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Research Article

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Identification of Conserved Orthologous Set Markers in Cultivated *Vigna radiata* (L.) *Wilczek*

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Abstract

FWith an aim to develop widely applicable conserved gene markers to identify and tag orthologous genes from related *Vigna* species to reveal phylogenetic relationships and the nature of genes conserved in genus *Vigna* across the evolution, low copy nuclear Conserved Ortholog Set (COS) genes were tested to explore the interspecific genetic relationship of *Vigna radiata* (L.) Wilczek with other species such as *V. mungo* (L.), *V. umbellata Thunb*., *V. angularis* Wild and *V. unguiculata* (L.) Walp. To detect COS regions, computational approach was followed utilizing the available expressed sequence tags (EST) database of *Vigna radiata* and its related species. The ESTs were processed to eliminate sequence repeats, contaminants and low-complexity sequences. Upon alignment with Soybean genome, only high quality ESTs were grouped into clusters from which consensus sequences representing putative genes are generated for each *Vigna* species. From 6443 ESTs, 2550 contigs were acquired to develop 230 primer pairs to amplify conserved orthologous sequences across *Vigna* species. Among 14 primer pairs used for validation with genotypes of *V. radiata*, 3 gave double bands (paralogs) and 9 produced single bands indicating single copy orthologs. This infers that DNA sequences identified by this comparative genomics approach would be of great use in analyzing genomic information of related, unexplored crop genomes to augment gene discovery and plant breeding in other legumes.

Keywords

Comparative genomics; Conserved orthologous set (COS) markers; ESTs; *Vigna*

Introduction

Legumes provide protein-rich food for a large part of the world's population hence the research on legumes is essential for the establishment of extensive genetic and genomic resources, which can accelerate the discovery of critical genes. Cultivated *Vigna* species are an important protein source in countries where people have limited access to food rich in protein [1]. Globally, *Vigna* species are cultivated as three main types such as Asian beans, African beans and American *Vigna*. The Asian *Vigna* is: moth bean (*V. aconitifolia* (Jacq.) Marechal), adzuki bean (*V. angularis* (Willd.) Ohwi and Ohashi), black gram (*V. mungo* L.), mungbean (*V. radiataL.*), rice bean (V. *umbellata* Thunb.); two African beans; bambara ground nut (*V. subterranean* L.) and cowpea (*Vigna unguiculata* (L.) Walp). One of the prerequisites for increasing yield of these legumes is the study on comparative genomics for better understanding of genome structure [2] which is essential for the establishment of extensive genetic and genomic resources to accelerate the discovery of critical to genes for crop improvement. Genetic improvement of *Vigna* species through conventional breeding has been slow due lack of exploitable genetic variability within the cultivated germplasm and also due to limited gene pool and sexual-incompatibility with wild and related species, the reservoir of desirable genes. Genome research in mungbean is still far behind the other major legume crops such as soybean, cowpea, and common bean, or even their relative but less important, adzuki bean. The genome study in mungbean and related *Vigna* species has been made possible by using genetic markers from other related legumes, and this trend will continue since only limited genetic resources are available for further study in this crop. The utility of marker assisted selections in improvement of *Vigna* species is limited largely due to limited marker polymorphism within the species and hence there is a scope to look for diversity in related crop species. Efforts are being made to develop high-throughput markers with greater resolution [3]. A high degree of similarity in the nucleotide sequences among green gram and other *Vigna* species reported in earlier studies by performing comparative genome analysis using DNA markers [4,5]. To alleviate the use of the wide genetic diversity present in wild relatives and landraces of crops, more information is needed on the organization and structure of their genes and genomes. Molecular markers linked to loci with important effects can facilitate the introgression of those traits into adapted germplasm. Agriculturally important traits captured during domestication are often coded by very limited number of loci with major phenotypic effects. These loci possibly have putative orthologous counterparts in other species [6] and therefore molecular markers, such as Conserved Orthologus Set (COS) markers are of great use in comparing genomic information between the phylogenetically related species within the genus or family. They are extremely useful for the analysis of genome evolution among closely and distantly related species within Leguminosae family. For a given group of species, a COS is formed by identifying a gene from each species that is orthologous to all other genes in the set. These markers are apparent single-copy evolutionary conserved genes in two or more species that share common ancestry (are orthologous) [7]. Information from the comparative genomic analyses within the genus *Vigna* will elucidate the genetics of domestication and enable the isolation of novel genes for use in breeding of mungbean germplasm in particular and *Vigna* in general. Generation of molecular level genetic data in *Vigna* has led to search for ways and means to utilize the existing plant genetic resources to support breeding challenges aiming at gainful applications in crop improvement. Molecular markers such as RAPD, AFLP [8] RFLP, ISSR [9,10], SSRs [11] and sequence tagged microsatellite site [12] have been used in mungbean to test their usefulness in genetic diversity among cultivars. During domestication, species of *Vigna* have got the agriculturally important traits coded by very limited number of loci with major phenotypic effects. It is common to find that these loci have putative orthologous counterparts in other species within

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the same genus and therefore molecular markers, such as Conserved Orthologous Set (COS) markers, are powerful in comparing genomic information across species. Orthologous genes are called so as they are related by common ancestry sharing the same function or activity and acquire homologous relationship across the speciation event.

COS sequences were first described by Tanksley et al. with identification of a subset of plant genes that have remained relatively stable in both sequence and copy number since the radiation of flowering plants from their last common ancestors [7]. This is the landmark work to enunciate utility of COS sequences in comparative genomics and phylogenetic studies. In a further refinement, Wu et al. [13] identified and annotated a large set of conserved, single-copy, putatively orthologous genes using a set of approaches of computational and phylogenetic algorithms to demonstrate the use of these new ortholog resources to elucidate issues related to comparative genomics, molecular systematics, and gene evolution studies in the euasterid clade. Mingai Li [14] developed widely applicable COS markers (pCOS) for phylogenetic reconstructions at low taxonomic level and found that these markers are highly informative in phylogenetic reconstruction of congeneric species. An understanding of conservation of genome structure among legume species is a prerequisite, to use existing wide genetic diversity present in landraces and wild relatives of legumes [2]. Since the last three decades, we have seen large advancement in linking plant genomes through comparative genetic maps, especially for species belonging to the same family [15]. In the present study, computational approach of finding COS markers in *Vigna* species, we have used ESTs for finding conserved regions as molecular markers constructed from ESTs since they are contained within an exon region of genes that are actually expressed. The present study was undertaken with the objectives of mining the large EST collections of *Vigna* species to identify non-redundant ESTs, design primer pairs across all four species for putative COS markers across the *Vigna* species and finally to test some of these markers in *V. radiata*. To define conserved genic regions between *V. radiata* and other related *Vigna* species with soybean genome (which is the most sequenced and is also a legume) orthologous groups that share among them were identified. Validation is done using some of the newly designed primers from contigs of *Vigna* species and proven their amplification in *V.radiata*.

