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COI Gene Variation of Pseudococcidae in Ornamental Plants Based on In Silico RFLP Method

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Abstract

Ornamental plants are susceptible to pest attacks that influence lowering the quality and quantity of plants, one of which is from the Pseudococcidae family. Pseudococcidae has many species that attack various types of plants due to genetic variation. Pseudococcidae pest detection methods are needed to develop prevention and treatment methods. The aim of the research is to analyze the genetic variation of the Pseudococcidae family based on the Cytochrome Oxidase Subunit I (COI) gene using the Random Fragment Length Polymorphism (RFLP) method in silico, so that it can become basic information for further research related to pest control through genetic approaches. The methods applied were screening restriction enzyme candidates to compare the restriction patterns of tested DNA sequence samples. Next, the in silico RFLP test had completed to analyze the nucleotide sequence using certain restriction enzymes to obtain polymorphism results in the form of differences in the length of the restriction fragments. The results of the research showed genetic variation (polymorphism) on the AluI (allele A1, A2, A3, A4, A5, A6, and A7 alleles) and BspHI (B1 and B2 alleles) restriction recognition site of the 12 COI gene sequences in PopSet: 262216777. This research shows that the restriction enzymes AluI and BspHI are able to identify genetic variations in the COI gene of the Pseudococcidae family. This information can help identify Pseudococcidae types and contribute to controlling Pseudococcidae pests on ornamental plants.

Keywords: AluI; BspHI; restriction enzymes; leaf pest; mealybug.

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INTRODUCTION

Ornamental plants are valued for their uniqueness in the shape, color, and fragrance of their stems, leaves, flowers, and fruits. The demand for these plants is increasing, constituting a notable business opportunity due to their economic potential (Pratomo and Andri, 2013). Ornamental plants can also serve as medicinal plants besides their main function for decorative purposes. For example, the cat whisker plant is famous as an ornamental species but is also recognized for its medicinal properties. The leaves of this plant contain orthosiphonin glycosides, which are beneficial for treating conditions such as rheumatism, coughs, colds, constipation, kidney stones, and diabetes (Lestari, 2016). Despite their benefits, ornamental plants are vulnerable to pest invasions and diseases, causing damage to their quality, yield, and aesthetic value (Monawati et al., 2021; Wahyuni, 2015). Among the pests that commonly affect ornamental plants are plant lice insects. Some species of plant lice can act as virus vectors and cause chlorosis in the leaves, resulting in the leaves appearing paler and yellowing (Marianah, 2020).

The mealybug, a member of the family Pseudococcidae, is generally considered to be a notorious pest that attacks ornamental plants. This family comprises many species, such as *Planococcus minor*, *Ferrisia virgata*, and *Dysmicoccus neobrevipes* (Trianto et al., 2020). *Ferrisia virgata* is known to invade many ornamental plants, such as elephant ear, frangipani, croton, red betel, and anthurium (Sartiami et al., 2011). Other species, *Paracoccus marginatus*, attack frangipani plants, resulting in yellowing and the drop of leaves, while two others species of Pseudococcidae family, *Planococcus ficus*

and *Planococcus citri*, are major pests that cause severe damage to host plants and greatly affect agricultural yields (Sumartayasa et al., 2021; Cavalieri et al., 2008). According to Wang et al. (2018), mealybugs infest many crops and ornamental plants, including papaya, cassava, orange, aubergine, frangipani, and acalypha. As stated by Rahmadani et al. (2021), mealybugs release honeydew, which leads to the development of sooty fungi that blacken the infected areas. Such invasions by the pest might cause defoliation and sometimes be deadly for the plants (Puig et al., 2021).

Different varieties of the Pseudococcidae family member may result from mutations that lead to genetic variations expressed as alleles (Girija and Dhavanel, 2015). These alleles code for different structures and functions of proteins, which may affect the physiology and development of an organism (Kilmaskossu, 2009). Dunham (2004) stated that a higher level of genetic variation within a population enhances individuals' ability to adapt to environmental changes. Accordingly, Oyarieme et al. (2019) stated that mutation, gene flow, genetic drift, and natural selection allow a population to develop new characters that are advantageous in particular environments. Thus, these mechanisms contribute to the genetic diversity observed within and between populations. Genetic variation or polymorphism may also provide the survival chances of a population under stable or threatened conditions (Rell et al., 2013). Therefore, polymorphism has various functions, such as controlling genetic diversity within the population, e.g., targeting mealybug pests. Yunianti et al. (2007) noted that knowledge of genetic variation or polymorphism in pests can determine the success of plant breeding programs.

