

## Research Paper

# Genetic diversity of a global collection of maize genetic resources in relation to their subspecies assignments, geographic origin, and drought tolerance

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The genetic diversity among an international collection of 40 maize accessions has been evaluated using DNA ISSR fingerprinting. Among the 180 ISSR markers scored by 15 primers, 161 markers (89.59%) were polymorphic and 19 were unique in 16 accessions. A cluster tree based on the average distance coefficients and the Dice similarity indices divided the accessions into three major groups, each including clusters of accessions assigned to their subspecies. However, a low level of genetic differentiation among the accessions was demonstrated by the STRUCTURE analysis of ISSR data in agreement with the low gene flow ( $N_m$ ) value among the accessions. A scatter diagram of the principal component analysis (PCA) based on ISSR data analysis revealed that the accessions were differentiated into three groups comparable to those produced by the cluster analysis, in which some accessions of the same subspecies showed a close similarity to each other. A scatter diagram of the principal coordinate analysis (PCoA) based on the drought tolerance indices (DTIs) showed that nine genetically similar accessions share drought tolerance characteristics; these include four of subsp. *indurata*, three of subsp. *everata*, and two of subsp. *indentata*. An abundance of unique ISSR alleles found in the 16 accessions, including the nine drought-tolerant accessions, represents rich untapped genetic resources and these accessions may be exploited in the future breeding of maize commercial lines.

**Key Words:** maize germplasm, molecular markers, genetic resources, abiotic stress.

## Introduction

Plant genetic resources (PGR) are plant materials of essential value for present and future generations of people. PGR have been recognized as essential sources of genotypic variation and are required for the future breeding of new crop varieties (Hammer 1998). Huge collections of PGR are available in hundreds of gene banks around the world, but only a little information on the extent of genetic variation in the traits is available for in situ conserved PGR. The landraces of PGR in the centers of crop diversity represent past and contemporary patterns of natural and farmer-mediated evolutionary forces. Successful in situ conservation of crop genetic resources depends on continuity of these evolutionary processes (Mercer and Perales 2010). Upon the characterization and utilization of PGR, genotype information, which can be effectively used for the develop-

ment of cultivars with high yield and good agronomic traits, pest and disease resistance, and/or adaptation to a broad range of environmental conditions that may prevail as a result of expected climate change, is becoming increasingly important (Battisti and Naylor 2009, Cooper *et al.* 2014, Howden *et al.* 2007, Jarvis *et al.* 2008).

Maize (*Zea mays* L.) is ranked third after wheat and rice in the world production of cereal crops and is widely cultivated throughout the globe in a wide range of agro-ecological environments (FAO 2019). The demand for maize global production as a source of food, forage, oil, and biofuel is increasing for the ever-increasing world human population. However, the number of maize landraces decreases in farmers' fields over time, threatening the availability of genetic resources for the future. A study on 93 maize landrace accessions from Morelos, Mexico, stored at the International Maize and Wheat Improvement Center (CIMMYT), showed that maize landrace cultivation had diminished over the last 50 years in the studied area (McLean-Rodríguez *et al.* 2019). Diversity among the maize germplasm is important for identifying parental lines for successful breeding programs and hybrid development. With the climate change scenarios, the majority of maize-

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producing areas will become warmer and drier, and these areas will be subjected to new maize diseases and pests that may lead to an alarming impact on maize production under the warmer climate with changing rainfall patterns (Edmeades 2013). The diversity of the maize germplasm is a sustainable source of alleles useful for the future challenge that may be imposed by abiotic stresses caused by climate changes.

The development of molecular markers to measure the relationships between plants and genetic diversity depends on polymorphisms found in DNA (Badr 2008, Mondini *et al.* 2009). Polymerase chain reaction (PCR)-based approaches are commonly used to assess the genetic diversity of maize genetic resources. Simple sequence repeats markers (SSR), also known as micro-satellites, were used to characterize and differentiate the Bulgarian maize germplasm collection (Kostova *et al.* 2006), the isolation-by-distance and altitude of maize landraces in the Western Highlands of Guatemala (Van Etten *et al.* 2008), and also for the assessment of genetic diversity among maize inbred genotypes developed in Italy (Losa *et al.* 2011). The phenotypic and SSR-based diversity of maize landraces in India, especially from the North East Himalayan region, was characterized by Sharma *et al.* (2010), and the variability of six morpho-physiological traits as well as SSR markers was used to differentiate 91 Indian genotypes by Kumar *et al.* (2012). SSR analysis was successfully employed to study genetic variation among the farmers' maize varieties and maize hybrids in Nigeria (Adeyemo and Omidiji 2019). In addition to SSR, the start codon targeted (SCoT) markers were also used to differentiate genotypes of old maize from Eastern European countries and Russia (Vivodík *et al.* 2016, 2017). Analyzing SCoT markers on eight inbred lines showed a consistency with their pedigree (Sadek and Ibrahim 2018).

Inter simple sequence repeat (ISSR) markers are regarded as reproducible and specific tools for genome fingerprinting (Bornet and Branchard 2001). The application of an adequate number of ISSR markers has gained acceptance for genetic diversity evaluation in maize. Carvalho *et al.* (2002) reported high levels of genetic variability in 81 maize landraces and varieties from different states of Brazil using ISSR markers. The high level of genetic variability was justified as the multiple origins of varieties associated with cultivation at different localities for several years. The results of Júnior *et al.* (2011) on the genetic diversity of maize genetic resources in Brazil demonstrated the separation and identification of the accessions of maize genotypes, but they also indicated that breeders in Brazil are using a germplasm of narrow diversity and called for greater attention to the selection of more distinct genotypes in breeding programs. The ISSR markers efficiently identified diverse genotypes of maize in Pakistan that may be used for breeding new varieties with distinct characteristics and the identified genotypes were recommended as parents for the future development of new cultivars (Muhammad

*et al.* 2017). The ISSR markers were successfully used to estimate the genetic diversity among different maize inbred lines (Amoon and Abdul-Hamed 2020).

Although increased maize production is a global demand for the ever-increasing world human population, the annual maize yield loss due to drought is too high and is likely to increase with the expected climate change (Ferguson 2019, Webber *et al.* 2018). With the weather expected to become generally drier and warmer, the situation may be further exacerbated as competition for water intensifies between human usage and crop irrigation (Lobell *et al.* 2014). Measures of drought tolerance based on germination and seedling traits under controlled conditions and drought stress have been used by a few researchers to identify candidate drought-tolerant genotypes. Meeks *et al.* (2013) reported that clear genotypic differences among 62 diverse maize inbred lines and hybrid testcrosses were observed approximately 13 and 18 days after planting, respectively, while the drought sensitivity of eight commercial hybrid lines with a reference inbred line was reported by Avramova *et al.* (2016). In addition, drought tolerance indices based on the response of seedling traits under stress conditions compared to the control have been recently applied to evaluate maize drought tolerance by Badr *et al.* (2020).

