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Selection of molecular markers associated with resistance to *Fusarium* wilt disease in chickpea (*Cicer arietinum* L.) using multivariate statistical techniques

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Abstract

Fusarium wilt is a destructive and widespread disease of chickpea caused by *Fusarium oxysporum* f.sp *ciceris*. A total of 40 unrelated genotypes of chickpea were classified into two distinct phenotypic groups as resistant or susceptible to F. *oxysporum* f.sp *ciceris*. Genotype selection was based on disease severity in chickpea following inoculation. Inter- simple-sequence-repeat (ISSR) marker profiles were generated for each individual and used in association studies to identify markers suitable for classifying the two pre-defined phenotypic classes. Nine ISSR primers were screened and optimized for detecting genetic diversity. From these primer combinations a total of 44 polymorphic clear bands out of a total of 61 (72.1%) were generated. Two multivariate statistical methods, Discriminant analysis and logistic regression were used to select informative markers, and to develop models that would classify the two phenotypic groups. Both discriminant analysis and logistic regression in *P* value equal at 0.03 selected three markers, UBC-864_{400bp}, UBC-811_{1250bp} and UBC-811_{650bp} that achieved 80% correct classification genotypes into phenotypic groups. Logistic regression and discriminant analysis achieved 100% correct classification of tolerant and susceptible phenotypes with 13 and 28 markers, respectively. To achieve higher correct classification rate using more informative marker, logistic regression were more efficient than discriminant analysis. Stepwise multiple regression analysis selected the same two markers, UBC-864_{400bp} and UBC-8111_{250bp} which selected with discriminant analysis and logistic regression methods. Our results suggest that these informative markers can be used to efficiently select for disease resistant individuals in a breeding population.

Abbreviations: ISSR: Inter Simple Sequence Repeats; MRA: Multiple regression analysis.
Keywords: Discriminant analysis, *Fusarium oxysporum* f.sp *ciceris,* ISSR, Logistic regression, Molecular markers, Multiple regression analysis (MRA), Multivariate statistical techniques .

Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes in many countries of Asia and Africa, cultivated mostly by the poor and subsistence farmers. In addition to its importance as a food crop, it is valued for sustainability and profitability of production systems (Sivaramakrishnan et al., 2002). *Fusarium* wilt (*F. oxysporum* f.sp *ciceris* (Padwick) Matuo and K.Sato) is one of the major yield limiting factors of chickpea (*Cicer arietinum* L.) (Dubey et al., 2007). It is difficult to manage the disease either through crop rotation or application of chemicals because of soil nature persistence and its capacity to survive for long time, even in the absence of host (Haware et al., 1996). The use of wilt resistance chickpea cultivars is the most effective and eco-friendly method of managing the disease (Sharma et al., 2005). Classical breeding programs for improving quantitative traits like resistance to pathogen require evaluation of large number of genotypes. This phenotypic evaluation for resistance to pathogen is difficult, expensive and affected by environmental factors. Alternative programs as a substitute for traditional procedures include use of molecular assisted selection. The ISSRs are PCR products obtained using primers based on dinucleotide, trinucleotide, tetranuclotide and pentanucleotide repeats (Zietkiewicz et al., 1994). The main advantage of ISSRs is that no sequence data for primer construction is needed (Kumar et al., 2009). This technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of crop species (Reddy et al., 2002) and cultivars such as palm (*Phoenix dactylifra* L.). banana cultivars (Rout et al., 2009) and wheat (*Triticum aestivum* L.). ISSR markers have been used to study genetic diversity and taxonomic relationship in the genus *Cicer*. Iruela et al. (2002) applied ISSR molecular markers to determine phylogenetic relationships in 14 species of the genus *Cicer*. Rajesh et al. (2002) used ISSR markers to evaluate the genetic diversity in six annual and seven perennial wild species of *Cicer*. Chowdhury et al. (2002) used ISSR markers to study their potential in determination of genetic relationships in chickpea cultivars and breeding lines. Rao et al. (2007) used ISSR molecular markers to detect of genetic relationship among 19 chickpea cultivars and five accessions of its wild progenitor *Cicer reticulatum* Ladizinsky. ISSR markers have been used in many marker-trait association studies such as productivity traits in silkworm (*Bombyx mori* L.) (Chatterjee and Mohandas, 2003), yield traits (number of branches, total shoot length, leaf Weight, internodal distance, leaf chlorophyll, protein, leaf moisture percentage) in mulberry (Vijayan et al., 2006),

