ns)	−0.059 (ns)
*tef*1 (haplogroup Blue)	2	3	0.257	0.26470	0.00069	−0.45365 (ns)	−0.504 (ns)
*tef*1 (haplogroup Purple)	1	2	0.149	0.14913	0.00039	−0.23647 (ns)	−0.152 (ns)

clusters: one including individuals from the Canary Islands and Morocco, as well as some from the southern Iberian Peninsula and the Balearic Islands, and the other including individuals collected in localities across the Mediterranean Basin, from the Iberian Peninsula to Azerbaijan (Figure 1c). Individuals with an admixed origin were found mainly in Morocco, the southern Iberian Peninsula and the Balearic Islands, where the geographical distribution of both clusters overlap.

The number of haplotypes inferred from the nrITS and *tef*1 datasets were 10 and 5 respectively. Haplotype parsimony networks of both loci further supported the genetic clustering obtained with STRUCTURE, and revealed two groups of haplotypes, or haplogroups, which are hereafter named 'Blue' and 'Purple' according to the graphical representation in Figure 1d,e. Haplogroup Blue in the nrITS genealogy was composed of four haplotypes distributed from the Middle East region to the Iberian Peninsula, with haplotype I being the most abundant and widespread (Figure 1d). nrITS haplogroup Purple was made up of six haplotypes, distributed in the Canary Islands, Morocco, the Iberian Peninsula (Granada and Almeria) and the Balearic Islands (Ibiza), with haplotype VI being the most frequent and widespread. In the *tef*1 haplotype genealogy, haplogroup Blue was composed of two haplotypes, haplotype I being the most frequent and widespread across the Mediterranean region (Figure 1e). *tef*1 haplogroup Purple was composed of three haplotypes which were distributed mainly in the western Mediterranean, Morocco and the Canary Islands, and haplotype III was the most frequent. Frequencies and the geographical distribution of the haplotypes are summarized in Figure 2c and Table S5 in Appendix S1.

3.2 | Estimation of divergence times

Date estimates for the divergence between *B. zoharyi* and its sister species *B. elegans*, and the diversification within *B. zoharyi*, were based on phylochronograms obtained using three secondary calibrations (Figures S1–S3 in Appendix S3). Mean ages, and their respective 95% highest posterior density, HPD, for both evolutionary events are summarized in Table 2 and graphically displayed in Figure 2a. Briefly, the dating analysis with the combined matrix of five loci representing the phylogeny of the *Caliciaceae* (Prieto & Wedin, 2017), the family to which *B. zoharyi* belongs, estimated the origin of this species between the late Eocene and the mid to late Miocene (mean age 25.25 Myr, 95% HPD 39.35–11.06 Myr). The diversification of *B. zoharyi* was dated back to the Pleistocene, with a mean estimate of 1.07 Myr (95% HPD 2.19–0.29). On the other hand, the analysis using the *Melanohalea* nrITS substitution rate established the divergence of *B. zoharyi* from *B. elegans* between the Miocene and the Pliocene with a mean estimate of 9.86 Myr (95% HPD 3.42–17.66), whereas *B. zoharyi* diversification started between the Mid-Late Miocene and the Pleistocene (mean 5.61 Myr, 95% HPD 11.14–0.84 Myr). By using the nrITS substitution rate of Erysiphales, the mean age for the divergence of *B. zoharyi* from *B. elegans* was set between the Oligocene and Late Miocene (mean age 16.73 Myr, 95% HPD 31.35–6.01), and the diversification of the target species began between the Early Miocene and Pleistocene (mean age 8.11 Myr, 95% HPD 18.38–1.52).

4 | DISCUSSION

4.1 | Origin of *Buellia zoharyi* in the Mediterranean

In this study, the three dating strategies collectively inferred the divergence of *B. zoharyi* from *B. elegans* between the Eocene and the Pliocene (39.35–3.42 Myr). Our results agree with estimated divergence dates for closely-related species in other genera of lichen-forming fungi (e.g. *Nephroma*, Sérusiaux, Villarreal, Wheeler, & Goffinet, 2011; *Oropogon*, Leavitt, Esslinger, & Lumbsch, 2012; *Mastodia*, Garrido-Benavent, Ríos, Fernández-Mendoza, & Pérez-Ortega, 2018; *Psora*, Leavitt, Westberg, et al., 2018). Furthermore, our estimated wide temporal window partly overlaps with the proposed time period for the origin of most gypsophile plant lineages starting in the latest Miocene (Moore, Mota, Douglas, Olvera, & Ochoterena, 2014). Altogether, these evidences suggest that diversification in these organisms might have been influenced by broadly similar historical and ecological factors.

The current association of *B. zoharyi* and *B. elegans* to gypsum and calcareous soils, respectively (Trinkaus & Mayrhofer, 2000),

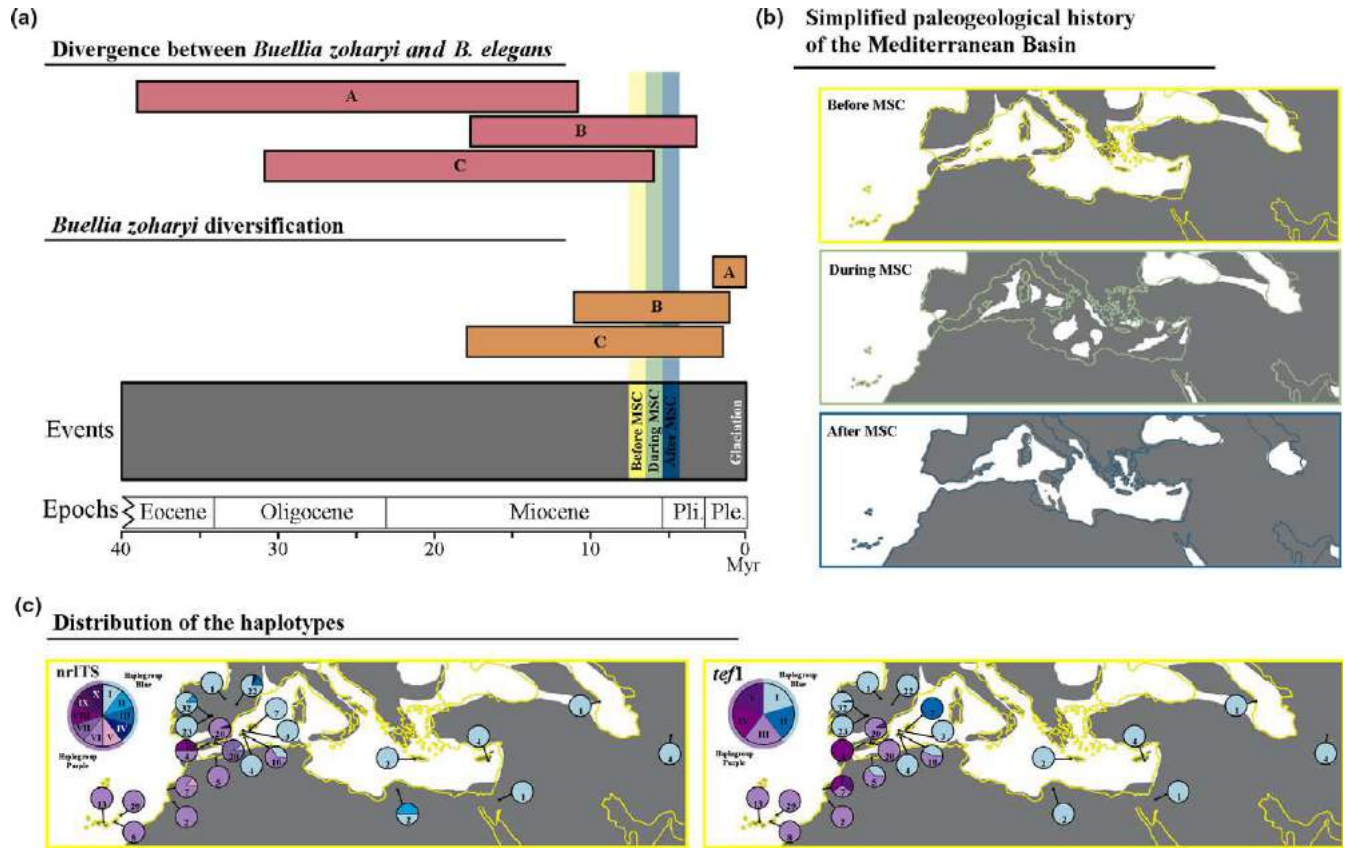

FIGURE 2 Origin and diversification of *Buellia zoharyi*, current spatial distribution of the species genetic diversity, and simplified geological history of the Mediterranean Basin. (a) The BEAST 95% high posterior density (HPD) age intervals for *B. zoharyi-B. elegans* divergence and *B. zoharyi* diversification obtained using (A) a secondary calibration on a phylogeny of *Caliciaceae*, and a nrITS substitution rate based on data from (B) the lichen-forming genus *Melanohalea* (Leavitt, Esslinger, Divakar, et al., 2012) and (C) the order Erysiphales (Takamatsu & Matsuda, 2004). (b) Simplified palaeogeological history of the Mediterranean Basin before the Messinian salinity crisis (MSC, yellow column), during the MSC (green) and after MSC (dark blue); setting of the main geological events occurring in the Mediterranean Basin since the Miocene in (a) and (b) follow Thompson (2005), and references therein; the current distribution of emerged lands in the Mediterranean Basin is represented by a coloured line. (c) Current spatial distribution of nrITS and *tef*1 haplotypes in *B. zoharyi* populations located on a representation of the Mediterranean Basin before the MSC; different haplotypes are coded with roman numerals (I–X) and different colours; the two shades of colour (purple and blue) correspond with the two genetic clusters inferred with STRUCTURE; the total number of individuals analysed in each population (*n*) is indicated within each circle [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Age estimates in million years (Myr) for the divergence and diversification of *Buellia zoharyi* obtained using three different dating strategies with BEAST

Event	Phylogeny of *Caliciacae* (Prieto & Wedin, 2017)	*Melanohalea* nrITS substitution rate[a]	Erysiphales nrITS substitution rate[b]	Temporal interval (epochs)
Buellia zoharyi divergence from its sister species, *B. elegans*	25.25 (39.35–11.06)	9.86 (17.66–3.42)	16.73 (31.35–6.01)	Eocene–Pliocene
Buellia zoharyi diversification	1.07 (2.19–0.29)	5.61 (11.14–0.84)	8.11 (18.38–1.52)	Miocene–Pleistocene

[a] 3.41×10^{-9} substitutions per site per year (Leavitt, Esslinger, & Lumbsch, 2012).
[b] 2.52×10^{-9} substitutions per site per year (Takamatsu & Matsuda, 2004).

indicates that their divergence was potentially promoted by an ecological niche shift. Examples of closely related species of lichen-forming fungi occupying distinct ecological niches are well-known (Brodo, 1973; Culberson, 1986; Rizzi & Giordani, 2013), although evidence for ecological speciation supported by molecular data is scarce. Recently, Leavitt, Westberg, et al. (2018) revealed a clade comprised exclusively of strictly gypsicolous *Psora saviczii* specimens within the core group of the more calcicolous *P. decipiens*, another typical component of BSCs. Incipient genetic differentiation was also detected at the intraspecific level between rock and bark populations of the widespread lichen *Xanthoria parietina* (Lindblom & Ekman, 2006). Other authors have suggested that the association of lichen-forming fungi to ecologically adapted photobionts in different geographical regions, or under different ecological conditions, may

potentially drive fungal speciation and affect the distribution of the fungal partner (Fernández-Mendoza et al., 2011; Ortiz-Álvarez, los Ríos, Fernández-Mendoza, Torralba-Burrial, & Pérez-Ortega, 2015; Peksa & Škaloud, 2011). Furthermore, diversification of xerophilous species in the *B. epigaea* group could have been enhanced by sympatric or allopatric processes resulting from climatic oscillations and mountain uplift starting in the Mid-Late Miocene which promoted aridification in large areas of the world (Miao, Herrmann, Wu, Yan, & Yang, 2012; Thompson, 2005; Zachos, Pagani, Sloan, Thomas, & Billups, 2001). These historical factors have been recently proposed as drivers of diversification in other groups of lichen-forming fungi (e.g. Leavitt, Kirika, et al., 2018; Leavitt, Westberg, et al., 2018).

Future work investigating key functional traits, including ecophysiological strategies as well as the range of associated photobionts, in members of the *B. epigaea* group will be necessary to understand the potential role of ecological specialization in the origin of *B. zoharyi* in the Mediterranean region. The incorporation of additional fossil evidence into analyses (Lücking & Nelsen, 2018) and the use of new methods for accommodating fossil uncertainty (Höhna et al., 2016) will assist in refining divergence time estimates and identifying more specific historical and ecological factors at the basis of the evolutionary history of this group of soil-dwelling taxa.

4.2 | Genetic diversity and diversification within *B. zoharyi*

A remarkable result of our study is the overall low genetic diversity shown by the target species, at least when compared with other crustose lichen-forming fungi with wider geographical distributions (e.g. Leavitt, Westberg, et al., 2018; Muggia, Pérez-Ortega, Fryday, Spribille, & Grube, 2014), or to the two foliose, epiphytic species with a similar Mediterranean–Macaronesian distribution *Parmelina carporrhizans* and *P. tiliacea* (Alors et al., 2017; Núñez-Zapata et al., 2015). We found that the majority of *B. zoharyi* populations from the Iberian Peninsula to Azerbaijan shared almost the same nrITS and *tef*1 haplotypes (haplogroup Blue, Figure 2c) whereas other populations in the southern Iberian Peninsula, North Africa and the Canary Islands were included in a different genetic cluster (haplogroup Purple, Figure 2c). This pattern of genetic clustering was more clearly shown in the STRUCTURE analysis, which further revealed admixed populations in Morocco, the Iberian Peninsula and the Balearic Islands. This region may configure a contact zone that has potentially sustained long-term levels of gene flow thanks to nearland bridges connecting the southern Iberian Peninsula and Morocco during the Late Miocene (the Betic-Rif arc, Krijgsman, Hilgen, Raffi, Sierro, & Wilson, 1999; Negro, De Sigoyer, Goffé, Saddiqi, & Villa, 2008), and recurrent direct dispersal across the Strait of Gibraltar in more recent times (Figure 2b). In fact, frequent dispersal through this strait, coupled with the presence of admixed populations in the Iberian Peninsula, has also been revealed for some vascular plants (Fernández-Mazuecos & Vargas, 2011; Migliore, Baumel, Juin, & Médail, 2012; Rodríguez-Sánchez, Pérez-Barrales, Ojeda, Vargas, & Arroyo, 2008).

The dispersal of *B. zoharyi* over medium to long distances can be accomplished by either meiotic ascospores produced in fertile thalli, or thallus fragments detached from sterile ones (Barreno, 1994; Casares & Llimona, 1983). Both types of reproductive strategy are suitable for long-distance dispersal, even across the Mediterranean Sea, based on evidence of other lichens showing widely disjunct populations (Alors et al., 2017; Fernández-Mendoza & Printzen, 2013; Garrido-Benavent et al., 2018). Moreover, increased connectivity among the Canary Islands, Africa and the Iberian Peninsula possibly occurred during Pleistocene glaciations, when the distance between the easternmost island (Fuerteventura) and the Moroccan coast was reduced to 60 km (Fernández-Palacios & Whittaker, 2008). Based on the above, it seems reasonable to suggest that the major factor limiting the effective dispersal of *B. zoharyi* is the existence of suitable edaphic conditions (i.e. gypsum soils).

Historical processes, including key geological events, potentially affected the current amount and distribution of the genetic diversity of *B. zoharyi* by acting on the availability of gypsiferous soils in the Mediterranean over time. Although age estimates are likely to be inaccurate due to the lack of more suitable calibration data, our approaches collectively suggest that *B. zoharyi* diversification took place during the late Miocene and Pleistocene (mean ages 8.11–1.07 Myr), a temporal period overlapping with the diversification of *P. carporrhizans* and *P. tiliacea* (mean ages 6.9–4.5, Nuñez-Zapata et al., 2017), as well as various species of Mediterranean vascular plants (reviewed in Feliner, 2014). The diversification of *B. zoharyi* is thought to have been greatly influenced by the Messinian salinity crisis (MSC, 5.96–5.33 Myr; Duggen, Hoernle, van den Bogaard, Rüpke, & Morgan, 2003), an event lasting over *c.* 600 Kyr which caused the almost complete desiccation of the Mediterranean Sea, producing a general and drastic increase in aridity around the Mediterranean Basin (Hsü et al., 1977; Thompson, 2005). The deposition of evaporites, and the later formation of gypsum-rich soils in this period of time (Duggen et al., 2003; Hsü et al., 1977; Krijgsman et al., 1999), presumably favoured the geographical expansion of *B. zoharyi* together with a likely increase in its genetic diversity, whereas the subsequent rapid flooding of the Mediterranean Basin (Zanclean Flood, *c.* 5.33 Myr) possibly had the opposite effect. Therefore, the current amount and distribution of the genetic diversity of *B. zoharyi* may still be reflecting the effects of this intricate Mediterranean geological past, and more recent events as the Quaternary climatic oscillations (Hewitt, 2004; Nuñez-Zapata et al., 2017; Thompson, 2005).

However, the observed distribution and levels of genetic diversity of *B. zoharyi* should be interpreted with caution as they are based on data of two loci. Future fieldwork and research must increase the sampling effort in the easternmost distribution range of the target species, and take advantage of high-throughput sequencing (e.g. genotyping by sequencing and genome-scale methods, reviewed in Werth, Miao, Jónsson, & Andrésson, 2015) to produce greater amounts of information. Inferences based on an extended dataset will provide valuable insights into population structure, including questions about how and when the two haplogroups first

segregated, the geographical limits of the contact zone, the historical directionality of gene flow, and the levels of among-population genetic differentiation which are predicted to be high for species inhabiting substrate archipelagos such as gypsum (Moore et al., 2014).

5 | CONCLUSIONS

The present phylogeographical study of *Buellia zoharyi* represents a convenient starting point for exploring the historical and ecological factors that potentially affected the evolution in space and time of gypsicolous, crustose lichen-forming fungi in the Mediterranean region. More generally, we consider that the outlined geological and climatic events occurring in the Mediterranean since the Miocene (e.g. the MSC and the Zanclean Flood) were pivotal in shaping the genetic diversity of the gypsophile biota in this region by influencing on the availability of gypsum soils and aridification in the region.

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DATA ACCESSIBILITY

DNA sequences: GenBank accessions MG384624–MG384638 and MG592314–MG592314.

BIOSKETCH

Salvador Chiva is a PhD student at the Universitat de València (Spain). His research is focused on terricolous lichens and the phycobionts involved in the formation of Biological Soil Crusts.

Author contributions: S.C, P.M, A.M and E.B designed the research and collected samples; S.C performed the experiments and assembled the datasets; S.C and I.G.B. analysed the data; E.B contributed to the funding of the study; S.C, I.G.B, P.M, A.M and E.B wrote the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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SUPPORTING INFORMATION

How did terricolous fungi originate in the Mediterranean region? A case study with a gypsicolous lichenized species.

Salvador Chiva, Isaac Garrido-Benavent, Patricia Moya, Arantzazu Molins and Eva Barreno

Appendix S1. Supplementary Tables and Figures.

The following supporting information in appendix S1 is available for this article:

Table S1. Samples used in this study, with details on collection data (country, region, locality, code, n, coordinates, collector (s), year) and herbarium code.

Table S2. Loci and primers used in this study.

Table S3. List of *Buellia zoharyi* lichen specimens, haplotypes and GenBank accessions numbers.

Table S4. GenBank accession numbers of samples used to build the nrITS dataset for the mycobiont phylogenetic analyses.

Table S5. nrITS and *tef*1 haplotypes of *Buellia zoharyi*, their frequencies and the locations of each haplogroup. Only sites that are polymorphic within our dataset are listed, with their respective base numbers in the complete alignment, starting at ITS1 in nrITS.

Figure S1 nrITS ML and BI phylogeny of *Buellioideae* (*Caliciaceae*, Ascomycota) including newly generated sequences of *B. zoharyi*, and rooted with *Xanthoria elegans*.

Table S1. Samples used in this study, with details on collection data and herbarium codes.

