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ACKNOWLEDGEMENTS. We thank Prof. M. S. Swaminathan, Prof. P. C. Kesavan and S. Arunachalam of M. S. Swaminathan Research Foundation for encouragement and support. Financial support provided by the India–Canada Environment Facility, New Delhi and Canadian International Development Agency and Ministry of Environment and Forests, Government of India is acknowledged. We thank the Tamil Nadu Forest Department for permitting us to undertake this study.

Received 26 December 2002; revised accepted 26 June 2003

Intraspecific variation in the internal transcribed spacer region of rDNA in black gram (*Vigna mungo* (L.) Hepper)

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rDNA internal transcribed spacer (ITS) region from *V. mungo* var *silvestris*, *Vigna trilobata*, *Vigna glabrescens* and diverse cultivars of *Vigna mungo*, were amplified and digested with twelve restriction enzymes. There was no size variation in the ITS region of the diverse cultivars and other *Vigna* species studied. The approximate length of the amplified product of the entire rDNA ITS region was found to be 650 bp. ITS1 consisted of 250 bp and ITS2 was 300 bp long. Restriction fragment length polymorphisms within species could not be detected in cultivated accessions of *V. mungo* for all the restriction enzymes tested whereas interspecific variation was found among *V. mungo* var *silvestris*, *V. trilobata* and *V. glabrescens*. Of the eleven restriction enzymes (*EcoRI*, *HindIII*, *PstI*, *SmaI*, *Sau3AI*, *TaqI*, *SacI*, *MspI*, *AluI*, *BamHI* and *HaeIII*) tested, seven endonucleases (*Sau3AI*, *TaqI*, *SacI*, *MspI*, *AluI*, *BamHI* and *HaeIII*) had restriction site in the ITS region, of which five were in ITS1 and two in ITS2 of cultivated species. Presence of *BamHI* restriction site which is unique to *Vigna* was not found to be methylated. The ITS2 of *V. glabrescens* and *V. trilobata* had restriction sites for *Sau3AI* and *AluI*, which are not found in *V. mungo* var *silvestris* and the cultivated varieties studied. *MspI* enzyme had restriction site specifically present in ITS2

of *V. trilobata*. No intraspecific variation was observed among widely distributed Indian cultivars of *V. mungo* and *V. mungo* var *silvestris*.

BLACK gram belongs to the subgenus *Ceratotropis* of the genus *Vigna*. The genus *Vigna* comprises eight subgenera and seven cultivated species, two of which are of African origin (subgenus *Vigna*) and five are Asiatic (subgenus *Ceratotropis*). The African group consists of cowpea (*V. unguiculata* (L.) Walp) and Bambara groundnut (*V. subterranea* (L.) Verdc.). The Asiatic group comprises green gram/mung bean (*V. radiata* (L.) Wilczek), black gram/urdbean (*V. mungo* (L.) Hepper), moth bean (*V. aconitifolia* (Jacq.) Marechal), adzuki bean (*V. angularis* (Willd)) and rice bean (*V. umbellata* (Thunb)). Black gram is considered to have been domesticated in India from its wild ancestral form *V. mungo* var *silvestris*¹. Recognition and exploitations of variations among genetically divergent groups of germplasm are fundamental in breeding and genetic engineering. Seed proteins have been used to find homology among the *Vigna* species from Asian and African origin². Intra and interspecific variations were studied in genus *Vigna* by RFLP³ and in subgenus *Ceratotropis* by RAPD⁴. Inter simple sequence repeat DNA polymorphism was used to distinguish taxa within the genus *Vigna*⁵.

Internal transcribed spacers are sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 25S rRNA coding regions (ITS2). Studies of restriction site variation in the ribosomal DNA (rDNA) in populations of animals and plants have shown that while coding regions are conserved, the spacer regions are variable⁶. These spacer sequences have a high evolution rate and are present in all known nuclear rRNA genes of eukaryotes^{7,8}. They are useful for phylogenetic analysis among related species and/or among populations within a species⁹.

