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ORIGINAL PAPER

Peyote identification on the basis of differences in morphology, mescaline content, and *trn*L/*trn*F sequence between *Lophophora williamsii* and *L. diffusa*

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Abstract Genus *Lophophora* (Cactaceae) has two species: *Lophophora williamsii* Coulter, which is called peyote, and *L. diffusa* Bravo. Although it was reported that *L. williamsii* contained mescaline and *L. diffusa* did not, we found *L. williamsii* specimens that did not contain mescaline. This finding indicated that the two species could not be differentiated in terms of mescaline content. Moreover, the relationship between mescaline content and morphology of the two species is also unknown. In this study, we attempted to clarify the difference in morphology, mescaline content, and DNA alignment of the chloroplast *trn*L/*trn*F region between *L. williamsii* and *L. diffusa*. As a result, *L. williamsii* specimens were classified into two groups. Group 1 had small protuberances on the epidermis, contained mescaline, and the analyzed region on the *trn*L/*trn*F sequence was 881 base pairs (bp) long in all except one (877 bp). Group 2 had large protuberances on the epidermis, did not contain mescaline, and the analyzed region was 893 bp long. On the other hand, *L. diffusa* had medium-sized protuberances on the epidermis, did not contain mescaline, and the analyzed region was 903 bp long. Also investigated was the potential application of the PCR–restriction fragment length polymorphism (RFLP) method as a means of identification based on the *trn*L/*trn*F sequence. By applying the PCR–RFLP method, the two species could be distinguished and *L. williamsii* specimens could be differentiated into group 1 and group 2.

Keywords *Lophophora* · Peyote · Cactaceae · Identification · Morphology · Mescaline · *trn*L/*trn*F sequence · PCR–RFLP

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Introduction

Lophophora is a flattened globose plant belonging to family Cactaceae. It is widely distributed in the highlands of middle Mexico through southern Texas in North America [1]. Because plants belonging to this genus show morphological variations, it is very difficult to classify them. As well as *L. williamsii* and *L. diffusa*, *L. fricii* Habermann and *L. jourdaniana* Habermann were reported in the past [2, 3]. But in the present classification, Lophophora is two species, *L. williamsii* and *L. diffusa* [3–7]. And in CITES Cactaceae Checklist [8], which is becoming the standard of the Washington Convention, it is classified into two species.

L. williamsii is called peyote in its place of origin and is popularly used in medicine or religious rites [3, 9]. As its

constituents, mescaline, a classic hallucinogen, and other alkaloids were reported [3, 10–13]. Mescaline and peyote are classified as Schedule I drugs that possess the greatest risk of abuse according to the US Controlled Substance Act [3] and their cultivation, purchase, possession, and sale are strictly controlled. In Japan, as *L. williamsii* is commercially available for cultivation and possession, it is easily misused as a narcotic drug. Clearly, it is necessary to regulate the possession of *L. williamsii*. However, there are no methods to identify *L. williamsii* correctly. Although it is reported that mescaline is present in only *L. williamsii*, it is unclear whether all *L. williamsii* species contain mescaline or not. It is difficult to identify *L. williamsii* on the basis of mescaline content alone.

Although *L. williamsii* is morphologically similar to *L. diffusa*, these two species can be distinguished by stem color, the presence or absence of ribs and furrows over stems, and the color of inner perianth segments (flowers) [3]. For example, *L. williamsii* has blue-green or sometimes reddish-green stems with ribs and furrows, and the flower is usually pink. On the other hand, *L. diffusa* has yellowish-green stems with no ribs, and the flower is usually white.

However, the relationships between morphology and mescaline content of the two species are unclear. Therefore, in this study, we microscopically observed the epidermis, measured mescaline content, and analyzed the DNA sequences of chloroplast *trn*L 5′ exon, *trn*L intron, *trn*L 3′ exon, intergenic spacer (IGS) between *trn*L 3′ exon and *trn*F, and *trn*F (*trn*L/*trn*F) region using *L. williamsii* and *L. diffusa* specimens.

We also studied the utility of the PCR–restriction fragment length polymorphisms (PCR–RFLP) method as a means of identification based on the *trn*L/*trn*F sequence. This PCR–RFLP method is expected to enable the identification of even dried or powdered *Lophophora* plants.

