Refinements for the amplification and sequencing of red algal DNA barcode and RedToL phylogenetic markers: a summary of current primers, profiles and strategies

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This review provides a comprehensive summary of the PCR primers and profiles currently in use in our laboratory for red algal DNA barcoding and phylogenetic research. While work focuses on florideophyte taxa, many of the markers have been applied successfully to the Bangiales, as well as other lineages previously assigned to the Bangiophyceae sensu lato. All of the primers currently in use with their respective amplification profiles and strategies are provided, which can include full fragment, overlapping fragments and what might best be called “informed overlapping fragments”, i.e., a fragment for a marker is amplified and sequenced for a taxon and those sequence data are then used to identify the best primers to amplify the remaining fragment(s) for that marker. We extend this strategy for the more variable markers with sequence from the external PCR primers used to “inform” the selection of internal sequencing primers. This summary will hopefully serve as a useful resource to systematists in the red algal community.

Key Words: DNA barcode; Florideophyceae; molecular markers; phylogeny; polymerase chain reaction; primers; RedToL

INTRODUCTION

There can be little question that molecular techniques have radically altered the face of phycological research especially in the fields of biodiversity and systematics (Maggs et al. 2007). Whereas in the early years researchers focused strongly on two commonly used markers, viz., the plastid rbcL (Freshwater et al. 1994) and the nuclear small subunit rDNA (Bird et al. 1992, Ragan et al. 1994, Saunders and Kraft 1994), a number of alternative markers have been developed for use with red algae from the population to the highest taxonomic levels (see Maggs et al. 2007, Table 6.1, for a list). Of particular importance in this regard have been the recent large-scale DNA barcoding (iBOL: http://ibol.org) (Saunders 2005) and Red Algal Tree of Life initiatives (RedToL: http://dblab.rutgers.edu/redtol/) (Vis et al. 2010). Both of these projects target a broader and more comprehensive taxonomic sampling than previous initiatives, challenging researchers to develop more universal amplification strategies for markers (e.g., Saunders and McDevit 2012). Not even widely used markers such as the nuclear SSU and large subunit rDNA (Harper and Saunders 2001b) or the plastid rbcL (Freshwater et al. 1994) have weathered these global initiatives without the need for refinement.

In this review, we focus strictly on the primers, profiles and strategies for the PCR amplification of markers currently in use in our lab (for a review of current collection and DNA extraction protocols see Saunders and McDevit 2012). Being partners in both the DNA barcode and Red-
Fig. 1. Overview of PCR / sequencing strategy for the nuclear SSU. The primers G09, G15, and G16 are for chromophytic algae, but are included for reference. Primer P1 puts in context the standard forward primer that we use for amplification of the internal transcribed spacer (ITS) region.

Fig. 2. Overview of PCR / sequencing strategy for the nuclear internal transcribed spacer (ITS).
ToL initiatives, we have been heavily involved in marker development and refinement to meet their respective objectives. As a result, numerous changes have been introduced to “tried and true” markers in an effort to improve universality.

In the following section each marker has a brief summary with a corresponding figure. We have tried to adhere to the same format for all figures—from top to bottom (examples refer to Fig. 1): marker name (e.g., small subunit ribosomal DNA), acronym (e.g., SSU), and approximate size (e.g., ~1,800 bp); a schematic of the fragment(s) typically amplified for each marker, their corresponding acronym / name and the primers used to amplify them when more than one fragment is an option (e.g., E', a fragment corresponding to the 5' end of the SSU is amplified with G01 and G14); a schematic showing the relative location of all primers along the marker (gene regions presented as wider boxes than adjacent spacers); and finally a listing of the actual PCR / sequencing primers. The typical primers used for PCR amplification are underlined for each marker, while M13 linkers and sequencing primers are indicated in bold italics type.

**NUCLEAR MARKERS**

**Small subunit ribosomal DNA (SSU, ~1,800 bp)**

The SSU was widely used in early studies of red algal phylogenetics (e.g., Bird et al. 1992, Ragan et al. 1994, Saunders and Kraft 1994) and is still useful for exploring deeper relationships (e.g., West et al. 2008) with Harper and Saunders (2001b, Fig. 2a) providing a summary of PCR primers and profiles to that time. In the past it was typical to amplify this gene as four (L, M, N, O) fragments, but with improvements in PCR reagents over the years we now routinely amplify this gene as two fragments (E', F) or as a single product (SSU full) (Fig. 1). The primers listed here remain unchanged from Harper and Saunders (2001b) and the specialty primers still serve the same purposes, viz., H1 or H2 replace G01 and HR replaces G10 for members of the Hildenbrandiales, in which fungal contamination (symbionts) is an issue, while G05 is a replacement for G10 in taxa for which the latter fails to give readable sequence data (Harper and Saunders 2001b). We no longer use all four internals to generate sequence data for the E' fragment and simply use one of the forward (G02 or G03) and one of the reverse (G10 or G11) primers (Fig. 1). Similarly, for the F fragment we typically acquire internal sequence data only for G06 and G08, data for G12 (G15) and G13 (G16) usually not necessary to generate full bidirectional reads (Fig. 1).

