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Universal plastid primers for Chlorophyta and Rhodophyta

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To date, the majority of molecular genetic studies in algae have utilized a fairly limited range of markers such as the plastid *rbc*L gene and spacer, the mitochondrial *cox*2-3 spacer or the nuclear ribosomal DNA and spacers. The lack of available markers has been particularly problematic in studies of within-species variation. Whilst microsatellites are now being developed in many algal species, there remains a need for universal markers that can be applied to a wide range of species. The increasing availability of complete plastid genome sequences for several algae has allowed us to develop two sets of universal primers, similar to those available in higher plants, for the amplification of coding and non-coding regions of the plastid genome in red and green algae. These markers are expected to be useful in a broad range of algal population genetic and phylogenetic studies.

Key words: Chlorophyta, chloroplast, genetic markers, plastid, Rhodophyta, universal primers

Introduction

The development of universal primers for the amplification of various regions of the chloroplast genome, particularly of non-coding DNA, has provided invaluable tools for evolutionary genetic studies in plants (Taberlet *et al*., 1991; Demesure *et al*., 1995; Dumolin-Lapegue *et al*., 1997). The original paper, describing three pairs of consensus primers for the amplification of the *trn*T – *trn*L intergenic region, the *trn*L intron and the *trn*L-*trn*F intergenic region in plant taxa ranging from bryophytes to angiosperms (Taberlet *et al*., 1991), has now been cited over 500 times. These universal plastid primers are routinely used in plant phylogenetic studies and particularly for population genetics since many of these regions, particularly the *trn*T – L – F intergenic regions and intron, display higher levels of sequence variation than more traditional markers such as the RuBisCO large subunit gene (*rbc*L; e.g. McDade & Moody, 1999).

In algae, phylogenetic studies have generally utilized either the *rbc*L gene of the plastid genome (e.g. Freshwater *et al*., 1994; McIvor *et al*., 2002) or the nuclear ribosomal DNA small subunit (SSU), and recently also some more variable domains of the large subunit (LSU; e.g. Harper & Saunders, 2001, 2002). For evolutionary studies at a finer scale, the lack of tools like the plant universal primers has been a handicap. The majority of phylogeographic studies have employed the internal transcribed spacers (ITS) in the ribosomal cistron of the nuclear genome (for review see Wattier & Maggs, 2001). The popularity of the ITS region can be attributed to the relatively high rate of nucleotide substitution, permitting comparison of relatively recently diverged taxa. In addition the ITS region can be readily PCR-amplified and sequenced with conserved primers positioned in the cistronic regions. However, many of the earlier studies used the suite of primers published by White *et al*. (1990) for fungi, and their lack of specificity often resulted in the amplification of spurious algal sequences (such as those investigated by Bown *et al*., 2003). Furthermore, the occurrence of intraindividual variation in ITS sequences means that cloning is often required prior to sequencing (Famà *et al*., 2000; Lange *et al*., 2002). Single-locus nuclear microsatellites have now been employed in population studies in red, green and brown algae, providing information on kinship, paternity and gene flow (e.g. van der Strate *et al*., 2002; Engel *et al*., 2002; Coyer *et al*., 2003). Their development has been very laborious, and in most cases they can be used only in the species for which they were defined, or for very closely related algae.

Two non-coding organellar DNA regions are widely used in algae. In many red and brown algae, the spacer between the genes for the large and small

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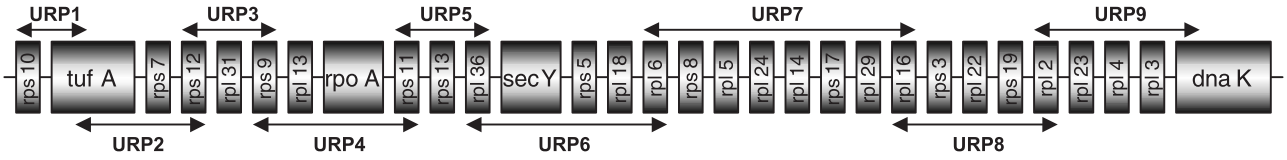