We demonstrate here the use of this new Ortholog resource to shed light on issues related to comparative genomics, molecular systematics and gene evolution studies in the legumes especially in *Vigna* genus. The present study was undertaken with the objectives of mining the large EST collections of *Vigna* species to identify non-redundant ESTs, design primer pairs across all four species for putative COS markers across the *Vigna* species and finally to test some of these markers in *V. radiata*. To define conserved genic regions between V. radiata and other related *Vigna* species with soybean genome (which is the most sequenced and is also a legume) orthologous groups that share among them were identified

Materials and Methods

EST datasets

The ESTs of all *Vigna* species in the study were searched and retrieved from NCBI (ftp://ftp.ncbi.nih.gov/blast/db/). Also the genome sequence data set of Soybean was downloaded and used as reference genome for identifying conserved genomic regions in *Vigna* species. EST

Processing and assembly

The repetitive and ambiguous sequences in the downloaded ESTs were first trimmed. Subsequently, ESTs with sequences <30 bp were omitted from the final data set. The processed EST sequence files were combined and assembled into contigs using the CAP3 program at both high and low stringency levels. The steps followed while EST analyses [16] are depicted in Figure 1.

Primer designing

The Conserved Primer 2.0 pipeline was implemented and the command line made it possible to design intron-flanking primer pairs or marker candidates for polymorphism discovery in a high-throughput manner and to use any genome size of the model species and any number of the ESTs as inputs without memory and speed restrictions. Processed ESTs are input for designing primers in batch (Tables 1-3) and got custom synthesized.

DNA Isolation

Genomic DNA was isolated using the CTAB isolation method from young leaves collected from plants of 40 mungbean varieties. DNA quantity is estimated by Nanodrop method and quality by running 2uL of genomic DNA solution mixed with 1 uL loading buffer on a 1% agarose gel. The DNA was then diluted to a concentration of 10ng per uL for PCR amplification.

PCR Amplification and marker analysis

To test the feasibility of using *Vigna* COS markers in mungbean, genomic DNA fragments from 40 varieties were amplified using 14 COS markers. PCRs were conducted in a 25-ml reaction volume each reaction consisted of 10 mM Tris-HCl (pH 9.0 at room temperature), 1.5 mM $MgCl_2$, 100 mM each of dNTPs, 0.1 mM each primer, 10 ng of genomic DNA template, and 1unit of Taq DNA polymerase. Reactions were heated at 94^{0}C for 4 min followed by 35 cycles of 1 min at 94^{0}C, 1 min at specific annealing temperature for each primer pairs and a 1-min extension at 72^{0}C. Final reactions were extended at 72^{0}C for 5 min. Amplification was performed in a programmable thermal controller. Following the amplification reactions, the PCR products were separated on 1.8% agarose gel and visualized using ethedium bromide staining (Figure 2). Successful amplification of COS markers are mentioned in table 2.

Results and Discussion

Approximately 6443 ESTs were processed and analyzed for the searching of COS in *Vigna* genus for redundancy minimization and assembling of sequences. The non-redundant ESTs analysed (Figure 1) were used for the development of specific intron-based markers. Soybean genomic sequence database as reference is used to predict intron positions in the EST sequences and then designed a pair of primers flanking the intron position. The Multiple sequence alignments, with the Soybean genomic sequence inferred intron position, facilitate design of PCR primers that anneal to conserved exon sequences and amplify across more diverged introns. A query EST was considered to be homologous to a subject-coding sequence only if there were at least 100 bp overlapping and 80% similarity between them. Only high-quality ESTs are grouped into clusters based on sequence similarity and assembly of clusters which would represent a putative gene, likewise consensus sequences representing putative genes are generated for each *Vigna* species in the study. We found four contigs in *Vigna* radiata from 829 processed ESTs, 33

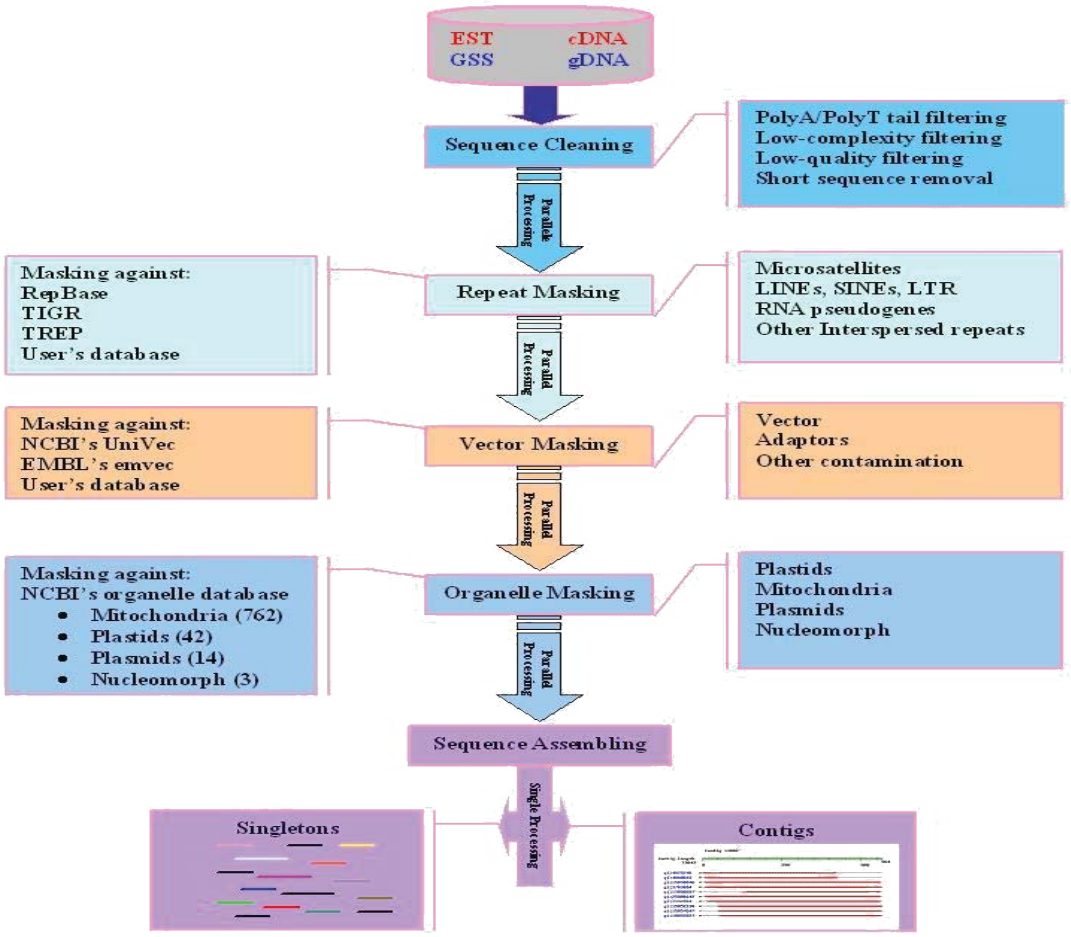

Figure 1: EST processing and Analysis.

