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Confirmation of mutation and genetic stability of the M4 generation of chili pepper's (*Capsicum frutescens* L.) Ethyl Methane Sulfonate (EMS) mutant based on morphological, physiological and molecular characters

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Abstract. *Arumingtyas EL, Atiaturrochmah, Kusnadi J. 2023. Confirmation of mutation and genetic stability of the M4 generation of chili pepper's (*Capsicum frutescens *L.) Ethyl Methane Sulfonate (EMS) mutant based on morphological, physiological and molecular characters. Biodiversitas 24: 531-538.* The initial generation of Ethyl Methane Sulfonate (EMS) random mutations usually still shows high variation due to allele segregation. This research aimed to confirm the genetic differences between the M4 generation of chili pepper (*Capsicum frutescens* L.) mutant resulted from EMS mutations (G7/01) and the initial line (G7), and the stability of the mutant based on morphological, physiological, and molecular characters. The morphological characters and the capsaicinoid content of the mutants G7/01 with the initial line (G7) were compared. The capsaicinoid content and fruit spiciness were measured by spectrophotometer λ 280 nm. Molecular characterization was conducted using Random Amplified Polymorphic DNA (RAPD) genetic markers. Based on the morphological characters, the G7/01 mutants have some superior properties compared to the initial line G7. All the G7/01 mutant plants contain higher capsaicinoid compounds than the initial line plants. The dendrogram developed based on RAPD profile showed that all the mutant plants were positioned apart from the initial line plant, suggesting that there are some genomic changes in the mutants compared to the initial line plants. All the mutant plants, except T1, showed insignificant variation in morphological characteristic, capsaicin content, and RAPD profile, indicating genetic stability.

Keywords: *Capsicum frutescens*, Ethyl Methane Sulfonate, mutant, RAPD, stability

INTRODUCTION

The genus *Capsicum* covers over 30 species (Tripodi and Kumar 2019). Among them, *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. pubescens* Ruiz & Pav., and *C. baccatum* L. have been highly cultivated in the world because of their high economic value (Arumingtyas et al. 2017; Olatunji and Afolayan 2019). *Capsicum frutescens*, known as chili pepper, is a horticultural plant with a pungent taste caused by its capsaicin content. This species is cultivated in the tropics and sub-tropics area (Carvalho et al. 2014). In Indonesia, small-size fruit variants of *C. frutescens* are recognized by their local name *cabai rawit*, and are the most widely cultivated and used. Among many types of chili pepper found in Indonesia, six genotypes found in the markets at Malang, East Java, namely G1, G2, G3, G4, G5, and G6 (Dwinianti et al. 2018) and one genotype, G7, originated from markets in Lombok Island, Indonesia. Due to the high consumer demand for chili, various new types of chili pepper have been developed and cultivated in various regions in Indonesia.

Plant breeders employed many ways. including introduction, hybridization, induced mutation, and genetic engineering to develop new superior varieties (Oladosu et al. 2016). Mutation is changing in genetic constituents that are inherited in an organism. Mutations have been reported to have a major role in increasing crop productivity and quality. More than 3,000 varieties or lines of 175 plant species have been successfully developed using mutation techniques (FAO/IAEA 2018; Mir et al. 2020). These mutants may be functional and can be used directly to meet human needs or can be a source of variation for breeding purposes. Among various types of chemical mutagens Ethyl Methane Sulfonate (EMS) is a chemical mutagen the most widely used since it is not mutagenic after being hydrolyzed.

Ethyl Methane Sulfonate (EMS) is an alkylating agent which causes large changes in chromosomes, DNA, and RNA by producing a transition of GC to AT and AT to GC mutations, insertion and deletion (Wei et al. 2021). Mutations and segregation will produce genetic variation and could be expressed in subsequent offspring (Arruvitasari 2016; Oladosu et al. 2016; Wei et al. 2021). The initial generation produced by EMS mutations (M1) generally still shows high variation due to allele segregation (Arruvitasari 2016; Oladosu et al. 2016). Subsequent selection after the process of mutation induction will produce stable mutants. In semi-dwarf rice, it was reported to achieve stability at F3 after the CRISPR/Cas9-target mutation (Apriana et al. 2021).

