* Hybridity Assessment of Chickpea Genotypes to Identify True Hybrids using SSR Markers

**Abstract-**Chickpea (Cicer arietinum L.) is one of the most important food legumes globally. However, its production is significantly affected by Ascochyta blight (AB), caused by Ascochyta rabiei, and Botrytis gray mold (BGM), caused by Botrytis cinerea. Breeding programs have been effective in developing disease-resistant cultivars, but closely related germplasm often lacks morphological markers required to confirm hybridity, a crucial step in breeding programs. This study aimed to identify SSR markers suitable for confirming true hybridity in chickpea.

Chickpea genotypes C214, PDG3, and JG14 were used as disease-susceptible parents, while GLWP61, GLWP147, and GLWP63 were used as disease-resistant parents. Four cross combinations—PDG3 × GLWP61, PDG3 × GLWP147, C214 × GLWP63, and JG14 × GLWP61—were performed. A total of 101 SSR markers were screened across the parents. Among them, 10 markers in PDG3 × GLWP147, 4 markers in C214 × GLWP63, 10 markers in PDG3 × GLWP61, and 10 markers in JG14 × GLWP61 showed parental polymorphism, leading to the identification of 21 unique polymorphic SSR markers. Notably, marker CaM2049 exhibited polymorphism across all crosses and was further utilized for hybridity assessment in F1 plants.

A total of 37 putative F1 plants from the four crosses were examined, and 33 plants (~89%) were confirmed as true hybrids. These results demonstrate that SSR markers are effective for molecular characterization and hybridity assessment in chickpea breeding programs.

**Keywords:** Chickpea, Ascochyta blight, Botrytis gray mold, Parental polymorphism, Hybridity, SSR markers.

**Introduction**

Among food legume crops, Chickpea (*Cicer arietinum L*.) stands at the third most important crop in the world after the common bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.). Chickpea is a self-pollinating diploid (2n=2x=16) member of the family Leguminosae and subfamily Papilideae. There are 43 species in genus *Cicer* among which 9 species are annuals, 33 are perennials, and one species remains unclassified (van der Maesen, 1987). (Ladizinsky and Adler, 1976) considered *C. reticulatum* as the wild progenitor of the cultivated chickpea and south eastern Turkey as the centre of its origin. Chickpea genome is approximately 738 MB long and contains around 28,269 genes (Varshney et al., 2013). It is cultivated for centuries and is widely grown in more than 50 countries across Asia and Europe. Asia has the highest chickpea cultivable area of 89.7% of the total, followed by 4.3% in Africa, 2.9% in America, 2.6% in Oceania and 0.4% in Europe. India accounts for 64% of the total chickpea production of the world and tops the list in terms of both total production and cultivable area (Samriti et al., 2020).For millions of people in under-developed nations, notably in South Asia, who are primarily vegetarian by choice or economic constrains, chickpea provides a significant protein source (Jukanti et al., 2011). Like many other agricultural crops, diseases pose a problem for chickpea crop. The three major diseases that seriously harm this crop of legumes are Ascochyta blight (AB), Fusarium wilt and Botrytis gray mold (BGM).

Ascochyta blight, caused by the fungal pathogen *Ascochyta rabiei*, is the primary and highly detrimental disease affecting chickpea crops. In Northern India, the Ascochyta blight can cause significant yield losses, reaching up to 100%. These losses are primarily attributed to environmental conditions such as cold temperatures (15-20°C) and high levels of humidity (>150 mm of rainfall). (Gaur et al., 1996) identified AB as the primary biotic constraint in chickpea production, leading to reduction in yield and quality. The fungus *Botrytis cinerea* Pers. ex. Fr. is responsible for another foliar disease known as botrytis gray mold (BGM). This disease is considered to be the second most significant foliar disease, next to AB, as stated by (Pande et al., 2006). Similar to AB, BGM can also result in total yield loss during the years having heavy rainfall and high humidity (Pande et al., 2006).

