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Vulcanochloris (Trebouxiales, Trebouxiophyceae), a new genus of lichen photobiont from La Palma, Canary Islands, Spain

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Abstract

This paper describes a new genus of lichen photobionts, *Vulcanochloris*, with three newly proposed species, *V. canariensis*, *V. guanchorum* and *V. symbiotica*. These algae have been discovered as photobionts of lichen *Stereocaulon vesuvianum* growing on slopes of volcanos and lava fields on La Palma, Canary Islands, Spain. Particular species, as well as the newly proposed genus, are delimited based on ITS rDNA, 18S rDNA and *rbcL* sequences, chloroplast morphology, and ultrastructural features. Phylogenetic analyses infer the genus *Vulcanochloris* as a member of Trebouxiophycean order Trebouxiales, in a sister relationship with the genus *Asterochloris*. Our data point to the similar lifestyle and morphology of these two genera; however, *Vulcanochloris* can be well distinguished by a unique formation of spherical incisions within the pyrenoid. Mycobiont specificity and geographical distribution of the newly proposed genus is further discussed.

Introduction

The class Trebouxiophyceae, originally circumscribed by ultrastructural features as Pleurastrophyceae, is currently defined phylogenetically, predominantly by a similarity in 18S rDNA sequence data. As presently conceived, the class comprises single-celled, colonial and multicellular algae living mainly in freshwater or terrestrial habitats (Leliaert *et al*. 2012). Many members of this class are able to make symbiotic relationships. For example, species of genera *Elliptochloris* Tschermak-Woess (1980b: 71) and *Chlorella* Beyerinck (1890: 758) have been reported as symbionts of invertebrates (Letsch *et al*. 2009, Hoshina *et al*. 2010). The class is generally known to comprise the majority of eukaryotic lichen symbionts, *i.e*., the phycobionts. The genera *Trebouxia* Puymaly (1924: 109), *Asterochloris* Tschermak-Woess (1980a: 291), *Coccomyxa* Schmidle (1901: 23) and *Myrmecia* Printz (1921: 13) are among the most common photobionts worldwide (Friedl & Büdel 2008, Tschermak-Woess 1988). However, due to a simple morphology and small cell sizes, diversity of Trebouxiophycean algae is still poorly understood. Indeed, a number of new species and genera are still being discovered (Hoshina *et al*. 2010, Neustupa *et al*. 2011, 2013, Gaysina *et al*. 2013). Many findings of new taxa could be expected also among lichen photobionts, mainly among "*Chlorella*-like" lichenized algae (Friedl & Bhattacharya 2002, Nyati *et al*. 2007, Thüs *et al*. 2011).

The Canary Islands are famous for their extraordinary diversity of vascular plants. Among the free-living algae attention was almost exclusively paid to marine representatives (Bouza *et al*. 2006, García-Jiménez *et al*. 2008, Cassano *et al*. 2012). The diversity of lichenized algae has been studied only marginally, as a part of the studies investigating the photobiont diversity of *Tephromela atra* (Hudson 1762: 445) Hafellner (1983: No. 297) (Muggia *et al*. 2010), *Ramalina farinacea* (Linnaeus 1753: 1146) Acharius (1810: 606) (Casano *et al*. 2011, Campo *et al*. 2013), *Lecanora rupicola* (Linnaeus 1767: 132) Zahlbruckner (1928: 525), *L. carpinea* (Linnaeus 1753: 1141) Vainio (1888: 23) (Blaha *et al*. 2006) and *Parmotrema pseudotinctorum* (Abbayes 1951: 973) Hale (1974: 338) (Molins *et al*. 2013). The Canary Islands are known to host a high diversity of lichens and lichenicolous organisms. The most recent checklist lists more than 1600 species for an area of just 7447 km^2 (Hernández Padrón & Pérez-Vargas 2010). One of the most abundant lichens of Canary Islands, *Stereocaulon vesuvianum* Persoon (1810: 19), has been subjected to a study investigating its role in rock weathering processes (Stretch & Viles 2002). However, no study has been performed to explore the photobiont diversity in this remarkable lichen species, so far.

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TABLE 1. Localities of specimens of *Stereocaulon vesuvianum* from La Palma, Canary Islands, Spain.

Specimen	Locality	Substrate	Elevation	GPS	Date	GenBank accession		
						*rbc*L	SSU rDNA	ITS rDNA
L1616	Volcán de San Antonio	lava stone	about 630	28.481944° N, 17.849444° W	16/10/2011	KR952309		KR952317
L1617	Volcán de San Antonio	lava stone	about 500	28.481389° N, 17.845556° W	16/10/2011			KR952318
L1618	Volcán de San Antonio	lava stone	about 500	28.481389° N, 17.845556° W	16/10/2011	KR952310	KR952314	KR952319
L1620	Volcán Teneguía	lava stone	about 330	28.473056° N, 17.847222° W	16/10/2011	KR952311	KR952315	KR952320
A72	Volcán de San Antonio	top of the volcano	589	28.485500° N, 17.849917° W	17/5/2013			KR952321
A73	Volcán de San Antonio	top of the volcano	589	28.485500° N, 17.849917° W	17/5/2013			KR952322
A74	Volcán de San Antonio	lava stone	575	28.487167° N, 17.849139° W	17/5/2013			KR952323
A75	Volcán de San Antonio	lava stone	550	28.486511° N, 17.849786° W	17/5/2013			KR952324
A77	foothill of Volcán de San Antonio	lava stone	399	28.477694° N, 17.850361° W	17/5/2013			KR952325
A78	Volcán Teneguía	lava	396	28.474722° N, 17.851028° W	17/5/2013			KR952326
A80	foothill of Volcán Teneguía	lava stone	188	28.464139° N, 17.845333° W	17/5/2013			KR952327
A97	3 km to the East of El Paso	rock on edge of lava field	849	28.653167° N, 17.851194° W	19/5/2013			KR952328
A98	3 km to the East of El Paso	lava field	860	28.652800° N, 17.851200° W	19/5/2013	KR952312		KR952329
A104	2.5 km to the north-east Puerto de Naos	lava field	400	28.604722° N, 17.895389° W	20/5/2013	KR952313	KR952316	KR952330
A105	2.5 km to the north-east Puerto de Naos	lava field	463	28.598806° N, 17.89338° W	20/5/2013			KR952331

During our recent investigation of lichen symbionts on slopes of volcanos and lava fields on La Palma, Canary Islands, we discovered a new photobiont lineage in several thalli of *Stereocaulon vesuvianum*. The main goal of this study is to describe this lineage as a new genus of Trebouxiophycean algae, *Vulcanochloris*, and to characterize its three newly proposed species, *Vulcanochloris canariensis*, *V. guanchorum* and *V. symbiotica*.