Research on EMS induced mutation in chili pepper showed that 0.01 % EMS gave the best response to an increase in the number of leaves and branches, as well as the capsaicin content compared to the control (Juliandari et al. 2017). Induced mutation with 0.01% EMS has produced several chili pepper mutants, one of which is genotype G7/01 mutant. Until the 3rd generation of the mutant's offspring variations was still observed, namely the total content of capsaicin which tends to be lower than the initial line, but not significantly different between each mutant (Arumingtyas et al. 2017). The G7/01 mutant plants tend to be more adaptive, resistant to environmental stress conditions and pests, as well as diseases (Arumingtyas et al. 2017). Considering these advantages and the indication of the stability of mutants-which is shown by the low variation - it is necessary to conduct further study on the M4 generation to ensure its genetic stability. This research aimed to confirm the genetic differences between the M4 generation of chili pepper mutant resulted from EMS mutations (G7/01) and the initial line (G7), and the stability of the mutant based on morphological, physiological, and molecular characters.

MATERIALS AND METHODS

Plant materials

This research used ten lines (T1, T2, T3, T9, T17, T20, T22, T28, T36, and T39) of the M4 generation of mutants G7/01 chili pepper (*C. frutescens*) arose from EMS mutation. Three initial lines (Lombok cultivar) plants were used in this research. The seeds of the initial and mutant types come from the collection of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, East Java, Indonesia.

Procedures

Morphological characterization

Morphological characterization was done when the plant entered the generative phase. Observation of the 3 initial line plants and 10 mutant plants was done qualitatively and quantitatively for 7 vegetative and 8 generative parameters based on chili pepper descriptors IPGRI, AVRDC and CATIE (1995). The vegetative characterization includes habitus, plant height, first dichotomous branching height, leaf length and width, internode length and number. The generative characterization includes the color of corolla, stamens color, fruit length, fruit diameter, stalk length, fruit weight, and seeds number.

Analysis of total capsaicinoid

Chili pepper fruits of initial and mutant plants harvested at 40 days post anthesis (DPA) were measured for the total capsaicinoid content and the level of pungency using the spectrophotometric method (Zamora et al. 2015). As much as 0.5 g of fresh chili pepper fruits were ground using a mortar and pestle and dissolved in 5 ml of ethanol. The extract was placed in a closed bottle, shaken for 10 minutes, and filtered using filtered paper. The extract was diluted 10 times using ethanol, then analyzed using UV/ Vis spectrophotometer with a wavelength of 280 nm. The capsaicinoid content was deducted from a standard curve developed based on pure capsaicin. The standard curve was constructed using capsaicin standard solution (Sigma Aldric) dissolved in ethanol with different concentrations of 0, 20, 40, 60, 80, and 100 ppm. Total capsaicinoid content was represented in mg/g FW and capsaicin pungency (SHU; Scoville Heat Unit).

DNA isolation and RAPD amplification

The young leaves of initial and mutant lines were used for genomic DNA isolation employing the CTAB method with slight modification (Doyle and Doyle 1987; Clark 1997). As much as 0.1 g of fresh leaves were ground within liquid nitrogen using a mortar and pestle. DNA was lyzed using 2% CTAB buffer (2% CTAB, 0.1 M tris Cl, 0.02 M EDTA, 1.4 M NaCl, PVP 2%, and 0.2% β-mercaptoethanol). DNA was purified using PCI (Phenol: Chloroform: Isoamyl Alcohol of 25:24:1) and CI (Chloroform: Isoamyl Alcohol of 24:1). DNA precipitation was done using Ammonium Acetate and Absolute Ethanol then it was incubated at -20°C for overnight (± 15 hours). DNA pellet was washed using 70% ethanol and then was eluted using TE buffer pH 7.6 (10 mM tris Cl and 1 mM EDTA), then the genomic DNA was stored at -20°C.

