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Phylogenetic relationships and genetic diversity of the USDA *Vigna* germplasm collection revealed by gene-derived markers and sequencing

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Summary

Phylogenetic relationships in the USDA *Vigna* germplasm collection are somewhat unclear and their genetic diversity has not been measured empirically. To reveal interspecific phylogenetic relationships and assess their genetic diversity, 48 accessions representing 12 *Vigna* species were selected, and 30 gene-derived markers from legumes were employed. Some high-quality amplicons were sequenced. Indels (insertion/deletions) were discovered from the sequence alignments that were specific identifiers for some *Vigna* species. With regard to revealing polymorphisms, intron-spanning markers were more effective than exon-derived markers. These gene-derived markers were more successful in revealing interspecific polymorphisms than intraspecific polymorphisms at both the DNA fragment and sequence levels. Two different dendrograms were generated from DNA fragment data and sequence data, respectively. The results from these two dendrograms supported each other and showed similar phylogenetic relationships among the *Vigna* species investigated. The accessions clustered into four main groups and 13 subgroups. Each subgroup represents a subgenus or a species. Phylogenetic analysis revealed that an accession might be misclassified in our collection. The putative misclassified accession was further supported by seed morphology. Limited intraspecific genetic diversity was revealed by these gene-derived markers and/or sequences. The USDA *Vigna* germplasm collection currently consists of multiple species with many accessions further classified into specific subspecies, but very few subspecies of the total subspecies available exist within the collection. Based on our results, more attention should be paid to the subspecies, wild forms and/or botanical varieties for future curation in order to expand the genetic diversity of *Vigna* germplasm in the USDA collection.

1. Introduction

Considerable confusion in the synonymy and classification of various *Vigna* species exists in the literature (Verdcourt, 1970; Fery, 1980). The genus *Vigna* comprises seven subgenera and more than 80 species. Some of the species adapt well to a wide range of environmental conditions (such as poor soils and drought) and have been domesticated to cultivated species (Faris, 1965; Verdcourt, 1970; Santalla *et al.*, 1998). Cultivated *Vigna* species are an important protein source in countries where people have limited access to food rich in protein (Singh, 2005). The main cultivated species worldwide include the following: five Asian beans: moth bean (*Vigna aconitifolia* (Jacq.) Marechal), azuki bean (*Vigna angularis* (Willd.) Ohwi and Ohashi), black gram (*Vigna mungo* L.), mung bean (*Vigna radiata* L.), rice bean (*Vigna umbellata* Thunb.); two African beans: bambara groundnut (*Vigna subterranea* L.) and cowpea (*Vigna unguiculata* (L.) Walp); and American *Vigna* beans (Jaaska & Jaaska, 1990; Jaaska, 1999, 2001).

Isoenzymes as biochemical markers have been used to assess genetic diversity and reveal phylogenetic relationships among *Vigna* species (Jaaska & Jaaska, 1990; Pasquet, 1999, 2000). The phylogenetic relationships and genetic diversity among and within *Vigna* species were first assessed by restriction

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Table 1. *Selected accessions from Vigna species*

No.	PI	Species	Identifier	Seed colour	Seed weight	Origin/collected location
1	633451	*Phaseolus vulgaris* L.	BAT 93	Tan (str)	19·2	USA
2	164419	*Vigna aconitifolia* Jacq.	8712	Brown (sol)	2·59	India
3	165479	*V. aconitifolia* Jacq.	8954	Brown (sol)	1·87	India
4	372355	*V. aconitifolia* Jacq.	Kitna	Brown (m)	2·22	Yemen
5	312898	*Vigna adenantha* G. Mey.	n/a	Brown	3·28	Argentina
6	319448	*V. adenantha* G. Mey.	22156	Black	2·1	Mexico
7	93815	*Vigna angularis* Willd.	n/a	Tan (sol)	9·59	China
8	157625	*V. angularis* Willd.	26	Red (sol)	8·78	Korea
9	360707	*V. angularis* Willd.	Treasure small bean	Red (sol)	8·17	China
10	416742	*V. angularis* Willd.	65-078-02570A	Red (sol)	8·16	Japan
11	527686	*V. angularis* var. *niponensis* Owhi & H. Ohashi	Bird bean	Brown (sol)	2·37	Korea
12	146800	*Vigna caracalla* L.	No. 842	Tan (sp)	1·93	South Africa
13	322588	*V. caracalla* L.	IRI 1289	Brown (sol)	5·22	Brazil
14	310294	*Vigna longifolia* Verdc.	298	Grey (m)	1·38	Brazil
15	292866	*Vigna luteola* Jacq.	C36-323	Brown (sp)	2·89	India
16	406329	*V. luteola* Jacq.	TVnu 2	Brown (sp)	2·79	Tanzania
17	164316	*Vigna mungo* L. var. *mungo*	OLANDU	Black (sp)	4	India
18	208462	*V. mungo* L. var. *mungo*	n/a	Black (sp)	4·32	Nepal
19	218104	*V. mungo* L. var. *mungo*	MASH	Black (sp)	5·61	Pakistan
20	305073	*V. mungo* L. var. *mungo*	5	Black (sp)	4·41	Thailand
21	292872	*Vigna oblongifolia* A. Rich.	C36-307	Brown (sol)	3·25	Nigeria
22	181585	*V. oblongifolia* A. Rich. var. *oblongifolia*	Wilmsii	Black (m)	2·57	South Africa
23	292868	*V. oblongifolia* A. Rich. var. *parviflora*	C36-328	Black (m)	2·27	India
24	406358	*V. oblongifolia* A. Rich. var. *parviflora*	TVnu 36	Brown (m)	0·79	Tanzania
25	164301	*Vigna radiata* L. var. *radiata*	PACHAPAYARU	Green (sol)	3·94	India
26	171435	*V. radiata* L. var. *radiata*	55	Green (sol)	4·36	China
27	381351	*V. radiata* L. var. *radiata*	IVau 65001	Yellow green (sol)	5·22	Nigeria
28	427064	*V. radiata* L. var. *radiata*	K-970	Green (sol)	2·92	Pakistan
29	240867	*Vigna subterranea* L.	Light reddish brown	Reddish brown (sol)	36·2	Uganda
30	245951	*V. subterranea* L.	Bereke	Reddish brown (sol)	56·9	Zimbabwe
31	378867	*V. subterranea* L.	Ditloo	Red (sol)	32	Nigeria
32	173933	*Vigna umbellata* Thunb.	Oorat	Red (sol)	4·57	India
33	208460	*V. umbellata* Thunb.	MASH	Tan (sol)	5·34	Nepal
34	247689	*V. umbellata* Thunb.	Rouge	Red (sol)	5·62	Zaire
35	275636	*V. umbellata* Thunb.	1422	Tan (sol)	4·87	India
36	291384	*Vigna unguiculata* L. ssp. *cylindrica*	276	Black (spec)	7·72	China
37	292883	*V. unguiculata* L. ssp. *dekindtiana*	C36-258	Black (spec)	1·99	Nigeria

