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Taxonomic Relationships in East Asian *Vicia* Species with Unijugate Leaves Based on Random Amplified Polymorphic DNA Markers

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Random amplified polymorphic DNA(RAPD) markers were investigated to clarify the taxonomic positions of *Vicia linearifolia* and *V. bifolia*, and to assess the genomic diversity among the 9 populations of *V. unijuga*, each of which represents a geographical variation or infraspecific taxa in southern Korea. These species are characterized by unijugate leaves in East Asia and have been controversial as to infra- or interspecific classification. The polymorphic markers among the populations examined were observed for fifteen decamer primers. The degree of band sharing was used to calculate genetic similarity between populations, and a phenogram using UPGMA cluster analysis was generated based on the Dice similarity coefficient. The taxa studied were divided into two main groups and the populations of *V. unijuga* were all grouped together in the phenogram. The genetic similarities of *V. unijuga* were very high among the populations and did not show distinctions between the infraspecific taxa, although the populations of Mt. Odae and adjacent areas in eastern Korea were different from others of the species. *V. linearifolia* fell within the range of the genomic variation among the populations of *V. unijuga*, while *V. bifolia* was grouped with *V. venosa* var. *cuspidata* having multijugate leaves rather than *V. unijuga*. The result from studying RAPD markers suggested that *V. linearifolia* should be integrated into *V. unijuga* and that species with unijugate leaves of *V. bifolia* and *V. unijuga* are polyphyletic.

Keywords: genomic diversity, RAPD, taxonomic relationships, *Vicia bifolia, V. unijuga*

INTRODUCTION

Among the species of *Vicia* in East Asia, the following three species are characterized by having the leaves with a pair of leaflets (unijugate); *V. unijuga* A. Br., *V. bifolia* Nakai and *V. linearifolia* Y.N. Lee which are all classified in the section *Vicilla* (Kupicha, 1976; Endo and Ohashi, 1996; Seok, 1997). The taxonomic position of the last two species, however, has been controversial. *V. bifolia* grows in C. Honshu of Japan and has been separated from *V. unijuga* by its large and marcescent bracts as against the small and deciduous of the latter as a species rank (Nakai, 1923; Ohashi, 1982; Ohwi, 1984), while the plant has also been treated as an infraspecific taxon of *V. unijuga*, i.e., var. *bracteata* Fran. et Sav. (Franchet and Savatier, 1879; Kitamura and Murata, 1961). Another species of *V. linearifolia* was recorded at Mt. Kyebang near Mt. Odae National Park in eastern Korea by the characteristics of narrow linear leaflets and whitish flowers (Lee, 1982). This species, however, was not clearly distinguished from *V. unijuga* because the leaflet shapes and flower colors of the species fell within the variations of those in the latter (Seok and Choi, 1997).

On the other hand, *V. unijuga* is widely distributed in the temperate regions of East Asia and has been separated into several infraspecific taxa on the basis of morphological variations such as leaflet shapes, inflorescence features, and flower colors (Maximowicz, 1873; Makino, 1908; Léveillé, 1913; Nakai, 1923 and 1935; Honda, 1939). The species, however, shows extreme variations in the diagnostic characters of the infraspecific taxa, and until recently we could recognize the following infraspecific taxa in *V. unijuga* based solely on studies of external morphologies; f. *albiflora* Nakai, var. *angustifolia*

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Makino, var. *apoda* Maxim. and var. *kaussanensis* H. Lév. (Seok, 1997; Seok and Choi, 1997).

We question whether the separation of *V. bifolia* or *V. linearifolia* from *V. unijuga* would be supported by DNA data and whether the morphological variations and the infraspecific classification of *V. unijuga* are associated with genomic diversity in southern Korea.

Random amplified polymorphic DNA (RAPD) analysis is based on the amplification of genomic DNA with a single primer of arbitrary nucleotide sequence and is a relatively easy tool for assessing genomic diversity (Williams *et al.*, 1990). Recently RAPD methods have been applied to resolve taxonomic questions between closely related groups (Demeke *et al.*, 1992; Buren *et al.*, 1994; Campos *et al.*, 1994; Stammers *et al.*, 1995; Lifante and Aguinagalde, 1996). It has also been used to study genomic diversity in the species of *Vicia* (Torres *et al.*, 1993; Gustafsson and Gustafsson, 1994).

