# DNA MARKER-ASSISTED AND MORPHOLOGICAL SELECTION ON BC3 GENOTYPES SHORTCUT THE INTROGRESSION OF CMV TOLERANCE GENES ON CHILI PEPPER

Catur Herison[*)], Sri Winarsih, Merakati Handayaningsih and Rustikawati

Faculty of Agriculture University of Bengkulu
Jl. WR Supratman Kandang Limun Bengkulu
[*)] Corresponding author Phone: +62- 736 21170 E-mail: herisoncatur@yahoo.com

## ABSTRACT

Superior hybrid and CMV tolerant cultivar development requires a pair of highly hetero-beltiosis parents and both of which are CMV tolerant. Gene introgression has to be accomplished if the tolerance does not exist in the parents. The objective of this research was to employ DNA markers and morphological traits to identify CMV tolerant individuals in BC3 that were the most similar to their recurrent parents in order to shortcut the backcrossing cycle in chili pepper (*Capsicum annuum* L.). This research used fifteen BC3 populations generated from crosses between PBC1354 and PBC378 hybrid parents and tolerant parents of C1024, C1042 and C1043. The BC3 populations were previously selected for their CMV tolerance and were characterized by RAPD technique as well as morphological traits. Selection by means of both RAPD markers and morphological traits identified BC3 individuals with 99.9% similarity to their respective recurrent parent. B3A24-20, B3A29-13, B3A29-22, B3B12-13, B3B37-9, B3B12-25, B3C16-5, B3C16-16, B3C34-18 genotypes were 99.9% similar to PBC378; meanwhile, B3D11-8, B3D11-17, B3D38-5, B3E12-17, B3E20-22, B3E31-19 were 99.9% similar to PBC1354. Those genotypes were both similar to their recurrent parents and tolerant to CMV. The employed strategy shortened CMV tolerance gene introgression through backcross breeding.

Keywords:   chili pepper, introgression of CMV tolerance, RAPD, backcross

## INTRODUCTION

Chili pepper (*Capsicum annuum* L.) is one of the most important vegetable crops in Indonesia. However, its national production has yet to fulfill the increasing demands due to the increase of population. For instance, in 2011, the government of Indonesia had to import chili product up to 25.593.900 ton (BPS, 2011). Many factors contribute to the low production level, and two most important of them are the genetic make-up of cultivars commonly grown by farmers and virus disease in the field.

Cucumber mosaic virus (CMV) is the most damaging among 45 known viruses that are present in chili pepper field in Indonesia (Duriat, 1996). Although quantitative yield loss was not well documented, the virus attacks potentially cause a significant total yield loss. In a controlled environment, CMV infection during seedling stage reduced fruit number and weight of 81,4% and 82,3%, respectively (Sari *et al.,* 1997). In Korea, a field survey on paprika producing area found that CMV was the most predominantly existing mosaic virus, followed by PepMoV, PMMoV, and TSWV (Mun *et al.,* 2008; Ryu *et al.,* 2009).

An alternative solution to cope with the problems is the use of high yielding and CMV tolerant hybrid cultivars. The development of CMV tolerant hybrid cultivars requires high heterobeltiosis couple of parents, both of which must be CMV tolerant. Thus, introgression of tolerance controlling genes is a compulsory step when the tolerance character does not exist in the parents.

In a breeding program, genes introgression is conventionally conducted by backcross approach employing merely morphological traits. In such a way, it takes several plant generations to complete the selection process. Moreover, when the desired character is controlled by recessive gene(s), such as the one(s) that control CMV tolerance (Herison *et al.*, 2004), many more plant generations are needed to complete a breeding program. Therefore, many efforts have been explored to find such the way to shortcut the

breeding program to become less time consuming and more economical.

Recently, DNA markers have been extensively used to assist many breeding programs, including breeding for biotic stress tolerance (Klein-Lankhorst *et al.,* 1991; Wechter *et al.,* 1995; Saidi and Warade, 2008). Marker assisted selection (MAS) has also been employed in an introgression of a high protein content trait into a high yielding wheat variety by a backcross method (Davies *et al.,* 2006).

