RESEARCH ARTICLE

Genetic Diversity Analysis in Chickpea (Cicer arietinum L) Genotypes Grown in Northwestern Algeria using Microsatellite Markers (SSR)

Bouri Amina1, 2, Mediouni Mohammed Rida3, 4, Ameur Ameur Abdelkader1, 5, Udupa Sripada1, Gaouar Souheil Bechir Semir2, 4, 5

ABSTRACT
The present study aimed to characterize a subset of 10 selected chickpea accessions (Cicer arietinum L) using SSR. The result indicated a presence of a total of 59 alleles. The genetic diversity at the 15 microsatellites loci was varied from 0.32 for TA22 to 0.78 for TA72 and TA117 with an average of 0.66. Polymorphic information content (PIC) values ranged from 0.27 to 0.74. This study also detected a high significant (P < 0.01) positive correlation between alleles per locus, gene diversity (H) and polymorphism information content (PIC). In the dendrogram and on the PCoA bi-plots, chickpea genotypes were adjoined according to their geographic origin, type of chickpea (Kabuli/ Desi). Nevertheless, the distribution of the different grouping through the factorial correspondence analysis (AFC) is due to the genetic variability.

Key words: Chickpea, Cicer arietinum, Genetic diversity, North West of Algeria, SSR.

INTRODUCTION
Chickpea (Cicer arietinum L, 2n=2x=16) is an important diploid self-pollinated pulse crop of the semi-arid tropics and ranks third in worldwide cultivation after pea and common bean. It is mainly cultivated in Indian subcontinent, Mediterranean region, West Asia, America and recently Australia. The genome size of chickpea is estimated to be 740Mb. (Arumuganathan and Earle, 1991).

However, even though its nutritional characteristics are similar to those of other important grain legumes such as field pea, chickpea is less used in animal feeding (Bampidis et al., 2009). The straw and dried roots of chickpea are used as fuel for cooking. Chickpea starch is suitable for textile sizing and in the manufacture of plywood (Bejiga et al., 2006). The leaves yield an indigo-like dye and have uses in traditional medicine Taylor et al. (2007).

In Algeria chickpea is one of the largest seed legumes, ranks second after the bean. Most of the cultivated area of this species is concentrated in the west of the country (MADR, 2018).

In order to improve the productivity of chickpea, the use of DNA based molecular markers has been proposed for marker assisted selection (MAS) , mapping of QTL (Quantitative Trait Loci), SSR and positional cloning of genes in chickpea. SSR markers have been employed to analyze genetic diversity and relationships in a number of crops (Ajbade et al. 2000, Raina et al. 2001, Bart et al. 2002, Srivastava et al. 2016, Babayeva et al. 2018, Syeda Asma Koinain et al . 2015). Simple sequence repeats (SSR) are short tandem repetitive DNA sequence with a repeat length of few (1-6) base pairs. These sequences are abundant, dispersed through the genome and are highly polymorphic in comparison with other molecular markers. Due to their short repeat length and limited interaction at individual loci, SSR markers were used in the present study to investigate the genetic polymorphism among chickpea cultivars. The extensive use of molecular markers in chickpea
genetics and breeding started only after the development of simple sequence repeat (SSR) markers. The multi-allelic and co dominant nature of these markers made them ideal for genomic studies and for use in plant breeding. The SSR markers have been developed from sequence information obtained from various sources, including genomic libraries (Hüttel et al. 1999, Winter et al. 1999, Sethy et al. 2006 a, b, Nayak et al. 2010). The published draft genome sequence of chickpea identified over 48,000 SSRs suitable for PCR primer design for use as genetic markers (Varshney et al., 2013).

The aim of this work was to detect the genetic structure, diversity and allelic variability included in a collection of 10 chickpea genotypes using 20 SSR markers. This work is the first one in Algeria in which we used SSR markers.

MATERIALS AND METHODS

Plant materials

Ten chickpea (Cicer arietinum L.) accessions comprised 3 landrace collected from different geographical locations of the north west of Algeria, one landrace origin from Italia and 6 breeding line genotypes introduced in Algeria and one other accession that we take back from Spain; were considered for the study of genetic diversity using SSR markers (Table 1).

