Indian J. Genet. Plant Breed., (2025); 85(4): 593-602
https://doi.org/10.31742/ISGPB.85.4.7
ISSN: 0975-6906
www.isgpb.org

RESEARCH ARTICLE

Folate profiling and characterization of sub-tropically adapted maize inbreds using folate-metabolism related genes

K. Victor Paul, Prafulla Kumar, Firoz Hossain, Vignesh Muthusamy, Subhra J. Mishra, Ashvinkumar Katral and Rajkumar U. Zunjare*

Abstract

Folates, also known as vitamin B9, are vital for the normal growth and development of humans. In this study, we assessed 48 specialty and biofortified maize inbred lines for folate content and characterized them using markers specific to 78 candidate genes governing folate metabolism. Folate content, measured as the sum of 5-formyl-tetrahydrofolate (5-FTHF) and 5-methyl-tetrahydrofolate (5-MTHF), varied widely from 21.4 to 98.0 µg/100g, with a mean of 55.7 µg/100 g. Analysis using 78 SSR markers indicated a high conservation of folate-related genes across the genotypes. A total of 116 alleles were detected with a mean of 2.27 alleles/locus (range: 2–5). Among the markers, Fo-SSR-17 had five alleles, showing the highest discriminating power. The mean polymorphism information content (PIC) was 0.36 with a range from 0.19 (Fo-SSR-62) to 0.59 (Fo-SSR-50). Diversity analysis grouped the 48 genotypes into three distinct clusters, with a dissimilarity coefficient ranging from 0.08 to 0.34 (mean: 0.22). Single marker analysis found four markers significantly associated with folate content. Further, the haplotype analysis identified Hap6 (ABBB), Hap8 (ABHB), and Hap5 (ABAA) as superior haplotypes exhibiting higher folate content with mean values of 87.6 µg/100 g, 83.7 µg/100 g and 79.5 µg/100 g, respectively. Identification of diverse high folate inbred lines of maize inbreds and superior haplotypes offers potential for use in maize folate biofortification programs. This is the first report on characterization using a candidate gene for folate accumulation in subtropical genetic background.

Keywords: Folate deficiency, Tetrahydrofolate, Specialty corn, Molecular diversity analysis, Folate biofortification.

Introduction

Malnutrition has become an emerging serious concern, particularly in the developing and underdeveloped countries of the world. Folates, which include tetrahydrofolate (THF) and its derivatives, are essentially required for proper growth and development of the fetus within mother's womb (Akwaa-Harrison et al. 2024). It plays a biologically important role in C1 metabolism that involves the biosynthesis of nucleotides, amino acid metabolism, and different methylation reactions along with DNA repair (Blancquaert et al. 2010). Folates are synthesized *de novo* in plants and other microorganisms, but not by mammals. Hence, humans must rely on dietary sources to meet their folate requirements (Strobbe and Van Der Straeten 2017). Folate deficiency (FD) refers to reduced folate concentrations in erythrocytes (<140 ng mL^{-1}) and/or blood plasma (<3 ng mL^{-1}) (Blount et al. 1997). FD is highly associated with disorders like neural tube defects (NTDs), which occur especially during early pregnancy. Folate biofortification, the enhancement of folates in staple food through crop breeding, offers an alternative and sustainable approach (Guo et al. 2019).

Maize (*Zea mays*) is one of the leading staple crops, along with rice and wheat. It is cultivated in around 165 countries, serving diverse roles based on consumer needs. The surge in maize production (1.163 billion tonnes in 2022) dominating over other major grains is attributed to the rapid shift towards hybrid cultivation, technological advances, yield increases, and area expansion (Erenstein et al. 2022). In India, maize is cultivated around 9.96 m ha with

Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India

***Corresponding Author:** Rajkumar U. Zunjare, Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India, E-Mail: raj_gpb@yahoo.com

How to cite this article: Paul K.V., Kumar P., Hossain F., Muthusamy V., Mishra S.J., Katral A. and Zunjare R.U. 2025. Folate profiling and characterization of sub-tropically adapted maize inbreds using folate-metabolism related genes. Indian J. Genet. Plant Breed. , **85**(4): 593-602.

Source of support: EMEQ project (EEQ/2019/000289) and IARI-ICAR, Govt. of India

Conflict of interest: None.

Received: Jan. 2025 **Revised:** Aug. 2025 **Accepted:** Sept. 2025

its production reaching 33.7 million tonnes (FAOSTAT 2022), ranking 6th position at the global level. Genetic improvement of maize for enhanced folate content could be accomplished by exploiting the genetic variation that naturally exists in the germplasm. Some of the international studies on diverse maize genotypes revealed phenotypic differences accounted for by the presence of genetic factors (Yang et al. 2011; Guo et al. 2019; Shahid et al. 2020; Islam et al. 2021). Analysis of folate variation between contrasting inbred lines has allowed identification and mapping of two major effect QTLs-*q5-F-THF-5a* and *q5-F-THF-5b*, encoding for S-adenosyl-L-methionine-dependent methyltransferase and transferase protein containing folic acid domain, respectively (Guo et al. 2019). A to G single-nucleotide substitution in the *CTM* gene in maize has led to a rise in young kernels of sweet corn hybrids (Lv et al. 2022). Furthermore, expression profiling of diverse maize genotypes revealed several key candidate genes affecting the accumulation of folate in maize genome (Lian et al. 2015; Guo et al. 2019; Song et al. 2021). In this study, we aimed to (i) assess a set of specialty and biofortified inbreds for total folate and characterize them using candidate genes governing folate metabolism, and (ii) provide key leads into folate biofortification by identification of specific haplotypes associated with high folate content.

Materials and methods

A total of 48 corn inbred lines were raised in two rows of 3 m length each and spacing of 75 cm × 15 cm, at the Experimental Farm, ICAR-IARI, New Delhi, under a randomized complete block design (RCBD) during the *spring* season (2024) (Table 1). The seed samples for folate analysis were collected at the maturity stage of the plants. The collected kernels from each inbred were cleaned and dried before being stored in a deep freezer (-80°C) until folate was extracted.

