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Abstract: Detection of phylogenetic relationships between chickpea cultivars and accessions of wild species Cicer reticulatum were investigated by using RAPD and ISSR markers. Nineteen cultivars of chickpea and five accessions of C. reticulatum were analysed. On an average, 6 bands per primer were observed in RAPD analysis and 11 bands per primer in ISSR analysis. In RAPD, the wild accessions shared 22.15 % common bands and 77.8 % polymorphic bands with chickpea cultivars, whereas they shared 20.3 % common bands and 79.6 % polymorphic bands in ISSR analysis. In RAPD analysis 48.3 % and 49.4 % common bands and 51.7 % and 50.5 % polymorphic bands were observed among wild accessions and chickpea cultivars, respectively. Similarly, 34.37 % and 43.75 % common bands and 65.63 % and 56.25 % polymorphic bands were found in ISSR analysis. The dendrogram developed by pooling all the data of RAPD and ISSR analysis revealed that the wild accessions and the ICCV lines showed similar pattern with the dendrogram of RAPD analysis. The ISSR analysis clearly indicated that even with six polymorphic

primers a reliable estimation of genetic diversity could be obtained, while with RAPD markers nearly 30 primers are required. Further, a large amount of genetic variation exists between chickpea cultivars and accessions of C. reticulatum, which can be used efficiently for gene tagging, and genome mapping of wild and cultivar crosses to introgress disease and insect resistance into the cultivated genotypes.

RAPD and ISSR fingerprinting in cultivated chickpea [*Cicer arietinum* (L.)] and wild species

Linga S. Rao[1], P. Usha Rani[2], P. S. Deshmukh[1], P. A. Kumar[2] & S. K. Panguluri[3] *

INTRODUCTION

Chickpea (*Cicer arietinum.* L), is the third most important pulse crop in the world and ranks first in the Indian sub continent and Mediterranean basin (FAO, 1996). The annual growth rate of chickpea production has been very slow at 1.9 % and yields have risen at the rate of only 0.6 % annually (Ratnaparkhe et al., 1998). Globally, the average yield is about 700 kg/ha, which is much below it's potential of 4,000 kg/ha. One of the main reasons for this is its susceptibility to various biotic and abiotic stresses (Singh et al., 1997). The development of cultivars tolerant to such stresses would be an ideal approach to overcome this problem. Another direct approach to obtain a higher yield in chickpea is the introgression of various yield-improving traits into cultivated germplasm. Screening of existing genetic diversity becomes the most crucial step in this regard.

Several different molecular methods are available for the identification of cultivars and genetic diversity. The polymerase chain reaction (PCR) methods using arbitrary primers have been widely utilized in the last 10 years. Random amplified polymorphic DNA (RAPD) is one of the most popular techniques, which has been used for measuring genetic diversity in several plant species, including chickpea (Collard et al., 2003) and lens (Duran et al., 2004). According to Ratnaparkhe et al. (1998), ISSR technique is more reliable than the RAPD and generates larger numbers of polymorphisms per primer. RAPD and ISSR markers are highly polymorphic and are useful in studies on genetic diversity in *Lupinus* spp (Talhinhas et al., 2003), and pigeonpea (Ratnaparkhe et al., 1998); phylogeny in chickpea (Iruela et al., 2002);

gene tagging in chickpea (Rajesh et al., 2002); genetic linkage mapping in *Lens* (Duran et al., 2004) and *Ananas* (Carlier et al., 2004) and evolutionary biology (Reddy et al., 2002). ISSR markers have already been used in chickpea (Fernandez et al., 2002 and Collard et al., 2003). RAPD and ISSR remain attractive options despite availability of other sophisticated techniques because they are easy, quick, simple and economical. Neither sequence information nor any prior genetic studies is required for this analysis.

To date there have been very few reports investigating the level of genetic variation between the accessions of *C. arietinum* and *C. reticulatum* for the generation of intraspecific or interspecific populations of chickpea. Previous research in genetic mapping of chickpea populations has utilized populations derived from interspecific crosses between chickpea and *C. reticulatum*, in order to detect polymorphisms between parents and marker segregation (Simon and Muehlbauer, 1997; Santra et al., 2000; Winter et al., 2000). Chickpea breeding aims at developing high yielding cultivars that combine long lasting resistance against *Ascochyta* blight and *Fusarium* wilt and with tolerance to abiotic stresses such as drought, cold and salinity. Use of RAPD and ISSR for genetic diversity analysis of Indian accessions has not been demonstrated. The present study was conducted to determine the genetic variability between and within *Cicer* species using RAPD and ISSR markers and to provide a better understanding of the phylogenetic relationships of the *Cicer* species.

