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Genetic Variation of Golden Silk Insect *Cricula trifenestrata* Helf. (Lepidoptera: Saturniidae) Using ISSR (*Inter Simple Sequence Repeats*) Molecular Marker

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a b s t r a c t

The aims of this research were to conduct a study on genetic variation *C. trifenestrata* collected from several sampling locations using ISSR (*Inter-Simple Sequence Repeat*) molecular markers. The applied methods of the research were to collect samples (*C. trifenestrata*), isolation DNA genome, and analysis of genetic variation using the ISSR-PCR method. Based on genetic variation analysis using 4 *primer*s (ISSR 1, ISSR 2, ISSR 6, dan ISSR 7), it is known that the level of genetic variation and polymorphism of *C. trifenestrata* is high with a percentage of 98.9%. The formed dendogram showed that the 29 studied samples of *C. trifenestrata* separate into two major groups at the similarity level of (0,60) 60%.

1. Introduction

The world textile industry has known silk as a high quality fabric base material for a long time. There are two kinds of silk-producing insects that are known to produce silk fibers that are *murbei* silk-producing insects (*Bombyx mori*) and non-*murbei* silk-producing insects or better known as wild silkmoths (Indrawan, 2002; Norbertus, 2010).

In Indonesia, at least five species are identified as wild silk-producing insects from Familia Saturniidae; they are Antheraea rosieri, Attacus atlas, Samia cynthia ricini, Cricula aleaezea, and Cricula trifenestrata. A. atlas, Antheraea pernyi, and C. trifenestrata were found in Yogyakarta and around the area in 1994-1995 (Situmorang, 1996). Among the wild silk-producing insects in Indonesia, C. trifenestrata, also known as gold silk-producing insects, is a superior species, because it has certain characteristics, which produces golden yellow silk cocoons that can be utilized into high-quality textile materials. The silk yarn produced by this species are very beautiful with exclusive yellow color (Norbertus, 2010; Tikader et al., 2014; Nindhia et al., 2014).

The development and utilization of wild silk-producing insects in Yogyakarta were begun in the 1990s, but it still needs further development (Akai, 1997). Based on information obtained from the villagers of Karang Tengah (Bantul), Dlingo (Bantul), Jurangombo (Magelang), Godokidul (Wonogiri), Playen (Gunung Kidul), Ngawen (Gunung Kidul) and Sidorejo), *ulat kipat* (a local name for C. trifenestrata larvae) is a pest that causes their leaves to be barren so that they infrequently eradicate them. Therefore, based on the information, there is a population of C. trifenestrata in the seven villages. Hence, the seven villages were selected as sampling sites.

Research on the morphological characterization of wild silk-producing insects, feed crops, habitats, and silk fibers have been conducted in Indonesia. However, at this time, there has been no research on the genetic variation of wild silk-producing insects. Differences in environmental conditions and differences in feed crops will affect the adaptation response of each species causing variations or polymorphisms that occur in the population as well as individuals in the population. Through genetic studies, information about the diversity between individuals within and between populations can be known, so the status of taxonomy and kinship relationships can be clearly identified.

A molecular marker is a technique that can be used for the analysis of genetic variation between population and individual variation in a population. ISSR (*Inter-simple Sequence Repeat*) which is a PCR (*Polymerase Chain Reaction*) - based marker, does not require information about genome sequences to be tested. It uses a single microsatellite *primer* in PCR reactions with the target of genomics multiple-locus to amplify *inter-simple sequence repeats* with different sizes (Wahyuni et al., 2004).

The ISSR (*Inter simple sequence repeat*) Molecular Marker is a simple method with the ability to detect high, reliable, and sensitive polymorphisms in detecting close-related individual variations (Reddy, et al., 1999; Williams *et al* 1999). This ISSR molecular marker amplifies the DNA segment between two 'repeat'. The ISSR region is a microsatellite part that does not encode protein (non coding region) (Widiastuti, Sobir & Suhartanto, 2013). The ISSR molecular markers demonstrate the potential not only to reveal variations between populations that are geographically separated, but also between individuals in the population (Jabbarzadeh et al., 2010; in Subositi, D., & Kasiamdari, R. S. 2010). The main advantage of this molecular marker is that it can analyze multiple loci in a single reaction. ISSR has been

widely used to detect genetic diversity and genetic coherence (Rucinska & Puchalski, 2011; Isshiki et al., 2008) as well as mutant identification (Campbell et al., 2011).

