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GENETIC DIVERSITY IN NINE CHICKPEA (*CICER ARIETINUM* L.) VARIETIES BASED ON DIFFERENT MOLECULAR MARKERS

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Abstract

Nine varieties (BARI Chola-1 to BC-9) of chickpea (*Cicer arietinum* L.) were studied with respect to isozymes, SDS-PAGE, amplified DNA produced by RAPD- and SSR-markers. The varieties could be characterized by SDS-PAGE bands on the basis of their location, size and intensity. Three isozyme systems, namely acid phosphatase, esterase and peroxidase were investigated of which esterase was found to be suitable for produced distinct polymorphic bands with 57.14% diversity. Ten RAPD primers were producing 74 bands with 93.24% polymorphisms which indicated highly diverse nature. In addition to polymorphism, 12 variety-specific RAPD fragments were identified. Ten SSR primer pairs were producing 20 distinct bands of which 11 were considered as polymorphic (55%). On the basis of RAPD and SSR analysis, BC-1 and BC-6 were placed in cluster-1 and the remaining varieties were placed in cluster-2. On the other hand, the combined data of three isozymes systems and SDS-PAGE made BC-8 and BC-9 were different and thus placed in cluster-2 while the other seven varieties were placed in cluster-1.

Introduction

Bangladesh Agricultural Research Institute (BARI) released nine varieties of *Cicer arietinum* L. (Mandal *et al.* 2011). These are characterized on the basis of their morphological features and yield production. This kind of characterization sometimes creates problem since phenotypic features are not always reliable. Successful breeding program depends on the complete knowledge and understanding of the genetic diversity within and among genetic resources of the available germplasms. This will enable plant breeders to choose parents that generate diverse populations for selection (Esmail *et al.* 2008).

Molecular markers are efficient tools to estimate the genetic diversity. Among the molecular markers isozymes systems and SDS-PAGE have been successfully applied for germplasm characterization (Netra and Prasad 2007). Three most widely used isozymes are acid phosphatase, esterase and peroxidase. These are being used in plants for various purposes, including taxonomic or genetic relationship studies (Iqbal *et al.* 2005).

DNA fingerprinting by RAPD is one of the molecular methods for characterizing germplasms. RAPD analysis is used to evaluate a diverse level of polymorphism in different crops such as chickpea 98.14% (Rasool 2013), chickpea 87% (Datta *et al.* 2010), *Brassica* 98.03% (Ghosh *et al.* 2009), eggplant 57.89% (Biswas *et al.* 2009), peanut 96% (Lang and Hang 2007), peanut 42.7% (Raina *et al.* 2001) and chilli 90% (Paran *et al.* 1998).

Simple sequence repeats (SSRs) are another class of molecular microsatellite marker based on tandem repeats of short (2 - 6 bp) DNA sequences (Litt and Lutty 1989). These repeat sequences are often highly polymorphic, even among closely related varieties. Due to the slippage mutations during DNA replication causing variation in the number of repeating units. SSR markers are generally reported to detect higher levels of polymorphism and to provide the molecular differentiation to facilitate routine diversity analysis and molecular breeding applications (Russel *et al.* 1997, Crouch *et al.* 1999).

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Although some molecular marker analyses of chickpea have been undertaken earlier, these were scattered and not exactly used for characterization. No attempt has been made earlier to analyze different molecular data for characterizing chickpea varieties. In the present study, different types molecular analysis *viz.* isozyme, SDS-PAGE, RAPD and SSR were carried out for the first time to characterize nine chickpea varieties released from BARI with a view to evaluating the genetic diversity and the phylogenetic relationship.

Materials and Methods

The seeds of nine varieties of chickpea (*Cicer arietinum* L.) *viz.* BC-1 (BARI Chola-1), BC-2, BC-3, BC-4, BC-5, BC-6, BC-7, BC-8 and BC-9 obtained from the Pulse Research Center (PRC), BARI were grown in the field of the Botanical garden, Department of Botany, University of Dhaka.

