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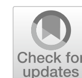

Molecular characterization of genetic diversity and similarity centers of safflower accessions with ISSR markers

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Abstract
Crop genetic resources are vital inputs in crop genetic improvement. In this study, genetic diversity, population structure, and similarity centers for 131 safflower accessions obtained from 28 countries were investigated using 12 ISSR markers. A sum of 201 ISSR bands were obtained among which 188 (93.844%) were found polymorphic. Mean polymorphism information content (0.448) and diversity parameters including mean effective number of alleles (1.655), mean Shannon's information index (0.557), mean expected heterozygosity (0.354), and mean overall gene diversity (0.377) showed a good level of genetic diversity in the studied safflower materials. Model-based structure, unweighted pair-group method with arithmetic means, and principal coordinate analysis clustered all accessions into three main populations; A, B, and C and an unclassified population. Accessions originated from Asian countries like Pakistan and Israel were found most diverse. Three accessions, Pakistan-11, Israel-1, and Pakistan-26, were found most genetically distant and might be used as parental sources for genetic combinations in safflower breeding activities. Analysis of molecular variance revealed highly significant differentiation among the identified populations and population × country combinations. The results presented in this work most probably supported the hypothesis of seven similarity centers of safflower but need to be validated with further confirmed investigations. The information provided herein is expected to be helpful for the scientific community interested in safflower breeding.

Keywords Genetic differentiation · Genetic resources · Molecular markers · Population structure · Safflower breeding

1 Introduction

The fast growth of the world's population requires sufficient availability of food in terms of calories and other nutrients (Long et al. 2015). Daily average of per capita available calories across the world was 2789 kcal in the year 2000, and it is believed that it will become 3130 kcal by year 2050 (FAO 2019), highlighting a steady increase in food demand paralleling the growth of world's population. A steady increase in the production of safflower has been observed during the last two decades to mainly meet the vegetable oil shortage. However, according to FAO's 2018–2027 predictions, the increase in oilseed production will amount 1.5% annually, implying a great drop relative to the last decade. Vegetable

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oil has one of the highest trade shares (41%) of production of all agricultural commodities. This share is expected to remain stable throughout the outlook period, with global vegetable oil exports reaching 96 Mt by 2027 (http://www.fao.org/docrep/i9166e/i9166e_Chapter4_Oilseeds.pdf). Scientists agree on the importance of increasing the crop yield instead of increasing area under the cultivation in the process of sustainable agriculture (Godfray et al. 2010). Therefore, in order to mitigate the vegetable oilseed shortage and avoid future demand problems, there is a need to focus on the breeding activities.

Safflower (*Carthamus tinctorius* L.) belongs to family Asteraceae and is self-pollinated having haploid genome size of about 1.4 GB with $2n=24$ chromosomes (Kumari et al. 2017). Safflower is a multipurpose crop cultivated on wide geographical zones all over the world for different purposes like dyes production, edible oil extraction, and several pharmaceutical utilizations (Weiss 2000; Ali et al. 2019a). Safflower oil production is lower compared to other oilseed crops, but still it is cultivated due to its potential of better adaptability to saline and drought conditions (Weiss 1983). Safflower has popularized due to its huge potential as biofuel crop in the recent years (Dordas and Sioulas 2009). Safflower has been used since pre-historic time, while archeological remains of *Carthamus* species were found 7500 years BC at sites of Syria (Marinova and Riehl 2009), indicating that safflower was distributed from these locations to connected geographies, i.e., Egypt, the Aegean, and southern Europe.

Safflower accessions revealing similarity based on various agro-morphological traits from different regions of the world are referred to as safflower similarity centers. Different researchers proposed various safflower similarity centers across the world, but still the number of actual similarity centers is ambiguous as ascertained by different molecular systems (Chapman et al. 2010). Seven safflower similarity centers were suggested by Knowles (1969) (1: Far East, 2: India-Pakistan, 3: Middle East, 4: Egypt, 5: Sudan, 6: Ethiopia, and 7: Europe). Similarly, ten similarity centers (1: Near East, 2: Iran/Afghanistan, 3: Turkey, 4: Egypt, 5: Ethiopia, 6: Sudan, 7: Far East, 8: India/Pakistan, 9: Europe, and 10: Kenya) were proposed by Ashri (1975); however, five similarity centers (1: Near East, 2: Iran and Afghanistan, Turkey, 3: Egypt, Ethiopia, (Sudan), 4: Far East, India/Pakistan, (Sudan), 5: Europe) were identified by Chapman et al. (2010). Still, this debate is ongoing and scientist did not come to single hypothesis about the similarity centers of safflower.

