Chapter 6

Primer Design for the Analysis of Closely Related Species: Application of Noncoding mtDNA and cpDNA Sequences

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Abstract

Noncoding regions of the chloroplast (cpDNA) and mitochondrial (mtDNA) genomes are commonly used in plant phylogenetic and population studies. Consensus primers, which are homologous to most coding regions, but amplify variable noncoding regions, are very useful for this purpose. However, high genetic diversity of plants poses a problem in developing molecular methods that require conserved DNA sequences between species.

This chapter describes the protocol for designing PCR primers suitable for analysis of closely related plant species. As an example, we used PCR primer design for cpDNA noncoding regions of the rye (*Secale*).

Key words Primer design, Noncoding sequences, Related species, Species identification, mtDNA, cpDNA1

1 Introduction

Molecular markers are an important tool in species classification as they can directly detect gene variations. In phylogenetic studies, it is very important to select appropriate sequences, depending on the taxonomic level at which the phylogenetic reconstruction is carried out. The primary choice regards highly variable rapidly evolving sequences. The more closely related are the tested subjects, the less variable the studied region should be. Hence, the relatively slow rate of evolution of certain sequences may exclude statistically significant analyses within a family or species, while determining the relationship between phylogenetically more distant species using slower evolving sequences can be very informative. Generally, coding sequences have a slower rate of evolution than noncoding sequences [1–5]. These regions accumulate more deletions/insertions or substitutions than coding regions, and may therefore be more suitable at the intergeneric or intrageneric level [6–8].

Noncoding regions of the chloroplast and mitochondrial genomes have been described as highly variable [6, 7, 9–16]. This has

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led to the design of "universal" primer pairs, which enable the amplification of noncoding regions separating two coding fragments in most plant species [17–20] (Table 1). The use of such consensus primers, which are homologous to most coding regions, but amplify variable noncoding regions is very useful in phylogenetic and population studies [19–21].

It has been shown that "universal" PCR primers can be effectively used to detect plant DNA from many taxonomic groups [12, 18]. However, it is often difficult to adapt one target region to all genetic varieties in different plant lines, which results in amplification failures in many species.

The current work presents the reassessment of PCR primers commonly used to amplify cpDNA and mtDNA regions in plants as well as the methods of developing primers adapted to the tested material. New primers were able to amplify the entire region in representative samples of 13 species and subspecies of the genus *Secale*. These primers can be used in various fields of plant research, including DNA barcoding, molecular ecology, metagenomics, or phylogenetic research.

2 Materials

2.1 PCR Evaluation

1. Primers can be ordered from different manufacturers (e.g., Genomed, IDT).
2. DNA from closely related plant species and subspecies: DNA can be isolated using various methods (*see* **Note 1**) or commercial kits (e.g., Maxwell® Tissue DNA Purification Kit (Promega)).
3. UV/Vis spectrophotometer (e.g., Nanodrop (Thermo Fisher)).
4. PCR reagents (e.g., ThermoFisher).
5. Thermocycler (e.g., BioRad).
6. Electrophoresis apparatus (e.g., TKBiotech).

2.2 Sequence Comparison and Primer Design

1. NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore).
2. Sequence alignment program (e.g., MEGA7 (https://megasoftware.net/)).
3. Primer analysis (e.g., OligoAnalyzer (https://eu.idtdna.com/calc/analyzer)).

Table 1
Primer list of sequences (5′–3′) for amplification of different regions of cpDNA and mtDNA and their references used in this study

Type of DNA	Locus	Direction	Sequence 5′–3′	Reference
cpDNA	*atpB-rbcL*	Forward	ACATCKA RTACKGGACCAA TAA	Chiang et al. [17]
		Reverse	AACACCAGCTTTRAA TCCAA	Chiang et al. [17]
	trnT (UGU)–*trnL* (UAA) exon	Forward	CATTACAAATGCGA TGCTCT	Taberlet et al. [18]
		Reverse	TCTACCGA TTTCGCCATATC	Taberlet et al. [18]
	trnL (UAA) intron	Forward	CGAAATCGG TAGACGCTACG	Taberlet et al. [18]
		Reverse	GGGGATAGAGGGAC TTGAAC	Taberlet et al. [18]
	trnD[tRNA–Asp(GUC)]-*trnT* [tRNA–Thr(GGU)]	Forward	ACCAATTGAACTACAA TCCC	Demesure et al. [19]
		Reverse	CCCTTTTAACTCAG TGGTAG	Demesure et al. [19]
mtDNA	*nad1* exon B-*nad1* exon C intron	Forward	GCATTACGATC TGCAGCTCA	Demesure et al. [19]
		Reverse	GGAGCTCGATTAG TTTCTGC	Demesure et al. [19]
	nad4/1-2	Forward	CAGTGGGTTGGTC TGGTAATG	Demesure et al. [19]
		Reverse	TCATATGGGCTAC TGAGGAG	Demesure et al. [19]
	nad4L-orf25	Forward	CTGTYTTTTCGCAC TTAGGC	Duminil et al. [22]
		Reverse	GTCCGRGGTACTA TTGCTGT	Duminil et al. [22]
	rps12-1/nad3-2	Forward	TTTCTTCTCTACCA TGACGA	Duminil et al. [22]
		Reverse	TGATCCYACTCGG TSTTCCT	Duminil et al. [22]
	rps12-2/nad3-1	Forward	ACCATATTTDGATC TGCCDC	Duminil et al. [22]
		Reverse	YACGATHGGATTTC TMTATG	Duminil et al. [22]
	rrn5/rrn18-1	Forward	GAGGTCGGAATGGGA TCGGG	Duminil et al. [22]
		Reverse	GGGTGAAGTCG TAACAAGGT	Duminil et al. [22]