Fig. 1. Diagram showing the universal rhodophyte primer (URP) pairs designed to amplify the 13–15 kb *rps*10 – *dna*K ribosomal protein gene cluster conserved across *Porphyra purpurea*, *Cyanidium caldarium* and *Cyanidioschyzon merolae* (not shown to scale).

subunits of RuBisCo (*rbc*L–*rbc*S spacer) can be amplified using primers originally designed for members of the Gracilariaceae and Gigartinales (Destombe & Douglas, 1991; Maggs *et al*., 1992). This marker has been developed as a target for PCR–SSCP (single-stranded conformational polymorphism) (Zuccarello *et al*., 1999). However, the RuBisCo spacer is short (typically 75–120 bp), limiting its value. For red algae, the mitochondrial *cox*2-3 spacer amplified by the primers of Zuccarello *et al*. (1999) is a potentially universal marker for non-coding DNA. The spacer, approximately 300–350 bp long, has proved informative in a range of systematic and phylogeographic studies in red algae (Gabrielsen *et al*., 2002; Marston and Villalard-Bohnsack, 2002; Zuccarello & West, 2002; Zuccarello *et al*., 2002).

Although the various markers mentioned above have proved to be informative in particular cases, often they are not appropriate e.g. they do not provide enough resolution (at the population level) or they are too variable and approaching saturation (at higher taxonomic levels). Increasingly, therefore, phylogenetic and phylogeographic studies require two or more sets of data, aimed at resolving different depths of evolutionary relationships (e.g. Bellorin *et al*., 2002; Draisma *et al*., 2002; Hayden *et al*., 2003).

There are several reasons why universal primers similar to the ones described for plants have not been developed for algae. Firstly, comparison of the available algal plastid genome sequences (see below) shows that they display high levels of structural and sequence diversity compared with plants, even within the divisions/phyla Chlorophyta (green algae) and Rhodophyta (red algae). Furthermore, such comparisons also show that there tends to be a lower percentage of intergenic/intronic DNA in algal genomes, particularly in the highly compact plastid genomes of rhodophytes (with certain exceptions e.g. the *rbc*L–*rbc*S spacer). Finally, the availability of algal plastid sequence data is limited: until fairly recently, the only complete plastid genome sequences available were those of the rhodophyte *Porphyra purpurea* (Reith & Munholland, 1995), the glaucocystophyte *Cyanophora paradoxa* (Stirewalt *et al*., 1995), the heterokont *Odontella sinensis* (Kowallik *et al*., 1995) and the chlorophyte *Chlorella vulgaris* (Wakasugi *et al*., 1997).

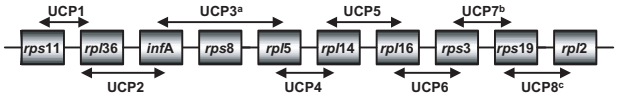

Fig. 2. Diagram showing the universal chlorophyte primer (UCP) pairs designed to amplify the ~5 kb *rps*11–*rpl*2 gene cluster conserved across *Nephroselmis olivacea, Chlorella vulgaris, Mesostigma viride* and *Chaetosphaeridium globosum* (not shown to scale). (a) Primer pair UPC3 also amplifies *ORF*54 which lies between *rps*8 and *rpl*5 in *Chlorella vulgaris*. (b) Primer pair UPC7 also amplifies the *rps*22 gene which lies between *rps*3 and *rps*19 in *Mesostigma viride* and *Chaetosphaeridium globosum*. (c) Primer pair UPC8 also amplifies *ORF*45 which lies between *rps*19 and *rpl*2 in *Chlorella vulgaris*.