The PCR-RAPD amplification process was started by primers screening. Twenty RAPD primers consisting of OPA, OPB, OPD, OPL, OPU, and OPW (Operon Technologies) were screened and ten selected primers were based on the ability to produce polymorphic DNA bands (Table 1). The PCR reaction mixture consisted of 1 µl DNA template (5-25 ng/µl), 2 µl primer (10 pmol), 3 µl ddH_2O, and 4 µl PCR master mix (Go Taq Green Master mix, Promega) with a total volume of 10 µl. The PCR program consisted of 1 cycle of predenaturation at 94°C for 5 minutes. Then, continue with 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. The amplification products were run for electrophoresis using 1% agarose gel at 60 V for 45 minutes and visualized using UV transilluminator.

Table 1. Primer RAPD using PCR technique

Primer	Sequence primer (5'-3')
OPW04	CAGAAGCGGA
OPD13	GGGGTGACGA
OPB11	GTAGACCCGT
OPL05	ACGCAGGCAC
OPB04	GGACTGGAGT
OPU10	ACCTCGGCAC
OPD19	CTGGGGACT
OPU19	GTCAGTGCGG
OPA1	CAGGCCCTTC
OPF9	CCAAGCTTCC

Data analysis

The data of quantitative characters were analyzed using Analysis of Variance (ANOVA) and Least Significant Difference (LSD) of T-paired test at 5% with Microsoft Excel and SPSS version 23 software. RAPD data were analyzed based on the presence of DNA amplified bands that appeared on the gel. Scoring is done based on the appearance of the band before the polymorphism analysis. Reproducible, well-resolved and unambiguous RAPD bands were scored as '1' for the presence and '0' for the absence of bands. A binary data matrix was tabulated for further analysis. Similarity analysis based on 15 morphological characters, one physiological character and RAPD data was done using The Unweighted Pair Group Method with Arithmetic Means (UPGMA) Paleontological Statistics (PAST) 3 manual (Hammer et al. 2001).

RESULTS AND DISCUSSION

Morphological description and variation

Morphological differences were observed in the mutants compared to the control/initial line plants. The initial line plant (G7) has shrub habitus and looks less dense than mutant G7/01 (Figure 1). The plant height and all of the components of plant height, such as the height of the first dichotomous branching, the number of nodes, and the length of internodes between the mutants and the initial line were almost similar (Table 2). However, there is a tendency for the mutant to have a higher value than the initial line.

The vegetative character that clearly shows the difference between mutants and other initials is leaf size and the number of internodes. The size of the mutant leaf (leaf length and width) was significantly larger than the initial line (Table 2). Changes in leaf size in mutants were also reported by Manzila et al. (2020). They found that the character of leaf length and width was a high heritability character. Although it was not statistically different, mutants G7/01 tended to be taller and stronger than the initial line (Figure 2). This might be caused by the number of internodes in the mutants which is more than in the initial line. The clear difference in high heritability characters indicates that there has been a genetic change from the initial line to a mutant. On the other hand, the generative characters were mostly similar (Table 2). A slight difference was shown by the length and diameter of the fruit, but it was not significant.

Table 2. Vegetative and generative characters of mutants and initial line

Characters	G7 (initial line) (n=3)	G7/01 (Mutant) (n=10)
Vegetative		
Habitus	Shrub	Shrub
Plant height (cm)	131.67 ± 7.64 [a]	127.00 ± 18.92 [a]
Dichotomous height (cm)	62.00 ± 4.36 [a]	61.98 ± 7.87 [a]
Leaf length (cm)	14.23 ± 2.05 [a]	17,79 ± 1.81[b]
Leaf width (cm)	5.43 ± 0.22 [a]	6.79 ± 0.82[b]
Internodes Length (cm)	3.83 ± 0.58 [a]	3.53 ± 0.54 [a]
Internodes number	16.00 ± 1.00 [a]	17.86 ± 1.38[b]
Generative		
Number of corollas	5 or 6	5 or 6
Color of corolla	greenish-white	greenish-white
Color of stamens	purplish stamens	purplish stamens
Fruit length (cm)	4.35 ± 0.12 [a]	4.38 ± 0.17 [a]
Diameter of fruit (cm)	0.75 ± 0.07 [a]	0.70 ± 0.11 [a]
Stalk length (cm)	2.47 ± 0.18 [a]	2.56 ± 0.21 [a]
Fruit weight (g)	1.11 ± 0.04 [a]	1.15 ± 0.05 [a]
Number of seeds	30.6 ± 2.4 [a]	31.6 ± 3.4 [a]

