Research Paper

Assessment of Genetic Purity of Chilli Hybrids Using Molecular Markers

W.A.R. Dhammika[1*], D.M.J.B. Senanayake[2], K.N. Kannangara[1], P.G.S.D. Gunasena[1], P.N. Deniyagedara[1], I.M.S.N. Ilangakoon[1]

[1] *Field Crops Research and Development Institute, Mahailuppallama, Sri Lanka*

[2] *Rice Research and Development Institute, Bathalagda, Ibbagamauwa, Sri Lanka*

**Corresponding author: dhammikamuna@gmail.com*

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Abstract

Genetic purity is an important criterion for quality assurance for hybrid seed production. Grow out test (GOT) based on morphological traits is the practice usually used to confirm hybrid purity. Identification of morphological differences is time consuming, laborious, costly and highly influenced by the environment. Simple sequence repeats are capable of distinguishing parents and F_1 hybrids. In this study the purity of two locally developed chilli hybrids namely; MICH HY1 and MICH HY2 were tested with their parents, inbred line MI *Waraniya* 1, inbred line *Galkiriyagama* selection and inbred line MI 1. Seven SSR markers were used to survey parental polymorphism of the hybrids MICH HY 1 and MICH HY 2 separately. Out of these SSR markers, three markers, designated as CAM5647F/ CAM5647R, JS223/JS224, JS 217/JS 218 were found to be effective in determining the genetic purity percentages of two chilli hybrids; MICH HY 1 and MICH HY2. Chilli hybrid MICH HY1 showed a hybridization percentage of 96.15% and MICH HY 2 94.0%. These findings are more reliable and can be used to substitute for grow out test. These findings can be used for seed certification of chilli hybrids MICH HY 1 and MICH HY 2. Further, these findings will help breeders and growers to identify non hybrid type mixtures in chilli hybrid seed lots of MICH HY1 and MICH HY2.

Keywords: Chilli hybrids, Genetic purity, Polymorphism, SSR markers

Introduction

Chilli (*Capsicum annuum* L.) is a vital commercial crop, cultivated as a vegetable, a spice, and for value-added processed products (Kumar and Rai, 2005). Approximately, more than 89% of chilli growing area in the world is inhabited by Asian continent countries (Pinto *et al*., 2016). Throughout the world, chilli is consumed fresh, dried or as powder form (El-Ghoraba *et al*., 2013). It is rich in proteins, lipids, carbohydrates, fibers, mineral salts (Ca, P, Fe) and in vitamins A, D_3, E, C, K, B_2 and B_{12} (El-Ghoraba *et al*., 2013). Chilli is widely cultivated in Sri Lanka as an important condiment. It is an indispensable spice crop, essentially used in every Sri Lankan cuisine due to its pungency, colour, taste and flavor. In the year 2018, chilli cultivation extent and production were 13,553 ha and 79,003 tons of green chilli, respectively. During the same year 52,849 tons of dry chilli was imported spending 111,798.54 million rupees (Agstat 2019). Open pollinated chilli varieties are widely cultivated in the country. Imported chilli hybrid varieties are also cultivated by farmers. However, most of the exotic hybrids have been rejected by the farmers due to their poor performances, especially low pungency and susceptibility to major pests and diseases (Kannangara *et al*., 2013). In year 2015 and 2017, two chilli hybrid varieties, MICH HY 1 and MICH HY 2 were released by the Field Crops Research and Development Institute of Sri Lanka as alternatives for exotic chilli varieties. Presently, these two chilli hybrids, MICH HY 1 and MICH HY 2 are gaining higher popularity because of their high yield performance than open pollinated varieties. Hence, hybrid chilli seed production is getting popularized among chilli seed production industry in the country. Production and distribution of high-quality seeds are important for high yield, quantity and quality wise and maintaining of genetic purity is an essential pre requisite for quality seed production. Conventionally characterization of hybrids is being done considering specific morphological and floral characters in plant grown to maturity through Grow Out Test (GOT).

