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Proteinase inhibitor polymorphism in the genus *Vigna* subgenus *Ceratotropis* and its biosystematic implications

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Summary

The diversity of components for four proteinase inhibitors found in species of the genus *Vigna* subgenus *Ceratotropis* are described. Trypsin, chymotrypsin, subtilisin and cysteine proteinase inhibitors were analyzed by isoelectric focusing followed by the gelatin replica method. Of these proteinase inhibitors, trypsin inhibitors showed most polymorphism both within and between species. Many trypsin inhibitor components were also active to chymotrypsin. Several accessions had very low levels or absence of some inhibitors, such as very low levels of trypsin inhibitor in two accessions of the *V. tenuicaulis* and absence of chymotrypsin inhibitors in *V. grandiflora* and *V. subramaniana.* Proteinase inhibitor polymorphism broadly agreed with the taxonomic system for the subgenus *Ceratotropis*. Based on inhibitor variation species analyzed could be divided into three groups which corresponding to sections *Aconitifoliae*, *Angulares* and *Ceratotropis*. Some species have very little variation in trypsin inhibitors despite wide distribution, such as, *V. radiata* and *V. reflexo-pilosa*. Accessions of other species showed considerable intraspecific variation for trypsin inhibitors, such as, *V. grandiflora*, *V. aconitifolia* and *V. stipulacea*. Proteinase inhibitor polymorphism provides an indication of the species that may have contributed a genome to the tetraploid species, *V. reflexo-pilosa*.

Abbreviations: CI – chymotrypsin inhibitor(s); CPI – cysteine proteinase inhibitor(s); IEF – isoelectric focusing; PF – photo film; SI – subtilisin inhibitor(s); SP – Servalyt precotes; TI – trypsin inhibitor(s)

Introduction

There are currently 21 species in the genus *Vigna* subgenus *Ceratotropis* of which seven are cultivated. The cultigens in the subgenus *Ceratotropis* are particularly important in Asian countries. Among these crops the most widely grown are mungbean (*V. radiata*), black gram (*V. mungo*) and rice bean (*V. umbellata*) in tropical countries of South and Southeast Asia and azuki bean (*V. angularis*) in East Asia. *V. reflexo-pilosa* var. *glabrescens*, *V. aconitifolia* and *V. trilobata* are grown on a limited area and are of local importance (Lawn, 1995).

The wild relatives of these cultigens are a source of useful genes for crop improvement. For example, resistance to the bruchid storage pest, *Callosobruchus chinensis* L. was found in the wild relative of mungbean *V. radiata* var. *sublobata* (Fujii & Miyazaki, 1987). This source of pest resistance has been used to develop mungbean resistant lines (Tomooka et al., 1992, 2000b).

Until recently, evaluation and use of wild species in the subgenus *Ceratotropis* has been hampered by, the poor understanding of the taxonomy of the subgenus and species relationships. Maréchal et al. (1978) clarified the *Phaseolus-Vigna* complex, however, they mentioned that species delimitation in the subgenus *Ceratotropis* was incomplete because of the insufficient number of herbarium specimens for the species from East and Southeast Asia. In addition there are

few simple key characters that enable *Ceratotropis* taxa to be distinguished. We use here the nomenclature of the most recent monograph of the subgenus *Ceratotropis* by Tomooka et al. (2002c).

Several chemotaxonomic and molecular taxonomic studies of the genus *Vigna*, including the subgenus *Ceratotropis*, have been reported which have clarified species relationships using isozymes (Jaaska & Jaaska, 1990), low molecular weight carbohydrate (Yasui et al., 1985), RFLPs (Fatokun et al., 1993), nuclear and chloroplast DNA (Doi et al., 2002; Vaillancourt & Weeden, 1993) RAPDs (Kaga et al., 1996; Tomooka et al., 1996) and AFLPs (Tomooka et al., 2002b). Only recent studies have been comprehensive due to germplasm collection of several species, previously not in genebanks, from South and Southeast Asia in recent years (Tomooka et al., 2000a).

The azuki bean is an important crop in Japan, therefore the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan, initiated a project to improve representation of wild species from the subgenus *Ceratotropis* in its genebank system (Tomooka et al., 2000a). As a consequence 19 species (24 taxa) are now conserved in the Japanese MAFF genebank system. Some of these materials were used to investigate species relationships based on proteinase inhibitors polymorphism.

Plant proteinase inhibitors can be used as genetic markers for plant diversity and evolutionary studies (Kollipara & Hymowitz, 1992; Konarev, 1987; 1994). Analysis of proteinase inhibitor spectra can be useful for resolving biosystematic problems. The component composition of inhibitors revealed by electrophoresis, or related methods, is an informative characteristic of the biological specificity of these proteins.

There are a number of reports in the literature related to inhibitors in *Vigna*. The majority of reports discuss trypsin (TI) and chymotrypsin (CI) inhibitors (Norioka et al., 1988; Ishikawa et al., 1985; Kiyohara et al., 1981; Wilson & Chen, 1983). A few reports deal with subtilisin inhibitors (SI) (Nozawa et al., 1989) and cysteine proteinase inhibitors (CPI) (Baumgartner & Chrispeels, 1977). However, there are no reports that have assessed the diversity of several inhibitor systems both within and among species of the genus *Vigna* subgenus *Ceratotropis*.

The objectives of the study reported here were:

1. To describe comparatively proteinase inhibitor variation in species of the subgenus *Ceratotropis*;
2. To interpret proteinase inhibitor variation in relation to taxonomic relationships and evolutionary trends within the subgenus *Ceratotropis*;
3. To identify potentially useful variation for crop improvement.

Materials and methods

Plant materials

Accessions used in this study included 16 species, belonging to the genus *Vigna* subgenus *Ceratotropis* and two species in subgenus *Vigna* (Table 1). This represents 16 out of the 21 species recognized in the most recent monograph of the subgenus *Ceratotropis* (Toomoka et al, 2002c). Taxa that were not available for analysis were *Vigna aridicola, V. dalzelliana*, *V. exilis*, *V. khandalensis*, *V. trilobata*, *V. trinervia* var. *bourneae*. Of accessions used *Vigna angularis*, *V. mungo, V. radiata* and *V. reflexo-pilosa* include two intra-specific taxa. Two species from the subgenus *Vigna*, *V. unguiculata* and *V. luteola,* were used for marker purposes. Each taxon was represented by between one and six accessions (Table 1).

