SEQUENCING NUCLEAR MARKERS IN FRESHWATER GREEN ALGAE: CHARA SUBSECTION WILLDENOWIA

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ABSTRACT

Chara subsection *Willdenowia* (Charophyta) is a monophyletic group within the Characeae, and has been considered one cosmopolitan species - *Chara zeylanica* - since the most recent revision of the group in 1965. Four gene chloroplast DNA sequences and other data strongly contradict the single-species hypothesis. However, a phylogenetically informative nuclear marker would be of value for this group. Sequencing data indicate that chloroplast DNA is maternally inherited, and so could represent an alternative phylotgenetic signal to a nuclear marker, particularly if hybridization between clades is taking place. An altered topology between a *Willdenowia* nuclear phylogeny and chloroplast phylogeny could indicate hybridizations are taking place and would better inform species circumscription for the group. The nuclear Internal Transcribed Spacer 2 (*ITS2*) marker has been suggested as a universal barcode for species identification and phylogenetics in plants, animals, and fungi. *ITS2* was sequenced for members of the *Willdenowia* and a phylogeny comprised of these and published sequences was compared with the chloroplast phylogeny.

Key words: **Characeae, Charophyta, chloroplast phylogeny, internal transcribed spacer, *ITS2*, nuclear phylogeny, *Willdenowia***

INTRODUCTION

The Characeae is a family of macrophytic green algae closely related to land plants (Lewis & McCourt, 2004). The group is globally distributed (except for Antarctica) and can be readily found in many freshwater lakes (Dodds, 2002). Even though these algae can dramatically influence nutrient loads and water quality where they occur (Kufel & Kufel, 2002; Kufel & Ozimek, 1994), a misleading and incomplete understanding of the species within this group makes well informed management strategies for these organisms and the ecosystems in which they reside difficult.

In the 1965 revision of the Characeae, 81 species were recognized, a number that was revised down from a previous estimate of 314 species (Wood & Imahori, 1965; Wood, 1964). This work resulted in morphologically, ecologically, and reproductively diverse species. Such is the case for *Chara* subsection *Willdenowia*, which was condensed from 19 species into just one species: *Chara zeylanica*. This amalgamation of the group was met with criticisms early on. It was shown for instance, that morphologically distinct members of this clade could not readily hybridize with one another, or in cases where they could, the offspring could not form viable zygotes (McCracken et al., 1966; Proctor & Wiman, 1971). These data suggested that there were reproductively isolated species within the *Willdenowia* that more or less corresponded with morphologically distinguishable characteristics.

With the advent of molecular systematics, it is now possible to examine the relationships within this group in greater detail. A four gene chloroplast phylogeny supports the separation of the *Willdenowia* into multiple species (Fig. 1). A phylogeny using nuclear markers is lacking for this group, but would be desirable to confirm phylogenies made from the maternally inherited chloroplast genes. It has been show in land plants that chloroplast and nuclear phylogenies do not always corroborate one another, and this is sometimes due to interspecific hybridization (Soltis

