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Transferability of newly developed genomic lentil SSR markers to *Cicer* species

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ABSTRACT

Development of microsatellite markers requires a great effort, expertise and research infrastructure. Therefore, cross genera or species transferability of already developed markers has constituted a significant alternative. In this study, transferability of newly developed 53 genomic lentil (*Lens culinaris* Medik.) SSR markers to 32 accessions of *Cicer* species including *C. arietinum* L., *C. bijugum* K.H. Rech., *C. echinospermum* P.H. Davis, *C. reticulatum* Ladiz., *C. pinnatifidium* Jaub. & Sp., *C. anatolicum* Alef. was investigated. Of these markers, 33.09% were found to be transferrable to *C. arietinum*, 37.7% to *C. echinospermum*, 35.8% to *C.reticulatum*, 39.6% to *C. bijugum*, 18.8% to *C. pinnatifidium* and finally 15.09% to *C. anatolicum* species. From these markers, 11.3% were found to be transferable to all *Cicer* species. Transferable 6 polymorphic SSR primers had 19 alleles in 32 accessions with a mean of 3.16 alleles per locus. Polymorphic information content (PIC) values varied between 0.375 (Lc_MCu21) and 0.587 (Lc_MCu53) with an average value of 0.528. Transferable SSR markers were thought to provide significant contributions to inter and intra-specific studies in *Cicer* species.

Key words: Chickpea, *Cicer*, Cross-transferability, Lens, Microsatellites, SSRs.

INTRODUCTION

Microsatellites or Simple sequence repeats (SSRs) are composed of DNA motifs with 1-6 nucleotides and tandem repeats and present in non-coding and coding sequence components of crop genomes. Microsatellites are widely employed in various genetic projects such as genetic diversity, population structure, phylogenetic relationships, construction of frame-work linkage maps, QTL interval mapping, map-based cloning of genes, marker-assisted selection (MAS) (Hendre *et al.,* 2007; Jha *et al.,* 2019) because of their abundance, multi-allelic nature, reproducibility, co-dominant inheritance, locus specific and good genome coverage (Powell *et al.,* 1996). Development of SSR markers is a costly and troublesome process. Such constraints limit the use of these markers in breeding programs of several crops (Gutierrez *et al.,* 2005). Identification of cross genera or species transferable loci will allow widespread use of SSRs and facilitate the performance of benchmarking genome studies.

SSR markers were proved transferable across taxa (Rossetto, 2001). Conserved homologous DNA flanking regions and primer binding sites in different genera have been reported between wheat and barley (Erpelding *et al.,* 1996), between *Malus* and *Pyrus* (Yamamoto *et al.,* 2001), among *Vitaceae* (Thomas and Scott, 1993; Rossetto *et al.,* 2002), among *Rosaceae* (Cipriani *et al.,* 1999; Downey and Iezzoni 2000; Sosinski *et al.,* 2000; *Dirlewanger et al.,* 2002), among *Betulacea* (Gurcan and Mehlenbacher, 2010), among *Vitaceae* and *Rosaceae* families (Decroocq *et al.,* 2003). In Fabaceae or *Leguminosae*, cross-species amplification was tested on soybean and other legume genera (Peakall *et al.,* 1998), *Medicago truncatula* and *Medicago* genus (Eujayle *et al.,* 2002), three additional legume species (*Pisum sativum*, *Phaseolus vulgaris* and *Glycine max*) (Baquerizo-Audiot *et al.,* 2001), *M. truncatula* and *Vicia faba*, *P. sativum*, *C. arietinum* (Gutierrez *et al.,* 2005), *Glycine max* and wild species of soybean (Hempel and Peakall, 2003), *C. arietinum* and wild species of chickpea (Choudhary *et al.,* 2009), *P. vulgaris* and *L. culinaris* to *Cajanus cajan* (Datta *et al.,* 2010a) *Vigna unguiculata* and other *Vigna* species (Gupta and Gopalakrishna, 2010), *L. culinaris* and wild species of lentil (Gupta *et al.,* 2016), *V. unguiculata*, *M. truncatula*, *Vigna radiata*, *C. arietinum* and *G. max* to *Lablab purpureus* L. (Shivakumar *et al.,* 2017).