Folate extraction and quantification

Folate extraction was performed using a di-enzyme extraction procedure as described by Shahid et al. (2020) with minor modifications. High-performance liquid chromatography (HPLC) was performed to detect folate filtered solution. Folates were separated using a UHPLC Dionex Ultimate 3000 RS pump. It consists of a C18 column (4.6 × 150 mm, 5 µm particle size). The mobile phases used were 33 mM phosphate buffer in phase A and acetonitrile in phase B. The dilutions of 5-methyltetrahydrofolate (5-MTHF) and 5-formyltetrahydrofolate (5-FTHF) standard, respectively (SIGMA Aldrich, USA), were used to make the standard curves. The folate concentration on a dry weight (DW) basis was estimated as the sum of 5-MTHF and 5-FTHF concentrations, as both these derivatives constitute more than 90% of the total folate level among the folate derivatives (Lian et al. 2015; Shan et al. 2019; Islam et al. 2021).

Marker genotyping

A total of 78 designed SSR (simple sequence repeat) primers using the BatchPrimer3 online tool were synthesized from Sigma Aldrich Pvt. Ltd. and used for genotyping. The gene ID of the candidate genes has been obtained from the studies of Lian et al. (2015), Guo et al. (2019), and Xiao et al. (2022). The genomic DNA was extracted from leaf samples using the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). DNA quantity was measured using a spectrophotometer (Bio-Tek Instruments USA). DNA quality was confirmed using 0.8% agarose gel electrophoresis, followed by dilution with Tris-EDTA solution, the final concentration for subsequent polymerase chain reaction (PCR) experiments. The PCR-mediated amplifications were carried out in a GenePro-Thermal Cycler with a final quantity of 20 µL. The PCR amplified products of each SSR marker were resolved by agarose gel electrophoresis. The amplicons have been classified as loci alleles and were scored manually.

Statistical analysis

Based on the presence or absence of bands, a binary data matrix of 1 or 0 for each marker was produced. The binary data matrix for the 78 gene-specific markers was used to obtain the mean PIC value, allele frequency and allele number using PowerMarker V3.0 (Liu and Muse 2005). To compare genotypes in pairs, genetic dissimilarity was computed using the coefficient of Jaccard's. The dendrogram was constructed and clusters were grouped using the neighbor-joining method from DARwin-6.0 (Perrier et al. 2003). The data for total folate content recorded was analyzed using the aov() function in R (Wickham 2016) in RStudio (R Development Core 2023). Statistical significance between the pair-wise group means of the clusters and sub-clusters for folate-related traits was analyzed using the *rstatix* package (Kassambara 2023) in RStudio, R software (R Development Core 2023). Further, the distribution of data points in each group, along with their group means, was visualized through boxplots, which were deduced using the *ggplot2* package (Wickham 2016) in RStudio, R software (R Development Core 2023).

Single marker analysis and identification of the best haplotype

The genotypic data of polymorphic markers were further subjected to single marker analysis (SMA) using the regression function in MS Office Excel (2019). The polymorphic markers with significant regression coefficient values based on SMA were considered for haplotype analysis. Different combinations of marker alleles of significant markers from SMA were considered to identify the superior haplotype(s) based on their corresponding phenotypic value. Different combinations of alleles in each haplotype have been represented with 'A' and 'B' and heterozygotes with 'H'. The bar graphs representing the

Table 1. Details of the diverse corn inbred lines used in the study

S. No.	Genotypes	Specialty type	Source Institution	Sl. No.	Genotypes	Specialty type	Source Institution
1	PMI-Q1	QPM	ICAR-IARI, New Delhi	25	PMI-PC104	Popcorn	ICAR-IARI, New Delhi
2	PMI-Q2	QPM	ICAR-IARI, New Delhi	26	MGU201Y	Popcorn	ICAR-IARI, New Delhi
3	HKI161	QPM	CCSHAU, Uchani	27	MGU202Y	Popcorn	ICAR-IARI, New Delhi
4	HKI163	QPM	CCSHAU, Uchani	28	MGU203W	Popcorn	ICAR-IARI, New Delhi
5	HKI193-1	QPM	CCSHAU, Uchani	29	IML127-1	Field corn	ICAR-IIMR, Ludhiana
6	PMI-PV1	QPM + Provitamin A	ICAR-IARI, New Delhi	30	MGU205R	Red coloured corn	ICAR-IARI, New Delhi
7	PMI-PV2	QPM + Provitamin A	ICAR-IARI, New Delhi	31	MGU206BTF	Coloured corn	ICAR-IARI, New Delhi
8	PMI-PV3	QPM + Provitamin A	ICAR-IARI, New Delhi	32	CML426	Field corn	CIMMYT, Mexico
9	PMI-PV4	QPM + Provitamin A	ICAR-IARI, New Delhi	33	MGU208B	Black coloured corn	ICAR-IARI, New Delhi
10	PMI-PV5	QPM + Provitamin A	ICAR-IARI, New Delhi	34	MGU209wx	Waxy corn	ICAR-IARI, New Delhi
11	PMI-PV6	QPM + Provitamin A	ICAR-IARI, New Delhi	35	MGU210wx	Waxy corn	ICAR-IARI, New Delhi
12	PMI-PV7	QPM + Provitamin A	ICAR-IARI, New Delhi	36	HKI1128	Field corn	CCSHAU, Hisar
13	PMI-PV8	QPM + Provitamin A	ICAR-IARI, New Delhi	37	MGU211wx	Waxy corn	ICAR-IARI, New Delhi
14	PMI-PV11	QPM + Provitamin A	ICAR-IARI, New Delhi	38	MGU212wx	Waxy corn	ICAR-IARI, New Delhi
15	PMI-PV12	QPM + Provitamin A	ICAR-IARI, New Delhi	30	MGU213wx	Waxy corn	ICAR-IARI, New Delhi
16	PMI-PVE101	QPM + Provitamin A + Provitamin E	ICAR-IARI, New Delhi	40	MGU214wx	Waxy corn	ICAR-IARI, New Delhi
17	PMI-PVE102	QPM + Provitamin A + Provitamin E	ICAR-IARI, New Delhi	41	MGU215ae	High amylose corn	ICAR-IARI, New Delhi
18	PMI-SWT016	Sweet corn	ICAR-IARI, New Delhi	42	MGU216ae	High amylose corn	ICAR-IARI, New Delhi
19	PMI-SWT017	Sweet corn	ICAR-IARI, New Delhi	43	MGU217ae	High amylose corn	ICAR-IARI, New Delhi
20	PMI-SWT019	Sweet corn	ICAR-IARI, New Delhi	44	MGU218ae	High amylose corn	ICAR-IARI, New Delhi
21	PMI-SWT020	Sweet corn	ICAR-IARI, New Delhi	45	MGU219Ho	High oil corn	ICAR-IARI, New Delhi
22	PMI-PC101	Popcorn	ICAR-IARI, New Delhi	46	MGU220Ho	High oil corn	ICAR-IARI, New Delhi
23	PMI-PC102	Popcorn	ICAR-IARI, New Delhi	47	MGU221Ho	High oil corn	ICAR-IARI, New Delhi
24	PMI-PC103	Popcorn	ICAR-IARI, New Delhi	48	MGU222Ho	High oil corn	ICAR-IARI, New Delhi