38	632910	*V. unguiculata* L. ssp. *pubescens*	TVnu 109	Yellow green (spec)	1·6	Tanzania
39	215659	*V. unguiculata* L. ssp. *sesquipedalis*	13738	Tan with red eye (sol)	11·6	India
40	419163	*V. unguiculata* L. ssp. *sesquipedalis*	Ching	Brown (str)	11·7	China
41	610582	*V. unguiculata* L. ssp. *sesquipedalis*	UCR 5438	Brown (sol)	15·4	Italy
42	632903	*V. unguiculata* L. ssp. *stenophylla*	TVnu 354	Tan (grey spec)	1·78	Zambia
43	632904	*V. unguiculata* L. ssp. *stenophylla*	TVnu 462	Tan (grey spec)	1·23	Botswana
44	582470	*V. unguiculata* L. ssp. *unguiculata*	UCR 776	White with black eye	10·6	Botswana
45	582469	*V. unguiculata* L. ssp. *unguiculata*	UCR 350	Tan/brown Holstein	20·6	Philippines
46	582578	*V. unguiculata* L. ssp. *unguiculata*	KVu 56 CRN	Brwon (sol)	12·8	Kenya
47	612607	*V. unguiculata* L. ssp. *unguiculata*	GC-86L-98	White with brown eye	20·9	Brazil
48	406390	*Vigna vexillata* L.	TVnu 74	Black (spec)	1·74	Rwanda

Letters in parentheses: m, mottle; sol, solid; spec, speckled; str, streaked.

fragment length polymorphism (RFLP) DNA markers (Fatokun *et al*., 1993), and then by random amplified polymorphic DNA (RAPD) (Kaga *et al*., 1996; Santalla *et al*., 1998; Lakhanpaul *et al*., 2000; Mimura *et al*., 2000; Xu *et al*., 2000; Amadou *et al*., 2001; Ba *et al*., 2004; Diouf & Hilu, 2005) and amplified fragment length polymorphism (AFLP) (Tomooka *et al*., 2002; Zong *et al*., 2003; Seehalak *et al*., 2006; Yoon *et al*., 2007) DNA markers. As more DNA sequence information is now available, internal transcribed spacer (ITS) sequences (Doi *et al*., 2002; Goel *et al*., 2002), simple sequence repeat (SSR) markers (Li *et al*., 2001; Kumar *et al*., 2002; Wang *et al*., 2004; Gillaspie *et al*., 2005) and DNA amplification fingerprinting (DAF) (Simon *et al*., 2007) were used for assessing the phylogenetic relationships and genetic diversity. Although various DNA markers have been developed from different *Vigna* species, there are neither a common nor a sufficient set of robust DNA markers available for evaluation of germplasm applicable to all *Vigna* species. Recently, gene-derived markers were developed across 15 legumes (Choi *et al*., 2006). Since these markers are derived from putative genes, they may be a good source to reveal phylogenetic relationships and assess genetic diversity among and within *Vigna* species.

The US germplasm resource for *Vigna* species is maintained at the USDA-ARS Plant Genetic Resources Conservation Unit (PGRCU) located at Griffin, GA, USA. The phylogenetic relationships in the USDA *Vigna* germplasm collection are unclear and their genetic diversity is unknown. Revealing the phylogenetic relationships and assessing genetic diversity will help develop strategies for better organization and management of existing as well as further acquisitions of *Vigna* germplasm. The objectives of the present study were to: (i) reveal phylogenetic relationships and assess genetic diversity of *Vigna* species in the USDA collection using gene-derived DNA markers, (ii) sequence amplicons generated from gene-derived primers to identify polymorphisms and (iii) evaluate the effectiveness of gene-derived markers in revealing the phylogenetic relationships and assessing genetic diversity among and within *Vigna* species.

2. Materials and methods

(i) *Plant materials and DNA extraction*

Taxonomic classifications of accessions used in the present study are based on the Germplasm Resources Information Network (GRIN; found at http://www.ars-grin.gov/npgs/index.html). Forty-eight accessions from several *Vigna* species (Table 1) were used in this experiment and all accessions were diploids containing

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11 pairs of chromosomes ($2n = 2x = 22$) (Singh, 2005). Among them, 12 accessions were from *V. unguiculata* L. (cowpea, representing six subspecies); five accessions from *V. angularis* Willd. (azuki bean); four accessions each from *V. radiata* L. (mung bean), *V. mungo* L. (black gram), *V. umbellata* Thunb. (rice bean) and *Vigna oblongifolia* A. Rich.; three accessions each from *V. aconitifolia* Jacq. (moth bean) and *V. subterranea* L. (bambara groundnut); two accessions each from *Vigna adenantha* G. Mey., *Vigna caracalla* L. and *V. luteola* Jacq.; and one accession each from *Vigna longifolia* Verdc. and *Vigna vexillata* L. One accession from *Phaseolus vulgaris* L. (common bean), which is closely related to the *Vigna* genus (Verdcourt, 1970), was also included as an outgroup in the present study. Leaf tissue samples were collected from plants grown in a greenhouse at Griffin, GA, USA. DNA was extracted from leaf tissue using an E.Z.N.A. Plant DNA Miniprep kit from Omega Bio-Tek (Doraville, GA, USA). The DNA was then diluted to 10 ng/μl and later used as a template for PCR.

(ii) *PCR and PCR product separation*

Thirty pairs of primers tested in mung bean (*V. radiata*) were selected from GenBank® (BV164338 to BV165946) based on published information (Choi *et al*., 2006) and are listed in Table 2. All PCR reactions, programmes and product separations on agarose gels were performed by following the method described previously by Wang *et al*. (2006).

(iii) *Allele sequencing*

Before sequencing, PCR products were checked on a 3% agarose gel to verify that only a single band was produced from each sample. The PCR product was treated with 1 μl of exonuclease I (10 units/μl) and 1 μl of shrimp alkaline phosphatase (1 unit/μl) (GE Healthcare, Piscataway, NJ, USA) for every 12 μl of PCR reaction to digest single-stranded DNA and cleave the 5′-phosphate, respectively. The PCR product was also cleaned with a Qiagen PCR cleanup kit (Qiagen, Valencia, CA, USA) to remove excess nucleotides, primers, enzymes and other impurities. Then, 1 μl of the cleaned product was run on an agarose gel with a quantitative marker (Invitrogen, Carlsbad, CA, USA) to determine product concentration and thus prepare the sample for sequencing. Sequencing reactions were prepared by following the instructions of the DTCS Quick Start sequencing kit (Beckman Coulter, Fullerton, CA, USA). The sample was sequenced bi-directionally and pUC18 was also sequenced as a positive control. Each sample was sequenced twice to verify the fidelity of the sequenced bases. Samples were injected and sequenced on a Beckman CEQ 8000 by using the LFR-1 method. The sequence module of the software package CEQ 8000 Genetic Analysis System version 8.0.52 from Beckman was used to call the bases after the sequencing was performed. The forward and reverse strands were edited and aligned using AlignIR version 2.0 (LI-COR, Lincoln, NE, USA).

(iv) *Morphology comparison*

Seeds were harvested from plants grown in a greenhouse at Griffin, GA, USA. Seeds mainly representing the accession were weighed. Seed-coat colour and size were scanned and recorded with a Hewlett-Packard Scanjet 7400C. In order to confirm the classification, more accessions from the same species or subspecies were requested from the Griffin seed store and compared regarding their seed morphology.

(v) *Data analysis*

Strong clear bands on the gel images were scored as either present (1) or absent (0) for DNA fragment analysis. The data were entered into a binary matrix for analysis. A distance matrix was created between all pairwise combinations by using the proportion of shared allele algorithm in the program MICROSAT v.1.5 (Minch *et al*., 1997). One hundred bootstrap replicates were generated and a neighbour-joining tree and a consensus tree were constructed using PHYLIP v.3.6 (Felsenstein, 2005). The trees were then viewed and printed using TreeView (Page, 1996).

