Phycobionts of some species of *Evernia* and *Ramalina*

Angelika TSCHAIKNER, Elisabeth INGOLIĆ, Andreas HOLZINGER & Georg GÄRTNER

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Several strains of the coccal green alga *Trebouxia* were isolated from two species of the lichen genus *Evernia* (*E. divaricata*, *E. prunastri*) and from five species of the lichen genus *Ramalina* (*R. capitata*, *R. farinacea*, *R. fraxinea*, *R. pollinaria*, *R. siliquosa*). Morphological characters of vegetative cells (cell shape and size, morphology of plastids, structure of pyrenoids) and asexual reproduction were investigated and used to identify the phycobionts. Light and transmission electron microscope investigations, as well as comparison of the isolated photobionts with literature and existing strains from the culture collection of algae at the Botanical Institute of the University in Innsbruck, led to an identification of three species of *Trebouxia*: *T. jamesii*, *T. arboricola* and *T. crenulata*. The most common photobiont encountered was *Trebouxia jamesii* for which additional observations on its morphological variability are presented.

Zusammenfassung: TSCHAIKNER, A., INGOLIĆ, E., HOLZINGER, A. & GÄRTNER, G. 2007. Phycobionten einiger Arten von *Evernia* und *Ramalina*. – Herzogia **20**: 53–60.

Aus zwei Arten der Flechtengattung *Evernia* (*E. divaricata*, *E. prunastri*) und aus fünf Arten der Flechtengattung *Ramalina* (*R. capitata*, *R. farinacea*, *R. fraxinea*, *R. pollinaria*, *R. siliquosa*) wurden Vertreter der coccalen Grünalgengattung *Trebouxia* als Phycobionten isoliert. Merkmale vegetativer Zellen wie Zellform und Zellgröße, Morphologie der Chloroplasten, Pyrenoidstrukturen und Reproduktion wurden untersucht. Die Artbestimmung erfolgte anhand von lichtmikroskopischen und TEM Untersuchungen, unter Zuhilfenahme von Literatur und Vergleich der Isolate mit Kulturen aus der Algenkulturensammlung des Botanischen Instituts der Universität Innsbruck. Aus den isolierten Phycobionten konnten folgende *Trebouxia*-Arten bestimmt werden: *T. jamesii*, *T. arboricola*, *T. crenulata*. Der am häufigsten vorkommende Phycobiont war *Trebouxia jamesii*, ergänzende Beobachtungen zu dieser Art und dessen morphologischer Variabilität werden behandelt.

Key words: Lichen algae, Chlorophytes, Trebouxiophyceae, chloroplast structure, pyrenoids.

Introduction

Since SCHWENDENER (1869) discovered the dual nature of lichens, the knowledge about the systematic affiliation of phycobionts is still incomplete. The time consuming isolation and specific cultivation methods are probably the main reasons for this.

About 44 genera of Cyanobacteria and Algae are known as lichen photobionts (TSCHERMAK–WOESS 1988), and the coccal green alga *Trebouxia* Puymaly is the most common among these (AHMADJIAN 1993).

According to GÄRTNER (1985a), FRIEDL (1989a), ETTL & GÄRTNER (1995) and BECK (2002) *Trebouxia* comprises about 26 species. Cytological characters of vegetative cells such as morphology of plastids, formation of autospores, structure of pyrenoids, visible under the light microscope, are used to distinguish the different *Trebouxia* species (e.g. ETTL & GÄRTNER 1984,

GÄRTNER 1985a). FRIEDL (1989a) investigated pyrenoid ultrastructure and established it as a taxonomic character, dividing *Trebouxia* into 8 groups. BECK (1999) used molecular methods (ITS sequence analyses) to investigate *Trebouxia* and found some morphological differences corresponding with molecular criteria. For future taxonomic studies only a combination of light microscopical and ultrastructural investigations and DNA sequence analyses seems to be useful.

The aim of the present study of the *Trebouxia* phycobionts of the lichen genera *Evernia* and *Ramalina* is to describe newly isolated strains, to give further information about their morphology and to elucidate their relationships.

