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Diversity of *Chroothece* (Rhodophyta, Stylonematales) including two new species

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ABSTRACT

Chroothece has been reported from a range of freshwater environments, including streams, shallow ponds, trickling water on cliffs and moist soils, mostly in Europe and North America. The identification of genera and species by morphology is difficult because of overlaps in critical characters. To help clarify diversity within the genus, samples from Spain and from other regions (UK and Guam, western Pacific) were compared. Ecological and morphological data from field and cultured material were correlated with molecular data (*rbc*L gene sequences) that differentiate two new species: *Chroothece thermalis* I. Chapuis, P. Sánchez, M. Aboal & O. Necchi Jr., sp. nov. in a thermal spring and *Chroothece lobata* M. Aboal, B. A. Whitton, I. Chapuis, P. Sánchez & O. Necchi Jr., sp. nov. in a semi-arid stream. The results suggest recognition of four species, *C. thermalis, C. lobata*, *C. richteriana* Hansgirg and *C. rupestris* Hansgirg, from Spain. Morphology and ecology are useful to help distinguish these species, but the genus needs further study for possible cryptic diversity.

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KEY WORDS *Chroothece*; cryptic diversity; ecology; phylogeny; *rbc*L; red algae; Spain; taxonomy

Introduction

The red algal genus *Chroothece* has been reported as a rare component of streams, shallow ponds, trickling water on cliffs and moist soils, mostly in Europe and North America (Eloranta *et al*., 2011; Sheath & Sherwood, 2011; Sheath & Vis, 2015). Some recent papers have provided more detailed information about its morphology and ecology (Pentecost *et al*., 2013; Aboal *et al*., 2014*a*) and the increasing number of reports suggests it has probably been overlooked in the past.

Chroothece was described by Hansgirg (1884) and the genus was later placed in the Porphyridiales (Bangiophyceae) and Porphyridiaceae, which include only unicells and mucilaginous colonies (Bourrelly, 1985). Hansgirg described *Chroodactylon* a year later (Hansgirg, 1885) and placed this genus in the Bangiophyceae (Goniotrichales, Goniotrichaceae) characterized by uniseriate or pluriseriate filaments, intercalary growth and presence/absence of branches (Bourrelly, 1985).

Morphological similarities between the genera are evident at the cellular level in spite of the contrasting cell arrangement: unicellular or colonial in *Chroothece* and pseudofilamentous (with cells usually not in contact inside a mucilaginous tube) in *Chroodactylon*. Both genera are currently placed in the Stylonematophyceae, Stylonematales and Stylonemataceae, which is considered a basal phylogenetic group of the Rhodophyta (Yoon *et al*., 2006).

The cells in both cases are ellipsoid or cylindrical with rounded poles, with a 3-D star-like, multi-lobed blue or bluish chloroplast and a big central pyrenoid surrounded by a mucilaginous envelope that may grow unilaterally. In *Chroothece* this can develop into a stalk, whereas it forms the mucilaginous tube in *Chroodactylon*. The pyrenoid ranges in colour from yellow-green to orange due to the numerous lipid droplets surrounding it (Aboal *et al*. 2014*b*). Once in culture it is difficult to differentiate the genera morphologically.

Seven species of *Chroothece* have been described: *C. richteriana* Hansgirg (Hansgirg, 1884), *C. rupestris* Hansgirg (Hansgirg, 1886), *C. willei* N. L. Gardner (Gardner, 1927), *C. mobilis* Pascher & Petrová (Pascher & Petrová, 1931), *C. littorinae* C. K. Tseng & M. Hua (Tseng 1984) *nomen invalidum, C. antarctica* (Wille) F. D. Ott (Ott, 2009) and *C. endophytica* (Lemmermann) F. D. Ott (Ott, 2009). Eloranta *et al*. (2011) reported only three species for Europe: *Chroothece mobilis, C. richteriana* and *C. rupestris*.

Just three species are recognized in *Chroodactylon*, two of which have been reported in Europe: *C. wolleanum* Hansgirg (Hansgirg, 1885), *C. ornatum* (C.

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Agardh) Basson (Basson, 1979) and *C. depressum* (G. Martens) V. Krishnamurthy, M.S. Balakrishnan & T. V. Desikachary (Guiry & Guiry, 2017).

Species identification is usually difficult even with ecological and morphological data (mainly cell size and shape) because of significant overlaps in features (Eloranta *et al.*, 2011; Sheath & Sherwood, 2011) and the lack of detailed studies for most species. However, the three European *Chroothece* species in the above list have appeared to be easy to differentiate by their characteristic environment according to Eloranta *et al.* (2011), with *C. richteriana* found on humid clay-rich soil at more or less saline sites, *C. rupestris* obtained from wet rocks and *C. mobilis* reported frequently from peat-bogs, although Pascher & Petrová (1931) in the diagnosis of the species stated '*Überzügen auf der Oberflache der mineralsalzhaltigen Moores der Soos bei Franzensbad*' (Overlay the surface of the salt marshes of the Soos near Franzensbad). The Pascher type specimen of *C. mobilis* was destroyed at the end of World War II (Y. Nemaková, pers. comm.) and the type locality was at least partially destroyed when a sugar beet factory was built there (Ott, 2009: 550, cited pers. comm. from B. Fott). No information is available about the collection site of the *C. mobilis* culture isolate (FD Ott 01363) used for molecular analysis (Yoon *et al.*, 2006).

