Original Research Paper

Comparison of Molecular Markers ISSR, SSR and RFLP to Study the Genetic Variation of Strawberry Cultivars

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Abstract: Strawberry is grown in Indonesia because it has potential high economic value. The purpose of this study was to investigate relationship of phenetic lineage and genetic variations in 12 strawberry cultivars using three different molecular marker methods, including ISSR, SSR and RFLP. Five primers of ISSR markers were used and they generated 53 bands with 44 polymorphic DNA bands. Percentage of polymorphic using ISSR method was 83%. Five primers of SSR markers were used and they gave 41 DNA bands with 36 polymorphic DNA bands. Percentage of polymorphic using SSR method was 87%. Four primers of PCR-RFLP markers and 3 enzymes were used and they had 20 DNA bands with 9 polymorphic DNA bands. Percentage of polymorphic PCR-RFLP method was 45%. Three dendrogram results based on each method indicated that Stroberi Hitam has smallest similarity index compared with other eleven cultivars. 'Californica' cultivar had the closest similarity with Stroberi Hitam. Data analysis of molecular character requires complementary and supporting data of other characters such as morphology, biochemistry and cytology so this will strengthen the lineage data of the organisms to be investigated. Molecular marker can be used as a good method for genetic variation analysis if it has some advantages such as high polymorphism, codominance and specific characteristics and wide distribution.

Keywords: Strawberry, *Fragaria* spp., Molecular Markers, Genetic Variation

Introduction

Strawberry (*Fragaria* spp.) is one of fruit plants grown almost all over the world because it has high economic value. In Indonesia, strawberry production increases from 19,132 tons in 2009 to 169,796 tons in 2012 but it decreased to 90,352 tons in 2013 and decreased again to 58,882 tons in 2014 (Directorate General of Horticulture of the Ministry of Agriculture, Indonesia, 2015). The decrease in strawberry production in Indonesia requires efforts to improve strawberry superior character in order to increase the strawberry productivity again. One of the efforts to improve strawberry superior character in Indonesia is made by more intensive plant breeding technique through study of genetic diversity. This is carried out to ensure that strawberry plant that has superior quality is available for cultivation.

The rapid development of DNA molecular marker technique has resulted in the increase of various techniques for genetic diversity analysis of an organism (Weising *et al*., 2005). Molecular marker data can be used to overcome the limitation of genetic variation analysis based on morphological, cytological, biochemical and physiological characters (Jonah *et al*., 2011). Some molecular markers such as Random Fragment Length Polymorphism (RFLP), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) are efficient techniques to detect genetic diversity (Chakravarthi and Naravaneni, 2006). Analysis using molecular marker is relatively more effective in plants with high level of variety such as strawberry (*Fragaria* spp.). Several techniques have technical differences such as the amount of DNAs needed, cost, polymorphism level and complexity (Semagn *et al*., 2006).

The purpose of this study was to investigate relationship of phenetic lineage and genetic variations in 12 strawberry cultivars using different molecular markers such as ISSR, SSR and RFLP.

Materials and Methods

Plant Materials

Twelve strawberry cultivars obtained from Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI), Malang, East Java and Strawberry Agrotourism in Banyuroto Village, Magelang, Central Java (Table 1).

DNA Isolation

About 12 Fifth leaves aged 3 to 4 weeks from each cultivar were cut using scissors and then inserted in sample box in cold condition. Samples were stored at -20°C. DNA was extracted from strawberry leaves of each cultivar using modified CTAB method (Doyle and Doyle, 1990).

Table 1: Strawberry cultivars used in the study

No.	Cultivars	Origin of sample
1	'Aerut'	ICSFRI, Malang
2	'Berastagi'	ICSFRI, Malang
3	'Dorit'	ICSFRI, Malang
4	'Deeprose'	ICSFRI, Malang
5	'Festival'	ICSFRI, Malang
6	'Oso Grande'	ICSFRI, Malang
7	'Oso Purbalingga'	ICSFRI, Malang
8	'Californica'	Banyuroto, Magelang
9	'Earlybrite'	Banyuroto, Magelang
10	'Rosalinda'	Banyuroto, Magelang
11	'Tristar'	Banyuroto, Magelang
12	Stroberi Hitam	Banyuroto, Magelang

DNA purity was calculated using spectrophotometer and DNA was stored at -20°C. The isolated DNA was then amplified using Polymerase Chain Reaction (PCR).