Materials and methods

Materials

Between January 2005 and May 2005, 20 *Lophophora* plants available commercially in Japan were purchased and cultivated. Of the 20 plants, 16 were *L. williamsii* (Lo-1 to 16) and four were *L. diffusa* (Lo-17 to 20) (Table 1). The plants were grown in an outdoor plastic greenhouse between spring and fall (mid April to mid October) and in-house during winter. Blooming period is from May to August, and individual variations were observed in the color and length of the flowers (inner perianth segments). The specimens were identified according to the classification method of Anderson [3].

Table 1 *Lophophora* plants used for experiments

Voucher no.	Scientific name[a]	Commercial name	Collection date	Means of acquisition
Lo-1	*Lophophora williamsii*	Ubatama	April 2005	Internet
Lo-2	*L. williamsii*	Ubatama	April 2005	Internet
Lo-3	*L. williamsii*	Ubatama	January 2005	Market (Mie Pref.)
Lo-4	*L. williamsii*	Ubatama	January 2005	Market (Mie Pref.)
Lo-5	*L. williamsii*	Ubatama	January 2005	Market (Mie Pref.)
Lo-6	*L. williamsii*	Ubatama	April 2005	Internet
Lo-7	*L. williamsii*	Ubatama	April 2005	Internet
Lo-8	*L. williamsii*	Ubatama	April 2005	Internet
Lo-9	*L. williamsii*	Ubatama	April 2005	Internet
Lo-10	*L. williamsii*	Ubatama	April 2005	Internet
Lo-11	*L. williamsii*	Ougataubatama	April 2005	Internet
Lo-12	*L. williamsii*	Kofukiubatama	March 2005	Internet
Lo-13	*L. williamsii*	Ougataubatama	April 2005	Internet
Lo-14	*L. williamsii*	Ginkangyoku	May 2005	Internet
Lo-15	*L. williamsii*	Ginkangyoku	May 2005	Internet
Lo-16	*L. williamsii*	Ginkangyoku	May 2005	Internet
Lo-17	*L. diffusa*	Difyusa	January 2005	Market (Mie Pref.)
Lo-18	*L. diffusa*	Difyusa	May 2005	Internet
Lo-19	*L. diffusa*	Difyusa	May 2005	Market (Mie Pref.)
Lo-20	*L. diffusa*	Difyusa	May 2005	Internet

[a] We identified above plants on the basis of morphology

All plant materials used in our experiments are preserved in the Tokyo Metropolitan Medicinal Plant Garden along with their voucher numbers.

Morphological observation

The number of stems, the diameter of the center stem, and the number of ribs over stems were determined. Stem cross section was observed with a stereoscopic microscope (Model SMZ1000, Nikon Corporation). The morphology of the stem epidermis was observed with a scanning electron microscope (SEM; Model S-800, Hitachi, Ltd.). The stem section was fixed on a pedestal and platinum and palladium were deposited by the ion sputtering method.

Analysis of contents

Preparation of solution for quantitative analysis

Fresh plant stems were freeze-dried and pulverized with a vibrating mill (Model TI-100, CMT Co. Ltd., Japan). Approximately 0.1 g of the specimen was extracted by ultrasonication with 20 mL of 0.1 N HCl for 20 min, and the mixture was centrifuged. Then, the supernatant was transferred to a 50-mL volumetric flask. The same process was performed on the residue, 0.1 N HCl was added to make a total volume of 50 mL, and the solution was filtered through a 0.2-μm filter to obtain the specimen solution.

Calculation of mescaline content

Mescaline isolated from *L. williamsii* was used as the reference standard. A calibration curve in the range of 0.0007–0.0414 μg was obtained by using the peak height at 205 nm, and mescaline content was calculated with a regression equation.

Analytical conditions

Ammonium formate (special grade; Wako Pure Chemical Industries, Ltd.) and other reagents of HPLC grade were used.

For analysis, we used Acquity UPLC/PDA (Waters), a BEH C18 column (1.7 μm, 2.1 × 50 mm) with column temperature set at 40°C, and water/acetonitrile mixture (950 mL:50 mL) containing 5 mM ammonium formate with 0.05% formic acid filtered through a 0.45-μm filter as eluent. The elution speed was 0.6 mL/min, one cycle was performed every 10 min, and PDA detection wavelength was 200–400 nm. Under these conditions, we also performed qualitative analysis on the basis of the retention time and the UV spectrum. The injection volume was 1 μL for both the test solution and the reference solution.