Thirty-two consensus sequences were imported into ClustalX (Thompson *et al*., 1997) and aligned for DNA sequencing data analysis. Low gap penalties (gap penalty = 10 and gap extension = 0·1) were applied using the slow and accurate pairwise alignment of ClustalX. The resulting alignment was evaluated for maximum pairwise identity. In general, high gap penalties are suitable for intraspecific data, whereas low gap penalties are suitable for interspecific data. The sequence data were imported into Phylip v.3.6 (Felsenstein, 2005) and maximum likelihood (DNAML) was employed. The program SEQBOOT from the PHYLIP package was used to perform bootstrapping with 100 replicates to test the stability of the clades. CONSENSE was used to create consensus trees from bootstrap replicates.

3. Results and discussion

(i) *Gene-derived markers, genetic diversity and phylogenetic relationships*

All 30 gene-derived primers amplified DNA fragment(s) from the accessions tested. Employed as DNA markers, 26 (86·7%) were polymorphic and

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Table 2. *Selected primers*

Name	Origin	Gene	Type	F-primer	R-primer	Size (bp)
BV164524	*Vigna radiata*	6DCS	IS	CTCACGAAAGCCATTGGAGCCAGCAACT	TGCCATGAGCATCAGCCAATTCTTTGA	263–311
BV164858	*V. radiata*	AAT	IS	TGCTTCACGATGCCACCAAAGCCCTTAGA	TCCGACATTAGGATCATCAAGTAGG	432–596
BV164871	*V. radiata*	ACL	IS	ATAAGGTCAAGACTGTATTTATGCCAACA	CTCATAACCCTTCCAAATGGCAATGGAAA	447–509
BV164954	*V. radiata*	BIPA	IS	GAGGAGTCTCACAAAGGATTGC	GGTTTTTCATGTTGTAGACATAGGTTTCA	363–405
BV165019	*V. radiata*	CPOX2	IS	GATAATGGCCTTGTTATGAATTACTACA	GCTCAGACAAGCTTCTTCTTGTGGA	253–286
BV165028[a]	*V. radiata*	CTP	ED	GTTTCGACCCGGACCACCATAATAGAAGTA	TGGAAATGACCATGTTCTAGGATACTGGC	303–316
BV165051	*V. radiata*	CYSK	IS	GGAATTGCTAAAGATGTTACAGAATTGA	AATGAGGACACTCTGTCCAGGTGTGA	310–423
BV165086	*V. radiata*	DK326	IS	GGAACCTGTGCACGACTCTTCAA	TAGACATTGGTACACCAGCATAGCAT	539–631
BV165128	*V. radiata*	ENOL	IS	CATCAAGGCCCGCCAGATCTTTGACA	TTGCACCAACCCCATTCATT	1087–1104
BV165137	*V. radiata*	EST763	STS	CACTCTAAAAAGGCCCAGAAGGTTTGACT	CTTATGACCAATAGTCTGTTCCACTC	348–369
BV165163	*V. radiata*	FENR	IS	ATGCTTATGCCAAAAGATCCAAATGC	CTCACAGCAAAGTCGAGCCTGAAGT	479–508
BV165196	*V. radiata*	GLNA	IS	GAATGGTGCTGGTGCTCACACA	TGGTGGTGTCTGCAATCATGGAAG	498–502
BV165202	*V. radiata*	GLP	ED	GACTCAACACACTTGGTATATCTTTGGCTC	TTATCCACTTGAAAGGCTTTGGTGAGAAC	278–356
BV165337[a]	*V. radiata*	MSU107	STS	ATATTGACGATAATGGCCATTCCTA	AGACTCTGCTGCTCCAGGTTCTAAT	491–518
BV165346	*V. radiata*	MSU141	STS	TTGATCAGCCACAGAAATATAAACCA	GCCTCCCACAAAGTAACAAGTTTC	636–652
BV165392	*V. radiata*	MSU72	STS	AGAAAGCAGTTGGGGAACATATTG	TCGTCTATATGCATTTCTGTGAGAC	392–415
BV165438	*V. radiata*	Ntlim1	IS	GAATGGCATTTGCAGGAACAACTCAGA	TGTCCCTTGTTCCACCAAACATACTTGA	382–450
BV165476	*V. radiata*	PDC	ED	GTTGGAAGCAGCAGTTGAGGCAGCA	CCAAATGCAGGTCCGTTAGCAATCACA	341–365
BV165482[a]	*V. radiata*	PEPCASE	ED	TGGCAGCTATATAAGGCTCAGGAG	GAAATACTCAACAAAACGTGGTTCCTTGA	323–350
BV165526	*V. radiata*	PNDKN	IS	GGCCGAACAAACTTTCATCATGATCA	CCAGGCTCGGATTGAGCAGGGTTTGT	553–694
BV165531	*V. radiata*	PP	IS	TGTCAGTGGATCATGAACCAACCACAGA	CTGCTTCTTCAGTGAGGCGCTTTGCTG	450–636
BV165543[a]	*V. radiata*	PPH	ED	ATGTTCCTTGCCATGGCAGATGCAAGA	CGCCCAGAAATGACATGATAAGAAATG	287–299
BV165627	*V. radiata*	RBCPO	ED	CCCCATCATGATGAGTGCTGGAGA	TTGAGAAATCGTTACCGGTGACAATGATG	273–280
BV165651	*V. radiata*	RNAH	IS	GCTTCCACCAGCTGATACACG	TTAGCCCTAGCAAGAATGTCACTG	670–727
BV165660	*V. radiata*	RNAR	IS	GTTTGGCAGATTGTTGGGGTGAAGA	GGTAGGGCAATTGATGCAAGGTTACACA	312–465
BV165710	*V. radiata*	SHMT	IS	ACCACAACTCACAAGTCACTTC	TTGCTGAGAACCTGCTCTTGGTATG	570–575
BV165732	*V. radiata*	SUSY	IS	TCGCAATGAACCACACAGATTTCA	GTCCAACCTTGCCATGGTGAAGATA	481–488
BV165766	*V. radiata*	TGFRIP	ED	ATTCTGATGAAAGGCCACGAGAGGCCA	CAAGCTTATCACCAACAGAGAAATCGA	323–353
BV165781	*V. radiata*	tRNLS	IS	GGTCTGCGAGCTGTTTTTGGAGAAG	GCAATTCCCTCCTCAGCTAAAAGTG	293–324
BV165813	*V. radiata*	TUP	IS	GAATGGGATGCTATGGGAAGTG	TGGATCAGTGGCACCATCTTTAT	745–754

[a] Markers were monomorphic for *Vigna* germplasm.
IS, intron-spanning marker; ED, exon-derived marker; STS, sequence-tagged site-derived marker.