The young leaves were collected from the field and homogenized in cold distilled water in an ice cold mortar pastel with liquid nitrogen and centrifuged at 4°C using 12000 rpm for 10 min. Supernatant was discarded and pellet used for isozyme and SDS-PAGE experiment. A mixture of 50 ml was freshly prepared by the following proportion of 49 ml of Na_2CO_3 (1%), 0.5 ml of $CuSO_4$ (1%) and 0.5 ml of Na-K titrate (2%). The quantity of proteins in various extracts was estimated following Lowery *et al.* (1951). Fifteen test tubes were marked as A, B, C, D, E, F and 1 to 9. These tubes contained materials in the following proportion: tube A: 1 ml of distilled water (control), tube B: 200 ml Bovine Serum Albumin (BSA) with 800 ml distilled water, tube C: 400 ml BSA with 600 ml distilled water, tube D: 600 ml BSA with 400 ml distilled water, tube E: 800 ml BSA with 200 ml distilled water, tube F: 1 ml BSA, tubes 1 to 9 (samples): 20 ml extract with 980 ml distilled water. Five ml of above mixture and 0.5 ml of 50% Folin-Ciocalteu's Phenol reagent were added to each test tube and kept for 30 m. Then spectrophotometer reading was taken for standard and sample by a spectrophotometer (UV-120-02). Three isoenzyme systems (namely esterase, acid phosphatase and peroxidase) and SDS-PAGE were used in the present investigation. These experiments were carried out following the method of Arüs and Orton (1983) and Laemmli (1970) on a Biorad Protean II system, respectively. Three separate methods of staining were used to detect the enzyme activity on the gels and SDS-PAGE methods of staining used for protein profiling. Before loading, the samples were diluted to the ratio of 2 : 1 with the sample buffer. Gel was run at 60 V until the tracking dye reached the separating gel and then at 200 V for 4 hrs. Sample containing 200 µg of proteins was loaded in the gel. After running, the gel was stained by different methods for 3 different types of enzyme localization. The banded gels were photographed quickly with 8 mega pixels canon power shot A720 model.

Leaves were harvested and total genomic DNA was extracted by using modified CTAB method (Doyle and Doyle 1987). DNA concentration was quantified through spectrophotometer (Analylikjena, Specord 50, Germany).

The PCR reaction mixture for 25 µl containing template DNA (25 ng) 2 µl, de-ionized distilled water 18.8 µl, Taq buffer A 10 × (Tris with 15 mM $MgCl_2$) 2.5 µl, primer (10 µM) 1.0 µl, dNTPs (2.5 mM) 0.5 µl and Taq DNA polymerase (5 U/µl) 0.2 µl. The PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denature 94ºC for 5 min, denature at 94ºC for 1 min, annealing at 34 - 36ºC for 30 sec, extension at 72ºC for 3 min and final extension at 72ºC for 5 min. In the present study, 10 oligonucleotide primers and 10 microsatellite primer pairs were used for RAPD and SSR assay, respectively (Tables 1, 2).

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing 10 µl ethidium bromide (10 mg/ml) and 100 ml 1×TAE buffer at 50 V and 100 mA for 1.0 h. Ladder DNA of 1 kb and 100 bp were

electrophoresed alongside the RAPD and SSR product as marker, respectively. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system. The PCR products were analyzed after gel electrophoresis. The photographs were critically discussed on the basis of presence (1) or absence (0), size of bands and overall polymorphism of bands. These were carried out for further investigation. The scores obtained using all parameters such as isozyme, SDS-PAGE, RAPD- and SSR- analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei's (1972) gene diversity, genetic distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the specimen using the computer program "POPGENE32" (Version 1.32) (Yeh *et al.* 1999).

Results and Discussion

Seven bands were found in esterase system. Besides common bands, a few specific bands were found in these varieties with esterase. Band number 1 (*Rf* value 0.243) was present in all samples except BC-8. On the other hand, band number 2 of BC-8 was much bigger than the other band with *Rf* value 0.326 (Fig. 1A). This band (band number 2) in BC-2 was the thinnest among nine varieties. Therefore, esterase banding profile showed distinct polymorphism among nine varieties. In contrast, no significant polymorphism was found in acid phosphatase and peroxidase banding profile (Fig. 1A). Sarker and Haque (1996) observed similar pattern in esterase and peroxidase systems. Therefore, activities of esterase would be a useful tool for characterizing chickpea varieties.