DNA analysis

Total DNA extraction and amplification with PCR method

Total DNA was extracted from approximately 20 mg of the specimen using a DNeasy Plant Mini Kit (QIAGEN). Amplification was performed with the PCR method under the following conditions: As the reaction solution, total DNA was used as template and 0.25 μM of each primer (trnL-cF, trnL-dR, trnL-eF, trnF-fR) [14] and GoTaq (Promega) was added to make a total reaction solution volume of 50 μL.

The primer sequences used were trnL-cF, 5′-CGAAAT CGGTAGACGCTACG-3′; trnL-dR, 5′-GGGGATAGAG GGACTTGAAC-3′; trnL-eF, 5′-GGTTCAAGTCCCTCT ATCCC-3′; and trnF-fR, 5′-TCGTGTCACCAGTTC-3′. A Thermal Cycler (TaKaRa Dice) was used for the reaction with hot-start at 94°C (2 min), followed by 94°C (1 min), 58°C (1 min), and 72°C (3 min), and the cycle was repeated 45 times. After the reaction, the amplification was confirmed by agarose gel electrophoresis.

trnL/trnF sequence analysis

After the cycle sequence reaction (ABI, BigDye Terminator v1.1) of the PCR amplified products purified with Wizard SV Gel and PCR Clean-Up System (Promega), *trn*L/*trn*F sequences (ABI PRISM 377) were analyzed and homology analysis among sequences was performed with software (AutoAssembler Program v1.3.0).

The sequences analyzed were registered in the DNA databank of Japan at the National Institute of Genetics (DDBJ). The accession numbers are as follows: AB362488 for *L. williamsii* (Lo-1 to 10, Lo-12, and Lo-13), AB362489 for *L. williamsii* (Lo-11), AB362490 for *L. williamsii* (Lo-14 to 16), and AB362491 for *L. diffusa* (Lo-17 to 20).

PCR–RFLP method

On the basis of the analyzed *trn*L/*trn*F sequences, the primers for PCR–RFLP were redesigned. The primer sequences were as follows: LpTL-5F, 5′-CCCGAACC CATACGTAATCC-3′; and LpTL-3R, 5′-GGGAAAAG CATTTTC GTCCG-3′. The reaction conditions were 94°C (2 min), followed by 94°C (1 min), 52°C (1 min), and 72°C (1 min), and the cycle was repeated 35 times. After the reaction, the amplification was confirmed by 1% agarose gel electrophoresis. Subsequent to the confirmation of amplification, restriction enzyme reaction was performed. For the restriction enzyme reaction, 5 μL of PCR product, 4 U of *Mse*I (New England Biolabs), NEBuffer, and BSA were added to make a total volume of 10 μL. Then, with

the Thermal Cycler, the reaction was performed at 37°C for 60 min and at 65°C for 20 min, and the fragments were confirmed by 2% agarose gel electrophoresis.

Results

Morphology of 20 *Lophophora* specimens and identification

The aerial parts of Lo-1 to 20 were all flattened globose. Stem color varied among individuals and the stem was either single or multiple (Fig. 1; Table 2).

Although the color of flowers in Lo-12 could not be observed due to pre-blooming, the plant could still be identified as *L. williamsii* on the basis of other morphological characteristics. Moreover, although the stems were yellowish-green in Lo-13 and grayish-green or yellowish-green in Lo-14 to 16, they were identified as *L. williamsii* on the basis of the ribs and furrows over the stems and the pale pink (Lo-13) to pink and deep pink (Lo-14 to 16) color of the flowers. On the other hand, although Lo-20 had green stems, since it lacked ribs and furrows over the stems and had white flowers, it was identified as *L. diffusa*.

The stems of *L. williamsii* (Lo-1 to 16) had four to 13 ribs specifically observed only in this species, while those