Isoelectric focusing (IEF) of proteins

Protein from 5–20 mg of ground seeds was extracted with a 10-fold volume of 20% glycerin for 1 hour at 20 °C. After centrifugation at 14000g the samples were kept in a freezer at –20 °C. For analysis of cysteine proteinase inhibitors, proteins were extracted with a 20-fold volume of 0.05M ammonium acetate. After centrifugation the supernatant was freeze dried and proteins were dissolved in 20% glycerin (two-fold to seed weight).

Trypsin, chymotrypsin and cysteine proteinases IEF was carried out in Servalyt precotes (SP) pH 3–10 gels 0.15 mm thin (Boehringer Ingelheim, Heidelberg) on a Multiphor II (Pharmacia) electrophoresis system with 50–100 mm distance between electrodes. Anode fluid 10 and cathode fluid 3 (Boehringer Ingelheim) served as electrode buffers for the Servalyt gels. For analysis of subtilisin inhibitors (SI) 5% polyacrylamide gel 0.3mm thin containing 2.5% ampholines pH 2.5–5 (Pharmacia, Uppsala, Sweden) with 180 mm distance between electrodes was used. 0.01 m sulphuric acid and cathode fluid 3 were used as electrode buffers for gel with ampholines. The samples (0.5–3 mg protein/ml) were applied to a gel with paper

Table 1. Accessions of *Vigna* used in the study

No[1]	Taxa abbreviation	Species name[2]	Former nomenclature or identification and accession. no. of source institute[3]	MAFF accession No.[4]	Origin
subgenus *Ceratotropis* section *Angulares*					
1	an-a	*V. angularis* (Willd.) Ohwi & Ohashi var. *angularis*		03031366	Japan
2	an-a	″		03013592	Japan
3	an-n	var. *nipponensis* (Ohwi) Ohwi & Ohashi		03028830	Japan
4	an-n	″		03033593	Korea
5	um-c	*V. umbellata* (Thunberg) Ohwi & Ohashi (cultivated)		03009006	Nepal
6	um-c	″		03025780	Thailand
7	um-w	″ (wild)		03031356	Thailand
8	min	*V. minima* (Roxb.) Ohwi & Ohashi		03028839	Thailand
9	riu	*V. riukiuensis* (Ohwi) Ohwi & Ohashi		03030456	Japan
10	riu	″		03028854	Taiwan, China
11	nak	*V. nakashimae* (Ohwi) Ohwi & Ohashi		03028850	Korea
12	nak	″		03028849	Japan
13	min	*V. minima* (Roxb.) Ohwi & Ohashi		03028840	Taiwan, China
14	hir	*V. hirtella* Ridley		03030505	Malaysia
15	hir	″		03031362	Thailand
16	hir	″	cf. *V.minima,* NI 1377	03033587	Thailand
17	hir	″	cf. *V.minima,* NI 1394	03033588	Thailand
18	nep	*V. nepalensis* Tateishi & Maxted	cf. *V.minima,* NI 970	03033574	India
19	nep	″	cf. *V.minima,* NI 971	03033575	India
20	ten	*V. tenuicaulis* N. Tomooka & Maxted		03031363	Thailand
21	ten	″		03031364	Thailand
22	nep	*V. nepalensis* Tateishi & Maxted		03031369	Bhutan
23	nep	″		03031370	Nepal
24	nep	″		03028851	Nepal
25	nep	″		03031368	Nepal
26	trin	*V. trinervia* (Heyne ex. Wight & Arnott) Tateishi & Maxted	*V. radiata* var. *sublobata*	03028858	Malaysia
27	rp-r	*V. reflexo-pilosa* Hayata var. *reflexo-pilosa*	*V. reflexo-pilosa*	03028860	Malaysia
28	rp-r	″	*V. reflexo-pilosa*	03030466	Japan
29	rp-g	var. *glabrescens*(Roxburgh) Tateishi & Maxted	*V. glabrescens,* V 1160	03031365	Philippines
30	rp-g	″	*V. glabrescens,* NI 1185	03033592	Philippines
subgenus *Ceratotropis* – section *Ceratotropis*					
31	ra-r	*V. radiata* (L.) Wilczek var. *radiata*		03025800	Japan
32	ra-r	″		00035043	Iran
33	ra-s	var. *sublobata*(Roxburgh) Verdcourt		03028846	Australia
34	ra-s	″		03028847	Madagascar
35	ra-s	″		03028845	India
36	ra-s	″		03033591	Australia
37	sub	*V. subramaniana* (Babu ex Raizada) Sharma	*V. radiata* var. *setulosa,* NI 1135	03033584	India
38	mun-m	*V. mungo* (L.) Hepper var. *mungo*		03031349	Thailand
39	mun-m	″		03019941	Pakistan
40	mun-m	″		03027092	Thailand
41	mun-s	var. *silvestris* Lukoki, Maréchal & Otoul		03028841	India
42	mun-s	″		03028842	India
43	mun-s	″		03028843	India
44	mun-s	″		03028844	India

Table 1. Continued

No[1]	Taxa abbreviation	Species name[2]	Former nomenclature or identification and accession. no. of source institute[3]	MAFF accession No.[4]	Origin
45	mun-s	″		03028848	Thailand
46	gra	*V. grandiflora* (Prain) Tateishi & Maxted	*V. radiata* var. *sublobata*	03028832	Thailand
47	gra	″	*V. radiata* var. *sublobata*	03031348	Thailand
subgenus *Ceratotropis* – section *Aconitifoliae*					
48	aco	*V. aconitifolia* (Jacq.) Maréchal		03020037	Pakistan
49	aco	″		03020041	Pakistan
50	sti	*V. stipulacea* Kuntze	*V. trilobata,* NI 251	03028856	India
51	sti	″	*V. trilobata,* NI 1030	03028855	India
subgenus *Vigna*					
52	ung	*V. unguiculata* (L.) Walpers		03033595	Tanzania
53	lut	*V. luteola* (Jacq.) Bentham		03033594	Tanzania

[1] Sample number referred to in text, tables and figures.
[2] Reidentified by authors following the nomenclature of Tomooka et al. (2002c).
[3] NI accessions were introduced from the Botanical Garden of Belgium, V 1160 from AVRDC.
[4] Accessions in the Ministry of Agriculture, Forestry and Fisheries (MAFF) genebank, Japan.

