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DNA BARCODING AND GENETIC DIVERSITY ASSESSMENT OF *INDIGOFERA LONGERACEMOSA* FROM JAVA, INDONESIA

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SUMMARY

The rediscovery of *Indigofera longeracemosa* Boiv. ex Baill. in Sleman in 2016 has since continued in its cultivation in various regions of Central Java and Yogyakarta. This study explored the molecular characteristics and genetic diversity of *I. longeracemosa* from Java. The replanting of specimens resulted in 32 accessions that underwent morphological characterization and genetic diversity using ISSR markers. Four accessions, selected for DNA barcoding, helped confirm their identity. Significant morphological variation was evident, particularly in leaf coloration and margins. Notably, samples from Srandakan and Trisik exhibited a deep bluish-green color in fresh leaves, whereas those from Grogol and Lemahireng displayed a bluish-green shade. The dried leaves from Srandakan and Trisik were dark gray, contrasting with the gray leaves from the other two locations. Undulate leaf margins were outstanding in the Srandakan samples. The ITS sequence analysis confirmed the identity of all samples as *I. longeracemosa*, revealing a closer relationship to Indian specimens than to specimens from Madagascar, differing by one nucleotide at position 408. Genetic diversity assessment indicated greater intrapopulation variation than interpopulation variation, highlighting substantial genetic diversity within *I. longeracemosa*. This study enhances our understanding of the species' morphological and genetic characteristics, contributing to its biodiversity and conservation efforts.

Keywords: *I. longeracemosa*, Fabaceae, genotypes, molecular markers, phylogenetic, morphological examination, molecular identification

Key findings: The presented study offers significant insights into species validation utilizing ITS for DNA barcoding in *I. longeracemosa*. The obtained genetic diversity data will support its conservation and plant breeding initiatives.

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INTRODUCTION

Indigofera longeracemosa Boiv. ex Baill. is a perennial herb belonging to the family Fabaceae. The species exhibited notable morphological traits, such as a broad canopy at the apex, with the main branch growing at an angle of 45°-90°. The young stem exhibits a reddish tint and features a zigzag growth pattern. The pods are cylindrical and erect, featuring a shiny surface, while the mature fruit is copper brown (Muzzazinah *et al*., 2015). For seven years, this extraordinary species, originally from Madagascar, has received its launch to numerous new areas globally. Its spread beyond its native environment underscores its adaptability and the increasing fascination with its distinctive traits and ecological importance. The four locations, Sukoharjo, Klaten, Bantul, and Kulon Progo, represent the extensively documented distribution regions in Java, Indonesia. In different regions, the morphological characteristics remained unchanged; however, the color of dry leaves on the upper and lower surfaces exhibited considerable variations. The Srandakan and Trisik ecotypes demonstrated a dark gray coloration in their dried leaves, while their fresh leaves displayed a dark bluish-green hue. In contrast, the Grogol and Lemahireng ecotypes were distinct with a gray tone in their dried leaves and a bluish-green shade in their fresh leaves.

Plants exhibit a range of adaptive responses to environmental variations, which include morphological, physiological, and genetic changes, as well as modifications in metabolic pathways (Kusano et al., 2011). Key environmental factors, such as soil composition, light intensity, and temperature, play a significant role in shaping plant metabolite production and, consequently, their morphological characteristics (Yang *et al*., 2018). Furthermore, altitude substantially impacts plant physiology and metabolic processes due to the considerable variations in air pressure, which can affect photosynthesis and other physiological functions (Dusenge *et al*., 2019).

The morphological variations observed in plants often reflect their underlying genetic diversity. These variations can manifest in various forms, including differences in leaf shape, size, and surface texture. Understanding whether these morphological traits arise from genetic plasticity or represent stable characteristics is crucial for accurate species identification. Morphological approaches can provide direct insights into the variations among different ecotypes, while molecular methods can elucidate the genetic basis for these differences (Sommer, 2020; Schneider, 2022).