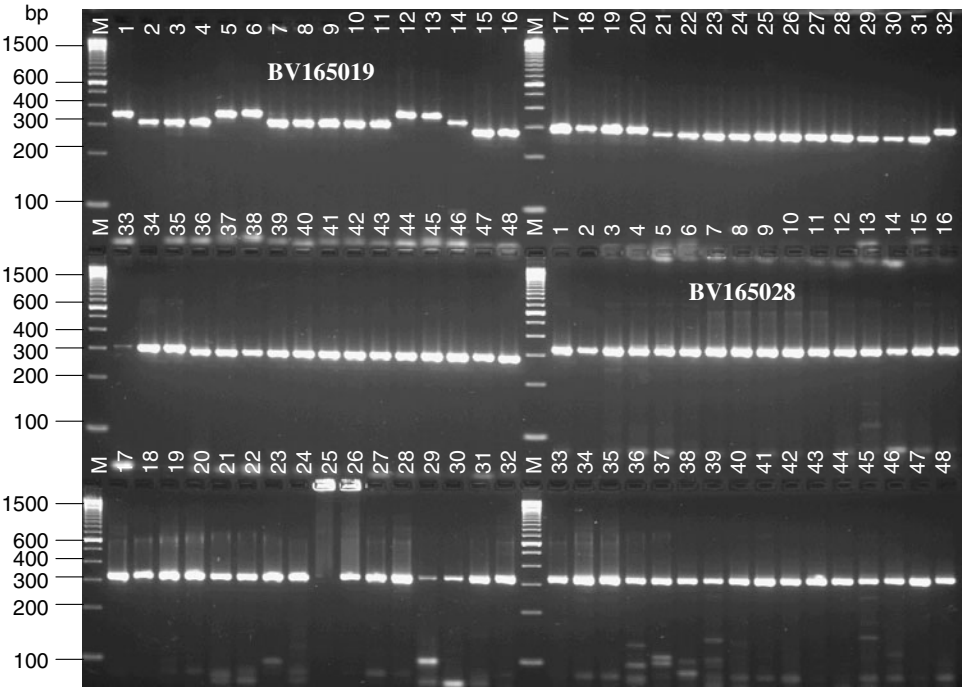

Fig. 1. Amplicons generated by PCR and separated by electrophoresis. Each well contains either 10 μl of molecular marker (100 bp ladder, 250 ng) or 12·5 μl of PCR products. The fragments were separated by electrophoresis on a 3% agarose gel. PCR products were generated with the primer pairs of BV165019 and BV165028 from *Vigna radiata*.

4 (13·3%) were monomorphic. There were no polymorphic markers identified within an accession. All of the markers derived from the intron-spanning (IS) regions were polymorphic, whereas only four of the seven markers from the exon-derived (ED) regions were polymorphic (Table 2). For example, marker BV165019 derived from the intron-spanning region of the gene cationic peroxidase 2 (COPX2) revealed interspecific polymorphisms but not intraspecific polymorphisms, whereas the marker BV 165028 from the exon-derived region of carboxyl-terminal peptidase (CTP) was monomorphic for all the accessions examined (Fig. 1). The results from gene-derived markers demonstrated that at the DNA fragment level, (i) intron-spanning markers were more effective in revealing polymorphisms than exon-derived markers and (ii) gene-derived markers were more effective in revealing polymorphisms among species than within species (Fig. 1 and Table 2). However, future studies should include more accessions from a single species to evaluate intraspecific polymorphism.

A total of 134 polymorphic bands (DNA fragments) were observed with an average of approximately five bands per marker, which were subsequently used for phylogenetic analysis. A dendrogram generated from gene-derived DNA marker data is shown in Fig. 2 and the accessions examined clustered into four main groups (American bean group, African bean group, Asian bean group and intermediate group). One accession (Plant Introduction number (PI) 633451) from *P. vulgaris*, which was used as the outgroup, was different from most of the accessions, but most similar (genetic distance of 0·027) to one accession (PI 146800) from *V. caracalla*. This was not surprising because *P. vulgaris* was taxonomically separated from the *Vigna* genus only since 1970 (Verdcourt, 1970).

Group I included three accessions: two accessions (PI 312898 and PI 319448) from *V. adenantha*, and one accession (PI 322588) from *V. caracalla*. Species of *V. adenantha* and *V. caracalla* belong to the same subgenus *Sigmoidotropis* (Piper) and were also classified as American beans using biochemical markers (Jaaska, 2001). Interestingly, PI 312898 was closely related (distance = 0·013) to PI 322588, which was from a different species (*V. caracalla*, supported by a 59% bootstrap value). Species within group I was closely related to *P. vulgaris*. This result was similar to previous phylogenetic analyses of the *Phaseolus–Vigna* complex (Jaaska, 2001; Goel *et al.*, 2002).

Group II contained only one accession (PI 310294), which belongs to *V. longifolia*. This group is designated as an intermediate group.

Group III contained 20 accessions (from PI 164419 to PI 171435 in Fig. 2) covering five species or five Asian beans, representing five subgroups. Accessions from each species formed a cluster. Three accessions classified as *V. aconitifolia* (moth bean) were examined. Two accessions (PI 165479 and PI 372355) clustered together with little genetic variation (genetic distance = 0·001) and were supported by a 66% bootstrap value. These accessions were different

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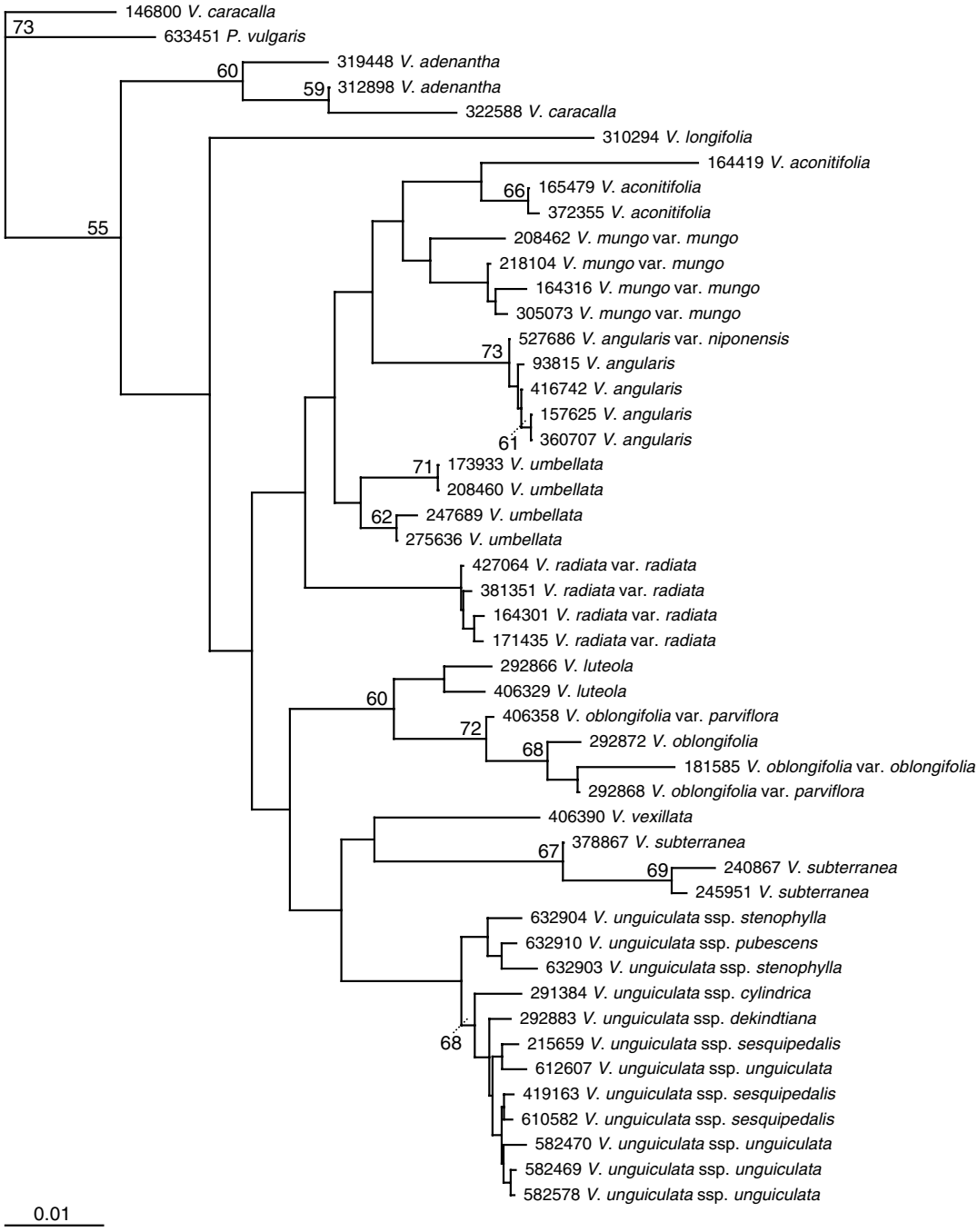

