Phylogenetic Differentiation of *Vigna* Species in Asia

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Abstract

The subgenus *Ceratotropis* of the genus *Vigna* which originated in Asia includes the cultigens mungbean, blackgram, adzuki bean, rice bean and moth bean. We collected their wild related species on the Ryukyu islands of Japan as well as in the northern part of Thailand and in Peninsular Malaysia.

Analysis of cross-compatibility using wild and cultivated *Ceratotropis* species showed that *V. nakashimae* and *V. riukiuensis* are cross-compatible with adzuki bean. Hybridization between adzuki bean and rice bean cannot be easily achieved. However, since *V. riukiuensis* is also cross-compatible with rice bean, *V. riukiuensis* could be useful as a bridge species between adzuki bean and rice bean.

V. reflexo-pilosa and *V. glabrescens* are both tetraploid species ($2n=44$) and are cross-compatible with each other. Cytogenetical analysis by interspeciffic hybridization involving diploid species showed that *V. glabrescens* has a homologous genome with rice bean but not with either mungbean or blackgram.

Isozyme analysis suggested that *V. reflexo-pilosa* originated from interspecific hybridization between *V. minima* and *V. trinervia* followed by natural chromosome doubling. *V. glabrescens* is considered to be derived from *V. reflexo-pilosa* as cultivated type with erect growth habit. Isozyme analysis also suggested that *V. trinervia* is phylogenetically intermediate between the "mungbean group" showing an epigeal germination habit and the "adzuki bean group" showing a hypogeal germination habit.

Introduction

The subgenus *Ceratotropis* of the genus *Vigna*, which is considered to have originated in Asia, includes five cultivated species, i. e. mungbean (*V. radiata*), blackgram (*V. mungo*), rice bean (*V. umbellata*), adzuki bean (*V. angularis*) and moth bean (*V. aconitifolia*). In addition to the cultivated species, about twenty wild species are described in monographs written by Marēchal *et al*. (1978) and Tateishi (1993).

The *Ceratotropis* species are diploid ($2n=22$, $2x$) except for *V. glabrescens* and *V. reflexo-pilosa* which are tetraploid ($2n=44$, $4x$). This subgenus forms a morphologically homogeneous group (Baudoin and Marēchal, 1988 ; Tateishi and Ohashi, 1990) and can be subdivided into two groups based on the seedling characteristics ; namely "mungbean group" which exhibits an epigeal germination habit and has primary leaves with a short petiole, and "adzuki bean group" which exhibits a hypogeal germination habit and has primary leaves with a long petiole. Mungbean, blackgram and moth bean belong to the mungbean group, while adzuki bean and rice bean belong to the adzuki bean group.

In the present paper we describe interspecific relationships between cultivated and wild *Ceratotropis* species, based on cross-compatibility, cytogenetical analysis of the interspecific hybrids, and analysis of isozyme banding patterns.

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Phylogenetic relationship between cultigens belonging to mungbean group

Mungbean, blackgram and their wild ancestral forms (*V. radiata* var. *sublobata* and *V. mungo* var. *silvestris*) are considered to form a closely related taxonomic group called *V. radiata-mungo* complex (Miyazaki, 1982). There are some reproductive barriers between mungbean and blackgram (Miyazaki, 1982). Hybrid plants can be successfully produced only when mungbean is used as a female parent.

As shown in Table 1, we produced successfully hybrids between mungbean and blackgram using many local varieties of mungbean as female parent and two varieties of blackgram as male parent at Chainat Field Crops Research Center, Thailand. The hybrids grew vigorously and produced numerous flowers continuously. However, they set pods rarely and only one seed was set in the pod (Table 1). Although a total of 2,510 flowers opened, only 25 pods set (1.0% pod setting) and only 25 F_2 seeds were obtained by open pollination without bagging. The percentage of pollen stainability was very low, ranging from 3.3% to 31.5%.

The hybrid between mungbean and blackgram exhibited irregular meiosis with a high frequency of univalent occurrence (see Egawa, 1988). Two to ten univalents were obereved. Irregular meiosis could be ascribed to low pollen fertility and low pod setting by open pollination in the hybrids.

The genomes of mungbean and blackgram are thus considered to be only partially homologous. They were first considered to consist of a single species (Verdecourt, 1970). However, the present results suggest that they are phylogenetically highly differentiated.

