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Molecular and morphological diversity in photobionts associated with *Micarea* s. str. (*Lecanorales*, Ascomycota)

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Abstract: Lichenization is a symbiotic ecological strategy that is widely distributed among the fungi, but in which the diversity of partners is relatively poorly known. Limited morphological diversity has hindered the recognition of true diversity in many lichen fungi, and also in their algal partners. In the temperate and boreal zones, the crustose microlichens are the most speciose but arguably the least studied, particularly in terms of their photobiont partners. In this study, we sampled eight species of *Micarea* s. str. collected from Europe, culturing and sequencing their green-algal partners using chloroplast (*rbc*L) and nuclear ribosomal (nucSSU) markers. All specimens collected in Great Britain were associated with members of *Coccomyxa* (including *Pseudococcomyxa*), but in the smaller sample of Ukrainian material, both *Coccomyxa* and *Elliptochloris* were found. This study extends the known range of fungal hosts for symbionts in the genus *Coccomyxa*, and supports earlier findings that a separate lineage of predominantly non-symbiotic *Coccomyxa* exists.

Key words: algae, *Coccomyxa*, *Elliptochloris*, lichen, *Pseudococcomyxa*

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Introduction

Lichenization is a symbiotic ecological strategy that is widely distributed among the fungi, and in which fungi are obligately or facultatively associated with a photosynthetic partner as a carbon source. In these morphologically and ecologically diverse symbioses, many of the fungi are both perennial and macroscopically visible, facilitating their study. Although the field of species interactions had long been limited by morphologically enigmatic photosynthetic partners (Kroken & Taylor 2000; Škaloud & Peksa 2010) and cryptic fungal lineages (Kroken & Taylor 2001; Leavitt *et al.* 2012; Lücking *et al.* 2014), molecular tools have allowed huge advances to be made in the recognition of patterns of association and the description of diversity of these and other symbiotic lineages (Grube & Kroken 2000; Letsch *et al.* 2009; Fernández-Martínez *et al.* 2013). A major theme in recent research on lichens has been the description of diversity encompassed within lichen symbioses and the elucidation of factors promoting and maintaining this diversity (Grube & Kroken 2000; Piercey-Normore & DePriest 2001; Yahr *et al.* 2006; Fernández-Mendoza *et al.* 2011; Werth 2012; del Campo *et al.* 2013; O'Brien 2013*a*, *b*).

Photobionts in lichens have been studied in only a very small number of the lichen fungi currently known (Honegger 2008; Voytsekhovich *et al.* 2011*b*), and genetic studies have until recently been mostly limited to those partnerships formed with the conspicuous macrolichen lineages (but see Beck *et al.* 1998; Beck 1999). However, most of the diversity of lichen fungi in any given area comes from the inconspicuous and often less tractable microlichens (e.g. nearly 80% of the well-studied British lichen mycota belong to the microlichens; Yahr *et al.* 2011),

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where it is expected that most of the photobiont diversity remains unexplored.

Recent work has begun to expand the pool of lichens studied to include these much more diverse species, and in less well investigated parts of the world (e.g. Thus *et al.* 2011; Ruprecht 2012; Muggia *et al.* 2014). Even in the relatively well-studied macrolichens, the genetic diversity at the strain level is higher than was once expected based on morphology alone, with many undescribed and morphologically cryptic lineages (Škaloud & Peksa 2010; Mansournia *et al.* 2012; Leavitt *et al.* 2013; Sadowska-Des *et al.* 2014). In general, it is suspected that the diversity of photobionts is much higher than is currently known.

The genus *Micarea* s. lat. has been shown using molecular data to be polyphyletic, consistent with suggestions from morphological studies in the monographs by Coppins (1983) and Czarnota (2007). In the former work, Coppins described three groups of species differentiated based on their photobionts: the small and distinctive 'micareoid' type found in 'most species' including the type, *Micarea prasina* Fr.; the larger and irregular cells in the *M. sylvicola* (Flot.) Vězda & Wirth group (including *M. bauschiana* (Körb) V. Wirth & Vězda, *M. lutulata* (Nyl.) Coppins, and *M. tuberculata* (Sommerf.) R. A. Anderson); and the large globose cells from *M. intrusa* (Th. Fr.) Coppins & Killias (Coppins 1983). He suggested that these latter two groups may require further taxonomic work. Indeed, more recently *M. intrusa* has been transferred to *Scoliciosporum* (Hafellner 2004), with which the similarities in both thallus and photobiont characters had already been noted. Along similar lines, the *M. sylvicola* group has recently been elevated to the generic level as *Brianaria*, in recognition of several important characters, including the different photobiont, which are now known to correlate with phylogenetic distinctiveness for this separate lineage in the *Psoraceae* (Ekman & Svensson 2014). Photobionts from *Brianaria* (*Psoraceae*) are irregular, larger, and have indistinct haustorial pegs, whereas those in *Micarea* s. str. (*Pilocarpaceae*) have so-called 'micareoid' algae, with more delicate haustorial connections and regular, smaller cells (Coppins 1983). Until very recently, the identity of the photobionts in any of these groups had not been studied systematically.

