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Assessment of genetic variability in maize cultivars (*Zea mays* L.) in Mahasarakham Province, Thailand based on ISSR analysis

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Abstract - Maize (*Zea mays* L.) is one of the most important cereals and most widely cultivated staple crops worldwide. Studying genetic diversity is necessary for varietal identification, better understanding of relationships, and conserving genetic resources. This study aimed to evaluate the genetic diversity of eight local maize cultivars sourced from Kantarawichai, Muang, Chiang Yuen, Na Chueak, and Yang Si Surat in Mahasarakham Province, Thailand. DNA profiling was conducted using 14 ISSR markers, revealing that 11 of these markers produced a total of 61 bands, with an average of 5.55 alleles per locus across the samples. Our findings demonstrated a high polymorphism rate of 86.85%. The genetic similarity coefficients ranged from 0.128 to 0.791, while PIC values varied from 0.12 to 0.44, averaging 0.34. ISSR markers exhibited significant discriminatory power in detecting genetic diversity. The dendrogram classified genotypes into three clusters; Cluster I comprised genotypes with yellow kernels, Cluster II included five genotypes divided into two subclusters—SCII-A with white kernels and SCII-B with mixed kernels—and Cluster III with one genotype featuring white kernels. This clustering based on kernel colors closely aligned with the ISSR profiles of the maize genetic resources, effectively distinguishing between groups with different kernel types. These results highlight the effective use of ISSR markers in assessing genetic diversity among local maize cultivars in Mahasarakham Prov-

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ince. This preliminary evaluation emphasizes the importance of conserving and utilizing local maize genetic resources to enhance regional maize cultivation and support future breeding programs.

Keywords: Cluster analysis, genetic diversity, ISSR markers, molecular characterization, DNA polymorphism

1. Introduction

Maize (*Zea mays* L.) is a vital staple crop globally consumed by humans and livestock, ranking third in production after wheat and rice. It plays a crucial economic role, supporting livelihoods worldwide (Erenstein et al., 2022). However, modern farming practices and selective breeding have narrowed. The genetic diversity in maize, increasing susceptibility to pests and environmental stress. Conserving genetic resources and promoting sustainable practices are essential to preserve and utilize maize's genetic variability effectively.

Assessing genetic diversity in maize involves morphological traits and molecular markers. While morphological markers offer insights, they are influenced by environmental factors, potentially skewing diversity assessments. Biochemical and cytological markers, though useful, have limited genome coverage (Hoxha et al., 2004; Yaman, 2022). In contrast, molecular markers like Simple Sequence Repeat (SSR), Randomly Amplified Polymorphic DNA (RAPD), Single Nucleotide Polymorphisms (SNP), and Inter Simple Sequence Repeats (ISSR) offer broader genome coverage and independence from environmental factors. These markers show a significant role in advancing our understanding of genetic diversity, population structures, and gene mapping (Amiteye, 2021).

Among these, ISSR markers stand out for their efficiency and reproducibility in targeting multiple SSR loci across the genome. Developed by Zietkiewicz et al. (1994), ISSR markers are renowned for their high reproducibility, polymorphic richness, and cost-effectiveness. They have been extensively applied in diverse studies including plant genetic diversity assessments (Lenka et al., 2015), cultivar identification (Dar et al., 2018; Zeyad et al., 2023), and gene mapping (Domenyuk et al., 2002), highlighting their utility in advancing maize breeding programs.

In Mahasarakham Province, local maize cultivars are traditionally cultivated by farmers across the region. However, there is a pressing need to systematically evaluate these cultivars to uncover and utilize their genetic diversity for future maize variety development. Previous studies on maize genetic diversity in Thailand or the region have often focused on commercial cultivars like sweet or waxy corn, potentially overlooking comprehensive assessments, particularly using molecular markers such as ISSR, for local cultivars. This study aims to (1) assess genetic variation among eight maize cultivar accessions sourced from four locations within Mahasarakham Province using ISSR markers, (2) determine the genetic relationships among these cultivars, and (3) identify the most genetically diverse local genotypes, which could serve as valuable candidates for future breeding programs aimed at developing improved maize varieties.