Information from GenBank sequences is sometimes unclear with some possible misidentifications. The *rbc*L sequences of *Chroothece richteriana* refer to strain 100.79 from SAG Culture Collection (University Göttingen) obtained from F.D. Ott. Ott (2009) stated 'I have collected *Chroothece* at Hamilton Falls, northwest of Austin, Texas. Directly behind and underneath the falls, can be found *Chroothece richteriana* growing in close association with numerous blue-green algae at the seeping fissures in Edward's limestone overhang', however, he did not provide any morphological details. The 133-year-old type specimen of *C. richteriana* from the Wien herbarium was examined and photographed, but lacked clear structural details and in addition was not useable for molecular analyses.

Chroothece is well-documented for the Iberian Peninsula (Chapuis *et al.*, 2014). Margalef (1955) reported *C. richteriana* from four localities in Cataluña and there are more recent records for the species in the Murcia Region (Aboal *et al.*, 2003) and the Tajo (Moreno *et al.*, 2013) and Ebro basins (Tomás *et al.*, 2013). *Chroothece rupestris* has been observed in several localities in north-east and south-east Spain (Aboal, 1989; Sabater *et al.*, 1989), including Ebro basin (Tomás, 2015).

The confusion about taxonomic limits led us to study further samples to provide a clearer understanding of environmental requirements, morphological variability and phylogenetic relationships. *Chroothece* and *Chroodactylon* from several localities of Spain, together with samples from UK and Guam, western Pacific (USA) were used. Interpretation is based on both culture studies and sequencing the plastid-encoded ribulose-1,5-bisphosphate carboxylase-oxygenase large subunit gene (*rbc*L).

Materials and methods

Sampling

Of the 508 localities visited throughout the Iberian Peninsula and Balearic Islands from 2011 to 2014 *Chroothece* was detected in only five samples. The list and geographic coordinates of the sites are compiled in Chapuis (2016). Samples were collected in both aquatic (mainly splash area) and subaerial habitats (Table 1). Samples from the UK and Guam (USA) are also incorporated in the present study. Material for molecular analysis was preserved in silica gel in the field or from cultures in the laboratory. Field material was transported to the laboratory in cool and dark conditions. There it was used to prepare culture isolates, preserved in Sass liquid (FAA, formalin, acetic acid, ethanol) (Sass, 1958) or pressed and dried. Samples for DNA analysis were dried in silica desiccant.

Cultures

Cultures were maintained on agarized (1.5% agar) SWES medium (SAG Culture Collection) at 20°C and 45 µmol photon m^{-2} s^{-1} in the Culture Service of Murcia University or the Department of Botany of Granada University.

Light microscopy

Material was studied with a LEICA DMRB microscope plus digital camera at the Microscopy Service of Murcia University. Measurements correspond to at least 20 cells.

SEM

Field samples were fixed *in situ* with glutaraldehyde and paraformaldehyde in cacodylate buffer, and subsequently post-fixed with OsO_4. *Chroothece lobata* samples were then critical point dehydrated and coated with gold-palladium before observation with a JEOL-6100, while *C. thermalis* samples were carbon coated and observed with a Zeiss SUPRA40VP scanning electron microscope.

Table 1. Environmental features of *Chroothece* sites.

	Boggle Hole Gorge, UK	Marbo Cave, Guam, USA	Santa Fe, Granada, Spain	Río Chícamo, Murcia, Spain	Río Algar, Alicante, Spain
Geographic coordinates	54°25′N 2°12′W	13° 29′13″N 144° 52′ 06″W	37° 9′27.5″N 3°45′ 6.7″W	38°24′97″N 1°00′ 18″W	38°39′33″N 0°5′ 45″W
Altitude (m)	400	11	675	200	247
Orientation	SW	NE	SW	S	SE
Geology	Limestone with travertines	Limestone	Detrital rocks	Conglomerates, sandstone	Limestone
Aerophytic	Yes	Yes	No	No	No
Aquatic	No	No	Yes	Yes	Yes
Freshwater seepage	Permanent or semipermanent	Permanent	Permanent	Permanent	Permanent
Water temperature (° C)	12.7	–	38.0	11.9–22.3	11.4–28.8
pH	7.9	–	7.0	8.2	8.0–8.9
Conductivity (μS cm^{-1})	656	–	>3,000	2,640–3,100)	281–1,250
SO_4-S (mg l^{-1})	6.0	–	–	199–448	25.0–225.7
NO_3-N (mg l^{-1})	0.4	–	0.5 (TDN)	1.7–6.2	3.8–10
FRP (mg l^{-1})	0.004	–	0.030 (TDP)	<0.004–0.07	0.04–0.36
Other habitat characteristics	–	Low irradiance	Hot spring	–	–
Chroothece species	*C. richteriana*	*C. mobilis*	*C. thermalis*	*C. lobata*	*C. lobata*
Reference	Pentecost *et al.* (2013)	This work	This work	Aboal *et al.* (2014*a*)	This work
GenBank accession numbers	KY962003	KY962004	KY962006	KY962005	KY962002

