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Research Article

Genetic Variations of Fennel (*Foeniculum vulgare* Mill.) Based on Inter-Simple Sequence Repeats (ISSR) Marker

Anshary Maruzy*, M. Bakti Samsu Adi, Devi Safrina, Aditya Dwi Permana Putra, Nur Rahmawati Wijaya, Dyah Subositi
Research center for Pharmaceutical Ingredient and Traditional Medicine, National Research and Innovation Agency, Bogor, West Java, Indonesia
*Email: anshary.maruzy@brin.go.id

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ABSTRACT
Foeniculum vulgare Mill., commonly known as fennel, has been utilized by Indonesian community for various purposes, from culinary to medicinal applications. The primary constituent of its chemical composition is the essential oil belonging to the monoterpene class, comprising more than 80% of its content. Genetic information plays a crucial role in determining the identity of a plant species. This information can also serve as a fundamental basis for conservation and plant breeding purposes. The aim of the research to determine the genetic diversity of F. vulgare. This study utilized fennel from six different locations. DNA examination of fennels used the Inter-Simple Sequence Repeats (ISSR) marker. Cluster analysis of binary data on DNA fragment scores used Jaccard similarity coefficient and UPGMA method as basis. The average polymorphism was 86.11%. The study indicated that the genetic variability of F. vulgare samples from the six locations fell within the moderate category. The results of this study open opportunities for further research in finding specific molecular markers to identify F. vulgare with its potential adulterant species.

Keywords: *Accession; DNA fingerprinting; Fennel; Foeniculum vulgare; Genetic mapping.*

Introduction

Foeniculum vulgare Mill. commonly known as fennel, which belongs to the Apiaceae family, is widely cultivated in Indonesia because of its benefits [1]. The community uses fennel in cooking, medicine, and as a topical oil [2]. Fennel seeds have nutritional value, phytochemicals, and pharmacological properties so it is widely used in culinary and medicinal purposes [3]. Almost all parts of the fennel plant, from roots to seeds, are used for various purposes [4].

Fennel contains essential oils with over 80% anethole and limonene [5], [6]. The oils mainly composed of monoterpenes [7]. These essential oils can be found throughout plant parts [8], [9]. This oil can kill bugs, fight germs, ease depression, and prevent cancer [10]–[15].

In Indonesia, there are at least two types of plants called fennel. They are very similar in appearance: *F. vulgare* (fennel) and *Anethum graveolens* (dill) [16]. Because of these similarities, using morphological data for characterization often leads to errors [17], to avoid errors, the technique that can be used is genetic mapping. Genetic mapping of fennel can ensure the right type or species. Counterfeiting can also misuse this high level of similarity, which can cause disadvantages to users [18]. Knowledge of genetic maps is also essential in fennel breeding activities. Based on genetic information, we can search for superior

accessions and crosses between accessions, to increase productivity based on the mix of these featured traits [19]–[22].

ISSR marker was able to differentiate between cultivars of fennel could reveal the genetic diversity of fennel [23]. Moreover quantitative morphological characters, essential oil components and ISSR markers could reveal the genetic diversity of the varieties between and within fennel populations [24]. Several fennel genetic diversity studies have been carried out. One of related research is the dendrogram from morphological data revealed that genotypes of fennel from different origins occurred in different groups, but local genotypes collected from Turkey occurred in the same group and subgroup, and this data strengthens improvement plant breeding programs on fennel [22], [25]. Based on previous studies, there has never been a study that has determined the genetic diversity of fennel in several locations in Indonesia. This research aims to determine the genetic diversity of *F. vulgare*from five places in Indonesia, and apply the ISSR markers. The result of this research hopely may enhance the knowledge of fennel genetics in Indonesia.

Materials and methods

Materials

F. vulgare seed samples were taken from five locations in Malang, Karanganyar, Boyolali, Bandung, and Padang (Figure 1 and Table 1). The seeds were sown in the experimental garden in Tawangmangu as a germplasm collection. Molecular markers were employed to analyze genetic variation in *F. vulgare* samples. The materials used in ISSR marker amplification were 2 µl DNA template, 0.8 µl primer (Table 2), 12.5 µl master mix, and 9.2 µl distilled water.

Methods

Sample collection

The leaf sample neither too young nor too old were collected DNA analysis with ISSR markers. We used silica gel containers to store specimens with accession codes. The collection of leaf is taken by stationery, scissors, ziplock plastic, and silica gel. Each site has two samples gathered from different plants.

