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RESEARCH PAPER

Genetic diversity of microsatellite alleles located at quantitative resistance loci for Ascochyta blight resistance in a global collection of chickpea germplasm

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Summary. A global collection of 43 chickpea (*Cicer arietinum* L.) genotypes, resistant and susceptible to Ascochyta blight caused by *Ascochyta rabiei* was evaluated for the disease under controlled conditions. In this study three known pathotypes (P-I, P-II, and P-III) were used to evaluate the reactions of this collection. The chickpea genotypes were also characterized using 14 microsatellite markers flanking the genomic regions associated with Ascochyta blight resistance quantitative trait loci (QTLs). Phenotyping results indicated that 27 genotypes were resistant to P-I, 14 to P-II, and five to P-III, revealing the possible erosion of resistance through the evolution of virulent pathogen pathotypes. The genetic diversity analysis revealed 67 alleles at 14 microsatellite loci with an average of 4.8 alleles per locus among the genotypes tested. Genetic similarity estimates differentiated four sub-clusters (A, B, C, and D) of the genotypes. However, none of sub-clusters were separated into resistant genotypes for a specific pathotype. The genetic diversity ranged from 0.48 to 0.80 which indicated that there is considerable variation in QTL regions associated with Ascochyta blight resistance among the collections of chickpea genotypes studied, as assessed using the hyper-variable microsatellite markers.

Key words: *Ascochyta rabiei*, *C. arietinum*, chickpea.

Introduction

Ascochyta blight caused by *Ascochyta rabiei* (Teleomorph: *Didymella rabiei*) is one of the major problems facing chickpea production worldwide and causes a huge loss of yield and quality – up to 100% in severely infected fields (Acikgoz *et al*., 1994). Seed treatment and foliar application of fungicides are often used for controlling this disease, but, unfortunately, they are still unsuccessful and uneconomical (Nene and Reddy, 1987; Atik *et al*., 2011). The use of resistant cultivars is considered the best option for long-term Ascochyta blight management.

A small number of chickpea genotypes have been reported to carry Ascochyta blight resistance genes and only five – ICC4475, ICC6328, ICC12004, ILC200, and ILC6482 – out of 19,343 accessions screened were resistant to the disease in repeated field and greenhouse evaluations at ICARDA, Syria (Singh and Reddy, 1993). Additional germplasm lines (ICC3996, ICC4475, and ICC12004) were also reported resistant against a number of *A. rabiei* isolates\ from the northwestern United States (Chen *et al*., 2004) and ILC72, ILC195, ILC200, ILC482, ILC3279, and ILC6482 were identified as cultivars with rate-reducing resistance to Ascochyta blight in comparison with a susceptible cultivar (Reddy and Singh, 1992; Singh *et al*., 1992). Most breeding programs worldwide have

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relied heavily on two kabuli genotypes, ILC72 and ILC3279, as sources for Ascochyta blight resistance (Crino, 1990; Muehlbauer *et al*., 1998a, 1998b; Muehlbauer and Kaiser, 2002; Millan *et al*., 2003; Muehlbauer *et al*., 2004; Rubio *et al*., 2004). However, it is important to characterize accessions from different germplasm sources that may contain new or different genes for Ascochyta blight resistance. This will allow breeders to pyramid resistance genes into one cultivar. However, no differential chickpea lines are identified to distinguish different Ascochyta blight resistance genes.

The pathogen shows high variability, and Ascochyta blight resistant chickpea cultivars have become susceptible in some countries. Variability of *A. rabiei* has been reported in Syria and other chickpea-growing countries; Reddy and Kabbabeh (1985) identified six races of *A. rabiei* isolates collected from Syria and Lebanon using 18 chickpea differentials, and later Udupa and Weigand (1997) grouped 47 isolates and the six races into three pathotypes based on differences in aggressiveness on three chickpea differentials. None of the pathotypes described by Udupa and Weigand (1997) were virulent on chickpea genotypes ICC 12004 and ICC 3996, and later more virulent isolates, which attack the two genotypes, were identified in an *A. rabiei* population collected in Syria (Bayaa *et al*., 2004; Imtiaz *et al*., 2011; Atik *et al*., 2013).

