Phylogenetic Relationship of Three Lettuce species (*Lactuca* spp.) based on their Molecular Profiles using RAPD Markers

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Abstract

***Lactuca* spp., Asteraceae, like: Green lettuce (L. *sativa*) is the most common type of lettuce grown, while the spiny (*L. serriola*) and wild lettuce (*L. virosa*)have not been cultivated so far in Indonesia. The current study explores those three species morphological and molecular characters. The data obtained are then used to elucidate the genetic relationship among the species. The genomic DNA was extracted from each sample and measured for their qualities and quantities. The extracted genomic DNA subjected to RAPD-PCR (Random Amplified Polymorphic DNA) usingeight primers as follows: OPF-1, OPF-2, OPF-3, OPF-4, OPC-11, OPAE-5, OPA-2, OPAM-7. The total number of DNA bands were evaluated for polymorphic or monomorphic patterns. The polymorphic bands were analysedusing the MEGA 6 software applying a maximum parsimony method to obtain the cladogram of the samples. The RAPD-PCR obtained a total of 91 amplicons, where 84 (92.3%) of the 91 amplicons were are polymorphic and 7 amplicons were monomorphic. Primers that produced the highest polymorphism are OPF-1, OPF-4, OPC-11, OPAE-5, OPA-2 (100%) and OPAM-7. OPAM-7.produced the highest number of DNA bands, 20 DNA bands).The relationship among these three species showed that L. *serriola* and L. *virosa* have the closest genetic distance with a bootstrap 100%. The cladogram following the maximum parsimony analysis grouped the species into two clads,i.e., clad I and clad II. Clad I consists of L. *serriola* and L. *virosa*, clad II consists of L. s*ativa* only**.

Key words: Asteraceae, genetic diversity, Lactuca, lettuce, phylogenetic tree, RAPD, Maximum Parsimony.

Introduction

In Indonesia, horticultures play a significant role, because of their high economic value in both local market and so export. In the meantime, the Indoneisa's demand of lettuce (*Lactuca spp.,*) is growing fast parallel with the presence of International chain restaurants like Mc Donald, and others, that serve this lettuce in their menus (Prasetyo & Lazuardi, 2019). On the other hand, Indonesia, also exported this crop to some countries as many as 47.920 tones in year 2018 and increased to 55.710 the following year, and so classified as need to be developed seriously for its high economic value (Samadi, 2019). Green lettuce, has been cultivated quite massively (*L. sativa*), however, it is different from the [(*L. serriola*) and (*L. virosa*)] (Abdul-Jalil, 2020). To grow well, the farmers cultivate this crop in a mixture of aged compost and other rich organic matter, in an area that gets abundance of sun kightwith a pH between 6.0 and 7.0. *Lactuca spp.,* belong to asteraceae that live mostly in a temperate zone (20^0C ± 2^0C) with about 12 H photoperiod and light intensity (400 µmol m^{-2} s^{-1}), colour spectrum (440-460 nm), temperature (20 ± 2 °C), and humidity (80 ± 5%). The nutritional solution in a hydroponic system must be carefully monitored, by checking certain essential parameters such as the following (average ideal values): pH (6.3 ± 0.4), electrical conductivity (1.8 ± 0.2 mS), dissolved oxygen (6 mg L−1), and temperature (18 ± 2 °C).

Most of the Indonesian farmers cultivate green lettuce to fulfill the market demands though this crop was originally from west Asia and America (Prasetio, 2013). In traditional medicament, some species of the Lactuca species such as the spiny lettuce (*L. serriola*) and wild lettuce (*Lactuca virosa*) are known to have medicinal compound and, therefore, were applied to cure some diseases (Mieslerova *et al*., 2013; Lebeda *et al*, 2021).

The genetic diversity of *Lactuca* genus might be detected through a *Polymerase Chain Reaction* (PCR) method, applying short primers called as *Random Amplified Polymorphic DNA* (RAPD) (Purnaningsih, 2013). This technique has proven to be a good molecular marker for analysis of genetic variability among the unknown plants (Istiqomah *et al*., 2016). However, this method is known as low reproducible method although it can still be addressed by setting a consistent PCR condition (Wijaya *et al*., 2009).