Table 1: Statistics of EST analysis for *Vigna* spp.

Species	No. of ESTs	Contigs	No. of Singletons	Predicted genes from contigs	No. of conserved markers found from ESTs
V.radiata	829	4	807	3	2
V.mungo	299	33	198	14	2
V.umbellata	3006	268	1946	95	78
V.unguiculata	2309	175	403	168	134

Table 2: List of conserved primers designed from contigs obtained using available ESTs of *Vigna* species and used for amplification in *V.radiata* genotypes.

SN	SEQ. ID	OLIGO	FORWARD PRIMER	REVERSE PRIMER	REFRENCE	SIZE(bp)	REMARKS
1	XET	47-FR	ACAAACGGTTGCATTTCA	ATGTTCCTTCCCATTAGGAT	Soybean chr03	298	No amplification
2	CSD	48-FR	ACAAACGGTTGCATTTCA	ATGTTCCTTCCCATTAGGAT	Soybean chr08	725	Double bands
3	AGA	49-FR	TTGAATGGAATTGCTGAGA	GTGAATTGACTTCAGCACAA	Soybean chr05	255	Single band
4	SP	50-FR	ACATGCCTAAAGTTGGTTCT	ATTTGGCTTTCCCTCTTT	Soybean chr05	407	Single band
5	CGGCS	51-FR	TTGAATGGAATTGCTGAGA	AATCCAATTCCCATTTCC	Soybean chr08	560	Single band
6	PK	52-FR	TCTTGAAAGAAATGCAGCAC	AAACCAGATACAAACGCTTC	Soybean chr03	732	Single band
7	angularis_CA908726	53-FR	AAGCGATTGCTGAATTGGAT	TCGGTGCTGCTTCTTTAATTT	Soybean chr08	239	Single band
8	CA908739_angularis	54-FR	TTGCTGAATTGGATACATTGG	GCGGCTTCTTTAATTTCATCA	Soybean chr08	236	Double bands
9	DY637441_angularis	55-FR	TTGCTGGAAATGCTTTGTGA	AACGGCATCGCTAACATAAA	Soybean chr05	899	No amplification
10	AB037239_angularis	56-FR	TATGCCGAAGAAATGCAATG	TGTTGGGTGCCATATAATGAA	Soybean chr08	695	Single band
11	mungo.Contig12	57-FR	AAGTCATTTGCACCAGGAA	GAAATTTATCCAAGCGCACA	Soybean chr03	383	Single band
12	umbellata_contig10	58-FR	ATCTTCAATTGCCCTCCATT	TCTTCATGAATGCAAACCAG	Soybean chr08	202	Single band
13	umbellata_contig16	59-FR	TGGTGTTTGCAAGGGAATTT	TTCTTCCCAATAGCATCAACA	Soybean chr08	604	Double bands
14	umbellata_contig44	60-FR	ACCCAAACAATGAGAAAGCA	CACAATCCTTGACAAATCCAAA	Soybean chr05	466	Single band

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Table 3: List of Conserved markers identified in the study.

Index	Seq ID	Orientation	Len	Seq	Prod Size	Seq Length	Reference	E-value
							G.max	2e-25/2e-16/1e-
1	FJ896375.1	Fwd	18	ACAAACGGTTGCATTTCA	298	1065	chr03	24
2	FJ896375.1	Rev	20	ATGTTCCTTCCCATTAGGAT				
							G.max	4e-28/e-147/e-
3	FJ896374.1	Fwd	18	ACAAACGGTTGCATTTCA	298	1451	Chr08	162
4	FJ896374.1	Rev	20	ATGTTCCTTCCCATTAGGAT				
							G.max	8e-25/1e-60/2e-
5	EU570914.1	Fwd	18	TTCCATTTGTGCTCTTGA	725	4890	Chr05	45
6	EU570914.1	Rev	18	TTTGGGTGCAATTCTTCA				
							G.max	6e-23/7e-17/8e-
7	HQ999996.1	Fwd	19	TTGAATGGAATTGCTGAGA	255	1202	Chr05	25
8	HQ999996.1	Rev	20	GTGAATTGACTTCAGCACAA				
							G.max	8e-25/1e-60/5e-
9	HQ999996.1_1	Fwd	20	ACATGCCTAAAGTTGGTTCT	407	4584	Chr08	43
10	HQ999996.1_1	Rev	18	ATTTGGCTTTCCCTCTTT				
							G.max	1e-63/4e-47/5e-
11	HQ999996.1_2	Fwd	19	TTGAATGGAATTGCTGAGA	560	6330	Chr05	61
12	HQ999996.1_2	Rev	18	AATCCAATTCCCATTTCC				
							G.max	8e-60/7e-46/6e-
13	AA080680_1	Fwd	18	TTGAAAGAAATGCAGCAC	732	1926	Chr08	51
14	AA080680_1	Rev	20	AAACCAGATACAAACGCTTC				
							G.max	
15	radiata.Contig4_1	Fwd	21	AAATGGACAACCCATTTACCA	862	1133	Chr08	1e-14/4e-11
16	radiata.Contig4_1	Rev	21	CATTCAAATGCTGAGATCGAA				
							G.max	3e-42/2e-27/3e-
17	mungo.Contig12	Fwd	20	AAAGTCATTTGCACCAGGAA	383	525	Chr09	39
18	mungo.Contig12	Rev	20	GAAATTTATCCAAGCGCACA				
							G.max	3e-34/1e-18/2e-
19	umbellata.Contig10	Fwd	20	ATCTTCAATTGCCCTCCATT	202	619	Chr08	16
20	umbellata.Contig10	Rev	21	TCTTCATGAATGCAAACCAGA				
							G.max	5e-27/2e-66/3e-
21	umbellata. Contig10_1	Fwd	20	ATCTTCAATTGCCCTCCATT	202	621	Chr04	59
22	umbellata. Contig10_1	Rev	21	TCTTCATGAATGCAAACCAGA				
							G.max	
23	umbellata.Contig16	Fwd	20	TGGTGTTTGCAAGGGAATTT	604	868	Chr03	8e-32/9e-44
24	umbellata.Contig16	Rev	21	TTCTTCCCAATAGCATCAACA				
							G.max	
25	umbellata. Contig16_1	Fwd	20	TGGTGTTTGCAAGGGAATTT	522	781	Chr07	6e-11/2e-11
26	umbellata. Contig16_1	Rev	21	TTCTTCCCAATAGCATCAACA				
							G.max	8e-16/1e-36/2e-
27	umbellata.Contig29	Fwd	22	AAGCATCTGAAATGGCAGAATA	1056	1088	Chr07	16
28	umbellata.Contig29	Rev	20	ATGTAAGGGCGTCAAATCAA				
							G.max	
29	umbellata.Contig33	Fwd	20	TGTGTTGCTGACAAATCCAA	1038	1263	Chr04	6e-13/1e-84
30	umbellata.Contig33	Rev	22	TTCATCCAATGTATCTGGCAAT				
							G.max	
31	umbellata.Contig44	Fwd	20	ACCCAAACAATGAGAAAGCA	319	466	Chr04	4e-33/1e-73
32	umbellata.Contig44	Rev	22	CACAATCCTTGACAAATCCAAA				
							G.max	
33	umbellata. Contig44_1	Fwd	20	ACCCAAACAATGAGAAAGCA	325	472	Chr07	2e-50/3e-98
34	umbellata. Contig44_1	Rev	22	CACAATCCTTGACAAATCCAAA				