resistance to mite in coconut (Shalini et al., 2007), thermal stress in polyvoltine silkworm (Srivastava et al., 2007), some biochemical traits in mulberry (*Morus* spp.) (Kar et al., 2008), resistance to dried-shrink disease in sea buckthorn (Raun et al., 2009), eleostearic content in Tung tree (*Vernicia fordii*) (Li et al., 2009), flag leaf senescence under water stressed conditions in wheat (*Triticum aestivum* L.). Ratnaparkhe et al. (1998 a,b) are the only reports on the use of ISSR molecular markers in finding markers linked to *Fusarium* wilt resistance gene in chickpea. In first mapping study using linkage analysis in recombinant inbred lines (RILs) population, they found UBC-855_{500} ISSR marker linked to the gene for resistance to *Fusarium* wilt race 4 (Ratnaparkhe et al., 1998a). In their next research they used the same method and introduced UBC-825_{1200} marker (Ratnaparkhe et al., 1998b).

New approach in molecular assisted selection using multivariate statistical techniques including discriminant and logistic regression has been described by Mcharo et al. (2010). Discriminant analysis and logistic regression were used extensively in molecular marker selection studies (Capdevielle, 2001; Fahima et al., 2002; Aluko, 2003; Mcharo, 2005; Mcharo et al. ,2005; Miano et al., 2008; Mcharo et al., 2010). In addition to the statistical technique described, MRA method have also been used in the identification of molecular markers that associated with various traits (Chatterjee and Mohandas, 2003; Shalini et al., 2007; kar et al., 2008; Raun et al., 2009). The objectives of present study were: (1) Using discriminant analysis and logistic regression to select ISSR informative molecular markers that can be used to select efficiently for *F. oxysporum* f.sp *ciceris* resistant individuals in a breeding population. (2) Comparing effectiveness of discriminant analysis, logistic regression and MRA in assigning of individuals to resistant and susceptible groups.

Results

After data transformation for disease severity we used analysis of variance to compare the isolates. ANOVA of disease severity after inoculation with *F. oxysporum* f.sp *ciceris* showed significance difference in disease severity between genotypes of chickpea for three isolates (Table 2).
Cluster analysis divided genotypes into two main groups based on disease severity after inoculation with three isolates of pathogen (Fig 1). Resistance and susceptible groups consist of 22 and 18 genotypes, respectively (Fig 1), (Table 1). Nine ISSR primers (Table 3) amplified 61 clear and reproducible band that 44 band (72.1%) were polymorphic. On average each primer produced 6.77 bands in total and 4.88 polymorphic bands. Primer UBC-841 produced 100 % polymorphic products, while primer $(ACTG)_4$ produced only 40% polymorphic products. In our genetic studies of chickpea, discriminant analysis and logistic regression would involve relating a genotype, as described by its molecular marker profile, with its disease resistance class or phenotypic group. Discriminant analysis by using the markers as the multiple variables would develop an optimum classification function that maximizes differences between the resistant and susceptible classes while minimizing differences among members of those two classes (Mcharo 2005). Three predictive markers (Table 4), (Fig 2), (Fig 3) were selected for genotypes classification using discriminant analysis in *P* values equal at 0.03. In table 4 the values of partial *R*-square for each marker indicating the impact of marker on the efficiency of the model. Low partial *R*-square indicating that marker has minimal impact on the power of model. Wilk's lambda indicating the power of model in each step that entry new marker in the model. The efficiency of predictive markers for classifying genotypes into phenotypic groups was evaluated by cross-validation as described by Lachenbruch and Mickey (1968). Three predictive markers achieved 80% correct classification that eight out of forty genotypes misclassified. Five genotypes (3, 9, 33, 34, and 37) that was evaluated resistance in phonotypical assay were misclassified into susceptible group. Three other genotypes (8, 14, and 40) that were belonging to the resistance phenotypic group misclassified in to the susceptible group. Logistic regression is a statistical technique that is frequently used to associate explanatory variables with a binary outcome (Ostir and Uchida, 2000). Logistic regression in *P* values equal at 0.03 selected three predictive markers that were the same with selected by discriminant analysis (Table 6). Decreasing values of AIC (Akaik Information Criterion) indicating that power of model increased (Table 6), (Table 7). Evaluation of logistic regression was performed using Hosmer and Lemeshow test (Hosmer et al., 1991). Results of goodness of fit test (chi-square=0.4271, df=3, Pr>chi-square=0.9346) indicated that data fitted well. Partition for the Hosmer and Lemeshow test consist of expected and observed value for each group and confirms results of goodness of fit test (data not shown). It is important to establish equilibrium between the number of markers used in the model and amount of information achieved. Our aim was to have as few markers possible to achieve highly accurate classification. We selected three predictive markers in *P* value=0.03 in both analytic method and achieved 80% correct classification (Table 5), (table 7). One of the objectives in this study was to compare two multivariate statistical methods, discriminant analysis and logistic regression.

