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Research Article

INTRA-SPESIFIC DIVERSITY OF BUTTERFLY PEA (*Clitoria ternatea* L.) REVEALED BY ISSR WITH INVARIABLE ITS RECORDS

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ARTICLE HIGLIGHTS

- The ITS region exhibits no genetic variation among butterfly pea genotypes.
- ISSR reveals high polymorphism, proving effective for genetic diversity analysis.
- White single and purple single genotypes show the greatest genetic distance.
- ISSR-6 ($(CT)_8TG$) demonstrates the highest polymorphism and reliability for assessment.
- The ITS sequences have been deposited in GenBank under the accession IDs PQ198055–PQ198061.

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ABSTRACT

Clitoria ternatea L., a perennial plant in the Fabaceae, is recognized for its resilience in tropical climates and its diverse applications in both culinary and medicinal fields. However, the limited exploration of its genetic diversity constrains breeding efforts aimed at improving desirable traits. This limitation highlights the need to optimize selection strategies, identify superior genotypes, and preserve valuable genetic resources for long-term conservation and crop enhancement. This study aimed to explore genetic variation using molecular markers to analyze *C. ternatea* genotypes based on petal architecture and color differences. To assess the genetic diversity of *C. ternatea*, Sanger sequencing of the Internal Transcribed Spacer (ITS) region and Inter-Simple Sequence Repeat (ISSR) markers were applied to seven wild populations from Sleman, Yogyakarta. The ITS region exhibited no genetic variation, indicating its conserved nature and limited ability to differentiate genotypes. In contrast, ISSR markers effectively detected genetic variation, identifying 62 polymorphic fragments out of 162 total bands. The highest genetic distance (0.297) was observed between the WS and PS genotypes, whereas the double-petal genotypes (WD and PD) displayed the closest phenetic relationship. Among the ISSR primers, UBC-808, UBC-812, and ISSR-6 exhibited high PIC and RP values, confirming their reliability in genetic diversity analysis. These results underscore the utility of ISSR markers as a robust tool for genetic diversity assessment, offering valuable insights for breeding programs and germplasm conservation in *C. ternatea*.

Keywords: butterfly pea, genetic distance, internal transcribed spacer, inter-simple sequence repeat, morphological variation

INTRODUCTION

Clitoria ternatea L. is a perennial plant from the Fabaceae, thrives in tropical climates and demonstrates resilience to environmental disturbances. It is widely recognized for its traditional Ayurvedic medicine and culinary applications (Ashraf *et al.* 2023; Maneechot *et al.* 2023). The flowers are used as supplements to enhance cognitive function (Oguis *et al.* 2019) and serve as a natural food coloring in rice cakes, tea, and various desserts (Chusak *et al.* 2018). Additionally, flower extracts have been reported to exhibit antibacterial, antioxidant, and antidiabetic properties (Rajamanickam *et al.* 2015; Jeyaraj *et al.* 2022).

Morphologically, *C. ternatea* flowers are solitary, grow in the axils, and have a characteristic papilionaceous structure. This species exhibits notable variations in petal morphology, with flowers having either single or double layers of

petals. In the single-layer type, the flower follows a papilionaceous structure, consisting of a standard petal (the largest), two wing petals, and two keel petals. In contrast, the double-layer type is characterized by enlarged wing and keel petals that reach the size of the standard petal, creating a layered appearance. The colors were distinct, ranging from white to blue and purple, corresponding to differences in anthocyanin composition (Suarna & Wijaya 2021; Surya *et al.* 2022).

Genetic variation plays a vital role in shaping species' traits and their capacity to adapt to changes in the environment (Mora *et al.* 2005). Molecular data-based identification techniques are particularly effective in describing species because they rely on stable genetic markers that are not influenced by environmental factors (Loureiro & Malfeito-Ferreira 2006; Elufioye & Badal 2017). A frequently used marker in genetic research is the Internal Transcribed Spacer (ITS) region, which is located between the 16S, 5.8S, and 26S regions of rDNA (Froeschke & Von Der Heyden 2014). Several studies have demonstrated the success of using ITS regions for assessing plant genetic diversity at the inter- and intraspecific levels (Tripathi *et al.* 2013; Sokołowska *et al.* 2022; Pere *et al.* 2023). ITS1 is generally more variable than ITS2 due to the presence of variable repeat units, making it particularly useful for species discrimination and effective in distinguishing various species in Fabaceae (Nolan & Cribb 2005; Vilas *et al.* 2005; Gao *et al.* 2010).