Many quantitative trait loci (QTL) analyses identified molecular markers linked to Ascochyta blight resistance genes and could be used to assess the diversity at the Ascochyta blight specific genomic regions and to measure genetic relationships among genotypes. Two major QTLs on LG 2, close to the GA16 and TA37 loci, control resistance to Ascochyta blight Pathotype I (Cho *et al*., 2004). Another QTL to Pathotype II is located on LG4 around SSR loci GAA47, TA130, TR20, TA72, TS72, and TA2 (Winter *et al*., 2000; Udupa and Baum, 2003; Cho *et al*., 2004). Cho *et al*. (2004) identified an additional SSR marker (TA46) that was strongly associated with the resistance derived from FLIP84-92C to Pathotype II. This marker explained between 59 and 69% of the variations for resistance using different isolates under controlled environments. Furthermore, loci TS12b and STMS28, on LG1 TS45, and TA3b, on LG2, were significantly associated with the disease reaction under controlled environments (Flandez-Galvez *et al*., 2003a, 2003b). In summary, QTLs contributing to *A. rabiei* (*Ar*) resistance were identified by many research groups – 14 *Ar* loci located on eight chickpea LGs, named as Ar_1a, Ar_2a, Ar_2b, Ar_2c, Ar_3a, Ar_3b, Ar_3c, Ar_4a, Ar_4b, Ar_5a, Ar_6a, Ar_6b, Ar_7a, and Ar_8a (Tekeoglu *et al*., 2002; Flandez- Galvez *et al*., 2003a, 2003b; Udupa and Baum, 2003; Cho *et al*., 2004; Iruela *et al*., 2006; Lichtenzveig *et al*., 2006; Tar'an *et al*., 2007; Anbessa *et al*., 2009: Kottapalli *et al*., 2009; Taleei *et al*., 2009).

This high degree of pathogenic variability demands continuous efforts to search for new sources of resistance and the deployment of these for chickpea improvement. In this study we used SSRs from previous mapping and QTL studies to evaluate the genetic relationships among 43 chickpea germplasm accessions differing in their reactions to Ascochyta blight and attempted to establish the relationship between different sources of Ascochyta blight resistance.

Materials and methods

The fungal cultures of *A. rabiei* pathotypes I, II, and III (P-I, P-II, P-III) reported by Udupa and Weigand (1997) were used in separate experiments in this study. The cultures were obtained from the Legume Pathology Laboratory at ICARDA. The experiments were laid out in randomized complete block design with two replications. Four healthy seeds of each of the 43 chickpea genotypes were germinated in a 15 cm diameter pot in a growth chamber (temperature 22°C and 12/12 hours light/dark). A spore suspension of *A. rabiei* with a concentration of 10^5 spores mL^{-1} was prepared in sterile distilled water using a 14-day old culture grown on chickpea dextrose agar and sprayed onto plants until runoff. The disease was scored when symptoms on the susceptible check (ILC-263) were observed. Scoring was based on an individual plant using a nine point rating scale (Singh and Reddy 1993), where 1, immune, no symptoms of disease; 2, few, very small lesions (<2 mm) on leaves and stems (1 to 2% of the plant area infected); 3, many small lesions (6 to 10% of the plant area infected); 4, many small and large lesions (26 to 50% of the plant area infected); 5, many small lesions on the stem; 6, many large lesions, lesions coalescing, stem girdled (76 to 90% of the plant area infected); 7, many small and large lesions, lesions coalescing, girdling stem breakage (>90% of the plant area infected), 8, almost dead plants; and 9, dead plants. The disease score for each genotype was averaged from 8 plants in two pots (4 plants per

