Easy strategy used to detect the genetic variability in chickpea (*Cicer arietinum* L.)

E. Valadez-Moctezuma¹ · A. J. Cabrera-Hidalgo¹

Received: 25 August 2017 / Revised: 17 April 2018 / Accepted: 3 May 2018 / Published online: 13 July 2018
© Prof. H.S. Srivastava Foundation for Science and Society 2018

Abstract A priority in the management and use of elite plant materials for breeding has been based on molecular markers or DNA sequencing of entire genomes, in order to perform genetic differentiation which is still quite costly. Chickpea (*Cicer arietinum*) is one of the species with genomic monotony and very low polymorphism, and its detection even with DNA markers has not been easy. In germplasm banks, the genetic distinction is a priority in order to use properly selected lines. In this study, 57 chickpea accessions from a germplasm bank were analyzed by using nrRAMP (non-radioactive Random Amplified Microsatellite Polymorphism) markers, and their genetic variability was determined. Our results showed DNA polymorphisms, which are enough to differentiate between the accessions and between *C. arietinum* and *Cicer reticulatum* (out-group); this last wild species is closely related to chickpea. We concluded that the nrRAMP technique was an effective and a highly useful method to assess the genetic diversity and variability among closely related plants, such as chickpea; in addition, this technique can be easily implemented in laboratories.

Keywords Chickpea · DNA markers · Genetic diversity · nrRAMP

Introduction

Many cultivars have been analyzed for various purposes using molecular markers. In the early 1990s, several techniques were developed to obtain genetic information from plants. However, not all techniques are efficient in all cultivars and there are species that are difficult, especially those that have high levels of selfing or clones. To estimate genetic diversity in plant populations, many PCR based markers are available, such as germplasm banks, and according to Keneni et al. (2012), the use of such markers is more reliable and repeatable compared to descriptors based on morphological characterization.

Cicer arietinum is a cultivar that has been the subject of an intense selection process for about a hundred years, which has generated genomic monotony (Robertson et al. 1997; Aslam et al. 2013). In this species, several breeding programs have been carried out to improve the genetic potential of the crop and to increase its yield, seed quality, disease resistance, and to improve its nutritional value. During this process, many genes responsible for desirable traits and alleles that are agriculturally favorable have been removed; thus, the result of domestication is the reduction of genetic diversity of the crop compared to its wild relatives (Doebley et al. 2006; Olsen and Wendel 2013).

The chickpea is a cultivated species where various strategies have been used for differentiation purposes. For example, biochemical markers have been used (Kazan and Muehlbauer 1991); but also genetic markers, such as amplified fragment length polymorphism (AFLP) (Nguyen et al. 2003), restriction fragment length polymorphisms

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12298-018-0548-x>) contains supplementary material, which is available to authorized users.

✉ E. Valadez-Moctezuma
evaladez@chapingo.mx

A. J. Cabrera-Hidalgo
ajcabrerahidalgo@gmail.com

¹ Laboratorio de Biología Molecular, Departamento de Fitotecnía, Universidad Autónoma Chapingo, Carr. México- Texcoco km 38.5, C.P. 56230 Chapingo, Edo. México, Mexico

(RFLP) (Udupa et al. 1993; Simon and Muehlbauer 1997), random amplified polymorphic DNA (RAPD) (Ahmad et al. 2010), etc have been used. Nevertheless, generally none of these methods has been efficient enough to show genetic variability due to high homozygosity among accessions of this cultivar, suggesting that *C. arietinum* contains extremely low polymorphism (Winter and Kahl 1995; Hüttel et al. 1999).

On the other hand, SSR markers are one of the most suitable for studying the diversity and genetic variability technique; however, a large number of markers are required to achieve differentiated cultivars like chickpea (Upadhyaya et al. 2008; Saeed et al. 2011).

The RAMP technique (Random Amplified Microsatellite Polymorphism) was reported by Wu et al. (1994) and Zietkiewicz et al. (1994) and has been successfully employed in genetic diversity studies; for example, in barley (Sánchez de la Hoz et al. 1996; Dávila et al. 1999), peach (Cheng et al. 2001), pomegranate (Lihua et al. 2013), *Kengyilia* species (Zhang et al. 2003) and *Leymus* species (Yang et al. 2006). The advantages of RAMP include easy polymorphism detection, wide distribution throughout the genome, as well as easy application and low cost; however, it is difficult to interpret and the technique has poor repeatability (Wu et al. 1994; Provan et al. 1999); nevertheless, the main limitation is particularly that it requires radioactivity. In this study, the aim was to check the availability of RAMP method in chickpea germplasms by estimating the genetic variation of the Mexican chickpea.