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Population differences in the length of the ITS have been found in a variety of plants^{10,11}. Since ITS are flanked by highly conserved coding regions, primers can be designed for the amplification of the first internal transcribed spacer region (ITS1) and the second internal transcribed spacer region (ITS2) by known sequences from *Glycine*¹². Although, ITS1 sequences from different families are virtually too diverse to unambiguously align, Jin-Shou Liu and Schardl¹³ reported the presence of conserved sequences in ITS1 region of many flowering plant species.

In *Vigna*, it will be interesting to compare the restriction digestion pattern of rDNA ITS region and to study their evolutionary changes during the selection of cultivars and evolution in the genus. Variation in the length of 5S and 18-25S ribosomal RNA unit has been used as a taxonomic tool in soybean⁹, *Lens* and *Cicer*¹⁴, pigeonpea¹⁵ and in *Vigna* species¹⁶. We report here the rDNA ITS variation patterns within diverse cultivars of *Vigna mungo*, *V. mungo* var *silvestris*, *V. trilobata* and *V. glabrescens*.

Accessions used in this study are described in Table 1. Genomic DNA was isolated from young leaves using the method of Dellaporta *et al.*¹⁷. DNA was quantified using DyNA Quant Fluorometer (Amersham Pharmacia Biotech, UK, Limited).

The primers used for amplifying the region containing the ITS1 and ITS2 were designed from the conserved sequences previously published for soybean and mungbean^{18,19}. The primer sequences were as follows: ITS1F (5'-AAGTCGTAACAAGGTTTCCGTAG-3'), ITS1R (5'-AAAGACTCGATGGTTCACG-3'), ITS2F (5'-TAGCG-

AAATGCGATA CTTGGT-3') and ITS2R (5'-GTTAGT-TTCTTTTCCTCC-3'). The primers were synthesized by Board of Radiation and Isotope Technology, Mumbai, India. ITS1F and ITS2R primers were used to amplify the entire ITS region. Amplifications were done in 30 µl reaction volume containing 1X *Taq* polymerase assay buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM MgCl₂ and 0.01% gelatin), 0.1 mM of each dNTP, 0.5 µM of both forward and reverse primer, 0.5 units of *Taq* polymerase (Bangalore Genei Pvt. Ltd, India) and 100 ng of DNA. Amplification was performed using Eppendorf Master Cycler gradient (Eppendorf Netheler-Hinz GMBH, Hamburg), programmed for an initial denaturation at 94°C for 4 min for one cycle, then 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 35 cycles with a 10 min final extension at 72°C. Amplified products were resolved in 2% agarose gel electrophoretically at 75 V, using 1X TBE buffer. A 100 bp DNA ladder (Bangalore Genei Pvt. Ltd, India), served as the standard molecular weight marker. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV light²⁰.

The restriction enzyme digestion analyses were performed using 15 µl of the amplified PCR product using 10 units of enzyme overnight. The following enzymes were used: *TaqI*, *Sau3A1*, *HaeIII*, *AluI*, *EcoRI*, *SmaI*, *BamHI*, *HindIII*, *PstI*, *MspI*, and *SacI*, as per the specifications of the manufacturers (Bangalore Genei Pvt. Ltd, India). The restriction fragments were size separated by electrophoresis on 2% agarose gel at 50 V for 3 h.

Seventeen accessions of *Vigna mungo* and three wild species representing a wide range of geographic origin and maturity group were tested for variations in rDNA ITS region by restriction digestion. The black gram accessions studied were chosen in an effort to sample the native diversity in the species, selected from geographically diverse regions and physiologically diverse accessions. Primers ITS1F and ITS2R were used to amplify the entire ITS region and was approximately 650 bp. There was no length variation in the PCR product from the seventeen cultivars of *V. mungo* and three *Vigna* species studied. The amplified product from all the samples had very similar molecular size (Figure 1). This would mean that during evolution and selection there has been no selection for or against ITS length variation.

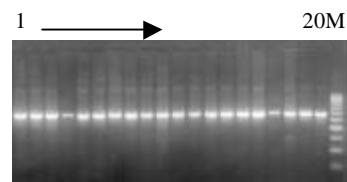


Figure 1. PCR amplified rDNA ITS region with ITS1F and ITS2R primers. M, marker 100 bp ladder; Lanes 1 to 20 correspond to the accessions in Table 1.

