Title: Distinguishing *Ophiopogon* and *Liriope* tubers based on DNA sequences

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Abstract

Ophiopogon japonicus is a herbaceous perennial plant in Liliaceae, and its tubers are used in traditional Japanese medicine as *Bakumondo* (麦門冬), prescribed for treating cough, sputum, and thirst. *Liriope* is a genus of ornamental plants related to *Ophiopogon*, and the tubers are used in folk medicine as well. Although tubers from both genera are traded in Korean and Chinese markets, only *O. japonicus* is defined as the plant of origin for *Bakumondo* in the Japanese Pharmacopoeia [1], and *Liriope* tubers cannot legally be used as *Bakumondo* in Japan. *Ophiopogon* plants can be distinguished clearly from *Liriope* by their fruit color and by the morphological

characteristics of their flowers. However, the tubers of both species are greatly similar, making it very difficult to differentiate the two genera by appearance of their tubers. We therefore investigated the most appropriate DNA regions to use for practical and accurate identification of *Ophiopogon* and *Liriope* tubers. The sequence of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene was found to be suitable for discriminating *Ophiopogon* and *Liriope* tubers. The identification procedure was simplified using restriction enzyme digestion of the amplified *rbc*L fragment. The detection limit for *Liriope* contamination was estimated by performing the procedure using mixed samples of powdered *Ophiopogon* and *Liriope* tubers.

Keywords: *Ophiopogon japonicus*, ophiopogon tuber, *Liriope* sp., DNA analysis, PCR-RFLP

Introduction

Bakumondo (麦門冬), a tuber derived from *Ophiopogon japonicus* (Liliaceae), is used as a traditional Japanese medicine for the treatment of cough, sputum, and thirst. Some species belonging to other genera are related to *O. japonicus*, such as *Liriope muscari*, and are used as folk medicines as well. Tubers of both *Ophiopogon* and *Liriope* are traded in Korean and Chinese markets. However, the Japanese Pharmacopoeia lists only *O. japonicus* as the plant of origin for *Bakumondo* [1], and it is not legal to sell and use *Liriope* tubers under this name. *Ophiopogon* plants can be clearly distinguished from *Liriope* by the morphological characteristics of their flowers and their fruit color; however, the leaves and tubers are very similar and are very difficult to differentiate by morphology. Thin layer chromatography (TLC) analyses are often used to detect characteristic compounds in identification of herbal medicines. Two homoisoflavonoids, methylophiopogonanone A and B, have been reported as the characteristic chemical constituents [2] of *Ophiopogon* tubers, although TLC analysis methods to detect these compounds have not yet been developed.

Nucleotide sequence-based methods [3,4,5], so-called restriction fragment-length polymorphism (PCR-RFLP) analysis, have also been developed to distinguish or identify plant species from which several natural medicines originate. These new

methods should be useful to identify the plant origin of and to detect contamination in natural medicines when morphological comparison is not possible. *Bakumondo* is a good example of a case where application of these new methods in combination with conventional methods will promote the appropriate use of natural medicines. Purity tests for natural medicines based on genetic information are described in the Japanese Pharmacopoeia [6]. Unlike identification according to morphological characteristics, this method using DNA sequences allows us to identify their original plant species of natural medicines without needing expertise in plant identification.

In the present study, DNA regions useful for discriminating between fresh and dried tubers of *Ophiopogon* and *Liriope* were explored. Analysis of these regions was then used to identify the plant species origin of fresh market *Bakumondo* and powdered samples.

Materials and Methods

1. Materials

Fresh whole plant ~~materials and market samples~~ of *Ophiopogon* and *Liriope* shown in Table 1 were collected from various part of Japan and provided by

Shoyakuhinshitsushudankai ~~(Tables 1 and 3)~~. Among these plant samples, the species of sample Nos. 1-7 were morphologically identified by Prof. Eiji Sakai, Gifu Pharmaceutical University, and that of sample Nos. 8-13 were identified by Mr. Tomonari Kanaya, respectively. The market samples and fresh plants shown in Table 3 were also provided by Shoyakuhinshitsushudankai. These samples were dealt with as *Bakumondo* in the Chinese market. Samples Nos. 24-32 and 36-38 were tubers, Nos. 33-35 were aerial parts, and Nos. 39-41 were fresh whole plant, whose origin were supposed from morphological information by Shoyakuhinshitsushudankai. The market samples previously used to identify origin of plant species were from Tsumura & Co. (Tokyo, Japan) (Table 2) [2]. They were identified as described in the report [2].

