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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

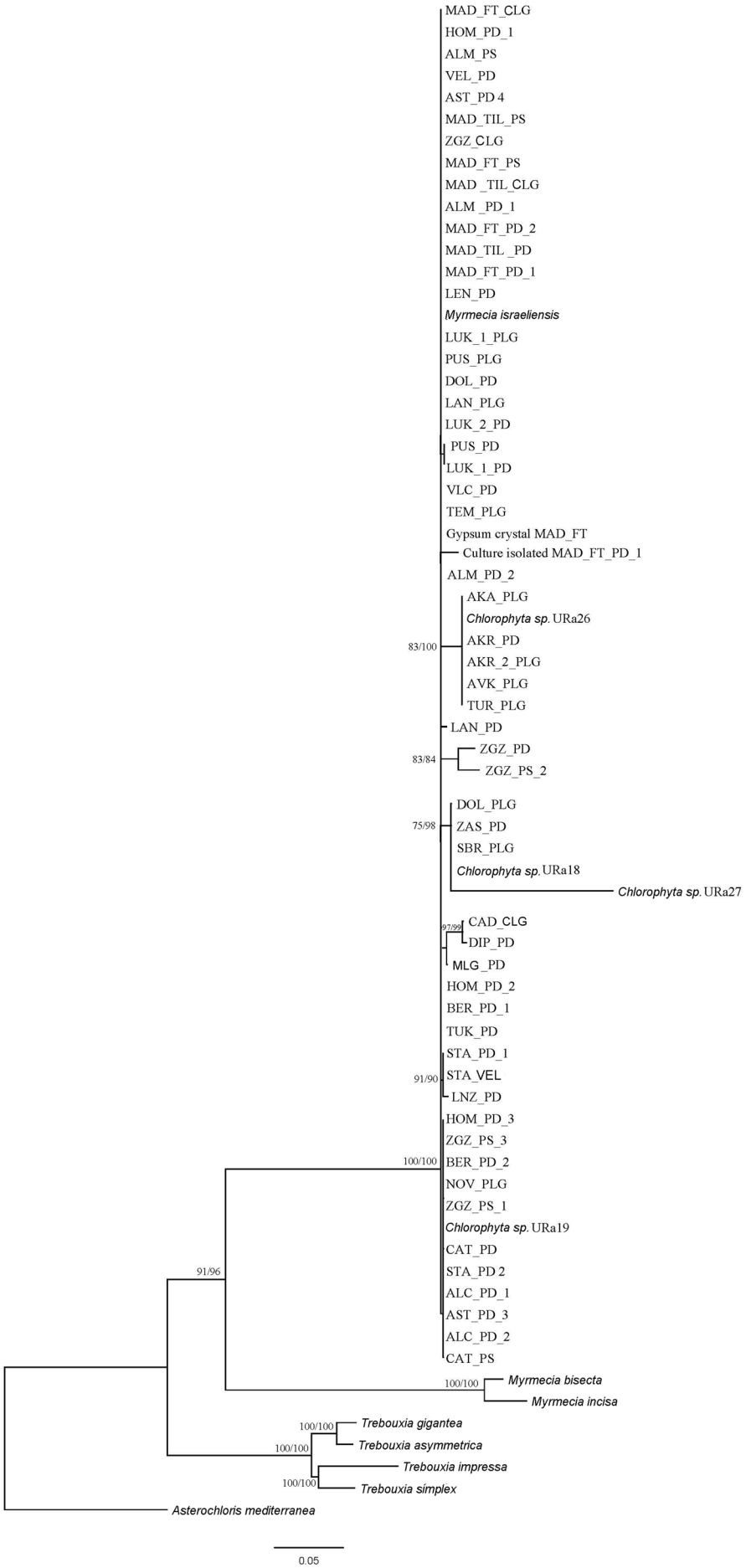
MAD_FT_CLG
HOM_PD_1
ALM_PS
VEL_PD
AST_PD 4
MAD_TIL_PS
ZGZ_CLG
MAD_FT_PS
MAD _TIL_CLG
ALM _PD_1
MAD_FT_PD_2
MAD_TIL _PD
MAD_FT_PD_1
LEN_PD
Myrmecia israeliensis
LUK_1_PLG
PUS_PLG
DOL_PD
LAN_PLG
LUK_2_PD
PUS_PD
LUK_1_PD
VLC_PD
TEM_PLG
Gypsum crystal MAD_FT
Culture isolated MAD_FT_PD_1
ALM_PD_2
AKA_PLG