Country	Region	Locality	Code	n	Coordinates	Collector(s), year	Herbarium code*
SPAIN	Canary Islands	Lanzarote, Los Valles	LA 1-20	20	29º 06' 28" N 13º 31' 38" W	Barreno & Molins, 2013	
		Lanzarote, Haria, Peñas del Chache	LA 21-23	3	29º 07' 31" N 13º 31' 25" W	Barreno & Molins, 2013	
		Lanzarote, Haria, Peñas del Chache	LA 24-29	6	29º 07' 31" N 13º 31' 25" W	Trinkaus & Grube, 1996	GZU 4-2000 (125), 32-2011(448), 13-2002 (450), 32-2011 (451, 658, 659)
		Tenerife, Igueste de San Andrés	TE 1-13	13	28º 31' 44" N 16º 08' 49" W	Santos, 2014	
		Fuerteventura, Betancuria	FU 1-7	7	28º 26' 12" N 14º 03' 12" W	Santos, 2016	
		Fuerteventura, Betancuria	FU 8	1	28º 26' 12" N 14º 03' 12" W	van den Boom, 2001	Boom Collection
	Aragón	Zaragoza, Bujaraloz	SA 1-20	20	41º 29' 31" N 0º 15' 31" W	Barreno, Chiva, Moya & Salvà, 2014	
		Zaragoza, Alfajarín	SA 21	1	—	Poelt, 1983	GZU 111-83(54)
		Zaragoza.	SA 22	1	—	Volk, 1957	GZU 6-P1(46)
	Navarra	Ablitas, LIC de Peña del Montecillo	NA 1	1	41º 57' 50" N 01º 38' 17" W	Etayo, 2012	Etayo Collection 27904
	Madrid	Madrid, Titulcia	MT 1-30	30	40º 07' 35" N 03º 33' 07" W	Barreno, Chiva, Molins & Salvà, 2012	
		Madrid, Ciempozuelos	MT 31	1	—	Crespo, —	GZU 234(52)
		Madrid, Ciempozuelos	MT 32	1	—	Crespo & Hafellner, 1980	GZU (43)
		Madrid, Fuentidueña	MF 1-23	23	40º 08' 16" N 03º 08' 31" W	Barreno, Chiva, Molins & Salvà, 2012	
	Andalucia	Almería, Tabernas, Venta de los Yesos	AT 1-20	20	37º 04' 58" N 02º 17' 21" W	Barreno, Casares, Chiva, Moya & Salvà, 2014	

Country	Region	Locality	Code	n	Coordinates	Collector(s), year	Herbarium code*
SPAIN	Andalucia	Almería, Sorbas	AS 1-20	20	37º 08' 44" N 2º 08' 43" W	Barreno, Casares, Chiva, Moya & Salvà, 2014	
		Granada, La Bernardilla	GR 1-4	4	36º 48' 36" N 3º 32' 20" W	Casares, 1980, 1982	GDA 2698, 2699, 2700, 2717
	Balearic Islands	Mallorca, Llucmajor, Cabo Blanco	MJ 1-6	6	39º 21' 52" N 2º 47' 16" E	Molins & Conesa, 2015	
		Mallorca, Llucmajor, Cabo Blanco	MJ 7	1	—	Kottitz, 1988	GZU 1-88(44)
		Ibiza, Punta Ses Portes	IB 1-5,9,10	6	38º 50' 05" N 1º 24' 14" E	Lluzar, 2015	
		Ibiza, Punta Ses Portes	IB 6-8	3	38º 49' 56" N 1º 24' 21" E	Atienza, 2008	VAL 27715
		Ibiza, Sa Conillera	CO 1-3	3	38º 58' 40" N 1º 12' 50" E	Atienza, 2008	VAL 30062
		Formentera, Racó de s'Argela	FO 1-3	3	38º 41' 06" N 01º 26' 44" E	Lluzar, 2015	
		Formentera, Racó de s'Argela	FO 4	1	—	Feige, 1998	B M-224465
MOROCCO	Oriental	Nador, River Nekor	MN 1-5	5	—	Casares, 1992	GDA 2742, 2755, 2767, 2774, 2806
	Marrakesh-Safi	Safi	MS 1-7	7	—	Casares, 1992	GDA 2868, 2891, 2906, 2912, 2913, 2914, 2958
	Souss – Massa	Ait Baha	MA 1-2	2	—	Egea, 1987	S L19093, B M-224464
AZERBAIJAN	Bakú	Qobustan	AZ 1	1	—	Jelínková & Vezda, 1976	S L19085
CYPRUS	Larnaca	Oroklini	CY 1-4	4	—	Vezda, 1987	S L19090, B 60-74973, 60-63757, M-224466
ISRAEL	Central Negev	Makhtesh Ramon	NE 1	1	30º 37' N 34º 54' E	Temina, 2000	Temina Collection

Country	Region	Locality	Code	n	Coordinates	Collector(s), year	Herbarium code*
LIBYA	Cyrenaica	Sidi Alli Kollan	LI 1	1	—	Anderberg,1982	S F127803
		Al Abyar	LI 2-3	2	32º 11' 26" N 20º 55' 05" E	Thor, 1982	S F127804, L9082
GREECE	Crete	Nomos	CR 1-2	2	35º 13' N 24º 10' E	Trinkaus & Mayrhofer, 1997	GZU 24-2011(560, 562)
IRAN	Golestan	Maraveh-Tappeh, Ghazan Ghayeh	IR 1-3	2	37º 57' 40" N 56º 01' 49" E	Maassoumi, Sohrabi & Safavi, 2003	B 60-133553, 60-133554, 60-133555
		Gonbad-e-Kavus, Gorgan	IR 4	1	37º 13' 00" N 54º 29' 47" E	Sipman, Sohrabi, Sochting & Zare, 2007	B 60-175403
	Ilam	Shirvan Chardavol, Chame Jangal área	IR 5	1	33º 45' N 46º 30' E	Valadbeigi, 2008	B 60-175565

*GZU: Herbarium GZU of the University of Graz (Austria). GDA: Herbario de la Universidad de Granada (Spain). VAL: Universitat de València (Spain), Colecciones de Criptogamas (VAL_Lich). S: Herbarium of the Swedish Museum of Natural History (Sweden). B: Herbarium of the Botanic Garden and Botanical Museum Berlin-Dahlem (Germany).

Table S2. Loci and primers used in this study. Newly designed primers are in bold. Primers are indicated as forward (F) or reverse (R)

Locus	Primer	Primer sequence 5' - 3'	References
nrITS	ITS1F (F)	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
	ITSBZR (R)	**GTCGTAACAAGGTAGCCGTA**	**This study**
	ITS4 (R)	TCCTCCGCTTATTGATATGC	White et al. (1990)
*tef*1	**EF1-BZF (F)**	**CTGTCCCATTCGTGCCCATTT**	**This study**
	EF1-1018F (F)	GAYTTCATCAAGAACATGAT	Stielow et al. (2015)
	EF1-1620R (R)	GACGTTGAADCCRACRTTGTC	
mtSSU	mtSSU1 (F)	AGCAGTGAGGAATATTGGTC	Zoller et al. (1999)
	mtSSU3R (R)	ATGTGGCACGTCTATAGCCC	
β-tubulin	Bt3LM (F)	GAACGTCTACTTCAACGAG	Myllys et al. (2001)
	Bt10LM (R)	TCGGAAGCAGCCATCATGTTCTT	
*rpb*2	7cF (F)	ATGGGYAARCAAGCYATGGG	Liu et al. (1999)
	3053R (R)	TGRATYTTRTCRTCSACCATRTG	Reeb et al. (2004)
nuLSU	5·8SR (F)	TCGATGAAGAACGCAGCG	Vilgalys & Hester (1990)
	JLT-1R (R)	TCCGGCACCTTAACCTCAC	Prieto & Wedin (2017)

Table S3. List of *Buellia zoharyi* specimens used in the present study with the following information: taxon name, nrITS and *tef*1 haplotype working codes, and GenBank accession numbers. DP, Doubles peaks detected (in this case, sequences were not used in any analysis and therefore they were not submitted to GenBank).

Taxon/Code	nrITS		*tef*1		Taxon/Code	nrITS		*tef*1	
	Haplotype	Nº accession	Haplotype	Nº accession		Haplotype	Nº accession	Haplotype	Nº accession
B. zoharyi/LA 1-29	VI	MG384632	III	MG384626	*B. zoharyi*/CO 1-3	I	MG384629	I	MG384624
B. zoharyi/TE 1-13	VI	MG384632	III	MG384626	*B. zoharyi*/FO 1-4	I	MG384629	I	MG384624
B. zoharyi/FU 1-8	VI	MG384632	III	MG384626	*B. zoharyi*/IB 1, 10, 3-5	VI	MG384632	IV	MG384627
B. zoharyi/SA 1-5, 7, 9, 12, 13, 15-22	I	MG384629	I	MG384624	*B. zoharyi*/IB-2	DP	n/a	n/a	n/a
					B. zoharyi/IB 6-8	I	MG384629	I	MG384624
B. zoharyi/SA 6, 8, 10, 14	III	MG384631	I	MG384624	*B. zoharyi*/IB-9	VI	MG384632	I	MG384624
B. zoharyi/SA 11	IV	MG384636	I	MG384624	*B. zoharyi*/MN 1-2	VI	MG384632	I	MG384624
B. zoharyi/MT 1-7, 9-17, 19, 20, 22-29, 32	I	MG384629	I	MG384624	*B. zoharyi*/MN 3-5	VI	MG384632	III	MG384626
					B. zoharyi/MS-1, 2	VI	MG384632	III	MG384626
B. zoharyi/MT 8, 18, 21, 30	II	MG384630	I	MG384624	*B. zoharyi*/MS 3-5	VI	MG384632	IV	MG384627
B. zoharyi/MT 31	I	MG384629	II	MG384625	*B. zoharyi*/MS-6	VI	MG384632	V	MG384628
B. zoharyi/MF 1-23	I	MG384629	I	MG384624	*B. zoharyi*/MS-7	VIII	MG384634	V	MG384628
B. zoharyi/NA 1	I	MG384629	I	MG384624	*B. zoharyi*/MA 1-2	VI	MG384632	IV	MG384627
B. zoharyi/AT 1-7, 9-17, 19, 20	VI	MG384632	III	MG384626	*B. zoharyi*/NE-1	I	MG384629	I	MG384624
					B. zoharyi/AZ-1	I	MG384629	I	MG384624
B. zoharyi/AT 8	VI	MG384632	V	MG384628	*B. zoharyi*/CY-1-4	I	MG384629	I	MG384624
B. zoharyi/AT 18	X	MG384638	V	MG384628	*B. zoharyi*/LI-1	DP	n/a	n/a	n/a
B. zoharyi/AS 1-12, 14-18, 20	VII	MG384635	III	MG384626	*B. zoharyi*/LI-2	II	MG384630	I	MG384624
B. zoharyi/AS 13, 19	I	MG384629	III	MG384626	*B. zoharyi*/LI-3	I	MG384629	I	MG384624
B. zoharyi/GR 1	V	MG384633	IV	MG384627	*B. zoharyi*/IR-1	DP	n/a	n/a	n/a
B. zoharyi/GR 2, 3	VI	MG384632	IV	MG384627	*B. zoharyi*/IR 2-4	I	MG384629	I	MG384624
B. zoharyi/GR 4	IX	MG384637	IV	MG384627	*B. zoharyi*/CR-1-2	I	MG384629	I	MG384624
B. zoharyi/MJ 1-7	I	MG384629	II	MG384625					

Table S4. GenBank accession numbers of samples used for constructing the nrITS dataset for the phylogenetic analysis of *B. zoharyi*.

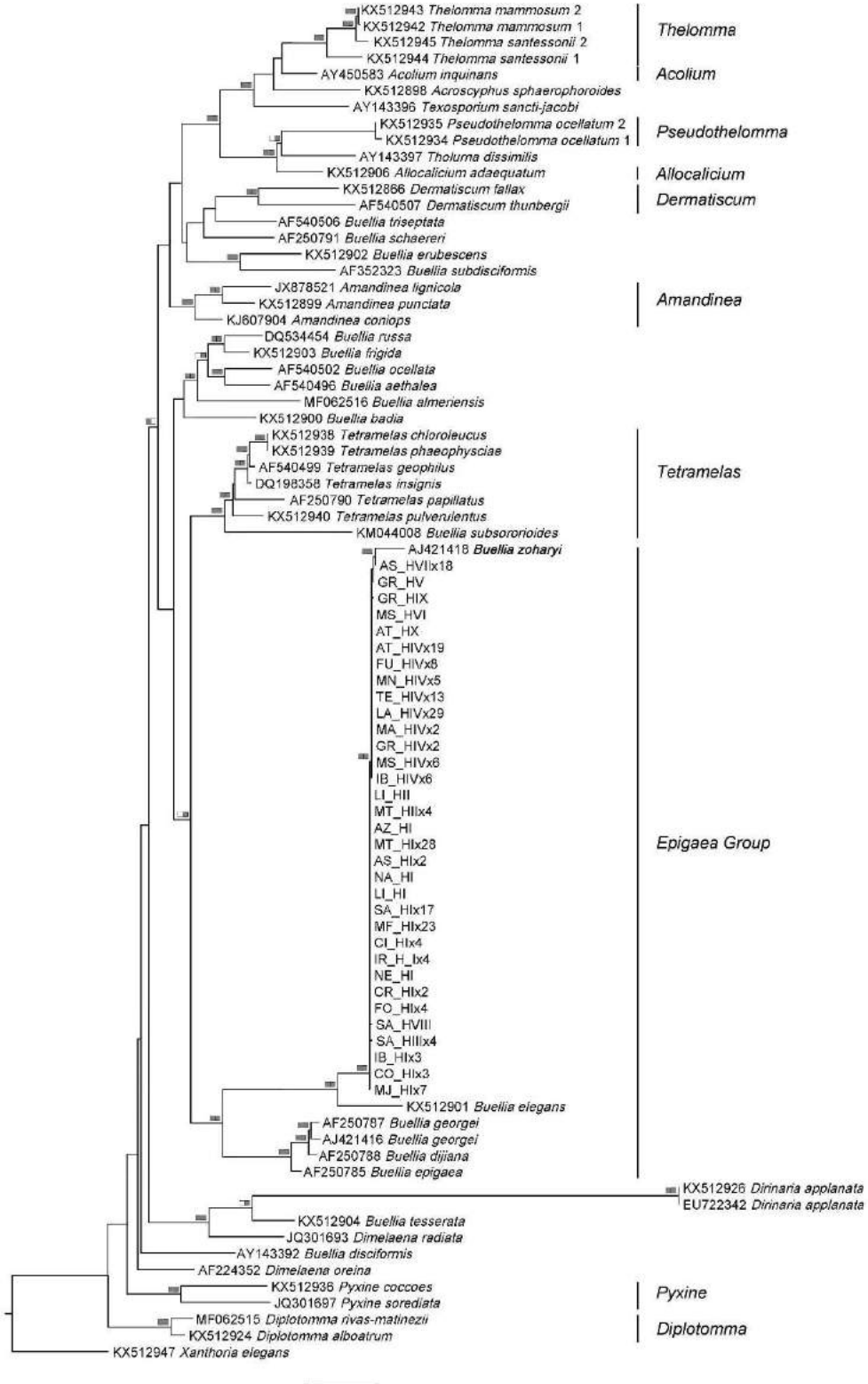
Taxon	GenBank accession number	Taxon	GenBank accession number	Taxon	GenBank accession number
Acolium inquinans	AY450583	*Buellia ocellata*	AF540502	*Pseudothelomma ocellatum* 2	KX512935
Acroscyphus sphaerophoroides	KX512898	*Buellia russa*	DQ534454	*Pyxine coccoes*	KX512936
Allocalicium adaequatum	KX512906	*Buellia schaereri*	AF250791	*Pyxine sorediata*	JQ301697
Amandinea coniops	KJ607904	*Buellia subdisciformis*	AF352323	*Tetramelas chloroleucus*	KX512938
Amandinea lignicola	JX878521	*Buellia subsororioides*	KM044008	*Tetramelas geophilus*	AF540499
Amandinea punctata	KX512899	*Buellia tesserata*	KX512904	*Tetramelas insignis*	DQ198358
Buellia aethalea	AF540496	*Buellia triseptata*	AF540506	*Tetramelas papillatus*	AF250790
Buellia almeriensis	MF062516	*Buellia zoharyi*	AJ421418	*Tetramelas phaeophysciae*	KX512939
Buellia badia	KX512900	*Dermatiscum fallax*	KX512866	*Tetramelas pulverulentus*	KX512940
Buellia dijiana	AF250788	*Dermatiscum thunbergii*	AF540507	*Texosporium sancti-jacobi*	AY143396
Buellia disciformis	AY143392	*Dimelaena oreina*	AF224352	*Thelomma mammosum* 1	KX512942
Buellia elegans	KX512901	*Dimelaena radiata*	JQ301693	*Thelomma mammosum* 2	KX512943
Buellia epigaea	AF250785	*Diplotomma alboatrum*	KX512924	*Thelomma santessonii* 1	KX512944
Buellia erubescens	KX512902	*Diplotomma rivas-martinezii*	MF062515	*Thelomma santessonii* 2	KX512945
Buellia frigida	KX512903	*Dirinaria applanata*	EU722342	*Tholurna dissimilis*	AY143397
Buellia georgei 1	AJ421416	*Dirinaria applanata*	KX512926	*Xanthoria elegans*	KX512947
Buellia georgei 2	AF250787	*Pseudothelomma ocellatum* 1	KX512934		

Table S5. nrITS and *tef*1 haplotypes of *Buellia zoharyi* and their frequencies. See Figure 1c, d for graphic details.

Haplotype working code for each locus		Loci, position (column) in the alignment, and nucleotide										Haplogroup purple *	Haplogroup blue*	Frequency (number of sequences collapsing to a particular haplotype)
nrITS	*tef*1	nrITS							*tef*1					
		035	089	093	113	131	175	269	104	270	272			
I	I	T	C	C	C	C	G	G	C	A	-		SA MT MF NA FO IB CO NE AZ CY LI IR CR	91
I	II	T	C	C	C	C	G	G	C	G	-		MJ MT	8
I	III	T	C	C	C	C	G	G	C	A	T	AS	AS	2
II	I	T	-	-	-	C	G	G	C	A	-		MT LI	5
III	I	T	C	-	-	C	G	G	C	A	-		SA	4
IV	I	T	C	C	C	C	G	C	C	A	-		SA	1
V	IV	T	C	C	C	T	C	G	T	A	T	GR		1
VI	I	T	C	C	C	T	G	G	C	A	-	IB MN	IB MN	3
VI	III	T	C	C	C	T	G	G	C	A	T	LA TE AT FU MN MA MS		75
VI	IV	T	C	C	C	T	G	G	T	A	T	GR IB MS		10
VI	V	T	C	C	C	T	G	G	C	G	T	AT MS		2
VII	III	C	C	C	C	T	C	G	C	A	T	AS		18
VIII	V	T	-	-	-	T	G	G	C	G	T	MS		1
IX	IV	T	C	T	C	T	G	G	T	A	T	GR		1
X	V	T	C	C	-	T	G	G	C	G	T	AT		1
														Total 223

*AS: Almería-Sorbas (Iberian Peninsula, Spain). AT: Almería-Tabernas (Iberian Peninsula, Spain). AZ: Azerbaijan. CO: Conillera (Balearic Islands, Spain). CY: Cyprus (Larnaca, Cyprus). CR: Crete (Greece). FO: Formentera (Balearic Islands, Spain). FU: Fuerteventura (Canary Islands, Spain). GR: Granada (Iberian Peninsula, Spain). IB: Ibiza (Balearic Islands, Spain). IR: Iran (Golestan and Ilam, Iran). LA: Lanzarote (Canary Islands, Spain). LI: Libya (Cyrenaica, Libya). MA: Morocco-Ait Baha (North Africa). MF: Madrid-Fuentidueña (Iberian Peninsula, Spain). MJ: Mallorca (Balearic Islands, Spain). MN: Morocco-Nador (North Africa). MS: Morocco-Safi (North Africa). MT: Madrid-Titulcia (Iberian Peninsula, Spain). NA: Navarra (Iberian Peninsula, Spain). NE: Negev Desert (Israel). SA: Zaragoza (Iberian Peninsula, Spain). TE: Tenerife (Canary Islands, Spain).

Figure S1. nrITS ML and BI phylogeny of *Buellioideae* (*Caliciaceae*, Ascomycota) including newly generated sequences of *B. zoharyi*, and rooted with *Xanthoria elegans*. Squares filled with grey represent significant statistical clade support obtained with PhyML (left square, bootstrapping probabilities ≥ 70%) and MrBayes (right square, posterior probabilities ≥ 0.95).



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SUPPORTING INFORMATION

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Appendix S2. Supplementary Material and Methods.

Phylogenetic analyses of *B. zoharyi* using nrITS data

A multiple sequence alignment was built including the newly obtained nrITS sequences together with sequences of species belonging to the subfamily Buellioideae (Caliciaceae, Ascomycota) included in a previous phylogeny of the group published in Prieto and Wedin (2017). Other nrITS sequences from different *Buellia* species not included in the previous phylogeny were also identified and downloaded from the GenBank. The compiled sequence dataset was first aligned using MAFFT v 7.308 (Katoh, Misawa, Kuma & Miyata, 2002; Katoh & Standley, 2013) and then adjusted manually using MEGA v 6 (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). Further edition of the resulting alignment was carried out in the program GBlocks v 0.91b (Castresana, 2000), and it involved the automatic removal of ambiguously aligned regions using the least stringent parameters, but allowing gaps in 50% of the sequences. The final alignment was 391 bp in length and included the three sub-regions ITS1, 5.8S and ITS2 (partial).

Subsequently, the most appropriate substitution model was estimated using the Akaike Information Criterion (AIC) as implemented in jModelTest v 2.1.4 (Darriba, Taboada,

Doallo & Posada, 2012). This analysis favoured the GTR+I+G over other substitution models.

Phylogenetic hypotheses were constructed using two alternative approaches: Maximum Likelihood (ML) and Bayesian Inference (BI). The ML analysis was done with RAxML v 8 (Stamatakis, 2014) using the GTRCAT substitution model and 1000 bootstrap pseudo-replicates to evaluate nodal support (Stamatakis, Hoover & Rougemont, 2008). The BI analysis was carried out with the software MrBayes v 3.2 (Ronquist et al., 2012). Two parallel, simultaneous six-chain runs were executed over 1 x 10^7 generations starting with a random tree, and sampling after every 100^{th} step. We discarded the first 25% of data as burn-in. The 50% majority-rule consensus tree and corresponding posterior probabilities were calculated from the remaining trees. Chain convergence was assessed by ensuring that average standard deviation of split frequency (ASDSF) values were below 0.01, and potential scale reduction factor (PSRF) values approached 1.00.

ML and BI analyses were run using the CIPRES Science Gateway v 3.3 web-portal (Miller, Pfeiffer & Schwartz, 2010). The phylogenetic tree was visualized in FigTree v 1.4.2 (available at http://tree.bio.ed.ac.uk/software/figtree/) and Photoshop CS5 was used for the artwork. Clades that received bootstrap support ≥ 70% in ML analyses and posterior probabilities (PP) ≥ 0.95 were regarded as significantly supported.

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Appendix S3 GenBank accession numbers of samples used in dating analyses, and chronograms obtained with BEAST depicting age estimates for the origin and diversification of *B. zoharyi*.