Material and Methods

The material was collected in October 2011 and May 2013 on La Palma (Canary Islands, Spain) on volcanos, lava fields and lava flows (Table 1). Photobionts were isolated by the thallus fragment method (Ahmadjian 1993) and cultivated as described in Peksa & Škaloud (2008).

For transmission electron microscopy (TEM) investigations, the samples were fixed for 2 h at 5 °C in 2% glutaraldehyde in 0.05 M phosphate buffer. Then, they were post-fixed for 2 h in 1% OsO_4 in 0.05 M phosphate buffer and for 12 h at 5 °C in 1% uranyl acetate solution. Then, the samples were dehydrated through an ethanol series and finally, they were embedded in Spurr's medium via isobutanol. Ultrathin sections, cut with a diamond knife were post-stained with lead citrate and examined using a JEOL 1011 transmission electron microscope.

Total genomic DNA was extracted from fragments of thalli following the modified CTAB protocol (Cubero *et al*. 1999), with minor modifications. Three molecular markers were amplified by PCR: nuclear ITS and 18S rDNA, and chloroplast *rbcL*. The internal transcribed spacer region (ITS1-5.8S-ITS2 rDNA) was amplified using the algal-specific primer nr-SSU-1780-5' (5'-CTG CGG AAG GAT CAT TGA TTC-3'; Piercey-Normore & DePriest 2001) and a universal primer ITS4-3' (5'-TCC TCC GCT TAT TGA TAT GC-3'; White *et al*. 1990). PCR amplification of the algal ITS began with an initial denaturation at 94 °C for 5 min, and was followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 2 min, with a final extension at 72 °C for 10 min. The amplification of *rbcL* region was performed as described by Thüs *et al*. (2011) using primers PRASF1-5' (5'-ATG GTT CCA CAA ACA GAA AC-3') and PRASR1-3' (5'-TTG TCA ATA GTA TCA AAT TC-3'; Sherwood *et al*. 2000) or a-ch-rbcL-203-5'-MPN-5' (5'-GAA TCW TCW ACW GGW ACT TGG ACW AC-3') and a-ch-rbcL-991-3'-MPN-3' (5'-CCT TCT ART TTA CCW ACA AC-3'; Nelsen *et al*. 2011). The amplification of 18S rDNA was performed as described by Thüs *et al*. (2011) using primers 18S F-5' (5'-AAC CTG GTT GAT CCT GCC AGT-3'; Katana *et al*. 2001) and newly designed 1650R-Astero-3' (5'-TCA CCA GCA CGT CCA AT-3') for first part of 18S rDNA region; and primers Al 1500af-5' (5'-GCG CGC TAC ACT GAT GC-3'; Helms *et al*. 2001) and ITS4-3' (5'-TCC TCC GCT TAT TGA TAT GC-3'; White *et al*. 1990) for second part of 18S rDNA region. PCR reactions were performed in a volume of 20 μL with Red Taq Polymerase (Sigma) as described by Peksa & Škaloud (2011) or with My Taq Polymerase (11.8 μL sterile Milli-Q Water, 4 μL 5x My Taq PCR buffer (Bioline), 0.5 μL of primers (25 $pM.mL^{-1}$), 0.2 μL My Taq HS DNA Polymerase (Bioline) (1 $U.mL^{-1}$), 3 μL of DNA (not quantified)). The PCR products were purified and sequenced at Macrogen in Seoul, Korea. The newly obtained sequences of the ITS rDNA, 18S rDNA and *rbcL* regions were deposited in GenBank with the accession numbers KR952309–KR952331.

Sequences of the *rbcL* gene were selected primarily based on the dataset of Fučíková *et al*. (2014b), and based on BLAST searches of our newly collected sequences. The outgroup was composed of ten representatives of order Chlorellales, which appears to be outside the Trebouxiophyceae according to recent research (Fučíková *et al*. 2014a). Alignment was produced manually by using MEGA6 (Tamura *et al*. 2013). Sequences of 18S rDNA were selected primarily based on the dataset of Škaloud *et al*. 2015. Ingroup comprises 36 sequences of representatives of Trebouxiales order, including three newly obtained sequences. *Lobosphaera incisa* (AY762602) was selected as outgroup. The sequences were aligned using MAFFT version 7 software (Katoh & Standley 2013) under the Q-INS-I strategy. The ITS rDNA data set consisted of 31 sequences: 15 newly obtained sequences from La Palma, three highly similar sequences from NCBI and 13 representatives of main lineages of *Asterochlosis* genus. Alignment was produced manually according to the secondary structures of ITS2 of *Asterochloris* (Škaloud & Peksa 2010) by using MEGA6 (Tamura *et al*. 2013).

The phylogenetic trees were inferred with Bayesian Inference (BI) by using MrBayes v. 3.2.2 (Huelsenbeck & Ronquist 2001), maximum likelihood (ML) analysis using GARLI v. 2.0 (Zwickl 2006), and maximum parsimony (MP) analysis using PAUP v. 4.0b10 (Swofford 2003), respectively. BI and ML analysis were carried out on a partitioned dataset to differentiate among individual *rbcL* codon positions or ITS1, 5.8 S and ITS2 rDNA regions. Substitution models were selected using the Bayesian information criterion (BIC) as implemented in JModelTest2 (Guindon & Gascuel 2003, Darriba *et al*. 2012): for the first *rbcL* codon position TIM1+I+Γ (gamma shape 0.8380), second *rbcL* codon position TVMef+I+Γ (gamma shape 0.3960), third *rbcL* codon position TVM+I+Γ (gamma shape 0.9800), 18S rDNA TrNef+I, ITS1 TrNef+Γ (gamma shape 1.0700), 5.8S JC, ITS2 TPM3+Γ (gamma shape 0.2540). ML analysis was carried out using default settings, five search replicates, and the automatic termination set at 10^5 generations. The MP analysis was performed using heuristic searches with 1000 random sequence addition replicates and random addition of sequences (the number was limited to 10^4 for each replicate). ML and MP bootstrap support values were obtained from 100 and 1000 bootstrap replicates, respectively. Only one search replicate was applied for the ML bootstrapping.

