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Assessment of Microsatellite Markers for Varieties Identification: A Case of Six Varieties of Maize Hybrids Sold in Tanzania

Godlove Sollo Malya[1], Christopher Jacob Kasanga[1], Luseko Amos Chilagane[2], Jevenary Nassoro Lukeye[3]

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ABSTRACT

Background: Varieties identification is essential for protecting seeds sold to farmers from counterfeiting. Currently, the grow-out test (GOT) is used for varieties identification. This test is constrained by its long assessment, environmental dependency and subjective nature. Alternative test/tests are needed. This study aimed to determine the utility of microsatellite markers as a complement to GOT in varieties identification.

Methods: In this study, six varieties of commercial maize hybrids released in Tanzania, herein coded as H1, H2, H3, H5, H6 and H9, were selected as candidates. For morphological evaluation, a field experiment was laid out in a complete randomized block design, followed by the collection of phenotypic data. The qualitative data were analyzed by one-way ANOVA in SPSS v.20 software, while qualitative data were summarized in a frequency distribution table. On the other hand, by conventional polymerase chain reaction (PCR), seven microsatellite markers were used to fingerprint the varieties. The PCR results were manually scored and transferred to GenAlEX v.6.5 and DARwin v.6. software for genetic distance and clustering analysis, respectively.

Result: Quantitative data showed significant variation in all the tested varieties at $p<0.05$, whereas 15 qualitative traits out of 21 showed phenotypic variations among the tested varieties. Furthermore, two microsatellite markers, namely phi080 and umc1071, out of the seven markers used in this study, proved useful in the fast and timely identification of two varieties (H1 and H2) out of six varieties. Nei's genetic distance ranged from zero to four; the highest measure of genetic distance was observed in H1, followed by H2 and the least measure was observed in both of the four varieties. Neighbor Joining clustered the varieties into three different populations that distinguished each of H1 and H2 from the rest of the four varieties. This study shows that microsatellite markers are a promising complement to GOT in varietal identification.

Key words: Fingerprinting, GOT, Maize hybrids, Microsatellite markers, Varieties identification.

INTRODUCTION

Maize (*Zea mays* L.) is among the top three most cultivated cereal crops in Africa. The crop is utilized as human food, animal feed and a source of income (Kabululu *et al.,* 2017). Maize is rich in nutrients, including protein, oil, fiber, vitamins A and B, starch and sugar. Sub-Saharan Africa (SSA) has around 40 million hectares of land where maize is farmed (Ayesiga *et al.,* 2023). In SSA, maize production was based on the use of traditional open-pollinated varieties (OPVs), characterized by low yields due to the severe succumbing of both biotic and abiotic factors (Mwase *et al.,* 2015; Tumwesigye *et al.,* 2024). The advancement in crop improvement programs by the public and private sectors has resulted in the generation of hybrid varieties that can withstand stresses, resulting in increased yields and food security (Crossa *et al.,* 2014). The suitability of these varieties attracted the attention of farmers over traditional OPVs, leading to their amplified demands (Wani *et al.,* 2017). These demands attempt to persuade some seed dealers to sell counterfeit seed varieties (Kahwili, 2020; Machibya *et al.,* 2021; Setimela *et al.,* 2016). Thus, the sale of counterfeit seeds has necessitated for the National Designated Authority (NDA) the need for quality control tests to oversee the authenticity of the hybrid seed varieties sold to farmers.

[1]Department of Microbiology, Parasitology and Biotechnology, Sokoine University of Agriculture, P. O. Box 3019, Morogoro, Tanzania.
[2]Department of Crop Science and Horticulture, Sokoine University of Agriculture, P.O. Box 3005, Chuo Kikuu, Morogoro, Tanzania.
[3]International Institute of Tropical Agriculture (IITA), P.O. Box 34441, Dar es Salaam, Tanzania.