MATERIALS AND METHODS

Plant Material

The plant material comprised of 24 genotypes of chickpea including 19 Pusa cultivars and five wild accessions of *C. reticulatum* (Table.1). All the above material was obtained from Pulse Research Laboratory, Indian Agricultural Research Institute, New Delhi, India.

Table.1: The accession, pedigree and characteristics of chickpea cultivars and its wild relatives taken for RAPD and ISSR analysis.

S.No	Accession	Pedigree	Plant type	Seed size (g/100 S)	Plant Characteristics
1	Pusa -256	(JG 62 X 850 – 3/27)X (L-550 X H-208)	Semi Erect	26.3	HY; WA; MM; MRD
2	Pusa- 362	(BG 203 X P 179)X (BG 303)	Erect	25	HY; WA; EM; RD
3	Pusa -372	(P 1231 X P 1265)	Semi spread	16.8	HY; WA; EM; RD
4	Pusa -391	(ICC 3935 X P 256)	Semi spread	20.4	HY; S SI; EM; MRD
5	Pusa -1003	(ICCV 32 X Rabat)	Semi spread	24.6	MY;SEI;EM; MRD
6	Pusa -1101	(P 256 X *C.reticulatum*) X (P 362)	Erect	23.6	HY; S NI; MM; RD
7	Pusa -1103	(P 256 X *C.reticulatum*) X (P 362)	Erect	23	HY; S NI; EM; RD
8	Pusa -1105	(C 104 X BG 1003) X (ICC 88503 X BG 1048)	Semi Erect	30	HY; WA; EM; MRD
14	BGD-72	(P 256 X E 100/ym) X (P 256)	Semi Erect	26.5	HY;WA;MEM; MRD, HDDST
15	BGD-112	(GL 84038 X BG 209) X (BG 212)	Semi Erect	17.6	HY;SSI;Green Seeded;MLM;
16	DG-36	(ICCV 92502 X Flip 91-80C) X (ICC 15578)	Semi Erect	31	HY;EM;RD, HDDST
17	DG-46	(ICCV 89314 X ICCV 88101)X (ICCV 92944)	Semi Erect	28.6	HY;EM;RD, HDDST
18	DG-51	(P 362) X (Avarodhi X WR 315)	Semi Erect	29.2	HY;EM;RD, HDDST
19	DG-63	(P 362) X (Avarodhi X P 212)	Semi Erect	29.6	HY;EM;RD, HDDST

20	DG-71	(P 362) X (Avarodhi X P 212)	Semi Erect	30.9	HY;EM;RD, HDDST
21	DG-72	(RSG 143-1 X ICC 12238) X (BG 365)	Semi Erect	31.3	HY;EM;RD, HDDST
22	ICCV-2	(L-550 X Gaumirchil)	Semi spread	25.6	HY; SSI; EM; RD
23	ICCV-10	(P 1231 X P 1265)	Semi spread	21	HY; WA; MEM; MRD
24	Annegiri	Selection from Land race (Karnataka)	Semi Erect	24.5	MY;SSI;MEM; SD
9	ICC 17121	NA	Spread	NA	NA
10	ICC 17123	NA	Spread	NA	NA
11	ICC 17124	NA	Spread	NA	NA
12	ICC 17160	NA	Spread	NA	NA
13	ICC 17163	NA	Spread	NA	NA

HY= High Yielding, MY= Medium Yielding, WA= Widely Adopted, SSI= Suitable for South India, SNI = Suitable for North India, SEI = Suitable for Eastern India, EM= Early in Maturity, MM= Medium in Maturity; MEM= Medium Early in Maturity, RD= Resistant to Diseases, MRD= Moderately Resistant to Diseases, SD= Susceptible to Diseases, HDDST= High Degree of Drought and Salinity Tolerance.

Plant DNA extraction and purification

Total plant genomic DNA was isolated following the protocol given by Doyle and Doyle (1987) as modified by Panguluri et al. (2005).