Materials and methods

Plant material

Accessions of 57 *Cicer arietinum* genotypes involving germplasm from Mexico and other geographical areas, like the International Center for Agricultural Research in the Dry Areas (ICARDA, Aleppo, Syria) and the Institute of International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Patancheru, India), were provided by the Experimental Field Culiacan Valley Center for Forestry and Agricultural Research (CEVACU), in Sinaloa, Mexico. The *Cicer reticulatum* species was included in the study as an out-group. All genotypes are shown in supplementary material.

DNA isolation and preliminary tests

The total DNA was obtained from 2.5 g of young leaves from five plants per accession, based on the protocol by Dellaporta et al. (1983). Initially, primers for Inter Simple Sequence Repeats (ISSR) (GACA)₄, (GATA)₄ and

(GGAT)₄ (Gibco-BRL[®]) and random primers G-02, G-03, G-09 and G-10 (Operon Technologies Inc., Alameda, CA, USA) were evaluated. The results from the previous evaluation indicated that RAPD did not generate enough information because the profiles were very scarce and extremely limited polymorphisms in chickpea (Table 1) were found, as was previously reported in this species (Ahmad et al. 2010; Winter and Kahl 1995; Udupa, et al. 1993). The ISSR markers were most informative compared to RAPD, achieving at least an inter-specific separation.

Development of the nrRAMP technique

The nrRAMP technique was developed using initiators for RAPD and ISSR markers. These classes of primers have significant advantages because they present important benefits for genotyping, such as their abundance and dispersion in the genomes; thus, they can detect variations and obtain information from DNA fingerprints of different lengths. Both types of primer combinations increase polymorphism detection (Valadez-Moctezuma et al. 2005).

To implement the nrRAMP technique (non-radioactive Random Amplified Microsatellite Polymorphism), the primers (GACA)₄ and (GATA)₄ were selected, which had shown clear and reproducible PCR amplification products, although uninformative when they were independently assessed. Each one of these primers was combined with each random primer, such as G-02, G-03, G-09 and G-10 in PCR. Each test was repeated at least twice. For this purpose, the annealing temperatures were optimized to ensure the functionality of the RAPD-ISSR primer pair selected. PCR was performed in a Perkin Elmer DNA Thermo cycler 480[®] (Perkin Elmer Corp., Norwalk, CT) in a reaction volume of 25 µL containing 50 ng genomic DNA, 200 µM dNTPs, 3 mM MgCl₂ buffer 1X PCR, 20 pM and 1.5 U of Taq polymerase (Gibco-BRL). The thermal program was 93 °C for 1 min, followed by 40 cycles of 93 °C, 20 s; 48 °C (for primer pairs containing (GACA)₄) and 40 °C (for primer pairs containing (GATA)₄), 1 min; 72 °C, 20 s; and a cycle of 72 °C, 10 min for final extension. To find the functional alignment temperature (T_M) for PCR, primers combination (GACA)₄ + G-02, G-03, G-09 or G-10 and (GATA)₄ + G-02, G-03, G-09 and G-10, were initially amplified at 35, 40 and 48 °C. Based on the banding profiles obtained and the reproducibility, the T_M used in the technique for nrRAMP couples containing (GACA)₄ was 48 °C, and for (GATA)₄ it was 40 °C.

Detection and analysis of amplified products

The amplified products were separated on agarose gels 1.4% (Separide[®] Gel Matrix, Gibco-BRL) in TAE buffer (40 mM Tris–HCl, pH 8.0; 20 mM sodium acetate, 2 mM

Table 1 Efficiency and characteristics of the RAPD, ISSR and nrRAMP primers used in the analysis of 57 chickpea accessions (*Cicer arietinum* L.)