Chlorophyta sp. URa26
83/100
AKR_PD
AKR_2_PLG
AVK_PLG
TUR_PLG
LAN_PD
83/84
ZGZ_PD
ZGZ_PS_2
DOL_PLG
75/98
ZAS_PD
SBR_PLG
Chlorophyta sp. URa18
Chlorophyta sp. URa27
97/99
CAD_CLG
DIP_PD
MLG _PD
HOM_PD_2
BER_PD_1
TUK_PD
STA_PD_1
91/90
STA_VEL
LNZ_PD
HOM_PD_3
ZGZ_PS_3
100/100
BER_PD_2
NOV_PLG
ZGZ_PS_1
Chlorophyta sp. URa19
CAT_PD
91/96
STA_PD 2
ALC_PD_1
AST_PD_3
ALC_PD_2
CAT_PS
100/100
Myrmecia bisecta
Myrmecia incisa
100/100
Trebouxia gigantea
100/100
Trebouxia asymmetrica
Trebouxia impressa
100/100
Trebouxia símplex
Asterochloris mediterranea
0.05

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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.

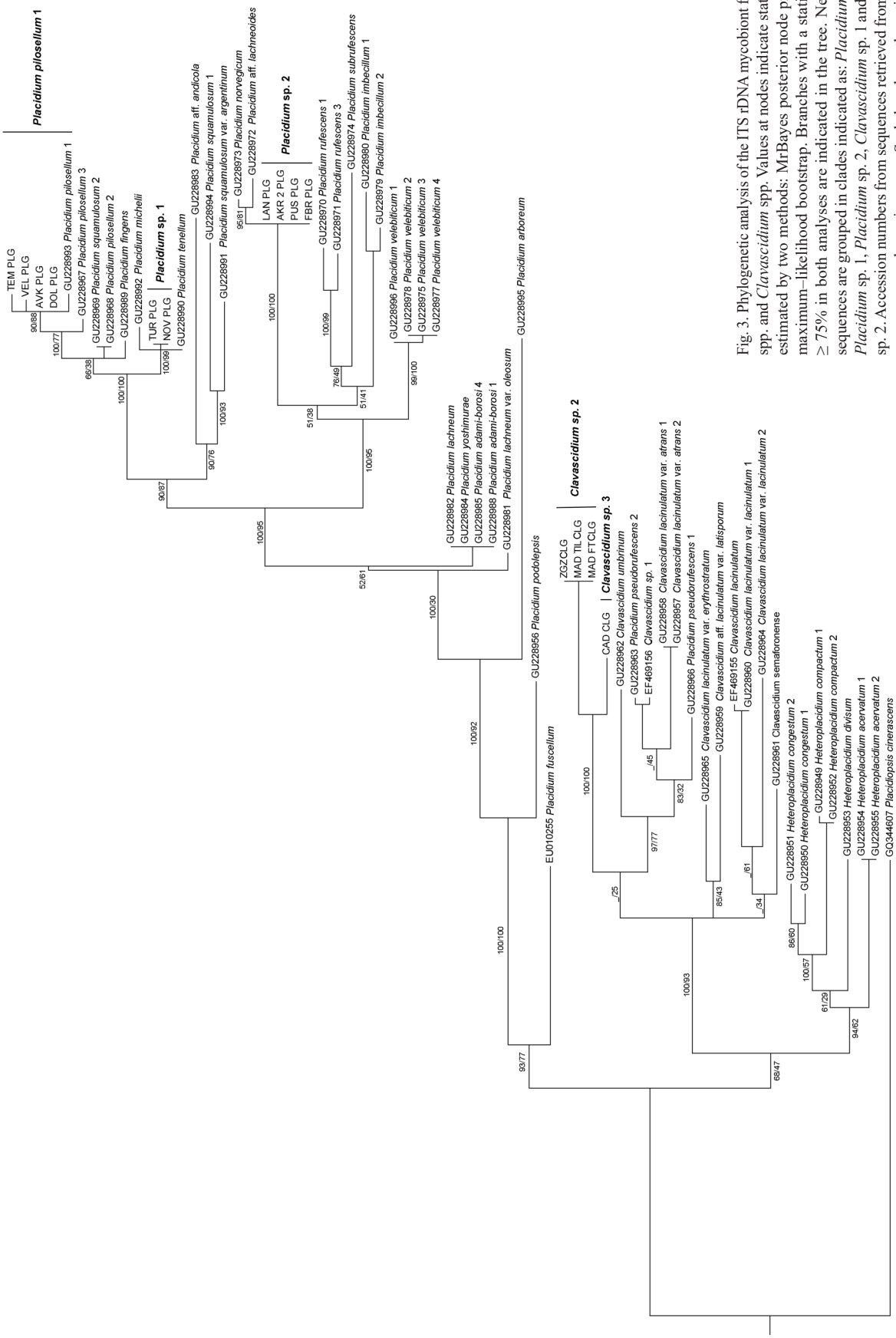

Fig. 3. Phylogenetic analysis of the ITS rDNA mycobiont from *Placidium* spp. and *Clavascidium* spp. Values at nodes indicate statistical support estimated by two methods: MrBayes posterior node probability