The following Supporting Information in Appendix S3 is available for this article:

Table S1 GenBank accession numbers of samples used for constructing the chronogram based on the phylogeny of *Caliciacae* published in Prieto and Wedin (2017).

Figure S1 Chronogram based on the phylogeny of *Caliciacae* published in Prieto and Wedin (2017) and including newly-obtained *B. zoharyi* data obtained with BEAST.

Figure S2 Chronogram providing a temporal framework for *B. zoharyi* origin and diversification, obtained using a *Melanohalea* nrITS substitution rate obtained with BEAST.

Figure S3 Chronogram providing a temporal framework for *B. zoharyi* origin and diversification, obtained using an Erysiphales nrITS substitution rate obtained with BEAST.

Table S1 GenBank accession numbers of samples used for constructing the chronogram based on the phylogeny of *Caliciacae* published in Prieto and Wedin (2017). Abbreviations: —, not available; mtSSU; *mcm7*; nrITS; nuLSU; β-tubulin.

Taxon	GenBank accession number				
	mtSSU	*mcm7*	nrITS	nuLSU	β-tubulin
Acolium inquinans	AY143404	JX000161	AY450583	AY453639	KX529023
Acolium karelicum	—	KX529045	KX512897	KX512879	—
Acroscyphus sphaerophoroides	KX512984	KX529029	KX512898	—	—
Allocalicium adaequatum	KX512986	—	KX512906	KX512859	KX528996
Amandinea coniops	KX512978	—	—	KX512865	KX528998
Amandinea punctata	AY143399	KX529025	KX512899	AY340536	—
Anaptychia ciliaris	AY143400	KX529054	AY143391	KX512894	—
Anaptychia runcinata	KX512977	KX529034	—	—	—
Baculifera remensa	KX512962	—	—	KX512881	—
Buellia badia	KX512963	—	KX512900	KX512880	KX529008
Buellia disciformis	JX000116	JX000152	AY143392	JX000082	—
Buellia dispersa	—	KX529035	—	—	—
Buellia elegans	KX512993	—	KX512901	—	KX528988
Buellia zoharyi SA2	MG592321	—	MG592314	MG592328	MG592346
Buellia zoharyi MT30	MG592322	—	MG592315	MG592329	MG592347
Buellia zoharyi SA6	MG592323	—	MG592316	MG592330	MG592348
Buellia zoharyi TE13	MG592324	—	MG592317	MG592331	MG592349
Buellia zoharyi AS20	MG592325	—	MG592318	MG592332	MG592350
Buellia zoharyi SA11	MG592326	—	MG592319	MG592333	MG592351
Buellia zoharyi AT18	MG592327	—	MG592320	MG592334	MG592352
Buellia erubescens	KX512969	—	KX512902	KX512874	KX529004
Buellia frigida	KX512992	—	KX512903	KX512852	—
Buellia tesserata	—	KX529050	KX512904	KX512885	—

Calicium abietinum	KX512971	KX529041	KX512905	KX512872	KX529003
Calicium adspersum	KX512949	KX529055	KX512907	KX512895	KX529022
Calicium chlorosporum 1	KX512956	KX529059	—	KX512892	—
Calicium chlorosporum 2	KX512955	—	—	—	—
Calicium corynellum	KX512985	KX529048	KX512908	KX512855	KX528989
Calicium denigratum	KX512965	KX529044	KX512909	KX512878	—
Calicium glaucellum	KX512980	KX529032	KX512910	KX512864	—
Calicium lecideinum	KX512961	KX529046	KX512911	KX512882	KX529009
Calicium lenticulare	KX512979	KX529033	KX512912	—	KX528997
Calicium montanum	—	—	KX529069	KX512853	—
Calicium nobile 1	KX512988	KX529060	KX512913	KX529070	—
Calicium nobile 2	KX512987	KX529061	KX512914	KX529071	—
Calicium notarisii	KX512960	KX529047	KX512915	KX512883	KX529011
Calicium pinicola 1	KX512972	KX529040	KX512916	KX512871	KX529015
Calicium pinicola 2	KX512991	KX529066	KX512917	KX512887	KX529014
Calicium quercinum	—	—	KX512918	KX512854	—
Calicium salicinum	KX512982	KX529027	KX512919	KX512861	KX528991
Calicium tigillare	JX000123	JX000162	JX000104	JX000088	KX529002
Calicium trabinellum	—	KX529026	KX512920	KX512858	KX528995
Calicium trachylioides	KX512959	KX529058	KX512933	KX529072	KX529018
Calicium verrucosum	—	KX529030	—	—	—
Calicium viride	AY584696	JX000153	HQ650703	AY340538	KX529013
Dermatiscum fallax	—	—	KX512921	KX512866	—
Dimelaena oreina	KX512976	KX529036	KX512922	KX512867	KX528999
Dimelaena radiata	—	KX529049	KX512923	KX512884	—
Diplotomma alboatrum	KX512966	KX529043	KX512924	KX512877	KX529007
Diplotomma venustum	KX512968	—	KX512925	—	KX529005
Dirinaria applanata	KX512990	—	KX512926	KX512856	—
Heterodermia speciosa	KX512975	KX529037	KX512927	KX512868	KX529000

Heterodermia vulgaris	KX512989	—	KX512928	KX512857	—
Phaeophyscia ciliata	KX512958	KX529051	KX512929	KX512886	KX529012
Phaeophyscia orbicularis	KX512967	—	KX512930	KX512876	—
Physcia aipolia	AY143406	KX529052	KX512931	AY300857	KX529021
Physcia tenella	KX512974	KX529038	KX512932	KX512869	—
Pseudothelomma ocellatum 1	KX512957	KX529062	KX512934	KX512862	KX529019
Pseudothelomma ocellatum 2	KX512952	KX529063	KX512935	KX512891	KX529020
Pseudothelomma occidentale	KX529073	KX529057	—	—	KX529074
Pyxine coccoes	KX512964	—	KX512936	—	KX529010
Pyxine sorediata	KX512973	KX529039	KX512937	KX512870	KX529001
Tetramelas chloroleuca	—	—	KX512938	KX512875	KX529006
Tetramelas phaeophysciae	—	—	KX512939	—	—
Tetramelas pulverulentus	KX512983	—	KX512940	KX512860	KX528990
Texosporium sancti-jacobi	KX512981	KX529031	KX512941	KX512863	KX528994
Thelomma mammosum 1	KX512954	KX529067	KX512942	KX512888	KX529016
Thelomma mammosum 2	KX512953	KX529065	KX512943	KX512851	KX529017
Thelomma santessonii 1	KX512951	KX529064	KX512944	KX512889	—
Thelomma santessonii 2	KX512950	—	KX512945	KX512890	—
Tholurna dissimilis	AY143407	KX529053	AY143397	KX512893	KX528992
Tornabea scutellifera	KX512970	KX529042	KX512946	KX512873	—
Xanthoria elegans	KX512948	KX529056	KX512947	KX512896	KX529024

Figure S1 Chronogram based on the phylogeny of *Caliciacae* published in Prieto and Wedin (2017) and including newly-obtained *B. zoharyi* data obtained with BEAST. Red stars on nodes indicate strong statistical support (PP > 0.95).

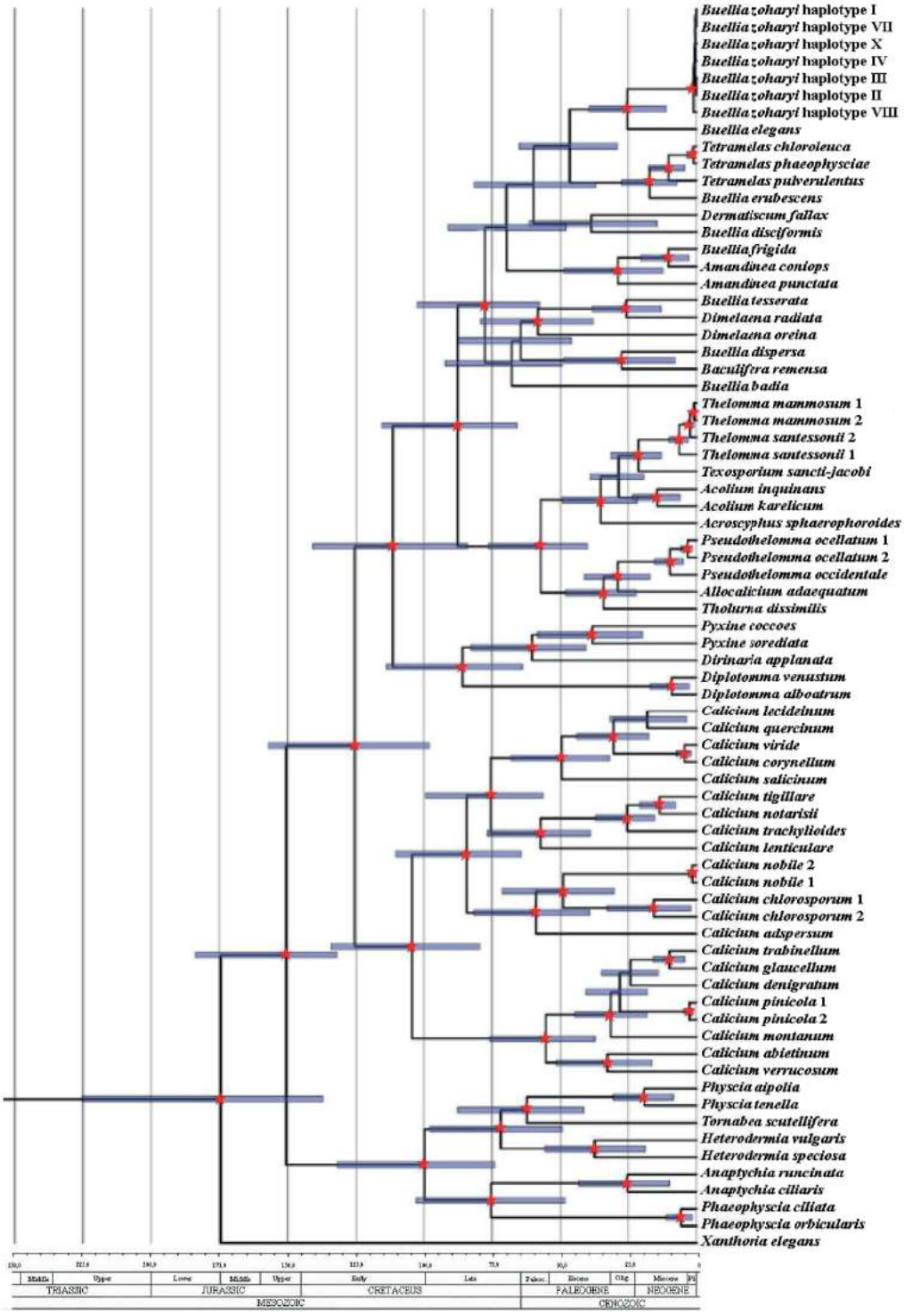


Figure S2 Chronogram providing a temporal framework for *B. zoharyi* origin and diversification, obtained using a *Melanohalea* nrITS substitution rate obtained with BEAST. Red stars on nodes indicate strong statistical support (PP > 0.95).

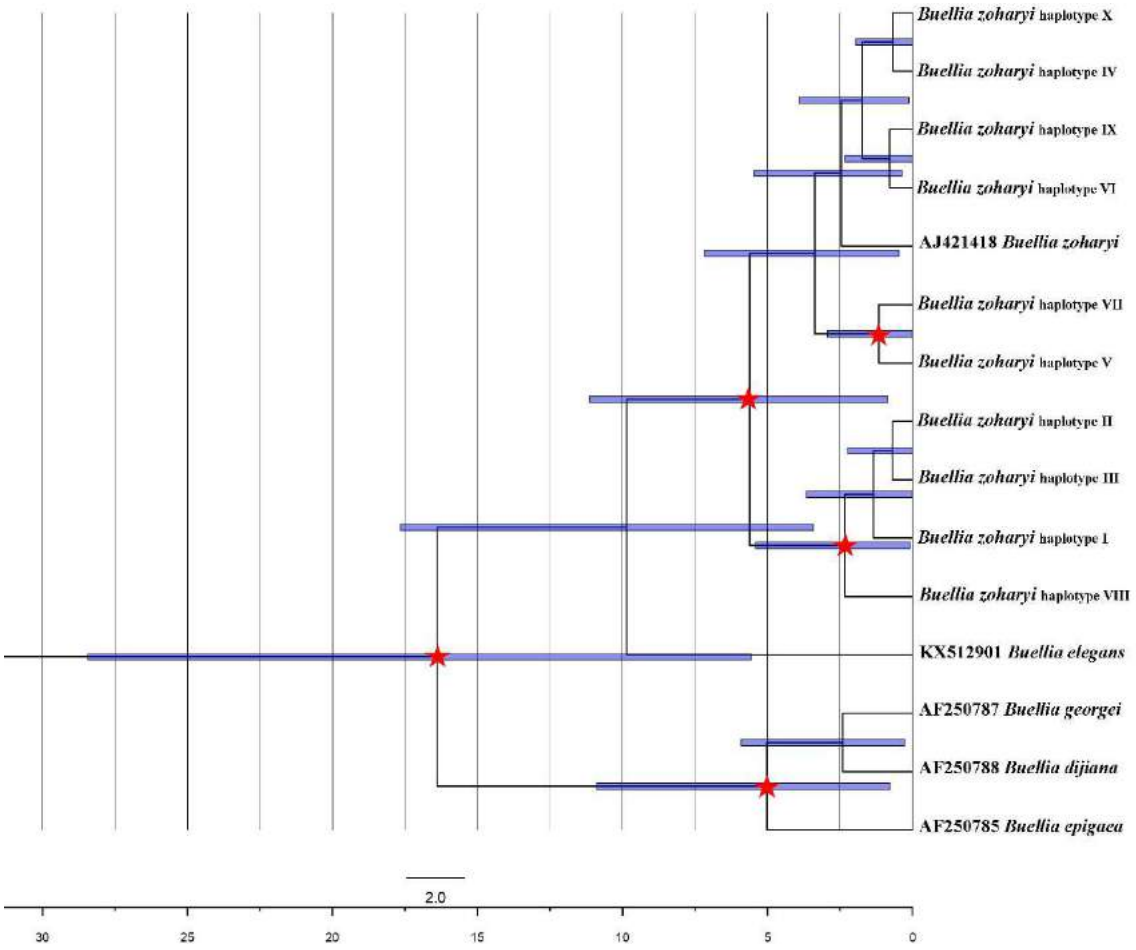
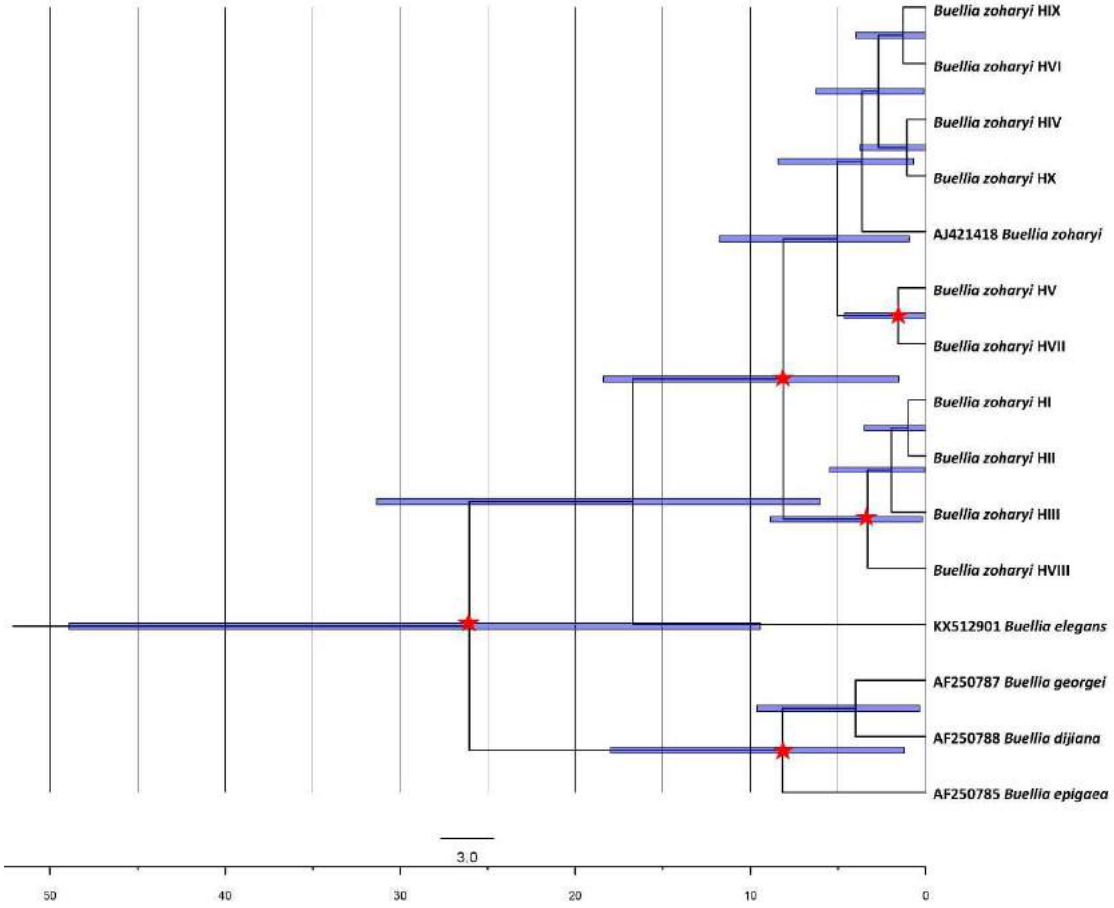


Figure S3 Chronogram providing a temporal framework for *B. zoharyi* origin and diversification, obtained using an Erysiphales nrITS substitution rate obtained with BEAST. Red stars on nodes indicate strong statistical support (PP > 0.95).



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3. Discusión general

4 DISCUSIÓN GENERAL

El término selectividad se utiliza en estudios que analizan los patrones de asociación de los componentes mayoritarios de las simbiosis liquénicas (micobionte/microalga). Este término se define como la frecuencia por la que un simbionte elige de entre un grupo (*pool*) de posibles acompañantes a uno de los componentes (Bowler y Rundel 1975; Hestmark 1992; Nash 2008). Esta selección es particularmente importante en los líquenes que se dispersan mediante esporas, ya que el establecimiento de un nuevo talo en ese nicho dependerá de la interacción con las microalgas adecuadas (Galun et al. 1968; Dal Grande et al. 2014). Entre otros factores, la compatibilidad entre los distintos componentes de los talos liquénicos está condicionada por la especificidad (posible rango taxonómico de socios aceptables) de cada uno de los simbiontes. Otros aspectos relacionados con el sustrato, biotipo, etc., podrían influir y ser claves para la selección de los simbiontes compatibles en cada nuevo talo liquénico. Estas interacciones y factores han sido poco estudiados en los líquenes de las biocostras.

En cada capítulo de esta tesis (Resultados) se han discutido, de manera independiente, los resultados sobre los patrones de asociación entre géneros de microalgas y un determinado grupo de líquenes de las biocostras. En cambio, esta discusión general trata de discutir los resultados de estos artículos en conjunto, para analizar, a nivel de comunidad, dichas interacciones (selectividad/especificidad) entre los simbiontes liquénicos de las biocostras. Se discutirán teniendo en cuenta los diferentes rangos taxonómicos, los patrones de selección (especificidad y selectividad) que podrían estar actuando entre los simbiontes, y las complejas relaciones que se establecen en estas comunidades liquénicas, tales como coexistencia, *algal switching* y transferencia de ficobiontes. También se toma en consideración la influencia que el sustrato, las estructuras de dispersión y el biotipo podrían ejercer sobre ellas.

Selectividad y especificidad entre las microalgas simbióticas y los micobiontes

Líquenes del género *Cladonia* y el microalga *Asterochloris mediterranea*

En la literatura se ha descrito ampliamente que la familia Cladoniaceae presenta un nivel de especificidad muy alto hacia las microalgas del género *Asterochloris* (Yahr et al. 2004, 2006; Nelsen y Gargas 2006; Beiggi y Piercey-Normore 2007;

Bačkor et al. 2010; Piercey-Normore et al. 2010; Škaloud et al. 2015, Steinová et al 2019). En R 3.1 (Moya et al. 2015) se analizaron ultraestructural y filogenéticamente las microalgas simbióticas presentes en distintas especies del género *Cladonia* de las biocostras. Estos líquenes también mostraron alta especificidad por el género *Asterochloris* como simbionte fotosintético, concretamente por la nueva especie descrita en ese mismo artículo *Asterochloris mediterranea* sp. nov. Barreno, Chiva, Moya et Škaloud.

En búsquedas realizadas por GenBank, se ha detectado la presencia de A. mediterranea en *Stereocaulon* (*S. vesuvianum*) y *Lepraria* (*L. nylanderiana* y *L. isidiata*) además de en las especies de *Cladonia* incluidas en nuestro estudio (Anexo I, Tabla 1). También, ha sido encontrada en el liquen *Diploschistes muscorum* (Wedin et al. direct submission GenBank 2015), lo que podría deberse al hecho de que este liquen presenta una fase parásita sobre especies de *Cladonia* durante la cual podría captar *A. mediterranea* para, posteriormente, en su fase independiente intercambiarla por alguna especie de *Trebouxia*, proceso ya descrito por Friedl (1987) y Piercey-Normore y DePriest (2001) (Anexo I, Tabla 1). Esta microalga parece ser la predominante en líquenes de los géneros *Cladonia*, *Stereocaulon* y *Lepraria* principalmente, al igual que el resto de especies del género *Asterochloris*. La alta afinidad de las especies de *Asterochloris* por líquenes de los géneros anteriormente mencionados, ha sido ampliamente estudiada en diversos trabajos (Peksa y Škaloud 2011; Steinová et al. 2019). Estos autores señalan que las relaciones entre los simbiontes no se producen al azar y que las preferencias ecológicas y las estrategias reproductivas son clave en dichas interacciones.