Safflower is known as an underutilized oilseed crop compared to other crops like soybean, rapeseed, and sunflower due to low oil content and seed yield as well as susceptibility to different diseases and insect pest attack limiting its quality and productivity (Zeinali 1999; Ali et al. 2019a). Current safflower local and traditional varieties comprise narrow genetic base; thus, assessment of the genetic diversity present in the safflower germplasm from different geographical zones will aid to provide valuable information necessary for the conservation and future utilization of safflower breeding programs (Yang et al. 2007). Safflower genetic diversity went to bottleneck during the process of domestication, which resulted in the significant decrease in its capability against the threatening environments, especially the environmental stresses (Yang et al. 2007; Mayerhofer et al. 2011). Genetic diversity serves as an effective tool and provides a rich source of variations (Baloch et al. 2017; Nadeem et al. 2018b). Diversity analyses in the germplasm possess great importance to plan an efficient and successful safflower breeding program (Mary and Gopalan 2006; Guliyev et al. 2018; Yildiz et al. 2019). Crop germplasm that consists of diverse desirable traits may be integrated in a better way during breeding programs in order to develop superior cultivars (Arystanbekkyzy et al. 2018; Yaldiz et al. 2018). Assessment of the crop genetic diversity is important for the germplasm conservation and also food security. Decreasing genetic diversity in the crop plants is one of the most important environmental concerns as outlined by the Food and Agriculture Organization (Castañeda-Álvarez et al. 2016). Limited information about genetic diversity within *C. tinctorius* and lack of efficient genomic tools has hampered the pace to improve economical traits during various safflower breeding programs. Need of the safflower genome molecular characterization and development of the efficient molecular markers has been recognized by a number of research groups (Amiri et al. 2001; Johnson et al. 2007).

Molecular markers overcome limitations present in the morphological and biochemical markers by detecting diversity at the DNA level (Erzurumlu et al. 2018; Nadeem et al. 2018a). It should be understood that different molecular markers have different characteristics, thus reflecting the different genetic diversity aspects (Talebi et al. 2012; Nadeem et al. 2018a). Safflower genetic diversity using several molecular markers like random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), iPBS-retrotransposon, and single nucleotide polymorphism (SNP) has been estimated (Johnson et al. 2007; Yang et al. 2007; Amini et al. 2008; Khan et al. 2009; Chapman et al. 2010; Lee et al. 2014; Pearl and Burke 2014; Ambreen et al. 2015, 2018; Kumar et al. 2015; Ali et al. 2019b). These researchers suggested the presence of good level of genetic diversity among different global safflower germplasm and also validate some of the similarity centers that were initially based on various morpho-agronomic traits (Johnson et al. 2007; Chapman et al. 2010; Pearl and Burke 2014; Kumar et al. 2015). Inter-simple sequence repeat (ISSR) primers based on di, tetra, or penta-nucleotide

repeats are routinely used for various purposes (Zietkiewicz et al. 1994). The advantages of simple procedure, low cost, high reproducibility, and excellent stability made ISSR primers suitable for the determination of genetic diversity analysis (Rawat et al. 2016; Hadian et al. 2017; Ekincialp et al. 2019), mapping studies (Casaoli et al. 2001; Cekic et al. 2011; Tanyolac 2003), and germplasm identification (Nagaoka and Ogihara 1997; Potter et al. 2002). ISSR markers have been effectively employed for the determination of genetic diversity and molecular characterization of different crops including; cluster bean (Ansari et al. 2016), Chickpea (Gautam et al. 2016), and *Brassica* (Khalil and El zayat 2019). Besides these crops, ISSR markers were also used for the genetic diversity and molecular characterization of safflower by various researchers (Yang et al. 2007; Sabzalian et al. 2009; Golkar et al. 2011; Bagmohammadi et al. 2012; Majidi and Zadhoush 2014; Houmanat et al. 2016) revealing its efficient potential for the germplasm characterization. The current study was aimed to determine the genetic diversity, population structure, and safflower similarity centers at molecular level using ISSR markers, and the useful information will be then used for the future safflower breeding programs by the scientific community.

2 Materials and methods

Plant materials and DNA isolation – One hundred and thirty-one safflower accessions collected from twenty-eight different countries were tested during the current study. The safflower accessions 94, 17, and 20 were provided by the United States Department of Agriculture (USDA), Plant Genetic Resources Institute (PGRI) Pakistan, and the Turkish Central Research Institute for Field Crops, respectively (Table 1). The safflower accessions provided by USDA (94 accessions) and PGRI (17 accessions) were landraces. The 20 Turkish safflower accessions were single plant selection among the international safflower germplasm obtained from the USDA and are candidate cultivars. Safflower seeds of each accession were sown at the research and experimental area of Bolu Abant Izzet Baysal University. The fresh, healthy, and young leaves from each accession were harvested for the DNA isolation and frozen at the temperature of − 80 °C in laboratory. Bulk of leaves from each accession was used for the DNA extraction following the CTAB protocol (Doyle and Doyle 1990) with slight modifications (Baloch et al. 2016). DNA concentration was estimated using agarose gel (0.8%) and was then confirmed with NanoDrop (DeNovix DS-11 FX, USA). Final DNA concentration of the 131 safflower accessions was adjusted to 5 ng μL^{-1} for the purpose of polymerase chain reactions. All samples were stored at the temperature of − 25 °C until PCR amplification.