							G.max	
35	umbellata.Contig48	Fwd	21	TCAATTGCATTTGTGGAAGAA	371	420	Chr01	4e-13/5e-25
36	umbellata.Contig48	Rev	21	CGAATATATTGCCAACCCAAA				
							G.max	3e-20/7e-15/5e-
37	umbellata. Contig48_1	Fwd	21	TCAATTGCATTTGTGGAAGAA	352	406	Chr01	28
38	umbellata. Contig48_1	Rev	21	CGAATATATTGCCAACCCAAA				
							G.max	4e-13/3e-11/8e-
39	umbellata. Contig48_2	Fwd	21	TCAATTGCATTTGTGGAAGAA	351	415	Chr04	24
40	umbellata. Contig48_2	Rev	21	CGAATATATTGCCAACCCAAA				
							G.max	
41	umbellata. Contig48_3	Fwd	21	TCAATTGCATTTGTGGAAGAA	351	407	Chr02	2e-12/3e-23
42	umbellata. Contig48_3	Rev	21	CGAATATATTGCCAACCCAAA				
							G.max	
43	umbellata. Contig48_5	Fwd	21	TCAATTGCATTTGTGGAAGAA	367	416	Chr08	8e-13/1e-24
44	umbellata. Contig48_5	Rev	21	CGAATATATTGCCAACCCAAA				
							G.max	
45	umbellata.Contig70	Fwd	21	AATGTTCCCGAAGATGACATT	670	844	Chr03	2e-19/3e-43
46	umbellata.Contig70	Rev	22	TTGTTCTTGGACAAGCATGTTA				
							G.max	
47	umbellata.Contig86	Fwd	20	ATGTTGGCATTCTTGGGATT	213	862	Chr04	2e-75/2e-87
48	umbellata.Contig86	Rev	22	TCATGCCCAAATCTATCTCAAA				
							G.max	
49	umbellata. Contig86_1	Fwd	20	ATGTTGGCATTCTTGGGATT	213	827	Chr09	7e-39/8e-11
50	umbellata. Contig86_1	Rev	22	TCATGCCCAAATCTATCTCAAA				
							G.max	5e-32/4e-23/1e-
51	umbellata.Contig89	Fwd	20	TTTGACGGCATTCAAAGTGA	221	293	Chr08	51
52	umbellata.Contig89	Rev	20	TCACCAACATTTCCAACGAT				
							G.max	
53	umbellata.Contig91	Fwd	22	TTCAATTGGTTTAATGCCAGAA	1008	1083	Chr03	7e-16/8e-34
54	umbellata.Contig91	Rev	22	TTCCACCATCATCAAATAATGC				
							G.max	
55	umbellata.Contig94	Fwd	22	TTTGTTTATGTTGTTGCTCGAA	436	596	Chr09	3e-20/3e-42
56	umbellata.Contig94	Rev	22	TTCAATATTTGCAGCTTCCTTG				
							G.max	1e-20/2e-34/2e-
57	umbellata. Contig94_1	Fwd	22	TTTGTTTATGTTGTTGCTCGAA	428	513	Chr03	43
58	umbellata. Contig94_1	Rev	22	TTCAATATTTGCAGCTTCCTTG				
59	umbellata. Contig110	Fwd	20	TCCAATCGGAAAGTGAACAA	878	977	*G.max*	2e-19/4e-17/1e-
							Chr01	38
60	umbellata. Contig110	Rev	21	TTCTCCATAAACGGAACCAAA				
							G.max	
61	umbellata. Contig110_1	Fwd	20	TCCAATCGGAAAGTGAACAA	805	904	Chr05	1e-36/5e-11
62	umbellata. Contig110_1	Rev	21	TTCTCCATAAACGGAACCAAA				
							G.max	
63	umbellata. Contig117	Fwd	20	ATTTGCGATATGGTGCGATT	446	662	Chr05	e-114/1e-18
64	umbellata. Contig117	Rev	20	TGCACCAATCAAACGTGAAA				

							G.max	
65	umbellata. Contig117_1	Fwd	20	ATTTGCGATATGGTGCGATT	446	645	Chr04	1e-61/4e-15
66	umbellata. Contig117_1	Rev	20	TGCACCAATCAAACGTGAAA				
							G.max	e-102/2e-97/e-
67	umbellata. Contig121	Fwd	20	AAAGACCATTTGCTGCCATT	261	998	Chr05	101
68	umbellata. Contig121	Rev	21	CAAATTTGTCTGCAATCACCA				
							G.max	e-110/2e-91/e-
69	umbellata. Contig122	Fwd	22	TTTCTTGTCCAATCCATAAGCA	739	913	Chr05	101
70	umbellata. Contig122	Rev	21	TCATTGGGATTGATCTTGGAA				
							G.max	e-107/1e-86/4e-
71	umbellata. Contig122_1	Fwd	22	TTTCTTGTCCAATCCATAAGCA	735	1992	Chr08	99
72	umbellata. Contig122_1	Rev	21	TCATTGGGATTGATCTTGGAA				
							G.max	e-102/2e-97/e-
73	umbellata. Contig122_2	Fwd	22	TTTCTTGTCCAATCCATAAGCA	740	1927	Chr08	103
74	umbellata. Contig122_2	Rev	21	TCATTGGGATTGATCTTGGAA				
							G.max	
75	umbellata. Contig122_3	Fwd	22	TTTCTTGTCCAATCCATAAGCA	710	887	Chr07	e-115/1e-18
76	umbellata. Contig122_3	Rev	21	TCATTGGGATTGATCTTGGAA				
							G.max	
77	umbellata. Contig125	Fwd	21	TTTCAATGGCCTTGATTTCAG	341	489	Chr04	1e-21/3e-22
78	umbellata. Contig125	Rev	20	TCGGGATAATTCTGCATTTG				
							G.max	8e-13/4e-27/6e-
79	umbellata. Contig127	Fwd	21	TTCATCATTCATTCTCCTCCA	204	866	Chr08	23
80	umbellata. Contig127	Rev	22	TGTGCACAATTTCTCTGTTTGT				
							G.max	
81	umbellata. Contig130	Fwd	20	TTGCAACGATGAAGAAAGGT	753	2237	Chr08	1e-40/4e-16
82	umbellata. Contig130	Rev	22	TCCAATGATACATTTGGAGGAA				
							G.max	6e-12/8e-36/1e-
83	umbellata. Contig130_2	Fwd	20	TTGCAACGATGAAGAAAGGT	740	886	Chr05	13
84	umbellata. Contig130_2	Rev	22	TCCAATGATACATTTGGAGGAA				
							G.max	
85	umbellata. Contig137	Fwd	20	TCCAGGCATTCTTTGTGAAA	498	969	Chr01	2e-14/e-107
86	umbellata. Contig137	Rev	21	TTTGGATTCACACCATGAACA				
							G.max	4e-14/2e-19/2e-
87	umbellata. Contig137_1	Fwd	21	TGCAATGTTCATGGTGTGAAT	203	762	Chr03	12
88	umbellata. Contig137_1	Rev	22	TTTCCAAACACATCCAACTTGA				
							G.max	1e-44/1e-20/2e-
89	umbellata. Contig140	Fwd	20	AAACCACCAATGTTCCACAA	329	2108	Chr07	34
90	umbellata. Contig140	Rev	21	TTTCAATGGAGGCTTTCTTCA				