Type of primer	Primers	T _M (°C)	Total bands	PB	PPB	Rp	PIC	MI
RAPD	G-02	*	2	**	–	–	–	–
	G-03	*	2	**	–	–	–	–
	G-09	*	3	**	–	–	–	–
	G-10	*	4	**	–	–	–	–
ISSR	(GACA) ₄	48	10	4	40	1.51	0.113	0.451
	(GATA) ₄	40	1	0	0	0.00	0.000	0.000
	(GGAT) ₄	48	5	3	60	0.74	0.126	0.737
nrRAMP	(GACA) ₄ /G-02	48	15	7	46.67	1.89	0.098	0.688
	(GACA) ₄ /G-03	48	15	2	13.33	0.70	0.033	0.067
	(GACA) ₄ /G-09	48	16	10	62.50	4.17	0.187	1.867
	(GACA) ₄ /G-10	48	8	0	0.000	0.00	0.000	0.000
	(GATA) ₄ /G-02	40	12	6	50.00	1.79	0.117	0.704
	(GATA) ₄ /G-03	40	13	4	30.77	1.61	0.085	0.341
	(GATA) ₄ /G-09	40	14	5	35.71	2.00	0.106	0.531
	(GATA) ₄ /G-10	40	15	4	26.67	2.53	0.094	0.378
	Means		13.50	4.75	33.21	1.84	0.090	0.572
	Total		108	38	265.65	14.7	0.722	4.577

*Annealing Temperature 35, 40 and 48 °C

**It was not evaluated in all germplasm

T_M = annealing temperature; PB polymorphic bands, PPB percentage of polymorphic bands, Rp resolving power, PIC polymorphic information content, MI marker index

EDTA). Molecular weight markers of 100 bp (Fermentas) and one kb (Gibco-BRL) were used. The electrophoresis was run at 80 V for 2.5 h, and the gels were stained with ethidium bromide (0.5 mg/mL) in TAE buffer. All gels were recorded and digitally analyzed using the Kodak Digital Science[®] DC 120 system (Eastman Kodak, Co., Rochester, NY, USA).

Data analysis

nrRAMP fragments of each accession were recorded in a binary matrix as qualitative characteristics for presence (1) or absence (0). Then, the data were processed with the NTSYSpc 2.2 (Exeter Software, Setauket, NY, USA) software, to generate a genetic distance matrix using the Dice coefficient (Dice 1945). Using the resulting matrix, dendrograms were constructed through a clustering method UPGMA (Unweighted Pair Group Method with Arithmetic mean algorithm) with 1000 bootstrapping repetitions. The efficiencies of the nrRAMP technique were contrasted with the results obtained with RAPD and ISSR through polymorphic information content (PIC), marker index (MI) and the resolving power (Rp). The percentage of polymorphic bands (PPB) was also calculated. The wild accession (*Cicer reticulatum*) was included in the analyses to compare the clusters.

Results

Preliminary tests

The results obtained using RAPD markers, as expected, were not satisfactory; they were obtained as very few DNA fragments (2–5), but they were also not informative to differentiate accessions (Table 1). The ISSR type markers obtained with primers (GACA)₄, (GATA)₄ and (GGAT)₄ also showed few bands defined in all the germplasm (10, 1 and 5, respectively) (Table 1). Low values of MI and PIC indicated that these primers were not efficient to make an intra-specific genetic differentiation between chickpea cultivars.

nrRAMP profiles

All combinations of primers used for the nrRAMP technique detected several polymorphisms in 57 chickpea accessions, except (GACA)₄ combined with G-10. Polymorphic bands could distinguish each accession; however, they did not do so for accessions 46 K and 56 K. This technique produced a total of 108 bands, of which 38 were polymorphic (35.18%) and 70 monomorphic (64.81%). The total number of bands per primer pair nrRAMP varied between 8 and 16 (Table 1). The polymorphism percentage ranged from 0.00% (GACA)₄/G-10 to 62.5% (GACA)₄/G-

Fig. 1 DNA profiles in 22 chickpea accessions (*Cicer arietinum* L.) separated in agarose gel 1.4%. The efficiency of the nrRAMP technique is shown: **A** with the primers (GATA)₄; **B** (GATA)₄/G-02;

C (GATA)₄/G-03. Some polymorphisms are indicated with arrows. M1 = 1 kb marker (GibcoBRL) and M2 = 100 bp marker (MBI Fermentas)

09), with an average of 33.21%. Rp values ranged from 0.00 (GACA)₄/G-10) to 4.17 (GACA)₄/G-09) with an average of 1.84 (Table 1). The primer (GATA)₄/G-09 was the most efficient to separate the chickpea's genotype, presenting the highest Rp values. The PIC ranged from 0.0 (GACA)₄/G-10) to 0.187 (GACA)₄/G-09) with an average of 0.090, and the marker index ranged from 0.0 (GACA)₄/G-10) to 1867 (GACA)₄/G-09) with average values of 0.572.