DNA barcoding is a widely employed technique for species identification, encompassing both known and unknown species. The methodology outlined is utilization for taxonomic identification of species via the analysis of DNA fragments sourced from diverse regions of plants (Hollingsworth *et al*., 2011; Tripathi *et al*., 2013; Moura *et al*., 2019). This study employed the internal transcribed spacer (ITS) as a molecular marker for DNA barcoding. Kress *et al*. (2005), Chase *et al*. (2007), and Kress and Erickson (2007) identified two widely used DNA barcode markers in plants, i.e., *rbc*L (coding region) and *psb*A-*trn*H IGS (non-coding region), with both sourced from chloroplast DNA (*cp*DNA).

Genetic diversity assessment through molecular markers is swift, cost-effective, and efficient. Molecular markers can also be favorable to evaluate the genetic diversity as well as specific traits within a species (Mable, 2019; Miles *et al*., 2019). Several molecular markers reached recognition, with the inter-simple sequence repeat (ISSR) being the most user-friendly due to its highest yield of polymorphic DNA bands, along with its efficiency, accuracy, and reproducibility (Talaat *et al*., 2013; Hariri *et al*., 2017; Kumar *et al*., 2018; Al-Hadeithi and Jasim, 2021).

The aim of this study is to utilize DNA barcoding, focusing on the internal transcribed spacer (ITS) as a molecular marker, to confirm the identity of *I. longeracemosa,* improve species identification, and evaluate the genetic diversity within *I. longeracemosa* populations

through the use of accessible ISSR markers. Additionally, the study hopes to offer insights into the genetic diversity and specific characteristics of *I. longeracemosa*, thereby enhancing one's understanding of its biodiversity and ecological importance.

MATERIALS AND METHODS

Sample collection and morphological observations

In the promising study, the use of leaves from various accessions of *I. longeracemosa* came from Central Java and Yogyakarta, Indonesia (Figure 1). *I. longeracemosa* accessions totaled 32 incurred cultivations across four villages in Central Java and Yogyakarta Provinces, Indonesia. These are Grogol (Sukoharjo, Central Java), Lemahireng (Klaten, Central Java), Srandakan (Yogyakarta), and Trisik (Yogyakarta) (Figure 2, Table 1). The fresh and healthy leaves' collection followed the methods outlined by Widjaja and Poerba (2004), with morphological observations using the guidelines established by Beentje (2016). The collected leaves from each accession gained storage in a plastic bag containing silica gel for subsequent DNA analysis. The color of fresh leaves, the adaxial surface of dried leaves, the abaxial surface of dried leaves, the leaf apex, base, margin, and surface, the shape of terminal and lateral leaflets, the color of young twigs and old twigs, and the direction of flowering underwent scrutiny.

DNA extraction, amplification, and visualization

Grinding the leaflet samples in a mortar had silica sand incorporated as a facilitating agent. The DNA extraction procedure continued following the guidelines established by the TianGen Plant Genomic DNA Kit (TIANGEN Biotech, Beijing). The DNA amplification of *I. longeracemosa* used ITS primers through the polymerase chain reaction (PCR) method. In this study, the selection of primers used employed the methodology outlined by Sun *et al*. (1994). The PCR comprised several distinct stages, i.e., pre-denaturation, denaturation, annealing, extension, and post-extension. The amplification process involved pre-denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 15 s. The final phase included post-extension at 72 °C for 5 min. The electrophoresis segment of this experiment