							G.max	
91	umbellata. Contig148	Fwd	20	CATTTCATCGAACAGTGCAA	414	2945	Chr02	9e-46/2e-28
92	umbellata. Contig148	Rev	22	AGCTCAACATCGGATTCAATTA				
							G.max	
93	umbellata. Contig152	Fwd	22	TGGAACCTTTGATGTTTCCATA	848	1195	Chr02	3e-28/4e-43
94	umbellata. Contig152	Rev	20	TCACATCCAACAGCAACAAA				
							G.max	
95	umbellata. Contig171	Fwd	21	TCCATGTGATTGCTTGTTTGA	430	810	Chr05	3e-44/2e-14
96	umbellata. Contig171	Rev	20	TTCATCGGTTCTTGGAGAAA				
							G.max	8e-38/e-119/1e-
97	unguiculata. Contig8	Fwd	21	TTAATCCCAAGGCCAAATCTT	820	1561	Chr06	67
98	unguiculata. Contig8	Rev	22	GGATGAGAATCATTCCAACAAA				
							G.max	
99	unguiculata. Contig8_1	Fwd	21	TTAATCCCAAGGCCAAATCTT	1117	1827	Chr01	4e-43/4e-15
100	unguiculata. Contig8_1	Rev	22	GGATGAGAATCATTCCAACAAA				
							G.max	
101	unguiculata. Contig8_2	Fwd	21	TTAATCCCAAGGCCAAATCTT	1081	1401	Chr02	4e-18/3e-22
102	unguiculata. Contig8_2	Rev	22	GGATGAGAATCATTCCAACAAA				
							G.max	
103	unguiculata. Contig9	Fwd	22	TTCCTGGATCGTCAAATTTCTT	1163	1250	Chr03	8e-63/4e-15
104	unguiculata. Contig9	Rev	20	AAACCATGTTTCGTCAACCA				
							G.max	
105	unguiculata. Contig13	Fwd	21	GCTTTGGTTTGAGGAATTTCA	467	1435	Chr04	2e-42/2e-54
106	unguiculata. Contig13	Rev	22	TGAAGAAATGGATTCATTGTGG				
							G.max	3e-22/2e-36/3e-
107	unguiculata. Contig14	Fwd	21	TTTCACTGCCAAGAAACTTGA	640	999	Chr06	13
108	unguiculata. Contig14	Rev	21	CAAGAAACAACCAACACGAAA				
							G.max	
109	unguiculata. Contig14_2	Fwd	21	TTTCACTGCCAAGAAACTTGA	1463	2134	Chr01	3e-22/8e-20
110	unguiculata. Contig14_2	Rev	22	CCAATATCCTTCAAAGCACAAA				
							G.max	
111	unguiculata. Contig14_3	Fwd	21	TTTCACTGCCAAGAAACTTGA	1080	1153	Chr02	2e-17/1e-15
112	unguiculata. Contig14_3	Rev	22	CCAATATCCTTCAAAGCACAAA				
							G.max	6e-67/2e-30/3e-
113	unguiculata. Contig16_1	Fwd	21	AAATTGGTGCGAGATTTCAAG	257	764	Chr04	16
114	unguiculata. Contig16_1	Rev	20	TTTCGGATTTCGATGGATTT				
							G.max	5e-58/2e-23/8e-
115	unguiculata. Contig16_2	Fwd	21	TCTTCCGAAACGATGAACATT	338	796	Chr06	20
116	unguiculata. Contig16_2	Rev	20	TTTCGGATTTCGATGGATTT				

							G.max	1e-46/9e-26/3e-
117	unguiculata. Contig16_3	Fwd	21	TCTTCCGAAACGATGAACATT	338	783	Chr06	22
118	unguiculata. Contig16_3	Rev	20	TTTCGGATTTCGATGGATTT				
							G.max	5e-49/9e-26/8e-
119	unguiculata. Contig16_4	Fwd	21	AAATTGGTGCGAGATTTCAAG	257	758	Chr06	20
120	unguiculata. Contig16_4	Rev	20	TTTCGGATTTCGATGGATTT				
							G.max	
121	unguiculata. Contig16_5	Fwd	21	AAATTGGTGCGAGATTTCAAG	257	756	Chr07	2e-11/5e-21
122	unguiculata. Contig16_5	Rev	20	TTTCGGATTTCGATGGATTT				
							G.max	4e-65/2e-70/5e-
123	unguiculata. Contig19	Fwd	20	CATGCCATGGAAATCATTCA	1500	1843	Chr02	15
124	unguiculata. Contig19	Rev	20	ATCAGCCAAACATTCAGCAA				
							G.max	
125	unguiculata. Contig19_1	Fwd	22	AAGACAATTGCTGAATGTTTGG	200	2037	Chr04	6e-67/4e-31
126	unguiculata. Contig19_1	Rev	21	CCTTGGCAACTCTTTCAATTT				
							G.max	
127	unguiculata. Contig24	Fwd	20	TCTTTGCCATTCACATGCTT	234	1603	Chr08	2e-14/8e-14
128	unguiculata. Contig24	Rev	20	TGTCGGATTTGATTGCTTGA				
							G.max	5e-53/1e-84/e-
129	unguiculata. Contig24_1	Fwd	20	TCAAGCAATCAAATCCGACA	1224	1247	Chr09	139
130	unguiculata. Contig24_1	Rev	22	CAATTTCAATCTTGGCATTCAA				
							G.max	
131	unguiculata. Contig25	Fwd	21	TGAATTTGCCTCCACTTTCAT	1460	1795	Chr02	4e-31/0.0
132	unguiculata. Contig25	Rev	20	TTCAATTGGGATCACAGCAT				
							G.max	
133	unguiculata. Contig33	Fwd	21	TCGTGGCAAACTTATGTTGAT	1478	1728	Chr08	9e-26/4e-65
134	unguiculata. Contig33	Rev	21	CATCATAAATGCCAATGACCA				
							G.max	6e-30/2e-29/e-
135	unguiculata. Contig33_1	Fwd	21	TCGTGGCAAACTTATGTTGAT	1145	1285	Chr05	103
136	unguiculata. Contig33_1	Rev	20	TTGTGCAGAAGCCAATGAAA				
							G.max	1e-24/1e-43/1e-
137	unguiculata. Contig33_2	Fwd	21	TGGTCATTGGCATTTATGATG	252	1811	Chr08	77
138	unguiculata. Contig33_2	Rev	22	CAAGCATCAAACACTTTCTTCA				
							G.max	
139	unguiculata. Contig37	Fwd	20	AATCGGCTCAAAGGTGAAAT	1097	1630	Chr07	2e-11/2e-11
140	unguiculata. Contig37	Rev	20	TTGCTTCCTGAAACGAAATG				
							G.max	1e-77/2e-23/2e-
141	unguiculata. Contig47	Fwd	20	AGCATTGCCAACGACAATAA	265	780	Chr06	17
142	unguiculata. Contig47	Rev	20	TTCGATATCGTTTCCATCCA				