The present study aims to evaluate the genetic diversity among 40 maize accessions from different parts of the world in relation to their subspecies (subsp.) delimitation, geographic origin, and drought tolerance as indicated by seedling traits related to drought stress.

## Materials and Methods

### *Plant materials and simulated drought application*

Seed materials of 40 maize (*Zea mays* L.) accessions were kindly provided by the Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany. The accessions ID, nomenclature, subspecies assignment, and geographical origin are listed in **Table 1**. The accessions were sampled to represent an international collection and include varying responses to drought tolerance as indicated by Badr *et al.* (2020). The seeds of all accessions were germinated, and the seedlings were grown as described by Badr *et al.* (2020) under simulated drought stress imposed by 10% of polyethylene glycol (PEG). Six shoot and root traits, i.e., shoot length (ShL), shoot fresh weight (ShFW), shoot dry weight (ShDW), root length (RL), root fresh weight (RFW), and root dry weight (RDW), were measured for the control plants and plants exposed to 10% of PEG 9 and 16 days after sowing. Drought tolerance indices (DTIs) were calculated for each measured trait as the percentage of the measured average under stress compared to the average of the control plants. Leaf samples of all accessions were collected and dried in airtight plastic containers between a one-inch-thick layer of silica gel for DNA extraction.

**Table 1.** List of examined IPK maize accession codes, taxonomic information and geographic origin

Serial	Accession ID	Accession subspecies/cultivar assignment	Country of origin
01	Zea 12	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Germany
02	Zea 242	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Germany
03	Zea 323	subsp. <i>everata</i> (Sturtev) Zhuk. var. <i>oryzoides</i> Körn.	Soviet Union
04	Zea 355	subsp. <i>saccharata</i> (Körn.) Zhuk var. <i>flavodulcis</i> Körn.	Soviet Union
05	Zea 382	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Romania
06	Zea 394	subsp. <i>indentata</i> (Sturtev) Zhuk. Ashoro Zairai.	Japan
07	Zea 487	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>rubropaleata</i> Körn.	Greece
08	Zea 630	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	China
09	Zea 633	subsp. <i>everata</i> (Sturtev) Zhuk. var. <i>oryzoides</i> Körn.	China
10	Zea 668	subsp. <i>indentata</i> (Sturtev) Zhuk. var. <i>xantodon</i> Alef.	Macedonia
11	Zea 677	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Hungary
12	Zea 711	subsp. <i>everata</i> (Sturtev) Zhuk. var. <i>oryzoides</i> Körn.	Czech Republic
13	Zea 1006	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Libya
14	Zea 1015	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Libya
15	Zea 1019	subsp. <i>everata</i> (Sturtev) Zhuk. var. <i>oryzoides</i> Körn.	Italy
16	Zea 1062	subsp. <i>indentata</i> (Sturtev) Zhuk. var. <i>flavorubra</i> Körn.	Korea
17	Zea 1102	subsp. <i>indurata</i> (Sturtev) Zhuk.	Korea
18	Zea 1114	subsp. <i>indurata</i> (Sturtev) Zhuk. Var. <i>aurantiaca</i> .	Italy
19	Zea 1121	subsp. <i>indurata</i> (Sturtev) Zhuk.	Austria
20	Zea 1224	subsp. <i>everata</i> (Sturtev) Zhuk. var. <i>gracillima</i> Körn.	Romania
21	Zea 3002	subsp. <i>indurata</i> (Sturtev) Zhuk.	Georgia
22	Zea 3065	subsp. <i>indentata</i> (Sturtev) Zhuk. var. <i>leucodon</i> Alef.	Georgia
23	Zea 3175	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>alba</i> Alef.	Georgia
24	Zea 3244	subsp. <i>everata</i> (Sturtev) Zhuk. var. <i>oxyornis</i> Körn.	Germany
25	Zea 3257	subsp. <i>indentata</i> (Sturtev) Zhuk. var. <i>xantodon</i> Alef.	Albania
26	Zea 3280	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Romania
27	Zea 3282	subsp. <i>indurata</i> (Sturtev) Zhuk. Oarzan.	Romania
28	Zea 3301	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Portugal
29	Zea 3324	subsp. <i>indentata</i> (Sturtev) Zhuk.	Albania
30	Zea 3325	subsp. <i>indentata</i> (Sturtev) Zhuk. var. <i>leucodon</i> Alef.	Albania
31	Zea 3346	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>caesia</i> Alef.	USA
32	Zea 3392	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>rubra</i> Bonaf.	Bulgaria
33	Zea 3400	subsp. <i>semindentata</i> Kuleshov.	Georgia
34	Zea 3424	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>rubropalata</i> Körn.	Italy
35	Zea 3425	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>vulgata</i> Körn.	Germany
36	Zea 3576	subsp. <i>indurata</i> (Sturtev) Zhuk. var. <i>alba</i> Alef.	Italy
37	Zea 3582	subsp. <i>semindentata</i> Kuleshov.	Kroatia
38	Zea 3602	subsp. <i>indentata</i> (Sturtev) Zhuk.	Turkey
39	Zea 3712	subsp. <i>everata</i> (Sturtev) Zhuk. var. <i>gracillima</i> Körn.	Georgia
40	EGIW 237	cultivar imported by the Agriculture Research Center	Egypt

### DNA extraction and ISSR profiling

The total genomic DNA was isolated from leaves using Zymo-Spin total DNA extraction kits (Zymo Research Cat# C1011, USA) according to manufacturer's protocol. The purified DNA was resolved on 1% agarose gel prepared in  $1 \times$  TAE (Tris-acetate-ethylenediaminetetra acetic acid) buffer containing 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide to check the DNA integrity. For ISSR profiling, the PCR was carried out in a Biometra thermal cycler using the 15 primers listed in **Table 2** in 25  $\mu\text{L}$  reaction volume. The PCR mix included the following: 30 ng of DNA, 0.5 U of *MyTaq* DNA polymerase and  $1 \times$  *Taq* polymerase buffer (BIOLINE Cat# BIO-21108), and 10  $\mu\text{M}$  of the corresponding primer. The

PCR profile started with 95°C for 4 min, followed by 37 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 sec, and an extension at 72°C for 2 min. A final extension at 72°C for 10 min was included. The ISSR PCR products were resolved in 1.2% (w/v) agarose gel in  $1 \times$  TAE buffer containing 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide. Ethidium-bromide-stained gel was visualized using a UV-transilluminator (Vilber Lourmat-Germany) and the images were captured with a Nikon COOLPIX L820 digital camera. The PCR reaction was repeated twice for each primer to confirm the reproducibility. Only reproducible primers were considered for the further analysis. ISSR markers were scored using Quantity One software version 4.6.2.70.