The objective of this research was to employ both molecular markers and morphological traits to shorten the introgression of CMV tolerance controlling gene(s) from donor parents into hybrid parents. The approach was to identify BC3 individuals with highest CMV tolerance similarity to their respective recurrent parent based on both molecular markers and morphological traits.

## MATERIALS AND METHODS

This research was conducted in the Greenhouse and Biotechnology Laboratory of Agronomy Department, the Faculty of Agriculture, Bengkulu University, and the molecular analysis was conducted in Molecular and Electrophoresis Laboratory of Agrohort Department, the Faculty of Agriculture, Bogor Agricultural University. It was carried out during the period of April to October 2010.

### A. DNA Profiling in Marker Assisted Back-crossing (MAB)

Markers that were used to assist selection in this study were the profiles of recurrent parents based on DNA markers. Plant genetic materials in this study were PBC378 and PBC1354 hybrid parents, and BC3 populations. Chemicals used for DNA extraction and analysis were REDExtract-N-Amp Plant PCR Kits XNAP (Sigma-Aldrich, USA), random decamer primers of OPE7, OPE15, OPE20, OPH5, OPH13 from OPERON Technology (Alameda, USA) and other chemicals for electrophoreses. Devices used in this analysis were Eppendorf micropipetters (0.5-2,5 $\mu$l, 10-100 $\mu$l, 100-1000 $\mu$l), Eppendorff micropipette tips, 2ml size plastic tubes, water bath, Sorvall RC-55 high sonic centrifuge (Dupont, USA), mini vacuum pump, DNA Gene Amp PCR system PE2400 Thermal Cycler (Perkin-Elmer, USA), electrophoresis apparatus, UV transluminator T 220, and digital camera.

### DNA Isolation

Plant DNA isolation was accomplished by a modified method of KIT XNAP (SIGMA, USA). Several leaf cuts of 0.5 cm x 0.5 cm put into a 2 ml plastic tube previously loaded with 100 $\mu$l extraction solution and incubated in 95°C water bath for 10 minutes. Subsequently, a 100 $\mu$l dilution solution was added into the tubes and the tube was shaken gently to optimize the extraction process. The liquid was moved into a new tube, added with 200 $\mu$l aqua bidestilata and 100 $\mu$l CIA (chloroform : isoamyl alcohol, 24:1), and then centrifuged at 10000 rpm and 4°C, for 10 minutes to separate the extracted DNA genome from other leaf tissue residues. The aliquot was transferred into a new tube and the DNA genome was precipitated with 1 ml 95% ethanol and incubated in a chamber set at 4°C, for 30 minutes. The mix with precipitated DNA was centrifuged at 10000 rpm, 4°C, for 10 minutes to separate the DNA genome from the extraction solution. The solution was discarded and the DNA pellet stuck on the bottom of the tube was vacuum dried. Lastly, the dry DNA pellet was dissolved with 100 $\mu$l sterilized ion free water and ready to be used for further analysis.

### RAPD Analysis

DNA amplification was performed with random primers that were able to amplify hot chili pepper DNA genome, following the RAPD tehnique of William *et al.* (1990) with some modification. An approximate amount of 10-25 ng DNA, XNAP amplification kit solution and respective random primer were loaded into a PCR tube and run in the PCR thermo cycler machine. The PCR cycles consisted of one pre cycle of 94°C for 5 minutes, 45 cycles of 5 sec 94°C denaturation, 30 sec MT- 4°C annealling, and 1 min 72°C elongation, and one stop PCR cycle of 72°C for 10 minutes. Electrophoresis was run on agars gel (0.8 w/v) in TAE buffer and the gel was stained in etidium bromide solution (0.5 mg/l) for 20 seconds. DNA bands were visualized by UV transluminator and documented by Canon D1000 digital camera.

In this research, as many DNA markers as possible were identified from each of PBC378 and PBC1354 recurrent parents. DNA profiling of

these recurrent parents were used in the marker assisting selection.