DNA-based molecular markers are other ideal tools for evaluating genetic diversity, breeding-related traits, and other mobile segments due to their neutrality and practicality (Bishoyi *et al.* 2014; Hasan *et al.* 2021; Aliabadi *et al.* 2023; Nidianti *et al.* 2023; Rosyidi *et al.* 2023). Inter Simple Sequence Repeats (ISSR) are a molecular marker technique that utilizes microsatellite sequences as primers to amplify regions between tandem repeats. ISSR markers are widely used due to their reliability, cost-effectiveness, rapid application, and high sensitivity in genetic analysis (McGlaughlin *et al.* 2002; Sarwat 2012; Rahali *et al.* 2022), and has been widely used to identify intraspecific variation (Zebarjadi *et al.* 2016; Nilkanta *et al.* 2017; Verma *et al.* 2017; Abdelaziz *et al.* 2020; Yusuf & Daryono 2021; Devi *et al.* 2024; Ahmed *et al.* 2022). ISSR cover a broad portion of the genome, include numerous polymorphic loci, and do not necessitate prior genomic knowledge (Liu *et al.* 2015; Tiwari *et al.* 2016).

Given the considerable variability in petal color and structural traits among *C. ternatea* genotypes, this study has been implemented to identify genetic variations and expand the knowledge base for breeding programs. Documenting this diversity is crucial for preserving genetic material that may otherwise be lost through breeding and cultivation, as well as for ensuring the continuity of information regarding species richness and existence.

MATERIALS AND METHODS

Genomic DNA Extraction

Seven distinct variants of *C. ternatea* were collected from a wild population in Sleman, Indonesia, each exhibiting different petal architecture and colors differences. Genomic DNA was extracted from 100 mg of frozen leaf samples with the Genomic DNA Mini Kit Plant (Geneaid, Taiwan). The concentration and purity of the DNA were evaluated using a NanoDrop Lite Plus spectrophotometer (Thermo Fisher Scientific, USA), with acceptable purity from 1.8 to 2.0 (Green & Sambrook 2012).

Amplification Process

PCR amplification was performed using the MyTaq HS RedMix (Bioline, US) protocol with several modifications. The total reaction volume was 20 µL, comprising 10 µL of MyTaq HS RedMix, 2 µL of primer, 2 µL of DNA sample at 50 ng/µL, and 6 µL of nuclease-free water. The amplification process was conducted in a T100 Thermal Cycler (Bio-Rad, US) over 40 cycles. The total reaction volume was 20 µL, consisting of 10 µL of MyTaq HS RedMix, 2 µL of primer, 2 µL of DNA sample (50 ng/µL), and 6 µL of nuclease-free water. Amplification was performed using a T100 Thermal Cycler (Bio-Rad, US) with 40 cycles. Each cycle involved a 45-second denaturation step at 95 °C, a 30-second annealing step at 45 - 50 °C, and a 1-minute elongation step at 72 °C. The protocol began with an initial denaturation at 95 °C for 3 minutes and ended with a final extension at 72 °C for 5 minutes. A universal ITS primer pair was employed, comprising ITS-u1 (5'-GGAAGKARAAGTCGTAACAAGG-3') and ITS-u4 (5'-RGTTTCTTTTCCTCCGCTTA-3') (Cheng *et al.* 2016). Details of the eighteen ISSR primers used are provided in Table 1.

Table 1 ISSR primer list in this study

No	Primer	Repeated motifs	Primer length, bp	Annealing Temperature, °C
1	UBC-807[a]	$(AG)_8T$	17	50.3
2	UBC-808[a]	$(AG)_8C$	17	50.3
3	UBC-809[a]	$(AG)_8G$	17	50.3
4	UBC-810[a]	$(GA)_8T$	17	47.5
5	UBC-811[a]	$(GA)_8C$	17	47.5
6	UBC-812[a]	$(GA)_8A$	17	50.3
7	UBC-815[a]	$(CT)_8G$	17	45.1
8	UBC-817[a]	$(CA)_8A$	17	50.3
9	UBC-818[a]	$(CA)_8G$	17	45.1
10	UBC-834[a]	$(AG)_8YT$	18	47.5
11	UBC-836[a]	$(AG)_8YA$	18	48.6
12	UBC-840[a]	$(GA)_8YT$	18	47.5
13	UBC-841[a]	$(GA)_8TC$	18	48.6
14	UBC-842[a]	$(GA)_8YG$	18	48.6
15	UBC-845[a]	$(CT)_8RG$	18	48.6
16	UBC-873[a]	$(GACA)_4$	16	48.6
17	UBC-880[a]	$(GGAGA)_3$	15	47.5
18	ISSR-6	$(CT)_8TG$	18	50.3

Notes: [a] = Obtained from the University of British Colombia; Y = pyrimidines; R = purines.