A recent study (Voytsekhovich *et al.* 2011*a*) investigated the algae from several species of fungi belonging to *Micarea* s. str. and found that the photobiont in most samples (8 of 11) belonged morphologically to the genus *Elliptochloris*, confirming very early work by Brunner (1985). One specimen contained *Pseudococcomyxa* sp. and another two accessions associated with several algal species simultaneously (Voytsekhovich *et al.* 2011*a*). *Pseudococcomyxa* has only rarely been reported as the photobiont of lichen fungi (Muggia *et al.* 2010), but this fact certainly reflects problems using morphology to distinguish *Pseudococcomyxa* from *Coccomyxa*, and recent work by Pröschold *et al.* demonstrated that the authentic strain of *P. simplex* SAG 216-9a belongs within the genus *Coccomyxa* in the *Elliptochloris* clade (2011). On the other hand, some species from the genus *Elliptochloris* (for example, *Elliptochloris bilobata* Tsch.-Woess) are widely recognized as symbionts in lichens (Tschermak-Woess 1985) and as free-living algae of sub-aerial habitats (Eliáš *et al.* 2008; Tsarenko 2011). Other species in the genus are recognized as photobionts in marine invertebrates (Letsch *et al.* 2009) or as free-living terrestrial algae (Ettl & Gärtner 1995). Using molecular data, *E. bilobata* has also been reported as the symbiont of several other lichens, including *Verrucaria sublobulata* Eitner ex Servít, using a combined morphological and molecular approach (Thüs *et al.* 2011). However, given the relatively wide variation in cell morphology contrasted with the relative paucity of discrete characters visible with light microscopy, a certain degree of unrecognized genetic variation may be expected in lichen photobionts, particularly based on results of other molecular studies of chlorophyte algae (e.g. Darienko *et al.* 2010; Škaloud & Peksa 2010; Thüs *et al.* 2011; Muggia *et al.* 2014).

This study was undertaken to examine the range of photobionts within *Micarea* s.

str., recognizing that several lineages of green algae are likely to be involved in the segregate genera (Coppins 1983), which we do not treat at this time. We used culturing techniques to isolate algae and sequenced cleaned, fresh specimens directly, in addition to obtaining sequences from uni-algal cultures from eight *Micarea* species in the *Pilocarpaceae*.

Methods

Sample collection

New samples were collected in 2013 from several locations in Great Britain (Table 1) and were immediately frozen to promote and prolong viability (Honegger 1999). These collections have been deposited in the herbarium of the Royal Botanic Garden Edinburgh (E). Four specimens of *Micarea* from Ukraine and the cultures arising from these were also examined (from Voytsekhovich *et al.* 2011*a*).

Species identification

Collections were identified using standard morphological characteristics and thin-layer chromatography (TLC) for separation of morphologically similar species in the *M. prasina* group. TLC was performed using solvent systems A and G, following standard procedures (Orange *et al.* 2001).

Culturing

All samples were washed thoroughly to remove superficial contamination by placing them in a muslin bag and running a jet of water over them for three hours. Samples for culturing were selected under a dissecting microscope to find a clean section of washed thallus which had no obvious epiphytes or other lichens nearby. For one specimen (AF4), direct inoculations were picked from the medulla of the washed thallus, and for the remaining specimens, a piece of thallus was ground between two microscope slides to form a suspension, from which algal cells with hyphal connections were selected on an inverted microscope (Ahmadjian 1967). All samples were inoculated onto Petri dishes containing agarized Bold's Basal Medium at 1·5%. Cultures were grown in growth cabinets under 12 h light/dark cycles at 15 °C. After the first inoculations, up to four separate colonies from each plate were maintained separately to test for heterogeneity in the original sample. Representative cultures were deposited in Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland.

