The Use of ISSR markers for clustering sesame genotypes based on geographical origin

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Abstract. Sesame (Sesamum indicum) germplasm in Indonesia is very diverse with morphological characteristics that looks very similar. Morphological characteristic is affected by the environment which has resulted into data inconsistencies. Genetic variations in Sesame germplasm markers such as Inter Simple Sequence Repeats (ISSR) could be used to analyze the genetic relationship. This study aimed to determine the genetic relationship between 81 accessions of sesame plants using ISSR markers. The method used in this study is descriptive qualitative with a sample of 81 sesame accessions. The primers were three ISSR primers, namely I2, T21, T22, and one RAPD primer, namely S1, as a comparison primer to the three of those. The analysis produced 29 DNA bands which 100% are polymorphic bands. Through phylogenetic analysis, the sesame collections clustered into seven major groups. There were clear separation of some accessions based on their geographical origin. ISSR markers can identify sesame germplasm diversity and provide helpful information for sesame breeding and germplasm management programs.

Keywords: Sesamum indicum, germplasm, clustering, phylogeny

1. Introduction
Sesame (Sesamum indicum) is an important and ancient oilseed crop belonging to Tubiflorae, the family Pedaliaceae [1]. Sesame seed contains high oil content (46 – 50%). The seed is rich of natural antioxidants such as sesamolin, sesamin, and sesame. It also has more than 80% unsaturated fatty acids and 20% proteins [2,3]. Sesame seeds and oil has many purposes. Sesame seed is for the production of paste, salads, and various food formulations. The oil is markedly different from other vegetable oil due to its high nutritional and therapeutic values [4]. The market demand for sesame seed and oil is continuously increasing because of the health benefits of sesame seed and oil [5]. Despite the increasing demand, the productivity of sesame is continuously decreasing. It is of great concern and that is caused mainly due to high yielding varieties is not available yet [6]. Sesame cultivars still have undesired characters including seed indehiscence, indeterminate growth habit, and non-uniform capsule ripening. That characters results in a very weak seed yield (300-400 kg/ha) [5,7,8]. In Indonesia, the sesame production is continuing to decline and the sesame consumption is provided by importing the commodity [9].

Genetic diversity in germplasm collections is a requirement for a breeding program to create high-yielding sesame varieties. Genetic diversity could be measured based on phenotype and genotype variation. There were several reports on morphological variation in sesame [6,10]. Assessment of genetic diversity based on morphological markers is affected by the environmental effect. Molecular-
based identification using Polymerase Chain Reaction (PCR) could be alternative and complement for identifying sesame germplasm genetic diversity [11]. One of the molecular markers to analyze plant genetic diversity is Inter Simple Sequence Repeats (ISSR). ISSR involved using micro-satellite sequence order that spread across the genome as primers in a polymerase chain reaction to produce multi-locus markers [12,13]. Researchers reported studies of diversity in sesame over the world using ISSR [13,14]. ISSR markers could detect the level of genetic diversity and group sesame collections.

Indonesian sesame collection is derived from a different locations in Indonesia. The collection also consisted of introductions from other regions (India, Australia, China, and South America) [10]. The diversity of germplasm collection was already assessed by morphological variation and used as material for breeding programs for creating high yielding varieties [10,15,16]. However, the report of genetic diversity and relationships based on morphological markers among Indonesian collection is not available yet. Geographical separation is usually considered as a vital parameter to collect germplasm even if it does not always predict genetic differences [13]. The aim of this study was to assess the genetic diversity and the relationship between sesame germplasm collections using ISSR markers.

2. Materials and method

2.1. Plant materials and DNA extraction

The plant materials used in this study were 81 sesame collections from Indonesian Sweeteners and Fiber Crops Research Institute (ISFCRI) (Table 1). Twenty seeds of each accession were germinated in a sand medium in a plastic bag and placed in a greenhouse. The germination process was carried out in Plant Breeding Department – ISFCRI. DNA extraction, amplification, and electrophoresis took place at Molecular Biology Laboratory - ISFCRI.