To compare alternative phylogenetic topologies, the one-tailed Shimodaira-Hasegawa nonparametric tests (SH tests; Shimodaira & Hasegawa 1999). For the tests, ML trees were calculated with specified topological constraints using GARLI v. 2.0. Thereafter, trees with topological constraints were compared with the optimal topology using the SH test statistics, inferred with the RELL bootstrap option, as implemented in PAUP v. 4.0b10.

Results

Phylum **Chlorophyta**
Class **Trebouxiophyceae**
Order **Trebouxiales**
Family **Trebouxiaceae**

Vulcanochloris Vančurová, Peksa, Němcová *et* Škaloud, *gen. nov.*

Vegetative cells spherical, rarely oval or oviform. Cell wall thin, seldom a flat local thickening of the cell wall can be observed. Rarely, the cell wall is slightly thickened along its entire surface. Cells contain a single asteroid chloroplast, with a distinct pyrenoid in its centre. The pyrenoid often contains one to several spherical incisions. Prior to aplano- and zoosporogenesis, the chloroplast flattens and assumes a parietal position. Asexual reproduction by 16–128 aplanospores or 64–128 zoospores. Zoospores naked, with two apical flagella and a simple basal chloroplast; stigma not observed. Mature aplanospores and zoospores liberated by rupturing of the mother cell wall. Lichen photobiont, so far found only in thalli of *Stereocaulon vesuvianum*. Morphologically similar to *Asterochloris*, from which it differs by the presence of spherical incisions in the pyrenoid matrix.

Type species:—*Vulcanochloris canariensis*, *sp. nov.* (see below)

Etymology:—From "Vulcanus" (L), Roman god of fire, and "chloris" (Gr.), meaning greenish-yellow. The name indicates that this algal genus was originally reported from a volcanic substrate.

Chloroplast morphology and ultrastructure:—The chloroplast is centrally located, axial, with variously arranged lobes reaching the cell periphery. Several chloroplast types can be recognized, as follows: i) a deeply lobed type, characterized by long lobes emerging directly from the thin chloroplast layer spreading around the pyrenoid ("Tieflappig Typ" *sensu* Gärtner 1985b; Figs. 1A, B); ii) a shallowly lobed type, which is similar to the previous type but differs in that the chloroplast lobes are shorter, emerging from the central mass of the chloroplast layer ("Normaltyp" *sensu* Gärtner 1985b; Figs. 1C, D); iii) a crenulate type, characterized by a central, massive chloroplast with a regularly nodulated surface ("Crenulater Typ" *sensu* Gärtner 1985a; Figs. 1E, F); and vi) an echinate type, distinguished by numerous thin radial lobes emerging uniformly from the central mass of the chloroplast layer (Figs. 1G, H). In the late ontogenetic stages, specifically prior to zoo- or aplanosporogenesis, the chloroplast transforms into the parietal type, with smooth, never lobed margins, which is followed by its division into numerous parts in preparation for asexual reproduction (Fig. 1I).

Large, distinct pyrenoid lies in the chloroplast centre (Figs. 1A, C, E, G). The pyrenoid is usually spherical, rarely irregularly elongated, surrounded by a high number of small starch grains (Figs. 1C, J). The pyrenoid is irregularly transversed by inclusions bearing a close structural resemblance to the chloroplast thylakoids (Figs. 1K, M). In some cases, the incisions are clearly lined by membranes (Figs. 1K, M). No pyrenoglobuli are associated with the thylakoid-like inclusions in the centre of the pyrenoid matrix. Instead, they are developed at the pyrenoid periphery (Figs. 1K, M). One to several electron-lucent, spherical to elongated regions are frequently formed within the pyrenoid matrix (Figs. 1N, O). Rarely, these regions may be associated with several pyrenoglobuli (Fig. 1N). Occasionally, a higher number (more than 8) of these electron-lucent regions are formed within the pyrenoid matrix (Fig. 1P). These regions probably correspond to spherical pyrenoid incisions observed in a light microscope (Figs. 1E, G, J).