2. Materials and methods

2.1 Seed material

In this study, we collected eight local maize samples from Mahasarakham Province, chosen for their traditional cultivation by local farmers and the limited prior genetic diversity studies on these cultivars. This study aims to provide a preliminary assessment and lay the groundwork for future research. The eight maize genotypes were obtained from farmer fields in various locations: Kantarawichai, Muang, Chiang Yuen, Na Chueak, and Yang Si Surat within Mahasarakham Province, Thailand. The maize collection sites, along with their respective GPS coordinates and altitude information, are shown in Table 1 and Figure 1. All seed samples were germinated in soil-filled culture trays in the greenhouse of the Department of Biology, Faculty of Science, Mahasarakham University. Young, fresh leaf samples were collected from three individual plants of each genotype at 14-15 days old.

Table 1. Sample number, accession code, and collection locations of the eight maize genotypes used in this study.

Sample no.	Accession code	Location	Coordinates (Latitude-N, Longitude-E)	Altitude (m asl.)
1	Acc.MK-1-Y	Ban Nong Ko, Khok Pra Subdistrict, Kantarawichai, Mahasarakham Province	16.33137, 103.34256	159.5
2	Acc.MK-2-W	Khok Pra Subdistrict, Kantarawichai, Mahasarakham Province	16.33029, 103.29110	152.7
3	Acc.MM-3-Y	Khwao istrict, Mueang Maha Sarakham District, Maha Sarakham	16.14546, 103.41108	142.7
4	Acc.MM-4-W	Waeng Nang Subdistrict, Mueang Mahasarakham District, Mahasarakham Province	16.16150, 103.28615	149.8
5	Acc.MC-5-Y	Kutong Subdistrict, Chiang Yuen District, Mahasarakham Province	16.44516, 102.96128	159.6
6	Acc.MC-6-W	Chiang Yuen District, Mahasarakham Province	16.40545, 103.10594	165.8
7	Acc.MN-7-W	Nong Mek Subdistrict, Na Chueak District, Mahasarakham Province	15.81283, 103.03645	157.9
8	Acc.MY-8-W	Yang Si Surat Subdistrict, Yang Si Surat District, Mahasarakham Province	15.69397, 103.10507	162.5

Note: Y = yellow kernel, W = white kernel

Figure 1. Sample collection sites used in the study. The location numbers (1-8) correspond to the collection sites for each maize accession listed in Table 1.

2.2 Genomic DNA isolation

The molecular analysis was performed in the Laboratory of Molecular Genetics, Department of Biology, the Faculty of Science, Mahasarakham University, Thailand. The Cetyl Trimethyl Ammonium Bromide method (Doyle & Doyle, 1990) was partially adapted and used for extracting genomic DNA from small amounts of young leaf tissue (Hormaza, 1999). Approximately 0.2 g of fresh young leaves were ground in liquid nitrogen, and the homogenate was added to 1 ml of extraction buffer [2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 1% PVP-40, pH 8.0] containing 1 µl of 2-mercaptoethanol, which was added just before use. The extract was incubated at 60°C for an hour with occasional swirling, then combined with an equal volume of chloroform: iso-amyl alcohol (24:1, v/v) and centrifuged at 13,000 g for 10 minutes. The aqueous phase was transferred to a fresh tube and combined with 2/3 volume of ice-cold isopropanol. The mixture was left at -20°C for 20 minutes and then centrifuged again at 13,000 g for 10 minutes. The resulting pellet was rinsed with 10 mM ammonium acetate in 76% ethanol, allowed to dry for 1 to 2 hours at room temperature, and dissolved in 40 µl TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). The DNA solution was treated with 10 mg of RNAse A and incubated at 37°C for 10 minutes to remove RNA. The extracted DNA was analyzed using 1% agarose gel electrophoresis, and its quantity was estimated by measuring the absorbance at 260 and 280 nm using a spectrophotometer. Based on the spectrophotometer readings, 50 ng/µl DNA solutions were prepared and used for PCR amplification.