In this study, we performed RAPD analysis to assess the extent of genomic diversity in *V. unijuga* while also comparing morphological variations and infraspecific classification to more precisely determine the taxonomic position of *V. bifolia* and *V. linearifolia*.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

Eleven individuals from eleven populations of the *Vicia* species having unijugate leaves in East Asia were included in this study, that is, nine of *V. unijuga*, one of *V. bifolia* and one of *V. linearifolia* (Table 1). For the comparison we included one species having multijugate leaves, i.e., *V. venosa* var. *cuspidata* Maxim. which is also classified in the section *Vicilla* (Kupicha, 1976; Endo and Ohashi, 1996) together with the unijugate species studied, and is similar to *V. unijuga* in gross morphology and growing habitat except the number of leaflets.

Among the infraspecific taxa recognized in *V. unijuga* (Seok, 1997; Seok and Choi, 1997) we could not examine the samples of var. *angustifolia* Makino distributed in C. Honshu of Japan and var. *apoda* in northern Korea to S. Ussuri in this study. On the other hand, to cover the geographical varieties and the infraspecific taxa of *V. unijuga* in southern Korea we employed nine representative populations of *V. unijuga*, that is, seven of var. *unijuga*, one of f. *albiflora* and one of var. *kaussanensis*. The last one is the type locality of the variety at Mt. Halla, Cheju Isl. Seven populations of var. *unijuga* were collected from distinct regions in S. Korea such as three populations from Kyounggi Province, two from Kangwon, one from Cheolla and one from Kyoungsang (Taegu-shi) respectively. Among the populations of var. *unijuga*, that of Mt. Cheonma (No. 1) corresponds to var. *ouensanensis* H. Lév. in having the characteristic of large leaflets, although it was treated as var. *unijuga* according to the infraspecific classification by Lee (1969) in this study. Because *V. unijuga* shows the greatest variation in external morphology at Mt. Odae and adjacent areas, including Mts. Odae and Kyebang of Prov. Kangwon in eastern Korea, more than one representative of each population of *V. linearifolia* and *V. venosa* var. *cuspidata* were collected from those regions. Also, three of *V. unijuga* were included from the region; two of var. *unijuga* and one of f. *albiflora* (Table 1).

Plant samples for RAPD analysis were transferred to the greenhouse from the field in small spots or the leaves were transported to the laboratory on ice or with silicagel and stored at -70°C until use. Voucher specimens used for this study are deposited

Table 1. Plant materials studied for RAPD analysis

No.	Taxon	Locality	Voucher
1.	*Vicia unijuga* var. *unijuga*	Korea, Prov. Kyonggi, Namyangju-gun, Mt. Cheonma	Seok 771 (IUI)
2.	*V. unijuga* var. *unijuga*	Korea, Prov. Kyonggi, Kimpo-gun, Mt. Munsu	Seok 907 (IUI)
3.	*V. unijuga* var. *unijuga*	Korea, Incheon-shi, Kanghwa-gun, Mt. Mani	Seok 804 (IUI)
4.	*V. unijuga* var. *unijuga*	Korea, Prov. Kangwon, Pyoungchang-gun, Mt. Kyebang	Seok 598 (IUI)
5.	*V. unijuga* var. *unijuga*	Korea, Prov. Kangwon, Pyoungchang-gun, Mt. Kyebang	Seok et Kim 636 (IUI)
6.	*V. unijuga* var. *unijuga*	Korea, Taegu-shi, Mt. Ap	Seok 894 (IUI)
7.	*V. unijuga* var. *unijuga*	Korea, Prov. Chollanam, Kwangyang-shi, Mt. Paekun	Seok 880 (IUI)
8.	*V. unijuga* f. *albiflora*	Korea, Prov. Kangwon, Pyoungchang-gun, Mt. Odae	Seok 708 (IUI)
9.	*V. unijuga* var. *kaussanensis*	Korea, Prov. Cheju, Sogwipo-shi, Mt. Halla	Seok 756 (IUI)
10.	*V. linearifolia*	Korea, Prov. Kangwon, Pyoungchang-gun, Mt. Odae	Seok 655 (IUI)
11.	*V. bifolia*	Japan, Tochigi Pref., Nikko-shi, Ogoragawa	Choi *et al.* 15010 (IUI)
12.	*V. venosa* var. *cuspidata*	Korea, Prov. Kangwon, Pyoungchang-gun, Mt. Odae	Seok 906 (IUI)

at Herbarium (IUI) of Inha University.