DNA Amplification

The DNA of each successfully isolated strawberry cultivar then used as template for amplification process. DNA amplification was performed with 3 different kinds of molecular markers namely ISSR, SSR and PCR-RFLP. Molecular markers used for ISSR (Debnath *et al.*, 2008), SSR (Gil-Ariza *et al.*, 2006) and PCR-RFLP (Kunihisa *et al.*, 2006) are shown in Table 2.

In ISSR method, DNA amplification was done on pre-denaturation temperature of 95°C for 10 minutes, denaturation temperature of 95°C for 30 seconds, annealing temperature of 43–49°C for 30 seconds, elongation temperature of 72°C for 2 minutes and post elongation temperature of 72°C for 5 minutes. In SSR method, DNA amplification was performed on pre-denaturation temperature of 95°C for 3 minutes, denaturation temperature of 95°C for 30 seconds, annealing temperature of 52 and 54°C for 30 seconds, elongation temperature of 72°C for 1 minute and post elongation temperature of 72°C for 1 minute. In RFLP method, DNA amplification was carried out on pre-denaturation temperature of 95°C for 3 min, denaturation temperature of 95°C for 30 sec, annealing temperature of 54 and 58°C for 30 sec, elongation temperature of 72°C for 1 min and post elongation temperature of 72°C for 1 min.

Table 2: Names and sequences of Primers used for each technique

	Primers		Primer Sequence 5'–3'
ISSR	UBC-817		CACACACACACACACAA
	UBC-823		TCTCTCTCTCTCTCTCC
	UBC-827		ACACACACACACACACG
	UBC-834		AGAGAGAGAGAGAGAGYT
	UBC-876		GATAGATAGACAGACA
SSR	ChFaM01	Forward	GGAGATTATGCACAAAATATAGAGA
		Reverse	CCAGAACTCCATCAGCCTCT
	ChFaM04	Forward	CCCAGCATATACTTTGCCGTA
		Reverse	TCCTTTCTTCATCCCCTCCT
	ChFaM07	Forward	AATATATTACTCATCCAATCTTGTC
		Reverse	AGATGGAGGGCTTGGAAGTT
	ChFaM23	Forward	AGGAGAAGACCGGCTGTGTA
		Reverse	TGCCTATAGCTGTGGCTGTG
	ChFaM29	Forward	ACTTCATCGCCAGAATGGTC
		Reverse	GCCATTCAATACACAATCCAA
RFLP	CT12	Forward	GTCAAACCTCTCACGAAACCACT
		Reverse	GTTRCTAAGAAAATGAAAGAGCTGATG
	OLP	Forward	TGTGTCCAAAACCGATCAGTATTGC
		Reverse	TCTTTCAGAGTGGTACGTACCCC
	F3H	Forward	TAATAGGGTCTAGGTGCGTGG
		Reverse	GAGTTCACTACKGCCTGGTGATC
	APX	Forward	GTGGTCACACCTTGGTGC
		Reverse	AGTATAATATTTAAGCAG\ AATGCAGACTTC

Table 3: Restriction Enzymes used and its cut-off point

No.	Restriction enzymes	Cut-Off point	Incubation temperature (°C)
1	*Taq*I	5' ↓GATC 3' 3' CTAG↑5'	37
2	*Mlu*I	5' A↓CGCG T 3' 3' T GCGC↑A 5'	37
3	*Taq*I	5' T↓CG A 3' 3' A GC↑T 5'	60

All methods used the denaturation, annealing and elongation phases repeatedly as much as 40 cycles. SSR amplification results were separated using 6% polyacrylamide gel. In PCR-RFLP, the amplified DNAs were cut using restriction enzyme (Table 3) with different incubation temperatures (Table 3).

