Identification of chickpea cultivars by microsatellite markers

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SUMMARY

Characterization of plant varieties is traditionally based on phenotypic observation. However, some varieties have very similar morphological characteristics, which make it difficult to distinguish between them. The present study employed 15 microsatellite markers distributed across all linkage groups (LG) of the chickpea genetic map to characterize 32 commercial chickpea cultivars and determine the usefulness of these markers for cultivar identification. These markers showed a high level of polymorphism; a total of 154 different alleles were detected, with a mean of 10·3 alleles per locus. The polymorphic information content (PIC) value ranged from 0·455 to 0·897. All the markers, with the exception of TA130, TA135 and TA144, were considered to be informative (PIC > 0·7), indicating their potential usefulness for cultivar identification. A subset of markers (TA186, TA200, TA106, TA113, TA117 and TA30) was sufficient to identify all the cultivars studied. In order to confirm their discriminatory power, 16 unreleased chickpea cultivars (V1–V16) were screened and all of them presented different patterns. Therefore, these microsatellites can be regarded as a reference set for chickpea cultivar identification and their profiles can be used as a DNA fingerprint for each registered cultivar, avoiding redundancy of identical cultivars as well as to protect breeders' rights.

INTRODUCTION

The chickpea (*Cicer arietinum* L.) is a self-pollinating diploid ($2n = 16$) cultivated species. It is the third most important pulse crop in the world after soybean and beans, covering an area of 11·5 million ha. In Europe, it ranks fourth in terms of area harvested and production, with Spain being the major producer (FAOSTAT 2009, http://faostat.fao.org). It is mainly used for food, and has unique physical characteristics, chemical composition and anti-nutritional components compared to other legumes (Ahlawat *et al.* 2007). Two distinct chickpea types, different in their morphology and processed in different ways, have been described: desi and kabuli. Desi chickpeas have purple flowers and small, dark, angular seeds with a higher fibre content. They are consumed largely in

India and Pakistan and eaten mainly as dhal or flour, but also used as a fresh immature green seed (Yadav *et al.* 2007). Kabuli chickpeas have white flowers and large, cream-coloured seeds; they are preferred in the Mediterranean basin and central Asia and are consumed mainly as a whole seed. This second type constitutes only *c.* 0·15 of global chickpea production, but good-quality large-seeded kabuli chickpeas are much sought after in the market and fetch prices three times higher than other chickpea cultivars.

Chickpea cultivars released in Europe are mainly of the kabuli type, but there is interest in some desi for animal feed. The identification of new cultivars has previously relied on phenological and morphological characteristics (van Gastel *et al.* 2007). However, discrimination among cultivars on the basis of these characteristics is influenced by environmental factors and requires large-scale growth experiments of mature plants under uniform conditions and very well-trained staff. Additionally, some genetically related cultivars

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are morphologically very similar and it is difficult to distinguish between them by visual comparison. DNA analysis could help differentiate genotypes accurately and may be of use in cultivar identification.

The development and widespread adoption of molecular markers for genotyping studies have provided a framework for studying genetic diversity and species relationships as well as varietal identification. For chickpea, various marker systems such as amplified fragment length polymorphism (AFLP), internal transcribed spacer (ITS), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and microsatellite markers such as simple-sequenced repeats (SSR) or sequenced tagged microsatellite sites (STMS) have been used for diversity analysis (Serret *et al*. 1997; Iruela *et al*. 2002; Nguyen *et al*. 2004; Sethy *et al*. 2006*a*,*b*; Singh *et al*. 2008). Currently, microsatellite markers are preferred for diversity assessments and cultivar identification due to their high level of polymorphism, repeatability and reproducibility among laboratories, and the possibility of automation. Concerted efforts have led to the identification and characterization of a large number of microsatellite markers in chickpea (Hüttel *et al*. 1999; Sethy *et al*. 2003; Lichtenzveig *et al*. 2005; Choudhary *et al*. 2006; Sethy *et al*. 2006*a*,*b*) and more recently expressed sequence tags (EST) containing SSR motifs have been developed by Choudhary *et al*. (2009). SSR markers have been used for diversity analysis within *C. arietinum* (Sethy *et al*. 2006*b*). Due to the high degree of conservation in the microsatellite flanking regions throughout the genus *Cicer* L., available microsatellite chickpea markers can be used for polymorphism detection in wild chickpea relatives (Choumane *et al*. 2000; Sethy *et al*. 2006*a*,*b*; Choudhary *et al*. 2009). Many of these markers have also been utilized for genome mapping (Millan *et al*. 2010), providing valuable information on their distribution in the chickpea genome.

