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Optimization of Conditions for Assessment of Genetic Diversity in Chickpea (*Cicer arietinum* L.) Using SSR Markers

Khalil Khamassi[1,2,3*] • Sahari Khoufi[1,2,3] • Ramzi Chaabane[1] • Jaime A. Teixeira da Silva[4] • M'barek Ben Naceur[1]

[1] Laboratoire de Biotechnologie et Physiologie Végétale, National Agronomic Research Institute of Tunisia, Hedi Karray Street, 2049 Ariana, Tunisia
[2] Institut National Agronomique de Tunis, Avenue Charles Nicolle Tunis 1082, Tunisia
[3] Banque Nationale de Gènes, Boulevard du Leader Yasser Arafat, Charguia 1, 1080 Tunisia
[4] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Ikenobe, Miki-cho, 761-0795, Japan

Corresponding author: ***khalilkhamassi@yahoo.com; khkobe@hotmail.com; khalil.khamassi@bng.nat.tn**

ABSTRACT

The present study was carried out to optimize SSR-PCR conditions for assessment of genetic diversity in chickpea (*Cicer arietinum* L.) through the use of microsatellite markers. Parameters optimized for 16 SSR markers included template DNA concentration, *Taq* polymerase, primers and $MgCl_2$ concentration. Optimal template DNA concentration with consistent results was 50 ng/µL, which corresponds to 100 ng for a 25-µL reaction volume. Optimal concentrations for primers, $MgCl_2$ and *Taq* polymerase were 2.5 mM and 1.5 mM, 1 U/µL respectively.

Keywords: chickpea, DNA, microsatellite optimization, PCR, SSR, template

INTRODUCTION

Chickpea (*Cicer arietinum* L.), one of the earliest domesticated grain legume crops (Van der Maesen 1972) that is important in semi-arid areas of Central, South and South-East Asia, Southern Europe, Northern and Eastern Africa, as well as the Americas and Australia, is third in terms of production, following dry beans and peas (FAOSTAT 2005). The breeding of this crop through the use of conventional techniques did not lead to expected results; marker-assisted selection allows the transfer of useful genetic information in a more precise and controlled way (Vural and Akcin 2011). Despite this, chickpea is considered to have minimal genetic polymorphism (Ahmad and Slinkard 1992; Udupa *et al*. 1993; Labdi *et al.* 1996); breeding crop programs in recent years, especially for chickpea, have been facilitated by the use of DNA molecular markers (Millan *et al.* 2006). According to Sethy (2006), several studies that used biochemical markers (Ahmad *et al.* 1992; Labdi *et al*. 1996) and molecular markers such as restriction fragment length polymorphism (RFLP) (Udupa *et al.* 1993) and random amplified polymorphism (RAPD) (Sant *et al.* 1999; Choudary *et al*. 2002; Iruela *et al.* 2002; Sudupak *et al.* 2002) failed to detect genetic diversity within chickpea. Indeed, the studies proved that microsatellites, which are abundant in the genome (San *et al.* 1999; Udupa *et al.* 1999; Udupa and Baum 2001), could be used for detecting genetic variation within chickpea species (Huttel *et al.* 1999; Udupa *et al.* 1999), and could be used in map construction (Winter *et al.* 1999, 2000; Flandez-Galvez *et al.* 2003). Microsatellites or simple sequence repeats (SSRs) are also considered to be the markers of choice for assessment of genetic diversity and practical plant breeding as they not only are co-dominant but also have the highest information content (Gupta and Varshney 2000).

The investigation of genetic diversity between some wild relatives of chickpea in comparison to the cultivated species '*arietinum*' led to the conclusion that inter simple sequence repeat (ISSR) is superior to RAPD for fingerprinting chickpea geneotypes; however, SSR markers, which are abundant in the plant genome, are considered to be the major source of genetic variation in quantitative traits (Vural and Akcin 2011). Recently, the "Generation Challenge Program", a project between two institutes, ICRISAT (Patancheru, India) and the International Center for Agricultural Research in the Dry Areas (ICARDA, Aleppo, Syria), provided the international community with a genotyping kit that could serve as a reference kit to compare between genetic diversity in other studies (Hoisington *et al.* 2007). Primers within that kit were used to investigate genetic variation within a Tunisian collection of chickpea and SSR primers could be effectively used to estimate the relationships between a local landrace and new lines as a new tool in breeding programs that for 20 years had utilized conventional selection methods (Khamassi *et al.* unpublished). This study forms part of a project on the assessment of genetic diversity in a Tunisian winter chickpea collection. PCR conditions were optimized for 35 SSR markers in chickpea.