mean performance of each haplotype were deduced using MS Office Excel (2019).

Results

Variation of folate among inbred lines

ANOVA for 5-MTHF, 5-FTHF, and total folate content among the 48 maize inbreds evaluated during the *spring* season 2024 at the Experimental Farm of ICAR-IARI, New Delhi, revealed significant variation among the genotypes. The genotypic effect remains the most substantial contributor to the observed variation. The average content of 5-MTHF across the genotypes was 14.5 µg/100 g, with the lowest value recorded at 3.9 µg/100 g (PMI-Q1) and the highest, 25.9 µg/100 g (PMI-PV7). For 5-FTHF, the average was 41.2 µg/100g, with values ranging from 12.3 µg/100 g (PMI-PC101) to 75.8 µg/100 g (PMI-SWT016). The total folate content

had an average of 55.7 µg/100 g, with a minimum of 21.4 µg/100 g (PMI-PC101) and a maximum of 98.0 µg/100 g (MGU213wx). The highest 5-MTHF content was observed in the inbred PMI-PV7, which recorded a value of 25.9 µg/100 g, followed by MGU213wx with 25.0 µg/100 g, and PMI-SWT020 with 23.4 µg/100 g. On the lower end of the spectrum, the inbred PMI-Q1 exhibited the lowest 5-MTHF content at 3.9 µg/100 g, with PMI-Q2 and PMI-PVE101 recording slightly higher values of 4.9 µg/100 g and 7.6 µg/100 g, respectively. For 5-FTHF content, PMI-SWT016 ranked with the highest recorded values of 75.5 µg/100 g, followed by PMI-SWT019 (73.7 µg/100g). MGU213wx also ranked high, with a 5-FTHF content of 73.0 µg/100 g. In contrast, the lowest 5-FTHF content was found in PMI-PC101, which recorded a value of 12.3 µg/100g, followed by IML127-1 at 12.9 µg/100 g and MGU216ae at 15.7 µg/100 g. For total folate content, MGU213wx emerged as the top-performing genotype with a value of 98.0 µg/100g. On the other hand, PMI-PC101 exhibited the lowest total folate content at 21.4 µg/100 g, followed by IML127-1 (21.5 µg/100 g) and PMI-Q1 (26.1 µg/100 g). The 5-FTHF is the predominant folate vitamer, constituting around 73.9% of the total folate content, while 5-MTHF comprises the remaining 26.1%.

Composition of folate among various maize types

The comparison of mean folate content among different specialty corn types with field corn revealed interesting patterns (Fig. 1). Coloured corn shows a lower mean folate content of 29.5 µg/100 g, with the range from 28.6 µg/100 g (MGU208B) to 30.1 µg/100 g (MGU205R), falling below the folate levels found in field corn. Field corn has an average folate content of 30.3 µg/100 g, ranging from 21.5 µg/100 g (IML127-1) to 40.2 µg/100 g (HKI1128). QPM corn shows a higher mean folate content of 46.8 µg/100g, and a high range from 26.1 µg/100 g (PMI-Q1) to 78.7 µg/100 g (HKI193-1). QPM + provitamin-A based genotype, the mean folate content was 65.47 µg/100 g, and shows a high range from 41.9 µg/100g (PMI-PV5) to 91.4 µg/100 g (PMI-PV7). While QPM combined with both provitamin-A and provitamin-E accumulates a folate mean content of 38.9 µg/100 g. Sweet corn exhibits the highest mean folate content among the corn types under study, averaging around 83.7 µg/100 g, and ranged from 68.3 µg/100g (PMI-SWT017) to 90.4 µg/100 g (PMI-SWT019), making it one of the most enriched types compared to field corn. Popcorn has a mean folate content of 46.2 µg/100 g, which is slightly higher than field corn, but not as enriched as other specialty corn types. Surprisingly, popcorn inbreds showed the highest dispersion for folate content, ranging from 21.4 µg/100 g (PMI-PC101) to 80.5 µg/100 g (MGU203W). Waxy corn demonstrated a high mean folate content of 79.0 µg/100 g, values ranging from 65.1 µg/100 g (MGU212wx) to 98.0 µg/100 g (MGU213wx). High amylose corn has a mean folate content of 43.9 µg/100 g, with values between 28.5 µg/100 g (MGU216ae) to 54.5 µg/100 g (MGU217ae). Similarly, high oil corn exhibited an average folate content of 54.8 µg/100 g, ranging from 35.9 µg/100 g (MGU220Ho) to 83.7 µg/100 g (MGU221Ho). Sweet corn and waxy corn inbreds are ranked with the highest mean folate contents, significantly surpassing the folate levels of field corn. While other types, like popcorn, high amylose corn, and high oil corn, show moderate to substantial improvements in folate levels compared to field corn.