A total number of 12 high molecular weight polypeptide bands were observed among nine varieties in SDS-PAGE. The position of each band tagged through labeling (Fig. 1A, Table 3). The low molecular weight bands were not reproducible, therefore not considered in the study. Although most of the bands were common in these varieties, three were polymorphic (*Rf* value 0.099, 0.275 and 0.507) revealing 25% (3 out of 12) polymorphism. The varieties also differed in respect of banding intensity. Band number 6 and 7 were much darker in BC-2 and BC-6, respectively. The band number 7 of BC-8 was so thick that could be easily isolated from the same band of different varieties. In addition, band number 9 was only present in BC-2, BC-6, BC-8 and BC-9 (Fig. 1A). Parker *et al.* (1998) had mentioned that SDS-PAGE was a powerful tool in studying population genetics. Since storage proteins (seed proteins) are not affected by environmental fluctuation, many workers used SDS-PAGE profiling technology as a reliable tool for authentic characterization of germplasm. Moreover, Jha and Ohri (1996) reported SDS-PAGE patterns as promising tool for distinguishing varieties of particular species. In contrast, other workers reported that variety identification was not possible with the SDS-PAGE method (Ahmad and Slinkard 1992, De Vries 1996). In this investigation, it was possible to characterize some chickpea varieties with SDS-PAGE protein markers and thus became a reliable tool for chickpea diversity study.

In this study, the variation among the chickpea varieties was assessed with RAPD markers. In total 35 primers were tested. Only ten RAPD primers were selected because they revealed multiband fingerprinting which were easily scorable and reproducible. A total of 74 DNA fragments were amplified with an average of 7.4 fragments per primer. Band size ranging from 250 to 10,000 bp of PCR amplification products scored for all primers (Fig. 1B, Table 1). Among the ten primers OPA-8 and primer-2 produced the highest number (10) of polymorphic bands (Fig. 1B). In contrast, the primer-23 generated the least number (4) of polymorphic bands (Fig. 1, Table 1B). Different light and bright bands were observed where light bands produced from low homology between the primer and the pairing site on the DNA strand (Thormann *et al*. 1994). Out

of the total 74 amplified fragments, only 5 were common and the remaining 69 (93.24%) were polymorphic indicating high level of diversification (Table 3). Several workers carried out RAPD analysis on chickpea. Rasool (2013) and Datta *et al*. (2010) found 98.14 and 87.00% polymorphism in chickpea genotypes, respectively. In contrast, only 14.56% polymorphic products with 10 polymorphic primers were observed among 29 elite chickpea varieties by Sant *et al*. (1999). Moreover, Sonnante *et al*. (1997) observed 25.5% polymorphic products among six chickpea accessions using 16 polymorphic primers. These results revealed that chickpea genotypes showed both high and low level of polymorphism. High level and low level RAPD polymorphisms suggested a wide range of diversification existing among different genotypes of chickpea. This information would be useful for improved breeding program of chickpea.

Table 1. List of RAPD markers generated from ten primers in nine varieties of *Cicer arietinum* L. (Source: Macrogen Incorporation Korea).

Primer codes	Sequences (5'—3')	No. of total bands	Size ranges (bp)	No. of common bands	No. of polymor-phic bands	Number and size of unique bands (bp)
OPA-1	CAG GCC CTT C	7	500 - 10000	0	7	--
OPA-3	AGT CAG CCA C	5	1100 - 5000	1	4	BC-1 (2500)
OPA-4	AAT CGG GCT G	9	500 - 3000	0	9	BC-1 (2500, 2200, 800, 625)
OPA-5	AGG GGT CTT G	8	1100 - 4000	0	8	BC-1 (2000), BC-4 (4000), BC-7 (1700, 1300)
OPA-7	GAA ACG GGT G	10	750 - 4000	0	10	BC-1 (1600), BC-6 (2000), BC-9 (4500)
OPA-8	GTG ACG TAG G	10	250 - 4000	0	10	--
Primer-2	GTT GCG ATC C	6	625 - 3000	0	6	--
Primer-12	GTA TGG GGC T	8	500 - 3000	4	4	--
Primer-19	GAT GAC CGC C	7	1500 - 4000	0	7	--
Primer-23	GTC AGG GCA A	4	500 - 2000	0	4	--
Total	Ten primers	74	250 - 10000	5	69	12