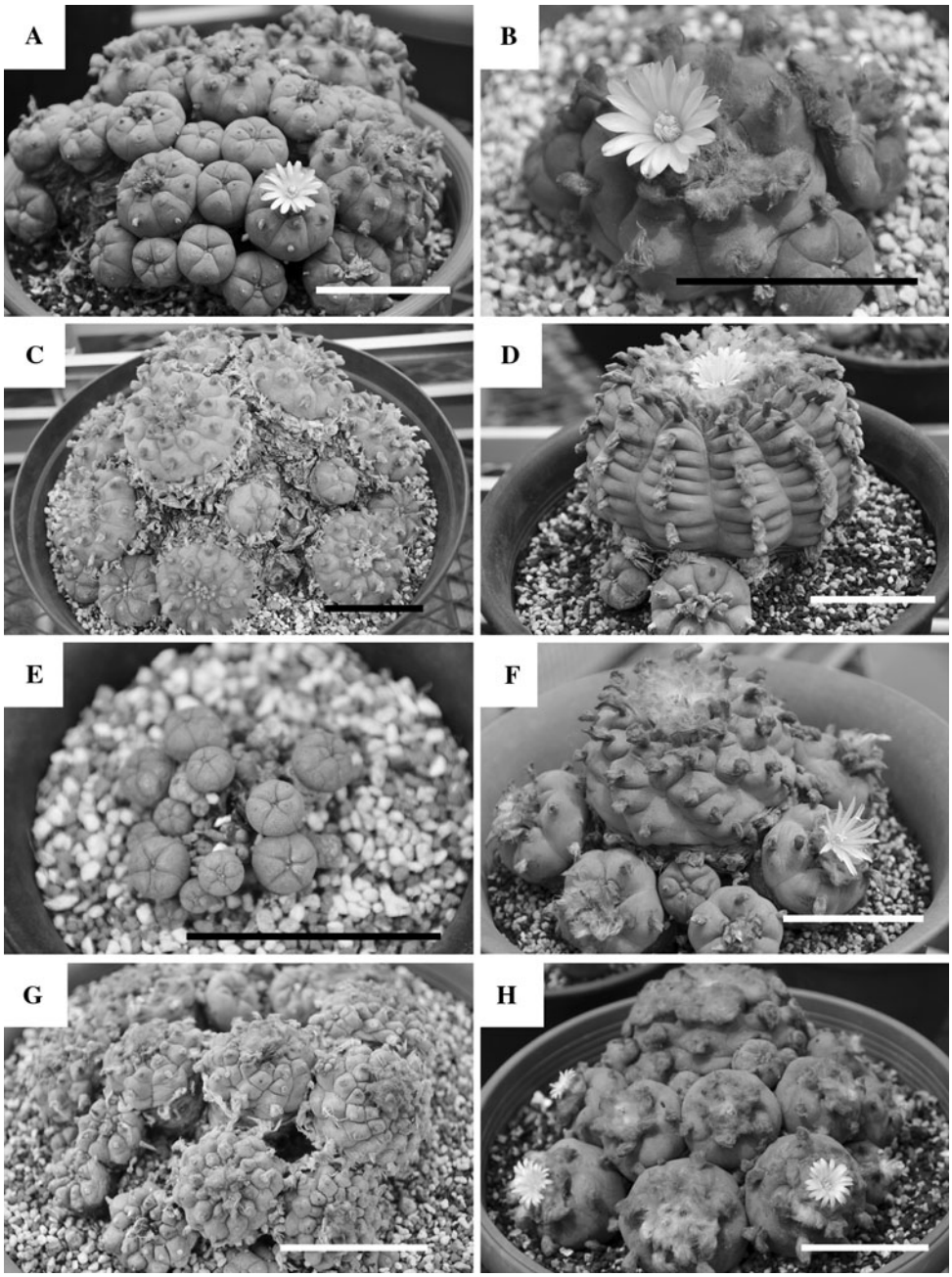

Fig. 1 Morphology of *Lophophora* plants. **a–g** *Lophophora williamsii*, **h** *L. diffusa*. **a** Lo-2, **b** Lo-3, **c** Lo-9, **d** Lo-11, **e** Lo-12, **f** Lo-13, **g** Lo-14, **h** Lo-18. *Bar* 5 cm