Total DNA was isolated from fresh or dried leaf tissue according to CTAB method (Doyle and Doyle, 1987), except that 2% PVP-40 was added in the extraction buffer to reduce phenolic compounds and polysaccharides. The extracted DNA was quantified by a Spectrophotometer, diluted to uniform concentrations of 1-2ng per μl, and stored at -20°C until use.

DNA Amplification

RAPD markers were amplified from total cellular DNA as described by Williams *et al.* (1990) with some modifications. One hundred 10-base oligonucleotide primers (purchased from The University of British Columbia, Nucleic Acid-Protein Service Unit; primer Nos. 1-100) were examined for PCR amplification in the present study. DNA amplification was conducted in 25 μl final volumes containing 2-5 ng total genomic DNA templates, 1.0 unit of Taq Polymerase (Boehringer Mannheim), 10mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μM each of dNTP, and 0.5 μM primer. The mixture was covered with a drop of mineral oil to prevent evaporation. The amplifications were performed using DNA Thermal Cyclers (Progene, Techne) programmed for 35 cycles of 1 min at 94°C to denature, 1 min at 37°C for annealing, and 2 min at 72°C for extension. A predenaturation step for 2 min at 94°C preceded the first cycle, and a final extension step for 7 min at 72°C followed the completion of 35 thermal cycles. In the preliminary survey, we applied a variety of annealing temperatures from 35°C to 41°C for each primer, but the amplification products did not strongly depend on these ranges of the annealing temperatures in the primers used. We therefore performed all amplifications at 37°C of annealing temperature in this study. Duplicate amplifications of each sample were run to estimate repeatability of the amplification process.

7 μl of each amplified product were electrophoresed in 1.5% agarose gel (70 V, 2.5 h) in TAE buffer, and visualized in the presence of ethidium bromide. The gels were photographed under UV light with Polaroid 667 film. λDNA/Hind III (Promega) and 100bp (Gibco BRL) DNA ladders were included as molecular weight markers on both outside lanes.

Data Analyses

The RAPD markers were scored as present or absent for amplification fragments. Each band was coded as: 0=no band, or 1=band present. Only markers that were clearly visible and detected for the replications performed for each primer were scored. A rectangular data matrix of each population for each primer was generated by coding the DNA bands as described above. Dice (1945) similarity coefficient index was used to obtain genetic similarity between the populations. The calculation is:

$$GSxy = 2Nxy/(Nx+Ny)$$

in which Nx and Ny are the number of bands for individual x and y, and Nxy is the number of shared bands for both individuals x and y. These algorithms consider only the presence of a shared band as a measure of similarity, but not the shared absence of fragments.

The NTSYS-pc package of computer programs (version 1.70; Rohlf, 1992) was used to generate the similarity coefficient and the phenogram using UPGMA cluster analysis.

RESULTS

One hundred primers were screened prior to the selection of thirty one primers. The remaining sixty nine primers failed to amplify DNA product or produced ambiguous fragments. Of the thirty one primers examined, fifteen were suitable to amplify polymorphic scorable bands for the plants studied (Table 2), while another eight produced monomorphic bands in all individuals and the other eight were too

Table 2. Sequences of 15 decamer primers used for generating polymorphic RAPD markers and number of amplification fragments detected

Primer No.	Primer sequences (5' to 3')	No. of amplified fragments	No. of polymorphic fragments
2	CCTGGGCTTG	8	7
3	CCTGGGCTTA	4	3
6	CCTGGGCCTA	4	3
7	CCTGGGGGTT	2	1
21	ACCGGGTTTC	5	3
34	CCGGCCCCAA	4	2
36	CCCCCCTTAG	2	2
42	TTAACCCGGC	4	3
48	TTAACGGGGA	2	2
70	GGGCACGCGA	3	2
77	GAGCACCAGG	6	2
79	GAGCTCGTGT	2	1
80	GTGCTCTAGA	2	1
82	GGGCCCGAGG	4	3
97	ATCTGCGAGC	5	1