Amplification profile: 94°C for 4 min; 38 cycles of 94°C for 30 s, 55°C annealing for 30 s, 72°C extension for 1 min 30 s; followed by 72°C final extension for 7 min.

**Internal transcribed spacer ribosomal DNA (ITS, ~650-1,100 bp)**

First investigated for red algae by Steane et al. (1991), the ITS marker is useful for species discrimination and detecting hybridization, in some cases population level analyses, and for resolving relationships among closely related taxa. Our default methodological reference for ITS amplification is Tai et al. (2001) or, more recently, Saunders and McDevit (2012) with our overall scheme first summarized in Harper and Saunders (2001b, Fig. 2c, and references therein). We amplify the ITS typically as a single fragment with the standard primers P1 and G4 (Fig. 2), although it is easy to amplify the ITS1 (P1 and R1) or ITS2 (P5 and G4) individually. We have encountered problems in using R1 with some taxa (Corallinales) (see Hind and Saunders 2013) and have developed a slightly degenerate (more universal) version of this primer (R1s) (Fig. 2). Moving forward this modified primer will likely be used in routine applications. The primers P1 and G4 are relatively universal and work on a wide variety of eukaryotes, which can be useful when trying to generate ITS data from divergent lineages, but detrimental for use in species prone to biological infestation. For example, recently we had trouble acquiring clean ITS data for Membranoptera spp. (Wynne and Saunders 2012) necessitating development of the more specific MEMR4 to replace G4 and similarly HarV4 for Neosiphonia (Savoie and Saunders unpublished). It is not unusual to have to develop primers to meet particular needs, a practice that occurs routinely in our laboratory (e.g., Druehl et al. 2005).

Amplification profile: 94°C for 2 min; 38 cycles of 94°C for 30 s, 50°C annealing for 45 s, 72°C extension for 2 min; followed by 72°C final extension for 5 min.

**Large subunit ribosomal DNA (LSU, ~2,700 bp)**

Freshwater and Bailey (1998) first used the LSU as a phylogenetic tool for red algae while Harper and Saunders (2001a) refined the marker by extending the portion of the gene that was amplified (summarized in Harper and Saunders 2001b, Fig. 2b). There have been a number of refinements since that time that have yet to be summarized in a review of this nature. Harper and Saunders
Large subunit ribosomal DNA (LSU, ~2,700 bp)

Fig. 3. Overview of PCR / sequencing strategy for the nuclear LSU, as well as the D2 / D3 barcode region. Primer G4 puts in context the standard reverse primer that we use for amplification of the internal transcribed spacer (ITS) region.

Elongation factor 2 (EF2, ~1,700 bp)

Fig. 4. Overview of PCR / sequencing strategy for the nuclear EF2.
success for both amplification with their external PCR, as ing this marker for additional taxa we have had mixed fragments (Le Gall and Saunders 2007, Fig. 1). In pursu-

product ~1,700 bp in length or as two shorter overlapping ceae. In that study this region was amplified as a single

Amplification profile: 94°C for 2 min; 5 cycles of 94°C for 30 s, 45°C annealing for 30 s, 72°C extension for 1 min; then 35 cycles of 94°C for 30 s, 46.5°C annealing for 30 s,

Amplification profile for two subfragments: 94°C for 4 min; 38 cycles of 94°C for 1 min, 50°C annealing for 1 min, 72°C extension for 2 min; followed by 72°C final extension for 7 min.

In all cases, we sequence with the external PCR primers and use those data to identify the best internal forward primer from the Mang1-13 series to use against our external reverse PCR primers to amplify the EF2-3’ fragment (Fig. 4). In all cases, we sequence with the PCR primers and use those data to select the best internal sequencing primers from the forward series Mang1-13 and Mang51-58 and from the reverse series Mang21-28 and Mang31-46 (Fig. 4).

Amplification profile for full fragment: 94°C for 4 min; 38 cycles of 94°C for 1 min, 50°C annealing for 1 min, 72°C extension for 2 min; followed by 72°C final extension for 7 min.

