Amplified Fragment Length Polymorphism (AFLP) analysis in *Cyamopsis* spp. for Genetic Diversity Study

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Abstract
The aim of present work is to study genetic diversity and phylogenetic relationship among 13 guar varieties from different germplasm conservation centers of India. A total of 64 primer combinations were tested and 24 primer combinations producing best DNA fingerprints were selected for final analysis. The AFLP marker gives keen amplification with 1,671 polymorphic bands and 62 unique bands ranging from 55 bp to 350 bp. Maximum numbers of unique bands were produced with E-ACT/M-CAG primer combination.

This analysis allows determining association with an undefined feature and helps in identification of these genotypes. The phylogenetic relationship was explained by a dendrogram generated with UPGMA method. These guar genotypes may be used to develop new varieties with significant improvement in quality and quantity of gum.

Keywords: Genetic diversity, AFLP, Dendrogram, Molecular markers, *Cyamopsis tetragonoloba.*

Introduction

Guar (*Cyamopsis tetragonoloba* [L.] Taub) is an annual, drought-tolerant plant of leguminosae family. It is one of the main crops of North Western India, South Eastern Pakistan and Oklahoma and Texas states of USA. Guar was traditionally used as a feed for livestock and poultry but now has become an industrially important crop due to the commercial applications of the gum present in its seeds.[8] Despite being a very important crop, it has not sought much attention of the molecular biologists.[2,18]

Recently there has been a considerable increase in the demand for guar gum due to its increased use in drilling of oil wells.[14] In order to meet this increasing demand, improved varieties of guar are required. For genetic improvement of guar. An overview of its genetic diversity is essential. Guar accessions have been conventionally characterized based on morphological traits.[4,13]

However morphological and biochemical characteristics are not sufficient to assess genetic diversity effectively as these are scarce and also vary with environmental fluctuations. Previous reports have shown that even when no morphological differences are present, the possibility of finding genetic difference is not ruled out.

The DNA segments which act as landmarks in genome analysis, prove to be very useful in diversity study and plant breeding. These segments, also known as molecular markers, are stable and can be detected in a tissue at any growth or developmental stage. For an effective and reliable germplasm characterization, a large number of molecular markers are required.

Several such markers like RFLP, RAPD, AFLP, SCAR, STS, SSR, SNP etc. have been described.[1,22] Genetic diversity in guar has been studied using RAPD markers[9,17], ISSR markers[9] and SSR markers.[10] However, the use of AFLP (amplified fragment length polymorphism) markers might prove to be a better option for such studies as it combines the reliability of RFLP and ease of RAPD markers.[20] This technique has also been used for the construction of genetic linkage map in various plants such as maize, alfalfa etc.[6,21] A genetic linkage map would further enhance research in this crop where genetic progress is very slow. The present study was carried out to optimize the AFLP primer combinations and use these combinations to understand genetic diversity in 13 accessions of *Cyamopsis* assisted with factor based analysis.

Material and Methods

Plant material and DNA extraction: The varieties used in the present study (Table 1) were obtained from Central Arid Zone Research Institute (CAZRI), Jodhpur, National Bureau of Plant Genetic Resources (NBPGR), New Delhi and Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), Hisar. Genomic DNA was isolated from young leaves using CTAB method as described by Khanuja et al[7] with some modifications and 500ng of DNA of each variety was used for AFLP analysis.

AFLP analysis: Genomic DNA was digested with two restriction endonucleases *Eco*RI and *Mse*I (NEB). AFLP procedure was followed according to the Applied Biosystems protocol. Restriction and ligation reactions were carried out in a single reaction tube simultaneously.[20] Eight fluorescent labeled *Eco*RI primers and eight unlabeled *Mse*I primers were used to make 64 combinations. The *Eco*RI primers contained three selective nucleotides with the sequence 5'-[Dye- Primer-Axx]-3' while the *Mse*I primers had the selective nucleotides starting with C, that is, 5'-[Primer-Cxx]-3'.