Research using ISSR molecular markers to look at genetic diversity or variation has been applied on some species of silk-producing insects, eg *S. Cynthia ricini* (Vijayan et al., 2006), B. mori (Reddy et al., 1999), and Anthereae mylitta (Chatterjee et al., 2004). It showed a genetic variation in individuals obtained from different sites. The ISSR markers used for analysis on *S. cynthia riccini* show that these molecular markers can be used to detect genetic variation between populations and individuals in the population (Vijayan et al., 2006). Such studies may serve as a basis for the study of genetic variations of gold silk-producing insects (C. trifenestrata) using these ISSR (Inter Simple Sequence Repeats) molecular markers.

Analysis of the population's genetic variation needs to be found so that taxonomic status and kinship relationships can be clearly identified and can be used as a basis to support species identification and characterization, which may be useful for the further development of cultivation.

2. Method

The research material used is *C. trifenestrata* samples collected from 7 village locations in Jogjakarta and Central Java.

Table 1. The Location of Sample-Taking, Feed Plants, Geographical Locations, and Height of Locations in Taking *C. trifenestrata* Samples

No	Picking-Locations	Sample Code	Feed Plants	Height (m dpl)	Astronomical Location	
					South Latitude	East Longitude
1	Village Godokidul, Wonogiri	W	Cashew	±247	07°52’36,9”	111°03’48,7”
2	Village Karang Tengah, Bantul	KT	Cashew	±271	07°56’04,8”	110°24’12,6”
3	Village Ngawen, Gunung Kidul	P	Cashew	±226	07°50’28,1”	110°41’27,3”
4	Village Playen, Gunung Kidul	T	Pomegranate	±222	07°55’26,2”	110°33’23,3”
5	Village Dlingo, Bantul	D	Pomegranate	±409	07°51’40,5”	110°28’18,2”
6	Village Sidorejo, Sleman	K	Avocado	±635	07°37’33,3”	110°25’27,8”
7	Village Jurang Ombo, Magelang	M	Avocado	±374	07°31’08,7”	110°12’27,9”

2.1 Identification and Morphological Observation of *C. trifenestrata*

Each sample of C. trifenestrata obtained was kept in the laboratory until it became an imago, then the imago was identified through the book the "Introduction of Insect Lessons" (Borror, 1992), *Moths of Thailand* (Pinratana & Lampe, 1990), and *Saturniidae Mundi* (d'Abrera, 1995). Identification was done with the aim of ensuring that the sample used is *C. trifenestrata.* The identification of the sample was done by looking at the shape of the antenna, the front wing venation and the rear wing venation, and the fenestra on the *C. trifenestrata* wing.

The morphological observations were conducted by looking at and comparing the *C. trifenestrata* samples obtained from the seven sampling sites based on wing color differences, wing span length, wingspan, and body size, to see the morphological variation in each sample.

2.2 DNA Isolation

The process of DNA isolation is done with a commercial DNA isolation kit (®Fermentas). The implementation procedure follows instructions from the Kit protocol (®Fermentas) (*GeneJET Genomic DNA Purification Kit*). Then, the quantification of DNA was conducted by knowing the absorbance of light at wavelength 260 nm to know the concentration and purity of DNA of isolation result.