DNA analysis

For DNA extraction, material was processed with a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), followed by DNA extraction using a DNeasy plant mini kit (Qiagen GmbH, Hilden, Germany) and a NucleoSpin plant II mini kit (Macherey-Nagel, Düren, Germany), according to the manufacturers' protocols. The partial sequence (1394 bp) of *rbc*L was amplified using the primers and cycles described by Vis *et al.* (1998), Ciniglia *et al.* (2004) and Stewart & Vis (2007). The amplification was performed on two fragments, using pairs of forward and reverse primers as indicated above. Two different amplification systems were used for PCR reactions: (1) puReTaq Ready-to-go PCR beads (GE HealthCare Life Sciences, Buckinghamshire, UK); (2) Top Taq Master Mix (Qiagen). We chose the PCR products of the system that worked best, as determined by the brightest band in the gel. The resulting PCR products were purified using the QIAquick PCR (Qiagen) or NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) according to manufacturers' protocols. Sequencing reactions were run using the ABI PRISM Big Dye v3.1 Terminator Cycle Sequencing Ready Reaction kit and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Sequence alignments were assembled in Geneious 7 (Kearse *et al.*, 2012).

For phylogenetic analyses of the *rbc*L data, a GTR + G was determined as the best-fit model of sequence evolution by the Akaike Information Criterion using jModelTest 2.1.4 (Darriba *et al.*, 2012). Maximum likelihood (ML) topologies and bootstrap values from 10 000 replicates were inferred using the Randomized Accelerated Maximum Likelihood graphic user interface (RAxMLGUI version 1.2; Silvestro & Michalak, 2011). Bayesian inference (BI) was performed in MrBayes 3.2 (Ronquist & Huelsenbeck, 2003) with three runs of five chains of Metropolis-coupled Markov Chain Monte Carlo for 10 000 000 generations; 500 000 chains were removed as burn-in prior to determining the posterior probabilities.

Five *rbc*L sequences were generated in this study, which were compared with 14 sequences obtained from GenBank (Table S1): one of *Chroothece*, three of *Chroodactylon* and 10 sequences of more distantly related taxa.

Results

*rbc*L *sequence analysis*

Phylogenetic analyses resulted in the same tree topologies for BI and ML, showing fully supported clades representing the genera *Chroothece* and *Chroodactylon* (Fig. 1). The *Chroothece* clade had two major clades with high support: (1) with sequences of two samples of this study (Alicante – KY962002 and 6332 – KY962005) interpreted as representing a single species because sequence divergence between these two samples was only 0.6% (8 bp); (2) with sequences of three samples from this study (6334, UK and West 4733) and DQ308430 (*C. mobilis*); sequence divergences among these samples ranged from 0.3 to 1.5% (4–19 bp). The latter clade contains three groups interpreted as representing distinct species: (1) sample 6334 (KY962006), with sequence divergences of 0.7–1.1% with the other species; (2) sample UK (KY962003), diverging by 0.8–1.5% (11–21 bp) from the remaining species; (3) samples West 4733 (KY962004) and DQ308430

