Coexistence of different intrathalline symbiotic algae and bacterial biofilms in the foliose Canarian lichen *Parmotrema pseudotinctorum*

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ABSTRACT

Parmotrema pseudotinctorum (des Abb.) Hale is a foliose lichen able to colonize large areas on rock surfaces in semiarid and warm localities in the Canary Islands. In this contribution, we investigate the phycobionts of this successful lichen under these extreme environmental conditions using ultrastructural and genetic methodologies. Two populations from La Gomera and La Palma islands were analyzed. After TEM analyses, three algal types were clearly distinguished in intrathalline symbiosis, provisionally named Ph1, Ph2, and Ph3. Two of them (Ph1 and Ph2) were *Trebouxia* showing a well visible pyrenoid *corticola*-type the chloroplast thylakoids being very different in both. The type Ph3 could be a taxon included in the genus *Asterochloris*. Our molecular approach consisted in sequencing two different DNA loci: a portion of the chloroplast *psb*A gene and nuclear ITS. Sequences of the *psb*A gene resulted in electrophoretograms showing double peaks when DNA extracted from the whole lichen thallus was used as template. Such double peaks were interpreted as single nucleotide polymorphisms (SNPs). This interpretation was confirmed by cloning. However, no intrathalline polymorphisms were detected among the nrITS sequences. Phylogenetic analyses on the basis of the *psbA* gene revealed three

distinct clades. It is likely that these clades corresponded to the the three different morphotypes revealed by TEM. One of these clades, was closely related to *T. corticola*, other was closely related to *Asterochloris glomerata* and the third did not grouped with any specific taxa. These results are the first piece of evidence that algal coexistence may even be established between species of different genera of the Trebouxiophyceae (*Asterochloris* and *Trebouxia* at least). Moreover, the ccoexistence of several microalgal taxa evidenced in this study appears as a consistent character among the populations of this foliose lichen. Further isolation and cultivation of the three different algal types and physiological studies should shed light on the ecological plasticity of the entire holobiont. Along with such variety of intrathalline coexisting algae, another unexpected result was the observation of an almost continuous layer of bacterial-communities coating the lower cortex in all the studied samples of *P. pseudotinctorum* the lower cortex was coated with an almost continuous layer of bacterial-communities. The function of these biofilms in the lichen symbiosis remains to be elucidated. The existence of such particular symbiosis involving different algal species and bacteria could be explained by an increased fitness in particular habitats or under specific environmental conditions.

Key words: Microalgae, phycobiont, Trebouxiophyceae, nuclear ITS, chloroplast *psb*A, anatomy, ultrastructure, ML, MP, NJ analyses, Canary Islands

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). The unique symbiogenetic phenotype of specific biological organization is the lichen thallus (holobiont). The relationships between syntrophic metabolism and morphogenesis in the emergence of novelty through physical association are made obvious in their thalli, and in addition the contribution of symbiogenesis to speciation and taxonomy in them is manifest, and their capacity for revival from severe desiccation is remarkable (Margulis & Barreno, 2003). Lichenization allows the partners to thrive in habitats that would otherwise be unavailable to either one on its own. The important functional interactions between

photobionts and mycobionts, and possibly other symbionts such as specific bacterial communities, suggest that these partners evolved simultaneously (Printzen *et al.et al.* 2012; del Campo *et al.et al.* 2013). Recently, our team has demonstrated the coexistence of several intrathalline phycobionts in some lichen thalli (del Campo *et al.* 2010a; Casano *et al.* 2011).

Parmotrema pseudotinctorum is a rosette-forming foliose lichen able to colonize large areas on volcanic rock surfaces, which may be slightly nitrophytic, in semiarid to very dry and warm localities in the Canary Islands, mainly in the infra- and thermo-canarian bioclimatic belts, surrounded predominantly by *Kleinio-Euphorbietea canariensis* (crassicaule) and *Rhamno-Oleetea cerasiformis* (xeric Mediterranean) vegetation, but it may occasionally be found in the driest areas of the laurisilva (*Visneo -Apollonion barbujanae*) (Barreno, pers. obs.; look the syntaxonomy at the bottom). In some sites, *P. pseudotinctorum* can reach a high biomass giving a significant trait to the general landscape.

Parmotrema pseudotinctorum (des Abb.) Hale has recently been recognized as an independent taxon of the beforehand confused isidiate species of *P. tinctorum* (Despr. ex Nyl.) Hale (Roca-Valiente *et al.* 2013). The results revealed two divergent and strongly supported monophyletic clades, in which isidium morphology was concordant with the molecular phylogenetic tree topology excluding *P. tinctorum* from the extant biota of the Canarian archipelago. In addition, very little is known about the genetic and ultrastructural diversity of the chlorobionts in the Canarian lichen flora.

The initial objective of this study was to investigate the physiological aspects of the phycobiont of this successful lichen in colonizing habitats under these extreme environmental conditions and provide insights into basic ecophysiological adaptations. Previous experimental studies indicate that the lichen association between photobionts and mycobionts increases tolerance to stress conditions (Kranner *et al.*, 2005; Kosugi *et al.*, 2009; Catalá *et al.*, 2010). There is some evidence that lichens thriving in stressful habitats can adjust their algal partner according to their presence in different habitats, following the concept of 'habitat-adapted symbiosis' (Rodriguez *et al.*, 2008). Algae are the primary producers in lichen thalli micro-ecosystems and physiological studies are also necessary to confirm our hypothesis that the coexistence of physiologically complementary photobionts within the same lichen thallus increases tolerance to changing and often stressful environments (Álvarez *et al.* 2011; del Hoyo *et al.* 2012). Nevertheless, when conducting preliminary assays to identify and isolate the phycobionts we obtained unexpected results. Sequences of the chloroplast *psb*A resulted in electrophoretograms showing double

peaks when DNA extracted from the whole lichen thallus was used as template. Such double peaks were interpreted as single nucleotide polymorphisms (SNPs). In addition, we clearly distinguished three algal types in intrathalline symbiosis by morphology-based characteristics using TEM, and moreover the lower cortex was coated with an almost continuous layer of bacterial-colonies. Accordingly, we decided to deal exclusively with architectural characteristics of lichen thalli and phycobionts using ultrastructural and genetic methodologies.

Conclusively, the aims of this interdisciplinary study of *Parmotrema pseudotinctorum* were: 1) identify the chlorobionts in populations from different islands; 2) test the coexistence of multiple intrathalline microalgae in individual thalli; 3) determine the types of mutualistic associations including bacterial biofilms.

MATERIALS AND METHODS

Sampling

Two representative populations of *Parmotrema pseudotinctorum* (des Abb.) Hale collected in the Canary Islands were included in the analysis and the photobiont genotypic and anatomical diversity within and between thalli were investigated. The samples from San Sebastián 28°05'04"N 17°07'13"W (La Gomera island) were collected on volcanic rocks by Ana Crespo and her research team (Universidad Complutense de Madrid, Spain), and preserved at -20ºC. The samples from the population at Los Cancajos 28°38'49"N 17°45'41"W (La Palma island) were used as fresh material to compare possible changes in algal anatomy. Some of these samples are preserved at -20ªC and other ones at 4ªC at the Eva Barreno laboratory.

Microscopy examinations

The confirmation of algal morphology and its presence inside each lichen thalli was assessed in several samples from each locality, taking both peripheral lobes and the median region of the thallus. Examinations were performed both by transmission electron microscopy (TEM) and light microscopy (LM). For TEM, pieces of rehydrated *P. pseudotinctorum* thalli 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 fixed with 2% OsO_4 in 0.01 M PBS, pH 7.4, for 2 h at 4°C. Thereafter, specimens were washed in 0.01 M PBS, pH 7.4, for 15 min and 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 in each step. The fixed and dehydrated samples were embedded in Spurr's resin according to the manufacturer's instructions. To mount the samples, 90 nm sections were cut with a diamond knife (DIATOME Ultra 45°) using an ultramicrotome (Ultratome Nova LKB Bromma), mounted on copper grids of 100 mesh and post-stained with "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, at the SCSIE Service of the University of Valencia.. For LM analyses, 1–2 mm sections were cut from samples embedded in Spurr's resin using a diamond knife (DIATOME Histo 45°) and an ultramicrotome (Ultratome Nova LKB Bromma). The sections were stained with 1% toluidine blue pH 7.2 and observed with an Olympus Provis AX 70 microscope equipped with an Olympus Camedia C-2000 Z camera.