Fig. 2. Neighbour-joining tree of *Vigna* species. Bootstrapping was performed with 100 replicates and values greater than 40 % were placed on the branches.

(genetic distance = 0·027) from accession PI 164419. Therefore, some genetic diversity was detected within moth bean. Four accessions of *V. mungo* var. *mungo* (black gram, from PI 208462 to PI 305073) were examined and they formed a distinct cluster. Five accessions of *V. angularis* (azuki bean, from PI 527686 to PI 360707) were examined and very little genetic difference was detected among these accessions as demonstrated by the short branches and small genetic distance values among these accessions ranging from 0·0008 to 0·0018. Furthermore, strong support for monophyly was apparent with the support of a 73 % bootstrap value. The accession, PI 527686 was previously determined to be the wild form (or progenitor) of cultivated azuki bean (Mimura *et al*., 2000) and the phylogeny supports this notion. Four accessions were examined within *V. umbellata* (rice beans). Accessions (PI 247689 and PI 275636) and (PI 173933 and PI 208460) formed two small clusters that were supported by 71 and 62 % bootstrap values, respectively.

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Four accessions of *V. radiata* (mung bean, PI 171435, PI 164301, PI 381351 and PI 427064) were examined and formed a small cluster. Very limited genetic diversity was detected in mung bean (genetic distance ranging from 0·0009 to 0·003). This result was consistent with the genetic diversity revealed by SSR markers in mung bean (Gillaspie *et al.*, unpublished results) and EcoTILLING (Barkley *et al.*, 2008), which may suggest a narrow genetic base for *V. radiata*. Low levels of genetic diversity were also revealed among 32 Indian mung bean cultivars (Lakhanpaul *et al.*, 2000). Based on branch nodes from our results, mung bean (*V. radiata*) was genetically very different from the other four beans.

Group IV contained 22 accessions covering five species (including two African beans: bambara groundnut and cowpea), representing five subgroups. Accessions from each species formed a cluster. The species within group IV originated in Africa and therefore this group was named the African bean group. Two accessions (PI 292866 and PI 406329) from *V. luteola* clustered with a genetic distance of 0·008. Four accessions were examined within *V. oblongifolia* and they formed a small cluster with the support of a 72% bootstrap value. However, PI 406358 was genetically similar to three other accessions with genetic distances ranging from 0·011 to 0·014 (PI 292868, PI 181585 and PI 292872; Fig. 2). Based on phylogenetic analysis, it seems that the *V. luteola* species was closely related (distance = 0·22–0·31) to *V. oblongifolia* with the support of a 60% bootstrap value. One accession (PI 406390) from *V. vexillata* clustered closely to *V. unguiculata* and *V. subterranea*, which was consistent with the early RFLP analysis (Fatokun *et al.*, 1993) and morphological observations (Bisht *et al.*, 2005). Three accessions (PI 378867, PI 240867 and PI 245951) were examined within *V. subterranea* (bambara groundnut) and they formed a small cluster with the support of a 67% bootstrap value. However, PI 240867 and PI 245951 were closely related genetically (distance = 0·005) with the support of a 69% bootstrap value and were distinct from PI 378867. In a previous study, genetic classification of *V. subterranea* accessions was related to geographic origin and accessions collected from Nigeria were very different from accessions collected from Zimbabwe (Amadou *et al.*, 2001). In the present study, PI 378867 collected from Nigeria was also very different from PI 245951 (genetic distance of 0·01) collected from Zimbabwe. Twelve accessions (PI 632904–PI 582578) from cowpea (*V. unguiculata*) clustered together, forming a subgroup. There were six subspecies examined within *V. unguiculata* (Table 1). The accession (PI 632910) from subspecies *pubescens* may be closely related (distance = 0·005–0·007) to the accessions (PI 632903 and PI 632904) from subspecies *stenophylla* and formed a small cluster. Four accessions (PI 582578, PI 582469, PI 582470 and PI 612607) from subspecies *unguiculata*, three accessions (PI 610582, PI 419163 and PI 215659) from subspecies *sesquipedalis*, one accession (PI 292883) from subspecies *dekindtiana* and one accession (PI 291384) from subspecies *cylindrica* formed a small cluster with the support of a 68% bootstrap value. Obviously, some genetic diversity was detected among these subspecies but compared with other species examined within group IV; the genetic diversity detected was minimal, as demonstrated by short branch lengths and low genetic distance among accessions ranging from 0·005 to 0·013 within *V. unguiculata*. This result was consistent with results from other studies (Li *et al.*, 2001; Diouf & Hilu, 2005) in which a narrow genetic base was also found in cowpea breeding lines and local varieties from Senegal. A single domestication event between wild and cultivated cowpea may be the explanation for the narrow genetic base within cowpea (Coulibaly *et al.*, 2002; Ba *et al.*, 2004). Another possible explanation is that the type of DNA markers employed may also affect the level of polymorphism revealed. To reveal genetic diversity of cowpea (*V. unguiculata* var. *unguiculata*), SSR, RAPD and gene-derived markers were used in two previous studies as well as in the present study, respectively. When 26 DAF primers were employed, 54 cowpea accessions (*V. unguiculata* var. *unguiculata*) were classified into separate groups (Simon *et al.*, 2007). DAF may be a highly efficient system for the generation of polymorphic DNA markers for revealing cowpea genetic diversity.

