Rapid and Sensitive Detection of *Lophophora williamsii* by Loop-Mediated Isothermal Amplification

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We have developed a convenient method for the detection of *Lophophora williamsii* using loop-mediated isothermal amplification (LAMP). We designed six species-specific primers for *L. williamsii*, including two loop primers. This *L. williamsii*-specific primer set was used for LAMP of total DNA extracted from *L. williamsii* and from *L. diffusa*. Real-time monitoring of LAMP was achieved by measuring turbidity due to the formation of magnesium pyrophosphate. Amplification occurred in samples mixed with total DNA from *L. williamsii*, but not in those mixed with total DNA from *L. diffusa*. We could also visually observe *L. williamsii* by adding fluorescent detection reagent to the reaction tube and exposing it to UV light. This new method amplified *L. williamsii* selectively and is expected to be applicable to the detection of peyote.

Key words *Lophophora williamsii*; Cactaceae; peyote; visual detection; loop-mediated isothermal amplification; *trnL* intron

In plants, nucleotide alignment of specific DNA regions, such as nuclear ribosomal RNA or chloroplast DNA, has been used for the determination of phylogenetic relationships and species classification.^{1–3)} Such DNA information has also been used to authenticate herbal medicine in pharmacognosy.^{4,5)} There have been attempts to use DNA polymorphism-based detection techniques—commonly employed in the life sciences—for the authentication of medicinal plants. Such techniques include amplification-refractory mutation system (ARMS),⁶⁾ polymerase chain reaction (PCR)-restriction fragment-length polymorphism (PCR-RFLP),⁷⁾ and Taq-Man assay.⁸⁾ However, not all techniques can be applied directly to plant materials because total DNA from plants contain many secondary metabolites and polysaccharides. In PCR amplification, difficulties are sometimes encountered when total DNA extracted from plant materials was used as template without preliminary preparation of the sample *via* purification or technical modification.^{9,10)} Moreover, the sequence selectivity of oligonucleotides such as PCR primers is decreased in reaction solutions that contain impurities. As many herbal medicines are derived from underground parts having high polysaccharide content, we saw the need to develop a new method to overcome this problem.

Loop-mediated isothermal amplification (LAMP) technique has been developed and used in the detection of microorganisms.^{11–13)} As LAMP employs 4 to 6 primers, it involves 4 to 6 reactive sites and 6 to 8 recognition regions, giving it increased sensitivity and specificity compared to typical PCR. Moreover, the isothermal reaction condition increases time efficiency compared to PCR, which requires three temperature steps. LAMP achieves detection in one to two hours. We have already observed that LAMP has good amplification ability in the detection and identification of *Curcuma* plants.¹⁴⁾ LAMP was successful even though *Curcuma* rhizomes have high polysaccharide content, which made extraction of total DNA difficult.

The genus *Lophophora*, Cactaceae, is distributed in Central and South America; *L. williamsii*, *L. diffusa*, and several other varieties are included in this genus.^{15–18)} The conven-

tion on international trade in endangered species of wild fauna and flora (CITES) checklist lists only two species (*L. williamsii* and *L. diffusa*).¹⁹⁾ Classifications of Genus *Lophophora* are unresolved because there are only minor morphological differences between them. Even *L. williamsii* and *L. diffusa* are classified as *L. williamsii*.²⁰⁾ In this manuscript, we have adopted the theory that these two species are different. *L. williamsii* is known by its common name, peyote. Because it contains mescaline, a psychedelic, its use is regulated in some countries. There should be the method to identify this species but there is not because it is very difficult to differentiate those two species morphologically. The species-specific detection methods are all the more nonexistent. Thus, we have analyzed the nucleotide alignment of the chloroplast *trnL* intron region in *L. williamsii* and *L. diffusa* and clarified the differences. Three sequence patterns were observed in *L. williamsii* (DNA Data Bank of Japan (DDBJ) accession Nos. AB362488, AB362489, and AB362490), and one pattern was observed in *L. diffusa* (AB362491). We found that these two species have different sequences in the *trnL* intron region. In the course of our investigation, we realized that PCR amplification using total DNA extracted from *Lophophora* plant as template was sometimes unsuccessful because of the polysaccharide content of the plants. We wanted to devise an amplification technique that is unaffected by impurities in the template. With this motivation, we adopted LAMP for the identification of *Lophophora* plants. This is the first report for the rapid detection of a psychedelic-containing plant by LAMP.