Morphology

Established cultures were examined to ensure replicate inoculations were homogeneous, and this examination was carried out using light microscopy during culture conditions, observing both vegetative as well as reproductive cells. Measurements were made using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with a differential interference contrast (DIC). The photographs were taken using an Olympus Z5060 camera.

Molecular methods

Material for extraction was chosen as follows: for the fungal partner, slices of cleaned apothecia were chosen and placed in an extraction tube; for the photobiont from the fresh samples, an apothecial slice was taken and examined under a compound microscope at ×400. There were algal cells present in the thallus material below the apothecia, and these were checked to ensure that they were all morphologically similar and had hyphal connections. Any parts of the material which were seen to contain epiphytic algae that did not have a hyphal connection were cut away and not included in the material for DNA extraction. For cultured algal material, two independent extractions were made from replicates of each established culture. A mixer-mill (Qiagen Tissuelyser II) was used to grind fungal samples in preparation for DNA extraction, for two 30 s cycles at 20 beats per s. For algal cultures, cells were scraped into tubes and ground with a mini-pestle in extraction buffer. Qiagen Plant MiniKits were used for extraction according to the manufacturer's instructions, but with an extended incubation time at the start (1 h at 65 °C), and reduced final elution volumes (50 µl). The DNA was quantified using Nanovue (GE), and DNA concentrations were standardized to *c.* 2–5 ng μl^{-1} across samples by vacuum centrifuge or dilution with sterile water.

Algal *rbc*L and nuclear small subunit ribosomal (nucSSU) DNA were amplified using PRASF1 and PRASR2 (Sherwood *et al.* 2000) and NS1 and NS4 (White *et al.* 1990), respectively, from both fresh collections and from cultures. Fungal amplification reactions each contained 2·5 µl of 10× NH4 reaction buffer (Bioline, London, UK), 2·5 mM $MgCl_2$ (Bioline), 0·2 mM dNTPs, 0·3 µM of each primer, 0·125 units of BIOTAQ DNA polymerase (Bioline), and 1 µl genomic DNA with double-distilled water for a total of 25 µl. The fungal mtSSU amplifications were performed with a programme of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 90 s, and 72 °C for 30 s, with a final 7 min at 72 °C. For algal *rbc*L and nucSSU amplifications, the same PCR reaction recipes were used except that primer concentrations were increased to 0·8 µM, and 5 µl of TBT-PAR (Samarakoon *et al.* 2013) was added. Cycling conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 45 s, 47 °C (*rbc*L) or 52 °C (nucSSU) for 90 s, and 72 °C for 120 s, with a final 7 min at 72 °C. Products from PCR amplifications were cleaned using ExoSAP-IT (Affymetrix, Carlsbad, California), according to the manufacturer's instructions.

Cycle sequencing was conducted with a BigDye Terminator v 3.1 100 Reaction Ready kit (Applied Biosystems, Carlsbad, California, USA) following the manufacturer's protocol and using the same primers as for the original PCR. Sequences were analyzed by

TABLE 1. *Specimens examined with collection details and newly deposited GenBank accession numbers..*