2.3 PCR of the ISSR technique

This work utilized fourteen ISSR primers from the University of British Columbia (UBC), selected based on previous research and following the protocol described by Borse et al. (2018). The primers used were UBC-811, UBC-817, UBC-818, UBC-825, UBC-827, UBC-834, UBC-810, UBC-813, UBC-815, UBC-819, and UBC-820 (see Table 2). The ISSR-PCR was conducted with a total reaction volume of 25 µl, containing 0.2 mM dNTP mix (Vivantis Technologies, Malaysia), 2 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Invitrogen, Brazil), 1X *Taq* polymerase buffer (Invitrogen, Brazil), 1.5 µM of each ISSR primer (Integrated DNA Technologies, Singapore), and 50 ng of genomic DNA. DNA amplifications were performed using a programmed thermal cycler (Applied Biosystems Veriti™, Singapore) with an initial denaturation at 95°C for 5 minutes, followed by 35

cycles of 95°C for 30 seconds, annealing at 45-54°C for 45 seconds, and extension at 72°C for 1 minute. A final extension at 72°C for 4 minutes was used to complete the primer extension. Each primer had a specified annealing temperature (Table 2). Gel electrophoresis in 1% agarose gel with 1X TBE buffer was used to separate the amplification products. DNA was stained with 0.5 µg/ml non-toxic DNA dye (RedSafe™, iNtRON Biotechnology, Inc., South Korea) and visualized using a UV-Vis transilluminator.

2.4 ISSR data analysis

The profiling pattern of the accessions developed by each ISSR primer was scored separately. The size of the amplified fragments was determined by comparing their migration distance to that of known size markers (100 bp DNA ladder). Bands produced by each ISSR primer were counted for every maize genotype. Band sizes were recorded, and monomorphic and polymorphic bands were identified among all genotypes for each primer. A base pair position was scored as '1' for the presence and '0' for the absence of a band at that position. The number of polymorphic and monomorphic bands were determined for each primer.

The parameters assessed included the total number of amplified bands (NTB), the number of polymorphic bands (NPB), the percentage of polymorphism (PP), the number of monomorphic bands (NMB), and the polymorphic information content (PIC). NTB was determined by counting the total number of clear DNA bands produced through PCR amplification. NPB was calculated by observing the different positions of DNA bands across all lanes, and PP was calculated as the ratio between NPB and NTB. The total number of amplified bands across all samples was used to determine NMB. The PIC value was estimated for each ISSR locus using the formula by Roldán-Ruiz et al. (2000): $PIC = 2fi(1 - fi)$, where fi is the frequency of the marker bands present and $1\text{-}fi$ is the frequency of the absent marker bands.

A binary matrix was constructed based on the DNA banding data. This matrix was then used to construct a dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method through the SAHN (Sequential, Hierarchical, Agglomerative, and Nested Clustering) module of the NTSYSpc 2.1 software (Rohlf, 2000) to illustrate the genetic relationships. A tree plot was also used to draw a cluster analysis tree.

3. Results and discussion

All isolated genomic DNAs were of sufficient quality to serve as PCR templates. The PCR amplification produced appropriate profiling patterns for the DNA samples of the eight maize genotypes. The band patterns produced by primer UBC-813 are depicted in Figure 1. Out of the 14 primers, 11 ISSR markers produced a total of 61 bands across the eight samples, of which 53 (86.89%) were polymorphic. Only the polymorphic bands were included in the final analysis.

In the analysis, a total of 61 different alleles were amplified across the 11 ISSR primers, resulting in an average of 5.55 ISSR alleles per locus. Among these primers, Primer UBC-815 produced the highest number of polymorphic alleles count with 10 bands, following closely, UBC-810 and UBC-817 yielded 8 polymorphic alleles

each while UBC-820 generated the lowest, with only one polymorphic allele. Notably, NPB presented 53 bands (86.89%), whereas NMB had 8 bands (13.11%), averaging 4.82 and 0.73 bands, respectively. The percentage of polymorphism (PP) varied from 20% (UBC-825) to 100% (UBC-817, UBC-818, UBC-827, UBC-824, UBC-815, UBC-819 and UBC-820), with an average polymorphism rate of 86.85%.

