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An assessment of proposed DNA barcodes in freshwater green algae

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Abstract – The development of DNA barcodes for green algae would aid in their accurate and rapid identification. This is particularly important for the green algae because in many species structural characters are few or difficult to observe. In this study we test the utility of several DNA barcode loci in seven distantly related species groups of freshwater charophytes and chlorophytes. The ITS rDNA and COI markers were the most variable, although COI was least applicable because it was successfully amplified and sequenced in only one test genus. The ITS1 and ITS2 rDNA regions each successfully amplified in only a subset of the charophytes. Both *rbcL* and *tufA* were moderately variable, although *tufA* may pose challenges in some lineages where the gene is encoded in the nucleus. The 18S rDNA and UPA were the least variable loci tested. Of the markers tested, *rbcL*, ITS2 and *tufA* (in chlorophytes) are the most promising for use as DNA barcodes. However, none of the loci tested were ideal for use across all tested lineages of green algae because of taxon-specific applicability, because none of the markers could distinguish closely related species in all groups tested, and because of overlap in the level of intraspecific and interspecific variation.

DNA barcode / green algae / Charophyceae / Chlorophyceae / Zygnematophyceae / 18S rDNA / COI / ITS rDNA / rbcL / tufA / UPA

Résumé – Le développement d'un marqueur code-barres ADN pour les algues vertes améliorerait leur identification fiable et rapide. Cette étape est particulièrement importante pour les algues vertes pour lesquelles les caractères structuraux sont peu nombreux ou difficiles à observer. Dans cette étude nous testons l'efficacité de plusieurs marqueurs de code-barres ADN au sein de sept groupes d'espèces dulçaquicoles, génétiquement distants et appartenant tant aux charophytes et qu'aux chlorophytes. Les marqueurs ITS des ADNr et COI étaient les plus variables, bien que le COI n'ait été amplifiable et séquençable que pour un seul genre teste. Les ITS1 et IITS2 ADNr n'ont été amplifiés avec succès que chez certaine charophytes. La *rbc*L et le *tufA* n'étaient que modérément variables, cependant *tufA* pourrait se révéler problématique dans certains groupes où il serait code par le génome nucléaire. Le 18S ADNr et l'UPA étaient les marqueurs les moins variables testés. Parmi les marqueurs testés, ITS2 et *tufA* (chez les chlorophytes) sont les marqueurs les plus prometteurs. Cependant, aucun des marqueurs testés s'est avéré le candidat idéal pour développer le code-barres ADN chez les algues vertes car aucun marqueur n'a pu délimiter des espèces proches dans tous les groupes testés, en raison notamment du chevauchement des distances intra et interspécifique.

Code-barres ADN / algues vertes / Charophyceae / Chlorophyceae / Zygnematophyceae / 18S rDNA / COI / ITS rDNA / rbcL / tufA / UPA

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INTRODUCTION

The green algae comprise an ancient and taxonomically diverse lineage that includes land plants (Lewis & McCourt, 2004; McCourt *et al.,* 2004; Hall & Delwiche, 2007; Pröschold & Leliaert, 2007; Becker & Marin, 2009). There are more than 14,000 species of green algae recognized on the basis of anatomical and ultrastructural characteristics (Norton *et al.,* 1996; Pröschold & Leliaert, 2007). The lineage includes many microscopic algal species as well as large green seaweeds and the macroscopic freshwater Charophyceae. Although green algae have a long history of study, they are still poorly understood taxonomically and phylogenetically. New species are discovered each year and molecular investigations have revealed many cryptic species and unexpected phylogenetic relationships.

Identification of green algae can be difficult because many species lack obvious structural features and some of the observable characteristics are variable within species. Often, diagnostic characteristics are only detectable using light or electron microscopy. Even in the macroscopic Charophyceae, some species can only be distinguished based on sexual state, gametangial position and mesospore membrane ornamentation — characteristics only apparent with magnification (Allen, 1888; Wood & Imahori, 1965; Sakayama *et al.,* 2004a; 2004b; Casanova & Karol, 2008). Microscopic green algae are identified based on the general shape of their vegetative cells, the shape and position of chloroplasts and pyrenoids, ultrastructural characteristics — such as the architecture of swimming cells — and developmental or life history stages (Bourrelly, 1990; Ettl & Gärtner, 1995; Watanabe & Floyd, 1996; Škaloud *et al.,* 2006). Although some species of microscopic green algae are easily recognizable, many require careful examination of actively growing cultured cells for identification. Because identification typically requires the use of a microscope, sometimes at very high magnification, taxonomy of green algae is somewhat inaccessible to non-specialists and rapid identification of some species even by microscopy is impossible. DNA barcodes would allow non-specialists to identify green algae consistently and rapidly regardless of life stage. They would also make it possible for researchers to identify suboptimal material and potentially recognize cryptic species or intraspecific variation correlated to geographic distribution.

The ideal DNA barcode would be sufficiently variable to distinguish among closely related species. The marker should be found in all taxa, amplify using universal primers and sequence cleanly. Finding an ideal molecular barcode for all green algae is complicated by the fact that green algae are an ancient and diverse lineage. Although little is known about the arrangement of their nuclear genomes it is now clear that organellar genomes in green algae vary considerably in content and organization (Turmel *et al.,* 2002; Bélanger *et al.,* 2006; Turmel *et al.,* 2007; 2009a; 2009b).

Molecular barcoding methods have been applied to land plants (e.g., Cbol Plant Working Group, 2009; Kelly *et al.,* 2010) and a number of algal groups including some green algae (e.g., O'Kelly *et al.,* 2010). DNA barcodes in the strict sense have not been widely used in green algae and no study has investigated the utility of proposed DNA markers in most lineages. In practice, however, researchers are using 'barcodes' — nearly all published work on microscopic green algae includes at least one molecular marker and taxonomists now routinely use DNA markers to distinguish species of green algae (e.g., Fawley *et al.,* 2004; 2005; Hegewald *et al.,* 2010).

Several loci have been proposed for algae and land plants. The 5' end of cytochrome oxidase I (COI) is widely used in animals (Ferri *et al.,* 2009; Wilson,

2010, and references therein) and some algal lineages including red algae (Sherwood *et al.,* 2008; Le Gall & Saunders, 2010), brown algae (McDevit & Saunders, 2010) and some diatoms (Evans *et al.,* 2007). However, COI can be difficult to amplify from some taxa (Moniz & Kaczmarska, 2009). In green algae, this gene can contain several introns (e.g., five in *Chaetosphaeridium,* Turmel *et al.*, 2002) although the position and number of introns in the gene are not known for most species.

The internal transcribed spacer (ITS) of the nuclear ribosomal operon also has been proposed as a DNA barcode for algae and land plants (e.g., Moniz & Kaczmarska, 2010) and has been widely used in species-level phylogenetics of green algae (Sakayama *et al.,* 2004a; Verbruggen *et al.,* 2006; Coleman, 2007; Mei *et al.,* 2007; Keller *et al.,* 2008; Sluiman *et al.,* 2008; O'Kelly *et al.,* 2010) and at deeper nodes (Hershkovitz & Lewis, 1996). The locus lies between the large and small nuclear ribosomal subunit genes and includes the 5.8S rDNA gene. The length of the ITS varies from a few hundred to more than a thousand base pairs depending on the taxon. Interpretation of ITS phylogenies is not always straightforward due to within-species variation, difficulties with alignment and determination of orthology (reviewed in Feliner & Rosseló, 2007; Poczai & Hyvõnen, 2010), although incorporation of information from secondary structure and compensatory base changes holds promise (Friedrich *et al.,* 2005; Wolf *et al.,* 2008; Coleman, 2009).

A third barcode has been recently proposed for plastid-containing organisms: a portion of the chloroplast ribosomal large subunit (23S), sometimes called the Universal Plastid Amplicon (UPA) (Sherwood & Presting, 2007). This locus has not been broadly sampled and recent investigations suggested that it is less variable than other markers, including other chloroplast markers (Sherwood *et al.,* 2008; Clarkston & Saunders, 2010).

Many different molecular markers have been proposed for use in land plants, especially angiosperms. In addition to the ITS region, plastid markers such as *matK*, *rpoB*, *rpoC1* and the *trnH-psbA* spacer have been proposed. One recent study suggested that *matK* and *rpoC1* are the most variable (Kelly *et al.,* 2010). The Cbol Plant Working Group (2009) recommended the use of *rbcL* and *matK* as the molecular barcodes for land plants. However *matK* is only found in a subset of charophytic plastid genomes (Turmel *et al.,* 2005; Lemieux *et al.,* 2007) and is completely absent from chlorophytes (Pombert *et al.*, 2005).

The plastid encoded *rbcL* gene has been widely used for phylogenetic inference in green algae. Saunders and Kucera (this volume) present data indicating that the 3' end of *rbcL* may be sufficiently variable to identify species of marine green algae. Truly universal primers are not available for *rbcL*, although some primers can be used on a wide range of taxa (Lewis *et al.,* 1997). Given that variable portions of the gene are small enough to be sequenced in a single reaction and a large amount of sequence data is available from a wide range of taxa through GenBank (http://www.ncbi.nlm.nih.gov/genbank/) it is possible to design lineage-specific primers.

Several additional molecular markers have been developed to address phylogenetic questions in various groups of green algae. One of these, *tufA*, is generally conserved and has been investigated in marine algae (Fama *et al.*, 2002). Although *tufA* may be useful as a DNA barcode in chlorophytes (Saunders & Kucera, this volume), the gene was transferred from the plastid to the nuclear genome in some lineages of charophytic algae and in land plants (Baldauf *et al.,* 1990) and in a few cases a copy is maintained in each genetic compartment.

In this study, we assessed the utility of the markers COI, ITS, UPA, *rbcL* and *tufA* in seven species groups of phylogenetically diverse green algae: the charophytes *Chara*, *Desmidium*, *Micrasterias* and *Nitella*, and the chlorophytes *Bracteacoccus*, *Chlorosarcinopsis* and *Scenedesmus*. In chlorophytes we also contrasted these results with information from 18S rDNA data. Species groups within these genera were used to test and quantify marker variability.