**Table 2.** List of selected ISSR primers and their codes, sequences, the number of amplified markers and percent of polymorphism for each primer in maize accessions\*

Ser	Primer code	Primer sequence (5'-3')	Types and number of ISSR markers			% Polymorphic	Size range bp
			Polymorphic	Unique	Total		
1	I5	(AG) <sup>8</sup> TG	10	0	10	100.0	1372-588
2	I10	(AC) <sup>8</sup> CA	17	3	20	85.00	2800-423
3	I14	(ACC) <sup>6</sup>	12	5	17	70.59	4000-330
4	I15	(AGC) <sup>6</sup>	–	–	–	–	–
5	I19	TACAGCAGCACAG	12	1	13	92.31	2500-378
6	I22	(AC) <sup>8</sup> G	10	1	11	90.90	1600-420
7	I25	(CT) <sup>8</sup> AG	5	1	6	83.33	2839-770
8	I29	(TG) <sup>8</sup> AA	–	–	–	–	–
9	812	(GAG) <sup>5</sup> AT	12	2	14	85.71	2312-237
10	844	(CT) <sup>8</sup> GC	14	2	16	87.50	2121-301
11	873	G(ACAG) <sup>3</sup> ACA	6	1	7	85.71	1317-327
12	885	(CGT)(ACT)(CGT)(GA) <sup>5</sup>	16	2	18	88.88	2673 386
13	889	(AGT)(CGT)(AGT)(AC) <sup>5</sup>	15	0	15	100.0	1641-255
14	891	(ACT)(ACG)(ACT)(TG) <sup>5</sup> T	18	1	19	94.73	1480-203
15	ISSR-5	(ACG) <sup>4</sup> GAC	14	0	14	100.0	1902-308
Total number of markers			161	19	180		
Average			12.38	1.46	13.85	89.59	

\* The overall genetic diversity values among the examined accessions of maize: total genetic diversity ( $H_T=0.3076$  and Shannon's index ( $N_m$ )=0.4681).

with reference to a Gene-Ruler 1 kb+ DNA ladder (Thermo Scientific SM1331) to determine the size of the ISSR markers.

The DNA bands on the ISSR fingerprinting gels were scored as (1) for presence and (0) for absence in the binary matrices for data analysis. The number of unique and polymorphic bands and the percentage of polymorphic bands for each primer and each accession were calculated. Genetic diversity among the 40 accessions was calculated based on the binary data from the amplified ISSR markers using two clustering methods: the Community Analysis Package-5 (CAP) (Seaby and Henderson 2007) to construct the average linkage distance based on the hierarchical grouping function (Ward 1963) and PAST software version 3.22 based on the paleontological statistics software developed by Hammer *et al.* (2001) to construct a similarity tree based on the Dice's similarity coefficient. Trees for the two cluster analyses were constructed using the unweighted pair group method with an arithmetic mean (UPGMA) algorithm. Gene flow ( $N_m$ ) was computed for each locus using POPGENE version 1.31 (Yeh *et al.* 1999). POPGENE was also used to obtain an estimate of the total genetic diversity for all the populations ( $H_T$ ).

The STRUCTURE program version 2.3.4 was used to assess the distinction of accessions based on their subsp. assignment (Pritchard *et al.* 2000). The number of genetically distinct clusters ( $k$ ) was set to vary from 1 to 7 (number of subsp.). The model was run for five independent simulations for each  $k$ , with a burn-in length of 50,000 and a run length of 100,000 iterations. The settings of a “no

admixture” model for dominant markers and uncorrelated allele frequencies among populations were presumed. The most likely number of clusters ( $k$ ) was estimated using Structure Harvester (Earl and von Holdt 2012). In addition, the principal component analysis (PCA) in the PAST software (Hammer *et al.* 2001) has been applied to produce a scatter diagram illustrating the grouping of accessions based on the ISSR polymorphism. The principal coordinates analysis (PCoA) was used to produce a scatter biplot to rank the accessions for their drought tolerance as estimated by the DTI of the measurements of the shoot and root traits.

## Results

### ISSR fingerprinting and genetic diversity statistics

Of the 15 primers used, 13 primers produced a total of 180 ISSR markers ranging in size from 4,000 bp to 203 bp (Tables 2, 3). Among them, 19 ISSR markers detected by ten primers were unique in 16 accessions. The remaining two primers (pr. I15 and pr. I29) failed to produce stable and reproducible markers. Fig. 1 illustrates examples of ISSR fingerprints that were amplified in the 40 maize accessions by Primer I14 with a sequence of (ACC)<sup>6</sup> (Fig. 1A), Primer I10 with (AC)<sup>8</sup>CA (Fig. 1B), and Primer 812 with (GAG)<sup>5</sup>AT (Fig. 1C). No monomorphic markers were recorded in any of the accessions. When some primers failed to produce ISSR markers in a few accessions, these have been regarded as missing data. In the comparison of the 180 ISSR markers, the number of markers recorded for

**Table 3.** Number and percentage of ISSR markers and the number of unique markers and their producing primers as well the average drought tolerance indices for the 40 maize accessions