2.3 DNA Amplification through ISSR *primers*

PCR reaction using *thermocycler* (Boeco) for 35 cycles. Pre-denaturation at 94 ° C for 2 minutes, followed by 35 cycles consisting of 30-second denaturation at 94 ° C, annealing of 1 minute at 40.2 ° C (ISSR 1), 50° C (ISSR 2), 52.3 ° C (ISSR 6), and 41.8 ° C (ISSR 7) and 2 minutes of elongation at 72° C. After 35 cycles completed, followed by PCR (post-elongation) 7 minutes at 72° C. The amplification results were separated through electrophoresis by using agarose gel (®SIGMA) 2% in TBE 1X buffer (Tris borate-EDTA) for 75 min at 50 V. Then, they were immersed in ethidium bromide solution for 30 min. The results of DNA fragment separation were observed with UV transilluminators and photographed using a digital camera. As a standard, 100 bp DNA ladder (Vivantis) is used to determine the size of the band of DNA amplification.

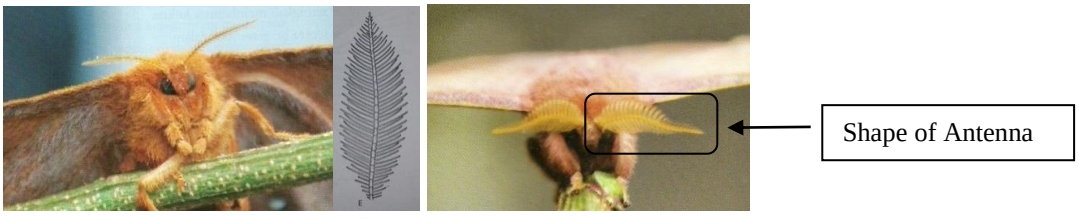
2.4 Data Analisis

The DNA amplification results with ISSR-PCR were analyzed with binary matrix data. The DNA fragments seen in the electrophoretic photo gel were scored 1, while the invisible DNA fragment was given a score of 0. Similarities were analyzed from the appared fragment of ISSR results. Then, the unweighted pair-group method using arithmetic average (UPGMA) method is used to produce a dendrogram that shows the genetic similarity between each individual. This analysis is done with *Numerical Taxonomy and Multivariate System (NTSY) Spc2.1 software program*. Formed dendogram was to know the similarity of *C. trifenestrata* by looking at the coefficient similarity and grouping that occurred.

3. Result and Discussion

3.1 Identification of *C. trifenestrata*

The sample identification was performed to ensure that the sample used in the study was *C. trifenestrata* species. *C. trifenestrata* belongs to the family of Saturniidae whose members are characterized, among others, having a relatively large wingspan, having a bipectinate antenna, a combed antenna; in male individuals the shape is wider than that of the female (Fig. 1), has a bright wing color, and has a transparent spot (fenestra) on its wings (Figure 2) (Borror, 1992). *C. trifenestrata* is a member of the Saturniinae subfamily with a characteristic that can be seen in the wing frame pattern (Fig. 3) (Borror, 1992). The wing frame pattern of heteroneura type, ie anterior wing frame is different from the posterior wing frame. Radial (R) on the anterior wing usually has five branches, but on the rear wing radial sector is not branched, and R1 is usually united with subcosta (SC). The disc cell (D) is formed in the center of the wing. The anal wing frame (A1) is united with A2. Three branches of the framework of the media (M) arise from the tip of the disk cell (D) on both wings, and the cubitus wing (Cu) wings branching in half.



(a) (b) (c)

Figure 1. The shape of Antenna on *C. trifenestrata*; (a) the female; (b) according to Borror, et al. (1992); (c) male

Figure 2. Stadium Imago *C. trifenestrata*; (a) Female imago; (b) Male imago; (c) three small transparent windows on the front wing