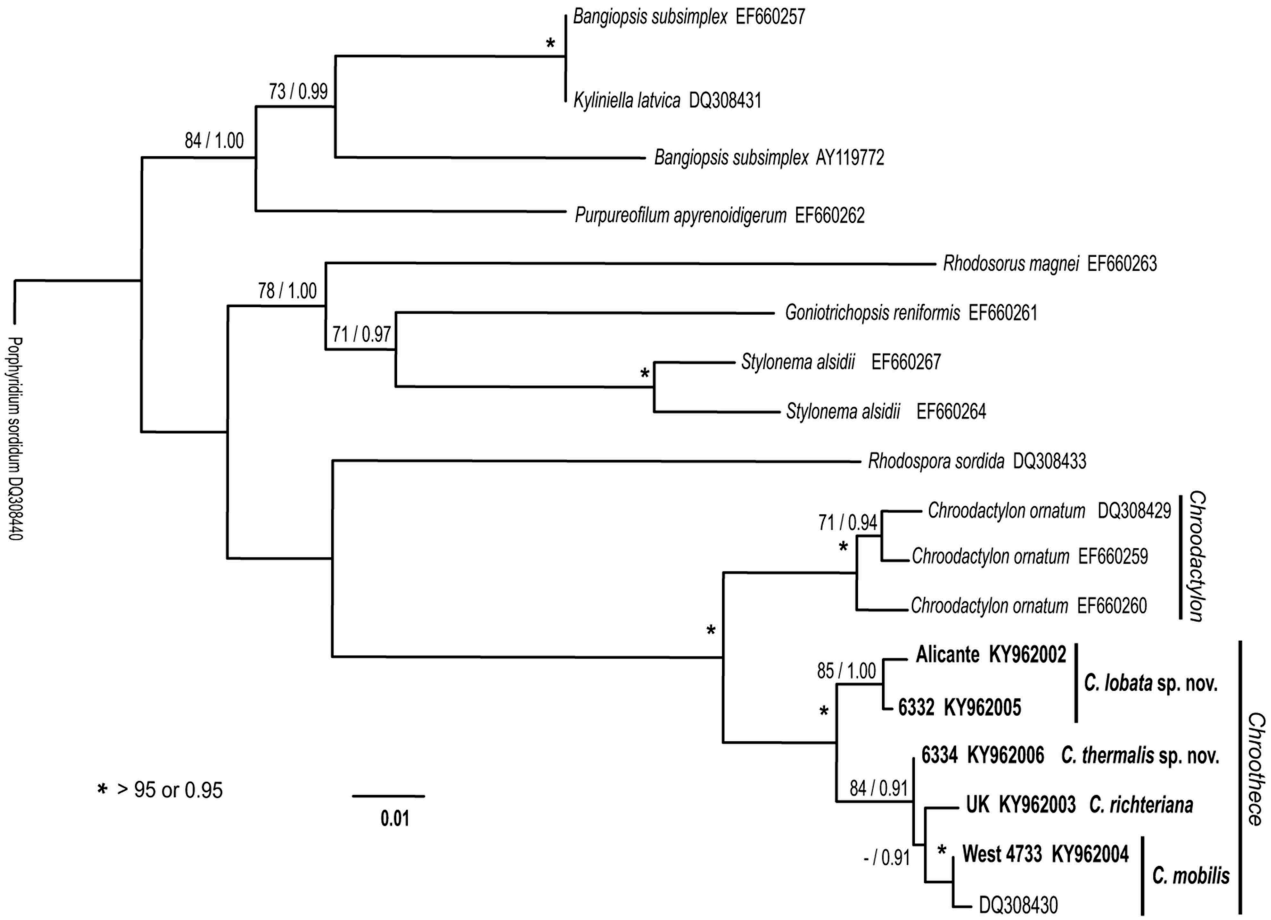
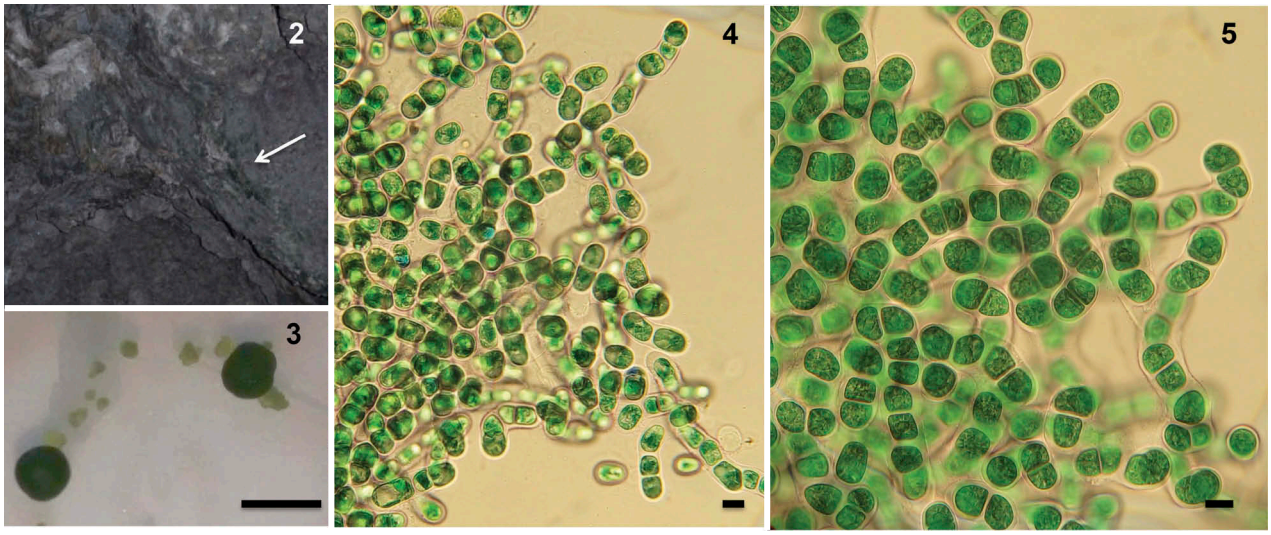