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Amplification profile for full fragment: 94°C for 4 min; 38 cycles of 94°C for 1 min, 50°C annealing for 1 min, 72°C extension for 2 min; followed by 72°C final extension for 7 min.

Le Gall and Saunders (2007) championed EF2 as a phy-

Amplification profile: 94°C for 5 min; 38 cycles of 94°C for 30 s, 50°C annealing for 30 s, 72°C extension for 2 min; followed by 72°C final extension for 7 min.

Elongation factor 2 (EF2, ~1,700 bp)

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Amplification profile: 94°C for 5 min; 38 cycles of 94°C for 30 s, 50°C annealing for 30 s, 72°C extension for 2 min; followed by 72°C final extension for 7 min.

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Elongation factor 2 (EF2, ~1,700 bp)

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Amplification profile: 94°C for 5 min; 38 cycles of 94°C for 30 s, 50°C annealing for 30 s, 72°C extension for 2 min; followed by 72°C final extension for 7 min.

Elongation factor 2 (EF2, ~1,700 bp)
Photosystem I P700 chlorophyll \( a \) apoprotein A1 (\( psaA \), ~1,600 bp)

For this marker the external PCR primers are from Yoon et al. (2004) and again we amplify it as a single fragment (Fig. 6). As with the previous markers, we generate sequence with the PCR primers and then identify the best internal forward and reverse primers from our newly developed series abF1-8 and abR1-6, respectively (Fig. 6).

Amplification profile: 94°C for 2 min; 5 cycles of 94°C for 30 s, 45°C annealing for 30 s, 72°C extension for 1 min; then 35 cycles of 94°C for 30 s, 46.5°C annealing for 30 s, 72°C extension for 1 min; followed by 72°C final extension for 7 min.

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Photosystem II thylakoid membrane protein D1 (psbA, ~950 bp)

This is an easy marker to amplify with the two external PCR primers developed by Yoon et al. (2002) and works broadly among florideophyte algae (Fig. 7). While their (loc. cit.) internal forward sequencing primer psbA500F also works widely, we have had poor sequence for some taxa using the internal reverse primer psbA600R. We thus sequence with external primers first and then pick the best internal reverse option among psbA600R and the newly designed psbArev1 and psbArev2.

Amplification profile: 94°C for 2 min; 5 cycles of 94°C for 30 s, 45°C annealing for 30 s, 72°C extension for 1 min; then 35 cycles of 94°C for 30 s, 46.5°C annealing for 30 s, 72°C extension for 1 min; followed by 72°C final extension for 7 min.

Plastid LSU (23S) domain V (UPA, ~370 bp)

Sherwood and Presting (2007) championed UPA as a universal barcode marker for the algae noting that a single primer pair can reliably recover sequences from a broad taxonomic range including green, red, and brown marine macroalgae, diatoms, and even cyanobacteria. Whereas we have had strong success with their forward primer p23SrV_f1, the reverse primer p23SrV_r1 can result in messy sequence data for some florideophytes. Clarkston and Saunders (2010) reasoned that a repeat near the 3’ end of this primer (AGAG) was the cause of this problem and dropped the final nucleotide in designing the modified primer p23SnewR (Fig. 8). We have found this an easily amplified marker from a wide variety of florideophytes with this modification. Being a short marker, no internal primers are necessary to generate full bidirectional reads (Fig. 8).
Ribulose-1,5-bisphosphate carboxylase large subunit (\textit{rbcL}, \textasciitilde1,350 bp)

\begin{center}
\begin{tabular}{c c}
\textbf{Forward primers} & \textbf{Reverse primers} \\
F57 & rbcLevNEW ACAAATGCTGCAGTGGCCCTTC \\
Ruf & GCTTGGATTGACAGGAGCTCT \\
Ruf & rbcLevNEW GAAATGCTTATGTGTATGCC \\
M13P & rbcLevNEW TACACGCTTTGTTCAGCAG \\
TLF1 & rbcLevNEW AGCTATGCTTTGATTATCA \\
TLF2 & rbcLevNEW GAGAGACAGTACATATAG \\
TLF3 & rbcLevNEW CAGTCGTATAGTACATA \\
TLF4 & rbcLevNEW TACAGTGATGTGATGATG \\
TLF5 & rbcLevNEW GAGAGACAGTACATATAG \\
AcroF1 & rbcLevNEW GAAATGCTTATGTGTATGCC \\
\end{tabular}
\end{center}

Fig. 9. Overview of PCR / sequencing strategy for the plastid \textit{rbcL}. For the DNA barcode region \textit{rbcL}-3P the dashed line indicates that we amplify the entire fragment, but use sequence from the external 3' PCR primer (rbcLevNEW) to identify the best internal forward primer (TLF1-5, ACROF1) to generate a full bidirectional read for only the 3' end of this gene (solid line).