MATERIALS AND METHODS

The chickpea germplasm used in this study that was obtained from the Field Crops Laboratory of the Institut National de la Recherche Agronomique de Tunisie (INRAT) consists of one Tunisian spring landrace 'Amdoun', six new lines introduced from ICARDA and varieties 'Bochra', 'Neyer', 'Beja1', 'Kasseb' and 'Chetoui' registered in the official catalogue of plant varieties of Tunisia. The elite seeds were hand sown on 26 November in one row 2 m long with an inter-row spacing of 50 cm and an inter-plant distance of 35 cm. Hand weeding was performed regularly as was the application of pesticides, namely λ-Cyalothrine at 25 g/l (Sygenta) and Deltamethrine at 25% (Bayer Crop Science). No irrigation or fertilization was required and the experiment was conducted under rainfed conditions. A total of 35 SSR markers were screened but only 16 were selected using a kit (kit project GCP: 2005-CB05j, Chickpea Molecular Marker Reference Kit, ICRISAT) (Hoisington *et al.* 2007). The forward and reverse primer sequences are described in **Table 1**.

All experiments listed next were repeated three times.

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Research Note

Table 1 Characteristics of primers (ICARDA-ICRISAT 2007).

Primer	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Source
CaSTMS21	CTACAGTCTTTTGTTCTTCTAGCTT	ATATTTTTTAAGAGGCTTTTGGTAG	Huttel 1999
TA27	GATAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	Winter 1999
TA71	CGATTTAACACAAAACACAAA	CCTATCCATTGTCATCTCGT	Winter 1999
TA116	AATTCAATGACGAATTTTTATAAGGG	AAAAAGAAAAGGGAAAAGTAGGTTTTA	Winter 1999
TA117	GAAAATCCCAAATTTTTCTTCTTCT	AACCTTATTTAAGAATATGAGAAACACA	Winter 1999
TA118	ACAAGTCACATGTGTTCTCAATA	GGAAAGGTTAAGAAATTTTACAATAC	Winter 1999
TA135	TGGTTGGAAATTGATGTTTT	GTGGTGTGAGCATAATTCAA	Winter 1999
TA200	TTTCTCCTCTACTATTATGATCACCAG	TTGAGAGGGTTAGAACTCATTATGTTT	Winter 1999
TAA58	CATTGCTTAAGAACCAAAATGG	CAATTTTACATCGACGTGTGC	Winter 1999
TaaSH	GGTAGACGCAAAAGAGTGGG	GCCACATTGACCAGGAATG	Winter 1999
TR2	GGCTTAGAGTTCAAAGAGAGAA	AACCAAGATTGGAAGTTGTG	Winter 1999
TR29	GCCCACTGAAAAATAAAAAG	ATTTGAACCTCAAGTTCTCG	Winter 1999
TR31	CTTAATCGCACATTTACTCTAAAATCA	ATCCATTAAAACACGGTTACCTATAAT	Winter 1999
NCPGR4	TTACAGCTTGTGCTCAG	AGTCAGATTCTTATCCGA	Sethy 2003
NCPGR12	CCTTGTTAGTGTGTATAGGT	GTAATGACCAAGTGAACA	Sethy 2003
NCPGR19	TCCATTGTAGCTTAGCTTAG	TCTTACTCTTAGCTTACCTCTT	Sethy 2003

DNA extraction and quantification

Genomic DNA was extracted according to the Ben Naceur (1998) protocol, which combines three methods described by Murray and Thompson (1980), Saghai-Maaroof *et al*. (1984) and Webb and Knap (1990), and is based on the CTAB method. DNA was quantified on a 0.8% agarose (Sigma-Aldrich, St. Louis, MI, USA) gel stained with 0.5 mg/ml ethidium bromide (EtBr). The gel was run at 70 V for 1.5 hr in 1X TBE buffer. DNA concentration was assessed by comparison to λ *Hind*III DNA ladder (Promega) (**Fig. 1**).