Líquenes escuamulosos y el microalga *Myrmecia israeliensis*

Como se ha reseñado en la Introducción, tradicionalmente las microalgas del género *Myrmecia* han sido descritas en modo de vida libre colonizando diferentes tipos de sustratos (p. ej. Hartmann et al.2009; Hallmann et al. 2013; R 3.2, Moya et al. 2018). Hasta el momento se han descrito formalmente nueve especies para este género (Guiry y Guiry 2019; Anexo II Listado A), y solo dos de ellas como simbiontes liquénicos: *M. biatorellae* y *M. israeliensis*.

Myrmecia biatorellae es el ficobionte de *Sarcogyne privigna*, de varias especies de la familia Verrucariaceae (*Placidium* spp. y *Verrucaria submersella*) y de *Psora* decipiens (Anexo I, Tabla 2). *M. israeliensis* y otros dos taxones de este género que todavía no han sido descritos (*Myrmecia* sp. URa20 y *Myrmecia* sp. URa23) se asocian como simbiontes predominantes a líquenes escuamulosos (Anexo I, Tabla 2).

En R 3.2, se ha constatado que *M. israeliensis* es el microalga predominante en los líquenes escuamulosos de las familias Verrucariaceae (*Clavascidium* spp. y *Placidium* spp.) y Psoraceae (*P. decipiens* y *P. saviczii*) que se desarrollan en las CBSs (Moya et al. 2018). Todos estos datos sugieren de nuevo, como sucedía en el caso de *Cladonia/A. mediterranea*, la presencia de un posible patrón de asociación entre *M. israeliensis* y los líquenes escuamulosos pertenecientes a las familias Psoraceae y Verrucariaceae.

Los líquenes crustáceos y las microalgas del género *Trebouxia*

Una vez analizados los líquenes que presentan microalgas de los géneros *Asterochloris* y *Myrmecia*, para obtener una visión completa de las comunidades de líquenes de las biocostras de yesos, era necesario incluir en el estudio los líquenes crustáceos. En R 3.3, se han analizado *Diploschistes diacapsis*, *Acarospora placodiiformis*, *A. nodulosa*, *Diplotomma rivas-martinezii* y *Rhizocarpon malenconianum*, y en todos ellos se han detectado como socios simbióticos predominantes a especies del género *Trebouxia*. En concreto, se han detectado cuatro taxones del clado 'A' de este género*: T. asymmetrica*, *T. cretacea*, *Trebouxia* sp. OTU A25 y *T. vagua* (Helms et al. 2001, 2003; Beck 2002). Por otro lado, también se ha detectado la presencia de taxones del clado 'A' en otros líquenes crustáceos como en *Gyalolechia* spp. (Schaper y Ott 2003).

También se han realizado búsquedas en GenBank para determinar qué especies de líquenes están asociadas a las cuatro *Trebouxia* detectadas en las biocostras. Como ocurre con muchas especies/linajes de *Trebouxia* estas cuatro especies podrían considerarse como generalistas, ya que una misma especie de microalga está asociada con un amplio rango de micobiontes (Muggia et al. 2017) (Anexo I, Tabla 3). Además, es importante resaltar que estos cuatro ficobiontes se detectan mayoritariamente en especies de líquenes terrícolas y saxícolas, pero no se han encontrado, hasta el momento, en líquenes epífitos (Anexo I, Tabla 3).

Se conocen muy pocos taxones de los géneros *Asterochloris*, *Myrmecia* o *Trebouxia* que se asocien exclusivamente a una única especie de liquen y viceversa, fenómeno que se ha denominado alta especificidad recíproca (Muggia et al. 2018). En el caso de *Trebouxia* sp. OTU A25 hasta el momento únicamente se había detectado asociada con el liquen saxícola *Xanthoparmelia maricopensis* (Leavitt et al. 2015) por lo que se consideró como un caso excepcional de alta especificidad recíproca. En este trabajo de tesis, se ha puesto en evidencia la presencia de dicho

taxón en varias especies de líquenes de las biocostras analizadas (R 3.3 y Chiva et al. 2016, 2018). Por tanto, en la medida de lo posible, cuando se analicen patrones de asociaciones simbióticas en líquenes, deberían incluirse un amplio rango de especies, de áreas, de sustratos y de tipos de climas antes de adjudicar categorías de especificidad.

Patrones de selección de microalgas

Variabilidad genética de los micobiontes

La diversidad genética de las poblaciones, se ha relacionado en muchos organismos con la capacidad para adaptarse a distintos ambientes y hacer frente a posibles cambios en el largo plazo (Jump et al. 2009; Rizvanovic et al. 2019; Toczydlowski y Waller 2019). Para analizar la posible relación entre la diversidad genética con la capacidad de colonización de distintos hábitats, se ha analizado la variabilidad genética de los micobiontes de nueve especies de líquenes con distribución muy variada y con diferentes requerimientos edáficos (*A. nodulosa*, *A. placodiiformis*, *B. zoharyi*, *C. foliacea*, *D. diacapsis*, *D. rivas–martinezii*, *P. decipiens*, *P. saviczii* y *R. malenconianum*). Aunque para alguna de las especies los datos de diversidad genética se indican en su capítulo correspondiente (p. ej. R 3.3), en la Figura D1 de esta discusión se muestra una representación gráfica de todas ellas, donde se observan valores dispares en cuanto a la variabilidad nucleotídica.

En especies con distribución cosmopolita, tales como C. *foliacea* o *P. decipiens*, es interesante señalar que, en nuestro estudio, la variabilidad nucleotídica del micobionte es muy elevada en comparación con otros líquenes de carácter gipsófito preferente, como ocurre con *A. nodulosa*, *D. diacapsis*, *P. saviczii* y *B. zoharyi* (Figura D1). Estos últimos, presentan su óptimo de cobertura y frecuencia sobre suelos de yesos, aunque puedan colonizar otros sustratos en áreas restringidas. Las especies *A. placodiiformis* y *D. rivas–martinezii* son gipsófitos exclusivos, ya que hasta ahora solo se les ha encontrado colonizando sustratos yesíferos (Barreno 1994; Molina et al. 2002). Estos últimos presentan todavía menor diversidad genética que los gipsófitos preferentes (Figura D1). En consecuencia, esto indicaría la posible existencia de una relación entre la variabilidad nucleotídica y la capacidad de colonización de diferentes sustratos, puesto que las especies de líquenes con menor preferencia hacia los yesos son los que muestran una mayor variabilidad genética y al contrario. Aunque los estudios sobre diversidad genética de micobionte/ficobiontes son relativamente frecuentes (Domaschke et al. 2012; Beck et al. 2019), los estudios de estas relaciones en líquenes gipsícolas son casi inexistentes.

Domaschke y coautores (2012) realizaron un análisis de la diversidad en poblaciones del liquen de amplia distribución *Cetraria aculeata*, incluyendo las regiones antárticas, antiboreales, templadas y árticas. Sus análisis revelan una disminución de la diversidad genética en las poblaciones aisladas de la Antártida, probablemente debida a un efecto fundador durante la colonización a larga distancia. Recientemente, Beck y coautores (2019) han analizado poblaciones también de la Antártida de líquenes del género *Placopsis* y han determinado que los haplotipos de los ficobiontes de este liquen, muestran diferentes preferencias ambientales posiblemente relacionadas con su capacidad para adaptarse a condiciones ambientales cambiantes y/o extremas. Estos datos de baja diversidad en micobiontes de poblaciones de la Antártida podrían extrapolarse a otras también aisladas como son las que ocurren crecen en las biocostras de yesos.

En general, la pérdida de diversidad genética suele reducir el potencial de adaptación de las poblaciones y aumenta el riesgo de extinción de las mismas. El riesgo de extinción, por lo tanto, parece aumentar cuando las especies están bajo un mayor estrés ambiental (Bijlsma et al. 2000; Reed et al. 2002) como podría ser la hiperespecificidad por un único tipo de sustrato.

Finalmente, como sugieren Domaschke y coautores (2012) para algunas de las poblaciones antárticas, la variabilidad genética reducida, podría ser un artefacto causado por un muestreo geográfico desigual o por un número reducido de individuos muestreados en cada población.

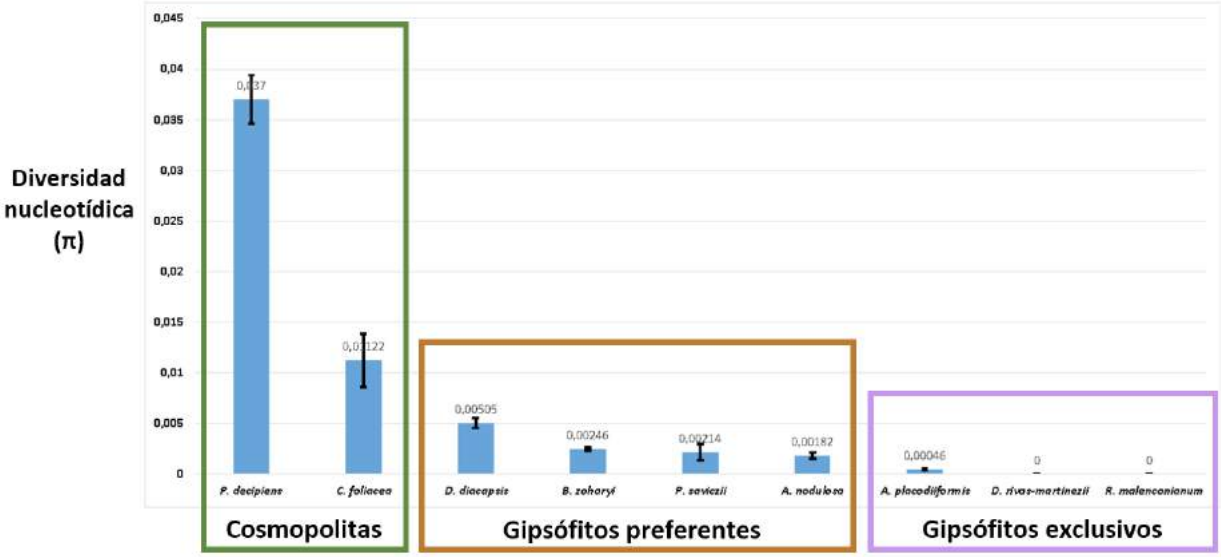

Figura D1. Representación mediante un gráfico de barras de la variabilidad nucleotídica en líquenes de las biocostras estudiadas. En verde se recuadran las especies cosmopolitas, en marrón las gipsófitas preferentes y en lila las gipsófitas exclusivas.

La relación entre la amplitud del área de distribución y la variabilidad genética ha sido ampliamente estudiada en plantas. Diversos estudios con plantas sugieren que en especies de gran distribución la diversidad genética detectada es mayor en comparación con las especies de distribución más relicta, al igual que sucede con las especies de líquenes analizadas en esta tesis (Zhang et al. 2005; Ikeda y Setoguchi 2007; Sosa et al. 2009; Ornelas et al. 2010; Pérez-Alquicira et al. 2010; Escobar et al. 2011; Martínez-Nieto et al. 2013; Aguirre-Liguori et al. 2014).

Las especies de plantas con restricciones edáficas han sido utilizadas como ejemplos para estudios sobre distribución, flujo genético, diversidad genética y diversificación (Moore et al. 2014; Salmerón-Sánchez et al. 2014a, b 2017). En R 3.4 (Chiva et al. 2019) se considera a *Buellia zoharyi* como especie modelo de liquen gipsófito de la cuenca mediterránea. En este trabajo se sugiere que los eventos acontecidos en esta cuenca, y que posiblemente afectaron a *B. zoharyi*, también pudieron ser clave en la especialización ecológica y la diversidad genética en otras especies mediterráneas con similares requerimientos. Aunque a principios de la crisis del Messiniense estos líquenes pudieron tener un periodo de expansión a través de la cuenca mediterránea, con la inundación Zancleana se perdieron grandes extensiones de sustratos yesíferos. Este evento redujo la conectividad entre las distintas localidades, y pudo provocar la pérdida de gran parte de la variabilidad nucleotídica de los líquenes gipsícolas. Posiblemente, las especies con gran afinidad por el sustrato de yesos como *A. placodiiformis*, *D. rivas-martinezii* y *R. malenconianum* se vieron afectadas por los mismos mecanismos que afectaron a *B. zoharyi*.

Biotipos liquénicos (arquitecturas de los talos)

Otro aspecto de los líquenes que resulta interesante estudiar es su morfología o biotipo, ya que la arquitectura de los talos podría estar relacionada con su capacidad para seleccionar determinadas microalgas y condicionar su interacción con el sustrato. En las biocostras liquénicas suelen convivir varias especies de líquenes con distintos biotipos, el más generalizado es el crustáceo, aunque también se encontran líquenes escuamulosos, foliáceos, leprariodes y dimórficos (Anexo I, Tabla 4).

Los resultados obtenidos en los artículos que se incluyen en R 3.1, R 3.2 y R 3.3 sugieren una correlación entre los biotipos presentes en los líquenes de las biocostras y los géneros de microalgas simbióticas. El biotipo crustáceo contiene como socios fotosintéticos especies del género *Trebouxia* (R 3.3; Chiva et al. 2016, 2018), el biotipo escuamuloso se asocia con microalgas del género *Myrmecia* (R 3.2), y los

líquenes dimórficos, foliáceos y l eprarioides con especies del género *Asterochloris* (R 3.1; Chiva 2012).

Estos resultados plantean si la selectividad que establecen ciertos líquenes por un determinado género de microalgas podría estar condicionada por la morfología del talo, y por las particularidades de cada grupo de microalgas. Aunque no son muy frecuentes los estudios sobre la morfología, organización y desarrollo de la arquitectura de los talos liquénicos, Sanders (2001) ha dedicado muchos esfuerzos para una precisa descripción de dichos aspectos. Otros autores (Colesie et al. 2017; Eriksson et al. 2018; Wan y Ellis 2019) han abordado el estudio de la morfología para analizar su influencia en la capacidad para retener agua, estos análisis evidencian que la morfología es un factor significativo en las diferencias entre especies de líquenes. Sin embargo, los estudios ultraestructurales y químicos más detallados sobre la relación entre los simbiontes liquénicos (microalga/micobionte) y el sustrato donde se desarrollan han sido los realizados por Souza-Egipsy y coautores (2002a, b). En estos trabajos se demuestra que la morfología del talo es un elemento clave en la forma en la que se relacionan con el sustrato, resultado que estaría en consonancia con los obtenidos en esta tesis.

Sustrato

Como se ha comentado anteriormente, el establecimiento de un nuevo talo depende de la presencia de los simbiontes adecuados en el *pool* de microalgas y de la captación e interacción exitosa con algunas de ellas. Por tanto, las características geoquímicas del sustrato donde se realizan los contactos entre micobiontes y ficobiontes son importantes para determinar tanto la presencia de las microalgas como para el desarrollo del talo.

Las comunidades liquénicas incluidas en esta tesis fueron recolectadas principalmente en localidades que presentaban biocostras de yesos. Sin embargo, en todos los artículos (Resultados) y trabajos generados en este estudio (Punto 6, Otras publicaciones) se incluyeron a modo de comparación individuos/ poblaciones provenientes de otros tipos de sustrato (Anexo I, Tabla 5).

En el caso de *Cladonia*/*A. mediterranea* se incluyeron un total de cuatro tipos diferentes de sustrato: yesoso, calcáreo, silíceo y volcánico (Anexo I, Tabla 5). En todas las especies de *Cladonia* analizadas, independientemente del tipo de sustrato

de la localidad, se detectó *A. mediterranea* como simbionte predominante. Por tanto, la presencia de esta microalga en los líquenes analizados no parece estar condicionada por las características del sustrato.

Tampoco se ha detectado influencia del sustrato en el establecimiento de *M. israeliensis* como simbionte en líquenes de las familias Psoraceae y Verrucariaceae (R 3.2).

Tres de las especies de *Trebouxia* encontradas en los líquenes crustáceos (*T. asymmetrica*, *T. cretacea* y *Trebouxia* sp. OTU A25), se han confirmado como microalgas predominantes en líquenes de suelos calcáreos, yesosos y volcánicos, y por lo tanto no parecen presentar especificidad por el sustrato (Anexo I, Tabla 5). Aunque *T. vagua* en esta tesis solo se ha detectado en líquenes crustáceos de las biocostras de yesos, búsquedas en GenBank revelan su presencia en líquenes terrícolas sobre otros sustratos, por lo que no podría establecerse una relación directa entre el tipo de sustrato y su presencia en este medio (Anexo I, Tabla 3). *Trebouxia* sp. "arnoldoii" se ha detectado únicamente en poblaciones de *B. zoharyi* en Tenerife, curiosamente en otras islas próximas con sustratos volcánicos, se han detectado otras especies de *Trebouxia* como ficobiontes en este liquen. Este dato podría ser un artefacto causado por el número reducido de individuos muestreados en cada población, quizá el sustrato o el micro-ambiente de esa localidad tinerfeña posea alguna peculiaridad ecológica que permita a este microalga ser específica de esa población.

La presencia de *A. mediterranea*, *M. israeliensis* y los otros taxones de *Trebouxia* encontrados no parece estar condicionada por el tipo de sustrato, sin embargo, las características geoquímicas y determinados componentes de los suelos podrían influir sobre la biodiversidad de las costras (p. ej. Rogers 1972; Eldridge y Tozer 1997; Ponzetti y McCune 2001; Eldridge 1996; Lalley et al. 2006; Lobel et al. 2006; Bowker y Belnap 2008; Root et al. 2011; Ochoa-Hueso et al. 2011) y por ello sobre la presencia/ausencia de determinadas especies de líquenes. El análisis de macro y microelementos incluidos en R 3.3 han mostrado picos significativos de hierro y estroncio en las biocostras de yesos Miocenos de las dos localidades muestreadas del centro de la Península Ibérica (Fuentidueña del Tajo y Titulcia), este hecho podría condicionar la composición del *pool* de microalgas y las especies de líquenes que se desarrollan. Ochoa-Hueso y coautores (2011) han asociado la presencia de hierro con una elevada cobertura liquénica, sin embargo Starks y Shubert (1979) asociaron la presencia de estroncio con una disminución en la abundancia y diversidad de determinadas algas.

Aunque nuestros resultados son preliminares, consideramos que los análisis sobre las propiedades del suelo no deben ser menospreciados en estudios sobre diversidad de microalgas, por sus posibles implicaciones en la composición del *pool* fotosintético.

Tipos de estructuras reproductoras y formas de dispersión

Otro aspecto clave en los estudios sobre los patrones de selectividad/ especificidad son los tipos de estructuras reproductivas y las formas de dispersión, tanto a corta como a larga distancia.

La dispersión de los líquenes de las biocostras incluidos en esta tesis, se puede producir mediante esporas con las que se propaga únicamente el micobionte (Gjerde et al. 2015). Estas esporas pueden ser sexuales (ascosporas generadas en ascomas) o asexuales (conidiosporas provenientes de picnidios) (Anexo I, Tabla 6). Además, es muy importante destacar la importancia de los pequeños fragmentos de talos para la dispersión conjunta alga/ hongo, especialmente en especies vagrantes o semivagrantes (Heinken 1999). Esta se produce por roturas generadas por la acción mecánica de viento, animales, etc., que permite la multiplicación vegetativa y rápida de ejemplares de un determinado liquen en la misma localidad. Los líquenes pueden usar otros tipos de propágulos especiales, por ejemplo isidios y/o soredios, en los que también participan las microalgas simbióticas, los cuales no son muy frecuentes en las comunidades que se han analizado (Heinken 1999; Büdel y Scheidegger 2008). La combinación de varias de estas estrategias es utilizada por la mayoría de los líquenes para aumentar la probabilidad de éxito en la colonización de nuevos espacios disponibles. En los líquenes de las biocostras sobre yesos una mayor capacidad de colonización y dispersión es muy importante, principalmente en el caso de especies gipsófitas (preferentes y/o exclusivas) las cuales dependen, en mayor o menor medida, de la presencia de yesos para su establecimiento.

Los líquenes de las CBSs asociados con microalgas predominantes de los géneros *Trebouxia* o *Myrmecia* suelen presentar apotecios (reproducción sexual) y algunos de ellos también picnidios (reproducción asexual). Ambas estructuras permiten la dispersión exclusiva del micobionte en largas distancias debido al pequeño tamaño y peso de sus esporas (Honegger et al. 2008; Gjerde et al. 2015). Si estas esporas llegan a un lugar con las condiciones apropiadas y se encuentran con las microalgas adecuadas se desarrollará un protoliquen (Bowler y Rundel 1975; Galun 1988,

Sanders y Lcking 2002). El origen de estas microalgas podría ser del *pool* local de la biocostra o de propágulos vegetativos liberados por otro liquen cercano (Dal Grande et al. 2014). En numerosos trabajos, se indica que las posibilidades de asentamiento en diferentes tipos de hábitats, de aquellos líquenes que tienen un amplio rango de amplitud ecológica, podría deberse a su capacidad de dispersión de esporas a larga distancia y de selección de microalgas apropiadas (Muñoz et al. 2004; Blaha et al. 2006; Insarova y Blagoveshchenskaya 2016). En estos líquenes, la fragmentación de los talos también puede ser un factor importante para la dispersión conjunta de micobionte y microalga/s asociada/s (Rosentreter 1993). Cabe destacar que únicamente los líquenes de las costras que se asocian con *Trebouxia* o *Myrmecia* presentan apotecios (o peritecios en el caso de líquenes escuamulosos), aunque con los datos que disponemos hasta el momento no podríamos asegurar la relación entre estas dos coincidencias (R 3.3).

