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Morphological and Molecular Diversity of Tunisian Chickpea

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Summary

The objective of this study was to evaluate the genetic diversity and relationships among 6 new improved lines, one spring landrace and 6 varieties of winter chickpea (*Cicer arietinum* L.). To achieve this, 16 polymorphic simple sequence repeats (SSRs) were used in the DNA analysis. A total of 554 bands were generated with an average of 2.16 detectable bands/primer/genotype. The within-genotype genetic dissimilarity coefficient ranged from 13.0 to 77.7. Cluster analysis indicated that most genotypes could be clustered into four groups according to their geographic origin, selection objectives or pedigree. Moreover, morphological analyses generated clusters similar to those generated by molecular studies. Indeed, a comparison of morphological and molecular data using the Mantel test showed a highly significant correlation ($r = 0.554$, $P < 10^{-5}$), indicating that SSR primers used in this study likely cover a vast area of the chickpea genome or might flank chromosome regions that control quantitative traits or resistance to Ascochyta blight or wilt. This also means that different combinations of alleles might reproduce similar morphological traits. In this sense, the two methods showed a low degree of variation among analysed genotypes, indicating the narrow genetic base of Tunisian chickpea germplasm. It also showed that the Tunisian chickpea collection should be increased by increasing diversity by importing new genotypes or by inducing mutations which could be used for future breeding programs.

Key words. chickpea – *Cicer arietinum* L. – Mantel test – morphological traits – SSR markers

Introduction

In Tunisia, farmers traditionally grow chickpea in spring. However, the lack of rainfall during the growth period results in low biomass and yield (Singh et al. 1984; Kanouni et al. 2011). Sowing is no longer in spring but rather in winter, resulting in higher and more stable productivity with improved water-use efficiency and an expand vegetative growth period, all of these as a result of research efforts. Under these conditions, plants benefit from adequate moisture conditions and yield can be improved up to 2 t ha^{-1} (Singh et al. 1992, 1995). Despite the success of this simple but effective technique, spring varieties can not be used in winter for several reasons. For example, their sensitivity to Ascochyta blight (AB) caused by *Ascochyta rabei* (Pass), and low temperatures, are the two major limitations preventing breeders around the world to shift the sowing date from spring to winter (Kanouni et al. 2011).

During the last 20 years and based on these results, a Tunisian chickpea improvement program in collaboration with ICARDA developed new winter chickpea varieties by either adapting ICARDA's entries or by improving their local population through crosses with other strongly performing foreign landraces. The first efforts made in the 1980 s resulted in the selection of the following three high-yielding varieties. Local 'Amdoun 1', which out-yields the old landrace 'Amdoun' by 10 %, is totally resistant to wilt but is recommended for spring sowing due to its sensitivity to AB (Halila and Harrabi 1990; Haddad et al. 1996). After that, two other varieties, 'Kasseb' and 'Chetoui', were selected from the ICARDA elite collection and adapted to the Tunisian environment after sowing for several years; they showed higher yield than 'Amdoun 1', a local (i.e. Tunisian) spring-type variety, but had moderate resistance to AB. Yet, the major constraint to the adoption of winter varieties is the size and weight of seed, which are at least 30 % smaller than 'Amdoun 1' seed

(Haddad et al. 1996). Despite this, in 2003, 'Bochra', 'Neyer' and 'Beja 1' were selected and added to the official Tunisian catalogue. 'Bochra' and 'Neyer' resulted from intense breeding in order to combine good resistance to fungal diseases and adaptation to harsh winters while 'Beja 1' is the result from a (('Amdoun 1' × ILC3279) × ILC200) cross. Moreover, 'Bochra's and 'Neyer's 1000-seed weight (1000-SW) vary between 340 and 360 g with a yield of 2.0 t ha^{-1} whereas 'Beja 1' has a 1000-SW as high as 380 g and a yield as high as 2.5 t ha^{-1}. These varieties fall within the preferred size by Tunisian consumers who prefer spring-type seeds with a 1000-SW between 450 and 500 g. After years of effort spent in the creation of winter varieties adapted to the Tunisian environment, it is now necessary to extract information regarding the genetic diversity of these germplasms. This is the primary function of this study.