(ii) *DNA sequencing, genetic diversity and phylogenetic relationships*

To detect polymorphism at the DNA sequence level, 32 amplicons generated from the marker BV165019 were sequenced and the sequence alignment is shown in Fig. 3. Possible sequence errors were identified at a primer site when the consensus sequences were constructed from forward and reverse reads. The first few base pairs of the sequences with possible errors were removed when the phylogenetic tree was generated from sequence data. The phylogenetic tree generated from sequence data is shown in Fig. 4. Comparing the sequence alignment of the common bean (*P. vulgaris*, PI 633451) with the remaining sequences derived from species within the genus *Vigna*, several small deletions or insertions (indels) were identified. This implies that indels may play an important role in differentiation and speciation (Fig. 3). According to the phylogenetic analysis from DNA bands (size of the DNA fragments), PI 322588 from *V. caracalla* formed a cluster with accessions (PI 312898 and PI 319448) from *V. adenantha*. Our sequence data further confirmed the above phylogenetic relationship. Within the

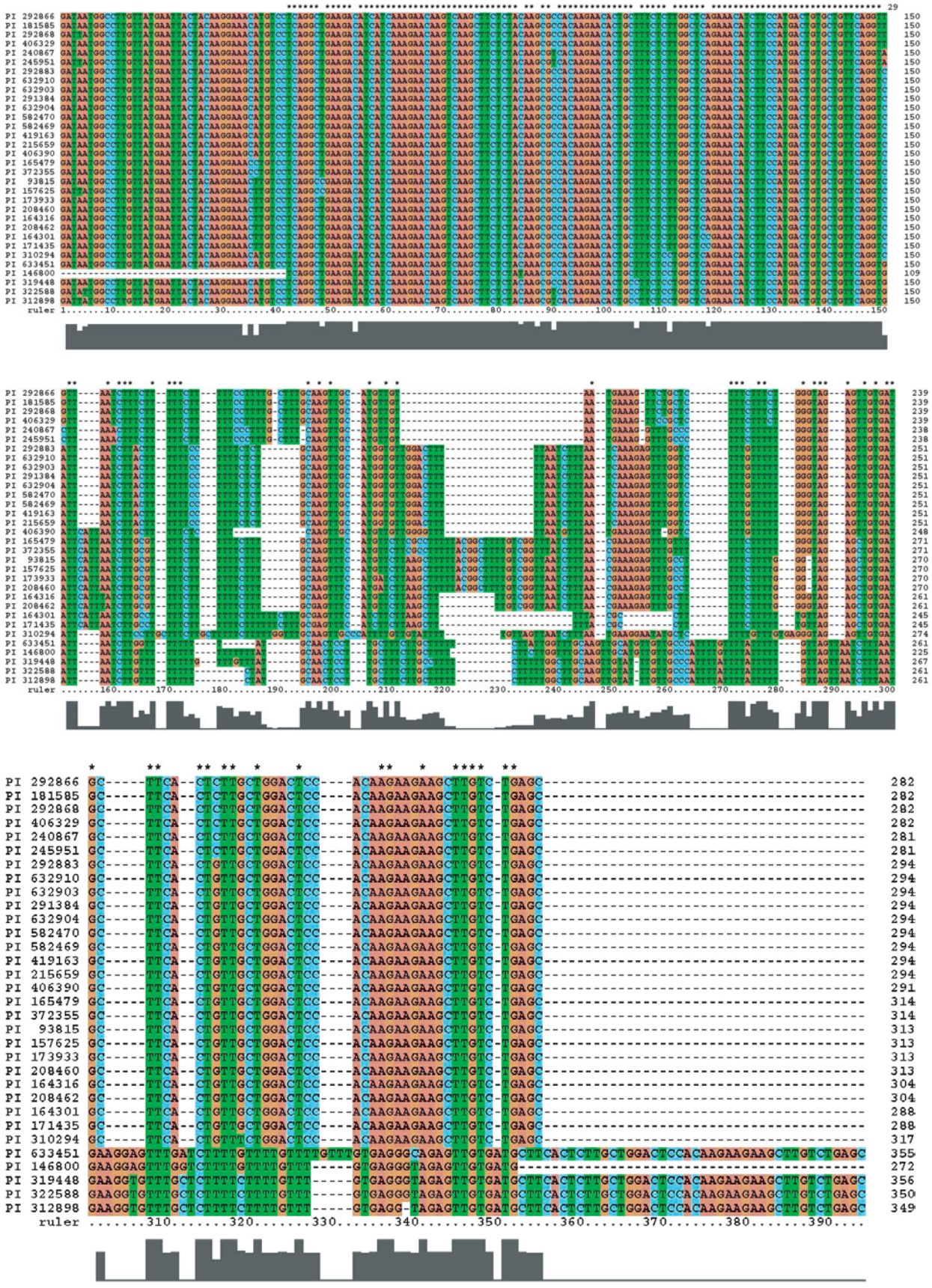

Fig. 3. Sequence alignment of gene-derived marker BV165019 alleles generated from selected *Vigna* accessions produced by using AlignIR version 2.0.

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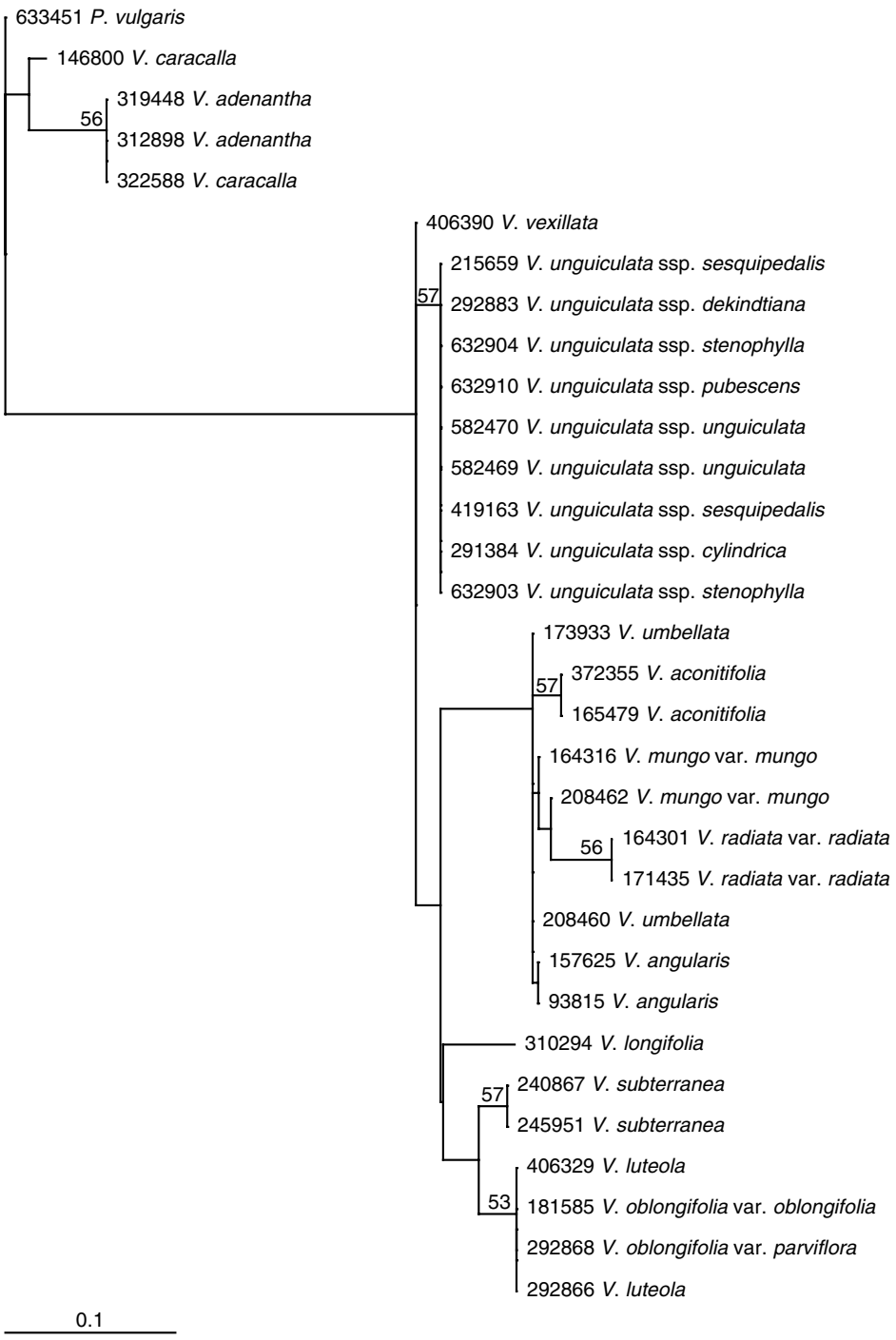

Fig. 4. Maximum likelihood tree of *Vigna* species generated from BV165019 amplicons. Bootstrapping was performed with 100 replicates and values greater than 40% were placed on the branches.

species of *V. adenantha*, there was a base pair deletion at position 340 observed between PI 319448 and PI 312898 (Fig. 3).

