

Cytogenetic diversity analysis of *Coix* species using ISSR markers

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ABSTRACT

The genus *Coix*, a wild relative of maize, belonging to tribe Maydeae of family Poaceae is represented by 3 species in India viz. *C. aquatica*, *C. gigantea* and *C. lacryma-jobi*. It is of wide occurrence in most of south-east Asian countries and of great economic importance as food, forage and medicinal plant. Even though the floras written on Grasses of India describe this genus to be comprised of 3 species, there is not only negligible morphological distinction among these, but at least 2 of them produce a range of semi fertile interspecific hybrids and all form various number of aneuploids. Some interspecific hybrids are suspected to have originated through apomixis. Formation of interspecific hybrids is rather contradictory to the very definition of a species Thus species delimitation poses a taxonomic problem for this genus. The single sequence repeats (SSR) micro satellites, present in nuclear and organelles DNA can be used as molecular markers and has wide ranging applications in the field of genetics including kinship and population studies. These Inter simple sequence repeats (ISSR) represent genome region between micro satellite loci. Sequences amplified by ISSR-PCR can be used for delimiting species.

Key words: *Coix*, Cytogenetic diversity, ISSR, phylogeny analysis.

INTRODUCTION

Coix is an oriental wild relative of maize. It belongs to tribe Maydeae of family Poaceae. It is widely distributed all over South-East Asian countries. In India also, its occurrence has been reported from almost all states, showing its adaptation to a variety of climatic and edaphic conditions. *Coix* has been extensively studied and exploited from food, fodder and medicine point of view, in countries like China, Japan, Thailand, Indonesia, Korea and Brazil etc. In India, its commercial value as a food crop has been totally neglected, though it has been studied here widely for its cytology and genetics.

The genus *Coix* is represented by three species viz. *Coix aquatica* with $2n = 10$ chromosomes, *C. gigantea* with $2n = 20$ chromosomes and *C. lacryma-jobi* with $2n = 20$ chromosomes. (Bor N.L., 1960) The species *C. aquatica* and *C. gigantea* were found to breed freely and spontaneously, in nature (Deshpande, 1986)

and also when controlled cross pollinations were carried out, producing a range of semi-fertile interspecific hybrids in a range from $2n = 11$ to $2n = 26$ (Naik, 1991). In addition to these, all three species have been reported to produce number of fertile aneuploids (Barve; 1983). Some aneuploids of *C. gigantea* and *C. lacryma-jobi* were shown to have been established and successfully competed with their diploid counterparts. (Barve S.S., 1983).

However, there are no clear cut morphological markers on the basis of which the species can be identified in the field. One has to rely upon the conventional cytological method such as study of meiotic prophase-I, especially diakinesis to ascertain their distinction as different species. When the meiotic behaviour of the chromosomes of interspecific hybrids between *C. aquatica* and *C. gigantea* was studied, the 5 fairly large and nearly metacentric chromosomes of *C. aquatica* formed heteromorphic pairs with the chromosomes of *C. gigantea* genome comprising of two large, four

medium sized and four small chromosomes. From a total of four smaller chromosomes of *C. gigantea*, it is difficult to say which of them are homologous they are of same size and shape. Some chromosomes were left as univalents. The fact that these species hybridize naturally is indicative of their close relationship. The specific delimitation of genus *Coix* has always posed a problem to taxonomists. All the vegetative characters such as annual vs. perennial habit, leaf breadth, glands on leaf surfaces etc., together with features of reproductive organs like margin of lower glumes of male spikelets, size and shape of the involucre of grain, seem to overlap and appear to be continuous in different species. This has caused much uncertainty with respect to diagnostic features for specific delimitation. It seems that conventional cytological methods should be supplemented with molecular data regarding the DNA analysis to confirm the taxonomic position of *Coix* species. Through this present investigation, an attempt has been made to explore, whether any molecular markers can be identified which might help in species delimitation in the genus *Coix*.