Material and methods

Isolation and purification

Studies of lichen algae require clonal, i.e. unialgal and sometimes axenic cultures for further taxonomic research. The cultivation of strains that originate from a single algal unit is required (AHMADJIAN 1993, ETTL & GÄRTNER 1995). During the present investigation lichen thalli from different substrates and locations (Table 1) were cleaned under running water and were shortly dipped in 3 % H_2O_2 to avoid contaminations by other free living algae from the thallus surface. Lichen thalli were cut into small pieces, and cross sections were transferred to petri dishes containing growth medium (BBM, BISCHOFF & BOLD 1963) solidified with 1 % agar. After 3–4 weeks a small amount of cells from a colony was transfered to a sterile petri-dish containing solidified BBM growth media. To clean algal units from associating contaminants (periphyton algae, fungal hyphae, bacteria), aggregates of cells (or single cell units) were moved over the whole surface of the medium with a sterile glass hook (with the end shaped in the form of a glass ball) under a Reichert Mak S stereo microscope. After 8–12 weeks growth unialgal axenic cultures were obtained. When the cultures were still contaminated the transfer processes were repeated.

Unialgal cultures from BBM-petri dishes were transferred into BBM agar slants and deposited in the Algal Culture Collection of the Botanical Institute in Innsbruck, Austria for maintenance. Cultures were maintained under standard conditions: 10–12 °C, 1200 lux and a 12 hours light : 12 hours dark regime. Samples of the investigated lichens were deposited in IB.

Light microscopy

Cells were examined with an Olympus BH-2 light microscope and recorded either with an Olympus PM-10 AK Automatic exposure Photomicrographic System and built-in camera, or with a CCD-IRIS Color Video Camera (Sony) and PICed Cora image analysis system (Jomesa Messsysteme GmbH). For isolation and purification a Reichert Mak S stereo microscope, a Wild M8 stereo microscope and an Olympus SZH 10 Research stereo microscope, if required with camcorder or a microscope camera (specified above), were used.

Cell walls and gelatinous sheath were stained with methylene blue, starch with Lugol's iodine solution and/or Chlorine-iodine, pyrenoids with Azocarmine-G, vacuoles with Neutral red and nuclei with Carmine acetic acid. Identifications were made on the basis of cell and colony morphology using standard authoritative references (GÄRTNER 1985a, FRIEDL 1989b, ETTL & GÄRTNER 1995).

Transmission electron microscopy

Algae were separated from agar and concentrated by short centrifugation. The pellet was mixed with 5 % low melting agarose (at 30 °C), then chilled over ice at 4 °C until solidified. The pellet was cut into pieces of about 1 mm^3 which were fixed in 2.5 % glutaraldehyde in cacodylate buffer (50 mM, pH = 7.0) for 1.5 hour and washed in cacodylate buffer for 1 hour partially following the method of HOLZINGER et al. 2004 (for strain AT 2, AT 11). Osmium fixation (1 % in cacodylate buffer) lasted for 12 hours at 4 °C. Samples were rinsed in cacodylate buffer for 1 hour, dehydrated in gradually increasing ethanol concentrations and embedded in modified Spurr's epoxy resin (SPURR 1969). Ultrathin sections were poststained in 2 % uranyl acetate and lead citrate (REYNOLDS 1963). Sections were viewed in a Zeiss 912 TEM (culture AT 2), or a Zeiss Libra 120 energy filter transmission electron microscope (EFTEM). Images were captured with a ProScan Slow Scan CCD camera system using iTEM© 5.0 Software from Soft Imaging System GmbH (culture AT 11) or a Tecnai 12 (FEI) equipped with a Gatan CCD camera (culture AT 6).