Molecular characterization of inbred lines and haplotype analysis

We characterized a set of 48 maize genotypes using 78 SSR markers designed from candidate genes involved in folate metabolism. Out of the 78 SSR markers evaluated, 30 were found to be polymorphic, while the remaining 48 were monomorphic, indicating that 38.46% of the markers exhibited variation among the genotypes, highlighting the presence of limited genetic diversity across the genotypes for the candidate genes. The polymorphic markers showed varying levels of genetic diversity and allele frequencies. The major allele frequency across the polymorphic markers ranged from 0.36 to 0.88, with an average of 0.63. Lower major allele frequency indicates a higher level of genetic diversity, as seen in markers like Fo-SSR-50 (0.36), which showed the highest diversity. The number of alleles detected per marker ranged from 2 to 5, with an average of 2.27. The marker Fo-SSR-17 exhibited the highest number of alleles (5), indicating a higher level of polymorphism. Gene diversity ranged from 0.22 to 0.66, with an average of 0.44. The highest gene diversity was observed in marker Fo-SSR-50 (0.66), suggesting it is a highly informative marker. The PIC value, which indicates the informativeness of a marker, ranged from 0.19 to 0.59, with an average of 0.36. The highest PIC value was recorded for marker Fo-SSR-50 (0.59). Among the 30 polymorphic SSR-markers analysed, five markers stand out for their high PIC and the highest number of alleles detected. These markers are Fo-SSR-50, with a PIC of 0.59 and 3 alleles detected; Fo-SSR-17, with a PIC of 0.53 and the highest number of alleles detected (5); Fo-SSR-20, with a PIC of 0.57 and 3 alleles detected; Fo-SSR-63, with a PIC of 0.58 and 3 alleles detected; and Fo-SSR-37, with a PIC of 0.54 and 3 alleles detected (Table 2). These SSR markers are of particular interest due to their high PIC values, indicating they are highly informative and useful for distinguishing between the different maize genotypes. Based on the allelic profile of these markers, the 48 genotypes were grouped into 14 distinct haplotypes. Superior haplotypes associated with higher folate content include Hap6 (ABBB), Hap8 (ABHB), and Hap5 (ABAA) with folate mean values of 87.62 µg/100g, 83.72 µg/100g and 79.53 µg/100g, respectively. Out of 30 polymorphic markers, significantly higher R^2 values were observed for SSR markers- Fo-SSR-53 (14.30), Fo-SSR-45 (10.77), Fo-SSR-52 (8.44) and Fo-SSR-31 (8.11) (Table 3).

Table 2. Summary statistics of the genotyping assay using gene-specific SSR markers

S. No.	Markers	Major allele frequency	No. of alleles detected	Gene diversity	PIC
1	Fo-SSR-2	0.50	2.00	0.50	0.38
2	Fo-SSR-3	0.51	2.00	0.50	0.37
3	Fo-SSR-6	0.56	2.00	0.49	0.37
4	Fo-SSR-7	0.50	2.00	0.50	0.38
5	Fo-SSR-8	0.83	2.00	0.28	0.24
6	Fo-SSR-14	0.52	2.00	0.50	0.37
7	Fo-SSR-16	0.50	2.00	0.50	0.38
8	Fo-SSR-17	0.63	5.00	0.56	0.53
9	Fo-SSR-20	0.41	3.00	0.64	0.57
10	Fo-SSR-21	0.78	2.00	0.35	0.29
11	Fo-SSR-31	0.58	2.00	0.49	0.37
12	Fo-SSR-32	0.49	3.00	0.58	0.49
13	Fo-SSR-34	0.87	2.00	0.22	0.20
14	Fo-SSR-37	0.45	3.00	0.62	0.54
15	Fo-SSR-39	0.86	2.00	0.24	0.21
16	Fo-SSR-41	0.57	2.00	0.49	0.37
17	Fo-SSR-43	0.85	2.00	0.25	0.22
18	Fo-SSR-45	0.66	2.00	0.45	0.35
19	Fo-SSR-50	0.36	3.00	0.66	0.59
20	Fo-SSR-52	0.77	2.00	0.35	0.29
21	Fo-SSR-53	0.85	2.00	0.25	0.22
22	Fo-SSR-57	0.82	2.00	0.29	0.25
23	Fo-SSR-59	0.85	2.00	0.25	0.22
24	Fo-SSR-62	0.88	2.00	0.22	0.19
25	Fo-SSR-63	0.42	3.00	0.65	0.58
26	Fo-SSR-66	0.50	2.00	0.50	0.38
27	Fo-SSR-68	0.57	2.00	0.49	0.37
28	Fo-SSR-72	0.67	2.00	0.44	0.34
29	Fo-SSR-75	0.67	2.00	0.44	0.35
30	Fo-SSR-78	0.52	2.00	0.50	0.37
Mean		0.63	2.27	0.44	0.36
Minimum		0.36	2.00	0.22	0.19
Maximum		0.88	5.00	0.66	0.59

PIC = Polymorphism information content; SSR = Simple Sequence Repeats

Cluster analysis of 48 genotypes

The dissimilarity matrix generated using Jaccard's coefficient was used to construct a neighbor-joining tree using the UPGMA (unweighted pair group method with arithmetic mean) algorithm, which grouped the selected set of 48 maize genotypes into three distinct clusters. The genetic