The genus *Cicer* has 49 taxa (Smykal *et al.,* 2015) species out of which *C. arietinum* L. is the only cultivated species which is an annual, self-pollinated diploid (2n=16) cool season legume crop with a haploid genome size of 713 Mbp (Varshney *et al.,* 2013). Draft genome sequences for both *desi* and *kabuli* chickpea types have already been completed and respectively 30 000 and 81 000 microsatellite markers were developed as *in-silico* (Varshney *et al.,* 2013; Jain *et al.,* 2013). In wild chickpea species, draft genome sequences were completed in *C. reticulatum* Ladiz., a progenitor of chickpea (Gupta *et al.,* 2017) and 4072 SSR markers were developed through transcription sequencing analysis (Jhanwar *et al.,* 2012). However, experimental validation of these microsatellite markers and identification of polymorphic ones is a troublesome and costly process. Besides, narrow genetic base of chickpea and non-

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polymorphic nature of majority of identified markers (~70%) hinder potential use of these markers in genotyping practices (Sethy *et al.,* 2006; Nayak *et al.,* 2010; Gaur *et al.,* 2011; Hiremath *et al.,* 2011; Gujaria *et al.,* 2011; Choudhary *et al.,* 2012, Rialch *et al.,* 2018, Suman *et al.,* 2018).

In this study, cross-transferability of 53 newly developed SSR markers in lentil (*Lens culinaris* Medik.) (Bakır and Kahraman, 2019) to *Cicer* species was investigated and their use for discerning its phylogeny was discussed. Present findings will have great contributions for increasing the number of polymorphic makers in chickpea and identification of interspecies candidate genes through legume comparative genomics studies.

MATERIALS AND METHODS

Plant material and DNA isolation: To determine transferability of SSR markers developed for lentil (*Lens culinaris* Medik.) to *Cicer* species including 18 accessions (15 registered and 3 local cultivars) of *C. arietinum* L., one accession of *C. bijugum* K.H. Rech., two accessions of *C. echinospermum* P.H. Davis, eight accessions of *C. reticulatum* Ladiz., one accession of *C. pinnatifidium* Jaub. & Sp., two accessions of *C. anatolicum* Alefeld (a total of 32 accessions) were used (Table 1).

Genomic DNA was isolated from fresh, young leaves of all accessions in accordance with the protocol described by Lefort *et al.* (1998). The quality and quantity of the extracted DNA were determined in NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis (0.8 %).

PCR amplification for transferability of SSRs: A total of 53 newly developed lentil SSR markers were used in this study (Bakır and Kahraman, 2019). PCR reactions were performed in a 15µl volume containing 90 ng genomic DNA, 10 µM of each primer, 0.2 mM of each dNTPs, 1X DreamTaq Green Buffer (includes $MgCl_2$ at a concentration of 2 mM) (Thermo Scientific, Waltham, MA, USA) and 0.5U DreamTaq DNA Polymerase (Thermo Scientific, Waltham, MA, USA). PCR was performed on the Bio-Rad thermocycler with an initial denaturation of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50-66 °C, 2 min at 72 °C and a final extension at 72°C for 10 min. The amplified products were analyzed through 3 % agarose gel electrophoresis.

Table 1: List of *Cicer* species used in this study.