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2. Extraction of total DNA

Total DNAs of plant specimens and market products were extracted using a DNeasy® Plant Mini Kit (QIAGEN, Valencia, CA).

Preparation of dried tubers for DNA extraction: tubers were cut transversely through the core of tubers into slices 2–3 mm thick and were soaked into 10 ml~~l~~ of distilled water for 2 h in a Petri dish. Weights of the imbibed tuber slices were measured, and a 180-mg portion of tissue was used for DNA extraction.

Preparation of mixed powder of *Ophiopogon* and *Liriope*: *Ophiopogon* and *Liriope* tubers were freeze-dried at −23 ºC for 24 h and then ground with a mortar and pestle. The Powdered tubers of *L.* ~~*iriope*~~ *muscari* (No. 3)~~tubers~~ were mixed with those of *O. japonicus* ~~*phiopogon*~~ (No. 1) ~~powder~~ at ratios of 1, 10, 30, 50, 90, or 99%. Samples of 80 mg of the mixed powders were used for DNA extraction.

3. Primers for polymerase chain reaction (PCR) of the ITS and *rbc*L region

We amplified two internal transcribed spacer (ITS) regions in this study: ITS 1, from nuclear ribosomal 18S–5.8S and ITS 2, from 5.8S–26S (hereafter, "ITS region"). ITS-5 (5′ -GGAAGTAAAAGTCGTAACAAGG-3′) was used as the forward primer for amplification and ITS-4 (5′ -TCCTCCGCTTATTGATATGC-3′) was used as the reverse primer for both *Ophiopogon* and *Liriope*. Two other primers were used for the selective amplification of the ITS region from *Ophiopogon* (GenBank accession No,): 21its-33-f (5′-CAACGGATCTCTTGGCTCTC-3′) as the forward primer and 21its-33-r (5′-AATTGTATCGACCGCCACTC-3′) as the reverse primer. Another primer pair was also designed for amplifying the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) sequence from chloroplast DNA: Af-2 (5′-CTTCCATTGTGGGTAATGTA-3′) as the forward primer and Ar-1

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(5′-GTTAAGTAATCATGCATTAC-3′) as the reverse primer.

4. Amplification of ITS and *rbc*L regions

PCR for amplifying the ITS and *rbc*L DNA sequences was performed in a reaction mixture of 30.0 µl~~L~~ containing DNA template (1.5 µl~~L~~), 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 0.2 µM forward and reverse primers, 5% DMSO, and Blend Taq (1.0 U~~units~~; Toyobo Co., Ltd., Osaka, Japan). Temperature cycling programs for PCR were specific to the sequences as shown below.

Amplification of the ITS region: initial denaturation at 94 ºC for 180 s, followed by 38 cycles of 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 45 s, and a final elongation at 72 ºC for 180 s.

Selective amplification of the ITS region from *Ophiopogon*: initial denaturation at 94 ºC for 180 s, followed by 38 cycles of 94 ºC for 30 s, 54 ºC for 30 s, 72 ºC for 30 s, and a final elongation at 72 ºC for 120 s.

Amplification of the *rbc*L region: initial denaturation at 94 ºC for 180 s, followed by 40 cycles of 94 ºC for 30 s, 48 ºC for 30 s, 72 ºC for 40 s, and a final elongation at 72 ºC for 180 s.

5. Sequence analysis of ITS regions

ITS amplicons were purified after agarose gel electrophoresis using the NucleoSpin® Extract II Kit (Macherey-Nagel, Düren, Germany). Purified ITS sequences were cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) and confirmed by BIO MATRIX RESEARCH, INC. (Chiba, Japan).

6. Analysis of the undigested *rbc*L fragment by *Hinc*II

The amplification products from the *Liriope* ITS region were digested with *Hinc*II (New England Biolabs, Ipswich, MA), and the resulting fragments were electrophoresed on a 1% agarose gel which was then stained with ethidium bromide. The fragments of interest were isolated from the agarose gel using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), cloned into pTA2 (Toyobo), and their sequences were confirmed by FASMAC Co., Ltd. (Kanagawa, Japan).

