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Genetic Variations and Phenetic Relationships of Hairy Bittercress (*Cardamine hirsuta* L.) Accession in Java based on *Inter-Simple Sequence Repeat*

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ABSTRACT

Cardamine hirsuta from the Family Brassicaceae is a potential plant that can be used as an object of comparative study with *Arabidopsis thaliana* (L.) Heynh. Plant comparative studies involving closely related species with similar traits are used to investigate the genetic pathways underlying morphological diversity within and between species. This comparative study approach is based on the natural variation of *C. hirsuta*. Genetic variation can be found at the molecular level, resulting in a more stable classification. Inter-Simple Sequence Repeat (ISSR) markers are frequently used to study the genetic variation of closely related species. The purpose of this study was to examine genetic variation using ISSR markers to determine variations and phenetic relationships of *C. hirsuta* accessions in Java (Indonesia). Molecular data were numerically analyzed with seven ISSR markers using the DNA fingerprinting technique, including polymorphism percentage and Polymorphic Information Content (PIC) value. The dendrogram construction uses the Jaccard coefficient with the UPGMA method, and the PIC uses the dominant marker equation. The results showed that the ISSR marker effectively estimated the variation of genetic accession of *C. hirsuta*, which was characterized by the degree of polymorphism formed. The seven ISSR markers formed 54 bands with an average polymorphism proportion of 58.70% and an average PIC value of 0.33. In the dendrogram based on molecular characters, 4 sub-clusters were formed at 92% similarity based on location, namely Cibodas (West Java), Kaliurang (Yogyakarta), Dieng Plateau (Central Java), and Senduro (East Java).

Keywords: *Cardamine hirsuta L.,Genotypic variation, ISSR, Phenetic analysis*

1. INTRODUCTION

Cardamine hirsuta L. is a species of the Brassicaceae family that is found throughout the world, including in Java, Indonesia [1]. It is widely known that *C. hirsuta* is closely related to *Arabidopsis thaliana* (L.) Heynh [2]. *C. hirsuta* and *A. thaliana* belong to lineage I in the Family Brassicaceae, along with *A. lyrata* and *Capsella rubella* and have complete genomes [3]. The close relationship between *C. hirsuta* and *A. thaliana* causes them to have similar characteristics, namely diploid species, small genome, short life cycle, capable of self-pollination, multiple offspring, and easy to cultivate on a large scale [4, 5].

In plants, the similarity of traits possessed by closely related species can be used to conduct comparative studies [3, 5]. The natural variation of *C. hirsuta* can be used as a comparative study object to investigate the genetic pathways underlying morphological diversity within and between species [3, 4, 5]. Genetic variations can be found at the morphological and molecular levels [6]. Morphological variations are frequently plastic and influenced by environmental factors [7], so molecular characters are needed for comparison data [8].

Molecular characters are able to detect the genetic variation of a species, resulting in a more stable classification system [9]. Inter-Simple Sequence Repeat (ISSR) is a type of molecular marker that is commonly used to study the genetic variation of closely related species [10]. ISSR markers are often used in variation identification research because they can produce high polymorphisms, identify variations at low taxonomic levels (genus and species), and generate dominant

markers [10, 11]. ISSR is a PCR-based technique amplifies DNA sequences between microsatellite regions in opposite directions [12]. The nucleotide length in ISSR markers is generally 16–25 bp in the form of di, tri-, tetra-, penta-nucleotide repeats targeting multiple genomic loci [13, 14].

ISSR molecular markers were used to identify genetic variation in the Family Brassicaceae such as *Brassica juncea*, *B. nigra*, *B. rapa*, and *B. arvensis* [15], *A. thaliana* natural populations in China [9], and *Brassica oleracea*, *B. carinata*, *B. napus*, and *A. thaliana* of the Columbia ecotype [16]. Taxonomic studies on genetic variation of *C. hirsuta* based on molecular characters in Indonesia have not been carried out. Previous research in the form of plant inventory data in Yogyakarta [17] and ethnobotany data in Kalimantan [18]. The research aimed to analyze genetic variation based on ISSR molecular markers to determine variations and phenetic relationships of accessions of *C. hirsuta* in Java (Indonesia).