Accessions	Species	Origin
Hasanbey	*C. arietinum*	Eastern Mediterranean Agricultural Research Institute, Adana, Turkey
Seckin	*C. arietinum*	Eastern Mediterranean Agricultural Research Institute, Adana, Turkey
Aksu	*C. arietinum*	Eastern Mediterranean Agricultural Research Institute, Adana, Turkey
Aslanbey	*C. arietinum*	Eastern Mediterranean Agricultural Research Institute, Adana, Turkey
Ilgaz	*C. arietinum*	Eastern Mediterranean Agricultural Research Institute, Adana, Turkey
Tavas	*C. arietinum*	Eastern Mediterranean Agricultural Research Institute, Adana, Turkey
Arda	*C. arietinum*	GAP International Agricultural Research and Training Center, Diyarbakir, Turkey
ILC 482	*C. arietinum*	GAP International Agricultural Research and Training Center, Diyarbakir, Turkey
Hisar	*C. arietinum*	Transitional Zone Agricultural Research Institute, Eskisehir, Turkey
Azkan	*C. arietinum*	Transitional Zone Agricultural Research Institute, Eskisehir, Turkey
Cakir	*C. arietinum*	Transitional Zone Agricultural Research Institute, Eskisehir, Turkey
Akça	*C. arietinum*	Transitional Zone Agricultural Research Institute, Eskisehir, Turkey
Gulumser	*C. arietinum*	Black Sea Agricultural Research Institute, Samsun, Turkey
Sezenbey	*C. arietinum*	Black Sea Agricultural Research Institute, Samsun, Turkey
Zuhal	*C. arietinum*	Black Sea Agricultural Research Institute, Samsun, Turkey
Orenli-1	*C. arietinum*	Adıyaman/Turkey
Tasan-1	*C. arietinum*	Kayseri/Turkey
Tasan-2	*C. arietinum*	Kayseri/Turkey
AWC-6	*C. bijugum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
AWC-306	*C. echinospermum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
AWC-307	*C.echinospermum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
AWC-605	*C. reticulatum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
AWC-609	*C. reticulatum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
AWC-610	*C. reticulatum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
AWC-613	*C. reticulatum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
AWC-614	*C. reticulatum*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
Bari	*C. reticulatum*	Faculty of Agriculture, Harran University, Sanliurfa, Turkey
Cudi	*C. reticulatum*	Faculty of Agriculture, Harran University, Sanliurfa, Turkey
Kesen	*C.reticulatum*	Faculty of Agriculture, Harran University, Sanliurfa, Turkey
AWC-503	*C. pinnatifidium*	Faculty of Agriculture, Akdeniz University, Antalya, Turkey
K621	*C. anatolicum*	Faculty of Agricultural Biotechnology, Erciyes University, Kayseri, Turkey
K630	*C. anatolicum*	Faculty of Agricultural Biotechnology, Erciyes University, Kayseri, Turkey

Identification polymorphic SSRs: To determine polymorphism ratios of the SSR markers of which the first scans were performed and successfully amplified, PCR reactions were performed in 32 accessions of *Cicer* species by using M13-tailed primer according to the methods described by Schuelke (2000). A tail (M13 universal sequence (-21), TGTAAAACGACGGCCAGT) was added to the 5' end of each forward primers. PCR amplifications were performed in 15 µl reaction mixture containing 90 ng genomic DNA, 0.1 µM of each SSR primer, 0.1 µM labelled M13 (-21) universal primer, 0.2 mM of each dNTPs, 1X DreamTaq Green Buffer (includes $MgCl_2$ at a concentration of 2 mM) (Thermo Scientific, Waltham, MA, USA) and 0.5U DreamTaq DNA Polymerase (Thermo Scientific, Waltham, MA, USA). The amplification program consisted of an initial step of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50-66 °C, 2 min at 72 °C, followed by 8 cycles of 1 min at 94°C, 1 min at 53°C, 2 min at 72°C, and a final extension of 10 min at 72°C. The M13 (-21) primer was 5'-fluorescently tagged with HEX, 6-FAM or ROX to facilitate multiplexing. A set of three PCR products (0.5 µl each) was mixed with 0.5 µl GeneScan-600 LIZ size standards (Applied Biosystems, Foster City, CA, USA) and 9.5 µl Hi-Di™ formamide (Applied Biosystems, Foster City, CA, USA) and denatured at 95 °C for 5 min, chilled on ice and electrophoresed on the Applied Biosystems Prism 3500 Genetic Analyzer System (Applied Biosystems, Foster City, CA, USA). GENEMAPPER software v5.0 (Applied Biosystems, Foster City, CA, USA) was used to determine fragment size.

Data analysis: Microsatellite diversity analyses were carried out at the locus level in a given species. For each locus, the expected heterozygosity (*He*), observed heterozygosity (*Ho*) and polymorphism information content (PIC) (Nei, 1973) were calculated with PowerMarker V3.025 software (Liu and Muse, 2005). The UPGMA (unweighted pair-group method using arithmetic average) were used to construct and draw a dendrogram from the genetic similarity matrix by using the MEGA6 (Tamura *et al.*, 2007) and PowerMarker software programs. Bootstrap analyses with 100 replicates were performed and a consensus tree was obtained to measure the confidence levels for the clusters.