The marker index of nrRAMPs was obtained in a good range, indicating that they are a powerful molecular system marker for the characterization of *C. arietinum* L. cultivars. Finally, (GACA)₄/G-09 and (GATA)₄/G-02 provided the most information on the genetic variation of chickpea genotypes under study.

Oligonucleotide (GATA)₄ only detected one evidently monomorphic band (Fig. 1A), while primers (GGAT)₄ and (GACA)₄ amplified five and 10 fragments, respectively, of

Fig. 2 Profiles nrRAMP of 57 chickpea accessions separated in agarose gel 1.4% using the combination of primers (GATA)₄/G-09. Arrows indicate some polymorphisms. Lines 1–57 = Accession's

chickpea (*C. arietinum* L.) and line 58 = *Cicer reticulatum*. M1 = 1 kb marker (GibcoBRL) and M2 = 100 bp marker (MBI Fermentas)

which three and four were polymorphic, representing 60 and 40% of total polymorphism. Although this percentage is apparently high, it really was not significant due to the low amount of amplified bands. With these primers, a low Rp (0.0–1.51) (Table 1) value was obtained. In our experience, this technique may be very informative when primer pairs to combine have similar alignment temperatures. However, it is always necessary to optimize the TM for alignment in PCR to obtain a pattern of more abundant and reproducible banding.

The Fig. 1 shows the fingerprints of 22 *C. arietinum* accessions using (GATA)₄ (panel A) and fingerprints of the same accessions with (GATA)₄/G-02 (panel B) and (GATA)₄/G-03 (panel C). Combination of primers for obtaining nrRAMP markers showed a higher amount of DNA bands and more polymorphisms. In Fig. 2, the fingerprints obtained with nrRAMP are shown; they were obtained with (GATA)₄/G-09 but in all chickpea germplasm analyzed, including *C. reticulatum*. The DNA profiles shown in this Figure were the most representative of nrRAMP technique in this study.

Fig. 3 UPGMA dendrogram for 57 accessions of chickpea with the similarity coefficient Nei and Li/Dice using nrRAMP markers. Grouping is defined at a distance of 0.89. *Cicer reticulatum* (Cret)

The dendrogram obtained with the 108 nrRAMP markers and grouped with UPGMA facilitated the discrimination among the 57 genotypes of chickpea (Fig. 3). UPGMA could establish interspecific and intraspecific differences with *C. reticulatum*, represented by several groups based on genetic distance.

The nrRAMP analysis separated all accessions of *C. arietinum* of *C. reticulatum* (wild species, outgroup). The largest group (Group A) separated the 41 accessions into four major subgroups (I–IV); the first subgroup was represented by 14 accessions Kabuli type and only one Desi (10D) type. They are characterized by upright growth habit, bipinnate leaf and cream color angular seed, and medium to large size, white flowers and wrinkled seed. The second subgroup (12 accessions) was mainly composed of Desi type accessions (eight), characterized by erect habit with bipinnate leaves, variable color angular seed, and small to medium-size, surface not smooth seed, flowers with pink and white tonalities. The third subgroup was characterized by containing a mixture of Kabuli (7) and Desi (6) chickpeas. This subgroup was distinguished for sharing upright and spreading growth, and bipinnate chickpea leaves. The other characteristics were highly variable. The subgroup four, represented only by the

was used as a comparative group. The UPGMA clustering was constructed using the NTSYSpc 2.2 software

genotype 30D had plants with bushy growth, bipinnate leaves, pink flowers, angular seeds with dark-brown color, small size and tuberculate surface.