3 Methods

3.1 DNA Isolation and PCR Evaluation

1. Isolate DNA (*see* **Note 1**).
2. Run PCR amplification of cpDNA and mtDNA using consensus primers (Table 1) (*see* **Note 2**).
3. Run PCR amplification of cpDNA in 25-μl reaction mixtures containing approximately 50–150 ng of genomic DNA template, 2.0–3.0 mM $MgCl_2$, 0.2–1.0 mM each dNTP, 0.1–1μM each primer, 0.1 mg BSA/ml, and 1 U of *Taq* DNA polymerase. Specifics of reaction conditions and components for amplification of each analyzed regions are given in Table 2.
4. Run PCR amplification of mtDNA in 25-μl reaction mixtures containing approximately 75–250 ng genomic DNA template, 2.5–4.0 mM $MgCl_2$, 0.1–0.2 mM each dNTP, 0.2–0.55μM each primer, 0.05 mg BSA/ml, and 1–1.5 U *Taq* DNA polymerase. Specifics of reaction conditions and components for amplification of each analyzed regions are given in Table 3.
5. Run electrophoresis of the obtained products on a 1.5% (m/v) agarose gel in a 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA, pH 8.3).
6. Sequence PCR products.

3.2 In Silico Evaluation

Built multiple sequence alignments.

1. Align sequences according to the instruction for the appropriate sequence alignment software (e.g., MEGA7) (*see* **Note 3**). An example of a multiple sequence alignment is shown in Fig. 1.
2. Select "Edit", "Select all", subsequently "Alignment" and "Align by ClustalW".
3. Select the most conserved regions and use them to design the primer (*see* **Note 4**).
4. Copy the 18–35 nucleotide segment from the 5′ strand of the consensus sequence and the 18–35 3′ nucleotide segment to the text file. Be sure to select a fragment that contains consensus sequences and adjacent variable sequences. These sequences will be assessed for potential use as 5′ and 3′ primers (*see* **Note 5**).
5. PCR primers evaluated in this example are shown in Fig. 2.
6. Evaluate potential primers using the free web analysis tool—OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer). First paste the 5′ primer into the program window. Default settings are used for all calculations. Select "Analyze". Record the results for the 5′ primer length, melting point, and GC content in the text file. To adjust melting temperature

Table 2
Thermocycling conditions for a PCR amplification of cpDNA noncoding (intron) regions [21]

	atpB-rbcL		*trnT* (UGU)-trnL (UAA) 5′exon		*trnL* (UAA) intron		*trnD* [tRNA–Asp(GUC)]-*trnT* [tRNA–Thr (GGU)]	
Phase of PCR	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**
Initial denaturation	94	4 min	94	3 min	94	3 min	94	4 min
Denaturation	92	45 s	94	1 min	94	1 min	92	45 s
Primer annealing	53.5	45 s	55	1 min	62	1 min	53.5	45 s
Primer extension	72	2 min	72	2 min	72	2 min	72	2 min
Final extension	72	10 min	72	10 min	72	10 min	72	10 min
Number of cycles	30		35		35		30	

Table 3
Thermocycling conditions for a PCR amplification of mtDNA noncoding (intron) regions [21]

	nad1 exon B *nad1* exon C intron		*nad4*/1-2		*nad4L-orf25*		*rps12-1/nad3*(2)		*rps12-1/nad3*(1)		*rrn5/rrn18-1*	
Phase of PCR	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**
Initial denaturation	94	1 min	94	1 min	94	1 min	94	12 min	94	12 min	94	12 min
Denaturation	94	45 s	94	45 s	94	45 s	94	45 s	94	45 s	94	45 s
Primer annealing	52	45 s	52	45 s	51.5	45 s	52	45 s	52	45 s	52	45 s
Primer extension	72	1 min	72	1 min	72	1 min	72	1 min	72	1 min	72	1 min
Final extension	72	10 min	72	10 min	72	10 min	72	10 min	72	10 min	72	10 min
Number of cycles	40		40		40		40		40		40	