MATERIALS AND METHODS

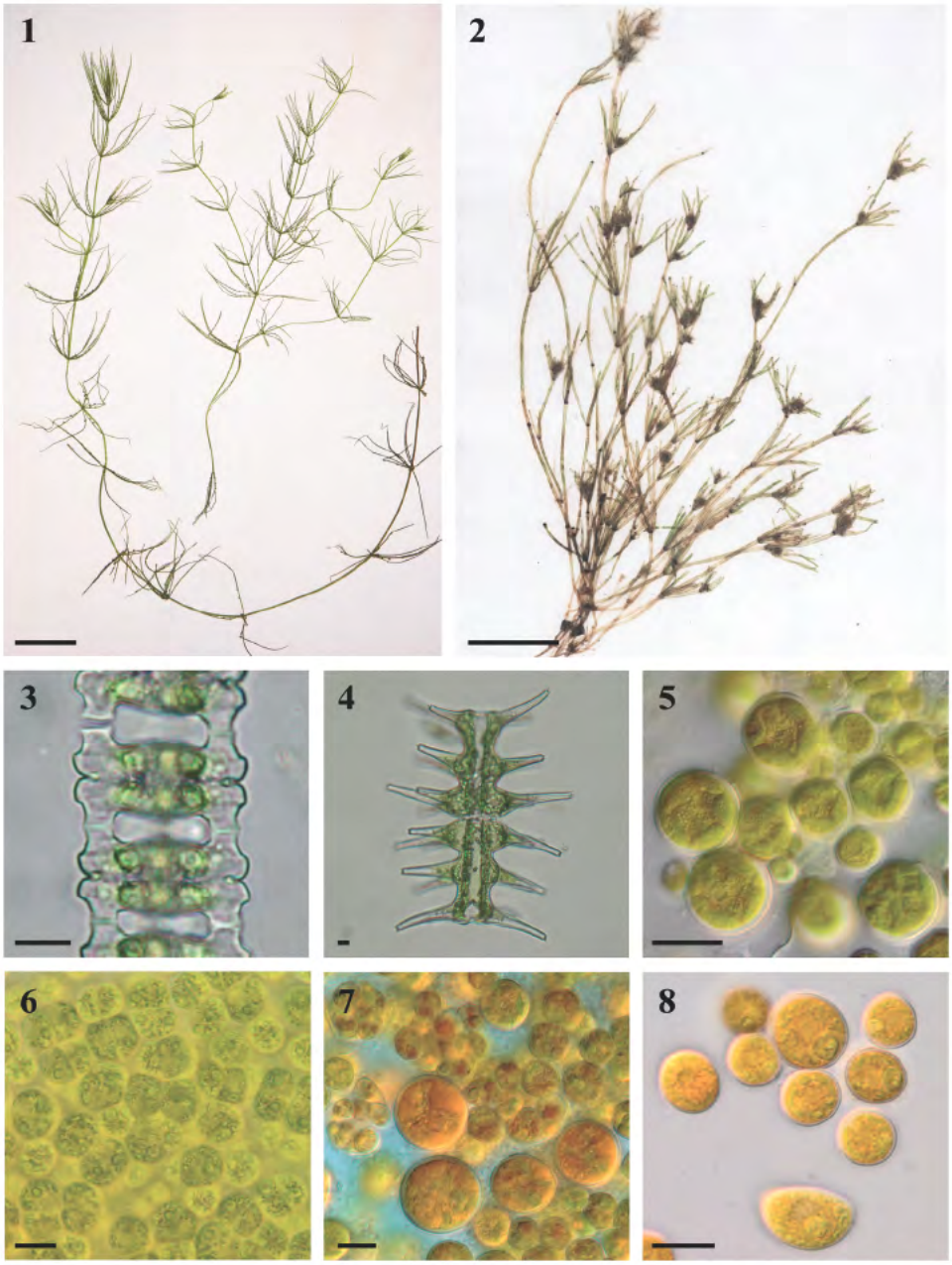
Taxon selection

Seven different species groups were used to test the utility of proposed DNA barcodes. These include four charophytic and three chlorophytic groups (Figs 1-8). Of these, *Chara* and *Nitella* are macroscopic and the rest are microscopic. Two of the microscopic groups belong to the species-rich desmids, algae that have varied and intricate structural characteristics. The remaining three groups contain microscopic and morphologically cryptic species. Although these are all freshwater or terrestrial species, they represent distantly related lineages of green algae. These groups also represent taxonomically difficult groups for which DNA barcodes would be a valuable aid to more accurate identification (described more fully below). A complete list of taxa with GenBank accession numbers is provided in Table 1.

Chara subsection *Willdenowia* — The genus *Chara* (Charophyceae) includes macroscopic freshwater algae with a complex thallus with whirls of branchlets at nodes and specialized reproductive structures. The internode is comprised of a single cell that may be naked or covered in a layer of cortical cells. The subsection *Willdenowia* (Fig. 1) includes those species whose branchlets are corticated except at the first (basal) segment, which remains ecorticate throughout the lifecycle (Wood & Imahori, 1965). The subsection is monophyletic (Karol, unpublished) and in many cases the species are known to be reproductively isolated (Proctor *et al.,* 1971; Proctor & Wiman, 1971). Eight species in the subsection were sampled (Table 1). Many of these species have overlapping distributions and can only be distinguished morphologically when reproductive structures are present.

Nitella hookeri species complex — This species group is comprised of several Australasian species of *Nitella* (Charophyceae). Previous work has shown these species to be closely related phylogenetically yet morphologically distinct (Casanova *et al.*, 2007). Seven named species and two unnamed species were sampled (Table 1). The unnamed species are structurally similar to *Nitella cristata* (Fig. 2) but are phylogenetically distinct (based on previous work). Both are from Australia while strains of *N. cristata* are from New Zealand (Table 1). One form is more robust than the other. Although difficult to identify, understanding species diversity and distribution in this group will provide valuable information about biogeographic patterns in Australia, New Zealand and the Pacific Islands.

Desmidium — The genus *Desmidium* (Zygnematophyceae) includes filamentous species that are angular in apical view. Species of this genus are common in freshwater habitats around the world. Five taxa in four species were sampled (Table 1). *Desmidium swartzii* and *D. pseudostreptonema* can be difficult to distinguish from *D. aptogonum* (Fig. 3) in some circumstances (Croasdale *et al.,*



Figs 1-8. Representatives of the seven study genera. **1.** *Chara foliolosa*, **2.** *Nitella* aff. *cristata*. **3.** *Desmidium aptogonum*, **4.** *Micrasterias muricata*, **5.** *Bracteacoccus cohaerans*, **6.** *Chlorosarcinopsis eremi*, **7.** *Scenedesmus rotundus*. **8.** *Scenedesmus deserticola*. Scale bars = 2.5 cm (Figs 1-2) and 10 µm (Figs 3-8).

Table 1

CHAROPHYTES	*Strain*	*Locality*	*ITS1*	*ITS2*	*rbcL*	*UPA*
CHAROPHYCEAE						
Chara drouetii (R. D. Wood) R. D. Wood	KGK0467, site 166	Hildago, Quintana Roo, Mexico	n/a	n/a	HQ380444	HQ380560
C. drouetii (R. D. Wood) R. D. Wood	V. W. Proctor Loc. 36	Outside town of Floris, San Luis, Guatemala	n/a	n/a	HQ380445	n/a
C. foliolosa Mühl. *ex* Willd.	KGK0061, V. W. Proctor X-883	0.5 mile South of Surl, NC, USA	n/a	HQ380616	HQ380446	n/a
C. foliolosa Mühl. *ex* Willd.	KGK0184	Clopper Lake, Montgomery Co., MD USA	n/a	HQ380617	HQ380447	n/a
C. foliolosa Mühl. *ex* Willd.	KGK0233	Small pond, Campache, Mexico	n/a	HQ380618	HQ380448	n/a
C. foliolosa Mühl. *ex* Willd.	KGK0333	Lake Erickson, Oneida Co., WI USA	n/a	n/a	HQ380449	HQ380561
C. foliolosa Mühl. *ex* Willd.	KGK0341, V. W. Proctor 579	Mrs. L's Playa, TX, USA	n/a	HQ380619	HQ380450	HQ380562
C. foliolosa Mühl. *ex* Willd.	KGK0393	Partin's Mill Pond, Wake Co., NC USA	n/a	HQ380620	HQ380451	HQ380563
C. foliolosa Mühl. *ex* Willd.	KGK0428	Lake Erickson, Oneida Co., WI USA	n/a	HQ380621	HQ380452	HQ380564
C. haitensis Turp.	KGK0029	Small pond, Manatí, Puerto Rico	n/a	HQ380622	HQ380453	n/a
C. haitensis Turp.	KGK0032	Jardin Botanico Nacional, Santa Domingo, Dominican Republic	n/a	HQ380623	HQ380454	n/a
C. haitensis Turp.	KGK0483, V. W. Proctor 173	Lake Silvituc, Campeche, Mexico	n/a	n/a	HQ380455	HQ380565
C. haitensis Turp.	V. W. Proctor S/T-076	Chiapas, Mexico	n/a	n/a	HQ380456	HQ380566
C. haitensis Turp.	V. W. Proctor TAMPS 80	Xicotemcatl, Tamalpais, Mexico	n/a	n/a	HQ380457	HQ380567
C. haitensis Turp.	V. W. Proctor X-900	Laguna San Felipe, Quintana Roo, Mexico	n/a	n/a	HQ380458	HQ380568
C. haitensis Turp.	V. W. Proctor X-901	Campeche, Mexico	n/a	HQ380624	HQ380459	HQ380569
C. haitensis Turp.	V. W. Proctor X-930	Everglades, Monroe Co., FL, USA	n/a	n/a	HQ380460	HQ380570
C. hydropitys A. Braun	KGK0213, V. W. Proctor 028	Chiapas weather station, Chiapas, Mexico	n/a	HQ380625	HQ380461	n/a
C. hydropitys A. Braun	KGK0340, V. W. Proctor 147	Los Chiles, Costa Rica	n/a	HQ380626	HQ380462	HQ380571
C. hydropitys A. Braun	KGK0466, V. W. Proctor Y-069	UNKNOWN	n/a	n/a	HQ380463	HQ380572

Table 1 *(cont'd)*

CHAROPHYTES	*Strain*	*Locality*	*ITS1*	*ITS2*	*rbcL*	*UPA*
C. hydropitys A. Braun	KGK0774	Lago Carite, Puerto Rico	n/a	n/a	HQ380464	n/a
C. kenoyeri Howe	TP118	Gatun Lake, Canal Zone, Panama	n/a	n/a	HQ380465	n/a
C. martiana A. Braun *ex* Wallm.	TP097, V. W. Proctor X-068	Parana River, Itapura, São Paulo, Brazil	n/a	n/a	HQ380466	n/a
C. martiana A. Braun *ex* Wallm.	V. W. Proctor X-952	Caracas Botanic Garden, Caracas, Venezuela	n/a	n/a	HQ380467	n/a
C. rusbyana Howe	LG	Unknown. Growing in culture, University of Wisconsin - Madison, USA	n/a	HQ380627	AF097169.1	HQ380573
C. rusbyana Howe	V. W. Proctor X-066	Entre Ríos, Argentina	n/a	n/a	AF097168.1	n/a
C. zeylanica Willd.	KGK0057b	Taihu Lake, Jiangsu, China	n/a	HQ380628	HQ380468	n/a
C. zeylanica Willd.	KGK0059c, V. W. Proctor Y-031	Lake Mattamuskeet, Hyde Co., NC, USA	n/a	HQ380629	HQ380469	n/a
C. zeylanica Willd.	KGK0062, V. W. Proctor X-853	Headwaters of Bechler River, WY, USA	n/a	HQ380630	HQ380470	n/a
C. zeylanica Willd.	KGK0307	West Beach Pond, Porter Co., IN, USA	n/a	n/a	HQ380471	n/a
C. zeylanica Willd.	KGK0342, V. W. Proctor 160	Chenote Chen-Ha, Yucatan, Mexico	n/a	n/a	HQ380472	HQ380574
C. zeylanica Willd.	KGK0343, V. W. Proctor 154	Lake Nicaragua, Nicaragua	n/a	n/a	HQ380473	HQ380575
C. zeylanica Willd.	KGK0344	San Solomon Springs, Reeves Co., TX USA	n/a	HQ380631	HQ380474	HQ380576
C. zeylanica Willd.	KGK0389	Lake Okeechobee, FL USA	n/a	HQ380632	HQ380475	HQ380577
C. zeylanica Willd.	KGK0403	Rice paddy, Sacramento Co., CA, USA	n/a	n/a	HQ380476	HQ380578
C. zeylanica Willd.	KGK0468, V. W. Proctor site 151	Lago Xiloá, Nicaragua	n/a	HQ380633	HQ380477	HQ380579
C. zeylanica Willd.	KGK0709	Audubon Park Lake, Orleans Parish, LA, USA	n/a	HQ380634	HQ380478	HQ380580
C. zeylanica Willd.	V. W. Proctor Loc. 32	Lago Isobal, Guatemala	n/a	n/a	HQ380479	n/a
C. zeylanica Willd.	V. W. Proctor X-315	Israel	n/a	n/a	HQ380480	HQ380581
C. zeylanica Willd. (sensu *C. vandalurensis* Sundaralingam)	V. W. Proctor X-574	Sri Lanka (Ceylon)	n/a	n/a	HQ380481	n/a
Nitella arthroglochin (A. Braun) M. T. Casanova	KGK0432	Currency Creek, South Australia, Australia	HQ380426	HQ380636	HQ380482	n/a