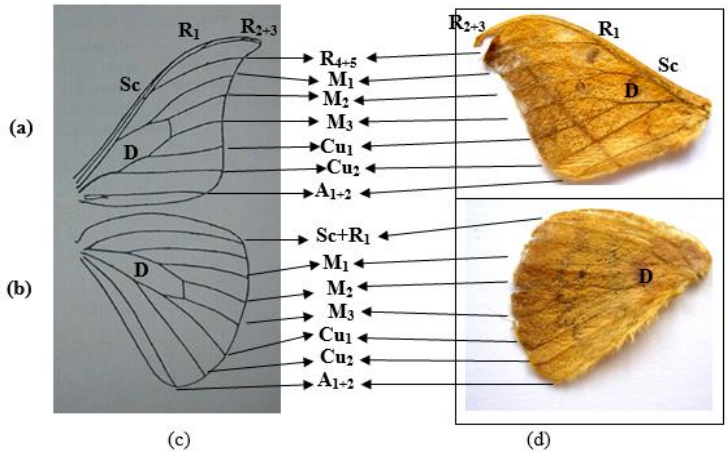


Figure 3. Skeletal patterns on the wings of *C. trifenestrata*; (a) anterior wing; (b) posterior wings; (c) according to Pinratana & Lampe (1990); (d) samples identified. Notes: D = Disk Cell; R = radial wing frame; Sc = subcosta; M = Frame of media wings; Cu = Cubitus; A = anal wing frame.

3.2 Morphology of *C. trifenestrata*

Morphological observations were conducted by looking at and comparing samples of *C. trifenestrata* obtained from the seven sampling sites based on wing color differences, wing span length, wingspan, and body size. The observation of morphological characters in *C. trifenestrata* samples showed a varied morphological appearance in each population (Table 2).

Based on Table 2, it can be seen that there are a color variation in wing size, and body size. Of the 29 samples studied, most of the samples were individuals of the female gender of 25 individuals with wing colors varying from brown (D-2, D-4, M-4, M-5, W-4, T-2, P-2, P-3, K-1), light brown (KT-2, M-1, K2), dark brown (KT-1, KT-3, KT-4, D-1, D-3, D-5, M-2, W3), yellowish brown (M-3), oranged brown (W-2), to reddish brown (KT-5), while in male individuals there is similarity of wing color that is brownish yellow (W- 1, T-1, T-3, P-1). These color and wing variations are individual variations that may occur due to random parental mating, resulting in varying breeds. Different populations, especially at different locations, have different levels of variation. These differences result from selection, genetic drift, mutation, and migration processes, leading to both genotype and phenotypic variations

shown by individuals in the population as well as between populations. Differences in variation can be seen from morphological characters to molecular characters such as DNA sequences, amino acids and proteins (Anggreini, 1998; Campbell et al., Frankham, et al., 2002).

Wings size and body size also show variation. The largest stretch of wings and body size is found in samples taken from Godokidul village, Wonogiri, which is a W-4 sample with a wingspan length of 10 cm and a body length of 3.3 cm. While the widest wingspan and smallest body size were found in P-3 samples (obtained at 3rd instar, then moved to be maintained in the laboratory) taken from Ngawen Village, Gunung Kidul, the length of the wingspan of 5 cm and the length of the body 1.6 cm. This is probably due to the process of moving the maintenance site, so it is suspected that P-3 individuals use most of their energy to perform the adaptation process. This is in line with what Moczek (2010) says, Individuals use most of their energies to adapt to new environmental conditions, and to maintain their homeostasis balances.

Based on the morphological observations of *C. trifenestrata* samples used, there appears to be variations in morphology, in samples from different regions and feed plants. It is suspected that these variations are individual variations that may be caused by differences in age, gender, genotype expression and individual adaptive responses to the environment (Frankham et al., 2002; Indrawan et al., 2007).

Table 2. Morphology Variation of *C. trifenestrata*

No.	Sample Location	Sample Code	Wings Length (cm)	Body Length (cm)	Colour
1.	Village Karang Tengah, Bantul	KT-1 ♀	8	2,2	Wings: dark brown Thorax: oranged-brown
		KT-2 ♀	7,3	2,5	Wings: light brown Thorax: yellow
		KT-3 ♀	7,5	2,2	Wings: dark brown Thorax: orange
		KT-4 ♀	8,5	2,7	Wings: dark brown Thorax: reddish brown
		KT-5 ♀	8	2,4	Wings: reddish brown Thorax: orange
					