For these reasons, molecular profiles of cultivars, even though still not used for distinctness, uniformity and stability (DUS), are recommended for plant breeders' rights protection by the International Union for the Protection of New Varieties of plants (UPOV). In fact, it has been suggested for many crops, such as soybean (Giancola *et al*. 2002), pepper (Kwon *et al*. 2005), corn (Gunjaca *et al*. 2008) and rice (Bonow *et al*. 2009), that molecular profiles associated with the description of a cultivar should be regarded as an official DNA fingerprint of accepted cultivars for the purpose of enforcing the rights granted to breeders (Bonow *et al*. 2009).

The molecular characterization of the chickpea cultivars released in Europe has not been reported until now. The present study aims to characterize Spanish commercial cultivars of chickpea by the use of microsatellite data and to determine the potential utility of these markers for cultivar characterization.

MATERIALS AND METHODS

Plant material and DNA extraction

In the first analysis, 32 registered chickpea (*C. arietinum* L.) cultivars developed in Spanish breeding programmes were used (Table 1). A second study was carried out with 16 unreleased cultivars, referred to as V1–V16. Both registered and unreleased cultivars were provided by the Spanish National Institute for Agricultural Research (INIA) in Seville (Spain).

DNA extraction was carried out in duplicate for each cultivar, using young leaves and the plant DNAzol® method (Invitrogen). The first extraction was from individual plants. A second extraction was performed in order to assess intra-cultivar homogeneity, from a mix of equal proportions of five plants per cultivar. DNA from the kabuli line ILC3279 was used as a control for amplification pattern and fragment size.

Microsatellite markers and amplification

The present study used 15 STMS markers developed by Winter *et al*. (1999) and Hüttel *et al*. (1999) and distributed through all LG of the chickpea genetic map (Millan *et al*. 2010) (Table 2). Forward primers were synthesized with fluorescent dyes 6FAM, HEX or NED (Applied Biosystems, UK.) at the 5′ ends. The amplification of STMS markers was performed by multiplex polymerase chain reaction (PCR) using QIAGEN® multiplex PCR kit (QIAGEN, Madrid). The total volume of reaction mixture was 5 μl, containing 15 ng genomic DNA, 1 × QIAGEN multiplex PCR master mix (containing HotStarTaq® DNA Polymerase, QIAGEN multiplex PCR buffer and dNTPs mix) and a mix of three primer pairs labelled with fluorescent dyes displayed in Table 2, at a concentration of 0·3 μM each. The thermal PCR profile used followed that of Winter *et al*. (1999), with adapted modifications to the multiplex PCR kit used. The PCR reactions were performed in a TGradient Biometra® thermocycler (Biometra GmbH, Göttingen, Germany) and consisted of an initial polymerase activation step of 15 min at 94 °C followed by 34 cycles of 20 s at 94 °C, 50 s at 55 °C and 50 s at 60 °C, concluding with a final extension step of 10 min at 60 °C. The PCR products were separated using an automatic capillary sequencer (ABI 3130 Genetic Analyzer Applied Biosystems, Madrid/ HITACHI, Madrid) at the Unit of Genomics of the Central Service for Research Support of the University of Córdoba (Spain). The size of the amplified bands was calculated based on an internal DNA standard (400HD-ROX, Applied Biosystems) with GeneScan 3.x software (Applied Biosystems) and the results were interpreted using the Genotyper 3.7 program from Applied Biosystems.

Table 1. *Registration code, pedigree and Institution or seed factory of chickpea* (C. arietinum L.) *commercial cultivars analysed*