However, a grow out test is environmental sensitive and it may alter the expression of specific morphological or physiological traits; thus alternative reliable methods to assess the genetic purity of seed samples are needed to help seed producers to assure high quality standards. Comparison between traditional GOT and DNA-based PCR analysis test to estimate F_1 hybrid purity reveals that GOT requires a complete season, is labour –intensive and sensitive to environmental changers thus, it is not totally a true to type method to analyze genetic purity (Pattanik *et al*., 2018). Due to these reasons GOT limit the availability of hybrid seeds for immediate cultivation. Under this situation molecular markers can be used as an effective tool for efficient selection of desired agronomic trait because they are based on plant genotype and they are independent of environment variation (Josthana *et al*., 2018).

Various molecular marker techniques such as Isozyme and Protein polymorphism (Ballester and Vicente, 1998; Sharmilla *et al*., 2011), Restriction Fragment Length

Polymorphism (RFLP) (Tanksley and Tones, 1981; Arush *et al*., 1985; Livneh, *et al*., 1990) have been applied to assess the genetic purity of hybrids. Among the molecular markers Simple Sequence Repeats (SSR) have high reproducibility and better use in germplasm characterization and genetic diversity analysis (Rajput *et al*., 2006). PCR-based SSR molecular markers are effective in ensuring genetic identity and purity of the hybrids and its parental lines (Padmanabha and Kiruthika, 2018).

The objectives of the present study were, to identify SSR markers for hybridity testing of MICH HY1 and MICH HY 2 chilli hybrids and to assess the genetic purity percentage of recommended chilli hybrids of MICH HY1 and MICH HY 2.

Materials And Methods

The laboratory experiment was conducted at the Biotechnology Division of the Field Crops Research and Development Institute (FCRDI), Maha-Iluppallama, Sri Lanka during 2020 *Yala* and 2020/2021 *Maha*. The seeds MICH HY 1 and MICH HY 2 along with their two parental inbreds (around 0.5g of seeds from each) were used for the study (Table 1). The study consists of two experiments, selection of polymorphic markers for the study and application of selected molecular markers for genetic purity testing.

Sample preparation and DNA extraction

Three weeks old immature, healthy fresh leaf samples were collected from chilli hybrids tested and inbred lines of their parents, inbred line MI *Waraniya* 1, inbred line *Galkiriyagama* selection and inbred line MI 1 for extraction of genomic DNA. The collected leaf samples were put into labeled polyethylene bags and stored in a – 80 °C freezer. Total DNA extraction was carried out using Cetyl Tri-methyl Ammonium Bromide (CTAB) extraction protocol, described by Murray and Thompson (1980) with slight modifications such as dissolving of precipitated DNA pellet with nuclease free water at 50 °C and further purification with 1M NaOH using absolute ethanol.

Testing of quality and quantity of DNA

The DNA yield was measured by using a UV-Visible spectrophotometer (Jenway, Genova Nano, UK) at 260 nm. DNA purity was determined by calculating the absorbance ratio A260/280. Further to assess quality and yield, electrophoresis was done for all the DNA samples in 0.8% agarose gel, stained with ethidium bromide (0.5 mg mL^{-1}) and amplified products were observed in a gel documentation system (Vilber,-Quantum, France). List of SSR primers, sequences and relevant information are mentioned in Table 2.

Table 1. Chilli hybrids (*C. annuum* L.) and their respective parents

Hybrid	Female parent	Male parent
MICH HY1	Inbred line *Galkiriyagama* Selection	Inbred line MI-*Waraniya* 1
MICH HY2	Inbred line MI-1	Inbred line MI-*Waraniya* 1

Polymerase chain reaction (PCR) and Gel visualization

PCR amplification reaction was carried out in a final volume of 15 µl containing 4.20 µl of 5 X reaction buffer, 1.40 µl of 25 mM $MgCl_2$, 0.70 µl of 10 mM dNTPs, 0.7 µl of 10mM primers (each), 0.25 µl of 5U/ µl *Taq* Polymerase and 2 µl of template DNA. Final volume was adjusted to 15 µl with sterilized distilled water. PCR cycle consisted of an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute. Primer annealing for 1 minute for each primer (various temperatures used are mentioned in Table 2) and primer extension at 72 °C for 2 minutes with a final extension of 72 °C for 10 minutes. Amplification was performed in an ABI Veriti 96 well thermal cycler (Life Technologies, New York, USA). The PCR products after the amplification process were visualized using 1.2% agarose gel and Poly Acrylamide Gel Electrophoresis (PAGE).