DNA isolation, amplification, cloning and sequencing

Four individuals per population were analyzed after a washing performed following Muggia *et al.* (2013). Samples from the population of La Gomera island were given a numerical code (3722-3723-3724-3725) and the samples from La Palma island an alphanumeric code (PAL1-PAL2-PAL3-PAL4). Total genomic DNA was isolated and purified using DNeasyTM Plant Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Two algal loci were amplified from genomic DNA from the lichen thallus using specific primers. Because nuclear and chloroplast genomes are inherited independently and show different evolutive patterns, we included loci from both genomes. As chloroplast genome marker we studied a region of the *psb*A gene encoding the D1 protein, by using the primers psbAchl1: and psbAchl2: (del Campo *et al.*, 2010c). We also amplified the nuclear locus encoding the nrITS RNA using the primer pair nr-SSU-1780 (Piercey-Normore & DePriest, 2001) and ITS4 (White *et al.*, 1990).

PCR reactions were performed in 50 µl using EmeraldAmp GT PCR Master Mix (Takara, Shiga, Japan). 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 contaminations. Negative controls, without DNA template, were included in every round of PCR amplifications to ensure against false-positive results caused by

contaminants in the reagents. The PCR program for amplifications comprised an initial denaturation at 94ºC, 2 min, and 30 cycles of 94ºC for 30 sec, 56ºC for 45 sec and 72ºC for 1 min, followed by a final elongation at 72ºC for 5 min. Amplifications were carried out on a 96-well SensoQuest labcycler (Progen Scientific Ltd., South Yorkshire, UK). The PCR products were separated on 2% agarose gels and purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Heathlcare Life Science, Buckinghamshire, England). The amplified PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, USA) per the manufacturer's instructions and sequenced with an ABI 3100 Genetic analyzer using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California).

Phylogenetic analyses

The DNA sequences were aligned and manually adjusted using ClustalX (Thompson *et al.*, 1997). Phylogenetic analyses were performed in MEGA 5.0 (Tamura *et al.*, 2011). Maximum likelihood (ML) was used, and results were corroborated with maximum parsimony (MP) and neighbor joining (NJ) analyses to ensure that results are not specific to parsimony analysis. The branches in the resulting phylogenies were tested for robustness with 1,000 bootstrap replicates (Hillis & Bull, 1993).

RESULTS AND DISCUSSION

Morphological and ultrastructural characterization of *Parmotrema pseudotinctorum* thallus show the intrathalline coexistence of different algae and bacterial biofilms

The observations were made by LM in all the samples, and 3722, PAL1 and PAL4 samples were selected for TEM analysis. *Parmotrema pseudotinctorum* is a grey green foliose lichen of medium size (6-12(16) cm) with rounded lobes, covered by abundant coarse isidia (figs. 1, 2, 5) located at central and middle areas. The lower cortex is dark and wavy showing scarce rhizines (figs. 1, 2) mostly located at the center. The thallus structure is typically heteromerous (figs. 1, 2). Epicortex more developed in the central areas (figs. 1, 2), the upper cortex is made up of a palisade plectenchyma 1–3 (–4) cell height, with small round cells of medium-size thickness walls shaped by the concurrence of parallel hyphae that extend beyond the medulla (figs. 1, 2, 5). The

medulla shows a bi-stratified structure, with abundant crystals (¿lecanoric acid?) near the lower cortex and without them in the stratum near the upper cortex, where hyphae are loosely arranged in a mainly horizontal direction (figs. 1, 2). Lower cortex prosoplectenchymatic with small round cells (3-4 height) of dark hyphal walls coated by an external layer showing high affinity for osmium (figs. 1, 2), which may indicate that it is composed of lipids. This lower cortex is outwardly covered by a thick, and more or less, continuous layer of bacterial-colonies which is an unusual characteristic (figs. 1, 2). Isidia are coarse and constricted at the base, during the ontogeny a plectenchymatic layer of dark hyphae layer develops, similar to those of the lower cortex, and colonies of bacteria adhere to it (fig. 5). The phycobiont layer is thin and irregular; the groups of algae are located immediately below the upper cortex and it is possible to find two or three types of phycobionts cells (figs. 1-5). These anatomical characteristics are similar to those published for *Parmotrema tinctorum* (Barbosa *et al.* 2009), except for the isidia (Roca-Valiente *et al.* 2013) and the lower cortex bacterial biofilms. In the marginal lobes two types of algae are found while in the median zones three types may be coexisting. When isidia begin to grow the three types of algae migrate inside (fig. 5) and an intense hyphal ramification takes place.

We were able to clearly distinguish three algal types in intrathalline symbiosis by morphology-based characteristics using TEM. The type 1-phycobiont (Ph1) shows a well visible central pyrenoid *corticola*-type (Friedl, 1989) (figs. 2, 3, 5) with very thin, unbranched tubules and a sinuous profile without pyrenoglobuli associated with the pyrenoid matrix; pyrenoglobuli are rather developed in the chloroplast stroma, close to the pyrenoid, the starch grains are dark and closely connected with the pyrenoid matrix, forming a starch sheath made up of a few large, curved plates adjacent to the pyrenoid, also more than one pyrenoid per cell was observed. The lamellae of the thylakoids are loosely arranged and deeply curved giving an undulating appearance to the chloroplast; mitochondria are abundant as well as the small peripheral vesicles. The association with the hyphae is made by simple contact. These ultrastructural characteristics seem to include this taxon in the group of *Trebouxia corticola* (Archibald) Gärtner *s. lat.*, but on the basis of Ohmura *et al.* (2006), this taxon seem to be an unknown species of this interesting group, already poorly known from ultrastructural and genetic aspects. As Ohmura *et al.* (2006) point out the taxa *T. galapagensis*, *T. higginsiae* and *T. usneae* are closely related to the *T. corticola* type and could be synonymies of it. The results offered here strongly indicate that this group of *Trebouxia* algae requires a deep taxonomic revision. In any case this morphotype could provisionally be named

as "*Trebouxia pseudocorticola*" (fig. fig. 3) and it seems to be the preponderant type in the samples 3722 (cryopreserved), PAL1, PAL4 (both fresh) we studied by TEM.

The type 2-phycobiont (Ph2) (figs. 2, 4, 5) should also be considered as included in the *Trebouxia corticola* group by the pyrenoid features with very thin, unbranched tubules of curved profile, without pyrenoglobuli associated with the pyrenoid matrix being rather developed in the chloroplast stroma, close to the pyrenoid; starch grains are closely connected with the pyrenoid matrix, forming a starch sheath made up of a few large, arched plates adjacent to the pyrenoid, but contrary to type-Ph1 these are bigger and of a white colour (fig. 4e). Nevertheless, the chloroplasts show very dense and tidy thylakoid membranes with some pyrenoglobules, and the secretion zone is evenly distributed; mitochondria are scarce, and at the periphery several large and electro-dense vesicle containing a deposit of electro-dense material (fig. 4a, d) are obvious even in LM observations (fig. 2). The interaction phycobiont-mycobiont is through a type 2 intraparietal haustorium (fig. 4f) according to Honegger (1991). This morphotype could not be directly related to any other known *Trebouxia* species, it can probably be included in one of the clades obtained by cloning techniques with the ITS and *psb*A markers (figs. 6, 7).

The type 3-phycobiont (Ph3) (fig. 5) is markedly different to the former types, no pyrenoids were observed, the pyrenoglobules are scant and spread, the chloroplast consists of loosely networked thylakoids with wavy lamellae; ribosomes concentrate around the scanty mitochondria; small vesicles of sparse grey content are abundant at the periphery, the secretion zone is irregular in thickness with a lateral enlargement (fig. 5b); the mycobiont-phycobiont interaction is through a type 2 intraparietal haustorium according to Honegger (1991). These features suggest that this morphotype could be a taxon included in the genus *Asterochloris* (Pavel & Skaloud 2011).