Although accessions from different subspecies within cowpea were sequenced, there were no sequence polymorphisms identified (Fig. 3). The accessions from cowpea formed a cluster with a bootstrap value of 57% and no variation among accessions. The low genetic diversity revealed within cowpea may be explained by a single domestication event (Pasquet, 1999). In comparison with cowpea accessions, *V. vexillata* (PI 406390) had a four-base-pair insertion (154–157), a large deletion (182–194) and one-base-pair deletion (259) (Fig. 3). Within the Asian bean group, two accessions from each species were sequenced and all sequenced Asian bean accessions formed a group (Fig. 4) with little diversity among the species within this group. Sequence variation (point

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mutation, insertion or deletion) was identified among these five species. However, only one point mutation (A/G) at position 196 was identified within the species *V. mungo* (Fig. 3). The species *V. longifolia* (PI 310294) within the African bean group was distinguished from other African bean accessions by several smaller deletions (Fig. 3). The species *V. subterranea* (PI 240867 and PI 245951) were separated from the species *V. luteola* and *V. oblongifolia*. There was a point mutation identified (from T to C) at position 258 within the species *V. luteola*. Overall, sequencing gene-derived amplicons detected more variation among species than within species, which was consistent with the results from detecting size difference of DNA fragments on an agarose gel. The topologies of the two dendrograms generated from DNA fragment data and DNA sequence data were very similar (Figs 2 and 4). The method of sequencing DNA amplicons for characterization of germplasm may be expensive, but the method for separating DNA fragments on agarose gels may require processing a large number of DNA markers to be effectively utilized in some genera.

(iii) *Seed morphological observation and phylogenetic relationships*

Seed morphology from 48 accessions was observed and recorded (shown in Fig. 5). The phylogenetic analysis showed that two accessions (PI 146800 and PI 322588) from *V. caracalla* were placed into different groups. Accession PI 146800 had small speckled tan colour seeds (1·93 g per 100 seeds), whereas PI 322588 had large solid brown seeds (5·22 g per 100 seeds) (Fig. 5 and Table 1). Furthermore, within 313 base pairs of the sequenced amplicons, eight point mutations were identified between these two accessions (Fig. 3). Given all the collected data, it is possible that these two accessions may belong to different species. Actually, the seed morphologies of PI 322588 (*V. caracalla*) and PI 312898 (*V. adenantha*) were very similar (Fig. 5) and these two accessions were also clustered into the same subgroup from the phylogenetic analysis of markers and sequence data (Figs 2 and 4). It is suspected that these two accessions may belong to the same species (*V. adenantha*). To confirm this speculation, more accessions classified as *V. adenantha* were requested from the Griffin seed store and their seed morphology was compared. The seed morphology of these two accessions and other accessions from the same species was very similar. Further confirmation of the possible misidentified accessions will include collecting more observational data (for example, seedling morphology, flowering characteristics and other traits) and experiments need to be conducted. Future work will include growing these accessions and collecting descriptor data to determine whether this accession has been misclassified or mislabelled during curation of this crop.

There were five accessions investigated from *V. angularis* for phylogenetic analysis. One of them (PI 527686) was the wild form, which was different from the other four accessions (Fig. 2). The seed size of PI 527686 (2·37 g per 100 seeds) was almost four times smaller than the cultivated form (from 8·16 to 9·59 g per 100 seeds, Table 1). From phylogenetic analysis, three *V. subterranea* accessions clustered into two distinct groups. The accession (PI 378867) was distinct from the remaining accessions (PI 240867 and PI 245951) (Fig. 2). The seed morphology was consistent with the phylogenetic analysis. The former accession had a red seed coat, whereas the latter two accessions had reddish brown seed coats.

Phylogenetic analysis from DNA fragment data classified 12 investigated cowpea accessions into two small clusters. The first small cluster contained three accessions (PI 632904, PI 632910 and PI 632903) and the second small cluster contained nine accessions (from PI 291384 to PI 582578) (Fig. 2). All accessions within the first cluster had small seeds (1·23, 1·6 and 1·78 g, respectively) and this cluster may be called the 'wild group'. All accessions except one within the second small cluster had large seeds (from 7·72 to 20·89 g) (Table 1 and Fig. 5) and this small cluster may be called the 'domesticated group'. The result from seed morphological observation was consistent with those from phylogenetic analysis. Our results demonstrated that phylogenetic analysis with morphological re-examination may provide a more complete approach to classify accessions or to examine misidentified accessions in a plant germplasm collection.

Very limited genetic variation (especially diversity within a species) was detected within the USDA *Vigna* germplasm collection based on the present and previous studies. Currently, some genetic gaps exist in the USDA *Vigna* collection. For example, on the botanical variety level, there are at least three varieties (var. *radiata*, var. *sublobata* and var. *setulosa*) available within *V. radiata* (Bisht *et al*., 2005). However, most accessions preserved in the USDA collection are from *V. radiata* var. *radiata*. Only one accession is maintained from *V. radiata* var. *sublobata*, while there are no accessions classified as *V. radiata* var. *setulosa* in the USDA collection. There are at least two botanical varieties (*V. mungo* var. *mungo* and *V. mungo* var. *silvestris*) within *V. mungo* (Seehalak *et al*., 2006), but only *V. mungo* var. *mungo* was collected and maintained as part of our collection. At the species level, some newly described ones (for example, *V. aridicola*, *V. exilis*, *V. nepalensis*, *V. tenuicaulis* and other species) are available (Tomooka *et al*., 2002). Tomooka *et al*. found that the species *V. aridicola* was closely related to *V. aconitifolia* (moth bean), *V. exilis* was