Genomic DNA extracted from the leaves from a healthy seedling using GeneAll exgene Plant SY mini kit (General bio-system, Korea). DNA quality was checked on 0.6 % agarose gels in TBE1x and electrophoresis at 70V for 45 min using 1 Kb DNA Ladder.

| Accession code | Origin | Accession code | Origin | Accession code | Origin |
|----------------|--------|----------------|--------|----------------|--------|
| Si-1           | No prior information | Si-60  | East Java | Si-68  | Sulawesi |
| Si-2           | No prior information | Si-69  | East Java | Si-70  | Sulawesi |
| Si-66          | No prior information | Si-31  | Central Java | Si-6   | South America |
| Si-67          | No prior information | Si-39  | Central Java | Si-64  | South America |
| Si-3           | East Java | Si-40  | Central Java | Si-7   | Australia |
| Si-13          | East Java | Si-41  | Central Java | Si-8   | Australia |
| Si-14          | East Java | Si-43  | Central Java | Si-9   | Australia |
| Si-15          | East Java | Si-44  | Central Java | Si-10  | Australia |
| Si-16          | East Java | Si-45  | Central Java | Si-11  | Australia |
| Si-17          | East Java | Si-46  | Central Java | Si-12  | Australia |
| Si-18          | East Java | Si-47H | Central Java | Si-33  | Asia (India) |
| Si-19          | East Java | Si-47P | Central Java | Si-35  | Asia (India) |
| Si-20          | East Java | Si-48  | Central Java | Si-36  | Asia (China) |
| Si-21          | East Java | Si-49  | Central Java | Si-50  | Asia (China) |
| Si-22          | East Java | Si-52  | Central Java | Si-51  | Asia (China) |
| Si-23          | East Java | Si-53  | Central Java | Si-71  | Asia (China) |
| Si-24          | East Java | Si-54  | Central Java | Si-73  | Asia (China) |
| Si-25          | East Java | Si-55  | Central Java | Si-63  | Asia (Japan) |
| Si-26          | East Java | Si-56  | Central Java | Si-76  | Asia (Thailand) |
| Si-27          | East Java | Si-77  | Central Java | Si-62  | Afrika |
| Si-32          | East Java | Si-4   | West Java | Si-65  | Afrika |

Table 1. List of 82 sesame germplasm and information of their origins.
2.2. ISSR markers and DNA amplification

The primers used in this study were three ISSR primers and one RAPD primer (Macrogen). The PCR reaction was performed using 20 µl reaction mixtures containing 1 µl (20 ng) genomic DNA, 4 µl 5x FIREPol Master Mix (Solis BioDyne), 1.2 µl ISSR/RAPD primer, and 13.8 µl nuclease-free water. The amplification was conducted using Thermal cycler (Bio-Rad T100™ Thermal cycler) with the PCR profile as follows: pre- denaturation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 39 - 56°C for 45 s, extension at 72°C for 1 min; and final extension at 72°C for 5 min. The PCR products were separated by electrophoresis (Bio-Rad) on 2% agarose gels staining with Gel red™ Nucleic Acid (Biontium) in 1x TBE buffer for 55 min at 85V. The gel is visualized using Gel Documentation (KETA GLX).

2.3. Scoring and data analysis

A data matrix was created by scoring ISSR and RAPD bands from gels as 1 and 0 for their presence and absence, respectively. The percentage of polymorphism was calculated from the number of polymorphic bands divided by the total number of bands. Polymorphic information content (PIC) characterizes the primers for their ability to differentiate the accessions. PIC was calculated. [17], as:

\[ PIC_i = \frac{1 - \sum j P_{ij}^2}{n - 1} \]

Where \( j \) is the primer concerned, \( n \) is the size of \( i \) bands, and \( P_{ij} \) is the frequency of marker I revealed by the primer \( j \) through the band sum.

The genetic diversity parameters were measured for allele number and expected heterozygosity/gene diversity (He). Genetic differentiation among populations was estimated using analysis of molecular variation (AMOVA) using GenAlEx [18]. A phylogenetic tree reveals the genetic relationship among the accessions using Nei’s genetic distance [19]. Measurement of genetic diversity parameters and genetic distance use Power Marker 3.25 [20]. A circular unrooted neighbor-joining tree with 1000 bootstrap replication was drawn using Mega 6.0 [21].

3. Results and discussion

3.1. Marker polymorphism and genetic diversity

The three ISSR primers and one RAPD primer amplified 29 bands with 7-8 bands for each primer (table 2, figure 1). All primers used in this study produced polymorphism (100%). The polymorphism information content (PIC) of the primers used ranged from 0.2109 (I2) to 0.3646 (T22), with an average of 0.2987. The PIC reveals the degree of the ability of the primer to differentiate the genotypes [14]. The PIC value of 0.2987 (almost 0.3) showed low to moderate ability of the primers to differentiate the Indonesian sesame collections. The PIC of the marker with values ranging from 0 to 1 and locus having PIC values near to 1 are more desirable [22,23]. However, the PIC of the primers used in this study is higher than that reported a PIC of 0.169 using 7 ISSR primers [14].