Information on genetic diversity between the green lettuce and its wild relationship is important in developing this plant for any purposes like vegetable crop and medical sources and further domestication of the wild lettuce ones (Yu *et al*., 2001; Khan *et al*., 2019); as well as ex situ conservation (El-Esawi*et al*., 2017; van Herwijnen & Manning 2017). The current study was aimed to profiling the genetic diversity of the green lettuce (*L. sativa*), spiny lettuce (*L. serriola*), and wild lettuce (*L. virosa*) following the morphological and molecular approaches.

Material and Methods

The current study used three different types of lettuce, which included green lettuce (*L. sativa*), spiny lettuce (*L. serriola*) and wild lettuce (*L. virosa*). The samples were initially described and identified for their morphological characters including their similarities and dissimilarities of their leaf, buds and costa. This step was done in the Laboratory of Plant Taxonomy. Meanwhile, the genetic diversity among them was done in the plant genetic laboratory by applying RAPD -PCR for their DNA bands polymorphims using 8 primers. Both laboratories belong the Faculty of Biology University of Jenderal Soedirman from September to October 2022. Following to the analyses of molecular characters among the samples, the data were then used to set up the cladogram of all samples an out group plant. Finally, the *Chrysanthemum morifolium*was clad as out group plants to make up them into two clads. The *C.morifoium*was chosen as out group of the samples because this plant belongs to the sam family Asteraceae

The RAPD-PCR used eight different primers namely: OPA-02, OPAM-07, OPC-11, OPF-1, OPF-2, OPF-3, OPF-4, and OPAM-5. For the PCR, the DNA on Lettuce were isolated using a GeneAid™ Plant DNA isolation mini kit. Following to this, the PCR was applied by mixing the solution of MyTaq PCR master mix, primer and nuclease-free water. The last step, visualization, was done in an agarose powder, mixed with TAE 1x buffer to make up a slab then added with loading dye and a 1 Kb DNA ladder of Thermofischer.

The morphological characters of *Lactuca* were observed in the Plant Taxonomy Laboratory but the molecular profiling was done in the Genetics and Molecular Biology Laboratory, bith are in the Faculty of Biology Unsoed from August to October 2022.

Design of the study

A random sampling technique was applied to obtain the samples of *Lactuca* spp. The samples were taken to the laboratory for validation and identification. Following to this, the samples were subjected for molecular analysis using a PCR technique.

Isolation of Genomic DNA

Genomic DNA of *Lactuca* spp. was isolated following to the protocol ofGeneAid™ plant

DNA isolation mini kit Lot 22605, as follows: 1 cm leaf was cut from each plant species then cleanse with 70% alcohol and dried in an open air before being grind. Add 400 µL GPX1 buffer and transfer the sample into 1.5 mL microtube then 5 µL RNAse to homogenize in 30 seconds and incubated for 15 minutes at 60°C and incubated for 3 minutes over the ice block. 100 µL GP2 buffer was also added to the tube containing the sample and homogenized. Sample was then transferred into 2 mL collection tube which completed with a filter column and centrifuged at 1000 x g for 8 minutes. Put them into a new 1.5 mL microtube. Add the GP3 buffer as much as 1.5 times the volume then transferred into a GD column to be centrifuged at 15000 x g fort 2 minutes. Add 400 µL W1 buffer and centrifuged at 15000 x g for 30 seconds then discarded the supernatant by flowing it slowly. Add 600 µL washing buffer into the GD column and centrifuged at 15000 x g for 30 seconds. After supernatant was thrown out, dry the GD column by centrifuging at 15000 x g for 3 minutes. GD column was set to a 2 mL collection tube, then add. 100 µL warmed elution buffer and left for 5 minutes and centrifuged at 15000 x g for 30 seconds.