and maximum–likelihood bootstrap. Branches with a statistical support ≥ 75% in both analyses 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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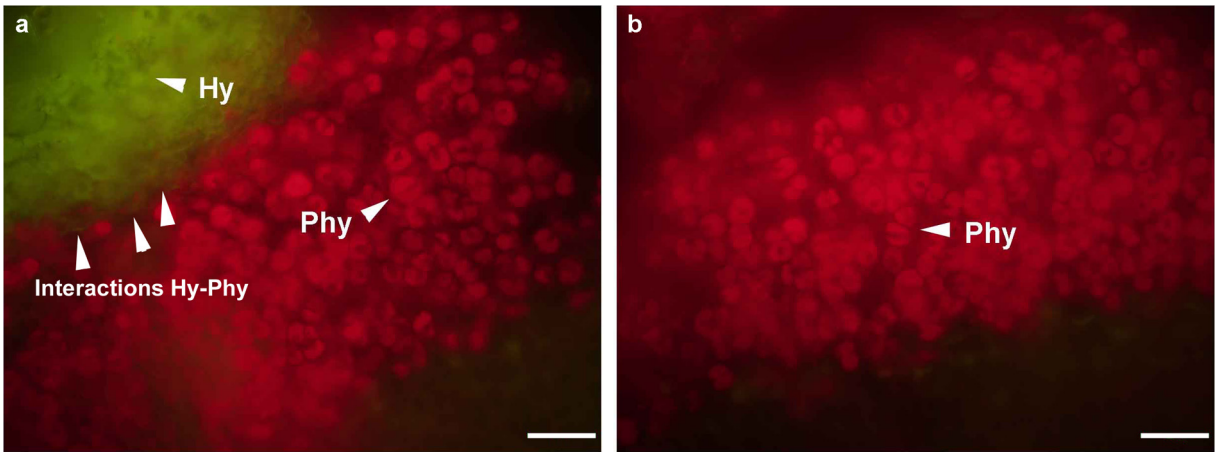


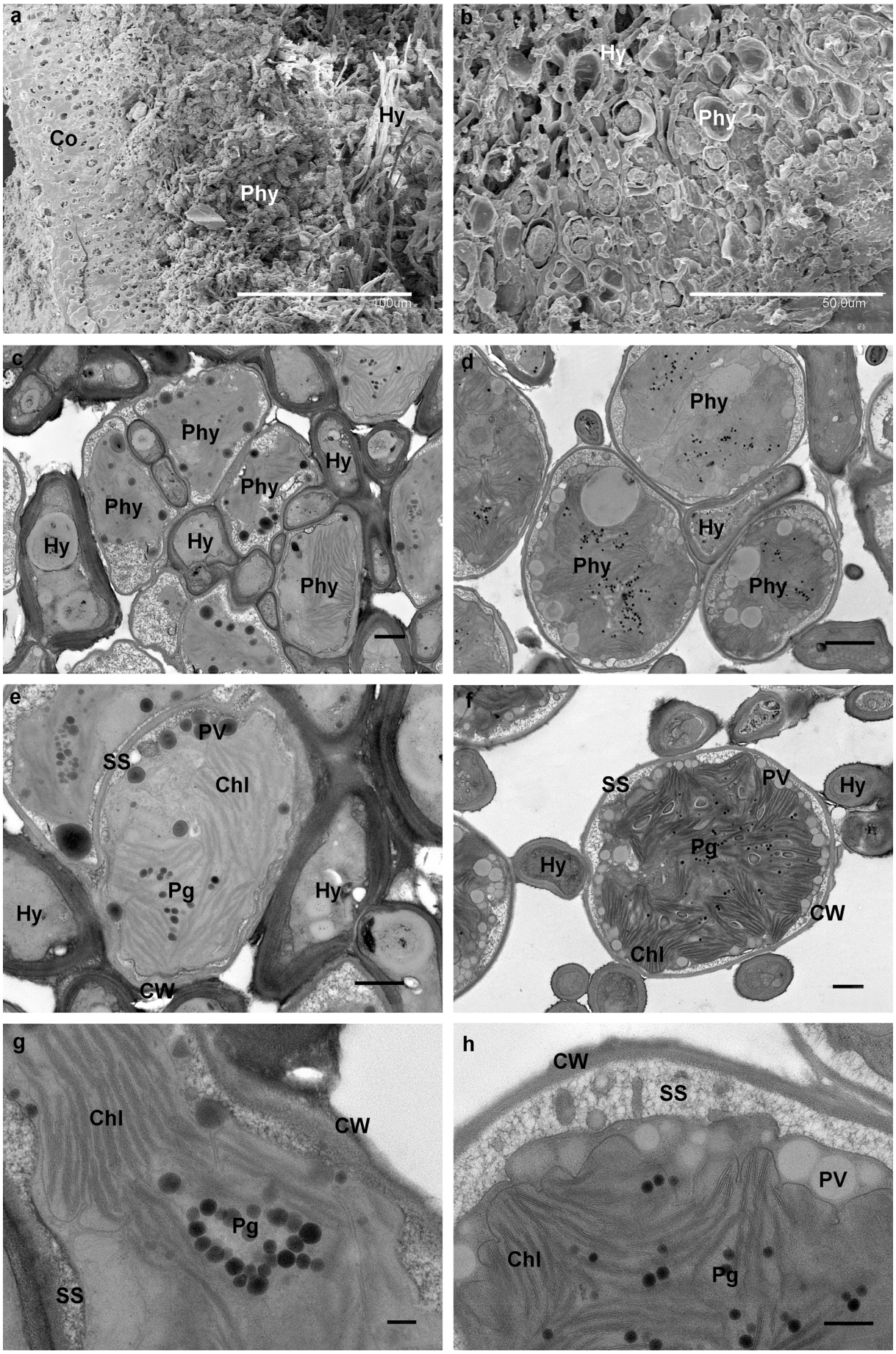
Fig. 4. Localization of *Myrmecia israeliensis* in squamules of *Psora decipiens* s. str. by FM. Abbreviations; Phy (Phycobionts) and Hy (Hyphae). Scale bar 20 μm.