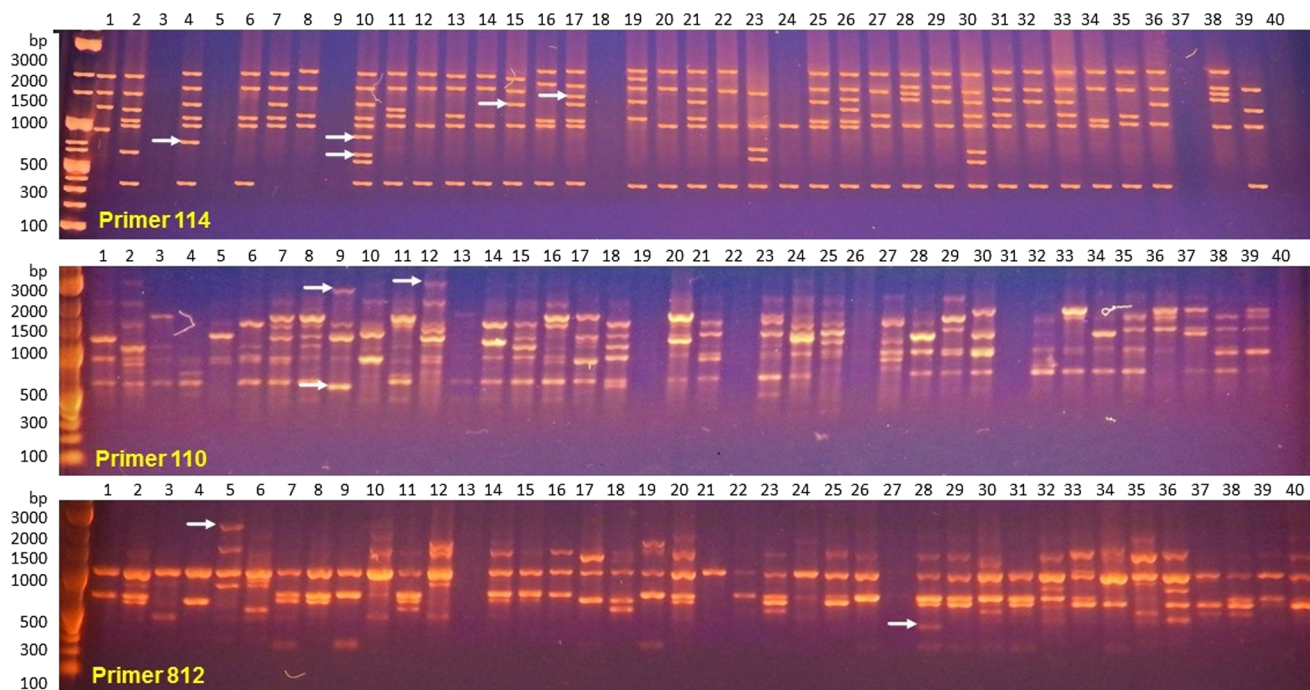
Serial	Accession ID	ISSR markers		Unique marker primers	Unique markers size in bp	DTIs average
		No. of alleles	%			
01	Zea 12	51	28.33			0.618
02	Zea 242	61	33.88			0.747
03	Zea 323	55	30.55	1-pr. 844	301	0.686
04	Zea 355	55	30.55	1-pr. I14	941	0.661
05	Zea 382	46	25.55	1-pr. 812	2312	0.742
06	Zea 394	59	32.77			0.686
07	Zea 487	57	31.66			0.637
08	Zea 630	58	32.22			0.715
09	Zea 633	43	23.88	2-pr. I10, 1-pr. I22	1750, 439-1320	0.690
10	Zea 668	65	36.11	2-pr. I14	1065, 584	0.690
11	Zea 677	65	33.88	1-pr. 885	386	0.757
12	Zea 711	61	36.11	1-pr. I10	1892	0.750
13	Zea 1006	58	32.22	1-pr. 873	1041	0.744
14	Zea 1015	56	32.22			0.737
15	Zea 1019	54	30.00	1-pr. I14	1660	0.770
16	Zea 1062	52	28.88	1-pr. I19	804	0.713
17	Zea 1102	60	33.33	1-pr. I14	2378	0.726
18	Zea 1114	51	28.33			0.682
19	Zea 1121	64	35.55	1-pr. I25	2839	0.636
20	Zea 1224	44	21.66			0.750
21	Zea 3002	49	27.22			0.696
22	Zea 3065	53	28.33			0.616
23	Zea 3175	55	30.55			0.673
24	Zea 3244	51	28.33			0.617
25	Zea 3257	57	31.66	1-pr. 891	891	0.602
26	Zea 3280	58	32.22	1-pr. 844	1828	0.661
27	Zea 3282	46	25.55			0.636
28	Zea 3301	49	27.22	1-pr. 812	429	0.656
29	Zea 3324	52	28.88			0.574
30	Zea 3325	52	28.88			0.650
31	Zea 3346	51	28.33			0.670
32	Zea 3392	53	28.33			0.615
33	Zea 3400	46	25.55			0.644
34	Zea 3424	52	28.88	1-pr. 885	1931	0.627
35	Zea 3425	47	26.11			0.663
36	Zea 3576	40	22.22			0.591
37	Zea 3582	43	23.88			0.573
38	Zea 3602	45	25.00			0.557
39	Zea 3712	44	24.44			0.725
40	EG IW 237	40	22.22			0.680
Average		52.25	29.04			0.672

each accession varies greatly between accessions; however, it was not associated with their taxonomic assignment to subspecies. It ranges from 40 markers in Zea 3576 and EGIW 237 to 65 in Zea 668 and Zea 677. The number of markers differs substantially between accessions and no correlation is evident between the number of markers and the subspecies delimitation of the accessions. Eight unique markers were scored in eight of the 21 accessions of subsp. *indurata* and six unique markers were scored in four of the seven accessions of subsp. *everata*. Four unique markers

were scored in three of the eight accessions of subsp. *indentata* and only one unique marker in Zea 355 of subsp. *saccharata* (Tables 1, 2).

The genetic diversity statistics (Tables 2, 3) indicate that the average percentage of polymorphic loci produced by the ISSR primers is generally high (89.59%). For the three primers: Primer I5 (AG)<sup>8</sup>TG, Primer 889 (AGT)(CGT)(AGT)(AC)<sup>5</sup>, and Primer ISSR-5 (ACG)<sup>4</sup>GAC, 100% polymorphism was recorded. On the other hand, the percentage of polymorphism for the other primers ranges from 94.73%





**Fig. 1.** Photographs illustrating ISSR fingerprints amplified in the 40 maize accessions by the three primers. (A) Primer 114 with primer sequence (ACC)<sup>6</sup>, (B) Primer 110 with (AC)<sup>8</sup>CA, and (C) Primer 812 with (GAG)<sup>5</sup>AT.

