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Molecular Characterization and Phylogenetic Relationships of Melon (*Cucumis melo* L. Meloni Cultivar) Based on Expression of *Andromonoecious* Gene

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ABSTRACT

Melon (*Cucumis melo* L.) is one of horticultural commodities with good economic prospect for melon farmers because of several advantages. 'Meloni' is result of crossing between 'Sun Lady-3' and 'PI 371795' by Genetics and Breeding Laboratory, Faculty of Biology, UGM. The 'Meloni' plant produces females flowers that expressed by *CmACS-7* gene and it can be expected that melon plants 'Meloni' included in the primitive cultivars, while 'Meloni' is a cultivation cultivar. The aim of this research is to study the relation of 'Meloni' kinship to primitive and modern cultivars. Characterization of the *CmACS-7* gene is begun by a total isolation process of RNA from a sample of melon plant flowers and detection using a specific primer. The cDNA bands obtained are sequenced and then phylogenetic analysis is performed based on *CmACS-7* gene expression using MEGA 7.0. Result of this study show that the presence of the *CmACS-7* gene in female and hermaphrodite flowers of 'Meloni', 'PI 371795', 'Sun Lady-3', and 'Hikapel'. Phylogenetic tree analyze show that 'Meloni' from female plant, 'PI 371795' and 'Sun Lady-3' have closely relation, while 'Hikapel' separated from other cultivars.

1. Introduction

Melon (*Cucumis melo* L.) is one of the horticultural commodities that has good economic prospects. Especially for melon farmers because most of the Indonesian people like melons very much because melon has many benefits. Moreover, the increasing of melon demand, melon fruit production is also increasing. Therefore, it is necessary to produce melon with highest quality, not only the resistant to disease but also has excellent fruit quality. The new and superior melons can be gained from crossing between two or more parental which also have superior qualities, so their qualities can be inherited.

The Genetics and Breeding Laboratory of Biology Faculty of UGM has conducted several crossing (hybridization) to produce new and superior melon cultivars. One of them is namely 'Meloni'. 'Meloni' is the result of a crossing between 'PI 371795' and 'Sun Lady-3'. Meloni's melon plant has the potential to compete with other cultivars which seeds are imported seeds. The shape of Meloni looks oval with a sweet, fragrant, and yellow color. However, 'Meloni' has male and female sex in one plant (*monoecious*), and the type of male flower and hermaphrodite in one plant (*andromonoecious*). The form of fruit which is produced by the two Meloni plants is different. Meloni's melon plant with the hermaphrodite and male flowers produces globular fruit shapes, while female and male Meloni melon plants produce elliptical fruit. The unique character of Meloni sees from the shape of the fruit which is elliptical, if the shape of the globular fruit is not a characteristic of 'Meloni'. The sex of flowers that express female flowers or hermaphrodites in melon plants is influenced by *andromonoecious* genes (*CmACS-7*). Active or not of *andromonoecious* genes (*CmACS-7*) will affect to the work of ethylene hormones. Ethylene is the main hormone that regulates the determination of sex in the melon plants flowers. Takashi and Jaffe (1984) plants with *gynoecious* sexual types produce more ethylene hormones when compared to *monoecious* plant sex types. The active *CmACS-7* enzyme is needed for the development of female flowers at monoecious, while the reduction

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of enzyme activity plays a role in formation of hermaphrodite flower in *andromonoecious* (Zhang *et al.* 2014).

CmACS11 and *CmACS-7* genes play a role in the development and formation of certain sex in flower organs. Both genes are members of the gene family 1-*aminoacyclopropane*-1 *carboxylix acid synthase* (ACS) which has an important role in ethylene biosynthesis pathways (Wang *et al.* 2002; Boualem *et al.* 2008, 2015). The interaction between the two genes namely *CmACS-7* and *CmWIP1* produces a series of sexual phenotypes. *CmACS-7* is expressed in the carpels to suppress the development of the stamen and *CmWIP1* epistatic against the *CmACS-7* by controlling the development of the carpel in where the *CmACS-7* is expressed. The expression of the *CmWIP1* gene causes carpel abortion and the lack of expression of *CmACS-7* suppresses the development of the stamen so, produce male flowers. The loss of mutations function in *CmWIP1* leads to the development of carpel and *CmACS-7* will be expressed on carpel to suppress the development of the stamen, so it will produce female flowers. If there is a loss of function mutations in the two genes, namely *CmACS-7* and *CmWIP1*, it will lead to the formation of carpels and stamen, so resulting that produce in hermaphrodite flowers (Zhang *et al.* 2014).