Results and Discussion

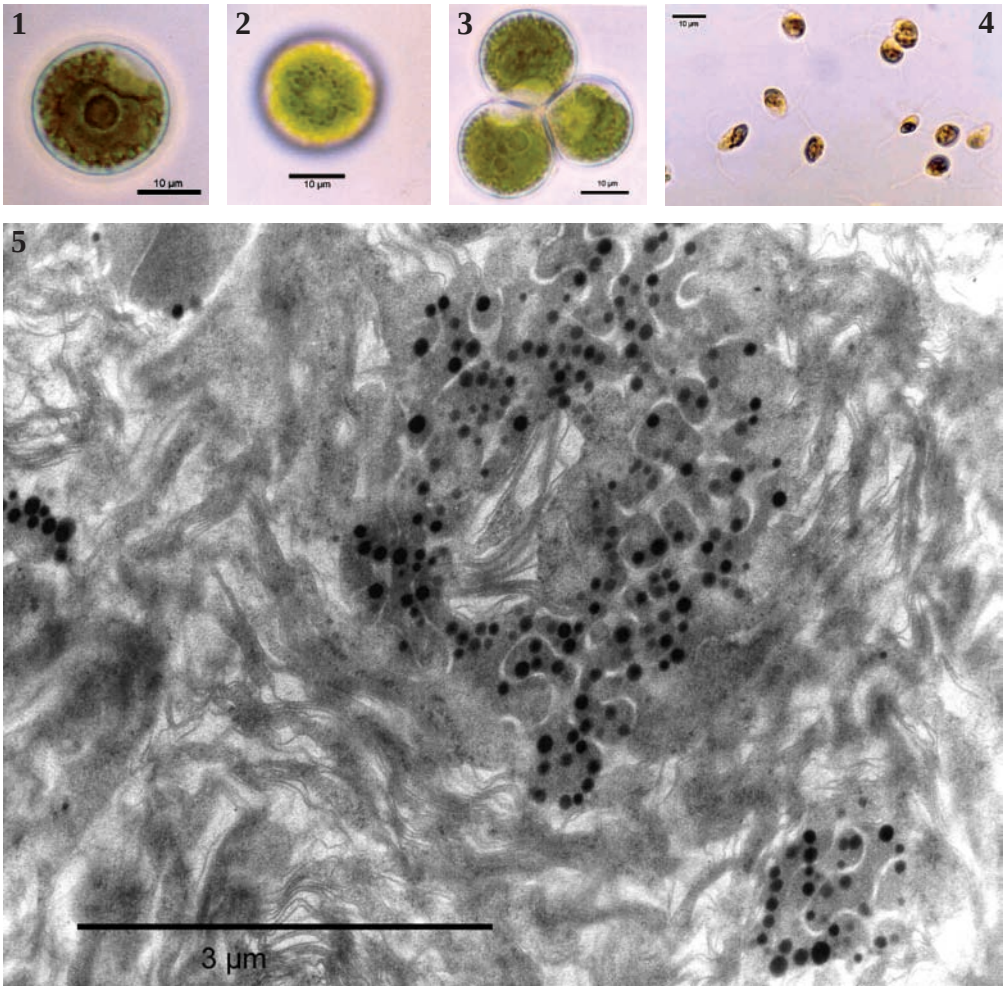
Trebouxia arboricola Puymaly (Figs 1–5)

Three phycobiont strains (Table 1), isolated from *Ramalina farinacea* (L.) Ach., *R. pollinaria* (Westr.) Ach., *R. siliquosa* (Huds.) A.L.Sm., showed good correspondence with the type strain[1] *Trebouxia arboricola* Puymaly (CCAP 219/1a = SAG 219-1a = IB 363, GÄRTNER 1985b). Cells had a regular, spherical form, 10–15–20 µm in diameter. However, in strain AT 8, cells occasionally had a slightly ovoid shape. No cell wall thickenings were seen in fresh cultures, in strain AT 8 and AT 10 rarely cap-like cell wall thickenings were observed. The chloroplast was very finely crinkled ("crenulate chloroplast structure", GÄRTNER 1985a). The well visible, round to angular pyrenoid was perforated by a sievelike structure (prolate slots), and starch was distributed throughout the chloroplast as fine grains. Cells with several pyrenoids were often visible. The pyrenoids belonged to the "arboricola-type" which is characterized by long invaginations meandering through the matrix (FRIEDL 1989a) (Fig. 5). Asexual reproduction was by 2–32 autospores, held together for some time by the extendable sporangial wall. In fresh culture zoospores, 6–10 µm long , 1.5–5 µm wide, tapering towards the apex and rounded posteriorly, with two flagella of equal length were frequently observed. However, in strain AT 8 zoospores were never found.

Table 1: Survey of isolated *Trebouxia*-phycobionts (T.) of *Evernia* and *Ramalina*.

Phycobiont	Lichen	Location	Strain
T. arboricola	*Ramalina farinacea*	Ramsau, Styria, Austria, bark of free standing *Fraxinus excelsior*	AT 6
T. arboricola	*Ramalina pollinaria*	Innerst, Tyrol, Austria, rock surface	AT 8
T. arboricola	*Ramalina siliquosa*	Ploumanach, France, coastal granitic rocks	AT 10
T. crenulata	*Ramalina capitata*	Obergurgl, Tyrol, Austria, rock surface	AT 5
T. jamesii	*Ramalina siliquosa*	Lofotes, near Moskenes, Norway, coastal rocks	AT 9
T. jamesii	*Ramalina siliquosa*	Rörö, Sweden, coastal granitic rocks	AT 11
T. jamesii	*Ramalina fraxinea*	Ånimskog, Sweden, bark of free standing *Fraxinus excelsior*	AT 7
T. jamesii	*Evernia prunastri*	Leutasch, Tyrol, Austria, bark of *Acer pseudoplatanus*	AT 3
T. jamesii	*Evernia prunastri*	Sellrain(valley), Tyrol, Austria, bark of *Picea abies*	AT 4
T. jamesii	*Evernia divaricata*	Walchen, Tyrol, Austria, bark of *Picea abies*	AT 1
T. jamesii	*Evernia divaricata*	St. Sigmund, Tyrol, Austria, bark of *Picea abies*	AT 2

[1] Type strain = authentic strain = original clones, which had been the basis for species diagnoses.