Evaluation of discriminant analysis by cross-validation indicated that 28 predictive markers needed to achieve 100% correct classification (Table 5). Logistic regression achieved 100% correct classification with 14 markers (Table 7). When the number of markers and their prediction rates are less than 100% correct classification, logistic regression give more efficient classification than discriminant analysis (Table 5, Table 7). To ascertain markers that selected with multivariate statistical techniques, logistic regression and discriminant analysis, we used stepwise MRA. Stepwise MRA selected two markers that associated with resistance to *F. oxysporum* f.sp *ciceris* (low disease severity) in chickpea genotypes (Table 8). The marker UBC-864_{400bp} showed maximum (-0.527) and highly significant (p=0.000, t=-3.818) correlation with disease severity. The standardized beta coefficient was also high (-0.527). The R^2, R^2 change, F change and significance of F change of two selected markers presented in Table 8. The first and second markers that selected with discriminant analysis and logistic regression also have been selected in MRA. Results of logistic regression and discriminant analysis were confirmed by stepwise MRA.

Discussion

The importance and need of chickpea cultivars resistant to *Fusarium* requires evaluation of germplasm to assist the future breeding programs. Hence, it is essential to characterize chickpea germplasm using markers like ISSR that associated with *Fusarium*. In the present study, out of the 13 primers used for PCR-studies, nine primers amplified

Table 1. Chickpea genotypes, disease severity and phenotypic classification for resistance to *Fusarium* wilt (*Fusarium oxysporum* f.sp *ciceris)*

Number	Name	Reaction group[a]	Disease severity[b]	Origin
1	FLIP-01-37c	R[a]	19.366	Line-ICARDA[c]
2	FLIP-01-45c	R	22.002	Line-ICARDA
3	FLIP-01-36c	R	27.590	Line-ICARDA
4	FLIP-02-09c	R	22.335	Line-ICARDA
5	FLIP-02-50c	R	26.536	Line-ICARDA
6	FLIP-02-23c	R	29.512	Line-ICARDA
7	FLIP-02-21c	R	29.078	Line-ICARDA
8	FLIP-03-45c	R	27.145	Line-ICARDA
9	FLIP-03-141c	R	33.430	Line-ICARDA
10	FLIP-01-49c	R	37.751	Line-ICARDA
11	FLIP-03-103c	R	37.422	Line-ICARDA
12	FLIP-02-39c	R	38.374	Line-ICARDA
13	FLIP-01-63c	R	39.664	Line-ICARDA
14	FLIP-03-53c	R	46.327	Line-ICARDA
15	FLIP-02-40c	R	48.979	Line-ICARDA
16	FLIP-01-47c	R	42.054	Line-ICARDA
17	FLIP-00-17c	R	45.915	Line-ICARDA
18	FLIP-02-07c	R	44.176	Line-ICARDA
19	FLIP-03-36c	R	48.999	Line-ICARDA
20	FLIP-02-03c	R	45.318	Line-ICARDA
21	FLIP-01-35c	R	43.007	Line-ICARDA
22	FLIP-01-02c	R	49.868	Line-ICARDA
23	FLIP-02-47c	S[a]	56.898	Line-ICARDA
24	FLIP-03-35c	S	59.191	Line-ICARDA
25	FLIP-01-34c	S	58.809	Line-ICARDA
26	FLIP-03-106c	S	63.342	Line-ICARDA
27	KC-217005	S	62.671	Native accession-Iran
28	KC-215012	S	59.313	Native accession-Iran
29	KC-215018	S	63.243	Native accession-Iran
30	KC-215025	S	62.936	Native accession-Iran
31	KC-215015	S	74.470	Native accession-Iran
32	KC-215028	S	67.450	Native accession-Iran
33	KC-217011	S	69.562	Native accession-Iran
34	KC-217012	S	67.215	Native accession-Iran
35	Kaka	S	90	Cultivar-Iran
36	Pirooz	S	65.802	Cultivar-Iran
37	ILC-482	S	72.576	Cultivar-ICARDA
38	Hashem	S	59.212	Cultivar-Iran
39	Arman	S	67.987	Cultivar-Iran
40	Biovenig	S	79.758	Cultivar-Iran