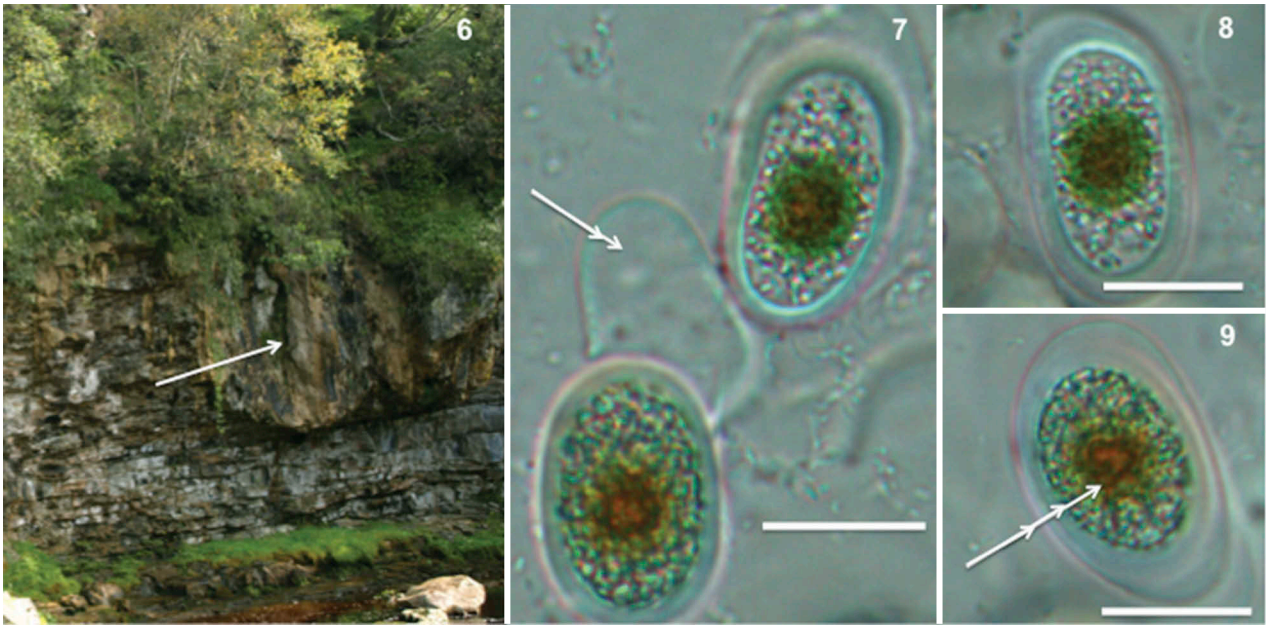
Fig. 1. ML phylogenetic tree based on *rbc*L sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian inference; nodes without values indicate BS < 70% and PP < 0.70. Newly generated sequences are shown in bold.

(divergence of 0.3%, 4 bp), differing by 0.8–1.4% (11–19 bp) from the other species of this clade.

The molecular evidence supports continued recognition of *Chroothece* as a distinct genus, with four species in the dataset, two representing new species, which are described below. *Chroothece/Chroodactylon* were detected at only five of the sample sites. The very small proportion of localities with specimens probably reflects the fact that isolated cells are easily overlooked when there is no conspicuous mat or colony.



Figs 2–5. *Chroothece mobilis* from Guam, USA. **Fig. 2**. Detail of the wall in the entrance of the Marbo cave with the mats. **Fig. 3**. Colonies on agar plate. **Figs 4–5**. Dividing cells forming pseudofilaments. Scale bar represents 1 cm in 3 and 10 μm in 4–5.



Figs 6–9. *Chroothece richteriana* from Boggle Hole Gorge, UK. **Fig. 6**. General view of the seepages (arrow). **Fig. 7**. Cells with cell wall remnants (double arrow) and large pyrenoids surrounded by lipid droplets (triple arrow). **Figs 8–9**. Cells in longitudinal section with large pyrenoids and fairly lamellated sheaths. Scale bar represents 10 μm.

Table 2. Morphological and ecological features of all *Chroothece* species reported for Spain and the others used in this molecular study.

	C. richteriana	*C. rupestris*	*C. thermalis* sp. nov.	*C. lobata* sp. nov.	*C. mobilis*
Cell length (μm)	15.0–33.0	9.0–25.0	15.0–29.0	11.0–31.0	23.7–38.5
Cell diameter (μm)	5.0–10.0	7.0–10.0	12.0–24.0	7.0–17.5	15.4–22.4
Wall width (μm)	2.0–4.0	1.5–2.0	3.0–5.0	1.2–2.5	1.6–6.6
Colony form	Mat to hemispheric	Mat to hemispheric	Mat	Hemispheric to lobate	Mat
Occurrence	Calcareous slightly saline streams	Freshwater streams	Thermal source	Calcareous slighty saline river	Cliff, cave wall with permanent or semipermanent seepage
References	Aboal *et al.* (2003); Moreno Alcaraz *et al.* (2013)	Aboal (1989); Tomás *et al.* (2013)	This work	Aboal *et al.* (2014*a*)	Pentecost *et al.* (2013), this work