pot). A leaf sample was collected from young tissue before inoculation, and the DNA was extracted according to the CTAB method (Weising *et al*., 1991). Fresh leaves from the seedlings were frozen in liquid nitrogen and ground into a fine powder, which was subsequently added to a 2 mL Eppendorf tube with 1 mL pre-warmed 2×CTAB buffer – 2% CTAB, 0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA). The suspension was mixed and incubated at 65°C for 30 minute. The suspension was cooled at room temperature (RT) for 5 minute, 1 mL chloroform-isoamyl alcohol (24:1) was added to the tube and the suspension gently mixed by shaking for 10 minute. The suspension was centrifuged at 4500 rpm (Beckmann YA-12) for 20 minute at RT and the supernatant transferred to a new tube. The DNA was precipitated with 700 µL of cold isopropanol. The DNA was transferred into a microcentrifuge tube and washed twice with a washing buffer (75% ethanol and 200 mM sodium acetate) for 20 minute. After air-drying for about 10 to 20 minute, the DNA was dissolved in 100 µL of 1×TE buffer – 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The 43 chickpea genotypes (Table 1) were characterized using 14 microsatellite markers flanking the genomic regions associated with Ascochyta blight resistance quantitative trait loci (Winter *et al*., 2000; Lichtenzveig *et al*., 2005). These SSR markers may be associated with seven genes for *A. rabiei* resistance (*Ar_2-a, Ar_2-c, Ar_3-c, Ar_4-a, Ar_4-b, Ar_6-b* and *Ar_8-a*) identified on five chickpea linkage groups.

The polymerase chain reactions (PCRs) were performed in a total reaction mixture of 20 µL containing: 50 ng of total genomic DNA (2 µL) as template, 1× PCR buffer (Roche, Manheim, Germany), 0.2 mM of dNTP PCR mix (Roche), 0.5 U of *Taq* DNA polymerase (Roche) and 10 pmol of each of the primers (forward and reverse primers). Amplifications were performed in an Thermocycler (Applied Biosystems) with the following conditions: a denaturation step of 5 minute at 95°C followed by 35 cycles of 15 s at 95°C, 15 s at 58°C and 30 s at 72°C, and a final extension step at 72°C for 5 minute. Amplification products were separated on 8% polyacrylamide gels stained by ethidium bromide. A 100-bp DNA ladder (Promega Corporation) was used as a size standard. The DNA banding patterns were visualized on an UV transilluminator and documented by using a Gel Documentation System (Alpha Innotech).

Data analysis

An analysis of variance (ANOVA) was performed using GenStat 12th edition. Analyses of microsatellite diversity were conducted at the locus and for each locus we estimated the number of alleles, range of fragment sizes, mean of fragment size, and genetic diversity using PowerMarker V3.25 (Nei, 1973; Liu and Muse, 2005). The genetic diversity ($\hat{D}$) was calculated based on the following equation:

$$\hat{D} = 1 - \sum_{i=1}^{k} x_i^2$$

where x_i is the relative frequency of the i^{th} allele of the SSR loci; k is the total number of loci.

The unweighted pair group method arithmetic average (UPGMA) was used to cluster the accessions studied. A cluster analysis and a bootstrap analysis (with 100 bootstrap samples) were performed using PAST software version 1.62 (Hammer *et al*., 2001).

Results

ANOVA analysis indicated no significant differences among replications while significant differences ($P \leq 0.001$) were observed among chickpea genotypes when inoculated by each of Ascochyta blight pathotypes P-I, P-II and P-III (Table 2). The results also indicated that 27 genotypes were resistant to P-I, 14 to P-II, and five to P-III (Table 1). Only four (9.3%) of the genotypes tested (CICA857, GENESIS510, ICC12004, and ICC3996) showed resistance to all three pathotypes.