Table 1
Details of guar genotypes used in the study for AFLP analysis

S.N.	Accession number	Place of procurement	Geographic origin	Branching /Non Branching	Pubescence	Accession type
1	M-83	CAZRI, Jodhpur	-	Non branching	Smooth	Vegetable variety
2	RGC-197	CAZRI, Jodhpur	-	Branching	Hairy	Gum producing variety
3	RGC-1002	CAZRI, Jodhpur	-	Branching	Hairy	Gum producing variety
4	RGC-1003	CAZRI, Jodhpur	-	Branching	Hairy	Gum producing variety
5	RGC-1055	CAZRI, Jodhpur	-	Branching	Hairy	Gum producing variety
6	RGC-1066	CAZRI, Jodhpur	-	Non branching	Hairy	Gum producing variety
7	IC-103323	NBPGR, New Delhi	Himmatnagar-Gujrat	Branching	Smooth	Landrace
8	IC-113304	NBPGR, New Delhi	Kachchh-Gujrat	Branching	Smooth	Landrace
9	IC-116751	NBPGR, New Delhi	Nagour-Rajasthan	Branching	Hairy	Landrace
10	IC-116767	NBPGR, New Delhi	Nagour-Rajasthan	Branching	Hairy	Landrace
11	IC-116958	NBPGR, New Delhi	-	Branching	Hairy	Landrace
12	*C. serrata*	CCSHAU, Hisar	Africa	Branching	Hairy	Wild
13	*C. senegalensis*	CCSHAU, Hisar	Africa	Branching	Hairy	Wild

The ‘‘explorer’’ gel for all 64 primer combinations was run using M-83 to determine the most responsive primer pairs (generating more number of fragments) for the guar genome. All 64 reactions were set up in 24 tubes by multiplexing.

The product of selective amplification was loaded on a 5% polyacrylamide gel on an ABI Prism 377 DNA sequencer (Applied Biosystems).[3] The selective amplification reaction product (1 µl) was mixed with 0.25 µl of loading buffer (ROX500 size standard) and 9.25 µl deionized formamide and loaded on the gel. Out of 64 primer combinations tested, 24 primer combinations (Table 2) producing best DNA fingerprints were chosen after analyzing the explorer gel.

All the accessions were then subjected to selective amplification with these primer combinations. Selective amplification products obtained with primers labeled with three different fluorescent dyes were pooled along with a loading buffer containing a size standard for loading as described in the Applied Biosystems manual.

Data analysis: The AFLP amplification profiles of 24 primer combinations (Table 2) obtained with 13 *Cyamopsis* accessions were scored as present (1) or absent (0) using Gene-Mapper analysis software (version 3.1; Applied Biosystems). The 1/0 data was further used for cluster analysis. A dendrogram was generated by NTSYSpc (version 2.02) which works on Unweighted Pair-Group Average (UPGMA) method using the similarity matrix by Jaccard’s similarity coefficient.[5] The distance matrix was constructed manually based on the number of polymorphic bands produced in each variety. To evaluate AFLP primer combinations, different important parameters were calculated i.e. polymorphic information content (PIC), diversity index (DI), resolving power (RP), marker index (MI) and effective multiplex ratio (EMR).

The PIC value for each primer was calculated by formula PICi= 2 fi (1-fi) where fi is the frequency of amplified fragment present and 1-fi is for fragments that were absent. The Rp value was calculated for each primer as $RP= \sum I_{b,}$ where $I_b= 1- [2*(0.5-p)]$ in which p is the proportion of the selected accession of the fragment.

The MI was calculated as per formula MI= PIC × EMR where EMR is the product of total number of loci per fragments per primer and the fraction of polymorphic loci fragments. The DI that represents genetic diversity of the selected varieties was calculated by $DI= 1- \sum Pi^2$ where Pi represents allele frequency.

Results

Thirteen *Cyamopsis* accessions including 6 commercial varieties, 5 landraces and 2 wild species were analyzed for diversity study using AFLP markers. A total of 1,671 polymorphic bands were obtained with 24 primer combinations (Table 2). E-ACC/M-CAT combination produced maximum number of bands i.e. 152 and E-AAG/M-CAG combination produced minimum number of bands i.e. 19. Unique bands were obtained in nine out of thirteen varieties which can be useful in molecular probing of the respective varieties (Table 3).