							G.max	4e-80/2e-23/8e-
143	unguiculata. Contig47_1	Fwd	20	AGCATTGCCAACGACAATAA	267	782	Chr06	20
144	unguiculata. Contig47_1	Rev	20	TTCGATATCGTTTCCATCCA				
							G.max	
145	unguiculata. Contig47_2	Fwd	20	AGCATTGCCAACGACAATAA	267	780	Chr04	4e-80/2e-23
146	unguiculata. Contig47_2	Rev	20	TTCGATATCGTTTCCATCCA				
							G.max	
147	unguiculata. Contig47_3	Fwd	20	AGCATTGCCAACGACAATAA	270	624	Chr07	2e-14/2e-11
148	unguiculata. Contig47_3	Rev	20	TTCGATATCGTTTCCATCCA				
							G.max	
149	unguiculata. Contig47_4	Fwd	20	AGCATTGCCAACGACAATAA	246	324	Chr08	6e-48/2e-42
150	unguiculata. Contig47_4	Rev	20	TTCGATATCGTTTCCATCCA				
151	unguiculata. Contig55	Fwd	20	AAATTGCATCATGCTCGTGT	1236	1279	*G.max*	5e-86/7e-82
							Chr04	
152	unguiculata. Contig55	Rev	22	TGATCTATAAATGGCAGCAACA				
							G.max	
153	unguiculata. Contig56	Fwd	20	TTTCAGCGAATTGTTGGAGT	1257	1544	Chr02	0.0/1e-24
154	unguiculata. Contig56	Rev	20	TTTGCTTCTTTCGATTCCTG				
							G.max	1e-31/1e-18/4e-
155	unguiculata. Contig63	Fwd	21	AAGGATGCCAAGAAGAAGAAA	289	1706	Chr08	16
156	unguiculata. Contig63	Rev	21	TCATTCCTGCCTTCAAAGAAA				
							G.max	4e-34/4e-25/6e-
157	unguiculata. Contig63_2	Fwd	21	AAGGATGCCAAGAAGAAGAAA	284	2261	Chr05	18
158	unguiculata. Contig63_2	Rev	21	TCATTCCTGCCTTCAAAGAAA				
							G.max	2e-39/9e-17/1e-
159	unguiculata. Contig63_3	Fwd	21	AAGGATGCCAAGAAGAAGAAA	289	1409	Chr05	15
160	unguiculata. Contig63_3	Rev	21	TCATTCCTGCCTTCAAAGAAA				
							G.max	1e-24/9e-26/5e-
161	unguiculata. Contig63_4	Fwd	21	AAGGATGCCAAGAAGAAGAAA	287	1242	Chr04	18
162	unguiculata. Contig63_4	Rev	21	TCATTCCTGCCTTCAAAGAAA				
							G.max	
163	unguiculata. Contig70	Fwd	20	TTCAAGGCAGGCAAATACAA	402	1776	Chr01	4e-22/e-132
164	unguiculata. Contig70	Rev	22	CAAAGCAACAGCATTGTCATAA				
							G.max	e-135/3e-13/1e-
165	unguiculata. Contig70_1	Fwd	20	TTCAAGGCAGGCAAATACAA	402	1304	Chr03	21
166	unguiculata. Contig70_1	Rev	22	CAAAGCAACAGCATTGTCATAA				
							G.max	
167	unguiculata. Contig72	Fwd	22	AAAGCCATGTTCTTCATCTTGA	298	598	Chr09	6e-39/1e-18
168	unguiculata. Contig72	Rev	20	TCCAAGCAAAGTCCCAATTT				