The genetic diversity analysis revealed 67 alleles at 14 microsatellite loci. These ranged from two at GAA47 to seven at TA2 and TA146, with an average of 4.8 alleles per locus among the tested genotypes (Table 3). The general mean of genetic diversity was relatively high (0.69) indicating a considerable diversity among Ascochyta blight resistant genotypes. However, the genetic diversity ranged from 0.48 (at locus GAA47, linked to resistant gene *Ar_4-a*) to 0.80 (at loci TR20 and TA146, linked to *Ar_4-b* and at locus GA20, linked to *Ar_2-a*).

Cluster analysis, based on genetic similarity estimates clearly delineated the genotypes into four major clusters, A with seven accessions, B with seven, C with 15, and D with 14 accessions (Figure 1). Accessions ILC5263 and ILC5894 in cluster (C) and GENSIS509 and GENESIS510 in cluster (D) showed

Table 1. Chickpea genotypes used in this study with seed type (K = kabuli, D = desi), status, origin and disease score in response to the three *Ascochyta rabiei* pathotypes I, II, and III. "R" in brackets indicates resistant varieties (disease score is less than 4).

Accession	Seed type	Status	Origin	Pathotype-I	Pathotype-II	Pathotype-III
ALMAZ	K	Cultivar	Australia	2.50 (R)	3.38 (R)	5.62
CDC Cabri	D	Cultivar	Canada	1.80 (R)	4.63	5.87
CDC Luna	K	Cultivar	Canada	3.10 (R)	1.50 (R)	5.58
CICA511	D	Cultivar	Australia	4.88	5.29	4.44
CICA512	D	Cultivar	Australia	4.88	5.58	5.75
CICA603	D	Cultivar	Australia	4.16	4.25	5.62
CICA857	K	Breeding line	Australia	1.00 (R)	2.50 (R)	3.90 (R)
FLIP94 -079C	K	Cultivar	ICARDA	2.50 (R)	4.72	5.25
FLIP94 -090C	K	Cultivar	ICARDA	1.30 (R)	4.88	4.75
FLIP97 -114C	K	Cultivar	ICARDA	4.75	5.38	4.87
FLIP98-1065	K	Breeding line	ICARDA	1.10 (R)	4.00	4.62
FLIPPER	D	Cultivar	Australia	3.00 (R)	1.80 (R)	6.71
GENESIS509	D	Cultivar	ICARDA	2.20 (R)	– *	2.9 (R)
GENESIS510	D	Cultivar	ICARDA	3.10 (R)	3.60 (R)	2.6 (R)
GENESIS836	D	Cultivar	India	4.38	4.38	5.62
HOWZAT	D	Cultivar	Australia	5.50	5.88	7.24
ICC 12004	D	Germplasm	Unknown	2.80 (R)	2.80 (R)	3.10 (R)
ICC 1963	D	Germplasm	Unknown	3.90 (R)	5.00	5.50
ICC 3996	D	Germplasm	India	3.30 (R)	2.60 (R)	3.80 (R)
ILC191	K	Germplasm	Russia	3.90 (R)	2.70 (R)	5.25
ILC194	K	Germplasm	Russia	4.50	4.88	5.93
ILC195	K	Germplasm	Russia	4.13	4.63	7.67
ILC196	K	Germplasm	Russia	4.27	6.28	5.00
ILC200	D	Germplasm	Russia	2.50 (R)	3 .00 (R)	5.88
ILC215	K	Germplasm	IRN	1.50 (R)	6.83	5.71
ILC263	K	Germplasm	Turkey	6.25	4.44	7.13
ILC2956	K	Germplasm	Former USSR	5.92	8.84	8.72
ILC3279	K	Germplasm	USSR	2.80 (R)	2.8 0 (R)	4.01
ILC482	K	Germplasm	Turkey	1.80 (R)	7.38	5.63
ILC5263	K	Germplasm	Unknown	2.70 (R)	2.30 (R)	6.25
ILC5894	K	Germplasm	Ukraine	3.10 (R)	2.20 (R)	6.01
ILC605	K	Germplasm	Algeria	6.27	9.03	7.41
ILC6260	K	Germplasm	Unknown	5.50	7.38	4.98