Agarose Gel Electrophoresis

The PCR products were separated by horizontal agarose gel electrophoresis on a 2% agarose gel (1st BASE, Singapore) stained with FloroSafe DNA stain (1st BASE, Singapore). Electrophoresis was carried out in 2% TBE buffer at 50 volts for 60 minutes. A 100 bp DNA ladder (Geneaid, Taiwan) was run simultaneously to estimate the molecular weights of the amplified products. The stained gels were subsequently visualized and documented with a GelDoc-UV transilluminator.

Sanger Sequencing and Bioinformatic Analysis

Sanger sequencing was analyzed at LPPT UGM, and the contig sequences were edited using GeneStudio v.2.2.0.0 (GeneStudio, Inc.). The cleaned sequences were subsequently analyzed using a BLAST search against the NCBI database. OPAL, integrated with MESQUITE (version 3.61), was used to align the contig sequences and accession sequence data retrieved from GenBank. Genetic distances were determined using MEGA-X (Kumar *et al.* 2018), and multiple sequence alignments were visualized with the MultAlin web-based software.

Binary Data Analysis

ISSR are dominant markers that provide bi-state (presence-absence) assessments (Shiran *et al.* 2007; Bishoyi *et al.* 2014). The recorded gels were scored for the absence (0) and presence (1) of bands, without considering band intensity, since each band occurrence with a specific molecular weight represents a locus (Ali *et al.* 2013). Band interpretation was performed using CorelDRAW software to define the operational taxonomical unit (OTU). The formula D = 1 – F, where F is the species similarity matrix value, was used to compute genetic distance (D) (Hillis *et al.* 1996). The Multi-Variate Statistical Package (MVSP) program was then used to create phenetic dendrograms based on the similarity matrix.

RESULTS AND DISCUSSION

Petals Observation

The flowers of *C. ternatea* exhibit a range of morphological variations, including petal architecture and colors differences (Bishoyi *et al.* 2014; Suarna & Wijaya 2021). Based on petal arrangement, we identified two different types: single and double petalloid (Fig. 1). In terms of color, there were four variations: white, light blue, light purple, and purple (Fig. 1). Petal colors have been recorded in several studies (Yeotkar *et al.* 2011; Ali *et al.* 2013; Bishoyi *et al.* 2014). Chemically,

petal color corresponds to its phytochemical composition, particularly anthocyanins (Jeyaraj *et al.* 2022), with no correlation to kaempferol content (Ali *et al.* 2013). Variations in anthocyanin concentration may influence the intensity of purple pigmentation, creating a color gradient ranging from white and blue to deep purple.

Invariable ITS Records

One of the most often utilized genetic markers in plant phylogenetics and DNA barcoding is the ITS region of nuclear ribosomal DNA (Poczai & Hyvönen 2010; Cheng *et al.* 2016). Due to its high sequence-level variability, conserved flanking regions, and quick coordinated evolution under comparable functional constraints, this region is helpful for resolving connections within genera and below species level (Vander Stappen *et al.* 2002; Poczai & Hyvönen 2010; Zhao *et al.* 2018).

The ITS sequences obtained from seven genotypes of *C. ternatea* were aligned to identify the types of mutations present. The alignment revealed two types of mutations: insertions/deletions (indels) and substitutions. An indel was observed at 56th nucleotide position (Fig. 2), where an additional nucleotide base T was present in the sample from Manipal, Karnataka, India, whereas the other samples exhibited a deletion at this position. Regarding substitution mutations, three specific changes were identified: A/G^{378}, T/C^{396}, and A/T^{491}, which distinguished the sample from Udupi, Karnataka, India from the others (Figs. 2 & 3).

Figure 1 Variations in flower petal of butterfly pea observed in this study

Notes: WS = Genotypes of white single; LBS = light blue single; LPS = light purple single; PS = purple single; LBD = light blue double; PD = purple double; and WD = white double; scale bar = 2 cm.