Table 2. Conditions for detection of proteinase inhibitors in *Vigna*

Inhibitors	Extraction (w/v)	Volume applied (μl)	Interval pH for IEF	Contact time of replica with gel (min)	Proteinase conc. (μg/ml)	Buffer	Incubation time on agarose (min)
Trypsin	1/10	0.5	3–10	3	2.5	0.2M Na_2HPO_4	20
Chymotrypsin	1/10	0.5	3–10	3 (10)[1]	17	0.2M Na_2HPO_4	35
Subtilisin	1/10	2	2.5–5	30	1.2	0.2M Na_2HPO_4	35
Cysteine proteinase (Papain)	1/20 [2]	4	3–10	45	1	O.2M Na_2HPO_4 pH 6.6 with 0.001M DTT	40

[1] Time for first and second replicas.
[2] Proteins were concentrated after extraction.

strips (between 0.5 × 1 mm and 10 × 1 mm) in volume of 0.5–4μl at 0.5 cm from the anode. For trypsin (TI) and chymotrypsin inhibitors (CI) 0.5μl and for SI 2μl of seed extract were applied to the gel. For cysteine proteinase inhibitors (CPI) (papain) 4μl of the concentrated seed proteins were applied to the gel (Table 2). Up to 48 samples were applied on one gel 125 mm wide. IEF in SP gel was conducted at a power of 4W, final voltage 2000 V and stopped at 1500–2400 Vh (for 125 × 125 mm gel). IEF in gel with ampholines was conducted for up to 6200 Vh. Cytochrome C with isoelectric point (pI) value of 10.65, horse myoglobin (7.3), whale myoglobin (8.3) and kit 9 (Serva) were used as pI markers.

Proteinase inhibitor detection

The modified gelatin replicas method (Konarev, 1986) was used for the detection of the proteinase inhibitors. After IEF of *Vigna* proteins photo film (PF) 'Foto 65' (Russia) was superimposed on a gel for 3–45 min (depending on the desired sensitivity of detection) at 20 °C. The first PF-replica was followed by a second one with duration of contact with the separating gel between two to three times longer, but not exceeding 1 hour. The PF with separated proteins imprinted was laid on a plate of 0.8% agarose gel (TAKARA, gel point 35 to 37 °C), containing proteinase and buffer with pH and composition optimal for that enzyme. For the preparation of the gel the proteinases were mixed with agarose solution cooled to 45 °C. Then the mixture was poured on Gel Bond film (Pharmacia) lying on the Multiphor II glass plate warmed to 45 °C and then cooled to 30 °C for consolidation. Subsequently PF was incubated with agarose gel at 45 °C for 20 to 40 min (depending on the activity of proteinase and desired sensitivity) on the plate of the Multiphor II. The gelatin of PF was not hydrolyzed in the zones where inhibitors were present. Conditions for detecting inhibitors are summarized (Table 2).

Analysis of inhibitor spectra

A mixture of two or three samples with contrasting inhibitor spectra was used as a control to determine band position in analyzed samples across each gel. Each position was named as 'p' combined with a number corresponding to the distance from one of the marker components in mm at standard conditions for IEF.

The composition of TI spectra of *Vigna* accessions was analyzed based on the results of five IEF separations of seed proteins. Two of them were carried out at 2400 Vh and three at 1500 Vh. The first variant gave sharper bands and was used as the standard to record band positions. The second variant gave improved separation of bands with higher pI. The mixture of protein extracts from two accessions with heterogeneous TI spectra (*V. umbellata* no. 6 and *V. unguiculata* no. 52) was used as a marker set for determination of positions of analyzed components. Positions of TI components on replicas were determined and expressed in mm from a marker component with position chosen as 0.

The scoring of CI components was performed as for TI. CI component analysis was based either on an original IEF separation for CI components or on secondary replicas from the same IEF gels originally analyzed for TI.

To score SI components the position of the most strongly detected component of SI of *V. angularis* was marked as p20. A mixture of three samples differing by SI spectra, namely of *V. angularis* (no. 3), *V. trinervia* (no. 26) and *V. luteola* (no. 53), was used as a control.

For improved resolution of single groups of components slightly different conditions were used (such parameters as amount of proteins, applying of samples near cathode or anode and concentration of papain were changed). Letters were used for designation of CPI components. For SI and CPI data on three or more separations were recorded.

For comparison between accessions, polymorphic bands were scored '1' for presence or '0' for absence. Genetic distances (Nei & Li, 1979) between two entries were computed as

$$\text{Genetic Distance} = 1 - [\ 2N / (N_i + N_j)\ ]$$

where N is the number of shared bands and N*i* and N*j* are the total number of bands for entry *i* and *j*. Genetic distances were used to construct a cluster phenogram by the UPGMA (average linkage) method (Phylip 3.5, procedure Neighbor, Felsenstein, 1993).