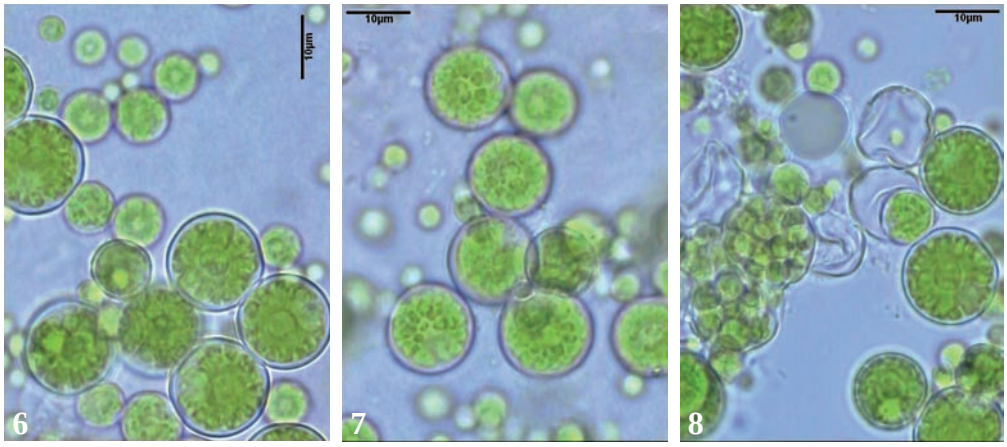


Figs 1–5: **1** – Vegetative cell with central pyrenoid of *Trebouxia arboricola*, strain AT 6 (living material). **2** – *Trebouxia arboricola*, strain AT 6, vegetative cell with crenulate chloroplast structure, surface view. **3** – Nearly fully developed autospores of *Trebouxia arboricola*, strain AT 6 (one autospore with 3 pyrenoids). **4** – *Trebouxia arboricola*, strain AT 6, zoospores stained with JKJ. **5** – Arboricola–type pyrenoids of *Trebouxia arboricola* in TEM, strain AT 6. Long thylakoids meandering through the matrix of one big and one small pyrenoid.

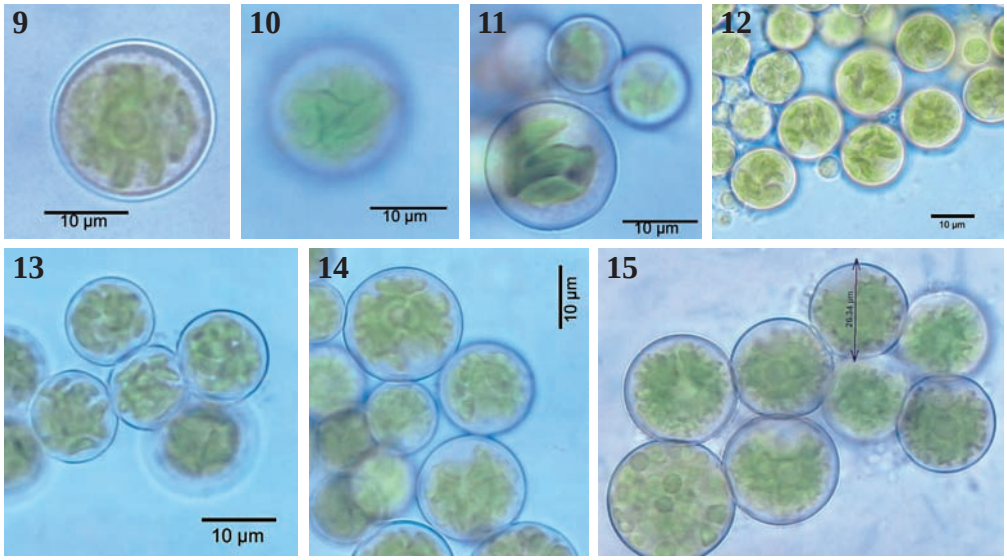
Trebouxia crenulata Archibald (Figs 6–8)

Strain AT 5, isolated from *Ramalina capitata* (Ach.) Nyl., was rather similar to the descriptions of the type strain *T. crenulata* Archibald (CCAP 219/2 = IB 359, GÄRTNER 1985b) in morphological characters like chloroplast structure, pyrenoid and cell size.