[a] Genotypes were grouped as resistance (R) or susceptible (S) based on disease severity of three isolates after arc-sin square root transformation using hierarchical cluster analysis. [b] Disease severity (0-100%) is the mean of disease severity of three isolates after arc-sin square root transformation based on percentage of affected foliage and wilt incidence. The lower the disease severity, the greater the resistance of the chickpea genotypes to *F. oxysporum* f.sp *ciceris* . [c] International Center for Agricultural Research in Dry Areas, Aleppo, Syria.

ISSR marker and showed polymorphism in chickpea genotypes. In this study we observed low level of ISSR marker polymorphism for chickpea genotypes. Compared to other pulse crops e.g. pea *(Pisum sativum* L.), lentil (*Lens culinaris* Medik) or grasspea (*Lathyrus sativus* L.), lower level of genetic polymorphism have been reported for chickpea (Chowdhury et al., 2002). Low level of genetic polymorphism in the cultivated chickpea germplasm was a major drawback in the development of closely linked molecular markers for different traits (Mayer et al., 1997). Chowdhury et al. (2002) determined genetic relationship among breeding lines and cultivars of chickpea with ISSR molecular. Results showed polymorphism varied from 11 to 50% with an average of 26% per primer. Rao et al. (2007) investigated genetic relationship among 19 cultivars of chickpea (*Cicer arietinum*) and five accessions of its wild progenitor (*Cicer reticulatum*) using RAPD and ISSR markers. ISSR analysis in chickpea cultivars shared 56.25% polymorphic band and 65.63% polymorphic band were observed among wild accessions. Rajesh et al. (2002) evaluated genetic diversity in annual and perennial wild species of *Cicer* using ISSR analysis. They observed an average 7.6 and 6.6 total and polymorphic band per primer, respectively. In this study discriminant analysis and logistic regression selected the same three predictive markers that suggesting markers selected, have a strong association with the phenotypic classification. Similar results have also been reported by by Miano et al. (2008). Using discriminant analysis and logistic regression they found four marker selected by two analytic method, have a strong association with resistance to SPVD (Sweet Potato Virus Disease) in sweet potato. Previous works showed that logistic regression are more efficient than discriminant analysis in classifying genotypes into phenotypic groups (Mcharo, 2005, Mcharo et

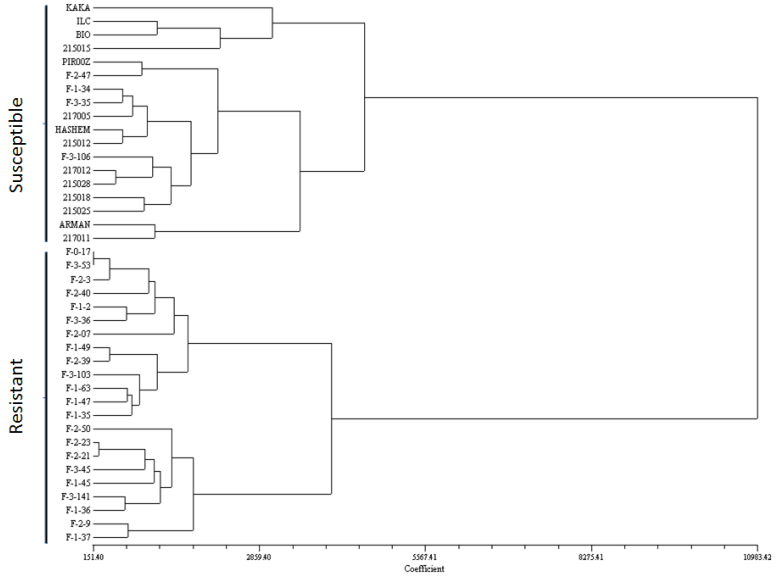

Fig 1. Dendrogram using the EUCLIDSQ (squared Euclidean) coefficient and UPGMA method based on disease severity in chickpea genotypes after inoculation with three isolates of *Fusarium oxysporum* f.sp *ciceris*