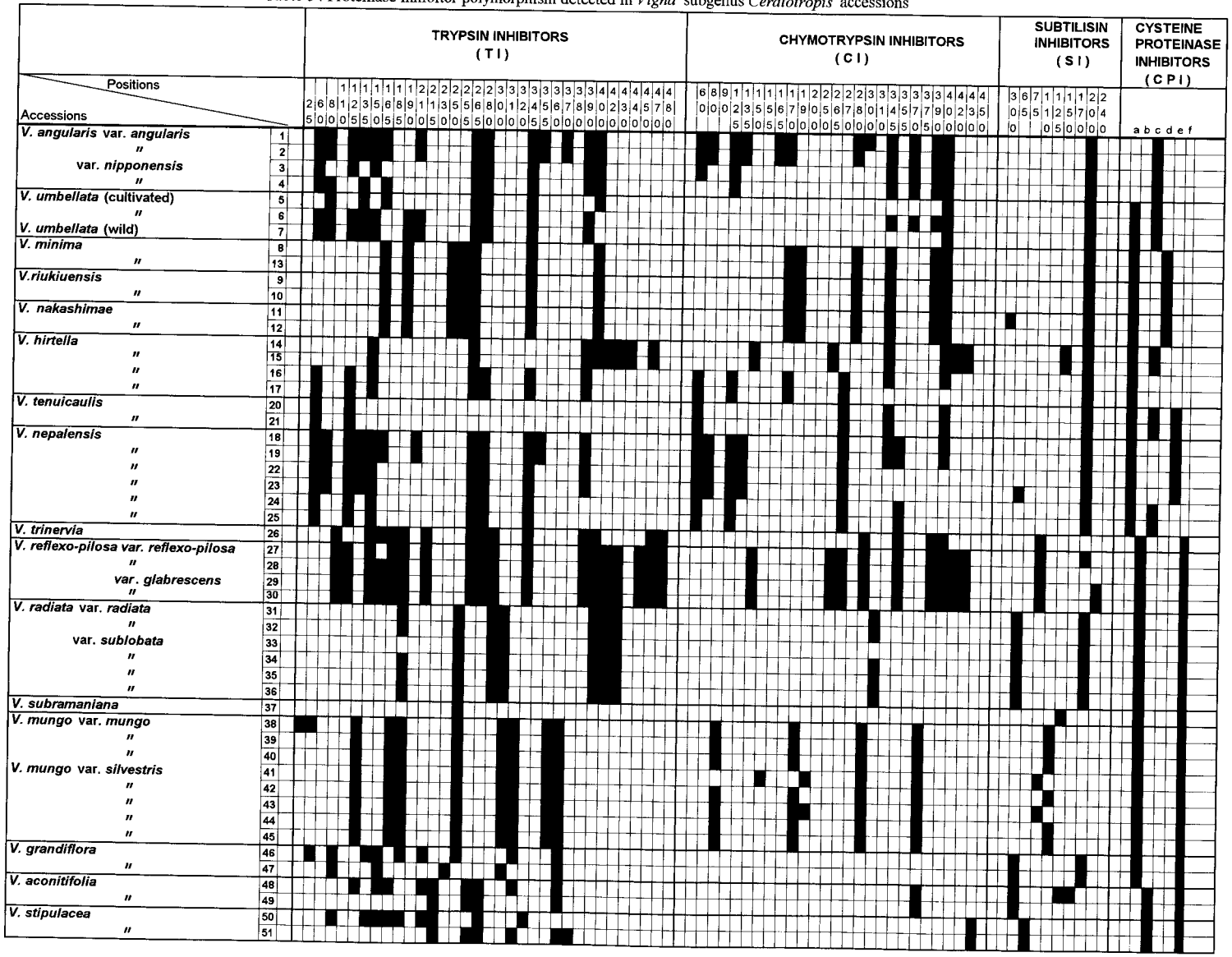
Results

Trypsin inhibitors (TI)

A high level of inter- and intra-specific polymorphism was detected for TI (Table 3). The average number of TI components per accession was 8. However, this varied from a single weakly detected component in *V. subramaniana* (no. 37) to 17 or 18 components in the two varieties of *V. reflexo-pilosa* (no. 27–30). TI components were found at 33 different positions.

The variation in TI components is shown (Figure 1, Table 3). For some species, such as, *V. minima, V. nakashimae, V. riukiuensis* and *V. tenuicaulis*, the TI band profile showed no intra-specific variation. *V. radiata* and *V. mungo* accessions had very little intraspecific variation. An accession of *V. radiata* var. *sublobata* (no. 33) does not have TI component [position (p)19.0 mm] and for *V. mungo* only no. 38 is different from the other accessions of this species with two high pI components (p25.0, p6.0). Other species showed considerable intraspecific variation. *V. angularis* var. *angularis* (no. 1, 2, cultigen) had 14 TI components compared to eight or nine in its closest wild relative *V. angularis* var. *nipponensis* (no.3, 4). *V. grandiflora*

Table 3. Proteinase inhibitor polymorphism detected in *Vigna* subgenus *Ceratotropis* accessions



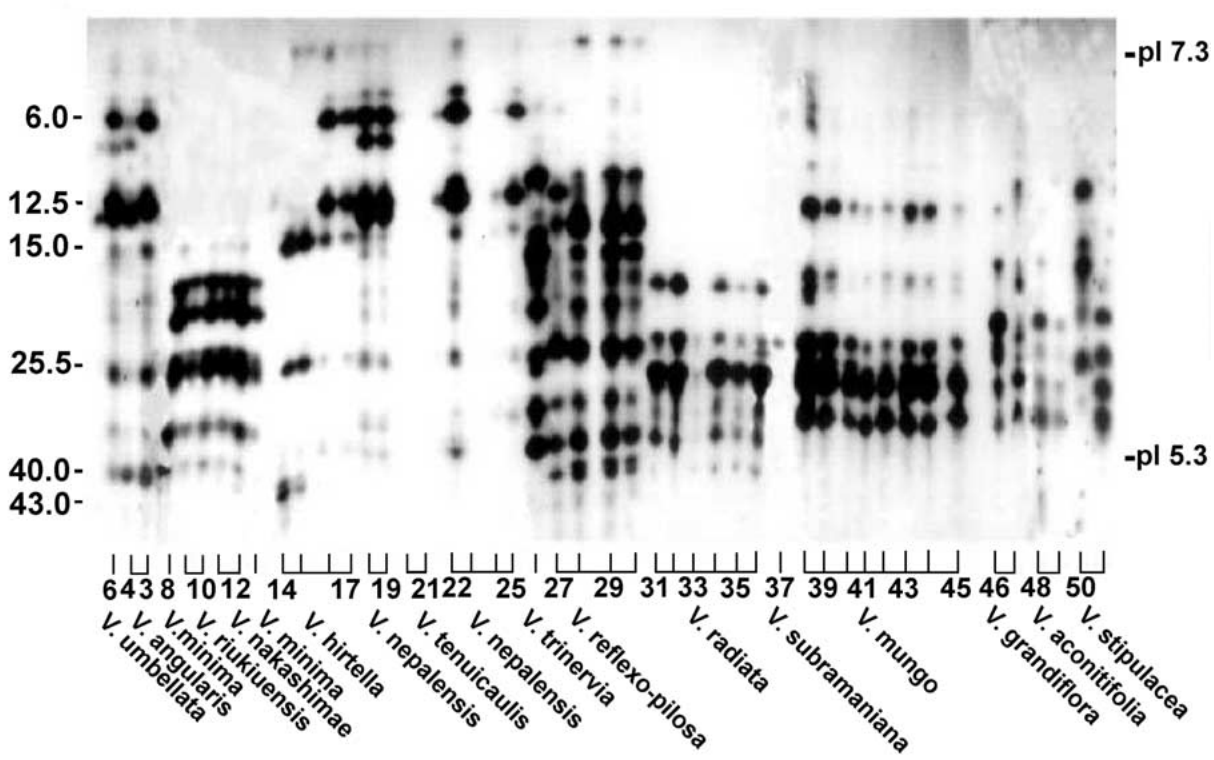


Figure 1. Trypsin inhibitor polymorphism in species of the genus *Vigna* subgenus *Ceratotropis*. Seed proteins were separated by IEF in Servalyt precotes gels (pH 3–10, 1500 Vh) and trypsin inhibitors were detected by the gelatin replicas method. pI 5.3 and 7.3 are positions of β-lactoglobulin and horse myoglobin according to isoelectric points. 6.0–43.0 are positions of trypsin inhibitor bands by nomenclature of authors. Lane 1, *V. umbellata*, no. 6; lanes 2 and 3, *V. angularis*, no. 4 and 3; other lanes, *V. minima*, no. 8 – *V. stipulacea* no. 51, are accessions of *Vigna* in order according to Table 1.