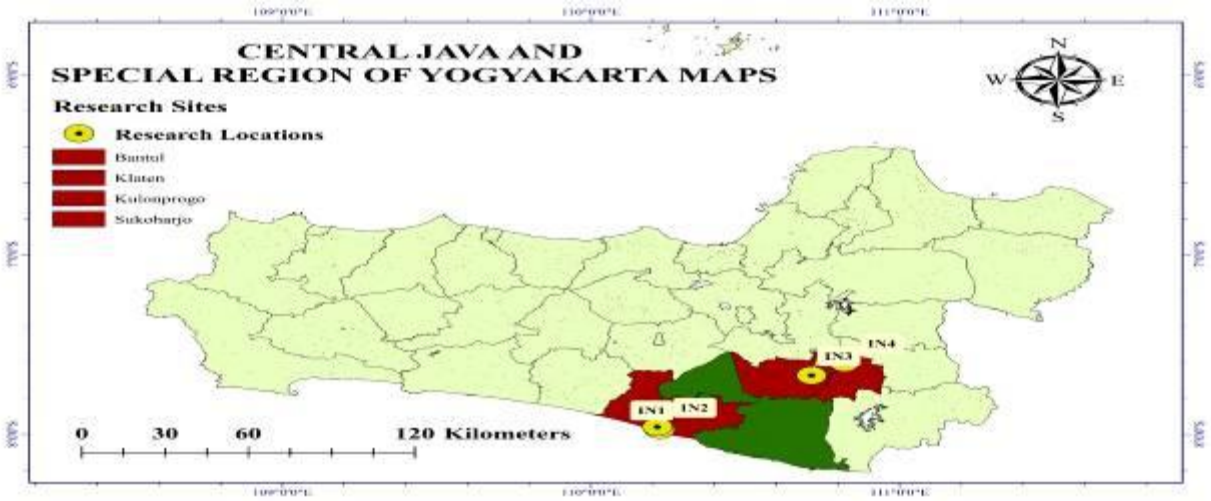

Figure 1. Locations of *Indigofera longeracemosa* sampling in Central Java and Yogyakarta. IN1 = Trisik (Yogyakarta), IN2 = Srandakan (Yogyakarta), IN3 = Lemahireng (Klaten, Central Java), and IN4 = Grogol (Sukoharjo, Central Java).

Figure 2. *Indigofera longeracemosa* grown in cultivation. A = habitat, B = branching, C = position and direction of flower growth, D = flowers, E = position of pods against stem, and F = pods and seeds.

Table 1. Sample code, locations, and morphological conditions of *Indigofera longeracemosa* samples used in the study.

No.	SampleCode	Locations	Morphological conditions			Sample Number
			Leaf Margin	Fresh Leaf Color	Dried Leaf Color	
1	C2N	Trisik	Entire	Dark bluish-green	Dark gray	4
2	C5N	Srandakan	Entire	Dark bluish-green	Dark gray	4
3	C5K	Srandakan	Undulate	Dark bluish-green	Dark gray	4
4	C6N	Lemahireng	Entire	Bluish-green	Gray	4
5	C7N	Grogol	Entire	Bluish-green	Gray	4
6	C8N	Srandakan	Entire	Dark bluish-green	Dark gray	4
7	K01	Srandakan	Undulate	Dark bluish-green	Dark gray	4
8	N01	Srandakan	Entire	Dark bluish-green	Dark gray	4

employed a 1% agarose gel subjected to an electrical potential of 100 volts for 1 h. The DNA amplification process utilized the PCR mixture with a total volume of 50 µl. The sequencing procedure took place at 1st Base in Singapore, employing the services of PT Genetika Science, Indonesia.

The DNA amplification for ISSR, when conducted, used 10 ISSR primers in a final volume of 10 µL, which included 10 ng of DNA template, 5 µM of ISSR primer, 6 µL of 2X MyTaq^{TM}HS Red Mix (Bioline, USA), and 1 µL of nuclease-free water. The protocol commenced with an initial denaturation at 94 °C for 1 min, succeeded by 35 cycles comprising denaturation at 94 °C for 15 s, annealing at 50 °C-58 °C for 15 s, and extension at 72 °C for 15 s, concluding with a final extension at 72 °C for 5 min (Table 2) (Lee *et al*., 2012; Mohammadzedeh *et al*., 2014; Dilsat-Yegenoglu and Sesli, 2017; György *et al*., 2020; Rao *et al*., 2020). The amplification products sustained electrophoresis for 90 min on a 1.2% agarose gel using 1X TAE buffer at 100 V. The gel, stained with one µL of FloroSafe (BIO-5170), underwent imaging using a UV-transilluminator GelDoc EZ Imager under UV light (Bio-Rad, USA).