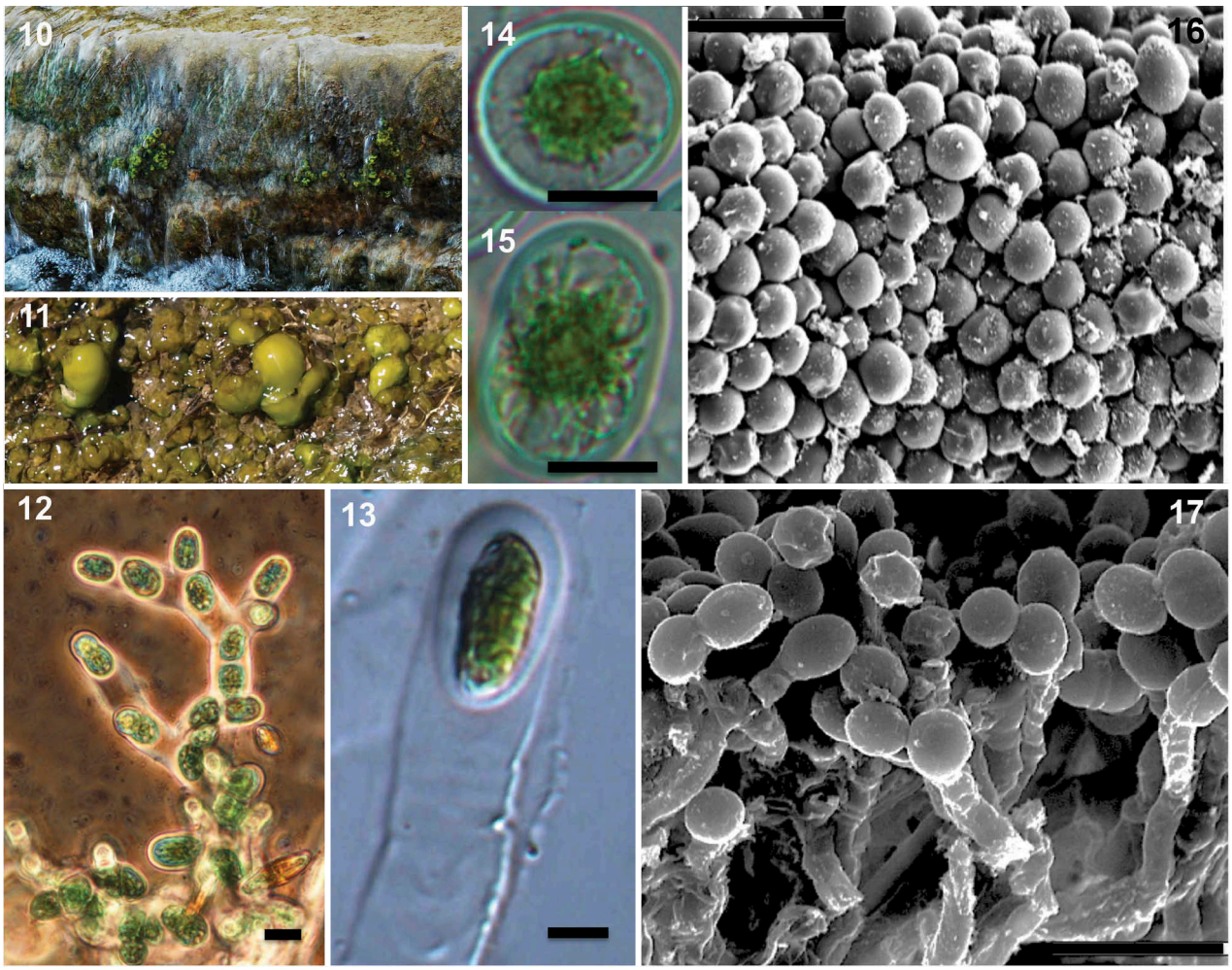
(nd= no data)

Both the *C. richteriana* sample from UK (Figs 2–5) and the *C. mobilis* sample from Guam (Figs 6–9) were collected on limestone cliffs with seepages, the former in a calcareous gorge together with cyanobacteria and desmids and the latter from a freshwater seepage area close to the sea with Chaetophorales and unicellular greens, diatoms and filamentous cyanobacteria on the cave roof in very low light near the Marbo Cave entrance. Their ecology and morphological ranges are shown in Tables 1 and 2.

The samples from Río Chícamo were originally identified as *C. richteriana* which was the closest taxon both morphologically and ecologically. However, molecular analysis showed a clear divergence from that species. The formation of hemispherical colonies several centimetres in diameter and the constant presence of stalks are distinct characters and it is proposed as a new species.

The mats in the thermal spring formed extended growths above the water level. The cell dimensions fall into the range of *C. mobilis*, but the external envelope appears undulate. This morphological character together with the ecology may differentiate it from the other species of the genus. Some of the samples formed mats underwater or, more frequently, at or immediately above the water level.

The Guam isolate (JW4733) did not have pseudofilaments in the field, but on the agar surface in culture variable pseudofilaments developed and look similar to those of *Chroodactylon* although molecular analysis places it in *Chroothece*. The pseudofilaments in Algar River field material were also attributed initially to *Chroodactylon* but identified as *Chroothece* by molecular analysis.



Figs 10–17. *Chroothece lobata* sp. nov. **Figs 10–11**. Colonies in the splash area. **Fig. 12**. Branched pseudofilament in culture. **Fig. 13**. Stalked cell. **Fig. 14**. Cross section of a cell. **Fig. 15**. Frontal view of the cell with multi-lobed stellate chloroplast. **Figs 16–17**. External and internal views of a colony with SEM. Scale bar represents 10 µm in 12–15 and 20 µm in 16–17.

Description of the new species

Chroothece lobata M. Aboal, B. A. Whitton, I. Chapuis, P. Sánchez & O. Necchi Jr., sp. nov.