FM examinations of squamules from the *Psora decipiens* s. str. sample (HOM_PD_1) revealed the interaction between *M. israeliensis* and 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


a
Co
Hy
Phy
100um
b
Hy
Phy
50.0um
c
Phy
Hy
Phy
Phy
Hy
Hy
Phy
d
Phy
Hy
Phy
Phy
e
PV
SS
Chl
Pg
Hy
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CW
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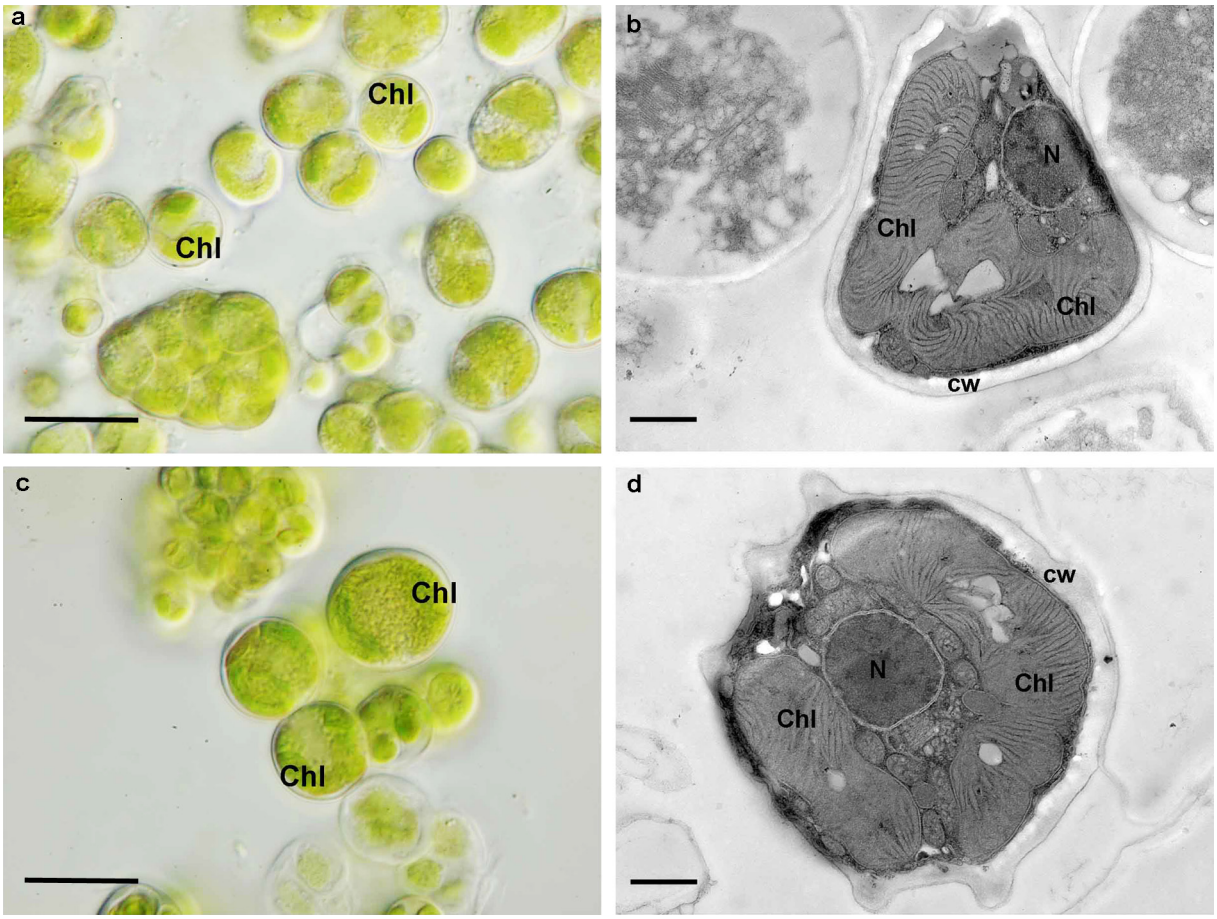

Fig. 6. *Myrmecia israeliensis* cells in isolated state by LM and TEM. Scale bar 800 nm. Abbreviations; Chl (Chloroplast), N (Nucleus), CW (Cell Wall).

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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