The polymorphism, PIC value, and genetic diversity parameters among Indonesian sesame collections are shown in table 3. The polymorphism of the ISSR marker among the populations ranged from 17.24% (African subpopulation) to 96.55% (East Java subpopulation), with an average of 49.52%. Kumar et al. [24] reported polymorphism of 24.71% (European subpopulation) to 92.55% (Indian subcontinent). The PIC values among the subpopulations showed accordance with the polymorphism value. The collection from East Java had the most allele numbers and diversity values,
as indicated by the highest value of gene diversity and PIC. Whereas the collection originated from Nusa Tenggara, Sulawesi and South Africa had the lowest diversity parameters value. These results might be due to the sample size of each subpopulation. The subpopulations from East Java, Central Java, Australia, and Asia had a bigger sample size than the others. Some researchers also reported that the population and the research sample influenced the allele numbers and the percentage of polymorphic loci [24,25,26].

Overall, the genetic diversity of Indonesian sesame collections showed a low value, as indicated by low gene diversity (0.1954±0.0791). This result was also reported by Kim et al. [7] for Korean sesame accessions, Mekonen et al. [27] for Ethiopia sesame accessions, and Kumar et al. [24] for worldwide sesame accessions using ISSR markers.

Table 2. Marker attribute information of 3 ISSR primers and one RAPD primer tested among 81 sesame accessions.

| Primers | Sequences (5’ – 3’) | Bands size (bp) | Total number of bands | Polymorphism (%) | PIC\(^a\) |
|---------|---------------------|-----------------|-----------------------|------------------|----------|
| I2      | (AG)\(_n\)YT        | 150 – 1000      | 7                     | 100              | 0.2109   |
| T21     | (AC)\(_n\)T         | 300 – 900       | 7                     | 100              | 0.2776   |
| T22     | (AC)\(_n\)C         | 350 – 800       | 7                     | 100              | 0.3643   |
| S1      | TGCCGAGCTG           | 150 – 800       | 8                     | 100              | 0.3039   |
|         |                     |                 | 29                    | 100 %            | 0.2987   |

\(^a\)Polymorphic information content

Figure 1. ISSR and RAPD banding patterns of 9 sesame accessions. Left lane – 100 bp DNA ladder, 11-19 denote accession code Si-11 to Si-19. a) primer I2; b) primer T21; c) primer S1 and d) primer L-1. Need to add the bands size in the marker line.
Table 3. Genetic diversity parameters among the subpopulation of Indonesian sesame collections.

| Population          | Sample size | Polymorphism (%) | PIC^a | Allele number | He^b |
|---------------------|-------------|------------------|-------|---------------|------|
| No information      | 4           | 48.28            | 0.1568| 1.4828        | 0.1983|
| East Java           | 25          | 96.55            | 0.2576| 1.9655        | 0.3193|
| Central Java        | 18          | 62.07            | 0.1594| 1.6552        | 0.1939|
| West Java           | 3           | 37.93            | 0.1311| 1.3793        | 0.1686|
| Nusa Tenggara       | 3           | 20.69            | 0.0715| 1.2069        | 0.0920|
| Sulawesi            | 3           | 27.59            | 0.0834| 1.2414        | 0.1073|
| South America       | 2           | 37.93            | 0.1293| 1.3448        | 0.1724|
| Australia           | 6           | 72.41            | 0.2250| 1.7241        | 0.2835|
| Asia                | 9           | 79.31            | 0.2478| 1.7391        | 0.3133|
| Africa              | 2           | 17.24            | 0.1568| 1.2424        | 0.1207|
| Released varieties  | 6           | 44.83            | 0.0905| 1.4483        | 0.1801|
| Mean                |             | 49.52            | 0.1540| 1.4984        | 0.1954|
| SD                  |             | 25.35            | 0.0645| 0.2526        | 0.0791|

^a Polymorphism Information Content  
b Expected heterozygosity/Gene diversity

3.2. Genetic differentiation and relationship

Analysis of molecular variance (AMOVA) reveals the genetic differentiation in sesame collections. The results showed high significant genetic differentiation (P<0.001) as indicated by a PhiPT/F_ST =0.292. (F_ST <0.05, low; 0.05≤ F_ST<0.15, medium; F_ST≥0.15, high [28]). The obvious significant differentiation was also shown the variation among groups that accounted for 29% of the total variation (Table 4). The variation among groups in this study is higher than that reported by other study (3%) [24]. The higher result might be due to the differences in the set of the populations of sesame germplasm in both studies.