Table 2. The ISSR primers used to amplify the DNA of *Indigofera longeracemosa.*

No.	Sequence Code	Primer Sequence (5'-3')	Tm (°C)	Reference
1	UBC 835	AGA GAG AGA GAG AGA GYC	58	György *et al*. (2020)
2	UBC 846	CAC ACA CAC ACA CAC AAT	50	Lee *et al*. (2012)
3	UBC 850	AGA GAG AGA GAG AGA GTT	55.4	Dilsat-Yegenoglu and Sesli (2017)
4	UBC 856	ACA CAC ACA CAC ACA CYA	50	Hariri *et al*. (2017)
5	UBC 873	GAC AGA CAG ACA GAC A	58	Rao *et al*. (2020)
6	UBC 878	GGA TGG ATG GAT GGA T	50	Rao *et al*. (2020)
7	UBC 880	GGA GAG AGG AGA	52.5	Mohammadzedeh *et al*. (2014)
8	UBC 881	GGG TGG GGT GGG GTG	58	Mohammadzedeh *et al*. (2014)

Phylogenetic tree construction

The sequencing data served to assemble contigs. The contigs' editing utilized the MEGA 11 program. The study used the Basic Local Alignment Search Tool (BLAST), specifically nucleotide BLAST, on the National Center for Biotechnology Information (NCBI) website, available at https://blast.ncbi.nlm.nih.gov. This methodology enables the quantification of genetic relatedness between a specific species and other species in the database (Stover and Cavalcanti, 2017). The selection of plant species using BLAST analysis involved multiple parameters, including maximum score, total score, query coverage, e-value, and percent identity. The hits obtained for the purpose of conducting a phylogenetic analysis engaged the MEGA 11 software (Tamura *et al*., 2021). The application of the Clustal-W method for multiple sequence alignment preceded the phylogenetic analysis, which used the Neighbor-Joining (NJ) approach with Kimura 2-parameters and 1000x bootstrap replications.

Nucleotide variations and genetic diversity analysis

The sequences' alignment ran with the Multalin V.5.4.1 (http://multalin.toulouse.inra.fr/multalin/), followed by analysis of the high and low consensus regions. The Multalin program version 5.4.1 helped examine variations in nucleotide frequencies and their positioning within the consensus region (Corpet, 1988). The DNA bands reached classification as either present (1) or absent (0). The genetic diversity parameters for *I. longeracemosa* and the stability of its morphological characters based on ISSR DNA fragments bore assessment using GenAlex version 6.501 (Peakall and Smouse, 2012).

RESULTS AND DISCUSSION

Phenotypic variations

The observed morphological characteristics of *I. longeracemosa* in different cultivation locations exhibited minor variations in the color of both fresh and dried leaves. Certain accessions, particularly those obtained from Srandakan and Trisik, showed a distinctive leaf morphology characterized by undulate leaflets (C5K and K01). Morphological differences may arise from selection, genetic drift, and phenotypic variations (Merilä and Hendry, 2014). Phenotypic plasticity is a significant mechanism that allows plants to adapt to their surrounding environment. Various environments may support plants exhibiting unique phenotypic plasticity mechanisms; thus, the adaptive mechanism may associate with the specific environments inhabited by the plants (Merilä and Hendry, 2014; Grenier *et al*., 2016).