Another unexpected result observed was that in all the studied samples of *P. pseudotinctorum* the lower cortex was coated with an almost continuous layer, similar to a biofilm, of bacterial-colonies showing different morphologies (figs. 1, 5) and establishing associations with modified hyphae growing from the cortex plectenchyma, even on the bottom and top of the isidia (fig. 1f, 5e). These cortical bacterial-colonies associated with upper cortex hyphae were detected earlier by our team in *Parmelia barrenoae, Ramalina farinacea, R. fraxinea, R. asahinae, Seirophora villosa* and some foliose Mexican lichens (Barreno *et al.* 2008; Barreno & Herrera-Campos, 2009; Royo *et al.* 2009; Catalá SG *et al.* 2012). The constricted isidium base may facilitate its

detachment from the thallus acting as efficient propagules for symbiosis perpetuation (Barbosa *et al.* 2009), and in the case of *P. pseudotinctorum* the isidia may also help to propagate some of the cortical bacterial associations. In recent years, several authors have studied the bacterial communities in lichens by sequencing complete thalli (summarized in Fernández-Mendoza *et al.* 2012) and indicate the original and high diversity of bacteria living in lichens, albeit that at present it is not possible to know which of them are preponderant in the cortical zones. The function of these biofilms in the lichen symbiosis should be elucidated on.

Genetic characterization of the *Trebouxiophyceae* algae coexisting within the same thallus of *Parmotrema pseudotinctorum*

The results obtained here from an anatomical perspective suggest that different species of *Trebouxia* or/and other Trebouxiophyceae genera coexist within a single *P. pseudotinctorum* thallus. To further characterize this possible intrathallline coexistence of different phycobionts, a second approach consisted of sequencing two different DNA loci, the chloroplast region *psb*A (encoding the D1 protein) and the nuclear internal transcribed spacer (nrITS) of the rRNA operon. Sequences of the *psb*A region from DNA extracted from the whole lichen thallus resulted in electrophoretograms showing double peaks in one of the eight thalli analyzed (PAL4). These double peaks were interpreted as single nucleotide polymorphisms (SNPs). Interestingly, the presence of polymorphic sequences has already been indicated in *Parmotrema tinctorum* (Despr. ex Nyl.) Hale (Ohmura *et al.*, 2006) although the author only hypothesized about the presence of different algae (i.e., the sample showing the polymorphism was removed from the analysis). If the analyzed gene was nuclear, such polymorphisms could be interpreted as presence of multiple and lightly different copies of the locus. However, in our case the analyzed gene is located within the chloroplast. This means that even in the case of duplication of the *psbA* gene, the copies would be identical by homologous recombination within the cpDNA. Thereby, our findings in this plastid-encoded gene suggest that such polymorphisms could represent the coexistence of different algae within each lichen thallus, presumably of different species (del Campo *et al.* 2010a, 2010b).

The presence of multiple algal strains or genotypes associated with a fungal species within a single thallus has already been reported, including *Evernia mesomorpha* (Piercey-Normore 2006), *Lecanora* (Blaha *et al.*, 2006) *Tephromela* (Muggia *et al.*, 2008, 2010), *Protoparmeliopsis muralis* (Guzow-Krzeminska 2006; Muggia *et al.*, 2013) or the sister species *Parmotrema tinctorum* (Ohmura *et al.*, 2006). In the lichens *Ramalina farinacea,*

R. fastigiata and *Tephromela atra* the same two different *Trebouxia* phycobionts (TR1 and TR9) coexist within each lichen thallus (Casano *et al.* 2011; del Campo *et al.* 2013). Such polymorphisms in the *psb*A gene were not clearly appreciated in the remaining populations sampled. Moreover, polymorphic sequences were not detected in the ITS region for any of the sampled populations. This result could likely be a consequence of differences in the abundance of the two algae within each thallus. So it seems that ITS sequence analyses could only reveal that of the predominant phycobiont, while the other alga or algae would remain undetected (del Campo *et al.,* 2010b; Muggia *et al.* 2011), because of intrinsic PCR constraints.

Electrophoretogram polymorphisms in PAL4 (La Palma island) allowed us to detect the presence of more than one sequence, but not to discern among the different sequences existing. Thus, a cloning strategy was performed to adequately separate the different sequences obtained. Moreover, in addition to the lack of polymorphism in the DNA sequences, the presence of different algal morphologies in the sample 3722 (La Gomera island) as described above (Figs.1, 2) pointed to the existence of different taxa -not only strains- which were not well detected with the direct sequencing. Consequently, we performed a detailed analysis of the two lichen thalli by re-amplifying primary amplification products from both lichens, which were then cloned in order to separate the different sequences existing in both loci.

The number of clones that could be obtained per individual loci varied depending on the efficiency of the cloning. In the case of *psb*A two clones were analyzed from 3722 and 16 from PAL4, whereas in nrITS 18 clones were analyzed from 3722 and eight from PAL4. In the cloned sequences, the length of the *psb*A was 255 bp in all the direct sequences and clones obtained. However, up to 62 mutations were detected among the different sequences, at the inter- and intra-population level and within the same individual thallus. In the case of the La Gomera samples the direct sequences of the four thalli (3722-3723-3724-3725) were identical, however two mutations resulted from clones 3722.3 and 3722.4, owing sequences undetected in any other thalli. This result should be taken with caution since mutations tale cooperation could be artifacts of the cloning process.

Of note was the fact that a higher sequence variation was detected in the La Palma population, showing many mutations which differ among individuals, there even being a high variability among the clones within a single individual (PAL4, fig. 6). Interestingly, the clone PAL4.11 appeared more closely related to *Asterochloris* than to *Trebouxia*. This fact along with the report of the Ph3-morphotype having characteristics of *Asterochloris,* suggest that this morphotype could be *Asterochloris* more than *Trebouxia* (Pavel & Skaloud 2011). Considering

that in *Ramalina fraxinea* at least six different *Trebouxia* phycobionts have been detected using TEM and the *psb*A markers (Catalá SG *et al.*2011, 2012 and unpublished results), it seems that the chloroplast region of the *psb*A gene encoding the D1 protein may be an accurate marker for identifying Trebouxiophyceae algae.

Regarding variation in the ITS, direct sequence variation resulted at inter- and intra- population level but, as indicated, no intrathallus polymorphism were detected. Besides this, polymorphic sequences in a single thallus were detected by cloning and confirming the observations of different algal morphologies (Figs.2, 5, 7). In the case of ITS, the length varied from 653 to 666 bp in all direct sequences and clones obtained. This size difference is due to the presence of indels. Similar to *psb*A, up to 84 mutations were found in the different sequences obtained with the ITS. The highest sequence variation was detected when comparing PAL1 and clones 3722.3 and 3722.13 with the remaining sequences.

Coexistence of several chlorobiont taxa evidenced in this study appears as a consistent character among the populations of this Canarian lichen. The presence of several species of algae within fruticulose and crustaceous individual lichen thalli was recently discovered (Casano *et al.* 2011; Muggia *et al.* 2011; del Campo *et al.* 2013) but in this work we also demonstrate this multisymbiosis occurrence in foliose lichens. If one of the morphotypes detected is definitely a member of *Asterochloris* it would be the first occasion showing that algal coexistence may even be established between the species of different genera of the Trebouxiophyceae. However, the confirmation of the high complexity in intrathalline algae observed in *P. pseudotinctorum* requires the isolation and propagation of the three morphotypes and genotypes in axenic cultures. This is a very time-expensive task and exceeds the limits of the present study, but we just begun the corresponding experiments in our laboratory as well as more cloning techniques.

Preliminary phylogenetic analysis of the symbiotic algae from *P. pseudotinctorum*

To investigate the phylogenetic relationships among the different phycobionts detected within a single lichen thallus, the DNA sequences generated were used to construct phylogenetic trees by inference methods (Neighbor-joining, NJ; maximum-likelihood, ML; and maximum-parsimony, MP) and the trees obtained were compared. The ML and NJ analyses resulted in the same well-supported clades as the MP. The ML tree is shown in both markers (figs. 6, 7). For comparison purposes, algal sequences from *Trebouxia* (*T. aggregata,* SAG219-1d; *T. asymmetrica,* SAG48.88; *T. corticola,* UTEX909; *T. jamesii,* UTEX2233; *T. potteri,* UTEX900 *T. showmanii,*

SAG2009), *Asterochloris* (*A. glomerata,* SAG 100.80; *A. phycobiontica,* SAG 26.81) algae along with *Dictyochloropsis reticulata* (SAG53.87), *Chlorella ellipsoidea* (SAG 211-1a) and two Streptophyta algae as outgroup (*Chara vulgaris*, accession NC_008097 and *Zygnema circumcarinatum*, accession NC_008117).