Generating chickpea cultivars with significant AB and BGM resistance has been a daunting task because of the lack of knowledge in tracking the genes for resistance in cultivated lines (Pande et al., 2006). More efforts are required to recognise new sources of chickpea resistance. Several researchers have studied the inheritance of genes that determine resistance or susceptibility to these two diseases (Bhardwaj et al., 2010). In the current era, molecular markers have provided a valuable means of determining the purity of F1 plants. These molecular markers are highly polymorphic, co-dominant, independent of environmental factors and accurately reflect the genetic makeup of a plant. By analyzing these markers, we can gain insights into the genomic constitution of a plant. Selection of superior plants from self- and cross-pollinated crops can be achieved using established molecular markers.

The yield of the commercial crop can be significantly reduced by about 100 kg/ha even with a slight loss of 1% in the purity of hybrid seed. Therefore, it is crucial that parents and their hybrids maintain genetic purity. In the conventional method, Grow-out Tests (GOT) are used for determining genetic purity which is based on assessment of plant morphological characters. This approach requires specific areas for the samples to grow and a longer time period (usually one complete season). This is the reason that scientists are turning towards the use of molecular marker for true hybridity of F1 plants. The main limitation in chickpea breeding program is availability of low number of polymorphic molecular markers, such as simple sequence repeats (SSRs). Hence, it is difficult to find polymorphic markers that are specific to particular genotypes. There is an urgent need for the identification of large number of polymorphic SSRs markers specific to the selected genotypes in order to provide impetus to the chickpea breeding programs. These can be then employed for the assessment of true hybridity. In the present work, we have analysed SSR markers that can be used to test true hybridity of F1 plants obtained from four crosses: PDG3 × GLWP61, PDG3 × GLWP147, C214 × GLWP63 and JG14 × GLWP61. Genotype C214, PDG3 and JG14 were AB and BGM disease susceptible parents and genotypes GLWP61, GLWP147 and GLWP63 AB and BGM disease resistant parents.

**Materials And Methods**

**Location**

The present investigation was carried out during 2019-2020 at Pulse Experimental Farm, Department of Plant Breeding & Genetics, PAU, Ludhiana, and all molecular experiments were conducted at School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana.

**Chickpea genotypes**

Female parental lines C214, PDG3, and JG14 as disease susceptible to Ascochyta blight (AB) and botrytis gray mold (BGM) and male parental lines GLWP61, GLWP147 and GLWP63 as disease resistant to AB and BGM were used. These genotypes were kindly provided by Pulse section of Department of Plant Breeding & Genetics, PAU, Ludhiana. During present investigation four crosses combinations were performed in chickpea comprising of PDG3 **×** GLWP61, PDG3 **×** GLWP147, C214 **×** GLWP63 and JG14 **×** GLWP61. Four putative F1 plants from Cross PDG3 **×** GLWP 61, seven putative F1 plants from cross PDG3 **×** GLWP147, ten putative F1 plants from cross C214 **×** GLWP63 and sixteen putative F1 plants from cross JG14 **×** GLWP61 were produced.

**Genomic DNA isolation**

The genomic DNA was isolated from the young leaves of parent and F1 plants according to Cetyl Trimethyl Ammonium Bromide (CTAB) method described by (Doyle and Doyle, 1987). Quantity and quality check (in terms of protein, RNA contamination, and DNA integrity) of the isolated genomic DNA was carried out through spectrophotometry and 0.8% agarose gel electrophoresis and the data were analyzed using a NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, USA). Total 101 markers were used for parental polymorphism survey.

**PCR**

The screening for polymorphism between parents was carried out using SSR markers. 101 SSR primer pairs as described by (Hüttel et al., 1999, Winter et al., 1999, Lichtenzveig et al., 2005, Nayak et al., 2010, Gaur et al., 2011, Gujaria et al., 2011 and Thudi et al., 2011) were used in this study. PCR amplification was performed in 12 μl reaction mixture containing 1µl each of forward and reverse primers (10 pmol/µl), 2 µl of genomic DNA (50 ng/µl), 6 µl of 2X PCR master mix (Takara) and 2 µl of nuclease-free water. PCR was carried out with an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at varying temperature for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 7 min. The annealing temperature varied as per primers in a temperature range of 45°C to 55°C. Touch-down PCR was also set up for the SSR markers with initial denaturation at 94°C for 5 min followed by followed by (i) 16 cycles of denaturation at 94°C for 45 sec, annealing at varying annealing temperature depending on melting temperature of the primers for 45 sec by lowering annealing temperature by 0.5°C/cycle and extension at 72 °C for 45 sec and (ii) 19 cycles of denaturation at 94°C for 45 sec, annealing at varying temperature for 45 sec and extension at 72°C for 45 sec. Final extension was carried out at 72°C for 7 min.