Data Analysis

The size of amplified DNA was determined using 100 bp and 20 bp markers (Promega). Binary matrix was made based on the bands of amplified DNA. The visualized bands were rated as (1) and non-visualized bands were rated as (0). The data were then prepared with Microsoft Excel 2013 software. Dendrogram construction was made using MVSP 3.1 software. Similarity Index was calculated using Percent Similarity method to estimate relationship among accessions. Dendrogram cluster was made using Unweighted Pair Group Method with Arithmetic Means (UPGMA).

Results and Discussion

Five primers of ISSR molecular markers generated 53 DNA bands, 9 of which were monomorphic DNA bands and the other 44 were polymorphic DNA bands (Fig 1). Percentage of the primer of polymorphic ISSR molecular marker was 83%. The more parts of DNA genomes complementary to the primer, the more DNA bands are produced. Five primers of SSR molecular markers generated 41 DNA bands, 5 of which were monomorphic DNA bands and the other 36 were polymorphic DNA bands. Percentage of polymorphic SSR primers was 87%. Furthermore, 4 primers of PCR-RFLP molecular markers and 3 enzymes generated 20 DNA bands, 9 of which were monomorphic DNA bands and the other 11 were polymorphic DNA bands (Fig. 3). Percentage of polymorphic RFLP primers was 45%.

Table (4) indicates that SSR molecular markers have the highest polymorphic percentage than ISSR and PCR-RFLP. This is because SSR molecular markers are codominant and can distinguish homozygous and heterozygous individuals. SSR markers can amplify DNA sequences at a locus by tandem consisting of 1 to6 bp repeatedly. A very abundant amount of SSRs in both eukaryotic and prokaryotic organisms and the number of its repetition show genetic difference or polymorphism level between individuals. SSR is found in both coding and non-coding DNA in a genome but more SSRs are found in non-coding region of DNA (Guan *et al.*, 2013). Regarding to PCR-RFLP band cutting results, polymorphism percentage found here was the lowest compared with other markers. This is because PCR-RFLP only amplified certain locus on DNA fragment of an individual. The resulting polymorphism also derived from the difference in certain sequences on the locus detected by restriction enzyme cutting results.

Regarding to the similarity index of ISSR markers, 'Oso Grande' and 'Oso Purbalingga' have similarity index of 95.385%, this is because 'Oso Purbalingga' cultivar represents 'Oso Grande' cultivar derived from Californica cultivated in Purbalingga, Central Java, so both have the highest similarity index. A slight difference in 'Oso Grande' and 'Oso Purbalingga' cultivars is caused by environmental adaptation in tropical region of Purbalingga, Central Java. In similarity index of 90.382%, 'Oso Grande' cultivar is clustered with 'Earlybrite', while in similarity index of 89.121%, it is clustered with 'Festival' because these cultivars have 'Rosalinda' parent. According to Chandler (2000), 'Festival' is the crossbreed between 'Rosalinda' and 'Oso Grande' while 'Earlybrite' is cultivar derived from the crossbreed between 'Rosalinda' and 'FL 90-38' on a research conducted in Florida.

Based on RFLP markers, the cultivars are clustered based on the origin of each cultivar. This relates to the nature of genetic material inheritance from the parent to its breed. 'Oso Grande,' 'Oso Purbalingga', 'Aerut', 'Brastagi', 'Californica', 'Earlybrite', 'Dorit', 'Deeprose', 'Rosalinda' and 'Tristar' Strawberries can be clustered into one phenetic cluster as the fragments of cutting results have similarity value of 96.02%. This can be associated with the origin of all cultivars. All of the cultivars are individuals belonging to *F.* x *ananassa* species. *F.* x *ananassa* is a hybrid of crossbreed between *F. virgiana* species from North American and *F. chiloensi* species from Chile. This shows that resistance gene sequence pattern of such ten cultivars is only slightly different from the restriction enzyme fragments used. The possibility of small difference in these cultivars is environmental factor. All primer pairs used in this study amplified gene that acts in resistance mechanism of pathogen attack and environmental stress. Extreme environmental stress may cause genetic mutation in the form of insertion and deletion. 'Californica' strawberry is different from the other ten cultivars because it only has fragment similarity of 92.02%.