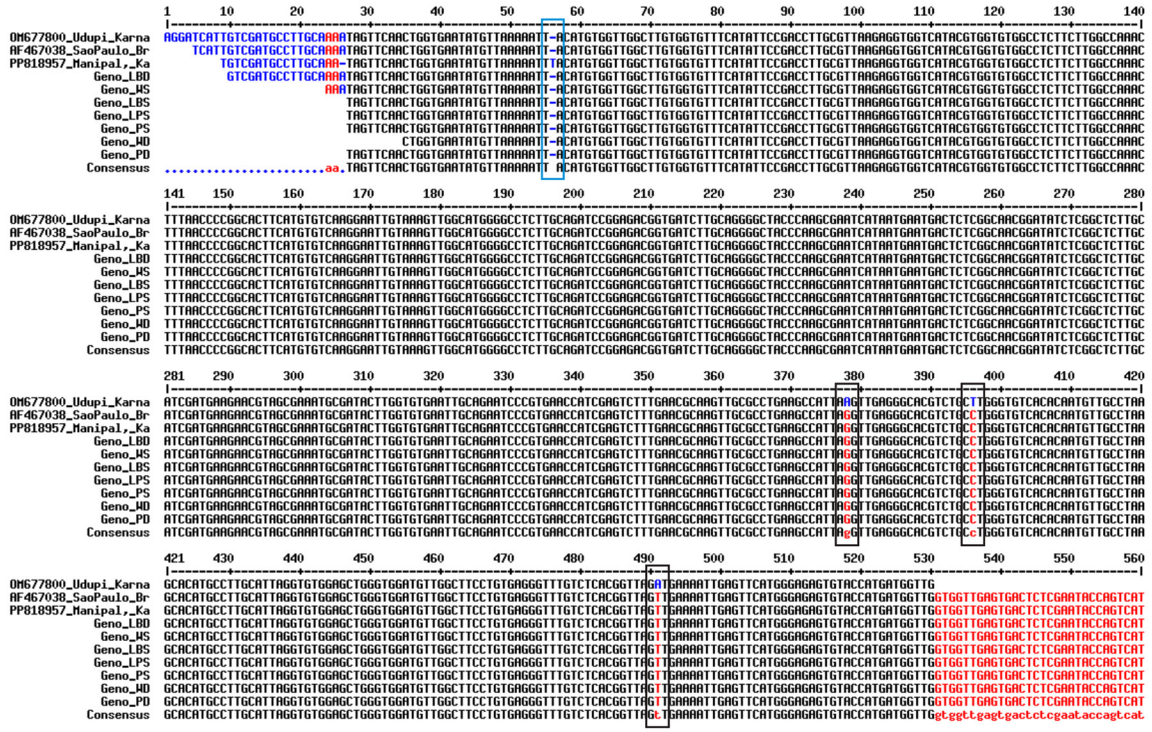

Figure 2 Multiple sequences alignment of internal transcribed spacer of butterfly pea

Notes: Black box represents substitution; blue box represents insertion/deletion mutational events; WS = Genotypes of white single; LBS = light blue single; LPS = light purple single; PS = purple single; LBD = light blue double; PD = purple double; and WD = white double.

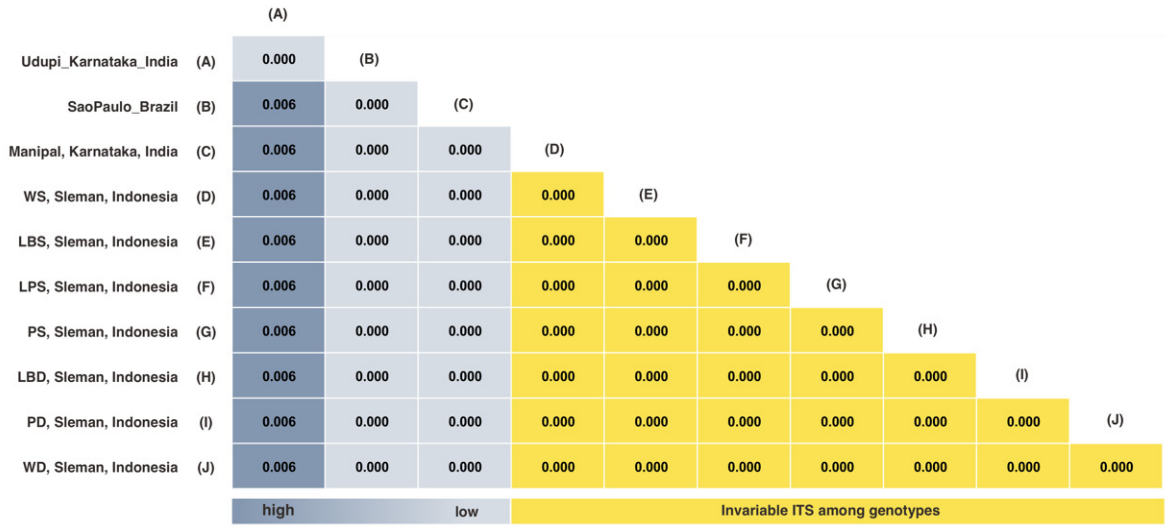

Figure 3 Genetic pairwise distance based on Kimura-2-parameter model

Notes: Yellow highlighted = Genotypes from Indonesia genotypes; grey highlighted = Genotypes from India and Brazil; WS = Genotypes of white single; LBS = light blue single; LPS = light purple single; PS = purple single; LBD = light blue double; PD = purple double; and WD = white double.