Knowledge about chickpea germplasm diversity and genetic relationships among breeding materials could be a valuable aid in crop improvement strategies. Such characterization would be helpful in the development of improved cultivars (Naghavi and Jahansouz 2005). It also has several applications and advantages, including gene bank management and association mapping studies. Gene bank management uses information on molecular diversity, including the maintenance of genetic diversity, increasing diversity through knowledge-based acquisition, reducing redundancy and creating new populations from association mapping studies (Varshney et al. 2007).

Recently, some efforts have been initiated to investigate variability in chickpea (Icrisat 2007) at the molecular level through the use of molecular markers. However, efforts have been limited and insufficient, even if some studies showed intra-specific diversity through the use of microsatellite fingerprinting (Hüttel et al. 1999; Udupa et al. 1999; Winter et al. 1999; Sethy et al. 2003). More recently, a simple sequence repeat (SSR) marker reference kit specifically designed for chickpea was developed (Hoisington et al. 2007), available on ICRISAT's web page (http://www.icrisat.org/gt-bt/Marker_Kits.htm). A more comprehensive study by Upadhyaya et al. (2008) and our comprehensive studies on optimization of SSR conditions (Khamassi et al. 2011) indicate that SSRs are powerful and effective markers for discriminating chickpea genotypes.

The objectives of the present work were to characterize the most widely cultivated Tunisian winter chickpea genotypes at the morphological level and to apply the recently developed ICRISAT SSR kit to estimate genetic diversity. Consequently, this process would help to identify new unknown lines of Tunisian winter chickpea and to link the result to their pedigree data and to their morphological and agronomic performance data.

Material and Methods

Plant material

The research material consist of 12 winter and one spring chickpea (*Cicer arietinum* L.) genotypes, the full complement of improved chickpea cultivars available in Tunisia and maintained in the Field Crops Laboratory of the National Institute of Agronomy Researches Tunis (INRAT). The collection and registration information is shown in Table 1. It

Table 1. Varieties and pedigree lines and their year of registration in the official Tunisian catalogue.

Varieties	Pedigree	Year
'Bochra'	ILC72 × ILC 215 (X80TH176)	1980
'Neyer'	ILC72 × ILC 215 (X80TH176)	1980
'Amdoun 1'	Be-sel-81	
'Kasseb'	ILC72 × ILC 215 (FLIP 83-46)	1983
'Chetoui'	ILC 3279	1980
'Béja 1'	(Amdoun 1 × ILC 3279) × ILC 200	2003
'Béja 2'	(Amdoun 1 × ILC 3279) × ILC 200	U
Line	Pedigree	Year
Line 1	X96TH61 = (FLIP93-176C × UC15) A3-W1-A2	1996
Line 2	X96TH61 = (FLIP93-176C × UC15) A5-W1-A2-A1	1996
Line 3	X24TH61 = (FLIP93-176C × UC15) A2-A1-A1	1996
Line 4	X96TH61 = (FLIP93-176C × UC15) A4-W2-A2	1996
Line 5	X24TH61 = (FLIP93-176C × UC15) A6-W1-A1	1996
Line 6	X96TH61 = (FLIP93-176C × UC15) A4-W2-A1	1996

ILC 3279: land race from Russia (URSS): an entry adapted to the Tunisian environment

A: mean one year test inoculation by Ascochyta blight. W: mean one year test inoculation by Fusarium wilt.

A3: means that plant number 3 is selected as to be tolerant or resistant to disease. x: cross. U: Unregistered in the Tunisian catalogue

consists of 'Amdoun 1' spring chickpea, selected by mass selection from the local landrace 'Amdoun', traditionally grown in Tunisia. Then there are two local improved varieties; 'Beja 1', registered in the official Tunisian catalogue and 'Beja 2', not yet registered. These were obtained by crossing 'Amdoun 1' to two Russian landraces entries. Finally, there are registered varieties; 'Bochra', 'Neyer', 'Chetoui' and 'Kasseb' together with six new improved lines in their last characterization year before registration.

Morphological characteristics

Each accession was sown by hand in 2 m long rows with a 50 cm inter-row spaced and 35 cm inter-plant distance. Observations were recorded for five selected plants for each genotype, based on the evaluation of 20 morphological and agronomical traits. The traits that varied (Table 3) were analysed. These were total plant length (TPL), number of days for 50% flowering (NDF), number of days to maturity (NDM), primary branching (Br1), secondary branching (Br2), total number of pods per plant (TNP-P), number of pods with one seed (NP1), number of pods with two seeds (NP2), total number of seeds per plant (TNSPP) and 1000-seed weight (1000-SW). All traits were measured and recorded using recommended scales as described by the International Union for the Protection of New Varieties (UPOV 2006) and (IBPGR, ICRISAT and ICARDA 1993) chickpea descriptors.

