Phylogenetic Differentiation of Tetraploid *Vigna* Species, *V. glabrescens* and *V. reflexo-pilosa*

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Abstract

The subgenus *Ceratotropis* of the genus *Vigna* which originated in Asia contains cultigens such as mungbean, adzuki bean, etc. The *Ceratotropis* species are diploid (2n=22, 2x) except for the occurrence of two tetraploid species, *V. glabrescens* and *V. reflexo-pilosa*. *V. glabrescens* exhibits resistance to major mungbean pests and diseases including powdery mildew, cucumber mosaic virus, bean fly (*Ophiomyia phaseoli*, *O. centrosematis* and *Melanagromyza soja*), etc., and is useful in mungbean improvement programs. In order to clarify the phylogenetic differentiation of the tetraploid species,

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we analysed the isozyme banding patterns of the *Ceratotropis* species. Isozyme analysis suggested that wild *V. reflexo-pilosa* originated from interspecific hybridization between *V. trinervia* and *V. minima* var. *minima* followed by spontaneous chromosome doubling. Isozyme analysis and interspecific hybridization experiments showed that *V. glabrescens* and *V. reflexo-pilosa* are phylogenetically closely related. *V. glabrescens* is thus considered to be derived from *V. reflexo-pilosa* as cultivated type with erect growth habit. The data obtained from interspecific hybridization experiments showed that *V. trinervia* was the seed parent rather than the pollen parent. *V. reflexo-pilosa*, *V. minima* var. *minima* and *V. trinervia* are also anticipated to be useful for breeding programs of *Ceratotropis* cultigens in addition to *V. glabrescens*, since they are involved in the speciation of *V. glabrescens*.

Additional key words: *Ceratotropis* species, *V. trinervia*, *V. minima* var. *minima*, isozyme analysis, interspecific hybridization

Introduction

The subgenus *Ceratotropis* of the genus *Vigna*, including cultivated mungbean, adzuki bean, etc., is considered to have originated in Asia. In addition to the cultivated species, the subgenus *Ceratotropis* also contains about 16 wild species[10, 14, 15] which are diploid (2n=22, 2x) except for the occurrence of two tetraploid species. One of the two tetraploid species, *V. glabrescens* was formerly treated as a variety of *V. radiata*, i.e., var. *glabra*[2, 10, 18]. One accession of *V. glabrescens* collected from the Philippines (USDA P.I. 207655; AVRDC V1160) regularly formed 22 bivalents without multivalents at the meiosis and was considered to be an amphidiploid[12]. Another tetraploid species, *V. reflexo-pilosa* is distributed from Japan (Ryukyu islands), to Taiwan, the Philippines, Malaysia, New-Guinea, Australia, etc.[14, 15] This species also shows the formation of 22 bivalents at the meiosis and is considered to be an amphidiploid[5].

V. glabrescens harbours resistance genes to major mungbean pests and diseases and is utilized in the mungbean improvement program at the Asian Vegetable Research and Development Center (AVRDC)[1, 9, 11, 19]. However, introduction of these valuable resistance genes from *V. glabrescens* into mungbean via interspecific hybridization had never been successful due to the interspecific hybrid sterility between them. Therefore, we attempted to clarify the genome constitution of this tetraploid species at the diploid level by cytogenetical analysis[4, 6] before the initiation of breeding programs.

In the present study, we report on the interspecific relationship of *V. glabrescens* with other species and also discuss the phylogenetic differentiation of *V. glabrescens* based on isozyme analysis and interspecific hybridization experiments.

Materials and Methods

1) Materials

The species used in the present study are listed in Table 1[3, 5, 7, 8, 16, 17].

2) Electrophoresis of isozymes

Crude extracts of isozymes were prepared by homogenizing a germinated seed 2 to 3 days after water-absorption using 0.02M Tris-HCI (pH 8.0) buffer solution containing 5% (W/V) of sucrose. The homogenate was centrifuged at 15,000 rpm for 10 minutes and 10 μl of the supernatant was used for electrophoresis. Electrophoresis was conducted at the rate of 15 mA for one polyacrylamide gel plate using a vertical mini-slab gel electrophoresis apparatus (ATTO Corporation, Japan). The separating gel with a gradient of acrylamide

Table 1. The subgenus *Ceratotropis* species used in the present isozyme analysis.