Phylogenetic relationship between cultigens belonging to adzuki bean group

Adzuki bean is cultivated mainly in Japan, Korea, Taiwan and China. Rice bean is widely cultivated in Southeast Asia, from southern China and Indonesia to eastern India.

When we crossed adzuki bean with rice bean as the female parent, pods developed normally and pod setting reached a value of 32%. However, these seeds were shrivelled and not viable. No pod setting was observed when rice bean was used as the seed parent. Adzuki bean and rice bean are thus strongly iso-

Table 1 Seed setting by open pollination and pollen stainability in F_1 hybrids between mungbean and blackgram

Cross-combination mungbean × blackgram	No. of plants	No. of flowers open pollinated*	No. of pods set	No. of F_2 seeds obtained	Pollen stainability
R 91 × U-Thong 2	3	88	1(1.1%)	1	18.5%
R 113 × 〃	1	72	0(0.0%)	0	10.3%
R 116 × 〃	2	79	3(3.8%)	3	23.1%
R 145 × 〃	1	54	1(1.9%)	1	28.5%
R 194 × 〃	2	70	1(1.4%)	1	–
R 312 × 〃	2	75	1(1.3%)	1	31.5%
R 460 × 〃	2	222	0(0.0%)	0	20.7%
R 466 × 〃	3	113	8**(7.1%)	8	18.2%
R 510 × 〃	2	129	1(0.8%)	1	13.2%
R 584 × 〃	2	13	0(0.0%)	0	–
R 603 × 〃	1	8	0(0.0%)	0	–
R 658 × 〃	1	88	0(0.0%)	0	21.4%
V 6085 × 〃	2	64	2(3.1%)	2	19.0%
IC 2 × 〃	1	19	0(0.0%)		9.1%
Total	25	1094	18(1.6%)	18	

* counted everyday between 8 th March and 6 th April (for 30 days)

* * two seeds out of eight were abortive

Table 1 (Continued) Seed setting by open pollination and pollen stainability in F_1 bybrids between mungbean and blackgram

Cross-combination mungbean× black-gram	No. of plants	No. of flowers open pollinated*	No. of pods set	No. of F_2 seeds obtained	Pollen stainability
R 81 × 219	4	293	3(1.0%)	3***	25.2%
R 88 × 〃	2	24	0(0.0%)	0	
R 91 × 〃	5	431	0(0.0%)	0	18.3%
R 95 × 〃	2	121	2(1.7%)	2	23.1%
R 100 × 〃	2	28	0(0.0%)	0	23.7%
R 113 × 〃	2	6	0(0.0%)	0	8.9%
R 145 × 〃	2	26	0(0.0%)	0	–
R 151 × 〃	3	101	1(1.0%)	1	30.2%
R 312 × 〃	2	116	0(0.0%)	0	24.1%
R 466 × 〃	1	60	0(0.0%)	0	19.6%
R 510 × 〃	1	21	1(4.8%)	1	21.3%
R 584 × 〃	1	20	0(0.0%)	0	13.1%
R 594 × 〃	2	49	0(0.0%)	0	–
R 658 × 〃	2	40	0(0.0%)	0	15.0%
R 668 × 〃	2	9	0(0.0%)	0	3.3%
R 669 × 〃	2	61	0(0.0%)	0	18.5%
440003 × 〃	2	10	0(0.0%)	0	11.6%
total	37	1416	7(0.5%)	7	

* * * One seed out of three was abortive.

lated reproductively (Fig. 1).

According to Ahn and Hartmann (1978), hybrids between adzuki bean and rice bean successfully obtained through embryo rescue regularly formed 11 bivalents at the meiosis and the pollen stainability was 76%. Chen *et al*. (1983) also reported a relatively high fertility of the hybrids. This evidence suggests that they share a homologous genome with each other in spite of the occurrence of a reproductive barrier between them.

Phylogenetic relationship between mungbean group and adzuki bean group

The hybrids between mungbean and rice bean grew vigorously and flowered profusely. The percentage of pollen stainability of the hybrids was 0 to 0.2%, and no F_2 seeds were obtained by open pollination (Egawa, 1988). Although more than 300 flowers were backcrossed with rice bean, no seeds could be obtained. Mungbean and rice bean are thus strongly isolated reproductively from each other. Meiosis of the hybrids between the two species was irregular (see Egawa, 1988). The number of univalents per cell ranged from 2 to 18. The configuration of 11 bivalents was not observed.