Table 1. Black gram accessions used in the study

Accessions	Zone/place of collection
AKU-4	CZ/Akola
Co-5	SZ/Coimbatore
TU 94-2	SZ/Trombay
TAU-1	CZ/Trombay
TPU-4	CZ/Trombay
<i>V. mungo</i> var <i>silvestris</i>	CZ/Trombay
LBG 17	SZ/Lam
LBG 402	SZ/Lam
Nayagarh	EZ/Nayagarh
TAU-5	CZ/Trombay
T9	NZ/Bariely
No. 55	CZ/Satpur plateau
Mash-1	NZ/Ludhiana
Shindekheda	CZ/Shindekheda
S-76	CZ/Trombay
EC 168200	AVRDC/Taiwan
Pusa 3	NZ/New Delhi
Pant 19	NZ/Pantnagar
<i>Vigna trilobata</i>	SZ/Lam
<i>Vigna glabrescens</i>	SZ/Coimbatore

SZ, south zone; NZ, north zone; CZ, central zone of India; AVRDC, Asian vegetable research and development center, Taiwan.

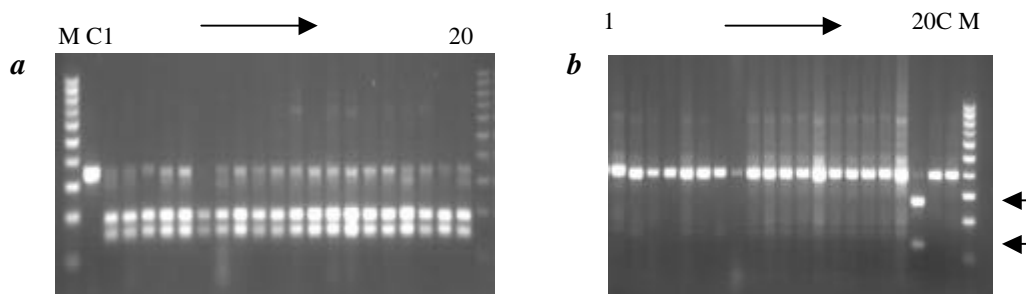


Figure 2. *a*, Restriction pattern of ITS1 amplified product with *MspI*. *b*, Restriction pattern of ITS2 region with *MspI*. M, marker 100 bp ladder; C, undigested control; Lanes 1 to 20 correspond to the accessions in Table 1.

Table 2. Restriction enzymes having site in rDNA ITS region of *Vigna mungo* cultivars, *V. trilobata* and *V. glabrescens*

Restriction enzymes	Cultivars of <i>V. mungo</i> and <i>V. mungo</i> var <i>silvestris</i>		<i>V. trilobata</i>		<i>V. glabrescens</i>	
	ITS1	ITS2	ITS1	ITS2	ITS1	ITS2
<i>TaqI</i>	+	+	+	+	+	+
<i>Sau3AI</i>	+	-	+	+	+	+
<i>SacI</i>	+	-	+	-	+	-
<i>BamHI</i>	+	-	+	-	+	-
<i>MspI</i>	+	-	+	+	+	-
<i>HaeIII</i>	-	+	-	+	-	+
<i>AluI</i>	-	-	-	+	-	+

+, - indicates presence and absence of restriction site.

