

Toru Nishizawa and Yasuyuki Watano : **Primer pairs suitable for PCR-SSCP analysis of chloroplast DNA in angiosperms**

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PCR-based genetic markers from chloroplast DNA (cpDNA) have been increasingly used at the intraspecific level for the study of phylogeography and population genetics, or at the hybridizing species complex level to detect interspecific gene flow. In contrast to animal mitochondrial DNA, however, cpDNA are characterized by a low mutation rate, which is a serious limitation to detect variations at intraspecific or closely-related species level. In order to overcome this limitation, universal primers that amplify non-coding regions are very useful (Dumolin-Lapegue et al. 1997).

PCR products amplified by universal primers are usually analyzed by direct-sequencing or restriction fragment length polymorphisms (RFLPs). DNA sequencing gives the most fundamental genetic information. However, most population level studies require large sample sizes. Obtaining DNA sequence data for all samples can be expensive and time-consuming. RFLPs are more effective for large samples. However, this analysis is not very sensitive because there is a reduced chance that a single base change will alter any particular cleavage sites. In some cases, therefore, even the search for a single polymorphism can be quite cumbersome.

Recently, highly sensitive electrophoretic techniques for detecting DNA sequence variation have been developed (Lessa and Applebaum 1993). Single-strand conformation polymorphism (SSCP) is one of these DNA screening techniques. SSCP is performed by denaturing dsDNA and fractionating the strands on non-denaturing polyacrylamide gel. Under appropriate electrophoretic conditions, SSCP can effectively distinguish DNA fragments that differ by as little as a single base substitution (Yap and MacGee 1994). It is expected that a combination

of SSCP and PCR with universal primers can significantly facilitate cpDNA marker development.

Despite its usefulness, SSCP has a limitation concerning the size of applicable DNA fragment. The DNA fragments shorter than 400 bp are commonly used to ensure effective detection of mutations (Yap and MacGee 1994). However, most universal cpDNA primers amplifying non-coding regions have been designed for the purpose of DNA sequencing or PCR-RFLPs, and thus the PCR products are typically longer than 1000 bp (Dumolin-Lapegue et al. 1997). Therefore, universal primers amplifying small DNA fragments are needed to use PCR-SSCP for cpDNA marker development. Here, we describe new primers suitable for PCR-SSCP analyses of cpDNA in angiosperms.

Materials and methods

We first calculated the length of introns or intergenic spacers throughout the large single-copy region of tobacco chloroplast genome (Shinozaki et al. 1986). Then, non-coding regions with suitable length (150-350 bp) were listed up as the candidates to be amplified. As for the relatively long non-coding regions, we searched the conserved sequences within introns or spacers by aligning the sequences of tobacco and rice cpDNAs (Hiratsuka et al. 1989). Primers were designed to be anchored within the coding regions flanking non-coding regions, or within the conserved sequences of non-coding regions, using DNASIS ver. 3.0 (Hitachi software engineering). The sequences selected by DNASIS were checked with Amplify ver. 1.2 (B. Engels, University of Wisconsin) by using tobacco and rice cpDNAs as target sequences.

The primers designed were further tested for their universality by practical amplification from

Table 1. Sequences of primers designed for PCR-SSCP of chloroplast genome in angiosperms

Code#	Primer 1	Position*	Primer 2	Position*	Size**	Comment
1	rps16/1 F	5340	CCCCTAGAAAACGTATAGGA	5751	CGAAGTAATGTCTAAACCCA	412 rps16 intron
2	rps16/2 F	5732	TGGGTTTAGACATYACTTTCG	6038	ATAGTCCATGATGGAGCTCG	307 rps16 intron
3	UCC/2 F	9515	GGTAAAAGTGTGATTCGTTC	9785	AICTTTCATCCATGGATCCT	271 trnG (UCC) intron
4	UCC/1 F	9895	ATATTGTTTGTAGCTCGGTGG	10148	GTTTCATCCGGCTCCCTTAT	254 trnG (UCC) intron
5	atpF/F	12687	TTCATTTGGCTCTCACGGCTC	12933	AAATGCTGAATCGACGACCTA	247 atpF intron
6	psbC-trnS/F	36867	TGAACCTGTTCTTTCCATGA	37172	GAACATTCGAGGTTCCGAAT	306 psbC-trnS (UGA), intergenic
7	trnG-fm/F	38083	TCTCTTTGCCAAGGAGAAGA	38394	ATAACTTGGAGTACACGGGT	312 trnG (GCC)-trnM (CAU), intergenic
8	trnV-M/F	54362	TGTAACCGAGTTGCTCTACC	54610	CTAACCTGAGTTAAGTAG	249 trnV (UAC)-trnM (CAU), intergenic
9	trnW-P/F	68327	GATTTGAACTAGCACATCC	69123	GATGTGGCCAGCTTGGTAG	297 trnW (CCA)-trnP (UGG), intergenic
10	petB/1 F	77517	AGAGATGGTTCTACTTCGTC	77846	TTCATACTAGAACCCAGATG	330 petB intron
11	petB/2 F	77834	GTCTAGTATGAATCTGAGG	78168	ACTTTTCATCTCGTACAGCTC	335 petB intron
12	petD-rpoA/F	80213	GGGCATTGGTGCACATATAC	80566	CAGCCAAAGAAATCTTATGA	354 petD-rpoA, intergenic
13	rpl16/F	84123	GTTTCTTCTCATCCAGCTCC	84438	GAAAGAGTCAATATTCCGCC	316 rpl16 intron

*Position of the 5'-end of each primer in the sequence of the tobacco chloroplast genome (ACCESSION Z00044, VERSION Z00044.1).