Ahn and Hartmann (1978) and Chen *et al*. (1983) concluded that adzuki bean is genetically distinct from mungbean and blackgram based on meiotic irregularities and sterility of the hybrids. It is thus considered that the mungbean group species and the adzuki bean group species are highly differentiated from each other.

Cross-compatibility between mungbean and *V. grandiflora*.

One accession of a wild *Ceratotropis* species belonging to the mungbean group was collected in Phisanulok, Thailand (Tomooka *et al*., 1991). According to Dr. Tateishi, Tohoku University (personal communication), this accession is morphologically consistent with the specimen of *Phaseolus sublobatus* var.

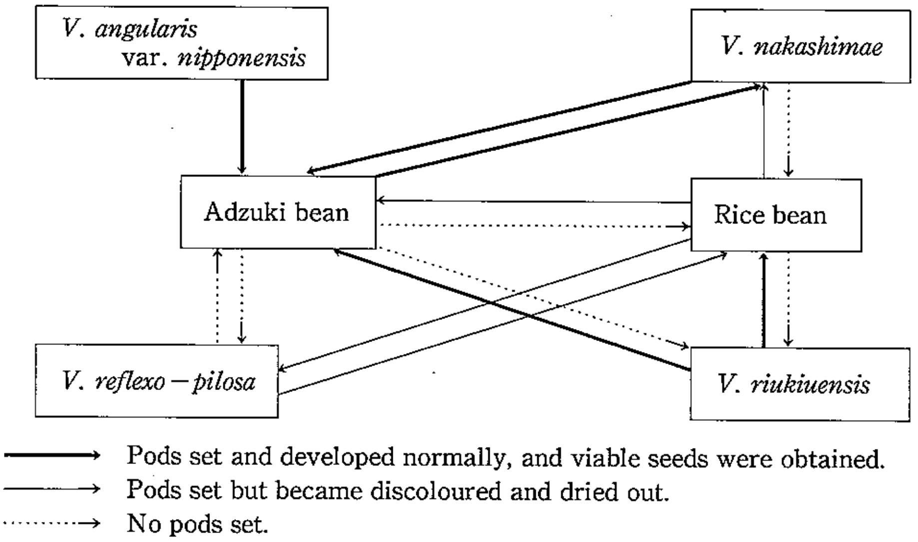

Fig. 1 Cross-compatibility of adzuki bean and rice bean with their wild relatives (Siriwardhane *et al*., 1991)

grandiflorus collected from Burma, for which he newly proposed the name of *V. grandiflora* (Tateishi, 1993).

When we crossed mungbean as female parent with the accession of *V. grandiflora*, 21.2% rate of pod setting was observed (Table 2). The hybrid pods developed normally at first, but ceased to develop and started to wilt 8 to 10 days after pollination. To rescue the hybrid plants, immature embryos were excised from the pods at 8 to 10 days after pollination and placed on White's medium supplemented with 200 mg/l of yeast extract. We cultured a total of 16 immature embryos. These embryos started to grow immediately after culture and 16 young seedlings were obtained (Table 2). We grew these seedlings in the growth chamber under a 16-hr daylight (30°C, 28,000 Lux) and 8-hr night (20°C) regime, but they all died a few weeks later due to poor root development. This observation suggests that *V. grandiflora* is reproductively isolated from mungbean and is not phylogenetically closely related to mungbean in spite of the morphological similarities of *V. grandiflora* to wild mungbean, *V. radiata* var. *sublobata* (Tomooka *et al*., 1991).

Cross-compatibility of cultigens belonging to adzuki bean group with the wild relatives

We collected wild *Ceratotropis* species belonging to the adzuki bean group, involving *V. riukiuensis*, *V. minima*, *V. trinervia* and *V. reflexo-pilosa*, in the Ryukyu islands, Japan, northern Thailand and Peninsular Malaysia (Egawa *et al*., 1990 ; Tomooka *et al*., 1991 ; Egawa *et al*., 1992). The species identfication was based on the key characters proposed by Tateishi (1984 ; 1993).