Quantitative and qualitative measurement of the genomic DNA

For qualitative analysis, the genomic DNA was visualized using the agarose gel. The DNA was measured at λ 260 nm, with a purity ratio of λ 260 - λ 280 nm. The measured genomic DNA was then subjected PCR analysis using an RAPD primers.

RAPD-PCR

The total volume for PCR reaction was 12.5 µL, of 6.25 µL MyTaq PCR mix, 1 µL DNA template, 1 µL primer, and 4.25 µL. The DNA amplification was run in a thermal cycler peqlab primus 250, which was set as the following: 1 cycle pre denaturation at 95°Cfor 2 minutes. After this step, the cycler runs for 35 cycles of denaturation at 95°C for 15 seconds and elongation for 15 seconds. The annealing temperature was set up at 38°C for 15 seconds and elongation at 72°C for 10 seconds plus final elongation 1 cycle at 72^0C for 5 minutes.

Electrophoresis

Visualization of the genomic DNA and the PCR products was done on an agarose gel. Before being visualized, the agarose gel was subjected for electrophoresis.An electrophoresis tank filled with TE 1x was set up as follows: 80 volt, 280 milli ampere for 35 minutes.A1% agarose gel was used to check the quality of the genomic DNA, while 2% agarose gel was used for all PCR products run. A total volume of 12.5 uL, consisting of 6.25 uL PCR mix, 1 uL DNA template, 1 uL Primer, and 4.25 NFW was used on the agarose gel electrophoresis. The appeared DNA fragments on the agar gel of a particular primer were used to determine the polymorphisms pattern. The gel patternswere captured by the camera. The fragments are called as polymorphic when they have more than 95% fragments at the same distance. The presence or absence of the fragment was then scored using a binary score (0/1) where score of 1 indicated DNA fragment present on the agar gel while score of 0 indicate absent of DNA fragment.

Data Analysis

The scored data was used to determine genetic diversity among the three species,*L. sativa*, *L. serriola*, dan *L. virosa*. The resulting banding pattern was analyzed using the MEGA-6 software (Molecular Evolutionary Genetics Analysis) applyinga maximum parsimonymethod. The species grouping was based on the kinship.

Results and Discussion

Morphological Characterisation of the *Lactuca* spp.

The morphological characteristic is a prerequisite for identification of the lettuce plants before genetic diversity analysis and scoring the accession numbers (Bermawie, 2005). The results of morphological diversity

among the three lettuce plants is presented in Table 1.

Species *L. sativa* has specific character on its leaf like orbicular leaf shape, rounded leaf apex, smooth-dentate leaf margin, leaf hairs absent, *sessile* leaf petiole, light green leaf upper surface, light green leaf lower surface, rugose leaf surface, rough costa lower surface, the colour of costa is white, many bud number on stem base (Figure 1).

Table 1: Morphological differences of *Lactuca sativa,L. serriola*, and *L. virosa.*

Species	*Lactuca sativa*	*L. serriola*	*L. virosa*
Bud number on stem base	Many	Many	Many
Leaf petiole	*sessile*	*Sessile*	*Sessile*
Leaf shape	*orbicular*	*Oblonceolate*	*Spathulate*
Costa color	White	White	Purple
Costa lower surface	Rough	Smooth	Rough
Leaf surface	*rugose*	*Rugose*	*Rugose*
Leaf apex	*rounded*	*Acute*	*Acuminate*
Leaf margin	Smooth-Dentate	Lobed	Dentate
Leaf hairs	None	Long	Short
Laef upper surface	Light green	Yellowish-light green	Greenish-Blue
Leaf lower surface	Light green	Light green	White

Figure 1. (A) Lettuce Plant, (B) The Upper Surface Leaf, (C) The Lower Surface Leaf, and (D) Leaf Margin.

Species *L. virosa* has specific character on its leaf like spathulate leaf shape, acuminate leaf apex, dentate leaf margin, short leaf hairs, *sessile* leaf petiole, greenish-blue leaf upper surface, white leaf lower surface, rugose leaf surface, rough costa lower surface, the color of costa is purple, many bud number on stem base (Figure 1).