2. MATERIALS AND METHODS

2.1. Plant materials

Table 1. Collection of Plant Samples

No.	Accession Code	Location	Altitude (m asl)
1.	CIB-001	Cibodas	1100
2.	CIB-002	Cibodas	1150
3.	CIB-003	Cibodas	1200
4.	KAL-001	Kaliurang	900
5.	KAL-002	Kaliurang	902
6.	DNG-001	Dieng Plateau	2080
7.	DNG-002	Dieng Plateau	2079
8.	DNG-003	Dieng Plateau	2090
9.	SEN-001	Senduro	2100
10.	SEN-002	Senduro	2124

In this study, a total of ten *C. hirsuta* accessions were used. *C. hirsuta* accessions were collected from four locations in Java, namely Cibodas (West Java), Kaliurang (Yogyakarta), Dieng Plateau (Central Java), and Senduro (East Java) using the exploration method. The authors marked the sampling location using the topographer mobile application. A sample of 2-3 populations was randomly selected from each location with different altitudes. Plant samples were taken as many as 3-5 individuals from different clumps as repetition. The total number of samples in this study was ten, with three from Cibodas (West Java), two from Kaliurang (Yogyakarta), three from the Dieng Plateau (Central Java), and two from Senduro (East Java). (Table 1). The study was conducted from December 2020 to June 2021.

2.2. DNA extraction, PCR amplification, and electrophoresis

The Genomic DNA Mini Kit Plant was used to isolate DNA from fresh leaf samples (GeneAid Biotech Ltd. Taiwan). The ratio of spectrophotometer readings at 260 nm/280 nm wavelength was used to determine the purity of DNA. PCR reactions were carried out in 25 µl containing 12.5 µl Bioline My Taq HS Red Mix, 2 µl ISSR Marker, 8.5 µl Nuclease Free Water, and 2 µl DNA template. Seven marker sets of UBC markers were used for ISSR analysis based on the study of Barth *et al*. [19], and Maraş Vanlıoğlu *et al.* [15] are listed in Table 2.

Table 2. ISSR markers used in this research

Marker	Sequence	*Annealing* Temperature (˚C)	*Size range* (bp)
UBC814	$(CT)_8A$	45	220-2500
UBC826	$(AC)_8C$	55	120-1200
UBC827	$(AC)_8G$	55	150-1300
UBC830	$(TG)_8G$	55	120-1350
UBC834	$(AG)_8YT$	49-52	190-1000
UBC835	$(AG)_8YC$	50-54	140-1200
UBC845	$(CT)_8RG$	48-52	230-2000

The following PCR profiles were used for each marker in a Bio-Rad thermocycler: one cycle pre-denaturing for 5 minutes at 94°C, followed by 35 cycles of denaturing for 1 minute at 94°C, annealing for 1 minute at recommended ISSR marker temperature, elongation for 1,5 minutes at 72°C and post elongation for 7 minutes at 72°C. A total of 5 µl of PCR product (ISSR) were separated by electrophoresis on 2% agar gel in 1.0x TBE buffer containing 4 µl FloroSafe DNA stain and running at 100 volts for 30 minutes using Electroporator. DNA ladder as much as 2 µl (EasyLadder I) was loaded to estimate the sizes of ISSR markers in base pairs. Gel Documentation visualized DNA bands.

2.3. Scoring and analysis

Based on the ISSR molecular marker, each fragment that appears was scored as present (1) or absent (0). The binary data matrix of ISSR was compiled by the seven markers of ten *C. hirsuta* accessions. Similarity coefficients were calculated with the Jaccard Coefficient. Based on similarity index data, the cluster method algorithm, Unweighted Pair-Group Method Using Arithmetic Average (UPGMA), was used to construct the dendrogram using Multivariate Statistical Program (MVSP) software version 3.2. The percentage value of each marker's polymorphism and PIC (Polymorphic Information Content) were calculated using the Microsoft Excel program. The PIC value is calculated using the dominant marker equation, namely PICi = 2fi (1-fi), where fi is the frequency of the amplified allele

(band appears) and (1-fi) is the frequency of the unamplified allele (the band does not appear) [20].