Table 1 *(cont'd)*

CHAROPHYTES	*Strain*	*Locality*	*ITS1*	*ITS2*	*rbcL*	*UPA*
N. claytonii M. T. Casanova	KGK0127	Lake Ohau, South Island, New Zealand	HQ380427	HQ380637	HQ380483	n/a
N. claytonii M. T. Casanova	KGK0185	Lake Wakatipu, South Island, New Zealand	HQ380428	HQ380638	HQ380484	n/a
N. claytonii M. T. Casanova	KGK0186	Lake Lyndon, South Island, New Zealand	n/a	HQ380639	HQ380485	n/a
N. claytonii M. T. Casanova	KGK0400	Lake Hawea, South Island, New Zealand	n/a	HQ380640	HQ380486	n/a
N. cristata A. Braun	KGK0090	Pond 2.0 km outside Woorndoo, Victoria, Australia	n/a	HQ380641	HQ380487	n/a
N. cristata A. Braun	KGK0120	Lake Taupo, North Island, New Zealand	n/a	HQ380642	HQ380488	n/a
N. cristata A. Braun	KGK0122	Lake Taupo, North Island, New Zealand	n/a	HQ380643	HQ380489	n/a
N. cristata A. Braun	KGK0123	Lake Taupo, North Island, New Zealand	n/a	HQ380644	HQ380490	n/a
N. cristata A. Braun	KGK0124	Lake Taupo, North Island, New Zealand	n/a	HQ380645	HQ380491	n/a
N. cristata A. Braun	KGK0125	Lake Rotoaira, North Island, New Zealand	n/a	n/a	HQ380492	n/a
N. cristata A. Braun	KGK0160	Carrot Lake, North Island, New Zealand	n/a	HQ380646	HQ380493	n/a
N. cristata A. Braun	KGK0161	Carrot Lake, North Island, New Zealand	n/a	HQ380647	HQ380494	n/a
N. cristata A. Braun	KGK0166	Lake Humuhumu, North Island, New Zealand	n/a	HQ380648	HQ380495	n/a
N. cristata A. Braun	KGK0183	Lake Moeraki, South Island, New Zealand	n/a	n/a	HQ380496	n/a
N. cristata A. Braun	KGK0277	Lake Brunner, South Island, New Zealand	n/a	HQ380649	HQ380497	n/a
N. cristata A. Braun	KGK0369	Ararat, Victoria, Australia	n/a	HQ380650	HQ380498	n/a
N. cristata var. ambigua A. Braun	KGK0799	Val Stajsic, Melbourne, Australia	n/a	HQ380635	n/a	n/a
N. aff. *cristata*	KGK0128	Barleyfields Lagoon, New South Wales, Australia	n/a	n/a	HQ380499	n/a
N. aff. *cristata*	KGK0129	Mother of Ducks Lagoon, New South Wales, Australia	HQ380429	HQ380651	HQ380500	n/a
N. hookeri A. Braun	KGK0121	Lake Rotoaira, North Island, New Zealand	n/a	HQ380652	HQ380501	n/a
N. masonae (R. D. Wood) M. T. Casanova	KGK0397	Lake Gunn, South Island, New Zealand	HQ380430	HQ380653	HQ380502	n/a
N. masonae (R. D. Wood) M. T. Casanova	KGK0401	Lake Okataina, North Island, New Zealand	n/a	n/a	HQ380503	n/a
N. aff. *cristata* (robust form)	KGK0444	Lake St. Claire, Tasmania, Australia	n/a	n/a	HQ380504	n/a
N. aff. *cristata* (robust form)	KGK0458	Great Lake, Tasmania, Australia	n/a	n/a	HQ380505	n/a
N. tricellularis (Nordst.) Nordst.	KGK0181	Lake Wanaka, South Island, New Zealand	HQ380431	n/a	HQ380506	n/a

Table 1 *(cont'd)*

CHAROPHYTES	*Strain*	*Locality*	*ITS1*	*ITS2*	*rbcL*	*UPA*
N. tricellularis (Nordst.) Nordst.	KGK0182	Lake Ellery, South Island, New Zealand	HQ380432	HQ380654	HQ380507	n/a
N. tricellularis (Nordst.) Nordst.	KGK0398	Lake Te Anau, South Island, New Zealand	HQ380433	HQ380655	HQ380508	n/a
N. tricellularis (Nordst.) Nordst.	KGK0399	Lake Pearson, South Island, New Zealand	n/a	HQ380656	HQ380509	n/a
ZYGNEMATOPHYCEAE						
Desmidium aptogonum Bréb. *ex* Ralfs	JH0042	Lake Artemesia, Prince Georges Co., MD, USA	HQ380385	n/a	EF371297	HQ380605
D. aptogonum Bréb. ex Ralfs	JH0112	Jyme Lake, Oneida Co., WI, USA	n/a	n/a	HQ380510	HQ380606
D. aptogonum Bréb. ex Ralfs	JH0121	Bug Lake, Oneida Co., WI, USA	HQ380386	n/a	HQ380511	n/a
D. aptogonum Bréb. ex Ralfs	JH0136	Hemlock Lake, Oneida Co., WI, USA	HQ380387	n/a	HQ380512	n/a
D. aptogonum Bréb. ex Ralfs	JH0195	Oxbow Lake, Iron Co., WI, USA	HQ380388	n/a	HQ380513	HQ380607
D. aptogonum Bréb. ex Ralfs	JH0328	Caroline Co., MD, USA	n/a	n/a	HQ380514	HQ380608
D. aptogonum Bréb. ex Ralfs	JH0385	Caroline Co., MD, USA	n/a	n/a	HQ380515	HQ380609
D. aptogonum Bréb. ex Ralfs	JH0387	Caroline Co., MD, USA	HQ380389	n/a	HQ380516	HQ380610
D. aptogonum Bréb. ex Ralfs	JH0571	Twin Lake, Bronx Co., NY, USA	HQ380390	n/a	HQ380517	HQ380611
D. aptogonum Bréb. ex Ralfs	JH0572	Twin Lake, Bronx Co., NY, USA	HQ380391	n/a	HQ380518	HQ380612
D. aptogonum Bréb. ex Ralfs	SVCK 108	Finnland	HQ380384	n/a	EF463091.1	HQ380604
D. aptogonum var. *ehrenbergii* Kütz.	JH0184	Lake Tomohawk, Oneida Co., WI, USA	HQ380392	n/a	EF371298	n/a
D. aptogonum var. *ehrenbergii* Kütz.	JH0188	Lake Tomohawk, Oneida Co., WI, USA	HQ380393	n/a	HQ380519	n/a
D. baileyi (Ralfs) Nordst.	JH0155	Bird Lake Road Bog, Oneida Co., WI, USA	n/a	n/a	HQ380526	n/a
D. baileyi (Ralfs) Nordst.	JH0223	Spencer Lake, Oneida Co., WI, USA	n/a	n/a	HQ380527	n/a
D. baileyi (Ralfs) Nordst.	JH0228	Spencer Lake, Oneida Co., WI, USA	HQ380400	n/a	EF371299	n/a
D. baileyi (Ralfs) Nordst.	JH0274	Spencer Lake Bog, Oneida Co., WI, USA	n/a	n/a	HQ380528	n/a
D. pseudostreptonema W.West *et* G. S. West	JH0482	Thailand	HQ380394	n/a	HQ380520	HQ380613
D. pseudostreptonema W.West *et* G. S. West	JH0495	Thailand	HQ380395	n/a	HQ380521	HQ380614
D. pseudostreptonema W.West *et* G. S. West	JH0513	Sorin, Thailand	HQ380396	n/a	HQ380522	HQ380615

Table 1 *(cont'd)*

CHAROPHYTES	*Strain*	*Locality*	*ITS1*	*ITS2*	*rbcL*	*UPA*
D. swartzii Ralfs	JH0122	Bug Lake, Oneida Co., WI, USA	HQ380397	n/a	HQ380523	n/a
D. swartzii Ralfs	JH0150	Bird Lake Road Bog, Oneida Co., WI, USA	HQ380398	n/a	HQ380524	n/a
D. swartzii Ralfs	JH0231	Swamp at Raven Trail, Oneida Co., WI, USA	HQ380399	n/a	HQ380525	n/a
Micrasterias apiculata Meneghini ex Ralfs	JH0067E	Pond along Benson Rd., Wake Co., NC, USA	HQ380407	n/a	HQ380537	HQ380589
M. apiculata Meneghini ex Ralfs	JH0200	Oxbow Lake, Iron Co., WI, USA	HQ380409	n/a	HQ380539	HQ380591
M. apiculata var. *fimbriata* (Ralfs) W.West *et* G. S. West	JH0105	Jyme Lake, Oneida Co., WI, USA	HQ380408	n/a	HQ380538	HQ380590
M. apiculata var. *fimbriata* (Ralfs) W.West *et* G. S. West	JH0024	Lake Tomohawk, Oneida Co., WI, USA	n/a	n/a	HQ380540	n/a
M. depauperata var. *wollei* J. Cushman	JH0260	Spencer Lake Bog, Oneida Co., WI, USA	HQ380420	n/a	HQ380553	HQ380598
M. depauperata var. *wollei* J. Cushman	JH0364	Spencer Lake, Oneida Co., WI, USA	HQ380421	n/a	HQ380554	HQ380599
M. foliacea Bailey *ex* Ralfs	JH0584	Lake Kanawauke (Lower), Orange Co., NY, USA	n/a	n/a	HQ380529	n/a
M. foliacea Bailey *ex* Ralfs	NIES 297	Higashihiroshima, Japan	n/a	n/a	EF371311	n/a
M. furcata Agardh ex Ralfs Bailey *ex* Ralfs	JH0020	Jyme Lake, Oneida Co., WI, USA	HQ380415	n/a	HQ380549	HQ380597
M. furcata Agardh ex Ralfs Bailey *ex* Ralfs	JH0108	Jyme Lake, Oneida Co., WI, USA	HQ380417	n/a	HQ380550	n/a
M. furcata Agardh ex Ralfs Bailey *ex* Ralfs	JH0134	Hemlock Lake, Oneida Co., WI, USA	HQ380418	n/a	HQ380551	n/a
M. furcata Agardh ex Ralfs Bailey *ex* Ralfs	JH0226	Spencer Lake, Oneida Co., WI, USA	HQ380419	n/a	HQ380552	n/a
M. furcata var. *dichotoma* (Wolle) Grönblad	JH0064	Elk Pond, Washington Co., OH, USA	HQ380416	n/a	EF371313	n/a
M. laticeps Nordst.	JH0239	Ink Pot Lake, Oneida Co., WI, USA	n/a	n/a	HQ380548	HQ380596
M. mahabuleshwarensis J. Hobson	JH0481	Thailand	HQ380402	n/a	HQ380532	n/a
M. muricata (Bailey) Ralfs	JH0119	Bug Lake, Oneida Co., WI, USA	HQ380403	n/a	HQ380533	n/a
M. muricata (Bailey) Ralfs	JH0120	Bug Lake, Oneida Co., WI, USA	HQ380404	n/a	HQ380534	n/a
M. muricata (Bailey) Ralfs	JH0179	Bird Lake Road Bog, Oneida Co., WI, USA	HQ380405	n/a	HQ380535	n/a
M. nordstedtiana Wolle	JH0564	Lake Te Ata, Orange Co., NY, USA	HQ380406	n/a	HQ380536	n/a
M. pinnatifida (Kützing) Ralfs	JH0106	Jyme Lake, Oneida Co., WI, USA	HQ380411	n/a	HQ380543	HQ380592
M. pinnatifida (Kützing) Ralfs	JH0227	Spencer Lake, Oneida Co., WI, USA	HQ380412	n/a	HQ380544	HQ380593
M. pinnatifida (Kützing) Ralfs	JH0494	Thailand	n/a	n/a	HQ380545	HQ380594

Table 1 *(cont'd)*

CHAROPHYTES	*Strain*	*Locality*	*ITS1*	*ITS2*	*rbcL*	*UPA*
M. pinnatifida (Kützing) Ralfs	JH0509	Sorin, Thailand	HQ380413	n/a	HQ380546	HQ380595
M. pinnatifida (Kützing) Ralfs	JH0512	Sorin, Thailand	HQ380414	n/a	HQ380547	n/a
M. radiosa Agardh *ex* Ralfs	JH0369	Spencer Lake, Oneida Co., WI, USA	n/a	n/a	HQ380530	HQ380588
M. radiosa Agardh *ex* Ralfs	JH0372	Spencer Lake Bog, Oneida Co., WI, USA	n/a	n/a	HQ380531	n/a
M. rotata (Greville) Ralfs	JH0095	Pond along Benson Rd., Wake Co., NC, USA	n/a	n/a	HQ380541	n/a
M. rotata (Greville) Ralfs	JH0128	Lake Tomohawk, Oneida Co., WI, USA	n/a	n/a	HQ380542	n/a
M. rotata (Greville) Ralfs	UTEX1941	BC, Canada	n/a	n/a	EF371312	n/a
M. truncata (Corda) Bréb. *ex* Ralfs	JH0017	Jyme Lake, Oneida Co., WI, USA	n/a	n/a	HQ380555	HQ380600
M. truncata (Corda) Bréb. *ex* Ralfs	JH0103	Jyme Lake, Oneida Co., WI, USA	HQ380422	n/a	HQ380556	HQ380601
M. truncata (Corda) Bréb. *ex* Ralfs	JH0141	Hemlock Lake, Oneida Co., WI, USA	HQ380423	n/a	HQ380557	n/a
M. truncata (Corda) Bréb. *ex* Ralfs	JH0242	Smith Fen, Cheboygan Co., MI, USA	HQ380424	n/a	HQ380558	HQ380602
M. truncata (Corda) Bréb. *ex* Ralfs	JH0258	Spencer Lake Bog, Oneida Co., WI, USA	HQ380425	n/a	n/a	HQ380603
M. truncata (Corda) Bréb. *ex* Ralfs	JH0642	Pico de Oeste, Puerto Rico	n/a	n/a	HQ380559	n/a

Note: Detailed locality information for BCP isolates at http://hydrodictyon.eeb.uconn.edu/bcp/