En las biocostras analizadas en esta tesis, los líquenes que establecen simbiosis con especies de *Asterochloris* (*A. mediterranea*) como microalga predominante carecen de apotecios o son muy raros (Anexo I, Tabla 6). En este caso, los líquenes utilizan las conidiosporas como propágulos asexuales, que facilita la dispersión exvlusiva del micobionte . De nuevo, como en el caso anterior, el establecimiento del nuevos talos dependerá de las posibilidades de captación de microalgas compatibles, ya sea por que estén disponibles en el sustrato o por los propágulos simbióticos liberados por otro liquen cercano (Galun et al. 1988; Dal Grande et al. 2014a). Curiosamente, en los microambientes más húmedos de las biocostras donde se encuentran los talos de *Cladonia* spp., también suele desarrollarse *Lepraria isidiata* que presenta la superficie cubierta por un elevado número de isidios (Vezda 1973; Maestre 2003; Crespo et al. 2006; Tretiach et al. 2009). En las costras de yesos analizadas *L. isidiata* también simbiotiza con *A. mediterranea* como predominante (Chiva 2012). Los propágulos (isidios) liberados por este liquen podrían suministrar *A. mediterranea* a las distintas especies de *Cladonia* que crecen en la misma comunidad. Además, estos liquenes usan como estrategia de dispersión conjunta la fragmentación de los talos al igual que ocurre con los que albergan *Trebouxia* y *Myrmecia* (Rosentreter 1993).

Las distintas estrategias de reproducción y dispersión son factores clave para determinar la diversidad de microalgas que simbiotizan en los líquenes. Es particularmente interesante que especies gipsícolas (exclusivas o preferentes), que se dispersan a larga distancia mediante esporas y requieren, por tanto, de una re-liquenización para poder establecerse, mantengan alta selectividad hacia sus socios fotosintéticos, incluso en ambientes tan extremos como el de las costras

biológicas del suelo. Estudios recientes de Steinová y coautores (2019) han constatado que varias especies del género *Cladonia* con reproducción asexual presentaban una alta especificidad por sus fotosimbiontes. En cambio, las especies de *Cladonia* que se reproducen sexualmente se asocian con diferentes microalgas, actuando como generalistas. La comunidad de líquenes incluidos en los análisis de biocostras en esta tesis parece más compleja en sus mecanismos de reproducción y dispersión dado que todavía no se han podio relacionar los patrones de selectividad/especificidad con dichos mecanismos.

Coexistencia de microalgas

Los estudios sobre diversidad de microalgas simbiontes en los talos de líquenes han sido abordados durante años utilizando, casi exclusivamente, la secuenciación de tipo Sanger (Sanger et al. 1977). Recientemente, diversos análisis de metabarcoding han puesto de manifiesto que existe una gran y casi desconocida diversidad de especies asociadas con las simbiosis liquénicas (Grube et al. 2015; Banchi et al. 2018; Gueidan et al. 2019; Wright et al. 2019), ya que el uso de técnicas de secuenciación masiva (HTS) permite detectar muchos más genotipos que con las técnicas de secuenciación convencional (Stewart y Cavanaugh 2009). En concreto, estas nuevas técnicas han posibilitado la constatación de la multiplicidad de microalgas dentro de un único talo (Muggia et al. 2013; Moya et al. 2017; Molins et al. 2018b), y ha obligado a revisar los términos de selectividad/especificidad. Con este objetivo, Paul y coautores (2018) han comparado el potencial de la tecnología Sanger y del HTS *metabarcoding* para inferir la diversidad de microalgas presentes en *Lasallia hispanica* y *L. pustulata*. En este trabajo, se asume que en la mayoría de estudios sobre diversidad de fotobiontes mediante Sanger, se detecta una única especie ya que sus electroforetogramas son únicos. Por ello, en muchos casos un porcentaje variable de especímenes que mostraban coexistencia ha podido ser obviado en bastantes estudios (Muggia et al. 2014; Voytsekhovich y Beck 2016; Leavitt et al. 2015), ya que los autores excluían de forma sistemática los ejemplares que mostraban electroforetogramas con doble picos o posiciones polimórficas.

Es importante destacar que, en los líquenes incluidos en R 3.3, el porcentaje de coexistencia abarca desde el 33% en *Rhizocarpon malenconianum* al 72% en *Acarospora nodulosa* y *Diplotomma rivas-martinezii*. Además, en Chiva y coautores (2016), se analizaron 11 localidades del liquen crustáceo *Buellia zoharyi* que incluían

a las poblaciones de Fuentidueña de Tajo y Titulcia, en las que se detectaron tres taxones de *Trebouxia* (*T. cretacea*, *T. asymmetrica* y *Trebouxia* sp. OTU A25). El análisis de coexistencia, mediante cebadores específicos, reveló un alto porcentaje, ya que en el 75% de los talos analizados se detectó la presencia de más de un ficobionte. En otros trabajos del grupo, se han detectado altos porcentajes de coexistencia en varias especies vagrantes y semivagrantes de *Circinaria* en matorrales continentales de la Península Ibérica (Molins et al. 2018a). Incluso se ha detectado en el liquen canario *Parmotrema pseudotinctorum* la coexistencia de algas de distintos géneros: *Asterochloris* spp. y *Trebouxia* spp. (Škaloud et al. 2018). El liquen modelo de experimentación, *Ramalina farinacea*, se ha usado en estudios de coexistencia y de respuestas diferenciales de los ficobiontes frente a estreses abióticos (del Campo 2010, 2013; García-Breijo et al. 2010; Casano et al. 2011; Moya et al. 2017), también en *R. fraxinea* aunque en menor profundidad (Català et al. 2016). Otros autores, han analizado la presencia de múltiples genotipos de fotobiontes en una misma areola o en un único lóbulo de los líquenes saxícolas *Tephromela atra* y *Protoparmeliopsis muralis* respectivamente (Muggia et al. 2008, 2013). La coexistencia de múltiples microalgas simbióticas en un mismo talo es un fenómeno frecuente en líquenes de distintas morfologías y que se desarrollan en diferentes tipos de sustratos y/o hábitats.

Sin embargo, hay otras especies de líquenes que no presentan coexistencia; como por ejemplo los líquenes foliáceos (*Cladonia* spp.) y escuamulosos (*Clavascidium* spp., *Placidium* spp., *Psora* spp.) estudiados en esta tesis donde solo se ha detectado como simbiontes predominantes *A. mediterranea* y *M. israeliensis*. Sin embargo, en estas especies no se han realizado todavía análisis adicionales de secuenciación masiva o con cebadores específicos; por tanto, surge la duda sobre si hay o no coexistencia debido a la limitación de las técnicas utilizadas.

Pese a estos datos, el fenómeno de la coexistencia y sus implicaciones en los estudios de diversidad continúan generando cierta controversia (Paul et al. 2018). La idoneidad del uso de la secuenciación Sanger para resolver cuestiones ecológicas y evolutivas en líquenes es incuestionable, pero es necesario precisar que la coexistencia puede variar según la especie de liquen y en algunos de ellos ser incluso "la norma", lo que se podido poner en evidencia en esta tesis. Además, el biotipo y el modo de vida de cada especie de liquen, podría favorecer la coexistencia de varias microalgas dentro de un talo, y estos factores deberían tenerse en cuenta cuando se plantean estudios sobre diversidad y patrones de asociación alga/hongo.

Switching y transferencia de microalgas

En una comunidad liquénica (*lichen guild*) es común que varias especies de líquenes posean/compartan las mismas microalgas como simbiontes, que constituyen su gremio de microalgas o *photobiont-mediated lichen guild* (Rikkinen et al. 2002). El proceso por el que ciertos líquenes son capaces de cambiar la especie de microalga predominante ya fue propuesto por Piercey-Normore y DePriest (2001) como *algal switching* (distintos géneros de hongos liquenizados comparten las mismas especies de algas). Este es un proceso ampliamente estudiado a lo largo de gradientes latitudinales y altitudinales, puesto que podría facilitar la adaptación de los líquenes a distintos entornos y a condiciones ambientales variables, debido a la nueva disponibilidad de algas en el *pool* y a los nuevos ambientes (Muggia et al. 2008; Fedrowitz et al. 2012; Vargas-Castillo y Beck 2012; Magain et al. 2017a; Dal Grande et al. 2018; Jüriado et al. 2019).

Además, el *algal switching*, está estrechamente relacionado con los conceptos de especificidad (posible rango taxonómico de socios admisibles) y selectividad (frecuencia de asociación entre socios compatibles). Estos patrones de asociación han sido ampliamente estudiados en distintas especies de un mismo género (Leavitt et al. 2015; Jüriado et al. 2019), y en esta tesis (R 3.3) se aborda por primera vez este fenómeno en una comunidad liquénica combinando el análisis "NGS" y Sanger. Las cuatro especies de *Trebouxia* encontradas (*T. asymmetrica*, *T. cretacea*, *Trebouxia* sp. OTU A25 y *T. vagua*) conformarían el *Trebouxia-mediated lichen guild* de esta comunidad liquénica en las biocostras de yesos que, a su vez, podrían participar en el *algal switching*. En esta comunidad, la flexibilidad de poder tener como microalga predominante cualquiera de las del gremio disponible, se ha observado en todos los líquenes crustáceos analizados (Figura D2) (R 3.3). Esta capacidad de cambiar de microalga predominante, en un mismo ambiente, posibilitaría que los líquenes de la biocostras se adapten a la variabilidad de las condiciones ambientes y, de esta manera, sobrevivir a las extremas condiciones de las costras biológicas de yesos.

Como ya hemos descrito anteriormente, en las comunidades liquénicas de las CBSs de yesos, se establecen relaciones parasitarias (o transitoriamente parasitarias). La combinación de técnicas de secuenciación (Sanger y "NGS") en R 3.3 evidencia que tanto el hospedador como los parásitos comparten el mismo gremio de microalgas simbióticas (*Trebouxia-mediated lichen guild).*

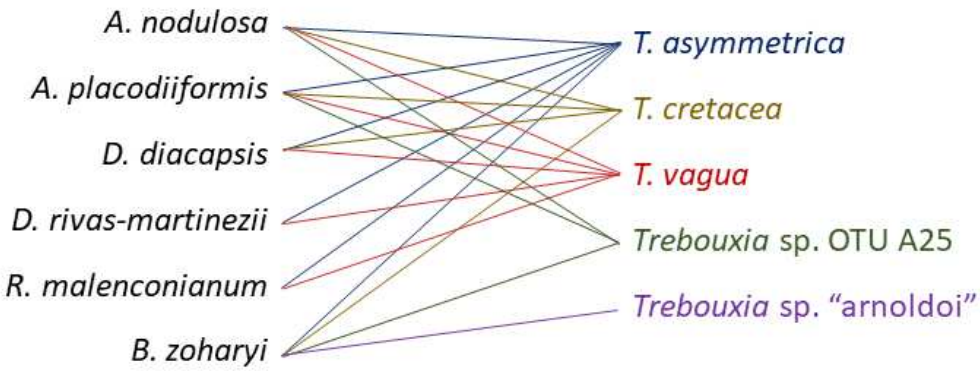

Figura D2. Diagrama de flechas en el que se representa el *algal switching* entre líquenes crustáceos de la CBSs de yesos (columna de la izquierda) y las especies de microalgas que constituyen el *Trebouxia-mediated lichen guild* (columna de la derecha).

Posiblemente, el hospedador (*Diploschistes diacapsis*) selecciona del sustrato estas microalgas, generando el *Trebouxia-mediated lichen guild* que estaría disponible para sus líquenes liquenícolas. En el caso de los líquenes "parásitos obligados" (*R. malenconianum*), la adquisición de microalgas, en los primeros estadios de desarrollo, se realiza de forma exclusiva a partir de las de su hospedador, por lo que ambos compartirían estas mismas microalgas. Sin embargo, especies como *A. nodulosa* y *A. placodiiformis* catalogadas como "parásitos transitorios" podrían a posteriori aceptar otras microalgas del *pool* que vive en el sustrato, ampliando así su diversidad en las etapas independientes (Ott et al. 1995; Dal Grande et al. 2014a).

En los individuos de *D. rivas-martinezii* analizados en R 3.3 únicamente se ha detectado *T. vagua* y *T. asymmetrica*. Esta liquen se desarrolla como saxícola en la misma comunidad que *Diploschistes diacapsis* y no presenta un estadio parásito, por lo que debe seleccionar sus especies compatibles del *pool* del sustrato y no de un hospedador. Aunque no se puede obviar que esta disminución de la diversidad encontrada para este liquen podría deberse a la ausencia de un análisis mediante secuenciación masiva o al bajo número de talos incluidos en el estudio.

Estos líquenes crustáceos con relaciones interespecíficas complejas (hospedador-parásito) son sistemas adecuados para analizar distintos patrones de asociación, ya que plantean un desafío para los conceptos clásicos de selectividad o especificidad.

4.1 Anexo I

Microalga	Liquen asociado	Autores
Asterochloris mediterranea	*Cladonia cervicornis*	R 3.1, Moya et al. 2015
	Cladonia foliacea	R 3.1, Moya et al. 2015
		Pino-Bodas et al. direct submission (FM205720 - FM205732)
	Cladonia fimbriata	Piercey-Normore y DePriest 2001
	Cladonia rangiformis	R 3.1, Moya et al. 2015
		Piercey-Normore y DePriest 2001
	Cladonia symphycarpa	Wedin et al. direct submission (KT215300, KT215301, KT215308, KT215309)
	Diploschistes muscorum	Wedin et al. direct submission (KT215303, KT215312)
	Lepraria isidiata	Chiva 2012
	Lepraria nylanderiana	Nelsen y Gargas 2008
	Stereocaulon vesuvianum	Vančurová et al. 2018

Tabla 1. Tabla con los resultados de la búsqueda en GenBank de los líquenes en los que se detecta el microalga *A. mediterranea*.

Microalga	Liquen asociado	Autores
Myrmecia biatorellae	*Sarcogyne privigna*	Perez-Ortega et al. 2012
	Placidium arboreum	Thüs et al. 2011
	Placidium lachneum	Thüs et al. 2011
	Psora decipiens	Williams et al. 2017
Myrmecia israeliensis	*Clavascidium* spp.	R 3.2, Moya et al. 2018
	Heteroplacidium imbricatum	Thüs et al. 2011
	Heteroplacidium contumescens	Thüs et al. 2011
	Placidium pilosellum	R 3.2, Moya et al. 2018
	Placidium sp.	R 3.2, Moya et al. 2018
	Placidium sp.1	R 3.2, Moya et al. 2018
	Placidium sp.2	R 3.2, Moya et al. 2018
	Placidium squamulosum	Thüs et al. 2011
	Placidium umbrinum	Thüs et al. 2011
	Psora decipiens	Ruprecht et al. 2014
		R 3.2, Moya et al. 2018
		Williams et al. 2017
	Psora saviczii	R 3.2, Moya et al. 2018
Myrmecia sp. URa20	*Psora decipiens*	Ruprecht et al. 2014
Myrmecia sp. URa23	*Psora decipiens*	Ruprecht et al. 2014

Tabla 2. Tabla con los resultados de la búsqueda en GenBank de los líquenes en los que se detectan los taxones simbióticos de *Myrmecia* (*M. biatorellae*, *M. israeliensis*, *Myrmecia* sp. URa20 y *Myrmecia* sp. URa23).

Microalga	Liquen asociado	Autores
Trebouxia asymmetrica	*Acarospora nodulosa*	R 3.3
	Acarospora placodiiformis	R 3.3
	Buellia zoharyi	Helms et al. 2001
		Chiva et al. 2016
	Caloplaca teicholyta	Voytsekhovich y Beck 2016
	Circinaria contorta	Voytsekhovich y Beck 2016
	Circinaria gyrosa	Molins et al. 2018b
	Circinaria hispida	Molins et al. 2018a, b
	Circinaria sp. *"oromediterranea"*	Molins et al. 2018b
	Circinaria sp. *"paramerae"*	Molins et al. 2018b
	Diploschistes diacapsis	UTEX 2507 y SAG 48.88, deposit. Friedl
		Friedl et al. 2000
		R 3.3
	Diplotomma rivas-martinezii	R 3.3
	Protoparmeliopsis muralis	Muggia et al. 2013b
		Guzow-Krzemińska 2006
		Guzow-Krzemińska y Stocker-Wörgötter 2013
	Psora decipiens	Ruprecht et al. 2014
	Rhizocarpon malenconianum	R 3.3
	Xanthoparmelia spp.	Leavitt et al. 2015
Trebouxia cretacea	*Acarospora nodulosa*	R 3.3
	Acarospora placodiiformis	R 3.3
	Aspicilia desertorum	Gasulla et al. 2010
		Voytsekhovich y Beck 2016
	Buellia elegans	Helms et al. 2001
	Buellia zoharyi	Chiva et al. 2016
	Circinaria gyrosa	Molins et al. 2018b
	Circinaria hispida	Molins et al. 2018a, b
	Circinaria sp. *"oromediterranea"*	Molins et al. 2018b
	Diploschistes diacapsis	R 3.3
	Diplotomma rivas-martinezii	R 3.3
	Gyalolechia fulgens	Ruprecht et al. 2014
	Lecidella stigmatea	Ruprecht et al. 2014
	Peltigera rufescens	Ruprecht et al. 2014
	Protoparmeliopsis garovaglii	Leavitt et al. 2015
	Protoparmeliopsis muralis	Guzow-Krzemińska 2006
		Guzow-Krzemińska y Stocker-Wörgötter 2013
	Psora decipiens	Ruprecht et al. 2014
	Rusavskia papillifera	Voytsekhovich y Beck 2016
	Squamarina lentigera	Ruprecht et al. 2014
	Rhizocarpon malenconianum	R 3.3
	Xanthoparmelia spp.	Leavitt et al. 2015
		Piercey-Normore 2009

Microalga	Liquen asociado	Autores
Trebouxia vagua	*Acarospora nodulosa*	R 3.3
	Acarospora placodiiformis	R 3.3
	Candelariella vitellina	Voytsekhovich y Beck 2016
	Circinaria contorta	Voytsekhovich y Beck 2016
	Circinaria gyrosa	Molins et al. 2018b
	Circinaria hispida	Molins et al. 2018a, b
	Circinaria sp. *"paramerae"*	Molins et al. 2018b
	Diploschistes diacapsis	Voytsekhovich y Beck 2016
		R 3.3
	Diploschistes muscorum	Wedin et al. direct submission (KT215313, KT215316, KT215317)
	Diplotomma rivas-martinezii	R 3.3
	Porpidia crustulata	Voytsekhovich y Beck 2016
	Rhizocarpon malenconianum	R 3.3
	Thamnolia sp. (lineage C)	Onuţ-Brännström et al. 2018
	Xanthoparmelia spp.	Leavitt et al. 2015
		Leavitt et al. 2013
Trebouxia sp. OTU A25	*Acarospora nodulosa*	R 3.3
	Acarospora placodiiformis	R 3.3
	Buellia zoharyi	Chiva et al. 2016
	Diploschistes diacapsis	R 3.3
	Diplotomma rivas-martinezii	R 3.3
	Rhizocarpon malenconianum	R 3.3
	Xanthoparmelia maricopensis	Leavitt et al. 2015

Tabla 3. Tabla con los resultados de la búsqueda en GenBank de los líquenes en los que se detectan *Trebouxia asymmetrica*, *T. cretacea*, *T. vagua* y *Trebouxia* sp. OTU A25.

Género microalga	Liquen asociado	Biotipo
Asterochloris	*Cladonia cervicornis*	Dimórfico A
	Cladonia rangiformis	
	Cladonia foliacea	Foliáceo B
	Lepraria isidiata	Leprarioide C
Myrmecia	*Psora decipiens*	Escuamuloso
	Psora saviczii	
	Clavascidium spp.	
	Placidium spp.	
Trebouxia	*Acarospora nodulosa*	Crustáceo
	Acarospora placodiiformis	
	Buellia zoharyi	
	Diploschistes diacapsis	
	Diplotomma rivas-martinezii	
	Rhizocarpon malenconianum	

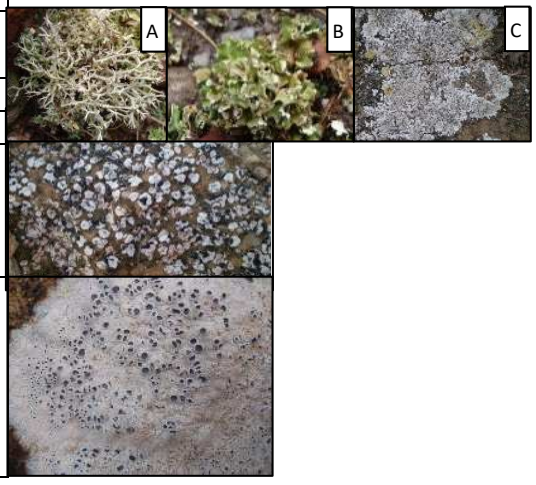

Tabla 4. Tabla en la que se relaciona cada género de microalga con los líquenes en los que se asocia y su biotipo liquénico. Imágenes: http://liquenesdealmeria.blogspot.com/

Género microalga	Especie microalga	Liquen asociado	Sustrato
Asterochloris	*A. mediterranea*	*Cladonia cervicornis*	Silíceo
		Cladonia foliacea	Calcáreo, silíceo y yesoso
		Cladonia rangiformis	Silíceo y volcánico
Myrmecia	*M. israeliensis*	*Clavascidium* spp.	Yesoso
		Placidium spp.	Calcáreo
		Psora decipiens	Calcáreo, volcánico y yesoso
		Psora saviczii	Yesoso
Trebouxia	*T. asymmetrica*	*Acarospora nodulosa*	Yesoso
		Acarospora placodiiformis	Yesoso
		Buellia zoharyi	Calcáreo, volcánico y yesoso
		Diplotomma rivas-martinezii	Yesoso
		Diploschistes diacapsis	Yesoso
		Rhizocarpon malenconianum	Yesoso
	T. cretacea	*Acarospora nodulosa*	Yesoso
		Acarospora placodiiformis	Yesoso
		Buellia zoharyi	Calcáreo, volcánico y yesoso
		Diploschistes diacapsis	Yesoso
	T. vagua	*Acarospora nodulosa*	Yesoso
		Acarospora placodiiformis	Yesoso
		Diplotomma rivas-martinezii	Yesoso
		Diploschistes diacapsis	Yesoso
		Rhizocarpon malenconianum	Yesoso
	T. sp. OTU A25	*Acarospora nodulosa*	Yesoso
		Acarospora placodiiformis	Yesoso
		Buellia zoharyi	Calcáreo y yesoso
	T. sp. "arnoldoi"	*Buellia zoharyi*	Volcánico

Tabla 5. Tabla en la que se relaciona cada género de microalga con su liquen asociado y el sustrato sobre el que se desarrolla.