**Expected size of the PCR product in tobacco.

Table 2. Tests of amplification for the total DNAs of nine species of angiosperms, one species of gymnosperms and one of ferns

Species	Code number of primer pair*												
	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>Magnolia praecoccissima</i> Koidz.	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ranunculus lyallii</i> Hook.f.	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Quercus serrata</i> Thunb.ex Murray	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Spinacia oleracea</i> L.	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Actinidia polygama</i> (Siebold et Zucc.) Planch.ex Maxim.	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Rubus odoratus</i> L.	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Lactuca sativa</i> L.	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Spiranthes sinensis</i> (Pers.) Ames var. <i>amoena</i> (M.Bieb.) E.Hara	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Commelina communis</i> L.	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Pinus parviflora</i> Siebold et Zucc.var. <i>pentaphylla</i> (Mayr)Henry	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Athyrium niponicum</i> (Mett.) Hance	-	-	-	-	-	-	-	-	-	-	-	-	-

*The code number of primer pair corresponds to the number of Table 1.

+ : good amplification ; - : no amplification.

the template DNAs of nine species of angiosperms, one species of gymnosperms and one of ferns (see Table 2). PCR reaction was conducted in a total volume of 25 μ l containing 20 ng of template DNA, 0.2 μ M of each primer, 0.2 mM of each dNTP, 1 \times Ex Taq buffer, and 0.625 unit of Ex Taq (TAKARA co., ltd.). The following cycling profile was used for all primer pairs: initial 3 min denaturation at 95°C, 25 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and a final 10 min extension at 72°C.

Results and discussion

Thirteen primer pairs qualified as universal primers are shown in Table 1. The results of amplification tests are shown in Table 2. Three

primer pairs (6, 8 and 9) were successful for all templates including *Pinus* and *Athyrium* (Fig. 1). Seven pairs (1, 2, 3, 4, 7, 11 and 13) were universal for angiosperms. The other three (5, 10 and 12) were unsuccessful only for one of nine species of angiosperms examined.

The coupling of SSCP and universal primers of cpDNA can be useful for various research objectives at the micro-evolutionary level. For example, Watano et al. (1995) has applied PCR-SSCP of cpDNA to develop species-specific marker of chloroplast genome, and has described the pattern of introgression of chloroplast genome between two pine species. Fujii et al. (1997) has utilized PCR-SSCP in order to survey intraspecific cpDNA variations of *Pedicularis chamissonis*. They first performed DNA sequencing on one plant from each population. Then, in order to check variation within each population, all samples were examined by PCR-SSCP. Effective application of PCR-SSCP may have much to offer to population biologists.

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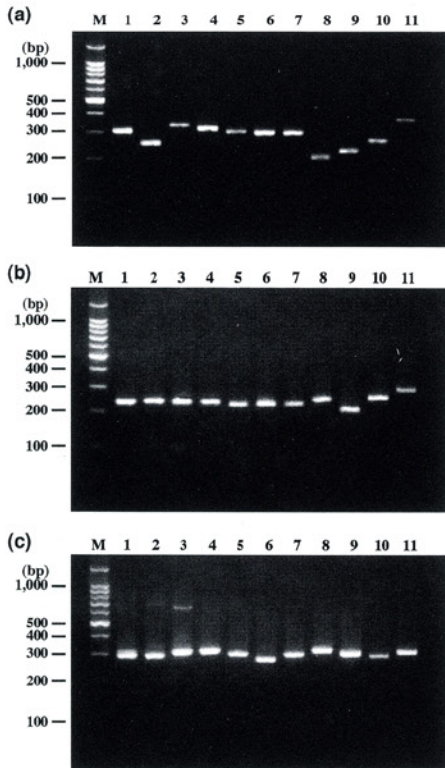


Fig.1. Agarose gel electrophoresis (3%) with PCR-amplified fragments of the eleven taxa. (a) : psbC-trnS; (b) : trnV-M; (c) : trnW-P. M: DNA size marker (100 bp DNA ladder, Promega) ; lane 1: *Magnolia praecocissima* ; lane 2: *Ranunculus lyalii* ; lane 3: *Quercus serrata* ; lane 4: *Spinacia oleracea* ; lane 5: *Actinidia polygama* ; lane 6: *Rubus odoratus* ; lane 7: *Lactuca sativa* ; lane 8: *Spiranthes sinensis* var. *amoena* ; lane 9: *Comelina communis* ; lane 10: *Pinus parviflora* var. *pentaphylla* ; lane 11: *Athyrium niponicum*.

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西沢 徹・綿野泰行：被子植物の葉緑体 DNA の PCR-SSCP 解析に適したプライマーセット

PCR-SSCP は、非常に感度がよく、しかも簡便な DNA の変異のスクリーニングの手段である。この方法を、被子植物の分類学的研究に応用するために、被子植物で広く PCR 増幅が可能なプライマーセットをタバコの塩基配列をもとに開発した。PCR-SSCP では、短い断片（約 400 bp 以下）でないと変異の検出感度が落ちるので、全て 300 bp 前後の長さの増幅産物になるように設計を行った。広い分類群で実際に増幅が可能かどうかを、被子 9 種・裸子 1 種・シダ 1 種について実際に調べた。設計した 13 種類のプライマーセットのうち 3 つは、全ての分類群で増幅に成功した。7 つは被子植物全てで増幅が認められた。残りの 3 つも、被子植物 9 種のうち、どれか 1 つの分類群で増幅されなかっただけであった。

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