Species name		2n= (ploidy)	No. of accessions	Location
V. angularis var. *nipponensis*	(wild adzuki bean)	22(2x)	3	Japan
V. umbellata (twining form)[1)]	(wild rice bean)	22(2x)	3	Thailand[4)]
V. nakashimae	(wild)	22(2x)	2	Japan, Korea
V. minima var. *minor*[2)]	(wild)	22(2x)	3	Japan[5)]
V. minima var. *minima*	(wild)	22(2x)	2	Malaysia[6)], Thailand[4)]
V. trinervia[3)]	(wild)	22(2x)	66	Malaysia[6)]
V. reflexo-pilosa	(wild)	44(4x)	10	Japan[5)], Malaysia[6)]
V. glabrescens	(cultivated)	44(4x)	1	Philippines[7)]

1) Tateishi and Ohashi (1990)[15)].
2) This species was formerly classified as *V. riukiuensis*.
3) Tateishi (1985)[14)].
4) Collected from Chiang Mai province (Tomooka 1991)[16)].
5) Collected from Irimote and Yonaguni islands, Okinawa (Egawa *et al.* 1990[5)]; Egawa *et al.* 1991[7)]).
6) Collected from Peninsular Malaysia (Egawa *et al.* 1992[8)]; Tomooka *et al.* 1993[17)]; Bujang *et al.* 1994[3)])
7) USDA P.I. 207655 (AVRDC V1160)

concentrations ranging from 7.5% to 15% (W/V) was used and the pH was adjusted to 9.0 (0.03M Tris-HCl). The acrylamide concentration of the stacking gel was 4.5% (W/V) with the pH adjusted to 7.0 (0.03M Tris-HCl). Variation of isozyme banding patterns was analysed for four isozymes, 6-phosphogluconate dehydrogenase (6PGDH), glutamate-oxaloacetate amino peptidase (GOT), shikimate dehydrogenase (SDH) and leucine amino peptidase (LAP) following the staining procedures shown in Table 2.

3) Interspecific hybridization experiments

The materials were grown in pots in a net house at the Chai Nat Field Crops Research Center, Chai Nat, Thailand. For crossing, unopened flower buds were emasculated on the day before opening. Thereafter, they were pollinated immediately and covered with paraffin-paper bags to prevent contamination. Open-pollinated flowers were removed to minimize the competition among pods. Pod setting was observed 8 to 10 days after pollination. Pods were harvested at maturity.

4) Pollen stainability

Pollen stainability was assessed based on the percentage of well-stained pollen grains with 1% acetocarmine solution. About 600 pollen grains were examined three times using flowers collected at different times.

Results

1) Isozyme analysis of the Ceratotropis *species*

As shown in Fig. 1, the diploid *Ceratotropis* species produced only one band for 6PGDH. We designated the banding pattern exhibited by *V. trinervia* and *V. angularis* var. *nipponensis* as "a" type (Table 3 and Fig. 5). Other diploid species except for these two species exhibited the "c" type banding pattern, which moved more slowly than the "a" type banding (Figs. 1 and 5). For GOT, the diploid species showed two banding patterns (Fig. 2). We designated them as "a" or "c" type (Fig. 5 and Table 3). Most of the diploid species exhibited the c type banding pattern (Table 3), while *V. trinervia* exhibited the "a" type banding pattern (Table 2). Both tetraploid species, *V. reflexo-pilosa* and *V. glabrescens*, exhibited the b type banding pattern for 6PGDH and GOT (Figs. 1, 2 and 5 and Table 3).

For SDH, we examined 22 accessions of *V. trinervia* and found that two banding patterns (a_1

Table 2. Procedures for isozyme staining

I) 6PGDH
1) take 100ml of 0.1M Tris-HCl, pH8.0 in a 100ml beaker
2) add 20mg of NBT
3) add 20mg of NADP
4) add about 40 mg of 6-phosphogluconic acid
5) add a small amount of PMS, and then transfer the solution to a container
6) immediately soak the gel in the solution
7) shake gently until bands appear
8) stop the enzyme reaction by washing the gel with plenty of tap water

II) GOT
1) take 100ml of 0.5M Tris-HCl, pH8.5 in a 100ml beaker
2) add 400mg of aspartic acid
3) add 200mg of α -ketoglutaric acid, and stir well for more than 15 minutes
4) add 2mg of pyridoxal 5 phosphate
5) add 400mg of Fast Blue BB salt and stir well
6) transfer the solution to a container
7) soak the gel in the solution and shake gently until bands appear
8) stop the enzyme reaction by washing the gel with plenty of tap water