Selection of SSR markers for genetic purity analysis

For the confirmation of selected SSR markers for genetic purity analysis, parents of the MICH HY1; inbred line MI *Waraniya* 1 and inbred line *Galkiriyagama* selection were subjected to PCR amplification with 7 molecular markers. Parents of MICH HY 2; inbred line MI 1 and *Galkiriyagama* selection were subjected to PCR amplification with 5 molecular markers to select polymorphic SSR markers for genetic purity testing of chilli hybrid MICH HY 1 and MICH HY2.

Table 2. List of SSR primers used for parental polymorphism studies of MICH HY1, MICH HY2 chili hybrids and their parental lines

JS No	Primer code	Primer sequence 5'-3'	Primer annealing temperature-°C	Expected product size bp	References
JS 112	GPMS161	CGAAATCCAATAAACGAGTGAAG	52	184-259	Thilahun *et al.*, (2013)
JS113		CCTGTGTGAACAAGTTTTCAGG			
JS 118	CAMS142	GAGCGCTTAAGTGGTCATAGG	52	251-269	Patel *et al.*, (2011)
JS119		CTACAACGCCCCAAAACAAT			
JS120	CAMS153	TGCACAAATATGAATCCCAAGA	49.2	225-265	Patel *et al.*, (2011)
JS121		AAGTCAGCAAACACATCTGACAA			
JS213	EPMS386	ACGCCAAGAAAATCATCTCC	49.2	122-170	Nagy *et al.*, (2007)
JS214		CCATTGCTGAAGAAAATGGG			
JS217	EPMS409	TCTCTCTCTACATCTCTCCGTTG	52	215-245	Nagy *et al.*, (2007)
JS218		TGTCGTTCGTCGACGTACTC			
JS223	CAK 24	AAACGTCATCACAGCCATCA	52	159	Nagy *et al.*, (2007)
JS224		CGTAACGCACCCTCTAGGAA			
JS286	CAMS5647	CGGATTCGGTTGAGTCGATA	52	190-240	Padmanabha and Kiruthika (2018)
JS287		GTGCTTTGGTTCGGTCTTTC			

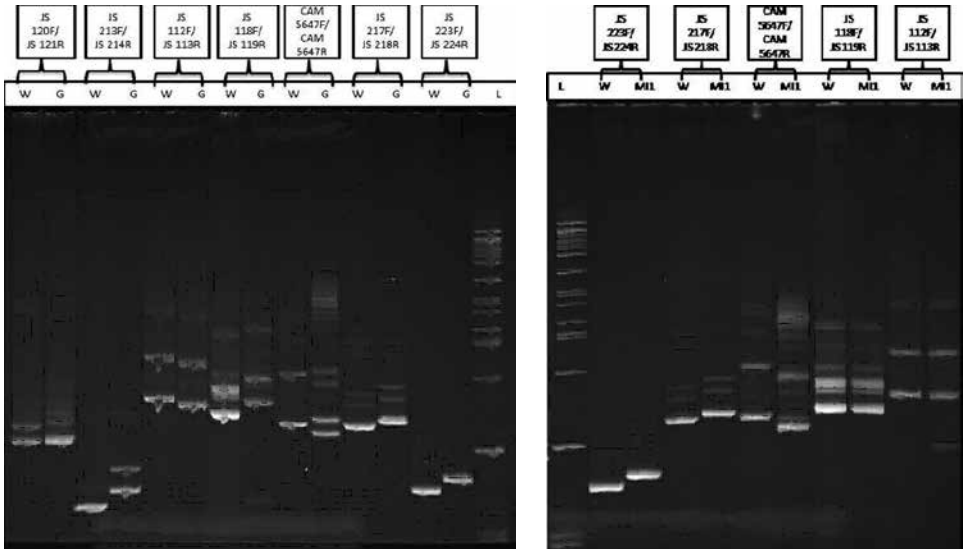
Two molecular markers each selected from polymorphism study namely JS223/JS224 and JS217/JS218, for MICH HY1 and JS217/ JS218 and CAM 5647F/ CAM 5647R for MICH HY2 were selected to assess genetic purity. Amplified products were compared with 100 bp DNA ladder and their accurate sizes were determined using Vilber Bio Vision Software (Vilber Lourmat, France). The banding pattern of gel was manually scored by visual observation to select polymorphic molecular markers. Hybrid purity of MICH HY1 and MICH HY2 was evaluated using the heterozygous banding pattern (co dominance), male specific and female specific alleles.