These accessions were grown in the randomized blocks at the research farms of the Department of Plant Breeding of the International Center for Agricultural Research in the Dry Areas - Morocco (ICARDA), the leaves of three to four weeks old seedlings were used for the present study.

DNA extraction and SSR analysis

The first step of any genetic characterization begins with the isolation of the DNA from the cells or tissues, once the extracted DNA has sufficient purity, other molecular biology techniques will have to be performed and this will depend on the purpose of the problematic.

Total genomic DNA was extracted from 2g fresh leaves of each genotype following the extraction protocol named Trimenthy Ammonium Methyl Bromide Protocol (CTAB 2X) (Saghai-Maroof et al. (1984), modified by Udupa et al. (1999).

The quality of genomic DNA was assessed in agarose gel (0.8%, TBE buffer) gel electrophoresis and spectrophotometry, respectively. The DNA quantification was also done by UV spectrophotometer (Nanodrop ND-1000, NanoDr Technologies). The DNA samples that showed a single band on agarose gel and had an A260 nm/A280 nm ratio of ~1.8 are chosen for the next steps for genetic analysis.

In the present study A total of 20 SSR markers initially were screened in the genotypes (TA113, TA27, TA46, TA22, TA76s, TA72, TA118, TA117, TA135, TA142 , TA116, TA130, TA58, TA64, TA14, TA28, TA21, TA71, TA206,TA200). The SSR markers have been developed from sequence information obtained from various sources, including genomic libraries (Hüttel et al. 1999, Winter et al. 1999, Sethy et al. 2006 a, b, Nayak et al. 2010).

PCR Amplification

PCR amplification was performed in a thermal cycler (BIORAD) using twenty SSR molecular markers. The amplification reaction is done using a reaction mixture of 10 μl, containing the DNA extract (1μl), specific complementary primers (1μl), Taq polymerase (0.025μl), the four deoxyribonucleotide triphosphates (1 μl), a buffer solution 5x (2μl) (Tris-Cl, KCl, (NH4 2SO4, MgCl2 (15mM), pH 8.7) and pure water (3.975 μl).

Amplifications were programmed for an initial step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, elongation at 60°C for 40 sec, followed by a final elongation step at 60°C for 7 min.

PCR products were analyzed on 4% polyacrylamide gels electrophoresis using TBE buffer and the amplification pattern was visualized using BET under UV light of (BIORAD GelDoc XR+). Band patterns for each of the microsatellites markers were recorded for each genotype by assigning a letter to each band.

Data analysis

Statistical estimation of all parameters of the genetic diversity were determined using the three software packages, Power Marker ver .3.25 (lie, 2005), GenAlex ver. 6.4 (Peakall and Smouse, 2006) and Poppgene 1.32 Yeh et al. (1997).

SSR markers were used for determining statistical parameters. The parameters obtained after screening 10

| Sample | Accessions | Origin | Biological status | Type |
|--------|------------|--------|-------------------|------|
| 1      | GARB (9) (GARBANSA) | Algeria | Landrace | Kabuli |
| 2      | CECE NERO | Italia | Landrace | Desi |
| 3      | BLED (6) | Algeria | Landrace | Kabuli |
| 4      | FLIP 8293 | Syria | breeding line introduced in Algeria | Kabuli |
| 5      | FLIP 8492 C | Syria | breeding line introduced in Algeria | Kabuli |
| 6      | ILC 482 | Turkey | breeding line introduced in Algeria | Desi |
| 7      | FLIP 9213 C | Syria | breeding line introduced in Algeria | Kabuli |
| 8      | FLIP 9393 C | Syria | breeding line introduced in Algeria | Kabuli |
| 9      | F10 38 | Algeria | Landrace | Kabuli |
| 10     | ESP 1 | Spain | breeding line | Kabuli |
chickpea genotypes at 15 SSR loci were: Allele number (Na), effective allele number (Ne), Shannon index (I), Theoretical Heterozygosity (H0), polymorphic information content (PIC), the index of genetic diversity (H), genetic distances Nei method (Nei et al. 1987), PCoA (Principal Coordinates Analysis (PCoA) and AFC (Factorial correspondence analysis).

Five markers were removed from the statistical analysis (TA14, TA28, TA21, TA71 and TA206). Because three of them were found to be non-polymorphic (TA21, TA71, TA206) and the two remaining did not give a result during PCR (TA14, TA28).