ISSR PCR amplifications – Ninety ISSR primers were initially screened using eight randomly selected accessions of safflower for PCR amplifications. Twelve ISSR primers were found most polymorphic by producing strong bands and were used for the amplification of PCR (Table 2). A total reaction volume of 25 µL for PCR amplifications were comprised of 4 ng ul^{-1} template DNA, 4 µL dNTPs (Thermo Scientific), 0.2 µL U Taq DNA polymerase (Thermo Scientific), 1 µL primer, 2.5 µL 1_X PCR buffer (Thermo Scientific), 2 µL MgCl2, and 11.3 µL distilled water. Reactions were performed in the sequence of denaturation at 94 °C for 3 min, subsequently followed by 30 denaturation cycles at 94 °C for 1 min, annealing temperature of 48–54 °C for one minute depending upon the primer, and a final extension for 10 min at 72 °C. Agarose gel 1.8% (w/v) containing 0.5_X TBE buffer was used for the electrophoreses of the amplified DNA fragments at a constant voltage of 120 V for 240 min. Ethidium bromide was used to perform the staining of the gel and then visualized using UV Imager Gel Doc XR + system (Bio-Rad, USA) light and photographed. A 100 bp + DNA ladder was used as a molecular weight marker.

Data analysis – Strong, unambiguous, and clear bands were used for the purpose of scoring, while vague bands were not selected as they could not be easily detected. ISSR markers are dominant markers and scored in a binary matrix as 1 for present or 0 for absent, respectively, of all the bands with relative to 100 bp + DNA ladder. PopGene ver. 1.32 (Yeh et al. 2000) was applied to compute genetic diversity indices like effective alleles number (Ne), Shannon's information index (I), and gene diversity (He) for individual ISSR markers (Table 3). Baloch et al. (2015) criteria were used to determine the polymorphism information content (PIC) for each ISSR marker. Pairwise genetic distance (GDj) was determined using R statistical software as measured by Jaccard's coefficient (Jaccard 1908). Analysis of molecular variance (AMOVA) was investigated using R statistical software considering variation among structure populations and structure populations within country (Table 4). The population structure was assessed using the Bayesian clustering model-based STRUCTURE software. The UPGMA and principle coordinate analysis (PCoA) were performed using R software to explore the level of diversity among 131 safflower accessions (Team 2013). Evanno et al. (2005) protocol was used through STRUCTURE software to determine the most suitable number of clusters (K subpopulations). We plotted the clusters number (K) against logarithm probability relative to standard deviation (ΔK). Assignment of the individual safflower accessions to the separate population was based on the membership coefficient magnitude being greater than or equal to 50% as outlined by Habyarimana (2016).

Table 1 List of 131 safflower accessions evaluated during current study for genetic diversity analysis at molecular level using 12 ISSR markers