Table 4: DNA polymorphisms generated by ISSR, SSR and RFLP markers

Molecular Marker	Number of DNA Bands		Mono-morphic DNA Bands	Poly- morphic DNA Bands	Percentage of Poly-morphism
ISSR	53	9	44	83%	
SSR	41	5	36	87%	
RFLP	20	11	9	45%	
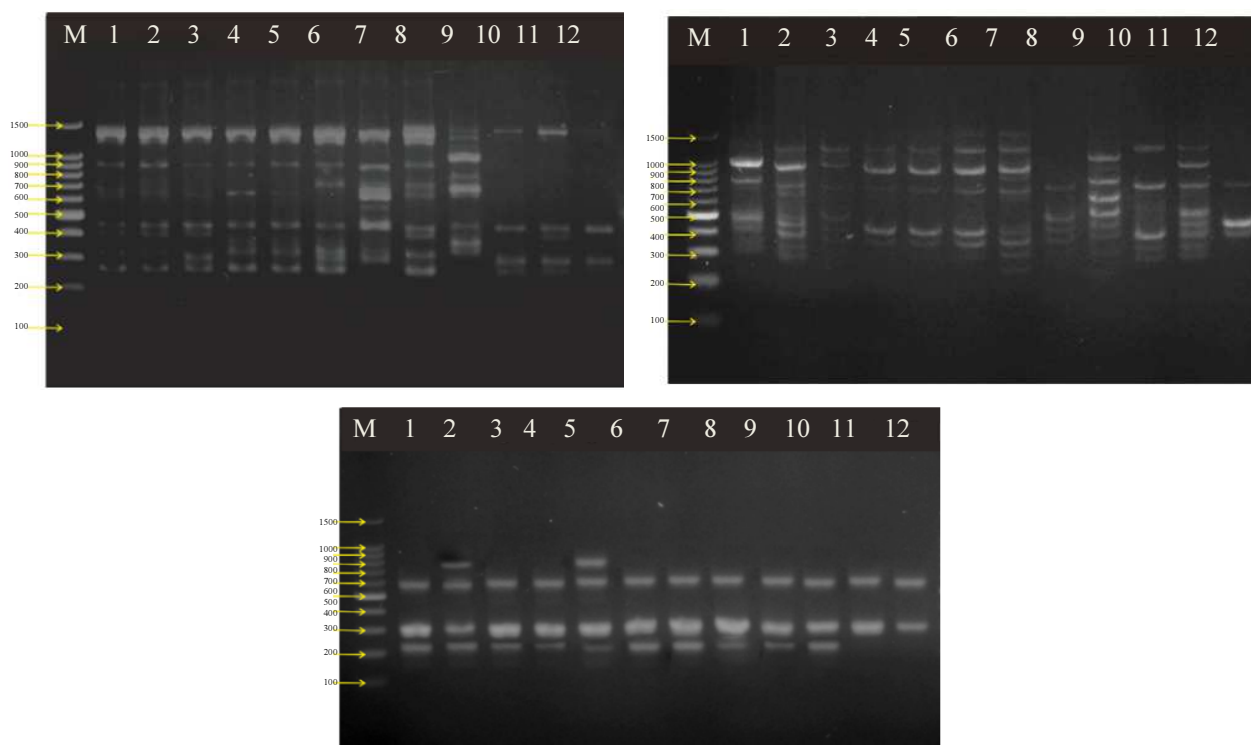
Fig. 1: Electrophoresis of 12 samples of strawberry (*Fragaria* spp.) cultivar using ISSR markers (a) UBC 823 primer (b) UBC 817 primer and (c) UBC 876 primer

Stroberi Hitam only has similarity index value of 77.47% of the other eleven cultivars. Both are different because they are classified into different species. Stroberi Hitam comes from *F. moschata* species while Californica Cultivar comes from *F. vesca* species. The species difference expectedly makes the amplified fragments in the four primers are also different. The twelve strawberry cultivars with RFLP markers were clustered based on the number of ploidy of each cultivar. Hancock *et al.* (2008) stated that the Aerut, Brastagi, Festival, Tristar, Earlybrite, Dorit, Deeprose, Rosalinda, Oso Grande and Oso Purbalingga Cultivars are octaploid individuals (2n=8x=56). Californica cultivar that is diploid individual (2n=2x=16) is different from the other ten cultivars, while Stroberi Hitam which is the most different cultivar from the other eleven cultivars is hexaploid individual (2n=6x=48). Ploidy difference among these cultivars is expectedly due to allopolyploidization event. Allopolyploidization is hybridization event between two different species, which are still closely associated, resulting in the breed (Janick and Moore, 1996).