In addition to polymorphism, 12 unique RAPD fragments were identified in nine chickpea varieties using ten different primer combinations. The term unique sequence means that the sequence found in a variety with a certain primer was absent in other varieties (Fig. 1B, Table 2). The unique bands were stable and specific for the respective varieties and thus could be used as a tool for characterization. In the earlier literature, there was no information about unique band except Rasool (2013) and Datta *et al*. (2010). The earlier authors considered all bands as polymorphic band. The unique band has a number of potential applications including the determination of variety purity, identification of mislabeled accessions and the establishment of property rights (plant variety protection and patenting).

Ten SSR primer pairs were used in this study. Each primer generated well-defined and reproducible polymorphic bands. The primer sequence, band size and banding pattern of nine chickpea varieties are presented in Table 2 and in Fig. 1C. The size of band ranged from 20 to 380

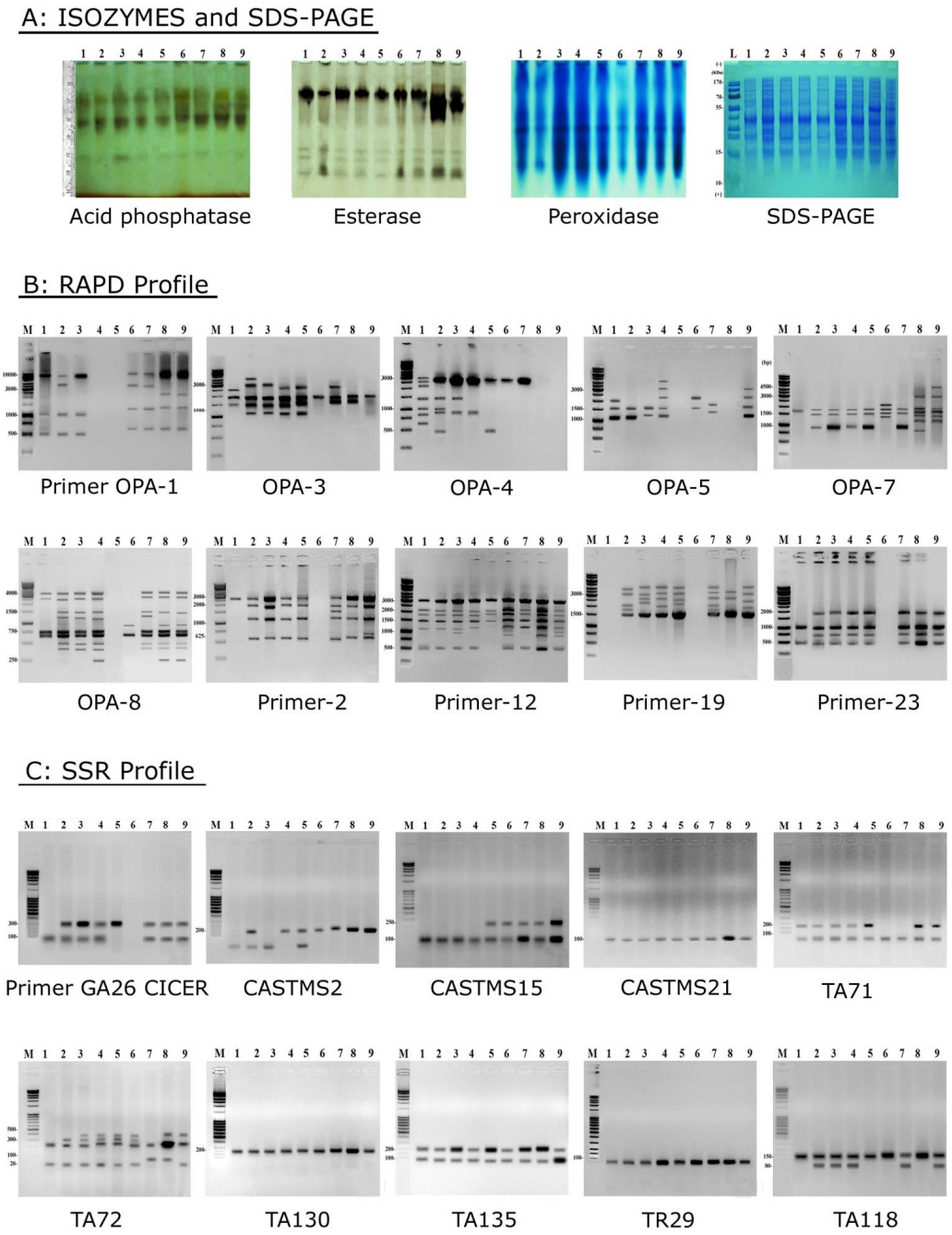

Fig. 1. Diversity in chickpea varieties based on isozyme activity, SDS-PAGE protein profile and amplified DNA profile generated by sets of RAPD and SSR primers. A: ISOZYMES and SDS-PAGE, B: RAPD profile, C: SSR profile. Chickpea varieties are marked as 1 to 9 in each gel. L and M are protein and DNA marker lanes, respectively.