## B. Markers Assisted and Morphological Selection

Genetic materials selected were individuals of 15 BC3 populations generated from crosses between hybrid parents (PBC1354, PBC378) and CMV tolerance donor parents of C1024, C1042 and C1043. They were BC3A (PBC378/[PBC378/ [PBC378/(PBC378/C1024)]]-11)-24, 25, 29; BC3B (PBC378/[PBC378/ [PBC378/(PBC378 /C1042)]] -6)-12, 37, 49; BC3C (PBC378/ [PBC378/[PBC378/(PBC378/C1043)]]-13)-16, 33, 34; BC3D (PBC1354/[PBC1354/ [PBC1354/ (PBC1354/C1-043)]]-18)-11, 13, 38 and BC3E (PBC1354/ [PBC1354/[PBC1354/ (PBC1354/ C1024)]]-4)-12, 20, 31. Five individuals from each BC3 popula-tion previously selected for CMV tolerance were used in this study. Those were the most tolerant to CMV infection among 498 BC3 individuals.

Chemicals and apparatus for DNA isolation, PCR runs, and electrophoreses were similar to the former study (point a.). DNA of each plant materials were extracted, amplified with random primers of OPE7, OPE15, OPE20, OPH5, OPH13 from OPERON Technology (USA) and run in electrophoresis. DNA profiles combined with morphological traits were used for similarity analysis to their recurrent parents by Cluster Analysis using Minitab 14. Three most similar individual to their parents among 15 member of each BC3 population were selected summing to a total of 15 individuals, that were used in further step. Those selected individuals were considered to be the improved hybrid parents with an additional character i.e. CMV tolerance.

## RESULTS AND DISCUSSION

All DNA samples of five most CMV tolerant individuals from each of 15 BC3 populations and their PBC378 and PBC1354 recurrent parents were isolated and amplified by random primers of OPE7, OPE15, OPE20, OPH5, OPH13 in RAPD technique. DNA extraction using REDExtract DNA Amp Kit (SIGMA, USA) with some modification made in the Biotechnology Laboratory of Agronomy Department, the Faculty of Agriculture, Bengkulu University, produced good quality of DNA genome for all individuals involved in the study, as indicated by good results in the subsequent amplification steps.

Amplification of 77 genomic DNAs by the selected random primers with RAPD technique produced 5-20 markers in each individuals variably in each primer and the markers sizes were in a range of 250 bp to1650 bp. Examples of the result of RAPD analysis were showed in Figure 1. The highest number of markers was produced by OPE-20, i.e. 20 markers, followed by OPH-5, OPE-7, OPH-13, and OPE-15, with 12, 10, 6 and 5 markers, respectively. Those markers segregated among individuals within BC3 populations. The marker sizes produced by each random primers were presented in Table 1.

Total number of markers yielded in the amplifications by the five random primers in all BC3 populations were 54 markers, and all of them formed the DNA profile of each BC3 individual. Several markers, i.e. those 350bp and 450bp bands produced by OPH13; 250bp and 350bp bands byf OPE20; 350bp band by OPE15, and 250bp band by OPE7, seemed to be the common bands for chili pepper, since they were exist in every individuals.

There was variation in band patterns among individuals within the BC3 populations. The segregation indicated that there was gene segregation although the plants had been formerly selected for morphological traits and CMV tolerance. Those segregating markers, therefore, were expected to be useful to select individuals most similar to their recurrent parent.