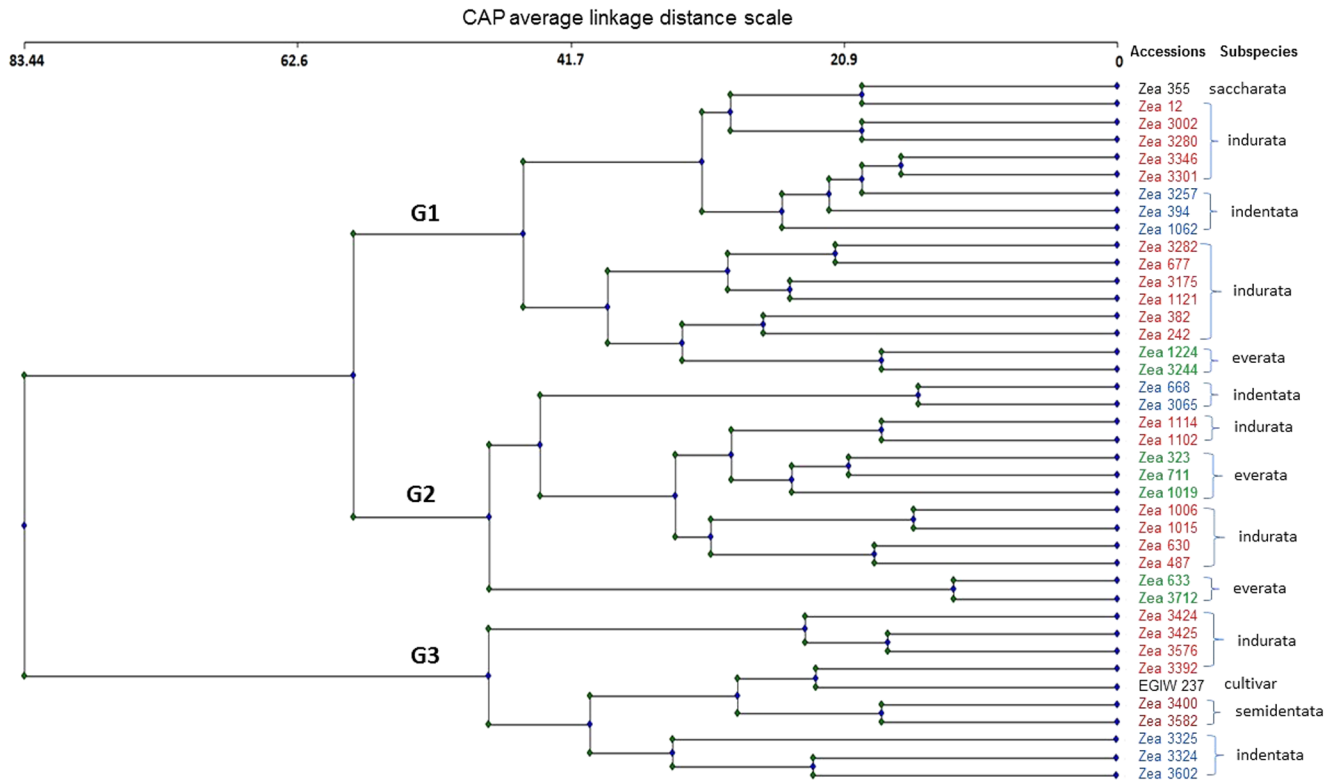
for Primer 891 (ACT)(ACG)(ACT)(TG)<sup>5</sup>T to 70.59% for Primer 114 (ACC)<sup>6</sup>. The latter primer produced five unique markers in four accessions including two in *Zea* 668 and one each in *Zea* 355, *Zea* 1019, and *Zea* 1102. Primer 110 produced three unique markers in two accessions including two markers in *Zea* 633 and one in *Zea* 711. Primers 812, 844, and 885 produced two unique bands and the other primers produced one unique band in different accessions (Table 3). The overall Nei's genetic diversity analysis showed that the total genetic diversity ( $H_T$ ) is 0.3076, indicating that the genetic diversity is distributed among the examined accessions. The gene flow index ( $N_m$ ) was low (0.468) indicating that the level of genetic differentiation among accessions is low.

### Genetic diversity analysis

As shown in the average linkage distance tree (hereafter called the CAP tree) constructed using CAP software based on the hierarchical grouping function (Fig. 2), the 40 maize accessions were divided into three main groups, (G1, G2, and G3). The codes of the 40 accessions of maize are as given in Table 1. In G1, two clusters of nine and eight accessions are distinguished, of which, the nine accessions cluster includes *Zea* 355 of subsp. *saccharata* (black), six accessions of subsp. *indurata* (red), and two accessions of subsp. *indentata* (blue). The eight-accessions cluster also includes six accessions of subsp. *indurata* (red) and two accessions (*Zea* 1224 and *Zea* 3244) of subsp. *everata* (green). In G2, two small clusters, each containing two accessions, are delimited from a major cluster in the middle

comprised of nine accessions. The first one includes *Zea* 668 and *Zea* 3065 of subsp. *indentata* and the other includes *Zea* 633 and *Zea* 3712 of subsp. *everata*. The nine accessions include two accessions of subsp. *indurata* (*Zea* 1114 and *Zea* 1102) that clustered with three accessions of subsp. *everata* (*Zea* 323, *Zea* 711, and *Zea* 1019) and another cluster of four accessions of subsp. *indurata* (*Zea* 1006, *Zea* 1015, *Zea* 630, and *Zea* 487). G3 is comprised of ten accessions, including four accessions of subsp. *indurata* (*Zea* 3424, *Zea* 3425, *Zea* 3576, and *Zea* 3392), the cultivar EG IW 237 (black), two accessions of subsp. *semidentata* (*Zea* 3400 and *Zea* 3582), and three accessions of subsp. *Indentata* (*Zea* 3325, *Zea* 3324, and *Zea* 3602).

In a cluster tree (hereafter called the PAST tree) constructed based on the Dice's similarity coefficients using the UPGMA algorithm of PAST software (Fig. 3), the 40 maize accessions were grouped in a similar clustering topology to the CAP tree (Fig. 2). However, the PAST tree is comprised of five groups. Two large groups, here marked as GE and GA, corresponded to G2 and G3 of the CAP tree, respectively. Three small groups, GB, GC, and GD, corresponded to G1 of the CAP tree. The GA of the PAST tree is comprised of 11 accessions, including nine of which were in G3 of the CAP tree. These include two accessions of subsp. *semidentata* (*Zea* 3400 and *Zea* 3582), three accessions of subsp. *indentata* (*Zea* 3324, *Zea* 3325, and *Zea* 3602, blue), the cultivar EGIW 237 (black), and also three accessions of subsp. *indurata* (*Zea* 3392, *Zea* 3424, and *Zea* 3576, red). The only accession of subsp. *saccharata* (*Zea* 355) was clustered with *Zea* 12 (red) in GA; however,



**Fig. 2.** UPGMA tree of the 40 examined accessions based on the average linkage distance calculated from the ISSR data. Three main groups, G1, G2, and G3, were observed.