Cultivar	Registration code	Pedigree	Institution or seed factory*
Amelia	19890268	Not known	SIA (Comunidad de Madrid)-INIA
Amparo	19890266	Not known	SIA (Comunidad de Madrid)-INIA
Angiano	140300	Not known	
Athenas	19900212	ILC72×CA2156	Koipesol Semillas S.A.-UCO-IFAPA
Ayala	19950078	Not known	(ARO) Volcani Center/Israel
Badil	20020302	Mass selection from ICARDA germplasm	SIA (Junta de Extremadura)
Bagdad	19900211	CA2156×ILC72	Koipesol Semillas S.A.-UCO-IFAPA
Bianka	19920127	Not known	Koipesol Semillas S.A.-UCO-IFAPA
Bonal	19930086	Mass selection from ICARDA germplasm	SIA (Junta de Extremadura)
Castúo	19870204	Mass selection from ICARDA germplasm	SIA (Junta de Extremadura)
Cavir	20030335	CA2269×ICCL81001	INIA-UCO-IFAPA
Chamad	19750002	Not known	
Duratón	19990205	Not known	ITACYL-INIA
Elvira	19950265	Not known	SIA (Comunidad de Madrid)-INIA
Eulalia	19809267	Not known	SIA (Comunidad de Madrid)-INIA
Fardón	19850082	Mass selection from ILC72	INIA-UCO-IFAPA
Inmaculada	19890264	Not known	SIA (Comunidad de Madrid)-INIA
Juano	19960172	ILC72×CA2156	INIA-UCO-IFAPA
Junco	20020306	Mass selection from ICARDA germplasm	SIA (Junta de Extremadura)
Kairo	19900214	ILC72×CA2156	Koipesol Semillas S.A.-UCO-IFAPA
Krema	19940099	Not known	Koipesol Semillas S.A.-UCO-IFAPA
Lechoso	19750004	Not known	Not known
Patio	19960170	ILC72×CA2156	INIA-UCO-IFAPA
Pedrosillano	19750003	Not known	Not known
Pilar	19890263	Not known	SIA (Comunidad de Madrid)-INIA
Pringao	19960171	ILC72×CA2156	INIA-UCO-IFAPA
Puchero	19850081	Mass selection from Spanish germplasm	INIA-UCO-IFAPA
Saborio	19960169	ILC72×CA2156	INIA-UCO-IFAPA
Solera	20010282	Not known	UCO
Tizón	19900187	Mass selection from ICARDA germplasm	SIA (Junta de Extremadura)
Zegri	19850079	Mass selection from ILC200	INIA-UCO-IFAPA
Zoco	20030332	(ICCL81001×CA2156)×ILC72	INIA-UCO-IFAPA

* SIA, Servicio de Investigación Agraria (Spain); INIA, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Madrid, Spain); UCO, Cordoba University (Spain); IFAPA, Instituto Andaluz de Investigación y Formación Agraria, Pesquera y de la Producción Ecológica (Junta de Andalucía, Spain); ITACYL, Instituto Tecnológico Agrario de Castilla y León (Junta de Castilla y León, Spain).

Statistical analysis

Frequencies of incidence of all polymorphic alleles for each STMS markers were calculated and used for determining statistical parameters. Confusion probability (Cj) and discriminating power (Dj) of each STMS were estimated according to Tessier *et al.* (1999) and polymorphic information content (PIC) following Botstein *et al.* (1980).

Alleles were scored as present (1) or absent (0) for each marker and a binary data matrix was created. Genetic distances between all pairwise combinations of the accessions were calculated using Jaccard´s coefficients of similarity. Grouping of the genotypes was determined by using the unweighted pair group method with arithmetic mean (UPGMA). The correlation coefficient between the similarity matrix and the

cophenetic values matrix was computed to test the goodness of fit of the cluster analysis. NTSYS-pc 2.02j software (Biostatistics Inc., USA, Rohlf 1998) was used for these statistical analyses.

RESULTS

All STMS markers analysed in the 32 released chickpea cultivars showed a high level of polymorphism, displaying a total of 154 different alleles with fragment size ranging from 171 to 297 bp (Table 3). The number of alleles per locus varied from 5 to 21, with an average value of 10·3 alleles. STMS TA130 and TA135 amplified the minimum number of alleles, 5 and 6, respectively, whereas the maximum was observed at TA186, with 21 different alleles. Of the

Table 2. *Microsatellites used in the molecular characterization of 48 (32 released and 16 unreleased) chickpea accessions*