Vegetative cells were spherical to mostly slightly oviform, 10–16 µm, without cell wall thickenings. The chloroplast was divided into numerous, regular, fine lobes which in surface view showed a pattern of fine warts. A single distinct large central, round to angular, pyrenoid occurred. Starch was deposited in fine grains throughout the chloroplast. Asexual reproduction involved 8–32 autospores and zoospores (3–5 µm wide and 6–8 µm long). Empty sporangia were often found.



Figs 6–8:
6 – *Trebouxia crenulata*, strain AT 5, vegetative cells in different stages of development.
7 – *Trebouxia crenulata*, strain AT 5, vegetative cells in surface view.
8 – *Trebouxia crenulata*, strain AT 5, vegetative cells, autosporangia, empty sporangial walls.



Figs 9–15:
9 – *Trebouxia jamesii*, strain AT 1, vegetative cell, deeply incised chloroplast.
10 – *Trebouxia jamesii*, strain AT 2, vegetative cell, chloroplast crinkled in large lobes, exhibiting fine chloroplast ribs in surface view.
11 – *Trebouxia jamesii*, strain AT 1, "parietal" stage.
12 – *Trebouxia jamesii*, strain AT 11, vegetative cells in surface view, chloroplast ribs.
13 – *Trebouxia jamesii*, strain AT 11, vegetative cells.
14 – *Trebouxia jamesii*, strain AT 1, vegetative cells.
15 – *Trebouxia jamesii*, strain AT 1, vegetative cells, autosporangium.

Trebouxia jamesii (Hildreth & Ahmadjian) Gärtner (Figs 9–17)

Seven phycobiont strains, isolated from *Evernia divaricata* (L.) Ach., *E. prunastri* (L.) Ach., *Ramalina fraxinea* (L.) Ach. and *R. siliquosa* (Table 1) were identified as *Trebouxia jamesii*. The morphology of only two strains (AT 4, AT 7) corresponded well with the descriptions of the type strain *Trebouxia jamesii* (strain UTEX 2233, formerly *Pseudotrebouxia jamesii* Hildreth & Ahmadjian, GÄRTNER 1985a). The pyrenoid was indistinct, often poorly visible and the chloroplast showed small ribs in surface view. Strains AT 3 and AT 9 were similar to the descriptions of the type strain, but the pyrenoid was well visible and of a bigger size than in the type strain. Further, cells of AT 3 only reached 10–15 µm in diameter. Very rarely, in cells isolated from *Ramalina siliquosa* (AT 9) two nuclei were observed. This seemed to be abnormal division phases. The morphology of chloroplasts in strain AT 11 also corresponded with that of the type strain, in that the chloroplast was incised and showed fine chloroplast ribs in surface view (only well visible in older cultures). However, in contrast to the type strain this isolate exhibited cells with one large and several satellite pyrenoids, and starch was concentrated in fine grains encircling the pyrenoid(s), sometimes resembling a starch sheath. Phycobionts isolated from *Evernia divaricata* (strain AT 1, AT 2) differed to some extent in the chloroplast structure from the other *Trebouxia jamesii* strains. The chloroplast was crinkled in large lobes, like windings, exhibiting an irregular pattern of panels or large ribs in surface view. Cells with several pyrenoids were often seen. In cells of strain AT 1 modifications in chloroplast structure prior to the first protoplast division were observed resulting in "parietal stages" of their chloroplasts (FRIEDL 1989b). The chloroplast margins were no longer crinkled and deeply incised, instead became smooth, and the chloroplasts themselves were closely positioned to the cell wall. The nucleus was located in the centre.