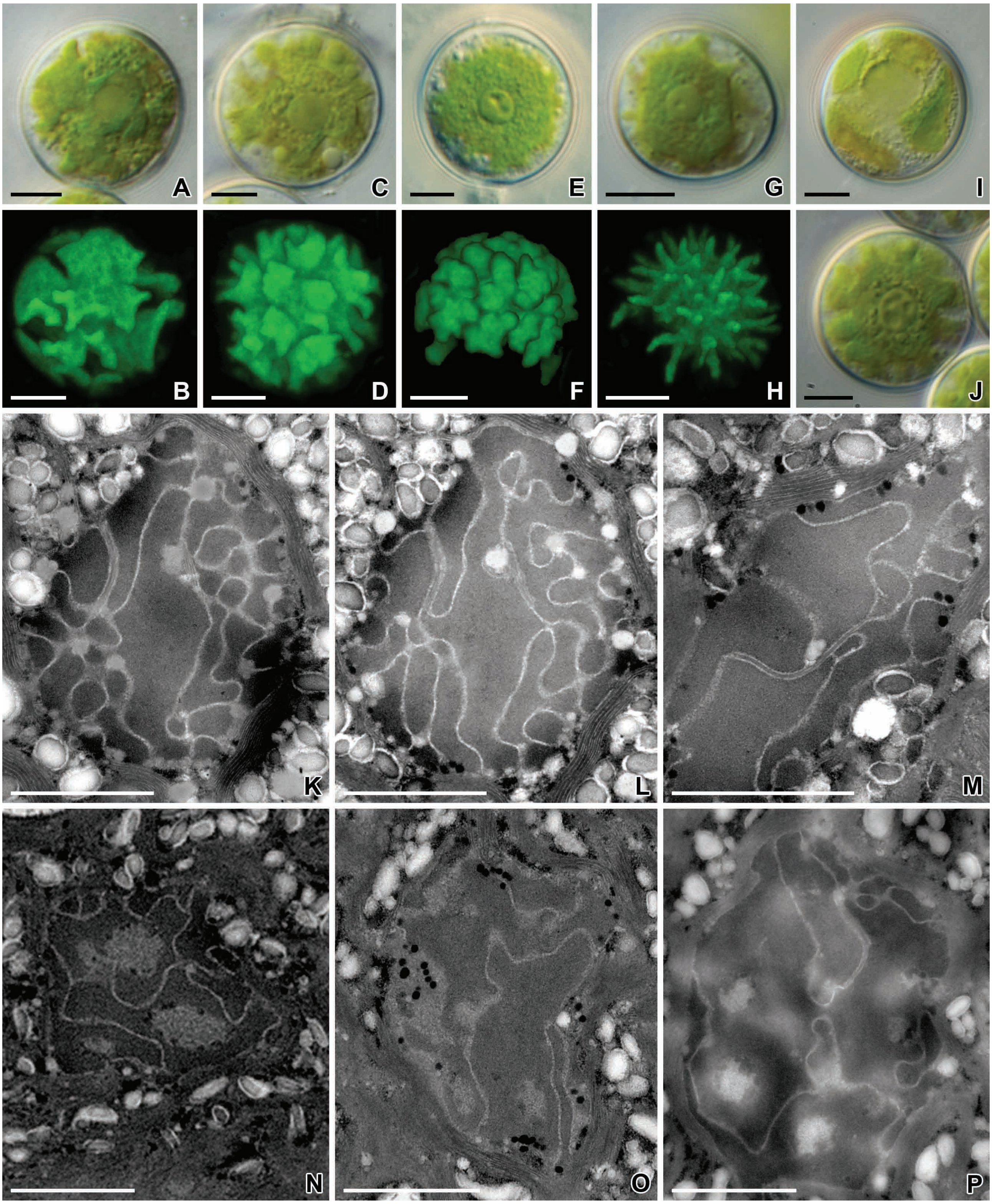

FIGURE 1. *Vulcanochloris canariensis*, *gen*. *et sp*. *nov*.. Chloroplast morphology and ultrastructure. A, B. A deeply lobed type of chloroplast. C, D. Shallowly lobed type of chloroplast. E, F. Crenulate type of chloroplast. G, H. Echinate type of chloroplast. I. Parietal type of chloroplast. J. High number of small starch grains surrounding the pyrenoid. K–M. Pyrenoid irregularly transversed by inclusions bearing a close structural resemblance to the chloroplast thylakoids. N, O. One to several electron-lucent, spherical to elongated regions frequently formed within the pyrenoid matrix. P. Higher number (more than 8) of the electron-lucent regions formed within the pyrenoid matrix. Scale bars = 5 µm (A–J); 1 µm (K–P).

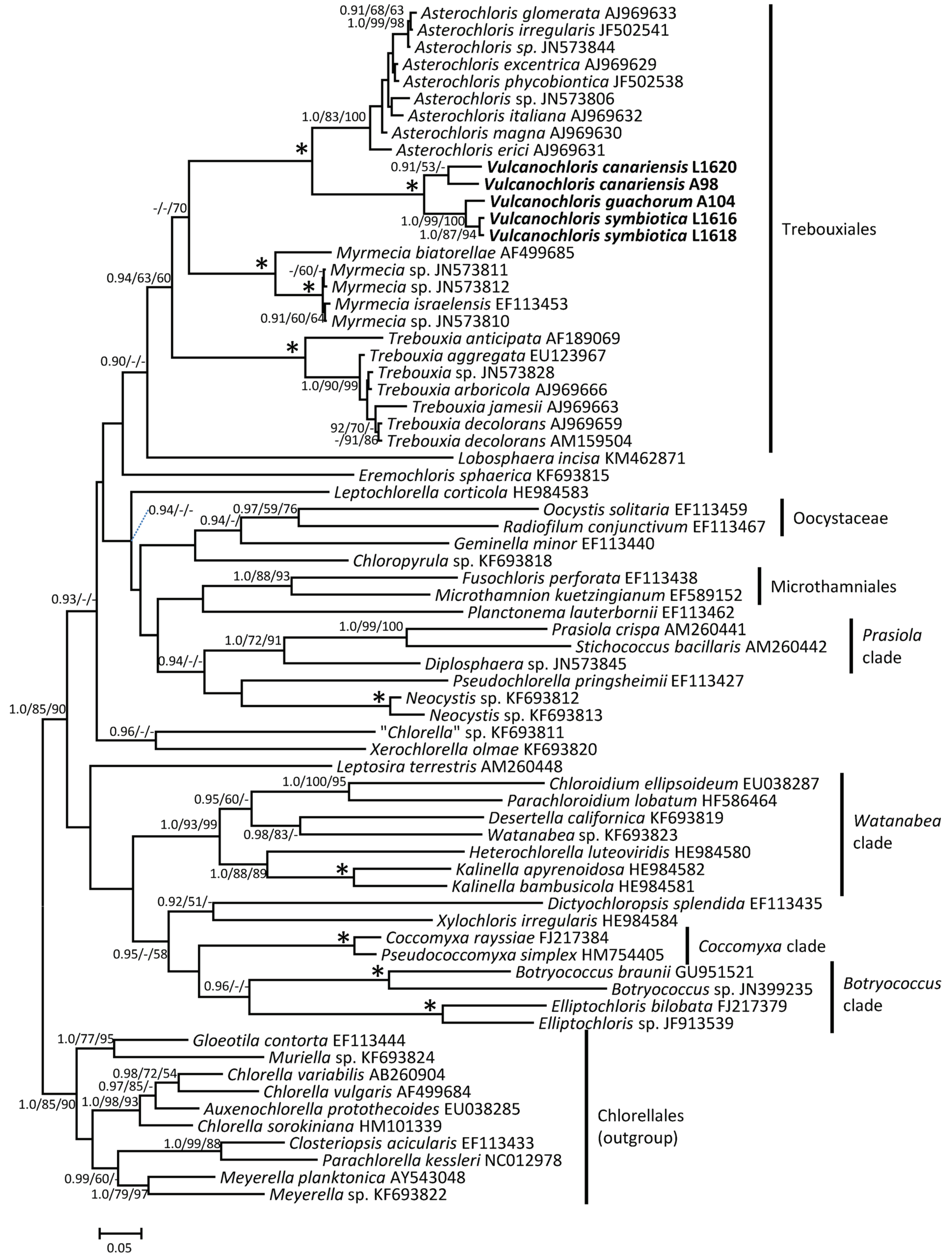

FIGURE 2. Bayesian analysis based on the *rbcL* dataset. Values at the nodes indicate statistical support estimated by three methods—MrBayes posterior-node probability (left), maximum-likelihood bootstrap (middle), and maximum parsimony bootstrap (right). Asterisk represents full support. Scale bar shows the estimated number of substitutions per site. Newly sequenced strains are marked in bold.