Table 3. Similarity coefficients matrix between the populations of *Vicia unijuga* and related three species obtained by Dice (1945)-Nei & Li (1979) index from RAPD data. Number of populations corresponds to that in Table 1.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	0.9714											
3	1.0000	0.9714										
4	0.9855	0.9855	0.9855									
5	0.8333	0.8611	0.8333	0.8451								
6	0.9855	0.9855	0.9855	1.0000	0.8451							
7	1.0000	0.9714	1.0000	0.9855	0.8333	0.9855						
8	0.8732	0.8732	0.8732	0.8571	0.9315	0.8571	0.8732					
9	0.9855	0.9855	0.9855	1.0000	0.8451	1.0000	0.9855	0.8571				
10	0.8732	0.8451	0.8732	0.8571	0.9041	0.8571	0.8732	0.9167	0.8571			
11	0.6667	0.6667	0.6667	0.6757	0.7013	0.6757	0.6667	0.6579	0.6757	0.6842		
12	0.7500	0.7500	0.7500	0.7619	0.7879	0.7619	0.7500	0.7692	0.7619	0.7692	0.7826	

complex to score. From the fifteen primers a total of 57 scorable bands were generated in sizes ranging from 300 to 2500 base pairs; 36 polymorphic products among the samples studied, and 21 monomorphic ones common to all samples.

A Dice similarity matrix from RAPD markers between the populations is presented in Table 3. A phenogram indicating taxonomic relationships among the populations of *V. unijuga* and other three species was generated by UPGMA cluster analysis based on the Dice similarity index (Fig. 1). The two main groups were distinguished by a mean similarity coefficient of 0.72. One of these groups, group C, included *V. bifolia* and *V. venosa* var. *cuspidata* and the similarity between the two species was 0.78. The other main group was divided into two subgroups, A and B, with a mean similarity of 0.86. One of them, subgroup B, included *V. linearifolia*, *V. unijuga* var. *unijuga* and f. *albiflora* with similarities of 0.91-0.94, which were all collected from Mt. Odae and adjacent areas in E. Korea. The remaining subgroup A clustered with 7 populations of *V. unijuga* which are all comprised in var. *unijuga* except var. *kaussanensis*. They showed high values of similarity coefficient of more than 0.98 to each other. The infraspecific taxa of *V. unijuga* such as var. *kaussanensis* and f. *albiflora* were not distinguished from var. *unijuga* in this

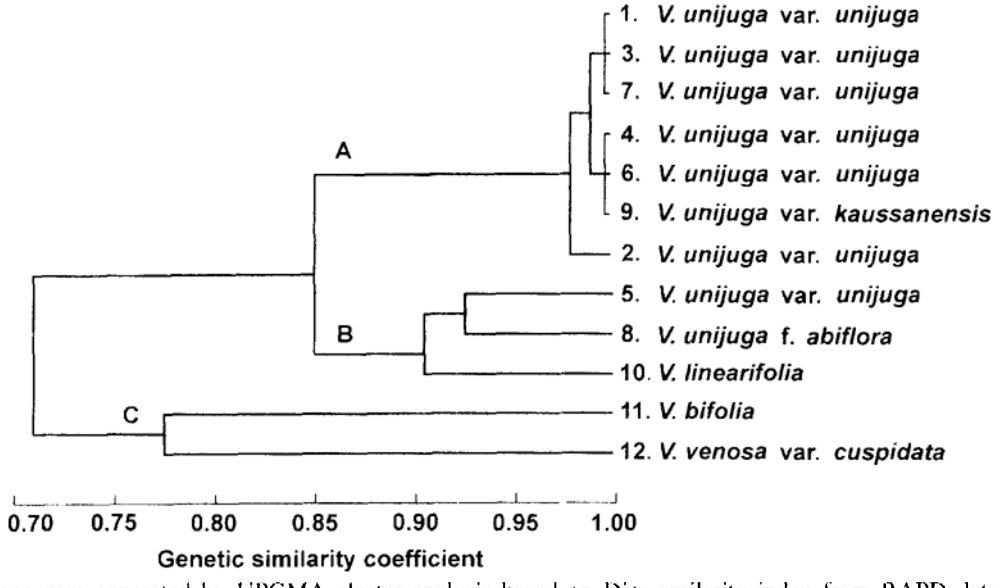

Fig. 1. A phenogram generated by UPGMA cluster analysis based on Dice similarity index from RAPD data, showing the relationships among the populations of *Vicia unijuga* and related three species in E. Asia. Their origins of populations are given in Table 1.