(*Continued*)

Table 1. *Continues.*

Accession	Seed type	Status	Origin	Pathotype-I	Pathotype-II	Pathotype-III
ILC6287	K	Germplasm	Unknown	1.30 (R)	5.43	4.72
ILC72	K	Germplasm	Unknown	2.00 (R)	4.50	6.25
ILC7795	K	Germplasm	Armenia	4.38	4.75	4.42
ILC182	K	Germplasm	Armenia	2.00 (R)	5.29	4.38
PBA HATTRICK	D	Cultivar	Australia	3.10 (R)	3.30 (R)	5.01
PBA PISTOL	D	Cultivar	Australia	6.00	6.13	5.25
PBA SLASHER	D	Cultivar	Australia	4.38	5.49	5.42
PCH15	D	Cultivar	Unknown	1.50 (R)	4.38	6.28
S050339	K	Cultivar	Unknown	1.20 (R)	4.00	4.56
YORKER	D	Cultivar	Unknown	3.80 (R)	2.00 (R)	4.63

* Missing value.

Table 2. ANOVA of the Ascochyta blight disease scale collected from 43 chickpea genotypes across two replications, against P-I, P-II, and P-III.

	df	MS	F	P
P-I				
Replications	1	5.746	2.39	
Varieties	42	17.699	7.38	<0.01
Residual error	282	2.399		
Total	325			
P-II				
Replications	1	0.411	0.12	
Varieties	41	25.789	7.39	<0.01
Residual error	263	3.496		
Total	304			
P-III				
Replications	1	1.501	0.56	
Varieties	42	13.176	4.93	<0.01
Residual error	261	2.673		
Total	304			

Df, degree of freedom; MS, mean of square; F, F value; *P*, probability.

similar genetic backgrounds. GENSIS509 and GENESIS510 are sister lines and have a similar disease reaction (3.2), whereas ILC5263 and ILC5894 showed low ratings of 3.8 and 4.4, respectively. The results showed that none of the clusters separated resistant genotypes for a specific pathotype. For instance, accessions CICA857, GENESIS510, and ICC12004, identified as resistant to P-III, were separated in different clusters (C, D, and A, respectively) indicating different genetic background and, most likely, different resistance genes to this pathotype with possible interaction among minor and major genes. However, of the 29 accessions clustered in C and D, only nine (approximately 31%) showed susceptibility and 69% were resistant to *A. rabiei* pathotype P-I, whereas eight (57.4%) of the 14 accessions clustered in A and B were susceptible to Ascochyta blight (Figure 1). Genotype CICA857 is a Kabuli breeding line from Australia and is derived from a cross between two ICARDA-developed resistant lines, S95342 (<4 severity rating; derived from FLIP84-79C X FLIP90-126C) and FLIP90-016C (Ascochyta blight rate <5; derived from ILC1919 x FLIP85-4C). ICC 12004 is a desi accession from India (<2 severity rating; derived from resistant line NEC 2861). GENESIS509 is an Australian breeding line derived from FLIP94-509 (derived from Ascochyta blight resistant lines ICC3996 X ILC5928). In our study, the resistant parent ICC3996 was also resistant to P-III and clustered in D.

Table 3. Variation at microsatellite loci used to study the genetic diversity of 43 chickpea genotypes, resistant and susceptible to Ascochyta blight.