CmACS-7 is an *andromonoecious* gene which controls the development of the stamen on female flowers, while *CmWIP1* is a gynoecious gene which controls the development of male flowers and it loses the function of these genes which can produce female plants flowers (Boualem 2009; Martin *et al.* 2009). Ethylene biosynthesis begins with the production of *ACC* (*1-aminocyclopropane-1-carboxylicacid*) from *S-adenosylmethionine* (SAM) with the help of an ACS enzyme (*1-aminocyclopropane-1 carboxylic acid synthase*). *ACC*, which has been produced will be formed into ethylene by *ACC* oxidase enzyme (Boualem 2015). Therefore, this research aims to look at the phylogenetic relationship of 'Meloni' with some cultivars which are considered primitive or modern based on the *andromonoecious* gene (*CmACS-7*).

2. Materials and Methods

2.1. Sample Preparation

The sample collected when the generative phase, it happened when the flowers appear on melon plants. Samples which were taken were female flowers on 'PI 371795', 'Sun Lady-3', 'Meloni' and hermaphrodite flowers on 'Sun Lady-3', 'Meloni' and 'Hikapel'. The number of samples which is taken were six flower samples including female flowers and hermaphrodites. Samples which have been taken are then stored in aluminum foil and immediately conducted RNA isolation. The part of the flower which is isolated is the part of the flower crown for the hermaphrodite and female flowers, while the male flower is the whole flower.

2.2. RNA Isolation and PCR Amplification

RNA was isolated from flower samples with *andromonoecious* sexual types and *monoecious* sexual types. The samples were isolated as much as 0.1 gram according to RNA protocol isolation kit, the GeneJet Fermentas Thermo Scientific brand with minor modification. The total RNA which has been obtained, then firstly synthesized into cDNA. cDNA is synthesized using the Toyobo brand RT PCR kit which contains the reverse transcriptase enzyme. The RT-PCR method which was conducted using two stages RT-PCR. The PCR reaction was performed with the forward primer specific and reverse which is presented in Table 1. The cDNA was amplified in 25 µl of the reaction which contained cDNA template, a primary forward and reverse, sterile ddH_2O, My Taq™ Extract-PCR kit (Bioline Reagent, UK). PCR amplification was carried out using T100 Thermal Cycler (BioRad) with several stages, namely 95°C pre-denaturation along 5 minutes, followed by 40 times PCR cycles consisting of denaturation at 95°C along 30 seconds, annealing at 60°C along1 minutes and extensions at 72°C along 2 minutes, then continued the post extension/final extension along 10 minutes at 72°C, and the reaction was stopped at 4°C. PCR products were detected using 2% agarose gel through electrophoresis and amplicon which was sent to 1st BASE (Singapore).

2.3. Sequences and Phylogenetic Analysis

Sequence results in the form chromatograms which is edited become sequences with GeneStudio

Table 1. Specific primers for detection of the *CmACS-7* gene with PCR

Primer name	Oligonucleotide squence	Target size
Forward primer	5'-CCGGATGTCTAGCTTTACGC-3'	±215 bp
Reverse primer	5'-ACAACGGGCTCAAATTCATC-3'	

v. 2.2 (GeneStudio, Inc.). The sequences are aligned and constructed become phylogenetic trees, then see the genetic distance using the MEGA 7.0 program (Kumar *et al.* 2016). The phylogenetic trees construction using maximum likelihood and neighbor-joining then bootstrapping is performed with 1,000 replications.

3. Results

This research used six samples of three types *monoecious* melon cultivars ('PI 371795', 'Sun Lady-3', and 'Meloni') and *andromonoecious* types ('Sun Lady-3', 'Meloni', and 'Hikapel'). In addition, *Cucumis sativus* is used as an outgroup using nucleotide sequences from *GenBank*. DNA bands from *andromnoecious* gene amplification were detected in female flowers and hermaphrodite from several melon cultivars, namely from female flowers 'PI 371795', 'Sun Lady-3', 'Meloni', and hermaphrodite flowers from 'Sun Lady-3', 'Meloni', and 'Hikapel' with a DNA band size of ±215 bp. PCR amplification results were observed in 2% agarose gel by electrophoresis and showed a single band (Figure 1) it is performed on all flowers in all cultivars. The amplification results can be done by sequencing method.

The nucleotide sequence is aligned to know the homology level of the nucleotide sequence from the six samples and one sample as an out-group.