RAPD Reaction

PCR amplification was performed as described by Williams et al. (1990) with some modifications. Sixty random decamer primers, twenty each of series OP (AS), OP (F) and OP (I) were obtained from Operon Technologies Ltd., (Alameda, CA, USA). 29 primers were used for analysis out of 60 RAPD primers. The reaction mixture (25 µl) contained 10X-assay buffer, 2.5 mM MgCl$_2$, 400 µM dNTP's (Bangalore Genei), 5.0 ρmoles of primer, 50 ng template DNA and 1 U of *Taq* DNA

Polymerase (Bangalore Genei). Amplification was carried out in a thermocycler (Biometra) for 40 cycles, each consisting of a denaturation step at 94^0 C for 1 min, annealing at 35^0 C for 1 min and an extension step at 72^0C for 2 min. An initial denaturation step at 94^0 C for 5 min and a final synthesis step of 8 min at 72^0 C were also included.

ISSR Reaction

PCR amplification was performed as described by Williams et al. (1990) with some modifications. Ten primers (UBC primers) were obtained. Six primers {UBC-112 (5^1 GACAGACAGACAGACA 3^1) (ISSR-1)}, UBC-864 {(5^1 ACTGACTGACTGACTG 3^1) (ISSR-2)}, UBC-841 {(5^1 GACACGACACGACACGACAC 3^1) (ISSR-5)}, UBC-810 {(5^1 TGGATGGATGGATGGA 3^1) (ISSR-6)}, UBC-827 {(GACTGACTGACTGACT)(ISSR-7)} and UBC-820 {(CACCACCACCACCAC) (ISSR-10)} were used for the analysis. Amplification was carried for 40 cycles, each consisting of a denaturation step at 94^0 C for 1 min, annealing at 45^0 C & 54^0 C for 1 min and an extension step at 72^0 C for 2 min. An initial denaturation step at 94^0 C for 5 min and a final synthesis step of 8 min at 72^0 C were also included.

Agarose gel electrophoresis for RAPD and ISSR

Reaction products were mixed with 4 μl of 6X loading dye (0.25% Bromophenol Blue, 0.25% Xylene Cyanol and 40% Sucrose, w/v), electrophoresed on 1.4% agarose gel, stained with ethidium bromide and visualized under UV light.

Data analysis for RAPD and ISSR

DNA fragment profiles representing a consensus of two independent replicates were scored in a binary fission with ‘0’ indicating the absence and ‘1’ indicating presence of band. Using the binary data, a similarity matrix was constructed using the

Jaccard coefficient, which was further subjected to UPGMA clustering analysis and a dendrogram was generated. A cophenetic matrix was constructed using the matrix that was used to generate the clusters. A correlation between the cophenetic matrix and the similarity matrix was determined by using MXCOMP module. All the above analysis was done using the software package NTSYS-PC (version 2.02e).

RESULTS AND DISCUSSION

Differences between genotypes with regard to agronomic, morphological and biochemical characters (e.g. storage proteins, isozymes) and molecular characteristics are either indirect or direct representations of differences at the DNA level and are therefore expected to provide information about genetic relationships. The assessment of genetic diversity is important not only for crop improvement but also for efficient management and conservation of germplasm resources. In the present study, nineteen cultivars of chickpea and five accessions of its wild relative *C. reticulatum* were analyzed by using 29 reproducible RAPD markers and 6 ISSR markers.

RAPD analysis

RAPD analysis revealed good polymorphism among chickpea cultivars. Sixty random (each twenty of OP- (AS), OP-(I) and OP-(F)) primers were taken and only 29 primers were found to be polymorphic. On an average 6 bands per primer and 74.4 % polymorphic bands were observed in a total of 176 bands. From RAPD data 49.4 % of common bands and 50.5 % of polymorphic bands (Fig.1) were observed among chickpea cultivars and 48.3 % of common bands and 51.7 % of polymorphic bands were observed among wild accessions. The primer OP (F)-3 elicited maximum number of bands (11) and OP (F)-12 showed the least number of bands (2).