Group B was composed by erect plants with bipinnate leaves, large angular seeds with creamy color, rough surface and white flowers. In this group, accessions 13D, 50D, 41D and 27D (Desi type) showed different characteristics compared to the rest of the group, which consisted of plants with angular and rounded medium to large seeds with brown coloring, and rough and smooth seed coat. The UPGMA clustering based on nrRAMP markers allows better separation of 57 chickpea genotypes, showing important intra- and inter-specific differences between the accessions studied.

Discussion

According to Wu et al. (1994), the RAMP technique combines the advantages of microsatellite markers (SSR) and RAPD type, proving to be potentially valuable in genetic diversity studies using molecular markers. These qualities in the combination of the primers used throughout this study, allowed more abundant and reproducible

banding profiles with regards to those obtained with RAPD and ISSR primers evaluated separately, but without radioactivity or acrylamide.

The RAMP type markers have been used in studies of genetic diversity in several important cultivars and fruit plants (Dávila et al. 1999; Cheng et al. 2001; Zhang et al. 2003; Yang et al. 2006; Lihua et al. 2013), but in *Fusarium* too (Alanís-Martínez et al. 2015), and by Avila-Treviño et al. (2017) who studied differentiation in plants propagated in vitro.

Currently, there are no reports of this technique for studies of genetic variability in chickpea; so this work is the first study of this nature in the evaluation of germplasm of this important legume in a macro-geographic level with very promising results.

The nrRAMP technique is successful, providing useful preliminary information at the species level; its use could support the selection phase preliminary to screening germplasm banks or large amounts of plant material to evaluate and maintain in-breeding programs. This will significantly reduce operating costs and time. Furthermore, this technique showed practical advantages such as simplicity, speed and applicability and ability to differentiate genotypes within a species with high levels of selfing such as chickpea.

The genotypes used in this study showed no relationship between genetic divergence and geographic origin, because accessions from one specific place were grouped into more than one cluster. Similar results were reported by Talebi and Rokhzadi (2013) for accessions of chickpea land races collected in the Province of ‘Kurdistan’, in northwestern Iran. Finally, accessions identified in this study as 46 K and 56 K (Kabuli type) showed identical nrRAMP profiles. This could be due to their close genetic relationship or because these two genotypes were classified as different when they are in fact the same.

Conclusions

The nrRAMP technique is effective and highly useful for evaluating the diversity and genetic similarity between species and chickpea accessions.