S. No	Genotype name	Accession No.	Donor organization	Location	Province/district	Country origin	Plant ID	Continent
G1	Israel-1	30548	USDA	–	–	Israel	P1-198990	Asia
G2	Romania-1	30549	USDA	–	–	Romania	P1-209287	Europe
G3	Morocco-1	30552	USDA	–	–	Morocco	P1-239042	Africa
G4	Egypt-1	30563	USDA	–	–	Egypt	P1-250082	Africa
G5	Pakistan-1	30564	USDA	–	–	Pakistan	P1-250194	Asia
G6	Pakistan-2	30565	USDA	–	–	Pakistan	P1-250201	Asia
G7	Pakistan-3	30567	USDA	–	–	Pakistan	P1-250345	Asia
G8	Pakistan-4	30568	USDA	–	–	Pakistan	P1-250346	Asia
G9	Pakistan-5	30569	USDA	–	–	Pakistan	P1-250351	Asia
G10	Pakistan-6	30570	USDA	–	–	Pakistan	P1-250353	Asia
G11	Pakistan-7	30573	USDA	–	–	Pakistan	P1-250481	Asia
G12	Egypt-2	30574	USDA	–	–	Egypt	P1-250528	Africa
G13	Egypt-3	30577	USDA	–	–	Egypt	P1-250532	Africa
G14	Egypt-4	30578	USDA	–	–	Egypt	P1-250540	Africa
G15	India-1	30579	USDA	–	–	India	P1-250601	Asia
G16	Egypt-4	30580	USDA	–	–	Egypt	P1-250605	Africa
G17	Egypt-6	30581	USDA	–	–	Egypt	P1-250608	Africa
G18	Iran-1	30588	USDA	–	–	Iran	P1-250720	Asia
G19	Jordan-1	30589	USDA	–	–	Jordan	P1-251284	Asia
G20	Jordan-2	30590	USDA	–	–	Jordan	P1-251285	Asia
G21	Israel-2	30594	USDA	–	–	Israel	P1-253386	Asia
G22	Spain-1	30595	USDA	–	–	Spain	P1-253388	Europe
G23	Spain-2	30596	USDA	–	–	Spain	P1-253391	Europe
G24	Spain-3	30597	USDA	–	–	Spain	P1-253394	Europe
G25	Spain-4	30598	USDA	–	–	Spain	P1-253395	Europe
G26	Portugal-1	30604	USDA	–	–	Portugal	P1-253553	Europe
G27	Portugal-2	30605	USDA	–	–	Portugal	P1-253556	Europe
G28	Morocco-2	30606	USDA	–	–	Morocco	P1-253560	Africa
G29	Portugal-3	30608	USDA	–	–	Portugal	P1-253564	Europe
G30	Portugal-4	30610	USDA	–	–	Portugal	P1-253569	Europe
G31	Portugal-5	30611	USDA	–	–	Portugal	P1-253571	Europe
G32	Iraq-1	30612	USDA	–	–	Iraq	P1-253761	Asia
G33	Iraq-2	30613	USDA	–	–	Iraq	P1-253762	Asia
G34	Afghanistan-1	30614	USDA	–	–	Afghanistan	P1-253764	Asia
G35	Israel-3	3015	USDA	–	–	Israel	P1-253892	Asia
G36	Syria-1	30616	USDA	–	–	Syria	P1-253898	Asia
G37	Syria-2	30617	USDA	–	–	Syria	P1-253900	Asia
G38	Portugal-6	30620	USDA	–	–	Portugal	P1-258412	Europe
G39	Uzbekistan-1	30623	USDA	–	–	Uzbekistan	P1-262435	Asia
G40	China-1	30624	USDA	–	–	China	P1-262452	Asia
G41	China-2	30625	USDA	–	–	China	P1-262453	Asia
G42	Iran-2	30631	USDA	–	–	Iran	P1-304444	Asia
G43	Iran-3	30633	USDA	–	–	Iran	P1-304448	Asia
G44	Turkey-1	30646	USDA	–	–	Turkey	P1-304498	Asia
G45	Turkey-2	30648	USDA	–	–	Turkey	P1-304502	Asia
G46	Turkey-3	30650	USDA	–	–	Turkey	P1-304504	Asia
G47	Turkey-4	30651	USDA	–	–	Turkey	P1-304505	Asia
G48	Afghanistan-2	30653	USDA	–	–	Afghanistan	P1-304592	Asia
G49	India-2	30662	USDA	–	–	India	P1-305195	Asia
G50	Russia-1	30663	USDA	–	–	Russia	P1-305535	Asia

Table 1 (continued)