Optimisation of PCR conditions

In order to determine the optimal template DNA concentration, seven different concentrations of template DNA in 25 µL (25, 50, 75, 100, 125, 150 and 200 ng/µl) were tested. Different concentration of $MgCl_2$ ranged from 0.5 to 2.5 mM. Five concentrations of *Taq* Polymerase (GOtaq, Promega) were tested: 1, 1.5, 2, 3 and 4 U/25 µl. The primer concentration used in our lab for SSR/microsatellite genotyping is 2.5 µM. Five concentrations ranging from 1.5 to 3 µM of forward and reverse primers were used to verify if this concentration gave the same results with chickpea germplasm, or not.

PCR amplification and electrophoresis

The amplification program was run in a thermocycler (Biometra UNO-Thermoblock; BIOTRON, Gottingen, Germany). It consisted of a pre-denaturation step at 94°C for 3 min followed by 35 amplification cycles (1 min denaturation at 94°C, 1 min annealing, and 2 min extension at 72°C) and a final post-extension step for 5 min at 72°C. PCR amplification products were electrophoresed in a 2% agarose gel containing EtBr only for the optimization experiments. After the optimization step, electrophoresis was performed in a 6% polyacrylamide gel (Promega) in addition to the use of a 100-bp DNA ladder (Promega). Amplified products were photographed using on a Bio-Rad 170-8170 Gel Doc XR System, (Bio-Rad, USA) after staining the gel with 0.5 mg/ml EtBr.

RESULTS AND DISCUSSION

Template DNA concentration

DNA at all concentrations could be detected except for 25 ng/µL template DNA (**Fig. 2**); optimal consistent results were observed with 50 ng/µL corresponding to 100 ng template DNA for a 25-µL reaction volume. Ahmed *et al,* (2009), who worked on optimization of PCR for the use of SSR in barely, reported that a high DNA concentration may decrease PCR efficiency due to the presence of contaminants during DNA preparation; those findings agreed with of those found by Kramer and Coen (2004). The template DNA concentration strongly influences the outcome of the reaction as the quality and quantity of DNA greatly affects the success of PCR (Rahman *et al.* 2008; Ahmed *et al.* 2009). Various studies conducted on chickpea genetic diver-

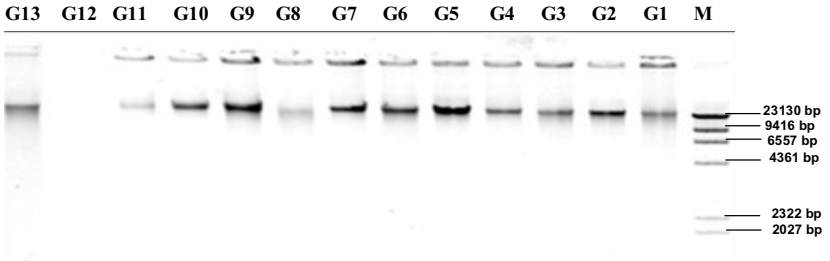

Fig. 1 DNA quantification in 0.8% agarose gel. M: λ *Hind*III marker; G1: 'Kasseb'; G2: 'Béja1'; G3: 'Chetoui'; G4: 'Amdoun'; G5: 'Béja2'; G6: 'Bochra'; G7: 'Neyer'. G8: lane 1, G9: lane 2; G10: lane 3; G11: lane 4; G12: lane 5; G13: lane 6.

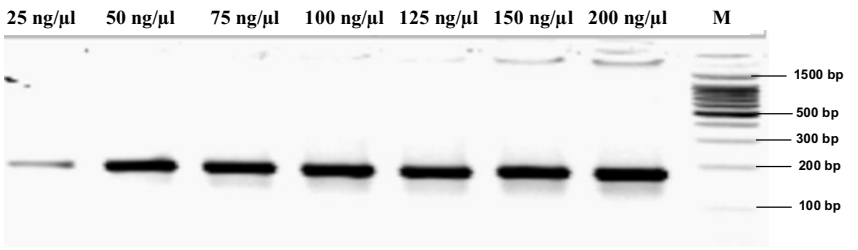

Fig. 2 Electrophoreses profile of the amplicons for genotype 5 'Beja2' in 2% agarose gel with SSR primer TR29. M: 100-bp marker, 25, 50, 75, 100, 125, 150 and 200 ng/µl DNA concentrations following PCR amplification.