Phylogenetic dendrogram designed using two statistical method, (UPGMA method (Unweighted Pair Group Method with Arithmetic and NJ method (Neighbor-joining)); and visualized using MEGA5 software (Tamura et al. 2011).

RESULTS AND DISCUSSION

Extraction of genomic DNA and quality test
The electrophoretic profile of the ten accessions on a 1.2% agarose gel revealed that all the extracted DNA samples are characterized by the presence of bands with a size greater than 21000 Pb and has different intensities which is explained by a difference of DNA concentration .The samples analyzed with the 260/280 ration show a value between 1.8 and 2. This allows us to say that our DNA is of good quality (not degraded) and can be used for the next analysis steps. (Fig 1).

DNA amplification by PCR
For this purpose 10 cultivars of chickpea were analyzed using 20 SSR markers. (TA113, TA27, TA46, TA22, TA76s, TA72, TA118, TA117, TA135, TA142, TA116, TA130, TA58, TA64, TA200, TA14, TA28, TA21, TA71, TA206). Five SSR markers did not give results of the amplification (PCR) and they were eliminated from this study (TA14, TA28, TA21, TA71 and TA206), three of them were found to be non-polymorphic (TA21, TA71, TA206) and the two remaining did not give a result during PCR (TA14, TA28). Since we analyzed a small number of SSR markers (20) and 15 of which were required (polymorphic), we relied on a selection of markers that were revealed to be polymorphic (a high level of polymorphism detected) in later work. Torutaeva et al. (2014) used 9 highly polymorphic SSR markers, as well as Sefera et al. (2011). Application of highly polymorphic markers will slightly overestimate genetic diversity in relation to randomly selected SSR loci.

Analysis of the amplification products by the primers studied gave the following electrophoretic profiles (Fig 2). The genetic diversity existing in those chickpea accessions have been identified by the electrophoretic profiles of the fifteen markers used which showed remarkable polymorphism; in the electrophoretic profiles we notice the presence of bands of different sizes due to the difference in base pair sizes of the amplified microsatellites. Each clear band in the gel represents an allele; so each primer gave a number of bands that matches the number of alleles.

SSR allelic polymorphism, genetic diversity and population structure
The fifteen polymorphic SSR loci analyzed produced fifty nine alleles; this number varies between two for the markers TA22 and TA76 to five for the markers TA27, TA130, TA200, TA72, TA58 and TA117 with an average of 3.93. The effective number of alleles (Ne) varies from 1.471 to 4.545 with an average of 3.229. (Table 2). These results were generally comparable to those of other studies. In Hajibarat et al. (2015) 48 accessions, were characterized with 10 SSR; they found from 2 to 4 alleles per locus which averaged to 3.1; Also in Hajibarat et al. (2014), 48 genotypes of chickpea were characterized with 38 SSR, they found from 2 to 7 allele per locus with an average of 3.05. Khan et al. (2010) and Ghaffari et al. (2014) have found the similar results which are close to our results. Keneni et al. (2012) found only 111 alleles when assessing the diversity of 155 chickpea accessions with 33 SSR markers, the average number of alleles per locus was 3.36. In Syed Gul et al. (2017) 82 genotypes of chickpea were studied with 8113 single nucleotide polymorphism markers (SNP) the average number of alleles was 14.779.

The major allele that represents the highest frequency (0.8) is found in accessions GAR8 (9), BLED (6), FLIP 8293 and FLIP 8492C, ILC 482 amplified by the TA22 primer. On the other hand, the major allele that generates the lowest
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Fig 2: Electrophoretic profile in polyacrylamide gel for 10 chickpea accessions after amplification by PCR.

Frequency (0.3) is found in the accessions GARB (9), ILC 482, F1038 amplified by primers TA72, in GAR (9), BLED (6) and F1038 amplified by TA58 and in GAR (9), ILC 482, F1038 amplified by TA117. The mean of the number of private allele is 3.933.

The number of alleles per locus showed a significant and positive relationship with both PIC ($r = 0.64$, $P < 0.01$) and gene diversity ($r = 0.68$, $P < 0.01$), Shannon’s information index ($) ranges from 0.5 (TA22) to 1.557 (TA72, TA117) with an average of 1.213. The index of gene diversity ($) ranged from 0.32 (TA22) to 0.78 (TA72, TA117) with an average of 0.66. PIC ranged from 0.22 (TA22) to 0.74 (TA72, TA117) with an average of 0.6.