Molecular analyses:—To evaluate both the phylogenetic position and genetic diversity of *Vulcanochloris*, we sequenced *rbcL* gene, 18S rDNA and ITS rDNA spacer for several isolates. The *rbcL* data set consists of 70 Trebouxiophycean taxa, with 1139 characters. All phylogenetic analyses (BI, ML, MP) resolved *Vulcanochloris* as a distinct clade within Trebouxiales, with full statistical support (Fig. 2). In addition, sister position of genera *Vulcanochloris* and *Asterochloris* was highly supported, as well, with a moderate to very strong support for the monophyly of the latter genus. To further evaluate the reciprocal monophyly of *Vulcanochloris* and *Asterochloris*, we performed several Shimodaira-Hasegawa nonparametric tests (SH tests) comparing the best tree with four optimal trees constrained for *Asterochloris* paraphyly. The four topological constraints each represented one of the paraphyletic trees obtained by the ML botstrapping, as follows: i) monophyly of *Vulcanochloris*, *A. erici*, *A. magna* and *A. phycobiontica*, ii) monophyly of *Vulcanochloris*, *A. erici* and *A. phycobiontica*, iii) monophyly of *Vulcanochloris* and *A. erici*, iv) monophyly of *Vulcanochloris*, *A. erici* and *A. magna*. Tree comparisons indicated that paraphyly of *Asterochloris* was a significantly worse interpretation of these data ($p < 0.001$, -ln for monophyly: 22,873.5, -ln for paraphyly: i) 22,905.5, ii) 22,902.9, iii) 22,893.4, iv) 22,893.4), supporting the reciprocal monophyly of genera *Asterochloris* and *Vulcanochloris*.

18S rDNA data set consisted of 37 sequences with 1776 characters, including three newly obtained *Vulcanochloris* sequences. Bayesian inference of the 18S rDNA and *rbcL* data yielded similar tree topologies, resolving *Vulcanochloris*, *Asterochloris*, *Trebouxia*, and *Myrmecia* as well-defined, distinct genera. In the 18S rDNA analysis (see Supplementary File 1), a clade of environmental sequences from soil samples (Lesaulnier *et al*. 2008) was additionally inferred. Comparison with other 18S rDNA sequences showed that six *Asterochloris* strains and two *Vulcanochloris* samples (A104, L1618) contained IB3 group I introns at position 516 relative to the *E. coli* coding region. The exon SSU rDNA sequences of samples A104 and L1618 were completely identical.

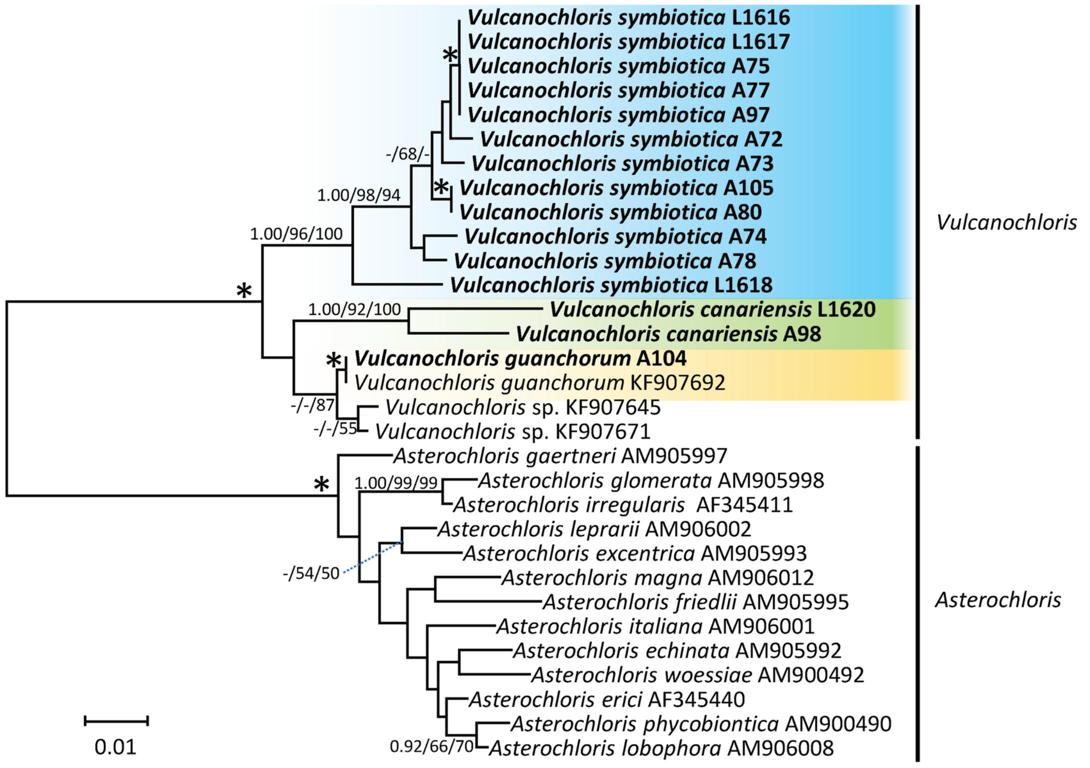

FIGURE 3. Bayesian analysis based on the ITS rDNA dataset. Values at the nodes indicate statistical support estimated by three methods—MrBayes posterior-node probability (left), maximum-likelihood bootstrap (middle), and maximum parsimony bootstrap (right). Asterisk represents full support. Scale bar shows the estimated number of substitutions per site. Newly sequenced strains are marked in bold.

ITS rDNA data set consisted of 31 sequences with 502 characters, including 15 newly obtained *Vulcanochloris* sequences, 13 *Asterochloris* sequences selected to encompass the entire diversity of this genus, and 3 additional sequences retrieved by BLAST searches at NCBI. The BI, ML, and MP phylogenetic analyses inferred from the ITS rDNA sequences resulted in highly similar phylogenetic trees, recognizing *Asterochloris* and *Vulcanochloris* as two distinct lineages, with full statistical support (Fig. 3). Newly obtained *Vulcanochloris* sequences formed three distinct lineages, here referred to as *V. symbiotica* sp. nov., *V. canariensis* sp. nov., and *V. guanchorum* sp. nov. *V. symbiotica* represents the most common lineage, containing 80% of all investigated isolates. This species was detected in all investigated localities. The second lineage, *V. canariensis*, consisted of two, genetically distinct isolates, A98 and L1620. The third lineage consisted of *V. guanchorum* isolate A104, and genetically identical sequence deposited in GenBank as "Chlorophyta sp. URa22" (KF907692). Finally, two additional sequences retrieved from GenBank as "*Asterochloris* sp. URa17" (KF907645, KF907671) were found to be members of the genus *Vulcanochloris*. However, their phylogenetic position, as well as the relationship among the three *Vulcanochloris* lineages, remain unresolved, though the *rbcL* and SSU rDNA phylogenetic analyses point to the close relationship of *V. guanchorum* and *V. symbiotica*.