STMS locus	Multiplex PCR*	Fluorescence dyes†	LG‡	Microsatellite motifs	Primer sequences	Expected size§
TA200	1	FAM	2	$(TTA)_{37}$	F:TTTCTCCTCTACTATTATGATCACCAG R:TTGAGAGGGTTAGAACTCATTATGTTT	296
TA113	1	HEX	1	$(TAA)_{26}$	F: TCTGCAAAAACTATTACGTTAATACCA R:TTGTGTGTAATGGATTGAGTATCTCTT	203
TA30	1	NED	1	$(TAA)_{18}TA(TAA)_{19}$	F:TCATTAAAATTCTATTGTCCTGTCCTT R:ATCGTTTTTCTAAACTAAATTGTGCAT	217
TA130	2	FAM	4	$(TAA)_{19}$	F:TCTTTCTTTGCTTCCAATGT R:GTAAATCCCACGAGAAATCAA	219
TA135	2	HEX	3	$(TAA)_{17}$	F:TGGTTGGAAATTGATGTTTT R:GTGGTGTGAGCATAATTCAA	192
TA59	2	NED	2	$(TAA)_{29}$	F:ATCTAAAGAGAAATCAAAATTGTCGAA R:GCAAATGTGAAGCATGTATAGATAAAG	258
TA11	3	FAM	5	$(TTA)_{17}$	F:CATGCCATAAACTCAATACAATACAAC R:TTCATTGAGGACAATGTGTAATTTAAG	230
TA186	3	HEX	4	$(TTA)_{40}$	F:ACAAAATTCTAAAAGTTCCTTCTACCA R:GTTGTTAGTCGAATAATTGAGAAAAAGA	249
TA5	3	NED	5	$(TTA)_{29}$	F:ATCATTTCAATTTCCTCAACTATGAAT R:TCGTTAACACGTAATTTCAAGTAAAGAT	205
TA78	4	FAM	7	$(TTA)_{30}$	F:CGGTAAATAAGTTTCCCTCC R:CATCGTGAATATTGAAGGGT	205
TA106	4	HEX	6	$(TAA)_{26}$	F:CGGATGGACTCAACTTTATC R:TGTCTGCATGTTGATCTGTT	248
TA14	4	NED	6	$(TAA)_{22}ATGA(TAA)_4T$ $(A)_3TGAT(AAT)_5ATT$ $(A)_3TGATAATAAAT$ $(GAT)_4(TAA)_5$	F:TGACTTGCTATTTAGGGAACA R:TGGCTAAAGACAATTAAAGTT	250
TA127	5	FAM	8	$(GTT)_5(ATT)_{23}$	F:AAATTGTAAGACTCTCATTTTTCTTTATT R:TCAAATTAACTACATCATGTCACACAC	243
TA144	5	HEX	8	$(TAA)_{27}$	F:TATTTTAATCCGGTGAATATTACCTT R:GTGGAGTCACTATCAACAATCATACAT	241
TA117	5	NED	7	$(ATT)_{52}$	F:GAAAATCCCAAATTTTTCTTCTTCT R:AACCTTATTTAAGAATATGAGAAACACA	248

* Identification of each multiplex PCR assay.
† FAM: 6-carboxyfluorescein; HEX: hexachloro-6-carboxyfluorescein; NED: 7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxyfluorescein.
‡ LG: linkage group.
§ Products expected size in line ILC3279.

Table 3. *Size range, number of alleles* (*rare, common and most frequent*), *confusion probability* (Cj), *discriminating power* (Dj) *and PIC observed in 32 chickpea cultivars studied with 15 STMS markers*

| STMS locus	Size range (bp)	No. of alleles	Rare alleles (<0·03)	Common alleles (0·03–0·2)	Most frequent alleles (>0·2)	Cj	Dj	PIC
TA130	212–224	5	1	3	1	0·53	0·47	0·46
TA135	171–199	6	1	4	1	0·40	0·60	0·59
TA144	224–257	9	4	4	1	0·30	0·70	0·68
TA59	228–255	9	3	4	2	0·21	0·79	0·77
TA5	179–210	10	4	3	3	0·19	0·81	0·79
TA14	245–278	8	2	5	1	0·19	0·81	0·79
TA78	190–233	10	2	7	1	0·17	0·83	0·81
TA11	223–263	9	3	3	3	0·17	0·83	0·81
TA127	219–241	8	1	5	2	0·16	0·84	0·82
TA30	196–223	9	3	4	2	0·15	0·85	0·83
TA117	211–263	15	6	8	1	0·14	0·86	0·84
TA113	178–211	12	4	7	1	0·14	0·86	0·84
TA106	213–260	11	3	7	1	0·13	0·87	0·85
TA200	264–297	12	3	9	0	0·11	0·89	0·87
TA186	174–251	21	9	11	1	0·09	0·91	0·90
Total		154	49	84	21			
Mean		10·3	3·3	5·6	1·4	0·206	0·793	0·775
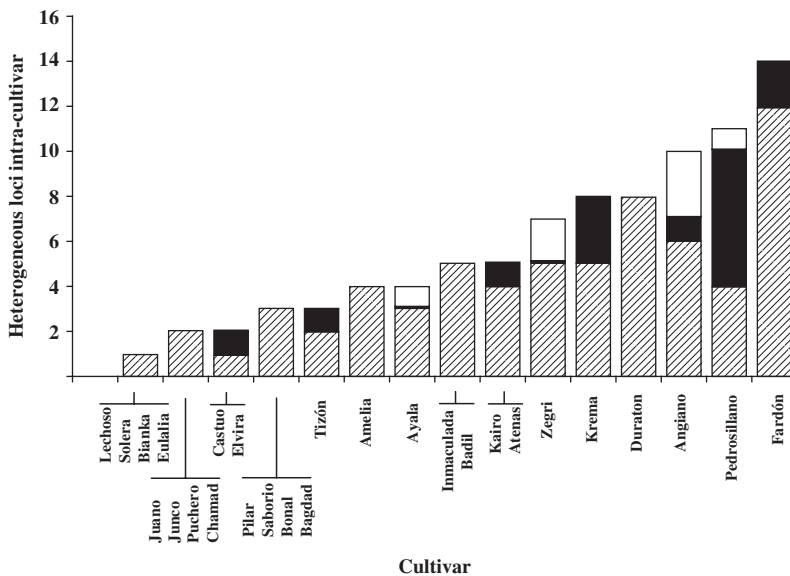
Fig. 1. Distribution of markers with 2 (▨), 3 (■) or 4 (□) alleles per locus in 32 released chickpea cultivars.