(no. 46, 47), *V. aconitifolia* (no. 48, 49) and *V. stipiculacea* (no. 50, 51) each showed intra-specific variation for TI components.

V. hirtella consists of two pairs of accessions having the same TI component composition. One pair (no. 14, 15) had eight components most of them having low pI. The second pair (no. 16, 17) had seven TI components of high pI with three of them also found in *V. hirtella* (no. 14, 15). *V. tenuicaulis* (no. 20, 21) had only two very weakly detected components of high pI also found in *V. hirtella* (no. 16, 17). *V. nepalensis* had twelve TI components of them six were found in all six accessions tested.

TI components in relation to the tetraploid species *V. reflexo-pilosa* were informative. The two varieties of *V. reflexo-pilosa*, var. *reflexo-pilosa* (no. 27, 28) and var. *glabrescens* (no. 29, 30) had almost identical TI component composition. Fourteen out of the 17 or 18 TI components in these two varieties were at the same position as TI components in *V. trinervia* (no. 26). Of the four TI components not shared with diploid *V. trinervia* two other species have two (p42.0, p43.0, *V. radiata* var. *radiata* and var. *sublobata* no. 31–36) or three (p42.0, 43.0, 47.0, *V. hirtella* no.14, 15) TI components at the same position.

Chymotrypsin inhibitors (CI)

64% of TI components had a component at a corresponding position when tested for CI (Table 3, Figure 2). This strongly suggests that these components are bifunctional. The total number of CI components detected in the 51 accessions of 16 species of the subgenus *Ceratotropis* tested was 26, seven fewer than TI components.

Five of the six *Vigna radiata* accessions had only one CI component but for *V. radiata* var. *sublobata* (no. 33) no CI components were detected. In *V. subramaniana* (no. 37) CI components were not detected. Eleven different TI components were found in *V. grandiflora* (no. 46, 47) but no CI components were detected.

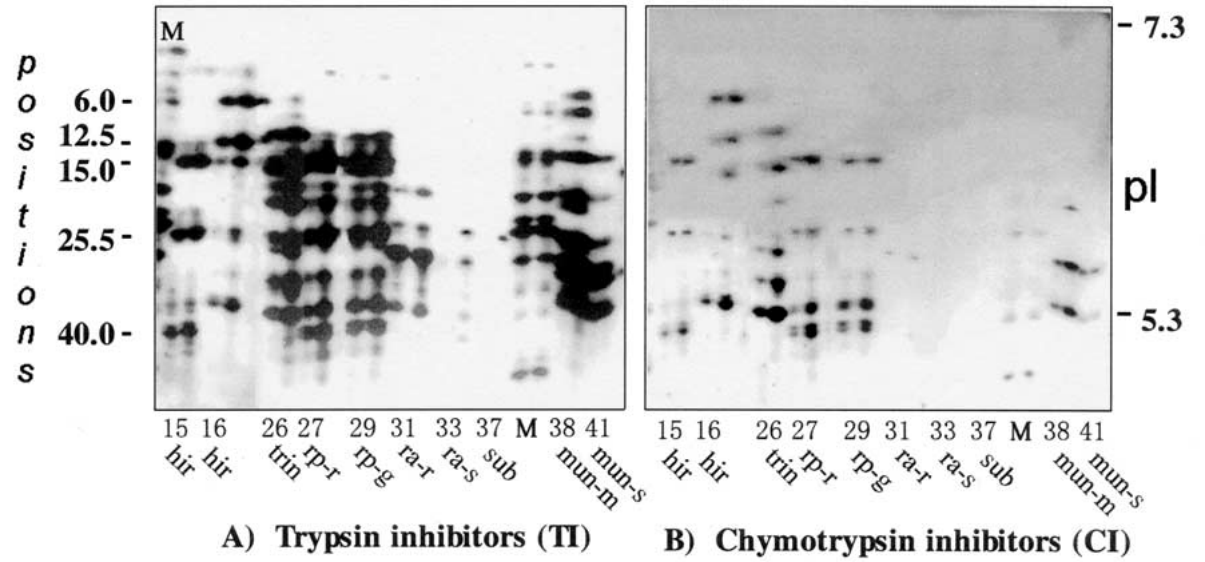


Figure 2. Polymorphism of trypsin and chymotrypsin inhibitors in *Vigna* subgenus *Ceratotropis* species. Two gelatin replicas obtained consecutively from the same gel after IEF of seed proteins in Servalyt precotes gel (pH 3–10, 2400 Vh) were developed by trypsin (A) and chymotrypsin (B). Two parallel lanes each of *V. hirtella*, no. 15; *V. hirtella*, no. 16; *V. trinervia*, no. 26; *V. reflexo-pilosa* var. *reflexo-pilosa* no. 27; *V. reflexo-pilosa* var. *glabrescens* no. 29; *V. radiata* var. *radiata* no. 31; *V. radiata* var. *sublobata*, no. 33; *V. subramaniana* no. 37; *V. mungo* var. *mungo* no. 38; *V. mungo* var. *silvestris* no. 41. m is a marker set of inhibitors (mixture of *V. umbellata* no. 6 and *V. unguiculata* no. 52). Taxa names are abbreviated as indicated in Table 1. The scales are the same as Figure 1.

No intra-specific variation was found for *V. aconitifolia* and *V. stipulacea* that showed intra-specific variation for TI components. *V. minima*, *V. nepalensis*, *V. reflexo-pilosa* var. *glabrescens*, and *V. riukiuensis* showed no intra-specific variation which was also the case with TI. Two accessions of *V. hirtella* (no. 14, 15) shared four CI components (p15.0, 26.5, 42.0, 43.0) only otherwise found in the tetraploid species *V. reflexo-pilosa*.

Subtilisin inhibitors (SI)

SI had lower pIs and less heterogeneity than TIs and CIs. Data on polymorphism of SI are summarized (Table 3). Between one and three SI components were found among the accessions examined. All accessions had one or two SI components except for one accession of *Vigna aconitifolia*. In *V. aconitifolia* (no. 48) three SI components were detected (Figure 3). The most commonly found SI component was at position p20.0 (approximately pI 4.3). Six species, *V. aconitifolia*, *V. hirtella*, *V. mungo, V. nakashimae, V. nepalensis* and *V. reflexo-pilosa* showed intraspecific variation for SI components.

Certain components were only found in one species or accession, such as components at positions p7.5 in *Vigna stipulacea* (no.50, 51) and p3.0 in *V. nakashimae* (no.11). The SI component at p12.5 was only found in *V. mungo* (no. 38–40, 42, 44, 45).