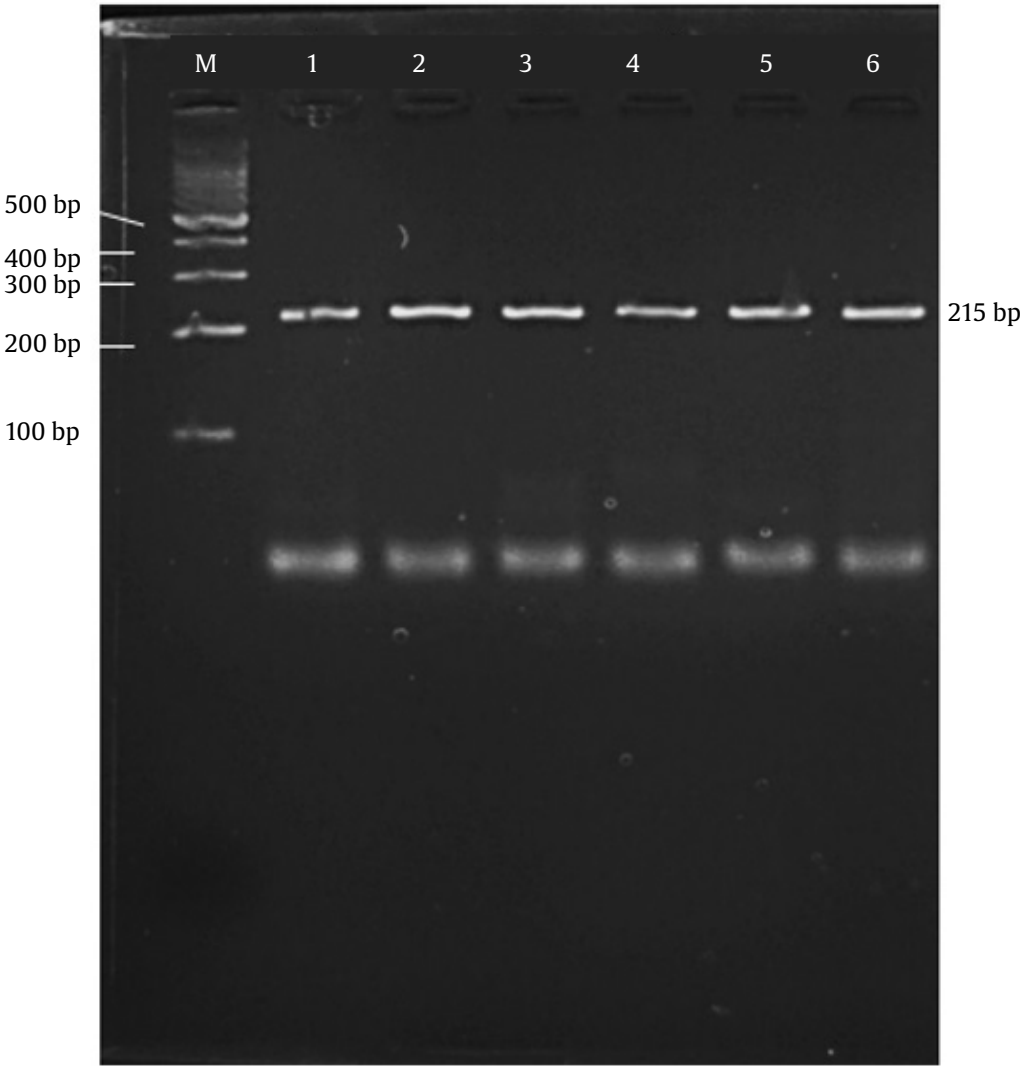

Figure 1. Results of *andromonoecious* gene amplification on female and hermaphrodite flowers in four melon plant cultivars with a length of ±215 bp. Description: (M) Marker 100 bp, (1) female flowers 'PI 371795', (2) female flowers 'Sun Lady 3', (3) female flowers 'Meloni', (4) hermaphrodite flowers 'Sun Lady 3', (5) hermaphrodite flowers 'Meloni', (6) 'Hikapel' hermaphrodite flowers

The alignment results show a gap of 11 gaps for all sample nucleotide sequences (including out-groups) and only 2 gaps has seen in melon cultivars. The construction of phylogenetic trees can be performed after the nucleotide sequence is aligned with the neighbor-joining method by repeating 1,000 times the Bootstrapping to support the analysis of phylogenetic trees. Moreover, it is also used to determine genetic distance based on the Kimura-2-parameter (K2P) model and reconstruct the maximum likelihood. Based on the construction results of phylogenetic trees of melon plants from various cultivars namely, 'PI 371795' female, 'Sun Lady-3' female, 'Meloni' female, 'Sun Lady-3' hermaphrodite, 'Meloni' hermaphrodite, and 'Hikapel' hermaphrodite to one clade (Figure 2). It shows that these cultivars are close relatives based on the andromonoecious gene (*CmACS-7*). When it is constructed with *Cucumis sativus* (outgroup), the melon cultivars separate from *Cucumis sativus*.