Table 1. List of *F. vulgare* accessions used for ISSR analysis.

No.	Code Accession	Location	Location altitude (m asl)	Annotation
1	JB1	Bandung	1,278	wild
2	JB2	Bandung	1,293	wild
3	SL1	Boyolali	1,500	cultivation
4	SL2	Boyolali	1,550	cultivation
5	TW1	Karanganyar	1,650	cultivation
6	TW2	Karanganyar	1,750	cultivation
7	MN1	Manoko (Bandung)	1,267	cultivation
8	MN2	Manoko (Bandung)	1,267	cultivation
9	BR1	Malang	2,098	wild
10	BR2	Malang	2,381	wild
11	PG1	Padang	1,053	wild
12	PG2	Padang	1,096	wild

DNA isolation

The DNA isolation process uses the DNA isolation kit protocol (Sigma GenElute Plant Genomic DNA Miniprep Kit). The quality of genomic DNA was checked qualitatively by the electrophoresis method. Spectrophotometric methods determine the purity and concentration of genomic DNA. To determine DNA purity, *F. vulgare* genomic DNA diluted 1,000X

was measured at absorbance wavelengths of 260 nm and 280 nm (λ260/280). Calculation of genomic DNA concentration uses the absorbance value of λ260 nm.

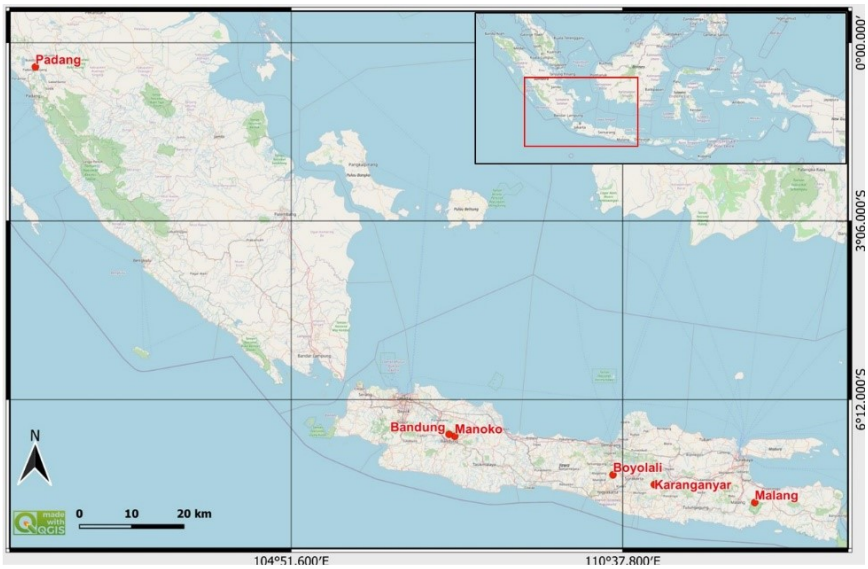

Figure 1. Map of *F. vulgare* sampling locations

Amplification of ISSR markers

The amplification procedure with the ISSR marker was 2 µl template DNA, 0.8 µL primer (25 ng), 12.5 µl Promega master mix, 9.2 µl distilled water to a volume of 25 µl. There are 6 primers used, primer PW2, PW3, TY2, TY5, E8 and E9, which have a sequence (5'-3') respectively: (GT)6CC, (AG)9C, (ACC)6G, (TG)8A, (CAG)5, and (CA)6GT. We used a Thermalcycler (BioRad C1000) with a pre-denaturation temperature of 95°C 3', denaturation temperature of 94°C 1', an initial annealing temperature of 50-51°C 5', extension temperature of 72°C 2', and final extension temperature of 72°C 8' for the

Data analysis

DNA fragments were scored manually as present (1) or absent (0) to generate binary data for cluster analysis. The Jaccard similarity coefficient and the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) method were used as basis for cluster analysis using the PAST version 4.13 program [26].