**Agarose and Polyacrylamide Gel Electrophoresis**

PCR products were analysed by running 2.5% agarose gel and 6% polyacrylamide gel depending upon the size range. 100 bp DNA ladder was used for estimating the size of the amplicons. Visualisation of the bands was achieved by staining the gel with ethidium bromide.

**Results**

**Parental polymorphism survey**

Identification of true hybrids is indispensable for any successful breeding program. The assessment of genetic variation within and between individuals or populations in a species can be readily accomplished through the utilization of various molecular markers. Microsatellite markers have gained popularity as a molecular marker system in various areas of genome analysis, such as genetic mapping (Paran et al., 1995) due to their advantageous characteristics, including abundance, hyper-variability, and high reproducibility. It is suitable for high throughput analysis for generating a large amount of data and can also detect significant allelic variations, even among closely related varieties. Therefore, SSR markers were used in the current study. Polymorphic markers were confirmed by analysing the PCR amplicons on agarose and polyacrylamide gel. A marker was classified as polymorphic if it produced discriminating banding pattern between parents (Figure 1). A total of 101 SSR markers were evaluated for polymorphism. Among the markers used, 10 markers showed parental polymorphism between cross PDG3 × GLWP147, 4 markers between C214 × GLWP63, 10 markers between PDG3 × GLWP61 and 10 markers between JG14 × GLWP61. The polymorphic marker CaM2049 was common in all the crosses.A total of 21 SSR makers were found to be polymorphic in four crosses. Markers showing polymorphism between parents are shown in Table 1. Therefore, marker CaM2049 was found very useful in hybridity assessments of F1 plants. Forward and reverse sequences along with annealing temperature of polymorphic markers are given in Table 2.

**Identification of true F1 hybrids**

Molecular markers are effective tools for the identification and evaluation of authentic F1 hybrids. Previous studies have reported the significant advantages of molecular markers over morphological or biochemical markers in assessing genetic purity in various crops. PCR markers have been effectively used for parentage verification, hybrid identification, and purity testing (Powell et al., 1996). Therefore, the markers which were found polymorphic were used for the identification of true F1 hybrids (Table 1). For confirming the genetic purity of hybrids, the banding patterns of polymorphic markers were compared with those of parents by running the PCR amplicons on agarose and polyacrylamide gel. The purity of the F1s was confirmed when they showed the presence of male and female-specific bands. Of the total 4 crosses, 37 putative F1 plants were screened for testing true hybridity in which 33 true F1 hybrids were obtained (Figure 2). The results of F1 hybridity test are also summarised in Table 3.

**Discussion**

With the climate change and growing world population, traditional plant breeding programs to generate resilient and high yielding crop varieties are crucial. In this regard, the identification and characterization of hybrid cultivars play a central role in improving varieties, producing seeds, and releasing them. To ensure successful crop production, it is essential to maintain the genetic purity of hybrid seeds. DNA markers have advantages over biochemical and morphological markers. Morphological markers are influenced by environmental factors and require substantial time and labour resources. Biochemical markers, such as protein and isozyme markers, are minimally influenced by environmental factors. However, their ability to distinguish closely related genotypes is limited due to a lack of polymorphism (Luchhese et al., 1999). DNA markers are advantageous over biochemical and morphological markers, making them valuable for hybrid confirmation.

Previous studies recommend the use of DNA-based markers in chickpea breeding programs to ensure the exclusion of selfed plants and to verify hybridity. The study found that chickpea breeders often include a large proportion (>50%) of selfed plants as hybrids in their breeding programs, which interferes with the goal of improving chickpea crops. Several researchers have conducted similar studies on hybridity confirmation. (Smitha et al., 2019) previously utilized SSR markers to confirm hybridity in chickpea. They identified 13 markers that were polymorphic for both the parents in crosses Super Annigeri-1 × BS 100B and Super Annigeri-1 × BS 72C2. However, only one marker ICCM0299 was able to detect the true hybridity. (Thakur et al., 2020) studied 51 SSR markers to confirm the hybridity of 80 putative F1 plants obtained from the cross between two lines of chickpea, ICC-16349 and GPF-2. Only one marker (TA 180) showed polymorphism among parents. The marker was further used to screen putative hybrids and it found 34 true hybrids among 80 putative hybrids, which accounts for 42.5%. However, in our study we found majority of the putative F1 plants to be true hybrids (89%).