RAPD Dendrogram

From the UPGMA dendrogram (Fig.3), it is discernible that the material taken for the analysis can be divided into two major clusters. Wild accessions are grouped into one cluster and all chickpea cultivars in another cluster, which is sub-divided into groups and subgroups. ICCV cultivars and Annegiri clustered into one group and all Pusa accessions clustered together in another group. The similarity coefficient varied from 0.84 to 0.96 in cultivars while it varied from 0.71 to 0.81 in wild accessions.

Although the *Cicer* species are predominantly self-pollinating, more variation was observed among them. The reason for this genetic variation could be that the specific accessions were heterozygous at some marker loci. Similar observations were reported by Simon and Muehlbauer (1997). Considerable variation was observed between wild accessions and cultivated chickpea (77.8 %) in RAPD analysis. As large amount of genetic variation exists between chickpea cultivars and its wild accessions, this can be used efficiently for gene tagging and genome mapping of wild and cultivar crosses to introgress disease and insect resistance into the cultivated genotypes.

ISSR analysis

Ten primers were taken for ISSR analysis and six primers (ISSR 1, ISSR 2, ISSR 5, ISSR 6, ISSR 7 and ISSR 10) were found to be polymorphic. On an average 11 bands per primer and 80% polymorphic bands were observed in a total of 64 bands. The wild species shared 20.3 % common bands and 79.6 % polymorphic bands with ISSR markers. 43.75 % common bands and 56.25 % polymorphic bands were found among chickpea varieties and 34.37 % common bands and 65.63 % polymorphic bands were found among wild accessions. Out of six polymorphic ISSR primers, ISSR-5 (Fig. 2) given the maximum bands (16) and ISSR-7 showed least number of bands (7).

ISSR Dendrogram

A close look at UPGMA dendrogram (Fig. 4) revealed that the material taken for analysis is divided into two major clusters. All wild accessions except ICC17163 are grouped together in the first cluster. Chickpea cultivars formed a major group in the second cluster. This group has been divided into two subgroups in which Pusa-1105 and Annegiri are together, whereas the other cultivars constitute a separate group. The similarity coefficient values for the cultivated chickpea lines in this UPGMA dendrogram range between 0.76 and 1.00, whereas the values range between 0.58 and 0.76 in case of wild accessions.

It was found that all the chickpea cultivars present in a cluster have similar pedigree or share a common parent. When compared to the RAPD dendrogram, the ISSR dendrogram showed more correlation with the pedigree data, which revealed that the ISSR markers are the most efficient marker system because of their capacity to elicit several informative bands from single amplification (a mean of 11 informative bands per primer). Similar observations were made by Fernandez et al. (2002) in barley and Qian et al. (2001) in rice. Earlier studies also reported that ISSR technique generates large number of polymorphisms in pigeonpea (Ratnaparkhe et al., 1998) and chickpea (Fernandez et al., 2002 and Collard et al., 2003).

RAPD and ISSR Dendrogram

To reduce the inaccuracies of the independent techniques, a dendrogram was developed by pooling the data of RAPD and ISSR analyses. Two major clusters were observed in this UPGMA dendrogram (Fig. 5). All the wild accessions grouped together into one major cluster whereas all the chickpea cultivars formed a second cluster. The second major cluster is again divided into many groups, subgroups and sub-subgroups. The similarity coefficient for the chickpea cultivars in the dendrogram varies from 0.8 to 0.94, whereas for wild accessions it ranges from 0.71 to 0.80. When the dendrogram was correlated with the pedigree data it was found that very few cultivars with common parents clustered together. The dendrogram showed similar clustering pattern for the wild accessions with that of RAPD, but in the case of cultivars, it showed much variation with the dendrograms of both RAPD and ISSR.

The chickpea cultivars DG-63 and DG-71 clustered together as they have similar pedigree. Similarly, Pusa-1101 and Pusa –1103 are in the same cluster as they have similar pedigree data. The degree of variation within *C. arietinum* and *C. reticulatum* accessions depended on the accessions. This observation was consistent with the study by Simon and Muehlbauer (1997), who detected variation within single *C. reticulatum* accession (PI 489777), used to generate an interspecific mapping population. The observed genetic variation within specific accessions could be due to the mixture of homozygous plants or the specific accessions were heterozygous at some marker loci. Although *Cicer* species are predominantly self-pollinating, a low level of outcrossing has been previously reported (Gowda, 1981).