Género microalga	Liquen asociado	Dispersión micobionte		Dispersión conjunta: micobionte-microalga	
		Vía apotecios (esporas sexuales)	Vía picnidios (esporas asexuales)	Vía estructuras especiales	Vía fragmentos del talo
Trebouxia	*Acarospora nodulosa*	+	+	—	+
	Acarospora placodiiformis	+	+	—	+
	Buellia zoharyi	+	—	—	+
	Diploschistes diacapsis	+	+	—	+
	Diplotomma rivas-martinezi	+	—	—	+
	Rhizocarpon malenconianum	+	—	—	+
Asterochloris	*Cladonia cervicornis*	— muy raro	+	—	+
	Cladonia foliacea	— muy raro	+	—	+
	Cladonia rangiformis	— muy raro	+	—	+
	Lepraria isidiata	—	—	+	+
Myrmecia	*Clavascidium* spp.	+peritecio		—	+
	Placidium spp.	+ peritecio		—	+
	Psora decipiens	+	+	—	+
	Psora saviczii	+	+	—	+

Tabla 6. Tabla en la que se relaciona cada género de microalga con su liquen asociado y sus estructuras de dispersión. Para *Clavascidium* spp. y *Placidium* spp. se representan las características principales pertenecientes al género ya que las especies aquí estudiadas todavía no han sido descritas, y por tanto, caracterizadas.

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5 Conclusiones

5 CONCLUSIONES

- Los líquenes del género *Cladonia* de las biocostras presentan especificidad hacia las microalgas del género *Asterochloris*, concretamente por *Asterochloris mediterranea*.

- La especie *Asterochloris mediterranea* también mostró alta selectividad por especies del género *Cladonia*.

- Se ha descrito una nueva especie de microalga simbiotica, *Asterochloris mediterranea* sp. nov. Barreno, Chiva, Moya et Škaloud.

- Los líquenes de las familias Verrucariaceae (*Clavascidium* spp. y *Placidium* spp.) y Psoraceae (*P. decipiens* y *P. saviczii*) exhiben un nivel de selectividad muy alto hacia las microalgas del género *Myrmecia*, concretamente por *Myrmecia israeliensis*.

- La especie *Myrmecia israeliensis* parece tener especificidad por los líquenes escuamulosos que se desarrollan en las CBSs.

- En los líquenes crustáceos; *Diploschistes diacapsis*, *Acarospora placodiiformis*, *A. nodulosa*, *Diplotomma rivas-martinezii* y *Rhizocarpon malenconianum*, se han detectado como socios simbióticos predominantes especies del género *Trebouxia*. En concreto, cuatro taxones del clado 'A': *T. asymmetrica*, *T. cretacea*, *Trebouxia* sp. OTU A25 y *T. vagua*.

- En especies con distribución cosmopolita como *C. foliacea* o *P. decipiens*, la variabilidad nucleotídica del micobionte es muy elevada en comparación con otros líquenes de carácter gipsófito preferente, tales como *A. nodulosa*, *D. diacapsis*, *P. saviczii* y *B. zoharyi*. Las gipsófitos exclusivos *A. placodiiformis* y *D. rivas–martinezii* presentan una diversidad genética menor que los gipsófitos preferentes o los cosmopolitas.

- Existe una relación entre la variabilidad nucleotídica del micobionte y la capacidad de colonización de distintos sustratos. Las especies de líquenes con menor preferencia hacia los yesos presentan mayor variabilidad genética y viceversa.

- La baja variabilidad genética actual en *B. zoharyi*, a lo largo de su actual área de distribución, puede estar reflejando los efectos de su pasado geológico, como la crisis Salina del Messiniense y la inundación Zancleana.

- Los líquenes de biotipo crustáceo contienen como socios fotosintéticos a especies del género *Trebouxia*. Los de biotipo escuamuloso, se asocia con microalgas del género *Myrmecia*. Los dimórficos, foliáceos y leprarioides eligen preferentemente especies del género *Asterochloris*.

- Existe una correlación entre los biotipos de los líquenes presentes en las biocostras y los géneros de microalgas con los que simbiotizan.

- No se ha detectado de los distintos tipos de sustratos analizados (calcáreos, silíceos, yesosos y volcánicos) tengan influencia en el establecimiento de una determinada microalga como simbionte predominante en los talos.

- No se ha podido establecer una relación entre los patrones de selectividad/ especificidad y las estructuras de reproducción/dispersión para el conjunto de los líquenes analizados en las biocostras.

- La coexistencia de múltiples ficobiontes en un mismo talo es un fenómeno frecuente en líquenes de distintas morfologías y que se desarrollan en diferentes tipos de sustratos.

- La arquitectura de los talos y el modo de vida de cada especie, podrían favorecer la coexistencia de varias microalgas dentro de un solo talo. Estos factores deberían tenerse en cuenta cuando se planteen estudios sobre diversidad y patrones de asociación alga/hongo.

- Los líquenes crustáceos de las CBSs, son capaces de asociarse con cualquiera de los cuatro taxones de *Trebouxia* disponibles en el *pool* del sustrato. Esta flexibilidad permite a esos líquenes adaptarse a las variaciones del ambiente y a las extremas condiciones ambientales de las biocostras.

- Los líquenes crustáceos con relaciones interespecíficas complejas como *D. diacapsis* y sus parásitos, son sistemas adecuados para analizar distintos patrones de asociación, ya que plantean un desafío para los conceptos clásicos de selectividad o especificidad.

6 DIFUSIÓN DE RESULTADOS

Microalgal selectivity patterns in biocrusts lichen communities

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Biocrusts are considered to be ecosystem pioneers in colonizing soil surfaces creating a consistent layer and binding soil particles due to their presence and activities. These biocrusts are particularly important in gypsum ecosystems which are normally dominated by lichens.
Different areas throughout the Iberian Peninsula show gypsum biocrusts, that are colonised by a well-developed terricolous lichen communities cohabiting in these areas: crustose species such as *Diploschistes diacapsis*, A*carospora placodiiformis*, *A. nodulosa* and *Buellia zoharyi*, occasionally accompanied by *Diplotomma rivas-martinezii* and *Rhizocarpon malenconianum*. A squamulose community composed of *Psora decipiens*, *P. saviczii*, *Clavascidium* spp. and *Placidium* spp.. Moreover, foliose (*Cladonia* spp.) covers microareas with an extra water.

We present a detailed study based on the myco/phycobiont relationships and its microalgal selectivity patterns. Barcode nrITS mycobiont analyses were performed to construct phylogenies, haplotype networks and biogeographical hypothesis. For the microalgae several nuclear and chloroplastic genetic markers were studied, moreover isolation and ultrastructural characterisation were included. Three different microalgal genera where detected in the community: *Trebouxia*, *Asterochloris* and *Myrmecia*. We suggest the morphology and/or growth type could be influencing the selection of each mycobiont for a particular microalgal genus.

(CGL2016-79158-P) (PROMETEO/2017/039).

THE COMPLEXITY OF LICHEN SYMBIOSIS: Novel interdisciplinary approaches from genomic to functional perspectives

SIMBIOLIQUEN

VALENCIA SPAIN

Biological Soil Crusts: myco/phycobiont relationships in terricolous lichen communities

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Biological soil crusts (biocrusts) are a mixture of organisms, both autotrophic and heterotrophic, which live on top or within the soil surface creating a consistent layer and binding soil particles due to their presence and activity (Belnap et al., 2003). They are considered to be ecosystem pioneers in colonize the soil surface. The lifestyle of lichens in biological soil crusts is remarkable due to the high level of morphological plasticity, photobiont switching and physiological adaptations (Green et al., 2018). These crusts are particularly notable in gypsum ecosystems which are normally dominated by lichens. They have a high conservation value due to their potential to form covers (sometimes >80%), their function, and their diversity (Ballesteros et al., 2017).

Different areas throughout the Iberian Peninsula showed gypsum biocrusts deposited in the late Miocene period, that are colonised by a well-developed terricolous lichen communities, mainly characterised by crustose species such as *Diploschistes diacapsis, Acarospora placodiiformis, A. nodulosa and Buellia zoharyi,* occasionally accompanied by *Diplotomma rivas-martinezii and Rhizocarpon malenconianum*. Also, a relevant squamulose community composed of *Psora decipiens, P. saviczii, Clavascidium* spp. *and Placidium* spp. are present in these Miocene gypsum areas. Moreover, foliose *Cladonia* spp. cover microareas with an extra water supply due to condensation.

In this study, the myco/phycobiont relationship for the complete lichen community has been analysed in detail. Barcode nrITS mycobiont analyses were performed to construct phylogenies, haplotype networks and biogeographical hypothesis. For the microalgae several nuclear and chloroplastic genetic markers were studied, moreover isolation and ultrastructural characterisation were included in the analyses. Three different microalgae genus where detected in the community: *Trebouxia*, *Asterochloris* and *Myrmecia*. Inthathalline coexistence was mainly detected in lichen species where the *Trebouxia* genus was the primarily microalgae. We suggest the morphology and/or growth type could be influencing the selection of each mycobiont for a particular microalgal genus.

Supported by the Ministerio de Economía y Competitividad (MINECO and FEDER, Spain) (CGL2016-391 79158-P), and the Prometeo Excellence in Research Program (Generalitat Valenciana, Spain) (PROMETEO/2017/039).

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SOCIEDAD ESPAÑOLA DE LIQUENOLOGÍA (SEL)
VIII JORNADA DE LIQUENOLOGÍA
SIERRA NEVADA
8 de septiembre de 2018

Relaciones entre el micobionte y el ficobionte de las comunidades liquénicas de la CBS

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Las costras biológicas del suelo son comunidades bióticas formadas por la estreacha asociación entre partículas del suelo, cianobacterias, algas, hongos, líquenes, hepáticas y briófitos. Se encuentran ampliamente distribuidas en muchos tipos de suelo, como en los de yesos miocenos. Las comunidades liquénicas de estos suelos están conformadas por especies en su mayoría de hábito gipsícola. El biotipo más generalizado es el crustáceo, que permite un estrecho contacto con el suelo. Además, en aquellos microambientes donde es posible un aporte extra de agua por condensación, pueden aparecer talos fruticulosos y/o foliáceos.

Se ha llevado a cabo un pormenorizado estudio sobre la diversidad genética del micobionte y los ficobiontes de *Acaropora nodulosa*, *A. placodiiformis*, *Buellia zoharyi*, *Cladonia convoluta*, *Clavascidium lacinulatum*, *Diploschistes diacapsis*, *Diplotomma rivas-martinezii*, *Psora decipiens*, *P. saviczii* y *Rhizocarpon malenconianum*. Para la correcta identificación del micobionte de los talos analizados se ha realizado un análisis filogenético utilizando la región barcode nrITS. En el caso de los ficobiontes se utilizaron marcadores moleculares nucleares y cloroplasticos en los 1 0 líquenes analizados. Se detectaron un total de7 tipos de microalgas diferentes. Algunos de estos ficobiontres son esclusivos de un único taxon, en cambio otros se pueden encontrar también en taxones difererentes.

(MINECO y FEDER, CGL2016-79158-P)(GVA, PROMETEO / 2017/039).

XXI SIMPOSIO DE BOTÁNICA CRIPTOGÁMICA

ARANJUEZ 20-24 JUNIO DE 2017

Universidad Rey Juan Carlos

BIODIVERSIDAD Y BIOGEOGRAFÍA-Presentaciones orales

BIO-O8. HINTS THAT DIFFERENT GEOLOGICAL EVENTS IN THE MEDITERRANEAN HAD INFLUENCE OVER THE DISTRIBUTION OF *BUELLIA ZOHARYI*

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Biological Soil Crusts (BSCs) are biologically modified soil surfaces composed of a combination of organisms which include lichens, bryophytes, liverworts, microalgae, cyanobacteria, and microfungi. BSCs are complex communities due to the many different organisms involved and functional aspects they provide. *Buellia zoharyi* Galun is a prevailing lichen species in xerophyte Mediterranean BSC. In the current literature, this lichen was described as highly specific in gypsum soils, but has also been found scarcely in other types of substrates such as volcanic soils and limestones under maritime influence. In this study, 24 different populations covering the entire range of this species in the field, from NW Africa to the Canary Islands and to Iran, were selected. A preliminary analysis was conducted from selected thalli using several molecular markers: nrITS DNA, mtSSU, ßtub, *rpb2* and TEF1α. Due to the results obtained, phylogenetic relationships between the samples were established using nrITS DNA. Moreover population analyses (haplotype networks, polymorphism statistics and neutrality tests) and molecular dating were performed with nrITS DNA and TEF1α. *B. zoharyi* makes up a monophyletic clade which is included by a recent study whitin the phylogeny of the *Caliciaceae*. The differential haplotype distribution obtained and the divergence time estimations could suggest that certain past geological events occurred in the Mediterranean region may drive the actual distribution of the populations of *B. zoharyi*.

XXI SIMPOSIO DE BOTÁNICA CRIPTOGÁMICA

ARANJUEZ 20-24 JUNIO DE 2017

Universidad Rey Juan Carlos

SISTEMÁTICA Y EVOLUCIÓN-Pósters

SIST-P2. *MYRMECIA ISRAELIENSIS* AS THE PRIMARY SYMBIOTIC MICROALGA IN TERRICOLOUS SQUAMULOSE LICHENS ACROSS EUROPE AND CANARY ISLANDS

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Myrmecia israelensis (S.Chantanachat & H.Bold) T.Friedl has been traditionally considered as a green coccoid free-living microalga. This microalga was suggested as the primary phycobiont in two related lichen genera *Placidium* and *Heteroplacidium*, and recently in *Psora decipiens*. However, due to the absence of nrITS DNA sequences (barcode information) these previous studies could not confirm this microalga as living in symbiotic state. The aim of this study was to settle the presence of *M. israelensis* as the primary microalga in different terricolous squamulose lichen species (*Psora* spp., *Placidium* spp. and *Clavascidium* spp.) by using both molecular and ultrastructural techniques, along to 31 localities from European and Canarian ecosystems. The lichen forming-fungi were identified using ITS rADN as barcode. Phycobiont phylogenetic analyses were made using both chloroplast (LSU rDNA) and nuclear (nrDNA ITS) molecular markers. In addition, fluorescence microscopy, transmission electron microscopy and scanning electron techniques were used to characterize *M. israelensis*. The mycobiont analyses for *P. decipiens* showed a surprisingly variability in this lichen species. Our results proved *M. israelensis* to be the primary symbiotic microalga in all the lichens analyzed, which its nrITS DNA barcode information was obtained for the first time by our team. Finally the presence of this microalga was verified using different microscopic observations. Combination of different techniques both molecular and microscopic allowed to the accurate identification of this symbiotic microalga, beforehand only known as free living.

Meeting of the *TREBOUXIA-WORKING* group

Trieste, Italy, 26-28 September 2016

Improved propagation method, rapid molecular identification and ultrastructural characterization as a multidisciplinary approach for *Trebouxia* species delimitation

Patricia MOYA[1], Salvador CHIVA[1], Arantxa MOLINS[1], Francisco GARCÍA-BREIJO[2], José REIG-ARMIÑANA[1], Eva BARRENO[1]

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The detection of new *Trebouxia* lineages is increasing dramatically, arising the need for an overall revision of taxonomic concepts to identify and delimit species boundaries. Multidisciplinary standardized approaches should be established in the lichenological community to avoid duplicating names.

An improved method for rapid propagation and molecular identification was designed to easily separate and identify the predominant microalgal diversity. Moreover, the coexistence of several phycobionts needs to be studied using comparative ultrastructural analyses in symbiotic and culture states, as some taxonomic features remain fairly stable both within the lichen thalli and in culture, and this would allow us to identify and distinguish them from other co-occurring species. The ability to correlate molecular and ultrastructural data represents a necessary improvement in clarifying the taxonomy of *Trebouxia* diversity both in the symbiotic and culture states.

Buellia zoharyi can be considered a model lichen for setting the boundaries when several *Trebouxia* lineages coexist. We have selected populations covering the entire range (from the Canary Islands, Mediterranean surrounding areas, and western Asia) of the species in the field. Phycobiont phylogenetic analyses were made using both chloroplast (LSU rDNA) and nuclear (nrDNA ITS) molecular markers. In addition, ultrastructural microscopic techniques were used to characterize each of the microalgae found. Our results evidence the presence throughout all populations of at least four different *Trebouxia* lineages. Furthermore, phycobiont coexistence events recovered in all the analyzed populations is strengthened by the presence of at least three lineages of co-occuring microalgae.

The integration of diverse techniques has led to considering symbiotic interactions more complex events, and to deal with lichen symbioses from a multifaceted point of view to efficiently reveal the hidden diversity of intrathalline microalgae inside a single thallus (phycobiont coexistence). (GVA, PROMETEOII/2013/021; MINECO, CGL2012-40058-C02-01; FEDER)

VII Jornadas de Liquenología, Ronda.

SOCIEDAD ESPAÑOLA DE LIQUENOLOGÍA (SEL)

Análisis de la estructura poblacional de *Buellia zoharyi*: liquen con baja diversidad genética y distribución geográfica amplia y disyunta

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Las costras biológicas del suelo (CBS) son comunidades bióticas formadas por la íntima asociación entre partículas del suelo, cianobacterias, algas, hongos, líquenes, hepáticas y briófitos, se encuentran ampliamente distribuidas en muchos tipos de suelo, aunque predominan en ambientes extremos. *Buellia zoharyi* Galun es un liquen característico de las CBS xerófilas mediterráneas, aunque tradicionalmente se ha descrito como una especie con alta especificidad por los suelos yesosos, también se localiza en otro tipo de sustratos, como volcánicos y calizos con influencia marítima.

Se ha llevado a cabo, por primera vez para esta especie, un profundo estudio sobre la diversidad genética del micobionte, incluyendo poblaciones que abarcan toda su área actual de distribución. En primer lugar, se realizó un análisis de individuos seleccionados de diferentes poblaciones, usando el marcador nuclear ribosomal ITS, el mitocondrial ribosomal mtSSU y fragmentos de los genes codificantes para la β-tub, *rpb2*, TEF1α. Los únicos marcadores moleculares informativos fueron ITS y TEF1α, aunque la variabilidad genética observada en este taxon fue escasa. Por tanto, los análisis filogenéticos, de diversidad genética y de variabilidad poblacional se realizaron para todos los individuos de todas las poblaciones con estos dos marcadores seleccionados.

Los análisis filogenéticos evidenciaron la robustez del taxón y de nuevo la escasa diversidad genética de la especie. Además, se observó una distribución desigual de los haplotipos encontrados en las distintas localidades que podría sugerir una relación entre dicha distribución y ciertos eventos geológicos ocurridos en el pasado.

(MINECO, CGL2012-40058-C02-01)(GVA, PROMETEOII / 2013/021).

Buellia zoharyi populations show noticeable microalgal diversity throughout their entire range of distribution

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Buellia zoharyi Galun is a widespread lichen on sun-exposed biological soil crusts (BSC) under xeric Mediterranean bioclimatic conditions. This species was said to be highly specific to gypsum soils, but it has also been found occasionally in a great variety of basic substrata. We have selected 24 different populations covering the entire range of the species in the field, from NW Africa to the Canary Islands and to Iran. The *B. zoharyi* mycobiont was identified using ITS rADN as barcode. Phycobiont phylogenetic analyses were made using both chloroplast (LSU rDNA) and nuclear (nrDNA ITS) molecular markers. In addition, ultrastructural microscopic techniques were used to characterize each of the microalgae found. Our results evidence the presence throughout all populations of at least four different *Trebouxia* lineages. Furthermore, phycobiont coexistence events occur in all the analyzed populations, strengthened by the presence of at least three taxa of intrathalline microalgae. In conclusion, these data seem to highlight that *Trebouxia* diversity inside a single thallus could be an advantageous event under extreme environmental conditions. (MINECO, CGL2012-40058-C02-01), FEDER, (GVA, PROMETEOII/2013/021)

Coexistence and microalgae diversity covering the entire range of distribution in *Buellia zoharyi* lichen populations

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Buellia zoharyi Galun is a terricolous lichen prevailing in Biological Soil Crusts (BSC) of xeric areas.
It seems to be specific to gypsum and carbonated soils.
The present study analyzes 202 lichen samples (including fresh and herbarium material) from 24 different populations covering the entire known distribution of *B. zoharyi* (Fig. 1). This lichen species shows an Irano-Turanian and North African Atlantic disjunt distribution.

Twenty-four *B. zoharyi* populations covering the entire range of the species.

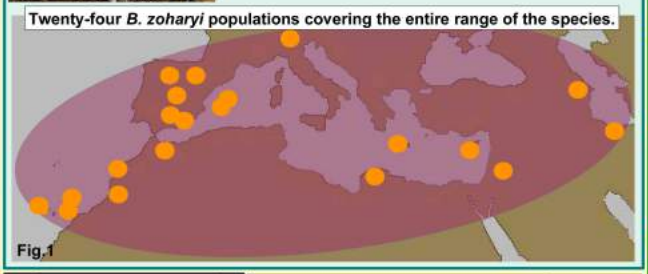
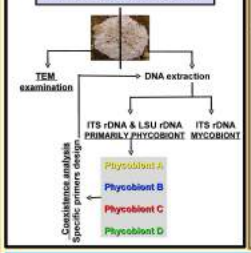


Fig.1

METHODOLOGY



MYCOBIONT IDENTIFICATION

Mycobiont ITS rDNA (Fig.2) phylogenetic analysis showed all the sequences grouped in an unique clade including the Genbank sequence of *Buellia zoharyi*, thus corroborating the precise lichen sample identification.