Above-mentioned genetic investigation, as well as detailed morphological analyses of all the studied *Vulcanochloris* strains, revealed the existence of three distinct species. Descriptions of these new taxa are provided below.

Vulcanochloris canariensis **Vančurová, Peksa, Němcová *et* Škaloud, *sp. nov.***

Vegetative cells spherical or oval, up to 21 μm in diameter (Figs. 4A–C). Cell wall thin, seldom a flat local thickening of the cell wall (up to 3 μm thick) can be distinguished (Fig. 4B). Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery (Fig. 4A). Mature cells exhibit a crenulate chloroplast, characterized by a central, massive chloroplast with a regularly nodulated surface (Figs. 4B–E). Rarely, the shallowly lobed chloroplast has been observed as well (Figs. 4F, G). The chloroplast contains one distinct, centrally positioned pyrenoid, frequently containing one to several spherical incisions (Figs. 4A–C, H). A number of small starch grains are distributed around the pyrenoid (Fig. 4C). Asexual reproduction by 16–64 aplanospores formed in spherical or ellipsoidal sporangia, often bearing a local cell wall thickening (Fig. 4I). Zoospores not observed.

Type:—SPAIN. Santa Cruz de Tenerife: La Palma, slope of Volcán Teneguía, 28.473056º N, 17.847222º W, 330 m a. s. l., *L. Vančurová, 16 October 2011* (holotype: CAUP!, cryopreserved photobiont cells isolated from the specimen L1620, deposited in the Culture Collection of Algae of the Charles University in Prague as the item TYPE-H 1016). Reference strain: CAUP H 1016.

Habitat:—In thalli of *Stereocaulon vesuvianum* growing on basalt lava stones and rocks.

Etymology:—The specific epitheton reflects the place of origin of all known samples (Canary Islands).

Vulcanochloris symbiotica **Vančurová, Peksa, Němcová *et* Škaloud, *sp. nov.***

Vegetative cells usually spherical, occasionally oval and oviform, up to 18 μm in diameter (Figs. 4J, K, N). Cell wall thin, seldom a flat local thickening of the cell wall (up to 3 μm thick) can be distinguished (Fig. 4J). Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery (Fig. 4J). Mature cells exhibit a broad range of chloroplast types, with a deeply lobed form being the mostly frequently observed (Figs. 4K–M). In addition, the shallowly lobed (Figs. 4N, O), crenulate (Figs. 4P, Q) and echinate chloroplast (Fig. 4R) is observed as well. Lobes of the deeply lobed chloroplast are not simply terminated, but extended to either irregular plates (Fig. 4L) or branched projections (Fig. 4M). The chloroplast contains one distinct pyrenoid located in its centre (Figs. 4K, N, P). The pyrenoid occasionally contains one to several spherical incisions (Fig. 4N). A number of small starch grains are distributed around the pyrenoid (Fig. 4K). Asexual reproduction by 32 aplanospores or 128 zoospores produced in spherical or ellipsoidal sporangia with diameters up to 22 μm (Figs. 4S, T). Zoospores drop-shaped, naked, with two apical flagella and a simple basal chloroplast, 7.0–7.5 μm long and 3–4 μm wide (Fig. 4U).

Type:—SPAIN. Santa Cruz de Tenerife: La Palma, top of Volcán de San Antonio, 28.485500º N, 17.849917º W, 589 m a.s.l., *L. Vančurová & J. Malíček, 17 May 2013* (holotype: CAUP!, cryopreserved photobiont cells isolated from the specimen A72, deposited in the Culture Collection of Algae of the Charles University in Prague as the item TYPE-H 1017). Reference strain: CAUP H 1017

Habitat:—In thalli of *Stereocaulon vesuvianum* growing on basalt lava stones and rocks.

Etymology:—The specific epitheton reflects symbiotic lifestyle of this alga.