Note: Analysis of Variance (ANOVA) using T-paired test significance (α=0.05). The similar notation showed no significantly different

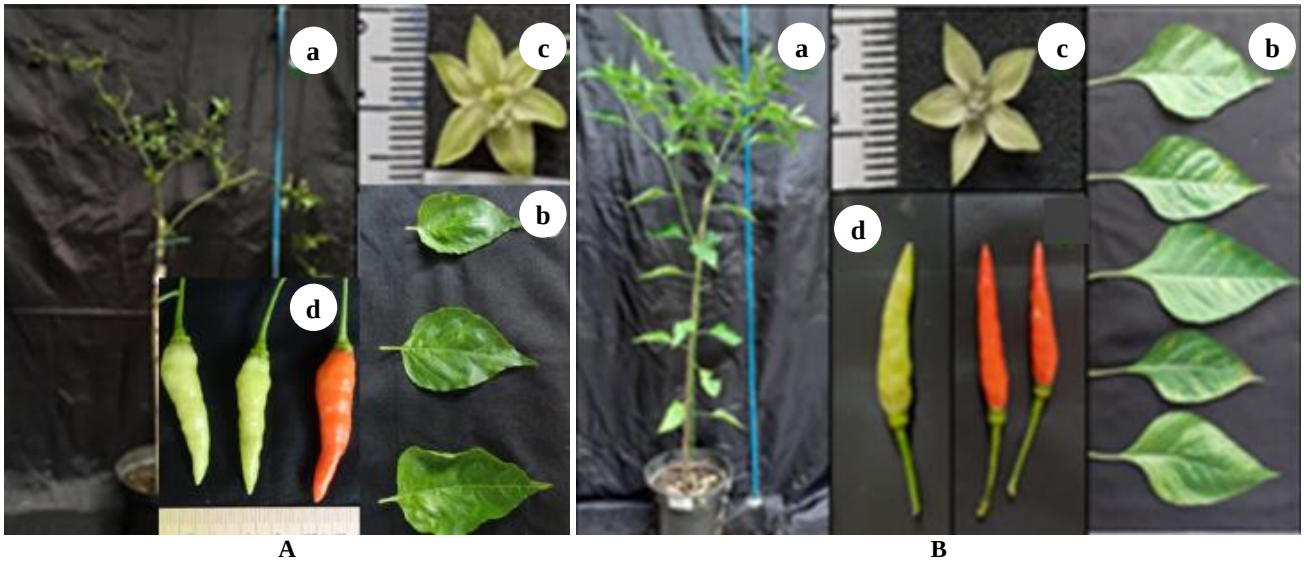

Figure 1. Morphological characters of *C. frutescens*: A. Initial Line (G7) and B. Mutants G7/01 M4 generation. Note: a. habitus, b. leaf, c. flower, d. fruit