Random Fragment Length Polymorphism (RFLP) is a common method used to evaluate genetic variation at the DNA sequence level. In this method, DNA variation is detected by comparing the band profiles produced through restriction digestion using particular enzymes on target DNA from different individuals (Zulfahmi, 2013). This technique employs specific restriction sites within an organism's genome that can be cut in the sugar-phosphate backbone without harming the base, based on the recognized sequence (Pandin, 2010). This analysis can also be performed in silico, which refers to experimental or testing activities carried out using computer simulations to predict actual conditions by bioinformatics tools. In silico studies can serve as preliminary research before moving on to other methods, such as in vivo and in vitro, offering insights, predictions, and research hypotheses (Achyar et al., 2021). Analyzing the genetic variation of the COI gene of Pseudococcidae in a laboratory setting is often time-consuming, requires high financial resources, and a larger workforce. Therefore, in silico studies can be a potential alternative (Megawati et al., 2016). According to Husain et al. (2016), molecular genetic diversity analysis, while more costly and complex, offers greater accuracy and precision because it can identify polymorphisms at the gene level, unlike morphological analysis. Polymorphism analysis through in silico studies can help identify potential restriction enzymes that serve as useful markers. Rung et al. (2009) have successfully employed RFLP to identify a unique recognition site in *Planacoccus minor* using the restriction enzyme BspHI, demonstrating that this method can effectively differentiate *Planacoccus minor* from other Pseudococcidae species.

Anantyarta (2017) explains that polymorphic properties are essential for genetic markers because these properties allow for the comparison of gene polymorphism among individuals within a studied population. The value of polymorphic information in a gene is directly related to the number of alleles at each locus; generally, a greater number of alleles results in a higher value of polymorphic information. Cytochrome Oxidase Subunit I (COI) is one gene that can serve as an effective genetic marker. Wang et al. (2011) utilized the COI gene to identify various insect species. Similarly, Ashfaq et al. (2010) employed partial nucleotide sequences from nuclear genes (elongation factor-1α and ribosomal DNA subunits 18S and 28S) along with mitochondrial COI sequences to characterize species within the Pseudococcidae family. Additionally, Cavalieri et al. (2008) found that the restriction enzyme HinfI can identify the genetic variation in the COI gene region of *Planacoccus ficus* and *Planacoccus citri*. Recent research also shows that three species of mealybugs, namely *Dysmicoccus neobrevipes, Maconellicoccus hirsutus*, and *Paracoccus marginatus*, can be identified based on the COI gene using the mPCR method (Xi et al., 2024).

Research on the genetic variation within the Pseudococcidae family is still less explored. This study is pivotal for analyzing genetic variation based on the COI gene using the RFLP method in silico, providing fundamental information for future research. The results of this study may be beneficial in reducing losses caused by pest species from this family, which can be addressed using a genetic approach to integrated biological control (Ningsih et al., 2021).

MATERIALS AND METHODS

This study was exploratory and descriptive research, which was conducted in silico from May to June 2023. We utilized bioinformatics analysis tools from http://insilico.ehu.es/restriction/ and https://www.benchling.com/ web servers. The primary material examined in this research was the Cytochrome Oxidase Subunit I (COI) gene sequence of Pseudococcidae, which was obtained from NCBI with GenBank ID: PopSet: 262216777 (refer to Table 1). This popset includes several species of mealybugs that commonly infest ornamental plants, such as *Ferrisia virgata*, *Planococcus minor*, and other related species.

Table 1. List of COI Genes of Pseudococcidae Popset: 262216777

Accession number	Species
GQ906767.1	*Planococcus lilacinus*
GQ906766.1	*Dysmicoccus lepelleyi*
GQ906765.1	*Ferrisia virgata*
GQ906764.1	*Dysmicoccus lepelleyi*
GQ906763.1	*Ferrisia virgata*
GQ906762.1	*Cataenococcus hispidus*
GQ906761.1	*Planococcus minor*
GQ906760.1	*Cataenococcus hispidus*
GQ906759.1	*Maconellicoccus hirsutus*
GQ906758.1	*Dysmicoccus lepelleyi*
GQ906757.1	*Dysmicoccus neobrevipes*
GQ906756.1	*Pseudococcus baliteus*

Restriction enzyme screening was conducted using a bioinformatics web server http://insilico.ehu.es/restriction. The comparison of restriction patterns across multiple DNA sequence samples was performed by utilizing the "*Compare restriction patterns of many sequences*" tool. Pseudococcidae COI gene sequences from PopSet: 262216777 were downloaded in FASTA format from NCBI and uploaded to the website to gather information on restriction enzymes that could recognize these sequences. After obtaining the alignment results, the option "*Only restriction enzymes with known bases (no N, R, Y...)*" was selected to ensure that only restriction enzymes with specific base recognition sites were considered. Finally, the "*Get the list restriction enzyme*" option was chosen to identify the restriction enzymes that are able to recognize and cut the twelve tested Pseudococcidae gene sequences.