Molecular Characterisation

Molecular analyzes was done by applying an RAPD-PCR method, the total numbers of DNA fragments were then used as binary numbers to develop a cladogram of the three lettuce plants kinship.

Isolation of the genomic DNA of *Lactuca* spp

To ensure the quality of the isolated genomic DNA, they were treated for an electrophorese and visualized on the agar gel. This step revealed some clear and thick DNA fragments except for the*L. Sativa* (Figure 2) because of its low quality (Millah *et al*., 2012). Nugroho *et al*. (2016) reminded there are some factors might affect the isolated genomic DNA quality like how to keep the fresh material before being isolated, the types of the organism tissue, the solution compositions, and the homogenization technique along the isolation processes.

Following to the checking step for the genomic DNA quality, the isolated genomic DNA were measured for their quantity and purity by using a nanodrop-spectrometer at λ 260/ λ 280 nm wavelengths for their ratio (Prayogo *et al*., 2020). Table 2 presents the ratio of the isolated genomic DNA vary between 1.755 and 1.796 which according to Tiwari *et al*. (2017), these ratio are lower than ideal for PCR. Furthermore, Prayogo *et al*. (2020), stated that the pure genomic DNA supposed to have ratio between 1.8 and 2.0. The ratio lower than 1.8 indicating that the genomic DNA is contaminated by either, carbohydrate, protein, or phenolic compounds; but when the ratio is over 2.0 the genomic DNA is contaminated by RNA. This contamination might due to practical errors as stated by Sari *et al*. (2014), the treatment of the samples during isolation processes will strongly affect the resulting of isolated genomic DNA.The concentration of genomic DNA for the three species, *L. seriola*, *L. virosa*, and *L. sativa*, ranged from 33.5 and 104 ng/µL (Table 2). The concentration of genomic DNA lower than 50 ng/µL is classified as poor that might be affected by some factors like temperature and time during the incubation period (Hutami *et al*., 2017; Triani, 2020).

The RAPD profiles of *Lactuca*spp

Eight different RAPD primers (Table 3) used in this study generated 91 amplicons sized of 90 to 1150 bp, and polymorphism pattern between 66.6 and 100% indicating that the genomic DNA samples have complementary sequences to these primers (Sulistyawati & Widyatmoko, 2017).The OPAM-7 primer, however, generated the most DNA fragments with a total of 20 bands where 16 bands of them were polymorphic and the rest 4 were monomorphic (Fig. 3). This was followed by the OPC-11,which produced 18 bands.The least band was showed by the OPF-2 primer with 3 bands (Fig. 4). The low number of DNA bands observed in the case of the OPF-2 primer can be attributed to suboptimal annealing temperature, the PCR mix composition, the purity of the genomic DNA and the total number of PCR cycles. Herman *et al*. (2018) showed that these factors might affect the appearance of the DNA bands on the agarose gel.

Figure 2. The Visualization Genom DNA Lactuca spp. and Chrysanthemum morifolium. (M = 1 kb DNA ladder, 1= The Results of Visualization of DNA Bands are Quite Clear, 2= The Results of Visualization of DNA Bands are Less Clear, A1 = L. serriola, A2 = L. serriola Duplo, B1 = L. virosa, B2 = L. virosa Duplo, C1 = L. sativa, C2 = L. sativa Duplo, D1 = C. morifolium, D2 = C. morifolium Duplo).