The higher the number of traits involved to identify the most similar BC3 individuals to their recurrent parent, the better the result would be. Therefore, in this research, besides molecular traits, morphological traits such as plant height, dichotomous height, total number of branches, total fruit number, fruit length and fruit weight, were also employed. An example of variation in fruit sizes in different BC3 populations is presented in Figure 2.
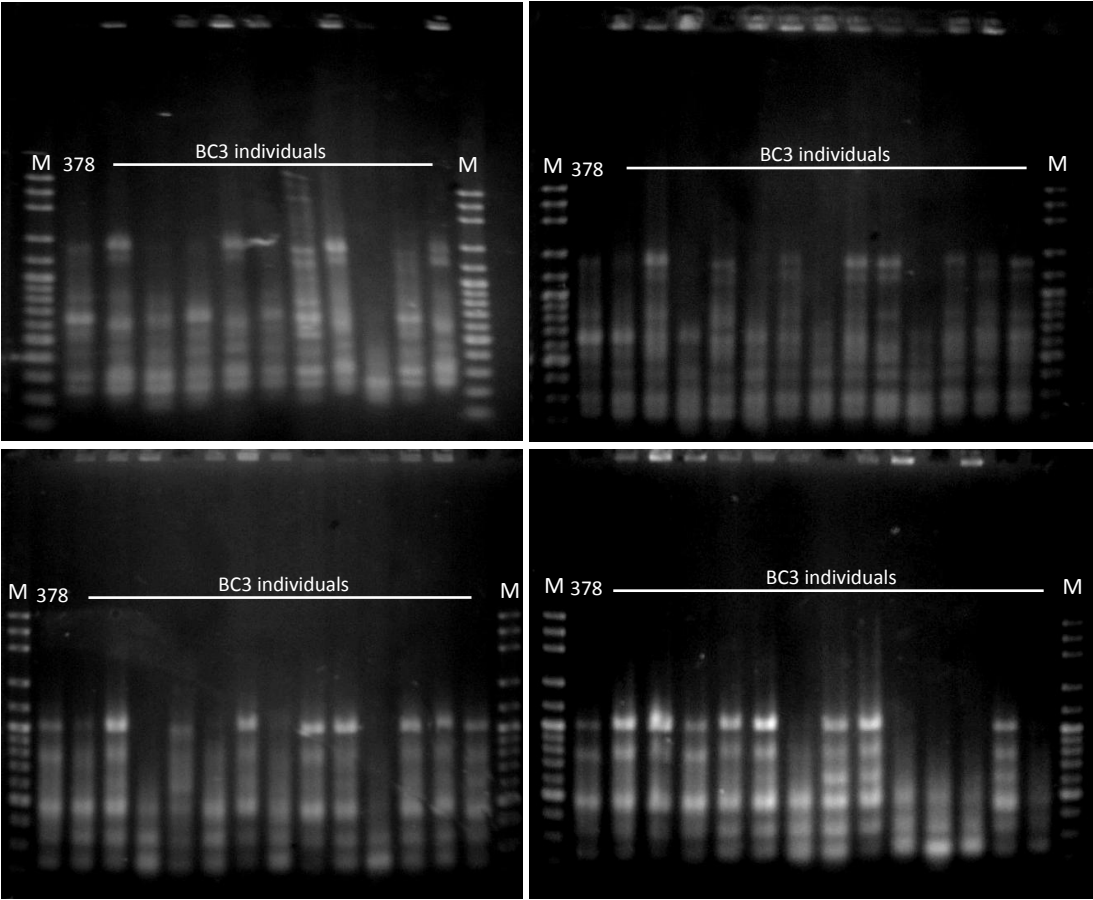
Figure 1. Examples of RAPD analyses with random primer OPE-21 (above) and OPE-7 (bottom) on BC3 population of chili pepper



Figure 2. Fruit size and shape variation among BC3 populations of chili pepper

Table 1. List of marker sizes (in bp) produced by each random primers employed in characterization on BC3 population of chili pepper

| No | Random Primer | | | | |
|----|----------|----------|----------|----------|-----------|
| | OPERON E-7 | OPERON E-15 | OPERON E-20 | OPERON H-5 | OPERON H-13 |
| 1 | OPE7-1050 | OPE15-800 | OPE20-1600 | OPH5-1000 | OPH13-750 |
| 2 | OPE7-1000 | OPE15-650 | OPE20-1400 | OPH5-800 | OPH13-600 |
| 3 | OPE7-900 | OPE15-550 | OPE20-1300 | OPH5-750 | OPH13-450 |
| 4 | OPE7-800 | OPE15-350 | OPE20-1200 | OPH5-700 | OPH13-350 |
| 5 | OPE7-700 | OPE15-200 | OPE20-1150 | OPH5-600 | OPH13-250 |
| 6 | OPE7-650 | | OPE20-1100 | OPH5-550 | OPH13-150 |
| 7 | OPE7-600 | | OPE20-1050 | OPH5-500 | |
| 8 | OPE7-500 | | OPE20-1000 | OPH5-450 | |
| 9 | OPE7-350 | | OPE20-900 | OPH5-400 | |
| 10 | OPE7-250 | | OPE20-800 | OPH5-350 | |
| 11 | | | OPE20-700 | OPH5-300 | |
| 12 | | | OPE20-650 | OPH5-250 | |
| 13 | | | OPE20-600 | | |
| 14 | | | OPE20-550 | | |
| 15 | | | OPE20-500 | | |
| 16 | | | OPE20-450 | | |
| 17 | | | OPE20-400 | | |
| 18 | | | OPE20-350 | | |
| 19 | | | OPE20-300 | | |
| 20 | | | OPE20-250 | | |