Corresponding Author: Godlove Sollo Malya, Department of Microbiology, Parasitology and Biotechnology, Sokoine University of Agriculture, P. O. Box 3019, Morogoro, Tanzania.
Email: gsollo64@gmail.com
ORCID: 0009-0004-3930-6004

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Currently, the quality control test to evaluate the authenticity of varieties relies on a field observation collectively known as the grow-out test (GOT). This test involves the assessment of different morphological traits of a particular variety in field conditions against the descriptor of that variety (Kumari *et al.,* 2022). Despite its

significance, GOT faces some drawbacks, such as taking a long time, needing relatively large space, being affected by environmental factors, being subjective and sometimes taking a whole cropping season (Bora *et al.,* 2016; Krishna *et al.,* 2020). Alternatively, molecular markers have the potential to resolve the inherent challenges accompanied by GOT. This is because they are not affected by environmental factors; the method has a short turnaround time and is relatively less costly (Aydin *et al.,* 2023).

The application of molecular markers in various genetic studies, such as genetic diversity, QTL mapping and marker-assisted selection (MAS) breeding, paved the way for DNA fingerprinting of crop varieties (Ramesh *et al.,* 2020). Different types of molecular markers based on PCR and non-PCR are used in such studies and these include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), microsatellite (SSR) and single nucleotide polymorphism (SNP). Among these molecular markers, microsatellite markers are preferred because they are co-dominantly inherited, multiallelic, highly polymorphic and very informative (Singh *et al.,* 2022).

Therefore, this study aimed to determine the utility of microsatellite markers in varieties identification as a complement to GOT. The microsatellite markers fingerprinting information generated from the selected maize hybrid varieties will serve as baseline data for efficient and timely identification of those varieties, thus protecting them from counterfeiting.

MATERIALS AND METHODS

Plant material

Six varieties of commercial maize hybrids released in Tanzania, coded H1, H2, H3, H5, H6 and H9, were acquired from public and private seed companies.

Study area

A field experiment was conducted in Arusha, Tanzania, at Madiira Farm, located at an altitude of 1250 m above sea level, at latitude 03°.22′S and longitude 36.48′E. The area has fertile and well drained loamy soil with a pH of 6.1. The region receives 20°C and 28°C of annual low and high temperatures, respectively. The region receives an average annual rainfall of 1180 mm. On the other hand, a laboratory experiment was conducted at the Nelson Mandela African Institution of Science and Technology (NM-AIST)-molecular biology laboratory.

Experimental design and layout

Six varieties of maize hybrids were laid out in a complete randomized block design with four replicates. Spacing was 0.75 m to 0.25 m of 2 plants per hole; after two weeks, thinning was done to leave one plant per hole. The experiment was conducted during the rainy season and planting was done on March 25, 2023. Agronomic practices were done uniformly in all plots.

Morphological data collection

11 quantitative and 21 qualitative traits (Table 1) were used to evaluate the level of variation among the varieties following the guidelines of the International Union for the Protection of New Varieties of Plants for Maize (UPOV, 2009).

DNA fingerprinting of maize hybrid varieties using microsatellite markers

DNA extraction

After two weeks from the planting date, the young leaf tissues of six to seven plants were sampled per variety grown in the field experiment. Total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as described by Jhansi *et al.* (2015).

Polymerase chain reaction (PCR)

This study used seven microsatellite markers that were obtained from the maize genomic database (https://www.maizegdb.org/) (Table 2). The target DNA amplification was done in a final volume of a PCR mixture of 25.0 µl, comprised of 12.5 µl of 2X one Taq PCR master mix, 0.5 µl of 100 µM each of forward and reverse primer, 9.5 µl nuclease-free water and 2.0 µl of 50 ng/µl template DNA. PCR products were resolved using gel electrophoresis, subsequently followed by scoring of bands.

Data analysis

Quantitative data were analyzed by a one-way analysis of variance (ANOVA) test in statistical package for social sciences (SPSS version 20).

The amplified PCR fragments from each polymorphic microsatellite marker were scored according to their band sizes, followed by analysis of Nei's genetic distance by using the GenAIEX software, version 6.5. Furthermore, the phylogenic tree of the six varieties of maize hybrids was constructed based on Neighbor Joining clustering using DARwin software, version 6.

RESULTS AND DISCUSSION

Morphological characterization of six varieties of maize hybrids

Morphological characterization is essential for variety identification. In the present study, 11 quantitative and 21 qualitative traits assessed during different plant growth stages. 11 qualitative traits showed significant variation among all six tested varieties (Table 3). Each variety tested in the present study was obtained from different seed companies; thus, the qualitative results confirm their different identity. The finding is in line with Mahmood *et al.* (2022), who morphologically assessed 20 maize inbred lines and found that all the varieties showed significant variation in their quantitative traits because those varieties were bred differently.