Molecular characterization

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 KNAPP (1990). Fresh chickpea leaves (500 mg) were ground in liquid nitrogen then transferred into a 2-ml polypropylene tube. Then one ml of freshly prepared extraction buffer (100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1.5 M NaCl, 2.5 % CTAB, 0.2 % β-mercaptoethanol (v/v) and 1 % polyvinylpyrrolidone-40 (PVP) (w/v)) was then added and mixed by gentle inversion.

The mixture was incubated in a 65 °C water bath for 30 min. An equal volume of phenol:chloroform (1:1) was added and mixed by gentle inversion for about 10 min. The mixture was spun at 13,000 rpm for 10 min at 25 °C. The upper clear aqueous layer was carefully transferred to another tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed by gentle inversion for about 10 min and spun at 13,000 rpm for 10 min at 25 °C. The upper clear aqueous layer was transferred to a new tube to which 0.1 volumes of 3 M sodium acetate, pH 5.2 and 2 volumes of 100 % chilled ethanol were added. The mixture was allowed to stand at room temperature for 30 min. Fibrous nucleic acid was gently scooped and transferred to a 1.5-ml microfuge tube. After centrifugation at the same conditions as mentioned above, the supernatant was discarded and the pellet was washed with 70 % ethanol. The pellet obtained was dried and dissolved in 100 µl of TE (10:1) buffer: pH 8, 100 mM Tris; 1.44 mM NaCl, 3 % CTAB, 20 mM EDTA and 1 % β-mercaptoethanol.

2 µl RNase A (10 mg ml^{-1}) was added and incubated at 37 °C for 30 min. The mixture was extracted with an equal volume of phenol:chloroform (1:1). The aqueous layer was transferred to a fresh 1.5-ml microfuge tube. A two-fold volume of 100 % chilled ethanol was added, mixed, kept at −20 °C for 20 min then spun at 13,000 rpm for 10 min at 25 °C. The pellet was washed with 70 % ethanol, dried for 15 min then dissolved in 100 µl TE buffer.

DNA was electrophoresed through a 0.8 % agarose gel (Sigma Aldrich), which contained ethidium bromide, at 70 V for 1.5 h in 0.5X TBE buffer. DNA concentration was estimated by comparison with a λ *Hind*III DNA ladder (Promega).

SSR analysis. Sixteen primer pairs were selected on the basis of a screening process from the 35 primer pairs of the chickpea molecular marker reference kit, ICRISAT (KHAMASSI et al. 2011). PCR amplification was carried out in a final volume of 25 µl containing 2 µl of genomic DNA solution (50 ng $µl^{-1}$), 5 µl of 5X Green Go *Taq* Reaction Buffer (Promega), 1.5 µl containing 25 mM Mg^{2+}, 2.5 µl of a 2.5 mM dNTP mix, 1 U of GOTaq DNA polymerase (Promega), 2.5 µl of a 2.5 µM solution of each forward and reverse primers and 10.3 µl of ultrapure water (UPW) (KHAMASSI et al. 2011; Table 2).

The amplification program was run in a thermocycler (Biometra UNO-Thermoblock; BIOTRON, Göttingen, 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.

The electrophoresis of PCR amplification products was performed in a 2 % agarose gel containing ethidium bromide only for three primers (NCPGR 4, NCPGR 12, NCPGR 19), which are dinucleotide repeat units. As for the other 13 primers, which are tri-, and a mix of mono- and tri-nucleotide repeat sequences, electrophoresis was performed in a 6 % polyacrylamide gel. DNA concentrations were estimated by comparison with 100-bp DNA ladder. The amplified product was visualized under UV light on a gel documentation system after staining the gel with 0.5 µg ml^{-1} ethidium bromide.

Data analysis

Fragments amplified by SSR primers were scored in binary format, with the presence of a band scored as 1 and the absence of a band scored as 0. The coefficient of genetic dissimilarity (GD) was calculated according to the formula of NEI and LI (1979). Based on the dissimilarity matrix, a

Table 2. Sequence of SSR markers Identification kit.