							G.max	2e-66/3e-16/5e-
169	unguiculata. Contig87	Fwd	22	TTTATTCGAGGAAGCAAAGTCA	255	2168	Chr05	27
170	unguiculata. Contig87	Rev	20	TGCATCCAAACGCTTAAACA				
							G.max	e-157/6e-19/6e-
171	unguiculata. Contig87_1	Fwd	22	TTTATTCGAGGAAGCAAAGTCA	254	1666	Chr07	28
172	unguiculata. Contig87_1	Rev	20	TGCATCCAAACGCTTAAACA				
							G.max	e-157/4e-26/2e-
173	unguiculata. Contig89	Fwd	21	AAAGTGCTTTGGGTTTGTGAA	1361	2190	Chr02	25
174	unguiculata. Contig89	Rev	20	CAAAGAAATTTGGCACAGGA				
							G.max	4e-37/e-114/5e-
175	unguiculata. Contig89_1	Fwd	21	AAAGTGCTTTGGGTTTGTGAA	1327	2157	Chr04	55
176	unguiculata. Contig89_1	Rev	20	CAAAGAAATTTGGCACAGGA				
							G.max	6e-27/e-106/3e-
177	unguiculata. Contig90	Fwd	20	ATTTACAAAGCCGTCCATGA	726	1547	Chr06	50
178	unguiculata. Contig90	Rev	20	TTCAAACCGACCAATGTGTT				
							G.max	
179	unguiculata. Contig90_1	Fwd	20	ATTTACAAAGCCGTCCATGA	711	1504	Chr09	1e-24/0.0
180	unguiculata. Contig90_1	Rev	20	TTCAAACCGACCAATGTGTT				
							G.max	
181	unguiculata. Contig91	Fwd	21	TTTGAAATGGCTTTGTCACTG	830	1151	Chr02	e-108/e-132
182	unguiculata. Contig91	Rev	22	TTTCAAGATCTTTGCACTGGTT				
							G.max	7e-39/2e-48/1e-
183	unguiculata. Contig98	Fwd	20	TTCCATTGTTCGTTTGACCA	439	1941	Chr05	58
184	unguiculata. Contig98	Rev	22	TGGTTTGTTGGCTTACCATAAA				
							G.max	5e-86/6e-30/5e-
185	unguiculata. Contig98_1	Fwd	20	TTCCATTGTTCGTTTGACCA	1134	1675	Chr02	61
186	unguiculata. Contig98_1	Rev	22	TGGTTTGTTGGCTTACCATAAA				
							G.max	
187	unguiculata. Contig105_1	Fwd	22	ATGAAATTCAAGGATGGGTACA	278	478	Chr07	3e-13/1e-15
188	unguiculata. Contig105_1	Rev	20	TTTAGGATCCCAATCAAGCA				
							G.max	2e-76/5e-21/2e-
189	unguiculata. Contig113	Fwd	20	AGCATTGCCAACGACAATAA	266	780	Chr06	17
190	unguiculata. Contig113	Rev	22	GTTCGATATCATTTCCATCCAA				
							G.max	7e-79/5e-21/8e-
191	unguiculata. Contig113_1	Fwd	20	AGCATTGCCAACGACAATAA	268	760	Chr06	20
192	unguiculata. Contig113_1	Rev	22	GTTCGATATCATTTCCATCCAA				
							G.max	
193	unguiculata. Contig113_2	Fwd	20	AGCATTGCCAACGACAATAA	268	758	Chr04	1e-37/5e-12
194	unguiculata. Contig113_2	Rev	22	GTTCGATATCATTTCCATCCAA				

							G.max	4e-46/3e-22/6e-
195	unguiculata. Contig113_4	Fwd	20	AGCATTGCCAACGACAATAA	271	624	Chr05	36
196	unguiculata. Contig113_4	Rev	22	GTTCGATATCATTTCCATCCAA				
							G.max	2e-14/e-108/2e-
197	unguiculata. Contig121	Fwd	20	TTTGCGAAGAAGGACTTCAA	201	1532	Chr05	35
198	unguiculata. Contig121	Rev	20	AGGAACAATTTCGGAAGGAA				
							G.max	
199	unguiculata. Contig121_1	Fwd	20	TTTGCGAAGAAGGACTTCAA	201	1783	Chr01	2e-14/5e-70
200	unguiculata. Contig121_1	Rev	20	AGGAACAATTTCGGAAGGAA				
							G.max	
201	unguiculata. Contig121_2	Fwd	20	TTTGCGAAGAAGGACTTCAA	201	397	Chr07	2e-22/6e-23
202	unguiculata. Contig121_2	Rev	20	AGGAACAATTTCGGAAGGAA				
							G.max	5e-27/1e-49/3e-
203	unguiculata. Contig126	Fwd	20	TTTGGGTTTGAGATTCCTGA	253	1596	Chr03 (	53
204	unguiculata. Contig126	Rev	20	TGAAGTTCGGTTATGCCAAT				
							G.max	3e-47/7e-48/e-
205	unguiculata. Contig138	Fwd	22	AATGAAGTCAACGAAATCTCCA	1253	1585	Chr03	144
206	unguiculata. Contig138	Rev	22	ATTCTCCTCATCCAAAGGATTT				
							G.max	1e-31/2e-33/e-
207	unguiculata. Contig138_1	Fwd	22	AATGAAGTCAACGAAATCTCCA	1039	1765	Chr02	116
208	unguiculata. Contig138_1	Rev	22	ATTCTCCTCATCCAAAGGATTT				
							G.max	9e-23/4e-34/e-
209	unguiculata. Contig138_2	Fwd	22	AATGAAGTCAACGAAATCTCCA	1047	1381	Chr02	135
210	unguiculata. Contig138_2	Rev	22	ATTCTCCTCATCCAAAGGATTT				
							G.max	9e-17/e-123/e-
211	unguiculata. Contig138_3	Fwd	22	AATGAAGTCAACGAAATCTCCA	883	1218	Chr07	111
212	unguiculata. Contig138_3	Rev	22	ATTCTCCTCATCCAAAGGATTT				
							G.max	
213	unguiculata. Contig142	Fwd	20	AAACGGCTTCAAACATTGGT	874	956	Chr02	6e-14/1e-33
214	unguiculata. Contig142	Rev	21	CCATAATCATTTGGGTTTCCA				
							G.max	1e-58/4e-43/2e-
215	unguiculata. Contig167	Fwd	20	TTTGGTTTGCCACTTCGTAA	255	1393	Chr05	42
216	unguiculata. Contig167	Rev	20	CAATGCCCGATTAATCTCAA				
							G.max	
217	unguiculata. Contig167_1	Fwd	20	TTTGGTTTGCCACTTCGTAA	250	1531	Chr04	3e-25/2e-11
218	unguiculata. Contig167_1	Rev	20	CAATGCCCGATTAATCTCAA				
							G.max	2e-23/e-114/2e-
219	unguiculata. Contig170	Fwd	21	TTAATCCCAAGGCCAAATCTT	819	1530	Chr06	51
220	unguiculata. Contig170	Rev	22	GATGAGAATCATTCCAACGAAA				

							G.max	
221	unguiculata. Contig170_1	Fwd	21	TTAATCCCAAGGCCAAATCTT	1116	1791	Chr01	5e-40/9e-20
222	unguiculata. Contig170_1	Rev	22	GATGAGAATCATTCCAACGAAA				
							G.max	
223	unguiculata. Contig170_2	Fwd	21	TTAATCCCAAGGCCAAATCTT	1080	1392	Chr02	3e-29/6e-18
224	unguiculata. Contig170_2	Rev	22	GATGAGAATCATTCCAACGAAA				
							G.max	
225	unguiculata. Contig175	Fwd	20	ATGCAGGTCACAATTGCTTT	323	685	Chr05	3e-19/1e-30
226	unguiculata. Contig175	Rev	22	TTGACTATTGATGGTTGGGTTT				
							G.max	
227	unguiculata. Contig175_1	Fwd	20	ATGCAGGTCACAATTGCTTT	911	1114	Chr04	2e-69/3e-40
228	unguiculata. Contig175_1	Rev	22	TTGACTATTGATGGTTGGGTTT				
							G.max	
229	unguiculata. Contig175_2	Fwd	20	ATGCAGGTCACAATTGCTTT	490	854	Chr04	2e-69/3e-40
230	unguiculata. Contig175_2	Rev	22	TTGACTATTGATGGTTGGGTTT				