Six qualitative traits (first leaf: anthocyanin coloration of sheath, first leaf: shape of apex, stem: degree of stem

zig-zag, ear: color of top of grain, ear: color of dorsal side of grain, ear: anthocyanin coloration of glumes of cob) exhibited monomorphism and couldn't discriminate between the studied varieties. These monomorphic traits were not useful in variety identification because they were commonly possessed in all the tested varieties. So far, some traits showed dimorphic and trimorphic characteristics (Table 4); these traits were able to distinguish at least one variety from among the other tested varieties and were found to be informative for variety identification. A similar finding is in accordance with Asati *et al.* (2023). In the present study, qualitative traits showed minimal variations among the test varieties when compared to quantitative traits. The minimal variation in qualitative traits among the tested varieties could be due to the related parental lines obtained during the breeding program.

DNA fingerprinting of six varieties of maize hybrids using microsatellite markers

In the present study, out of seven microsatellite markers screened, two markers (phi080 and umc1071) were polymorphic and the other five markers (umc1064, umc1013, umc1746, umc1136 and phi076) produced monomorphic bands. A microsatellite marker (phi080) produced a unique banding pattern of two alleles per locus with a base pair of 120 and 150 to identify H2 from the rest of the five hybrids, which had similar banding patterns (Fig 1A). Furthermore, the microsatellite marker umc1071 produced a unique

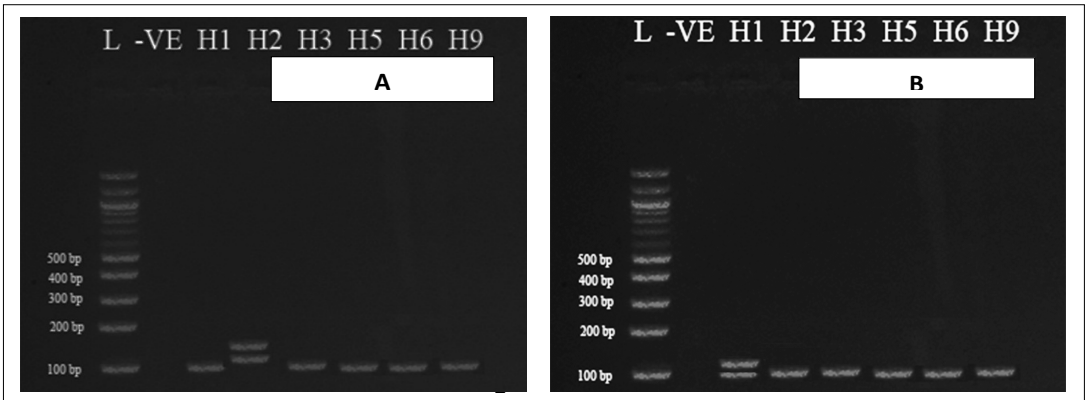
Table 1: Morphological traits observed during varieties identification per UPOV guidelines.