Collection Code	Species	Locality	Collector	Collection Date	DNA Vouchers	GenBank Accession Numbers	
						Algal *rbcL*	Algal SSU
AF4	*Micarea byssacea*	Scotland, W. Perthshire, Castle Campbell; 56·1731°N, 3·6754°W	*A. Florence*	3.i.2013	EDNA13-0030208	–	KT253175 (C)
AF10	*M. lignaria*	Scotland, E. Lothian, Traprain Law; 55·9828°N, 2·6519°W	*A. Florence*	7.ii.2013	EDNA13-0032078	KT253165 (F) KT253162 (C)	KT253183 (F) KT253181 (C)
AF11	*M. leprosula*	Scotland, E. Lothian, Traprain Law; 55·9828°N, 2·6519°W	*A. Florence*	7.ii.2013	EDNA13-0032079	KT253160 (C)	KT253179 (C)
AF17	*M. prasina* s. lat.	Scotland, E. Lothian, Woodhall Dean, Spott; 55·9828°N, 2·6519°W	*A. Florence*	12.ii.2013	EDNA13-0032085	–	KT253182 (F) KT253174 (C)
AF20	*M. byssacea*	Scotland, E. Lothian, Woodhall Dean, Spott; 55·9828°N, 2·6519°W	*A. Florence*	12.ii.2013	EDNA13-0032088	–	KT253172 (C)
AF21	*M. prasina*	Scotland, E. Lothian, Woodhall Dean, Spott; 55·9828°N, 2·6519°W	*A. Florence*	12.ii.2013	EDNA13-0032089	KT253158 (C)	KT253176 (C)
AF22	*M. denigrata*	Scotland, E. Lothian, Woodhall Dean, Spott; 55·9828°N, 2·6519°W	*A. Florence*	12.ii.2013	EDNA13-0032090	KT253168 (F) KT253163 (C)	KT253177 (C)
AF23	*M. denigrata*	England, Cambridgeshire, Buff Wood, Hatley St George; 52·1335°N, 0·1313°W	*M. Powell* 2890	10.ii.2013	EDNA13-0032091	KT253164 (F) KT253161 (C)	KT253171 (F) KT253178 (C)
AF24	*M. nitschkeana*	England, Berkshire, Ashdown Estate, Middle Wood; 51·5362°N, 1·5977°W	*M. Powell* 2882	12.ii.2013	EDNA13-0032092	KT253167 (F) KT253159 (C)	KT253180 (C)
AF25	*M. micrococca*	England, Cambridgeshire, Buff Wood, Hatley St George; 52·1335°N, 0·1313°W	*M. Powell* 2888	12.ii.2013	EDNA13-0032093	–	KT253173 (C)
AV s. n.	*M. prasina* s. str.	Ukraine, Crimea, Karadag Nature Reserve, Mt Svyata; 44·9407°N, 35·2309°E	*A. Voyt-sekhovitch*	18.v.2012	EDNA14-0036096	KT253156 (C)	KT253169 (C)
AV12	*M. prasina* s. str.	Ukraine, Transcarpathian District, Tiachivsky Region, near Posich; 48·3510°N, 23·7326 °E	*L. Dymytrova*	05.x.2009	EDNA14-0036093	KT253166 (C)	–

TABLE 1. *Continued*

Collection Code	Species	Locality	Collector	Collection Date	DNA Vouchers	GenBank Accession Numbers	
						Algal *rbcL*	Algal SSU
AV14	*M. peliocarpa*	Ukraine, Transcarpathian District, Tiachivsky Region, near Posich; 48·3515°N, 23·7321°E	*L. Dymytrova et al.*	05.x.2009	EDNA14-0036094	KT253157 (C)	KT253170 (C)
AV16	*M. prasina*	Ukraine, Donetsk District, Shakhtars'ky Region, near Petriv'ske: 44·9407°N, 35·2309°E	*O. Nadyeina*	18.iv.2006	EDNA14-0036095	–	–

Letters in parentheses following GenBank numbers indicate if sequences are direct from fresh material (F) or from cultures (C).

The GenePool (University of Edinburgh, Edinburgh, UK), and electropherograms were visually inspected and edited using Geneious (v 6.1.4, Biomatters). All sequences were subjected to megaBLAST searches (NCBI) to ensure forward and reverse sequences represented the same strains. Alignments were initially generated by adding newly assembled sequences and those with high BLAST matches from GenBank to matrices generated by Thüs *et al.* (2011). Three separate alignments were created: *rbc*L, nucSSU and combined matrices. Datasets for both *rbc*L and nucSSU were analyzed independently and checked for conflicting support values on the branches prior to creation of a combined alignment. Sequences included in all analyses are listed in Appendix 1 (see Supplementary Material Appendix S1, available on-line). Alignments were exported to Mesquite (Maddison & Maddison 2011), where they were examined by eye and ambiguous sites and introns (in nucSSU) were delimited and removed. Phylogenetic analysis was completed using both maximum likelihood (ML) using partitioned models for *rbc*L (codon positions 1, 2 and 3) and combined matrices (nucSSU, codon position 1, 2, and 3), and non-partitioned for nucSSU data alone. ML analysis was performed using RaxML HPC Black Box on the Cipres Web Portal (v 7.2+), which uses the GTRGAMMA model for both bootstrapping and inference of the most-likely tree. The combined dataset was also analyzed using MrBayes v.3.1.2 (BI; Huelsenbeck & Ronquist 2001) using 4 partitions (SSU plus three codon positions) and best-fit models as determined by jModeltest v.2.1.3 under the AICc criterion (Darriba *et al.* 2012). Four chains were used in each of two runs of 10 million generations each, sampling every 1000 generations. Convergence and stationarity were assessed using diagnostics in Tracer v1.6 (Rambaut *et al.* 2014), including examination of the potential scale reduction factor and average standard deviation of split frequencies.