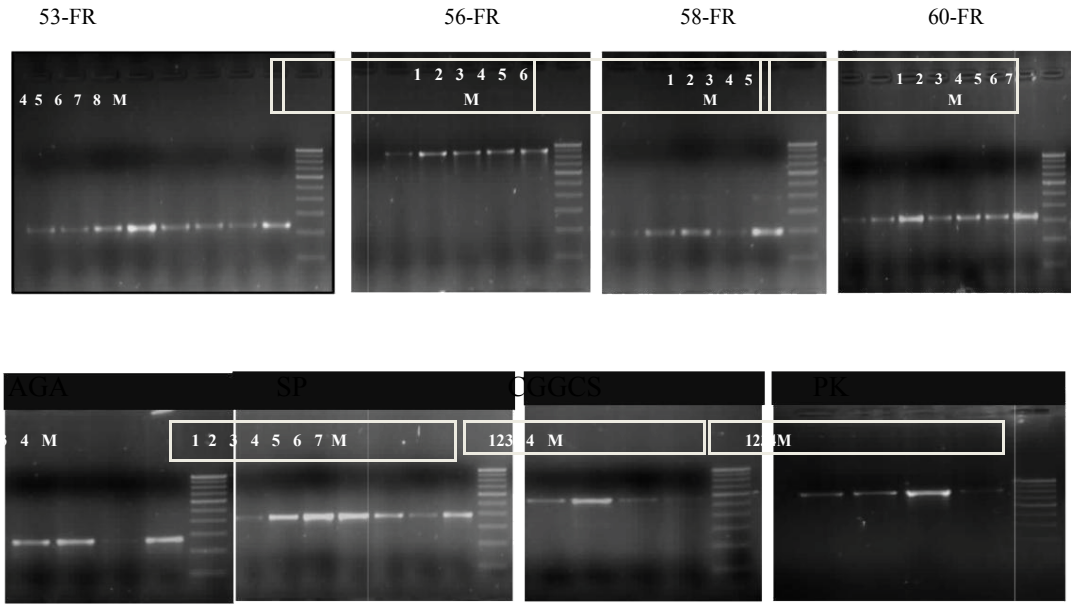

Figure 2: Representative patterns of DNA Amplification of single copy genes in *V.radiata*.

contigs for *V. mungo* from 299 processed ESTs, 268 contigs out of 3006 ESTs of *V. umbellata,* and 175 contigs from 2309 ESTs belonging to *V. unguiculata* (Table 1). These high-quality ESTs are grouped into 'clusters' based on sequence similarity. The maximum informative consensus sequences generated by assembling these clusters represent a putative gene. The output of all *Vigna* EST processing pipeline is the list of putative genes belonging to respective *Vigna* species providing list of 230 conserved primers. Of these, 14 markers were used to validate the COS markers generated with amplification of genomic DNA from 40 genotypes of *Vigna radiata* in Indian collections.

The successful amplification revealed the mixed type of result such as single band and multiple bands. To confirm the amplification, repeated reactions were carried for multiple/double banded amplicons and there was no change in such reactions even after changing the required components and thermal cycling conditions of PCR reaction. Three primer pairs (CSD-48FR, CA908739-54FR and umbellata_contig16-59FR) produced double or multiple bands which indicates that they probably multi gene loci and hence were not considered. Primer–genotype combinations that gave what appeared to be single bands on agarose gels were noted which are likely to be noted as single copy genes are considered as orthologs. Primers that produced single bands with genotypes of *V. radiata* varieties were 53-FR, 56-FR, 58-FR, 60-FR, AGA, SP, CGGCS and PK (Figure 2). Of these, the primer pair 57-FR was derived from contig derived from

ESTs belonging to *Vigna mungo*, whereas 58-FR and 60-FR were from contig of *V. umbellata*. This infers that the there is a possibility of these kind of markers derived from available genomic information (ESTs) of related species can amplify in *V.radiata.* Finally we have found that amplification of single copy genes attained were from 11 primer pairs (Figure 2).

Molecular (COS) markers in comparative genomic studies within *Vigna*

Though much progress has been made in the genomics of *Vigna* species, yet it is still far behind that in other grain legumes like common bean and soybean. Most of the cultivated *Vigna* species have a narrow genetic base resulting in limited marker polymorphism within the germplasm. Due to this major limitation, most of the genetic linkage maps in *Vigna* species have been constructed using inter-specific or inter-sub specific crosses to increase the level of polymorphism. The use of COS markers as a starting point for marker development was motivated by their expected low copy number in the genomes of various legume species for which genomic information is not abundant. A major challenge for comparative legume genomics is to translate information gained from model species into improvements in crop legumes. The complexity of that challenge may well be defined by the structural and functional similarities and dissimilarities among these very fascinating genomes. Agriculturally important traits captured during domestication are often coded by very limited number of loci with major phenotypic effects. It is common to find that these loci have putative orthologous counterparts (Orthologous Set markers) in other species and therefore such molecular markers are powerful in comparing genomic information across species.

The present study aimed at identifying the COS markers through computational approach and validating some of them in wet-lab screening lead us to use of this new ortholog resource can shed light on issues related to comparative genomics, molecular systematics, and gene evolution studies in the *Vigna* genus. COS markers thus selected can further be taken for characterization to test their applicability for phylogenetic studies in Asiatic *Vigna* species as COS markers are evolutionary conserved single-copy genes of great use in constructing syntenic genetic maps among species. The COS markers reported here will be useful for comparative mapping at the family level and may help to establish the syntenic relationship between genomes of different *Vigna* species, allowing a picture of chromosome evolution. In this study, a set of 230 COS markers were identified using ESTs of four *Vigna* species and some amplified in *V.radiata* can serve as anchor markers for a syntenic map of other *Vigna* species. This study forms the basis for a number of significant outcome for genomics of *Vigna* in general: (1) the genic markers developed here may be used across *Vigna* species to determine patterns of chromosomal evolution, as argued previously for markers with defined utility [17], and to characterize syntenic relationships between V. radiata and other related species under cultivation; (2) with the aid of shared anchor markers, the *Vigna* map created may be integrated with all existing legume maps containing various important domestication traits; (3) the high levels of syntenic relationships if detected between these species will enable the future identification of tightly linked markers for direct marker-assisted trait selection and future map-based isolation of candidate genes.

COS markers conserved in legume species can be used in phylogenetic studies and in the identification of conserved non-coding regions or to make comparative maps of major crop species. Although the amount of genetic data available for plants is increasing exponentially, most of the work is being done in just a few species. The identification of a set of such markers common to a variety of species within genus would allow researchers studying less well-characterized plant species to take advantage of gains being made in legume species such as Medicago, soybean and other model or reference plant species. Because ESTs give an idea of the genes expressed in an organism, and because EST data are abundant for a variety of species, ESTs are ideal starting material for the identification of genes conserved among species.

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