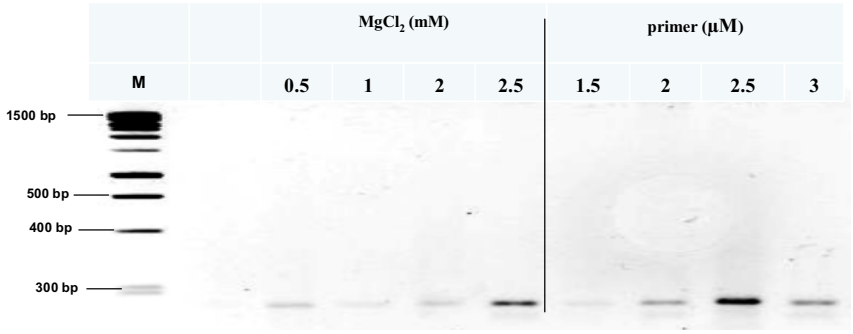

Fig. 3 Variable concentrations of forward and reverse primers and $MgCl_2$, electrophoresis profiles of the amplicons for genotype 5 'Béja2' in 2% agarose gel with primer TR29.

sity with SSR markers used different template DNA concentrations: 30 ng for a 10-µL reaction volume (Choudary *et al.* 2006), 25 ng for a 20-µL reaction volume (Jomova *et al.* 2005), 50 ng for a 20-µL reaction volume (Winter *et al.* 1999), 20 ng for a 20-µL reaction volume (Bhagyawantn and Srivastava 2008), and 60 ng for a 25-µL reaction volume using ISSR markers (Vural and Akcin 2010).

Optimization of $MgCl_2$ and *Taq* Polymerase concentration

Mg ions bind tighly to the phosphate sugar backbone of nucleotides and nucleic acids, thus variation in the concentration of $MgCl_2$ has strong effects on nucleic acid interaction (Rahman *et al*. 2002). For chickpea, $MgCl_2$ concentration was 1.5 mM (Winter *et al*. 1999; Jomova *et al.* 2005; Vural *et al*. 2010), or 1-4 mM (Upadhyaya *et al.* 2008). Mg^{2+} ions influence the enzymatic activity of *Taq* polymerase. In fact, $MgCl_2$ gave poor amplification in RAPD analysis of *Urginia indica* germplasm due to inadequate *Taq* polymerase activity (Harini *et al.* 2008). Rahman *et al.* (2000) and Ahmed *et al*. (2009), who optimized SSR in a conifer and in barely, respectively tested 0.5-3.5 and 1.5-3.5 mM $MgCl_2$, respectively and observed best results with 1 mM and 2.5 $MgCl_2$, respectively. However, in this study, the results were positive for 2.5 µM of primers and 2.5 mM of $MgCl_2$ (**Fig. 3**).

Taq polymerase concentration ranging from 2 to 2.5 U is normally used in 100 µl final volume (Rehman *et al*. 2002). According to Vural *et al.* (2010), who worked on PCR optimization for assessing genetic diversity of chickpea by RAPD and ISSR and Harini *et al* (2008), who worked on optimizing RAPD in *Urginia indica*, a high concentration *of Taq* polymerase resulted in decreased specificity and background (smear) formation upon gel electrophoresis. Upadhyaya *et al.* (2008) used 0.4 U (Qiagen *Taq* Applied biosystem) for the same primers that we used in this study for chickpea with touch down PCR methodology, although for our study best amplification was possible with 2 U/µl of Go *Taq* polymerase (Promega) (**Fig. 4**).