Several SI components were common to several species such as the components at position p6.5, 11.0 and 20.0 found in four, three and eleven species, respectively.

Cysteine proteinase inhibitors (CPI)

Six different CPI components were detected (Table 3, Figure 4 'a'–'f'). Each accession had one (12%), two (86%) or three (4%) distinct CPI components. The CPI variation is less than other inhibitors studied and suggests it is a more stable class of inhibitors. Intraspecific variation was detected for *Vigna hirtella* and *V. nepalensis*. CPI variation appears to be helpful in explaining the broad evolutionary trends in the subgenus *Ceratotropis* (discussed below).

Discussion

Recent taxonomic studies of the genus *Vigna* subgenus *Ceratotropis* has resulted in four new species being described, two of them (*V. nepalensis, V. tenuicaulis*) were included in this study (Tateishi and

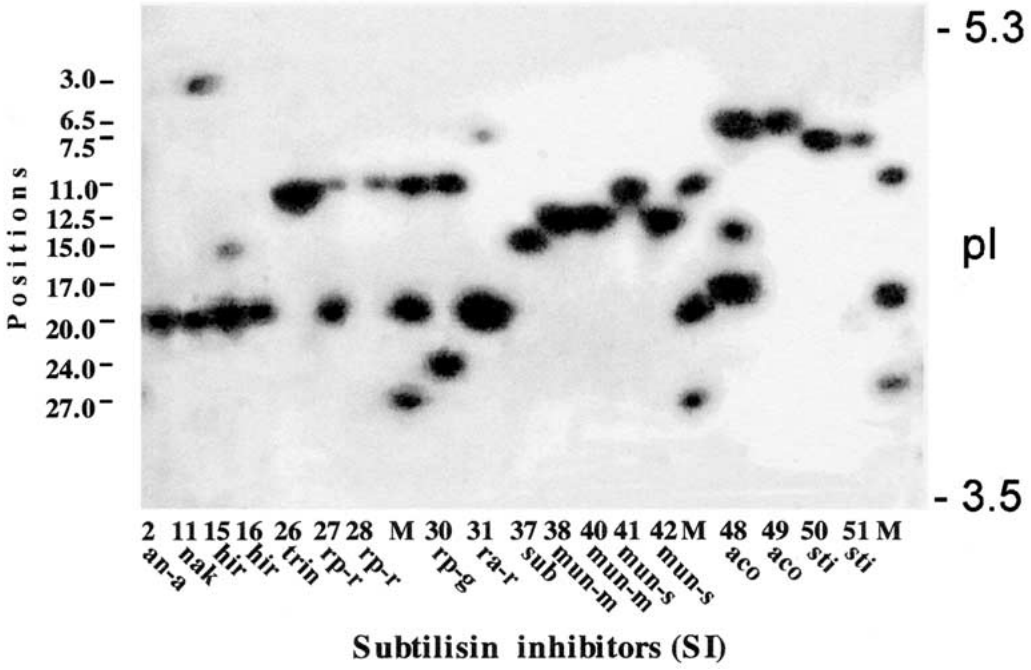


Figure 3. Polymorphism of subtilisin inhibitors in species of the genus *Vigna* subgenus *Ceratotropis*. IEF of seed proteins in pH interval 3.5—5.3. Gelatin replica obtained from the IEF gel was developed by subtilisin. Lane 1 to 21 are single lanes each of the following accessions listed in Table 1: *V. angularis*, no. 2; *V. nakashimae* no. 11; *V. hirtella* no. 15 and 16; *V. trinervia* no. 26; *V. reflexo-pilosa* var. *reflexo-pilosa* no. 27 and 28; *V. reflexo-pilosa* var. *glabrescens* no. 30; *V. radiata* var. *radiata* no.31; *V. subramaniana* 37; *V. mungo* var. *mungo*, no. 38, 40; *V. mungo* var. *silvestris*, 41 and 42; *V. aconitifolia* no. 48 and 49; *V. stipulacea* no. 50 and 51. M – marker set of inhibitors (mixture of seed proteins of *V. angularis* no. 3, *V. trinervia* no. 26 and *V. luteola* no. 53).

Maxted, 2002; Tomooka et al., 2002a). In addition, three sections have been recognized in the subgenus *Ceratotropis*, sections *Aconitifoliae*, *Angulares* and *Ceratotropis* (Tomooka et al., 2002a). Studies using molecular approaches support the subgenus *Ceratotropis* consisting of three major groups that correspond to these three sections (Doi et al., 2002; Tomooka et al., 2002b).

In the present study all known species in the subgenus *Ceratotropis* were analyzed except *Vigna aridicola*, *V. dalzelliana, V. exilis, V. trilobata* and *V. khandalensis*. Based on cluster analysis of inhibitor profiles a phenogram was constructed (Figure 5). Accessions form three clusters that correspond to the three sections in the subgenus. This phenogram is broadly in agreement with results of evolutionary relationships in the subgenus *Ceratotropis* based on morphological characteristics (Tateishi, 1996) and molecular analyses (Doi et al., 2002; Tomooka et al., 2002b).

Based on the phenogram in section *Angulares* three clusters of accessions can be discerned (Figure 5). A1 consists of *Vigna angularis*, *V. hirtella*, *V. nepalensis, V. tenuicaulis* and *V. umbellata*. *Vigna minima*, *V. nakashimae* and *V. riukiuensis* form a distinct cluster, A2. The tetraploid species *V. reflexo-pilosa* forms a distinct cluster with *V. trinervia* and two accessions of the *V. hirtella*. Within section *Ceratotropis* two groups are discerned, cluster B1 (*V. radiata, V. grandiflora, V. subramaniana*) and cluster B2 (*V. mungo*). These two groups correspond to the two evolutionary lines found in this section using molecular and biochemical techniques (Doi et al., 2002; Jaaska & Jaaska, 1990; Kaga et al., 1996) (Figure 5).