In order to develop of good mapping population for the studying of the inheritance pattern or mapping of gene of trait of interest, it is crucial to ascertain hybrid purity. For instance, (Solanki et al., 2010) studied genetic improvement of wilt resistance in lentil. It may be possible if the genetics of this disease is known and a correct segregating population is available. To achieve this, it is essential that crossed seeds should be true hybrids. They also conducted hybridity analysis of lentil F1 plants which were cross of five genotypes. 60 RAPD and 35 SSR markers were used for survey of parental polymorphism, among which 20 RAPD and 10 SSRs were found polymorphism among both parents. These were also used to test of true hybridity of 24 F1 plants of lentil and 5 plants were confirmed true hybrids.

**Conclusions**

Selfing is a common phenomenon in chickpea. Therefore, it is important to identify true hybrids using molecular markers. Additionally, it would save the money, effort and time required to replace erroneously segregating population. The findings of the present study demonstrated that SSR markers are highly polymorphic and more effective for molecular characterization and for confirmation of true hybridity of F1 plants. The present work has identified 21 polymorphic SSR markers among different crosses that will be significantly useful in further study of hybridity assessment. This can also be advantageous for diversity analysis and other molecular analyses. In comparison to conventional approaches, it will also advance the breeding techniques.

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**Tables**

**Table 1.** List of polymorphic SSR markers found between chickpea parents involved in crosses.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sl. No.** | **Cross** | **Polymorphic primers** | **Number of polymorphic markers** |
| 1 | PDG3 **×** GLWP61 | GA108, CGMM076, NCPGR242, NCPGR232, CaM2049, ICCM0289, CGMM007, ICCM0245, CGMM024, ICCM0272b | 10 |
| 2 | PDG3 **×** GLWP147 | TA96, TA196, GA16, CaM2049, GAA40, CaSTMS11, CaM0594, H1I16, CGMM010, NCPGR141 | 10 |
| 3 | C214 **×** GLWP63 | GA8, CaM2049, H1I16, GA16 | 4 |
| 4 | JG14 **×** GLWP61 | GA16, CaM2049, ICCM0272b, CGMM007, GA6, ICCM0245, CGMM024, ICCM0289, CGMM076, GA108 | 10 |