MATERIALS AND METHODS

Plant Materials Eleven specimens of *Lophophora williamsii* J. M. COULTER and four specimens of *Lophophora diffusa* BRAVO from the Medicinal Plant Garden, Tokyo Metropolitan Institute of Public Health were used (Table 1). We identified the materials according to Anderson's morphological classification.¹⁵⁾

Designing LAMP Primers We designed two sets of

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Table 1. Plant Materials Used in This Study and Initial Rise Time of LAMP

Species	Voucher No.	Initial rise time of LAMP (min) ^{b)}		Total DNA concentration (ng/ μ l)	Mescaline contents (mg/g) ^{c)}	DDBJ accession No. of <i>trmL</i> intron region
		Primer set for <i>L. williamsii</i>	Primer set for <i>L. diffusa</i>			
<i>Lophophora williamsii</i> J. M. COULTER	Lo-2	47.7–63.2	—	7.8	48.3	AB362488
	Lo-3	49.8–59.3	—	9.3	22.2	AB362488
	Lo-6	49.1–58.3	—	16.9	26.6	AB362488
	Lo-7	53.7–59.2	—	8.0	38.2	AB362488
	Lo-8	50.5–54.9	—	6.5	24.6	AB362488
	Lo-9	53.7–56.3	—	9.3	29.4	AB362488
	Lo-10	49.7–61.2	—	7.0	30.7	AB362488
	Lo-11	50.5–58.9	—	11.1	35.4	AB362489
	(<i>L. williamsii</i> var. <i>decipiens</i> CROIZ.) ^{a)}	Lo-14	70.6–87.1	7.3	N.D. ^{d)}	AB362490
	(<i>L. williamsii</i> var. <i>decipiens</i> CROIZ.) ^{a)}	Lo-15	77.6–84.3	7.1	N.D. ^{d)}	AB362490
	(<i>L. williamsii</i> var. <i>decipiens</i> CROIZ.) ^{a)}	Lo-16	76.5–87.3	7.1	N.D. ^{d)}	AB362490
<i>Lophophora diffusa</i> BRAVO	Lo-17	—	75.8–82.4	10.8	N.D. ^{d)}	AB362491
	Lo-18	—	68.9–77.6	11.9	N.D. ^{d)}	AB362491
	Lo-19	—	79.6–82.4	11.8	N.D. ^{d)}	AB362491
	Lo-20	—	74.6–81.1	15.1	N.D. ^{d)}	AB362491

a) These samples were identified as *L. williamsii* in this study but were identified as *L. williamsii* var. *decipiens* in the literature.^{16,18)} b) Results are given as the ranges of three examinations. c) Mescaline content was determined by HPLC, and details were described in another report (submitted). d) Not detected.

species-specific primers for *L. williamsii* and *L. diffusa* (Fig. 1). LPw-F3, LPw-B3, LPw-FIP, LPw-BIP, LP-LF and LPw-LB were for *L. williamsii*, and LPd-F3, LPd-B3, LPd-FIP, LPd-BIP, LP-LF and LPd-LB were for *L. diffusa*. The primer sequences are shown in Table 2.

Real-Time Monitoring of Turbidity and Detection in LAMP of *Lophophora* Plants Total DNA was extracted from 20–50 mg of plant material using a DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. DNA concentrations were measured with a DU640 spectrophotometer (Beckman). LAMP was carried out with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd.) according to the appendix with modification. Briefly, 25 μ l of a reaction mixture containing 1.0 μ l of genomic DNA, 12.5 μ l of 2 $\times$ Reaction Mix, 8 units of *Bst* DNA polymerase, 0.5 μ l of Fluorescent Detection Reagent, 10 pmol each of LPw-F3 (LPd-F3) and LPw-B3 (LPd-B3), 80 pmol each of LPw-FIP (LPd-FIP) and LPw-BIP (LPd-BIP), and 40 pmol each of LP-LF and LPw-LB (LPd-LB), was incubated at 60 °C (58 °C, for a set of primers for *L. diffusa*) for 90 or 120 min and then heated at 80 °C for 5 min. Real-time turbidity was monitored with Realoop-30 (Moritex Co., Ltd., Tokyo). Initial rise times were calculated by Realoop-30 from the amplification curves. After the reaction, the tubes were irradiated under a UV lamp (254 nm) to observe fluorescence, and 5 μ l of reaction mixture was electrophoresed in a 2% agarose gel containing GelRed (Biotium). Each experiment was conducted independently in triplicate runs. To confirm the sequences of the amplified products, the reaction solution was digested with *MseI* (NEB) and was electrophoresed.

RESULT AND DISCUSSION

Designing LAMP Primers First, we designed six primers for LAMP, including two loop primers based on the sequence from DDBJ accession No. AB362488, for the purpose of amplifying only *L. williamsii* DNA (Fig. 1, Table 2).

Two outer primers (LPw-F3 and LPw-B3), two inner primers (LPw-FIP and LPw-BIP), and two loop primers (LP-LF and LPw-LB) were employed in the reaction, and amplification occurred only when all primers were annealed to the target DNA. There are three nucleotide patterns in *L. williamsii*, and differences were observed. Compared to the topmost sequence (AB362488), four base pairs deletion were observed in Lo-11, and one base pair substitution in region w-F1c and ten base pairs insertion were observed in Lo-14, Lo-15, and Lo-16. The other seven regions (w-F3, F2, LFc, w-B1c, w-LBc, B2, and w-B3) were shared among the three groups. One of the inner primers, LPw-FIP, was designed based on regions w-F1c and F2, so this primer did not completely anneal with Lo-14 through Lo-16. However, LPw-FIP was well matched with Lo-11 (Fig. 2). On the other hand, differences were noted in five regions between the topmost sequence (AB362488) and the sequence of *L. diffusa* (AB362491); there were two base pairs insertion in w-F3, two base pair deletions and one base pair substitution in w-F1c, eight base pairs insertion from nucleotide position 191 to 198, one base pair substitution in w-B1c, thirteen base pairs insertion in w-LBc, three base pair substitutions in w-B3 and one base pair substitution on nucleotide position 335. We speculated that the set of primers designed for *L. williamsii* would not anneal to the sequence of *L. diffusa*. As a result, we could design an optimal primer set for *L. williamsii*. Moreover, we also designed a set of primers for *L. diffusa* to check the selectivity of the set of primers for *L. williamsii*. The set of primers for *L. diffusa* consists of LPd-F3, LPd-B3, LPd-FIP, LPd-BIP, LP-LF and LPd-LB (Table 2). The principles of LAMP are explained in detail by Notomi *et al.* and Nagamine *et al.*^{11,21)}