The phylogram of fig. 6 shows several samples within the *Trebouxia* clade, most of them closely related to *T. corticola* with a support value of 98 and one of them (PAL2) more closely related to other *Trebouxia* species (*T. showmaniii* and *T. asymmetica*) with a support value of 89. The phylogram also shows a sample (PAL4.11) more closely related to *Asterochloris* with a support of 87. These results are congruent with the presence of three different morphotypes revealed by the diagnostic characteristics observed with TEM. A third clade outside of *Trebouxia* and *Asterochloris* with a support of 96 can be distinguished. The algae represented in this clade, seem to belong to another genus than *Trebouxia* or *Asterochloris*. We must further determine if these samples are symbiotic chlorobionts within *P. pseudotinctorum* or may be present on the surface of the thallus despite the washing performance. As a whole this preliminary phylogenetic tree denoted that one of the phycobionts detected in *Parmotrema pseudotinctorum* seems to be closely related to, but clearly distinct from, *Trebouxia corticola,* whereas another (i.e. detected in PAL4) could match species to be included in algal genera different from *Trebouxia* or with unknown *Trebouxia* species. The large polymorphism detected in PAL2, even wider than that of PAL4, severely hinders the acquisition of all the clones representing the detected polymorphism.

In the case of the ITS (fig. 7), besides the outgroup and close relative species included in the *psb*A tree, several accessions of *Trebouxia corticola* sequences were incorporated, available in Genbank from Ohmura *et al.* (2006) and Mansournia *et al.* (unpublished data) which were obtained from the sister species *Parmotrema tinctorum*. All the PAL direct sequences and clones except PAL1, and all the La Gomera island sequences and clones, clustered together and separated from any other closest relative or outgroup sequences (99% bootstrap), but related to *T. corticola*. On the other hand, most Genbank sequences of *T. corticola* group together and joined to PAL1 (74% bootstrap); whereas clones 4 and 13 of 3722 sample fall into a separated clade (98% bootstrap) closer to the species of *Trebouxia* used as outgroups. In conclusion, our preliminary study suggests that the three algae analyzed in the individual thalli could be taxa which were unknown beforehand.

Altogether, our different approaches suggest that *P. pseudotinctorum* thalli show a new model of complex multiple symbioses in foliose lichens with at least three endosymbiont algal species coexisting in each individual. There is evidence that the acquisition of such multiple taxa of photobionts can increase the fitness of the whole

symbiotic association, the holobiont (Casano *et al.* 2011; Peksa & Škaloud, 2011; del Hoyo *et al.* 2011; Álvarez *et al.* 2012; del Campo *et al.* 2013). Therefore, the existence of such particular symbiosis involving different algal species could be explained by an increased fitness in particular habitats or under specific environmental conditions. Future investigation related to the quantification of the benefits of such symbiosis compared to single-algal symbiosis, might shed light on this issue. A possible cause of the presence of multiple algal genotypes or species is because this may confer advantages in the lichen's ability to respond to environmental changes or to occupy diverse microenvironments (Piercey-Normore 2006; Casano *et al.* 2011; del Hoyo *et al.* 2012). It is also important to note that ecological factors, especially climate, may have an impact on phycobiont selection (Beck *et al.* 2002; Yahr *et al.* 2006; Fernández-Mendoza *et al.* 2011; Peksa & Škaloud 2011, Ruprecht *et al.*, 2012).

As pointed out in the introduction *P. pseudotinctorum* is a foliose lichen able to colonize large areas on rock surfaces in arid or very dry and warm localities, principally in the infra- and thermo-canarian bioclimatic belts (Rivas-Martínez *et cols*. 2011). Probably, the multiple symbioses with several taxa of algae may help to overcome the ecophysiological constraints derived from the extreme environmental conditions of the habitat. Of equal importance for the adaptation to desiccation/ rehydration cycles and effective photosynthetic rates in these habitats may be the anatomy of the thalli, where the wavy lower cortex with hyphal walls surrounded by lipids (affinity for osmium) and the impressive layer of different bacterial cells must play an important role. Moreover, the palisade plectenchyma in the upper cortex, the numerous isidia and medulla characteristics also must contribute (Honegger 1991; Valladares & Sancho, 1995; Divakar *et al.* 2007) to the successful growth of this lichen in the Canary Islands.

The high complexity in intrathalline algae observed in *P. pseudotinctorum* requires the analysis of a larger number of thalli and a wider and more exhaustive sampling, including a higher number of populations to corroborate the preliminary results of the present study in which molecular data seems to support the so different phycobiont morphotypes detected by TEM. Moreover, in spite of the accuracy of the plastid *psb*A gene, the scoring of several new molecular markers should help in explaining the remarkable characteristics shown by *P. pseudotinctorum*. The isolation and propagation of its microalgae in axenic cultures would clarify the taxonomic identification and the physiological performance of the phycobionts studied here, shedding light on the ecological plasticity of the entire holobiont.

Syntaxonomic schema of plant vegetation (Rivas-Martínez et cols. 2011).

Cl. ***Kleinio nerifoliae-Euphorbietea canariensis*** (Rivas Goday & Esteve 1965) Santos in Anales Inst. Bot. Cavanilles 33: 252.1976

Cl. ***Rhamno crenulatae-Oleetea cerasiformis*** Santos ex Rivas-Martínez in Mem. Veg. España: 196. 1987 *nom. inv.*

Cl. ***Pruno hixae-Lauretea novocanariensis*** Oberdorfer 1965 corr. Rivas-Martínez, T.E. Díaz. Fernández-González, Izco, Loidi, Lousa & Penas in Itinera Geobot. 15(1): 241. 2002

Ord. ***Pruno hixae-Lauretalia novocanariensis*** Oberdorfer ex. Rivas-Martínez, Arnáiz, Barreno & A. Crespo 1977 corr. Rivas-Martínez, T.E. Díaz. Fernández-González, Izco, Loidi, Lousa & Penas in Itinera Geobot. 15(1): 241. 2002

Al. ***Visneo mocanerae-Apollonion barbujanae*** Rivas Martínez in Capelo, J.C. Costa, Lousa, Fontinha, Jardim, Sequeira & Rivas Martínez in Silva Lusitana 7(2): 266.2000

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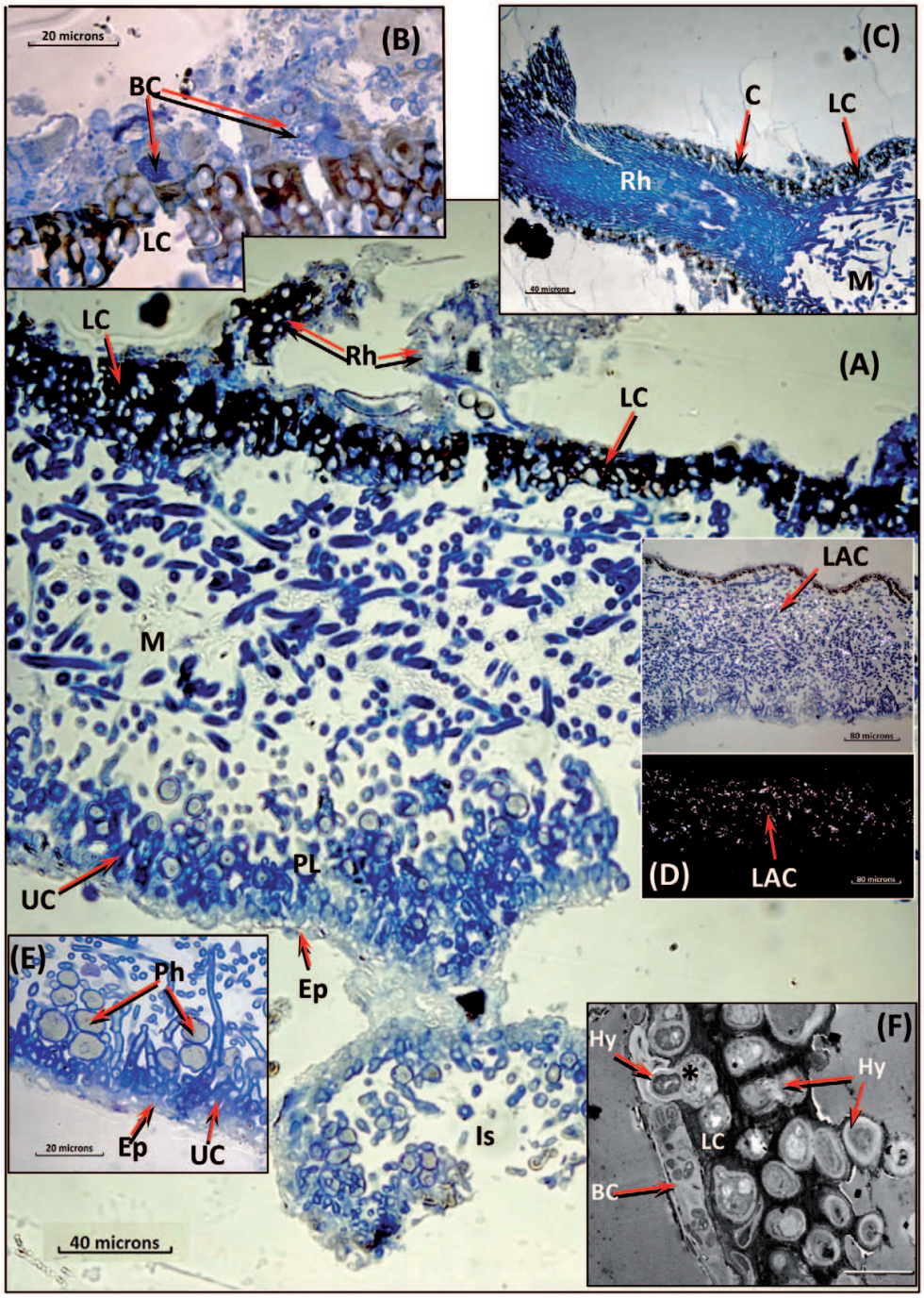