Attempts to use the plant universal chloroplast primers in algae have met with extremely limited success – a few work in green algae (Taberlet *et al*., 1991) but none have been successfully amplified in red algae due to the degree of divergence between streptophytes and rhodophytes and high levels of plastid genome rearrangement (Ohta *et al*., 1997; Wattier *et al*., 2001). Advances in DNA sequencing methodologies and sequence analysis have led to a vast increase in information available in DNA sequence databanks. The establishment of genome sequencing projects in a variety of organisms has resulted in a proliferation of complete chloroplast genome sequences from a range of plants and algae including the rhodophytes *Cyanidium caldarium* (Glockner *et al*., 2000) and *Cyanidioschyzon merolae* (Ohta *et al*., 2003), the chlorophytes *Nephroselmis olivacea* (Turmel *et al*., 1999) and *Mesostigma viride* (Lemieux *et al*., 2000), the charophyte *Chaetosphaeridium globosum* (Turmel *et al*., 2002) and the cryptophyte *Guillardia theta* (Douglas & Penny, 1999). Consequently, it is now possible to use comparative genomics to develop novel tools for molecular analyses in algae. We have utilized such an approach to develop two sets of universal primers for the amplification of coding and non-coding sections of the plastid genome in red and green algae.

Table 1. Universal rhodophyte primers (see Fig. 1 for details of loci)

Locus	Primers	*Porphyra purpurea*		*Cyanidium caldarium*		*Cyanidioschyzon merolae*		Annealing temperature
		Size[a]	Non-coding[b]	Size	Non-coding	Size	Non-coding	
URP1	GAATCTTTATTTACATGWGG AGAAGAAGGWGGAAGACAT	464 bp	15 bp	468 bp	7 bp	440 bp	15 bp	52°C
URP2	ATGTCTTCCWCCTTCTTCT CATAATWTWCAAGAGCATTC	1772 bp	150 bp	1751 bp	127 bp	1598 bp	27 bp	52°C
URP3	GAATGCTCTTGWAWTTATG GAAAGCWAGAAAAGCWCC	579 bp	96 bp	592 bp	96 bp	502 bp	8 bp	50°C
URP3	TGWGGWGCTTTTCTWGCTT CAAAAAAAGGWAGWCCATTTG	2333 bp	221 bp	2074 bp	22 bp	1840 bp	8 bp	54°C
URP5	CAAATGGWGTWCCTTTTTTTG AAGCATAAGCAAAGACAAGG	617 bp	85 bp	621 bp	44 bp	563 bp	3 bp	56°C
URP6	GTCTTTGTTTATGTTTWGGATT TTATAAGGGWAAAGGAATTCG	2529 bp	218 bp	2404 bp	186 bp	1929 bp	9 bp	56°C
URP7	CGAATTCCTTTWCCTTTATAA ATGGGHTCWGGKAAAGG	2804 bp	179 bp	2783 bp	257 bp	2335 bp	–	50°C
URP8	CCTTTMCCWGADCCCAT GTAGATCATCCKCATGG	1958 bp	86 bp	1912 bp	197 bp	1760 bp	57 bp	50°C
URP9	CCATGMGGATGATCTAC CTTGDGCHGAAATTTCTTC	3006 bp	297 bp	2927 bp	242 bp	2366 bp	37 bp	50°C

[a] Size of PCR product.
[b] Total non-coding DNA in PCR product.

Table 2. Universal chlorophyte primers (see Fig. 2 for details of loci)

Locus	Primers	*Nephroselmis olivacea*		*Chlorella vulgaris*		*Mesostigma viride*		*Chaetosphaeridium globosum*		Annealing temperature
		Size[a]	Non-coding[b]	Size	Non-coding	Size	Non-coding	Size	Non-coding	
UCP1	CAAGCWCCDGCAGAAGACC CCMAAACATAAACAAMSWCAGG	384 bp	207 bp	444 bp	266 bp	284 bp	106 bp	247 bp	68 bp	54°C
UCP2	CCTTGWCKTTGTTTATGTTTKGG GCTCATGTYTCHGGBAAAATWCG	391 bp	147 bp	281 bp	37 bp	329 bp	91 bp	132 bp	45 bp	56°C
UCP3	CGWATTTTVCCDGAGATATGGGC ATGTATGCKTTTTTAGATCGT	824 bp	47 bp	1341 bp	377 bp	1048 bp	282 bp	1076 bp	124 bp	50°C
UCP4	ACGATCTAAAAAMGCATACAT AATTGTWTCDTTDGCACCDGAAGT	367 bp	32 bp	371 bp	35 bp	421 bp	86 bp	367 bp	31 bp	50°C
UCP5	ACTTCHGGTGCHAGHGAWATAATT GAAACHCGDATGGGDTCKGG	818 bp	290 bp	1308 bp	778 bp	795 bp	259 bp	598 bp	56 bp	54°C
UCP6	CCMGAHCCCATHCGDGTTTC GGBMGHTTWAATGGHGCHGAWAT	712 bp	260 bp	679 bp	136 bp	513 bp	81 bp	931 bp	92 bp	56°C
UCP7	ATWTCDGCDCCATTWAGDCKVCC ATGGTWGGWCAWAAATTDGGTGAGTTT	571 bp	14 bp	523 bp	7 bp	1003 bp	66 bp	1084 bp	43 bp	56°C
UCP8	AAATTCGCCHAGTTTWTGWCCWATCAT GCHCAAHTDGTDGCNAAAGAGGG	582 bp	14 bp	807 bp	101 bp	649 bp	76 bp	635 bp	43 bp	56°C