dissimilarity among the parental pairs ranged from 0.08 (between MGU218ae and MGU203W) to 0.34 (between PMI-PC103 and MGU211wx), with an average dissimilarity value of 0.22 across all genotypes, indicating a high degree of genetic similarity. Cluster-A comprised 17 genotypes, with 10 genotypes forming sub-cluster A1, including MGU219Ho, MGU217ae, MGU215ae, MGU214wx, MGU213wx, MGU212Y, PMI-PV7, PMI-PV5, PMI-PV3, and MGU221Ho. Sub-cluster A2 contained 7 genotypes: MGU220Ho, MGU216ae, MGU218ae, MGU203W, MGU222Ho, and HKI193-1. Cluster-B consisted of 16 inbreds, divided into sub-cluster B1 with 10 genotypes: PMI-SWT016, PMI-PV11, MGU208B, MGU205R, PMI-SWT020, PMI-SWT019, PMI-PV12, PMI-PV8, PMI-PV2, and HKI163. Sub-cluster B2 included 6 genotypes: PMI-PC103, PMI-PC102, PMI101, PMI-PC104, and MGU201Y. Cluster-C was further divided into two sub-clusters, with cluster C1 comprising 10 genotypes: MGU211wx, IML127-1, PMI-PV1, PMI-PVE101, PMI-Q2, PMIQ1, PMI-PV4, MGU212wx, HKI1128, and CML426. Cluster C2 contained 5 genotypes: MGU206BTF, PMI-PVE102, MGU210wx, MGU209wx, and HKI161 (Fig. 2). The clustering pattern revealed that various corn types were dispersed across the clusters. However, certain types tended to group together within the same clusters. Sweet corn genotypes were primarily found in sub-cluster B1, popcorn genotypes were mainly grouped in sub-cluster B2, and waxy corn genotypes were predominantly located in sub-clusters A1 and C2. The analyses showed that the inbred were distributed in all four quadrangles, signifying their limited genetic variability.

Cluster-wise folate-traits comparison

We studied whether there were any significant differences among the genotypic clusters formed based on genotyping patterns. The analysis of folate-related traits across different clusters and sub-clusters of maize inbreds provides a comprehensive understanding of the genetic potential for folate accumulation. When comparing the clusters, cluster A ranked the highest among the bulk mean values across all three traits: 16.9 µg/100g for 5-MTHF, 44.7 µg/100g for 5-FTHF, and 61.7 µg/100g for total folate content. In contrast, cluster-B showed slightly lower values, with bulk means of 14.1 µg/100g for 5-MTHF, 43.9 µg/100g for 5-FTHF, and 58.1 µg/100g for total folate. Cluster-C exhibited the lowest means, with 12.2 µg/100g for 5-MTHF, 34.1 µg/100g for 5-FTHF, and 46.3 µg/100g for total folate content (Fig. 3). This indicates that genotypes within cluster-A have a stronger potential for folate accumulation. Although cluster-B trails behind cluster-A, it still demonstrates good folate accumulation, particularly in sweet corn inbreds, while cluster-C genotypes are lower in folate content; they may offer consistency and stability in folate accumulation, particularly in waxy corn types.

Within cluster-A, sub-cluster-A1 outperformed A2 in total folate content with a mean of 67.9 µg/100g compared to 50.3 µg/100g in A2. In addition, A1 had a relatively higher 5-MTHF and 5-FTHF mean (18.0 µg/100g and 49.9 µg/100g, respectively) than A2 (15.0 µg/100g and 35.2 µg/100g, respectively). In cluster-B, sub-cluster-B1 significantly outperformed B2 across all traits, with B1's mean total folate content at 64.8 µg/100g compared to 43.1 µg/100g in B2. B1 also had higher 5-MTHF (15.1 µg/100g) and 5-FTHF (49.9 µg/100g) levels. Cluster-C showed a marked difference between its sub-clusters. Sub-cluster-C2 demonstrated higher mean values with 14.8 µg/100g for 5-MTHF, 37.3 µg/100g for 5-FTHF, and 52.1 µg/100g for total folate content. These values are notably higher than those of C1, suggesting that C2, particularly with its waxy corn types, possesses greater potential for folate biofortification. Taken together, cluster-A, especially sub-cluster A2, holds the most promise for total folate accumulation. Sub-cluster-B1, is another valuable source for high folate levels, especially in sweet corn. Sub-cluster-C2, leading in folate content, presented a promising option for waxy corn types, despite its overall lower means.

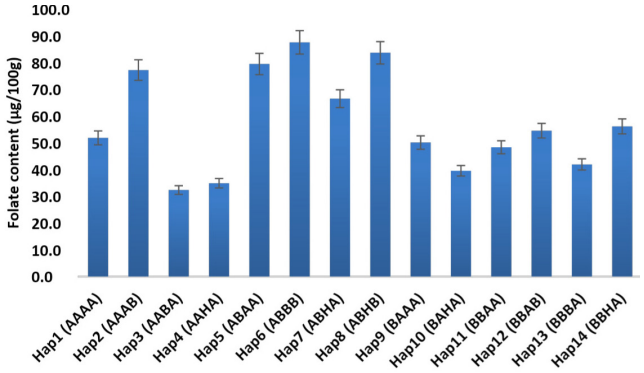

Fig. 1. Comparison of folate means among different haplotypes. Each haplotype is arranged in the order of SSR markers- Fo-SSR-31, Fo-SSR-45, Fo-SSR-52 and Fo-SSR-53. Banding pattern for each marker: Fo-SSR-31: A-130 bp/130 bp, B-140 bp/140 bp; Fo-SSR-45: A- 140 bp/140 bp, B-160 bp/160 bp; Fo-SSR-52: A-150 bp/150 bp, B-120 bp/120 bp; H-150 bp/120 bp; Fo-SSR-53: A- 120 bp/120 bp, B-140 bp/140 bp.