Table 1 *(cont'd)*

CHLOROPHYTES	*Strain*	*Locality*	*cox1*	*ITS1-ITS2*	*rbcL*	*UPA*	*tufA*	*18S*
CHLOROPHYCEAE								
Bracteacoccus cohaerens Bischoff *et* Bold	UTEX 1272	Dripping Springs, TX, USA	HQ246328	HQ246421	GQ985402	HQ246414	HQ246382	GQ985406
B. cohaerens Bischoff *et* Bold	CAUP H3802	»eské Středohoří, Czech Republic	HQ246329	HQ246422	HQ246362	HQ246415	HQ246383	HQ246323
B. cohaerens Bischoff *et* Bold	SAG SO5-1	Soebatsfontain, South Africa	HQ246330	HQ246423	HQ246363	HQ246416	HQ246384	HQ246325
B. pseudominor Bischoff *et* Bold	UTEX 1247	Hudspeth County, TX, USA	n/a	HQ246424	GQ985397	HQ246417	HQ246385	GQ985405
B. pseudominor Bischoff *et* Bold	BCP- WJT71-VFNP15	Joshua Tree National park, Riverside County, CA, USA	n/a	HQ246425	HQ246364	HQ246418	HQ246386	HQ246324
B. pseudominor Bischoff *et* Bold	SAG 2272	Otjiamongombe, Namibia	n/a	HQ246426	HQ246365	HQ246419	HQ246387	HQ246326
B. giganteus Bischoff *et* Bold	UTEX 1251	Enchanted Rock, TX, USA	HQ246331	HQ246427	EF113414	HQ246420	HQ246388	U63099
B. giganteus Bischoff *et* Bold	ATA 1-4-CV4	Atacama Desert, Chile	HQ246332	HQ246428	HQ246366	n/a	HQ246389	HQ246327

Table 1 *(cont'd)*

CHLOROPHYTES	*Strain*	*Locality*	*cox1*	*ITS1-ITS2*	*rbcL*	*UPA*	*tufA*	*18S*
Chlorosarcinopsis sp. A	ZA1-1	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	HQ246429	HQ246333	HQ246390	n/a	HQ246304
Chlorosarcinopsis sp. A	ZA1-8	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	HQ246430	HQ246334	n/a	n/a	HQ246305
Chlorosarcinopsis sp. A	ZA1-10	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	HQ246431	HQ246335	HQ246391	HQ246367	HQ246306
Chlorosarcinopsis sp. A	ZA2-1	3.75 K N of Vanrhynsdorp along R27, South Africa (S 31° 34.778', E 18° 47.228')	n/a	HQ246432	HQ246336	HQ246392	n/a	HQ246307
Chlorosarcinopsis sp. A	ZA3-2	30 K S of Newerus along N7, South Africa (S 31°18.084', E 18° 34.472')	n/a	HQ246433	HQ246337	HQ246393	n/a	HQ246308
Chlorosarcinopsis sp. A	ZA3-3	30 K S of Newerus along N7, South Africa (S 31°18.084', E 18° 34.472')	n/a	HQ246434	HQ246338	HQ246394	n/a	HQ246309
Chlorosarcinopsis sp. A	ZA6-1	5.25 K S of Nuy along R60, South Africa (S 33° 45.149', E 19° 43.327')	n/a	HQ246435	HQ246339	HQ246395	HQ246368	HQ246310
Chlorosarcinopsis sp. A	ZA6-2	5.25 K S of Nuy along R60, South Africa (S 33° 45.149', E 19° 43.327')	n/a	HQ246436	HQ246340	HQ246396	n/a	HQ246311
Chlorosarcinopsis sp. A	BCP- EM1-VF1	Mojave National Preserve, San Bernardino County, CA, USA	n/a	HQ246437	HQ246341	n/a	n/a	AY271673
Chlorosarcinopsis eremi Chantanachat *et* Bold	UTEX 1186	Phoenix, AZ, USA	n/a	HQ246438	HQ246342	HQ246397	HQ246369	AB218706
C. eremi Chantanachat *et* Bold	BCP- SEV3-VF14	Sevilleta LTER, Socorro Co., NM, USA	n/a	HQ246439	HQ246343	HQ246398	n/a	AF513371
C. eremi Chantanachat *et* Bold	BCP- SEV2-VF1	Sevilleta LTER, Socorro Co., NM, USA	n/a	HQ246440	HQ246344	HQ246399	HQ246370	AF516678
C. eremi Chantanachat *et* Bold	BCP-JT1-VF20	Joshua Tree National Park, Pinto Basin, Riverside County, CA, USA	n/a	HQ246441	HQ246345	HQ246400	n/a	HQ246312

Table 1 *(cont'd)*

CHLOROPHYTES	*Strain*	*Locality*	*cox1*	*ITS1-ITS2*	*rbcL*	*UPA*	*tufA*	*18S*
C. eremi Chantanachat *et* Bold	BCP- FI5L-VF16	Fort Irwin, CA, USA	n/a	HQ246442	HQ246346	HQ246401	n/a	HQ246313
C. eremi Chantanachat *et* Bold	BCP- JO1-VF2	Jornada Experimental Range, Dona Ana Co., NM, USA	n/a	HQ246443	HQ246347	HQ246402	n/a	HQ246314
C. eremi Chantanachat *et* Bold	BCP- JT1-VF8	Joshua Tree National Park, Pinto Basin, Riverside County, CA, USA	n/a	HQ246444	HQ246348	HQ246403	n/a	HQ246315
C. eremi Chantanachat *et* Bold	BCP- JT1-VF80	Joshua Tree National Park, Pinto Basin, Riverside County, CA, USA	n/a	HQ246445	HQ246349	HQ246404	n/a	HQ246316
Scenedesmus rotundus H. C. Wood	BCP- SEV3-VF49	Sevilleta LTER, Socorro Co., NM, USA	n/a	AY510465	HQ246350	HQ246405	HQ246371	AF513373
S. rotundus H. C. Wood	BCP- SEV3-VF4	Sevilleta LTER, Socorro Co., NM, USA	n/a	HQ246446	HQ246351	HQ246406	HQ246372	HQ246317
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- LG2-VF16	Sierra San Pedro Martir of Baja California, Mexico	n/a	AY510468	HQ246352	n/a	HQ246373	AF513372
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- LG2-VF34	Sierra San Pedro Martir of Baja California, Mexico	n/a	AY510467	n/a	n/a	n/a	AY510458
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- MX7-VF7	Baja California, Mexico.	n/a	AY510469	n/a	n/a	n/a	AY510459
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- MX15-VF11	Baja California, Mexico.	n/a	HQ246447	HQ246353	n/a	n/a	HQ246318
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	ZA1-2	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	HQ246448	HQ246354	HQ246407	HQ246374	HQ246319
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	ZA1-4	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	n/a	HQ246355	HQ246408	n/a	HQ246320
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	ZA1-5	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	HQ246449	HQ246356	HQ246409	HQ246375	HQ246321
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	ZA1-7	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	HQ246450	HQ246357	n/a	HQ246376	HQ246322
S. deserticola L.A. Lewis *et* V.R. Flechtner	BCP- EM2-VF30	Mojave National Preserve, San Bernardino County, CA, USA	n/a	AY510470	HQ246358	HQ246410	HQ246377	AY510460
S. deserticola L.A. Lewis & V.R. Flechtner	BCP-EM2-VF3	Mojave National Preserve, San Bernardino County, CA, USA	n/a	AY510474	HQ246359	HQ246411	HQ246378	AY410461

Table 1 *(cont'd)*

CHLOROPHYTES	*Strain*	*Locality*	*cox1*	*ITS1-ITS2*	*rbcL*	*UPA*	*tufA*	*18S*
S. deserticola L.A. Lewis & V.R. Flechtner	BCP-SNI-2	San Nicholas Island, CA, USA	n/a	AY510471	HQ246360	HQ246412	HQ246379	AY510462
C. eremi Chantanachat *et* Bold	BCP- SEV3-VF14	Sevilleta LTER, Socorro Co., NM, USA	n/a	HQ246439	HQ246343	HQ246398	n/a	AF513371
C. eremi Chantanachat *et* Bold	BCP- SEV2-VF1	Sevilleta LTER, Socorro Co., NM, USA	n/a	HQ246440	HQ246344	HQ246399	HQ246370	AF516678
C. eremi Chantanachat *et* Bold	BCP-JT1-VF20	Joshua Tree National Park, Pinto Basin, Riverside County, CA, USA	n/a	HQ246441	HQ246345	HQ246400	n/a	HQ246312
C. eremi Chantanachat *et* Bold	BCP- FI5L-VF16	Fort Irwin, CA, USA	n/a	HQ246442	HQ246346	HQ246401	n/a	HQ246313
C. eremi Chantanachat *et* Bold	BCP-JO1-VF2	Jornada Experimental Range, Dona Ana Co., NM, USA	n/a	HQ246443	HQ246347	HQ246402	n/a	HQ246314
C. eremi Chantanachat *et* Bold	BCP- JT1-VF8	Joshua Tree National Park, Pinto Basin, Riverside County, CA, USA	n/a	HQ246444	HQ246348	HQ246403	n/a	HQ246315
C. eremi Chantanachat *et* Bold	BCP- JT1-VF80	Joshua Tree National Park, Pinto Basin, Riverside County, CA, USA	n/a	HQ246445	HQ246349	HQ246404	n/a	HQ246316
Scenedesmus rotundus H. C. Wood	BCP- SEV3-VF49	Sevilleta LTER, Socorro Co., NM, USA	n/a	AY510465	HQ246350	HQ246405	HQ246371	AF513373
S. rotundus H. C. Wood	BCP- SEV3-VF4	Sevilleta LTER, Socorro Co., NM, USA	n/a	HQ246446	HQ246351	HQ246406	HQ246372	HQ246317
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- LG2-VF16	Sierra San Pedro Martir of Baja California, Mexico	n/a	AY510468	HQ246352	n/a	HQ246373	AF513372
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- LG2-VF34	Sierra San Pedro Martir of Baja California, Mexico	n/a	AY510467	n/a	n/a	n/a	AY510458
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- MX7-VF7	Baja California, Mexico.	n/a	AY510469	n/a	n/a	n/a	AY510459
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	BCP- MX15-VF11	Baja California, Mexico.	n/a	HQ246447	HQ246353	n/a	n/a	HQ246318
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	ZA1-2	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	HQ246448	HQ246354	HQ246407	HQ246374	HQ246319
S. bajacalifornicus L.A. Lewis *et* V.R. Flechtner	ZA1-4	6 K N of Klawer along N7, South Africa (S 31°44.163', E 18° 39.660')	n/a	n/a	HQ246355	HQ246408	n/a	HQ246320

1983). Some distantly related species with cylindrical cells that were historically included in *Desmidium* (e.g. *Didymoprium grevillei* Ralfs) were excluded (Gontcharov *et al.,* 2003; Hall *et al.,* 2008b).

Micrasterias – Species of the genus *Micrasterias* (Zygnematophyceae) are among the most recognizable microscopic green algae. Their cells are compressed with a deep medial incision that divides the cell into two halves (semicells) each of which is lobed and often dissected. Fourteen taxa in twelve species were sampled (Table 1). Although both *M. apiculata* var. *fimbriata* and *M. furcata* var. *dichotoma* have been considered as species distinct from the nominal variety by some authors (Ralfs, 1848; Wolle, 1884), they were treated as conspecific with their nominal varieties in our analyses. In general, species of *Micrasterias* are easily recognized, however there is considerable variation within taxonomic characters and occasionally cells are encountered that are difficult to identify. In this study, strain JH0024 is structurally similar to *M. apiculata* var. *fimbriata*, however it is phylogenetically related to *M. rotata* and was treated as conspecific with the latter species when calculating distances. *Micrasterias muricata* (Fig. 4) and *M. nordstedtiana* are very closely related (Hall, unpublished) and yet morphologically distinct and easily recognized. They serve as a test case for distinguishing closely related species using molecular barcodes.

Bracteacoccus – The spherical cells of *Bracteacoccus* (Chlorophyceae), when observed at maturity, possess multiple plate-like parietal plastids and multiple nuclei, lack pyrenoids and occasionally reproduce by formation of zoospores with two flagella of slightly unequal length. All known representatives of *Bracteacoccus* are terrestrial and are found in soils worldwide, ranging from polar to tropical habitats. The genus contains fourteen described species and two subspecific taxa, although some of these taxa are now being transferred to other genera (Fučíková *et al.*, unpub.). Within *Bracteacoccus*, subtle morphological differences found in zoospores are used to distinguish among species (Bischoff & Bold, 1963). *Bracteacoccus cohaerens* (Fig. 5), *B. giganteus*, and *B. pseudominor* are impossible to tell apart without observing the full life cycle, particularly zoospores, which often are not readily produced. As a result, morphological characterization can be difficult.