bp (Table 2). Out of ten, the primer pair TA72 produced highest number (4) of polymorphic bands while the primer pair CASTMS21, TA130, TA135 and TR29 did not generate any polymorphic band (Fig. 1C, Table 2). In total, ten primer pairs produced 20 distinct bands of which 11 were considered as polymorphic and thus showed moderate level of polymorphisms (55%) (Table 3). Datta *et al*. (2010) studied 93% average polymorphism among chickpea and pigeonpea genotypes. Like RAPD, a wide range of SSR polymorphism indicated broad diversity within chickpea varieties.

Table 2. SSR markers generated from ten primer pairs in nine varieties of *Cicer arietinum* L. (Source: Macrogen Incorporation Korea).

Primer codes	Sequences (5'—3')	No. of total bands	Size ranges (bp)	No. of common bands	Polymor-phic bands
GA26 CICER	F-GTGCAGAGCATCATGCGATGCTCAAG ACATCT GCCA R-TCATACTCAACAAATTCATTTCCC	2	80 - 300	0	2
CASTMS2	F-ATTTTACTTTACTACTTTTTTCCTTTC R-AATAAATGGAGTGTAAATTTCATGTA	2	50 - 200	0	2
CASTMS15	F-CTTGTGAATTCATATTTACTTATAGAT R-ATCCGTAATTTAAGGTAGGTTAAAATA	2	70 - 250	1	1
CASTMS21	F- CTACAGTCTTTTGTTCTTCTAGCTT R-ATATTTTTTAAGAGGCTTTTGGTAG	1	80 - 100	1	0
TA71	F- CGATTTAACACAAAACACAAA R- CCTATCCATTGTCATCTCGT	2	50 - 175	1	1
TA72	F-GAAAGATTTAAAAGATTTTCCACGTTA R-TTAGAAGCATATTGTTGGGATAAGAGT	5	20 - 380	1	4
TA130	F- TCTTTCTTTGCTTCCAATGT R- GTAAATCCCACGAGAAATCAA	1	170 - 200	1	0
TA135	F- TGGTTGGAAATTGATGTTTT R- GTGGTGTGAGCATAATTCAA	2	80 - 200	2	0
TR29	F-GCCCACTGAAAAATAAAAAG R- ATTTGAACCTCAAGTTCTCG	1	50 - 60	1	0
TA118	F-ACAAGTCACATGTGTTCTCAATA R-GGAAAGGTTAAGAAATTTTACAATAC	2	40 - 140	1	1
Total	Ten primer pairs	20	20 - 380	9	11

Table 3. Comparative banding pattern obtained from different molecular methods.