Fig. 1.- ***Parmotrema pseudotinctorum*** **thallus architecture**.(A). Transversal section across the heteromerous structure with rhizines (**Rh**) and an isidium (**Is**) (B). Detail showing the prosoplectenchymatic and wavy lower cortex (**LC**) with dark hyphal walls -affinity for osmium- and coated with an almost continuous layer of bacterial communities (**BC**). (C). Detail showing the presence of a cortical layer (**C**) covering a rhizine (**Rh**). (D). Top image: Detail showing bistratified structure, with crystals (**LAC**) in the medulla (**M**) near the lower cortex and without them in strata near the upper cortex. Bottom image: Thallus transversal section visualized under polarized light showing the presence of crystals in the medullary lower part. (E). Detail showing the epicortex (**Ep**), upper cortex (**UC**) of palisade plectenchyma and phycobiont layer (**PhL**). (F) Bacterial-colonies coat overlying lower cortex (**LC**) by TEM, Bar: 5 µm. Abbreviations: **Hy**, hyphae. (C) Obtained from fresh sample PAL4, the other photomicrographies from fresh sample PAL1.

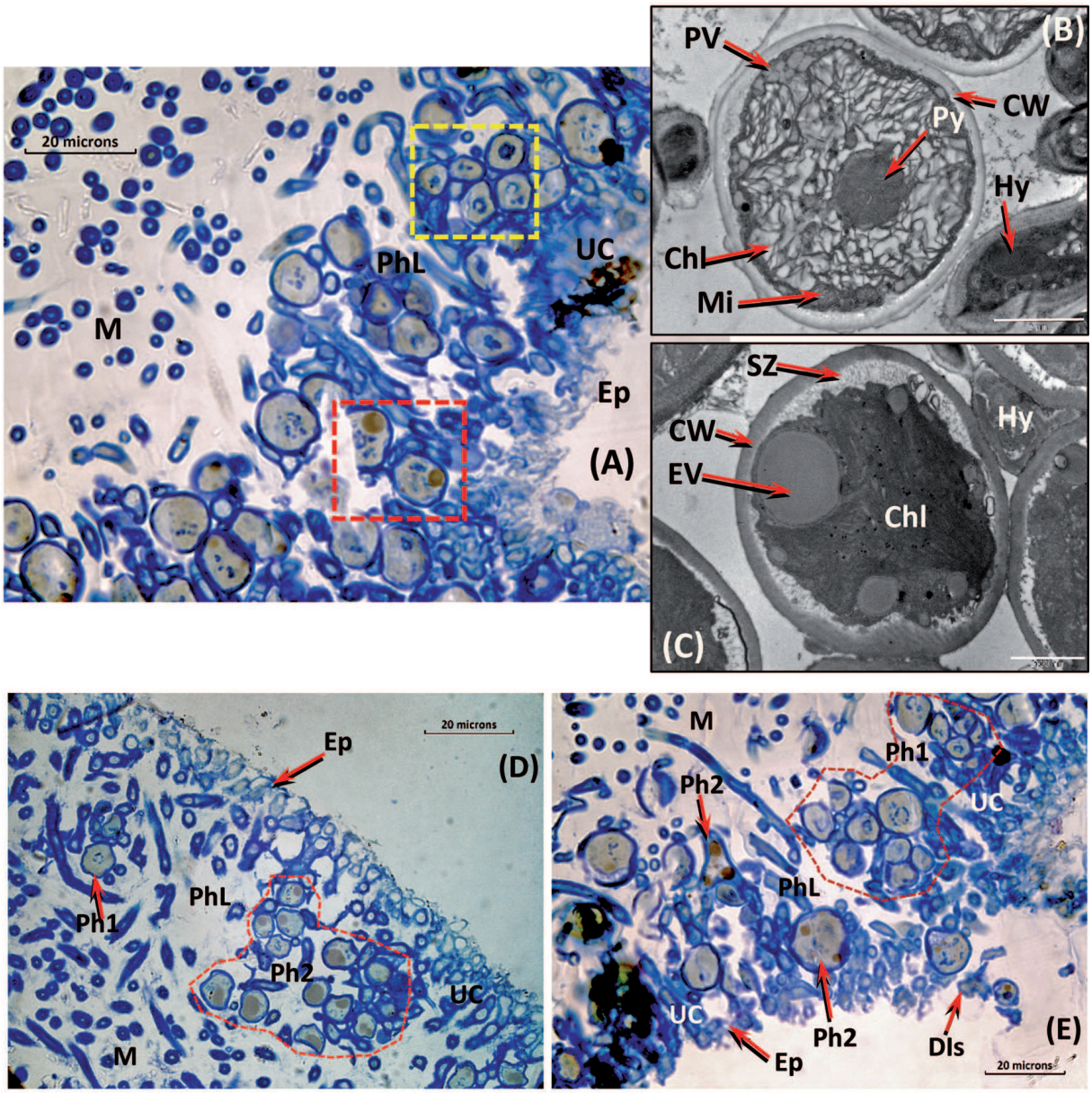

Fig. 2.- ***Parmotrema pseudotinctorum.*****Thallus structure from two samples by LM and phycobionts by TEM**. (A). Cross section of the thallus median region by LM (sample PAL4, fresh). Detail of the palisade plectenchyma upper cortex (**UC**) and phycobiont layer (**PhL**). Two types of phycobionts (yellow and red squares) coexist (**Ph1** and **Ph2**). (B) Phycobiont-type 1 (**Ph1)** (yellow square) showing a lax chloroplast (**Chl**) with a central pyrenoid (**Py**) *corticola-type*, abundant mitochondria (**Mi**) and small peripheral vesicles (**PV**). TEM, Bar: 2 µm. (C) Phycobiont-type 2 (**Ph2**) (red square) showing a dense chloroplast (**Chl**) with large electrodense vesicles (**EV**) and an irregular and considerable secretion zone (**SZ**). TEM, Bar: 2 µm. (D) Cross section of the thallus peripheral lobe by LM (sample 3722, cryopreserved), showing medulla (**M**), upper cortex (**UC**) and phycobiont layer (**PhL**). This layer has two types of phycobiont cells (**Ph1** and **Ph2**) located immediately below the upper cortex. The type 2-phycobionts- seem to be more abundant than those of type 1. (E) Cross sections of the thallus median region by LM (sample PAL4), medulla (**M**), upper cortex (**UC**) and phycobiont layer (**PhL**). This layer has two types of phycobiont cells (**Ph1** and **Ph2**) but the type 1-phycobionts- seem to be more abundant than those of type 2. Abbreviations: **Ep**, epicortex; **DIs**, developing isidium; **M,** medulla; **Hy,** hypha, **CW,** cell wall**.**