Diagnosis: Colonies hemispherical or lobate, usually occurring in the splash zone (Fig. 10), blue-green to yellow-orange when viewed macroscopically (Fig. 11). In transverse section, the inner part of the colonies is occupied by stalks and the cells form a single layer in the outer part (Figs 16–17). Cells ellipsoid, 19.6 (11.0–31.1) µm long and 11.9 (7.0–17.4) µm in diameter. Blue-green multi-lobed 3-D stellate chloroplast with a central pyrenoid (Figs 14–15). Mucilaginous sheath 1.8 (1.2–2.2) µm wide, smooth.

Genbank sequences: KY962002, KY962005.

Holotype: MUB-ALGAE-2076, Murcia University Herbarium. Collectors: Iara Chapuis, Pedro Sánchez and Marina Aboal, 14/02/2014. Río Chícamo, Umbría, Murcia, Spain.

Isotype: GDA-A-6332, Granada University Herbarium.

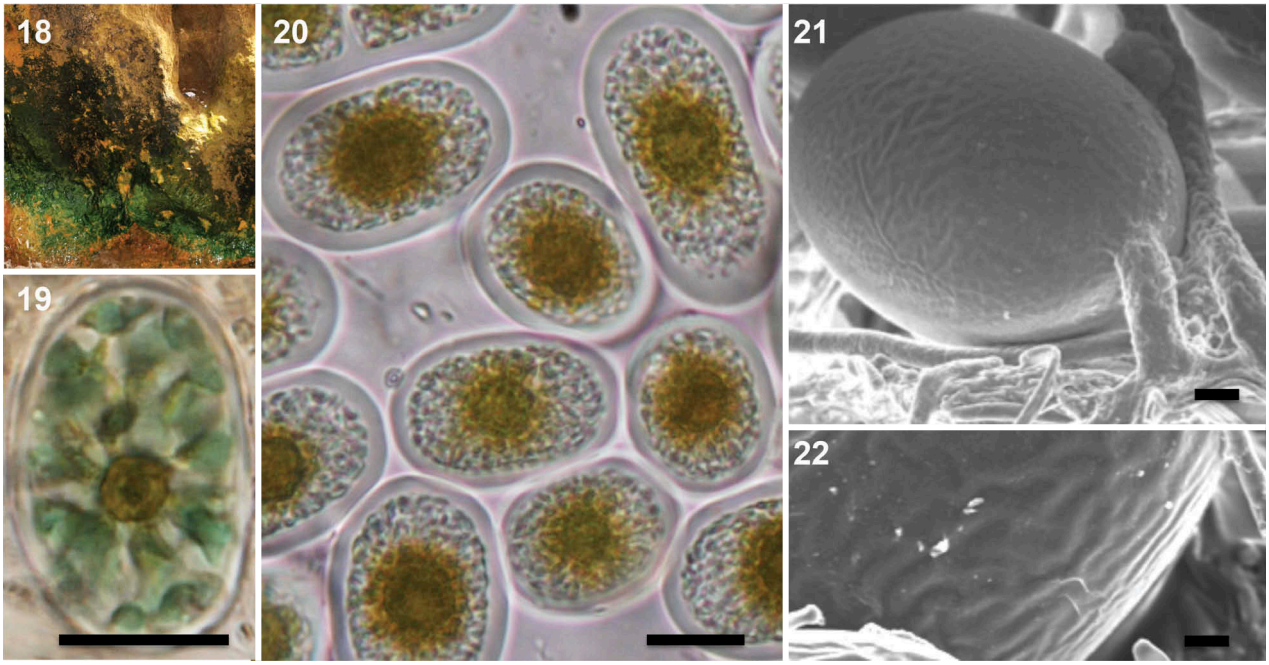
Paratype: MUB-ALGAE 5830, Murcia University. Collectors: Pedro García and Marina Aboal, 25/10/2015. Fuentes del río Algar, Alicante, Spain.

Type locality: In the calcareous and slightly saline river Chícamo. Umbría, Murcia, Spain (38°24′97″N, 1°00′18″W).

Etymology: *lobata* refers to the lobed colonies.

Description

Transverse cell binary divisions were frequent in cultures and sometimes cells remained associated in tubes, sometimes branched. Stalks could be branched (Figs 13, 17). Formation of hemispherical colonies was frequent on solid agar and in liquid medium. The development of large cells was interpreted as akinete formation and their germination to form short pseudofilaments of 3–4 cells was frequent (Fig. 12). Occasionally branching filaments resembling *Chroodactylon* were also observed in the field



Figs 18–22. *Chroothece thermalis* sp. nov. **Fig. 18**. Mats above the water level. **Fig. 19**. Detail of a cell with the star-like chloroplasts and central pyrenoid surrounded by lipid droplets. **Fig. 20**. Cells with big pyrenoids and accumulation of oil droplets. **Fig. 21**. Stalked cell with undulate external wall (SEM). **Fig. 22**. Detail of wall undulation (SEM). Scale bar represents 10 μm.

(Algar river) and identified after sequencing. The main morphological and ecological characters are compared with all the studied species in Table 2.

Chroothece thermalis I. Chapuis, P. Sánchez, M. Aboal & O. Necchi Jr., sp. nov.