Amplification profile: 94°C for 2 min; 35 cycles of 94°C for 20 s, 55°C annealing for 30 s, 72°C extension for 30 s; followed by 72°C final extension for 10 min.

Ribulose-1,5-bisphosphate carboxylase large subunit (\textit{rbcL}, \textasciitilde1,350 bp)

This marker is widely used in florideophyte phylogenetic and barcoding studies and was first applied broadly to these algae by Freshwater et al. (1994). We still routinely use the forward PCR primer F57 (Freshwater and Rueness 1994), but also trial the M13-linked Ruf and RUfn for problematic taxa (Fig. 9). These last two primers are linked with one of the available M13 variants (note that the M13 variant here is different from that included below for COI-5P, but both should work; just make sure the M13 variants used for sequencing are a match for those employed in your primer). Thus sequencing employs only the linker portion of the primer when these are used in PCR (Fig. 9). We have also developed an M13 linked reverse primer (RUr), but typically find that our modification of Vis and Sheath’s (1999) \textit{rbcL} \textit{R} (rbcLevNEW) works for most red algae (Fig. 9). A key innovation here is to generate sequence data from the PCR primers and then identify the best internal primers from the forward and reverse series TLF1-5 (plus ACROF1) and TLR1-7, respectively (Fig. 9). This approach results in clean and full bidirectional reads for this marker allowing for the generation of quality sequences.

We are using \textit{rbcL}-3P (\textasciitilde785 bp) as a secondary DNA barcode marker (Fig. 9), however, owing to the lack of an internal “universal” primer, we amplify the entire fragment, sequence with the external reverse PCR primer and then use those data to identify the best internal forward primer (ACROF1, TLF1-5) to give a bidirectional read for only the 3’ end of the marker.

Amplification profile: 95°C for 2 min; 35 cycles of 93°C for 2 min, 47°C annealing for 1 min, 72°C extension for 2 min; followed by 72°C final extension for 2 min.

\textbf{MITOCHONDRIAL MARKERS}

\textit{Cytochrome c oxidase subunit 1 DNA barcode region (COI-5P, \textasciitilde664 bp)}

Saunders and McDevitt (2012) have recently reviewed the DNA barcode marker COI-5P in detail, but cautioned that primer advances were constantly being developed. So it is that an update is already due, largely improving on their earlier primers by adding variants of M13 linkers to the standard primers (Fig. 10). We now have two forward and two reverse primers that in varied combinations generate PCR product for most red algae. Owing to the short length of this fragment, bidirectional reads are accomplished without the need for internal primers (Fig. 10).

Amplification profile: 94°C for 2 min; 5 cycles of 94°C for 30 s, 45°C annealing for 30 s, 72°C extension for 1 min;
then 35 cycles of 94°C for 30 s, 46.5°C annealing for 30 s, 72°C extension for 1 min; followed by 72°C final extension for 7 min.

**Cytochrome c oxidase subunit 1 extended fragment (COI, ~1,232 bp)**

Saunders (2008) argued that COI-5P had phylogenetic signal for use among closely related taxa and we wanted to expand on this usefulness by generating a larger COI fragment (Fig. 11). Either the external forward M13-linked primers outlined previously (Fig. 10) or the unlinked variants reported with this marker are used in combination with the external reverse primers from the CoTR1-6 series for amplification (Fig. 11). We then sequence with the PCR primers and determine the best internal primers for

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**Fig. 10.** Overview of PCR / sequencing strategy for the mitochondrial COI-5P barcode marker. The order in which primer combinations are trialed for taxa is indicated from the first (best) to fourth option.

**Fig. 11.** Overview of PCR / sequencing strategy for the mitochondrial COI extended fragment.
and reverse (COBR1-5) sequencing primers (Fig. 12).
Amplification profile: 95°C for 1 min; 35 cycles of 93°C for 30 s, 42°C annealing for 30 s, 72°C extension for 1 min; followed by 72°C final extension for 7 min.

Cytochrome oxidase subunit 2-3 intergenic spacer (cox 2-3, ~350-400 bp)

This relative newcomer to red algal studies is amplified with the novel external primers CB44F and CB1006R designed by Ga Hun Boo (laboratory of Prof. Sung Min Boo, a RedToL partner at Chungnam National University, South Korea). Sequence is then generated with these same primers to identify the best internal forward (COBF1-4) and reverse (COBR1-5) sequencing primers (Fig. 12).
Amplification profile: 94°C for 4 min; 35 cycles of 94°C for 1 min, 45°C annealing for 30 s, 72°C extension for 1 min; followed by 72°C final extension for 7 min.