Chlorosarcinopsis eremi species complex – The genus *Chlorosarcinopsis* (Chlorophyceae) commonly forms packets of 2-8 cells and it is found in soils or damp locations. Watanabe *et al.* (2006) demonstrated the non-monophyly of this genus. Several species, including *Chlorosarcinopsis eremi* (Fig. 6), were shown to be outside the phylogenetic lineage containing the type species of *Chlorosarcinopsis*. A new genus has not yet been designated for *C. eremi*. During a broad scale survey of green algae from desert soils several strains were isolated that are phylogenetically related to *C. eremi*. To determine if these strains represent one or more species, a phylogenetic study using several different barcode markers was conducted (Lewis *et al.,* unpublished). Eight isolates of *C. eremi* from five different locations in arid western North America were included. Nine isolates were also included of a related yet unnamed species, *Chlorosarcinopsis* sp. A, collected from four locations in South Africa and one location in North America (localities at Biotic Crust Project, http://hydrodictyon.eeb.uconn.edu/bcp/). *Chlorosarcinopsis eremi* and *Chlorosarcinopsis* sp. A are not distinguishable using light microscopy yet are always monophyletic and distinct in phylogenetic analyses.

Scenedesmus – Best known for its numerous planktonic species (Prescott, 1962; Hegewald & Silva, 1988), the freshwater genus *Scenedesmus* (Chlorophyceae) also contains three newly described yet cryptic species, *S. rotundus* (Fig. 7), *S. deserticola* (Fig. 8), *S. bajacalifornicus*, from western North American

desert soils designated based on divergent ITS2 data (Lewis & Flechtner, 2004). This study included additional isolates from different geographic locations, in order to improve the estimation of within and among species variation for these species.

DNA extraction and amplification

DNA was extracted using previously described methods (Hall *et al.*, 2008a) or using the Qiagen DNEasy Plant Extraction kit (Qiagen Inc., Valencia, CA, USA). When possible, published universal primers were tested. If these were unsuccessful, published lineage-specific primers were used or new primers were designed from the available published data (Table 2). Several different primer pairs and amplification procedures were optimized to maximize DNA amplification. Fragments were amplified using a single round of PCR with the exception of *rbcL* in Charophyceae in which the product of the first round of amplification was used as the template for a second round of amplification using Charophyceae-specific primers. This was done to minimize contamination from epiphytes. The most successful primers and parameter sets are presented in Table 3. Both *rbcL* and 18S required internal sequencing primers to obtain full-length sequences. These gene fragments were sequenced at the University of Washington Genome

Table 2. Primers

Primer	*Sequence*	*Reference*
GazF1	TCAACAAATCATAAAGATATTGG	Saunders, 2005
GazR1	ACTTCTGGATGTCCAAAAAAYCA	Saunders, 2005
cox1.50F	TGGTCTGGTGTWATWGCTAC	NEW
cox1.650R	TCACCWCCACCWGCWGGC	NEW
ITS 36F	GGCGCTGTGAGAAGTTCATTGAACC	NEW
ITS IR	TTCTGCAATTCACACTACGTATCGC	NEW
ITS IF	CAACTCTCGGCAAACGGATATCTTG	NEW
ITS R	GGTTGGTCCCGACCGATCTGAGGTC	NEW
ITS1	AGGAGAAGTCGTAACAAGGT	White *et al.*, 1990 (modified)
ITS4	TCCTCCGCTTATTGATATGC	White *et al.*, 1990
rbcL Z1	ATGTCACCACAAACAGAAACTAAAGCAAGT	McCourt *et al.*, 2000
rbcL RH1	ATGTCACCACAAACAGAAACTAAAGC	McCourt *et al.*, 2000
rbcL 1385	AATTCAAATTTAATTTCTTTCC	McCourt *et al.*, 2000
rbcL 295F	GCATATGTTGCTTATCCTCTT	NEW
rbcL 972R	ATCACCACCAGAAAGACGAAG	NEW
rbcL M28	GGTGTTGGATTTAAAGCTGGTGT	McManus & Lewis (accepted)
rbcL M1390R	CTTTCAAAYTTCACAAGCAGCAG	McManus & Lewis (accepted)
tufA F	TGAAACAGAAMAWCGTCATTATGC	Fama *et al.*, 2002
tufA R	CCTTCNCGAATMGCRAAWCGC	Fama *et al.*, 2003
tufA.50F	TGGATGGTGCTATTYTAGTTG	NEW
tufA.870R	ATAGTGTCRCCTGGCATAGC	NEW
p23SrV_f1	GGACAGAAAGACCCTATGAA	Sherwood & Presting, 2006
p23SrV_r1	TCAGCCTGTTATCCCTAGAG	Sherwood & Presting, 2006
SSU1	TGGTTGATCCTGCCAGTAG	Shoup & Lewis, 2003
SSU2	TGATCCTTCCGCAGGTTCAC	Shoup & Lewis, 2003

Table 3. Parameters used for PCR

cox1	**Forward**	**Reverse**	**Ta C**	**tS**	**Note**
Bracteacoccus	cox1.50F	cox1.650R	54	120	fresh DNA needed
Chlorosarcinopsis	GazF1	GazR1	54	120	
Scenedesmus	GazF1	GazR1	54	120	
ITS1	**Forward**	**Reverse**	**Ta C**	**tS**	**Note**
Chara, Nitella	ITS 36F*	ITS IR	55	45	DMSO
Desmidium, Micrasterias	ITS 36F*	ITS IR	55	45	DMSO
ITS2	**Forward**	**Reverse**	**Ta C**	**tS**	**Note**
Bracteacoccus	ITS1	ITS4	52	105	DMSO
Chara, Nitella	ITS IF	ITS R	55	45	DMSO
Chlorosarcinopsis	ITS1	ITS4	52	105	DMSO
Desmidium, Micrasterias	ITS IF	ITS R	55	45	DMSO
Scenedesmus	ITS1	ITS4	52	105	DMSO
rbcL	**Forward**	**Reverse**	**Ta C**	**tS**	
Bracteacoccus	rbcL M28	rbcL M1390	56	135	
Chara, Nitella	RH1	1385R	48	45	
	RH1	972R	48	30	
	295F	1385R	48	30	
Chlorosarcinopsis	rbcL M28	rbcL M1390	56	135	
Desmidium, Micrasterias	Z1	1385R	48	45	
Scenedesmus	rbcL M28	rbcL M1390	56	135	
tufA	**Forward**	**Reverse**	**Ta C**	**tS**	
Bracteacoccus	tufAF	tufAR	54	135	
	tufA.50F	TufA.870R	54	135	
Chlorosarcinopsis	tufAF	tufAR	54	135	
	tufA.50F	tufAR	54	135	
Scenedesmus	tufAF	tufAR	54	135	
UPA	**Forward**	**Reverse**	**Ta C**	**tS**	**Note**
Bracteacoccus	p23SrV_f1	p23SrV_r1	50	45	DMSO
Chara, Nitella	p23SrV_f1	p23SrV_r1	50	45	
Chlorosarcinopsis	p23SrV_f1	p23SrV_r1	50	45	DMSO
Desmidium, Micrasterias	p23SrV_f1	p23SrV_r1	50	45	
Scenedesmus	p23SrV_f1	p23SrV_r1	50	45	DMSO
18S	**Forward**	**Reverse**	**Ta C**	**tS**	**Note**
Bracteacoccus	SSU1	SSU2	54	135	DMSO
Chlorosarcinopsis	SSU1	SSU2	54	135	DMSO
Scenedesmus	SSU1	SSU2	54	135	DMSO

Notes: Forward, forward primer; Reverse, reverse primer; Ta C, annealing temperature in degrees Centigrade; tS, extension time in seconds. DMSO indicates that DMSO was added to the PCR mix.
* This primer site lies inside the 18S rDNA and amplifies an intron known to occur in the 18S rDNA of many Zygnematophyceae (Besendahl & Bhattacharya, 1999).

Center (Seattle, WA) or on an ABI 3100 or 3130xl in the EEB Department at the University of Connecticut (Storrs, CT). The reads were assembled and edited in Sequencher v. 4.9 (GeneCodes, Ann Arbor, MI). Sequences for each genus were aligned using ClustalX (Larkin *et al.*, 2007) and the alignments manually edited in MacClade 4.06 (Maddison & Maddison, 2000). Alignments are available in TreeBASE (www.treebase.org) submission number S11032. Intra- and interspecific distances (using Kimura 2-Parameter distances) were calculated for each species group in PAUP* 4 (Swofford, 2003) or Mesquite v. 2.5 (Maddison & Maddison, 2008).

RESULTS

Five different loci were tested for their use as DNA barcodes in seven species groups of freshwater green algae. Of these, COI, ITS and UPA are the loci most frequently proposed as barcodes for algae. For charophytes, primers were designed for the entire COI region and for the two largest exons based on available mitochondrial genome data (Turmel *et al.*, 2003). In spite of several attempts, different primer combinations and various PCR conditions, all attempts to amplify the COI gene from charophytes were unsuccessful. Fragments of the COI gene were successfully amplified from *Bracteacoccus*, but there were no successful amplifications of *Chlorosarcinopsis* or *Scenedesmus* using either a set of primers designed for red algae or a set of more taxon-specific primers (Table 2). In *Bracteacoccus*, the COI region was the most variable among species of all loci studied although ITS2 had similar levels of variation (Table 4, Fig. 9).

Portions of the ITS region were successfully sequenced from all groups although results varied among taxa. Within charophytes, ITS1 amplified from *Desmidium* and *Micrasterias* while ITS2 was more easily amplified from *Chara* and *Nitella*. In *Desmidium* and *Micrasterias* considerable length variation was found in ITS1 sequences. In *Desmidium*, large portions of the alignment were excluded because of uncertain homology. In *Micrasterias*, the ITS1 sequences were determined to be too variable to be confidently aligned across the genus. Sequences were aligned by species and intraspecific distances were calculated.

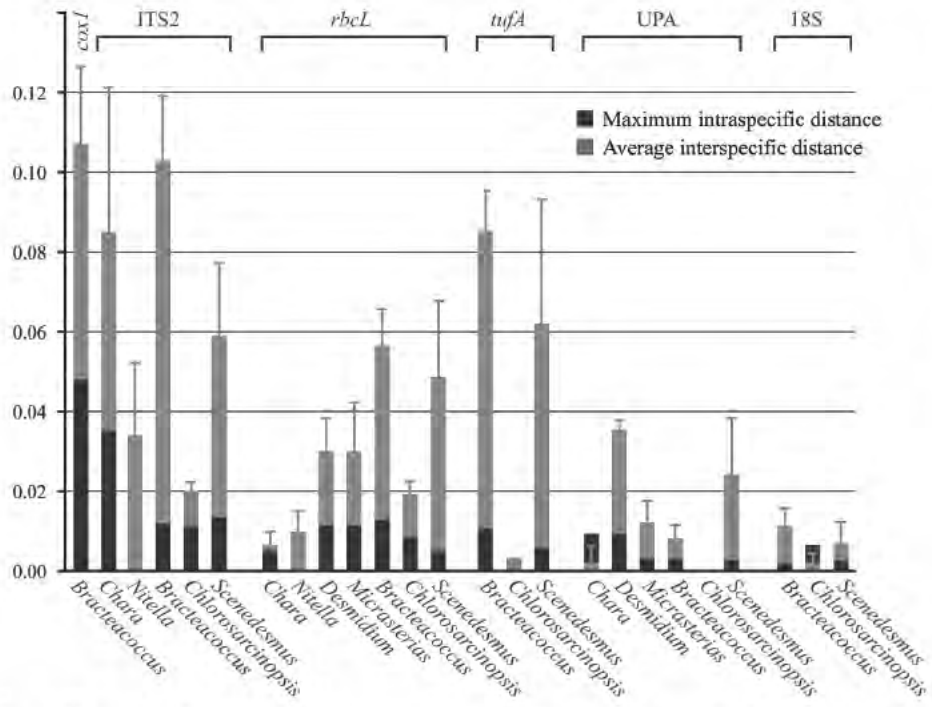


Fig. 9. Histogram showing the maximum intraspecific Kimura 2-Parameter distance (black bars) and average interspecific distance (gray bars) for each marker by species group. Error bars on the average interspecific distance represent one standard deviation.