S. No	Genotype name	Accession No.	Donor organization	Location	Province/district	Country origin	Plant ID	Continent
G51	India-3	30673	USDA	–	–	India	P1-306926	Asia
G52	India-4	30674	USDA	–	–	India	P1-306941	Asia
G53	India-5	30677	USDA	–	–	India	P1-306976	Asia
G54	Kazakhstan-1	30681	USDA	–	–	Kazakhstan	P1-314650	Asia
G55	Turkey-5	30688	USDA	–	–	Turkey	P1-340086	Asia
G56	Argentina-1	30695	USDA	–	–	Argentina	P1-367833	America
G57	Uzbekistan-2	30696	USDA	–	–	Uzbekistan	P1-369846	Asia
G58	Uzbekistan-3	30697	USDA	–	–	Uzbekistan	P1-369853	Asia
G59	Syria-3	30700	USDA	–	–	Syria	P1-386174	Asia
G60	Thailand-1	30701	USDA	–	–	Thailand	P1-387821	Asia
G61	Iran-4	30713	USDA	–	–	Iran	P1-405958	Asia
G62	Iran-5	30718	USDA	–	–	Iran	P1-405967	Asia
G63	Bangladesh-1	31509	USDA	–	–	Bangladesh	PI-401472	Asia
G64	Bangladesh-2	31510	USDA	–	–	Bangladesh	PI-401478	Asia
G65	Bangladesh-3	31511	USDA	–	–	Bangladesh	PI-401480	Asia
G66	India-6	33538	USDA	–	–	India	PI 199878	Asia
G67	Afghanistan-3	33541	USDA	–	–	Afghanistan	PI 220647	Asia
G68	Australia-1	33542	USDA	–	–	Australia	PI 235660	Oceania
G69	Turkey-6	33543	USDA	–	–	Turkey	PI 237538	Asia
G70	Pakistan-8	33547	USDA	–	–	Pakistan	PI 250474	Asia
G71	Pakistan-9	33548	USDA	–	–	Pakistan	PI 250478	Asia
G72	Iran-6	33556	USDA	–	–	Iran	PI 250840	Asia
G73	Jordan-3	33559	USDA	–	–	Jordan	PI 251265	Asia
G74	Jordan-4	33560	USDA	–	–	Jordan	PI 251267	Asia
G75	Jordan-5	33561	USDA	–	–	Jordan	PI 251268	Asia
G76	Israel-4	33564	USDA	–	–	Israel	PI 251290	Asia
G77	Turkey-7	33565	USDA	–	–	Turkey	PI 251978	Asia
G78	Turkey-8	33567	USDA	–	–	Turkey	PI 251984	Asia
G79	Austria-1	33568	USDA	–	–	Austria	PI 253519	Europe
G80	Hungary-1	33575	USDA	–	–	Hungary	PI 288983	Europe
G81	Libya-1	33608	USDA	–	–	Libya	PI 393499	Africa
G82	Bangladesh-4	33609	USDA	–	–	Bangladesh	PI 401470	Asia
G83	Iran-7	33621	USDA	–	–	Iran	PI 406010	Asia
G84	Turkey-9	33627	USDA	–	–	Turkey	PI 406701	Asia
G85	Turkey-10	33628	USDA	–	–	Turkey	PI 406702	Asia
G86	Pakistan-10	33635	USDA	–	–	Pakistan	PI 426521	Asia
G87	China-3	33638	USDA	–	–	China	PI 543979	Asia
G88	China-4	33639	USDA	–	–	China	PI 543982	Asia
G89	China-5	33642	USDA	–	–	China	PI 544001	Asia
G90	China-6	33651	USDA	–	–	China	PI 568809	Asia
G91	China-7	33661	USDA	–	–	China	PI 568874	Asia
G92	France-1	33662	USDA	–	–	France	PI 576985	Europe
G93	Austria-2	33670	USDA	–	–	Austria	BVAL-901352	Europe
G94	Pakistan-11	Check	PGRI-Pakistan	–	–	Pakistan	Thori-78	Asia
G95	Pakistan-12	16266	PGRI-Pakistan	Jacobabad	Sindh	Pakistan	–	Asia
G96	Pakistan-13	16267	PGRI-Pakistan	Shikarpur	Sindh	Pakistan	–	Asia
G97	Pakistan-14	16268	PGRI-Pakistan	Shikarpur	Sindh	Pakistan	–	Asia
G98	Pakistan-15	16269	PGRI-Pakistan	Larkana	Sindh	Pakistan	–	Asia
G99	Pakistan-16	16270	PGRI-Pakistan	Larkana	Sindh	Pakistan	–	Asia
G100	Pakistan-17	16355	PGRI-Pakistan	Dadu	Sindh	Pakistan	–	Asia

Table 1 (continued)