Cluster analysis based on both molecular and morphological traits showed that individuals on BC3 populations generated from crosses between PBC378 recurrent parent and the CMV tolerance donor parent (C1024), i.e. BC3A-24, BC3A-25, BC3A-29, formed two clusters with 99.3% similarity level among them, meaning that all 15 individuals analyzed were basically similar to each other. The first group consisted of 3 individuals and the second group 13 individuals including the PBC378 recurrent parent. In the second group, there were 4 individuals with >99.9% genetic similarity to their recurrent parent. These were BC3A-29-13, BC3A-29-22, BC3A-24-6 and BC3A-24-20, in order from the highest to the lowest (Figure 3).

In BC3 population generated from a cross between PBC378 hybrid parent and the CMV tolerance donor (C1042), i.e. BC3B-12, BC3B-37and BC3B-49, the cluster analysis grouped all individuals into two groups with a genetic difference of 1.82%. The first group consisted of 5 individuals with a genetic similarity of 99.9%. The second group was composed of 11 genotypes, including PBC378 recurrent parent, with a genetic similarity of 99.6%. There were 3 genotypes that were most similar to PBC378; they were BC3B-12-13, BC3B-12-25 and BC3B-37-9, with a genetic similarity of 99.9% (Figure 4).
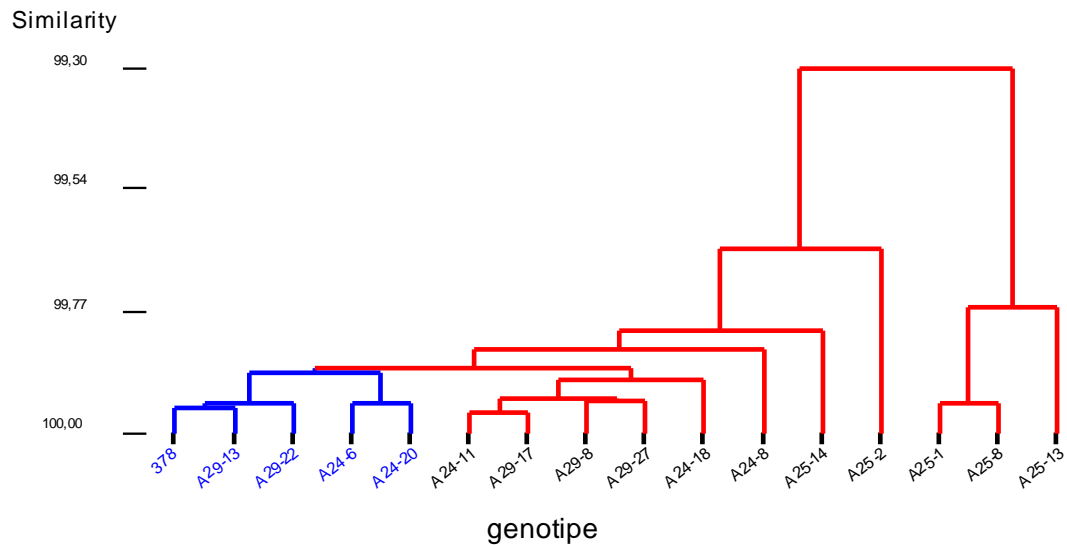
Figure 3.  Dendrogram of genetic similarity of BC3A-24, BC3A-25, BC3A-29 to their recurrent parent PBC378, based on molecular and morphological traits in chili pepper
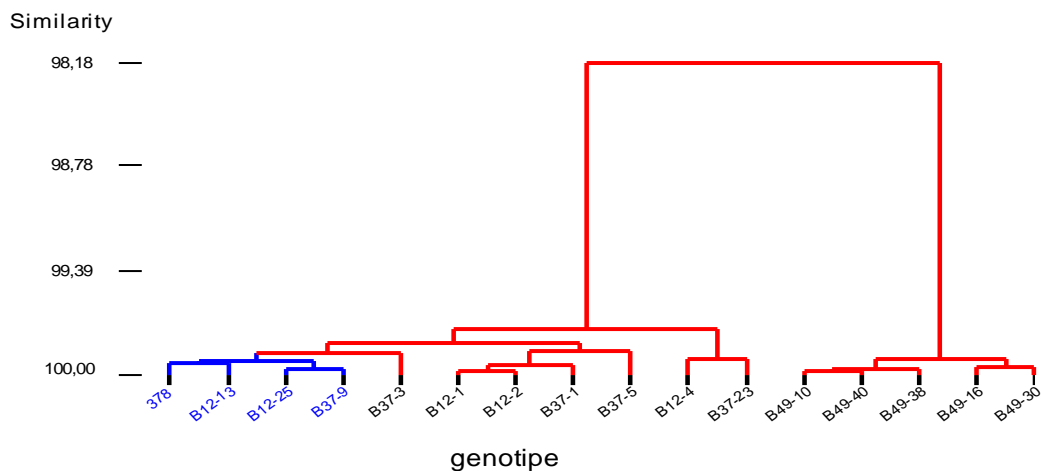