These findings suggested a degree of genetic variation among the analyzed genotypes, particularly in samples from Karnataka, India, which exhibited both indels and substitutions that differentiate them from other populations. The presence of an additional T nucleotide in the Manipal sample and the three substitution mutations in the Udupi sample indicated localized genetic divergence, possibly due to geographic isolation, environmental adaptation, or selective pressures.

Conversely, the identical ITS sequences observed in the seven genotypes and the São Paulo, Brazil samples suggested a high level of genetic conservation in this region, implying a shared evolutionary lineage or limited genetic differentiation over time. This could be due to recent common ancestry, low mutation rates in the ITS region, or historical seed exchange and cultivation practices that maintain genetic uniformity.

Moreover, ITS sequence data for *C. ternatea* remains highly limited in GenBank, restricting comprehensive comparative analyses. Nevertheless, an alignment-based analysis of all available ITS sequences, despite reducing the overall sequence length, revealed no genetic distance among the examined accessions (data not shown). This further reinforces the hypothesis that the ITS region in *C. ternatea* is highly conserved across different populations. Further phylogenetic studies incorporating additional molecular markers and broader geographic sampling would be necessary to better understand the genetic diversity and evolutionary history of this species. The sequence data from this study has been submitted to GenBank and assigned accession numbers PQ198055–PQ198061 for WS, LBS, LPS, PS, LBD, PD, and WD, respectively.

The universal ITS primer (ITS-u1/ITS-u4) employed in this study amplifies the ITS1-ITS2, and is suitable for over 95% of plants groups (Cheng *et al.* 2016). Numerous studies have already proven the efficiency of ITS in explaining phylogenetic connections and revealing interspecific genetic diversity within the Fabaceae (Ainouche & Bayer 1999; Vander Stappen *et al.* 2002; Gao *et al.* 2010; Wu *et al.* 2013; Xu *et al.* 2017).

In terms of their application in elucidating intraspecific relationships, ITS regions can reveal sufficient sequence divergence to assess genetic diversity in Korean native *Lilium* (Sultana *et al.* 2011), while ITS2 has been reported to show significantly higher intraspecific divergence in discriminating Chinese *Zanthoxylum* species (Zhao *et al.* 2018).

Nevertheless, some research findings indicated that ITS markers exhibit low intraspecific genetic diversity. This is exemplified by the Thar Desert endemic population of *Commiphora wightii* (Haque *et al.* 2009), as well as populations of *Aquilaria malaccensis* and *Aquilaria hirta* in Peninsular Malaysia, where no intraspecific variation was detected (Lee *et al.* 2018). Therefore, ITS markers are not linked to genes encoding morphological traits, further reinforcing the lack of correlation between morphological and genetic variation found in this study. The absence of ITS polymorphism among *C. ternatea* genotypes suggested a high level of sequence conservation, indicating that other genetic regions, such as functional genes or regulatory elements, may be responsible for the observed morphological differences. We overcame this obstacle by using ISSR molecular markers, which are essential for evaluating genetic stability in diverse breeding lines and for describing genetic variation both within and between species (Porth & El-Kassaby 2014; Aristya *et al.* 2019; Hasan *et al.* 2021; Yusuf *et al.* 2023).

Assessment of Polymorphism by ISSR

With an average of nine fragments generated from each of the eighteen ISSR primers, a total of 162 fragments with sizes ranging from 188 bp to 1583 bp were obtained (Table 2). A total of 62 polymorphic fragments were found among them, with an average of 3.61 polymorphic fragments for each primer. Each primer has a different number of polymorphic bands, ranging from 1 to 14. Specifically, the primer UBC-836 displayed eight monomorphic bands and no polymorphic bands, indicating a 0% polymorphism rate (Table 2). ISSR-6 had the largest polymorphism information content (PIC) value, measuring 93.33%, and a resolving power (RP) value of 10.00. This demonstrates the primer's usefulness in genetic variation research and its capacity to identify *C. ternatea* genotypes. The 18 ISSR primers produced an RP value of 3.23 and a PIC value of 0.12 on average.