III) SDH
1) take 100ml of 0.1M Tris-HCl, pH8.5 in a 100ml beaker
2) add about 20mg of shikimic acid
3) add 5mg of NADP
4) add 5mg of NBT
5) add 2mg of PMS, and then immediately transfer the solution to the container
6) put the gel in the solution and shake gently until bands appear
7) stop the enzyme reaction by washing the gel with plenty of tap water

IV) LAP
1) take about 5mg of L-leucine β -naphthylamide in a 100ml beaker
2) add 1ml of N, N-dimethylformamide to dissolve it
3) add 100ml of 0.1M phosphate buffer solution, pH7.1 in the beaker and then transfer the solution to a container
4) soak the gel in the solution for 20 minutes, and then discard the solution
5) take 100ml of 1M phosphate buffer solution, pH7.1 in a beaker
6) add 100mg of Fast Black K salt, and dissolve well by stirring and transfer the solution to a container
7) soak the gel in the solution and shake until bands appear
8) stop the enzyme reaction by washing the gel with plenty of tap water

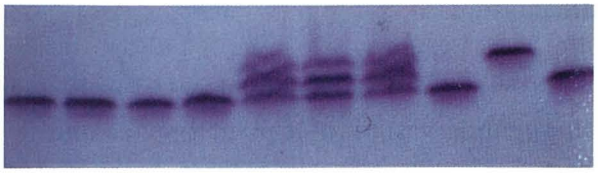
Table 3. The banding patterns of three isozymes in the *Ceratotropis* species

Species	6PGDH	GOT	LAP
V. angularis var. *nipponensis*	a(3)[1)]	c(2)	c(3)
V. umbellata (twining form)	c(3)	c(3)	a(1);c(1)
V. nakashimae	c(2)	c(2)	c(2)
V. minima var. *minor*	c(3)	c(3)	c(2)
V. minima var. *minima*	c(2)	c(2)	c(1)
V. trinervia	a(23)	a(23)	a(65)
V. reflexo-pilosa	b(6)	b(1)	b(10)
V. glabrescens	b(1)	b(1)	b(1)

1)Figure in parenthesis shows the number of accessions examined electrophoretically.

and a_2 types) occurred[8)] as shown in Figs. 3 and 5. All the five accessions of *V. reflexo-pilosa* examined electrophoretically exhibited the b type[8)] (Figs. 3 and 5). *V. minima* var. *minima* exhibited the c type banding pattern[8)] (Fig. 5).

We analyzed 65 accessions of *V. trinervia* and 10 accessions of *V. reflexo-pilosa* for LAP and found that these species exhibited polymorphism for the banding patterns[8)] (Fig. 4). However, these species did not exhibit intraspecific variation for the banding patterns in the zone A. All the accessions of *V. trinervia* produced one band in this zone which we designated as "a" type and all

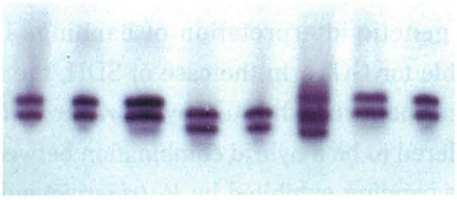


1 2 3 4 5 6 7 8 9 10

Fig. 1. The banding pattern of 6PGDH. 1 to 4, 8 and 10; *V. trinervia*, 5 to 7; *V. reflexo- pilosa* and 9; *V. minima* var. *minima*.

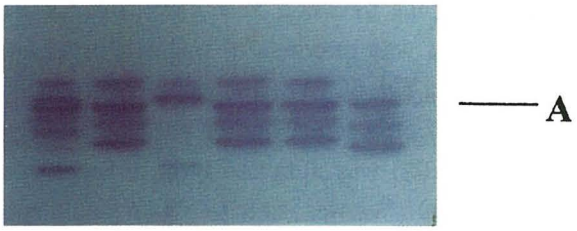
1 2 3 4 5 6 7 8 9 10

Fig. 2. The banding pattern of GOT. 1 to 7; *V. trinervia*, 8; *V. reflexo-pilosa*, 9; *V. glabrescens* and 10; *V. minima* var. *minima*.



1 2 3 4 5 6 7 8

Fig. 3. The banding pattern of SDH. 1 to 5 and 7 to 8; *V. trinervia* and 6; *V. reflexo-pilosa*. *V. trinervia* exhibited polymorphism for SDH. Two banding patterns (a_1 and a_2 types) were observed.