154 alleles detected in the chickpea cultivars, 49 were classified as 'rare' because of their low frequency (<0·03), 84 as 'common' (0·03–0·20) and 21 were considered the 'most frequent' alleles (>0·2) (Table 3). Rare and common alleles were detected at all 15 STMS loci studied. Rare alleles per locus ranged from 1 to 4 at all STMS loci analysed except at TA117 and TA186, which showed 6 and 9 alleles, respectively. The number of common alleles per locus were higher, ranging from three (TA130, TA5 and TA11) to 11

(TA186). With regard to the most frequent alleles, one or two were detected at most of the STMS loci except for TA5 and TA11, at which three were detected, and TA200, at which none were detected (Table 3). High values of discriminating power ($Dj \geqslant 0\cdot79$) and $PIC \geqslant 0\cdot77$, and low values of confusion probability ($Cj \leqslant 0\cdot21$) were obtained for all the markers evaluated except for TA130, TA135 and TA144 (Table 3). The most polymorphic marker in the present study was TA186 with a PIC value of 0·897 and 21 alleles
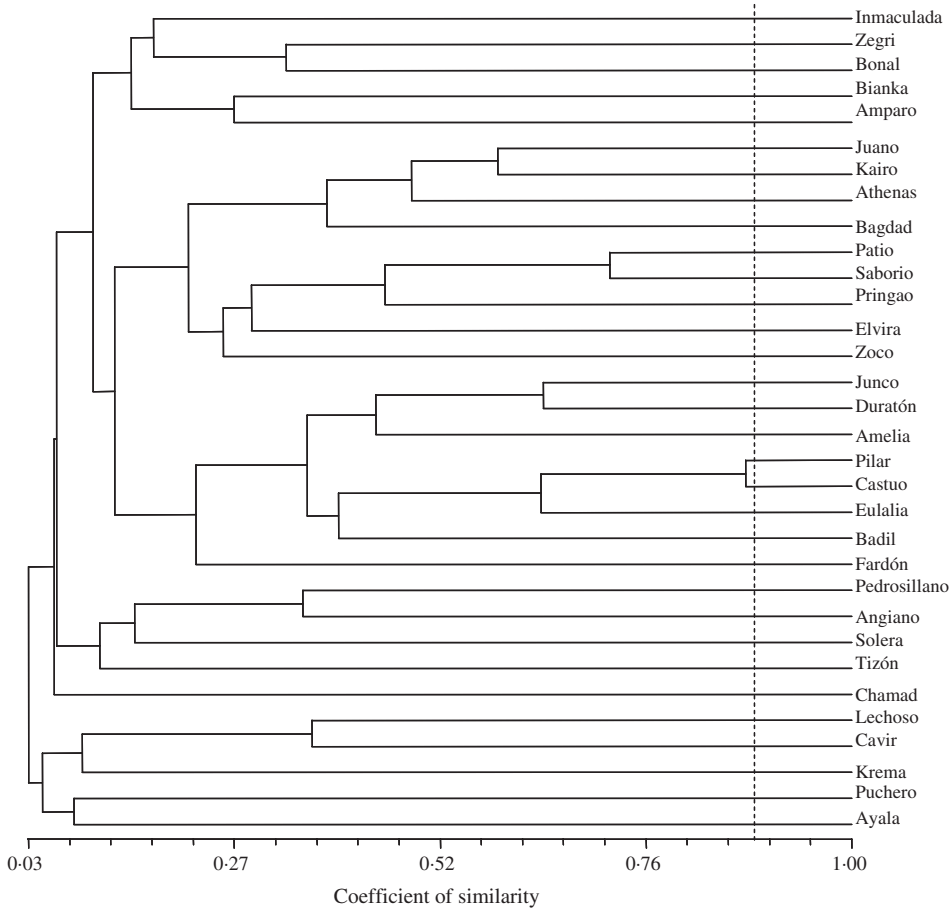
Fig. 2. UPGMA dendrogram obtained from cluster analysis of 32 released chickpea cultivars based on Jaccard's coefficient of similarity using the six STMS markers selected for their high PIC value. The vertical dashed line indicates the limit to the right of which lines are regarded as similar.