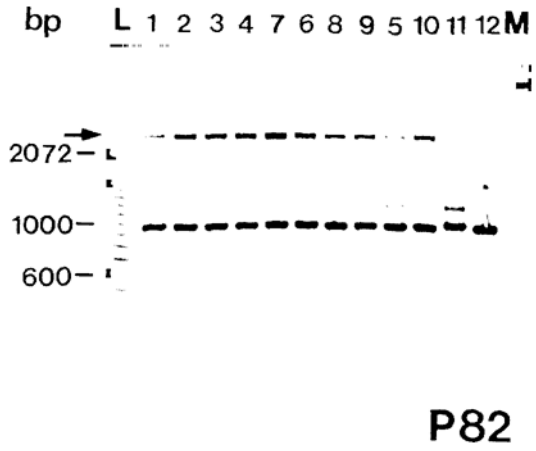

Fig. 2. RAPD patterns obtained with primer number 82. Arrow indicates the genomic marker shared in *Vicia unijuga* and *V. linearifolia* which is not detected in *V. bifolia* and *V. venosa* var. *cuspidata*. The DNA molecular weight markers are λDNA/*Hin*dIII (right) and 100 bp DNA Ladder (left). Lane assignments are as follows: Lanes 1 to 9; *V. unijuga*, 10; *V. linearifolia*, 11; *V. bifolia*, 12; *V. venosa* var. *cuspidata*. Population identity of 1 to 9 lanes corresponds to that in Table 1.

RAPD analysis. The genetic variation among the individuals in same population was not examined in this study, because very low genetic differences were revealed even among the geographical populations in *V. unijuga* var. *unijuga* as shown in Table 2 and Fig. 1.

Among the 36 polymorphic bands detected, *V. linearifolia* and *V. unijuga* shared 5 RAPD markers which were not detected in *V. bifolia* and *V. venosa* var. *cuspidata* included group C, the approximate band sizes of the markers were 1700 base pairs (bp) in primer No. 2 (P2), 500 bp in P6, 1500 bp in P48, 1800 bp in P70 and 2500 bp in P82 (Fig. 2), respectively.

DISCUSSION

RAPD analysis was applied for studying taxonomic relationships among the East Asian species of *Vicia* with unijugate leaves. Fifteen primers screened for this study which produced polymorphic bands among the samples, showed high percentages of G+C content ranging from 50% to 90%, with a mean of 67%. Because only the clear bands which were consistently detected for the PCR replications were scored, a relatively small number of bands per each primer, 2-8, was used in this analysis (Table 2).

In the phenogram generated by RAPD data, the populations of *V. unijuga* were all grouped together and showed high values of similarity coefficient, 0.83-1.00, to each other (Fig. 1) as compared with the results from other closely related groups studied (Buren *et al.*, 1994; Stammers *et al.*, 1995; Lifante and Aguinagalde, 1996). Among the infraspecific taxa of *V. unijuga*, var. *ouensanensis*, which was recorded in Korea basically by the characteristics of leaflet shapes (Léveillé, 1913), could not be distinguished from var. *unijuga* because of the variation in its diagnostic character of the leaflet shapes (Lee, 1969; Seok and Choi, 1997). The population (No. 1) corresponding to the infraspecific taxon in the characteristics of leaflet shapes was also not separable from var. *unijuga* by RAPD analysis. It was interesting that although *V. unijuga* var. *kaussanensis* is isolated at the highland of Mt. Halla of Cheju Island and differs from other infraspecific taxa in external morphologies such as the size of leaflets and inflorescences (Seok and Choi, 1997), the taxon also could not be distinguished from the other populations of *V. unijuga* by RAPD analysis. These results showed that the high level of genetic similarity among the populations of *V. unijuga* was not consistent with a great variation in external morphology or for separating it into the infraspecific taxa.

