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

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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	CCCCCTAGAAACGTATAGGA	rps16/1 R	5751	CGAAGTAATGTCTAAACCCA	412	*rps16* intron
2	rps16/2 F	5732	TGGGTTTAGACATTACTTCG	rps16/2 R	6038	ATAGTCCATGATGGAGCTCG	307	*rps16* intron
3	UCC/2 F	9515	GGTAAAAGTGTGATTCGTTC	UCC/2 R	9785	ATCTTCATCCATGGATCCT	271	*trnG* (UCC) intron
4	UCC/1 F	9895	ATATTGTTTTAGCTCGGTGG	UCC/1 R	10148	GTTTCATTCGGCTCCTTTAT	254	*trnG* (UCC) intron
5	atpF/F	12687	TTCATTTGGCTCTCACGCTC	atpF/R	12933	AATGCTGAATCGACGACCTA	247	*atpF* intron
6	psbC-trnS/F	36867	TGAACCTGTTCTTTCCATGA	psbC-trnS/R	37172	GAACTATCGAGGGTTCGAAT	306	*psbC-trnS* (UGA), intergenic
7	trnG-fM/F	38083	TCTCTTTGCCAAGGAGAAGA	trnG-fM/R	38394	ATAACCTTGAGGTCACGGGT	312	*trnG* (GCC)-*trnfM* (CAU), intergenic
8	trnV-M/F	54362	TGTAAACGAGTTGCTCTACC	trnV-M/R	54610	CTAACCACTGAGTTAAGTAG	249	*trnV* (UAC)-*trnM* (CAU), intergenic
9	trnW-P/F	68827	GATTTGAACCTACGACATCG	trnW-P/R	69123	GATGTGGCGCAGCTTGGTAG	297	*trnW* (CCA)-*trnP* (UGG), intergenic
10	petB/1 F	77517	AGAGATGGTTCTACTTCGTC	petB/1 R	77846	TTCATACTAGAACCACGATG	330	*petB* intron
11	petB/2 F	77834	GTTCTAGTATGAATCTGAGG	petB/2 R	78168	ACTTTCATCTCGTACAGCTC	335	*petB* intron
12	petD-rpoA/F	80213	GGGCATTGGTGCAACATTAC	petD-rpoA/R	80566	CAGCCAAGAAGATCTTATGA	354	*petD-rpoA*, intergenic
13	rpl16/F	84123	GTTTCTTCTCATCCAGCTCC	rpl16/R	84438	GAAAGAGTCAATATTCGCCC	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	Family	Code number of primer pair*												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Magnolia praecocissima Koidz.	Magnoliaceae	+	+	+	+	+	+	+	+	+	+	+	+	+
Ranunculus lyallii Hook.f.	Ranunculaceae	+	+	+	+	+	+	+	+	+	+	+	+	+
Quercus serrata Thunb.ex Murray	Fagaceae	+	+	+	+	+	+	+	+	+	+	+	+	+
Spinacia oleracea L.	Chenopodiaceae	+	+	+	+	+	+	+	+	+	+	+	—	+
Actinidia polygama (Siebold et Zucc.) Planch.ex Maxim.	Actinidiaceae	+	+	+	+	+	+	+	+	+	+	+	+	+
Rubus odoratus L.	Rosaceae	+	+	+	+	—	+	+	+	+	+	+	+	+
Lactuca sativa L.	Asteraceae	+	+	+	+	+	+	+	+	+	+	+	+	+
Spiranthes sinensis (Pers.) Ames var. *amoena* (M.Bieb.) H.Hara	Orchidaceae	+	+	+	+	+	+	+	+	+	+	+	+	+
Commelina communis L.	Commelinaceae	+	+	+	+	+	+	+	+	+	—	+	+	+
Pinus parviflora Siebold et Zucc.var.*pentaphylla* (Mayr) Henry	Pinaceae	—	—	—	—	+	+	+	+	+	—	—	—	+
Athyrium niponicum (Mett.) Hance	Woodsiaceae	—	—	—	+	—	+	—	+	+	—	—	—	—

*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 μl containing 20 ng of template DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1×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℃, 25 cycles of 1 min at 94℃, 1 min at 55℃ and 1 min at 72℃, and a final 10 min extension at 72℃.

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.

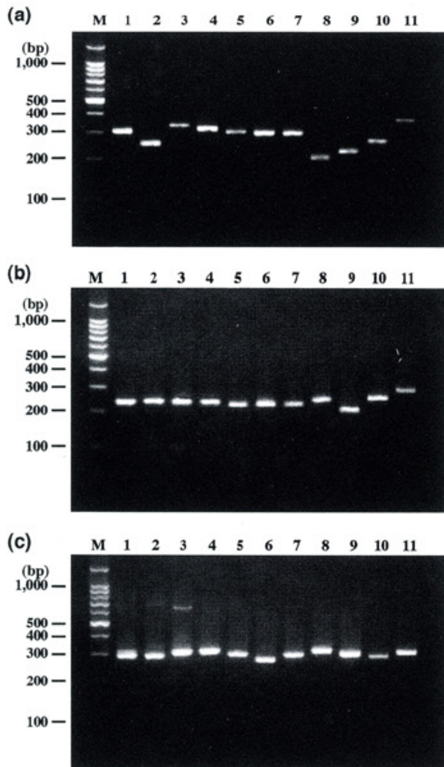

Fig.1. Agarose gel electrophoresis (3%) with PCR-amplified fragments of the eleven taxa. (a): psbC-trnS; (b): trnV-M; (c): trnW-P. M: DNA size marker (100 bp DNA ladder, Promega); lane 1: *Magnolia praecocissima*; lane 2: *Ranunculus lyallii*; lane 3: *Quercus serrata*; lane 4: *Spinacia oleracea*; lane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

References

Dumolin-Lapegue, S., Pemonge, M. H. and Petit, R. J. 1997. An enlarged set of consensus primers for the study of organelle DNA in plants. Mol. Ecol. **6**: 393–397.

Fujii, N., Ueda, K., Watano, Y. and Shimizu, T. 1997. Intraspecific sequence variation of chloroplast DNA in *Pedicularis chamissonis* Steven (Scrophulariaceae) and geographical structuring of the Japanese "alpine" species. J. Plant Res. **110**: 195–207.

Hiratsuka, J., Shimada, H., Whittier, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.R., Meng, B. Y., Li, Y. Q., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K. and Sugiura, M. 1989. The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol. Gen. Genet. **217**: 185–194.

Lessa, E. P. and Applebaum, G. 1993. Screening technique for detecting allelic variation in DNA sequences. Mol. Ecol. **2**: 119–129.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N.,

Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B. Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H. and Sugiura,M. 1986. The complete nucleotide sequence of tobacco chloroplast genome : its gene organization and expression. EMBO J. **5** : 2043–2049.

Watano, Y., Imazu, M. and Shimizu, T. 1995. Chloroplast DNA typing by PCR-SSCP in the *Pinus pumila*—*P. parviflora* var. *pentaphylla* complex (Pinaceae). J. Plant Res. **108** : 493–499.

Yap, E. P. H. and MacGee, J. O. 1994. Non-isotopic single-strand conformation polymorphism (SSCP) analysis of PCR products. Griffin, H. G. and Griffin, A. M. (eds.) PCR Technology : Current innovations, pp. 165–177. CRC Press, Boca Raton.

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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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