The SSR markers showed that 'Oso Grande', 'Oso Purbalingga', 'Rosalinda', 'Festival', 'Tristar' and 'Earlybrite' strawberry cultivars are clustered into one cluster namely B3 with similarity value of 75.34%. This result is supported by research conducted by Chandler *et al.* (1997; 2000), reported that the 'Earlybrite', 'Oso Grande', 'Rosalinda' and 'Festival' strawberry cultivars have the same parent namely 'Pajaro' cultivar (Fig. 2). 'Dorit', 'Rosalinda', 'Aerut' and 'Berastagi' cultivars were clustered with similarity value of 73%. 'Aerut' and 'Berastagi' cultivars have high similarity index of 82.93%.

The results of dendrogram based on each molecular marker (Fig. 4) show that Stroberi Hitam has the smallest similarity index than the other eleven cultivars, while 'Californica' cultivar has the closest similarity with Stroberi Hitam. Stroberi Hitam is classified as *F. Moschata* species, while 'Californica' cultivar is classified as *F. vesca* species. Both are not hybrid species of crossbreed result like the other ten cultivars.

Cytological data showed that *F. moschata* has hexaploid chromosomes, while *F. vesca* has diploid chromosomes and *F.* x *ananassa* has octaploid chromosomes (Hancock *et al*., 2008). Other cultivars such as 'Oso Grande', 'Oso Purbalingga', 'Rosalinda', 'Deeprose', 'Dorit', 'Earlybrite', 'Tristar', 'Festival', 'Brastagi' and 'Aerut' are classified in *F.* x *ananassa* species. This strawberry cluster is different or separated based on the number of Stroberi Hitam chromosomes (*F. moschata*) classified as hexaploid strawberry (2n=6x=42), while 'Californica' cultivar (*F. vesca*) is classified as diploid strawberry (2n=14) and the cultivars of 'Dorit', 'Tristar', 'Aerut', 'Brastagi', 'Deeprose', 'Festival', 'Oso Grande', 'Oso Purbalingga', 'Earlybrite' and 'Rosalinda' (*F.* x *ananassa*) are classified as strawberry with octoploid chromosomes (8n=56) (Darrow, 1966; Nathewet *et al*., 2010). According to Singh (2010), a taxon is said to belong to one genus if it has similarity index of ≥ 65% and ≥ 85% for species and the greater the similarity index the closer the lineage among varieties or cultivars. A variety or cultivar is said to have close similarity or lower level of diversity and variation if it has similarity level of ≥85%.