The mean Shannon’s index value found in our study was 1.213 which is comparable to the result given by Hajibarat *et al.* (2015) ‘1.26’ and definitely higher than the value given by Syed Gul *et al.* (2017) ‘0.043’. On the other hand, the mean PIC value was 0.77 in Hajibarat *et al.* (2014) and 0.7 in Hajibarat *et al.* (2015) which is comparable with our data (0.6); but Keneni *et al.* (2012) found a PIC =0.412 which is lower than our value. The same ascertainment was observed in Rookiwal *et al.* (2014) who have used 651 SNP markers to estimate the genetic diversity of 94 genotypes of chickpea the mean PIC value was 0.23 and also in Udita *et al.* (2018) using SNP markers to characterize 92 genotypes of chickpea, the mean value of the PIC was 0.45.

We can say after obtaining our result that the two primers
TA12, TA117 are qualified as highly informative, since the Polymorphism Information Content (PIC) index is at 0.74 and Shannon’s information index (I) is at 1.557. Similar results were found by Bharadwaj et al. (2011) (TA117 PIC=0.562 and TA72PIC=0.634) and Torutaeva et al. (2014) TA117 (PIC=0.9; I=2.53). However, in Hajibarat et al. (2014) TA 72 gives a low level of polymorphism (PIC=0.27, I=0.50). But in Hajibarat et al. (2014) TA 22 generates a high value of PIC (0.58). A high PIC value in chickpea microsatellite analysis was also reported by Udura et al. (1999), Upadhaya et al. (2008) and Bharadwaj et al. (2011) who attributed this to polymorphism of TA motif in chickpea.

All loci detected expected heterozygosity (H) ranged from 0.320 (TA22) to 0.780 (TA72, TA117), which averaged to 0.658 over all loci. Which is similar to the result found in Hajibarat et al. (2014) and relatively higher than values reported by Upadhaya et al.(2008), Saeed et al. (2011) and Hajibarat et al. (2015) respectively. We revealed an average value of 3.933 of the number of private allele (No) which is higher than value reported by Torutaeva et al. (2014).

The analysis of the different SSR profiles by the genetic distances (Nei, 1987) was calculated using the Power Marker software (Table 3) and subsequently to build a dendrogram that perfectly illustrates the probable genetic relations between the ten chickpea accessions (Fig 3).

The value of the genetic distance varies between 0.00 and 1 (Table 3). The smallest distance was observed between several accessions as an example: 0.43 (GARB (9)) and FLIP 8293); 0.47 (FLIP 9213C and BLED (6)); (FLIP 8293 and CECE NERO), 0.93 between (ESP1 and FLIP 9393C).

The genetic distance tree made using cluster analysis by the UPGMA method clearly delineated the genotypes in two major groups (A and B) (Fig 3). In group A the accessions

| Markers | Alleles Number (Na) | Ne | Allele major frequency | Ho | He | PIC | Gene Diversity (H) | I |
|---------|-------------------|----|------------------------|----|----|-----|-------------------|---|
| TA113   | 3                 | 2,632 | 0.5 | 0,000 | 0,620 | 0.55 | 0.62 | 1,030 |
| TA27   | 5                 | 3,846 | 0.4 | 0,000 | 0,740 | 0.70 | 0.74 | 1,471 |
| TA64   | 4                 | 3,240 | 0.44 | 0,000 | 0,691 | 0.64 | 0.69 | 1,273 |
| TA22   | 2                 | 1,471 | 0.8 | 0,000 | 0,320 | 0,27 | 0.32 | 0,500 |
| TA130  | 5                 | 3,571 | 0.4 | 0,000 | 0,720 | 0.68 | 0.72 | 1,418 |
| TA135  | 4                 | 3,333 | 0.4 | 0,000 | 0.700 | 0.65 | 0.70 | 1,280 |
| TA200  | 5                 | 3,846 | 0.4 | 0,000 | 0.740 | 0.70 | 0.74 | 1,471 |
| TA46   | 4                 | 3,333 | 0.4 | 0,000 | 0.700 | 0.65 | 0.70 | 1,280 |
| TA72   | 5                 | 4,545 | 0.3 | 0,000 | 0,780 | 0.74 | 0.78 | 1,557 |
| TA142  | 4                 | 3,333 | 0.4 | 0,000 | 0,700 | 0.65 | 0.70 | 1,280 |
| TA58   | 5                 | 4,167 | 0.3 | 0,000 | 0,760 | 0.72 | 0.76 | 1,505 |
| TA76s  | 2                 | 1,923 | 0.6 | 0,000 | 0,480 | 0.36 | 0.48 | 0.673 |
| TA117  | 5                 | 4,545 | 0.3 | 0,000 | 0,780 | 0.74 | 0.78 | 1,557 |
| TA116  | 3                 | 2,273 | 0.6 | 0,000 | 0,560 | 0.50 | 0.56 | 0.950 |
| TA118  | 3                 | 2,381 | 0.5 | 0,000 | 0,580 | 0.49 | 0.58 | 0,943 |
| Total  | 59                | -       | -       | 0.45| 0,00 | 0,658 | 0.60 | 0.66 | 1,213 |
| Mean   | 3.93              | 3,229 | 0.45 | 0,00 | 0,658 | 0.60 | 0.66 | 1,213 |