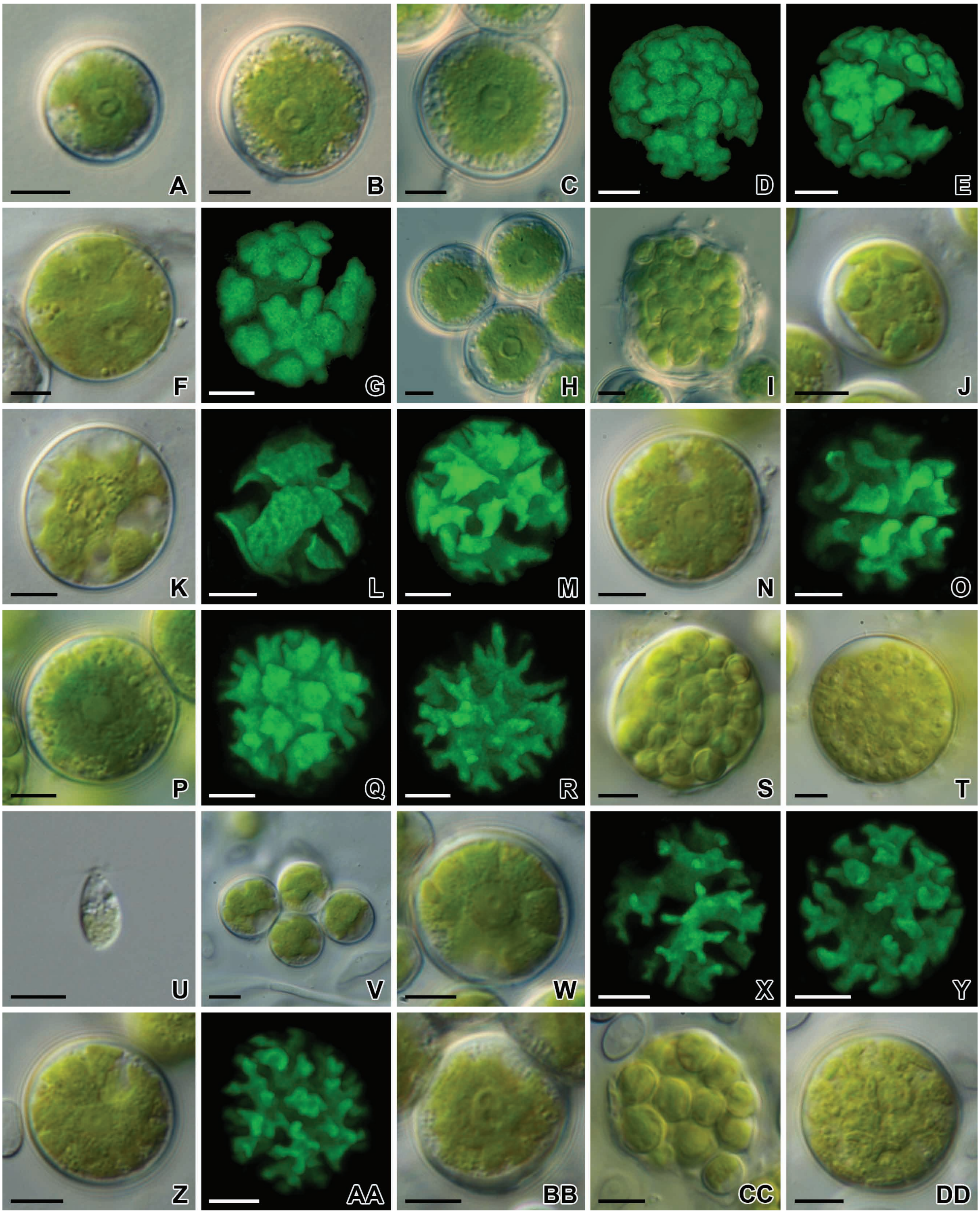

FIGURE 4. *Vulcanochloris*, *gen. nov*. Morphology in a light or a confocal microscope. A–I. *Vulcanochloris canariensis*, *sp. nov*. A–C. Spherical or oval vegetative cells. B–E. Crenulate chloroplast. F−G. Shallowly lobed chloroplast. A−C, H. One distinct, centrally positioned pyrenoid, frequently containing one to several spherical incisions. I. Asexual reproduction by 16–64 aplanospores formed in spherical or ellipsoidal sporangia. J−U. *Vulcanochloris symbiotica sp. nov*. J, K, N. Spherical, oval and oviform vegetative cells. K−M. Deeply lobed chloroplast. N, O. Shallowly lobed chloroplast. P, Q. Crenulate chloroplast. R. Echinate chloroplast. K, N, P. One distinct pyrenoid located in its centre. N. One to several spherical incisions in pyrenoid. S, T. Asexual reproduction by 32 aplanospores or 128 zoospores produced in spherical or ellipsoidal sporangia. U. Zoospores drop-shaped, naked, with two apical flagella and a simple basal chloroplast. V−DD. *Vulcanochloris guanchorum*, *sp. nov*. V, W. Spherical, occasionally oval vegetative cells. V. Chloroplast in young cells in the central position with several lobes spreading towards the cells periphery. W−Y. Deeply lobed chloroplast. Z−AA. Shallowly lobed chloroplast. BB. One distinct, centrally positioned pyrenoid, often containing one to several spherical incisions. CC, DD. Asexual reproduction by 16−32 aplanospores or 64−128 zoospores produced in spherical or ellipsoidal sporangia. Scale bars = 5 µm.

Vulcanochloris guanchorum **Vančurová, Peksa, Němcová *et* Škaloud, *sp. nov*.**

Vegetative cells spherical, occasionally oval, up to 20 µm in diameter (Figs. 4V, W). Cell wall thin, seldom a flat local thickening of the cell wall (up to 3.5 µm thick) can be distinguished. Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading towards the cell's periphery (Fig. 4V). Mature cells exhibit either a deeply lobed (Figs. 4W–Y) or a shallowly lobed chloroplast (Figs. 4Z, AA), simply terminated at their ends. The chloroplast contains one distinct, centrally positioned pyrenoid, often containing one to several spherical incisions (Fig. 4BB). A number of small starch grains are distributed around the pyrenoid. Asexual reproduction by 16−32 aplanospores or 64−128 zoospores produced in spherical or ellipsoidal sporangia (Figs. 4CC, DD). Zoospores drop-shaped, naked, with two apical flagella and a simple basal chloroplast, ca 6.5 µm long and 3 µm wide.

Type:—SPAIN. Santa Cruz de Tenerife: La Palma, lava field of Volcán de San Juan, 2.5 km to the north-east Puerto de Naos, 28.604722º N, 17.895389º W, 400 m a.s.l., *L. Vančurová & J. Malíček*, *20 May 2013* (holotype: CAUP!, cryopreserved photobiont cells isolated from the specimen A104, deposited in the Culture Collection of Algae of the Charles University in Prague as the item TYPE-H 1018). Reference strain: CAUP H 1018

Habitat:—In thalli of *Stereocaulon vesuvianum* growing on basalt lava stones and rocks.

Etymology:—The species is named after the Guanches, aboriginal Berber inhabitants of the Canary Islands.

Discussion

Recent phylogenetic studies provide a wide evidence of unsuspected, often morphologically cryptic, diversity in coccoid green algae (Leliaert *et al*. 2014). In the green algal class Trebouxiophyceae, numerous genus-level revisions, transfers, splits, and new taxa descriptions have been published during the last 15 years (Neustupa *et al*. 2011, 2013, Gaysina *et al*. 2013, Fučíková *et al*. 2014b). In this paper, we are adding a new piece to the puzzle of understanding the real diversity within the Trebouxiophyceae, by describing a new genus of coccoid green algae, *Vulcanochloris*.

According to our *rbcL* phylogenetic analysis, *Vulcanochloris* comprises a distinct genus within the order Trebouxiales (Fig. 2), in a sister position to the genus *Asterochloris*. These findings are in accordance with our 18S rDNA phylogenetic analysis (see Supplementary File 1), indicating a close relationship of these two genera. Nevertheless, this genetic similarity does not contradict resolving *Asterochloris* and *Vulcanochloris* as two distinct genera, since the exon 18S rDNA sequences were shown to evolve extremely slowly in *Asterochloris* (Škaloud *et al*. 2015). Morphologically, these two genera are highly similar, specifically in the formation of axial, lobed chloroplast type, the chloroplast transformation prior to sporogenesis, and production of a high number of daughter cells (aplanospores). This similarity could be even conceptualized as the ground to regard the newly discovered lineage as a new, yet distinct, species within the genus *Asterochloris*. However, a specific pyrenoid structure (see below) and substantial genetic divergence of ITS rDNA sequences (Fig. 3) warrants describing *Vulcanochloris* as a distinct genus.