Primers	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Linkage	Source*
CaSTMS21	CTACAGTCTTTTGTTCTTCTAGCTT	ATATTTTTTAAGAGGCTTTTGGTAG	LG6	[1]
TA27	GATAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	LG7	[2]
TA71	CGATTTAACACAAAACACAAA	CCTATCCATTGTCATCTCGT	LG3	[2]
TA116	AATTCAATGACGAATTTTTATAAGGG	AAAAAGAAAAGGGAAAAGTAGGTTTTA	NA	[2]
TA117	GAAAATCCCAAATTTTTCTTCTTCT	AACCTTATTTAAGAATATGAGAAACACA	NA	[2]
TA118	ACAAGTCACATGTGTTCTCAATA	GGAAAGGTTAAGAAATTTTACAATAC	NA	[2]
TA135	TGGTTGGAAATTGATGTTTT	GTGGTGTGAGCATAATTCAA	LG1	[2]
TA200	TTTCTCCTCTACTATTATGATCACCAG	TTGAGAGGGTTAGAACTCATTATGTTT	NA	[2]
TAA58	CATTGCTTAAGAACCAAAATGG	CAATTTTACATCGACGTGTGC	LG5	[2]
TaaSH	GGTAGACGCAAAAGAGTGGG	GCCACATTGACCAGGAATG	LG3	[2]
TR2	GGCTTAGAGTTCAAAGAGAGAA	AACCAAGATTGGAAGTTGTG	LG1	[2]
TR29	GCCCACTGAAAAATAAAAAG	ATTTGAACCTCAAGTTCTCG	LG3	[2]
TR31	CTTAATCGCACATTTACTCTAAAATCA	ATCCATTAAAACACGGTTACCTATAAT	LG1	[2]
NCPGR4	TTACAGCTTGTGCTCAG	AGTCAGATTCTTATCCGA	NA	[3]
NCPGR12	CCTTGTTAGTGTGTATAGGT	GTAATGACCAAGTGAACA	NA	[3]
NCPGR19	TCCATTGTAGCTTAGCTTAG	TCTTACTCTTAGCTTACCTCTT	NA	[3]

* Sources: [1] Huttel et al. 1999; [2] Winter et al. 1999; [3] Sethy et al. 2003

Table 3. Morphological traits used to calculate morphological distance.

Abbreviation	Trait
AC	Anthocyanic pigmentation
Ph	Presence of hair
PT	Width of the plant
FC	Coloration of the flower
TG	Size of the pod
GFS	Shape of the seed
GST	Texture of the seed
TPL	Total plant length
GLB	Length of the beak of the pod
GLP	Length of the peduncle of the pod
NDF	Number of days for 50 % flowering
NDM	Number of days for maturity
Br1	Primary branching
Br2	Secondary branching
TNP-P	Total number of pods per plant
NP1	Number of pods with 1 seed (NP1)
NP2	Number of pods with 2 seeds (NP2)
NEP	Number of empty pods
1000-SW	1000-Seed weight
TNSP	Total number of seeds per plant

dendrogram showing genetic relationships between genotypes was constructed using the Unweighted Pair Group Method Arithmetic Average (UPGMA) (Sneath and Sokal 1973).

To measure the informativeness of markers, the polymorphism information content (PIC) was calculated according to Anderson et al. (1993) according to the formula:

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of the *jth* allele for marker i and n is the number of marker alleles for marker i.

Comparison of molecular and morphological data

Simple regression (r) and Spearman rank correlation (rs) coefficients between molecular and morphological matrices were determined. The P-value for the rs coefficient was calculated based on its respective asymptotic distribution (Kendall and Stuart 1979). Correspondence between the two matrices as well as between their corresponding cophenetic matrices was tested with the Mantel (*Z*) statistic (Mantel 1967). Significance of the *Z* value was determined by comparing the observed *Z* values with a critical *Z* value obtained by calculating *Z* for one matrix with 7000 permuted variants of the second matrix. All computations were performed with an appropriate procedure of the NTSYS-pc version 2.2 software (Roehlf 1993).

Results

Morphological analysis

Results for morphological traits are summarized in Table 4. Highly significant variation ($P < 0.01$) was found among

Table 4. Analyses of variance (ANOVA) for quantitative traits.