3. RESULTS AND DISCUSSION

3.1. Analysis of polymorphism by ISSR

The resulting ISSR profiles of seven markers for ten accessions of *C. hirsuta* are shown in Figure 1. The seven markers produced reproducible polymorphic bands ranging from 250 to 2000 bp, with 54 amplified bands. Table 3 shows the amplified polymorphic bands and the percentage of polymorphic bands produced by each marker. The total bands produced 54 bands, of which 31 (57.40%) are polymorphic bands, and 23 (42.60%) are monomorphic bands. The highest percentage of polymorphisms was produced by marker UBC 826 with 80%, while the lowest percentage was produced by markers UBC 814, UBC 830, and UBC 835 with 50%. The analysis results showed that the level of polymorphism of all markers was quite high, with an average polymorphism percentage of 58.70%. The results are in accordance with Fang & Roose [12] that the ISSR marker can produce high polymorphism. Polymorphisms in a genome, one of which is caused by deletions and insertions, can cause genetic variations within species [21].

The most significant PIC value was generated by UBC 814 and UBC 845 markers with 0.43, while the lowest was generated by UBC 826 with 0.25 (Table 3). PIC represents the effectiveness of markers in showing polymorphisms of a population that depend on the number and frequency of alleles [22]. The ISSR marker is the dominant marker, so the maximum PIC value is 0.5 [23]. The PIC value close to 0.5 indicates that the marker is more informative and effective [22, 23].

The amplified band size ranged from 250-2000 bp (Table 3). The band size produced in this study is slightly different from the band size in previous studies, where the band size ranges from 120-2500 bp [15, 19]. The UBC 814 marker resulted in a lower band size, while the UBC 826, UBC 827, UBC 830, and UBC 835 markers were higher than previous studies. The UBC 834 and UBC 845 markers have nearly the same band size. This is because the ISSR marker in this study can also be used for different species in the Family Brassicaceae, such as *A. thaliana*, *B. juncea*, *B. nigra*, *B. rapa*, and *B. arvensis*.

3.2. Genetic variation of C. hirsuta

The similarity matrix between accessions based on the Jaccard Coefficient is shown in Table 4, with the UPGMA cluster analysis method forming a dendrogram as shown in Figure 2. The dendrogram revealed that 86%