4. Discussion

This experiment shows that the *andromonoecious* (*CmACS-7*) gene found in melons and *Cucumis sativus* has a not too close relationship. Although separated or not in the same clade, the *CmACS-7* gene in melon is become homology which associated with the *CsACS-7* gene in *Cucumis sativus*. *Cucumis sativus* plants have three main genes that play a role in sex formation of flowers, namely Female (F), *Androecious* (a), and *Monoecious* (M). Female (F) controls females, FF has *gynoecious* characteristic, while *Androecious* (a) increases male genitalia, aaff genotypes are *androecious*, and *monoecious* (M) acts as stamen suppressants on shoots determined to develop carpel. Dominant alleles only allow the formation of female flowers without stamens, as well as male flowers, while for mm will form bisexual. It is different from the case in melons which are generally controlled by two main genes in determining gender, namely

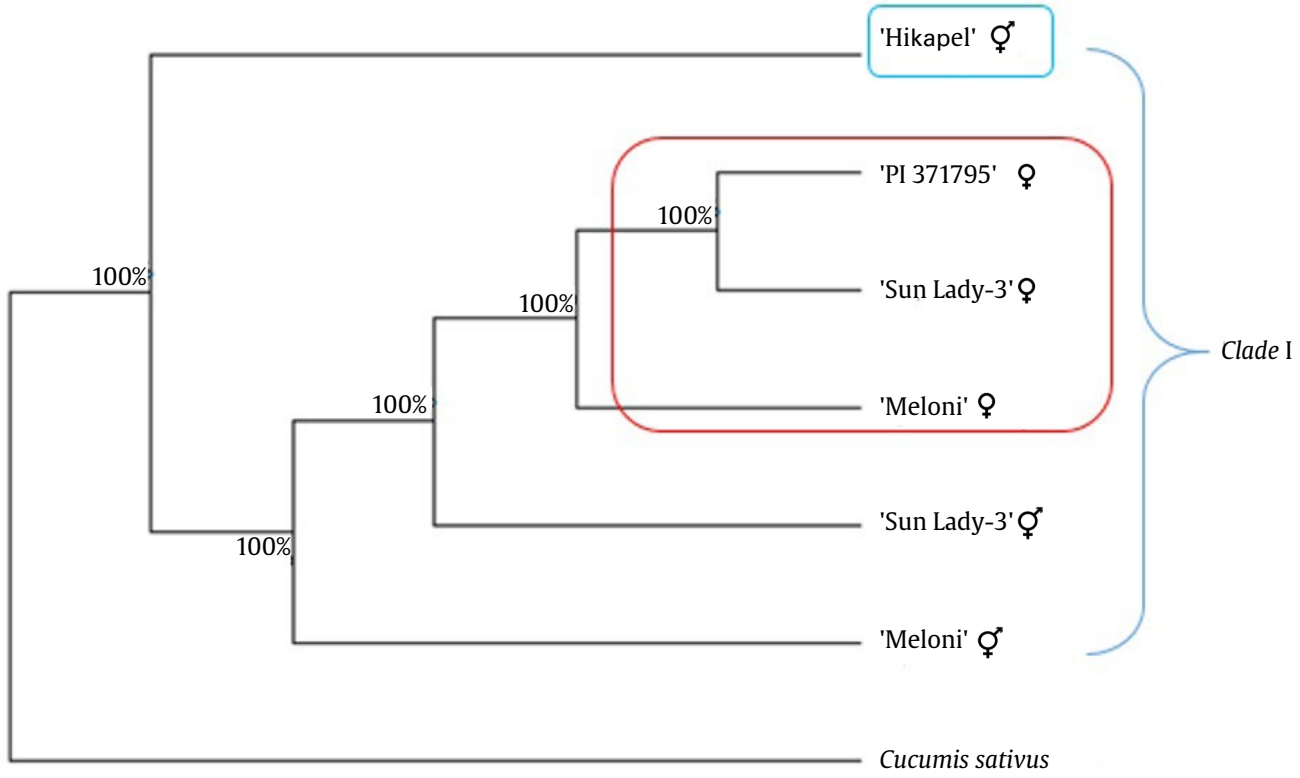

Figure 2. Phylogenetic trees based on the sequence of *andromonoecious* gene fragments (*CmACS-7*) in various melon cultivars and *Cucumis sativus* (outgroup)

andromonoecious (a) and *gynoecious* (g) (Boualem *et al.* 2009).