Molecular methods	No. of total bands	No. of common Bands	No. of polymorphic bands	Polymorphism (%)
Isozymes	14	6	8	57.14
SDS-PAGE	12	9	3	25.00
RAPD	74	5	69	93.24
SSR	20	9	11	55.00

The values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among nine varieties of chickpea were computed. The combined data of three isozyme systems and SDS-PAGE made BC-8 and BC-9 distinct and thus placed in cluster 2 (C_2) with 0.0392 genetic distance (Fig. 2a). The cluster made from RAPD- and SSR-marker analysis

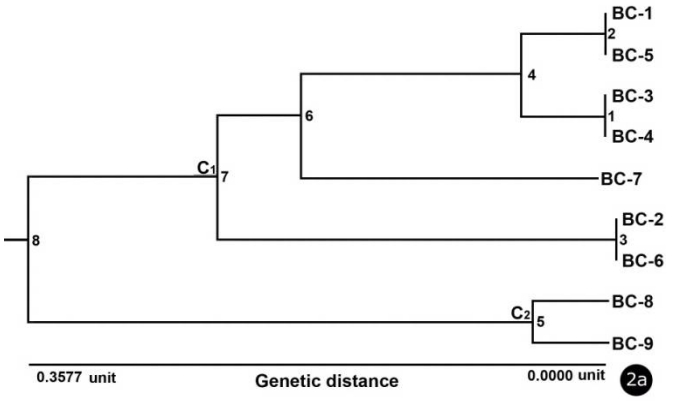

Fig. 2a. UPGMA dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation among nine varieties of *Cicer arietinum* L. by three isozyme systems and SDS-PAGE data analysis.

placed BC-1 and BC-6 in cluster 1 (C_1) with 0.4658 genetic distance. The lowest genetic distance was found in BC-2 and BC-3 (0.1366) while BC-8 and BC-9 showed second lowest genetic distance (0.1738) thus made sub cluster $C_{2.1}$ and $C_{2.2}$, respectively (Fig. 2b).

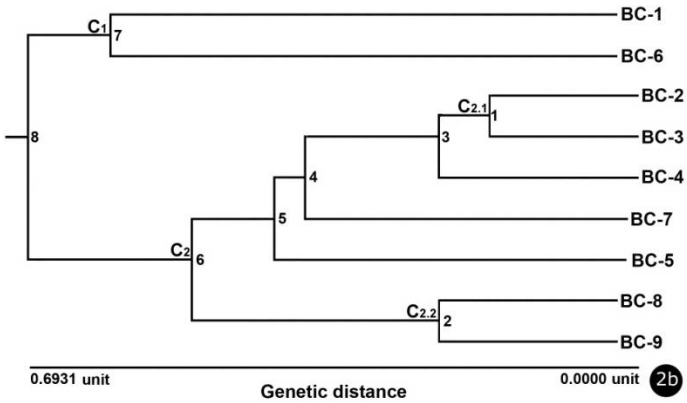

Fig. 2b. UPGMA dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation among nine varieties of *Cicer arietinum* L. by RAPD and SSR marker data analysis.

The difference between the highest and the lowest value of genetic distance revealed the wide range of variability persisting among the nine chickpea varieties. High genetic distance values between variety pairs were found due to difference in genetic constituent (Thormann *et al.* 1994). The varieties of lowest genetic distance can be used as parental source for breeding line to improve chickpea varieties.

The varieties BC-8 and BC-9 were distinct from the other seven chickpea varieties in various morphological and agronomical aspects. Among nine varieties only BC-8 showed white color flower whereas other eight showed pink color flower. Seed coat color of BC-8 was also white. Seed size of BC-8 and BC-9 was larger than other seven varieties. These two varieties were highly disease resistant and their production rate was also higher than other chickpea varieties (Mandal *et al.* 2011). According to isozymes and SDS-PAGE protein profiling analysis, varieties BC-8 and

BC-9 were different from other seven varieties and the dendrogram placed these two varieties in separate cluster (Fig. 2a). In contrast, according to RAPD and SSR-fingerprinting analysis, BC-1 and BC-6 were distant from other seven varieties and placed in separate cluster and variety BC-8 and BC-9 were closely related with 7 varieties and placed in sub-cluster $C_{2.2}$ (Fig. 2b). Therefore, it has been possible to determine the genetic diversity among nine chickpea varieties based on isozymes, SDS-PAGE, RAPD and SSR analysis.

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