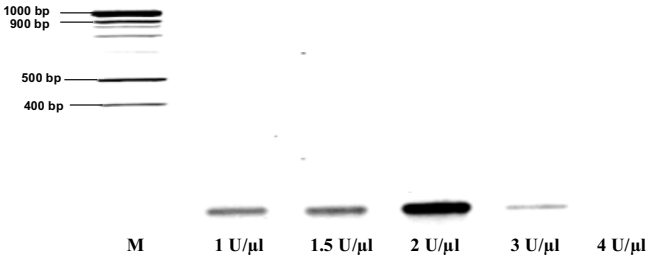

Fig. 4 Variable concentrations of *Taq* polymerase, electrophoresis profile of the amplicons for genotype 5 'Beja2' on a 2% agarose gel with SSR primer TR29. M: 1-kb ladder.

SSR marker selection

The use of the optimum reaction mixture wich combined all chosen concentrations of DNA, *Taq* Polymerase and $MgCl_2$ led to good amplification (**Table 2**): bands were sharp, polymorphism was clear both on a 2% agarose gel (**Fig. 5**) and on a 6% polyacrylaminde gel (**Fig. 6**). Genetic diversity was tested after optimizing the PCR reaction mix which gave good results for 16/35 primers. Polymorphism could be detected in both polyacrylamide and agarose gels; 8 primers gave monomorphic bands in the agarose gel (Ta72, Ta28, Ta14, Castms21, Ta113, Ta130, Ta22, NCPGR 24; **Fig. 7**).

The use of SSR markers in chickpea is a useful tool since many of those markers, such as TA110, flank a single resistance gene to *Fusarium oxysporom* (foc5) race 5 which has been mapped to LG 2 or linked to some quantitative trait loci (QTLs) associated with resitance to *Ascochyta* such as TR58 and TS82 (Iruela *et al*. 2007). Bhagyawantn and Srivastava (2008) indicated that the utility of ISSR markers lies in converting them into sequence-tagged microsatellites sites (STMS) in marker-aided selection. Previous studies in chickpea genetic diversity, which were based on RAPD, RFLP, amplified fragment length polymorphism (AFLP), ISSR and SSR, showed that large diversity was

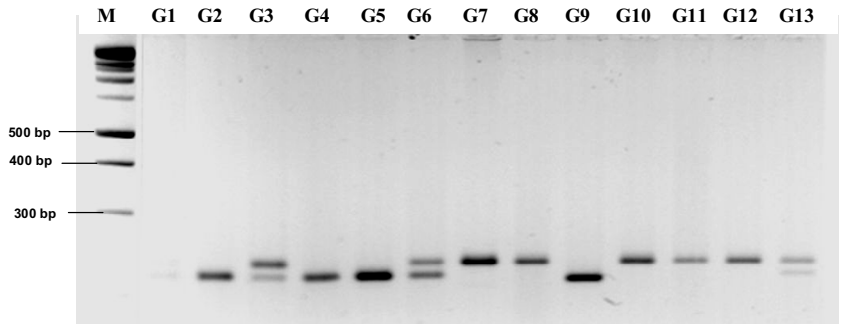

Fig. 5 Profile of the electrophoresis product of DNA amplification of different chickpea genotypes by SSR primer NCPGR12 on a 2% agarose gel. M: 100-bp ladder. Lanes 1-5 = chickpea samples. G1: 'Kasseb'; G2: 'Béja1'; G3: 'Chetoui'; G4: 'Amdoun'; G5: 'Béja2'; G6: 'Bochra'; G7: 'Neyer'. G8: lane 1, G9: lane 2; G10: lane 3; G11: lane 4; G12: lane 5; G13: lane 6.