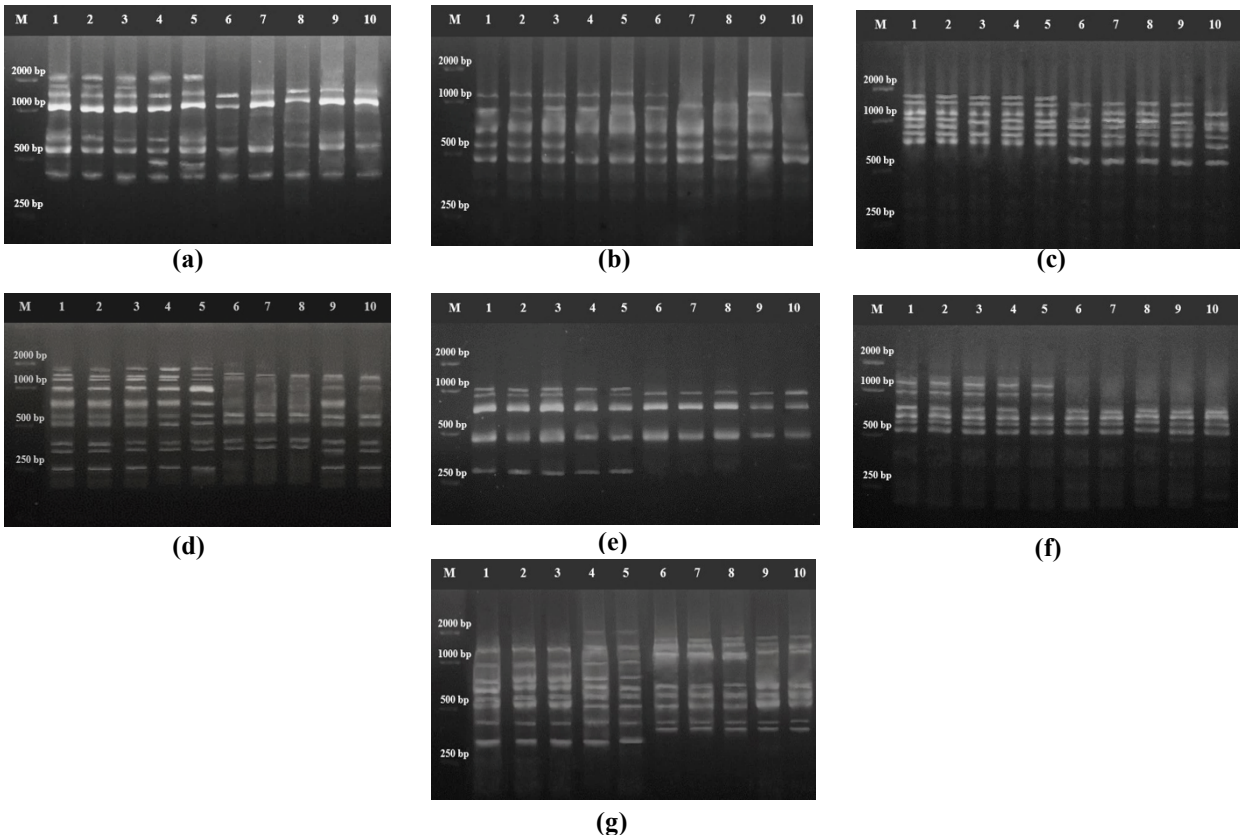

Figure 1. ISSR marker profiles of *C. hirsuta*: **(a)** UBC 814 **(b)** UBC 826 **(c)** UBC 827 **(d)** UBC 830 **(e)** UBC 834 **(f)** UBC 835 **(g)** UBC 845

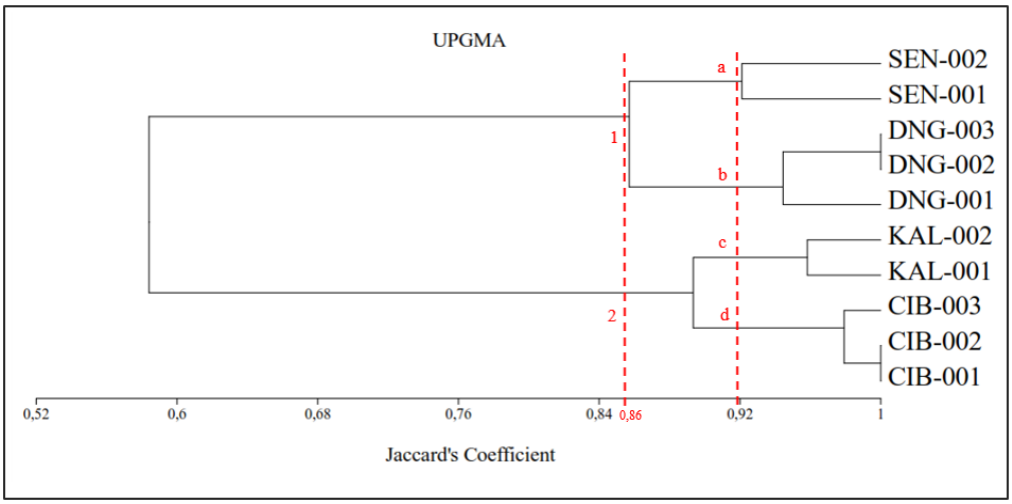

Figure 2. Dendrogram of *C. hirsuta* accession phenetic relationship based on molecular character (Note: CIB = Cibodas, KAL = Kaliurang, DNG = Dieng Plateau, SEN = Senduro

of the *C. hirsuta* accessions formed two major clusters based on the phenon line., Cluster I and Cluster II, with a similarity matrix ranging from 0,584-1,000.

The dendrogram of accessions of *C. hirsuta* from four locations in Java on separation with a phenon line of 0.86 or 86% formed two main clusters, namely Cluster 1 and Cluster 2. Cluster 1 included all accessions from Senduro (East Java) and the Dieng Plateau (Central Java), while Cluster 2 included all accessions from Yogyakarta and Cibodas (West Java). The clusters formed each form a sub-cluster at 0.92 or 92% phenone line separation. Cluster 1 forms sub-cluster A originating from Senduro, East Java (SEN-001 and SEN-002) and sub-cluster B originates from the Dieng Plateau, Central Java (DNG-001, DNG-002, DNG-003). Cluster 2 forms sub-cluster C originating from Kaliurang, Yogyakarta (KAL-001, KAL-002) and sub-cluster D originating from Cibodas, West Java (CIB-001, CIB-002, CIB-003).