Figure 4.  Dendrogram of genetic similarity of BC3B-12, BC3B-37, BC3B-49 to their recurrent parent PBC378, based on molecular and morphological traits in chili pepper

Cluster analysis on the BC3 population of a PBC378xC1043 cross resulted in two groups with a dissimilarity level of 1.18%. The first group consisted of only one member, namely C33-13 genotype, and the second group comprised of 15 genotypes, including PBC378 recurrent parent.  There were four genotypes that were most similar to the recurrent parent with a similarity level of 99,80%, namely BC3C-16-5, BC3C-16-16, BC3C-34-18 and BC3C-34-28 (Figure 5).
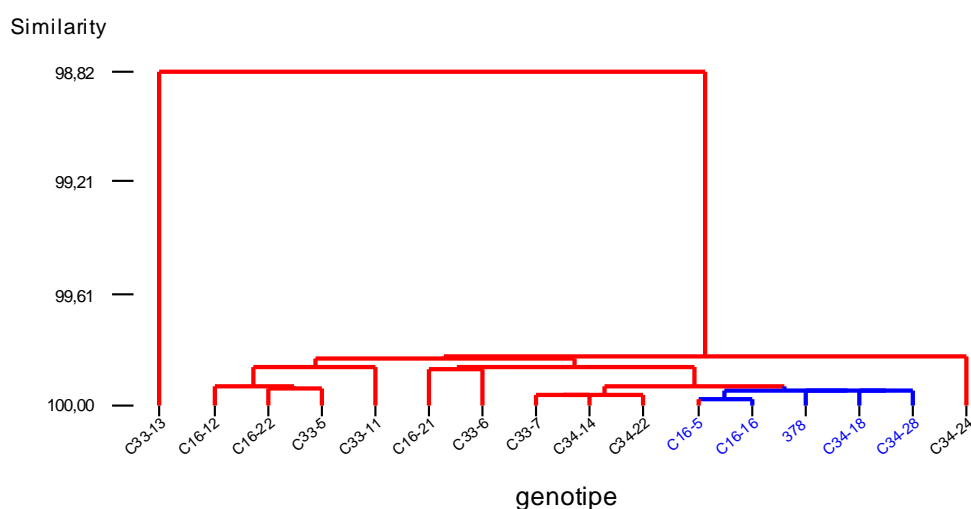
Figure 5. Dendrogram of genetic similarity of BC3C-16, BC3C-33, and BC3C-34 to their recurrent parent PBC378, based on molecular and morphological traits in chili pepper

A cluster analysis on BC3 population generated from a cross between PBC1354 hybrid parent and C1043 donor parent resulted in three groups, with similarity levels within each group higher than 99.45%. The first group consisted of 5 genotypes, the second group consisted of 1 genotype, and the third group was composed of 10 genotypes including the PBC1534 recurrent parent. There were two genotypes, i.e. BC3D-11-17 dan BC3D-38-5, that were most similar to PBC1354 with a similarity level of 99.8% (Figure 6).