identified. Based on PIC values obtained, most STMS, with the exception of TA130, TA135 and TA144, were considered as informative markers (PIC > 0·7), indicating the potential use of this set of STMS markers for cultivar identification (Table 3).

The 15 STMS markers were screened against five individuals of each cultivar to assess intra-cultivar homogeneity. Only five cultivars (Patio, Pringao, Zoco, Cavir and Amparo) showed a single allele for each STMS locus and consequently these could be considered as homozygous pure lines. However, in the remaining 27 cultivars intra-cultivar heterogeneity for at least one STMS locus was found (Fig. 1). The number of different alleles found per STMS locus within cultivars ranged between 2 and 4. Fardón, Pedrosillano and Angiano were the cultivars that showed the highest number of STMS that displayed intra-cultivar heterogeneity: 14, 11 and 10 markers, respectively. Each of these had 2–4 different alleles,

indicating a low level of homogeneity within these three accessions (Fig. 1).

Cultivar identification

An important aim in cultivar identification is to distinguish between genotypes to avoid redundancy of identical cultivars. According to the UPGMA results, the 15 STMS markers employed in the present study made it possible to discriminate between the 32 chickpea cultivars. Moreover, just six of these markers (TA186, TA200, TA106, TA113, TA117 and TA30) were sufficient to discriminate between all the cultivars at a similarity level of 0·88 (Fig. 2). These STMS were selected on the basis of their discrimination capacity with highest PIC and *Dj* values. Amplification patterns of the 32 genotypes using the six STMS could be considered as a fingerprint for these cultivars (Table 4).

Table 4. *Allelic patterns (in bp) of 32 chickpea cultivars obtained with six STMS primers chosen on account of their discrimination values, PIC > 0·82, discriminating power (Dj > 0·84) and confusion probability (Cj < 0·15)*

Cultivar	TA186	TA200	TA106	TA113	TA117	TA30
Amelia	214, 248	276, 291	229	194, 199	248	220
Amparo	211, 242	291	232, 241	199	260	220
Angiano	193, 196	279, 282	235	178, 184	248, 251, 257	202
Athenas	174, 211, 239, 245	282, 294	229, 250	199	217, 248	202, 220
Ayala	199	270, 273	235, 238	199	254	205
Badil	214, 248	276, 291	229, 238, 241	197, 199	245	199
Bagdad	174, 239	282, 285	244	194	248	202
Bianka	214, 251	291	244, 260	199	257	202
Bonal	177, 211	291, 294	235	190, 194	214	220
Castúo	214, 248	273, 276	244	197	239	208
Cavir	183, 226	282	244	202	223	205
Chamad	177, 232	264	244	202	248	202
Duratón	214, 248	276, 297	213	199	248	208, 220
Elvira	214, 248	294	253	194	254	223
Eulalia	214, 248	276	244	197	239	208, 211
Fardón	202, 214, 248	279, 291	232	188, 199, 203	245, 248, 260	205, 220
Inmaculada	193, 229	291, 294	232	199	263	208, 220
Juano	174, 214, 239	294	232	194	248	202
Junco	214, 248	276	241, 244	199	248	205, 208
Kairo	174, 239	294	241, 244	190, 194, 199	217, 248	202
Krema	186, 217	267, 270	235	188	233	199
Lechoso	183, 226	267	244	203	257	205
Patio	214, 245	282	225, 238	194	248	220
Pedrosillano	193, 196	279, 282, 285	232	184, 188, 190	211, 245	202, 205, 208
Pilar	214, 248	276	244	197	239	208
Pringao	214, 245	294	232, 244	199	248	202
Puchero	245	270	244	181	242	214
Saborio	214, 245	294	232, 235	194	248	220
Solera	193	282	232	188, 199	226	211
Tizón	180, 18	276, 279, 282	244	208, 211	226	196
Zegri	211, 242	285, 288, 291, 294	232, 244	190	248	208
Zoco	214, 245	282	244	184	214	211