al., 2010). Discriminant analysis in SAS is based on Bayesian hypothesis testing while logistic regression is maximum likelihood based using frequentist hypothesis testing (Mcharo et al., 2010). This difference may account for choosing number of marker to achieve correct classification. In addition these two multivariate statistical methods have other differences. In this study we used markers as dichotomous explanatory variables. We used nonparametric discriminant analysis for classification of binary variables which are a restriction for discriminant analysis that has a normality assumption of variables. However, logistic regression easily handles the dichotomous explanatory variables. Error rate as part of the results is not calculated in SAS for logistic regression, therefore we used new version of SPSS (SPSS/PASW18) that capable to calculate error rate for logistic regression. SPSS calculate percentage of correct classification. Using these statistical methods similar results were reported for other traits. Mcharo et al. 2005 selected seven and four AFLP markers by discriminant analysis in p=0.05 that achieved 88.7% and 88.04% correct classification sweetpotato genotypes into root-knot nematode resistance groups. Using logistic regression in p=0.03 they selected seven different AFLP markers in sweetpotato populations. Mcharo (2005) selected six and four AFLP markers that associated with resistance to SPCSV (Sweet Potato Chlorotic Stunt Virus) and SPFMV (Sweet Potato Feathery Mottle Virus) in sweet potato genotypes using discriminant analysis at significance levels of $p \leq 0.03$ for SPCSV and $p \leq 0.02$ for SPFMV, respectively. Selected markers achieved 92% and 96% correct classification genotypes into phenotypic classes for SPCSV and SPFMV. Logistic regression selected seven and six markers that associated with phenotypic classification for resistance to SPCSV and SPFMV at significance level of $p \leq 0.05$ for SPCSV and $p \leq 0.02$, respectively. Despite low level of polymorphism using ISSR marker in this study three predictive markers in P value=0.03 in both discriminant analysis and logistic regression achieved 80% correct classification of chickpea genotypes. These results suggest the possibility of using logistic regression and discriminant analysis to select predictor markers that predict phenotypic classes of new genotypes without phenotypic evaluation for *Fusarium* wilt of chickpea. Using Molecular markers revealing high level of polymorphisms such as SSR may led to finding better marker for classification of susceptible and resistance genotypes of chickpea. Our research provided an opportunity to use multivariate statistical techniques, discriminant analysis and logistic regression on the small size unrelated populations to select informative ISSR marker for *Fusarium* wilt disease in chickpea. These ISSR marker may used for quick selection during the early stages of chickpea breeding program.

Materials and methods

Plant materials and phenotypic evaluation

For this study we used 40 genotypes including lines, native accessions and cultivars of chickpea. The number, name and origin of genotypes are shown in Table 1. We presume that lines, native accessions and cultivars used in this study consists of unrelated genotypes and therefore did not have any population structure. We have provided 26 lines from Dry Land Agricultural Research Sub-Institute, Kermanshah, Iran and eight native accessions from Seed and Plant Improvement Institute, Karaj, Iran and six cultivars were provided from Agricultural and Natural Resources Research Center of Kurdistan, Sanandaj, Iran. Genotypes were evaluated for reaction to the disease in sick pots at greenhouse of Agriculture Faculty, Kurdistan University, Sanandaj, Iran. We used three isolates of *F. oxysporum* f.sp *ciceris* that were isolated and maintained in research plant pathology laboratory, Agriculture Faculty, Kurdistan University, Sanandaj, Iran. The cultures of *F. oxysporum* f.sp

Table 2. Analysis of variance for disease severity in chickpea genotypes after inoculation with three isolates of *Fusarium oxysporum* f.sp *ciceris*

Isolate	*Df*[a]	Mean square	*F* value[b]
F3	39	926.37	9.96**
F6	39	1104.02	14.57**
F10	39	944.57	10.5**

Data were transformed using arc-sin square root transformation before analysis. [a] Degrees of freedom. [b] Variance ratio (** $p<0.01$)

Table 3. Primers used and number of ISSR bands.

Primer	Sequence 5´→3´	Total ISSR bands	Polymorphic bands	Polymorphism (%)
UBC-873	GACAGACAGACAGACA	6	4	66.6%
UBC-811	GAGAGAGAGAGAGAGAC	7	5	71.4%
UBC-809	AGAGAGAGAGAGAGAGG	7	5	71.4%
UBC-808	AGAGAGAGAGAGAGAGC	8	5	62.5%
UBC-841	GACACGACACGACACGACAC	8	8	100%
UBC-864	ATGATGATGATGATGATG	7	5	71.4%
UBC-880	GGAGAGGAGAGGAGAGGAGA	6	5	83.3%
UBC-868	GAAGAAGAAGAAGAAGAA	7	5	71.4%
$(ACTG)_4$	ACTGACTGACTGACTG	5	2	40%
Total		61	44	

Table 4. STEPDISC (step discriminant) selection for DNA markers associated with resistance to *Fusarium* wilt (*Fusarium oxysporum* f.sp *ciceris)* in chickpea genotypes (p=0.03).