1 2 3 4 5 6

Fig. 4. The banding pattern of LAP. 1; *V. reflexo-pilosa*, 2 and 4 to 6; *V. trinervia*, 3; *V. minima* var. *minima*. *V. reflexo-pilosa* and *V. trinervia* exhibited polymorphism for the banding pattern. However, no intraspecific variation was observed in the zone A.

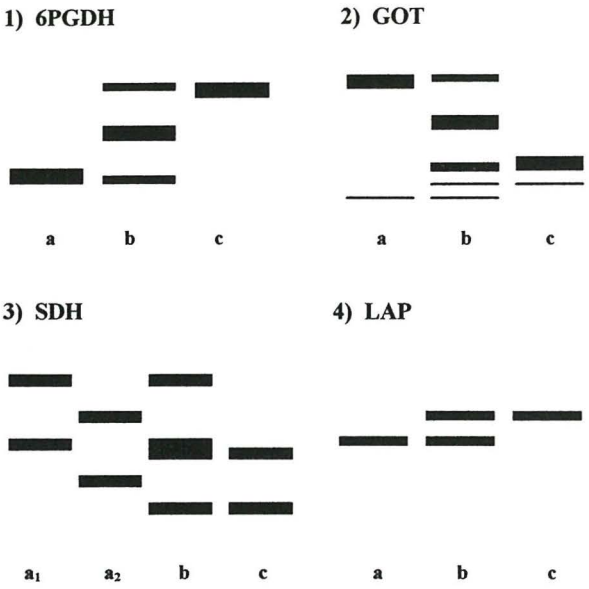


Fig. 5. Zymogram phenotypes for four isozymes of *V. trinervia* (a type), *V. reflexo-pilosa* and *V. glabrescens* (b type) and *V. minima* var. *minima* (c type). *V. trinervia* exhibited two types of banding pattern in SDH (a_1 and a_2 types). The b type banding exhibited by *V. reflexo-pilosa* and *V. glabrescens* was considered to be a hybrid combination between the a_1 type exhibited by *V. trinervia* and the c type exhibited by *V. minima* var. *minima*.

the accessions of *V. reflexo-pilosa* produced two bands in the zone ("b" type) as shown in Fig. 4. One accession of *V. minima* var. *minima* exhibited the other pattern which we designated as "c" type (Fig. 4 and Table 3).

2) Interspecific hybridization among V. minima *var.* minima, V. trinervia, V. reflexo-pilosa *and* V. glabrescens

When *V. minima* var. *minima* was crossed with *V. trinervia* as seed parent, a higher percentage of pod setting (10.8%) was obtained than when it was crossed as pollen parent (3.5%) (Table 4). However, the pods from *V. minima* var. *minima*×*V. trinervia* became discoloured 8 to 10 days after pollination and dried out. The cross was successful only when *V. trinervia* was used as seed parent. The pod growth was normal and hybrid seeds showed a normal appearance. Although the seeds were smaller and thinner than the seed parent (*V. trinervia*), they were viable. We were able to obtain hybrid plants and found that they were completely sterile (Table 4). Pollen stainability of the hybrids was 0%.

The cross between *V. reflexo-pilosa* and *V. glabrescens* was successful in both directions (Table 4 and Fig. 6). When *V. glabrescens* was used as seed parent, a much higher percentage of pod setting (60.0%) was obtained than in the reverse cross (15.4%). We were able to obtain viable seeds from the crosses in both directions. The size of the hybrid seeds was the same as that of the seed parent. Pollen stainability of the hybrids was relatively high ranging from 49.5 to 91.7% (Tables 4 and 5) and the hybrids normally produced F_2 seeds.

Fig. 6. Hybrid plant between *V. glabrescens* and *V. reflexo-pilosa*. *V. glabrescens* exhibits an erect growth habit although *V. reflexo-pilosa* exhibits a twining growth habit. The growth habit of the hybrid between them was intermediate.

Discussion

Isozyme analysis is a useful tool for estimating the genetic variation of plant species and for detecting interspecific relationships. In the present study, we analysed by polyacrylamide gel electrophoresis the banding patterns of four isozymes using wild *Ceratotropis* species. As shown in Fig. 5 and Table 3, *V. reflexo-pilosa* showed the same banding pattern as *V. glabrescens* for 6PGDH, GOT, SDH and LAP (in the zone A).