CONCLUSIONS

The present investigation demonstrates the potential of RAPD and ISSR fingerprinting in detecting polymorphism among chickpea cultivars and wild accessions. The ISSR analysis clearly indicated that even with six polymorphic primers reliable estimation of genetic diversity could be obtained, while nearly 30 primers are required for RAPD. Moreover, RAPD can cause genotyping errors due to competition in the amplification of all RAPD fragments.

The markers generated by ISSR and RAPD assays can provide practical information for the management of genetic resources. For the selection of good parental material in breeding programs the genetic data produced through ISSR can be used to correlate with the relationship measures based on pedigree data and morphological traits to minimize the individual inaccuracies in chickpea. Further, large amount of genetic variation which exists between chickpea cultivars and its wild accessions can be used efficiently for gene tagging and genome mapping of wild and cultivar crosses to introgress the disease and insect resistance into the cultivated genotypes.

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Figure legends

Fig.1. Agarose gel showing the amplified product using OP (AS)- 12 primer.

M; I kb ladder, Lane 1-8; Pusa-256, Pusa-362, Pusa-372, Pusa-391, Pusa1003, Pusa-1101, Pusa-1103 and Pusa-1105, Lane 9-13; ICC 17121, ICC 17123, ICC 177124, ICC 17160 and ICC 17163, Lane 14-24; BGD-72, BGD-112, DG-36, DG-46, DG-51, DG-63, DG-71, DG-72, ICCV-2, ICCV-10 and Annegiri.

Fig.2. Agarose gel showing the amplified product using ISSR-5 primer.

M; I kb ladder, Lane 1-8; Pusa-256, Pusa-362, Pusa-372, Pusa-391, Pusa1003, Pusa-1101, Pusa-1103 and Pusa-1105, Lane 9-13; ICC 17121, ICC 17123, ICC 177124, ICC 17160 and ICC 17163, Lane 14-24; BGD-72, BGD-112, DG-36, DG-46, DG-51, DG-63, DG-71, DG-72, ICCV-2, ICCV-10 and Annegiri.

Fig.3. UPGMA dendrogram of the cultivated chickpea and its wild accessions using RAPD marker.

Fig.4. UPGMA dendrogram of the cultivated chickpea and its wild accessions using ISSR marker.

Fig.5. UPGMA dendrogram of the cultivated chickpea and its wild accessions using both ISSR and RAPD markers.

Table.2. RAPD and ISSR primers selected from OP (Operon) and UBC set, respectively.

Primer	TNB	PWCA	%PWCA	PWA	%PWA	PCA	%PCA
RAPD'S							
OP(AS)-2	7	7	100	5	71.4	3	42.8
OP(AS)-3	8	5	62.5	3	37.5	4	50
OP(AS)-4	7	5	71.4	5	71.4	1	14.2
OP(AS)-5	4	0	0	0	0	0	0
OP(AS)-7	7	5	71.4	0	0	5	71.4
OP(AS)-8	7	3	42.8	2	28.5	2	28.5
OP(AS)-11	8	5	62.5	3	37.5	3	37.5
OP(AS)-12	7	5	71.4	4	57.1	1	14.2
OP(AS)-15	6	1	16.6	1	16.6	0	0
OP(AS)-16	5	3	60	2	40	2	40
OP(AS)-19	7	4	57.1	3	42.8	3	42.8
OP(F)-1	6	5	83.3	5	83.3	4	66.6
OP(F)-2	6	5	83.3	5	83.3	1	16.6
OP(F)-3	11	10	90.9	9	81.8	3	27.2
OP(F)-5	7	7	100	4	57.1	5	71.4
OP(F)-6	3	1	33.3	0	0	1	33.3
OP(F)-7	6	6	100	0	0	6	100
OP(F)-8	6	5	83.3	4	66.6	4	66.6
OP(F)-9	5	3	60	0	0	3	60
OP(F)-10	5	5	100	5	100	5	100
OP(F)-11	3	3	100	3	100	2	66.6
OP(F)-12	2	2	100	2	100	0	0
OP(I)-2	7	5	71.4	4	57.1	0	0
OP(I)-3	6	6	100	2	33.3	5	83.3
OP(I)-4	5	5	100	4	80	5	100
OP(I)-5	7	5	71.4	3	42.8	4	57.1
OP(I)-7	10	8	80	7	70	2	20
OP(I)-16	2	2	100	1	50	2	100
OP(I)-17	6	5	83.3	4	66.6	0	0
ISSR's							
ISSR-1	11	11	100	10	90.9	8	72.7
ISSR-2	9	9	100	8	88.8	6	66.6
ISSR-5	16	5	31.25	4	25	2	12.5
ISSR-6	10	8	80	7	70	4	40
ISSR-7	11	11	100	10	90.9	10	90.9
ISSR-10	7	7	100	5	71.4	6	85.7