Out of 24 selective primer combinations, E-ACT/M-CAG primer combination produced maximum number i.e. 20 of unique bands. Maximum number of unique bands i.e. 34 was obtained in M-83. RGC-1066 produced 11 unique bands.

Table 2
Summary of AFLP banding pattern obtained in 13 guar accessions

S. N.	Primer combination	Number of polymorphic bands	Number of unique bands	Accession in which unique band is present
1	E-AAG/M-CAA	88	8	M-83 and RGC- 197
2	E-ACT/M-CAA	106	0	-
3	E-AAC/M-CAA	116	0	-
4	E-AGG/M-CAA	128	1	M-83
5	E-ACA/M-CAA	67	1	*C. serrata*
6	E-ACC/M-CAA	73	3	M-83 and RGC- 1002
7	E-AAG/M-CAC	27	3	M-83 and RGC- 197
8	E-ACT/M-CAC	84	0	-
9	E-AAC/M-CAC	38	0	-
10	E-AGG/M-CAC	100	3	M-83
11	E-ACA/M-CAC	62	2	M-83
12	E-ACC/M-CAC	132	2	M-83
13	E-AAG/M-CAG	19	2	RGC-1055
14	E-ACT/M-CAG	52	18	M-83, RGC-1066, RGC-197 and IC-116958
15	E-AAC/M-CAG	29	2	RGC-197
16	E-AAG/M-CAT	20	1	M-83
17	E-ACT/M-CAT	60	2	M-83
18	E-AAC/M-CAT	102	2	RGC-1066
19	E-AGG/M-CAT	50	3	IC-103323, IC-116767 and RGC-1055
20	E-ACA/M-CAT	96	2	M-83
21	E-ACC/M-CAT	152	5	M-83
22	E-AAG/M-CTA	-	-	-
23	E-ACT/M-CTA	70	2	M-83 and *C. serrata*
24	E-AAC/M-CTA	-	-	-
Total		1671	62	

Table 3
Molecular probing of guar accessions

S.N.	Accession No.	Number of unique bands	Band Size	Primer combination used
1	M-83	34	51.29 - 341.02	Several
2	RGC-197	8	57.27 - 196.12	E-AAG/M-CAA , E-AAG/M-CAC, E-ACT/M-CAG and E-AAC/M-CAG
3	RGC-1002	1	116.36	E-ACC/M-CAA
4	RGC-1003	0	-	-
5	RGC-1055	3	56.98, 155.67 and 188.47	E-AAG/M-CAG and E-AGG/M-CAT
6	RGC-1066	11	54.43 – 332.29	E-ACT/M-CAG and E-AAC/M-CAT
7	IC-103323	1	91.56	E-AGG/M-CAT
8	IC-103304	0	-	-
9	IC-116751	0	-	-
10	IC-116767	1	214.41	E-AGG/M-CAT
11	IC-116958	1	55.95	E-ACT/M-CAG
12	*C. serrata*	2	55.51 and 100.42	E-ACA/M-CAA and E-ACT/M-CTA
13	*C. senegalensis*	0	-	-
Total		62		

A distance matrix was prepared on the basis of number of polymorphic bands obtained in each variety. The distance matrix from information of 24 primer combinations was used to generate a dendrogram (Figure 1) to get an overview of the relatedness of the guar varieties used in this study. The primer 2 was found with highest polymorphism with high PIC value of 0.308 and RP as 6.030 (Table 5).

Table 4
Summary of polymorphism in amplified fragments

	Amplified	Polymorphic
PRIMER I	83	36
PRIMER II	51	23
PRIMER III	51	22
PRIMER IV	65	30
PRIMER V	49	23
PRIMER VI	46	20
PRIMER VII	80	36
PRIMER VIII	32	14

Table 5
Different AFLP parameters

	MI	EMR	PIC	RP
PRIMER I	8.676	36	0.241	4.527
PRIMER II	7.084	23	0.308	6.030
PRIMER III	3.916	22	0.178	3.094
PRIMER IV	8.58	30	0.286	5.690
PRIMER V	4.209	23	0.183	3.261
PRIMER VI	4.88	20	0.244	4.997
PRIMER VII	6.804	36	0.189	5.05
PRIMER VIII	2.282	14	0.163	3.062