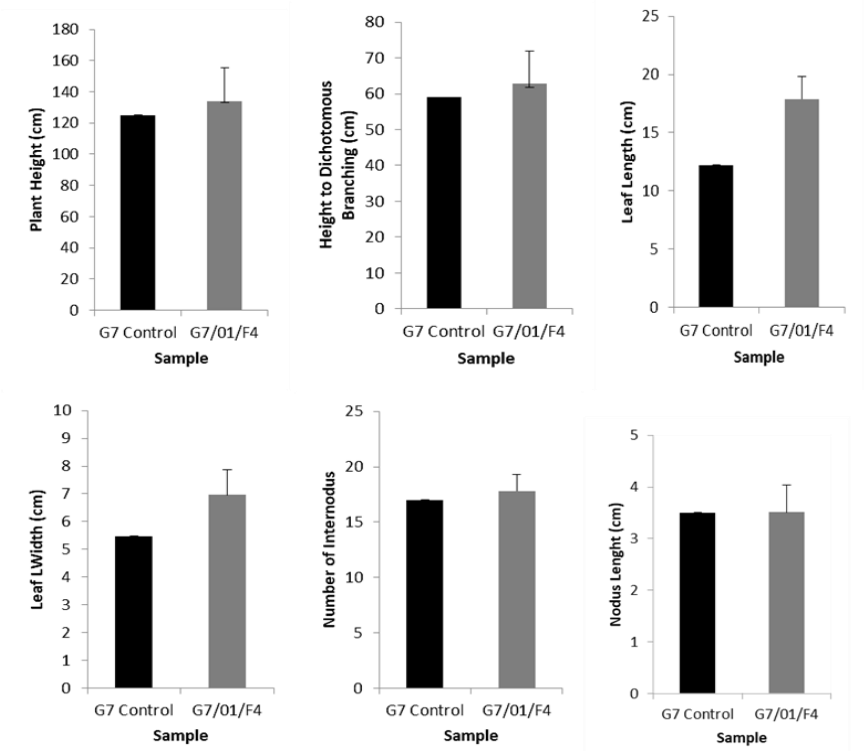

Figure 2. Variation morphological of *C. frutescens* initial line (G7) (n=3) and genotype (G7/01) M4 generation (n=10) in vegetative organs (notation shows a significant difference)

Research on mutation induction using EMS has reported many persistent morphological changes in mutants. The resulting mutations include stunted plants, fewer branches, narrower leaves, and an increased number of corollas (Manzila et al. 2020). Changes due to EMS mutation on plant habitus, fruit, and leaf character were also reported in *C. annuum* (Loewe and William 2010; Pharmawati 2018). Mutations that occur in leaves include changes in leaf color due to reduced chlorophyll content, changes in leaf thickness and size related to the amount of xylem and collenchyma tissue. The mutant leaves contained thicker palisade and spongy tissue than the wild type (Arisha et al. 2015). Of the various characters that change due to EMS mutation induction, the leaf character seems to be one of the characters that have a high probability of being inherited because it is controlled by genes.

Comparison of characters between mutants, showing the uniformity between them. The low standard deviation of all the vegetative characters shown by the mutant plants demonstrated the low variations that occurred between mutants (Figure 2). Quite low standard deviation of each character was also evidenced for generative characters, although the standard deviation shown by the mutant plant is slightly larger than the initial line. This result indicates that a further selection process is still needed to obtain truly uniform mutants. Mutation is a random process that causes the formation of new alleles as a source of character variability in mutant plant populations, so it is necessary to have a selection process to select the desired characters. The selection that follows is a very decisive process in the success of producing the desired variety. The result of the mutation will be selected to produce a superior pure line (Goyal et al. 2012). The selected character will be preserved and inherited by the next generation and spread throughout the population.

Among the generative characters of chili plants that characterize a type is the shape of the fruit. Of the ten mutant plants observed, they showed uniformity in the shape of the fruit they produced. All the mutant plants bear fruit with the same color, texture, and shape. The fruit has a pointed tip and a slightly wavy surface texture (Figure 3). This indicated that the mutant G7/01 has already started to approach stability.

Given the many vegetative and generative characters of the mutant plants that show uniformity, it can be stated that the G7/01 mutant is approaching stability. The low diversity among mutants indicates that the mutants are approaching stability (Baye et al. 2011). Genetic stability occurs when almost every homozygous locus and all individuals have homogeneous genotypes and phenotypes (Leitão 2012). If there are differences caused by environmental factors, then they will not be inherited by their offspring. Genetically stable mutant plants can be released as superior varieties after going through the stages of the selection and testing process. The selection is conducted until the minimum of 3rd or M4 generation (Oladosu et al. 2016).

Compared to the initial line, the M4 generation of genotype G7/01 has a denser appearance, strong habitus, and more branches. Furthermore, the success of flower and fruit formation in mutants was higher than in the initial line. The better performance of mutant plants indicates that the regulation of metabolic activities which are genetically encoded by these plants is better than the initial line.

Empirically, the better characters of mutants G7/01 seem to increase their performance. They also showed better resistance to unfavorable weather and environmental conditions.