The effectiveness of ISSR depends on the compatibility of each primer sequence with the target genome. The bands produced reflect the presence of specific microsatellites in the genome and guide the amplification process to generate

inter-microsatellite regions. It is highly probable that different amplified bands can be produced using the same ISSR primer sequences. For instance, similar ISSR primers studied by (Vijayan & Chatterjee 2003) in mulberry produced fewer scored bands compared to our study in *C. ternatea*, though both were lower than in *Mucuna pruriens* (Devi *et al.* 2024). This is consistent with the low amplification results produced by UBC-807, UBC-808, UBC-841, UBC-842, UBC-873, and UBC-880 in our study. The percentage of polymorphism is also influenced by the sample size, which correlates with the extent of the analyzed gene pool. Such variations were also observed in many Fabaceae species during ISSR-based analyses (Dos Santos Araújo *et al.* 2016; Bhadkaria *et al.* 2020; Ojuederie *et al.* 2020; Qahtan 2021; Reddy *et al.* 2022; Aliabadi *et al.* 2023; Helal *et al.* 2023).

Identification based on ISSR markers in different populations of *C. ternatea* produced Jaccard's coefficient similarity index from 0.80 to 0.98 (Bishoyi *et al.* 2014). In our study, the use of the informative primer ISSR-16 enhanced the ability to identify genetic variation among *C. ternatea* genotypes. Three identical repeated primer sequences—$(AG)_8C$, $(AG)_8G$, and $(CT)_8G$—were observed in this study, Bishoyi *et al.* (2014), and Nurhasanah *et al.* (2023). The observed results for the repeated motifs $(CT)_8G$ and $(AG)_8C$ by Bishoyi *et al.* (2014) were closely related to our study in terms of polymorphism rate, resolving power, and PIC values. The PIC value of $(AG)_8C$, around 0.20, was also similar to Nurhasanah *et al.* (2023). For the motif $(AG)_8G$, we identified a higher polymorphism rate and PIC value, although the RP value was lower than Bishoyi *et al.* (2014) and Nurhasanah *et al.* (2023). Another method for analyzing genetic variation is the use of Simple Sequence Repeats (SSR), which can provide allelic information among genotypes (Gebhardt 2007). SSR analysis has clearly validated the existence of allelic differences between blue and white flower color genotypes, based on the markers CcM2763 and CcM0830 (Naik *et al.* 2020).

Table 2 Amplified fragments and polymorphism derived from 18 ISSRs

Primer	NB	PB	PR (%)	FS	PIC	RP
UBC-807	7	1	14.29	380-1185	0.07	0.857
UBC-808	10	5	50.00	200-916	0.20	5.143
UBC-809	10	4	40.00	446-1158	0.13	3.143
UBC-810	8	1	12.50	267-1046	0.06	0.857
UBC-811	10	4	40.00	249-1117	0.16	4.000
UBC-812	11	8	72.73	278-1492	0.24	6.571
UBC-815	13	5	38.46	237-1583	0.14	7.143
UBC-817	10	5	50.00	427-1412	0.14	5.429
UBC-818	9	1	11.11	189-1096	0.03	0.286
UBC-834	9	2	22.22	188-1383	0.05	2.000
UBC-836	8	0	0.00	204-1038	0.00	0.000
UBC-840	7	3	42.86	199-1235	0.10	3.714
UBC-841	5	1	20.00	233-1183	0.05	0.286
UBC-842	6	2	33.33	370-1051	0.15	2.571
UBC-845	6	2	33.33	286-965	0.12	2.571
UBC-873	8	4	50.00	321-1259	0.17	2.286
UBC-880	10	3	30.00	222-1442	0.10	1.429
ISSR-6	15	14	93.33	340-1472	0.31	10.00
Total	**162**	**65**				
Mean		**3.61**	**36.34**		**0.12**	**3.24**

Notes: NB = No. of scored bands; PB = No. of polymorphic bands; PR = polymorphic rate; FS = fragment size; PIC = polymorphic information content; RP = resolving power.