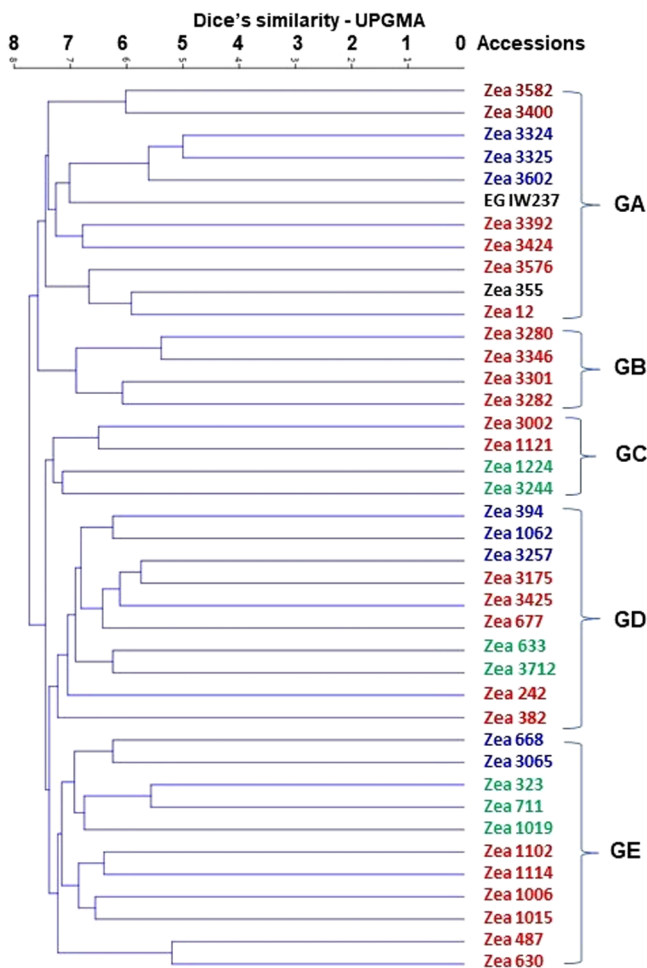
both were assigned to G1 of the CAP tree. GB and GC in the PAST tree (Fig. 3) were two small groups each comprised of four accessions, GB includes four accessions of subsp. *indurata* and GC is comprised of two accessions of subsp. *indurata* (Zea 3002 and Zea 1121) and two accessions of subsp. *everata* (Zea 1224 and Zea 3244). GD is comprised of ten accessions: three accessions of subsp. *indentata* (blue), three accessions of subsp. *indurata* (red), Zea 633 and Zea 3712 of subsp. *everata*, and Zea 242 and Zea 382 of subsp. *indurata* to form two small clusters. In GE, Zea 668 and Zea 3065 of subsp. *indentata*, Zea 323, Zea 711, and Zea 1019 of subsp. *everata* form one cluster and four accessions of subsp. *indurata* (Zea 1102, Zea 1114, Zea 1006, and Zea 1015) form another one, and the two accessions, Zea 487 and Zea 630, of subsp. *indurata* are also assigned to this cluster.

The result of the STRUCTURE analysis classified the 40 accessions as an admixture not assigned to groups and showed as approximately one population with unclear differentiation of accessions (Fig. 4). This demonstrates high genetic similarity among the accessions, as the 40 maize accessions are not distinguished into genetically distinct clusters that are either compatible with their subspecies assignment or with their origin. This finding may reflect a common gene pool for the 40 maize accessions that was supported by the high Dice similarity coefficients between the accessions as expressed in Fig. 3.

The genetic relationship of the 40 maize accessions was

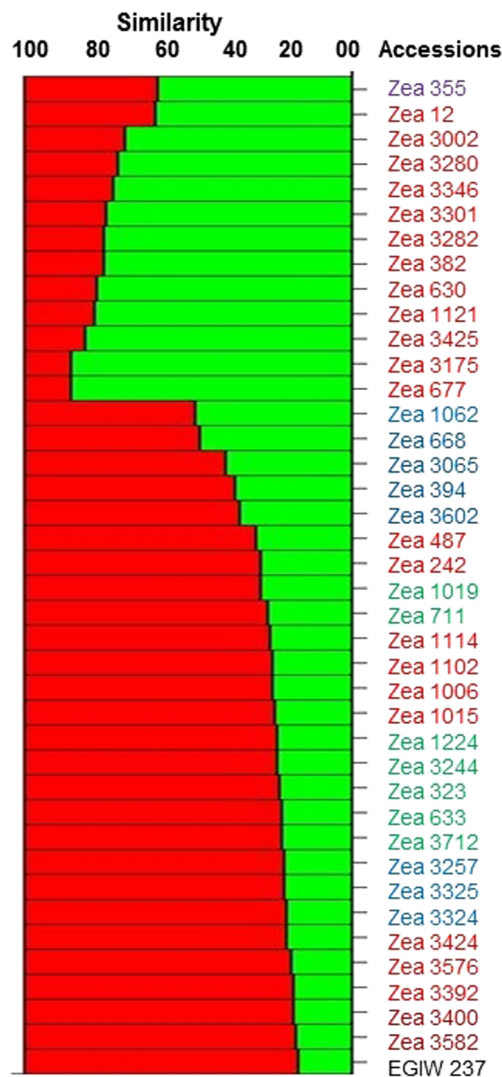
also expressed by a PCA scatter diagram, based on the ISSR data analysis, using the software PAST which ranks the accessions by the two first axes (PC1 and PC2) of the PCA (Fig. 5). In this diagram, the accessions are displayed in three groups generally congruent with their clustering in Fig. 2 and Fig. 3 with few differences. Some accessions clustered in G1 of Fig. 2 are grouped in the circle of G2 in Fig. 5, particularly Zea 12, Zea 382, and Zea 677 of subsp. *indurata* and Zea 394 and Zea 1062 of subsp. *indentata*. Zea 1102 and Zea 1114 of subsp. *indurata* and Zea 3065 of subsp. *indentata* clustered in G2 in Fig. 2 are circled in G1 (Fig. 5). Meanwhile, the grouping of Zea 382, Zea 394, Zea 677, and Zea 1062 circled in G2 (Fig. 5) is compatible with their clustering in G1 (Fig. 2). On the other hand, the scattering of accessions Zea 633 and Zea 3712 of subsp. *everata* (Fig. 5) is congruent with their distinction as a separate cluster in both Fig. 2 and Fig. 3. Interestingly, the grouping of accessions in G3 is in full agreement with their clustering in G3 of the CAP tree illustrated in Fig. 2.