TNB; Total number of bands, PWCA; Polymorphic between wild and cultivated accessions, PWA; Polymorphic among wild accessions and PCA; Polymorphic among cultivated accessions.

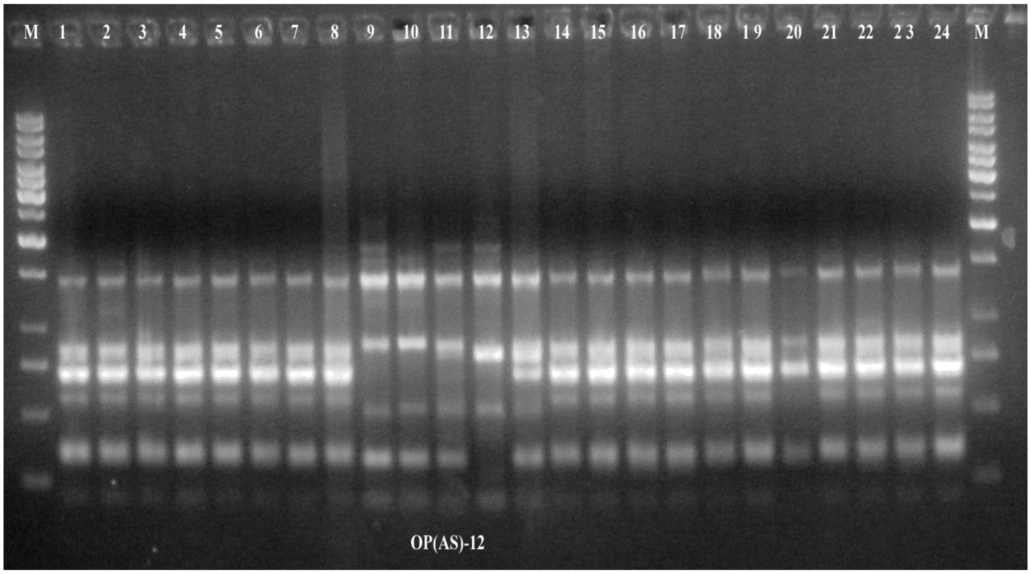

Fig. 1.

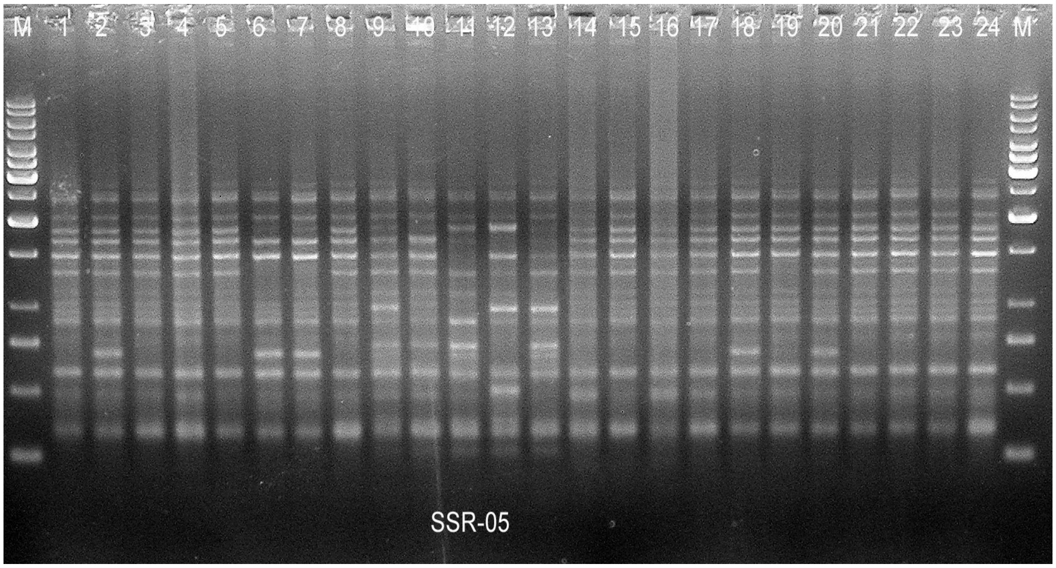

Fig. 2.