Several algal sequences generated from freshly extracted tissues produced only short sequences with high background (AF11F) or were not matched by the sequence from photobiont in culture (AF4F) and were excluded from the final analysis, although the data generated for these was used in preliminary analyses to check likely group membership.

Results

We examined algal photobionts from 14 specimens of *Micarea* s. str. using both sequence-based and morphological identification. All the British photobionts matched *Coccomyxa*, or what has been referred to previously as *Pseudococcomyxa* (AF17, AF25, AV12) in morphology (Fig. 1). The Ukrainian material was identified by Voytsekhovich *et al.* (2011*a*). The cultures from both AV12 and AV14 varied in morphology, with those represented in this study corresponding to

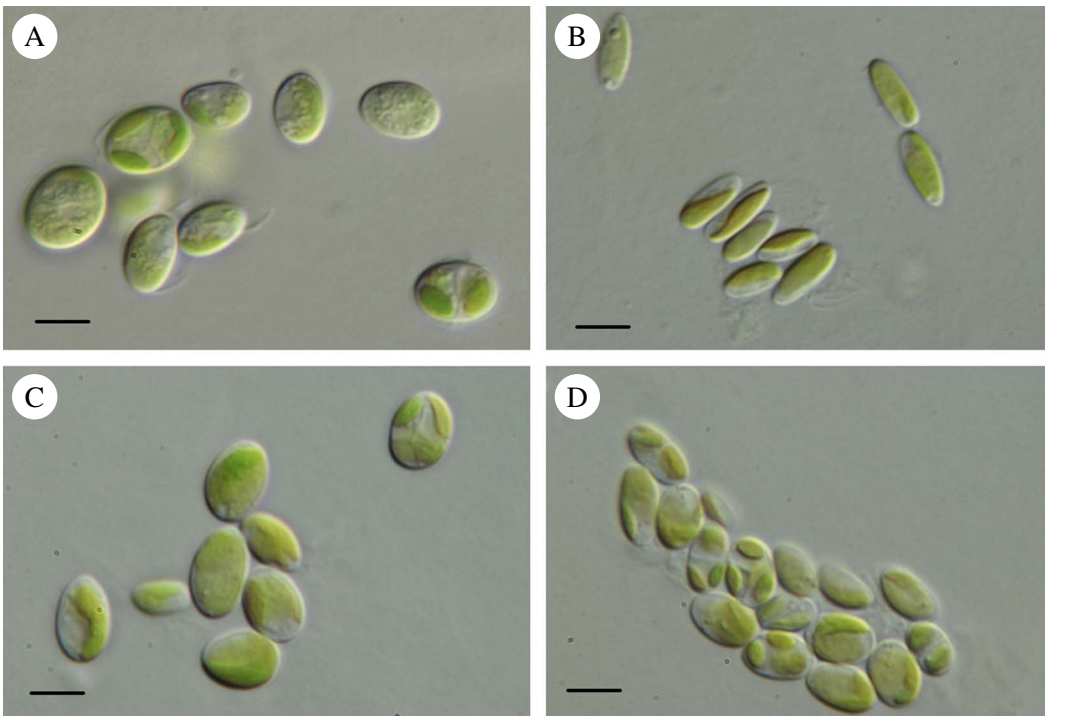

FIG. 1. Light micrographs of photobionts belonging to the *Coccomyxa* clade. A, *Coccomyxa* cf. *olivacea* 078 (AF10); B, *Coccomyxa simplex* 085 (AF17); C, *Coccomyxa simplex* 093 (AF23); D, *Coccomyxa* sp. 208 (AF4). Scales: = 10 μm.

Coccomyxa when young, but fitting *Elliptochloris* when more mature. A single Ukrainian photobiont matched *Elliptochloris bilobata* (AV s. n.).

Twelve new *rbc*L sequences and 17 new nucSSU sequences were generated for this study (Table 1). The *rbc*L alignment was 39 taxa and 1299 positions, with 467 parsimony-informative and 762 constant positions. The nucSSU alignment included 55 taxa and 3987 aligned positions, of which 2331 were excluded, resulting in 216 parsimony-informative and 1306 constant sites. BLAST searches and preliminary analyses showed that all photobiont sequences recovered belong in the *Trebouxiophyceae*, with most closely matching sequences of *Coccomyxa* in *rbc*L and nucSSU. In phylogenetic reconstructions of relationships among photobiont sequences using ML analyses, no conflicts were found between topologies comparing both gene regions individually; therefore, a combined analysis with 64 taxa, 5286 positions (2331 excluded), and 4 partitions was undertaken. In BI analysis, convergence and stationarity were confirmed with all sample sizes exceeding 1000 in Tracer v1.6 (Rambaut *et al.* 2014), potential scale reduction factor not greater than 1·005, and average standard deviation of split frequencies not greater than 0·005.