S. No	Genotype name	Accession No.	Donor organization	Location	Province/district	Country origin	Plant ID	Continent
G101	Pakistan-18	16356	PGRI-Pakistan	Dadu	Sindh	Pakistan	–	Asia
G102	Pakistan-19	16357	PGRI-Pakistan	Karachi	Sindh	Pakistan	–	Asia
G103	Pakistan-20	16358	PGRI-Pakistan	Karachi	Sindh	Pakistan	–	Asia
G104	Pakistan-21	16359	PGRI-Pakistan	Gilgit	GB	Pakistan	–	Asia
G105	Pakistan-22	19233	PGRI-Pakistan	Gilgit	GB	Pakistan	–	Asia
G106	Pakistan-23	20920	PGRI-Pakistan	Islamabad	Federal Areas	Pakistan	–	Asia
G107	Pakistan-24	21933	PGRI-Pakistan	Karachi	Sindh	Pakistan	–	Asia
G108	Pakistan-25	24779	PGRI-Pakistan	Quetta	Balochistan	Pakistan	–	Asia
G109	Pakistan-26	27549	PGRI-Pakistan	Hyderabad	Sindh	Pakistan	–	Asia
G110	Pakistan-27	30698	PGRI-Pakistan	Hyderabad	Sindh	Pakistan	–	Asia
G111	Pakistan-28	35803	PGRI-Pakistan	Gakooch	Gilgit/Baltistan	Pakistan	–	Asia
G112	Afghanistan-4	7-T	CRIFC-Turkey	–	–	Afghanistan	–	Asia
G113	Afghanistan-5	9-T	CRIFC-Turkey	–	–	Afghanistan	–	Asia
G114	China-8	27-T	CRIFC-Turkey	–	–	China	–	Asia
G115	China-9	29-T	CRIFC-Turkey	–	–	China	–	Asia
G116	Turkey-11	36-T	CRIFC-Turkey	–	Tarme	Turkey	–	Asia
G117	Turkey-12	37-T	CRIFC-Turkey	–	Tarme	Turkey	–	Asia
G118	Turkey-13	57-T	CRIFC-Turkey	–	Elbistan	Turkey	–	Asia
G119	Turkey-14	58-T	CRIFC-Turkey	–	Elbistan	Turkey	–	Asia
G120	Canada-1	74-T	CRIFC-Turkey	–	–	Canada	–	America
G121	Canada-2	75-T	CRIFC-Turkey	–	–	Canada	–	America
G122	USA-1	80-T	CRIFC-Turkey	–	Montana	USA	–	America
G123	Iran-8	116-T	CRIFC-Turkey	–	–	Iran	–	Asia
G124	USA-2	130-T	CRIFC-Turkey	–	–	USA	–	America
G125	USA-3	132-T	CRIFC-Turkey	–	–	USA	–	America
G126	Turkey-15	134-T	CRIFC-Turkey	–	Tarme	Turkey	–	Asia
G127	USA-4	149-T	CRIFC-Turkey	–	İdoha	USA	–	America
G128	Iran-9	152-T	CRIFC-Turkey	–	–	Iran	–	Asia
G129	USA-5	153-T	CRIFC-Turkey	–	İdoha	USA	–	America
G130	Iran-10	177-T	CRIFC-Turkey	–	–	Iran	–	Asia
G131	Turkey-16	277-T	CRIFC-Turkey	–	Tarme	Turkey	–	Asia

USDA United States Department of Agriculture, *PGRI* Plant Genetic Resources Institute, *CRIFC* Central Research Institute for Field Crop, –, Not known

3 Results

ISSR marker analysis and genetic diversity – Twelve most polymorphic ISSR primers produced a total of 201 scorable bands having average of 16.75 bands per primer using 131 safflower accessions. Among 201 ISSR bands, 188 (93.844%) were identified polymorphic having average of 15.67 bands per primer (Table 3). Primer ISSR809 displayed the highest number of total (22) and polymorphic (21) bands, while a lowest number of total (11) and polymorphic (10) bands were found with primer ISSR868. Diversity parameters like mean polymorphism information content, mean effective number of alleles, mean Nei's gene diversity, mean Shannon's information index, and mean expected heterozygosity were, respectively, 0.448, 1.655, 0.377, 0.557, and 0.354 among the 12 ISSR primers using 131 safflower accessions (Table 3). The primer ISSR868 was the most informative by revealing a good amount of polymorphism information content (0.592), effective number of alleles (1.849), Nei's gene diversity (0.454), Shannon's information index (0.645), and expected heterozygosity (0.441), while the primer ISSR810 was least informative by exhibiting low values of polymorphism information content (0.274), effective number of alleles (1.458), Nei's gene diversity (0.282), Shannon's information index (0.436), and expected heterozygosity (0.253).

Table 2 Sequence and annealing temperature of 12 ISSR primers used to determine genetic diversity among 131 safflower accessions

Primer name	Sequence	Annealing temperature (°C)
ISSR809	AGAGAGAGAGAGAGAGG	52
ISSR810	GAGAGAGAGAGAGAGAT	52
ISSR811	GAGAGAGAGAGAGAGAC	53
ISSR812	GAGAGAGAGAGAGAGAA	52
ISSR817	CACACACACACACACAA	51
ISSR818	CACACACACACACACAG	53
ISSR819	GTGTGTGTGTGTGTGTA	53
ISSR827	ACACACACACACACACG	52
ISSR830	TGTGTGTGTGTGTGTGG	53
ISSR834	AGAGAGAGAGAGAGAGYT	52
ISSR840	GAGAGAGAGAGAGAGAYT	52
ISSR868	GAAGAAGAAGAAGAAGAA	53
ISSR809	AGAGAGAGAGAGAGAGG	52

Pairwise genetic distance with the Jaccard coefficient was computed among the 131 safflower accessions in order to understand the picture of genetic diversity more clearly. The mean genetic distance among 131 accessions was found 0.336. Accessions Pakistan-11 and Israel-1 revealed highest genetic distance (0.816), while accessions USA-5 and Iran-10 showed lowest genetic distance (0.063). Analysis of molecular variance (AMOVA) resulted in highly significant effects of model-based structure ($P = 0.001$) and model-based structure × country combination ($P = 0.003$) on genetic differentiation (Table 4).