7. Digestion with restriction enzyme

Ten microliters from the 30 μl~~l~~ PCR reaction volume for *rbc*L were analyzed by agarose gel electrophoresis, and the remaining volume (20 μl~~l~~) of the *rbc*L PCR reaction volume was purified using the NucleoSpin Gel and PCR Clean-up followed

by digestion with 10–30 U of *Hinc*II at 37 ºC for 2 h (total reaction volume was 11.75–50.0 µl~~l~~).

Results and discussion

1. Distinguish *Liriope* from *Ophiopogon* based on ITS regions

~~Based on the ITS sequences from *Ophiopogon* and *Liriope* (GenBank accession Nos. EU930852–EU930857), p~~Primers ITS-5 and ITS-4 were ~~designed~~ used to amplify the ITS regions from *Ophiopogon* and *Liriope*~~these genera~~. Various amplicon sizes were apparent on agarose gels of the PCR products (Fig. 1). Fragments of about 680 bp, which was the calculated size of the ITS fragment from these genera (*O. japonicus*: GenBank accession Nos. EU930852–EU930854; *L. muscari*: Nos. EU930855–EU930856; *L. spicata* var. *prolifera*: No. EU930857), were selected and cloned for the sequence analyses. The sequences of the cloned amplicons varied significantly, and in some cases, several types of sequences were cloned from a single sample (GenBank accession Nos. – were isolated from sample No. 1 and Nos. – were from sample No. 3).

A primer set (21its-33-f and 21its-33-r) designed to amplify the part of the ITS region

from *Ophiopogon* (expected fragment size: 334 bp), but not from *Liriope*, was tested. This primer set amplified the ITS regions of both *Ophiopogon* and *Liriope* (Fig. 2), so it was not possible to distinguish *Ophiopogon* from *Liriope* based on the presence or absence of amplicons from this region. *Ophiopogon* and *Liriope* plants have long been cultivated for medicinal and ornamental purposes, and it is possible that this history of cultivation has caused the accumulation of different types of mutations in their respective ITS regions. These results suggest that the ITS regions are not suitable for discrimination of *Ophiopogon* and *Liriope*.

2. Distinguishing *Liriope* from *Ophiopogon* based on *rbc*L regions

Using similar protocols as those used to amplify and analyze the ITS region, the *rbc*L regions of *Ophiopogon* and *Liriope* were amplified and analyzed. Primers were designed based on the sequences deposited in the GenBank database (*O. jaburan*: GenBank accession Nos. AB029840, AB113256 and AB113257; *O. japonicus*:~~,~~ Nos. AB029841, AB113250 and ~~–~~AB113251; *O. intermedius*: No. AB113252; *O. bodinieri*: No. AB113253; *O. planiscapus* No. AB113254; *O. chingii*: No. AB113255; *L. spicata*: Nos. AB113257 and AB113258; *L. minor*: No. AB113259; *L. muscari*: Nos. AB113260 and~~,~~ Z77271). The PCR products obtained were the correct size for *rbc*L as indicated by

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sequence data in the database (ca. 460 bp, Fig. 3).

The base at position 266 from the 5′ end of the *rbc*L region is a guanine in *Ophiopogon*, and the corresponding base in *Liriope* is a thymine (Fig. 4). This difference indicated that the amplicon of this region derived from *Liriope* would be digested by *Hinc*II to generate two fragments, and that the corresponding sequence from *Ophiopogon* would not be digested. Actually, a long, undigested fragment was observed on agarose gels after digestion of *Ophiopogon* PCR products, and two short, digested fragments (266 and 194 bp) were detected after digestion of *Liriope* PCR products (Fig. 5). Accordingly, *Ophiopogon* and *Liriope* could be distinguished by *Hinc*II digestion of the amplified *rbc*L fragments (Fig. 5).

3. Application of the developed method involving *Hinc*II digestion of *rbc*L fragments to market samples

Ten samples of tubers that were previously analyzed based on their DNA sequences (five were derived from *Ophiopogon* and the other five were from *Liriope*, Table 2 [2]) were analyzed using the *Hinc*II-digestion method developed here, which confirmed the results based on sequence data comparison (Fig. 6). However, the amplicons of *rbc*L regions from dried *Liriope* tubers (Nos. 19–23) were not completely digested with

*Hinc*II, and three fragments were generated (460 bp, 266 bp, 194 bp; Fig. 6). In contrast, the amplicons from the *Ophiopogon* samples (Nos. 14–18) were not digested by *Hinc*II (Fig. 6).