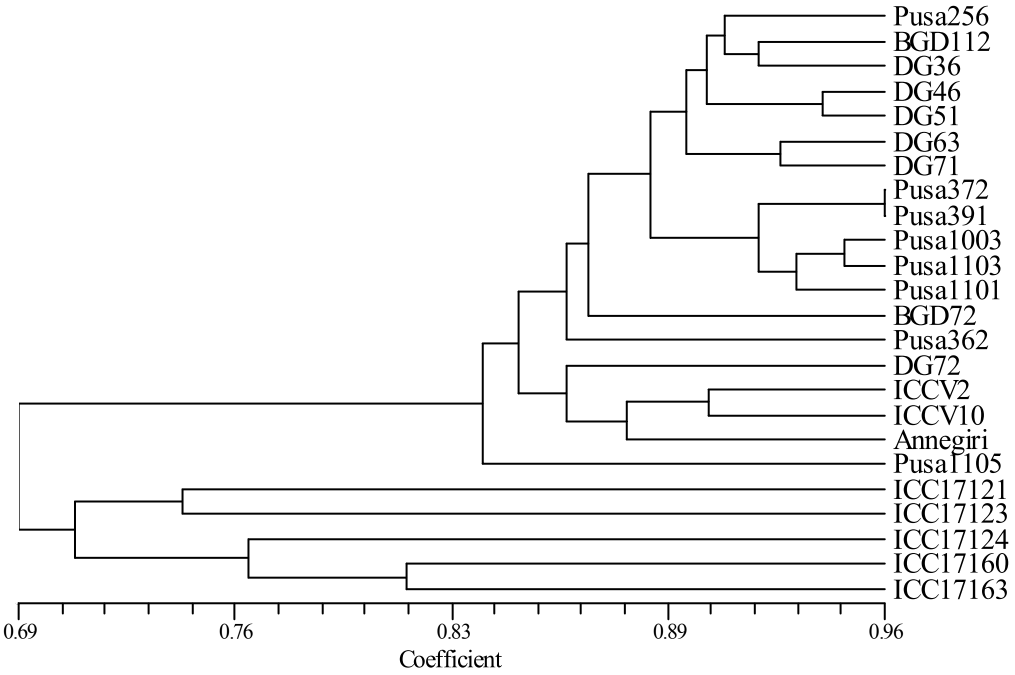
Pusa256
BGD112
DG36
DG46
DG51
DG63
DG71
Pusa372
Pusa391
Pusa1003
Pusa1103
Pusa1101
BGD72
Pusa362
DG72
ICCV2
ICCV10
Annegiri
Pusa1105
ICC17121
ICC17123
ICC17124
ICC17160
ICC17163
0.69
0.76
0.83
0.89
0.96
Coefficient

Fig. 3.