The presence and structure of pyrenoids represents an important feature in delimitation of green algal genera and species (Ettl & Gärtner 1995, Pröschold & Leliaert 2007). In addition, the ultrastructure of pyrenoids has been shown to be phylogenetically informative. Before the application of molecular techniques, pyrenoid ultrastructure has been used as one of the most important features to trace the evolutionary history of coccoid green algae. For example, in the genus *Chlorella*, pyrenoid ultrastructure has been applied to separate the species into several evolutionary coherent groups (Ikeda & Takeda 1995, Kalina & Punčochářová 1987). Later, molecular phylogenetic investigations corroborated this separation, showing the polyphyletic origin of *Chlorella* species (Huss *et al*. 1999). According to the present knowledge, the traditionally conceived genus *Chlorella* forms at least 10 particular lineages corresponding to different genera (Škaloud *et al*. 2014). In fact, many of these lineages were previously shown to differ ultrastructurally. For example, whereas true *Chorella* species possess a pyrenoid bisected by a pair of thylakoids, pyrenoids of *Heterochlorella* Neustupa *et al*. (2009: 167) and *Chloroidium* (Krüger 1906: 94) Darienko *et al*. (2010: 189) are bisected by up to four stacked thylakoids or by many single undulating thylakoids, respectively (Ikeda & Takeda 1995, Němcová & Kalina 2000).

Similarly, a substantial variability in pyrenoid ultrastructure has been documented in the genus *Trebouxia* (Fisher & Lang 1971), a close relative of *Vulcanochloris*. According to the arrangements and forms of thylakoid lamellae within the pyrenoid matrix, Friedl (1989) separated particular *Trebouxia* species into the eight natural groups. Later investigations showed a large congruence of the ultrastructural and molecular data (Helms 2003, Nyati 2006). Indeed, three groups of species have been even shown to form a separate genus *Asterochloris* (Škaloud & Peksa 2010). The pyrenoid ultrastructure found in *Vulcanochloris* cannot be assigned to any of the pyrenoid types previously described by Friedl (1989). The most prominent feature distinguishing *Vulcanochloris* from all other investigated taxa is the presence of electron-lucent, spherical to elongated regions formed within the pyrenoid matrix. To our knowledge, this pyrenoid ultrastructure, as well as the formation of spherical pyrenoid incisions observed in light microscope, was never reported for any other green algal taxa.

The genetic diversity within the genus *Vulcanochloris* is very high, fully comparable with the sister genus *Asterochloris* actually comprising 13 distinct species (Škaloud *et al*. 2015). Just for illustration, ITS rDNA sequences of *Vulcanochloris* from La Palma (Canary Islands, Spain) diverged each other 0.02–5.07%. Considering this substantial genetic diversity and morphological differentiation of particular lineages, we proposed the description of three species within the newly described genus *Vulcanochloris*. *Vulcanochloris symbiotica* is the most variable in the chloroplast morphology. Deeply lobed form is the most frequently observed. Furthermore, the shallowly lobed, crenulate and echinate chloroplast is observed as well. Lobes of the deeply lobed chloroplast are not simply terminated, but extended to either irregular plates or branched projections. On the contrary, lobes of chloroplasts of *V*. *guanchorum* are simply terminated at their ends. Finally, *V*. *canariensis* possess exclusively crenulate chloroplast. Chloroplast morphology has been recognized as one of the most important features to distinguish species within the related genera *Asterochloris* and *Trebouxia* (Helms 2003, Škaloud & Peksa 2010, Škaloud *et al*. 2015). Therefore, we consider the combination of the above-mentioned morphological differences with molecular data as a gold standard to delimit species boundaries in the newly proposed genus *Vulcanochloris*.

The species of *Vulcanochloris* belong to a few green algae described directly from lichens and known only in a lichenized form, similarly to two of the most recently described phycobionts—the sister genus *Asterochloris* represented by *A. phycobiontica* Tschermak-Woess (1980a: 291), and *Elliptochloris bilobata* Tschermak-Woess (1980b: 71). Interestingly, *Asterochloris* was several times recorded from *Stereocaulon* Hoffmann (1796: 128), including *S. vesuvianum*, which is the exclusive mycobiont of *Vulcanochloris* algae (Nelsen & Gargas 2006, Peksa & Škaloud 2011). Such sharing of the same mycobiont is certainly enabled by the close relationship between both algal genera. On the Canary Islands, the association of *S. vesuvianum* with *Vulcanochloris* instead of *Asterochloris* could represent a local adaptation to the harsh conditions on the lava stones and rocks. Besides *Stereocaulon*, *Asterochloris* is associated with many other taxa of lichen-forming fungi (Škaloud & Peksa 2010). Therefore, we consider the high specificity of *Vulcanochloris* algae to *Stereocaulon* as not definitive. Interestingly, some thalli of *Parmotrema pseudotinctorum* from the island of La Palma were associated with the phycobiont clone PAL4.11, closely related to *Asterochloris* (Molins *et al*. 2013). This clone could, in fact, very probably represent the newly proposed genus *Vulcanochloris*. However, since the authors used the *psb*A gene sequences to characterize genetically the phycobiont clones, we cannot compare their findings with our data, and thus confirm the presence of *Vulcanochloris* in *Parmotrema* lichens.

We report here a common occurrence of *Vulcanochloris* on La Palma. However, one lineage closely related to *V. guanchorum* was recently discovered at limestone localities in Germany and Sweden (Ruprecht *et al*. 2014), rejecting a putative endemic nature of *Vulcanochloris* on the Canary Islands. Although the biogeography of microorganisms has become a highly discussed topic (Caron 2009), investigations dealing with the biogeography of symbiotic protists are still very scarce. The population studies on lichenized *Trebouxia* species indicated that the distribution of particular genotypes is particularly shaped by either climatic factors (Fernández-Mendoza *et al*. 2011) or distribution patterns of mycobiont partners (Buckley *et al*. 2014). The single study dealing with the biogeography of *Asterochloris* photobionts indicated generally cosmopolitan distribution of species (Řídká *et al*. 2014). The existence of two lineages endemic to India has been explained by specific climatic or habitat preferences rather than by the historic factors. Accordingly, we expect that occurrence of *Vulcanochloris* is similarly associated with specific conditions common on La Palma, but rare in other areas.

Future work should therefore include follow-up investigations designed to evaluate whether *Vulcanochloris* occurs on volcanic localities in the rest of the world, and whether it associates exclusively with *Stereocaulon vesuvianum*, with other mycobionts, or even occurs as a free-living alga.

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