Variabel	Average	SD	Minimum	Maximum	CV	MS	F value	Pr > F	Sign.
pH	63.26	9.82229	40	86	10.89	308.87	6.51	< 0.0001	***
Br1	1.66	0.69094	1	3	38.06	0.81	2.03	0.0396	*
Br2	3.63	1.90040	2	9	33.38	12.89	8.78	< 0.0001	***
NPP	45.95	22.96834	10	102	35.82	1639.23	6.05	< 0.0001	***
NG1	33.21	21.68128	6	95	50.17	1303.41	4.69	< 0.0001	***
NG2	7.92	4.09767	2	20	51.20	18.21	1.11	0.3749	ns
NSSP	4.61	3.34333	0	14	68.62	16.14	1.61	0.1177	ns
1000-SW	337.80	40.53130	230	430	6.25	68.27	15.29	< 0.0001	***
TNSP	49.06	24.16213	12	106	36.67	1710.91	5.29	< 0.0001	***

SD: standard deviation; CV: coefficient of variation; MS: mean square; Sign.: Significance
Ns: Not significant; *: Significant (P < 0.05); **: Highly significant (P < 0.01); ***: Very highly significant (P < 0.001)

genotypes for the following traits: TPL, Br2, TNP-P, the number of single-seed pod (NSSP), and 1000-SW. In addition, significant variation (P < 0.05) was detected for Br1 and TNSPP. The dendrogram generated from the agro-morphological traits assigned the collection to four main groups (Fig. 2). The first cluster includes 'Chetoui and 'Kasseb' while 'Amdoun 1', 'Beja 1' and 'Beja 2' formed the second. The third group was divided into two sub-groups: in the first were 'Bochra' and 'Neyer' while in the second were the new lines: 'line 5' and 'line 6'. The last group contains two sub-groups: i) 'line 1' and ii) 'line 2' and 'line 3'.

Molecular studies

The 16 selected pairs of primers generated clear patterns with high polymorphism (Table 4) Amplification products generated 16 distinct polymorphic profiles. A total of 554 reproducible bands were scored, 450 of which were polymorphic translating into 81 % polymorphism with an average of 2.16 alleles per locus (Table 4).

The studied SSR sequences were comprised of di-, tri-, and a mixture of mono- and tri-nucleotide repeat sequences (Table 4). The lowest polymorphism was observed for a nucleotide repeat primer, NCPGR4. In contrast, highest polymorphism was noticed for tri- and mixed-nucleotide repeat primers (e.g., TA116). Song et al. (2002) proposed that the level of polymorphism would be higher when the motifs comprised three or four nucleotides, as observed in our study. Nagoaka and Ogihara (1997), studying hexaploid wheat, also observed maximum polymorphism when tetra-nucleotide primer repeats were used. The number of bands and the PIC/primer (Table 6), which are affected by accession, primer sequence and protocol conditions, were variable. In our study, the PIC varied from 0.593 (NCPGR4) to 0.898 (TA116) with an average of 0.72. Most markers, except for TR2, TR31 and NCPGR4, were highly polymorphic. There was partial agreement with some values reported by Upadhyaya et al. (2008, 2011).

Correlations between dissimilarity matrices

A moderately high correlation (r = 0.554; P = 0.001) was found between the molecular data and the morphological data. The significant correlation indicates that these two independent sets of data likely reflect the same pattern of genetic diversity. This validates the use of molecular markers to characterise diversity in Tunisian chickpea germplasm.

Discussion

Morphological analysis

The analysis of variance of the quantitative traits (Table 4) shows that all but two traits were significantly different between accessions. Those two traits (NP2 and NEP) are both components of total TNSP, showing that genetic variation in TNSP is determined almost entirely by NP1. No analysis of qualitative traits was necessary, since there was no variation either between or within accessions for these traits in this experiment. The dendrogram based on the agro-morphological traits (Fig. 1) shows that the new 'lines' tend to cluster apart from the established varieties grown in Tunisia. The exception is 'line 4', which clusters with 'Beja 2'. However, Amdoun 1 is a parent of both 'Beja 1' and 'Beja 2' and is an ancestral line of 'line 4'. In addition, 'Beja 1' and 'Beja 2' are two sister genotypes having the same pedigree [(Amdoun1 × ILC3279) × ILC200] (Table 2). The dendogram is therefore broadly consistent with what is known of the origin of the lines. In fact, the first group was formed by varieties 'Chetoui' ILC3972 and 'Kasseb' (ILC72 × ILC 215) that share some