To verify whether there were any sequence variation in the ITS1 or ITS2 region, amplification was done with primers ITS1F and ITS1R as well as primer ITS2F and ITS2 R so as to separately amplify ITS1 and ITS2 regions respectively. ITS1 was found to be 250 bp and ITS2 of 300 bp. These PCR products were digested with 11 restriction endonucleases, *EcoRI*, *HindIII*, *PstI*, *SmaI*, *Sau3AI*, *TaqI*, *SacI*, *MspI*, *AluI*, *BamHI* and *HaeIII*, of which seven endonucleases (*Sau3AI*, *TaqI*, *SacI*, *MspI*, *AluI*, *BamHI* and *HaeIII*) had restriction sites in the ITS region. Restriction digestion results with different enzymes are summarized in Table 2. The enzyme *TaqI* had one site in both ITS1 and ITS2 of all the materials studied. It showed size polymorphism in the ITS1 region of *V. glabrescens*. *Sau3AI* had a site in both ITS1 and ITS2 of *V. trilobata* and *V. glabrescens* but no site in the ITS2 region of *V. mungo* cultivars and *V. mungo* var *silvestris*. *SacI* and *BamHI* enzyme site was restricted to ITS-1 region of all the samples studied. The presence of *BamHI* restriction site in the ITS region observed in the study appears to be unique to *Vigna* as it has not been reported in other legumes^{14,21}. However, this enzyme site was found to be methylated in the ITS region of mung bean²¹.

The enzyme *MspI* had a site in ITS1 region of all the species studied. In *V. trilobata* *MspI* had a site in ITS2

region also (Figure 2 *a, b*). *HaeIII* enzyme had restriction site in the ITS2 region of all the samples studied. *HaeIII* also showed size polymorphism after cleavage in both *V. trilobata* and *V. glabrescens* (200 and 100 bp), respectively (Figure 3). The enzyme *AluI* did not have any restriction site in either ITS1 or ITS2 of the *V. mungo* cultivars or *V. mungo* var *silvestris*. However, it did have a site in the ITS2 region of *V. trilobata* and *V. glabrescens*. The cleaved product showed size polymorphism between the latter species since the products were 190 and 110 bp, respectively (Figure 4).

The present study has shown that the digestion of PCR amplified rDNA ITS region with restriction endonucleases *Sau3AI*, *TaqI*, *SacI*, *MspI*, *AluI*, *BamHI* and *HaeIII* did not detect any intraspecific variation among the widely distributed cultivars. However, interspecific variation was found in different *Vigna* species. The ITS2 of *V. glabrescens* and *V. trilobata* had restriction sites for *Sau3AI* and *AluI*, which are not found in *V. mungo* var *silvestris* and the cultivated varieties studied. *MspI* enzyme had restriction site specifically present in ITS2 of *V. trilobata*. Homology in the nucleotide sequence of 5.8S rRNA genes of closely related species and the presence of varying degree of non-homology in the ITS1 and ITS2 region of the unrelated species makes the ITS region especially useful for quantifying relatedness among species²². Relatively low level of intraspecific length and sequence polymorphisms of 5S repeat unit was observed in *Solanum* species²³.

In the present study *HindIII* enzyme did not digest the ITS region of *Vigna* sp. Inability of this enzyme in cutting rRNA unit of most of the *Lens* accessions was attributed to methylation²⁴, since *HindIII* will not cleave DNA if the cytosine residue in its recognition sequence AAGCTT is methylated. Alternatively, this site may also be absent from the species¹⁴. Molecular differences in an evolutionarily relevant DNA region that correlate to a certain extent with morphological character suggest that *V. glabrescens* and *V. trilobata* species have their own evolutionary rate due to the isolated location and concerted evolution²⁵ and irregular crossing over²⁶. *V. silvestris* produced the same restriction pattern as cultivated

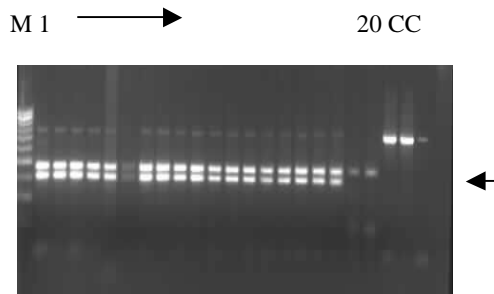


Figure 3. Full rDNA ITS region digested with *Hae*III enzyme. M, marker 100 bp ladder; C, undigested control; Lanes 1 to 20 correspond to the accessions in Table 1.