Table 2 Morphology, mescaline content, and genetic type of *Lophophora* plants

Voucher no.	Scientific name	Color of flower (inner perianth segments)	Epidermis of stem		Number of stems	Diameter of center stem (mm)	Number of ribs over center stem	Mescaline content (mg/g)	Genetic type[a]
			Color	Protuberance (SEM image)					
Lo-1	*L. williamsii*	Pale pink	Blue-green	Small	5	96.5	13	37.2	A
Lo-2	(Group 1)	Pale pink	Blue-green	Small	21	62.0	11	48.3	A
Lo-3		Pale pink	Blue-green	Small	5	59.3	8	22.2	A
Lo-4		Pale pink	Blue-green	Small	5	63.7	10	42.7	A
Lo-5		Pale pink	Blue-green	Small	1	83.0	13	38.5	A
Lo-6		Pale pink	Blue-green	Small	11	48.0	8	26.6	A
Lo-7		Pale pink	Blue-green	Small	8	81.0	13	38.2	A
Lo-8		Pale pink	Blue-green	Small	7	70.2	13	24.6	A
Lo-9		Pale pink	Blue-green	Small	11	63.5	11	29.4	A
Lo-10		Pale pink	Blue-green	Small	8	86.2	13	30.7	A
Lo-11		Pale pink	Blue-green	Small	3	123.7	12	35.4	B
Lo-12		–[b]	Blue-green	Small	8	10.6	4	25.0	A
Lo-13		Pale pink	Yellow-green	Small	7	90.5	8	12.7	A
Lo-14	*L. williamsii*	Deep pink	Gray-yellow-green	Large	12	51.7	13	ND	C
Lo-15	(Group 2)	Pink	Gray-green	Large	21	52.7	12	ND	C
Lo-16		Deep pink	Gray-yellow-green	Large	21	55.6	10	ND	C
Lo-17	*L. diffusa*	White	Yellow-green	Medium	1	40.9	–	ND	D
Lo-18		White	Yellow-green	Medium	13	87.5	–	ND	D
Lo-19		White	Yellow-green	Medium	1	90.8	–	ND	D
Lo-20		White	Green	Medium	10	70.1	–	ND	D

The accession numbers are as follows: AB362488 for genotype A, AB362489 for genotype B, AB362490 for genotype C, and AB362491 for genotype D

[a] We registered the sequence analyzed in the DNA databank of Japan at the National Institute of Genetics (DDBJ)

[b] No flowers grew during the observation period

of *L. diffusa* (Lo-17 to 20) did not have such ribs (Table 2). *L. diffusa* had either a flat surface (Lo-17 and 20) or a wart-like convex and dimples (Lo-18 and 19).

Stereoscopic microscopy showed that the stem surface had a marble pattern with mixed green, pale green, light brown, and white colors (Fig. 2a, d, g), and was covered with semitransparent epidermis (Fig. 2b, e, h).

SEM observation revealed that the stem epidermis of all plants was covered with minute nipple-like protuberances that could be classified into three types. Typical examples are shown in Fig. 2.

Lo-3 (Fig. 2c) had sporadic, small protuberances (diameter 12.5–16.7 μm) on gentle ribs and Lo-1, Lo-2, and Lo-4 to 13 showed similar results. Lo-16 (Fig. 2f) had densely populated large protuberances (diameter 62.5–66.7 μm) that were more similar to Lo-18 than to Lo-3 (Fig. 2i), and Lo-14 and 15 showed similar morphology. Lo-18 had densely populated protuberances (diameter 33.3–37.5 μm) that were smaller than those of Lo-16, and Lo-17, 19, and 20 showed similar morphology. These observations indicated that among the *L. williamsii* specimens, Lo-1 to 13 and Lo-14 to 16 had different morphological types, and the morphology of Lo-14 to 16 was similar to that of *L. diffusa* (Lo-17 to 20).

Quantification of mescaline

Among the Lo-1 to 16 specimens of *L. williamsii*, Lo-1 to 13 contained 12.7 to 48.3 mg/g mescaline, while Lo-14 to 16 did not. Lo-17 to 20 specimens of *L. diffusa* did not contain mescaline either (Table 2).

*trn*L/*trn*F sequence analysis

The analyzed length of the *trn*L/*trn*F region varied from 877 to 903 bp: 881 bp in genotype A, 877 bp in genotype B, 893 bp in genotype C, and 903 bp in genotype D (Fig. 3). The sequences of genotypes A and B were the same except for the 4-bp deletion at nucleotide positions 187 to 190 in genotype B. Compared with genotype A, genotype C differed in the 10-bp insertion from 191 to 200, the 2-bp insertion from 628 to 629, and three base substitutions at 180, 591, and 876. In the case of *L. diffusa*, genotype D differed from genotype A in the 2-bp insertion