References

- Ahmad F, Khan AI, Awan FS, Sadia B, Sadaqat HA, Bahadur S (2010) Genetic diversity of chickpea (*Cicer arietinum* L.) germplasm in Pakistan as revealed by RAPD analysis. *Gen Mol Res* 9:1414–1420
- Alanís-Martínez I, Medina-Mendoza C, Marban-Mendoza N, Valadez-Moctezuma E (2015) Técnicas moleculares clásicas para la diferenciación de formas especiales de *Fusarium oxysporum*. *Revista Mexicana de Micología* 42:1–7
- Aslam M, Maqbool MA, Akhtar S, Faisal W (2013) Estimation of genetic variability and association among different physiological traits related to biotic stress (*Fusarium oxysporum* l.) in chickpea. *J Anim Plant Sci* 23:1679–1685
- Avila-Treviño JA, Muñoz-Alemán JM, Pérez-Molphe-Balch E, Rodríguez-Sahagún A, Morales-Domínguez JF (2017) *In vitro* propagation from bud and apex explants of *Moringa oleifera* and evaluation of the genetic stability with RAMP marker. *S Afr J Bot* 108:149–156
- Cheng HY, Yang WC, Hsiao JY (2001) Genetic diversity and relationship among peach cultivars based on Random Amplified Microsatellite Polymorphism (RAMP). *Bot Bull Acad Sin* 42:201–206
- Dávila JA, Loarce Y, Ferrer E (1999) Molecular characterization and genetic mapping of random amplified microsatellite polymorphism in barley. *Theor Appl Genet* 98:265–273
- Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA miniprep: version II. *Plant Mol Biol Report* 1:19–21
- Dice LR (1945) Measures of the amount of ecological association between species. *Ecology* 26:297–302
- Doebley JF, Gaut BS, Smith BD (2006) The molecular genetics of crop domestication. *Cell* 127:1309–1321
- Hüttel B, Winter P, Weising K, Choumane W, Weigand F, Kahl G (1999) Sequence-tagged microsatellite site markers for chickpea (*Cicer arietinum* L.). *Genome* 42:210–217
- Kazan K, Muehlbauer FJ (1991) Allozyme variation and phylogeny in annual species of *Cicer* (Leguminosae). *Plant Syst Evol* 175:11–21
- Keneni G, Bekele E, Imtiaz M, Dagne K, Getu E, Assefa F (2012) Genetic diversity and population structure of Ethiopian chickpea (*Cicer arietinum* L.) germplasm accessions from different geographical origins as revealed by microsatellite markers. *Plant Mol Biol Report* 30:654–665
- Lihua Z, Mingyang L, Guangze C, Tianchun P, Chenghai S (2013) Assessment of the genetic diversity and genetic relationships of pomegranate (*Punica granatum* L.) in China using RAMP markers. *Sci Hortic* 151:63–67
- Nguyen TT, Taylor PWJ, Redden RJ, Ford R (2003) Genetic diversity estimates in *Cicer* using AFLP analysis. *Plant Breed* 123:173–179
- Olsen KM, Wendel JF (2013) A bountiful harvest: genomic insights into crop domestication phenotypes. *Annu Rev Plant Biol* 64:47–70
- Provan J, Thomas WTB, Forster BP, Powel W (1999) Copia-SSR: a simple marker technique which can be used on total genomic DNA. *Genome* 42:363–366
- Robertson LD, Ocampo B, Singh KB (1997) Morphological variation in wild annual *Cicer* species in comparison to the cultigen. *Euphytica* 95:309–319
- Saeed A, Hovsepyan H, Darvishzadeh R, Imtiaz M, Panguluri SK, Nazaryan R (2011) Genetic diversity of Iranian accessions, improved lines of chickpea (*Cicer arietinum* L.) and their wild relatives by using simple sequence repeats. *Plant Mol Biol Report* 29:848–858
- Sánchez de la Hoz MP, Dávila JA, Loarce Y, Ferrer E (1996) Simple sequence repeat primers used in polymerase chain reaction amplifications to study genetic diversity in barley. *Genome* 39:112–117
- Simon CJ, Muehlbauer FJ (1997) Construction of a chickpea linkage map and its comparison with maps of pea and lentil. *J Hered* 88:115–119
- Talebi R, Rokhzadi A (2013) Genetic diversity and interrelationships between agronomic traits in landrace chickpea accessions

- collected from ‘Kurdistan’ province, North-west of Iran. *Int J Agric Crop Sci* 5:2203–2209
- Udupa SM, Sharma A, Sharma RP, Pai RA (1993) Narrow genetic variability in *Cicer arietinum* L. as revealed by RFLP analysis. *J Plant Biochem Biotechnol* 2:83–86
- Upadhyaya HD, Dwivedi SL, Baum M, Varshney RK, Udupa SM, Gowda CLL, Hoisington D, Singh S (2008) Genetic structure, diversity, and allelic richness in composite collection and reference set in chickpea (*Cicer arietinum* L.). *BMC Plant Biol* 8:106–118
- Valadez-Moctezuma E, Kahl G, Rubluo-Islas A, Arreguín de los Monteros R (2005) Optimización de las huellas de DNA obtenidas con RAPDs y MP-PCR mediante la técnica nrRAMP. *Revista Chapingo Serie Horticultura* 11:351–356
- Winter P, Kahl G (1995) Molecular marker technologies for plant improvement. *World J Microbiol Biotechnol* 11:438–448
- Wu K, Jones R, Danneberger L, Scolnik PA (1994) Detection of microsatellite polymorphisms without cloning. *Nucleic Acid Res* 22:3257–3258
- Yang RW, Zhou YH, Zhang Y, Zheng YL, Ding CB (2006) The genetic diversity among *Leymus* species based on random amplified microsatellite polymorphism (RAMP). *Genet Resour Crop Evol* 53:139–144
- Zhang L, Zhou Y, Wei Y, Zheng Y, Liu S (2003) Relationships among *Kengyilia* species based on random amplified microsatellite polymorphism (RAMP). *High Technol Lett* 4:28–33
- Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176–183