RESULTS AND DISCUSSIOIN

Transferability of SSR markers: To test the transferability of lentil SSR markers to *Cicer* species, 53 SSR markers were analyzed by using 32 accessions of 6 different *Cicer* species. Of 53 SSR markers, 18 SSR markers (33.09%) were found to be transferable to cultivated chickpea (*C. arietinum*), 20 SSR markers (37.7%) to *C. echinospermum*, 19 SSR markers (35.8%) to *C.reticulatum*, 21 SSR markers (39.6%) to *C. bijugum*, 10 SSR markers (18.8%) to *C. pinnatifidium* and finally 8 SSR markers (15.09%) to *C. anatolicum*. Besides them, 7 SSR markers (13.2 %) (Lc_MCu1, Lc_MCu4, Lc_MCu11, Lc_MCu21, Lc_MCu31, Lc_MCu51a, Lc_MCu53) were found to be to all *Cicer* species and 24 SSR markers (45.2 %) were found to be transferable to none of the *Cicer* species. Except for *C. bijugum*, Lc_MCu2 marker worked on the other species (Table 2).

Polymorphism of SSR markers: To test the transferability of 53 lentil primers to *Cicer* species, PCR products were initially run in 3% agarose gel. At the end of agarose gel assessments, 14 SSR markers yielding clean bands between 100-400 bp were loaded and analyzed in Applied Biosystems Prism 3500 Genetic Analyzer System (Applied Biosystems, Foster City, CA, USA) and analyzed. Of these markers, Lc_MCu1, Lc_MCu2, Lc_MCu4, Lc_MCu21, Lc_MCu31, Lc_MCu53 primers were identified as polymorphic. Based on fragment analysis, Lc_MCu11 marker was not assessed since it was monomorphic, Lc_MCu3, Lc_MCu9, Lc_MCu51a markers were not assessed since they did not have multiple peak images, Lc_MCu20, Lc_MCu38a, Lc_MCu46a, Lc_MCu50a primers were not assessed since they did not yield any peak images.

Transferable 6 polymorphic SSR primers had 19 alleles in 32 accessions of *Cicer* species and the number of alleles per locus varied between 2 to 4 with an average value of 3.16 (Table 3). Expected heterozygosity ratios of 6 polymorphic SSR primers varied from 0.500 (Lc_MCu21) to 0.661 (Lc_MCu53) with an average value of 0.661. The heterozygosity ratios were found between 1.000 (Lc_MCu21) and 0.781 (Lc_MCu1) with an average value of 0.490. Polymorphic information content (PIC) values ranged from 0.375 (Lc_MCu21) to 0.587 (Lc_MCu53) with an average value of 0.528.

Transferability of lentil markers to *Cicer* species indicated that flanking regions of these microsatellites had sufficient immunity for locus amplification. Similar transferability ratios of lentil markers to chickpea were also reported in previous studies carried out with different legume species. Gutierrez *et al.,* (2005) reported 40%, 36.4% and 37.6% transferability of *M. truncatula* microsatellites to *V. faba*, *C. arietinum* and *P. sativum*, respectively. Choumane *et al.* (2004) observed 54.4% sequences conservation among chickpea, lentil and pea. Gutierrez *et al.* (2005) observed 40%, 36.3%, 37.6% transferability of *M. truncatula* microsatellites to *V. faba*, *C. arietinum* and *P. sativum*. Choudhary *et al*. (2009) observed cross-species transferability ranging from 68.3 to 96.6% across 6 annual *Cicer* species and from 29.4 to 61.7% across 7 seven legumes genera consisting of *P. mungo, P. sativum, G. max, T. alexandrinum, L. esculenta, C. cajan, M. truncatula*. Datta *et al.* (2010b) observed 46% transferability of chickpea microsatellites to *C. cajan*. In another study, Reddy *et al* (2010) reported the transferability of microsatellites on lentil accessions as 36.0%, 62.0% and 25.0% for *M. truncatula*, *T. pratense* and *P. sativum*-derived markers, respectively. Pandian *et al.* (2000) tested the transferability of chickpea primers to 3 legumes including *L.culinaris, Vicia cracca, and P. sativum* and they observed 5%, 3% and 18%

transferability of chickpea microsatellites to *L.culinaris*, *Vicia cracca, and P. sativum*, respectively. Datta *et al.* (2010a) reported 9.3 % transferability of lentil microsatellites to *C. cajan*. Variation in SSR transferability depends on factors including genetic and genomic nature of microsatellites, the number of markers tested, amplification

Table 2: Cross amplification of lentil SSR markers in *Cicer* species.