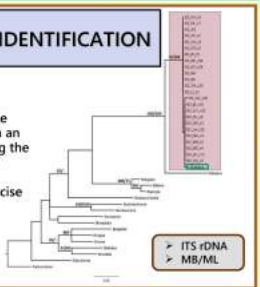


Fig.2

PHYLOGENETIC ANALYSIS of MICROALGAE

Primarily phycobiont distribution

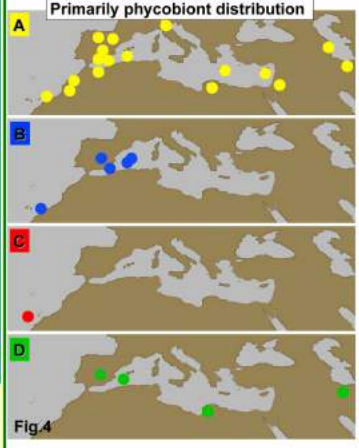


Fig.4

Phylogenetic analysis (Fig. 3) of the primarily phycobiont (ITS rDNA & LSU rDNA concatenated) detected in each sample, showed four clades termed as: Phycobiont A, Phycobiont B, Phycobiont C and Phycobiont D.

The range of the distribution of each primarily phycobiont detected are shown in Fig. 4.

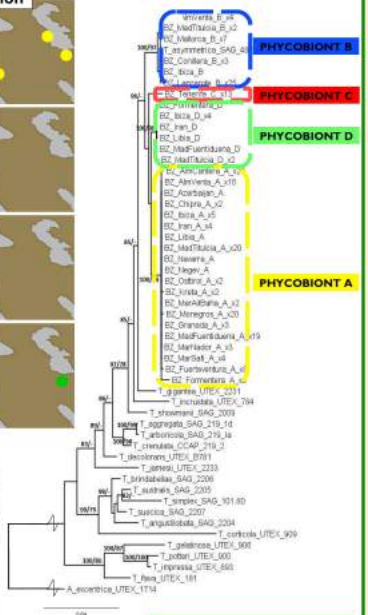
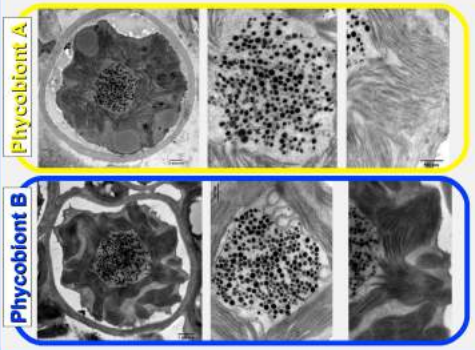


Fig.3

➢ITS rDNA & LSU rDNA concatenated
➢MB/ML

ULTRASTRUCTURAL COMPARATIVE ANALYSIS



One thallus per phycobiont-type was selected to perform trasmission electron microscopic (TEM) examinations. Selected samples have not shown coexistence events by Sanger sequencing. We asume that the microalgae ultrastructural characteristics match with the primarily phycobiont detected in this thallus.

Pyrenoid type in Phycobionts A, B, C and D matches the gigantea-type described by Friedl (1989), but showed characteristic differences of each type.

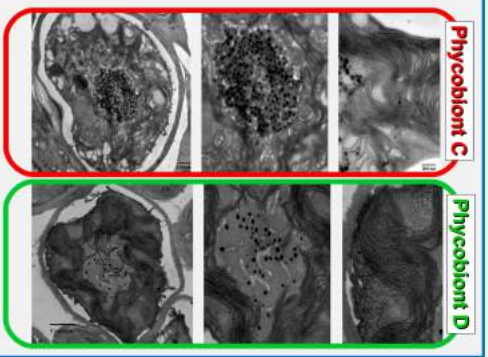
Our results suggest that these phycobionts could be distinguished by chloroplast morphologies:

Phycobiont A showed a lobed chloroplast with grouped thylakoid membranes but it was unpackaged.

Phycobiont B showed a deeply ramified chloroplast with grouped thylakoid membranes in stacks similar to those of the grana of vascular plants.

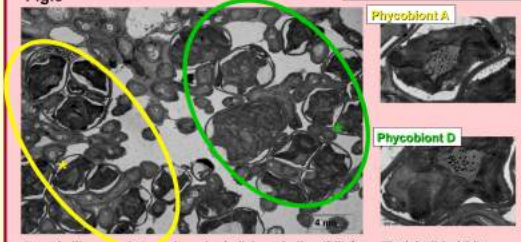
Phycobiont C showed the thylakoids loosely organized in the chloroplast.

Phycobiont D showed the thylakoids strongly organized in the chloroplast, but not grouped.



MICROALGAL COEXISTENCE

Fig.5



Intrathalline coexistence in a single lichen thallus (D7) from Titulcia (Madrid) between phycobiont A and D detected by TEM. * Detail of each phycobiont type detected.

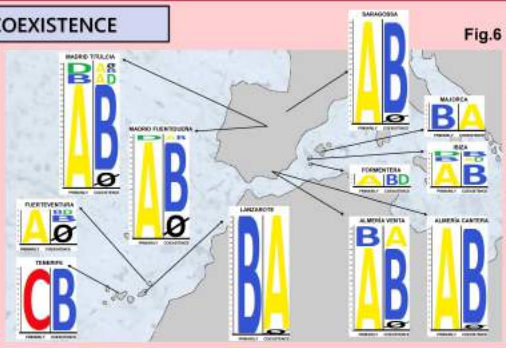


Fig.6

The specific primers were tested in 163 fresh samples (Fig.6).

Coexistence between two or three phycobionts were detected in 145 thalli, the remaining thalli only presented one phycobiont.

Phycobionts A and B appeared coexisting covering the entire range distribution of the species, except in Tenerife were Phycobiont C appeared restricted and only coexisting with Phycobiont B.

Phycobiont D appears only in a few samples.

La scheme of FUERTEVENTURA.
This population contained 7 lichen samples.
All samples showed Phycobiont A as PRIMARILY, in 4 samples coexistence was not detected (/Ø), 2 showed coexistence A/B and one showed coexistence A/BD.

References: Friedl (1989) Comparative ultrastructure of pyrenoids in *Trebouxia* (Microthamniales, Chlorophyta). – Plant Syst. Evol. 164: 145–159.

ACKNOWLEDGEMENTS: (MINECO. CGL2012-40058-C02-01). FEDER. (GVA. PROMETEOII/2013/021).

XX SIMPÓSIO DE BOTÂNICA CRIPTOGÂMICA
PORTO, 22 A 25 DE JULHO DE 2015

Coexistence and prevalence of symbiotic microalgae in *Buellia zoharyi* lichen: are substrata and/or biogeographic barriers involved?

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Biological soil crusts (BSC) frequently cover open spaces in xeric territories and are set up by highly specialized communities of lichens, mosses, bacteria and fungi. Lichens are common components of these communities, adapted to extreme environmental conditions.
Scientists are reviewing lichen characterization; these organisms are more complex than the marriage between a mycobiont and a phycobiont or cyanobiont, because non-phototrophic lichenic bacteria seem to also be implied in the symbiogenesis of thalli. Additional complexity was reported inside a single lichen thallus by the intrathalline coexistence of different algal species and/or genera. This process could be advantageous under extreme environmental conditions, and might be a more common phenomenon in ecologically adaptive lichens.
Buellia zoharyi Galun, a lichen species prevailing in the BSC, is said to be highly specific to gypsum soils, but it has also occasionally been found in other types of substrata.
We have selected six different populations, three on Miocene gypsum soils from the Iberian Peninsula (Almería, Madrid and Zaragoza) and three others from other substrata: two of them growing on volcanic soil in the Canary Islands (Lanzarote and Tenerife) and one other on calcareous soil in the Balearic Islands (Mallorca).
The *B. zoharyi* mycobiont was identified using ITS rADN as barcode. Phycobiont phylogenetic analyses were made using both chloroplast (LSU rDNA) and nuclear (nrDNA ITS) molecular markers. In addition, ultrastructural microscopic techniques were used to characterize each phycobiont found.
Our results suggest the presence of at least three different *Trebouxia* lineages. Furthermore, phycobiont coexistence events occur in all the populations analyzed, corroborated by the presence of at least two taxa of intrathalline symbiotic microalgae. The presence of a specific type of Trebouxia lineage as the prevailing phycobiont in each population, and the detection in Tenerife of a unique lineage which was not detected in the other locations, points out that the phycobiont distribution could be biased by the type of substrata or/and biogeographic barriers. In conclusion, these unexpected data seem to highlight these unique habitats as target areas for preservation.

(MINECO, CGL2012-40058-C02-01), FEDER, (GVA, PROMETEOII/2013/021).

EMBO 2015 - ABSTRACTS OF THE POSTERS

Complexity of lichen symbiosis: genomic diversity and coexistence of various green microalgae in the epigeous lichen *Buellia zoharyi*

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Lichens illustrate how individuality in eukaryotes is composite, complex and multi-dimensional. Lichen thalli are complex holobionts, which have originated from cyclical symbiosis involving a fungus, one or several photoautotrophs-green microalgae, cyanobacteria or both, and abundant non-photosynthetic bacteria. Lichenization allows the partners to thrive in extreme environmental conditions.

Buellia zoharyi Galun is a lichen prevailing in Biological Soil Crusts (BSC) in xeric areas. It seems to be specific to gypsum and carbonated soils. Six populations were selected, three on Miocene gypsum soils from the Iberian Peninsula and three more growing in other substrata: two from the Canary Islands and another one from the Balearic Islands. Also, herbarium samples from Morocco and Greece were included. The *B. zoharyi* mycobiont was identified using ITS rDNA as barcode. Green microalgae phylogenetic analyses were made using both chloroplast (LSU rDNA) and nuclear (nrDNA ITS) molecular markers. In addition, ultrastructural microscopic techniques were used to characterize each phycobiont found.

Our results suggest the presence of at least three different *Trebouxia* lineages. Furthermore, phycobiont coexistence inside a single thallus occurs in all the populations analyzed, defined by the presence of at least two taxa of intrathalline symbiotic microalgae. The intrathalline coexistence of different algal species and/or genera inside a single lichen thallus has recently been reported by our team. This process could be advantageous under extreme environmental conditions, and might be a common phenomenon in ecologically adaptive lichens, as in the case of *B. zoharyi.*

(MINECO, CGL2012-40058-C02-01), FEDER, (GVA, PROMETEOII/2013/021).

Complexity of lichen symbioses: genomic diversity and coexistence of various green microalgae in the epigeous lichen Buellia zoharyi

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Localities included in the study: three on Miocene gypsum soils from the Iberian Peninsula and three more growing in other substrata: two from the Canary Islands (volcanic) and another one from the Balearic Islands (calcareous). Also, herbarium samples from Morocco, Cyprus, Greece,and Fuerteventura were included.

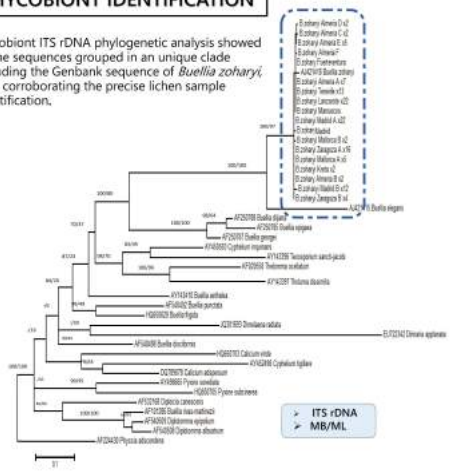
Buellia zoharyi Galun is a terricolous lichen prevailing in Biological Soil Crusts (BSC) of xeric areas. It seems to be specific to gypsum and carbonated soils

METHODOLOGY

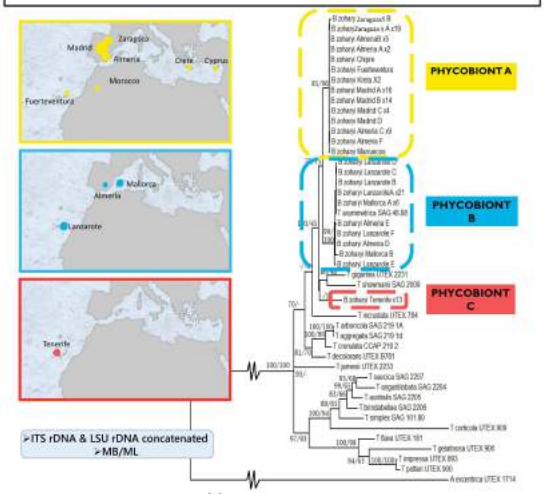
The most frequent methodology followed in lichen studies can be seen on this scheme: first, washing the lichen thalli to avoid contamination then DNA extraction and usually nuclear ITS PCR to identify the phycobiont and the mycobiont by Sanger sequencing. We decided to divide each thallus into two sections to avoid biasing each section and we used one section of the thallus to perform transmission electron microscopy (TEM) examinations.

MYCOBIONT IDENTIFICATION

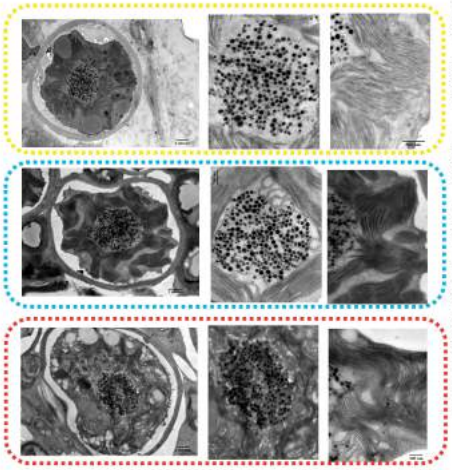
Mycobiont ITS rDNA phylogenetic analysis showed all the sequences grouped in an unique clade including the Genbank sequence of *Buellia zoharyi*, thus corroborating the precise lichen sample identification.



PHYLOGENETIC ANALYSIS of MICROALGAE (phycobionts)



ULTRASTRUCTURAL COMPARISONS



Phylogenetic analysis of the microalgae -using nrITS DNA and LSU rDNA markers- showed three well supported clades named; Phycobiont A, Phycobiont B and Phycobiont C.

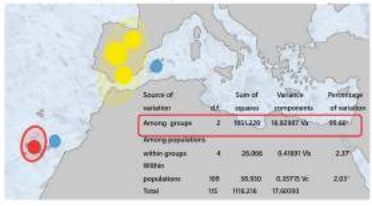
Phycobionts A and B show a wide range distribution meanwhile Phycobiont C appears to be restricted to Tenerife island.

We selected one thallus per phycobiont-type to perform trasmission electron microscopic (TEM) examinations.

Pyrenoid type in Phycobionts A, B and C matches into the gigantea-type described by Friedl.

Our results suggest that these phycobionts could be distinguished by chloroplast morphologies:
Phycobiont A showed a lobed chloroplast with thylakoid membranes grouped in stacks similar to those of the grana of vascular plants.
Phycobiont B showed a deeply ramified chloroplast.
Phycobiont C showed the thylakoids loosely organized in the chloroplast.

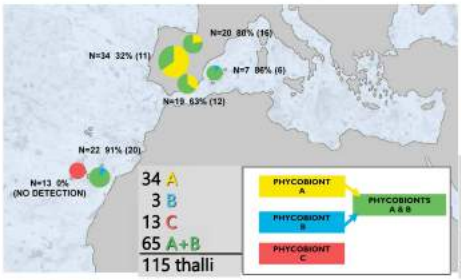
POPULATION ANALYSIS of PHYCOBIONTS (AMOVA) by the type of MICROALGAE DETECTED



Sequences were separated (groups) by the phycobiont-type detected: A, B or C

AMOVA analysis showed that phycobiont-type have influence in population variability. These results agree with the previous inferred from phylogenetic relationships that is, these microalgae (A, B and C) could represent different lineages.

MICROALGAL COEXISTENCE



We designed specific primers to detect each phycobiont. We tested these three primer pairs in all the 115 thalli
More than the 50% of thalli showed coexistence betwenn phycobionts A and B (highlighted in green)
Phycobiont A was the predominant in the Iberian Peninsula populations, Phycobiont B was the predominant in Lanzarote and Mallorca islands
No coexistence events were detected in Tenerife samples

ACKNOWLEDGEMENTS: (MINECO, CGL2012-40058-C02-01), FEDER, (GVA, PROMETEOII/2013/021).

EMBO Conference
Comparative Genomics of Eukaryotic Microorganisms: Complexity Patterns in Eukaryotic Genomes
19 – 24 October 2013 | Sant Feliu de Guixols, Spain

Patricia Moya, Salvador Chiva and Eva Barreno

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Coexistence and switching of chlorobionts (symbiotic microalgae) between Lichens and Lichenicolous lichens

Biological soil crusts (BSC) frequently cover open spaces between the scarce plant vegetation in arid territories. BSC are set up by highly specialized communities of lichens, mosses, bacteria and fungi. Lichens are common components of these communities adapted to extreme enviromental conditions. Lichenicolous lichens are not rare in these habitats which have parasitic phases in their life cycle when growing over other crustose lichens. Taking over an existing thallus provides a strategy for successful establishment of lichens in colonizing spaces available on soils. Previous works suggest that fungi have a specific range of compatible chlorobionts and select algal strains correlated with habitat conditions. Recent studies have discovered that switching among green algal strains is also a common phenomenon in lichens, and the number of selectable microalgal partners is variable among fungal species. Additional complexity was discovered in *Ramalina farinacea,* where the coexistence of two algal taxa appears as a consistent character among the populations of this lichen (Casano et al. 2011). Coexistence of physiologically different algal phenotypes could be advantageous under extreme environmental conditions, and might be a more common phenomenon in ecologically adaptive lichens.

BSC in two semiarid Miocene gypsum localities were analysed in terms of specificity and selectivity between the phycobiont relationships *Acarospora nodulosa*, *A. placodiiformis*, *Rhizocarpon malenconianum* and *Diplotomma rivas-martinezii,* lichenicolous lichens that begin to grow on *Diploschistes diacapsis* becoming independent later on. We compared nrDNA ITS sequences for both symbionts (mycobionts and phycobionts) and LSU rDNA (only in algae) to asses the degree of variability and specificity of both symbionts. This is the first time that nrDNA ITS sequences for *A. nodulosa*, *A. placodiiformis* and *R. malenconianum* have been studied. The chlorobionts in this community formed a strongly supported monophyletic group within the genus *Trebouxia* but, two distinct and well-supported algal clades were distinguished. One of them was clustered in the clade of *Trebouxia asymmetrica* and the other one in the *Trebouxia sp.* Ura3 (isolated from the antarctic *Lecidea cancriformis*). Every one of the five lichen and lichenicolous species studied here allocates these two algal strains even living together.

Our results suggest the coexistence of at least two different *Trebouxia* taxa, and show now the four lichenicolous lichens can share and steal its phycobionts in an event called algal switching from *D. diacapsis* to the other lichens which begin its development in it and, clearly show both events of coexistence and algal switching together. It is possible o hypothesize that extreme environmental conditions in the habitat could influence in both symbiotic partners and could select their algal strains according to its tolerance and

COEXISTENCE AND ALGAL SWITCHING OF CHLOROBIONTS (SYMBIOTIC MICROALGAE) BETWEEN LICHENS AND LICHENICOLOUS LICHENS

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INTRODUCTION

Biological soil crusts (BSC) frequently cover open spaces between the scarce plant vegetation in arid territories. Lichens are common components of these BSC adapted to extreme enviromental conditions, and also Lichenicolous lichens which have parasitic phases in their life cycle when growing over other crustose lichens. Taking over an existing thallus provides a strategy for successful establishment of lichens in colonizing spaces available on soils. This event occurs with 4 species of lichenicolous lichens (*Acarospora nodulosa*, *Acarospora placodiiformis*, *Rhizocarpon malenconianum* and *Diplotomma rivas-martinezii*) that begin to grow on the crustose lichen *Diploschistes diacapsis*.

Previous works suggest that fungi have a specific range of compatible chlorobionts and select algal strains correlated with habitat conditions. Recent studies have discovered that switching among green algal strains is also a common phenomenon in lichens (Piercey-Normore and DePriest), and the number of selectable microalgal partners is variable among fungal species. Additional complexity was discovered in *Ramalina farinacea*, where the coexistence of two algal taxa appears as a consistent character among the populations of this lichen (Casano et al. 2011). Coexistence of physiologically different algal phenotypes could be advantageous under extreme environmental conditions, and might be a more common phenomenon in ecologically adaptive lichens.

OBJECTIVES

*Analyze two semiarid Miocene gypsum areas in terms of specificity and selectivity relationship between the phycobiont of *Acarospora nodulosa*, *A. placodiiformis*, *Rhizocarpon malenconianum*, *Diplotomma rivas-martinezii*, and *Diploschistes diacapsis*.

*Compare nrDNA ITS sequences for both symbionts (mycobionts and photobionts) and LSU rDNA (only in algae) to asses the degree of variability and specificity of both symbionts.

*Obtain for the first time nrDNA ITS sequences for *A.nodulosa*, *A.placodiiformis* and *R. malenconianum*.

RELATIONSHIP BETWEEN SPECIES

A: General view of a biological soil crust (BSC) in a semiarid Miocene gypsum area.