Using these materials we analyzed the cross-compatibility of adzuki bean and rice bean with their wild relative. The results are illustrated in Fig. 1 (Siriwardhane *et al*., 1991). The cross of *V. nakashimae* with adzuki bean was successful in both directions and viable seeds were produced, while the cross with rice bean was not successful. *V. riukiuensis* is cross-compatible with adzuki bean and rice bean only when *V. riukiuensis* is used as male parent and the hybrid seeds showed normal germination. These results suggest that *V. nakashimae* and *V. riukiuensis* are phylogenetically closely related to adzuki bean.

Wild species belonging to the adzuki bean group, involving *V. angularis* var. *nipponensis* (wild ancestral form of adzuki bean), *V. nakashimae*, *V. riukiuensis*, *V. minima*, are cross-compatible with each other and are considered to share a homologous genome (unpublished data). They are not highly differentiated compared with the mungbean group species, indicating that the phylogenetic differentiation of the adzuki bean group is more recent than that of the mungbean group.

Table 2 Results of interspecific hybridization between mungbean and *Vigna grandiflora*

Cross - combination	No. of flowers pollinated	No. of pods set	No. of embryos obtained
Chainat 60 × *V. grandiflora*	4	0	0
R 60 × 〃	3	0	0
R 61 × 〃	5	1(20.0%)	2
R 243 × 〃	5	1(20.0%)	2
R 303 × 〃	16	5(31.3%)	25*
total	33	7(21.2%)	29

* 16 embryos out of 25 were cultured.

Phylogenetic relationship between two tetraploid *Vigna* species, *V. glabrescens* and *V. reflexo-pilosa*

A natural tetraploid *Vigna* accession was collected from the Philippines. This accession (P. I. 207655, AVRDC V 1160) designated as *V. glabrescens* regularly formed 22 bivalents without multivalents at meiosis and is considered to be an amphidiploid (Swindell *et al*.,1973).

Another tetraploid species, *V. reflexo-pilosa* is widely distributed from South China to Malaysia, and also occurs in the Ryukyu island, Japan and Taiwan. This species also shows the formation of 22 bivalents at meiosis and is considered to be an amphidiploid (Egawa *et al*., 1990). *V. reflexo-pilosa* is cross-compatible with *V. glabrescens*. They readily produced fertile hybrids when crossed with each other (Tomooka *et al*., 1991).

These two species belong to the adzuki bean group. Judging from the morphological similarities of seeds and primary leaves, the same ploidy level (2n=44, 4x) and the high level of hybrid fertility between them, they are phylogenetically closely related to each other and share a homologous genome constitution.

Cytogenetical analysis of *V. glabrescens* by interspecific hybridization involving diploid cultigens

V. glabrescens harbours resistance genes to major mungbean pests and diseases and is currently utilized in the mungbean improvement program at AVRDC (Fernandez and Shanmugasundarum, 1988). However, the introduction of these valuable resistance genes from *V. glabrescens* to mungbean via interspecific hybridization was unsuccessful due to the hybrid sterility. To detect the genome constitution of *V. glabrescens*, we produced interspecific hybrids with the diploid cultigens, mungbean, blackgram, adzuki bean and rice bean using the embryo rescue technique and analyzed the pattern of meiotic chromosome pairing of the hybrids (Egawa *et al*., 1988).

The hybrids grew vigorously and flowered profusely under short day conditions at Tsukuba, Japan. The low frequency of bivalent formation and occurrence of loosely-paired rod bivalents in *V. glabrescens* × mungbean and *V. glabrescens* × blackgram hybrids indicated that the genome of mungbean and blackgram is not included in *V. glabrescens*. In contrast, 11 bivalents were observed in the hybrids (2n=33) from *V. glabrescens* × rice bean with an average configuration of 11.1 univalents + 10.9 bivalents (7.5 rings + 3.4 rods). Moreover, most of the bivalents observed were closely associated (see Egawa *et al*., 1988). This finding indicates that one of the two genomes of *V. glabrescens* is derived from rice bean, adzuki bean or a wild species which has a homologous genome with rice bean and adzuki bean.