2.	Village Dlingo, Bantul	D-1 ♀	7,9	2,8	Wings: dark brown Thorax: oranged-brown
		D-2 ♀	6	1,5	Wings: brown Thorax: yellowish brown
		D-3 ♀	9	2,7	Wings: dark brown Thorax: oranged-brown
		D-4 ♀	6,5	2	Wings: brown Thorax: yellowish brown
		D-5 ♀	8,2	3	Wings: dark brown Thorax: yellowish
					
3.	Village Jurang-ombo, Magelang	M-1 ♀	7,8	2,7	Wings: light brown Thorax: yellow
		M-2 ♀	8	2,2	Wings: dark brown Thorax: orange
		M-3 ♀	8,5	2,4	Wings: yellowish brown Thorax: yellow
		M-4 ♀	8,2	3,2	Wings: brown Thorax: yellow
		M-5 ♀	8,6	2,8	Wings: brown Thorax: orange
					
4.	Village Godo-kidul, Wonogiri	W-1 ♂	8,5	2,4	Wings: brownish yellow Thorax: brownish yellow
		W-2 ♀	9,3	2,5	Wings: oranged brown Thorax: orange
		W-3 ♀	7	1,7	Wings: dark brown Thorax: reddish brown
		W-4 ♀	10	3,3	Wings: brown Thorax: orange
		W-5 ♀	8	2,2	Wings: dark brown Thorax: orange
					
5.	Village Playen, Gunung Kidul	T-1 ♂	7	2,2	Wings: brownish yellow Thorax: brownish yellow
		T-2 ♀	8,3	2,1	Wings: brown Thorax: orange
		T-3 ♂	7	2	Wings: brownish yellow Thorax: brownish yellow
					

6.	Village Ngawen, Gunung Kidul	P-1 ♂	5,8	1,5	Wings: brownish yellow Thorax: brownish yellow
		P-2 ♀	6,8	1,6	Wings: brown Thorax: orange
		P-3 ♀	5	1,8	Wings: brown Thorax: yellowish
					
7.	Village Sidorejo, Sleman	K-1 ♀	8,7	2,1	Wings: brown Thorax: orange
		K-2 ♀	7,7	2,4	Wings p: light brown Thorax: yellow
		K-3 ♀	8,2	2,7	Wings: dark brown Thorax: reddish
					

3.3 Analysis of Amplification results of *C. trifenestrata* through ISSR Molecular Marker

The electrophoresis results of PCR-ISSR using 4 *primer*s resulted in a total of 91 DNA DNA bands. The number of polymorphic DNA bands and the monomorphic DNA bands as well as the percentage of polymorphism are presented in Table 3. Based on the results of electrophoresis, it shows that the amplified DNA bands produced by each *primer* have varying amounts. The resulting band difference occurs because each individual has a different nucleotide sequence. This is in line with what William (1990) has said, each individual has a different nucleotide sequence, so that some fragments in an individual are amplified while others are not. Amplification with an ISSR *primer* occurs only when two microsatellites, the same recurrent sequence, in reverse orientation, are located close enough for each other to allow sequences as to be amplified (Pharmawati, 2009). The DNA bands formed are considered as one character representing a DNA locus. DNA bands of the same size are considered as a single locus (Agisimanto & Supriyanto, 2007).

Overall, there are 21-24 DNA bands formed on each *primer* with a size between 200-3000 bp. The most DNA bands are formed by ISSR 6 *primers* between 250-3000 bp. Then, It is followed by ISSR 2 and ISSR 7 *primers* that make up 23 DNA bands of 200-2500 bp. The fewest *primers* that form DNA bands are the *primer* of ISSR 1 of 21 DNA bands, but all of the bands are polymorphic DNA bands. The average percentage of polymorphic DNA bands of DNA amplification using 4 *primer*s is 98.9%.