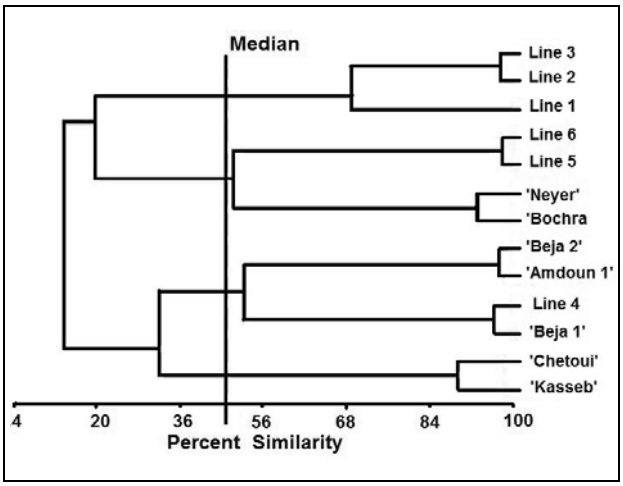

Fig. 1. Dendrogram resulting from Median cluster analysis of 13 chickpea genotypes and based on percent similarity calculated with 16 agronomic and morphological traits.

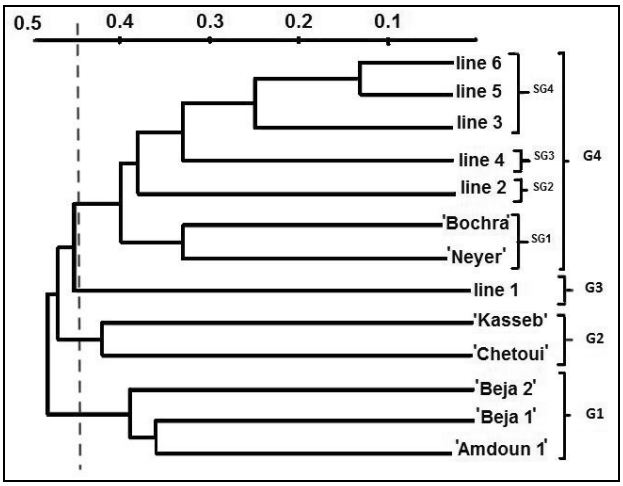

Fig. 2. Dendrogram of dissimilarity resulting from UPGMA cluster analysis of 13 chickpea genotypes and based on data of 16 microsatellite primer pairs. G: group, SG: subgroup.

common traits (1000-SW, NDF), which could be due to the similarity of the ancestor (Kasseb ILC 72) with 'Chetoui' (ILC3972), which are two imported genotypes from 'Russia Caucaso'. The same occurs in the second group formed by 'line 4' and varieties 'Amdoun 1', 'Beja 1' and 'Beja 2'. The latter three varieties share some morphological traits in common with 'Amdoun 1'. In fact, high coefficients of correlation were recorded for number of pods/branch, seeds/pod, yield/plant, seeds/plant, pods/plant and branches/plant, as described by Naghavi and Jahansouz (2005), who worked on other Kabuli varieties. The third group consists of two sub-groups: the first one formed by varieties 'Bochra' and 'Neyer', which share morphological traits with 'line 5'; 'line 6' formed the second sub-group. The last group contains two sub-groups: the first formed by 'line 1', the second by 'line 2' and 'line 3'.

Molecular studies

The SSR markers used in this study are effective in discriminating local Tunisian chickpea germplasm. They are also a valuable tool for assessing genetic diversity levels. In this study, the dendrogram obtained by these SSR markers (Fig. 2) is broadly similar to that obtained from the morphological traits. This is particularly true for the named varieties. The relationship between the new lines and the names varieties varies, however. The reason for this is unknown but might be a result of the selection process by which the lines were produced and which included screening for resistance to biotic stress with inoculation by AB and Fusarium wilt.

The dendrogram in Fig. 2 shows four groups: Group 1 contains 'Beja 1', 'Amdoun 1' and 'Beja 2'. Group 2 consists of 'Kasseb' and 'Chetoui' with 42 % GD (Table 5). Group 3 is formed by 'Line 1'. Group 4 contains 'Bochra', 'Neyer' and lines 2, 3, 4, 5, and 6 with 40 % of the dissimilarity coefficient (Table 6). This cluster includes several sub-groups. The first is formed by 'Bochra' and 'Neyer' with 33 % GD (Table 6). The second sub-group is made up of 'line 2' and 'line 4' while the third sub-group contains 'lines 3', '5' and '6'.