Random Fragment Length Polymorphism (RFLP) was performed in silico using the bioinformatics web server https://www.benchling.com/. Kanaya and Achyar (2023) stated that the RFLP method could help to compare the DNA band profiles resulting from cutting by restriction enzymes against the target DNA or gene sequence. The bands formed have different fragment lengths, indicating various mutations in an organism. In silico RFLP can be used to analyze nucleotide sequences using certain restriction enzymes to obtain polymorphism data in the form of differences in the length of the restriction or cutting fragments (Yeriska et al., 2021). The RFLP begun by uploading the COI Pseudococcidae gene sequence of PopSet: 262216777 into the "*projects*" folder. Next, one of the gene sequences was selected for restriction by selecting the scissors icon on the bar on the right. The "*new digest*" tab will appear in this option, then in the "*find enzyme*" feature, the previously determined restriction enzyme was selected based on the screening results and literature review, then "*run digest*" to perform the restriction. The restriction results will be obtained in the "*digest*" menu, which contains information on the cutting point, fragment length, and cutting type. In the "*virtual digest*" menu, an electrophoregram visualization of the previously selected restriction enzyme cutting results was obtained. These steps were carried out on each sequence tested.

The restriction results of the COI gene sequence of Pseudococcidae in PopSet: 262216777, which was visualized on the electrophoregram, were analyzed by observing the polymorphism or genetic variation that occurred. DNA polymorphism was observed based on fragment length diversity resulting from restriction enzyme cutting (Maylinda et al., 2015). The results of cutting restriction enzymes with different fragment lengths indicated different alleles. Iza (2017) explained that a population's high polymorphism can be interpreted as the variation of alleles and specific characteristics of the population is relatively high. Polymorphism is determined based on allele

frequency or the percentage of fragment presence. If there are two or more alleles in the population with the highest allele frequency equal to or less than 0.99 (99%), then it indicates that a locus is polymorphic. Conversely, if there is only one allele with a very high allele frequency or close to 1 (100%), then it indicates that a locus is monomorphic (Nei, 1987).

RESULTS

Based on the results of data processing obtained from the site http://insilico.ehu.es/restriction/, it is known that several candidates of restriction enzymes have recognition sites in 12 COI gene sequences in PopSet: 262216777. This study used two restriction enzymes, namely AluI and BspHI, which were chosen because they showed variations in cutting sites in all gene sequences in PopSet: 262216777. In silico RFLP was carried out on the site https://www.benchling.com/ (Hasanah et al., 2023). The results of in silico RFLP were visualized by virtual gel electrophoresis. Figure 1 shows the results of restriction using the AluI enzyme and Figure 2 shows the results of restriction using the BspHI enzyme.

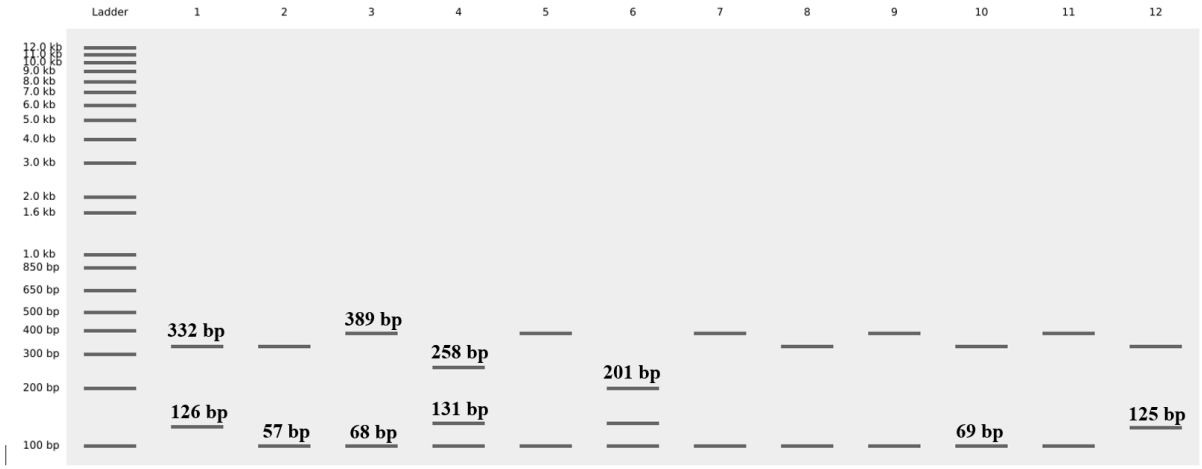

Figure 1. In silico restriction electrophoregram with AluI enzyme. Ladder Life kb plus, (1) GQ906756.1, (2) GQ906757.1, (3) GQ906758.1, (4) GQ906759.1, (5) GQ906760.1, (6) GQ906761.1, (7) GQ906762.1, (8) GQ906763.1, (9) GQ906764.1, (10) GQ906765.1, (11) GQ906766.1, (12) GQ906767.1