The combined analysis of *Micarea* photobionts shows that a single photobiont from *Micarea prasina* collected in the Ukraine belongs to *Elliptochloris*, whereas all other studied photobionts from *Micarea* belong to a well-supported clade including *Coccomyxa*, hereafter referred to as the *Coccomyxa* clade (arrow in Fig. 2), with 98% bootstrap support and 100% posterior probability. Two well-supported groups were resolved in ML within this clade, with one group predominantly comprised of symbiotic strains from this

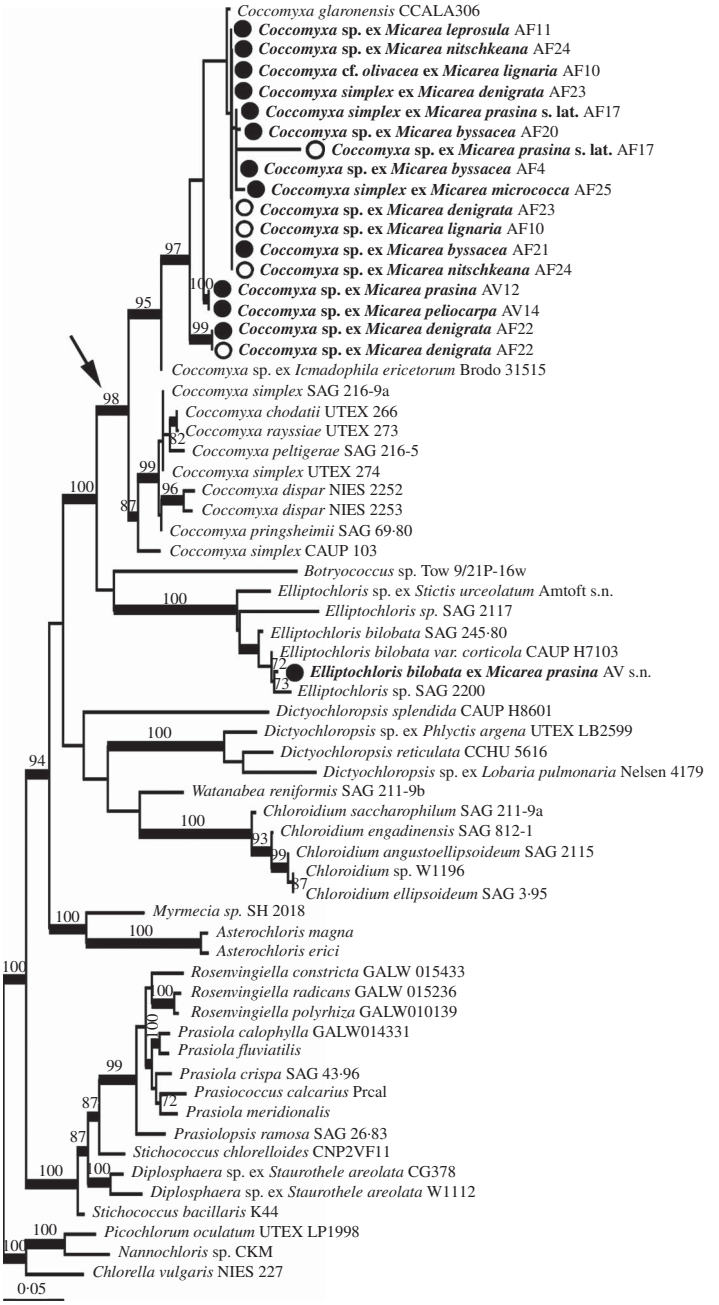

FIG. 2. Maximum likelihood phylogeny from combined *rbc*L and nucSSU of *Micarea* photobionts. Morphological determinations of studied strains are labelled on the terminals according to *Micarea* species with which they were associated, and the collection code for the specimen. Bootstrap support is shown above nodes for values above 70%. Support for nodes from ML analysis but without support from BI is shown to the right of nodes. Thickened branches indicate support over 0·97 in BI. Terminal names in bold show strains studied here. Open circles depict sequences derived from direct amplification of algae from fresh material, closed circles for photobionts sequenced from cultures and arrow indicates the *Coccomyxa* clade.