V. linearifolia is restricted at Mt. Kyebang near Mt. Odae in eastern Korea and has been distinguished from *V. unijuga* by its narrow linear leaflets and whitish flowers (Lee, 1982). The leaflet shape of the species, however, showed a clinal variation with that of *V. unijuga* var. *angustifolia* Makino (Seok, 1997), and the individuals of two taxa were grouped together in the principal components analysis based on morphological characters (Seok and Choi, 1997). Furthermore, the whitish flowers are also observed in *V. unijuga* f. *albiflora* growing in the region with the species. The similarity coefficients between *V. linearifolia* and the populations (Nos. 5 & 8) of *V. unijuga* from Mt. Odae and adjacent areas were very high, 0.91-0.94, in RAPD markers and these values fall within the range of similarity, 0.83-1.00, among the infraspecific taxa or the geographical populations in *V. unijuga*. Therefore, RAPD and morphological (Seok and Choi, 1997) data indicate that the separation of *V. linearifolia* from *V. unijuga* as species rank is no longer significant and that the plant is one of the populations showing extreme variation of leaflet shapes in *V. unijuga*. Furthermore, the taxon was almost the same as *V. unijuga* in the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA as sequence divergences 0.0 % (Seok

et al., unpublished).

All subgroup B were populations of Mt. Odae and adjacent areas of *V. unijuga* and *V. linearifolia*, where the species shows the greatest diversity of morphological variations such as leaflet shape and flower colors. Incidentally, the two populations of *V. unijuga* included in subgroup B are related to *V. linearifolia* in morphology, that is, f. *albiflora* in having whitish flowers and the other population of var. *unijuga* (No. 5) in narrow leaflets, respectively.

V. bifolia has been treated as a distinct species (Ohashi, 1982; Ohwi, 1984) or merged into *V. unijuga* (Franchet and Savatier, 1879; Kitamura and Murata, 1961). The controversy on the taxonomic position of the taxon is basically owing to that the taxonomic implication of the diagnostic characters of bracts and the number of leaflets was uncertain in this group. In the phenogram from RAPD data, *V. bifolia* was clearly separated from *V. unijuga* and *V. linearifolia* having unijugate leaves, while clustered together with the multijugate species of *V. venosa* var. *cuspidata*. The mean similarity coefficient value, 0.67 (0.66-0.70), between *V. bifolia* and *V. unijuga* was lower than that between *V. venosa* var. cuspidata and *V. unijuga*, 0.76 (0.75-0.79). This result from RAPD analysis is also in accord with the DNA sequence data of the ITS regions. In the sequence divergences of the ITS regions calculated by the Kimura two-parameter method, *V. bifolia* was more similar to *V. japonica*, 0.2%, which also has multijugate leaves, than to *V. unijuga*, 1.8%, or to *V. linearifolia*, 1.6% (Seok *et al.*, unpublished). The RAPD markers and ITS sequences support that *V. bifolia* retains a species rank. Furthermore, *V. bifolia* differs in having ovate or lanceolate stipules as against semi-sagittate in *V. unijuga* var. *unijuga* in addition to the known difference of bracts (Seok, 1997).

The mature leaves are usually multijugate in the genus *Vicia* (Kupicha, 1977; Endo and Ohashi, 1997) and multijugate leaves is pleisiomorphic to not multijugate (including unijugate of apomorphic) in Vicieae, Cicereae and Trifolieae (Endo and Ohashi, 1997). Therefore, the species with unijugate leaves in *Vicia* can be assumed to be evolved from the ancestral species having multijugate leaves. The DNA data from RAPD markers and ITS sequences, however, showed that *V. bifolia* was more related to the species with multijugate leaves than *V. unijuga*. It suggested that the reduction of leaflet number resulted from parallel evolution, and that it has occurred more than once in the course of evolution for this group. Consequently, East Asian species having unijugate leaves of *V. bifolia* and *V. unijuga* are polyphyletic.

The number of DNA polymorphisms that can be detected with RAPD analysis is higher than with traditional methods (Comincini *et al.*, 1996). RAPD data were useful to clarify the taxonomic position of *V. bifolia* and *V. linearifolia* which have been controversial because of the limited number of morphological characteristics available for classification.

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