Table 2: Quality and quantity of the genomic DNA of *Lactuca serriola., L. virosa* and *L. sativa.*

Sample	Duplo	λ 260/ λ 280 Absorbance ratio	Concentration (ng/µL)
Lactucaserriola	1	1.766	41.5
	2	1.755	43
Average			42.25
Lactuca virosa	1	1.796	102
	2	1.793	104
Average			103
Lactuca sativa	1	1.763	33.5
	2	1.795	35
Average			34.25

Phylogenetic relationship between *Lactuca*spp. and *Chrysanthemum morifolium*

A phylogenetic relationship between the three species is presented in figure 5. The maximum parsimony showed that the Consistency Index (CI) of the species and outgroup was 0.83, the Retention Index (RI) of 0.8 and Retention Consistency (RC) of 0.73 at the bootstrap 1000X. Hidayanti (2021), stated that the value that closer to 1 was quite high to determine the resolution and consistency of the phylogenetic tree. Furthermore, Sari *et al*. (2014), showed that the Consistency Index (CI) of 1 shows a character changes in the DNA bases pair with a parsimony character, meanwhile theRetention Index (RI) around 1 shows a complete and consistent character with the phylogeny. Thus the high CI and RI values determine the high parsimony (Hidayanti, 2021).

Table 3: Total numbers of DNA bands produced by the eight Primers.

Primer	Jumlah Total Pita	Pita Monomorpik	Pita Polimorpik	PersentasePolimorpik (%)
OPA-2	11	0	11	100
OPAM-7	20	4	16	80
OPC-11	18	0	18	100
OPF-1	6	0	6	100
OPF-2	3	1	2	66,6
OPF-3	9	2	7	77,7
OPF-4	14	0	14	100
OPAE-5	10	0	10	100
Total	91	7	84	86,597

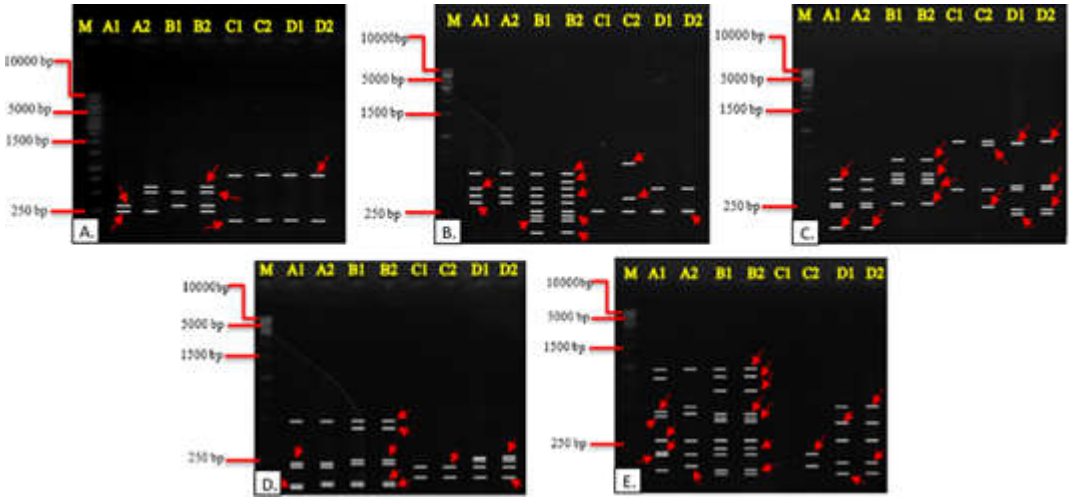

Figure 3. Amplification of the RAPD-PCR of the Lactuca spp. and Chrysanthemum morifolium with their polymorphism pattern values 100% shown by (A) OPF-1, (B) OPA-2, (C) OPF-4, (D) OPAE-5, (E) OPC-11.