Table 3: Genetic distance between the 10 accessions studied.

|                | BLED (6) | ESP1 | F1038 | FLIP 8492C | FLIP 9393C | FLIP 9213C | FLIP 8293 | GARB (9) | ILC482 | CECE NERO |
|----------------|----------|------|-------|------------|------------|------------|-----------|----------|--------|-----------|
| BLED (6)       | 0,00     | 0,67 | 0,80  | 0,60       | 0,73       | 0,47       | 0,67      | 0,57     | 0,87   | 0,87      |
| ESP1           | 0,67     | 0,00 | 0,80  | 0,60       | 0,60       | 0,80       | 0,80      | 0,79     | 0,67   | 0,93      |
| F1038          | 0,80     | 0,80 | 0,00  | 0,80       | 0,73       | 1,00       | 0,73      | 0,71     | 0,80   | 0,87      |
| FLIP 8492C     | 0,60     | 0,60 | 0,80  | 0,00       | 0,73       | 0,80       | 0,93      | 0,79     | 0,80   | 0,87      |
| FLIP 9393C     | 0,73     | 0,60 | 0,73  | 0,73       | 0,00       | 0,67       | 0,80      | 0,64     | 0,80   | 0,87      |
| FLIP 9213C     | 0,47     | 0,80 | 1,00  | 0,80       | 0,67       | 0,00       | 0,67      | 0,50     | 0,80   | 0,73      |
| FLIP 8293      | 0,67     | 0,80 | 0,73  | 0,93       | 0,80       | 0,67       | 0,00      | 0,43     | 0,67   | 0,47      |
| GARB (9)       | 0,57     | 0,79 | 0,71  | 0,79       | 0,64       | 0,50       | 0,43      | 0,00     | 0,71   | 0,71      |
| ILC482         | 0,87     | 0,67 | 0,80  | 0,80       | 0,80       | 0,67       | 0,71      | 0,00     | 0,60   | 0,60      |
| CECE NERO      | 0,87     | 0,93 | 0,87  | 0,87       | 0,87       | 0,73       | 0,47      | 0,71     | 0,60   | 0,00      |
numbered six was clearly differentiated from three clusters; Cluster I contained two accessions (GARB (9), FLIP 82 93) of which one is an Algerian landrace accession grouped with a breeding line; Cluster II included two genotypes (CECE NERO and ILC 482) of which the first CECE NERO is an Italian landrace, the second is an Algerian landrace. In Cluster III we find two genotypes (FLIP9213C and F1038) which the first is a breeding line; the second is an Algerian landrace. However group B included Cluster IV containing two genotypes (BLED (6) and FLIP 8492C) of which the first is an Algerian landrace the second is a breeding line. While cluster V includes the two remaining genotypes (FLIP 9393C, ESP1) of which both of them are breeding line.