A high PIC value suggests that these primers are effective in distinguishing between different genotypes. In this study, the PIC values varied from 0.12 to 0.44 across the primers, with UBC-827 suggesting the highest PIC value, followed by UBC-819. In contrast, UBC-825 indicated the lowest PIC value among the primers analyzed, the average PIC value was moderate at 0.34. (Table 2).

Table 2. List of ISSR markers, band variation, and PIC values for the 14 ISSR markers utilized in this study.

Markers	*Sequence (5'- 3')*	*NTB*	NPB	NMB	PP	*PIC*
UBC 811	GAG AGA GAG AGA GAG AC	4	3	1	75	0.27
UBC 817	CAC ACA CAC ACA CAC AA	8	8	0	100	0.37
UBC 818	CACACACACACACACAG	3	3	0	100	0.38
UBC 825	ACA CAC ACA CAC ACA CT	5	1	4	20	0.12
UBC 827	ACA CAC ACA CAC ACA CG	5	5	0	100	0.44
UBC-834	AGA GAG AGA GAG AGA GYT	6	6	0	100	0.41
UBC-810	GAG AGA GAG AGA GAG AT	9	8	1	88.88	0.33
UBC-813	CTC TCT CTC TCT CTC TT	7	5	2	71.43	0.31
UBC-815	CTC TCT CTC TCT CTC TG	10	10	0	100	0.32
UBC-819	GTG TGT GTG TGT GTG TA	3	3	0	100	0.42
UBC-820	GTG TGT GTG TGT GTG TC	1	1	0	100	0.41
Total		**61**	**53**	**8**		
Mean		**5.55**	**4.82**	**0.73**	**86.85**	**0.34**

Note: B, D, H, R, V, Y = IUB nucleotide code
: NTB = number of total bands, NPB = number of polymorphic bands, PP = percentage of polymorphism, NMB = number of monomorphic bands, PIC = polymorphic information content

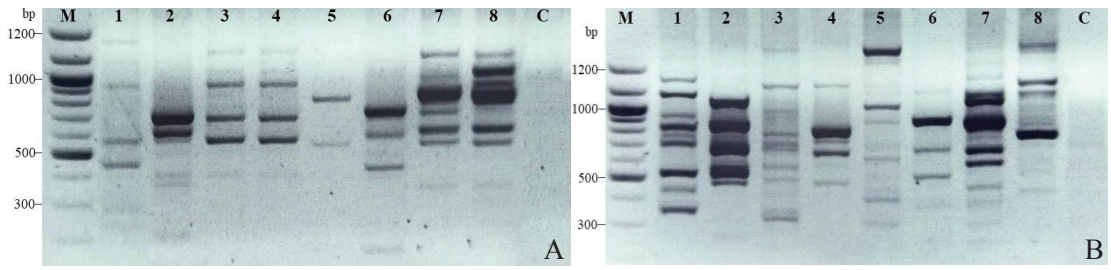

Figure 1. Results of DNA amplification of the eight local maize genotypes using ISSR primers (A) UBC-834 and (B) UBC-815 are shown. Lanes 1-8 correspond to the DNA profiles of maize samples 1-8, as referenced in Table 1. Lane C represents the negative control, and lane M contains the DNA molecular weight marker.

Table 3. Genetic similarity among 8 local maize genotypes based on the 14 ISSR markers.

Sample no.	1	2	3	4	5	6	7	8
1	1.000							
2	0.439	1.000						
3	0.661	0.579	1.000					
4	0.522	0.777	0.422	1.000				
5	0.470	0.418	0.326	0.479	1.000			
6	0.524	0.343	0.461	**0.791**	0.413	1.000		
7	0.320	0.520	0.595	0.313	0.683	0.324	1.000	
8	**0.128**	0.340	0.348	0.320	0.268	0.239	0.287	1.000

Note: Sample no. refer to Table 1

A genetic similarity matrix was established for the eight maize accessions using the method outlined by Randi et al. (1989). The genetic similarity coefficients among the local maize genotypes ranged from 0.128 to 0.791 (Table 3). The highest similarity value, 0.791, was observed between genotypes 6 (Acc.MC-6-W) and 4 (Acc.MM-4-W), indicating a close genetic relationship. Conversely, the lowest similarity value, 0.128, was found between genotypes 8 (Acc.MY-8-W) and 1 (Acc.MK-1-Y), suggesting a significant genetic divergence between these two genotypes.