Diagnosis: Blue-green or yellowish mats above water level (Fig. 18). Cells cylindrical, 22.5 (15.0–29.0) μm long and 17 (12.0–24.0) μm in diameter. Stellate blue-green chloroplast with a central pyrenoid (Fig. 19). Mucilaginous sheath 3.5 (2.5–4.0) μm wide, slightly undulate. Stalks, only visible clearly after staining with ink, approximately the same width as the cells (Figs 20–22). The margin of the stalks is transversely striated and appears wavy. Binary division frequent. No pseudofilaments have been observed. Daughter cells separate early, well before reaching their maximum size. No significant morphological variations were observed in cultures.

Genbank sequence: KY962006.

Holotype: 6334, Granada University Herbarium, Santa Fe, Granada, Spain; 26/10/2012.

Isotype: MUB-ALGAE 5835, Murcia Herbarium.

Type locality: Collected in a thermal spring, above water level, Santa Fe, Granada, Spain (37° 9'27.5"N, 3°45'6.7"W).

Etymology: *thermalis* refers to living in thermal waters.

The main morphological and ecological characters are compared with the other studied species in Table 2.

Discussion

Molecular evidence supported the separation of *Chroothece* from *Chroodactylon*, although the number of species and diagnostic characters remain to be defined. Molecular studies on Stylonematophyceae based on *rbc*L sequences are scarce and fragmentary and thus do not allow comparisons of intraspecific ranges. Thus, we will consider *rbc*L data from other more distantly related groups, i.e. Compsopogonophyceae and Bangiophyceae. Ramírez *et al.* (2014) reported intraspecific variation within *Porphyra* and *Pyropia* (Bangiophyceae) from Chilean populations as 0–0.7 and 0–0.8%, respectively. Necchi *et al.* (2013) found that intraspecific diversity within *Compsopogon* (Compsopogonophyceae) was very low globally (0.1–0.7%). Intraspecific variation within *Chroothece* was determined only for two species and fits within those ranges: *C. mobilis* (0.3%) and *C. lobata* (0.6%). In contrast, interspecific ranges were higher (0.7–3.3%) with a clear disjunction between intra- and interspecific variations. These data validate the proposed species delimitation in the genus *Chroothece* with the recognition of four species.

These genera are typically separated by their cell arrangement (Eloranta *et al.*, 2011; Necchi, 2016):

unicells or small colonies in *Chroothece* and pseudofilaments in *Chroodactylon*. Short pseudofilamentous stages, branched or unbranched, were often observed in *Chroothece* species in culture and sometimes in the field. It is still not clear if the overlapping is only gradual, i.e. shorter pseudofilaments in *Chroothece* in comparison to longer ones in *Chroodactylon*, or if there is a more defined limit between them. More information is needed before it is clear whether other morphological characters can be applied to separate these genera.

Seven species are currently accepted in *Chroothece* (Guiry & Guiry, 2017), of which six are unequivocally reported from freshwater, aerophytic or non-marine habitats. The present study adds two more species, raising the number to nine. The typical morphology of *Chroothece* in nature may vary from epiphytic pseudofilaments to epilithic hemispherical, lobate colonies in the case of *C. lobata*. The cell size of this species is in the range of variability of *C. richteriana* and the colonies resemble those described by Rieth (1973) from Cuba. Even though he stated that the material probably belong to a new taxon he did not describe it formally.

The morphological and morphometric characters of *C. thermalis* are in the range of variability of *C. mobilis*, but it differs clearly in habitat, wall ornamentation and DNA sequence divergence. It is the only species of *Chroothece* reported from a thermal spring.

In spite of their great morphological similarity the molecular segregation of *Chroothece* and *Chroodactylon* has been demonstrated. Two studies suggest this might be reinforced by some biochemical attributes. Karsten *et al.* (2003) reported that digeneasides were absent only in *Chroothece* among Stylonematophyceae while *Chroodactylon* synthesizes a specific type of agar with sulphated galactans (Cabrera *et al.*, 2014). The latter has not yet been investigated in *Chroothece*. Both genera may have lost the capacity to synthesize phycoerythrin as its absence has been reported in *Chroothece* (Aboal *et al.* 2014*b*) and *Chroodactylon* (Chapman, 1966). However, Pentecost *et al.* (2013) state that the chloroplast may even be red in *C. richteriana*. The phycobilins presence and expression in both genera should be further investigated.

Morphology alone does not permit the identification of the species, although ecology is probably a useful additional character in association with molecular data.

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Chris Lobban helpfully provided the field collection of *Chroothece mobilis* from Marbo Cave, Guam.

Author contributions

M. Aboal: original concept, culturing strains, study of *C. lobata* material, drafting and editing manuscript; I. Chapuis: DNA extraction and analysis of molecular data; M.O. Paiano: DNA extraction and analysis of molecular data; P. Sánchez: study of *C. thermalis* material; J. A. West study of *C. mobilis* and drafting; B.A. Whitton study of *C. richteriana* material and drafting; O. Necchi Jr.: Analysis of molecular data and phylogenetic analysis, drafting.

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No potential conflict of interest was reported by the authors.

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