The candidate for a donor species of another genome, which is different from rice bean, adzuki bean or their wild relatives but has a sufficiently high cross-compatibility with them to produce an amphidiploid, needs to be identified among the wild related species belonging to the adzuki bean group, since *V. glabrescens* also belongs to the adzuki bean group.

Phylogenetic differentiation of *V. glabrescens* detected by isozyme analysis

Isozyme analysis is a useful tool for estimating the genetic variation of plant species and for detecting interspecific relationships. We analyzed the banding patterns of several isozymes for *Ceratotropis* species by polyacrylamide gel electrophoresis (Egawa *et al*., 1992). Here we describe the results of the analysis for 34 accessions of *V. trinervia*, one of *V. minima* and 7 of *V. reflexo-pilosa* collected from Peninsular Malaysia (Fig. 2) in addition to *V. glabrescens*. The name of *V. trinervia* was newly proposed by Tateishi lately (Tateishi, 1993).

For GOT, 6 PGDH, SDH, *V. reflexo-pilosa* showed the same banding pattern as *V. glabrescens*. The pattern was intermediate between *V. trinervia* and *V. minima* (Fig. 3). Although we have not yet analyzed the banding patterns genetically, the genetic interpretation of the bandings described below is plausible.

For 6 PGDH, the bands which appeared in *V. trinervia* and *V. minima* are controlled by two alleles at the same locus. This enzyme is dimeric and the middle band which appeared only in the tetraploid species is a heterozygote between the upper and lower bands. The same genetic interpretation of bandings is also plausible for GOT. In the case of SDH, the c type banding pattern of *V. reflexo-pilosa* is considered to be a hybrid combination between the a type banding exhibited by *V. trinervia* and the d type exhibited by *V. minima*. These evidences suggest that *V. trinervia* and *V. minima* should be genome donor species to *V. reflexo-pilosa* and *V. glabrescens*.

V. nakashimae and *V. riukiuensis* exhibited the same banding patterns as *V. minima* for 6 PGDH and GOT (see next section). *V. nakashimae* is distributed in Korea, North China and the northern part fo Kyushu district, Japan. The distribution of *V. riukiuensis* is restricted to the Ryukyu islands, Japan and Taiwan. These two species are excluded from the candidate of the genome donor to *V. reflexo-pilosa* from the view point of geographical distribution.

Considering the above-mentioned lines of evidence, the phylogenetic differentiation of *V. reflexo-pilosa* and *V. glabrescens* as illustrated in Fig. 4 is plausible. This figure shows that *V. reflexo-pilosa* originated

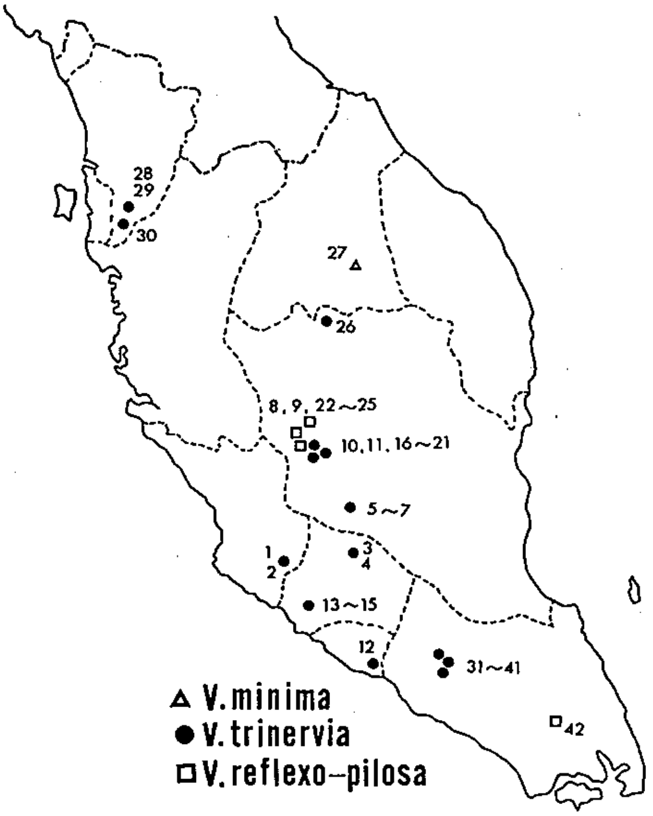

Fig. 2 Collection of wild *Ceratotropis* species in Peninsular Malaysia (Egawa *el al*., 1992)