B: Detail of the relationship between *Diploschistes diacapsis*(B1) and its lichelicolous lichens; *Acarospora nodulosa* (B2) and *Acarospora placodiiformis*(B3)

C: Relationship between *Diplotomma rivas-martinezii* (C1) and *Diploschistes diacapsis* (C2)

D: Relationship between *Rhizocarpon malenconianum* (D1) and *Diploschistes diacapsis* (D2)

CHLOROBIONT SWITCHING

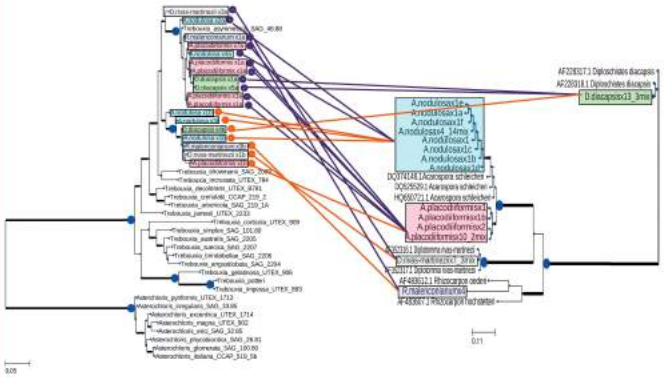


Fig1:Maximun likelihood tree based on the concatenated nrDNA ITS+LSU rDNA for photobionts and only for nrDNA ITS for mycobionts. Blue circle at the nodes indicate statistical support estimated by bootstrap with 100% values, and highlighted lines values with >70%. Each lichen specie is indicate in both trees with the same colour. Number of samples analyzed are indicate following by an x, we also indicate the number of samples that showed both photobionts in the same PCR that were excluded for contructing this tree (number_mix). Photobionts type A similar to *Trebouxia asymmetrica* are indicated in purple and the new detected Photobiont type B in orange.

CHLOROBIONT COEXISTENCE

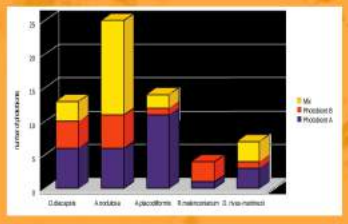


Fig 2: Photobiont diversity associated with the five species. Photobiont type A is indicated in purple and type B in orange. Samples that showed both photobionts are indicated in yellow.

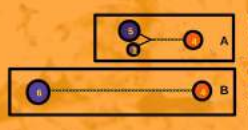


Fig 3: Haplotype network for LSU rDNA (A) and nrDNA ITS (B) for *D. diacapsis*. Photobionts A and B are indicated in purple and orange respectively. Number of haplotypes are indicated within the dots. Each yellow circle represents one mutational step.

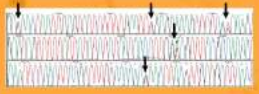


Fig 4: Electrophoretogram of a LSU rDNA PCR for a *D. diacapsis* sample that showed mix of photobionts in the same PCR. Arrows indicate doble peaks position.

Table 1: Parsed unique BLAST hits for each photobiont type with E value=0.0 and with host and country tag complete at the GenBank

CONCLUSIONS:

- Sequence in both genes with doble peaks (Fig 4) revealed the coexistence of photobiont A and B in the same thallus (Fig 2 in yellow), haplotype network defined each photobiont separate by 9 and 41 mutational steps (Fig 3).
- Chlorobiont switching events occurs in all species (Fig 1) probably due to the parasitic relationship between them.
- The same degree of photobiont selectivity was detected for all lichens, preference for one specific photobiont seems not to be detected. Both photobiont A and B appeared in all lichen species. Presence of apotecia in all species could influence in the way of dispersion and the maintenance of both photobionts together.
- Probably these photobionts could resist advers environmental conditions related with temperature. Both photobionts specially type B appear in lichens in extreme cold areas.
- ***A. nodulosa*, *A. placodiiformis* and *R. malenconianum* was defined in a well supported clades**

References: Casano L.M., et al., Environ Microbiol 13 (3), 806-818. 2001 / Piercey-Normore and DePriest., Am J of Botany 88 (8), 1490-1498. 2001

Acknowledgements: This study was funded by the Spanish Ministry of Economy and Innovation (MINECO CGL2012-40058-C02-01/02), FEDER and the Generalitat Valenciana (PROMETEO 021/2013 GVA). Sequencing was performed in the Servicio de Secuenciación de ADN, IBMCP- Universidad Politécnica de Valencia.

ESPECIFICIDAD Y VARIABILIDAD DE LOS FICOBIONTES IDENTIFICADOS EN LÍQUENES DE COMUNIDADES GIPSÍCOLAS

Trabajo Fin de Máster

Salvador Chiva Natividad

Visto Bueno de las directoras del

Trabajo de Fin de Master:

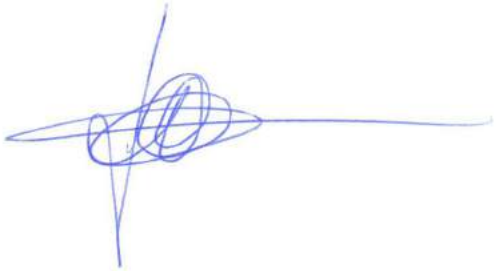
ESPECIFICIDAD Y VARIABILIDAD DE LOS FICOBIONTES IDENTIFICADOS EN LÍQUENES DE COMUNIDADES GIPSÍCOLAS

Realizado por:

J. SALVADOR CHIVA NATIVIDAD

DRA. EVA BARRENO RODRÍGUEZ
Prof. Catedrática de Botánica
Universitat de València

DRA. PATRICIA MOYA GAY
Contratada Técnico Superior
Universitat de València



Noviembre 2012

El Tribunal Evaluador acordó otorgar la calificación de Sobresaliente (9) a el Trabajo de Fin de Máster realizado por el estudiante J. Salvador Chiva Natividad

RESUMEN TFM

ESPECIFICIDAD Y VARIABILIDAD DE LOS FICOBIONTES IDENTIFICADOS EN LÍQUENES DE COMUNIDADES GIPSÍCOLAS.

Los afloramientos de yesos del Mioceno en la Península Ibérica constituyen para las plantas, musgos y líquenes un sustrato que parece haber favorecido en las especies que lo colonizan ciertos procesos de especiación ya que hay un elevado número de endemismos.

Se puede encontrar yesos miocenos en distintas zonas de la Península Ibérica: Valle de Ebro (Monegros), Murcia, Almería, Granada y varias zonas del centro de España. Las comunidades de líquenes presentes en esta zona han sido analizadas por diversos autores; Llimona, 1974, Barreno, 1975; Crespo y Barreno, 1975; Gutiérrez-Carretero y Casares-Porcel, 2011.

En concreto los líquenes gipsícolas de las Cuenca del Tajo y del Tajuña han sido ampliamente analizados tanto por lo que respecta a la diversidad de taxones y de comunidades por Barreno (1975), aunque en el momento en que se realizaron estos estudios no se disponía de análisis ultraestructurales ni moleculares.

En este trabajo se estudian determinadas especies de líquenes presentes en dos poblaciones (Fuentidueña y Titulcia) enclavadas en estos afloramientos de yesos miocenos. Se ha hecho especial énfasis en el estudio de las algas simbiontes, para analizar su especificidad y variabilidad en estas comunidades liquénicas sometidas a ambientes extremos tanto por la climatología como por las especiales características del sustrato. Ya que la textura, composición mineral y de microelementos de estos sustratos pueden ser también factores clave en su desarrollo. También se han realizado análisis moleculares con marcadores específicos para la correcta identificación de los micobiontes.

En varias de las especies analizadas se han obtenido, para los marcadores moleculares utilizados, sorprendentes resultados; como por ejemplo en el caso de *Buellia zoharyi*, donde tanto el micobionte como el fobionte forman clados independientes y separados del resto de secuencias del GenBank, lo que podría indicar un posible proceso de especiación. Lo mismos puede decirse para el caso de los taxones encontrados del género *Ramalina*, donde tanto los hongos como las algas parecen ser especies hasta ahora no descritas en la literatura. No menos interesante ha sido el resultado de los ficobiontes de *Diploschistes diacapsis* y de las especies de *Acarospora* que nacen primero como parásitas de este liquen y luego se independizan, ya que *D. diacapsis* está considerad como "donador" de ficobiontes para otros líquenes. En este caso además, se ha encontrado que es muy probable que alberguen más de un tipo de ficobionte, similar a lo encontrado en estudios previos de *Ramalina fraxinea, R. farinacea, R. fastigiata o Trephromela atra* (del Campo et al., 2012, Casano et al., 2011).

Pese a la importancia de estos hábitats ninguna de las especies se encuentra incluida en las Listas Rojas de especies amenazadas o en peligro de extinción. Además los ambientes yesíferos carecen de figuras de protección especial. Sin embargo, la preparación de una Lista Roja preliminar de la Comunidad Valenciana (Atienza y Segarra, 2000) si que propone algunas especies comunes en los afloramientos gipsícolas como: *Acarospora placodiiformis* (en peligro), *A. nodulosa var. reagens* (vulnerable), Diploschistes diacapsis (menor riesgo), *Fulgensia desertorum* (vulnerable), *F. poeltii* (en peligro), *Lecidea circinarioides* (en peligro), *Psora saviczii* (en peligro). Cabe señalar que otro estudio sobre la flora de briofitos y líquenes de los afloramientos yesíferos del SE de España, indicó que 17 taxones de líquenes y briofitos son raros o endémicos, que viven exclusivamente o casi exclusivamente sobre suelos de yeso (Guerra et al. 1995). Lo que corrobora la importancia del estudio con técnicas actuales y la preservación de estas comunidades.

CONCLUSIONES

Es la primera vez que se secuencian marcadores moleculares para los micobiontes: *Acarospora nodulosa, A. sp. amarillenta, A. placodiiformis, Buellia zoharyi, Psora saviczii, Ramalina sp. pl., Rhizocarpon malenconianum*.

Es la primera vez que se secuencian marcadores moleculares para los ficobiontes (ITS rADN y LSU rADN (23S)), de todos los taxones de este estudio. Se han detectado 3 géneros: *Trebouxia*, *Asterochloris* y *Myrmecia*.

Es la primera vez que se dispone de análisis ultraestructurales (MO, MEB) de los talos de estas mismas especies.

Se ha detectado en *Diploschistes diacapsis*, *Acarospora nodulosa*, *A. sp. amarillenta, A. placodiiformis* y *Rhizocarpon malenconianum*, componentes de las costras liquénicas y que presentan relaciones interespecíficas a lo largo de su ciclo vital, la posible coexistencia de 2 tipos de ficobionte. *D. diacapsis* actuaría como un "donador" de ficobiontes para las otras especies que inician su desarrollo sobre su talo.

Diplotomma rivas-martinezii que solo crece en cristales de yeso, también presenta los mismos tipos de ficobiontes que en el caso anterior. Este fenómeno fue descrito por primera vez en nuestro grupo.

En *Buellia zoharyi*, tanto el micobionte como el ficobionte forman clados independientes y separados del resto de secuencias, lo que podría indicar un posible proceso de especiación. Todas las *Buellia* de talo lobulado y terrícola podrían configurar un nuevo género y sus ficobiontes una nueva especie de *Trebouxia* del complejo *asymmetrica*.

En los taxones encontrados del género *Ramalina* tanto los hongos como las algas parecen ser especies hasta ahora no descritas en la literatura.

En el caso de *Psora decipiens* y *P. saviczii,* el ficobionte ha sido claramente identificado como *Myrmecia israeliensis*, hasta ahora solo conocida como de vida libre.

El ficobionte presente tanto en *Cladonia convoluta* como en *Lepraria isidiata* ha resultado ser una especie no descrita del género *Asterochloris*.

Estos novedosos, complejos e inesperados resultados plantean numerosas hipótesis y amplían la perspectiva para futuras líneas de trabajo. Además, parecen corroborar la importancia que puede tener un sustrato tan característico sobre la diversificación de la flora liquénica y de sus ficobiontes.

7 ANEXO II

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A) Listado y nomenclatura de las especies de los géneros *Asterochloris*, *Myrmecia y Trebouxia* aceptadas taxonómicamente (Guiry y Guiry 2019).

Asterochloris

Asterochloris echinata Škaloud & Peksa
Asterochloris erici (Ahmadjian) Škaloud & Peksa
Asterochloris excentrica (Archibald) Škaloud & Peksa
Asterochloris friedlii Škaloud & Peksa
Asterochloris gaertneri Škaloud & Peksa
Asterochloris glomerata (Warén) Škaloud & Peksa
Asterochloris irregularis (Hildreth & Ahmadjian) Škaloud & Peksa
Asterochloris italiana (Archibald) Škaloud & Peksa
Asterochloris leprarii Škaloud & Peksa
Asterochloris lobophora Škaloud & Peksa
Asterochloris magnus (Archibald) Škaloud & Peksa
Asterochloris mediterranea E.Barreno, S.Chiva, P.Moya & P.Škaloud
Asterochloris phycobiontica E.Tschermak-Woess
Asterochloris pyriformis Thüs et al.
Asterochloris sejongensis Kim & Shin
Asterochloris woessiae Škaloud & Peksa

Myrmecia

Myrmecia aquatica G.M.Smith
Myrmecia astigmatica Vinatzer
Myrmecia biatorellae J.B.Petersen
Myrmecia bisecta Reisigl
Myrmecia globosa Printz- type
Myrmecia irregularis (J.B.Petersen) Ettl & Gärtner
Myrmecia israeliensis (S.Chantanachat & H.Bold) T.Friedl
Myrmecia macronucleata (Deason) V.M.Andreeva
Myrmecia pyriformis J.B.Petersen

Trebouxia

Trebouxia aggregata (Archibald) Gärtner
Trebouxia angustilobata Beck P
Trebouxia anticipata Ahmadjian ex Archibald
Trebouxia arboricola Puymaly- type
Trebouxia asymmetrica Friedl & Gärtner
Trebouxia australis Beck U
Trebouxia brindabellae Beck U
Trebouxia cladoniae (Chodat) G.M.Smith
Trebouxia corticola (P.A.Archibald) Gärtner
Trebouxia crenulata Archibald
Trebouxia crespoana Barreno, Molins, Moya & Škaloud
Trebouxia cretacea Voytsekhovich & Beck
Trebouxia decolorans Ahmadjian
Trebouxia flava P.A.Archibald
Trebouxia galapagensis (Hildreth & Ahmadjian) Gärtner
Trebouxia gelatinosa Ahmadjian ex Archibald
Trebouxia gigantea (Hildreth & Ahmadjian) Gärtner
Trebouxia higginsiae (Hildreth & Ahmadjian) Gärtner
Trebouxia impressa Ahmadjian
Trebouxia incrustata Ahmadjian ex Gärtner
Trebouxia jamesii (Hildreth & Ahmadjian) Gärtner
Trebouxia potteri Ahmadjian ex Gärtner
Trebouxia showmanii (Hildreth & Ahmadjian) Gärtner
Trebouxia simplex Tschermak-Woess
Trebouxia solaris Voytsekhovich & Beck
Trebouxia suecica Beck P
Trebouxia usneae (Hildreth & Ahmadjian) Gärtner
Trebouxia vagua Voytsekhovich & Beck

B) Listado y nomenclatura de los taxones citados

Acarospora clauzadeana (Llimona) Casares & Hafellner 1992
Acarospora nodulosa (Dufour) Hue 1909
Acarospora placodiiformis H. Magn. 1956
Aspicilia desertorum (Kremp.) Mereschk. 1911
Asterochloris glomerata (Warén) Škaloud & Peksa 2010
Asterochloris irregularis (Hildreth & Ahmadjian) Škaloud & Peksa 2010
Asterochloris mediterranea Barreno, Chiva, Moya & Škaloud 2015
Asterochloris Tschermak-Woess 1980
Buellia almeriensis Llimona 1973
Buellia elegans Poelt 1974
Buellia zoharyi Galun 1970
Caloplaca inconnexa (Nyl.) Zahlbr. 1930
Caloplaca teicholyta (Ach.) J. Steiner 1895
Candelariella aurella (Hoffm.) Zahlbr. 1928
Cetraria aculeata (Schreb.) Fr. 1826
Chlorophyceae Wille in Warming 1884
Chlorophyta Reichenbach 1834
Circinaria contorta (Hoffm.) A. Nordin, Savić & Tibell 2010
Circinaria gyrosa Sohrabi, Sipman, Volk. John & V.J. Rico 2012
Circinaria hispida (Mereschk.) A. Nordin, Savić & Tibell 2010
Circinaria hoffmanniana (S. Ekman & Fröberg ex R. Sant.) A. Nordin 2016
Circinaria Link 1809
Cladonia cervicornis (Ach.) Flot. 1849
Cladonia fimbriata (L.) Fr. 1831
Cladonia foliacea (Huds.) Willd. 1787
Cladonia P. Browne 1756
Cladonia pocillum (Ach.) O.J. Rich. 1877
Cladonia rangiformis Hoffm. 1796
Cladonia symphycarpa (Ach.) Fr. 1826
Cladoniaceae Zenker 1827
Clavascidium Breuss 1996
Coccomyxa Schmidle, 1901
Degelia Arv. & D.J. Galloway 1981
Diploschistes diacapsis (Ach.) Lumbsch 1988
Diploschistes muscorum (Scop.) R. Sant. 1980
Diploschistes ocellatus var. *almeriensis* Llimona 1974

Diplotomma rivas–martinezii (Barreno & A. Crespo) Barreno & A. Crespo 2000
Elliptochloris Tschermak-Woess 1980
Fulgensia poeltii Llimona 1974
Gyalolechia bracteata (Hoffm.) A. Massal. 1852
Gyalolechia fulgens (Sw.) Søchting, Frödén & Arup 2013
Gyalolechia fulgida (Nyl.) Søchting, Frödén & Arup 2013
Gyalolechia Trevis. 1851
Heteroplacidium Breuss 1996
Heteroplacidium contumescens (Nyl.) Breuss 1996
Heteroplacidium imbricatum (Nyl.) Breuss 1996
Lasallia pustulata (L.) Mérat 1821
Lecanographa amylacea (Ehrh. ex Pers.) Egea & Torrente 1994
Lecanoraceae Körb. 1855
Lecidea circinarioides Casares & Hafellner 1994
Lecidea gypsicola Llimona 1973
Lecidella stigmatea (Ach.) Hertel & Leuckert 1969
Lepraria Ach. 1803
Lepraria isidiata (Llimona) Llimona & A. Crespo 2004
Lepraria nylanderiana Kümmerl. & Leuckert 1995
Micarea Fr. 1825
Micarea peliocarpa (Anzi) Coppins & R. Sant. 1979
Myriolecis crenulata (Ach.) Śliwa, Zhao Xin & Lumbsch 2015
Myriolecis dispersa (Pers.) Śliwa, Zhao Xin & Lumbsch 2015
Myrmecia biatorellae Petersen 1956
Myrmecia israeliensis (S.Chantanachat & H.Bold) T.Friedl 1995
Myrmecia Printz (1921)
Nephroma Ach. 1809
Nostoc Vaucher ex Bornet & Flahault 1886
Parmeliaceae F. Berchtold & J. Presl 1820
Parmotrema tinctorum (Despr. ex Nyl.) Hale 1974
Peltigera rufescens (Weiss) Humb. 1793
Placidium A. Massal. 1855
Placidium arboreum (Schwein. ex E. Michener) Lendemer 2004
Placidium lachneum (Ach.) B. de Lesd. 1932
Placidium pilosellum (Breuss) Breuss 1996
Placidium squamulosum (Ach.) Breuss 1996
Placidium umbrinum (Breuss) M. Prieto & Breuss 2009
Placopsis (Nyl.) Linds. 1866

Porpidia crustulata (Ach.) Hertel & Knoph 1984
Prasinophyceae Christensen ex Silva 1980
Protoparmeliopsis garovaglii (Körb.) Arup, Zhao Xin & Lumbsch 2015
Protoparmeliopsis muralis (Schreb.) M. Choisy 1929
Pseudococcomyxa Korshikov 1953
Psora decipiens (Hedw.) Hoffm. 1794
Psora Hoffm. 1789
Psora saviczii (Tomin) Follmann & A. Crespo 1975
Psoraceae Zahlbr. 1898
Ramalina farinacea (L.) Ach. 1810
Rhizocarpon malenconianum (Llimona & Werner) Hafellner & H. Mayrhofer 1977
Romjularia lurida (Ach.) Timdal 2007
Rusavskia papillifera (Vain.) S.Y. Kondr. & Kärnefelt 2003
Sarcogyne privigna (Ach.) A. Massal. 1854
Schizoxylon albescens Gilenstam, H. Döring & Wedin 2006
Seirophora lacunosa (Rupr.) Frödén 2004
Squamarina cartilaginea (With.) P. James 1980
Squamarina lentigera (Weber) Poelt 1958
Stereocaulon (Schreb.) Schrad. 1794
Stereocaulon vesuvianum Pers. 1811
Thalloidima sedifolium (Scop.) Kistenich, Timdal, Bendiksby & S. Ekman 2018
Thamnolia Ach. ex Schaer. 1850
Toninia A. Massal. 1852
Trebouxia arboricola Puymaly 1924
Trebouxia asymmetrica Friedl & Gärtner 1988
Trebouxia corticola (P.A.Archibald) Gärtner 1985
Trebouxia cretacea Voytsekhovich & Beck 2015
Trebouxia galapagensis (Hildreth & Ahmadjian) Gärtner 1985
Trebouxia impressa Ahmadjian 1960
Trebouxia Puymaly (1924)
Trebouxia showmanii (Hildreth & Ahmadjian) Gärtner 1985
Trebouxia simplex Tschermak-Woess 1978
Trebouxia vagua Voytsekhovich & Beck 2015
Trebouxiaceae Friedl 1995
Trebouxiales Friedl 1995
Trebouxiophyceae Friedl 1995
Trentepohlia C. Martius, 1817
Ulvophyceae Stewart & Mattox 1978

Umbilicaria spodochroa Ehrh. ex Hoffm. 1796
Verrucaria submersella Servít 1954
Verrucariaceae Eschw. 1824
Xanthoparmelia (Vain.) Hale 1974
Xanthoparmelia maricopensis T.H. Nash & Elix 1986

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