UPOV Character No	Character description (Morphological traits)	DSA	TO
1	First leaf: Anthocyanin coloration of sheath	Seedling	VS
2	First leaf: shape of apex	Seedling	VS
3	Foliage: Intensity of green color	Inflorescence visible	VS
4	Leaf: Undulation of margin of blade	Inflorescence visible	VS
5	Leaf: Angle between blade and stem	Anthesis	VS
6	Leaf: Curvature of blade	Anthesis	VS
7	Stem: Degree of stem zig-zag	Anthesis	VS
8	Tassel: Time of anthesis	Anthesis	MS
9	Tassel: Anthocyanin coloration at base of glume	Anthesis	VS
10	Tasse: Anthocyanin coloration of glumes excluding base	Anthesis	VS
11	Tassel: Anthocyanin colouration of anthers	Anthesis	VS
12	Tassel: Angle between main axis and lateral branches	Anthesis	VS
13	Tassel: Curvature of lateral branches	Anthesis	VS
14	Tassel: Number of primary lateral branches	Anthesis to milky	MS
15	Ear: Time of silk emergence	Anthesis	MS
16	Ear: Anthocyanin colouration of silks	Anthesis	VS
17	Stem: Anthocyanin colouration of brace roots	Anthesis to milky	VS
18	Tassel: Density of spikelets	Anthesis to watery ripe	VS
19	Leaf: Anthocyanin coloration of sheath	Watery ripe to milky	VS
20	Stem: Anthocyanin colouration of internodes	Watery ripe to milky	VS
21	Tassel: Length of main axis above lowest lateral branch	Watery ripe to milky	MS
22	Tassel: Length of main axis above highest lateral branch	Watery ripe to milky	MS
23	Tassel: Length of lateral branch	Watery ripe to milky	MS
24.2	Plant: Length	Milky to dough development	MS
25	Plant: Ratio height of insertion of peduncle of upper ear to plant length	Milky to dough development	MS
26	Leaf: Width of blade	Milky to dough development	MS
27	Peduncle: Length	Milky to dough development	MS
28	Ear: Length	After harvest	MS
36	Ear: Type of grain	After harvest	VS
38	Ear: Colour of top of grain	After harvest	VS
39	Ear: Colour of dorsal side of grain	After harvest	VS
41	Ear: Anthocyanin colouration of glumes of cob	After harvest	VS

Legend: DSA = Developmental stage of assessment, TO = Type of observation, VS = Visual assessment of a number of individual plants or part of plants, MS = Metric measurement of a number of individual plants or parts of plants.

Table 2: List of microsatellite markers used in this study.

Marker	Oligo sequence	Repeat motif	Annealing Temperature (°C)	Expected band size (bp)
umc1064	GTGGGTTTTGTCTGTAGGGTGGTA			
	TCCATCCACTCGACTTAAGAGTCC	(CT)8	57.38	100-130
umc1013	TAATGTGTCCATACGGTGGTGG			
	AGCTGGCTAGTCTCAGGCACTC	(GA)9	56.07	180-200
umc1746	ACACGAGCATCCTACATCCTCCTA			
	ACCTTGCCTGTCCTTCTTTCTCTT	(CAC)4	56.53	104-130
umc1071	AGGAAGACACGAGAGACACCGTAG			
	GTGGTTGTCGAGTTCGTCGTATT	(TACGA)5	57.18	100-130
phi080	CACCCGATGCAACTTGCGTAGAT			
	CGTCACGTTCCACGACATCAC	AGGAG	56.07	120-150
umc1136	CTCTCGTCTCATCACCTTTCCCT			
	CTGCATACAGACATCCAACCAAAG	(GCA)5	56.37	142
phi076	TTCTTCCGCGGCTTCAATTTGACC			
	GCATCAGGACCCGCAGAGTC	AGCGGG	60.00	160-174



Legend: Image A and B represent amplification results of phi080 and umc1071 markers, respectively. L= ladder (1kb), -Ve = Negative control, H1= Hybrid one, H2= Hybrid two, H3= hybrid three, H5= Hybrid five, H6= Hybrid six and H9= Hybrid nine.

Fig 1: Microsatellite results of six varieties of maize hybrids after amplification using phi080 and umc1071 markers.

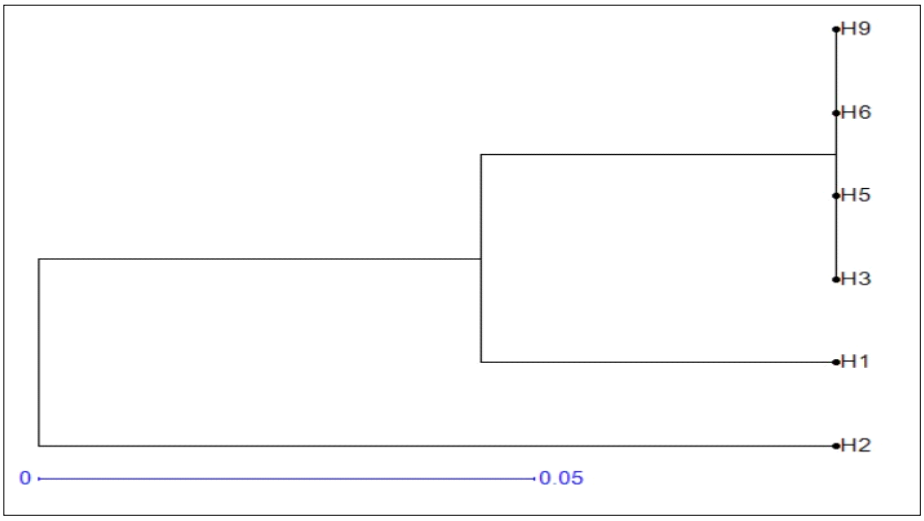


Fig 2: Unweighted Neighbor Joining dendrogram for six varieties of maize hybrids.