Results and Discussion

Figure 2 shows the amplification performed on 12 fennel accessions using six primer. The (GT)6CC primer produces PCR procedure to last 39 cycles. The mixture was then put into a Thermalcycler (BioRad C1000) with a predenaturation program of 95°C 3', denaturation 94°C 1', primary annealing 50-51°C 50", extension 72°C 2', final extension 72°C 8'. Afterward, the amplification results were seen through electrophoresis at a voltage of 85 volts, 400 mA, for 600 minutes on a 2% agarose gel containing 2.5-3 µl of SYBR-safe green. Electrophoresis used Thermo Scientific GeneRuler 100 bp plus DNA Ladder as a comparison. Visualization of electrophoresis results used UV light on the Gel Documentation System (BioRad XR+).

ten fragments measuring 250-1250 bp, and the (AG)9C primer generates four fragments measuring 600-1000 bp. The primer (ACC)6G produces five fragments measuring 350-900 bp and the primer (TG)8A produces three fragments measuring 800-1500 bp. The primer (CAG)5 gets 11 fragments measuring 100-925 bp, and the primer (CA)6GT produces five fragments measuring 300-900 bp. Amplification of 12 fennel accession genomes with six ISSR primers produced 38 DNA fragments (Table 2). The six ISSR primer used in this study brought out high

levels of polymorphism that vary from 66.66% to 100%. Six primers produced 38 DNA fragments ranging from 100 to 1.500 bp. The two primer in this study, ISSR PW3 and ISSR E8, with 5'-3'sequence (AG) 9C and (CAG) 5 respectively, yielded 100% polymorphism. With a polymorphism of 66.66%, the ISSR TY5 primer yielded the lowest results. The average polymorphism was 86.11% (Table 2). In addition, the average genetic similarity coefficient of *F. vulgare* accessions in this study was 0.522. Previousstudies employing molecular markers have classified the genetic variation of *F. vulgare* accessions as moderate. [27] classified the genetic similarity coefficient as 0.39 low, 0.7 moderate, and 0.92 high. According to [24], this species has a high degree of gene variety and a tendency to cross across, particularly in chromosomes, which significantly adds to population variability. The fennel ribosomal gene cluster is on a homologous pair. This moderate variation is possible, considering that fennel is an introduced plant in Indonesia. Humans were more influential at the beginning of their distribution and have now adapted locally [28].

Table 2. ISSR marker amplification results

No	Primers	Fragment length (bp)	No. of band	Polymorphic band (%)
1	PW2	250-1250	10	9 (90%)
2	PW3	600-1000	4	4 (100%)
3	TY2	350-900	5	4 (80%)
4	TY5	800-1500	3	2 (66.66%)
5	E8	100-925	11	11 (100%)
6	E9	300-900	5	4 (80%)
	Total		**38**	
	Average			**5.67 (86.11%)**

The dendrogram in Figure 3 shows the formation of two main clusters, namely clusters A and B, with a genetic similarity of 0.328. This dendrogram is very good at representing similarities between samples. The indication is that the cophenetic coefficient value is 0.92. The function of the cophenetic coefficient is to measure the distance in the classification of data sets and the efficiency of grouping techniques. Valued from 0 to 1, where if the coefficient value is higher (closer to 1), it means the grouping and distance are more accurate [29]. Cluster A includes eleven accessions divided into two sub-clusters, while cluster B only has one accession. The genetic similarity of the 12 *F. vulgare* accessions in this study ranged from 0.328 to 0.913, based on the Jaccard similarity coefficient (Table 3). Table 3 presents the correlation coefficient values between accessions from all research samples. Cluster B contains accession JB1, while Cluster A includes two subclusters, A1 and A2. Subcluster A.1 consists of accession JB2, while subcluster A.2 includes accessions TW1, TW2, MN1, MN2, SL2, SL2, BR1, BR2, PG1, and PG2. Accessions SL1 and SL2 have the highest similarity, with a similarity coefficient 0.913. Apart from that, accessions TW1-TW2 and PG1-PG2 are also grouped in one branch according to their location of origin. Some of the high levels of similarity in these accessions are due to low intraspecific variation in chromosome number and structure [30], [31]. Close locations and the same ancestral source result in the fertilization process of individuals in the population [32]. Fertilization contributes to population differentiation and indicates differentiation at different geographic and environmental scales [33], [34].