Dendogram has clearly delineated the genotypes into two major groups A and B and has relatively demonstrated the existence of a well-defined pattern of relationships between geographical origins, type of accession of chickpea (Kabuli type or Desi type) and genetic diversity. The primary grouping (A) appears to follow type of accession in which we find three clusters, the second cluster (II) contains only two genotypes (ILC 482 and CECE NERO) belonging to Desi type. On the other hand the two remaining clusters (I and III) contain four different genotypes (GARB 9, FLIP 8293, FLIP 9213C and F1038) belonging to Kabuli type. According to this regrouping, the geographical origin of Desi and Kabuli are different; so Desi type’s origin is southern Asia and Ethiopia while Kabuli type’s origin is from Mediterranean region. It is obvious that genotypes of the same geographical origin are typically divided into two clusters; so cluster I and III are separated because of the genetic distance existing between chickpea genotypes. It is obvious that the separation of the genotypes which belong to the same type or the same geographical origin is due to the genetic distance existing between them, so it is the case of the

Fig 3: Dendrogram generated using UPGMA with arithmetic average analysis, showing relationships between 10 chickpea genotypes.

Fig 4: Scatter plot of the first and second principal coordinates (PCoA), after an analysis of genetic diversity derived from 15 microsatellite loci in 10 accessions of chickpea.
Fig 5: Factorial correspondence analysis (AFC) derived from 15 SSR loci in 10 accessions of chickpea.
cluster I and III (4 genotypes of the Kabuli type (GARB 9, FLIP 8293, FLIP 9213C and F1038) were separated into two different cluster I and III. The secondary grouping (B) comprising only the genotypes belonging to the Kabuli type and originating from the Mediterranean region. The separation of the two groups (IV and V) is therefore only due to genetic, in cluster IV contains (BLED and FLIP 8492C) and in cluster V (FLIP 9393C and ESP1); the fact that two accessions contained in the same cluster means that the genetic distance between them is reduced.

A principal coordinate analysis PCoA was also conducted as can be seen in Fig 4. This PCoA reflect 52.69% of the total information generated by this molecular study, this value means that our PCoA is acceptable at the statistical level.

There is no clear separation of the populations, but we can see that we can find two groups the first included (GARB (9), FLIP 8293, CECE NERO and ILC 482, FLIP9213C and F1038) whereas the second group contained (BLED (6), FLIP 8492C, FLIP 9393C and. ESP1). A few populations share the same coordinate values. A similar pattern was observed for the UPGMA dendrogram (group A and group B) (Fig 3).

In the dendrogram and on the PCoA bi-plots, chickpea genotypes were adjoined according to their geographic origin, type of chickpea (Kabuli or desi) and genetic diversity. These results indicate the movement of seeds around the country. The same type of building clusters was found in Hajibarat et al. (2014) and Bharadwaj et al. (2011).

Factorial correspondence analysis (AFC) reveals a rate of 49.13% of the genetic variability included in the 10 genotypes of chickpeas studied. This rate is subdivided into 3 axes of AFC (Fig 5). The first axe accounted for 17.79% whereas the second and the third axes accounted for 16.41% and 14.93% of the total genetic variation, respectively. We can find six different groups; The first included three breeding line (FLIP 8492C, FLIP 9393C and ESP1); the second contained a breeding line (ILC482) and an Italian landrace (CECE NERO).The third group restrained one Algerian landrace (GARB (9) and a breeding line (FLIP 9213C). However three genotypes were completely separated two Algerian landrace (BLES (6) and F1038) and a breeding line FLIP 9213C.

The distribution of the different grouping through the factorial correspondence analysis (AFC) is due to the genetic variability hidden in ten genotypes of chickpea; some of which are breeding line, others are landrace (containing a high genetic diversity) as we know Chickpea is a self-pollinated crop, fertilization occurs before flowering and out crossing is reported to be rare, less than 2% (Tayyar et al., 1996).
CONCLUSION
The existence of genetic diversity in any species is essential for any genetic improvement research, the determination, detection and evaluation of this genetic variability is necessary before proceeding to this approach. We report here a moderate study undertaken in 10 chickpea genotypes to characterize them through genetic structure and allelic diversity, using 20 SSR markers.

Diversity analysis using 15 SSR markers produced 59 alleles, with an average of three alleles per marker. This suggested the presence of considerable polymorphism at the studied microsatellite loci and revealed a moderate level of genetic diversity in the existing chickpea germplasm. So this relatively rich genetic diversity of chickpea genotypes could be considerate as an interesting source for further breeding purposes aimed on improving this crop and must be followed by other more elaborate work.

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