Most variation was found within *Vigna hirtella*. Based on the analysis of all the inhibitor data presented in the phenogram, two pairs of accessions are present in the *V. hirtella* complex accessions analyzed (Figure 5). One pair from northern Thailand shows affinity to *V. angularis*, *V. nepalensis, V. tenuicaulis* and*V. umbellata*. The second pair of *V. hirtella* accessions consists of no. 14 from Malaysia and no. 15 from northern Thailand. The morphological characteristics of this pair of accessions (no. 14, 15) are quite distinct having small stipules and rather short bracteoles that resembles *V. minima* (N. Tomooka, personal ob-

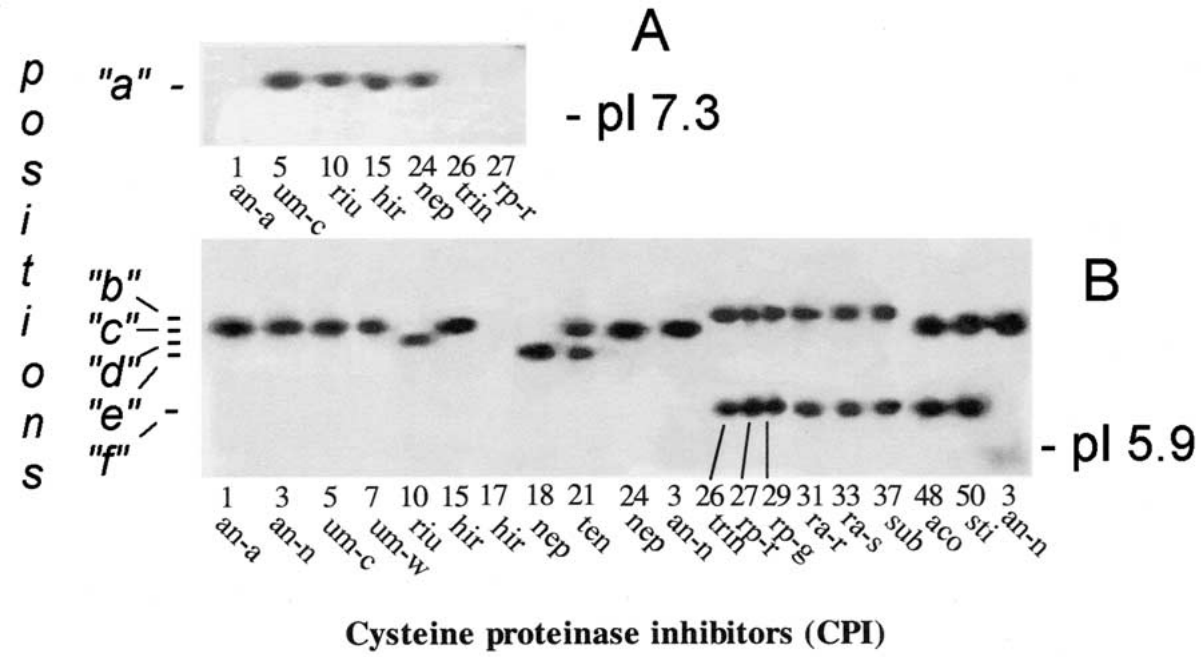


Figure 4. Polymorphism of cysteine proteinase inhibitors in species of the genus *Vigna* subgenus *Ceratotropis*. Figure is based on two separations, A and B, in Servalyt precotes pH 3–10 gel. Inhibitors were detected on gelatin replicas developed by papain. Lanes 1 to 7 are single lanes each of the following species listed in Table 1: *V. angularis* var. *angularis*, no. 1; *V. umbellata* no. 5; *V. riukiuensis* no. 10; *V. hirtella* no. 15; *V. nepalensis* no. 24; *V. trinervia* no. 26; *V. reflexo-pilosa* var. *reflexo-pilosa* no. 27. Lanes 1 to 20 show single lanes of the following species listed in Table 1: *V. angularis* var. *angularis*, no. 1; *V. angularis* var. *nipponensis*, no. 3; *V. umbellata* no.5 and 7; *V. riukiuensis,* no. 10; *V. hirtella* no. 15, 17, *V.nepalensis* no.18; *V. tenuicaulis* no. 21; *V. nepalensis* no. 24; *V. angularis* var. *nipponensis*, no. 3; *V. trinervia* no. 26; *V. reflexo-pilosa* var. *reflexo-pilosa* no. 27; *V. reflexo-pilosa* var. *glabrescens* no. 29; *V. radiata* var. *radiata* no. 31, *V. radiata* var. *sublobata*, no. 33; *V. subramaniana* no. 37; *V. aconitifolia* no. 48; *V. stipulacea* no. 50; *V. angularis* var. *nipponensis*, no. 3. 'a', 'b', 'c', 'd', 'e' and 'f' – positions of bands.

servation). However, based on inhibitor pattern this group differs both from other accessions of the *V. hirtella* and *V. minima*. These accessions of *V. hirtella* seems to be more closely related to *V. trinervia* and the tetraploid *V. reflexo-pilosa*. The *V. hirtella* deserves increased attention by germplasm collectors and in further biosystematic studies.

Vigna trinervia has been confused with *V. radiata* var. *sublobata* (Tateishi and Maxted, 2002). However, based on the profile of inhibitors, *V. trinervia* appears to be a distinct species. Tateishi (1996) considered the morphological and growth habit characteristics of *V. trinervia* to be intermediate between section *Angulares* and section *Ceratotropis*. Recent molecular analyses have also shown *V. trinervia* to be intermediate among sections (Doi et al., 2002; Tomooka et al., 2002b). *V. trinervia* has reticulate endocarp remnant covering the seed coat similar to the species of section *Ceratotropis* whereas its seedling development is similar to section *Angulares* species being hypogeous with cordate first leaf pair with long petiole. *V. trinervia* has TI and CI spectra similar to section *Angulares* species but the SI and CPI spectra are similar to section *Ceratotropis*. *V. trinervia* may have the potential to act as bridging species in breeding to facilitate hybridization between species of the two sections.

Prior to recent nomenclature changes in the subgenus *Ceratotropis* (Tomooka and Maxted, 2002a) the two varieties of *Vigna reflexo-pilosa*, var. *reflexo-pilosa* and var. *glabrescens*, were recognized as separate species, *V. reflexo-pilosa* and *V. glabrescens* (Maréchal et al., 1978). Their inhibitor profiles suggest that these taxa are very closely related and support their treatment as varieties within *V. reflexo-pilosa*. This is also supported by isozyme analysis of these two taxa (Egawa et al., 1996a).