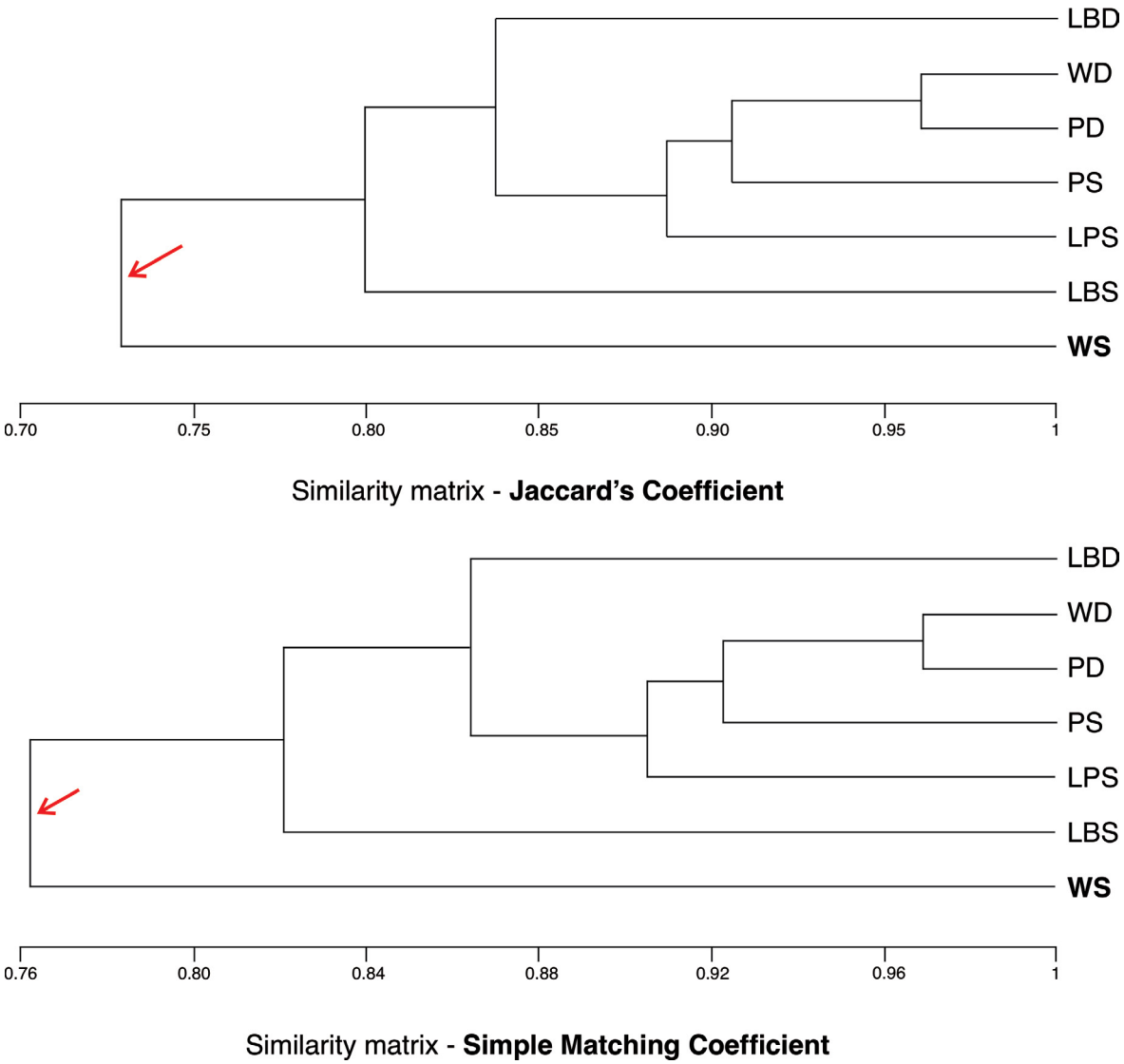

Figure 4 Phenetic dendrogram of seven genotype of butterfly pea based on (A) Jaccard's and (B) simple matching coefficient