In accordance with the observed most suitable goodness of fit ($K = 3$), the Bayesian clustering model implemented in STRUCTURE software divided the evaluated safflower accessions into three main populations; 47 accessions (35.88%) in the population A (green), 19 accessions (14.50%) in the population B (red), 64 accessions (48.86%) in the population C (blue), and 1 accession (0.76%) in an unclassified population (Fig. 1). The UPGMA clustering divided 131 safflower accessions into three main populations and an unclassified population corresponding to the populations identified using the model-based structure (Fig. 2). PCoA divided all accessions into three populations; A, B, and C and an unclassified population which were similar to structure based clustering (Fig. 3).

Table 3 Diversity parameters computed to evaluate genetic diversity among 131 safflower accessions using 12 ISSR primers

Primer	Total bands	Polymorphic bands	Polymorphism (%)	PIC	ne[a]	h[b]	I[c]	Ht
ISSR809	22	21	95.455	0.426	1.633	0.371	0.563	0.340
ISSR810	20	16	80.000	0.274	1.458	0.282	0.436	0.253
ISSR811	15	13	86.667	0.334	1.555	0.338	0.513	0.328
ISSR812	19	18	94.737	0.454	1.651	0.388	0.574	0.386
ISSR817	17	17	100.000	0.505	1.780	0.420	0.605	0.398
ISSR818	12	12	100.000	0.445	1.565	0.341	0.516	0.291
ISSR819	18	16	88.889	0.360	1.626	0.358	0.531	0.338
ISSR827	19	19	100.000	0.489	1.696	0.396	0.580	0.374
ISSR830	14	14	100.000	0.334	1.563	0.341	0.514	0.327
ISSR834	19	17	89.474	0.585	1.716	0.408	0.596	0.370
ISSR840	15	15	100.000	0.577	1.773	0.428	0.617	0.403
ISSR868	11	10	90.909	0.592	1.849	0.454	0.645	0.441
Mean	201	188	93.844	0.448	1.655	0.377	0.557	0.354

[a]ne = effective number of alleles; [b]h = Nei's (1973) gene diversity; [c]I = Shannon's information index; Ht = expected heterozygosity

Table 4 Analysis of molecular variance (AMOVA) revealing genetic diversity among structure populations and STRU CTURE populations within country

Source of variation	*df*	SS	MS	F Model	RSq	P value
Clusters (populations)	3	436.8	145.592	6.4063	0.12616	0.001***
Cluster: country	44	1139.1	25.889	1.1392	0.32902	0.003**
Residuals	83	1886.3	22.726	NA	0.54482	NA
Total	130	3462.2	NA	NA	1	NA

**Significance at the 0.1% nominal level and ***corresponds to significance at the 0.05% nominal level

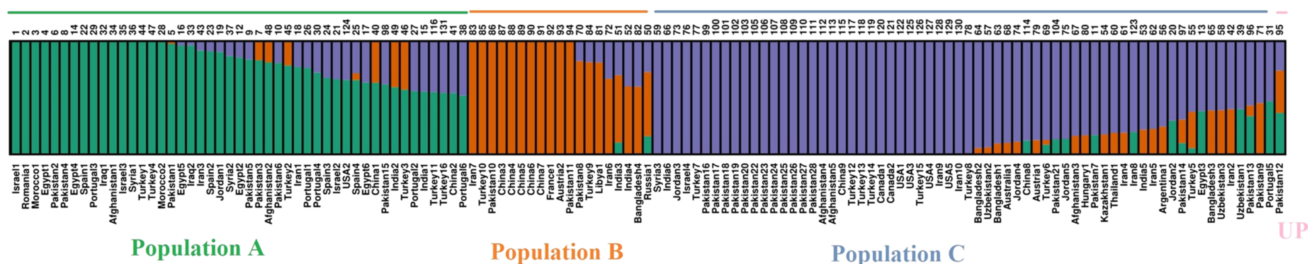

Fig. 1 Structure-based clustering among 131 safflower accessions using 12 ISSR primers

Pop A
Pop B
Pop C
Unclassified

Fig. 2 UPGMA-based clustering among 131 safflower accessions using 12 ISSR primers

4 Discussion

The knowledge on the partition of the genetic variation that existed in crop gene pools is helpful to describe the evolution of crop lineages and also disclose the unexplored sources of variation that enhance future crop improvement efforts (Tanksley and McCouch 1997; Yamasaki et al. 2005). Until now, population genetic analysis regarding safflower gene pool has not been fully exploited, and also the hypothesis

