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AGAROSE-RESOLVABLE SSR MARKERS BASED ON ddRADSeq IN CHICKPEA

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Abstract: Exploitation of genetic diversity is essential for sustainable crop production. Molecular markers have potential to reach these goals in more rapid and efficient manner. Here, we developed genomic SSR markers from chickpea with the use of ddRADSeq data. 1,396 SSR regions with an average of 530 SSR/Mb in the whole genome were successfully identified. Considering different types of repeats, dinucleotides were the most frequent type accounting for 62.03% of the total SSR regions identified, followed by trinucleotides (25.50%) and tetranucleotides (4.58%). The AT/TA motif was greatly characterized among dinucleotide repeats, and it was also the most common type in the chickpea genome accounting for 36.5% of the total SSR regions identified, followed by AG/GA (139) and TC/CT (135) among dinucleotide motifs. Considering their genomic distribution and simple visualization on agarose gels, we examined SSR regions of 10 bp and longer for identification of SSR markers. A total of 10 SSR markers were successfully designed and resulted in successful polymorphic bands among chickpea genotypes. Consequently, the results show that ddRADSeq is effective for marker development and these markers might be valuable for biodiversity studies, marker-assisted selection (MAS) and linkage map construction in chickpea.

Keywords: Chickpea, ddRADSeq, Marker, SSR, Sequencing

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1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the most significant legume crops in the family Fabaceae, subfamily Faboideae (van der Maesen et al., 2007). *C. arietinum* is the only cultivated species of the genus *Cicer*. The cultivated chickpea is considered to be evolved from its wild ancestor, *C. reticulatum* Ladiz by selection (van der Maesen, 1987). Chickpea is mainly cultivated in India, Australia, Ethiopia, Türkiye and Myanmar (FAOSTAT, 2022). It was globally grown in 14.8 million ha and world chickpea production was about 18.1 million tons in 2022 (FAOSTAT, 2022). Chickpea is a diploid (2n =2x = 16) and self-pollinated plant having a genome size of approximately 740 Mb (Varshney et al., 2013). It is high in carbohydrates (60-65%), plant-based protein (20-22%) and fat (6%), and a good source of vitamins (vitamin A, B, folate, and thiamine) and minerals (iron, potassium and zinc) (Gaur et al., 2016). It also plays significant role in the soil fertility enrichment and crop rotation because of its capacity to fix the atmospheric nitrogen (Herridge et al., 1993).

The chickpea production has been influenced by many environmental factors in the worldwide (Sari et al., 2022). Because genetic diversity in the cultivated chickpea was limited, an attempt to increase production has not been sufficient (Roorkiwal et al., 2014). The prime objective of chickpea breeding is improving high-yield and high-quality varieties. Molecular-marker assisted breeding have potential to reach these goals in more rapid and efficient manner. Molecular markers have the potential to reveal the genetic diversity among genotypes (Cui et al., 2017) and also efficient tools for biodiversity studies, segregation analysis, construction of genetic physical and genetic maps as well as transcript profiling (Singh et al., 2010). So far, random amplified polymorphic DNA (RAPD) (Iruela et al., 2002; Talebi et al., 2008), amplified fragment length polymorphism (AFLP) (Nguyen et al., 2004; Shan et al., 2007), simple sequence repeat (SSR) (Sethy et al., 2006), inter simple sequence repeat (ISSR) (Sudupak, 2004; Amirmoradi et al., 2012; Aggarwal et al., 2015) and internal transcribed spacer (ITS) (Singh et al., 2008) have been conducted to identify genetic diversity in chickpea. In recent years, the advances of high-throughput sequencing techniques (or next-generation sequencing (NGS)) have prompted the identification of high-quality markers such as simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers in various natural or mapping populations of chickpea (Gujaria et al., 2011; Gaur et al., 2012; Kujur et al., 2015; Sari et al., 2023). Opposite to SNP markers, SSRs are very simple and practical. They are also greatly informative, abundant in the genome, multiallelic and locus specificity, and have been widely utilized because of the co-dominance and

highly reproducible nature for plant breeding applications (Davey et al., 2011; Sakiyama et al., 2014). Several number of SSR markers have also been applied in genetic diversity studies (Seyedimoradi et al., 2019), linkage mapping (Ahmad et al., 2014) and QTL analysis for the identification of candidate genes (Jha et al., 2019). Nevertheless, the identification of markers might be restricted due to the narrow genetic base in chickpea (Sethy et al., 2006; Gujaria et al., 2011). So, it is crucial to find alternative markers for chickpea genomics-assisted breeding applications.