In order to evaluate the usefulness and efficiency of these six markers, they were used to analyse 16 new, unreleased chickpea cultivars (V1–V16). Similarity values among the 48 accessions (32 released plus 16 unreleased accessions) were low, except for the pair V1–V2 with a value of 0·90 and V12–Eulalia and Pilar–Castúo (0·88 in both pairs). The dendrogram obtained from the UPGMA analysis, based on the Jaccard similarity index, showed that all cultivars (released and unreleased) can be distinguished from the six selected markers (Fig. 3). A high cophenetic correlation was obtained ($r = 0·86$) indicating a good fit between the similarity matrix and the tree (respectively, the input and the output of the clustering method).

DISCUSSION

The present study aimed at characterizing and identifying chickpea cultivars using STMS and determining the potential utility of these markers for this purpose. Microsatellites are one of the most suitable and reliable marker systems for genetic diversity and genetic characterization of cultivars because of their power to detect polymorphisms in very closely related genotypes (Serret *et al.* 1997; Sethy *et al.* 2006*a*, *b*; Imtiaz *et al.* 2008). Moreover, these markers have a high amenability to automation and to being used in multi-pooling technologies. Multiplex PCR assays were developed for amplification of the 15 STMS employed in the present study, improving the efficiency and reducing the time and cost of analysing markers for cultivar identification compared to individual PCR analysis. Accordingly, it highlights the usefulness of STMS markers for multiplex PCR and capillary electrophoresis.

The 32 released chickpea cultivars were characterized using 15 STMS markers located through all eight LG of the chickpea map, with a distribution of two markers per LG, except for LG3 which is represented by only one. These markers provided uniform coverage of the chickpea genome, satisfying
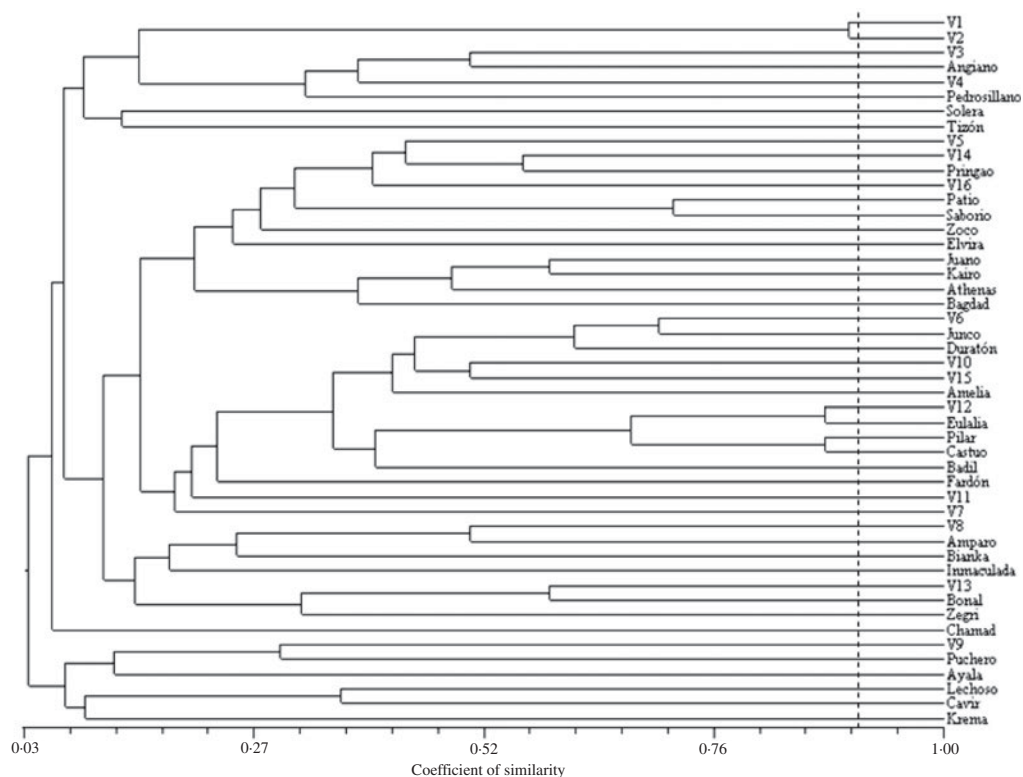
Fig. 3. UPGMA dendrogram obtained from cluster analysis of 48 chickpea cultivars (32 released and 16 unreleased) based on Jaccard's coefficient of similarity using the six STMS markers selected for their high PIC value. The vertical dashed line indicates the limit below which lines are regarded as similar.