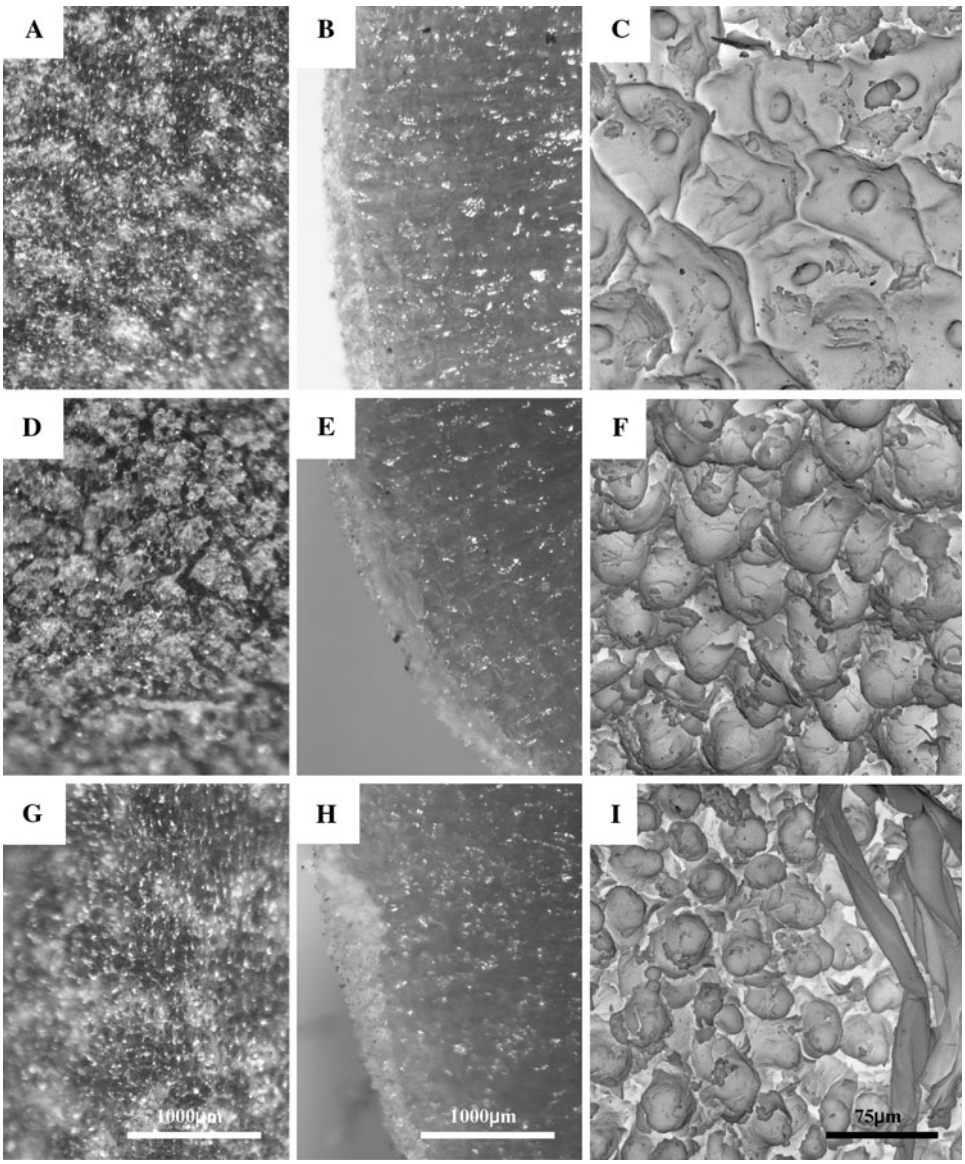

Fig. 2 Epidermal morphology of *Lophophora* plants by microscopy and SEM. **a**–**f** *Lophophora williamsii*, **g**–**i** *L. diffusa*. **a**, **b** Lo-9; **c** Lo-3; **d**, **e** Lo-14; **f** Lo-16; **g**–**i** Lo-18. **a**, **d**, **g** epidermis; **b**, **e**, **h** stem cross section; **c**, **f**, **i** nipple-like protuberance on epidermis

from 96 to 97, the 13-bp insertion from 241 to 253, the 1-bp insertion at 628, the deletions at 172, 174, and 199–200, and the base substitutions at 180, 306, 309, 332, 335, 517, 591, and 876.

PCR–RFLP method

PCR–RFLP was performed by using redesigned primer sets LpTL-5F and LpTL-3R. By way of the PCR–RFLP method using LpTL-5F and LpTL-3R, we expected to obtain the amplified products of 506 bp for genotype A, 502 bp for genotype B, and 518 bp for genotype C in *L. williamsii*, and 526 bp for *L. diffusa* (genotype D) (Fig. 4). The PCR products of genotypes A and B possessed one restriction site by *Mse*I and were digested into two fragments: 256 and 250 bp in genotype A, and 252 and 250 bp in genotype B. In a similar manner, there were two restriction sites in genotype C and the formation of three fragments—266, 164, and 88 bp—was expected. For *L. diffusa* (genotype D), there were four restriction sites and the formation of five fragments—152, 123, 89, 75, and 87 bp—was expected. When electrophoresis was performed on the reaction solution, the expected fragment pattern was obtained, as shown in Fig. 5. In fact, experiments showed that the reaction proceeded as expected (Fig. 5). One fragment found in the electrophoresis image of group A (from lanes 1 to 13) was similar in length to one fragment found in the electrophoresis image of group B. Three fragments were detected in group C (from lanes 14 to 16). In group D, about three fragments were obtained (89, 75, and 87 bp). From the differences in patterns, it was possible to clearly distinguish *L. williamsii* from *L. diffusa* species and their sequence types.