study and symbionts from *Ginkgo biloba* L. (*C. glaronensis* CCALA306, Tremouillaux-Guiller & Huss 2007) and *Icmadophila ericetorum* (L.) Zahlbr. (supported with 97% bootstrap in ML, and with PP at 100% in BI). The other group predominantly contained free-living strains, including sequences from strains representing the types of both *Coccomyxa* (*C. dispar* Schmidle) and *Pseudococcomyxa* (*P. simplex* (Mainx) Fott), *C. peltigerae* Warèn (symbiont of *Peltigera*) and *C. rayssiae* Chodat & Jaag (free-living) (supported with 87% bootstrap and 100% in BI). In both the predominantly symbiotic and free-living clades, strains with *Coccomyxa* and *Pseudococcomyxa* morphology were mixed. Topologies in BI and ML were congruent.

The phylogenetic analysis strongly supports the conclusion that the three strains with morphology corresponding to *Pseudococcomyxa* (AF17, AF25 and AV12) are not distinct from *Coccomyxa*, although no sequences could be obtained from cultures of AF17. One specimen (AV14) reported to have a photobiont corresponding to *Elliptochloris reniformis* (S.Watanabe) Ettl & Gärtner (No. 14, Voytsekhovich *et al.* 2011*a*) also groups with these *Coccomyxa* sequences. No sequences could be obtained from the specimen (AV16; No. 16, Voytsekhovich *et al.* 2011*a*) reported with *E. subsphaerica* (Reisigl) Ettl & Gärtner as photobiont.

In all but one case from the five specimens (AF4, AF10, AF22, AF23, AF24) where sequences were obtained from both fresh and cultured material (shown as open vs closed circles, respectively, in Fig. 2), the sequences obtained were identical or very closely related, strengthening the case for the algae identified being the dominant photobionts. In the case of AF4, the sequence from fresh material only poorly matched with *Stichococcus* / *Diplosphaera* according to BLAST and was not included in further analysis, whereas the cultured photobiont belonged to *Coccomyxa*. We interpret the partial sequence from the fresh material as an epiphytic alga.

Representative cultures of both the *Coccomyxa* and the *Elliptochloris* clades have been deposited in CCAP with the following numbers: CCAP 216/27 *Coccomyxa* sp. AF23, and CCAP 240/2 *Elliptochloris bilobata* AV s. n., respectively.

Discussion

Until now, the evolutionary position of 'micareoid algae' has been unknown. With the combined perspectives of morphological and molecular data (Voytsekhovich *et al.* 2011*a* and this study), it is clear that the typical micareoid algae belong to the '*Choricystis* clade' of the *Trebouxiophyceae* (Eliáš *et al.* 2008; Leliaert *et al.* 2012), which includes the genera *Choricystis*, *Botryococcus*, and *Coccomyxa*, among others. The majority of photobionts from eight species of *Micarea* belong to *Coccomyxa* (including *Pseudococcomyxa*), with *Elliptochloris* also occasionally present (Voytsekhovich *et al.* 2011*a*; this study). Our data support previous findings that symbiotic and free-living strains of *Coccomyxa* are largely well separated phylogenetically (Zoller & Lutzoni 2003; Pröschold *et al.* 2011). Further studies of these strains are required to identify the strains to species, for example using ITS-2, recently proposed as a barcode marker for the genus (Darienko *et al.* 2015).

Both fresh and cultured algae were targeted for sequencing, but obtaining clean algal sequence reads from fresh material proved challenging, typically with only a few hundred bases of high quality for both *rbc*L and nucSSU. In contrast, algal cultures proved easier to sequence. As great care was taken to clean specimens and begin algal cultures from homogeneous algal populations liberated from within thallus granules, we are confident that the algae studied are the dominant ones in the thallus. Identical sequences were also obtained from each of the original multiple isolates per culture. Observations of algal populations obtained by squashing carefully-cleaned and selected granules showed that cells were apparently homogenous and some, though few, maintained hyphal connections.

However, from the combined morphological and molecular perspective offered here, it is clear that *Coccomyxa* and strains previously referred to as *Pseudococcomyxa* are

intermixed in a single clade using combined *rbc*L and nucSSU data; they are also very similar morphologically, and both *Coccomyxa* and *Pseudococcomyxa* are characterized as having a mucilaginous envelope or mucilage on the tip of the cell (Tsarenko 2011). In *Coccomyxa*, this feature varies with environmental and culture conditions (e.g. Darienko *et al.* 2015), and must be interpreted with care. For example, the mucilaginous envelope is not apparent surrounding individual cells on solid media, but is consistently produced in liquid media for most strains of *Coccomyxa* in culture (Fig. 1B). The mucilage cap on the tip (one pole) of the cell is very fragile, but can be clearly observed after staining with methylene blue and black ink. On the other hand, the type species *C. dispar* is known to produce a clear mucilaginous layer only in the free-living or lichenized state and not in culture (AV, pers. obs.).