**Table 2:** Description of polymorphic markers and their annealing temperature

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sl. No.** | **Name of the Oligo** | **Primer sequence**  **(5′<------Sequence--------->3′)** | **Annealing temperature (°C)** | **Reference** |
|  |
| 1 | CaM2049F | CCCTTTGGAAAGAGAGGAGG | 45 | Thudi et al., 2011 |
| CaM2049R | AAGCCGATTCTTGGGACTTT |
| 2 | CGMM024F | GAGGTTCCCGATCCTTCTTC | 45 | Gujaria et al., 2011 |
| CGMM024R | AGCCCTCATCAAACCATCAG |
| 3 | H1I16F | GACATGAAATTCGGTGCATT | 55°C and 45°C*\** | Lichtenzveig et al., 2005 |
| H1I16R | AACGCCCTAAACCTCTTGGT |
| 4 | TA96F | TGTTTTGGAGAAGAGTGATTC | 45 | Winter et al., 1999 |
| TA96R | TGTGCATGCAAATTCTTACT |
| 5 | TA196F | TCTTTTTAAATTTCATTATGAAAATACAAATTATA | 45 | Winter et al., 1999 |
| TA196R | CCTCGGGAGAGGTAAATGTAATTTC |
| 6 | NCPGR232F | GGACCGAATGTCCATAAATC | 50 | Gaur et al., 2011 |
| NCPGR232R | TCTTTTAGGACCCAATGGAG |
| 7 | NCPGR242F | TCGTCATATCCACCCGATAA | 50 | Gaur et al., 2011 |
| NCPGR242R | TGGATAATGGTGCGAAAGAA |
| 8 | GA6F | ATTTTTCTCCGGTGTTGCAC | 45 | Winter et al., 1999 |
| GA6R | AAACGACAGAGAGTGGCGAT |
| 9 | GA16F | CACCTCGTACCATGGTTTCTG | 55°C and 45°C*\** | Winter et al., 1999 |
| GA16R | TAAATTTCATCCTCTCCGGC |
| 10 | CaSTMS11F | GTATCTACTTGTAATATTCTCTTCTCT | 55°C and 45°C*\** | Hüttel et al., 1999 |
| CaSTMS11R | ATATCATAAACCCCCCAC |
| 11 | NCPGR141F | ACTCAAAAGACAGCAAAGCA | 55 | Gaur et al., 2011 |
| NCPGR141R | AGCTTAGAGCACTCACATGC |
| 12 | CGMM007F | TCAAAATCCTGGTGGAGGTC | 55 | Gujaria et al., 2011 |
| CGM0007R | TGCCACTGCTGGTAAAGAGA |
| 13 | CGMM010F | TCAGAATCCCCATTAGTGCAG | 55°C and 45°C*\** | Gujaria et al., 2011 |
| CGMM010R | GCTTTGGGATAGGATTTCCAG |
| 14 | CGMM076F | GGAAGCAGCCATCTAAGGAT | 58°C and 55°C*\*\** | Gujaria et al., 2011 |
| CGMM076R | CCAAACCTCAAATAGGGGTC |
| 15 | GA108F | GTTTGTGATGGAGGAAGCGT | 55 | Winter et al., 1999 |
| GA108R | GCCGCATAGCATTGGTAAGT |
| 16 | GA8F | GCTCTAAAGGGAAGGCGATT | 55°C and 45°C*\** | Winter et al., 1999 |
| GA8R | AACCACCAAAGTTCCCCAG |
| 17 | GAA 40F | TTGACGCAGAGAACTCTCAA | 55°C and 45°C*\** | Winter et al., 1999 |
| GAA40R | ATTGGTGTGATGGGTGGATT |
| 18 | CaM0594F | GGCTTCACGGGAAAAATGT | 55°C and 45°C*\** | Thudi et al., 2011 |
| CaM0594R | TGAGGTGACAGGCGTAATGA |
| 19 | ICCM0245F | GCGGCTGGTTTAAGAGTGAG | 55 | Nayak et al., 2010 |
| ICCM0245R | CCAACACGACCCAAATCAAT |
| 20 | ICCM0289F | CAGCCTCCATGGCATAGATAA | 55°C and 47°C*\*\*\** | Nayak et al., 2010 |
| ICCM0289R | TGCTTGAATGAGTGCAACAA |
| 21 | ICCM0272bF | CGCGGTTGAGTTAGAGTGGT | 55°C and 47°C*\*\*\** | Nayak et al., 2010 |
| ICCM0272bR | CAAATCGGGGATTTTGTTTG |  |

*\**Touch-down PCR: 16 cycles at 55°C by lowering annealing temperature by 0.5°C/cycle and 19 cycles at 45°C

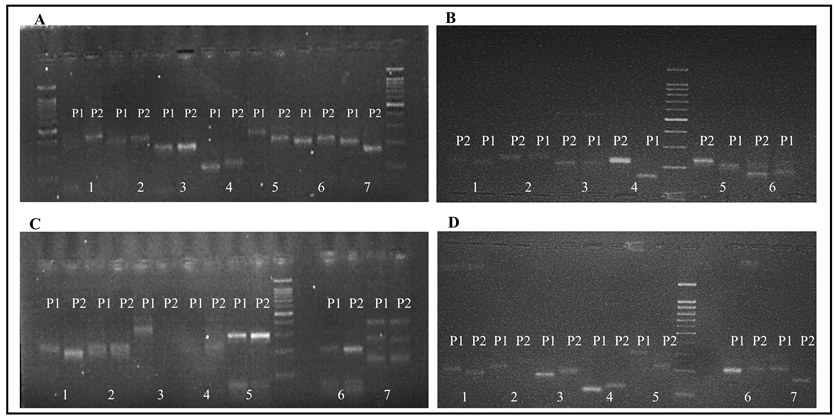
*\*\** Touch-down PCR: 16 cycles at 58°C by lowering annealing temperature by 0.5°C/cycle and 19 cycles at 55°C