Although we have not yet analyzed the banding patterns of F_2 seeds, the genetic interpretation of the bandings described below is plausible. For 6PGDH, the bands which appear in *V. trinervia* and *V. minima* var. *minima* are controlled by two alleles at the same locus. This enzyme is dimeric and the middle band between the upper and lower bands which appears only in the tetraploid species must be a heterodimer. The same genetic interpretation of bandings is also plausible for GOT. In the case of SDH, the b type banding pattern exhibited by *V. reflexo-pilosa* is considered to be a hybrid combination between the a_1 type banding exhibited by *V. trinervia* and the c type exhibited by *V. minima* var. *minima*. For LAP, the isozyme in the zone A is a monomer and the bands exhibited by tetraploid species should show the hybrid pattern between the a and c types. These data suggest that *V. trinervia* and *V. minima* var. *minima* should be genome donor species to *V. reflexo-pilosa* and *V. glabrescens*.

Table 4. Interspecific hybridization between *V. trinervia*, *V. minima* var. *minima*, *V. reflexo-pilosa* and *V. glabrescens* and pollen stainability of the F_1 hybrids

Cross-combination	No. of flowers pollinated	No. of pods set(%)	No. of viable seeds obtained	Pollen stainability
V. minima var. *minima*×*V. trinervia*	74	8(10.8)	0	-
V. trinervia×*V. minima* var. *minima*	293	11(3.8)	56	0.0%
V. reflexo-pilosa×*V. trinervia*	133	43(32.3)	0	-
V. glabrescens×*V. trinervia*	24	20(83.3)	0	-
V. reflexo-pilosa×*V. minima* var. *minima*	69	6(8.7)	0	-
V. glabrescens×*V. reflexo-pilosa*	10	6(60.0)	18	79.4%[1)]
V. reflexo-pilosa×*V. glabrescens*	13	2(15.4)	4	49.5%[1)]

1) also see Table 5.

Other diploid species, *V. umbellata*, *V. nakashimae* and *V. minima* var. *minor* (formerly classified as *V. riukiuensis*), also exhibited the same banding pattern as that of *V. minima* var. *minima* (c type in Table 3). Moreover, *V. nakashimae* and *V. minima* var. *minor* are morphologically closely related to *V. minima* var. *minima*. These species, however, may not be the parental species of *V. reflexo-pilosa* and *V. glabrescens* based on their geographical distribution (*V. nakashimae* and *V. minima* var. *minor*) and morphology of seed and primary leaves (*V. umbellata*). *V. nakashimae* occurs in Korea, North China and the northern part of Kyushu district, Japan while the distribution of *V. minima* var. *minor* is restricted to the Ryukyu islands, Japan and Taiwan[13]. On the other hand, *V. minima* var. *minima* is sympatric with both *V. trinervia* and *V. reflexo-pilosa* in Thailand and Peninsular Malaysia[3, 8, 17]. *V. minima* var. *minima*, *V. trinervia* and *V. reflexo-pilosa* have oblong to round-shaped seeds and heart-shaped primary leaves, while *V. umbellata* has cylinder-shaped seeds and lanceolate primary leaves.

As shown in Table 4, *V. reflexo-pilosa* is cross-compatible with *V. glabrescens*. They readily produced fertile hybrids when crossed with each other (Fig. 6). The pollen stainability of the hybrids between them was relatively high (Table 5). Based on the morphological similarities of seeds and primary leaves, the same ploidy level (2n=44, 4x) and the high level of hybrid fertility between them, it is concluded that they are phylogenetically closely related to each other and share a homologous genome constitution. Moreover, they exhibited the same banding pattern (Table 3). Thus the present study demonstrated that *V. reflexo-pilosa* and *V. glabrescens* are not two distinct species but a single species involving two different varieties.

Against this background, the phylogenetic differentiation of tetraploid species, *V. reflexo-pilosa* and *V. glabrescens*, illustrated in Fig. 7 is plausible. This figure shows that wild *V. reflexo-pilosa* originated from interspecific hybridization between *V. trinervia* and *V. minima* var. *minima*. Since the hybrids between *V. trinervia* and *V. minima* var. *minima* are completely sterile (Table 4), it is anticipated that they produced seeds as a result of spontaneous chromosome doubling and thereby produce tetraploid populations which evolve to *V. reflexo-pilosa*. *V. glabrescens* was derived from *V. reflexo-pilosa* as cultivated type with erect growth habit.