the criterion proposed by UPOV (http://www.upov.int) with regard to the genome distribution of the markers for DNA profiles of cultivars. All STMS revealed high levels of polymorphism and some of them (TA5, TA11, TA14, TA78, TA106, TA113, TA117, TA130, TA135, TA144 and TA200) have been employed to study chickpea collections in previous studies (Tar'an *et al.* 2007; Imtiaz *et al.* 2008; Upadhyaya *et al.* 2008; Jomová *et al.* 2009), although the number of alleles per STMS locus and PIC values were not always consistent with the results obtained in the present study. The marker TA130 showed the lowest PIC value in the present assay (0·455), and also presented a low PIC value (0·600) in a study including 182 cultivated chickpea accessions (Tar'an *et al.* 2007). Therefore, TA130 may not be a good marker for the identification of chickpea commercial cultivars. However, this marker showed a higher PIC (*c.* 0·80) when wild species were included in diversity analysis (Imtiaz *et al.* 2008; Upadhyaya *et al.* 2008). In general, PIC has higher values when *C. arietinum* wild relatives are included in the plant material, reaching PIC values close to 1 (Imtiaz *et al.* 2008; Upadhyaya *et al.* 2008).

Moreover, as would be expected, the PIC value depends on the size of the collection used in each experiment.

Five plants per cultivar were analysed in the present study, so that genotypic homogeneity within the accessions could be tested. In theory, a cultivar should be homogeneous, being defined as a set of cultivated plants that can be distinguished from any other by the expression of at least one characteristic and must be uniform and have stability (DUS) (UPOV 1991). Moreover, chickpea is a self-pollinated crop and registered cultivars are expected to display phenotypic uniformity. The level of uniformity required is determined by the breeder and seed regulatory agencies and until now the registration of cultivars has been based mainly on morphological characteristics. However, the present study found variability within cultivars at the genotype level in some of the 32 accessions examined, which could be attributed to a high rate of mutation at SSR loci (Udupa & Baum 2001). In chickpea, a high degree of allelic variation exists at microsatellites loci (Udupa *et al.* 1999), indicating that the mutation rate at these loci is high. A small degree

of variability within cultivars was also described in chickpea by Chowdhury *et al.* (2002) using RAPD and inter-simple sequence repeats (ISSR) markers.

In spite of the variability found within cultivars, all of them could be discriminated. An important aim in cultivar identification is to distinguish between genotypes to avoid redundancy of identical accessions or duplicates. Only 6 out of the 15 STMS markers employed in the present study were needed to discriminate between the 48 chickpea accessions (32 registered cultivars and 16 unreleased chickpea accessions). These six STMS markers, selected for their high discrimination value, have been validated with the 16 unreleased chickpea accessions included in the second part of the current study. In spite of the fact that V1 and V2, V12 and Eulalia, Pilar and Castúo presented high levels of similarity with each other (0·90, 0·88 and 0·88, respectively), it was still possible to discriminate among the 48 accessions, indicating the usefulness of these markers for cultivar identification in this chickpea collection. Moreover, four (TA106, TA113, TA117 and TA200) out of these six STMS markers have been used in previous genetic diversity studies (Imtiaz *et al.* 2008; Upadhyaya *et al.* 2008) and are considered to be highly polymorphic and suited to the diversity analysis of chickpea collections.

With the exception of Tizón and V16, which are desi chickpeas, all the cultivars analysed in the present study are kabuli. At the molecular level, the kabuli-type groups are clearly distinct from the desi type of chickpea (Iruela *et al.* 2002). Both types have been reported to represent different genetic backgrounds (Moreno & Cubero 1978; Gil & Cubero 1993). Therefore, taking into account that kabuli represents a different aspect of the chickpea genetic background, the six STMS markers identified in the current study could be considered as a reference set for chickpea cultivar identification.

In crops such as soybean (Giancola *et al.* 2002), pepper (Kwon *et al.* 2005), corn (Gunjaca *et al.* 2008) and rice (Bonow *et al.* 2009), molecular profiles, associated with the description of a cultivar have been used to enforce the rights granted to breeders. Amplification patterns of the chickpea cultivars analysed in the present study can be used to identify cultivars and could be regarded as an official fingerprint to be used by the Community Plant Variety Office (CPVO; www.cpvo.europa.eu), UPOV or by national registers to distinguish with accuracy any chickpea accession from the accepted cultivars. The marker profile could be a powerful tool for the characterization of chickpea accessions, avoiding redundancy of identical cultivars and protecting breeders' rights.

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