A PCoA biplot illustrating the classification of maize accessions based on the DTI values of the seedling traits measured 9 and 16 days after sowing is shown in Fig. 6. Eigenvectors generated by the PCoA were used to rank the accessions for their drought tolerance and the biplot is constructed by plotting PC1 and PC2, which account for the maximum variability of the measured traits. A higher impact was scored for the DTI of the root and shoot fresh weight and dry weight, particularly the RDW1, ShDW1,



**Fig. 3.** UPGMA tree of the 40 examined accessions based on Dice's similarity coefficients calculated from the ISSR data. Five groups, GA, GB, GC, GD, and GE, were observed.

RL1, ShL1, and RL2 as well as RFW2, ShFW2, ShL2, and LL; where 1 denotes measurements performed 9 days after sowing, 2 denotes measurements 16 days after sowing, and LL was measured in three-week-old seedlings. The values of the grand DTI for the accessions, which is the mean value of all DTIs of the measured traits, are given in **Table 3**. The values of DTI ranged between 0.55 for Zea 3602 of subsp. *indentata* to 0.77 for Zea 1019 of subsp. *everata* with an average value of 0.672. In brief, DTIs higher than 0.72 were observed at 10 accessions; these include six accessions of subsp. *indurata* (Zea 242, Zea 382, Zea 677, Zea 1006, Zea 1015, and Zea 1102) and four accessions of subsp. *everata* (Zea 711, Zea 1019, Zea 1224, and Zea 3712). These accessions, except for Zea 677, were grouped as G1 in **Fig. 6** with the other six accessions, including Zea 1114 and Zea 1121 (subsp. *indurata*), Zea 323, and Zea 633 (subsp. *everata*) and Zea 394 and Zea 668 (subsp. *indentata*). Most of these accessions were grouped together based on the analysis of ISSR data indicating the association of drought tolerance with genotype. This association was confirmed as ten accessions in G2 of



**Fig. 4.** Population structure analysis showing an admixture of ISSR alleles in the 40 maize accessions using the STRUCTURE software.

the CAP tree (**Fig. 2**), nine accessions are in GE of the PAST tree (**Fig. 3**), and nine accessions in G2 of the scatter diagram (**Fig. 5**).

**Fig. 5** and **Fig. 6** illustrate that the nine genetically similar accessions sharing the characteristic of drought tolerance include four of subsp. *indurata* (Zea 242, Zea 382, Zea 1006, and Zea 1015), three of subsp. *everata* (Zea 323, Zea 711, and Zea 1019) and two of subsp. *indentata* (Zea 394 and Zea 668). The other three accessions of subsp. *indurata* and two of subsp. *everata* also show high drought tolerance (**Figs. 5, 6**). On the other hand, nine other accessions had DTIs lower than 0.62, including three accessions of subsp. *indurata* (Zea 12, Zea 3392, and Zea 3576), four accessions of subsp. *indentata* (Zea 3065, Zea 3257, Zea 3324, and Zea 3602), one accession of subsp. *everata* (Zea 3244), and Zea 3582 of subsp. *semidentata*. All of these accessions are clearly grouped together in the G2 circle of **Fig. 6** with Zea 355 of subsp. *saccharata* and Zea 3280 of





of maize populations of the CIMMYT collection in Mexico (Berilli *et al.* 2011). Of the 140 alleles produced by the 13 primers, 81.4% were polymorphic and 18.6% monomorphic. A much lower polymorphism of 36.46% was produced by ten ISSR primers in six maize inbred lines in Egypt (El-Hosary and El-Akkad 2015). Also, much lower polymorphism was detected in 20 old maize genotypes from the former Soviet Union and countries in Eastern Europe using five Start codon Targeted (SCoT) markers (Vivodík *et al.* 2017). These primers produced 29 fragments across 20 maize genotypes, of which 22 fragments (77.9%) were polymorphic with an average of 4.4 polymorphic fragments per primer, which is very low compared to the average of 12.38 per primer in the current study. Meanwhile, 108 amplification products were generated with 17 ISSR primers in 50 plants representing 10 cultivars with an average of 6.35 fragments per primer including 83 (75.2%) polymorphic fragments (Dar *et al.* 2018). The high proportions of polymorphism in the examined 40 international maize accessions in comparison to the lower polymorphism levels in other material is expected since the 40 accessions used in the current study represent an international collection of maize accessions of different subspecies, while the materials used by Dar *et al.* (2018) represent less widespread collections such as Brazil and Eastern Europe.

The percentage of polymorphic markers ranged from 22.22% in Zea 3576 and the cultivar EGIW 237 to 36.11% in Zea 668 and Zea 711 with an average of 29.04%. Similar percentages of polymorphic markers are found in accessions of different subspecies and geographic origins. In addition to the 161 polymorphic markers produced by the 13 primers in the 40 accessions, 19 unique markers were produced by the ten primers in 16 accessions indicating an abundance of unique alleles. These accessions may be exploited for future crop improvement since untapped accessions with unique alleles are a great reservoir of genetic resources for maize improvement. Maize from the Algerian Desert was found to harbor a wide genetic diversity offering a source of novel/unique alleles useful for maize breeding programs to face the ongoing and future major challenge of climate changes (Aci *et al.* 2018). Based on the number and distribution of unique markers in the accessions, it is worth noting their high frequency in subsp. *everata*: six unique markers out of 19 (6/19) distributed within three of a total of seven accessions of subsp. *everata* (3/7 accessions). This is in comparison to (4/19) of subsp. *indentata* distributed in (3/8) accessions, (8/19) of subsp. *indurata* in (8/21) accessions, and one unique marker was also found in Zea 355 of subsp. *saccharata*. It is also noteworthy that five unique markers in the accessions of subsp. *indurata* are scored in var. *vulgata* from Europe and Libya and all six unique markers observed in accessions of subsp. *everata* belong to var. *oryzoides*. Unique SSR alleles were reported in different germplasm collections of maize; a nontemperate maize germplasm, landraces, open pollinated varieties, and inbred lines (Warburton *et al.* 2008). An

abundance of unique SSR alleles were also present for each SSR locus of 20 farmers' maizes collected from three selected states in Nigeria (Adeyemo and Omidiji 2019).