Cytochrome oxidase subunit 2-3 intergenic spacer (cox 2-3, ~350-400 bp)

This marker was first used in red algae by Zuccarello et al. (1999) and is a useful tool at the population level. We have only started to use this system and have not had to develop novel primers to date–so far the original primers of Zuccarello et al. have worked on a broad spectrum of taxa (Fig. 13).
Amplification profile: 94°C for 2 min; 5 cycles of 93°C for 1 min, 45°C annealing for 1 min, 72°C extension for 1 min; then 30 cycles of 93°C for 30 s, 55°C annealing for 30 s, 72°C extension for 30 s; followed by 72°C final extension for 7 min.

DISCUSSION

With the current rise in popularity and utility of powerful next generation technologies it is understandable that some of our colleagues may question the usefulness of summarizing single marker amplification protocols. Indeed there are currently three red algal genomes available in GenBank (Cyanidioschyzon, Galdieria, and Chondrus) with two more nearing completion (Porphyridium and Porphyra), while 16 additional species are in progress as part of the RedToL project (H.-S. Yoon personal communication). However, in our opinion there is still considerable value in being able to assign an unknown biological specimen to a genetic species with a simple PCR amplification and a single sequence read (both strands are not needed for routine identifications). We also see the value, at least for the near future, of targeted multi-gene phylogenetics, which will facilitate the inclusions of hundreds (e.g., RedToL is targeting ~650 species) rather than dozens of taxa in phylogenetic analyses. We thus hope that this summary will be of use to our colleagues.

A guiding principle for our approach to marker development and use is that primers are affordable and relatively easy to design and / or select once some sequence data are in hand. We thus routinely circumvent contamination and amplification problems by acquiring whatever portion of a marker we can for a specimen. Those data then “inform” our next steps with regards to amplification and or sequencing using primers currently in our lab or newly designed to meet a special need. An example is found in Clayden and Saunders (2010) in which LSU sequences for palmarialean taxa were compared to the host halymeniacean data to facilitate the development of the novel primers rsr1 and rsf1 for use with T01N and T15, respectively (Fig. 3). These primers were then used to amplify this marker as two overlapping fragments for the epi-endophyte Rhodonematella subimmersa (Setchell & N. L. Gardner) S. L. Clayden & G. W. Saunders from a bulk DNA extraction with its host.

We have extended this philosophy to markers for which relatively conservative external PCR primers have been developed, but for which “universal” internal sequencing primers remain elusive [i.e., EF2 (Fig. 4), psaA (Fig. 5), psaB (Fig. 6), psbA (Fig. 7), rbcL (Fig. 9), COI (Fig. 11) and COB (Fig. 12)]. In all of these cases we amplify and sequence with the PCR primers and then use those resulting data to “inform” our choice of the best internal sequencing primers to use from the options. This approach has greatly improved our overall success at getting quality, full bidirectional reads for these markers, all of which tend to lack internal conservative regions for “universal” primer design. In short, users should not be constrained by the fragments and primers as presented here and should explore alternative fragments and novel primers where necessary to circumvent problems of no amplification or contamination (e.g., Lane and Saunders 2005, Wynne and Saunders 2012).

However, one word of caution is necessary whenever markers are amplified as a series of fragments—always be wary of inadvertently generating chimeric sequences (e.g., for the LSU the X and Z fragments may be from your target species, but the Y fragment has amplified from a contaminant on or in the sample). We have encountered chimeric sequences in our own data and those of colleagues between various red and brown algae and have even uncovered mixed alga / invert sequences. This is going to happen, the critical point is to catch it when it does. The problem is obvious in the more variable markers where the fragments have a significant overlap (e.g., EF2 and rbcL) (Figs 4 & 9, respectively), but can be more difficult to detect in markers for which fragment overlap is short and usually corresponds to conservative regions of the markers (e.g., SSU and LSU, respectively) (Figs 1 & 3). We routinely generate trees for the various fragments to look for phylogenetic consistency among fragments for an isolate prior to combining them to generate a complete sequence. In addition, as GenBank becomes more populated with red algal sequences, simply blasting the fragments when you suspect a problem can highlight a chimeric sequence. On that last note, even markers amplified as a single fragment can be from contaminating organisms rather than the target species—a blast through the various databases such as GenBank (BOLD for COI-5P; http://www.boldsystems.org) is typically a prudent endeavor.

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REFERENCES