With an aim of enhancing the marker repository and development of the breeder friendly markers in chickpea, the present study focused on identification and development of SSR markers with the use of double digest restriction site-associated DNA sequencing (ddRADSeq) data from 20 chickpea accessions compared with a reference genome sequence. The developed markers were also tested on chickpea germplasm with a simple and low-cost agarose gel electrophoresis.

2. Materials and Methods

2.1. Plant Material

A total of 20 chickpea accessions from nine different regions in Africa, America, Asia, and Europe were evaluated for ddRADSeq analysis in this study (Table 1). The highest number of accessions was from India (8), followed by Spain (3), Türkiye (2), Russian Federation (2), Mexico (2), Iran (1), the United States (1) and Ethiopia (1). The seeds of each accession in the collection were sown in pots for DNA analysis.

Table 1. List of the chickpea genotypes used ddRADseq analysis

No	Species	Kabuli/Desi	Gen bank Number/Name	Origin	Continent
1	*Cicer arietinum*	Kabuli	ILC 200	Russian Fed	Europe
2	*Cicer arietinum*	Desi	ICC 552	India	Asia
3	*Cicer arietinum*	Kabuli	ILC 3507	Spain	Europe
4	*Cicer arietinum*	Desi	ICC 506	India	Asia
5	*Cicer arietinum*	Desi	ICC 988	Mexico	America
6	*Cicer arietinum*	Desi	ICC 5912	India	Asia
7	*Cicer arietinum*	Kabuli	ILC 3500	Spain	Europe
8	*Cicer arietinum*	Desi	ICC 5714	India	Asia
9	*Cicer arietinum*	Desi	ICC 8325	India	Asia
10	*Cicer arietinum*	Desi	ICC 5434	India	Asia
11	*Cicer arietinum*	Desi	ICC 7509	Iran	Asia
12	*Cicer arietinum*	Desi	ICC 4929	India	Asia
13	*Cicer arietinum*	Desi	ICC 12031	Mexico	America
14	*Cicer arietinum*	Desi	ICC 10301	USA	America
15	*Cicer arietinum*	Kabuli	Hasanbey	Türkiye	Europe
16	*Cicer arietinum*	Kabuli	CA 2969	Spain	Europe
17	*Cicer arietinum*	Desi	ICC 1069	Russian Fed	Europe
18	*Cicer arietinum*	Desi	ICC 8262	Türkiye	Europe
19	*Cicer arietinum*	Desi	ICC 533	India	Asia
20	*Cicer arietinum*	Desi	ICC 8617	Ethiopia	Africa

2.2. DNA Extraction

Extraction of genomic DNA was conducted using the cetyltrimethylammonium bromide (CTAB) method given by Doyle and Doyle (1990) with some modifications such as the use of extra chloroform:isoamyl alcohol and 70% ethanol cleaning steps to increase DNA purity. Quality of extracted DNA was checked on a 1% agarose gel.

2.3. Library Preparation, Sequencing and SSR Identification

The ddRADseq library involved using a modified version of the protocol from Peterson et al. (2012). Main difference was that we used different restriction enzymes. Briefly, for the library preparation, we digested ~200 ng DNA per sample with two restriction enzymes, VspI (AseI, Thermo Fisher) and EcoRI (methylation sensitive, Thermo Fisher), and ligated P1 and P2 adapters to the fragments' restriction ends. Before ligation, Ampure XP beads (Beckman Coulter Genomics) were used to clean digestion products. After ligation, 15 cycles of PCR amplification with genotype specific indexed primers were performed. The PCR products were visualized on an agarose gel and combined and equalized in concentration. The genomic library with insert size of 300–450 bp was run on Illumina HiSeq platform using the 2x150 bp paired-end sequencing protocol. The ddRAD sequencing data of 20 accessions have been deposited in the National Center for Biotechnology Information (NCBI) Sequence-Read Archive (SRA) database with the accession number of PRJNA1064701.