Table 3. The Number of DNA Bands Resulted from Amplification and Polymorphic Percentage

No	*Primer*	Nukleotida Order (5'-3')	Total Number of Bands	Number of Polymorphic Bands	Number of Monomorphic Bands	Polymorphic Percentage (%)
1.	ISSR 1	AGAGAGAGAGAGAG AGT	21	21	0	100
2.	ISSR 2	AGAGAGAGAGAGAG AGG	23	23	0	100
3.	ISSR 6	ACACACACACACAC ACACT	24	24	0	100
4.	ISSR 7	GGAGAGGAGAGGAG A	23	22	1	95,6
Total			91	90	1	395,6
Average			22,75	22,5	0,25	98,9

A primer that gives the best amplification results that can form the highest number of DNA bands with the highest polymorphism level are the ISSR 1, ISSR 2, and ISSR 6 *primers*. A number of DNA bands and polymorphism level successfully formed by ISSR 1, ISSR 2 and ISSR 6 *primer*s are the higher than the other successfully established by another *primer*.

3.4 Analysis of Grouping Results of *C. trifenestrata*

Based on a clustering analysis of all the molecular character data, the dendogram yields similar coefficients ranging from 0.60 to 0.93 (Figure 4). Dendogram of *C. trifenestrata* which were formed based on a molecular character in this research have coefficient similarity value of 0.60-0.93 or has similarity level between 60% -93%, and can be grouped into 2 large groups: group A and B (Figure 4). In both groups, it appears that most individuals of *C. trifenestrata*, both fror the Central Coral, Wonogiri, Dlingo, Magelang, Playen, Ngawen, or Kaliurang popula ions, are grouped and blended. These individuals clustered at a similarity level above 60% (0.60). This result is greater than what has been conducted by Neog (2010) which states that individuals will cluster in two clusters at a similarity level of 50%, and can be expressed as the same species. The degree of similarity of *C. trifenestrata* in this study refers to molecular character.

In group A, it appears that most individuals of *C. trifenestrata* are grouped and blended. while in Cluster B, it consists of KT-2 and KT-5 derived from Karang Tengah Village and D-4 and D-1 individuals originating from Dlingo Village. morphologically, individuals of KT-2 and KT-5 have the same wing color of reddish brown, unlike KT-1, KT-3, and KT-4 individuals who have dark brown wings. So from the dendogram, it can be seen that KT-1, KT-3, and KT-4 are separated from KT-2 and KT-5, although the samples are taken at the

same location. It can also be seen from coefficient similarity value where KT-1, KT-3, and KT-4 group, have high coefficient similarity value that is above 8,0, whereas coefficient similarity of KT-1, KT-3, and KT -4 with individuals of KT-2 and KT-5 are lower than 8.0 so it looks separated on the dendogram.