Traditionally, classification of the various subgenera, species, and subspecies is based primarily on morphological attributes. However, these traits may not be significantly distinct and usually require growing plants to maturity prior to identification. Moreover, morphological characters may be unstable due to environmental influences. Over the years, the methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits. Several DNA marker systems are now common use in diversity studies of plants. The most commonly used marker systems are restriction fragment length polymorphism (RFLP) (Soller and Beckmann, 1983), random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990), amplified fragment length polymorphism (AFLP) (Vos *et al.* 1995), inter simple sequence repeats (ISSRs) (Zietkiewicz *et al.* 1994) and microsatellites or simple sequence repeats (SSRs). Among them to characterize DNA variation patterns within species and among closely related taxa in *Vigna* species have been RAPD (Ba *et al.*

2004; Dikshit *et al.* 2007), AFLP (Sivaprakash *et al.* 2004; Fang *et al.* 2007), RFLP (Kaga *et al.* 2000), ISSR (Ajibade *et al.* 2000), SSRs and sequence tagged microsatellite site (STMS) (Phansak *et al.* 2005). Inter SSR (ISSR) fingerprinting was developed such that no sequence knowledge was required. Primers based on a repeat sequence and the resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping. PCR products are radiolabelled or PCR incorporation, and separated on a polyacrylamide sequencing gel prior to autoradiographic visualization concluded that ISSR would be a better tool than RAPD for phylogenetic studies. Nagaoka & Ogihara (1997) have also reported that the ISSR primers produced several times more information than RAPD markers in wheat.

MATERIAL AND METHODS

Karyotypic Analysis

Chromosome Preparation

The seed material of *C. auatica*, *C. gigantea* and *C. lacryma-jobi* was collected from Junnar in Pune district, Ambe ghat near Kolhapur district and Shivaji university campus, Kolhapur, and Thailand respectively. The seeds were sown in large earthen pots on the terrace, in the month of June. The immature male racemes were fixed in Carnoy's fluid (1: 3 acetic alcohol) for 24 hours and then transferred in straw-yellow colored Ferric chloride solution for mordanting. The anthers were squashed in 1 % acetocarmine to make cytological preparations for the study of meiosis, especially, to confirm their chromosome number.

DNA Amplification condition and gel electrophoresis

DNA Isolation

Leaf samples of all the three species and four samples were used for DNA isolation and genetic diversity. Genomic DNA was extracted from 4 individual *Coix* samples according to standard protocol. Isolation of genomic DNA from plant leaf samples was carried out using CTAB method (Doyle & Doyle, 1987)

DNA Quantification

Quantitative analysis of isolated DNA was

carried out using standard Spectrophotometric method for nucleic acid quantification. It was done by Spectrophotometric Assay. The ratio of OD_{260}/OD_{280} was determined to assess the purity of the sample.

PCR

PCR technique has promoted the development of a range of molecular assay systems which detect polymorphism at molecular level. In this study we used the most widely adopted PCR based ISSR marker technology for characterizing the amongst the Coix sample.

The PCR amplification was carried out in 200 μ l reaction mixture tube containing 10 \times PCR buffer (2.0 μ l), 20 ng of genomic DNA (3 μ l), 2 μ l of UBC ISSR primer (3 pmol/ μ l), 10 mM dNTPs (2.0 μ l), 3U/ μ l *Taq* DNA polymerase (0.4 μ l), Tween 20 (0.2 μ l). The final volume was made to 20 μ l by adding SMQ. PCR reactions were carried out in a Finnzyme make thermal cyclor.

PCR reactions were carried out in a Finnzyme make thermal cyclor using ISSR primers with di and tri nucleotides. ISSR primer sets were ordered from University of British Columbia (UBC). Fourteen ISSR primers were used for initial screening. Out of fourteen primers, 05 primers gave the amplification (in terms of repeatability, scorability) were selected for identification. The selected 4 primers were 14-23 mers. UBC Primer number 813, 814, 815, 819 and 821 showed the genetic variability.

PCR amplified product separated on 1% agarose gel. Gel stained using ethidium bromide and visualized under the UV Transilluminator.

For each sample, each fragment / band that was amplified using ISSR primers was treated as a unit rearrangement in genome. The primers which were given scorable and consistently reproducible amplicons were considered. The gel pictures were taken and documented to computer by using Alpha Imager gel documentation system and size of each amplicon was measured by using Alpha Imager Software with respect to standard molecular weight DNA ladder and molecular weight of each of the potential specific bands was calculated using the software program Alpha Imager.

Data Analysis

Marker index for ISSR markers was calculated in order to characterize the capacity of each primer to detect polymorphic loci among the genotypes. It is the sum total of the polymorphism information content (PIC) values of all the markers produced by a particular primer.