In a cross between PBC1354 recurrent parent and C1043 donor parent, cluster analysis yielded three genotype groups differing approximately 1.21% of total characteristics. The first group was composed of 1 genotype, the second group consisted of 2 genotypes, and the third group comprised of 13 genotypes, including PBC1354 recurrent parent. Within the third group, there were 12 individuals that were most similar to the recurrent parent with a similarity level of 99.80% (Figure 7). Those 12 genotypes were basically suitable for the next selection steps. However, to represent all previous generation evenly, three genotypes were randomly selected; they were BC3E-12-17, BC3E-20-22, and BC3E-31-19.

The previous research showed that some BC2 individuals resembled their recurrent parent with the similarity level in a range of 77 to 87% based on 38 molecular markers and 5 morphological traits (Herison *et al.*, 2010). In BC3 generation, based on 53 molecular markers and 5 morphological traits, the similarity level of selected individuals was higher than 99.9%. The results indicated that the procedure was effective in backcross selection. The main objective of a backcross selection is to find individual offsprings that are most similar to the recurrent parent, but with additional trait(s) concerned. In this study, those individuals were identified in BC3, not only highly similar to their recurrent parent but also resistant to CMV. Those individuals, in further steps, were self pollinated to produce a segregation population for CMV resistance and subsequently selected for the most resistant ones, meaning that they were homozygous for CMV resistance gene(s).

Molecular markers were proven useful to shorten selection cycles in plant breeding programs. One of the molecular techniques frequently used by plant breeders was random amplified polymorphic DNA (RAPD) (Waldron *et al.*, 2002). Molecular markers can detect plant variability at DNA genome level. For instance, RAPD markers produced through DNA amplification by randomly sequenced olygonucleotides (primers) of 10 bps in size were able to form traits useful in early generation selection. Moreover, molecular markers have been demonstrated to shorten a breeding program to transfer stripe disease resistance gene in rice (Yao *et al.*, 2011).
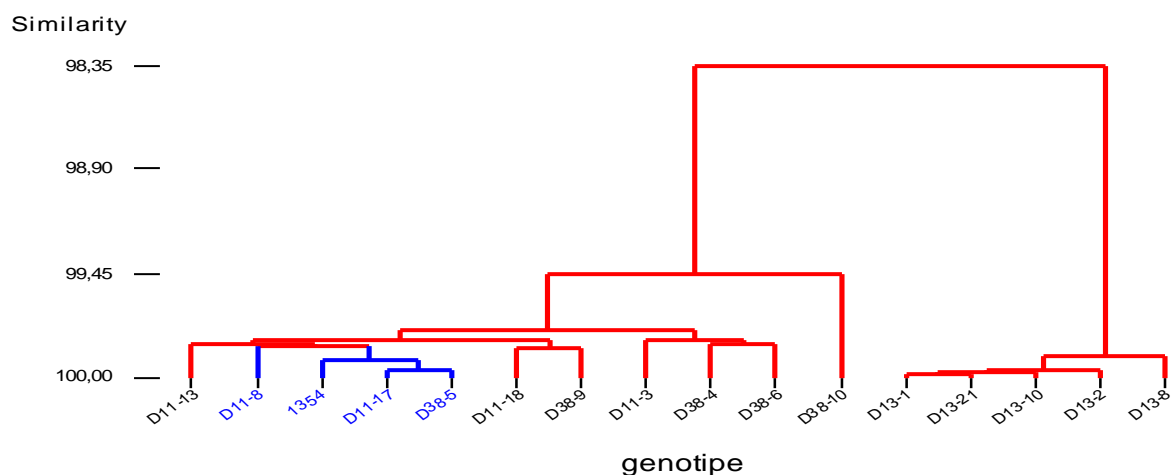
Figure 6. Dendrogram of genetic similarity of BC3D-11, BC3D-13, BC3D-38 to their recurrent parent PBC1354, based on molecular and morphological traits in chili pepper
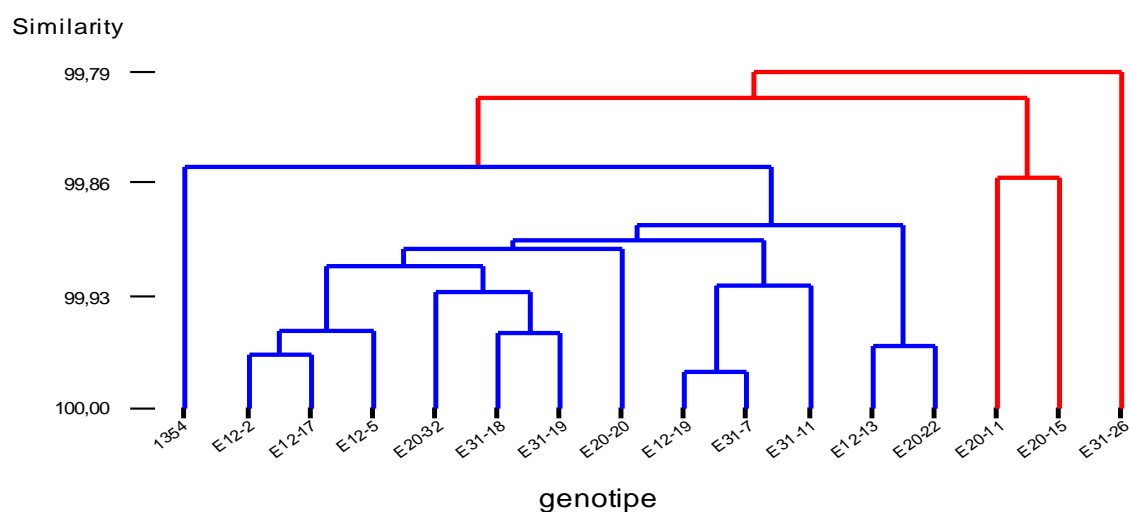
Figure 7. Dendrogram of genetic similarity of BC3E-12, BC3E-20, and BC3E-31to their recurrent parent PBC1354, based on molecular and morphological traits in chili pepper