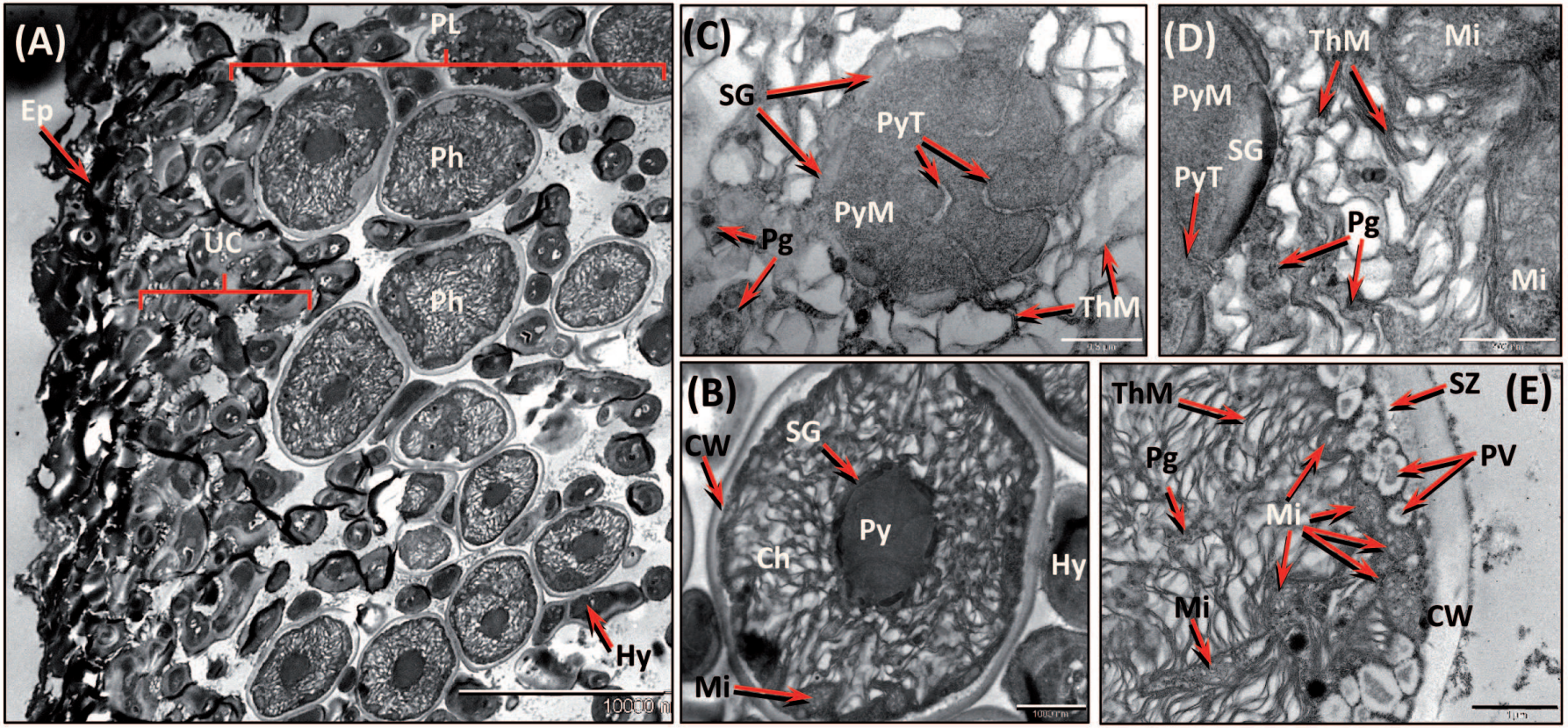

Fig. 3.- ***Parmotrema pseudotinctorum.*****Cross section of a peripheral lobe and phycobionts of** ***Trebouxia sp. corticola*** **gp.(Ph1) by TEM**. (A) Thallus detail showing the epicortex (**Ep**), the upper cortex (**UC**), and the phycobiont layer (**PhL**). Bar: 10 µm.(B) Phycobiont-type 1 (**Ph1)** showing a pyrenoid (**Py**) *corticola-type*, and abundant mitochondria (**Mi**). The chloroplast (**Chl**) thylakoid membranes are very lax. Bar: 1 µm. (C) Detail of a pyrenoid withvery thin, unbranched tubules (**PyT**) and a sinuous profile.There are no pyrenoglobuli (**Pg**) associated with the pyrenoid matrix (**PyM**).Pyrenoglobuli are rather developed in the chloroplast stroma, near the pyrenoid. Starch grains (**SG**) are dark and closely connected with the pyrenoid matrix, shaping a starch sheath made up by a few large, curved plates adjacent to the pyrenoid. Bar: 0.5 µm. (D) Chloroplast detail showing the thylakoid membranes (**ThM**) and the pyrenoglobules (**Pg**).Bar: 0.5 µm. (E) Detail showing numerous mitochondria and small peripheral vesicles (**PV**) with grey content. Bar: 1 µm. Abbreviations: cell wall, **CW**; hypha, **Hy**. From fresh sample PAL4.