profile with two alleles per locus with a base pair of 100 and 130 to identify H1 from the rest of the five hybrids, which produced similar bands (Fig 1B). Markers phi080 and umc1071 are highly polymorphic, as they evidently distinguished two varieties from the other varieties. The two varieties (H1 and H2) are proven to have high genetic variations compared to the other varieties; this makes them advantageous for easy identification. Therefore, variety H1 can be identified by marker umc1071, while variety H2 can be identified by marker phi080. This finding is comparable to Sharma *et al.* (2014). The similar banding patterns in H3, H5, H6 and H9 varieties could be due to a narrow genetic base among *Zea mays* or a small number of polymorphic markers used in the present study as the high number of polymorphic markers used during DNA fingerprinting increases the chance of representing the whole maize genome (Jayasooriya *et al.,* 2013).

Nei's genetic distance

Nei's genetic distance (Table 5) among six varieties of maize hybrids ranged from zero to four. The highest measure of genetic distance was four, followed by three, which was observed in H1 and H2, respectively. This verifies the genetic dissimilarity between each of H1 and H2 and the rest of the four varieties. The genetic dissimilarity is vital in the identification of different varieties (Jayasooriya *et al.,* 2013). The least genetic distance measure was zero, which was observed in both H3, H5, H6 and H9 (Table 2). A genetic distance of zero in H3, H5, H6 and H9 indicates the common ancestors among these varieties. This result is consistent with the findings of Yu *et al.* (2012), who found the minimum genetic distance approaching zero among six genotypes of wax maize and revealed that those genotypes were the sibling lines from the same pedigree.

Neighbor Joining clustering

The unweighted Neighbor Joining tree (Fig 2) made clusters of three populations from six varieties of maize hybrids. Each of the two hybrids, namely H1 and H2, clustered away from the other four varieties (H3, H5, H6 and H9), which clustered together, forming the same population. The different clustering of each of the two varieties (H1 and H2) from the rest of the four varieties indicates the genetic variations between the two varieties and from the rest of the four varieties that clustered together; this supports the unambiguous fingerprinting of H1 and H2 from the rest of the other four hybrids. Four varieties (H3, H5, H6 and H9) clustered together, revealing their relativeness and possibly confirming that they bled from similar germplasm, resulting in the possession of the same alleles in all four varieties. Similar clustering was reported by Kumar *et al.* (2022).

The microsatellite marker results in the present study agree with those of GOT; therefore, the bridge of both methods was crucial, though GOT took a relatively long time, approximately six months and it was labor-intensive. The same was noted by Zhang *et al.* (2023), who complemented

Table 3: Analysis of differences between six varieties of maize hybrid using quantitative traits.