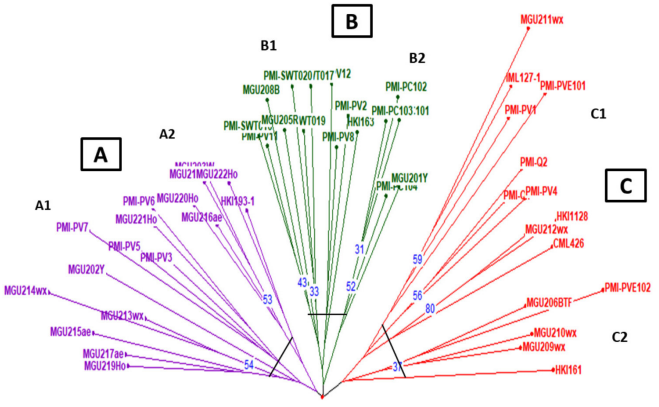

Fig. 2. SSR genotyping based cluster diagram among the 48 maize genotypes

Table 3. Summary statistics of single marker analysis in markers showing polymorphism

S. No.	Marker	Regression coefficient	R^2 (%)
1.	Fo-SSR-2	-9.87	1.55
2.	Fo-SSR-3	2.16	0.89
3.	Fo-SSR-6	-14.41	4.43
4.	Fo-SSR-7	28.41	3.22
5.	Fo-SSR-8	-0.75	0.06
6.	Fo-SSR-14	1.05	0.01
7.	Fo-SSR-16	-4.83	0.37
8.	Fo-SSR-17	-25.32	2.56
9.	Fo-SSR-20	-3.07	1.45
10.	Fo-SSR-21	-2.32	0.65
11.	Fo-SSR-31	6.54*	8.11
12.	Fo-SSR-32	4.85	2.56
13.	Fo-SSR-34	2.51	0.46
14.	Fo-SSR-37	3.90	1.48
15.	Fo-SSR-39	-1.96	0.27
16.	Fo-SSR-41	3.33	1.62
17.	Fo-SSR-43	7.51	4.57
18.	Fo-SSR-45	-7.76*	10.77
19.	Fo-SSR-50	0.78	0.11
20.	Fo-SSR-52	9.75*	8.44
21.	Fo-SSR-53	-12.12**	14.30
22.	Fo-SSR-57	0.63	0.03
23.	Fo-SSR-59	-2.67	0.69
24.	Fo-SSR-62	8.58	2.69
25.	Fo-SSR-63	-0.45	0.03
26.	Fo-SSR-66	-14.11	3.17
27.	Fo-SSR-68	-3.21	2.70
28.	Fo-SSR-72	-2.57	0.79
29.	Fo-SSR-75	4.67	2.13
30.	Fo-SSR-78	-12.19	5.12

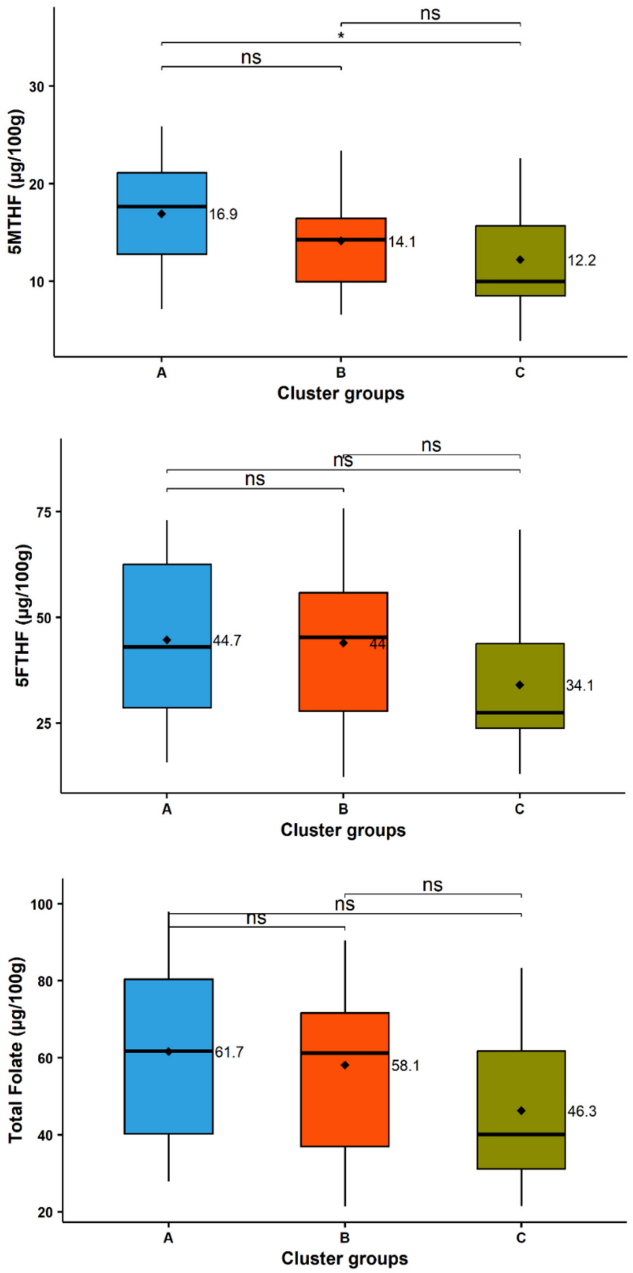

Fig. 3. Comparison of mean values of folate-traits across the various sub-clusters

Discussion

The maize inbreds exhibited significant genotypic effects on 5-MTHF, 5-FTHF, and total folate content. This indicated strong genetic control over folate accumulation, highlighting the potential for selecting and breeding maize genotypes with enhanced folate profiles. ANOVA revealed highly significant genotypic effects for all three traits. A higher proportion of 5-FTHF (73.9%) levels as compared to 5-MTHF (26.1%) suggests a metabolic shift favouring 5-FTHF accumulation during later stages of kernel development. Previously, glutamate metabolism, pyruvate metabolism and serine/glycine metabolism pathways were involved in stage-specific variation in folate content (Lian et al. 2022). The present study reports the folate wide range of 21.4 µg/100g to 98.0 µg/100g in the present panel of maize inbreds. Similarly, wide variation for folate content among 11 hybrids (Reynolds et al. 2005), waxy inbreds (Shan et al. 2019; Islam et al. 2021), segregating populations (Guo et al. 2019) and sweet corn inbreds (Xiao et al. 2022) was also reported. The presence of variation accounted for by genetic factors for folate in the sub-tropically adapted maize genotypes identified here provides an ample scope for its improvement

by breeding approaches.