Difference in the number of strawberry ploidy can be caused by hybridization among species with different genomes causing the difference in number of chromosomes or commonly called as allopolyploid. Allopolyploid leads to phenotypic change caused by the transfer of gene among species and chromosome substitution in order to acquire new or combined characteristics of both species. The low similarity of *F. moschata*, *F. vesca* and *F.* x *ananassa* is also caused by morphological difference of the three species. *F. moschata* has blackish red colored fruit, while *F. vesca* and *F.* x *ananassa* have bright red colored fruit (Janick and Moore, 1996).
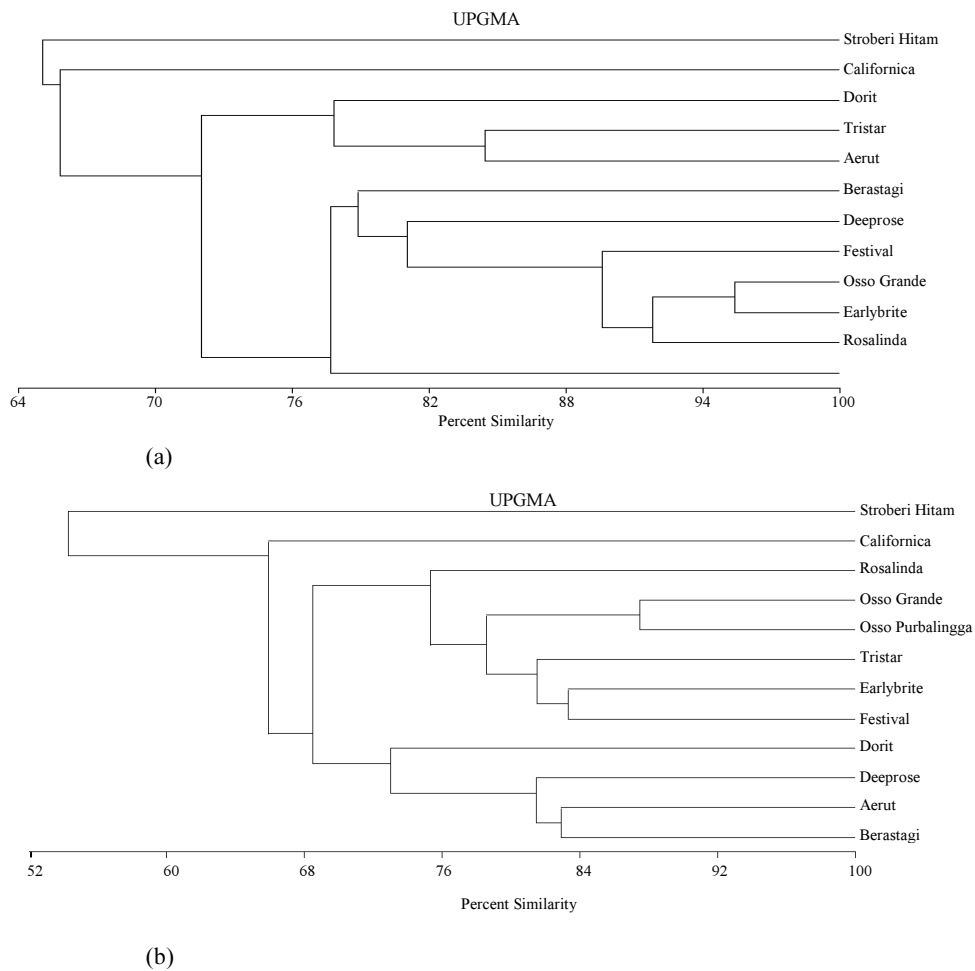
Data analysis of molecular characters requires complementary and supporting data of other characters such as morphology, biochemistry and cytology in order to strenghten the lineage data of the