Phylogenetic analysis

The amplification of the ITS region led to successful DNA amplification. The obtained results aligned with the intended objective. The DNA bands associated with the phosphorescent internal transcribed spacer (ITS) sequence measured approximately 900 base pairs (bp) (Kumari *et al*., 2018; Aprilianingsih *et al*., 2022). The amplification method was effective in its conduct, followed by sequencing to

acquire the requisite nucleotide base sequence for BLAST analysis on the NCBI website. The phylogenetic tree construction relied on the BLAST analysis of *I. longeracemosa* sequences, which exhibited high similarity scores ranging from 99.45% to 100% when compared to the *I. longeracemosa* entries in the NCBI database. This remarkable level of sequence similarity strongly supports the monophyly of the sampled specimens, indicating that they all share a common ancestor, being more closely related to each other than to any other species. The study encompasses 66 species of *Indigofera*, made possible by the availability of genetic material from the *Indigofera* genus, especially the *I. longeracemosa* species available in the NCBI database. The taxa identified through BLAST analysis aided in constructing the phylogenetic tree.

The ITS region is commonly suitable for analyzing the genetic sequences in plant research. The DNA barcode region possesses unique and effective characteristics. The disparities in discrimination can reach substantiation by evaluating the variances. The ITS sequences employed in the phylogenetic tree reconstruction highlighted the effectiveness of these primers in significantly differentiating the species, accurately confirming the identity of the specimens in this study as *I. longeracemosa*. Notably, these specimens grouped within the same clade as those from the NCBI database (Figure 3). Bolson *et al*.'s (2015) findings enunciated that the primers used in their research achieved the highest success rate in identification and resolution. The internal transcribed spacer (ITS) region was successful for extensive application for molecular identification due to its significant discriminatory power (Kress *et al*., 2005; Gu *et al*., 2011; Jiang *et al*., 2020).

A comparative analysis of four sequences of *I. longeracemosa* samples, along with sequences from the NCBI database, identified two nucleotide variations at position 408 (T/C) within the sequence alignment (Figure 4). Based on the sequence comparison, all samples from Java are identical to those from India (OP324635), differing by just one nucleotide from the specimen from Madagascar (AY124764). This suggests that the introduction of *I. longeracemosa* in Java likely originated from India.

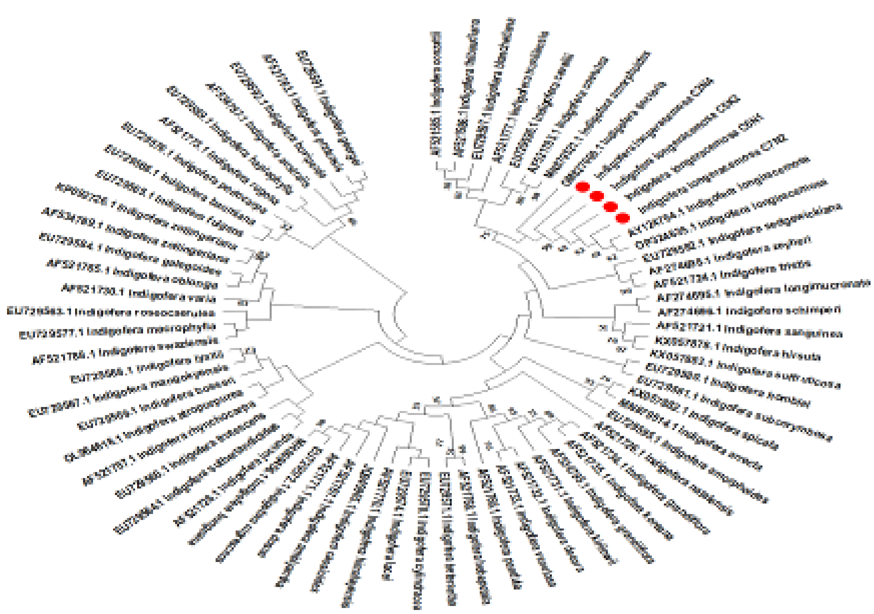

Figure 3. The position of *Indigofera longeracemosa* from Java, Indonesia on the phylogenetic tree using the internal transcribed spacer sequence.