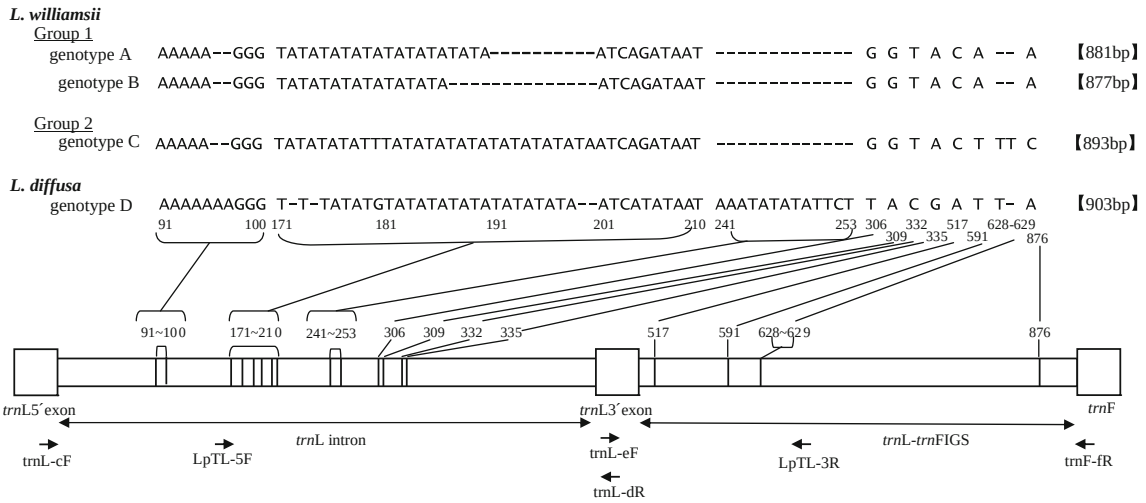

Fig. 3 Comparison of *trn*L/*trn*F sequences among analyzed *Lophophora* plants. *Hyphens* (-) denote alignment gaps. *Numerals under sequences* indicate aligned nucleotide positions from the 5′-end of the analyzed region. *Numerals in parentheses* indicate the whole length of each sequence. trnL-cF, trnL-dR, trnL-eF, and trnF-fR are primers for PCR. LpTL-5F and LpTL-3R are primers for PCR–RFLP

Discussion

Our study revealed that *L. williamsii* could be divided into two groups: Lo-1 to 13 and Lo-14 to 16. Using SEM, we found that the former (group 1) had small protuberances and the stem color was bluish-green. Group 1 specimens contained mescaline and all of them showed genotype A in the *trn*L/*trn*F region except one (Lo-11) that showed genotype B. We also found that the latter (group 2) had large protuberances and the stem color turned yellowish-green from green tinged with gray. Group 2 specimens contained no mescaline and showed genotype C in the *trn*L/*trn*F region. *L. diffusa* had green to yellowish-green stems with medium-sized protuberances on the epidermis. *L. diffusa* also contained no mescaline and showed genotype D.

It was reported that *L. williamsii* contained mescaline, but that *L. diffusa* did not [15, 16]; however, it was unknown whether that *L. williamsii* was within the wide classification that included *L. fricii*.

In this study, we clarified for the first time that there are two groups of *L. williamsii*, one with mescaline (group 1) and the other without it (group 2), and that *L. diffusa* contained no mescaline.