Results and Discussion

All the tested primers successfully amplified with hybrid parents, inbred line *Galkiriyagama* selection, inbred line MI 1 and inbred line MI *Waraniya* 1. Among the amplified seven molecular markers studied, four molecular markers JS213/JS 214, JS118/JS119, CAM 5647F/ CAM 5647R and JS223/JS224 were polymorphic for parents of MICH HY1 (Figure 1).

Further, out of five molecular markers studied, three molecular markers JS223/ JS224, JS217/JS218 and CAM5647F/ CAM5647R were polymorphic for parents of MICH HY2 (Figure 1).

Out of 51 samples of MICH HY1 screened with the molecular marker JS223/JS224, only two samples exhibited homozygous banding pattern for its parental inbred line *Galkiriyagama* selection and inbred line MI *Waraniya* 1 (Figures 2 & 3).



A **B**

Figure1. Discrimination of parental polymorphism of hybrid parents (A) MICH HY1 (inbred line MI Waraniya 1(W) and inbred line Galkiriyagama selection (G)) using molecular markers JS120/JS121, JS213/JS214, JS112/JS113, JS118/JS119, CAM 5647F/ CAM 5647R, JS217/JS218 & JS223/JS224, 100bp ladder (L), (B) MICH HY2 (inbred line MI Waraniya 1 and inbred line MI 1) using molecular markers JS223/JS224, JS217/JS218, CAM5647F/CAM5647R, JS118/JS119, JS112/JS113), 100bp ladder (L)

Further, out of fifty samples tested of MICH HY2 Chilli hybrid variety, three samples were behaved as it's parental line of inbred line MI 1. (Figures 4 & 5). The molecular results revealed that, genetic purity was 96.15% in MICH HY1 and 94% in MICH HY2. Therefore, SSR marker CAM5647F/ CAM5647R, JS 223/JS224 and JS217/JS 218 can be used to asses genetic purity of commercial grown two chilli hybrids MICH HY1 and MICH HY2 (Table 3, Figures 2, 3, 4 & 5).

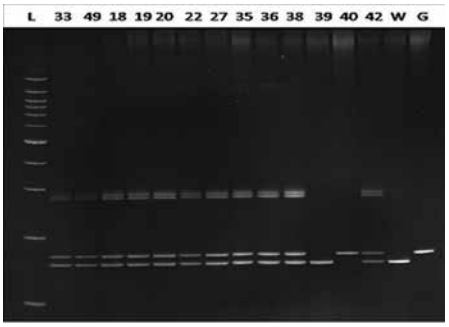


Figure 2. SSR marker JS 223/ JS 224 PCR amplification results of MICH HY 1 individual plants - 33, 49, 18, 19, 20, 22, 27,35, 36 ,38, 39,40,42, W- inbred line MI Waraniya 1, G-inbred line MI Waraniya1 Galkiriyagama selection, L-100bp ladder