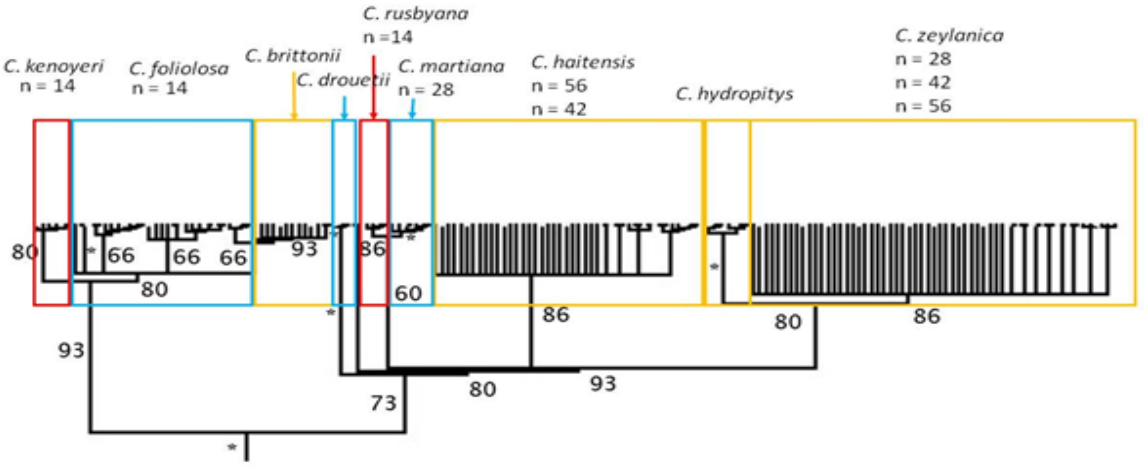

Figure 1: A chloroplast four gene RaxML phylogeny using the genes *rbcL, psbC, atpB, ndhD*. Colored boxes indicate previously recognized species within the *Willdenowia.* Red boxes indicate dioecious taxa, blue indicate sejoined taxa, and yellow indicate conjoined taxa. Chromosome counts are included where known. Bootstrap support (BS) values greater than 50 are included, BS values of 100 are represented by asterices.

& Kuzoff, 1995). For this study, the internal transcribed spacer 2 region of the nuclear ribosomal operon is used to construct a nuclear phylogeny for comparison with the chloroplast four-gene phylogeny. The *ITS2* region has been suggested as a universal barcode for plants, animals, and fungi (Yao et al., 2010), but as of yet, no phylogeny has been constructed for *Willdenowia*.

MATERIALS AND METHODS

Published *ITS2* primers were used to PCR amplify the *ITS2* portion of the nuclear ribosomal operon of *Chara* subsection *Willdenowia* (Fig. 2; Hall et al., 2010). PCR products were purified using the QIAquick PCR purification kit. PCR products were run on 1% agarose gels. Sequencing of purified PCR product was carried out by GENEWIZ (genewiz.com).

Alignments were made using Geneious 9.1.3 (Kearse et al., 2012). Phylogenies were constructed using RaxML-HPC2 in the CIPRES web portal (Miller et al., 2010) using *Klebsormidium ITS2* as an outgroup for the nuclear phylogeny and the other Characeae genera as outgroups for the chloroplast phylogeny.

DNA samples and live material were kindly provided by the New York Botanical Garden.

Figure 2: Part of the nuclear encoded ribosomal operon is shown. The 5.8S and 26S ribosomal genes are shown in orange and are separated by *ITS2* in blue. Primer locations (red arrows) used for this study were located at the 5' ends of the 5.8S and 26S segments.

RESULTS

ITS2 was successfully PCR amplified and sequenced for members of species-clades *C. foliolosa*, *C. brittonii*, *C. rusbyana*, *C. hydropitys*, and *C. zeylanica* (Fig. 3). There was an 89 % pairwise identity across the alignment for the resulting sequences. The nuclear *ITS2* phylogeny shared a similar topology to the chloroplast phylogeny, with no well supported rearrangements between groups (Fig. 4). The two *C. rusbyana ITS2* sequences were surprisingly different (30 polymorphic sites between the two sequences, data not shown) and in the *ITS2* phylogeny were paraphyletic with each other, despite having come from a clonally propogated individual harvested years apart (Fig. 4). NCBI blast results indicate that the *Willdenowia* do not have significant sequence similarity with *ITS2* sequences of related taxa, including members of the Characeae with published *ITS2* sequences such as *Nitella* (data not shown).