The nucleotide alignments of the chloroplast *rpl*16 regions of *L. williamsii* and *L. diffusa* were reported [17]. In that report, however, only one specimen each of *L. williamsii* and *L. diffusa* was used and no morphological information about the specimens was available. Moreover, it was difficult to obtain the PCR amplified products of the *rpl*16 region successfully. In this regard, the chloroplast *trn*L/*trn*F region was newly studied. Interestingly, the four genotypes in the *trn*L/*trn*F region were related to

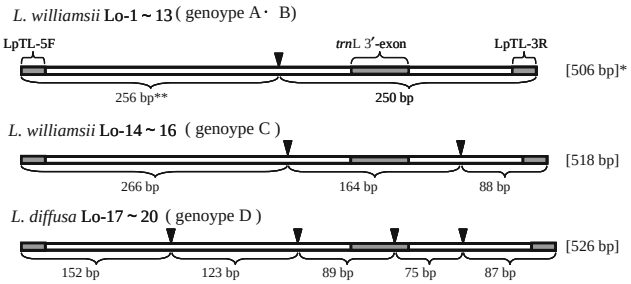

Fig. 4 Diagrams of restriction sites using the restriction enzyme *Mse*I on *trn*L/*trn*F region. PCR products, amplified using DNAs from *Lophophora* plants as template and primers (LpTL-5F and LpTL-3R), were digested with *Mse*I. *Numbers on the right side in brackets* indicate lengths of PCR products, and *numbers under each diagram* indicate lengths of digested fragments. *Closed inverted triangles* indicate restriction sites. *502 bp in Lo-11, **252 bp in Lo-11

morphology or mescaline content: genotypes A and B corresponded to group 1, genotype C corresponded to group 2, and genotype D corresponded to *L. diffusa*. It is possible to identify *L. williamsii* (genotypes A, B, and C) and *L. diffusa* (genotype D), and classify *Lophophora* plants containing mescaline (genotypes A and B) or not containing mescaline (genotypes C and D) if the genotypes in the *trn*L/*trn*F region of *Lophophora* plants were analyzed.

Furthermore, by applying the PCR–RFLP method on the basis of sequence differences in the *trn*L/*trn*F region, we were able to distinguish *L. williamsii* from *L. diffusa*—without having to analyze their *trn*L/*trn*F sequences—and to distinguish the type containing mescaline from that not containing mescaline in *L. williamsii*.

As mescaline is a legally regulated substance, it is important to identify species that contain it. Here, we

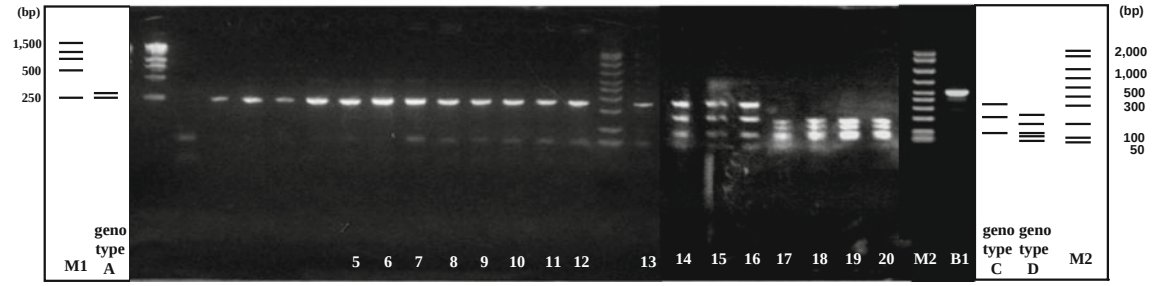

Fig. 5 Diagram of expected fragment pattern of each species after digestion with *Mse*I and agarose gel electrophoresis images of PCR–RFLP before and after digestion. *Lane M1* 1 kb DNA Ladder (Promega), *lane M2* 50–2,000 bp DNA ladder (BIO-RAD); *A*, *L. williamsii* (genotypes A·B); *C*, *L. williamsii* (genotype C); *D*, *L. diffusa* (genotype D). *Lane N lanes 1–20*, image after digestion; *lane B1*, image before digestion. *Lane N* and *lanes 1–20*, image after digestion; *lane B1* image before digestion. *Lane N*, DNA (−); *lanes 1–13*, *L. williamsii* Lo-1 to 13 (genotype A·B); *lanes 14–16*, *L. williamsii* Lo-14 to 16 (genotype C); *lanes 17–20*, *L. diffusa* Lo-17 to 20 (genotype D); *lane B1*, *L. williamsii* Lo-1 (genotype A). The amplification fragment in the neighborhood of 50 bp seems to be associated with the primer non-amplification

clarified the difference between *L. williamsii* and *L. diffusa* in terms of morphology, mescaline content, and *trn*L/*trn*F sequence. The PCR–RFLP method is applicable to even dry and powdered *Lophophora* plants.

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