For bioinformatic analysis, the raw data were demultiplexed with Je (v1.2) (Girardot et al., 2016). Quality control and preprocessing of FASTQ files were done using fastp (Chen et al., 2018), and reads were trimmed by removing bases with quality score with an

average Phred score less than 15. The cleaned data were mapped to kabuli reference genome 1.0 (Varshney et al., 2013) using Bowtie 2 (v2.2.6) (Langmead and Salzberg, 2012). Variant calling was performed in Freebayes (Galaxy Version 1.1.0.46-0) (Garrison and Marth, 2012) with genotype specific individual 'alignment files in BAM format' by selecting following parameters: simple diploid calling with filtering, and coverage of 20X. SNPs were removed from the variant files using VCFfilter (Galaxy Version 1.0.0). The separate .vcf files containing insertions and deletions were merged into a single data file. The combined variant file was organized in Microsoft Excel in order to remove duplicated regions and arrange the SSRs by sizes. SSR regions were visualized with Integrated Genome Browser (IGB) (Freese et al., 2016) using BAM files of the genotypes and the chickpea reference genome.

2.4. Primer Design and PCR Amplification

To develop the SSR markers, flanking sequences of the identified SSRs were extracted as the target sequence based on the chickpea reference genome by using IGB software. For designing forward and reverse primers, Primer3Plus (Untergasser et al., 2007; https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used with the following characteristics: optimal length of primers of 18–27 bp, melting temperature (Tm) between 50 and 60 °C, 30–70% GC, and PCR amplicons of 150–500 bp. The primer pairs were checked for possible duplication using IGB software. The designed primers were later controlled for possible matches of with other loci in the genome. All markers were termed as CA-D(I)-X-XXX format, where "CA" stands for chickpea, "D" and "I" for deletion and insertion, "X" for chromosome number, and "XXX" for start of the chromosomal position.

10 SSR markers which were evenly distributed on each chromosome, were selected from the designed primers pairs to be validated on 20 chickpea germplasm. For PCR analysis, a total volume of 20 reaction mix was used, which included 1 µL of genomic DNA, 1 µL of 10× PCR buffer, 2.5 mM $MgCl_2$, 0.3 µL of 10 mM dNTP mix, 0.3 µL of each primer (10 µM), 0.2 µL of Taq DNA polymerase (5 U/µL), and ddH_2O. The PCR reaction was conducted with the following conditions: 95 ^{0}C for 5 min; followed by 29 cycles of 95 ^{0}C for 20 s, 60 ^{0}C for 40 s, and 72 ^{0}C for 40 s; 7 cycles of 95 ^{0}C for 20 s, 55 ^{0}C for 40 s, and 72 ^{0}C for 40 s; and extension at 72 ^{0}C for 7 min (Sari et al., 2023). The PCR products were visualized on 3% agarose gels and recorded as codominant data, with genotypes by fragment size.

3. Results

A total 349.86 M raw sequence reads of 20 chickpea accessions with the mean of 3.68 M was generated from the Illumina HiSeq platform. The guanine-cytosine (GC) content of the reads was 38%. Using variant calling pipeline, 1,396 microsatellites were identified among the accessions, with an average of 530 SSR/Mb. Motifs ranged from 2 to 17 bp in length. Considering different types of repeats, dinucleotide motifs were the most frequent type corresponding to 62.03% of the total SSR regions identified, followed by trinucleotides (25.50%) and tetranucleotides (4.58%), while octa-nucleotide motifs were the rarest repeat (1.46 %) (Figure 1).