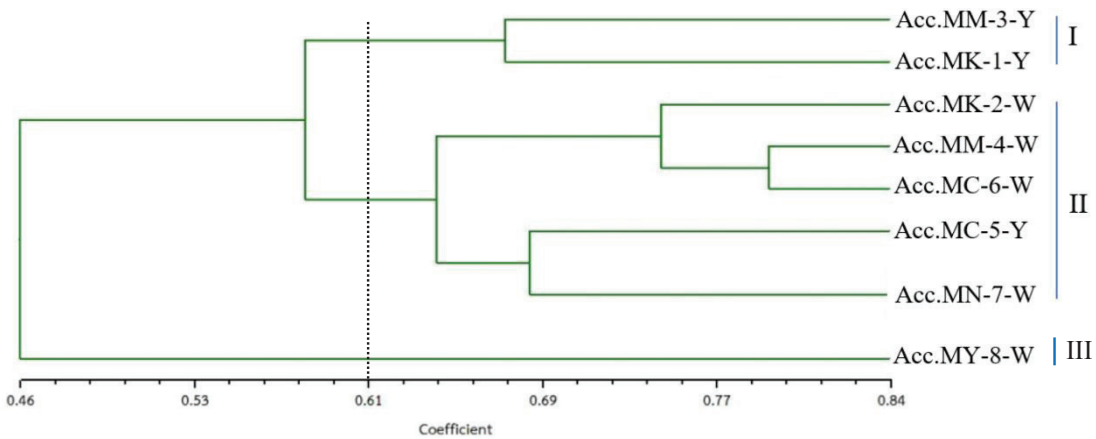

Figure 2. UPGMA dendrogram of 8 maize genotypes based on ISSR marker data. The accessions are grouped into three clusters: Clusters I, II, and III.

The cluster analysis based on genetic similarity values resulted in a dendrogram with three major clusters at a similarity coefficient of 0.61 (Figure 2). Cluster I consisted of two local maize genotypes, Acc.MK-1-Y and Acc.MM-3-Y, both with yellow kernels, representing 25% of the total accessions investigated. Cluster

II comprised five genotypes, accounting for 62.5% of the total. Cluster II was further divided into two subclusters: SCII-A included three white-kernel accessions (Acc.MK-2-W, Acc.MM-4-W, and Acc. MC-6-W), while SCII-B contained two accessions with mixed kernel colors (Acc. MC-5-Y and Acc.MM-7-W). Additionally, a single outlier accession (Acc.MY-8-W) with white kernels was separated from the main grouping into Cluster III, representing 12.5% of the total genotypes.

Evaluating genetic diversity is essential for conserving and breeding maize germplasm to develop ideal crop varieties for current and future needs. ISSR primers, which are longer and bind to conserved areas between SSR regions, offer advantages such as simplicity and high reproducibility (Amiteye, 2021). The ISSR method is effective in distinguishing genotypes with high genetic similarity due to the high mutation rate commonly seen in ISSR loci. marker has been widely used in maize genetic diversity studies. For instance, Uslan and Jannah (2020) used ISSR fingerprinting to assess the genetic diversity of local maize cultivars from South Amarasi, Kupang District, Indonesia. Similarly, Yani et al. (2022) analyzed the genetic diversity of local corn cultivars from the same region using ISSR markers, suggesting the effectiveness of this method in revealing genetic variation among local maize cultivars.

The high polymorphism rate of ISSR markers indicates their effectiveness in assessing genetic diversity among genotypes (Rini et al., 2023). Our study showed a polymorphism rate of 86.85%, which is comparable to the 88.9% polymorphism rate reported by do Amaral Júnior et al. (2011) among maize cultivars. Similarly, Yani et al. (2022) observed an 86.09% polymorphism rate in eight local maize cultivars, and Mukhlif et al. (2023) reported an 82.4% polymorphism rate in ten inbred lines of maize. These studies underscore the utility of ISSR markers in revealing genetic variation among maize genotypes.