Table 4. Analysis of molecular variance (AMOVA) for 81 accessions analyzed as eleven groups based on geographical regions.

| Source          | Df  | SS     | MS     | Est.Var. | %     | F-statistic | P-value |
|-----------------|-----|--------|--------|----------|-------|-------------|---------|
| Among groups    | 10  | 151.987| 15.199 | 1.670    | 29    | ^aPhiPT=0.292 | 0.001   |
| Within groups   | 70  | 283.593| 4.051  | 4.051    | 71    |             |         |
| Total           | 80  | 435.580| 5.721  |          | 100   |             |         |

^a Measure of genetic differentiation among subgroups to the total populations

We constructed a circular unrooted neighbor-joining tree using a frequency-based distance method to know the relationships of sesame accessions under study (Figure 2). The tree clustered the accessions into seven groups with mixed accessions as shown in Table 5. Group 1 mainly consisted of accessions from Central Java and East Java. Indonesian sesame released varieties clustered in group 2 and 3. Whereas group 4 mainly consisted of accessions from East Java and one accession with no prior origin information. Probably this accession also originated from a region in East Java. Group 5 consisted of accessions from Nusa Tenggara and Sulawesi and two accessions from West Java and one accession from South America. Group 6 consisted of an admixture from Asia, Africa, and Australia, whereas group 7 consisted of accessions from Asia, Australia, West Java, and East Java.

There was a clear separation of some accessions based on geographical origin. All accessions from
Central Java were clustered in group 1. All accessions from East Java were also clustered together in group 4, except accessions from the Nganjuk region clustered in group 1. The grouping of accessions from East Java and Central Java clustered together since the locations are adjacent and located on the same island. Singh et al. [22] also reported a similar pattern of mixed grouping of sesame accession originated from adjacent locations in India and stated that there might be an exchange of breeding materials across the regions. Surprisingly, we found that the accession from West Java separated from accessions from Central Java and East Java, although they are in the same island, namely Java island. Instead, the West Java’s accessions clustered together with the accessions from Nusa Tenggara and Sulawesi that had further distance and located in different island. This result suggested that there was an exchange of breeding materials between regions that had more distance. However, to prove this notion, we need to conduct further research with more germplasm samples.

The ISSR markers in this study could also be used to assign the accessions with no prior information to the representative groups, i.e., S1-66 and Si-67 that joined group 1 with accessions from Central Java and Si-2 that joined group 4 with East Java accessions. The diverse accessions from Indonesia with accessions from other countries showed that geographical separation did not generally result in greater genetic distances, possibly because sesame has been introduced into many countries and materials from widely separate locations have been exchanged [7].

**Table 5.** List of the members of the group of sesame accessions as viewed in neighbor-joining tree.

| Group | Origin of the accessions | Accession number | Total accessions |
|-------|--------------------------|------------------|------------------|
| 1     | Central Java             | 18               |                  |
|       | East Java                | 6                |                  |
|       | Asia (China and Thailand)| 2                | 29               |
|       | Australia                | 1                |                  |
|       | No information           | 2                |                  |
|       | East Java                | 4                |                  |
|       | Released varieties (SBR1, SBR4, Winas 2)| 3|                  |
| 2     | Australia                | 2                | 12               |
|       | South America (Venezuela)| 1                |                  |
|       | Asia (China)             | 1                |                  |
|       | No information           | 1                |                  |
|       | Released varieties (SBR2, SBR3, Winas 1)| 3| 4               |
| 3     | East Java                | 1                |                  |
|       | East Java                | 13               | 14               |
|       | No information           | 1                |                  |
|       | Nusa Tenggara           | 3                |                  |
|       | Sulawesi                | 3                | 9                |
| 4     | West Java                | 2                |                  |
|       | South America (Paraguay)| 1                |                  |
| 5     | Asia (China, India, Japan)| 3|                  |
| 6     | Brown                    | 2                | 6                |
|       | Australia                | 1                |                  |
|       | Asia                     | 3                |                  |
| 7     | Australia                | 2                | 6                |
|       | East Java                | 1                |                  |
|       | West Java                | 1                |                  |
**Figure 2.** Unrooted NJ tree showing a genetic relationship among sesame collections. The tree was constructed based on Nei’s genetic distance with 1000 bootstrap replications. Each color of branches represents the groups: G1 – G7. Each color of the circle at the end of the branch shows the origin as stated in Table 1.: Red = East Java, Green = Central Java, Light blue = West Java, Blue = Nusa Tenggara, Light green = Sulawesi, Yellow = South America, Purple = Australia, Tosca = Asia, Brown = Africa, and Pink = Released varieties.
4. Conclusions
In the present study, ISSR markers could reveal genetic diversity and relationships among Indonesian sesame collections. Based on these markers analysis, Indonesian sesame accessions were categorized as having low genetic diversity. Furthermore, the phylogenetic tree showed a clear separation of some accessions based on their geographical origin. This study’s information is valuable to properly manage sesame germplasm, and benefits Indonesian sesame breeding programs.

Acknowledgment
The authors would like to thank the Indonesian Agency for Agricultural Research and Development for research funding through DIPA/2019 of the Indonesian Sweeteners and Fiber Crops Research Institute.

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