Meanwhile, the grouping of JB1 and JB2 accessions does not suit their same

location. They are not in the same cluster. Likewise, MN1 and MN2 are not similar to JB1 and JB2, even though they come from Lembang, West Java. Furthermore, the Malang accessions BR1 and BR2 do not belong to the same close branch. In this case, a similar location does not appear as a factor in grouping accessions. Several things that can cause this condition include landrace and impure [35], [36]. Other factors, such as the extensive structural variation of the mitochondrial genome and the polymorphism, have an influence [37], [38]. In addition, environmental factors such as water stress also affect different cultivars or accessions, and they cause various physiological and biological changes in fennel accessions or cultivars [39].

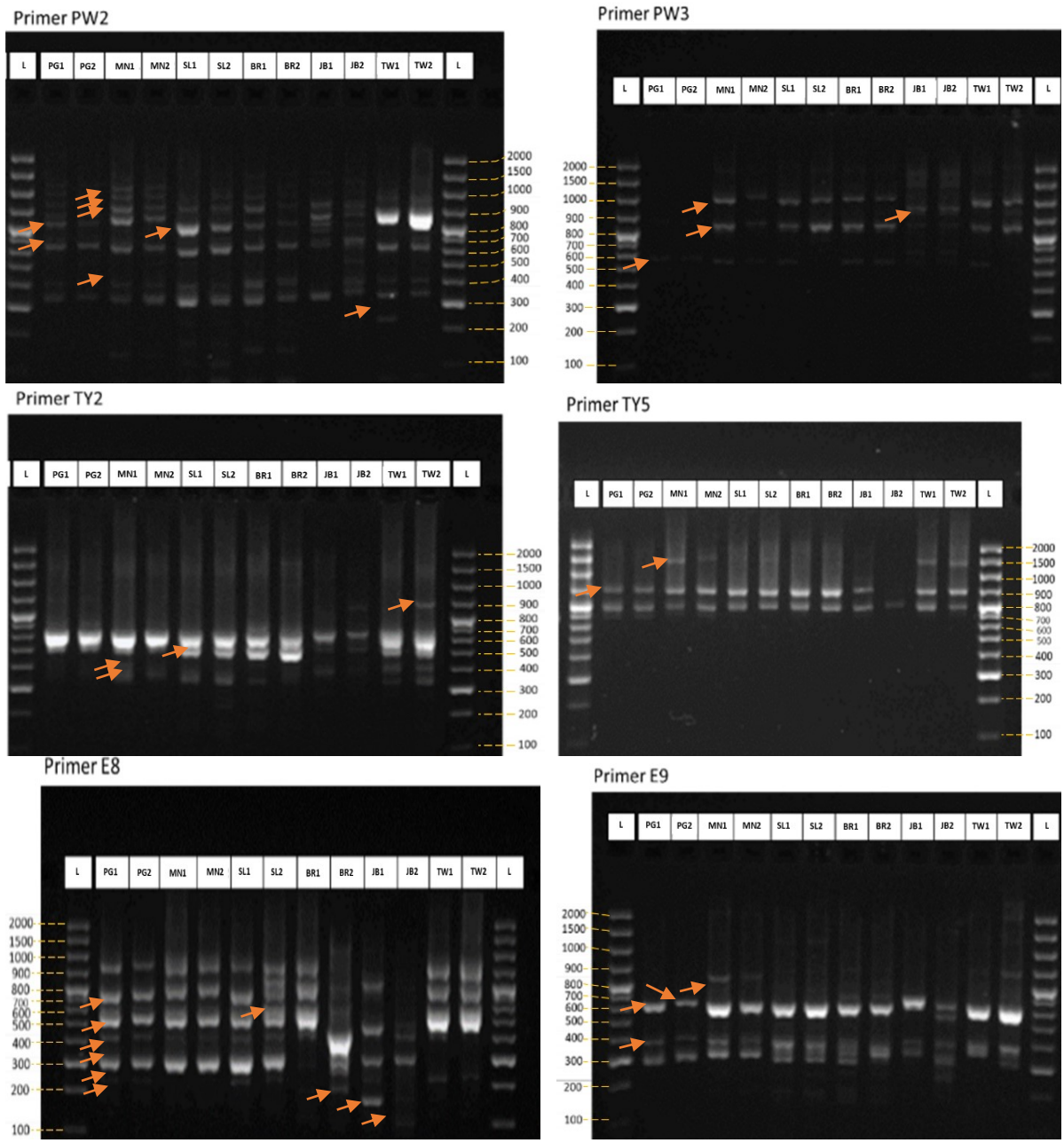

Figure 2. DNA fingerprinting of *F. vulgare* accessions showing the polymorphic presence of six primers. L= Marker, PG1= Padang 1, PG2= Padang 2, MN1= Manoko 1, MN2= Manoko 2, SL1= Boyolali 1, SL2= Boyolali 2, BR1= Malang 1, BR2= Malang 2, JB1= Bandung 1, JB2= Bandung 2, TW1= Karanganyar 1, TW2= Karanganyar 2.