[a] Size of PCR product.
[b] Total non-coding DNA in PCR product.

Materials and methods

Primer design

Since the plastid genomes of red and green algae are so divergent (Ohta *et al*., 1997), it is unlikely that truly universal primers could be developed that would amplify in both Rhodophyta and Chlorophyta. We therefore decided to design separate sets of primers for each phylum. Universal primers for rhodophytes were designed by a comparative analysis of the complete plastid genome sequences of *Porphyra purpurea* (Roth) C. Agardh (GenBank accession number U38804; Reith & Munholland, 1995), *Cyanidium caldarium* Geitler (AF022106; Glockner *et al*., 2000) and *Cyanidioschyzon merolae* De Luca, Taddei *et* Varano (AB005283; Ohta *et al*., 2003). For chlorophytes, the complete plastid sequences of *Chlorella vulgaris* (AB001684; Wakasugi *et al*., 1997), *Nephroselmis olivacea* Stein (AF137379; Turmel *et al*., 1999), *Mesostigma viride* Lauterborn (AF166114; Lemieux *et al*., 2000) and *Chaetosphaeridium globosum* (Nordstedt) Klebahn (AF494278; Turmel *et al*., 2002) were used. A search for syntenic regions of the genomes revealed that the cluster of genes containing mainly ribosomal proteins between *rps*10 and *dna*K was conserved across all three rhodophytes (Fig. 1) and that the gene cluster between *rps*11 and *rpl*2 was conserved in chlorophytes (Figure 2). Sequences of individual genes were edited using the ASSEMBLE program (Genetics Computer Group, Wisconsin, USA) and aligned using the BioEdit software package (V5.0.9; www.mbio.ncsu.edu/BioEdit/bioedit.html). For rhodophytes, nine overlapping pairs of primers designed to amplify the complete cluster of 30 genes (13 – 17 kb) are shown in Table 1. For chlorophytes, eight pairs of primers (Table 2) were designed to amplify the cluster of 10 – 12 genes ($\sim$5 kb).

Primer utility

To assess the cross-species utility of the rhodophyte primers, they were tested on the following species: *Porphyra purpurea* (Bangiales), *Rhodochorton purpureum* (Lightfoot) Rosenvinge (Acrochaetiales), *Phycodrys rubens* (L.) Batters (Ceramiales), *Corallina officinalis* L. (Corallinales), *Gelidium pulchellum* (Turner) Kützing (Gelidiales), *Mastocarpus stellatus* (Stackhouse) Guiry (Gigartinales), *Palmaria palmata* (L.) O. Kuntze (Palmariales) and *Plocamium cartilagineum* (L.) P. Dixon (Plocamiales). The chlorophyte primers were tested on *Dunaliella salina* Teodoresco (Chlorophyceae), *Ulva lactuca* L. (Ulvophyceae), *Codium fragile* (Suringar) Hariot (Ulvophyceae) and *Coleochaete orbicularis* Pringsheim (Charophyceae). Total DNA was extracted either by the phenol-chloroform protocol of Wattier *et al*. (2000) or using DNeasy Mini-Preps (Quiagen, Hilden). PCR was carried out on a MWG Primus thermal cycler using the following parameters: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48 – 56°C (see Tables 1 and 2 for optimum annealing temperatures) for 1 min, extension at 72°C for 1 – 5 min (1 min per kb of expected sized product) and a final extension at 72°C for 5 min. PCR was carried out in a total volume of 10 μl containing 100 ng DNA, 5 pmol of forward and reverse primers, 1 × PCR reaction buffer (5 mM Tris-HCl [pH9.1], 1.6 mM $[NH_4]_2SO_4$, 15 μg μl^{-1} BSA), 2.5 mM $MgCl_2$ and 1 U *Taq* polymerase (Genetix). PCR products were resolved on 2% agarose gels and visualised by ethidium bromide staining.