organisms to be studied. Molecular markers can be used as the effective and efficient method based on the genotype of an organism that is more stable and is not easily affected by the environmental factors (Benor *et al*., 2008). Molecular markers can be used as good method for genetic variation analysis if it has several advantages such as high polymorphism, codominance and specific characteristics and wide distribution.
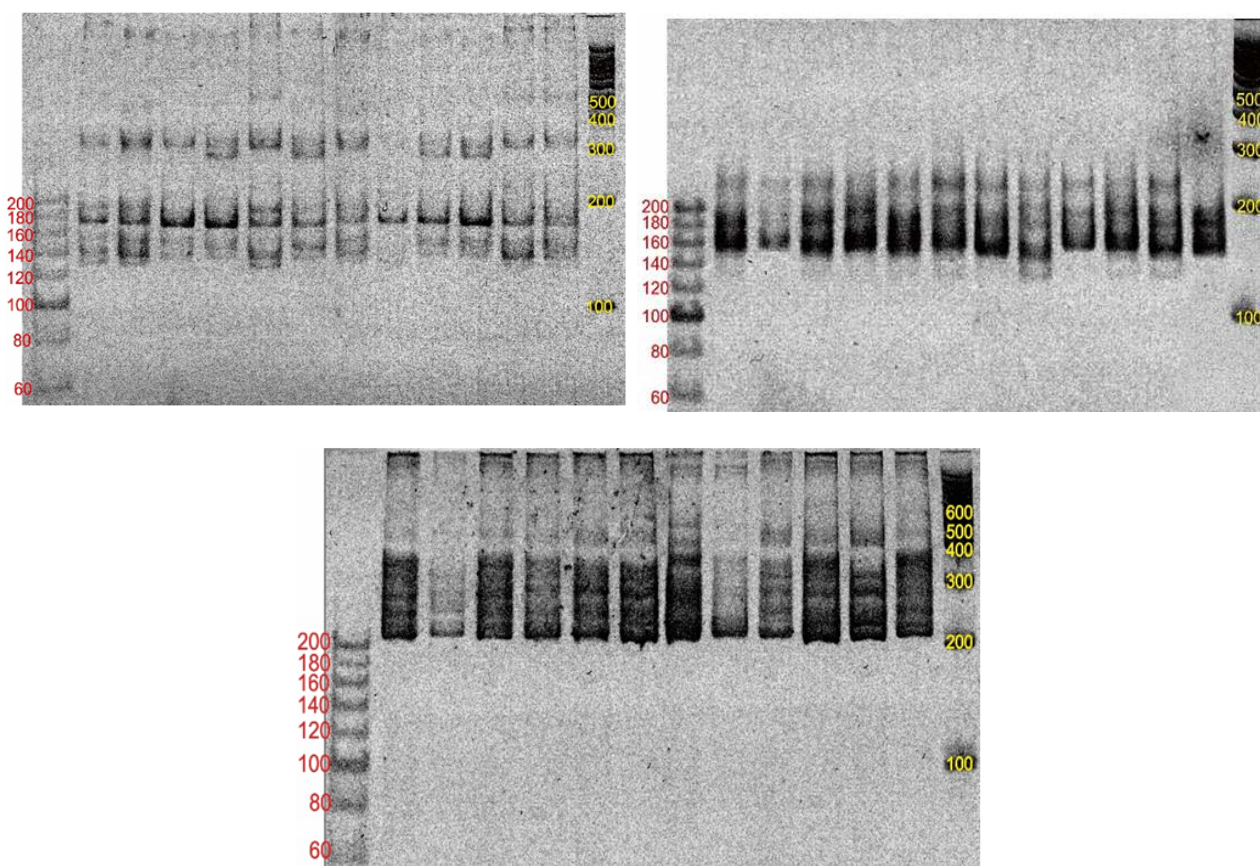
Fig. 2: Electrophoresis of 12 samples of strawberry (*Fragaria* spp.) cultivar using SSR markers (a) ChFaM04 primer (b) ChFaM07 primer and (c) ChFaM01 prime
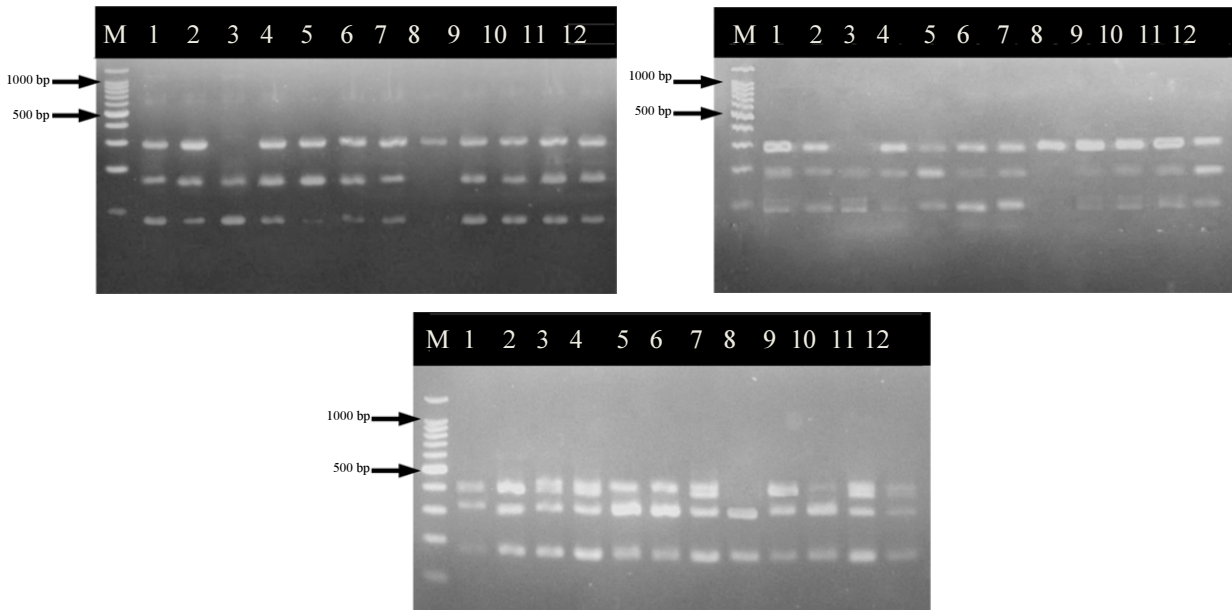
Fig. 3: Electrophoresis of 12 samples of strawberry (*Fragaria* spp.) cultivar using PCR-RFLP cutting result (a) CTI2-*Mlu*I 4 primer, (b) CTI2-*Mbo*I primer and (c) APx-*Taq*I primer
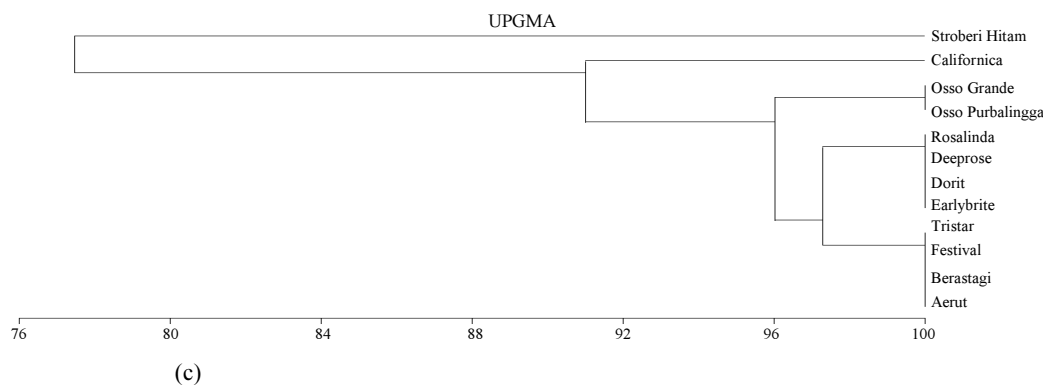