Accessions with codes SEN-001 and SEN-002 (sub-cluster A) had a similarity value of 92.10%. In comparison, accessions DNG-002 and DNG-003 had a similarity value of 100% and formed a sub-cluster with DNG-001 (sub-cluster B) with a value of similarity 94.40%. Accessions with codes KAL-001 and KAL-002 (sub-cluster C) had a similarity value of 95.80%. In comparison, accessions CIB-001 and CIB-002 had a similarity value of 100% and formed a sub-cluster with CIB-003 (sub-cluster D) with a value of similarity of 97.90%. The overall similarity value is in the range of 58.40%–100%.

The clustering result based on molecular characters are slightly different from morphological characters. Accessions with codes DNG-002 and DNG-003 and CIB-001 and CIB-002 merged based on molecular characters but separated based on morphological characters [24]. This is because the morphological characters that separate the accessions are quantitative characters and the color of plant organs. These characters are plastic characters that environmental factors can influence due to adaptation to different habitats [6, 7].

C. hirsuta has low genetic variation because it is a self-compatible annual plant [4]. *C. hirsuta* has a self-pollinating autonomous system with low crossover rates inside and outside its home area [25, 26]. In addition, species in the genus *Cardamine* are plants that produce cleistogamous flowers [4, 27]. Cleistogamous flowers are made with a cleistogamy mating system. Cleistogamy means a closed flower structure with an obligate self-pollination system [27].

Various environmental factors can influence cleistogamous flower production in plants such as light intensity [28], photoperiodism [29], availability of water

Table 3. Polymorphic band markers from ten *C. hirsuta* accession

No.	Marker	Σ ISSR fragment	Σ polymorphic fragment	% polymorphism	PIC	Fragment size
1.	UBC 814	8	4	50,00	0,43	440–2000 bp
2.	UBC 826	5	4	80,00	0,25	470–1250 bp
3.	UBC 827	8	5	62,50	0,27	480–1700 bp
4.	UBC 830	10	5	50,00	0,32	250–1700 bp
5.	UBC 834	5	3	60,00	0,34	350–1000 bp
6.	UBC 835	6	3	50,00	0,30	500–1300 bp
7.	UBC 845	12	7	58,33	0,43	300–2000 bp

Table 4. ISSR analysis revealed a similarity matrix between *C. hirsuta* accession.

	CIB-001	CIB-002	CIB-003	KAL-001	KAL-002	DNG-001	DNG-002	DNG-003	SEN-001	SEN-002
CIB-001	1,000									
CIB-002	1,000	1,000								
CIB-003	0,979	0,979	1,000							
KAL-001	0,900	0,900	0,880	1,000						
KAL-002	0,900	0,900	0,880	0,958	1,000					
DNG-001	0,596	0,596	0,577	0,547	0,547	1,000				
DNG-002	0,596	0,596	0,577	0,547	0,547	0,944	1,000			
DNG-003	0,596	0,596	0,577	0,547	0,547	0,944	1,000	1,000		
SEN-001	0,654	0,654	0,635	0,604	0,604	0,872	0,872	0,872	1,000	
SEN-002	0,596	0,596	0,577	0,547	0,547	0,842	0,842	0,842	0,921	1,000

[30], and availability of nutrient [31]. *Cardamine cokaiensis* Yahara, for example, can produce cleistogamous flowers with reduced lateral stamens when seeds are subjected to prolonged cooling in Japan [27].

The advantage of cleistogamous flowers is that plants can still reproduce even though there are few or no pollinators. In some species, this type of flower production requires less energy, so the energy allocation can be diverted to produce more seeds with higher fitness. Consistent processes can eliminate recessive alleles in the population. Reproductive weaknesses in cleistogamous flowers are decreased genetic variation, increased genetic drift, and competition between offspring scattered around the parent plant [32].

Genetic variation of *C. hirsuta* could be analyzed using ISSR markers with a quite high percentage of polymorphism (50-80%). Cluster analysis with dendrogram showed that *C. hirsuta* formed four sub-clusters grouped based on the sampling location.

AUTHORS' CONTRIBUTION

The research was created by T.A., P., and B.D.S. TA gathered and analyzed data, as well as wrote the manuscript. P and BDS reviewed, revised, proofread, and oversaw the complete manuscript.

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