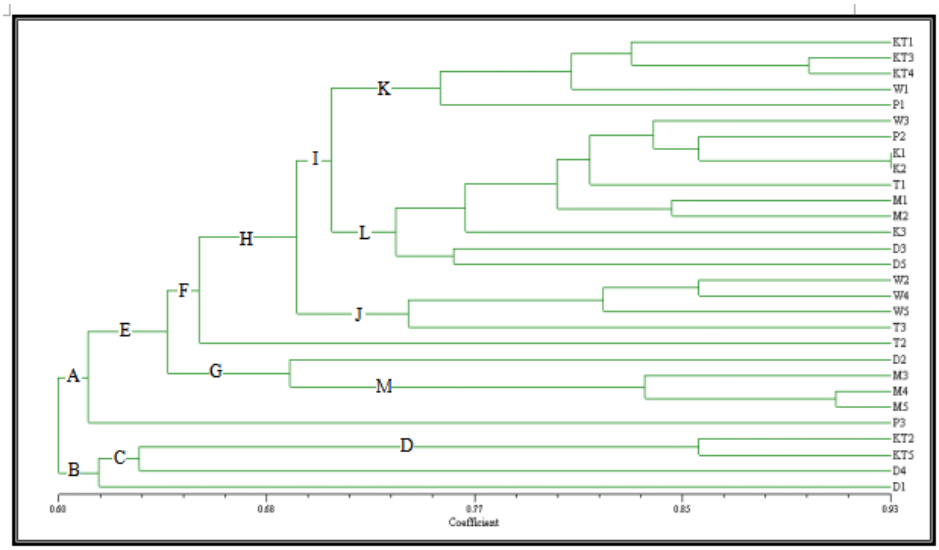


Figure 4. Dendogram of *C. trifenestrata*

In group A, P-3 individuals appear to separate from other individuals. This can be correlated to the coefficient value of similarity that is seen that individual P-3 has a somewhat lower coefficient similarity than other individuals, which is below 6.9, so the dendogram looks separated. Moreover, from the morphology it is known that P-3 individuals are individuals with the widest wingspan width compared to other individuals.

Individuals in group A formed group E on a coefficient of similarity of 61% (0.61). Group E formed two groups, F and G at 64% similarity (0.64). Group F includes 20 individuals consisting of individuals from the villages of Karang Tengah, Dlingo, Godokidul and Ngawen, Playen, Jurangombo, and Sidorejo, while group G consists of two individuals from Dlingo Village, D-2 and individuals from Jurangombo Village, namely M-3, M-4, and M-5.

In group F, it formed group H, which is divided into two groups: group I and J. In group A, individuals of W-2, W-4, and W-5 group, can also be seen from their high coefficient similarity value that is above 8.0. Group I is divided into two groups: K and L. Group K consists of individuals from Karang Tengah Village that are KT-1, KT-3, and KT-4 which have the same wing color morphology that is dark brown, as well as one individual from

Godokidul Village, which is W-1, and one individual from Ngawen Village which is P-1. These individuals are clustered because they have a high similarity coefficient value and are expected to have the same morphology as well as the same feed crop ie cashew nuts. Group L includes 9 individuals consisting of individuals from Dlingo Village (D-3 and D-5), Godokidul (W-3), Ngawen (T-1), Playen (P-2), Jurangombo (M -1 and M-2), and Sidorejo (K-1, K-2, and K-3). According to Frankham et al (2002), individuals with the same genotype can have different phenotypes, and otherwise individuals with the same phenotype can have different genotypes. This is due to the interaction between genotype and environmental factors. The statement is also reinforced by Jakaria et al (2001) who examined the interaction between genotype and environmental factors in *B. mori*, suggesting that differences in environmental conditions do not necessarily cause different genotypes in silkworms, since different environmental conditions may show the same genotype . Grouping into one cluster signifies a high degree of similarity between the DNA locus shown by the similarity coefficient (Yulita, 2013).

The similarity coefficient obtained by dendogram of 60% can be related to polymorphic locus percentage of 98.9%. The higher the polymorphic percentage indicates the higher the genetic variation that occurs. Therefore, it is believed that there is a high genetic variation in these *C. trifenestrata* individuals. Genetic variation can occur due to changes in DNA nucleotides. These changes may affect the phenotype of an organism or affect individual adaptation responses to a particular environment. In general, the genetic diversity of a population can occur due to mutations, recombination, or gene migration from one place to another (Suryanto, 2003).

Based on the dendogram, individual of *C. trifenestrata* from Karang Tengah, Wonogiri, Dlingo, Magelang, Playen, Ngawen, and Kaliurang populations can be assumed to be the same species. Besides, it can be presumed that the current *C.trifenestrata* was originally a large population. These large populations subsequently experienced habitat fragmentation due to the development of urban human civilization which became a limitation for these populations, thus indicating the existence of genetic variation in small populations that are the result of fragmentation (Frankham et al., 2002).

4. Conclusion

The result of DNA amplification using 4 *primer*s of ISSR resulted in a percentage of polymorphism of 98.9% indicating that the level of genetic variation between samples of

studied *C. trifenestrata* was high and characterized by variation of appearance frequency of DNA band on each polymorphic locus. Based on the dendogram that was formed, it showed that *C. trifenestrata* which were taken from seven sites have a close similarity. Further research needs to be done with more samples and use of other molecular markers, as well as a more diverse *primer* to see polymorphism.

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