The molecular characterization of inbreds showed that, out of 78 SSRs, a total of 48 markers were found to be monomorphic and only 30 markers were polymorphic, revealing the conservation of these genomic sequences among the genotypes studied. This indicated that the genes involved in folate metabolism are critical for the growth of the plants. Gorelova et al. (2019) showed that the number of genes, localization and the structure of the isoforms encoded by folate genes were highly conserved across algae and land plants. Furthermore, Lian et al. (2015) showed folate metabolism-related enzymes encoded by genes are conserved in maize and millets. Most of the SSRs developed from candidate genes governing a critical step in folate biosynthesis were monomorphic. For example, the markers Fo-SSR-1, Fo-SSR-70, Fo-SSR-71, and Fo-SSR-72 were designed based on the *GTPCH1* (GRMZM2G062420) gene, which catalyses the first step of pterin synthesis, i.e., converts GTP to dihydroneopterin triphosphate, the rate-limiting step of folate biosynthesis (Storozhenko et al. 2005). Out of these four markers, three (Fo-SSR-1, Fo-SSR-70, Fo-SSR-71) were monomorphic while Fo-SSR-72 was polymorphic. The mean genetic dissimilarity of the parental pairs across all genotypes was 0.22, indicating high genetic similarity among the selected genotypes for the folate-related genes. Notably, all four sweet corn lines evaluated in the study were grouped in sub-cluster B1, while popcorn genotypes were grouped in sub-cluster B2. This shows the similarity of folate-related genes among specific kinds of corn types. Though other types of specialty corn and biofortified types spread across the various clusters, the overall dissimilarity among the genotypes was low.

Among the 30 polymorphic SSRs, five markers, Fo-SSR-17 (0.53), Fo-SSR-20 (0.57), Fo-SSR-37 (0.54), Fo-SSR-50 (0.59) and Fo-SSR-63 (0.58) possessed PIC values greater than 0.50. The identification of these polymorphic markers with high PIC values is crucial for the genetic mapping of genes for folate quality improvement in maize (Lv et al. 2022). PIC mean value of 0.19 again indicates the low amount of genetic diversity of folate-related genes. The variation in major allele frequency across markers also provides insights into allele distribution. The study successfully identified 30 polymorphic SSR markers that can be utilized for genetic diversity analysis, QTL mapping, and marker-assisted selection for maize breeding programs focused on folate metabolism (Guo et al. 2019; Lian et al. 2022, Lv et al. 2022). We thought it would be interesting to see if the genotypes grouped in one cluster are significantly deviating from the genotypes of another cluster or not. The mean of 5-FTHF and folate content of genotypes within each major cluster was not significantly distinct from each other, except for 5-MTHF, which is significant between cluster A and -C. This suggested that high and low inbreds spread across all the clusters. To be more specific, when we compared the mean among the different sub-clusters, we found that cluster-A1 was significantly deviating from sub-cluster-C1 for 5-MTHF only, while no significance was observed for 5-FTHF and folate content among the sub-clusters. Genes amplified by these gene-specific primers are involved in encoding different enzymes, like the axial regulator of YABBY2 protein, protein binding domain, and E3 ubiquitin-protein ligase PUB23 enzyme (Xiao et al. 2022). Most of their gene functions align with markers showing high PIC values, emphasizing their proximity to genes/QTLs involved in folate accumulation, allowing us to map the genomic regions involved in folate accumulation. In addition, haplotype analysis of these four significant markers revealed the effect of the allele pattern of each marker and its combinations on folate accumulation. Among the superior haplotypes, both Fo-SSR-53 and Fo-SSR-45 show the same pattern. In haplotype ABBB, while substitution of the B allele of Fo-SSR-52 (120 bp/120 bp)with heterozygous allele 'H' (150 bp/ 120 bp) resulted in reduction of folate content by 4.45%, substitution of both 'B' alleles of Fo-SSR-52 and Fo-SSR-31 (140 bp/ 140 bp) with 'A' alleles of Fo-SSR-52 and Fo-SSR-31 (130 bp/ 130 bp) led to 9.23% decline in total folate content.

Considering the genotypic and phenotypic dissimilarities, heterotic crosses among the genotypes of each group, such as MGU202Y × MGU203W, HKI161 × HKI163, PMI-PV1 × PMI-PV6, MGU219Ho × MGU221Ho, MGU210wx × MGU214wx, MGU217ae × MGU218ae, PMI-SWT016 × PMI-SWT020 and IML127-1 × HKI1128, can be attempted for hybrid development. These crosses can also be used to generate segregating populations to map QTLs/genes responsible for higher folate content in maize. Besides, waxy corn types and high folate lines identified in the present study can be used as donors for the future maize folate-biofortification programme.

Authors' contribution

Conceptualization of research (RUZ); Designing of the experiments (KVP, PK, SJM); Contribution of experimental materials (FH, VM, RUZ); Execution of field/lab experiments and data collection (KVP, PK, RUZ); Analysis of data and interpretation (AK, KVP); Preparation of the manuscript (KVP, RUZ, FH, VM).

Acknowledgments

First author sincerely thanks the ICAR for providing JRF during the M.Sc. programme. The authors acknowledge the IARI, New Delhi, for the funding provided for the study. Financial support received from the EMEQ project (EEQ/2019/000289) is also gratefully acknowledged. The extensive help of Mr. Stuti Kiran, technical officer, especially during pollination and collection of samples, is greatly appreciated.