Table 4. Comparison of barcode marker characteristics in freshwater green algae

COI	**n**	**PRIMER**	**SEQ**	**NUC**	**MAX**	**MIN**	**INTRA**	**INTER**
Chara	40 (8)	TS	0	n/a	n/a	n/a	n/a	n/a
Nitella	30 (9)	TS	0	n/a	n/a	n/a	n/a	n/a
Desmidium	23 (4)	TS	0	n/a	n/a	n/a	n/a	n/a
Micrasterias	38 (12)	TS	0	n/a	n/a	n/a	n/a	n/a
Bracteacoccus	8 (3)	TS	5	588	0.0478	0.0874	0.0163 ± 0.021	0.1070 ± 0.019
Chlorosarcinopsis	7 (2)	TS	0	n/a	n/a	n/a	n/a	n/a
Scenedesmus	5 (2)	TS	0	n/a	n/a	n/a	n/a	n/a
ITS1	**n**	**PRIMER**	**SEQ**	**NUC**	**MAX**	**MIN**	**INTRA**	**INTER**
Chara	40 (8)	UNIV, TS	n/a	n/a	n/a	n/a	n/a	n/a
Nitella	30 (9)	UNIV, TS	8	381	0.0000	0.0000	0.0000 ± 0.000	0.0120 ± 0.018
Desmidium	23 (4)	UNIV	17	342	0.0643	0.2228	0.0339 ± 0.018	0.3399 ± 0.083
Micrasterias	38 (12)	UNIV	23	486	0.0821	n/a	0.0311 ± 0.033	n/a
Bracteacoccus	8 (3)	UNIV	8	312	0.0812	n/a	0.0565 ± 0.029	n/a
Chlorosarcinopsis	17 (2)	UNIV	17	255	0.0206	0.0159	0.0056 ± 0.005	0.0240 ± 0.005
Scenedesmus	14 (3)	UNIV	14	184	0.0165	0.0692	0.0055 ± 0.005	0.1778 ± 0.125
ITS2	**n**	**PRIMER**	**SEQ**	**NUC**	**MAX**	**MIN**	**INTRA**	**INTER**
Chara	40 (8)	UNIV, TS	20	354	0.0352	0.0232	0.0132 ± 0.011	0.0848 ± 0.036
Nitella	30 (9)	UNIV, TS	22	207	0.0000	0.0000	0.0000 ± 0.000	0.0338 ± 0.018
Desmidium	23 (4)	UNIV	n/a	n/a	n/a	n/a	n/a	n/a
Micrasterias	38 (12)	UNIV	n/a	n/a	n/a	n/a	n/a	n/a
Bracteacoccus	8 (3)	UNIV	8	413	0.0117	0.1319	0.0040 ± 0.002	0.1026 ± 0.016
Chlorosarcinopsis	17 (2)	UNIV	17	419	0.0112	0.0174	0.0021 ± 0.002	0.0200 ± 0.002
Scenedesmus	14 (3)	UNIV	14	380	0.0133	0.0381	0.0046 ± 0.004	0.0588 ± 0.018
rbcL	**n**	**PRIMER**	**SEQ**	**NUC**	**MAX**	**MIN**	**INTRA**	**INTER**
Chara	40 (8)	TS	40	1353	0.0052	0.0022	0.0006 ± 0.001	0.0065 ± 0.003
Nitella	30 (9)	TS	28	1353	0.0000	0.0000	0.0000 ± 0.000	0.0097 ± 0.005
Desmidium	23 (4)	TS	23	1239	0.0114	0.0172	0.0055 ± 0.004	0.0299 ± 0.008
Micrasterias	38 (12)	TS	34	1239	0.0114	0.0000	0.0024 ± 0.003	0.0296 ± 0.012
Bracteacoccus	8 (3)	UNIV, TS	8	1217	0.0127	0.0437	0.0078 ± 0.003	0.0563 ± 0.009
Chlorosarcinopsis	17 (2)	UNIV, TS	17	1258	0.0084	0.0139	0.0025 ± 0.003	0.0192 ± 0.003
Scenedesmus	13 (3)	UNIV, TS	12	1324	0.0048	0.0287	0.0017 ± 0.002	0.0483 ± 0.019
tufA	**n**	**PRIMER**	**SEQ**	**NUC**	**MAX**	**MIN**	**INTRA**	**INTER**
Bracteacoccus	8 (3)	UNIV, TS	8	670	0.0105	0.0674	0.0067 ± 0.004	0.0851 ± 0.010
Chlorosarcinopsis	5 (2)	UNIV, TS	4	631	0.0000	0.0032	0.0000 ± 0.000	0.0032 ± 0.000
Scenedesmus	13 (3)	UNIV	11	890	0.0057	0.0312	0.0015 ± 0.002	0.0619 ± 0.031
UPA	**n**	**PRIMER**	**SEQ**	**NUC**	**MAX**	**MIN**	**INTRA**	**INTER**
Chara	40 (8)	UNIV	22	325	0.0093	0.0000	0.0002 ± 0.001	0.0019 ± 0.004
Nitella	30 (9)	UNIV	n/a	n/a	n/a	n/a	n/a	n/a
Desmidium	23 (4)	UNIV	12	334	0.0091	0.0339	0.0023 ± 0.003	0.0353 ± 0.002
Micrasterias	38 (12)	UNIV	16	328	0.0031	0.0000	0.0006 ± 0.001	0.0121 ± 0.005
Bracteacoccus	7 (3)	UNIV	7	331	0.0030	0.0061	0.0010 ± 0.002	0.0081 ± 0,003
Chlorosarcinopsis	16 (2)	UNIV	15	347	0.0000	0.0000	0.0000 ± 0.000	0.0000 ± 0.000
Scenedesmus	11 (3)	UNIV	9	375	0.0027	0.0081	0.0011 ± 0.001	0.0240 ± 0.014
18S	**n**	**PRIMER**	**SEQ**	**NUC**	**MAX**	**MIN**	**INTRA**	**INTER**
Bracteacoccus	8 (3)	UNIV, TS	8	1708	0.0018	0.0077	0.0006 ± 0.004	0.0113 ± 0.004
Chlorosarcinopsis	17 (2)	UNIV, TS	3	1748	0.0064	0.0006	0.0008 ± 0.001	0.0020 ± 0.002
Scenedesmus	15 (3)	UNIV, TS	15	1684	0.0024	0.0024	0.0008 ± 0.001	0.0069 ± 0.005

Notes: n, number of strains tested, number of species in parentheses; UNIV, universal primers; TS, taxon-specific primers; SEQ, number of sequences obtained; NUC, number of usable nucleotides in the alignment; MAX, highest intraspecific distance; MIN, lowest interspecific distance; INTRA, average intraspecific distance ± 1 standard deviation; INTER, average intraspecific variation ± 1 standard deviation.

Notably, ITS1 data were sufficiently variable to distinguish between *M. muricata* and *M. nordstedtiana* whereas other markers were not. The ITS1 data also revealed high levels of variation in *M. pinnatifida* and *M. truncata*. By contrast, ITS1 was not significantly more variable than other loci in *Nitella*, however, this result is based on relatively few sequences (Table 4). In *Chara* and *Nitella*, the ITS2 marker had average pairwise distances at least twice as great as in any other marker. Even so, ITS2 was not sufficiently variable to distinguish among the closely related species *N. claytonii*, *N. masonae* and *N. tricellularis,* which all shared identical primary sequence (as in all markers tested). Among the chlorophytes, both ITS1 and ITS2 were successfully amplified, often as a single amplicon, and sequenced with universal primers (White *et al.*, 1990), although taxon-specific primers were needed in some instances. The ITS1 region in the three chlorophytic genera contained more variation than the ITS2 region, but it was also more difficult to assess homology of sites in ITS1. This was especially true in *Bracteacoccus*. In *Bracteacoccus*, ITS2 showed less intraspecific variation than either *rbcL* or *tufA* and more interspecific variation. In *Chlorosarcinopsis*, ITS2 provided levels of variation similar to *rbcL* but much greater than *tufA*, although this comparison was based on fewer *tufA* sequences in this genus. In *Scenedesmus*, the ITS2 region showed more intraspecific variation than *rbcL* or *tufA* but less interspecific variation than *tufA*.

The UPA amplified successfully from many strains. However, in charophytes sequences were sometimes of poor quality resulting in short reads or unusable sequence. Only about half of the strains of *Chara*, *Desmidium* and *Micrasterias* were successfully sequenced. In *Nitella*, the universal primers most often amplified non-target regions of the *Nitella* plastid genome (not shown). The UPA primers amplified the target fragment in all *Bracteacoccus* and a majority of the *Chlorosarcinopsis* and *Scenedesmus* isolates. In these taxa the UPA primers provided clean sequence and intraspecific variation was low. However, interspecific distances in *Bracteacoccus* were lower than for any other marker except 18S and there was no variation across all strains of *Chlorosarcinopsis* tested (Table 4).

Taxon-specific primers were designed for *rbcL* and sequencing success rates were very high. This gene fragment was sufficiently variable to resolve all species-level relationships except one species pair that was distinguished by ITS1 (*Micrasterias muricata* and *M. nordstedtiana*) and several species of *Nitella* (*N. claytonii*, *N. hookeri*, *N. masonae* and *N. tricellularis*). In *Nitella* and *Micrasterias*, there was considerable overlap in intra- and interspecific distances (Table 4). Amplification and sequencing of *rbcL* in chlorophytes was nearly always successful using a small number of primers. In two isolates of *Scenedesmus*, introns were found in the *rbcL* gene, making it necessary to design new primers to sequence the exons. Unlike the charophytes, no overlap in intra- and interspecific distances was observed in *rbcL* data in chlorophytes.

The *tufA* gene was generally easily surveyed in chlorophytes using a set of universal primers. For two chlorophytic genera, *Bracteacoccus* and *Scenedesmus*, the degree and pattern of variation observed in *tufA* and *rbcL* data were similar, although *tufA* was slightly more variable among species (Table 4). Fewer *Chlorosarcinopsis* isolates were tested using *tufA* primers, although these sequences yielded very low levels of intra- and interspecific variation.

In chlorophytes, 18S rDNA showed similar patterns to those found in the other markers examined. In *Bracteacoccus*, 18S rDNA performed well as a species-level marker, with interspecific distances three times greater than intraspecific distances, although variation levels overall were considerably lower

than those observed in any of the other examined markers (Fig. 9). Species delimitation based on 18S rDNA was the most problematic in *Chlorosarcinopsis*, where the average divergence within one of the test species, *C. eremi,* exceeded the average interspecific divergence (Fig. 9). In other markers, intraspecific variation within *Chlorosarcinopsis* had high standard error, but mostly due to greater variation within *Chlorosarcinopsis* sp. A (data not shown).

DISCUSSION

Both traditional morphological taxonomy and DNA barcoding focus on identification and classification of species. The two methods are reciprocally illuminating: well-defined morphological species groups are used to test the efficacy of molecular markers (for barcoding or phylogenetics) and often molecular markers reveal unexpected relationships or lead taxonomists to species, strains or specimens requiring more careful examination. This study was designed to test the utility of markers that have been proposed as DNA barcodes in previously unstudied species groups. Some of these species groups were selected because of their known phylogenetic relationships. Others were selected because they contained taxonomically challenging species relationships that had not been resolved by the molecular markers typically used for phylogenetics. We sampled species from six genera in three diverse lineages (classes) of green algae: the Charophyceae, Chlorophyceae and Zygnematophyceae. The species groups represent a wide range of structural complexity including coccoid unicells, filaments and thalloid species with specialized reproductive structures.

Taxon selection was also influenced by the availability of strains, particularly for the microscopic species. While it is typical for studies of macroalgae to include multiple representatives of each species in taxonomic studies, microalgal species are more often represented by a single strain (although this trend is changing). This study included as many strains of each species as were readily available. The number of strains per species tested ranged from one to eleven with an average number of four. Our taxonomic selection allowed us to test markers for the ideal barcoding qualities: universality (of occurrence and primer sites), ease of amplification and sequencing, ability to distinguish among closely related species, and greater inter- than intraspecific distances.

Although 18S and UPA have 'universal' primers, these loci were found to be the least variable (Table 4). The UPA primers were designed to amplify all phototrophic algae and cyanobacteria (Sherwood & Presting, 2007), but amplicons from *Desmidium* and *Micrasterias* did not sequence cleanly and in *Nitella* non-target regions of the plastid genome were amplified. A fragment of the plastid gene *tufA* was also amplified from *Bracteacoccus*, *Chlorosarcinopsis* and *Scenedesmus* using universal primers. Although this locus holds promise for species-level identification in chlorophytes (also see Saunders & Kucera, this volume), the gene *tufA* was transferred to the nuclear genome before the divergence of some charophytic lineages resulting in paralogs or pseudogenes (Baldauf *et al.,* 1990; Turmel *et al.,* 2005) that may complicate species identification in some lineages.