Tolerance to CMV in hot chili pepper was controlled by recessive genes (Herison *et al.,* 2004). The difficulty in introgression of such genes by backcrossing was due to the need of a progeny test in every selection step to find individuals that were most similar to their recurrent parent but with additional considered traits (Fehr, 1987). This made the selection process expensive and time consuming. In this study, RAPD markers were useful to shorten the backcross selection cycles to transfer CMV tolerance gene(s) from a donor parent into the recurrent parents without progeny tests. This was due to the ability of molecular markers to select unnoticeable traits (Saidi and Warade, 2008). The recurrent parent characteristics were well identified in BC3 population with the help of RAPD markers and morphological traits.

Molecular markers enabled to show similarities and dissimilarities among genotypes based on many more loci than morphological traits alone so that the selection became more accurate. Molecular markers were very useful tools to reveal the recurrent parent characteristics in early selection generation (Ribaut and Hoisington, 1998).

Other researches also showed that molecular markers could be used to identify recurrent parent characteristics in early BC generations. Molecular markers were used successfully in wheat as the main selection tools in introgression of genes that conferred a high protein content trait into a superior wheat cultivar by backcross method (Davies *et al.*, 2006). In rice, molecular markers were used to assist introgression of diseases resistance traits from undesirable wild species by backcross selection. Molecular markers were also useful for introgression of gene(s) from indica into japonica variety of rice, so that new rice cultivars with combination of desirable traits from both rice varieties were developed (Rahman *et al.*, 2008). Using molecular markers, an increased selection gain and a decreased selection cost were achieved in a snap bean breeding program to develop a cultivar resistant to white fungus diseases (Ender *et al.*, 2008). In bell pepper, molecular markers were successfully employed in introgression of genes for resistance to *Phytophtora* (Thabuis *et al.*, 2004). Marker-assisted backcrossing (MABC) possesses a very good prospect to develop new plant cultivars (Ribaut *et al.,* 2010).

## CONCLUSIONS AND SUGGESTIONS

Employing both molecular markers and morphological traits shortcut introgression of CMV tolerance controlling gene(s) through backcross breeding program. Selected individuals of BC3 populations were not only tolerant to CMV but also highly similar to their recurrent parents.Genotype B3A24-20, B3A29-13, B3A29-22, B3B12-13, B3B37-9, B3B12-25, B3C16-5, B3C16-16, B3C34-18 were highly similar to recurrent parent PBC378, and  B3D11-8, B3D11-17, B3D38-5, B3E12-17, B3E20-22, B3E31-19  were highly resemble recurrent parent PBC1354.

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