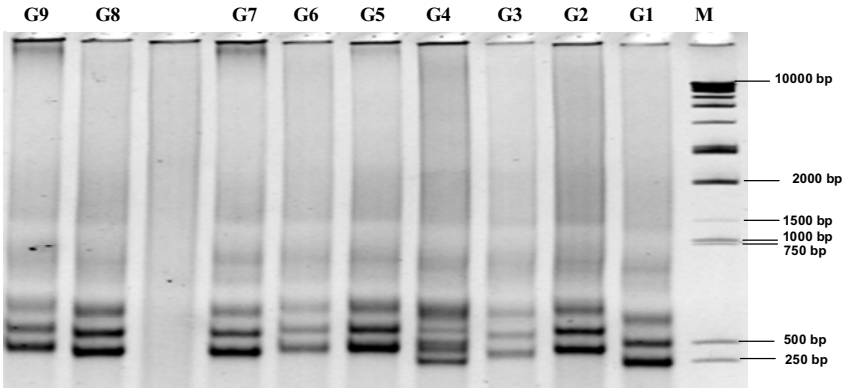

Fig. 6 Profile of the electrophoresis products of DNA amplification of different chickpea genotypes by SSR primer TA117 on a 6% polyacrylamide gel. M: 1-kb ladder. Lanes 1-2 = chickpea samples. G1: 'Kasseb'; G2: 'Béja1'; G3: 'Chetoui'; G4: 'Amdoun'; G5: 'Béja2'; G6: 'Bochra'; G7: 'Neyer'. G8: lane 1, G9: lane 2.

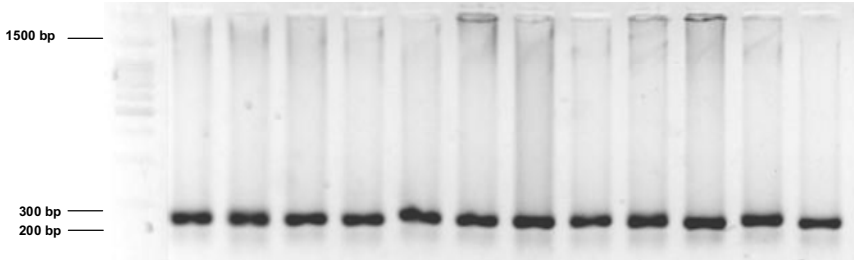

Fig. 7 Profile of the electrophoresis products of DNA amplification of different chickpea genotypes by SSR primer TA22 on a 2% agarose gel. M: 100-bp ladder. Lanes 1-6 = chickpea samples. G1: 'Kasseb'; G2: 'Béja1'; G3: 'Chetoui'; G4: 'Amdoun'; G5: 'Béja2'; G6: 'Bochra'; G7: 'Neyer'. G8: lane 1, G9: lane 2; G10: lane 3; G11: lane 4; G12: lane 5; G13: lane 6.

Table 2 Final optimized SSR mix.

Component	Concentration	Volume (µl)
DNA	50 ng /µl	2
Buffer	5 X	5
dNTP	2 mM	2.5
Primer (Forward)	2.5 µM	2.5
Primer (Reverse)	2.5 µM	2.5
Taq polymerase (Promega)	1 U/µl	0.2
$MgCl_2$	2.5 mM	1.5
H_2O		8.8

found in wild cicer with lower polymorphism in the cultivated species, *Cicer arietinum* (Sharma *et al.* 1995; Shan *et al.* 2005; Upadhyaya *et al.* 2008). Several efforts were made to discover and characterize new SSR marker in chickpea, as reported by Winter *et al.* (1999) and Varshney *et al*. (2007). Despite the limited studies based on SSR markers to study molecular diversity in chickpea, sufficient polymorphism was found which permitted the construction of a genetic linkage map and the identification of useful QTLs (Winter *et al.* 2000; Iruela *et al*. 2007).

The 'Generation Challenging Program' consists of the assessment of genetic diversity of 3000 chickpea accessions with the same SSR primers described in this manuscript; those markers have been recommended for use in investigation in genetic diversity in chickpea germplasm. Upadhyaya *et al*. (2008) reported that 48 SSR markers detected a total of 1683 alleles in 2915 chickpea accessions with an average of 35 alleles/locus and a polymorphism information content (PIC) ranging from 0.467 to 0.974 with an average of 0.854.

According to the final project report the use of this kit has started and 20 primers selected from the 35 initial selection also used in this study were used for genotyping the USDA core chickpea collection (Hoisington *et al.* 2007). Moreover, we used 16 primers described in that paper to assess genetic diversity in a Tunisian collection of chickpea, which provided us an opportunity to detect a useful tool in breeding and marker-assisted selection of chickpea genotypes in the future (Khamassi *et al.* unpublished).

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