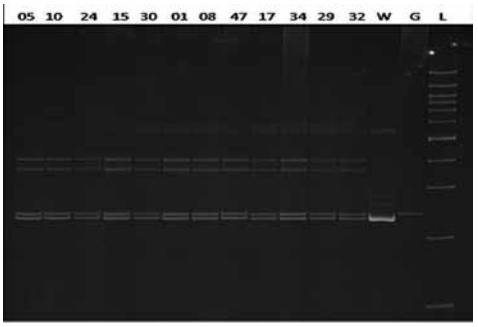


Figure 3. SSR marker JS 217/JS 218 PCR amplification results of MICH HY 1 individual plants- 10, 24, 1, 30, 01, 06, 47, 17, 34, 29, 32, W- inbrd line MI Waraniya 1, G-inbred line Galkiriyagama selection, L- 100bp ladder

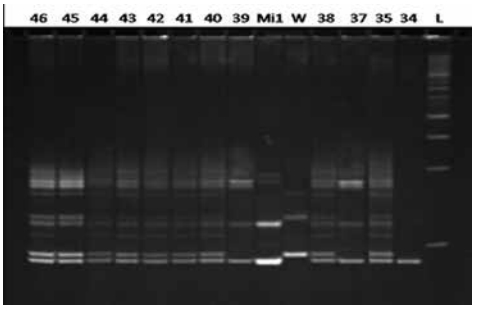


Figure 4. SSR marker CAM 5647F/ CAM 5647R PCR amplification results of MICH HY2 individual plants of 46, 45, 44, 43 ,42 .40, 39, 38, 37, 35, 34, individual plants of MICH HY01 , inbred line MI 1, inbred line MI Waraniya 1, L-100bp ladder

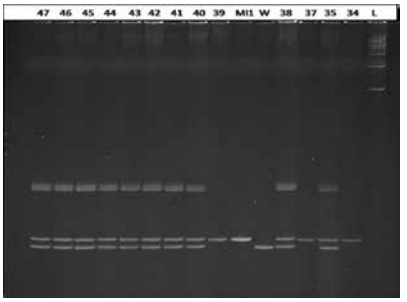


Figure 5. SSR marker JS 223 / JS224 PCR amplification results of MICH HY2 individual plants. 47, 46, 45, 44, 43, 42, 40, 39, 38, 37, 35, 34, inbred line MI 1, Inbred line MI Waraniya 1, L- 100bp ladder

Table 3. Genetic purity of MICH HY1 and MICH HY2 hybrids determined by SSR makers

No	Precise results	MICH HY 1	MICH HY 2
1	No of plants screened	51	50
2	No of heterozygous for both allele	50	47
3	No of homozygous for female parent	01	03
4	No of homozygous for male parent	01	0
5	Non amplified bands	0	0
6	Genetic purity percentage	96.15%	94.00%

Note: Results confirmed with two sets of SSR markers (JS 223/ JS 224 and JS 217/JS 218) for MICH HY1 and two sets of SSR markers (CAM 5647F/ CAM 5647R and JS 223/JS224) for MICH HY2.

Table 4. Genetic purity of MICH HY1 and MICH HY2 hybrids determined by SSR makers

No	Precise results	MICH HY 1	MICH HY 2
1	No of plants screened	51	50
2	No of heterozygous for both allele	50	47
3	No of homozygous for female parent	01	03
4	No of homozygous for male parent	01	0
5	Non amplified bands	0	0
6	Genetic purity percentage	96.15%	94.00%

Note: Results confirmed with two sets of SSR markers (JS 223/ JS 224 and JS 217/JS 218) for MICH HY1 and two sets of SSR markers (CAM 5647F/ CAM 5647R and JS 223/JS224) for MICH HY2.

Conclusions

The main target of the research was, to develop a molecular method to identify genetic purity percentage of locally produced chilli hybrid MICH HY 1 and MICH HY 2. SSR molecular marker JS 223/JS224 and JS 217/JS 218 could be effectively applied to test the genetic identity and purity of Chilli hybrid MICH HY 1 reported as 96.15%. Genetic identity and purity of Chilli hybrid MICH HY 2 could be assessed using SSR molecular marker CAM 5647F/ CAM 5647R and JS 223/JS224 reported as 94%. These findings will be helpful to maintaining genetic purity in a hybrid seed lot by separating non hybrid type seeds mixtures in plant breeding as well as in seed production.

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