Universal primers amplified ITS1 and ITS2 from chlorophytes but were not as successful in charophytes. Taxon-specific primers for ITS1 and ITS2 were designed for Charophyceae, although the success rate was still rather low: ITS1

only amplified from eight strains of *Nitella* and ITS2 amplified from about half of the samples of *Chara* and *Nitella*. Only ITS1 amplified successfully from *Desmidium* and *Micrasterias*. Other primers may have been more successful. For example, other studies have successfully amplified ITS from strains of *Micrasterias* (Neustupa *et al.,* 2010) and *Nitella* (Sakayama *et al.,* 2004a) using a combination of taxon-specific and universal primers. In this study, ITS2 amplified easily from Charophyceae but often sequenced poorly. This could be due to the secondary structure of the molecule or sequencing parameters or both, but it could also be the result of contamination. Contamination is an important factor to consider when using universal primers (e.g., Zhang *et al.,* 1997), especially for macroalgae. Charophyceae are often covered in microscopic epiphytic or endophytic algae including other green algae (e.g., Cimino & Delwiche, 2002). Although apparently epiphyte-free portions were selected for DNA extraction, the possibility of contaminating DNA cannot be eliminated. Amplification of contaminating DNA from most epiphytes could be eliminated by the use of taxon-specific primers. Particularly in field-collected material, this could be an advantage.

An effective molecular barcode must be able to distinguish among closely related species and have lower levels of intraspecific variation than interspecific variation. None of the markers tested met these criteria in all groups. None was sufficiently variable to distinguish among the closely related species of the *Nitella tricellularis* species group. Only ITS1 was variable enough to distinguish between *Micrasterias muricata* and the morphologically distinct *M. nordstedtiana*. Consequently some intraspecific distances were higher than some interspecific distances in all markers where data for *Micrasterias* and *Nitella* were obtained (Table 4). The overlap of intra- and interspecific distances was also observed in some (but not all) other species groups in all markers except *tufA* (which was not sampled in charophytes). Among chlorophytes, there was no overlap in intraspecific and interspecific distances in *rbcL*, similar to the results of Fawley *et al.* (2010).

Without test cases, identifying markers that were sufficiently variable would have been more subjective due to differences of scale between lineages and markers. When comparing pairwise distances of a single marker, interspecific distances in one species group were often more than two times greater than interspecific distances in another (Table 4). In a few cases, intraspecific distances in one species group were on the same scale as interspecific distances in another. As a result, a particular locus may be sufficiently variable to distinguish between closely related species in one group but insufficient in other groups of green algae.

The apparent disparity in intraspecific and interspecific distances among the species groups may reflect evolutionary history, differences in rates of evolution, our incomplete taxonomic knowledge or a combination of these and other unknown factors. The test cases are examples of extraordinarily low interspecific distances. However, there were relatively few species that show high levels of intraspecific variation: *Chara zeylanica*, *C. foliolosa*, *Desmidium aptogonum*, *Micrasterias pinnatifida* and *M. truncata*. In the case of *C. zeylanica* and *C. foliolosa*, these strains may have been incompletely identified. The most recent global monograph of this group classified several species as synonyms, varieties or forms of *Chara zeylanica* (Wood & Imahori, 1965). It is possible that the molecular data are consistent with an older, narrower species concept (especially for strains of *C. foliolosa* and *C. zeylanica*) and that these species have been named in the literature. However, we cannot eliminate other possibilities such as the role of geographic variation. *Desmidium aptogonum*, *M. pinnatifida* and *M. truncata* are all relatively common and cosmopolitan species. Variation within these species may reflect cryptic or pseudocryptic species or geographic variation.

In the case of *Desmidium aptogonum*, intraspecific variation may also be due to taxonomy. Many subspecific taxa have been assigned to *D. aptogonum* including variety *ehrenbergii*. The level of variation and phylogenetic position of the variety *ehrenbergii* are consistent with its treatment as either a variety of the nominal species or a distinct species. A similar pattern was observed in *Micrasterias furcata* and variety *dichotoma*. Treating these two varieties as distinct species would affect the average values of intra and interspecific distances within their respective genera, but would not affect the overall patterns of variation observed among loci.

Our data suggest that 18S, UPA and COI would be poor choices for a DNA barcode in green algae: 18S and UPA were insufficiently variable and COI did not amplify from most taxa. Of the loci tested, ITS, *rbcL* and *tufA* (in chlorophytes) were all sufficiently variable to distinguish among most species tested and these could be amplified with relatively few primer sets. However, the *rbcL* fragment required internal sequencing primers, which would greatly increase the cost of sequencing this locus as a molecular barcode. None of the markers could distinguish all species and none had truly universal primers. This is especially the case in charophytes, which had considerable overlap in the level of intra- and interspecific variation in both ITS and *rbcL*. None of the loci tested are ideal as a molecular barcode for charophytes although *rbcL* could be used with the caveat that some species cannot be distinguished using this marker alone. It seems likely that more than one locus may be needed to meet the criteria of universality and species-level variability. However, the possibility remains that other loci will be identified which will better serve as molecular barcodes for green algae, particularly charophytes.

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REFERENCES

ALLEN T.F., 1888 — *Characeae of America*. New York, John C. Rankin, Jr., 40 p.

BALDAUF S.L., MANHART J.R. & PALMER J.D., 1990 — Different fates of the chloroplast *tufA* gene following transfer to the nucleus in green algae. *Proceedings of the national academy of science* 87 (14): 5317-5321.

BECKER B. & MARIN B., 2009 — Streptophyte algae and the origin of embryophytes. *Annals of botany* 103 (7): 999-1004.

BÉLANGER A.-S., BROUARD J.-S., CHARLEBOIS P., OTIS C., LEMIEUX C. & TURMEL M., 2006 — Distinctive architecture of the chloroplast genome in the chlorophycean green alga *Stigeoclonium helveticum*. *Molecular genetics and genomics* 276 (5): 464-477.

BESENDAHL A. & BHATTACHARYA D., 1999 — Evolutionary analyses of small-subunit rDNA coding regions and the 1506 group I introns of the Zygnematales (Charophyceae, Streptophyta). *Journal of phycology* 35 (3): 560-569.

BISCHOFF H.W. & BOLD H.C., 1963 — Phycological Studies IV. Some soil algae from Enchanted Rocks and related algal species. *University of Texas Publications*. 6318.

BOURRELLY P., 1990 — *Les Algues Vertes*. Paris, Société Nouvelle des éditions Boubée, 572 p.

CASANOVA M.T. & KAROL K.G., 2008 — Monoecious *Nitella* species (Characeae, Charophyta) from south-eastern mainland Australia, including *Nitella paludigena sp. nov*. *Australian systematic botany* 21 (3): 201-216.

CASANOVA M.T., WINTON M D.D., KAROL K.G. & CLAYTON J.S., 2007 — *Nitella hooker*i A. Braun (Characeae, Charophyceae) in New Zealand and Australia; implications for endemism, speciation and biogeography. *Charophytes* 1 (1): 2-18.

CBOL PLANT WORKING GROUP, HOLLINGSWORTH P.M., FORREST L.L., SPOUGE J.L., HAJIBABAEI M., RATNASINGHAM S., VAN DER BANK M., CHASE M. W., COWAN R.S., ERICKSON D.L., FAZEKAS A.J., GRAHAM S.W., JAMES K.E., KIM K.-J., KRESS W.J., SCHNEIDER H., VAN ALPHENSTAHL J., BARRETT S.C.H., VAN DEN BERG C., BOGARIN D., BURGESS K.S., CAMERON K.M., CARINE M., CHACÓN J., CLARK A., CLARKSON J.J., CONRAD F., DEVEY D.S., FORD C.S., HEDDERSON T.A. J., HOLLINGSWORTH M.L., HUSBAND B.C., KELLY L.J., KESANAKURTI P.R., KIM J.S., KIM Y.-D., LAHAYE R., LEE H.-L., LONG D.G., MADRIÑÁN S., MAURIN O., MEUSNIER I., NEWMASTER S.G., PARK C.-W., PERCY D.M., PETERSEN G., RICHARDSON J.E., SALAZAR G.A., SAVOLAINEN V., SEBERG O., WILKINSON M.J., YI D.-K. & LITTLE D.P., 2009 — A DNA barcode for land plants. *Proceedings of the national academy of sciences* 106 (31): 12794-12797.

CIMINO M.T. & DELWICHE C. F., 2002 — Molecular and morphological data identify a cryptic species complex in endophytic members of the genus *Coleochaete* Brébisson (Charophyta; Coleochaetaceae). *Journal of phycology* 38 (6): 1213-1221.

CLARKSTON B.E. & SAUNDERS G.W., 2010 — A comparison of two DNA barcode markers for species discrimination in the red algal family Kallymeniaceae (Gigartinales, Florideophyceae), with a description of *Euthora timburtonii* sp. nov. *Botany* 88 (2): 119-131.

COLEMAN A.W., 2007 — Pan-eukaryote ITS2 homologies revealed by RNA secondary structure. *Nucleic acids research* 35 (10): 3322-3329.

COLEMAN A.W., 2009 — Is there a molecular key to the level of "biological species" in eukaryotes? A DNA guide. *Molecular phylogenetics and evolution* 50 (1): 197-203.

CROASDALE H., BICUDO C.E. M. & PRESCOTT G.W., 1983 — *A Synopsis of North American Desmids. Pt. II Desmidaceae: Placodermae sec. 5 filamentous genera.* Lincoln and London, University of Nebraska Press, 117 p.

ETTL H. & GÄRTNER G., 1995 — *Syllabus der Boden-, Luft- und Flechtenalgen*. Stuttgart, Jena and New York, Gustav Fischer Verlag, 721 p.

EVANS K.M., WORTLEY A.H. & MANN D.G., 2007 — An assessment of potential diatom "barcode" genes (*cox*1, *rbcL*, 18S and ITS rDNA) and their effectiveness in determining relationships in *Sellaphora* (Bacillariophyta). *Protist* 158 (3): 349-364.

FAMA P., WYSOR B., KOOISTRA W.H.F.C. & ZUCCARELLO G.C., 2002 — Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tufA* gene. *Journal of phycology* 38 (5): 1040-1050.

FAWLEY M.W., FAWLEY K.P. & BUCCHEIM M.A., 2004 — Molecular diversity among communities of freshwater microchlorophytes. *Microbial ecology* 48 (4): 489-499.

FAWLEY M.W., FAWLEY K.P. & OWEN H.A., 2005 — Diversity and ecology of small coccoid green algae from Lake Itasca, Minnesota, USA, including *Meyerella planktonica, gen. et sp. nov*. (Trebouxiophyceae, Chlorophyta). *Phycologia* 44 (1): 35-48.

FAWLEY M.W., FAWLEY K.P. & HEGEWALD E., 2010 — Taxonomy of *Desmodesmus serratus* (Chlorophyceae, Chlorophyta) and related taxa on the basis of morphological and DNA sequence data. *Phycologia* (in press).

FELINER G.N. & ROSSELÓ J.A., 2007 — Better the devil to know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. *Molecular phylogenetics and evolution* 44 (2): 911-919.

FERRI E., BARBUTO M., BAIN O., GALIMBERTI A., UNI S., GUERRERO R., FERTÉ H., BANDI C., MARTIN C. & CASIRAGHI M., 2009 — Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Frontiers in zoology* 6: 1.

FRIEDRICH J., DANDEKAR T., WOLF M. & MÜLLER T., 2005 — ProfDist. A tool for the construction of large phylogenetic trees based on profile distances. *Bioinformatics* 21 (9): 2108-2109.

GONTCHAROV A.A., MARIN B. & MELKONIAN M., 2003 — Molecular phylogeny of conjugating green algae (Zygnemophyceae, Streptophyta) inferred from SSU rDNA sequence comparisons. *Journal of molecular evolution* 56 (1): 89-104.

HALL J.D. & DELWICHE C.F., 2007 — In the shadow of giants; systematics of the charophyte green algae. *In*: Brodie J. & Lewis J. (eds), *Unraveling the algae: the past, present, and future of algal systematics*. Boca Raton, Systematics Association/CRC Press, pp. 155-169.

HALL J.D., KAROL K.G., MCCOURT R.M. & DELWICHE C.F., 2008a — Phylogeny of conjugating green algae based on chloroplast and mitochondrial nucleotide sequence data. *Journal of phycology* 44 (2): 467-477.

HALL J.D., MCCOURT R.M. & DELWICHE C.F., 2008b — Patterns of cell division in the filamentous Desmidiaceae, close green algal relatives of land plants. *American Journal of botany* 95 (6): 1-12.

HEGEWALD E. & SILVA P.C., 1988 — *Annotated catalogue of Scenedesmus and nomenclaturally related genera, including original descriptions and figures.* Berlin, Stuttgart, E. Schweizerbart. 587 p.