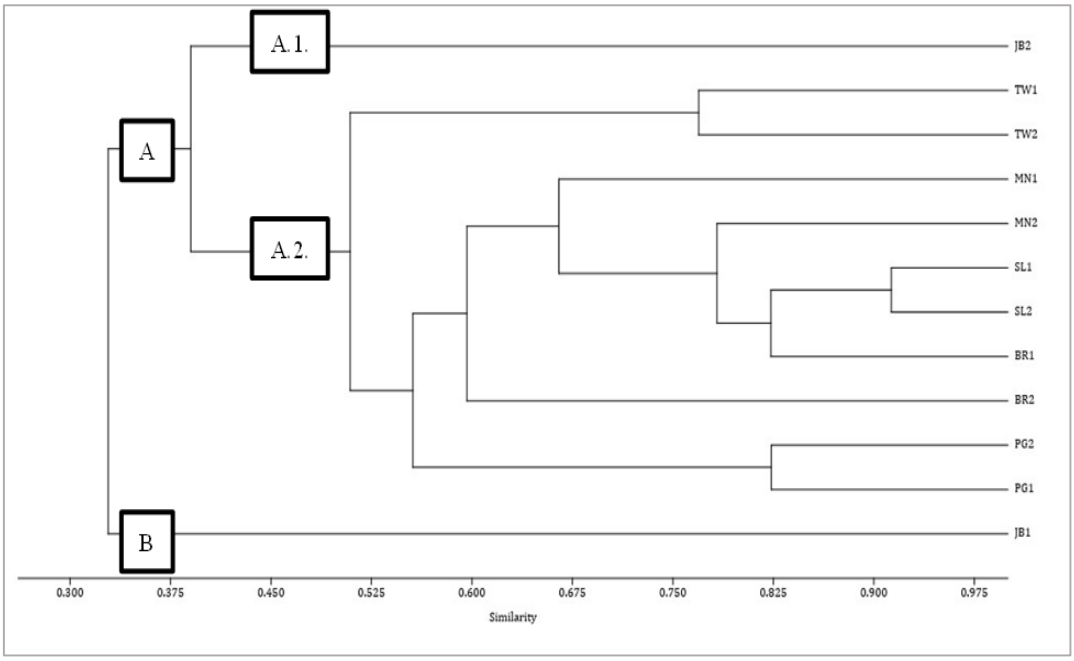

Figure 3. Dendrogram showing the grouping of *F. vulgare* accessions with a cophenetic correlation of 0.92.

Information on genetic variations of *F. vulgare* from Sumatra and Java, based on ISSR in this study, is newly published information. The information in the data supports standardizing raw materials for traditional medicine. It serves as a source of preliminary information about prospective accession cultivation for improved varieties. The clustering results in the dendrogram show that the JB1 and JB2 accessions are separated from other accessions which may show unique and distinctive genetic characters, and apart from that, the accessions with the codes TW, SL, and PG are also grouped separately and may have their own genetic uniqueness. However, in the future there needs to be morphological and phytochemical confirmation to confirm which accessions are superior morphologically, genetically and phytochemically. We can employ ISSR molecular markers for the molecular identification of *F. vulgare* to authenticate raw materials used in traditional medicine.

Conclusions

The dendrogram shows that the grouping of *F. vulgare* accessions is not entirely based on the same location of origin, but there are several accessions grouped according to location of origin, such as accessions with the codes TW, SL, and PG. Accessions that are not very clustered or separated from other accessions, such as JB1 and JB2, and two accessions that cluster according to location of origin, such as accessions with codes TW, SL, and PG can have the potential to become genetically unique accessions. *F. vulgare* samples from several locations in Sumatra and Java have moderate genetic variation according to ISSR marker analysis. The results of this study open opportunities for further research in finding specific molecular markers to identify *F. vulgare* with its potential adulterant species. This study, which shows evidence of genetic variation in *F. vulgare*, also provides a basis for the conservation and development of superior accessions.

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