Market samples of *Bakumondo* (Nos. 24–41, Table 3) were examined using the *Hinc*II-digestion method. Fourteen out of 18 samples were identified as *Ophiopogon* and the other four were identified as *Liriope* (Fig. 7). Among these eighteen samples, three samples that had been assumed to be *Ophiopogon* according to their morphological characteristics (Nos. 39–41) were found to be *Liriope* by the *Hinc*II-digestion method, and three others that had been supposed to be *Liriope* (Nos. 33–35) were found to be *Ophiopogon*. These results indicated that the comparison of morphological characteristics should be accompanied by other methods of species identification to correctly identify samples.

4. Application of the developed *Hinc*II-digestion method for *rbc*L fragments in mixed powders of *Ophiopogon* and *Liriope*

Powdered *Ophiopogon* (No. 1) and *Liriope* (No. 3) tubers were proportionally mixed and analyzed by PCR to detect *Liriope* contamination in the *Ophiopogon* powders. The detection limit for *Liriope* according to the developed method using *Hinc*II digestion of

the PCR product was 10% in a mixture of powders from the two genera. Two digested fragments (266 and 194 bp) of *rbc*L from *Liriope* were difficult to detect in a mixture containing 1% *Liriope*, but were detectable in a mixture containing 30% *Liriope* (Fig. 8). The digested fragments of a 10% *Liriope* mixture were barely detectable with under the same conditions as described in the Materials and Methods section of the present report. However, the digested fragments amplified from the 10% *Liriope* mixture were detectable with a modified PCR method that included increasing amplification cycles to 50, and doubling the total volume of the PCR reaction mixture used in the *Hinc*II digestion (data not shown). Accordingly, the detection limit for *Liriope* with the *Hinc*II-digestion method under the conditions described here is approximately 10% contamination in a mixed sample. Modification of the PCR conditions was sometimes necessary to visualize digestion products in samples containing less than 50% *Liriope*. Detection of the short, digested fragments on agarose gels was low quantities of DNA as templates for PCR, and the mass of PCR products could be increased by modifying the PCR protocols.

5. Analyses of undigested fragments of *rbc*L from *Liriope* in *Hinc*II digests

Undigested fragments were observed in the *Hinc*II-digested PCR products of *Liriope*

samples on agarose gels (Fig. 6 and 7). These undigested fragments were thought to be the result of incomplete digestion, so the *Hinc*II enzymes and reaction conditions were optimized. Varied parameters such as reaction time and units of enzyme were tested, and enzymes from different providers were tried. However, the undigested fragments were still not digested under these varied conditions.

To check the sequence of undigested fragments, the fragments from sample Nos. 40 and 41 (466 bp) were isolated from agarose gels, ligated into a vector, and sequenced (Fig. 9). The sequences of the clones from a sample with an undigested fragment did differ from those that could be digested. One clone, r2-2 (GenBank accession No.) from sample No. 40 whose sequence is nearly identical to rbcL from *L. spicata* (GenBank accession No. KF671518), had a mutation at position 268 that disrupts the *Hinc*II recognition site, whereas clones r2-1 and r2-3 (GenBank accession Nos.) from sample No. 40 were identical to the ~~*Liriope*~~ *L. spicata* *rbc*L sequence ~~registered in the GenBank database~~. Another clone, r3-3 (GenBank accession No.) from sample No. 41, was similar~~identical~~ to the registered ~~*Liriope*~~ *L. spicata* *rbc*L sequence in the database (GenBank accession No. KF671518) with 98.9% nucleotide identity and harbored the *Hinc*II recognition sequence~~, while~~. On the other hand, those of clones r3-1 and r3-6 (GenBank accession Nos.) showed the