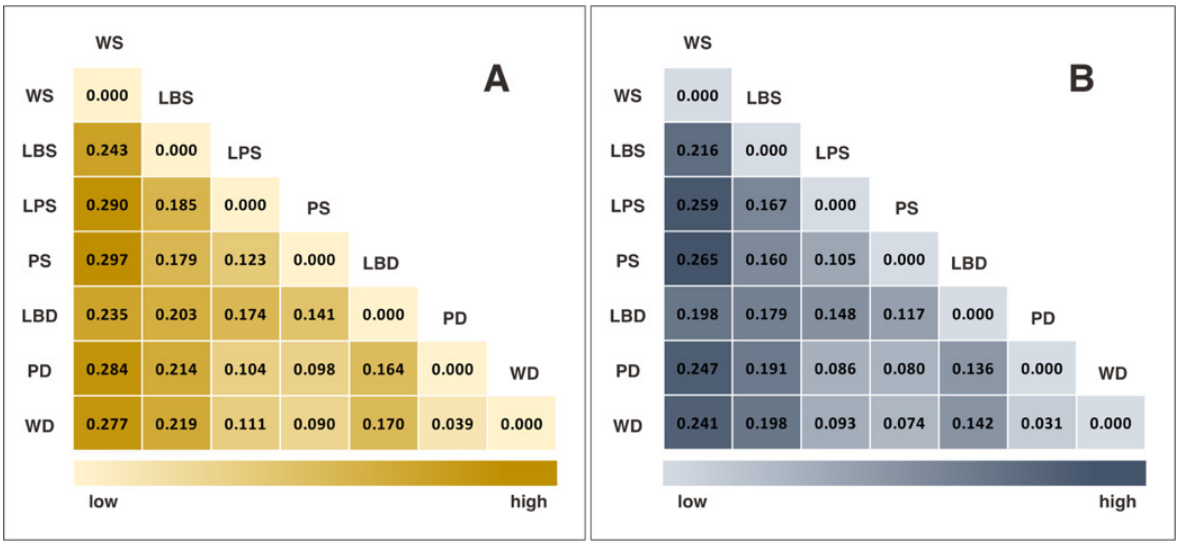

Figure 5 Figure 5 Genetic distance of seven genotype of butterfly pea based on (A) Jaccard's and (B) simple matching coefficient

Notes: WS = Genotypes of white single (WS); LBS = light blue single; LPS = light purple single; PS = purple single; LBD = light blue double; PD = purple double; and WD = white double (WD).

The dendrogram plots constructed from 18 ISSR primers did not reveal specific clades, instead showing random plotting due to the very low genetic variance among *C. ternatea* genotypes. Two methods for measuring similarity, Jaccard's (SJ) and simple matching (SSM) coefficients, produced similar results (Fig. 4).

The red line in Figure 4, indicates the lowest similarity percentage among the white single genotype compared to the other genotypes, with 73% and 76% similarity according to SJ and SSM, respectively. A higher similarity percentage, ≥ 80%, was observed among genotypes marked with a blue line in Figure 4. The highest similarity percentage among *C. ternatea* genotypes was between the white double and purple double genotypes, with similarity values of 96% and 97% for SJ and SSM, respectively.

According to Jaccard's coefficient, the greatest genetic distance was observed between the white single (WS) and purple single (PS) genotypes, with a value of 0.297 (Fig. 5A). This was followed by genetic distances between white single (WS) and the light purple single (LPS), purple double (PD), white double (WD), and light blue single (LBS) genotypes, with values of 0.290, 0.284, 0.277, and 0.243, respectively. A similar trend was observed when using the Simple Matching Coefficient, though the dissimilarity values were generally lower compared to those derived from Jaccard's method (Fig. 5B).

ISSR markers are dominant and arbitrary, amplifying random regions of the genome without targeting specific genes responsible for morphological traits. As a result, the genetic variation detected does not necessarily correspond to the observed morphological differences. This lack of correlation suggests that while phenotypic variation in *C. ternatea* may be influenced by genetic factors, it is likely regulated by specific loci that are not captured by ISSR markers. Additionally, environmental factors and epigenetic modifications could contribute to the observed morphological diversity, further decoupling it from the genetic profiles revealed in this study.

CONCLUSION

ITS sequencing revealed no genetic variation, as all 7 genotypes in this study exhibited identical sequences with no detected indels or substitutions, confirming the conserved nature of this region and its inefficacy in assessing genetic diversity. ISSR markers demonstrated high efficacy, identifying 62 polymorphic fragments out of 162 total bands, indicating substantial genetic variation within the population. The greatest genetic distance (0.297) was observed between the white single and purple single genotypes. The double-petal genotypes (WD and PD) exhibited the closest phenetic relationship. Among the ISSR primers tested, ISSR-6 exhibited the highest polymorphism (93.33%) and resolving power (10.00), further reinforcing its reliability for genetic diversity assessment. No direct correlation

was observed between ISSR polymorphism and petal morphology. The detected genetic diversity provides essential baseline data for *C. ternatea* germplasm conservation, ensuring the preservation of diverse genetic resources with potential applications in future breeding programs. ISSR markers offer a valuable tool for genetic diversity analysis, and their integration with advanced molecular approaches, such as SNP genotyping or gene expression analysis, can further enhance the identification of genetic loci associated with petal color and structure.

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