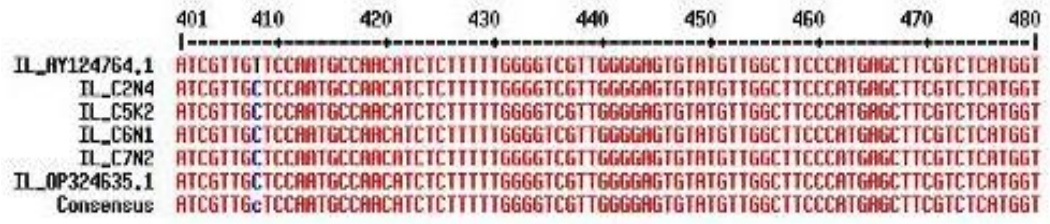

Figure 4. The multiple alignment of the cultivated *Indigofera longeracemosa* samples from Java and GenBank accessions reveals the presence of nucleotide variation at position 408.

Table 3. Summary of the amplified ISSR bands and polymorphisms in genetic analysis.

No.	Primer Code	Band Size (bp)	Scored Bands	Polymorphic Bands	Polymorphism (%)
1	UBC 835	200-1200	9	9	100
2	UBC 846	250-800	7	2	29
3	UBC 850	200-1300	4	4	100
4	UBC 856	400-2000	5	5	100
5	UBC 873	250-2000	11	11	100
6	UBC 878	600-1500	6	6	100
7	UBC 880	250-600	3	1	33
8	UBC 881	400-1500	7	7	100
Average					83

The ITS recognition rates were significantly high across the four samples, indicating its potential as a candidate for species identification within the *Indigofera* genus. Furthermore, the effectiveness of the markers and procedures employed was contingent upon the sample size. By studying singleton species, the inherent limitations heighten the likelihood of generating biased results (Jamdade *et al*., 2021, 2022).

For molecular identification and conservation of endangered species, the appropriate selection of sample size and accurate application of DNA barcoding technology can establish a strong foundation. The study was in the initial efforts to employ specific DNA barcode markers and evaluate the efficacy of DNA barcoding in the precise documentation of cultivated species originating in Indonesia. The dataset generated from this study will play a vital role in establishing a comprehensive reference library. Additionally, it will encourage the active engagement of other researchers in investigating the genetic potential of the current germplasm for a wide range of applications.

Genetic diversity

The evaluated ISSR primers effectively amplified the *I. longeracemosa* DNA, yielding diverse patterns, quantities, and band sizes. A total of 53 bands (ranging from 200 to 2000 bp) attained amplification using eight ISSR primers (Table 3). Compared to earlier studies on *I. tinctoria* assessment (Hariri *et al*., 2017), the observed band number and polymorphism were slightly lower, ranging from 29% to 100%, and appeared contrary to the previous range (50%–100%). Polymorphic bands are in closer association with simple sequence repeats, which are prevalent in eukaryotic genomes, resulting in the beneficial characteristics of the ISSR feature (Alhasnawi *et al*., 2015; Teeluck *et al*., 2016).

The primers UBC 880 and UBC 850 yielded the least and the most bands, respectively. The limited number of bands might be evident due to insufficient complementary DNA. The existence of multiple complementary primer sequences showed no consistent association with a high percentage of band polymorphism (Amiteye, 2021). The