Variation of capsaicinoid content

The capsaicin content is one of the specific and important phenotypic characteristics found in chili pepper (Dwinianti et al. 2018; Sahid et al. 2020). Capsaicin is a secondary metabolite of the alkaloid group produced in the phenylpropanoid and fatty acid pathways. The pathway of phenylalanine is a precursor to produce vanillylamine derivatives. While the fatty acid, valine or leucine pathways are precursors to produce 8-metil-6 nonenoat (Kehie et al. 2014).

The total capsaicinoid content of the M4 generation chili pepper mutant induced by EMS 0.01%, G7/01 significantly increased compared to the initial line. From the previous experiment, the initial line has a low total capsaicinoid content of 15.76 ± 1.97 mg/g FW and a total pungency level 252,133.34 ± 31,473.53 SHU (Juliandari et al. 2017). G7/01 has a capsaicinoid content of up to 193.33 ± 5.54 mg/g FW and a total pungency level of up to 3,076,338.00 ± 89,300.00 SHU (Table 3). The initial line and the mutant G7/01 M4 have a spiciness level of more than 80.000 SHU and are categorized as very spicy (Tanaka et al. 2016). The differences in total capsaicinoid content and total pungency level (SHU) may be influenced by the role and function of regulatory genes as well as structural genes in capsaicinoid biosynthesis. Capsaicinoid regulatory code by *Pun1* gene (Ogawa et al. 2015). Mutation followed by segregation processes, causes changes in the physiological process (Arruvitasari 2016). When the mutation occurs in the capsaicin pathway it can increase the total capsaicinoid content and total pungency level.

The G7/01 mutants had almost the same capsaicin content and spiciness level, around 500,000 to 900,000 SHU, except for the G7/01 T10 strain, which had a very high level of spiciness. G7/01 mutants had capsaicin content and various levels of spiciness, around 500,000 to 900,000 SHU, except for the G7/01 T10 strain, which had a very high level of spiciness. Based on the level of spiciness of this mutant chili, it can be seen that the mutant is not completely stable. However, some literature states that the level of spiciness of chili is strongly influenced by the environment (Rahman and Inden 2012; Lee et al. 2017; Montalvo et al. 2021). On the other hand, other studies have shown that chilies with small fruits, such as some varieties of *C. frutescens* with high spiciness show consistent capsaicinoid production in different environments (Gurung et al. 2011). In this study, the environment where chili grows was uniform. The question is how much environmental differences can change the capsaicin content. Thus, in the next study, it is necessary to confirm whether it is true that the variation in capsaicin content/level of spiciness that varies in the G7/01 mutant is indeed the influence of gene variations or whether it is due to environmental factors.

Polymorphism of mutant G7/01 and initial line based on RAPD genetic markers

RAPD is able to reveal the presence of polymorphisms in a genome through its ability to differentially amplify complementary regions with primary sequences (Amiteye 2021). With this technique, differences in the sequence in the genome can be detected from the amplification results that are different in terms of size and number of amplified fragments. The PCR successfully amplified the genome of the chili pepper studied. The RAPD bands profile showed polymorphic and monomorphic bands. Mutant plants T1, T17, dan T28 consistently showed significant differences in the RAPD band pattern compared to other mutants and their initial line. T1 mutant plants showed the most significant difference in amplification results compared to the others, even though some of the primers used did not show amplification at all when amplified with primers OPB13 (Figure 4), OPU10, OPU19, and OPW04. In contrast, the other mutants T2, T3, T9, T20, T22, T36, and T39 showed almost the same banding pattern which reflected the similarity of the DNA genome sequence. The similarity of the genome sequences of some of these mutants indicates the stability of the mutants. The same thing was shown by Prasad et al. (2013) who found that there were variations in the RAPD's band profile associated with morphological variations of chili peppers collected from Andhra Pradesh India, while the similarity of the band profile was related to the similarity of several morphological characters such as flower color, fruit size, and fruit orientation. Rêgo et al. (2011) also found that RAPD markers were effective in detecting the genetic diversity among 29 hot pepper accessions from Brazil.