Based on the results of in silico restriction with the AluI enzyme on 12 Pseudococcidae COI gene sequences, it showed the presence of genetic variation with the formation of seven allele variations (Figure 1; Table 2).

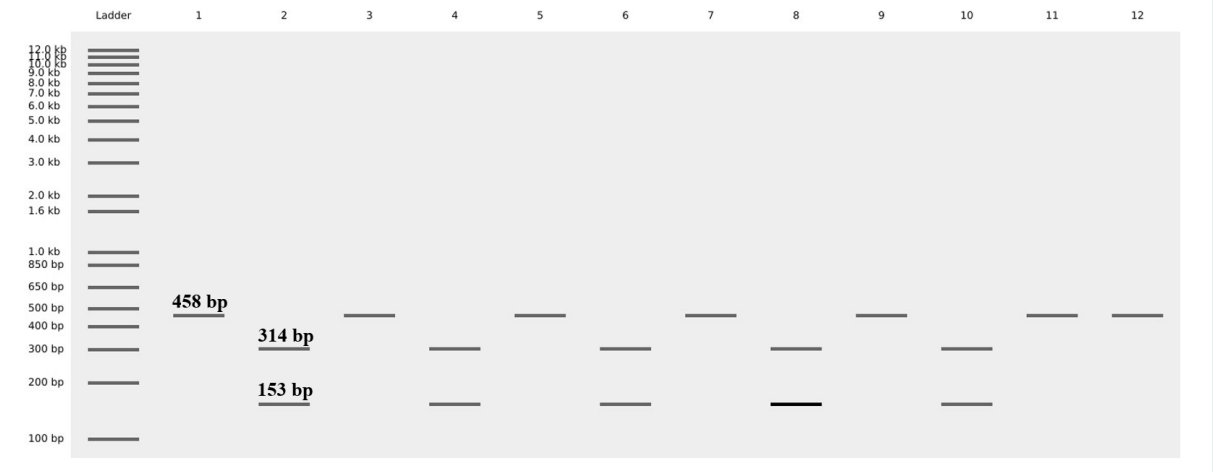

Figure 2. In-silico restriction electrophoregram with BspHI enzyme. Ladder Life kb plus, (1) GQ906756.1, (2) GQ906757.1, (3) GQ906758.1, (4) GQ906759.1, (5) GQ906760.1, (6) GQ906761.1, (7) GQ906762.1, (8) GQ906763.1, (9) GQ906764.1, (10) GQ906765.1, (11) GQ906766.1, (12) GQ906767.1.

The fragment pattern with the BspHI enzyme in silico restriction on 12 Pseudococcidae COI gene sequences showed genetic variation with the formation of two allele variations (Figure 2; Table 2).

Table 2. COI gene allele frequencies in Pseudococcidae based on in silico RFLP results

Restriction Enzyme	Recognition Site	Fragment Size (bp)	Allele	Sequence Sample	Number of Fragments Presence (N = 12)	Fragment Presence Percentage	Allele Frequency
AluI	AG'CT	332 126	A1	1	1	8.33%	0.0833
		332 57	A2	2, 8, and 10	3	25%	0.2500
		389 68	A3	3, 5, 7, 11	4	33.33%	0.3333
		258 131 68	A4	4	1	8.33%	0.0833
		57 201 131	A5	6	1	8.33%	0.0833
		389 69	A6	9	1	8.33%	0.0833
		332 125	A7	12	1	8.33%	0.0833
BspHI	T'CATG_A	458	B1	1, 3, 5, 7, 9, 11, 12	7	58.33%	0.5833
		314 153	B2	2, 4, 6, 8, 10	5	41.67%	0.4167

DISCUSSION

AluI recognizes the AG'CT site with a blunt end (symmetric) and the BspHI enzyme recognizes T'CATG_A with a sticky end (asymmetric). AluI is a restriction enzyme derived from *Arthrobacter luteus* and has the AluBI isoschizomer. The AluI restriction enzyme has previously been used to characterize invasive mealybugs (Pseudococcidae) based on DNA (Ashfaq et al., 2010). The BspHI enzyme comes from *Bacillus* sp. and has the isoschizomers CciI, PagI, and RcaI. Rung et al. (2009) used the BspHI enzyme in their study, which showed that BspHI has a unique recognition site in *Planacoccus minor* so that it can be used to distinguish it from other Pseudococcidae. Based on this, the AluI enzyme that recognizes the AG'CT site and the BspHI enzyme that recognizes the T'CATG_A site were selected to identify genetic variations in 12 gene sequences in PopSet: 262216777.