(a)

(b)

66

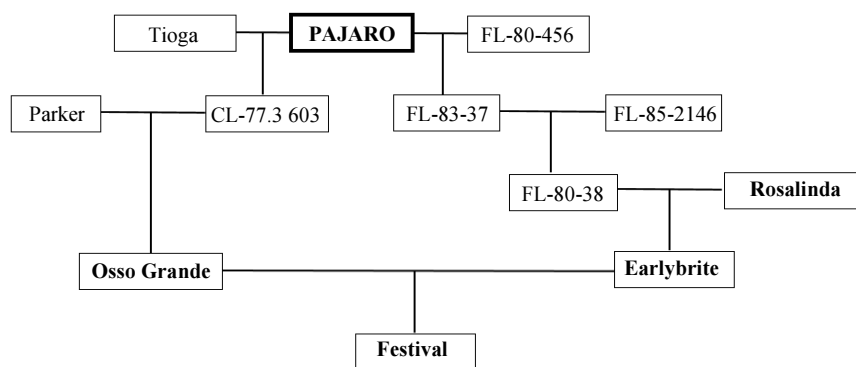**Fig. 4:** Dendrogram results of 12 strawberry cultivars of (a) ISSR, (b) SSR and (c) RFLP

Fig. 5: Diagram of the lineage of Oso Grande, Earlybrite, Rosalinda and Festival strawberry cultivars (Voth *et al.*, 1989; Chandler, 1997; 2000)

Conclusion

Stroberi Hitam is the most different cultivar compared with the other eleven cultivars based on the three molecular markers. Data analysis of molecular characters requires complementary and supporting data of other characters such as morphology, biochemistry and cytology in order to strenghten the lineage data of the organisms studied. Molecular markers can be used as the effective and efficient method based on the genotype of an organism that is more stable and is not easily affected by the environmental factors.

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Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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