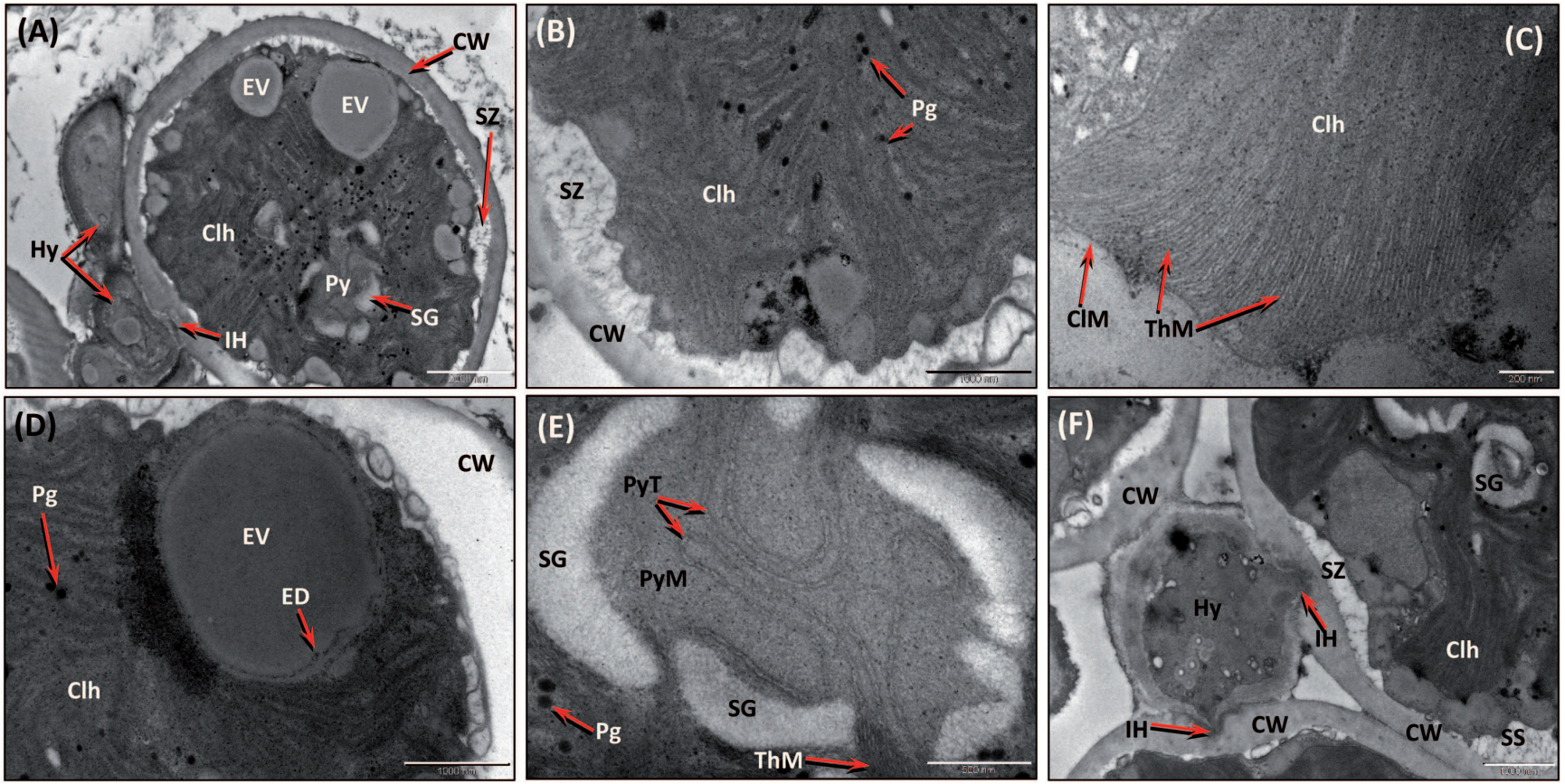

Fig. 4.- ***Parmotrema pseudotinctorum.*****Cross section of a thallus median region and type 2-phycobionts (Ph2) by TEM**. (A) Phycobionttype 2 (**Ph2)**. Bar: 2 µm. (B) Detail ofchloroplast (**Chl**) showing dense thylakoid membranes (**ThM**) with some pyrenoglobules (**Pg**) and the secretionzone (**SZ**). Bar: 1 µm. (C) Chloroplast (**Chl**) showingvery denseand tidy thylakoid membranes (**ThM)**. Bar: 0.2 µm. (D) Detail of an electro-dense vesicle (**EV**) showing a depositof electro-dense material (**ED**) atthe periphery. Bar: 1 µm. (E) Detail of a pyrenoid (**Py**) *corticola-type* withvery thin, unbranched tubules (**PyT**) of sinuous profile.There are nopyrenoglobuli (Pg) associated with the pyrenoid matrix (**PyM**).Pyrenoglobuli are rather developed in the chloroplast stroma, adjacent to the pyrenoid. Starch grains (**SG**) are closely connected with the pyrenoid matrix, shaping a starch sheath made up of a few large, curved plates adjacent to the pyrenoid. Bar: 0.5 µm. (F) Detail of an interaction phycobiont-mycobiont done by a type 2 intraparietalhaustorium (**IH**) (according to Honegger). Bar: 1 µm. Abbreviations: cell wall, **CW**; hypha, **Hy**; thylakoid membrane, **ThM**; chloroplast membrane, **ClM**.From fresh sample PAL4.

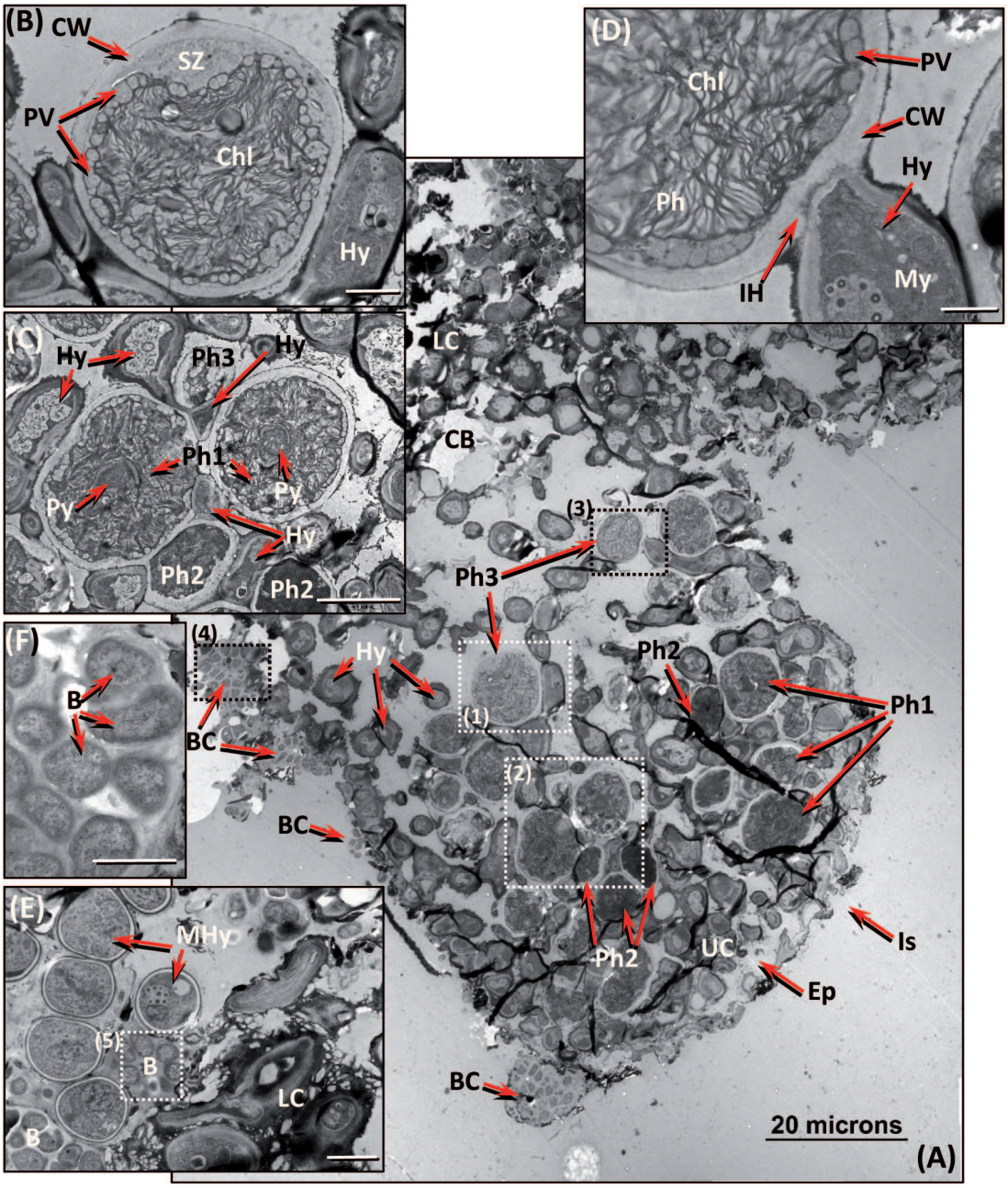

Fig. 5.- Isidium structure in *Parmotrema pseudotinctorum* (A). TEM photomicrography of a mature isidium (**Is**) showing three types of phycobiont cells (**Ph1, Ph2** and **Ph3**) spreading immediately below the upper cortex (**UC**), medullary hyphae (**Hy**) intrusions into the isidium center, and the constricted base (**CB**) with a lower cortex (**LC**) in development. Details highlighted in dashed square 1, 2, 3, and 4 are shown in Figures B, C, D and E respectively. (**BC**: bacterial communities, **Ep**: epicortex). Bar: 20 µm. (B) Phycobiont-type 3 (**Ph3**) showing a lax chloroplast (**Chl**) and abundant small peripheral vesicles (**PV**) with sparse content. Bar: 2 µm. (**CW**: cellular wall; **SZ**: irregular secretion zone). (C) Detail of hyphae (**Hy**) interacting simultaneously with phycobionts type 1 (**Ph1**) (apparent pyrenoid (**Py**) *corticola-type*), type 2 (**Ph2)**and type 3 (**Ph3**). Bar: 5µm. (D) Detail of a mycobiont (**My**)-phycobiont type 3 (**Ph3**) interaction of an intraparietal haustorium (type 2 according to Honegger). Bar: 1 µm. (E) Detail of a biofilm with abundant bacteria (**B**) interacting with modified hyphae (**MHy**) of the mycobiont near lower cortex (**LC**) of the isidium. Dashed square 5 is magnified in figure F. Bar: 2 µm. (F) Detail of bacteria (**B**). Bar: 1 µm.From fresh sample PAL1).