Results and discussion

As recently as July 2001, Wattier *et al*. (2001) stated that '... the evolutionary diversity of the Rhodophyta is so great that the possibility of designing universal primers for them is very limited.' Thanks to recent advances in whole-genome sequencing, comparative approaches now offer real scope for developing truly universal markers in both red and green algae. We have used such an approach to develop a suite of primers to amplify the 13 – 17 kb *rps*10-*dna*K gene cluster in all rhodophytes and another to amplify the 5 kb *rps*11-*rpl*2 cluster in chlorophytes. The new primers reported here

Table 3. Cross species amplification of rhodophyte universal primers. Ppu – *Porphyra purpurea*; Rpu – *Rhodochorton purpureum*; Pru – *Phycodrys rubens*; Cof – *Corallina officinalis*; Gpu – *Gelidium pulchellum*; Mst – *Mastocarpus stellatus*; Ppa – *Palmaria palmata*; Pca – *Plocamium cartilagineum*

	Species							
Locus	Ppu	Rpu	Pru	Cof	Gpu	Mst	Ppa	Pca
URP1	✓	✓	✓	✓	✓	✓	✓	✓
URP2	✓	–	✓	✓	✓	✓	✓	–
URP3	✓	✓	✓	–	–	–	✓	✓
URP4	✓	–	–	✓	–	✓	✓	–
URP5	✓	✓	–	–	✓	✓	✓	✓
URP6	✓	✓	–	✓	–	✓	✓	–
URP7	✓	✓	–	✓	✓	–	–	–
URP8		–	–	–	–	✓	✓	✓
URP9	✓	✓	–	✓	–	✓	–	–

represent much-needed additional tools for population and evolutionary studies in both red and green algae.

The amplification success of primer pairs varied between 50% and 100% in the species tested in this study and generated PCR products of the expected size, based on the previously published complete plastid genome sequences (Tables 3 and 4). As with all universal primers, some degree of largely empirical work is necessary to optimize reaction conditions in different taxa, particularly with those primers that amplify larger PCR products. Under the correct conditions, however, most of the primer pairs amplified a single band in each species studied (see Tables 3 and 4). Whilst it may take some effort to optimise PCR conditions for all primers, it is possible to use them to amplify a product in the species under study, sequence the product and then develop potentially more robust species-specific or clade-specific primers for subsequent analyses (e.g. to minimise the problem of amplifying products from epiphytic algae or, conversely, the hosts of parasites or endophytes). Furthermore, since the universal chlorophyte primers tend to amplify shorter products between contiguous genes, it is possible to 'skip' a gene and use a combination of primers (e.g. use the forward primer for UCP1 and the reverse primer for UCP2) to amplify larger regions, particularly if it proves difficult to optimize a specific pair of primers for the alga(e) in question.