The three lines in Group 1 are closely related, as explained earlier with GDs between 'Amdoun 1' and its two progeny lines of 35.5 % (Table 6).

Group 2 consisted of 'Kasseb' and 'Chetoui' with a GD of 42 %. To further explain, 'Chetoui' has in its pedigree the Russian 'Caucaso' landrace ILC3279, which has been adapted to the Tunisian environment. 'Kasseb' (ILC72 × ILC215)'s parent ILC72 is considered to be genetically identical to ILC3279 as revealed by ISSR markers (Iruela et al. 2002). In addition, ILC72 and ILC3279 originate from the same area, namely the Russian Caucasus. Furthermore, these lines share some common morphological traits: TNPP, NNP1, length of beak pod (GLB), TNSP, TNSP, TNSP, pod size (TG), NEP and seed shape (GFS).

Group 3 is formed by 'Line 1' only, which has the following pedigree (X96TH61-A4W1A1 = (FLIP93-176CXUC15)). This genotype was selected after inoculation by AB: 'A' for the first year, by Wilt 'W' for the second year and by AB 'A' for the third year again. This resistance induced in 'line 1' and the weakness of many agronomic parameters (late maturity date, plant height (PH), TG and TNSP) singles it out from the other genotypes.

Group 4 consisted of 4 sub-groups, the first one formed by 'Bochra' (ILC72 XS ILC215) and 'Neyer' (ILC72 XS ILC215) with 77 % similarity. These lines come from the same cross held in 1980 at Tel el Hadya (ICARDA). They were introduced to Tunisia as F_8s and tested against biotic stress before being registered in the official catalog.

Table 5. Characteristics of SSR markers used in this study with the number of alleles, the PIC value and annealing temperature.

Primer	Sequence	aT (°C)	Total allele	P allele	PIC	Allele (bp)	Upadhyaya et al. 2008	
							Allele (bp)	PIC
CaSTMS21	(TAT) 25	59	7	7	0.756	100–200	100–200	0.465
TA27	(TAA) 21	59	6	6	0.805	200–300	200–300	0.891
TA71	(AAT) 32	59	6	6	0.820	200–300	150–300	0.942
TA116	(TAA) 5n (A) 3 (TAA) 20	59	12	12	0.898	100–350	150–350	0.838
TA117	(ATT) 52	56	7	7	0.780	200–300	200–300	0.929
TA118	(TAA) 45	59	9	9	0.840	< 250	< 250	0.949
TA135	(TAA) 17	56	8	8	0.831	100–300	150–300	0.850
TA200	(TTA) 37	59	8	8	0.842	250–350	250–300	0.916
TAA58	(AAT) 41	56	6	6	0.768	200–300	200–300	0.955
TaaSH	(TAA) 40	59	7	7	0.819	400–500	400–500	0.930
TR2	(TTA) 36	56	3	3	0.603	200–300	200–300	NA
TR29	(TAA) 8n (TAA) 32	59	7	7	0.845	200–300	170–250	0.915
TR31	(TAA) 20n (A) 5 (TAA) 9	59	3	3	0.640	100–200	180–200	0.843
NCPGR4	(CT) 16	56	3	3	0.593	100–200	150–200	0.605
NCPGR12	(CT) 35	56	4	4	0.709	200–300	200–270	0.813
NCPGR19	(GA) 19	56	6	6	0.810	250–350	250–310	0.591

P: Polymorphic; PIC: polymorphic information content

Table 6. SSR dissimilarity matrix.

Variety/ line	'Kasseb'	'Beja 1'	'Chetoui'	'Amd. 1'	'Beja 2'	'Bochra'	'Neyer'	Line 1	Line 2	Line 3	Line 4	Line 5	Line 6
'Kasseb'	0.00												
'Beja 1'	60.53	0.00											
'Chetoui'	42.10	50.00	0.00										
'Amdoun 1'	48.68	35.53	40.79	0.00									
'Beja 2'	42.10	42.10	50.00	35.53	0.00								
'Bochra'	51.32	40.79	46.05	47.37	46.05	0.00							
'Neyer'	55.26	47.37	36.84	38.16	39.47	32.89	0.00						
Line 1	52.63	36.84	47.37	56.58	60.53	43.42	44.74	0.00					
Line 2	55.26	50.00	47.37	56.58	42.10	40.79	44.74	42.10	0.00				
Line 3	53.95	51.32	38.16	47.37	43.42	50.00	35.53	43.42	38.16	0.00			
Line 4	38.16	56.58	48.68	52.63	48.68	42.10	43.42	51.32	38.16	39.47	0.00		
Line 5	48.68	48.68	40.79	47.37	38.16	39.47	27.63	46.05	38.16	26.32	28.95	0.00	
Line 6	48.68	48.68	46.05	52.63	43.42	42.10	38.16	43.42	38.16	23.68	31.58	13.16	0.00

'Amd. 1': 'Amdoun 1'

These two varieties share the same agronomic characters: TG, NDF, NDM and 1000-SW.