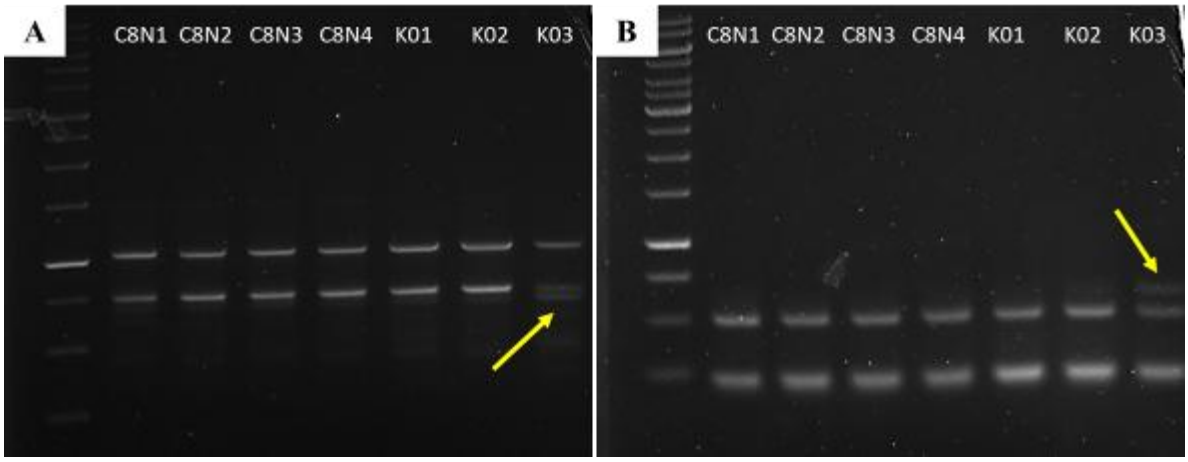

Figure 5. A partially displayed polymorphic bands of the primers UBC 856 and UBC 880. The potential promising bands for determining the undulate leaflet variation, as pointed by the yellow arrow.

Table 4. Total band patterns for binary data by the leaflet character group.

Leaflet Type	Entire	Undulate
Number of Different Bands	47	39
Number of Different Bands > or =5%	38	39
Number of Unique Bands to a Single Population	14	6
Mean Diversity (h)	0.184 (0.024)	0.191 (0.029)
Mean Unbiased Diversity (uh)	0.192 (0.025)	0.218 (0.033)

presented results indicated that a minimal quantity of amplified DNA in *I. longeracemosa* resulted in complete polymorphism. Complementary sequences, including the primer UBC 850, may exhibit lower abundance; however, they can display the diverse band lengths within the genome. Multiple bands appeared to identify the undulate leaflet variations in certain samples (KO3 and KO4), as evidenced by the bands amplified by the primers UBC 856 and UBC 880 (Figure 5). Six potential unique bands were also noticeable in *I. longeracemosa* exhibiting undulate leaves (Table 4). Further research may be necessary to confirm the consistency of the unique band patterns across the generations.

The observed genetic values among the *I. longeracemosa* populations reveal significant insights into the genetic diversity and structure of this species. The recorded highest genetic value of 0.29 emerged at the Trisik-Lemahireng location, while the lowest value of 0.08 appeared at Trisik-Srandakan (Table 5). This disparity in genetic values can refer to several influencing factors. One potential factor contributing to the high genetic value at Trisik-Lemahireng could be the presence of a more diverse habitat or ecological conditions that support a wider range of genetic variation. This location may have experienced less environmental disturbance, allowing for greater population stability and genetic exchange among individuals. Additionally, factors such as historical population size, migration patterns, and reproductive strategies may have facilitated the accumulation of genetic diversity in this area.

Conversely, the low genetic value observed at Trisik-Srandakan may indicate a more homogenous environment with limited ecological niches, which could restrict the genetic variation within the population. This location could have experienced higher levels of environmental stress or fragmentation, leading to a reduced gene flow and increased inbreeding. The minimal genetic distance values among populations from adjacent

Table 5. The genetic distance of *Indigofera longeracemosa* among the sampling location.