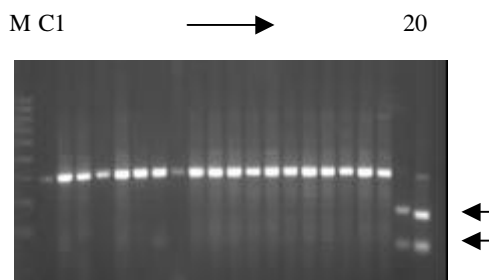


Figure 4. Restriction pattern of ITS2 amplified product with *Alu*I enzyme. M, marker 100 bp ladder; C, undigested control; Lanes 1 to 20 correspond to the accessions in Table 1.

species for all enzymes, confirming the hypothesis that it is the progenitor of cultivated *Vigna mungo* and domesticated in India¹. Genes controlling traits associated with the evolution of *Vigna* species from their primitive types have been reported by Fatokun *et al.*³. Similar response to domestication was observed in *V. mungo*²⁷.

In general all cultivars developed and released for different geographical locations did not show any variation in the ITS region. Conserved sequences in internal transcribed spacers have been observed in *Glycine*¹² and in other plant nuclear rRNA genes¹³. However, variation was found to exist among *V. mungo* var *silvestris*, *V. trilobata* and *V. glabrescens*. Different regions of the rDNA evolve at different rates and provide different levels of resolution for addressing specific questions at appropriate taxonomic levels. Repeated gene families like rDNA genes including spacer regions are subject to concerted evolution²⁵, which means variation in the rDNA family is homogenized among the repeating units within an individual and among members of a population. This phenomenon was postulated to occur through mechanisms like unequal crossing over and biased gene conversion²⁶. Variation in interspecific population suggested that there are at least two separately evolving populations within *Vigna* species. Genetic difference between cultivated and wild species extends far beyond rDNA and there is lim-

ited gene flow between the species of *Vigna*. The wild *Vigna* species exist in isolation, often as small populations and between which gene flow would be predicted to be low, a situation that could well lead to evolutionary divergence of genes²⁸. It has been suggested that the spacer sequences may accumulate mutations in the form of base substitution, duplication, deletions and insertions²⁹ and chromosomal rearrangements in genomes³⁰. No evidence of intraspecific variation was found in the rDNA ITS region of geographically widely distributed cultivars of black gram.

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Received 28 April 2003; revised accepted 18 June 2003

Equation-of-state study of copper using laser-induced shocks near 10 Mbar pressure and revalidation of theoretical modelling

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Laser-driven shock wave experiments have been performed to determine equation of state (EOS) of copper using impedance matching technique in the pressure range 8–11 Mbar. A 2J/200 ps Nd : YAG laser beam is used to induce dynamic shocks in aluminium foil (reference material) and Al–Cu layered targets. EOS of copper is obtained at shock pressures of 8.9 Mbar and 10.4 Mbar with a pressure enhancement of ~1.66 at Al–Cu interface. The experimental data points are consistent with the predictions of the EOS model based on first principle theory and are also in close agreement with the simulation results obtained using one-dimensional radiation hydro-code MULTI that uses SESAME data tables for EOS and opacity values.

EQUATION-of-state (EOS) of a material at high pressures is an important input parameter for astrophysics, geophysics, inertial confinement fusion and hydrodynamic codes used for the simulation of fission, fusion devices. The EOS data up to 5 Mbar pressure is obtained with high explosive loading facility or using a high-pressure gas gun¹. The pressure above 10 Mbar in the past had been obtained from underground nuclear explosions, but these measurements are difficult due to high cost and require large experimental configurations^{2–4}. The efforts in the recent past reveal that laser-driven shock wave technique can be employed for achieving shock pressures of 10–40 Mbar within 15–20% accuracy in the laboratory conditions^{5,6}. Recently, experiments using indirect drive method measures the shock pressure within an accuracy of 3–4% (ref. 7). With these developments it appears that laser-driven shocks can be used for the generation of accurate high-pressure data. These data can be utilized as a testing ground for the first principle theoretical models that is used for generating the EOS data in the pressure region not yet accessible experimentally. In this paper we present the extension of laser-driven shock wave experiments performed at CAT, Indore, to determine the EOS of copper (Cu) between 8 and 11 Mbar using impedance

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