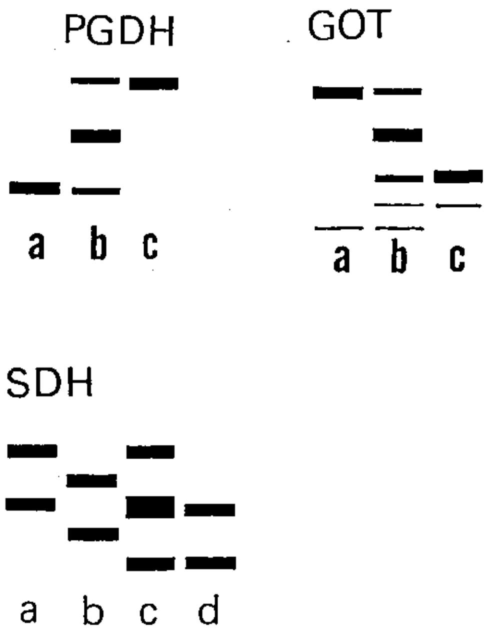

Fig. 3 Zymogram phenotypes for 3 isozymes of *V. trinervia*, *V. reflexo-pilosa* and *V. minima*.

PGDH : a = *V. trinervia*, b = *V. reflexo-pilosa*, c = *V. minima*
GOT : a = *V. trinervia*, b = *V. reflexo-pilosa*, c = *V. minima*
SDH : a, b = *V. trinervia*, b = *V. reflexo-pilosa*, c = *V. minima*

from interspecific hybridization between *V. trinervia* and *V. minima* followed by natural chromosome doubling. *V. glabrescens* was derived from *V. reflexo-pilosa* as cultivated type with erect growth habit.

Phylogenetic relationship of *V. trinervia* with the adzuki bean group and mungbean group detected by isozyme analysis

We carried out isozyme electrophoresis for GOT and 6 PGDH of mungbean group species (1 accession of mungbean, 3 of *V. radiata* var. *sublobata*, 1 of blackgram and 1 of *V. grandiflora*) and adzuki bean group species (1 of rice bean, 3 of *V. angularis* var. *nipponensis*, 2 of *V. nakashimae* and 3 of *V. riukiuensis*). For GOT and 6 PGDH, all the accessions belonging to the adzuki bean group (except for *V. angularis* var. *nipponensis* in the case of 6 PGDH) exhibited the same banding pattern as *V. minima* (c type in Fig. 3), while all the accessions belonging to the mungbean group exhibited the same banding pattern as *V. trinervia* (a type in Fig. 3). The two groups were thus found to be differentiated genetically. Although *V. trinervia* belongs to the adzuki bean group based on the seedling morphology, this species exhibited the same bandings as the mungbean group. These results suggest that *V. trinervia* may be phylogenetically intermediate between the adzuki bean group and the mungbean group and could be a bridge species between the two groups.

V. minima
× ⟶ *V. reflexo-pilosa* ⟶ *V. glabrescens*
V. trinervia cultivation
*interspecific hybridization
followed by natural chromosome
doubling

Fig. 4 Phylogenetic differentiation of *V. glabrescens*

The *Ceratotropis* species form a morphologically homogeneous group and gene flow occurs within this group through interspecific hybridization. Wild *Ceratotropis* species should be considered to be primary gene pools for the breeding of the cultigens. However, wild *Ceratotropis* species have never been collected intensively, evaluated and preserved as genetic resources. Considering the genetic erosion occurring rapidly on a world-wide scale, it is important to collect wild *Ceratotropis* species from the areas covering their wide range of geographical distribution.

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Discussion

Kikuchi, F. (Japan): Recently Dr. Morishima has indicated that the genetic erosion of wild species of rice is proceeding rapidly. Do you have similar evidence of genetic erosion of *Vigna* species or mungbean?

Answer: We have a similar experience based on the explorations carried out in Japan, Peninsular Malaysia and the norhtern part of Thailand for the collection of *Vigna* germplasm. When we visited the same collection sites two or three times, we noticed that the sites had disappeared even in mountainous areas due to the widening of roads and building construction.