Marker entry step	Marker[a]	Partial $R^{2,b}$	*F* value	Pr>F^c	Wilk's Lambda[d]	Pr<lambda[e]
1	UBC-864_{400bp}	0.245	12.34	0.0012	0.754	0.0012
2	UBC-811_{1250bp}	0.141	6.11	0.0182	0.647	0.0003
3	UBC-811_{650bp}	0.167	7.23	0.0108	0.539	<0.0001

[a] Name of marker consist of first part that is primer name and second part that is molecular weight (base pair). [b] Partial R^2 is the marginal variability accounted for by a variable when all others are already included in model. [c] This is the p-value associated with the F value of a given marker in the model. [d] Wilk's Lambda is the likelihood ratio measure of marker's contribution to the discriminatory power of model. [e] The p-value associated with the likelihood ratio test (Wilks' Lambda)

ciceris were maintained on Potato Dextrose Agar (PDA) medium. The experiments were conducted in a completely randomized design with three replications. Separate experiments were performed for each *F. oxysporum* f.sp *ciceris* isolate. The inoculums were prepared with Corn Meal Sand (CMS) mixture (Trapero-Casas and Jiménez-Díaz, 1985) in conical flasks and incubated for 21 days at room temperature (Gowda et al., 2009). The infected CMS mixture was mixed thoroughly with autoclaved soil mixture (clay loam, sand, peat 1:1:1 V/V) at 1:12 (W/W) and transformed to pots according to Brinda and Ravikumar (2005). Similar pots with no infested CMS were used as control. For each experiment, seeds of genotypes were surface sterilized with 70% alcohol and sown in the sick pots. The symptoms of disease were recorded at daily intervals after three weeks after sowing date. Disease severities (0-100%) were determined based on percentage of affected foliage (Jiménez-Gasco et al., 2001) and wilt incidence (Gowda et al., 2009). To validate the experiments, the pathogen was isolated from stem of infected plants for each isolate to determine the occurrence of vascular infections. Stems were cut into 5-10 mm large pieces, surface disinfected in 0.2% NaCLO for two min (Navas-Cortés et al., 2000) then plated on PDA and incubated at room temperature. Disease severity data was transformed using arc-sin square root transformation before analysis. Difference among the genotypes for disease severity after inoculation with *F. oxysporum* f.sp *ciceris* was evaluated with Analysis of variance (ANOVA) using SAS program (SAS Institute Inc 2001) for each isolates. Phenotypic grouping of genotypes was conducted by hierarchical cluster analysis using NTSYS pc ver.2.02 (numerical taxonomy and multivariate analysis system) (Rohlf, 2000). Distance matrix measured by squared Euclidean distance based on the disease severity after arc-sin square root transformation with three isolates of *F. oxysporum* f.sp *ciceris* using SIMINT model that computes various similarity or dissimilarity indices for interval measure (continuous) data (Rohlf, 2000). Clustering of genotypes was done based on distance matrix using UPGMA (Unweighted Pair Group Method with Arithmetic average) algorithm (Sneath and Sokal, 1973) using the SAHN model that performs the sequential, agglomerative, hierarchical and nested clustering methods (Rohlf, 2000). The clustering result was used to construct a dendrogram by the TREE model (Rohlf, 2000).

DNA extraction, PCR methods and gel electrophoresis

Genomic DNA was extracted from young leaf tissue using the CTAB extraction method according to Saghai-maroof et al. (1984) with some minor modifications. Quantity and quality of the isolated DNA was measured by spectrophotometer (Biochrom, UK) and visualized using 1% agarose gel electrophoresis.Amplification reaction was carried out in a total volume 20 µl with 100 mM Tris-HCl PH = 8.3, 500 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 unit of Taq DNA polymerase, 5.0 picomoles of corresponding primer and 20 ng DNA template using thermal cycler (icycler, BIORAD, U.S.A). After initial denaturation at 94 ˚C for 4 min, 40 cycles of PCR were conducted where each cycle consist of 30 sec denaturation at 92 ˚C, 40 sec annealing (annealing temperature optimized for each primer according to theoretical T_m ˚C for hybridization, 45-54) and 90 sec extension at 72 ˚C with a final extension five min at