The data obtained by scoring the ISSR profiles with different primers individually as well as collectively were subjected to the construction of similarity matrix using Neighbor-joining tree construction method of Nei and Li/Dice. The similarity values were used for cluster analysis. Data analysis was done using Free Tree and Tree View bioinformatics software.

RESULTS AND DISCUSSION

Genetic diversity analysis at cytological level

Total of 70 seeds were sown. The results of germination and chromosome number analysis based on conventional cytological methods were as per the following table:

No. of seeds Sown	No. of seeds Germinated	Plants obtained, with chromosome numbers		
		2n=10	2n=18	2n=20
80	76	03	43	30

Sample 1

Out of total 70 seeds sown, 3 plants showed chromosome no. 2n=10 with 5 bivalents at diakinesis (Fig. 1). All chromosomes are metacentric and fairly large. The rest of meiotic behavior of the chromosomes was normal. This chromosome number was denoted as sample 1.

Sample 2

Out of total 62 seeds that germinated, 26 plants showed chromosome number 2n=18 (Fig.2). These belonged to *Coix gigantea*. These seeds were collected from Junnar (western Maharashtra). Initially, the populations of *Coix gigantea*, collected from Taluka Purander, of Dist. Pune and from

nearby places of it showed all plants with chromosome nos. $2n=20$. The plants had showed habit. These 20 chromosomes formed 10 perfectly paired bivalents. When the seeds of these plants collected and raised in the botanical garden of Dr.B.A.M.University, Aurangabad, around the year 1975, gradually number of aneuploids were reported from the natural populations of *Coix gigantea*. First cytological evidence of non-disjunction of a bivalent (Sapre and Barve, 1985) was reported. This non-disjunction in $2n=20$, resulted in producing plants of *C. gigantea* with chromosome no. $2n=18$, $2n=19$ and, $2n=21$ (nullisomic, monosomic and trisomic, respectively). In subsequent years some more aneuploids with chromosome numbers ranging from $2n=18$ to $2n=26$ (Sapre and Barve, 1983) were also reported. The nullisomics with $2n=18$, were not only found to be fertile but also to establish themselves to such an extent that they have completely replaced the $2n=20$ (normal diploid chromosome no.) from the population.

In the present investigation also, seeds collected and plants raised from wild population of *C. gigantea*, surprisingly showed $2n=18$ chromosome number in 43 plants, out of total 46 plants, indicating that, in wild populations also, nullisomics have replaced the diploids with $2n=20$ chromosome number. The genome of $2n=18$ *C. gigantea* comprises of 1 pair, 4 medium sized and 4 small sized pairs of chromosomes. One pair of large sized chromosomes got eliminated as a result of meiotic non-disjunction. These plants with chromosome no. $2n=18$ were denoted as sample no.2

Sample 3

Some plants of the *C. gigantea* population, on cytological screening, showed $2n=20$ chromosome number, with 10 bivalents. These were denoted as sample no.3

Sample 4

Seeds were also acquired, by post, from Thailand. On cytological screening of the population raised from these seeds, all 30 plants showed chromosome no. $2n=20$ with most of the pollen mother cells showing 10 bivalents and in few of them, 8 II + 1IV (Fig.3). These plants were denoted as Sample-4.



Fig. 1: Sample 1, *Coix aquatica* $2n=10$

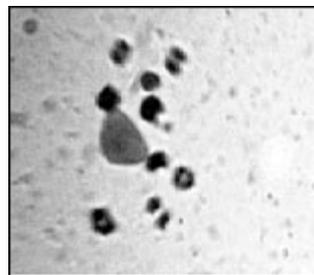


Fig. 2: Sample 2, *Coix gigantea* $2n=18$



Fig. 3: Sample 4, *Coix lacryma-jobi* $2n=20$

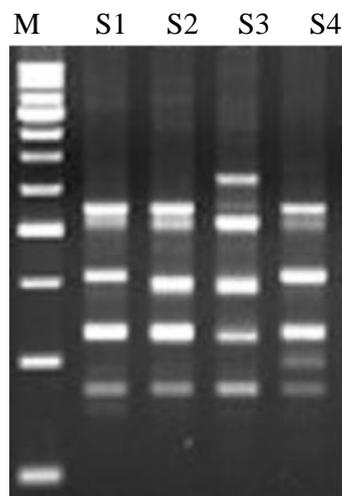
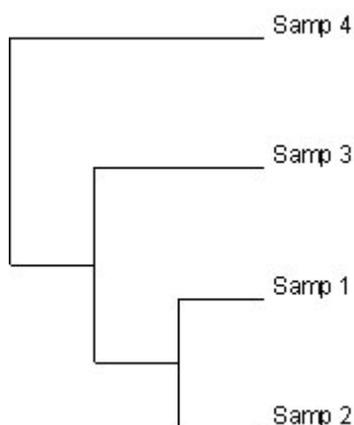