The various regions amplified by these primers display a range of levels of sequence divergence between the species from which they were designed. Pairwise sequence identities ranged from 40% (UCP7, *Nephroselmis olivacea* vs *Chaetosphaeridium globosum* and *Chlorella vulgaris* vs *Chaetosphaeridium globosum*) to 69% (UCP2, *Mesostigma viride* vs *Chaetosphaeridium globosum*; Tables 5 and 6), suggesting that different primer pairs can be

Table 4. Cross species amplification of chlorophyte universal primers. Dsa – *Dunaliella salina*; Ula – *Ulva lactuca*; Cfr – *Codium fragile*; Cor – *Coleochaete orbicularis*

	Species			
Locus	Dsa	Ula	Cfr	Cor
UCP1	✓	–	–	✓
UCP2	–	✓	✓	–
UCP3	✓	✓	✓	✓
UCP4	✓	–	–	✓
UCP5	✓	✓	✓	✓
UCP6	–	✓	✓	✓
UCP7	–	✓	✓	–
UCP8	✓	–	✓	–

Table 5. Pairwise sequence similarity by locus for rhodophyte universal primers

Locus	*Porphyra purpurea* vs *Cyanidium caldarium*	*Porphyra purpurea* vs *Cyanidioschyzon merolae*	*Cyanidium caldarium* vs *Cyanidioschyzon merolae*	Average
URP1	0.67	0.59	0.56	0.61
URP2	0.65	0.62	0.60	0.62
URP3	0.61	0.50	0.48	0.53
URP4	0.46	0.47	0.43	0.45
URP5	0.48	0.52	0.54	0.51
URP6	0.47	0.39	0.38	0.41
URP7	0.53	0.42	0.40	0.45
URP8	0.54	0.48	0.46	0.49
URP9	0.48	0.38	0.37	0.41

Table 6. Pairwise similarity by locus for chlorophyte universal plastid primers (*N. o.* – *Nephroselmis olivacea; C. v.* – *Chlorella vulgaris; M. v.* – *Mesostigma viride; C. g.* – *Chaetosphaeridium globosum*.)

Locus	*N. o.* vs *C. v.*	*N. o.* vs *M. v.*	*N. o.* vs *C. g.*	*C. v.* vs *M. v.*	*C. v.* vs *C. g.*	*M. v.* vs *C. g.*	Average
UCP1	0.43	0.48	0.53	0.51	0.54	0.57	0.51
UCP2	0.45	0.43	0.61	0.48	0.68	0.69	0.56
UCP3	0.42	0.53	0.44	0.44	0.44	0.56	0.46
UCP4	0.59	0.65	0.62	0.59	0.62	0.67	0.62
UCP5	0.45	0.53	0.56	0.51	0.56	0.58	0.52
UCP6	0.47	0.50	0.47	0.53	0.47	0.52	0.49
UCP7	0.43	0.56	0.40	0.45	0.40	0.45	0.45
UCP8	0.52	0.55	0.49	0.49	0.49	0.61	0.54

used to provide the desired degree of resolution appropriate for the taxonomic level under study. For example, less conserved loci may be useful for population level studies whereas the primers that amplify the more conserved regions would be better suited to analysis at the specific or generic level. Since the amount of non-coding DNA amplified by each primer pair varies greatly across taxa, however, it is not feasible to calculate levels of divergence separately for coding and non-coding regions.

Although a gene cluster identical to that found in rhodophytes is also present in the secondary plastid genomes of *Odontella sinensis* and *Guillardia theta*, higher levels of sequence divergence between rhodophytes and those algae with secondary red plastids (including heterokonts, haptophytes and cryptophytes; Stoebe & Kowallik, 1999) mean that it is unlikely that these primers will amplify as successfully in these taxa as as in rhodophytes. Because of this, it may be feasible to use a similar approach based on the plastid genome sequences of *Porphyra purpurea, Cyanidium caldarium, Odontella sinensis* and *Guillardia theta* to design a comparable set of primers for use in lineages that acquired red-type plastids via secondary endosymbiotic events. Interestingly, examination of the plastid genome of the glaucocystophyte *Cyanophora paradoxa* reveals that the same cluster appears to have been 'split' into four and redistributed around the genome but, again, it is unlikely that our primers will amplify in glaucocystophytes.

In summary, we have developed a novel suite of primers that provide potentially valuable tools for both population and phylogenetic studies in red and green algae. As the existing repertoire of markers is extremely limited, the effort of optimising universal primers in individual studies is greatly outweighed by the benefits of having new markers, particularly where existing ones were unsuitable.

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