UPOV no.	Traits	Hybrids					
		H1	H2	H3	H5	H6	H9
8	Tassel: Time of anthesis (Days)	68.25±1.81	57.75±1.12	74±0.71	77.50±1.19	88.25±2.06	97.25±1.12
14	Tassel: Number of primary lateral branches	15.25±0.48	11.25±0.48	13.25±0.85	13.25±0.96	18.00±0.41	12.75±0.48
15	Ear: Time of silk emergence (Days).	78.25±6.10	70.25±0.48	83.25±1.38	90.00±0.41	99.00±0.91	109.50±0.65
21	Tassel: Length of main axis above lowest lateral branch	36.68±0.59	34.35±0.95	41.15±1.04	42.38±0.65	39.00±0.38	39.36±0.63
22	Tassel: Length of main axis above highest lateral branch	26.00±0.45	19.88±0.28	26.65±0.47	27.58±0.58	25.45±0.42	29.83±0.30
23	Tassel: Length of lateral branch	11.68±0.57	14.00±0.62	14.50±0.81	14.53±0.16	13.63±0.47	11.80±0.51
24.2	Plant: Length	269.38±6.93	242.23±3.51	256.23±9.65	268.00±3.25	265.05±8.18	280.38±3.71
25	Plant: Ratio height of insertion of peduncle of upper ear to plant length	0.54±0.01	0.58±0.008	0.59±0.007	0.55±0.09	0.54±0.009	0.57±0.006
26	Leaf: Width of blade	9.45±0.18	10.25±0.13	9.32±0.09	9.32±0.13	10.28±0.07	9.81±0.10
27	Peduncle: Length	17.60±0.22	12.56±0.22	17.73±0.26	13.48±0.50	11.95±0.41	20.28±0.50
28	Ear: Length	20.55±0.21	18.50±0.28	19.10±0.24	20.40±0.41	20.25±0.52	19.73±0.20

Table 4: Frequency distribution table for analysis of differences between six varieties of maize hybrids using qualitative traits.

UPOV no.	Plant traits	Observation	No. of varieties	Frequency (%)
1	First leaf: Anthocyanin coloration of sheath	Purple	6	100
2	First leaf: shape of apex	Pointed	6	100
3	Foliage: intensity of green color	Green	4	66.7
		Dark green	2	33.3
4	Leaf: Undulation of margin of blade	Absent/Very weak	1	16.7
		Intermediate	1	16.7
		Strong	4	66.7
5	Leaf: Angle between blade and stem	Very small	1	16.7
		Medium	5	83.3
6	Leaf: Curvature of blade	Very slightly curved	1	16.7
		Slightly curved	1	16.7
		Moderate curved	4	66.7
7	Stem: Degree of stem zig-zag	Absent/Very slight	6	100
9	Tassel: Anthocyanin coloration at base of glume	White	1	16.7
		Light purple	2	33.3
		Purple	3	50
10	Tassel: Anthocyanin coloration of glumes	Green	1	16.7
	excluding base	Light purple	4	66.7
		Purple	1	16.7
11	Tassel: Anthocyanin coloration of anthers	Cream	4	66.7
		Purple	2	33.3
12	Tassel: Angle between main axis and	Very small	3	50
	lateral branches	Small	2	33.3
		Medium	1	16.7
13	Tassel: Curvature of lateral branches	Very Slightly curved	3	50
		Slightly curved	1	16.7
		Moderate curved	2	33.3
16	Ear: Anthocyanin coloration of silks	Cream	1	16.7
		Light purple	1	16.7
		Purple	4	66.7
17	Stem: Anthocyanin coloration of brace roots	Green	2	33.3
		Purple	4	66.7
18	Tassel: Density of spikelet's	Medium	2	33.3
		Moderate dense	4	66.7
19	Leaf: Anthocyanin coloration of sheath	Light purple	2	33.3
		Purple	1	16.7
		Green	3	50
20	Stem: Anthocyanin coloration of internodes	Green	4	66.7
		Purple	2	33.3
36	Ear: Type of grain	Dent	1	16.7
		Pop	3	50
		Intermediate	1	16.7
		Dent like	1	16.7
38	Ear: Color of top of grain	White	6	100
39	Ear: Color of dorsal side of grain	White	6	100
41	Ear: Anthocyanin coloration of glumes of cob	White	6	100

Table 5: Estimate of genetic distance among six varieties of maize hybrids.

H1	H2	H3	H5	H6	H9	
0						H1
4	0					H2
1	3	0				H3
1	3	0	0			H5
1	3	0	0	0		H6
1	3	0	0	0	0	H9

the SNP results with morphological analysis when working with tomato varieties.

CONCLUSION

The application of microsatellite markers complemented with morphological characters enabled successful fingerprinting of two commercial varieties of maize hybrids, herein coded as H1 and H2, out of the six varieties included in this study. Two markers, namely phi080 and umc1071, could be used for fast and timely varietal identification of H1 and H2. This study provides evidence and baseline data for consideration of the incorporation of these markers in varieties identification. Since a small sample size was used in terms of varieties, this study recommends that future research should focus on increasing the number of varieties.

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Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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