Table 3. Total capsaicinoid content and total pungency level in chili pepper Initial line G7 and mutant G7/01

Sample	Total Capsaicinoid (mg/g) FW	Total Pungency Level (x 103 SHU)
K	15.76 ± 1,97a	252.13 ± 31.4a
T1	59.86 ± 2.25b	962.10 ± 36.30b
T2	61.18 ± 13.0b	985.10 ± 210.2b
T3	60.45 ± 4.26b	937.31 ± 68.73b
T9	49.57 ± 1.15bc	798.15± 18.58bc
T17	33.16 ± 0.66c	533.95 ± 10.76c
T20	32.06 ± 0.82c	516.26 ± 13.3c
T22	42.06 ± 0.15c	677.26 ± 91.93c
T28	40.63 ± 0.19c	654.26 ± 13.27c
T36	44.55 ± 5.84c	717.36 ± 94.05c
T39	191.07 ± 5.54d	3,076.34 ± 89.30d

Note: Mean value ± SD based on Analysis of Variance (ANOVA) using T-paired test significance (α 0.05). A similar notation showed that of Initial line and G7/01 M4 generation EMS treatments were not significantly different

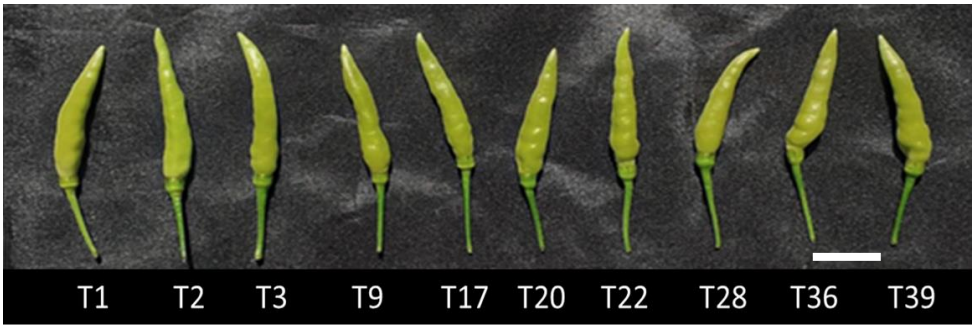

Figure 3. Young fruit of 10 plants of the M4 generation of mutant G7/01, T1-T39: Mutant number. Bar = 2 cm

The percentage of polymorphic bands is 86.4% and monomorphic band is 13.6% (Table 4). This shows that the RAPD markers used have a high level of polymorphism. Polymorphic bands are the representation of polymorphism in DNA between samples and populations based on the result of the amplification of locus in the DNA strand (Kumari and Thakur 2014).

A high level of polymorphism between plants indicated many variations in their genome. EMS mutation causes alkylation of DNA base which converts the AT base to GC, produce changing in DNA sequence (Yan et al. 2021). The high level of polymorphism in RAPD markers indicates that the mutant plants still have a segregation process. This finding supports the observed distinct and striking morphological characteristics of some mutant plants. For instance, T1 mutant plants can rarely be amplified using several primers.

Dendrogram generated by morphological, physiological, and RAPD genetic markers

Compilation of the differences and similarities of the RAPD bands as well as the fifteen morphological and one physiological characters that have been discussed previously resulted in a dendrogram tree showing variations between the mutants and their initial line as well as between the mutants studied (Figure 5). The ability of RAPD to classify chili pepper varieties that vary in terms of their morphology was also reported by Prasad et al. (2013). RAPD profile similarity above 50% indicates high morphological similarity between the varieties studied.

Table 4. Total of polymorphic and monomorphic bands result of amplification PCR RAPD

Primer	Polymorphic band	Monomorphic band	Σ bands
OPA-01	6	0	6
OPA-02	1	2	3
OPA-11	6	0	6
OPB-04	5	2	7
OPD-13	4	0	4
OPF-09	3	1	4
OPL-05	7	0	7
OPU-10	2	0	2
OPU-19	6	1	7
OPW-04	3	0	3
Total	38	6	44
Percentage	86.4%	13.6%	100%