*\*\*\** Touch-down PCR: 16 cycles at 55°C by lowering annealing temperature by 0.5°C/cycle and 19 cycles at 47°C

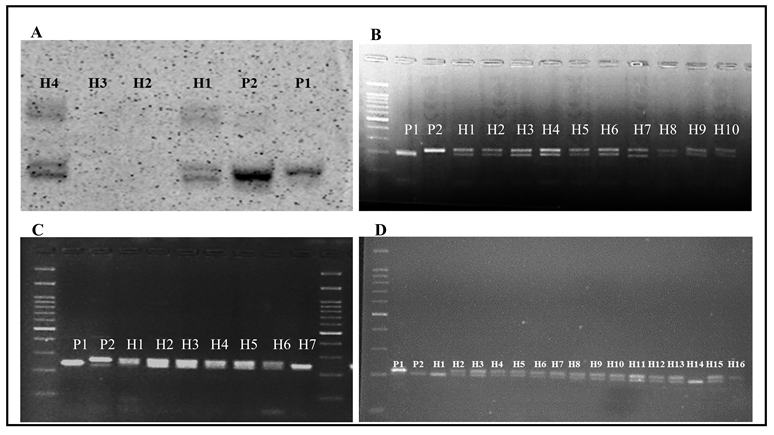
**Table 3.** Summary of F1 hybridity test of the plants obtained in 4 crosses.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sl. No.** | **Cross (P1 × P2)** | **Number of putative F1 plants** | **Number of true hybrids** | **Amplicon size with marker CaM2049** | |
| **P1** | **P2** |
| 1 | PDG3 **×** GLWP61 | 4 | 2 | 250 bp | 230 bp |
| 2 | PDG3 **×** GLWP147 | 7 | 6 | 240 bp | 260 bp |
| 3 | C214 **×** GLWP63 | 10 | 10 | 200bp | 215 bp |
| 4 | JG14 **×** GLWP61 | 16 | 15 | 220bp | 205 bp |
| **Total** | | **37** | **33** |  | |

**Figures**



**Figure 1.** Parental polymorphic analysis of SSR markers was done by running 2.5% agarose gel. P1 is susceptible and P2 is resistant parent. (A) P1: C214, P2: GLWP 63, 1-7: monomorphic and polymorphic SSR markers; 1: GA8, 2: TR10, 3: CGMM010, 4: CGMM069, 5: CAGM001, 6: H1I16 and 7: CGMM024. (B) P1: PDG3; P2: GLWP61; 1-7: monomorphic and polymorphic SSR markers; 1: CGMM076, 2: NCPGR242, 3: GA16, 4: NCPGR232, 5: CaM2049, 6: CGMM024 and 7: ICCM0272b. (C) P1: JG14, P2: GLWP61, 1-7: monomorphic and polymorphic SSR markers; 1: CaM2049, 2: TR1, 3: GA16, 4: GA6, 5: H1I16, 6: CGMM007 and 7: ICCM0272b (D) P1: PDG3, P2: GLWP147, 1-6: monomorphic and polymorphic SSR markers; 1: CaM2049, 2: TA18, 3: ICCM0272b, 4: CGMM010, 5: NCPGR141 and 6: GA16.



**Figure 2.** Hybridity test of F1 plants was done using polymorphic marker CaM2049 and PCR amplicons were resolved on 6% polyacrylamide gel (A) and 2.5% agarose gel (B-D). (A) In the cross PDG3 × GLWP61, 4 hybrids (H3- H6) were checked and 2 were found to be true hybrids. (B) In the cross C214 × GLWP63, 10 hybrids (H1- H10) were checked and all were found to be true hybrids. (C) In the cross PDG3 × GLWP147, 7 hybrids (H1- H7) were checked and all were found to be true hybrids except H7. (D) In the cross JG14 × GLWP61, 16 hybrids (H1- H16) were checked and all were found to be true hybrids except H16. P1 is susceptible and P2 is resistant parent. 100 bp step-up DNA ladder was used for size comparisons.