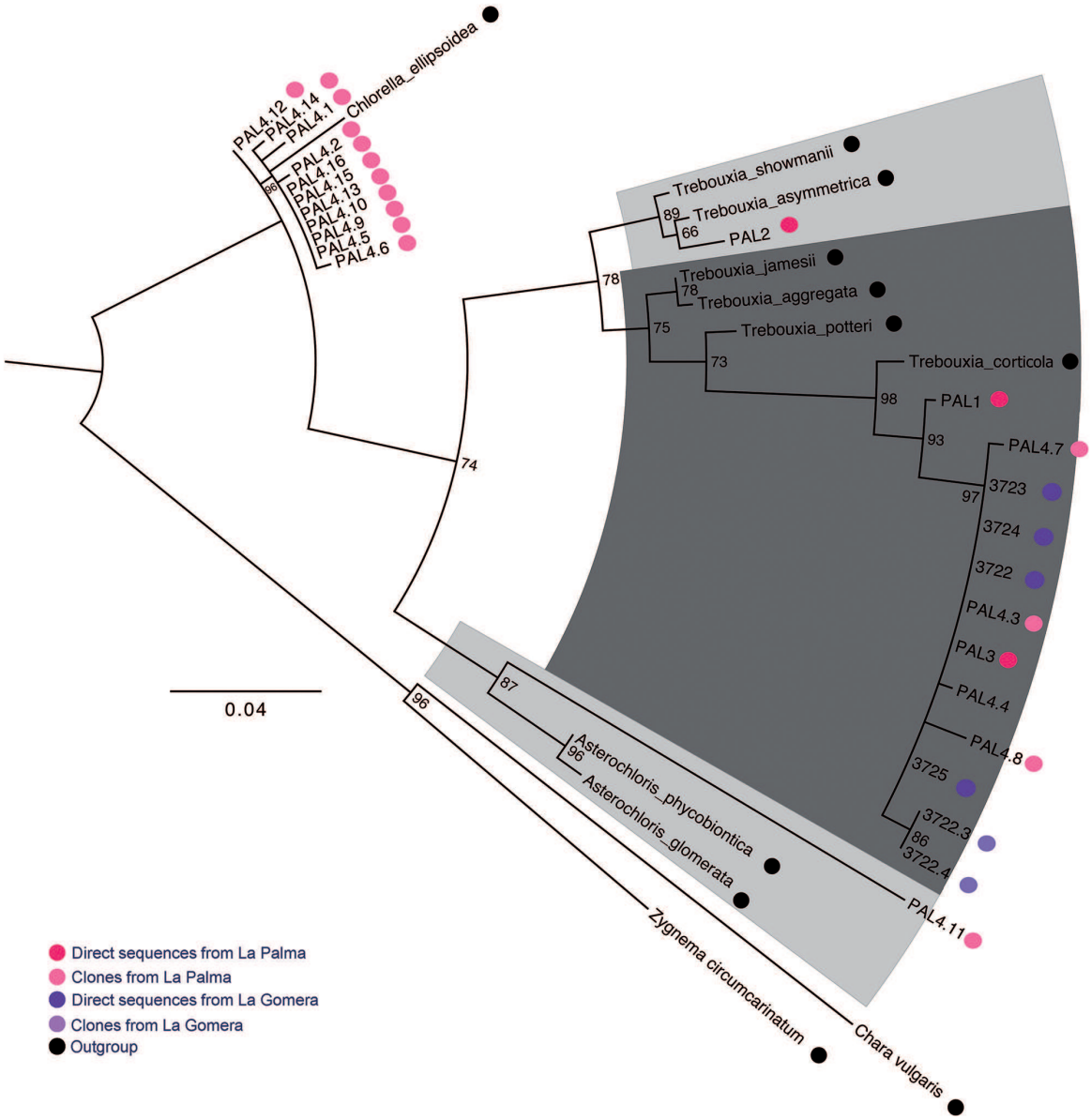

Fig. 6.- ML Phylogram based on the analysis of *psb*A gene sequences in specimens of Parmotrema *pseudotinctorum* phycobionts.The branches in the resulting phylogenies were tested for robustness with 1,000 bootstrap replicates. Samples from La Gomera island, direct sequences: 3722-3723-3724-3725 and clones: 3722.3-3722.4. Samples from La Palma island, direct sequences: PAL1-PAL3 and clones: PAL4.1, PAL4.2,…, PAL4.16. Several strains retrieved from GenBank were used as outgroup: *Asterochloris glomerata*,, *A. phycobiontica*, *Chara vulgaris, Chlorella ellipsoidea, Trebouxia aggregata*, *Trebouxia asymmetrica*, *Trebouxia corticola*, *Trebouxia jamesii*, *Trebouxia potteri, Trebouxia showmanii* and *Zygnema circumcarinatum* are reported with their NCBI accession numbers. Numbers at bold branches indicate bootstrap values.

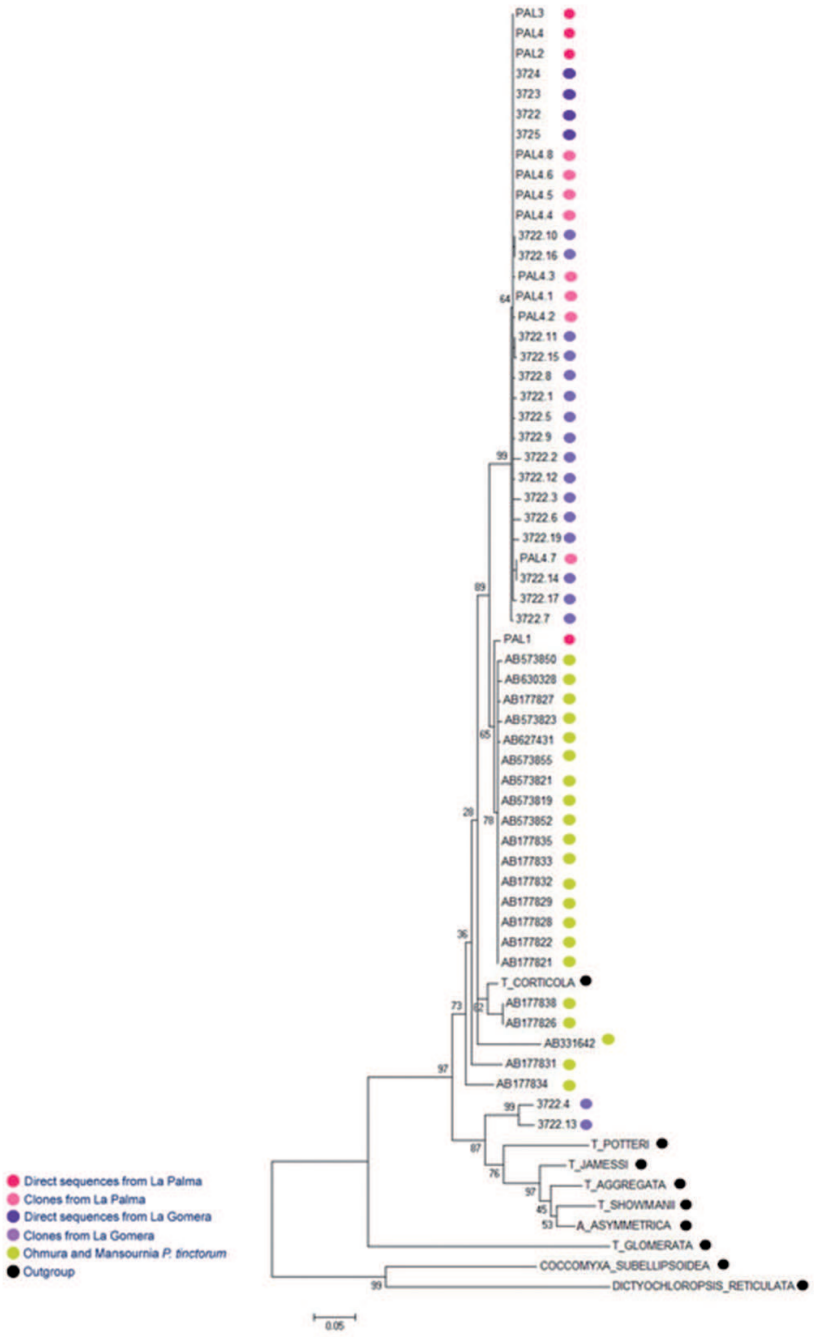

Fig. 7.- ML Phylogram based on the analysis of ITS gene sequences in specimens of *Parmotrema pseudotinctorum* phycobionts. The branches in the resulting phylogenies were tested for robustness with 1,000 bootstrap replicates. Samples from La Gomera, direct sequences: 3722-3723-3724-3725 and clones: 3722.1, 3722.2,…, 3722.18. Samples from La Palma, direct sequences: PAL1-PAL2-PAL3-PAL4 and clones: PAL4.1, PAL4.2,..., PAL4.8. Several strains retrieved from GenBank were used as out-group: *Asterochloris glomerata*, *Coccomyxa subellipsoidea*, *Dictyochloropsis reticulata*, *Trebouxia aggregata*, *Trebouxia asymmetrica*, *Trebouxia corticola*, *Trebouxia jamesii*, *Trebouxia potteri* and *Trebouxia showmanii* and *Trebouxia corticola* and are reported with their NCBI accession number. Numbers at bold branches indicate bootstrap values.