As shown in Table 4, the cross between *V.*

Table 5. Pollen stainability of the interspecific hybrids between *V. glabrescens* and *V. reflexo-pilosa*

Cross combination[1] accession#	No. of seeds sown	No. of seeds germinated	Pollen stainability
V1160×#9	2	2	71.5%
V1160×#24	1	1	89.9%
V1160×#25	2	2	91.7%
V1160×#76	4	4	64.8%
#9×V1160	2	2	49.5%
(#25×#9)[2]	2	2	(86.2%)
(V1160)			(96.4%)

1) V1160 is *V.glabrescens*. All other accessions are *V. reflexo-pilosa* collected from Peninsular Malaysia (Egawa *et al.* 1990[8]; Bujang *et al.* 1994[3]).
2) intraspecific hybrid of *V. reflexo-pilosa*

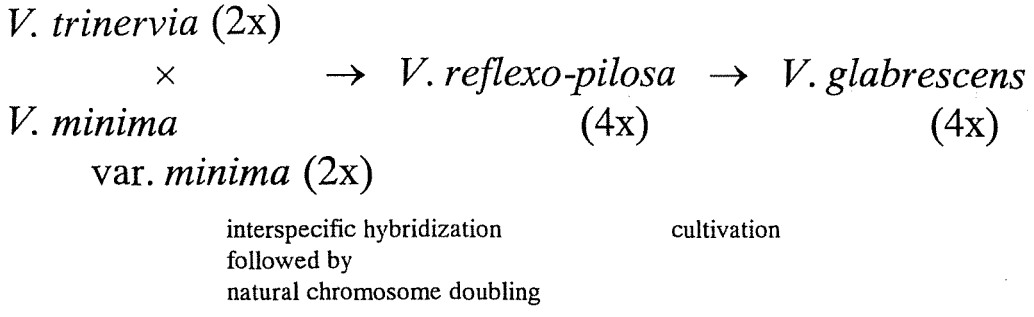


Fig. 7. Phylogenetic differentiation of tetraploid *V. reflexo-pilosa* and *V. glabrescens*

minima var. *minima* and *V. trinervia* was successful only when *V. trinervia* was used as seed parent. Although the value of pod setting was very low (3.5%), pod growth was normal and hybrid seeds were viable. These results suggest that *V. trinervia* should be the seed parent rather than the pollen parent.

As described previously, *V. glabrescens* is resistant to major mungbean pests and diseases such as powdery mildew and cucumber mosaic virus[19], bean fly (*Ophiomyia phaseoli*, *O. centrosematis* and *Melanagromyza soja*)[1] and is used in the mungbean improvement program at AVRDC[9, 11]. However, only a small number of accessions of *V. glabrescens* is now available. It is also anticipated that *V. reflexo-pilosa*, *V. minima* var. *minima* and *V. trinervia* will be useful for the breeding program of *Ceratotropis* cultigens in addition to *V. glabrescens*, since they are involved in the speciation of *V. glabrescens*.

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ササゲ属4倍体種，*Vigna glabrescens* と *V. reflexo-pilosa* の系統分化

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摘　要

ササゲ属（*Vigna*）のアズキ亜属（*Ceratotropis*）は，アジアに起原した豆類で，リョクトウ，アズキなどの重要な栽培種を含んでいる。アズキ亜属の種の多くは，2n＝22の2倍体であるが，2種の4倍体，*V. reflexo-pilosa*（野生種）と *V. glabrescens*（栽培種）も存在する。*V. glabrescens* は，主要なリョクトウ病害虫に対して抵抗性を示すのでアズキ亜属栽培種の品種改良のための育種素材として重要であり，AVRDC（アジア蔬菜研究開発センター）においてリョクトウ育種計画に利用されている。

本研究は，この4倍体と他の2倍体種との類縁関係を明らかにする目的でいくつかのアイソザイムのバンドパターンを分析し，種間雑種作出をこころみた。その結果，*V. reflexo-pilosa* は，2倍体野生種，*V. minima* var. *minima* と *V. trinervia* の間の種間交雑とそれにつづく自然染色体倍加により生じたことが示唆された。*V. reflexo-pilosa* と *V. glabrescens* の間では，雑種の作出が容易であり，雑種の稔性が高い。またこの両種が示すアイソザイムのバンドパターンは同一であり，遺伝的に極めて近縁であることが明らかとなった。このことから *V. glabrescens* は *V. reflexo-pilosa* から直立型の生育習性をもつ栽培型として生じたことが示唆された。

キーワード：アズキ亜属，*V. trinervia*，*V. minima* var. *minima*，アイソザイム分析，種間雑種作出

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