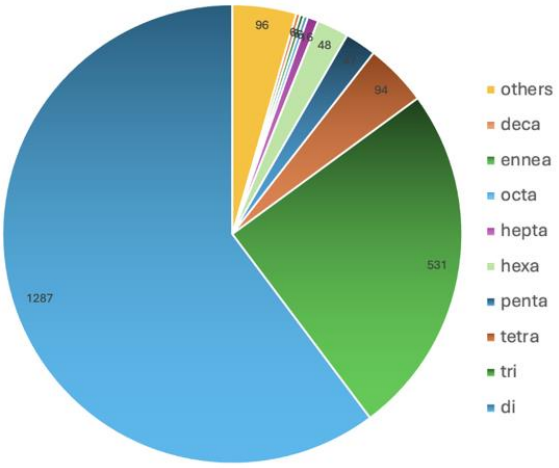

Figure 1. Distribution of SSRs in different classes.

All the SSRs distributed across all 8 chromosomes (Figure 2), with a correlation between the frequency of microsatellite loci and the chromosome size. For instance, chromosome 4 had the greatest frequency of 19.48 %, it was also one of the largest chromosomes. The greatest number of microsatellites was occurred on chromosome 4 (272), followed by chromosomes 6 (213) and 5 (199), and the smallest number of SSR was observed on chromosome 8 (60) (Figure 2).

The investigation of nucleotide composition characteristics showed that some motifs were more common than others. For instance, the AT/TA motif greatly characterized among dinucleotide repeats, and it was also the most common type in the chickpea genome accounting for 36.5% of the total SSR regions identified, followed by AG/GA (139) and TC/CT (135) among dinucleotide motifs. Trinucleotides (25.50%) and tetranucleotides (4.58%) were other abundant repeat types in chickpea genome. Among trinucleotides, the AAT/ATT (184) was the most abundant followed by GAA/CTT (68), whereas, among tetranucleotides, TAAA/TTTA (28) were most abundant type.

Considering their genomic distribution and simple visualization on agarose gels, we examined SSR regions of 10 bp and longer for identification of SSR markers (Table 2). A total of 10 SSR regions was successfully designed. The SSRs were dispersed across the 8 chromosomes. The chromosomal position, size information and repeat size of SSR regions were shown in Table 2. The longest polymorphic repeat (28 bp) was in chromosome 7 (physical position: 3887904), followed by chromosome 1 (physical position: 26908497), chromosome 2 (physical position: 35938514) and chromosome 5 (physical position: 38571762) with 18, followed by chromosome 2 (physical position:

36011349) with 16. Figure 3 showed the PCR aplicons which were generated with these primers. Annotation analysis of SSRs showed their highest frequency in intergenic regions (66.6%), followed by coding sequences (CDS) (22.2%), and exons (22.2%) (Table 3).

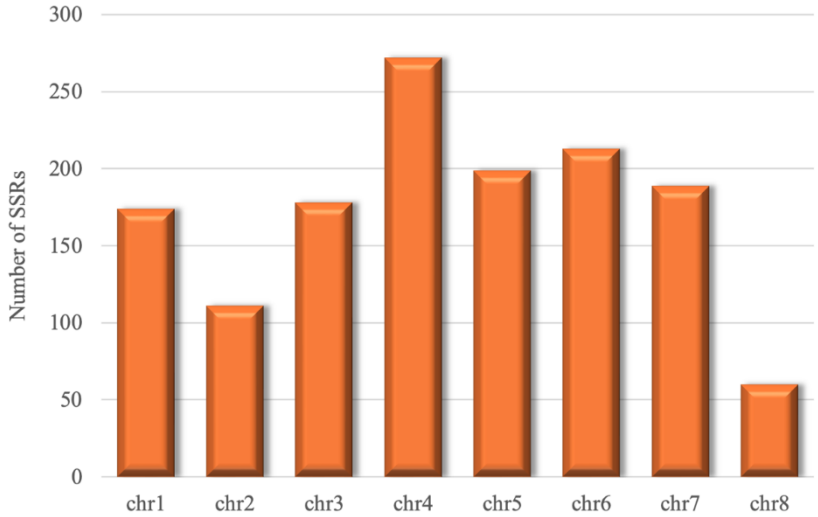

Figure 2. Distribution of all SSRs on 8 chromosomes (chr1-chr8) of chickpea.