The second sub-group consists of 'line 2' and 'line 4' which have respectively the following pedigree [X96TH61-A5W1A2A1 = (FLIP93-176C XS UC15)] and [X96TH61-A4W1A1 = (FLIP93-176C XS UC15)]. These two lines may be clustered due to the fact that they were selected for the same objectives (resistance to Fusarium wilt and anthracnose).

The third sub-group contains 'lines 3, 5 and 6' which have the [X24TH61-A2A1A1 = (FLIP93-176CXUC15)], [X24 TH61-A4W1A1 = (FLIP93-176CXUC15)] and [X96TH61-

A4W2A1 = (FLIP92-159CXULC1278)] pedigrees, respectively. 'Line 3' and 'line 5' have the same pedigree but 'line 6' is more related to 'line 5' with GD < 15 % (Table 5). Lines 5 and 6 were selected for the same objective, too (one year of inoculation by AB, second year by Fusarium wilt and a third year by AB again). This distribution could therefore be explained by adaptation of these two lines to abiotic stress. They also share the same agro-morphological traits (TG, PH, NDF, NDM, TNP-P and 1000-SW).

In most cases, more than one band in each locus was obtained with a single primer pair (Fig. 2). According to WINTER et al. (1999), these bands were derived from the same locus since they segregated identically. The occurrence of multiple bands from the same locus is also observed with STMS in other crops, which could be explained by the presence of cryptic sites up-and-down stream of, as well as in between the specific primer binding sites. Furthermore, according to HAUGE and LITT (1993) and LITT et al. (1993), shadow bands, DNA polymerase slippage products or stutter bands are the names used to refer to the PCR amplification results that differ in size from the main allele by multiples of the repeat unit size. The moderately high correlation (r = 0. 0.554; P = 0.001) between morphological distances and those of SSR markers may be explained by the properties of SSR markers being associated with the chromosomal region selected by a particular environment (SAGHAI-MAROOF et al. 1984; MEYER et al. 2000). Moreover, SSR markers used in this study could be associated with phenotypic variation of agronomic traits, in agreement with GUPTA and MUEHLBAUER (2006). In fact, according to that study, some SSR markers such as TA117 (linked to LG7) or R2 (unlinked) are associated with 100-SW and PH, respectively.

Conclusion

UPADHYAYA et al. (2008) stated that chickpea, like other legumes, has a narrow genetic base despite of the large collection of chickpea germplasm which is available. The SSR kit that we used in the current study is described by those authors as being useful for allele mining, association genetics, mapping and cloning of gene(s) and in applied breeding to broaden the genetic base of chickpea. We have validated the use of this SSR kit for the Tunisian chickpea breeding program.

This study provides proof that the available Tunisian chickpea genotypes present a narrow genetic diversity and that new lines like 'line 5' and 'line 6' are very similar to the two sister varieties 'Bochra' and 'Neyer'. New genotypes that would present an important agronomic and morphological performance for a future breeding program should be discovered. Moreover, the reliability of SSR markers in genotyping and evaluating the relationships between genotypes has been confirmed (KHAMASSI et al. 2011). This tool could be useful for selecting a plant candidate for a chickpea breeding program. The establishment of a significant relationship between SSR-based polymorphism and morphological traits shows that SSR markers are powerful enough for selecting suitable candidate plants for a breeding program, serving as a reliable tool for marker-assisted selection (MAS).

Correlation between SSR markers and agro-morphological traits. The dendograms in Fig. 1 and 2, describing relationships among accessions based on agro-morphological traits and SSR markers respectively, are broadly similar. In fact, the distances between the accessions calculated by the two methods show a positive significant correlation coefficient (r = 0.544). For this reason, in the future, accessions of Tunisian germplasm can be screened for diversity and relationships to existing lines using these markers rather than by time consuming evaluation of agro-morphological traits.

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