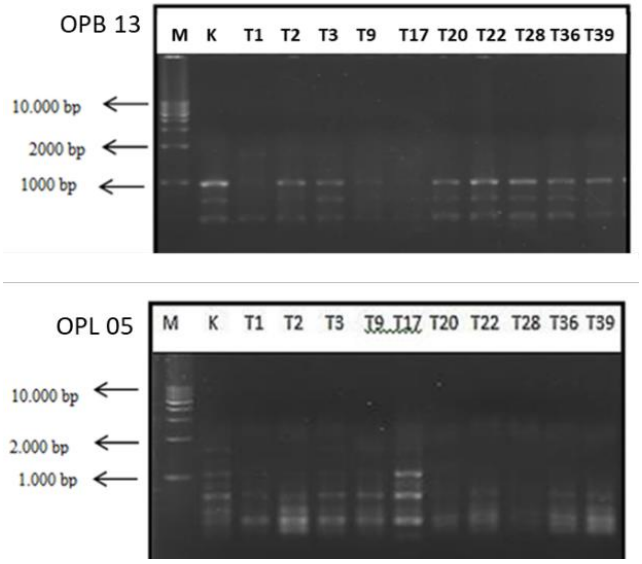

Figure 4. RAPD pattern of 10 mutant plants and the initial line using OPB 13 and OPL 05 primers

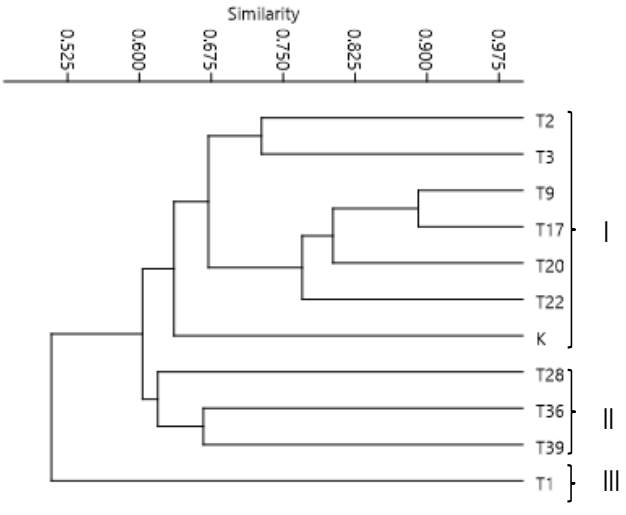

Figure 5. Dendrogram based on morphological, physiological (capsaicin content), and molecular characters

In this study, the dendrogram showed 3 clusters. Cluster I and II with a similarity value of 0.625 (62.5%) consisted of mutants that have an almost similar appearance but have some superior character compared to the initial line (K). However, Cluster III was separated at less than 52.5%, consisting of only one plant, T1, that differs greatly from the initial line and other mutants and forms a separate cluster (Figure 5). Based on the morphological, physiological, and molecular characters, the G7/01 mutant plant showed clear evidence of changes in the genotype and gene expression of the initial line. The observed G7/01 mutant plants showed a trend towards stability, especially when viewed from the character of the fruit, although variations were still found. Mutants T1, T28, T36, and T39 have the potential as superior strain candidates. These mutants show clear molecular differences and contain 3-12 times more capsaicin than the initial line. The use of RAPD to study the extent to which variation occurs among mutation-induced mutants was also carried out by Arumingtyas and Ahyar (2022). In this study, mutants induced by gamma rays formed 4 clusters at a similarity limit of 80%. Thus, depending on the population of chili plants studied, the limits of clustering similarity based on molecular data such as RAPD, which correlates with morphological data, may vary.

In conclusion, based on the morphological characters, the M4 generation of EMS mutations (G7/01) chili peppers G7/01 mutants have some superior properties compared to the initial line G7. All the G7/01 mutant plants contain higher capsaicinoid compounds than the Initial line plants. The dendrogram developed based on the RAPD profile showed that all the mutant plants were positioned apart from the Initial line plant, suggesting that there are some genomic changes in the mutants compared to the Initial line plants. Those mutants showed the indication of genetic stability showed by no significant morphological characteristic variation observed between mutant plants. All the mutant plants, except T1, showed insignificant variation in morphological characteristics, capsaicin content, and RAPD profile, indicating genetic stability.

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