Contributing to *A. rabiei* (*Ar*) resistance	Linkage group	Marker	Number of observations	Allele number	Gene diversity	Heterozygosity
-	-	H5H-02	43	4	0.65	0.05
Ar_2a	2	GA20	44	6	0.80	0.00
	2	GA16	36	5	0.65	0.00
Ar_2c	2	TA103	40	5	0.64	0.00
	2	TA200	34	4	0.72	0.00
	2	TA37	40	5	0.78	0.00
Ar_3c	3	TA34	37	4	0.62	0.03
Ar_4a	4	GAA47	44	2	0.48	0.07
Ar_4b	4	TA2	40	7	0.74	0.00
	4	TR20	36	6	0.80	0.00
	4	TA146	43	7	0.78	0.00
Ar_6b	6	TA80	41	5	0.75	0.02
Ar_8a	8	TA3	42	3	0.64	0.02
	8	TS45	43	4	0.61	0.00
		Mean	40.21	4.79	0.69	0.01

Discussion

The results indicated 27 genotypes were resistant to P-I, 14 to P-II, and five to P-III (Table 1). The five (13.9%) accessions showing resistance to P-III, which is reported to be one of the most virulent pathotypes known for chickpea, were CICA857, GENESIS509, GENESIS510, ICC12004, and ICC3996. Except for GENESIS509, which did not germinate in the P-II experiment, these accessions (as expected) were also resistant to P-I and P-II. In the present study, 59% of the genotypes were resistant to P-I, a result similar to the 54% observed by Tar'an *et al*. (2007), who evaluated the genetic relationships of 37 chickpea germplasm accessions differing in reaction to Ascochyta blight. They used isolate ar68-2001 which was collected from cv. Sanford from a commercial production field in Saskatchewan in 2001. Of the 37 used by Tar'an *et al*. (2007) only five accessions – ICC3996, ICC12004, ILC72, ILC2956, and ILC3279 – were used in the present study. However, it is unknown whether this isolate belonged to P-I, as in our experiment, or a different one. Recently, a new pathotype, P-IV reported by Imtiaz (2011) showed that all these accessions were susceptible, indicating this new pathotype with increased aggressiveness compared to the current *A. rabiei* pathotypes has overcome the resistance in these cultivars.

The current study provides an illustration of allele diversity at SSR loci associated with QTLs for Ascochyta blight resistance across a diverse collection of chickpea accessions. The 14 microsatellites used in this study that are linked to seven QTLs for *A. rabiei* resistance (Ar_2-*a*, Ar_2-*c*, Ar_3-*c*, Ar_4-*a*, Ar_4-*b*, Ar_6-*b* and Ar_8-*a*) on the five chickpea linkage groups showed high diversity (0.80) at Ar2-a and low diversity (0.48) at Ar4-a. The hierarchical clustering based on these SSR alleles enabled us to differentiate four major sub-clusters of these chickpea accessions differing in reaction to Ascochyta blight, but none of sub-clusters corresponded to resistant genotypes for a specific pathotype. These clusters also varied to those reported by Tar'an *et al*. (2007) who used 17 SSR