The close relationship found here between *Elliptochloris* and *Coccomyxa* is supported both by their sharing fungal symbionts (*Micarea*) and by molecular studies (e.g. Eliáš *et al.* 2008; Letsch & Lewis 2012). Morphologically, these two algal genera are similar, but are usually clearly differentiated morphologically since *Elliptochloris* has generally spherical vegetative cells, two types of autospores, and consistently lacks mucilage layers, whereas cells are oval/kidney-shaped in *Coccomyxa*, have a single type of autospore, and have mucilage production (Ettl & Gärtner 1995). However, it can be difficult to assign strains to genera even with cultured material due to wide morphological variation, particularly in the case of *E. reniformis*. Reviewing the literature on symbiotic partners, it is clear that the morphology of algae in the *Choricystis* group (*sensu* Leliaert *et al.* 2012) may present considerable challenges until enough sequenced reference strains are available for development of more robust hypotheses.

In addition, more than a single photobiont lineage may exist in lichen thalli (Piercey-Normore 2006; Mansournia *et al.* 2012; Park *et al.* 2014), and sometimes might be expected (Casano *et al.* 2011). Two photobionts from *M. melanoloba* (= *M. prasina*) AV12 were reported in Voytsekhovich *et al.* (2011*a*), *E. subsphaerica* and *Pseudococcomyxa* sp.; only the *Pseudococcomyxa* strain was available for sequencing. Likewise, both *E. reniformis* and *E. subsphaerica* were reported from *M. peliocarpa* AV14, but only the former was available for sequencing. This strain appears morphologically very distinct, but the presence of a single type of autospore (of elongated shape) and the formation of an irregular cell shape can justify classification of this strain into the genus *Coccomyxa*. Despite careful cleaning methods, the difficulty in direct amplification of photobionts could be a result of either closely attached epibionts, or the presence of more than a single photobiont.

The *Micarea* species included in this study are all members of the *Pilocarpaceae*, either related to the type species of *Micarea prasina* (e.g. *M. byssacea* (Th. Fr.) Czarnota *et al.*, and *M. micrococca* (Körb.) Gams ex Coppins), or in a separate clade related to *M. denigrata* (Fr.) Hedl. (e.g. *M. nitschkeana* (J. Lahm ex Rabenh.) Harm., *M. leprosula* (Th. Fr.) Coppins & A. Fletcher, *M. lignaria* (Ach.) Hedl. and *M. peliocarpa* (Anzi) Coppins & R. Sant.; Andersen & Ekman 2005). The recently segregated genus *Brianaria* was not studied here, though earlier morphological observations point to different photobionts as members of that symbiotic association (Coppins 1983).

The diversity of species interactions has been suggested to be one of the major forces driving diversification in diverse lineages, from insect pollinators of flowering plants (Thompson 2009) to specialization of fungal endophytes on lichens (Arnold *et al.* 2009). The evolutionary mechanisms of some species interactions have been studied in detail for only relatively few systems, permitting inferences about processes driving these patterns. In most groups, carefully planned studies of ecologically- and taxonomically-stratified samples are lacking, meaning that for the majority of descriptive studies so far completed, testable inferences for what mechanisms might be at work are impossible (e.g. Mueller 2012). Nevertheless, patterns of association between symbionts are being amassed at an increasing rate, and trends

among studies can be compared. For example, reciprocal specificity (*sensu* Smith & Douglas 1987) between fungi and their photobionts tends to be rare (Otálora *et al.* 2010), and most fungi can associate with several related strains of their photobiont hosts, which may be adapted to their ecological setting rather than their fungal host. In this first sequence-based glimpse at the diversity of photobionts within *Micarea*, patterns of association seem to be supporting the general trends of genus-level specificity of fungi for photobionts (i.e. *Micarea* with the *Elliptochloris* clade, *sensu* Pröschold *et al.* 2011), low specificity within fungal species, and unrecognized diversity of photobionts. The detailed genetic structure of this association, including symbiotic specificity, requires a carefully conceived study.

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Supplementary Material

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0024282915000341

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