Table 2. Information about SSRs identified in this study

Marker Name	Chromosome	Physical Position	Size (bp)	Repeat
CA-SSR-1-269	chr1	26908497	18	(ATA)
CA-SSR-1-221	chr1	2218745	14	(AATTTTC)
CA-SSR-2-359	chr2	35938514	18	(AGA)
CA-SSR-2-360	chr2	36011349	16	(CT)
CA-SSR-4-343	chr4	34309035	15	(TTA)
CA-SSR-5-385	chr5	38571762	18	(TG)
CA-SSR-7-388	chr7	3887904	28	(CT)
CA-SSR-7-630	chr7	6301545	15	(AATAT)
CA-SSR-7-165	chr7	16500535	12	(TAAAAA)
CA-SSR-8-101	chr8	10131288	12	(TAACCC)

Table 3. The primer sequences of the 10 SSR markers developed and used in this study

Marker Name	CHR	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	PL	Locus Location *
CA-SSR-1-269	chr1	AATGAATGAATTTATGGAACAATTT	GGCACTCCCTCCATTTAGAA	258	intergenic region
CA-SSR-1-221	chr1	CAAGATGTGCTAAAGCTTACAAAA	CCAAGGAATGGGAAAGGACT	180	intergenic region
CA-SSR-2-359	chr2	TTTCATTGCTAGGACCACCA	CTTTGTTCCTTTCCGGTCTG	191	CDS
CA-SSR-2-360	chr2	AACGCCATCCCTAATCGTC	TGAGGAACCCTAAGCATACAAA	180	exon
CA-SSR-4-343	chr4	TCTCTCATTATTATTCTTCCGACA	ATGGTCGTTTTCGGAACTTG	302	intergenic region
CA-SSR-5-385	chr5	TTCCTTGCTTTGCAGATCTTT	TCCGGTAGGGATAAAAGCAA	183	CDS
CA-SSR-7-388	chr7	GAAAGCGCAGGGAATATAACA	CACAACACAACGGAATGGAG	173	intergenic region
CA-SSR-7-630	chr7	TCTTCAAACAATGGTCCTCAGA	TCCACCGCGTTAGTCTTTCT	442	intergenic region
CA-SSR-7-165	chr7	GCTTACCGGAATCAGACCAA	AAAATCGAGAAAATGCTAATATCAAAA	169	intergenic region
CA-SSR-8-101	chr8	TAATGGCGAACAGAACACGA	CCGTACGGTTGGTAAGGAAA	199	exon

CHR= chromosome, PL= product length (bp).

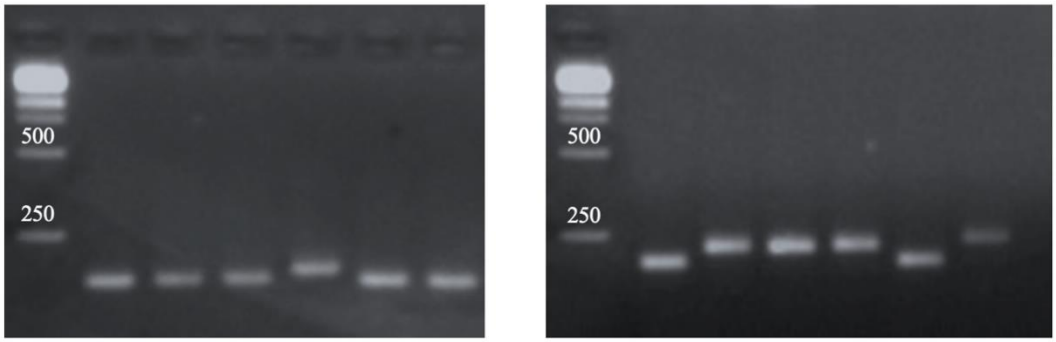

Figure 3. Amplification of chickpea DNAs with use of selected markers (Ladder 1 kb).