highest similarity with~~were the same as the~~ that of *O~~phiopogon~~. mairei* *rbc*L (GenBank accession No. KJ745600) with 95.9% and 95.2% identity, and their nucleotide sequences position from 264 to 269 (the *Hinc*II recognition sequence in *Liriope rbc*L) were identical to those of *Ophiopogon rbc*L (Fig. 9). Clones r3-2, r3-4, and r3-5 (GenBank accession Nos.) were different from both the *Ophiopogon* and *Liriope rbc*L sequences and had lost the *Hinc*II recognition sequence GTYRAC caused by substitution of adenine for the nucleotide position at 266 (Fig. 9). However, the sequence of clones r3-2, r3-4, and r3-5 showed the highest similarity with that~~were most similar to those~~ of *O~~phiopogon~~ and ~~Liriope~~. tonkinensis* *rbc*L (GenBank accession No. KF671510), at 92.4%, 92.4%, and 94.3% identity, respectively. These results showed that PCR of the *rbc*L region from *Liriope*/*Ophiopogon* generated various sequences, so that undigested fragments remained in some samples after *Hinc*II digestion. The undigested fragments also included *Hinc*II-digestible fragments, though the reason for incomplete digestion is unknown. However, no PCR products from *Ophiopogon* samples were digested by *Hinc*II; therefore, any digested *rbc*L fragments would indicate the existence of *Liriope* samples, or their hybrids, in *Ophiopogon* samples.

Conclusion

Bakumondo, which legally must be produced from only *O. japonicus~~phiopogon~~* in Japan, but which may be produced from *Ophiopogon* or *Liriope* in other countries, is difficult to identify morphologically. Using the method based on comparison DNA sequences described here, the two species could be clearly distinguished. One drawback of the method was that there is an undigested fragment in *Liriope* samples. However, this method could be combined with other methods to serve as a primary contamination check for *Bakumondo*.

The origin of some market samples from China used in the present study as predicted by their morphological characteristics differed from that determined using the newer DNA sequence-based method. It is therefore important that comparison of morphological characteristics should be accompanied by other methods of species identification to correctly identify sample origins. No TLC methods for discriminating between *Ophiopogon* and *Liriope* have yet been established. However, at present the method developed in this study could easily distinguish *Ophiopogon* from *Liriope* and does not require expertise in morphology-based identification plant identification, making it useful for identifying the origin of *Bakumondo* samples.

Acknowledgment

We are very grateful to “Shoyakuhinshitsushudankai” and TSUMURA & CO. for providing tuber and plant samples of *Ophiopogon* and *Liriope*. We thank Ms. S. Oguri and Ms. S. Orimichi for their assistance in DNA extraction from *Ophiopogon* and *Liriope* samples.

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Tables

Table 1. Fresh whole p~~P~~lant materials used in this study. Plant species were first identified based on their morphological characteristics.

Sample No.	Voucher	Identified original species	Japanese name	Provider
1	4978	*O. japonicus*	ジャノヒゲ	Shoyakuhinshitsu-shudankai
2	4979	*O. chekiangensis*	セッコウリュウノヒゲ	
3	4980	*L. muscari*	ヤブラン	
4	4981	*L. muscari*	ヤブラン	
5	4982	*L. minor*	ヒメヤブラン	
6	4983	*L. spicata*	リュウキュウヤブラン	
7	4984	*O. jaburan*	ノシラン	
8	4985	*O. japonicus*	ジャノヒゲ	
9	4986	*O. planiscapus*	オオバジャノヒゲ	
10	4987	*L. muscari*	ヤブラン	
11	4988	*O. japonicus*	ジャノヒゲ	
12	4989	*L. minor*	ヒメヤブラン	
13	4990	*O. jaburan*	ノシラン	

Table 2. *Ophiopogon* and *Liriope* tubers previously identified using DNA sequences [2].

Sample No.	Voucher	Identified genus	Provider
14	THS57174	*Ophiopogon*	Tsumura & Co.
15	THS61693	*Ophiopogon*	
16	THS62837	*Ophiopogon*	
17	THS65063	*Ophiopogon*	
18	THS67248	*Ophiopogon*	
19	THS57177	*Liriope*	
20	THS59959	*Liriope*	
21	THS60074	*Liriope*	
22	THS62900	*Liriope*	
23	THS67770	*Liriope*	

Table 3. ~~*Ophiopogon* and *Liriope* tubers~~*Bakumondo* purchased in the Chinese market and fresh plants from the field used in this study