Real-Time Monitoring of Turbidity in LAMP of *Lophophora* Plants Turbidity derived from magnesium pyrophosphate was monitored in real time with Realoop-30. Amplifications using a set of primers for *L. williamsii* and total DNA extracted from eleven samples of *L. williamsii* were detected between 47.7 and 87.3 min (Fig. 3A, Table 1). In contrast, no amplification was observed when the template

Fig. 1. Nucleotide Differences between *L. williamsii* and *L. diffusa* in Part of the *trnL* Intron Region and Locations of Primers Designed for LAMP

Double-stranded DNA sequences from nucleotide position 51 to 350 of the *trnL* intron region are shown. Dotted boxes indicated recognition site of *MseI* restriction enzyme. Numbers in italics above the sequences indicate aligned nucleotide positions. The shaded regions indicate nucleotide differences. Hyphens (–) denote alignment gaps. Boxed sequences indicate the primer sequences regions for primers.

Table 2. Primers Used in This Study

Name ^{a)}	Sequence (5'–3')																		
LPw-F3	CTT	CCA	TCA	AAA	CTC	CAA	TAA	AAA	AGG										
LPd-F3	CTT	CCA	TCA	AAA	CTC	CAA	TAA	AAA	AAA	GG									
LPw-B3	CCA	TTT	TTT	CAT	AGA	AAT	CTT	TTC	ACT	TC									
LPd-B3	CCG	TTT	TTT	CAT	AGA	AAT	CTT	TTC	ATT	TA									
LPw-FIP	TAT	ATA	TAT	ATA	AGG	ATT	ACG	TAT	GGG	TTA	TAC	GTG	TAG	TGA	ACT	ATA	TC		
LPd-FIP	CAT	ATA	AAT	AAG	GAT	TAC	GTA	TGG	GTT	ATA	CGT	GTA	GTG	AAC	TAT	ATC			
LPw-BIP	GAT	AAT	CTG	AAT	TCT	ACT	TTA	CTA	AAA	TTT	CTA	TTT	AGA	TAG	AAT	TAT	CC		
LPd-BIP	TAT	AAT	CTG	AAT	TCT	ACT	TTA	CTA	AAA	TTT	CTA	TTT	AGA	TAG	AAT	TAT	CC		
LP-LF	TTC	GGG	TTG	TCT	TTA	ATC													
LPw-LB	GAA	TAT	GAA	ATA	TAT	ATT	CTA	AAA	TGG										
LPd-LB	GAA	TAT	GAA	ATA	TAT	ATT	CTA	AAT	ATA										

^{a)} Names beginning with the letters “LPw” are primers that anneal to the sequence of *L. williamsii*; “LPd” indicates annealing to *L. diffusa*, and “LP” indicates annealing to both species.

Fig. 4. Visual Inspection (A₁, B₁) and Agarose Gel Electrophoresis Profiles (A₂, B₂) of LAMP Products, and Electrophoresis Profiles (A₃, B₃) after *Mse*I Digestions

Images using primers specific for *L. williamsii* (A) and for *L. diffusa* (B); visual inspection was conducted under a UV lamp (254 nm). Lane M indicates 1 kb DNA ladder (Promega).

Fig. 5. Real-Time Monitoring of LAMP Using Turbidity Measurements at Different DNA Concentrations

LAMP using a set of *L. williamsii*-specific primers and genomic DNA of Lo-6 as template. Concentrations of template DNA were 10.0 ng, 5.0 ng, 1.0 ng, 0.5 ng, 0.1 ng, and 0.05 ng per tube.

Moreover, no special equipment, such as a turbidity detector, is required because we have determined the optimal primer sequences and reaction conditions. This method requires only a water bath for incubation and a UV lamp for detection.

As *L. williamsii* contains mescaline, its use is regulated in some countries. Although the presence or absence of mescaline can easily be checked by chromatography, it is difficult to identify the species because not all *L. williamsii* contain mescaline. Chemotaxonomic identification of *L. williamsii* seems insufficient. DNA sequences of chloroplast *trnL* intron region in *Lophophora* plants were revealed to be beneficial for identification and showed a good correlation with mescaline content. Based on those sequences, rapid and sensitive detection of *L. williamsii* by LAMP was achieved.

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