SSR marker	*Cicer arietinum*	*Cicer bijugum*	*Cicer echinospermum*	*Cicer reticulatum*	*Cicer pinnatifidium*	*Cicer anatolicum*
Lc_MCu1	+	+	+	+	+	+
Lc_MCu2	+	-	+	+	+	+
Lc_MCu3	-	-	+	+	-	-
Lc_MCu4	+	+	+	+	+	+
Lc_MCu5a	+	+	-	-	-	
Lc_MCu6	-	-	-	-	-	-
Lc_MCu7	-	-	-	-	-	-
Lc_MCu8a	-	-	-	-	-	-
Lc_MCu9	+	+	+	+	-	-
Lc_MCu10	-	-	-	-	-	-
Lc_MCu11	+	+	+	+	+	+
Lc_MCu12b	-	-	-	-	-	-
Lc_MCu13a	-	-	-	-	-	-
Lc_MCu14b	-	-	-	-	-	-
Lc_MCu15	-	+	+	-	-	-
Lc_MCu16b	-	-	-	-	-	-
Lc_MCu17	-	-	-	-	-	-
Lc_MCu18	+	-	-	-	-	-
Lc_MCu19	-	-	-	-	-	-
Lc_MCu20	+	-	+	+	-	-
Lc_MCu21	+	+	+	+	+	+
Lc_MCu22	-	-	-	-	-	-
Lc_MCu23a	-	-	-	-	-	-
Lc_MCu24	+	-	-	-	-	-
Lc_MCu25	-	-	-	-	-	-
Lc_MCu26a	-	-	-	-	-	-
Lc_MCu27a	-	-	-	-	-	-
Lc_MCu28	-	+	-	-	-	-
Lc_MCu29a	-	+	-	-	-	-
Lc_MCu30a	-	-	-	-	-	-
Lc_MCu31	+	+	+	+	+	+
Lc_MCu32a	-	-	-	-	-	-
Lc_MCu33	-	+	+	-	-	-
Lc_MCu34a	-	-	-	-	-	-
Lc_MCu35	-	-	-	-	-	-
Lc_MCu36a	+	-	+	-	-	-
Lc_MCu37	-	-	-	-	-	-
Lc_MCu38a	-	-	-	+	-	-
Lc_MCu39ab	-	+	+	-	-	-
Lc_MCu40	-	-	-	-	-	-
Lc_MCu41a	-	-	-	-	-	-
Lc_MCu42	-	+	+	-	+	-
Lc_MCu43	-	-	-	-	-	-
Lc_MCu44	-	-	-	-	-	-
Lc_MCu45a	+	+	+	+	-	-
Lc_MCu46a	+	+	-	+	-	-
Lc_MCu47a	-	+	-	+	+	-
Lc_MCu48a	+	-	-	+	-	-
Lc_MCu49a	-	+	+	+	-	-
Lc_MCu50a	+	+	+	+	-	-
Lc_MCu51a	+	+	+	+	+	+
Lc_MCu52a	-	+	+	+	-	-
Lc_MCu53	+	+	+	+	+	+

Table 3: Genetic parameters for SSR primers number of alleles (n), expected heterozygosity (He) and observed heterozygosity (Ho), PIC (Polymorphism Information Content).

Locus	n	*He*	*Ho*	PIC
Lc_MCu1	3	0.601	0.781	0.516
Lc_MCu2	3	0.635	0.625	0.561
Lc_MCu4	4	0.632	0.125	0.560
Lc_MCu21	2	0.500	1.000	0.375
Lc_MCu31	4	0.635	0.125	0.571
Lc_MCu53	3	0.661	0.281	0.587
Total	19	3.664	2.938	3.171
Mean	3.166	0.611	0.490	0.528

conditions and plant species involved. Across genera and beyond transferability ratios of genomic microsatellites were about two-times less as compared to genic microsatellites (Peakall *et al.,* 1998; Roa *et al.,* 2000; Gutierrez *et al.,* 2005).

Just because of narrow genetic structure of chickpea, it was reported that majority of developed SSR primers did not exhibited polymorphism (Sethy *et al.,* 2006; Nayak *et al.,* 2010; Gaur *et al.,* 2011; Hiremath *et al.,* 2011 Gujaria *et al.,* 2011; Choudhary *et al.,* 2012). The polymorphism ratio of lentil markers to *Cicer* species was identified as 11.3%. When these newly developed lentil primers were tested on lentil, polymorphism ratio was identified as 58.4%. Of the markers exhibiting polymorphism on both species, 19.355 were common (Bakır and Kahraman, 2019).

In brief, transferability of newly developed 53 genomic lentil SSR markers to chickpea was tested in this study. Transferable SSR markers were thought to provide significant contributions for inter and intra-specific and comparative genomic studies to be carried out with *Cicer* species.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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