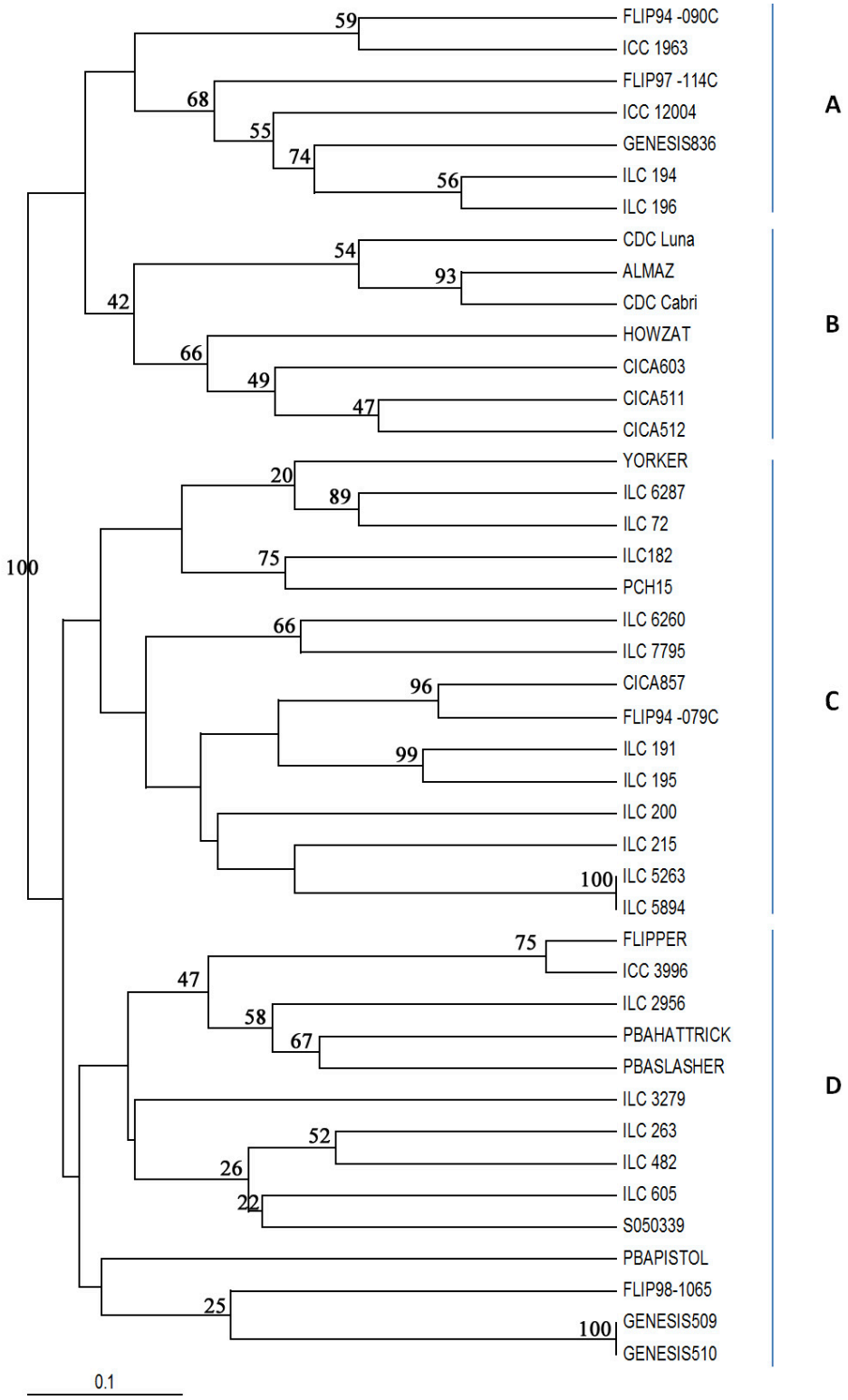

Figure 1. Dendrogram showing different groups of chickpea genotypes, resistant and susceptible to Ascochyta blight. The groups are denoted on the right side as A or B, C and D. Bootstrap values of above 20% are indicated at the nodes.

loci associated with QTLs, some of which were also included here. For example, they found that ILC72 and ILC3279, which have been widely used as sources of Ascochyta blight resistance around the world, were grouped in one cluster. In contrast, our results showed that these two accessions were grouped in different sub-clusters – ILC72 in sub-cluster C and ILC3279 in sub-cluster D.

Accessions CICA857, GENESIS510, ICC12004, and ICC3996 showed resistance to P-I, P-II, and P-III and were distantly related based on the SSRs linked to QTL regions, but two of them (GENESIS510 and ICC3996) grouped in sub-cluster D.

Several potential sources of resistance from germplasms or lines from different geographical origins could be used in combination with adapted varieties to develop better and possibly more durable resistance to Ascochyta blight. For example, CICA857 from Australia and ICC3996 from India both showed resistance to P-III, so would be valuable parents. Although the none of the alleles in this study identified association to a specific pathotype, the current analyses provided information on genotypes with distinct genetic backgrounds at genomic regions associated with the QTL for Ascochyta blight resistance, and these sources of resistance could still be used to broaden the genetic base for the newer cultivars by pyramiding different Ascochyta blight resistance genes using genotypes from different sources.

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