4. Discussion

Extreme weather events such as rains, floods droughts, heat waves, freezes and, acidification, and emergence of new infectious diseases appears as a result of climate change (Singh et al., 2023). Exploiting the natural variations from germplasm resources to develop climate-resilient crops is one of the main objects of plant breeding. Marker-assisted breeding is a rapid and efficient tool to characterize genetic diversity in plants (Hasan et al., 2021). During recent decades, the development of molecular markers based on PCR such as RAPD, SSR, AFLP, STS, SNP, etc. have led to genetic resources utilization in chickpea (Shan et al., 2007; Talebi et al., 2008; Sari et al., 2022). Among the markers, SSRs play an important role as molecular markers owing to their high polymorphic, co-dominant and multi-allelic nature (Khajuria et al., 2015). In addition, the advent of NGS facilitated SSR identification in chickpea. ddRADseq is a popular tool used for SSR discovery and genotyping. It is based on the genome reduced representation by digestion with two restriction enzymes (Peterson et al., 2012). In this study, we used ddRADseq to identify SSRs markers. As a result of the study, 1,396 SSR regions with an average of 530 SSR/Mb in the whole genome (1,396 SSRs in a genome size of 740 Mbp) were successfully identified. Our SSR frequency was similar to cucumber (552 SSR/Mb; Cavagnaro et al., 2010), lower than rice 807 SSR/MB; Lawson and Zhang, 2006), and higher than melon (109 SSR/Mb; Zhu et al., 2016), and wheat (163 SSR/Mb) (Huo et al., 2008). Sequencing method, the number of genotypes, or bioinformatic parameters during the variant calling might cause these differences in the SSR frequency.

Among the 1,396 SSRs identified in chickpea genome, dinucleotides were the most frequent type (62.03%). Trinucleotides were the second most common type account for 25.50% and followed by tetranucleotides (4.58%). These results were similar to those found in *Stevia rebaudiana* by Kaur et al. (2015). Interestingly, it was reported that trinucleotide repeats were the most abundant in safflower (Ahmadi and Ahmadikhah, 2022), pea (Gong et al., 2010), soybean (Hisano et al., 2007). Mononucleotide repeats were reported to be the most abundant in lentil (Bhati et al., 2015), *Brachypodium* (Sonah et al., 2011) and faba bean (Abuzayed et al., 2017).

Overall, The AT/TA motif was largely characterized among dinucleotide repeats, and it was the most common type in the chickpea genome, which result was similar to flax Cloutier et al., (2009). On the other hand, GC motif was very limited among all repeats. This result agreed with that of Wang et al. (1994) and Tangphatsornruang et al. (2009), who indicated GC-rich repeats as the rarest type in several plant species.

All the SSRs distributed across all 8 chromosomes, with a correlation between the chromosome size and the frequency of microsatellite loci. For instance, chromosome 4 had the largest frequency SSRs, while it was one of the greatest chromosomes. Chromosome 8 had the smallest size in chickpea, the least density of SSRs was obtained from chromosome 8. These findings are confirmed by previous studies of chickpea describing a large number of markers in chromosome 4 (Varshney et al., 2014; Jaganathan et al., 2015; Srivastava et al., 2016; Thudi et al., 2016). In contrast, Singh et al. (2023) reported that there was no correlation between the frequency of SSRs and the chromosome size in pomelo.

In molecular breeding, agarose gel-based markers with breeder-friendly genotyping appear to be better more than SNP or KASP markers from NGS technologies (Hu et al., 2020). For this reason, we developed 10 agarose-resolvable markers resulting successful polymorphic bands among chickpea genotypes. Consequently, this provides an effective method for ddRADSeq library preparation and scripts for SSR identification, resulting in 100% efficiency in PCR amplicons. Annotation analysis revealed the highest frequency of SSRs in intergenic regions (66.6%) (Table 3), similar to the results in different crops (Grover et al., 2007; Parida et al., 2009; Liu et al., 2013). Parida et al. (2015) indicated the efficiency of polymorphic SSRs derived from non-coding areas in chickpea.

5. Conclusion

The development of NGS technologies has prompted the discovery of high-quality genome-derived markers. SSRs are one of the most popular molecular markers in breeding studies due to its worthy desirable genetic

features. In the present study, we developed 10 SSR markers using ddRADSeq that might play an important role for chickpea genetic and genomic studies. Efficiency of these markers has also been tested on 20 different chickpea accessions.

Author Contributions

The percentage of the author(s) contributions is presented below. The author reviewed and approved the final version of the manuscript.

	D.S.
C	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100
FA	100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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