HEGEWALD E., WOLF M., KELLER A., FRIEDL T. & KRIENITZ L., 2010 — ITS2 sequence-structure phylogeny in the Scenedesmaceae with special reference to *Coelastrum* (Chlorophyta, Chlorophyceae), including the new genera *Comasiella* and *Pectinodesmus*. *Phycologia* 49 (4): 325-335.

HERSHKOVITZ A. & LEWIS L.A., 1996 — Deep-level diagnostic value of the rDNA-ITS region. *Molecular biology and evolution* 13 (9): 1276-1295.

KELLER A., SCHLEICHER T., FÖRSTER F., RUDERISCH B., DANDEKAR T., MÜLLER T. & WOLF M., 2008 — ITS2 data corroborate a monophyletic chlorophycean DO-group (Sphaeropleales). *BMC Evolutionary biology* 8: 218.

KELLY L.J., AMEKA G.K. & CHASE M.W., 2010 — DNA barcoding of African Podostemaceae (river-weeds): a test of proposed barcode regions. *Taxon* 59 (1): 251-260.

LARKIN M.A., BLACKSHIELDS G., BROWN N.P., CHENNA R., MCGETTIGAN P.A., MCWILLIAM H., VALENTIN F., WALLACE I.M., WILM A., LOPEZ R., THOMPSON J.D., GIBSON T.J. & HIGGINS D.G., 2007 — ClustalW and ClustalX version 2. *Bioinformatics* 23 (21): 2947-2948.

LE GALL L. & SAUNDERS G.W., 2010 — DNA barcoding is a powerful tool to uncover algal diversity: a case study of the Phyllophoraceae (Gigartinales, Rhodophyta) in the Canadian flora. *Journal of phycology* 46 (2): 374-389.

LEMIEUX C., OTIS C. & TURMEL M., 2007 — A clade uniting the green algae *Mesostigma viride* and *Chlorokybus atmophyticus* represents the deepest branch of the Streptophyta in chloroplast genome-based phylogenies. *BMC Biology* 5: 2.

LEWIS L.A., MISHLER B.D. & VILGALYS R., 1997 — Phylogenetic relationships of the liverworts (Hepaticae), a basal embryophyte lineage, inferred from nucleotide sequence data of the chloroplast gene *rbcL*. *Molecular phylogenetics and evolution* 7 (3): 377-393.

LEWIS L.A. & FLECHTNER V.R., 2004 — Cryptic species of *Scenedesmus* (Chlorophyta) from desert soil communities of western North America. *Journal of phycology* 40 (6): 1127-1137.

LEWIS L.A. & MCCOURT R.M., 2004 — Green algae and the origin of land plants. *American journal of botany* 91 (10): 1535-1556.

MADDISON D.R. & MADDISON W.P., 2000 — MACCLADE 4, analysis of phylogenetic character and evolution. Sunderland, MA, Sinauer Associates.

MADDISON W.P. & MADDISON D.R., 2008 — Mesquite: a modular system for evolutionary analysis.

MCCOURT R.M., DELWICHE C.F. & KAROL K.G., 2004 — Charophyte algae and land plant origins. *Ecology and evolution* 19 (12): 661-666.

MCDEVIT D.C. & SAUNDERS G.W., 2010 — A DNA barcode examination of the Laminariaceae (Phaeophyceae) in Canada reveals novel biogeographical and evolutionary insights. *Phycologia* 49 (3): 235-248.

MCMANUS H.A. & LEWIS L.A. accepted — Molecular phylogenetic relationships in the freshwater family Hydrodictyaceae (Sphaeropleales, Chlorophyceae), with emphasis on *Pediastrum duplex*. *Journal of phycology*.

MEI H., LUO W., LIU G.X. & HU Z.Y., 2007 — Phylogeny of Oedogoniales (Chlorophyceae, Chlorophyta) inferred from 18S rDNA sequences with emphasis on the relationships in the genus *Oedogonium* based on ITS-2 sequences. *Plant systematics and evolution* 265 (3-4): 179-191.

MONIZ M.B.J. & KACZMARSKA I., 2009 — Barcoding diatoms: is there a good marker? *Molecular ecology resources* 9: 65-74.

MONIZ M.B.J. & KACZMARSKA I., 2010 — Barcoding of diatoms: nuclear encoded ITS revisited. *Protist* 161 (1): 7-34.

NEUSTUPA J., ŠKALOUD P. & ŠŤASTNÝ J., 2010 — The molecular phylogenetic and geometric morphometric evaluation of *Micrasterias crux-melitensis*/*M. radians* species complex. *Journal of phycology* 46 (4): 703-714.

NORTON T.A., MELKONIAN M. & ANDERSEN R.A., 1996 — Algal biodiversity. *Phycologia* 35 (4): 308-326.

O'KELLY C.J., KURIHARA A., SHIPLEY T.C. & SHERWOOD A.R., 2010 — Molecular assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the Hawaiian Islands. *Journal of phycology* 46 (4): 728-735.

POCZAI P. & HYVÖNEN J., 2010 — Nuclear ribosomal spacer regions in plant phylogenetics: problems and prospects. *Molecular biology reports* 4 (4): 1897-1912.

POMBERT J.-F., OTIS C., LEMIEUX C. & TURMEL M., 2005 — The chloroplast genome sequnce of the green alga *Pseudendoclonium akinetum* (Ulvophyceae) reveals unusual structural features and new insights into the branching order of chlorophyte lineages. *Molecular biology and evolution* 22 (9): 1903-1918.

PRESCOTT G.W., 1962 — *Algae of the Western Great Lakes Area*. Dubuque, Iowa, WM.C. Brown Company Publishers, 977 p.

PROCTOR V.W., GRIFFIN III D. G. & HOTCHKISS A.T., 1971 — A synopsis of the genus *Chara*, series *Gymnobasalia* (subsection *Willdenowia* RDW). *American journal of botany* 58 (10): 894-901.

PROCTOR V.W. & WIMAN F.H., 1971 — An experimental approach to the systematics of the monoecious-conjoined members of the genus *Chara*, series *Gymnobasalia*. *American journal of botany* 58 (10): 885-893.

PRÖSCHOLD T. & LELIAERT F., 2007 — Systematics of the green algae: conflict of classic and modern approaches. *In*: Brodie J. & Lewis J. (eds), *Unraveling the algae: the past, present, and future of algal systematics*. Boca Raton, Systematics association/CRC Press, pp. 123-153.

RALFS J., 1848 — *British Desmidieae*. Richard and John E. Taylor, London. 226 p.

SAKAYAMA H., HARA Y., ARAI S., SATO H. & NOZAKI H., 2004a — Phylogenetic analyses of *Nitella* subgenus *Tieffallenia* (Charales, Charophyceae) using nuclear ribosomal DNA internal transcribed spacer sequences. *Phycologia* 43 (6): 672-681.

SAKAYAMA H., HARA Y. & NOZAKI H., 2004b — Taxonomic re-examination of six species of *Nitella* (Charales, Charophyceae) from Asia, and phylogenetic relationships within the genus based on *rbc*L and *atp*B gene sequences. *Phycologia* 43 (1): 91-104.

SAUNDERS G.W., 2005 — Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philosophical transactions of the royal society. B.* 360 (1462): 1879-1888.

SHERWOOD A.R. & PRESTING G.G., 2007 — Universal primers amplify a 23S rDNA plastid marker in eukaryotic algae and cyanobacteria. *Journal of phycology* 43 (3): 605-608.

SHERWOOD A.R., VIS M.L., ENTWISLE T. J., NECCHI O., JR. & PRESTING G.G., 2008 — Contrasting intra versus interspecies DNA sequence variation for representatives of the Batrachospermales (Rhodophyta): insights from a DNA barcoding approach. *Phycological research* 56 (4): 269-279.

SHOUP S. & LEWIS L.A., 2003 — Polyphyletic origin of parallel basal bodies in swimming cells of chlorophycean green algae (Chlorophyta). *Journal of phycology* 39 (4): 789-796.

ŠKALOUD P., NEUSTUPA J., RADOCHOVÁ B. & KUBÍNOVÁ L., 2006 — Confocal microscopy of chloroplast morphology and ontogeny in three strains of *Dictyochloropsis* (Trebouxiophyceae, Chlorophyta). *Phycologia* 44 (3): 261-269.

SLUIMAN H.J., GUIHAL C. & MUDIMU O., 2008 — Assessing phylogenetic affinities and species delimitations in Klebsormidiales (Streptophyta): nuclear-encoded rDNA phylogenies and its secondary structure models in *Klebsormidium*, *Hormidiella*, and *Entransia*. *Journal of phycology* 44 (1): 183-195.

SWOFFORD D.L., 2003 — PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, MA, Sinauer Associates.

TURMEL M., OTIS C. & LEMIEUX C., 2002 — The chloroplast and mitochondrial genome sequences of the charophyte *Chaetosphaeridium globosum*: insights into the timing of the events that restructured organelle DNAs within the green algal lineage the led to land plants. *Proceedings of the national academy of science* 99 (17): 11275-11280.

TURMEL M., OTIS C. & LEMIEUX C., 2003 — The mitochondrial genome of *Chara vulgaris*: Insights into the mitochondrial DNA architecture of the last common ancestor of green algae and land plants. *The plant cell* 15 (8): 1888-1903.

TURMEL M., OTIS C. & LEMIEUX C., 2005 — The complete chloroplast DNA sequences of the charophycean green algae *Staurastrum* and *Zygnema* reveal that the chloroplst genome underwent extensive changes during the evolution of the Zygnematales. *BMC Biology* 3: 22.

TURMEL M., GAGNON M.-C., O'KELLY C. J., OTIS C. & LEMIEUX C., 2009a — The chloroplast genomes of the green algae *Pyramimonas*, *Monomastix* and *Pycnococcus* shed new light on the evolutionary history of prasinophytes and the origin of the secondary chloroplasts of euglenoids. *Molecular biology and evolution* 26 (3): 631-648.

TURMEL M., OTIS C. & LEMIEUX C., 2009b — The chloroplast genomes of the green algae *Pedinomonas minor*, *Parachlorella kessleri*, and *Oocystis solitaria* reveal a shared ancestry between the Pedinomonadales and Chlorellales. *Molecular biology and evolution* 26 (10): 2317-2331.

TURMEL M., POMBERT J.-F., CHARLEBOIS P., OTIS C. & LEMIEUX C., 2007 — The green algal ancestry of land plants as revealed by the chloroplast genome. *International journal of plant science* 168 (5): 679-689.

VERBRUGGEN H., DE CLERCK O., N'YEURT A.D.R., SPALDING H. & VROOM P. S., 2006 — Phylogeny and taxonomy of *Halimeda incrassata*, including descriptions of *H. kanaloana* and *H. heteromorpha spp. nov*. *European journal of phycology* 41 (3): 337-362.

WATANABE S. & FLOYD G.L., 1996 — Consideration on the systematics of coccoid green algae and related organisms based on the ultrastructure of swarmers. *In*: Chaudhary B. R. & Agrawal S. B. (eds), *Cytology, genetics and molecular biology of algae.* Amsterdam, Netherlands, SPB Academic Publishers, pp. 1-19.

WATANABE S., MITSUI K., NAKAYAMA T. & INOUYE I., 2006 — Phylogenetic relationships and taxonomy of sarcinoid green algae: *Chlorosarcinopsis*, *Desmotetra*, *Sarcinochlamys gen. nov*., *Neochlorosarcina*, and *Chlorosphaeropsis* (Chlorophyceae, Chlorophyta). *Journal of phycology* 42 (3): 679-695.

WHITE T.J., BRUNS T., LEE S. & TAYLOR J., 1990 — Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. *In*: Innis M. A., Gelfand D. H., Sninsky J. J. & White T. J. (eds), *PCR Protocols: A Guide to Methods and Applications*. New York, Academic Press, pp. 315-322.

WILSON J.J., 2010 — Assessing the value of DNA barcodes and other priority gene regions for molecular phylogenetics of Lepidoptera. *PlosONE* 5: e10525.

WOLF M., RUDERISCH B., DANDEKAR T., SCHULTZ J. & MÜLLER T. 2008 — ProfDistS: (Profile-) Distance based phylogeny on sequence-structure alignments. *Bioinformatics* 24 (9): 2401-2402.

WOLLE F., 1884 — *Desmids of the United States, and list of American Pediastrums*. Bethleham, Moravian Publication Office, 168 p.

WOOD R D. & IMAHORI K., 1965 — *A Revision of the Characeae*. Weinheim,Verlag von J. Cramer, 904 p.

ZHANG W., WENDEL J.F. & CLARK L. G., 1997 — Bamboozled again! Inadvertent isolation of fungal rDNA sequences from bamboos (Poaceae, Bambusoideae). *Molecular phylogenetics and evolution* 8 (2): 205-217.