Sample No.	Voucher	Predicted genus	Locality	Year
24	4991	*Ophiopogon*	Sichuan (四川省)	2009
25	4992	*Ophiopogon*	Sichuan (四川省)	2007
26	4993	*Ophiopogon*	Sichuan (四川省)	2010
27	4994	*Ophiopogon*	Sichuan (四川省)	2008
28	4995	*Ophiopogon*	Sichuan (四川省)	2008
29	4996	*Ophiopogon*	Sichuan (四川省)	2008
30	4997	*Liriope*	Sichuan (四川省)	—
31	4998	*Ophiopogon*	Guizhou (貴州省), ornamental	—
32	4999	*Ophiopogon*	Guangxi (広西自治区), ornamental	—
33	5000	*Liriope*	Hubei (湖北省)	2006
34	5001	*Liriope*	Hubei (湖北省)	2007
35	5002	*Liriope*	Hubei (湖北省)	2008
36	5003	*Ophiopogon*	Sichuan (四川省)	2007
37	5004	*Ophiopogon*	Sichuan (四川省)	2008
38	5005	*Ophiopogon*	Sichuan (四川省)	2008
39	5006	*Ophiopogon*	Hubei	—
(Fresh plant)			(湖北省，河北源発薬業有限公司)	
40	5007	*Ophiopogon*	Hubei	—
(Fresh plant)			(湖北省，河北源発薬業有限公司)	
41	5008	*Ophiopogon*	Hubei	—
(Fresh plant)			(湖北省，河北源発薬業有限公司)	

Figure legends

Fig. 1. Amplification products from the ITS regions of *Ophiopogon* and *Liriope* analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. The numbers correspond to sample Nos. shown in Tables 1 and 3. Lane M1: 100-bp DNA ladder.

Fig. 2. The amplified ITS regions of samples amplified using the specific primer pair targeting the ITS sequence of *Ophiopogon*. The fragments were electrophoresed on 1% agarose and stained with ethidium bromide. The numbers correspond to sample Nos. shown in Table 1. Lane M12: 1200-bp DNA ladder.

Fig. 3. Amplification products from *rbc*L regions of *Ophiopogon* and *Liriope* analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. The numbers correspond to the sample Nos. shown in Table 1. Lane M12: 1200-bp DNA ladder.

Fig. 4. Alignment of the *rbc*L nucleotide sequences for the regions amplified from *Ophiopogon* and *Liriope* that were deposited in GenBank database. GenBank Accession Nos. are shown to the left of each lane. The open square indicates the sequence

recognized by *Hinc*II.

Fig. 5. Fragments produced by the restriction enzyme digestion (*Hinc*II) of the amplified *rbc*L region derived from fresh plant materials. Products were electrophoresed on 1% agarose and stained with ethidium bromide. The numbers correspond to the sample Nos. shown in Table 1. Lane M1 shows a 100-bp DNA ladder. Lanes marked with an “n” show *rbc*L amplification products without digestion, and those marked with a “d” are the products of restriction enzyme digestion.

Fig. 6. Fragments produced by restriction enzyme digestion (*Hinc*II) of the amplified *rbc*L region derived from market products. Products were electrophoresed in 1% agarose and stained with ethidium bromide. The numbers correspond to the sample Nos. shown in Table 2. Lane M1 shows a 100-bp DNA ladder. Lanes marked with an “n” show *rbc*L amplification products, and those marked with a “d” are the products of restriction enzyme digestion.

Fig. 7. Fragments produced by restriction enzyme digestion (*Hinc*II) of the amplified *rbc*L region derived from market products and fresh samples (1, 2, and 3). Products

were electrophoresed on 1% agarose and stained with ethidium bromide. The numbers correspond to the sample Nos. shown in Table 2. Lane M1 shows a 100-bp DNA ladder. Lanes marked with an “n” show the *rbc*L amplification products, and those marked with a “d” are the products of restriction enzyme digestion.

Fig. 8. Fragments produced by the restriction enzyme digestion (*Hinc*II) of amplified *rbc*L region derived from mixed powder samples (*Ophiopogon* and *Liriope*). Products were electrophoresed on 1% agarose and stained with ethidium bromide. Lane M1 shows a 100-bp DNA ladder. Lanes marked with “n” show the *rbc*L amplification products, and those marked with “d” are the products after restriction enzyme digestion. The proportion of *Liriope* powder in each sample is shown as a percentage (%).

Fig. 9. Alignment of the nucleotide sequences of the undigested fragments of the amplified *rbc*L regions from No. 2 and 3. Clones r2-1, r2-2, and r2-3 were cloned from sample No. 2, and the rest of the clones (r3-1 to r3-6) were obtained from sample No. 3. The recognition sequence for *Hinc*II is indicated by an open square.