Locations	Trisik	Srandakan	Lemahireng	Grogol
Trisik	**			
Srandakan	0.082	**		
Lemahireng	0.291	0.181	**	
Grogol	0.220	0.142	0.168	**

Table 6. Molecular variation within *Indigofera longeracemosa* among the sampling locations.

Locations	N	Na	Ne	I	H	P%
Trisik	4	0.642 ± 0.115	1.136 ± 0.035	0.127 ± 0.033	0.085 ± 0.022	0.113 ± 0.029
Srandakan	20	1.509 ± 0.103	1.288 ± 0.045	0.283 ± 0.035	0.180 ± 0.024	0.190 ± 0.026
Lemahireng	4	1.038 ± 0.117	1.302 ± 0.056	0.237 ± 0.043	0.165 ± 0.030	0.220 ± 0.040
Grogol	4	0.736 ± 0.108	1.132 ± 0.037	0.119 ± 0.032	0.080 ± 0.022	0.107 ± 0.029
Average	8	0.981 ± 0.060	1.215 ± 0.023	0.192 ± 0.018	0.128 ± 0.013	0.157 ± 0.016

Note: N = number of specimen, Na = number of alleles, Ne = effective number of alleles, I = Shannon's information index, H = observed heterozygosity, P% = percentage of polymorphic loci.

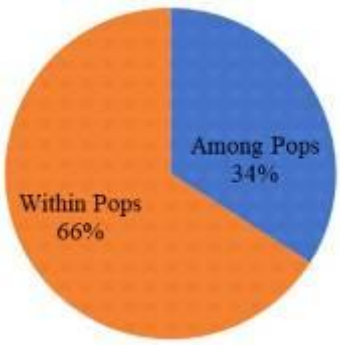

Figure 6. Molecular variation of *Indigofera longeracemosa* within and among the populations.

locations further support this notion, suggesting that the populations are genetically similar and may have limited opportunities for genetic exchange (Rodriguez *et al*., 2016).

Furthermore, the analysis of genetic structure (Table 6) implies that populations from Srandakan exhibited the highest gene diversity and Shannon's information index values (h = 0.180, I = 0.283), while populations from Grogol displayed the lowest values (h = 0.080, I = 0.119). The results suggest that while Srandakan has a higher level of genetic diversity, the overall genetic structure could still have gotten influences from factors such as habitat connectivity and environmental pressures. The contrasting values between these locations highlight the importance of habitat quality and ecological dynamics in shaping the genetic landscape of *I. longeracemosa* populations.

The genetic diversity of *I. longeracemosa* was significant (P=83%) (Table 3). The AMOVA results indicated that genetic variation among the populations (66%) exceeds the genetic variation within populations (Figure 6). This study demonstrated that the genetic composition of *I. longeracemosa* varies between ecotypes in Central Java and Yogyakarta Province, Indonesia. With the proximity of the four populations, further research should continue in a broader area, akin to prior studies on *I. tinctoria* in the Java and Madura islands, Indonesia (Hariri *et al*., 2017). Moreover, although *I. longeracemosa* is a self-pollinating species exhibiting reduced genetic variation

within its population, an alternative explanation is that the populations cultivated in the locations Grogol, Lemahireng, Srandakan, and Trisik possess the relevant genetic diversity sustained within each population (Zhang *et al*., 2017).

CONCLUSIONS

These investigations emphasize the phenotypic plasticity and genetic diversity of *I. longeracemosa* across the various cultivation sites in Central Java and Yogyakarta Province, Indonesia. Differences in leaf margin (entire and undulate) and coloration indicated how the species adjusted to various environmental factors. The analysis of the ITS region revealed its efficacy for accurate species identification, with considerable sequence comparability reinforcing its application in conservation efforts. Analysis using ISSR demonstrated that the genetic diversity among the populations surpassed those found within populations, with the Srandakan populations displaying the greatest gene diversity. The potential study will serve as a crucial reference for conservation and breeding initiatives and highlights the necessity for additional exploration in wider areas.

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