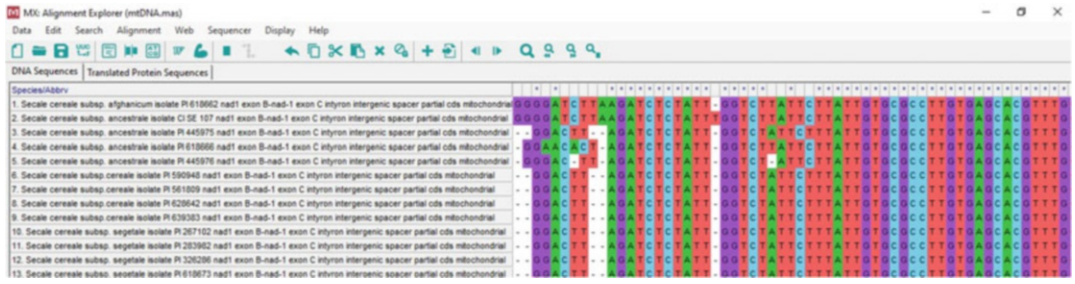

Fig. 1 An example of a multiple sequence alignment

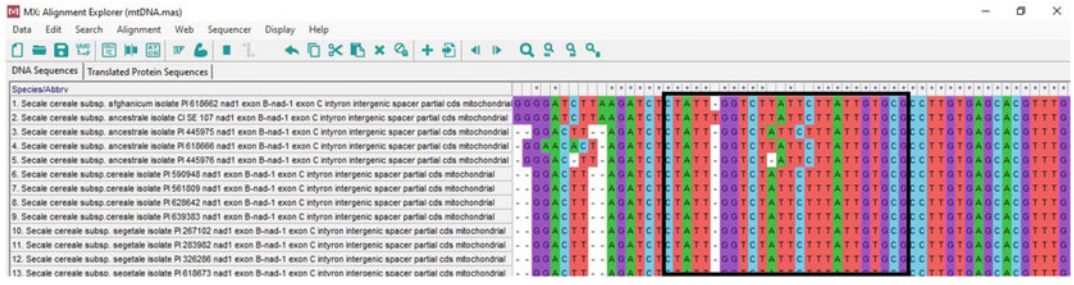

Fig. 2 PCR primers evaluated in this example

(if necessary), remove or add one or more bases to the end of the primer (*see* **Note 6**).

7. Test primers for secondary structure formation, such as homodimers and heterodimers. Perform "hairpin", "self-dimer", and "hetero-dimer" analysis on the 5′ primer. Record the results (*see* **Note 7**).
8. If primers meet in silico criteria, order the primers and test them by performing PCR.

3.3 Optimization of PCR Conditions

A number of parameters affect amplification efficiency, including primer and magnesium ion concentrations.

1. Change primer concentration and forward to reverse primer ratio or DNA concentration (*see* **Note 8**).
2. Test another polymerase or commercial PCR kit (*see* **Note 9**).

4 Notes

1. Fresh coleoptiles and leaves (etiolated), collected approximately 5–7 days after sowing on sterile plates with a blotting paper, are best suited for DNA extraction from plant tissues. The highest isolate concentration can be obtained using traditional methods (e.g., CTAB). Best purity – by using commercial kits (e.g., DNAeasy Plant Kit—Wizard® Genomic DNA Promega).

2. Concentration was adjusted to ~50 ng/μl for the amplification of noncoding (intron) regions.
3. Sequence alignment must be performed for each region separately.
4. Select conserved regions used as primer binding sites that flank the variable regions commonly used in phylogenetics and population genetics studies [12].
5. Common mistake during reverse primer design is ordering the reverse primer in the 5′–3′ direction without changing nucleotides into reverse complement.
6. The use of higher primer melting temperatures will promote more specific primer binding to the template. If Tm is too low (52–53 °C), more bases can be added to the primer (preferably more C or G).
7. Hairpin melting temperature should be significantly lower than primer annealing temperature in a PCR in which the hairpin is denatured.
8. 0.2 or 1.0 mM each primer and DNA concentrations of 50–150 ng can be used. 0.1 mg/ml BSA can optionally be used.
9. In this example, *Taq* polymerase was used.

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