References

Akwaa Harrison O., Ifie I., Nkwonta C., Dzandu B. A., Gattor A. O., Adimado E. E., Odoi K. K., Aziavor B., Saalia F. K. and Steiner-Asiedu M. 2024. Knowledge, awareness, and use of folic acid among women of childbearing age living in a peri-urban community in Ghana: a cross-sectional survey. BMC Pregnancy Child B., **24**(1): 241. https://doi.org/10.1186/s12884-024-06408-z

Blancquaert D., Storozhenko S., Loizeau K., De Steur H., De Brouwer V., Viaene J. and Van Der Straeten D. 2010. Folates and folic acid: From fundamental research toward sustainable health. Crit. Rev. Plant Sci., **29**(1): 14-35. https://doi.org/10.1080/07352680903436283

Blount B. C., Mack M. M., Wehr C. M., MacGregor J. T., Hiatt R. A., Wang G., Wickramasinghe S. N., Everson R. B. and Ames B. N. 1997. Folate deficiency causes uracil mis-incorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc. Natl. Acad. Sci. USA., **94**(7): 3290-3295. https://doi.org/10.1073/pnas.94.7.3290

Erenstein O., Jaleta M., Sonder K., Mottaleb K.A. and Prasanna B.M. 2022. Global maize production, consumption and trade: trends and R&D implications. Food Secur., **14**: 1295-1319. https://doi.org/10.1007/s12571-022-01288-7

FAOSTAT 2022. Food and Agriculture Organization of United Nations.https://www.fao.org/faostat/en/#data/QCL/visualize

Gorelova V., Bastien O., De Clerck O., Lespinats S., Rébeillé F.and Van Der Straeten D. 2019. Evolution of folate biosynthesis and metabolism across algae and land plant lineages. Sci. Reports, **9**(1): 5731. https://doi.org/10.1038/s41598-019-42146-5

Guo W., Lian T., Wang B., Guan J., Yuan D., Wang H., Safiul Azam F. M., Wan X., Wang W., Liang Q., Wang H., Tu J., Zhang C. and Jiang L. 2019. Genetic mapping of folate QTLs using a segregated population in maize. J. Integr. Plant Biol., **61**(6): 675-690. https://doi.org/10.1111/jipb.12811

Islam S., Liu J., Jiang L., Zhang C. and Liang Q. 2021. Folate content in fresh corn: Effects of harvest time, storage and cooking methods. J. Food Compos. Anal., **103**: 104123. https://doi.org/10.1016/j.jfca.2021.104123

Kassambara A. 2023. rstatix: Pipe-Friendly framework for basic statistical tests. R package version 0.7.2. https://rpkgs.datanovia.com/rstatix/

Lian T., Guo W., Chen M., Li J., Liang Q., Liu F., Meng H., Xu B., Chen J., Zhang C. and Jiang L. 2015. Genome-wide identification and transcriptional analysis of folate metabolism-related genes in maize kernels. BMC Plant Biol., **15**: 204. https://doi.org/10.1186/s12870-015-0578-2

Lian T., Wang X., Li S., Jiang H., Zhang C., Wang H. and Jiang L. 2022. Comparative transcriptome analysis reveals mechanisms of folate accumulation in maize grains. Inter. J. Mol. Sci., **23**(3): 1708. https://doi.org/10.3390/ijms23031708

Liu K. and Muse S.V. 2005. PowerMarker: An integrated analysis environment for genetic marker analysis. Bioinformatics, **21**: 2128-2129. https://doi.org/10.1093/bioinformatics/bti282

Lv G., Chen X., Ying D., Li J., Fan Y., Wang B. and Fang R. 2022. Marker-assisted pyramiding of γ-tocopherol methyltransferase and glutamate formiminotransferase genes for development of biofortified sweet corn hybrids. Peer. J., **10**: e13629. https://doi.org/10.7717/peerj.13629

Murray M. G. and Thompson W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., **8**(19): 4321-4325. https://doi.org/10.1093/nar/8.19.4321

Perrier X., Flori A. and Bonnot F. 2003. Data analysis methods. wwvln: Hamon, P, Seguin, M, Perrier, X and Glaszmann, JC (eds) Genetic diversity of cultivated tropical plants. Montpellier: Enfield Science Publishers 43-76.

R Core Team 2023. R: A language and Environment for Statistical Computing . R foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.

Reynolds T.L., Nemeth M.A., Glenn K.C., Ridley W.P. and Astwood J.D. 2005. Natural variability of metabolites in maize grain: differences due to genetic background. J. Agric. Food Chem., **53**(26): 10061-10067. https://doi.org/10.1021/jf051635q

Shahid M, Lian T, Wan X, Jiang L, Han L, Zhang C and Liang Q 2020. Folate monoglutamate in cereal grains: Evaluation of extraction techniques and determination by LC-MS/MS. J. Food Compos. Anal., **91**(14): 103510. https://doi.org/10.1016/j.jfca.2020.103510

Shan Q., Liu J., Li W., Wang H., Hu X., Li T., Hu J., Guo X. and Liu R.H. 2019. Comprehensive evaluation of biosynthesis, accumulation, regulation of folate and vitamin C in waxy maize (*Zea mays* L. var. ceratina) with kernel development. J. Cereal Sci., **87**: 215-224. https://doi.org/10.1016/j.jcs.2019.04.003

Song L., Yu D., Zheng H., Wu G., Sun Y., Li P., Wang J., Wang C., Lv B. and Tang X. 2021. Weighted gene co-expression network analysis unveils gene networks regulating folate biosynthesis in maize endosperm. 3 Biotech., **11**(441): https://doi.org/10.1007/s13205-021-02974-7

Storozhenko S., Ravanel S., Zhang G., Rébeillé F., Lambert W. E. and Straeten D.V. 2005. Folate enhancement in staple crops by metabolic engineering. Trends Food Sci. Technol., **16**: 271-281. https://doi.org/10.1016/j.tifs.2005.03.007

Strobbe S. and Van Der Straeten D. 2017. Folate biofortification in food crops. Curr. Opin. Biotechnol., **44**: 202-211. https://doi.org/10.1016/j.copbio.2016.12.003

Wickham H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. https://doi.org/10.1007/978-3-319-24277-4

Xiao Y., Yu Y., Xie L., Li K., Guo X., Li G., Liu J., Li G. and Hu J. 2022. A genome wide association study of folates in sweet corn kernels. Front. Plant Sci., **13**: 1004455. https://doi.org/10.3389/fpls.2022.1004455

Yang X., Gao S., Xu S., Zhang Z., Prasanna B.M., Li L., Li J. and Yan J. 2011. Characterization of a global germplasm collection and its potential utilization for analysis of complex quantitative traits in maize. Molecular Breed., **28**: 511-526. https://doi.org/10.1007/s11032-010-9500-7