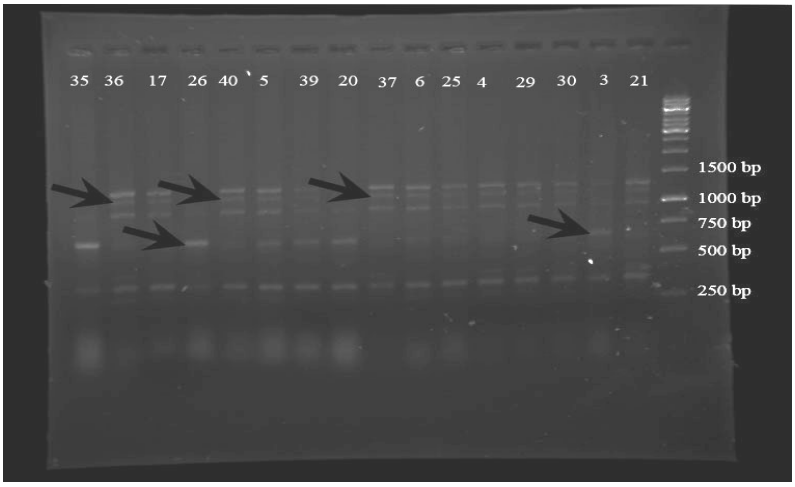

Fig 2. Amplification profile of ISSR primer UBC 811 in chickpea genotypes with GeneRuler^{TM} 1Kb DNA Ladder, ready-to-use. UBC-811_{1250bp} and UBC-811_{650bp} are shown in figure. The genotypes Number are presented in Table 1.

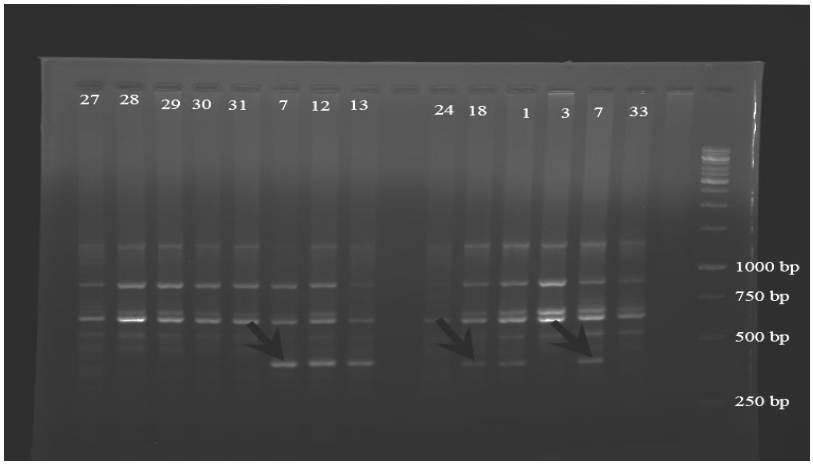

Fig 3. Amplification profile of ISSR primer UBC 864 in chickpea genotypes with GeneRuler^{TM} 1Kb DNA Ladder, ready-to-use. UBC-864_{400bp} is shown in figure. The genotypes Number are presented in Table 1

72 ˚C. We selected 13 primers from primer set (UBC primer set #9) of the University of British Columbia, Biotechnology laboratory, Vancouver, Canada:(www.michaelsmith.ubc.ca/ services/NAPS/Primer_Sets) based on previous work on chickpea. Nine ISSR primers were selected based on polymorphism and robustness of the bands obtained. All the generated patterns were repeated twice in order to verify reproducibility. Products of PCR reaction were mixed with four µliter of 6X loading dye (0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF and 40% Sucrose, W/V), separated on 1.5% agarose gel in 1X TBE buffer (Tris 0.89 M, EDTA 2mM, Boric Acid 0.89 M, PH= 8.3), stained with ethidium bromide and visualized under U.V light. ISSR products were scored as the presence (1) or absence (0) of bands and a binary matrix was constructed. Only ISSR fragments that could be scored unambiguously were included in the analysis.

Discriminant analysis and logistic regression

We were selected the most informative markers from original set of ISSR generated molecular markers, using discriminant analysis and logistic regression that previously described by Mcharo et al. 2010. STEPDISC procedure (SAS Institute Inc 2001) was used to perform stepwise discriminant analysis. The stepwise and forward selection options in STEPDISC procedure were used to select markers to be included in the classification model. The selected markers then used in discriminant analysis, DISCRIM option (SAS Institute Inc 2001) to construct and validate a phenotypic group prediction model. The performance of the discriminant criterion was evaluated by group-specific error-count estimates during cross-validation. The error estimator gives the proportion of misclassified observation in each group. Total error, from which we derive percent correct classification, is the

Table 5. Rate of correct classification of chickpea genotypes into *Fusarium* wilt *(Fusarium oxysporum* f.sp *ciceris)* resistance groups after cross-validation in discriminant analysis.