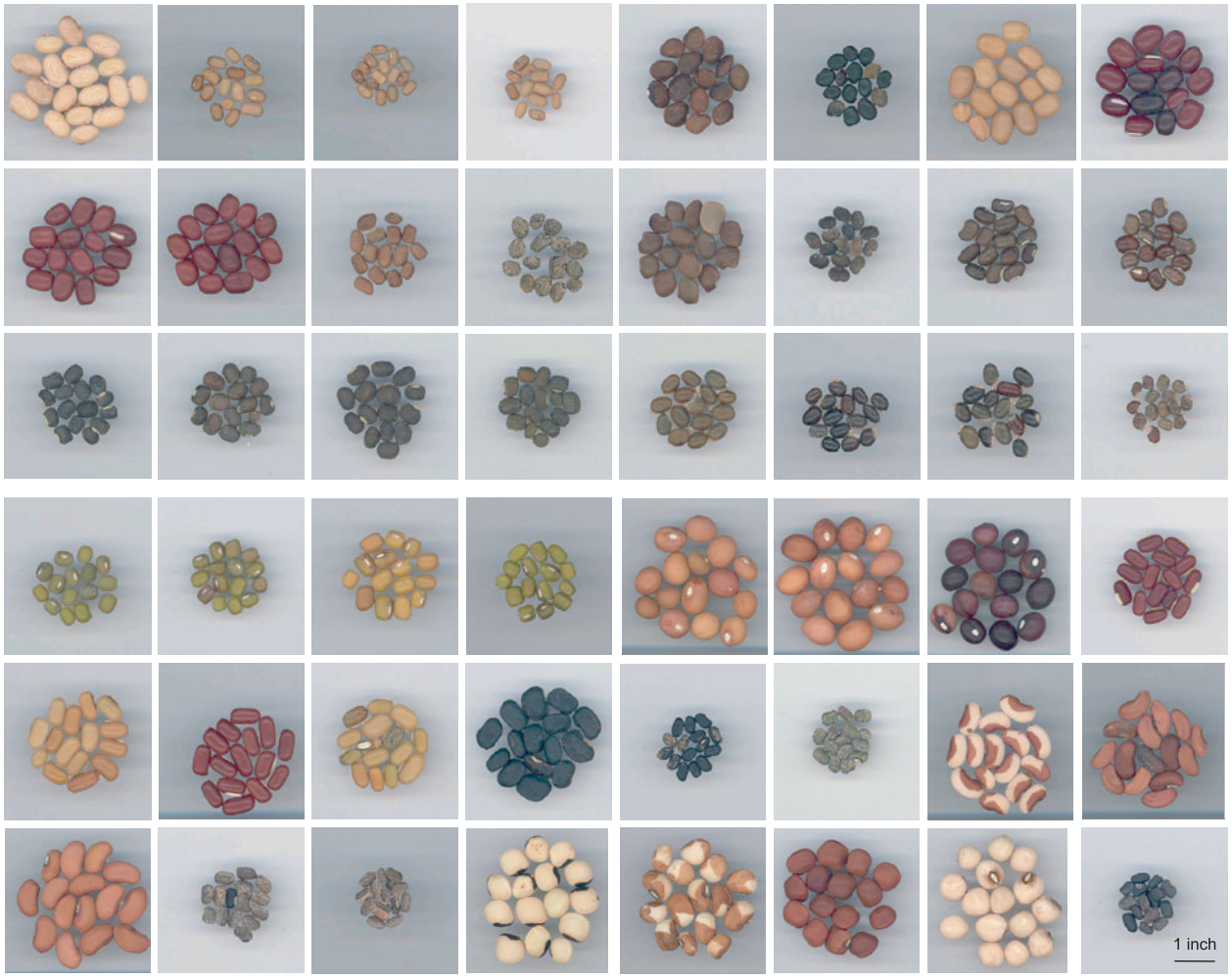

Fig. 5. Seed-coat colours from one common bean accession (*Phaseolus vulgaris* L.) and 47 *Vigna* accessions. From left to right: the eight accessions of the first row are PI 633451 (*P. vulgaris* L.), PI 164419 (*Vigna aconitifolia* Jacq.), PI 165479 (*V. aconitifolia* Jacq.), PI 372355 (*V. aconitifolia* Jacq.), PI 312898 (*Vigna adenantha* G. Mey.), PI 319448 (*V. adenantha* G. Mey.), PI 93815 (*Vigna angularis* Willd.) and PI 157625 (*V. angularis* Willd.); the eight accessions of the second row are PI 360707 (*V. angularis* Willd.), PI 416742 (*V. angularis* Willd.), PI 527686 (*V. angularis* var. *niponensis* Owhi & H. Ohashi), PI 146800 (*Vigna caracalla* L.), PI 322588 (*V. caracalla* L.), PI 310294 (*Vigna longifolia* Verdc.), PI 292866 (*Vigna luteola* Jacq.) and PI 406329 (*V. luteola* Jacq.); the eight accessions of the third row are PI 164316 (*Vigna mungo* L. var. *mungo*), PI 208462 (*V. mungo* L. var. *mungo*), PI 218104 (*V. mungo* L. var. *mungo*), PI 305073 (*V. mungo* L. var. *mungo*), PI 292872 (*Vigna oblongifolia* A. Rich.), PI 181585 (*V. oblongifolia* A. Rich. var. *oblongifolia*), PI 292868 (*V. oblongifolia* A. Rich. var. *parviflora*) and PI 406358 (*V. oblongifolia* A. Rich. var. *parviflora*); the eight accessions of the fourth row are PI 164301 (*Vigna radiata* L. var. *radiata*), PI 171435 (*V. radiata* L. var. *radiata*), PI 381351 (*V. radiata* L. var. *radiata*), PI 427064 (*V. radiata* L. var. *radiata*), PI 240867 (*Vigna subterranea* L.), PI 245951 (*V. subterranea* L.), PI 378867 (*V. subterranea* L.) and PI 173933 (*Vigna umbellata* Thunb.); the eight accessions of the fifth row are PI 208460 (*V. umbellata* Thunb.), PI 247689 (*V. umbellata* Thunb.), PI 275636 (*V. umbellata* Thunb.), PI 291384 (*Vigna unguiculata* L. ssp. *cylindrica*), PI 292883 (*V. unguiculata* L. ssp. *dekindtiana*), PI 632910 (*V. unguiculata* L. ssp. *pubescens*), PI 215659 (*V. unguiculata* L. ssp. *sesquipedalis*) and PI 419163 (*V. unguiculata* L. ssp. *sesquipedalis*); the eight accessions of the sixth row are PI 610582 (*V. unguiculata* L. ssp. *sesquipedalis*), PI 632903 (*V. unguiculata* L. ssp. *stenophylla*), PI 632904 (*V. unguiculata* L. ssp. *stenophylla*), PI 582470 (*V. unguiculata* L. ssp. *unguiculata*), PI 582469 (*V. unguiculata* L. ssp. *unguiculata*), PI 582578 (*V. unguiculata* L. ssp. *unguiculata*), PI 612607 (*V. unguiculata* L. ssp. *unguiculata*) and PI 406390 (*Vigna vexillata* L.), respectively. The bar for seed size represents 1 inch.

closely related to *V. umbellata* (rice bean), and both *V. nepalensis* and *V. tenuicaulis* were closely related to *V. angularis* (azuki bean). Although these newly described species could be potentially important for improving the cultivated species, none of them have yet been added to the USDA *Vigna* germplasm collection.

In conclusion, gene-derived markers are efficient to reveal phylogenetic relationships. Forty-seven *Vigna* accessions have been classified into four notable groups. Gene-derived markers are more effective at revealing polymorphism among species than within species. A few polymorphisms were identified within species by sequencing amplicons generated from

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gene-derived primers. The classification from DNA fragment analysis was consistent with the classification from DNA sequence analysis. Moreover, the genetic classification was supported by seed morphological observation. There was limited genetic diversity within the current USDA *Vigna* germplasm collection. In order to expand the genetic base of the USDA *Vigna* germplasm, new botanical varieties, subspecies and species need to be added to the USDA collection by germplasm curation and exchanges.

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