Genetic diversity and marker utility in this study were assessed using PIC values, which measure marker informativeness based on allele numbers and frequencies. Our PIC values ranged from 0.12 to 0.44, with UBC-827 being the most suitable for genetic diversity analysis. According to Botstein et al. (1980), a PIC value of ≥ 0.5 is highly informative, $0.5 > \text{PIC} \geq 0.25$ is moderately informative, and $\text{PIC} < 0.25$ is minimally informative (Tran et al., 2022). Our moderate PIC values were higher than those reported by Muhammad et al. (2017) but lower than those reported by Feroz et al. (2022). To gain more genetic information, it is recommended to increase the number of primers or use alternative DNA markers such as SSR (Islam et al., 2023), SNP (Dube et al., 2023), InDel, or SCoT (Vivodík et al., 2017). Higher PIC values indicate better allele discrimination, emphasizing their importance in genetic diversity studies.

The results revealed significant genetic variation among the genotypes, as indicated by the diverse range of similarity coefficient values. Muhammad et al. (2017) reported a wide range of similarity coefficients from 0.118 to 0.888, indicating high diversity among germplasm resources. In contrast, Feroz et al. (2022) Observed a genetic similarity index varying from 0.15 to 0.45, indicating a lower

variability range than this study. Additionally, Uslan and Jannah (2020) observed similarity coefficients ranging from 0.004 to 0.700 in eight local corn cultivars using ISSR markers. Our results, showing genetic similarity coefficients ranging from 0.128 to 0.791, Were comparable to those of Uslan and Jannah (2020), indicating a degree of genetic similarity among the accessions and suggesting relatively similar levels of genetic diversity.

The genetic relationships among the 8 local maize accessions revealed three clusters. Cluster I included genotypes with yellow kernels, while Cluster II comprised 5 genotypes, further divided into two subclusters: SCII-A with white kernels and SCII-B with mixed kernels. Cluster III consisted of one genotype with white kernels. The clustering based on kernel colors closely corresponded with the ISSR profiles of the maize genetic resources, effectively distinguishing between groups of white and yellow kernels, though some were mixed. Interestingly, the ISSR technique did not differentiate maize genotypes based on their regional origins of collection. Furthermore, previous studies on maize conducted by Muhammad et al. (2017), Uslan an Jannah (2020), and Yani et al. (2022) have similarly found genetically close relationships among maize accessions within the same cluster using ISSR markers.

Genetic diversity is crucial for conservation and breeding efforts, as it elucidates the processes influencing genetic variation, which underpin hierarchical biology (Zulfahmi, 2013). This foundational knowledge supports conservation initiatives, breeding programs, and the sustainable use of genetic resources. Our study assessed the genetic variation among eight local maize cultivar accessions from Mahasarakham Province using ISSR markers. The findings revealed significant genetic diversity and distinct genetic relationships among the cultivars. The identified genetically diverse genotypes are valuable candidates for future breeding programs aimed at developing improved maize varieties. This preliminary evaluation underscores the importance of conserving and utilizing local maize genetic resources to enhance regional maize cultivation.

5. Conclusion

This study revealed significant genetic variability among eight local maize genotypes using 14 ISSR markers. The results demonstrated a high polymorphism rate, with PIC values confirming the markers' efficacy in assessing genetic diversity. Similarity coefficients indicated varying degrees of genetic similarity among the accessions, suggesting that the genetically diverse genotypes are valuable candidates for future breeding programs. UPGMA clustering analysis grouped the genotypes into three clusters corresponding to kernel color traits. These findings enhance our understanding of genetic diversity among local maize accessions in Mahasarakham Province. This initial assessment highlights the critical need to conserve and harness the genetic diversity of local maize resources to improve maize cultivation regionally.

Declaration

The authors declare that this work has no conflict of interest.

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