Formerly *Vigna grandiflora* was confused with *V. radiata* var. *sublobata* (Tateishi & Maxted, 2002). Niyomdham (1992) treated *V. grandiflora* as a variety of *V. radiata*, *V. radiata* var. *grandiflora*. Differences between the inhibitor spectra of *V. radiata* and *V. grandiflora* are clear and supports species level distinc-

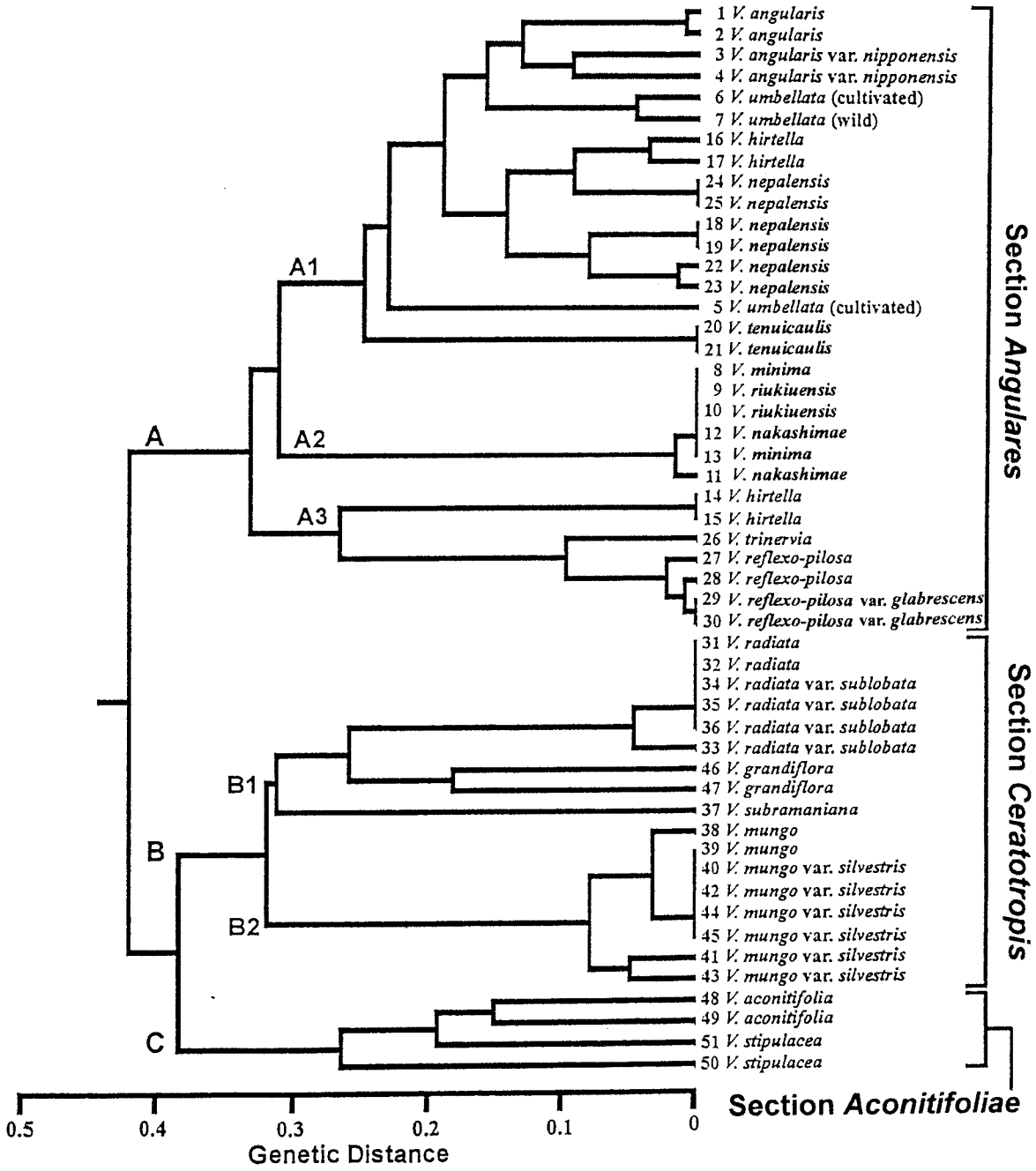


Figure 5. Phenogram constructed by the UPGMA (average linkage) method using genetic distances obtained from polymorphic proteinase inhibitor band data for 51 accessions of 13 species in the genus *Vigna* subgenus *Ceratotropis*. Numbers correspond to list in Table 1.

tion although these two species appear to be closely related.

Vigna reflexo-pilosa has two CPI bands 'b' and 'f' which are found in 5 diploid species *V. trinervia*, *V. radiata, V. subramaniana V. mungo* and *V. grandiflora* (Table 3). Based on TI variation *V. trinervia* is very similar to *V. reflexo-pilosa*. This result agrees with isozyme analysis by Egawa et al. (1996b) who suggested that *V. trinervia* was a likely genome donor to the tetraploids. Egawa et al. (1996b) also suggested that *V. hirtella* no. 14 (identified by Egawa et al. as *V. minima*) was the other likely genome donor. Based on TI and CI bands our results support the hypothesis that *V. hirtella* (particularly no. 14, 15) as the other possible genome donor and cluster analysis aligned *V. hirtella* (no. 14, 15) with *V. trinervia* and *V. reflexo-pilosa* (A3 cluster in Figure 5).

Several accessions analyzed had an absence or very low levels of some inhibitors. CI were not detected in *Vigna radiata* var. *sublobata* (no. 33) and *V. subramaniana* (no. 37), (Table 3, Figure 2B, 33, 37). In *V. tenuicaulis* (no. 20, 21) and *V. subramaniana* (no. 37) the amount of TI detected was very low (Figure 1, Figure 2A, 37). Since these inhibitors can have an adverse effect on crop quality these accessions may be useful for developing varieties of the *Vigna* subgenus *Ceratotropis* cultigens with low level or absence of these inhibitors (Hymowitz, 1980). *V. radiata* var. *sublobata* is cross compatible with *V. radiata* var. *radiata* (mungbean), while the *V. tenuicaulis* (no. 20) is cross compatible with *V. angularis* var. *angularis* (azuki bean) and *V. umbellata* (rice bean) (Miyazaki et al., 1984; Tomooka & Egawa, 1996). Recent results of evaluation of seeds for insect resistance have shown that *V. tenuicaulis* (no. 20) and *V. subramaniana* (no. 37), in spite of the low activity of some proteinase inhibitors (TI and CI), have complete resistance to *Callosobruchus chinensis* (azuki bean weevil) and to *C. maculatus* (cowpea weevil), which are serious storage pests of *Vigna* cultigens (Tomooka et al., 2000b).

Proteinase inhibitors analyzed during this study indicate that the TI and CI of *Vigna* are useful for analyzing diversity both within and between species of *Vigna* subgenus *Ceratotropis*. SI and CPI are more useful for analysis of diversity among species and groups of species in subgenus *Ceratotropis*. Variation revealed and approaches elaborated in this study could be useful in relation to taxonomy of other legumes and breeding for pathogen and pest resistance and improved nutritional value.

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