The results of in silico restriction with AluI enzyme on 12 COI gene sequences of Pseudococcidae showed genetic variation with the formation of seven allele variations (Figure 1; Table 2). Allele A1 produces two DNA bands measuring 332 bp and 126 bp; allele A2 produces two bands measuring 332 bp and 57 bp; allele A3 produces two DNA bands measuring 389 bp and 68 bp, allele A4 produces three DNA bands measuring 258 bp, 131 bp, and 68 bp; allele A5 produces three DNA bands measuring 57 bp, 201 bp, and 131 bp; allele A6 produces two DNA bands measuring 389 bp and 69 bp; allele A7 produces two DNA bands measuring 332 bp and 125 bp. The appearance of DNA bands, as visualized in the electrophoregram results, indicated that the AluI restriction enzyme recognizes the cutting sites. Based on the analysis results, the frequency of the A3 allele was higher than the frequency of other alleles. This was indicated by the percentage of the presence of the A3 allele fragment, which was 33.33%, which appears more than the percentage of the presence of the A2 allele fragment, which was 25%, or other alleles, which was 8.33% (Table 2).

The restriction pattern with the BspHI enzyme on 12 COI gene sequences of Pseudococcidae showed genetic variation with the formation of two allele variations (Figure 2; Table 2). Allele B1 produces one DNA band measuring 458 bp. Allele B2 produces two DNA bands measuring 314 bp and 153 bp. The appearance of DNA bands, as visualized in the electrophoregram results, indicated that the BspHI restriction enzyme recognizes these cutting sites. Based on the analysis results, the frequency of allele B1 was higher than the frequency of allele B2. This was indicated by the percentage of presence of allele B1 fragments, which was 58.33%, which appears more than the percentage of presence of allele B2 fragments, which was 41.67%.

Based on the results of the RFLP analysis carried out in silico, the results obtained showed that there was genetic variation (polymorphism) at the recognition site that had been recognized by

the AluI and BspHI restriction enzymes from 12 COI gene sequences in PopSet: 262216777. Accordingly, previous studies also used RFLP to analyze polymorphism of nucleotide sequences using certain restriction enzymes (Yeriska et al., 2021). Nei (1987) stated that a locus is considered polymorphic if it has two or more alleles in the population with the highest allele frequency ≤ 0.99 (99%) and a locus is considered monomorphic if there is only one allele with a frequency approaching or reaching 100%. Azizah et al. (2015) also emphasized that a polymorphic locus has allele variation within the population, whereas if there is no variation, it is considered a monomorphic locus.

Hebert et al. (2003) stated that Cytochrome Oxidase Subunit I (COI) is very effective in DNA-based species identification systems. Bucklin et al. (2003) also stated that the COI gene has a high sequence mutation rate and can show interspecies sequence divergence. This research showed that the COI gene can detect genetic variation in several species within the Pseudococcidae family through recognition by restriction digestion. Previous research by Cavalieri et al. (2008) also showed that RFLP analysis in the COI gene region could detect polymorphisms so that it can distinguish populations of two mealybugs, *Planacoccus ficus* and *Planacoccus citri,* within the Pseudococcidae family.

Information on genetic variation in the COI gene sequence of Pseudococcidae conducted in silico is fundamental data that is needed to validate through further laboratory research. The results of the study can help identify the type of mealybug pests of the Pseudococcidae family through the selection of biocontrol agents as an integrated pest control effort (Cavalieri et al., 2008). According to Parikesit et al. (2017), a bioinformatics approach in applied biology can be used for pest management and protection of plant varieties. Nevertheless, further research is needed to prove the effectiveness of restriction enzymes in determining genetic variation values with specific cutting characteristics in the RFLP.

CONCLUSION

The results of the analysis of genetic variation of the COI gene of Pseudococcidae using the RFLP method in silico in this study showed that there was genetic variation (polymorphism) at the AluI enzyme restriction recognition site (alleles A1, A2, A3, A4, A5, A6, and A7) and BspHI (alleles B1 and B2) of 12 COI gene sequences in PopSet: 262216777.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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