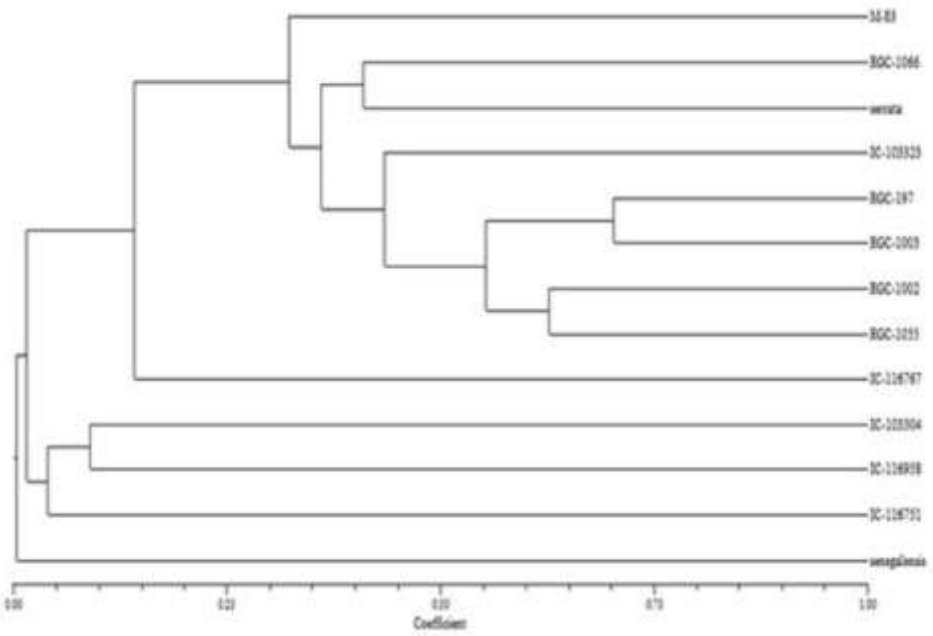

Figure 1: Dendrogram obtained for 13 *Cyamopsis* varieties with UPGMA based on Jaccard's coefficient

Discussion

AFLP technique has been frequently used in diversity analysis in plants,[16,19] species identification[12] and various other applications. The present study focuses on the utility of AFLP markers for studying the genetic diversity within and among landraces, commercial varieties and wild species of *Cyamopsis*.

The two varieties that produced unique bands also have various unique physical and biological characters. M-83, unlike others, is a vegetable variety and not a gum producing variety. RGC- 1066 also produced several unique bands which can be supported by the fact that it is a non- branching variety producing a large number of pods per plant.

Dendrogram showed two major clusters and an out-group with *C. senegalensis* which is a wild species. Landraces and commercial varieties formed separate clusters except a few exceptions. Landraces were not grouped according to their geographic location indicating that they cannot be classified

on geographical basis. Similar conclusions were drawn by Patil et al[16] when Kenyan field bean genotypes were clustered along with Indian genotypes.

High PIC and RP value of primer 2 may be due to association of this marker with chromosomes of guar. This marker can be useful in genetic improvement of the different species of guar in germplasm bank. Menz et al[11] have given marker association with genomic coverage to provide gene classification strategies. If AFLP generated by Pst I and Eco RI and restriction sites are arranged in close proximity to centromere, that region would be given more weightage than others. But markers are well proven to study genetic differences covering entire genome. EST-SSR and RAPD markers of cluster bean based genetic diversity were explored and revealed similar polymorphism in selected varieties.

In our study M-83, which is a vegetable variety, formed a different subgroup. RGC-1066 and *C. serrata* formed another subgroup. Another subgroup was formed by the 4 commercial varieties which also support the fact that they are morphologically similar (Table 1). The second cluster IC- 103304 and IC-116958, which are similar in some of their physical characters, formed a subgroup with IC-116751 lying out of the group, which is in accordance with the morphological characteristics.

Conclusion

M-83, RGC-197 and RGC-1066 show relatively high number of unique AFLP that may be associated with particular chromosome. The unique bands obtained in this study can be further sequenced for the development of SCAR markers. The germplasm of selected varieties is with good genetic diversity and may be used in generation of new variety of guar with improved gum production ability.

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