The clustering of the 40 maize accessions in both the average distance coefficient CAP tree (Fig. 2) and the Dice similarity index of the PAST tree (Fig. 3) is generally not in full agreement with their taxonomic assignment to subspecies and varieties or with their country of origin. This observation appears to agree with the low level of genetic differentiation between accessions as demonstrated by the STRUCTURE analysis (Fig. 4). However, careful inspection of clustered accessions shows clusters of accessions from the same subspecies in the main groups of the accessions as recognized in the cluster analyses of ISSR data indicating that the genetic diversity is distributed among the examined accessions in agreement with the value of 0.3076 for the genetic diversity ( $H_T$ ) in the 40 accessions. In the average distance CAP tree (Fig. 2), the 21 accessions of subsp. *indurata* (red) are recognized as five clusters; two clusters of 11 accessions in G1, two of six accessions in G2, and one of four accessions in G3. The eight accessions of subsp. *indentata* (blue) are recognized as three clusters; one of two accessions (Zea 668 and Zea 3065) in G2 and two clusters of three accessions each in G1 (Zea 3257, Zea 394, and Zea 1062) and in G3 (Zea 3324, Zea 3325, and Zea 3602). The seven accessions of subsp. *everata* are recognized as three clusters, one of Zea 1224 and Zea 3244 in G1 and the two clusters of Zea 323, Zea 711, and Zea 1019, as well as Zea 633 and Zea 3712 in G2. In the PAST tree (Fig. 3), similar clusters of accessions were observed particularly for subsp. *indentata* (blue) and subsp. *everata*. In both trees, two accessions, Zea 3400 and Zea 3582, of subsp. *semidentata* are always clustered together whereas Zea 355 of subsp. *saccharata* was clustered with Zea 12 of subsp. *indurata*. The PCA scatter diagram based on the ISSR data (Fig. 5) displayed the 40 accessions in three groups (G1, G2, and G3) in agreement with their clustering in the average distance CAP tree. However, accessions Zea 12, Zea 382, and Zea 677 of subsp. *indurata* and Zea 394 and Zea 1062 of subsp. *indentata* are grouped in the circle of G2, while Zea 1102 and Zea 1114 of subsp. *indurata* and Zea 3065 of subsp. *indentata* are circled in G1. The grouping of all these accessions is compatible with their clustering in the Dice similarity index PAST tree, except for Zea 12 (Fig. 3).

The use of a PCoA biplot and clustering methods for comparisons of drought tolerance in maize has also been found to be effective in screening for stress tolerance (Avramova *et al.* 2016, Hefny *et al.* 2017). In the current study, the display of accessions in the PCA agrees with their ranking based on the value of the grand DTI values. The PCoA biplot constructed by plotting PC1 and PC2, which account for the maximum variability of DTIs of the measured seedling traits showed high DTI of the root and shoot fresh and dry weight particularly for RDW1, ShDW1, RL1, ShL1, R12, as well as RFW2, RFW2, ShL2, and LL.

The most contributing drought-tolerance DTIs to the affinity of accessions in the PCA biplot are those of the shoot and root traits. The accessions with high DTIs of the examined accessions generally agree with those identified by Badr *et al.* (2020) and the drought tolerance of Zea 1006 from Libya was clearly indicated by the measurements of chlorophyll fluorescence and leaf relative water content compared to other ten maize genotypes from Egypt, Europe, Russia, and the USA (Badr and Brüggemann 2020). Screening for candidate drought-tolerant genotypes using seedling traits under controlled conditions and drought stress also identified maize inbred lines and hybrids (Avramova *et al.* 2016, Meeks *et al.* 2013).

Ten accessions, including six accessions of subsp. *indurata* and four accessions of subsp. *everata*, revealed high DTIs. Nine accessions share both a genetic affinity and higher drought tolerance compared to the other accessions; these include four of subsp. *indurata*, (Zea 242, Zea 382, Zea 1006, and Zea 1015), three of subsp. *everata* (Zea 323, Zea 711, and Zea 1019), and two of subsp. *indentata* (Zea 394 and Zea 668). Another three accessions of subsp. *indurata* and three of subsp. *everata* showed high drought tolerance. On the other hand, three accessions of subsp. *indurata*, four accessions of subsp. *indentata*, one accession of subsp. *everata*, and Zea 3582 of subsp. *semidentata* are clearly grouped together with Zea 355 of subsp. *saccharata* and Zea 3280 of subsp. *indurata* (Fig. 6). Almost all these accessions were also grouped together based the analysis of the ISSR data (Fig. 2). The drought tolerant accessions of subsp. *indurata* (Zea 242, Zea 382, Zea 677, Zea 1006, Zea 1015, and Zea 1102) were grouped with Zea 12, Zea 487, and Zea 630 of the same subspecies based on ISSR data analysis. In subsp. *indentata*, no accessions were among the top ten drought tolerant accessions and four accessions were among the ten accessions with a DTI less than 0.62; these are Zea 3065, Zea 3257, Zea 3324, and Zea 3602. Drought indices based on the response of seedling traits under stress conditions can predict the stability of yield under drought in different environments and genetic backgrounds in order to identify accessions with the potential for higher grain yield for genotype selection and for the breeding of commercial lines/cultivars (Abdel-Ghani *et al.* 2013, Grzesiak *et al.* 2012) and have been useful as a cost effective and quick method to screen for maize drought tolerance (Avramova *et al.* 2016, Badr *et al.* 2020, Meeks *et al.* 2013).

In conclusion, the clustering analysis of ISSR data using the average distance coefficient and Dice similarity index divided the accessions into three major groups showing the assignment of some accessions to their subspecies and varieties or geographic origin in spite of the low level of genetic differentiation of accessions as demonstrated by the STRUCTURE analysis in agreement with the low gene flow (Nm) value among accessions. In the PCA scatter diagram based on the ISSR data and DTIs, small sets of accessions of the same subspecies showed a close genetic

relationship to each other. Six accessions of subsp. *indurata* (Zea 242, Zea 382, Zea 677, Zea 1006, Zea 1015, and Zea 1102) were identified as drought stress tolerant. On the other hand, Zea 12, Zea 487, and Zea 630 of the same subspecies were grouped with the above accessions based on the ISSR data analysis. Six accessions of subsp. *everata* (Zea 323, Zea 633, Zea 711, Zea 1019, Zea 1224, and Zea 3712) were also identified as tolerant accessions, but only Zea 711 and Zea 1019 were grouped with Zea 323 of the same subspecies based on the ISSR data. Unique markers recorded in subsp. *indurata* are more common in var. *vulgata* from Europe and Libya, while all the unique markers observed in subsp. *everata* are of var. *oryzoides*. An abundance of unique alleles in the examined germplasm may be regarded as a reservoir of genetic resources that may be exploited for the future development of breeding lines/cultivars.

### Author Contribution Statement

Conceptualization, A. Badr and A. Börner; resources, A. Börner; methodology and investigation, H.H.E and E.R.S.; formal analysis, visualization, and validation, E.R.S, H.H.E., and A. Badr; writing original draft, H.H.E and E.R.S; reviewing and editing, A. Badr and A. Börner. Both A. Badr and A. Börner are corresponding authors at the request of their affiliations as a condition to support open access publication

All authors have read and agreed to the published version of the manuscript.

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