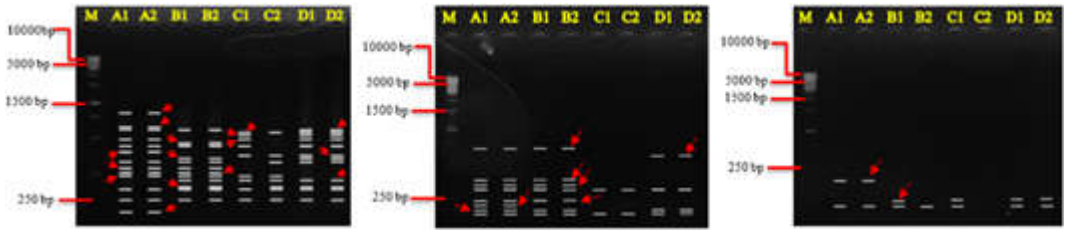

Figure 4. Amplification of the RAPD-PCR of the Lactuca spp. and Chrysanthemum morifolium with their polymorphism pattern values vary between 66.6 and 80% (A) OPAM-7 (B) OPF-3, (C) OPF-2.

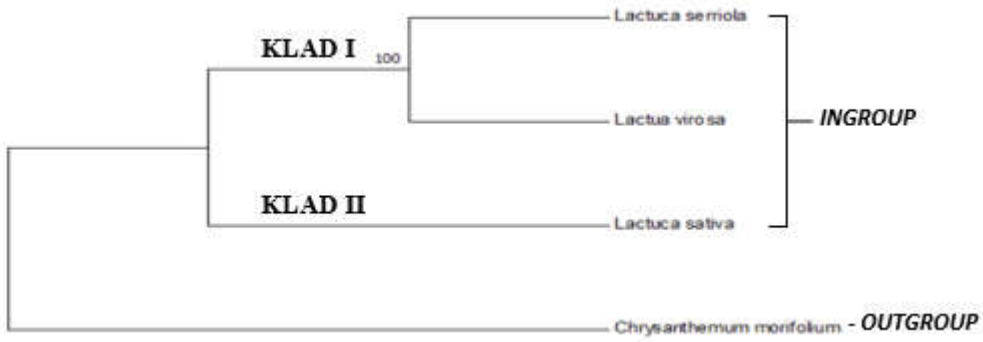

Figure 5. The phylogenetic relationship among the *Lactuca*spp. and *Chrysanthemum morifolium* using a Maximum parsimony.

The strength of the phylogenetic tree nodes were checked with thebootstrap 1000X, where the higher interval confidence of the bootsrap means the higher confidence of the phylogenetic tree (Bousquet *et al*., 1992). The bootstrapvalue between 70 and 100 means the cladogram might have a change on its node but when the score is < 70 means the possibility of nodes change is extremely high, and the nodes position could change quite often (Rosdiani *et al*., 2013).

The maximum parsimonyanalyzes done in the current study produced two different main clads,i.e., cladI consisting of *L. serriola* and *L. virosa,* with the bootstrapscore of 100%,and clad II has a monophyletic of *L. sativa* (Figure 3). Hidayanti, (2021) stated

that the bootstrapscore of 100% determines that the possibility of node changes is very low. It might then concluded that the *L. serriola* and *L. virosa*are sister plants. Rosidiani *et al*. (2013), the monophyletic clad has a very close relationship and came from the common ancestor. Furthermore, Hidayat *et al*. (2008), stated the formation monophyletic clad noted in this study suggested that the three samples of *Lactuca* spp., have similar material genetic and so biochemical compounds from the ancestor. Grouping the *L. serriola* and *L. virosa*in pone clad was due to those two species have very close relationship as revealed also in the RAPD-PCR results (Figure 4).

Conclusion

The RAPD-PCR profile of three*Lactuca* spp. consisting of *L. serriola*, *L. virosa*, and *L. sativa,* and *Chrysanthemum morifolium* an out group plant applying eight different RAPD primers generated 91 amplicons with 84 of the amplicons been polymorphic and 7 bands monomorphic. The genetic diversity among the green lettuce (*L. sativa*), spiny lettuce (*L. serriola*), and wild lettuce (*L. virosa*) showed that the three species vary in their morphological characteristics such leaves, leaf's edges, leaf's hair and the color of leaf's upper surface. However, the maximum parsimonyanalyzes with the bootstrap score of 1000X of the three species show that they are having a close relationship.

Acknowledgment

The authors would like to pass their gratitude to any parties who helped the authors to complete this paper.

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