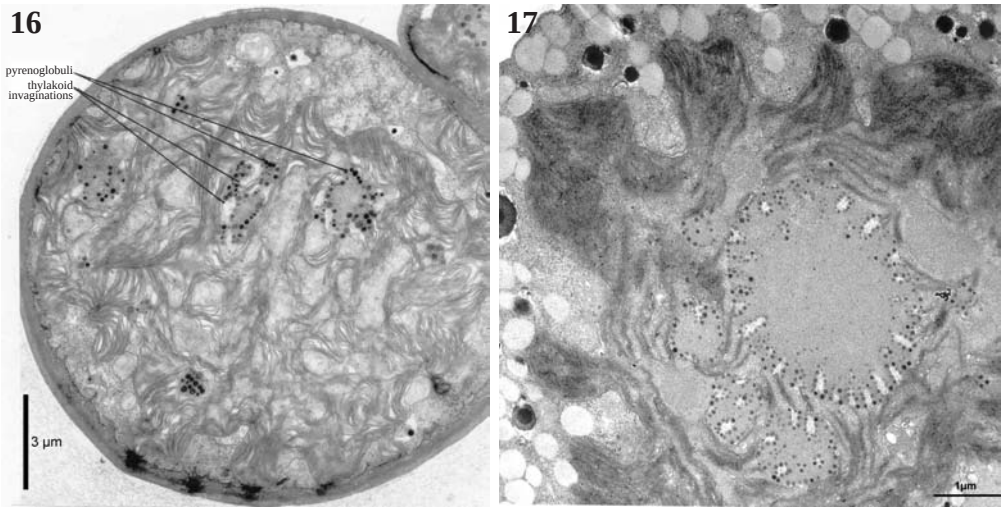
Vegetative cells of strain AT 2 and AT 11 were investigated with transmission electron microscopy (Figs 16, 17) which revealed that the pyrenoids of *Trebouxia jamesii* belong to the "impressa" type (FRIEDL 1989a). This pyrenoid type is characterised by straight, unbranched thylakoid invaginations, which appear long or short, depending on the orientation of section (FRIEDL 1989a).

Trebouxia jamesii was the most frequent phycobiont in the investigated *Ramalina* and *Evernia* species. All isolates from *Evernia prunastri* and *E. divaricata* (from different locations and substrates) could be assigned to *Trebouxia jamesii*. This suggests that this alga is the favoured phycobiont of those lichens, but whether *Evernia* exclusively uses *T. jamesii* as a photobiont is doubtful, not only because too few lichen samples have been investigated, also because MEISCH (1981) found *Trebouxia excentrica* Archibald as a phycobiont in *E. prunastri* and *E. divaricata*. *Trebouxia jamesii* seems to be a morphologically very variable species, only the typical chloroplast ribs were observed in all isolates belonging to this species.

FRIEDL (1989a) concluded on the basis of ultrastructural investigations that *T. jamesii* and *T. simplex* Tschermak-Woess were identical in all characters and therefore were conspecific.

BECK (1999) described a new subspecies, *Trebouxia jamesii* subsp. *angustilobata* A.Beck, differing from the typical *T. jamesii* by a crenulate chloroplast. Morphological differences were found corresponding to molecular differences (ITS sequence analyses). The ITS sequence data did not support the placement of *T. jamesii* in the *Trebouxia simplex* group, instead it was found to belong to the *T. arboricola* group (BECK 2002).

Due to aberrant morphological properties a placement of *T. jamesii* in the *T. arboricola* group in our opinion is not objectionable and we concur with the conclusions of BECK (2002: 80).



Figs 16, 17: **16** – Impressa – type pyrenoid of *T. jamesii*, strain AT 2, phycobiont of *Evernia divaricata*. Straight thylakoid invaginations. **17** – Same pyrenoid structures as in Fig. 16., seen in *T. jamesii*, strain AT 11, phycobiont of *Ramalina siliquosa*.

On that account it seems inappropriate and premature to create new species of *Trebouxia*, even if isolates differ in some properties, until its morphological and genetic variability is better known.

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Addresses of the authors

Angelika Tschaikner & Georg Gärtner, Institut für Botanik, Abteilung Systematik, Palynologie und Geobotanik, Leopold Franzens Universität Innsbruck, Sternwartestr. 15, A-6020 Innsbruck, Austria. E-mail: angelika.tschaikner@uibk.ac.at, georg.gaertner@uibk.ac.at

Andreas Holzinger, Institut für Botanik, Abteilung Physiologie und Zellphysiologie Alpiner Pflanzen, Leopold Franzens Universität Innsbruck, Sternwartestr. 15, A-6020 Innsbruck, Austria. E-mail: andreas.holzinger@uibk.ac.at

Elisabeth Ingolić, Forschungsinstitut für Elektronenmikroskopie und Feinstrukturforschung der Technischen Universität Graz, Steyrergasse 17, A-8010 Graz, Austria.
E-mail: elisabeth.ingolic@felmi-zfe.at