Number of predictor markers	Resistance group error rate	Susceptible group error rate	Total group error rate
3	0.272	0.222	0.247
4	0.227	0.166	0.197
11	0.136	0.111	0.123
16	0.09	0.055	0.073
27	0.045	0.055	0.05
28	0[a]	0[a]	0[a]

[a] The rate of misclassified genotypes in group

Table 6. Logistic regression selection for DNA markers associated with resistance to *Fusarium* wilt (*Fusarium oxysporum* f.sp *ciceris*) in chickpea genotypes (p=0.03).

Marker entry step	Marker[a]	Estimate (β)	AIC	χ^2 score[b]	pr> χ^2
0	intercept	6.156			
1	UBC-864_{400bp}	-3.028	48.838	9.808	0.0017
2	UBC-811_{1250bp}	-2.489	45.246	5.560	0.0184
3	UBC-811_{650bp}	-2.880	41.203	5.970	0.0145

[a] Name of marker consist of first part that is primer name and second part that is molecular weight (base pair). [b] χ^2 score is the largest significant score for marker not in model to be included in the model

Table 7. Percentages of correct classification of chickpea genotypes into *Fusarium* wilt (*Fusarium oxysporum* f.sp *ciceris*) resistance groups for logistic regression.

Number of predictor markers	correct classification in resistance group	correct classification in susceptible group	Total correct classification	Akaike Information Criterion (AIC)
3	72.7%	88.9%	80%	41.203
4	86.4%	83.3%	85%	39.603
5	90.9%	83.3l%	87.5%	37.130
6	95.5%	83.3%	90%	36.280
7	100%	83.3%	92.5%	35.828
8	100%	88.9%	95%	33.387
9	100%	94.4%	97.5%	33.118
13	100%	100%	100%	28.209
14	100%	100%	100%	30.210

Table 8. Analysis of variance (ANOVA) and coefficients for dependent variable of stepwise multiple regression analysis (MRA) for association of ISSR molecular markers with resistance to *Fusarium* wilt (*Fusarium oxysporum* f.sp *ciceris*) in chickpea genotypes.

Markers[a]	Sum of squares	Mean square	*F* value	*P* value	standardized coefficient beta	*t* value	*P* value	*r*
UBC-864_{400bp}	3370.657	3370.657	14.576	0.000	-0.527	-3.818	0.000	-0.527
UBC-811_{1250bp}	5580.788	2790.394	15.698	0.000	-0.429	-3.526	0.001	-0.678

Marker of previous step is included in the succeeding step. [a] Name of marker consist of first part that is primer name and second part that is molecular weight (base pair).

weighted mean of error estimates of two phenotypic groups (Mcharo 2005). LOGISIC procedure (SAS Institute Inc 2001) was used to carry out logistic regression to selected markers that significantly accounted for phenotypic variation with forward selection option. Significance level to include a marker for both discriminant analysis and logistic regression was set at P=0.03. Higher probability value was selected more molecular markers without considerable increasing in efficiency of the prediction. For calculate error rate for logistic regression we used SPSS/PASW version 18, statistical software package (SPSS Institute Inc 2009).

Multiple regression analysis

Pearson's correlation coefficient between each ISSR marker and the disease severity performed using SPSS/PASW version 18. We divided all ISSR markers into two groups, one with positive correlation and the other with negative correlation with disease severity. The lower the disease severity, the greater the resistance of the chickpea genotypes to *F. oxysporum* f.sp *ciceris* and in contrast the higher disease severity indicated the susceptibility to *F. oxysporum* f.sp *ciceris* in chickpea genotypes consequently we using negative ISSR molecular markers as independent variable in MRA. The association between ISSR molecular markers and resistance to *F. oxysporum* f.sp *ciceris* was estimated through stepwise MRA with SPSS/PASW version 18. The disease severity was treated as a dependent variable and the negative ISSR markers were treated as independent variable. *F* values with 0.045 and 0.099 probabilities were used to enter and remove independent variables from regression equation, respectively (Affifi and Clark, 1984; Roy and Bargmann, 1957).

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