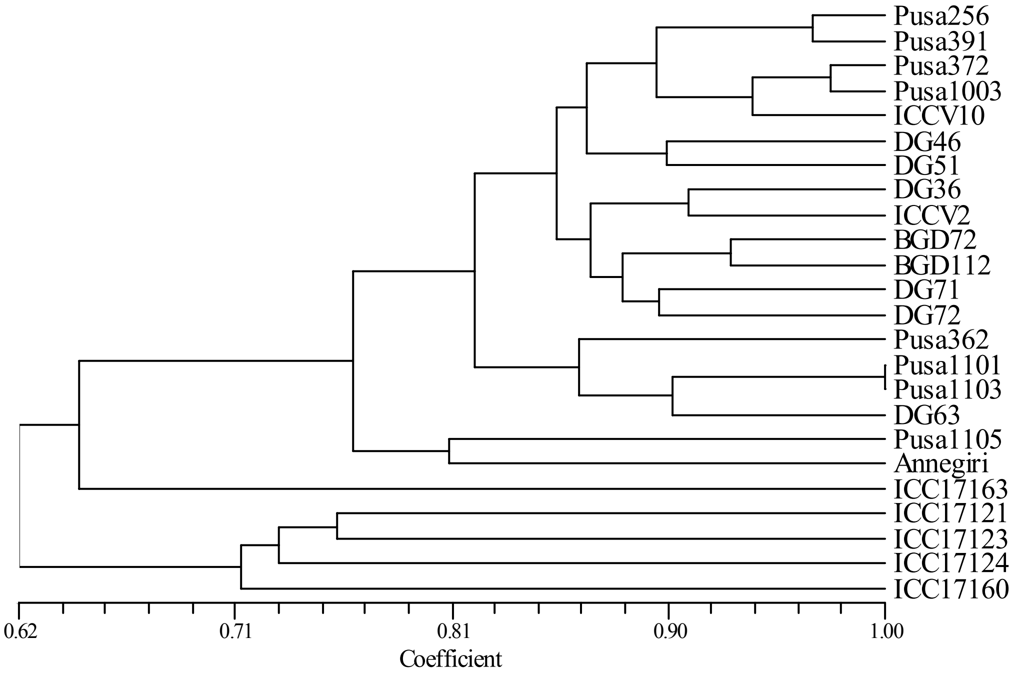
Pusa256
Pusa391
Pusa372
Pusa1003
ICCV10
DG46
DG51
DG36
ICCV2
BGD72
BGD112
DG71
DG72
Pusa362
Pusa1101
Pusa1103
DG63
Pusa1105
Annegiri
ICC17163
ICC17121
ICC17123
ICC17124
ICC17160
0.62
0.71
0.81
0.90
1.00
Coefficient

Fig. 4.

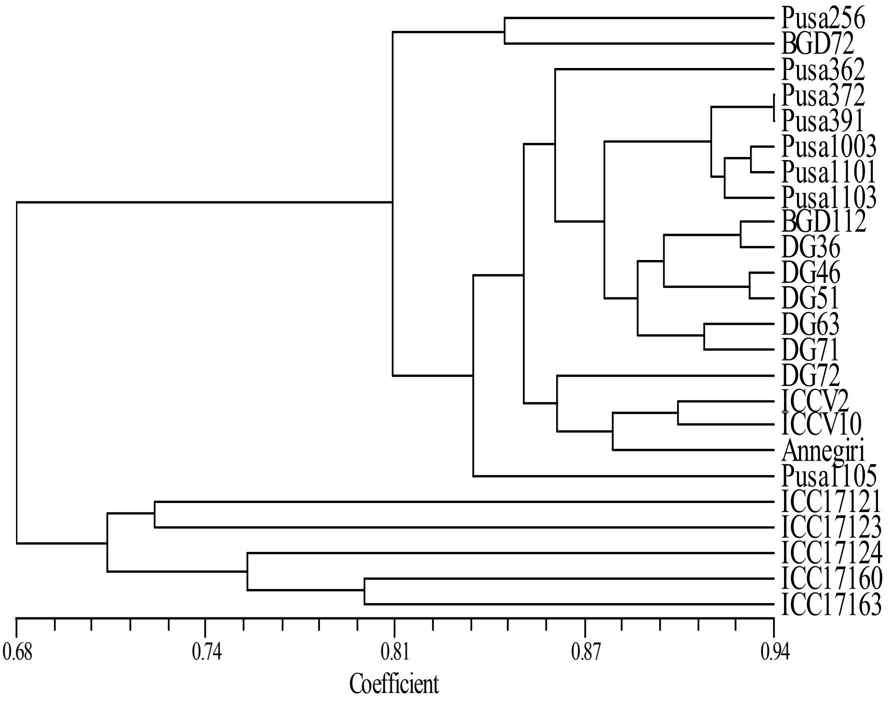
Pusa256
BGD72
Pusa362
Pusa372
Pusa391
Pusa1003
Pusa1101
Pusa1103
BGD112
DG36
DG46
DG51
DG63
DG71
DG72
ICCV2
ICCV10
Annegiri
Pusa1105
ICC17121
ICC17123
ICC17124
ICC17160
ICC17163
0.68
0.74
0.81
0.87
0.94
Coefficient

Fig. 5.