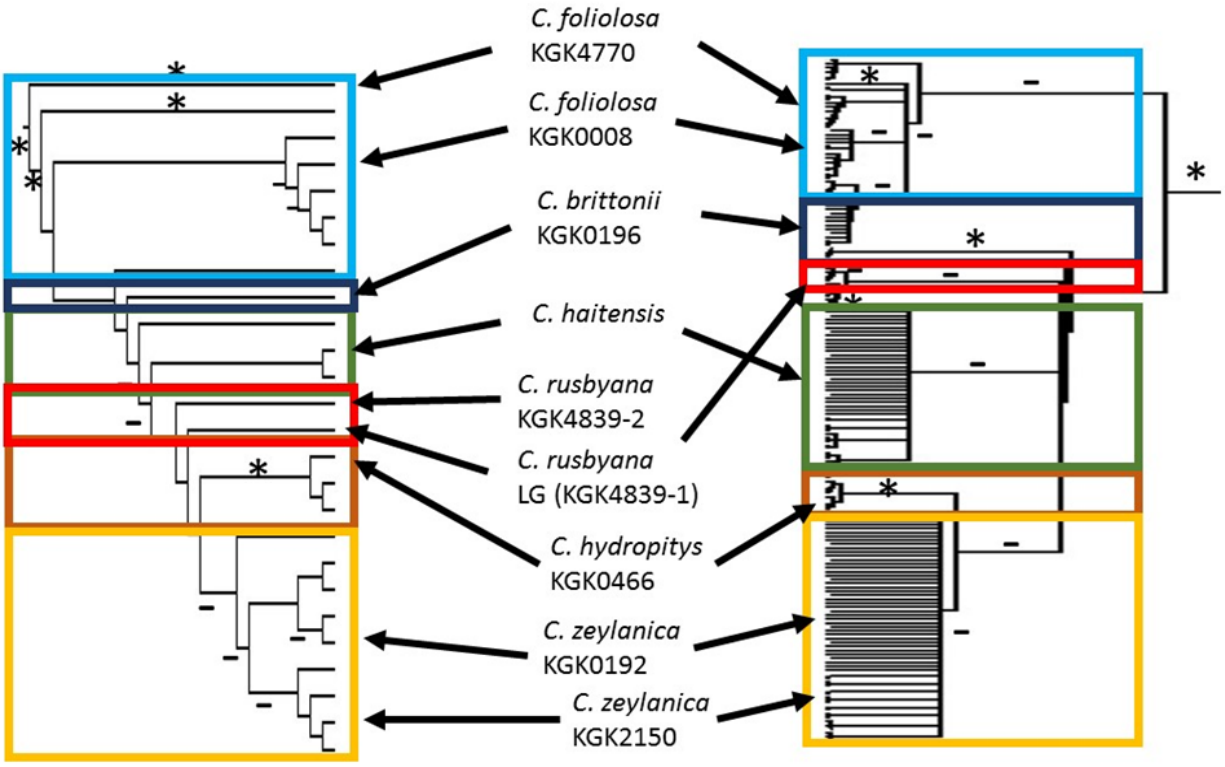

Figure 4: comparison of the nuclear *ITS2* (left) and chloroplast four gene (right) phylogenies. The major species-clades from the chloroplast phylogeny and their corresponding taxa in the *ITS2* nuclear phylogeny are boxed with corresponding colors. Though the *C. haitensis* group changes position with *C. rusbyana* in the two phylogenies, this does not appear to be a strongly supported rearrangement in either case. Published sequences are used to fill out the *ITS2* phylogeny (Hall et al., 2010). Notably, *C. rusbyana*, a cloned and resequenced individual represented as KGK4839-2 and KGK4839-1 have 30 SNPs with respect to one another, possibly due to multiple alleles for this locus. This causes them to appear paraphyletic in the nuclear *ITS2* phylogeny. Bootstrap support values indicated: asterices (*) = 100, hyphens (-) = 50-100, (blank) < 50.

DISCUSSION

The nuclear *ITS2* phylogeny corresponded well to the chloroplast phylogeny and supported a multiple species treatment of *Chara* subsection *Willdenowia*. Despite the lower sample size of the *ITS2* phylogeny compared to the chloroplast phylogeny, well supported clades (*C. hydropitys*, some *C. foliolosa)* were still identified, suggesting a high level of discrimination for this marker. No major rearrangements between the nuclear and the chloroplast phylogenies were observed (Fig. 4), suggesting hybridization between these major clades within *Willdenowia* does not occur. This is consistent with attempted crosses between these clades that either did not produce viable zygotes, or individuals that were not viable (Proctor & Wiman, 1971). It is surprising that the clonal samples of *C. rusbyana* showed such a high dissimilarity. Examination of the sequencing trace files for the newly sequenced specimen (KGK4839-2) revealed double peaks at multiple sites across the sequence (data not shown). This is consistent with and may suggest multiple alleles are represented in the sequencing results. *Chara* is known to be haploid,

with many presumed instances of genome doubling (Casanova, 2015, Fig. 1), so it is not clear whether multiple alleles of the *ITS2* segment are present on one chromosome or multiple. The possible paralogous nature of the *ITS2* segment would make it less desirable as a phylogenetic tool for *Chara* subsection *Willdenowia*, but its uniqueness would nevertheless serve as a reliable barcode for placing unknown specimens within this group.

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