Fig. 4: ISSR Marker Profile of *Coix* species generated by primer UBC19. Lane M – 100 bp DNA Ladder, S1-*C. aquatica*, S2-*C. gigantea*, S3-*C. lacryma-jobi*, S4-*C. lacryma-jobi* (Thailand)

SSR markers diversity among Coix

Amplification of genomic DNA of 04 samples using ISSR marker analysis, yielded total amplified 24 fragments. ISSR analysis yielded 24 fragments that could be scored, out of which 01 was polymorphic while the remaining were 23 were monomorphic in nature.

In ISSR analysis number of amplified fragments ranged from 4 to 8 and which varied in size from 255 bp to 1500 bp. UBC primer No. 814 showed the lowest bands while the primer number 821 produced the highest molecular weight bands. Out of the 24 amplified bands produced, only 01 band was polymorphic in nature with 4.16% polymorphism. An average of 1.04% polymorphic fragments per primer was observed. Percentage of polymorphism ranged from 0% (with UBC primer 814, 815 and 821) to 14.28% (with UBC primer 819) (Fig. 4). The PCR amplification using ISSR primers gave rise to



reproducible amplification products. The complete data was based on a total of 24 amplicons produced. A Dendrogram analysis was carried out by using bioinformatics phylogeny tool Free Tree and Tree View of DNA fingerprint analysis (Fig. 5). Distance similarity matrix was calculated by using Neighbour-joining tree construction method of Nei and Li/Dice which ranged from 0.0000 to 0.01587 (Table 1).

DISCUSSION

The present investigations showed that during the cytological studies of the Sample 1 of *Coix aquatica*, out of total 70 plants, 3 plants showed chromosome number. $2n=10$ with 5 bivalents at diakinesis. In Sample 2, the wild population of *C. gigantea*, surprisingly showed $2n=18$ chromosome number while few nullisomics have replaced the diploids with $2n=20$ chromosome number. The investigations of Sample 3, *C. gigantea* population, on cytological screening, showed $2n=20$ chromosome number, with 10 bivalents. While Sample 4 *C. gigantea* collected from Thailand showed chromosome number $2n=20$ with most of the pollen mother cells showing 10 bivalents and in few of them, 8 II + 1IV. This cytological analysis shows that *C. lacryma-jobi* there is instability in the chromosome number of the Coix and this varies from $2n=10$ to $2n=20$. Also the nullisomic condition was observed.

The molecular investigations couldn't show any such variation at molecular level with the primers used. The bands produced by UBC primer number 814, 815 and 821 does not showed any confirmatory polymorphism. While only primer number 819 has

Table 2: Dendrogram generated using ISSR primers showing relationship between Coix species. Samp 1-*C. aquatica*, Samp 2-*C. gigantea*, Samp 3-*C. lacryma-jobi*, Samp 4-*C. lacryma-jobi* (Thailand)

	<i>C. aquatica</i>	<i>C. gigantea</i> ($2n=18$)	<i>C. gigantea</i> , ($2n=20$)	<i>C. lacryma-jobi</i> (Thailand)
<i>C. aquatica</i>		0.00000	0.01587	0.00000
<i>C. gigantea</i> , $2n=18$	0.00000		0.01587	0.00000
<i>C. gigantea</i> , $2n=20$	0.01587	0.01587		0.01587
<i>C. lacryma-jobi</i> (Thailand)	0.00000	0.00000	0.01587	

Table No. 1 Distance Similarity Matrix:
Neighbor-joining tree construction method of Nei and Li/Dice.

given the polymorphic band. The percentage of polymorphism was not found to be satisfactory. According to the banding pattern with the primers used, the Dendrogram produced showed the evolutionary order of all the four samples.

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