Previous research showed that the similarity between the *CmACS-7* and *CsACS-7* genes, namely the nucleotide sequence of DNA amplification had 3 exons and 2 introns. Moreover, based on phylogenetic trees which representing twelve ACS sequences Arabidopsis and cucumber showed between *CSACS-2* and *CmACS-7* having great similarities and tied to *AtACS-7*. It is possible that the *CsACS-7* is an orthology cucumber from *CmACS-7* (Boualem *et al.* 2009). The separation of *Cucumis sativus* as an out-group based on the *andromonoecious* gene is possible because between cucumber and melon has deviated more than 10 years ago. However, it is no implied that *andromonoecious* genes in cucumbers and melons are different and cannot combine when based on phylogenetic trees with *andromonoecious* genes from other species, such as Boualem *et al.* (2009). Indeed, *cucumis sativus* was chosen as an out-group in this research.

Melon cultivars which merge into one clade because of their low genetic distance, it can also be seen that the closeness between cultivars, in this case the phylogenetic relationship. Based on Figure 2 the female flower from 'PI 371795' is closely related to the female flower 'Sun Lady-3'. Furthermore, the female flower 'Meloni' is related to both of them. This is possible to happend because 'Meloni' is the result of a crossing between 'PI 371795' and 'Sun Lady-3'. 'PI 371795' only has two types of flowers in one plant, namely male and female flowers, while the 'Sun Lady-3' melon plant has two types of plants. The first 'Sun Lady-3' melon plant has female and male flowers in one plant, while the second has the hermaphrodite and male flowers in one plant. In conclusion, the *andromonoecious* gene (*CmACS-7*) in female 'Meloni' is closely related to both of them. The close relationship between female 'Meloni' and 'PI 371795' and 'female Sun Lady-3' is possible because the *CmACS-7* gene is derived from their parents.

Subsequently followed by 'Sun Lady-3' hermaphrodite and 'Meloni' hermaphrodite which is closely relation. It is possible because the gene that encodes the formation of the hermaphrodite genitals in the 'Meloni' melon which is a hermaphrodite and male flower in one plant derived from the hermaphrodite 'Sun Lady-3' broodstock. Meanwhile for 'Hikapel' which only has one sex, namely hermaphrodite and males in one plant which seems to be separated. It was possible because 'Hikapel' was already a more modern cultivar compared to other cultivars. In addition, it is also supported that 'Hikapel' is a descendant of the parental 'Aromatic Hikadi' and 'Sun Lady-3', which 'Sun Lady-3' also has the sex of hermaphrodite flowers.

Gender determination involves the *CmACS-7* gene by taking the role in ethylene biosynthesis at the early stages of the melon development pistil flowers, thus are triggering the capture of stamens during female flower development, and producing *monoecious* plants. Hormones are the main key in determining the sex of melons, in this case the dominant role is ethylene. Ethylene biosynthesis start with the conversion of *S-Adenosylmethionine* (S-Adomet) to *1-aminocyclopropane-1carboxylate* (*ACC*) with the help of *ACC* synthase enzyme. Then, *ACC* is oxidized to ethylene by *ACC* oxidase (ACO). Mutations in the *CmACS-7* gene reduce the ethylene production in pistil flowers which produce hermaphrodite and *andromonoecious* plants (Fuentes *et al.* 2017). The *CmACS-7* gene actively suppresses the development of stamens in female flowers. Mutations in the gene *1-aminocyclopropane-1-carboxylic acid synthase* (ACS), *CmACS-7* cause loss of ACS enzyme activity which catalyzes the rate-limiting step in ethylene biosynthesis. Ethylene is very needed and supportive for the formation of female flowers, if the ACS enzyme activity is disrupted due to mutations, the stamens which should be suppressed in female flowers which cannot be controlled. As a result the stamens will be expressed on the female flower which causes the formation of hermaphrodite flowers. In addition, some external factors can modify sex in melons, such as the environment, mineral nutrition, temperature, water regime, light intensity, photoperiod, mechanical trauma, mechanical trauma, and plant regulators application (Girek *et al.* 2013). High temperatures and long day conditions can encourage male flower formation, while low temperatures and short-day conditions encourage feminization (Yamasaki *et al.* 2005; Li *et al.* 2012).

ACS-7 gene is the first regulator which is found in melons, and the homologous cucumber is *CsACS-2*, whereas in watermelon is *CIACS-7*, and in the pumpkin the *CmACS-7* gene is homogeneous with *CpACS27A* (Boualem *et al.* 2008, 2009; Martinez *et al.* 2014; Ji *et al.* 2015; Boualem *et al.* 2016; Manzano *et al.* 2016).

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