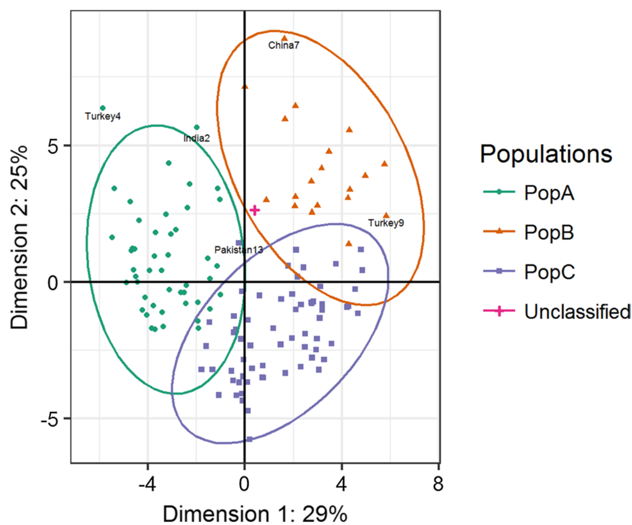

Fig. 3 Principal coordinate analysis (PCoA) among 131 safflower accessions using 12 ISSR primers

of Knowles (1969) and Ashri (1975) about the safflower similarity centers is still unclear at the genetic level. Our data presented herein strongly supported the Knowles (1969) hypothesis proposing seven similarity centers. Very few attempts have been done to investigate the total spectrum of variation in global safflower germplasm at the DNA level. Genetic diversity characterization within safflower gene pools is vital for its development and improvement. Our results about mean polymorphism (93.844%) was higher to that of Houmanat et al. (2016), as they found mean polymorphism of 63.38% using ISSR markers evaluating a safflower set of 55 accessions. Similarly, Golkar et al. (2011) reported lower polymorphism (70%) than ours using ISSR markers. Polymorphism information content (PIC) is a widely used metric of the usefulness of molecular markers (Anderson et al. 1993). Higher PIC (0.448) was obtained in the current study in comparison to Talebi and Abhari (2016). They evaluated 25 safflower accessions using 13 ISSR markers. Moreover, Houmanat et al. (2016) revealed lower PIC value (0.23) than us using ISSR markers in safflower. The presence of higher number of effective alleles revealed the availability of maximum level of genetic diversity and is always desirable. We obtained higher effective number of alleles (1.458–1.849) than that of Sung et al. (2010) (1.02–1.09). Obtaining superiority of various diversity parameters in this study than the previous results might be due to the difference of the experimental materials used in the current assessment and also the difference of the ISSR markers used. Shannon's information index usually distinguishes the level of available genetic diversity in a population, combining abundance and evenness. Kumar et al. (2015) observed lower Shannon's information index (0.24–0.44) contrary to our observation (0.436–0.645) using AFLP markers. It is a clear indication of the presence of higher level of genetic diversity in the studied safflower accessions with genetic variants evenly distributed throughout the population. Wodajo et al. (2015) reported lower mean Shannon's information index (0.46) than us (0.557) using ISSR markers. Our results about expected heterozygosity (0.354) are supported by Lee et al. (2014) as they revealed similar expected heterozygosity (0.386). Wodajo et al. (2015) studied 70 safflower accessions using ISSR markers and found Nei's gene diversity of 0.30, which is lesser than the value (0.377) obtained in this study. Diversity parameters revealed the presence of higher genetic variability in the studied materials suggesting the studied safflower accessions can provide useful building blocks for future breeding programs to enhance safflower productivity. Also, the ISSR markers used in this evaluation should be used for the genetic diversity investigation as these markers exhibited higher diversity levels.

The evaluation of pairwise genetic distance showed a mean of 0.336, with the highest genetic distance between accessions Pakistan-11 and Israel-1, followed by Pakistan-26 and Israel-1 with respective distance values of 0.816 and 0.808. Greater similarity was found between USA-5 and Iran-10 accessions showing least genetic distance of 0.063. One understandable reason behind the presence of maximum genetic similarity might be due to their origin from the common parents. The three most diverse safflower accessions (Pakistan-11, Israel-1, and Pakistan-26) identified during the current study can be recommended as a candidate parents for future breeding programs. The analysis of molecular variance (AMOVA) was used to determine the pattern of the partition of the total gene diversity among and within populations and to assess genetic differentiation. AMOVA showed that most of genetic structure was explained by variations among populations and the genetic populations within countries (Table 4).

The model-based structure application proved more robust and informative in previous investigations (Bouchet et al. 2012; Nadeem et al. 2018b; Ali et al. 2019b). Structure was therefore used in this work as a benchmark for clustering algorithms. The studied 131 safflower accessions were clearly separated into three main populations: A, B, and C and an unclassified population using structure (Fig. 1). Population A consists of 47 accessions originated from Israel (3 accessions), Romania (1 accession), Morocco (2 accessions), Egypt (5 accessions), Pakistan (7 accessions), Spain (4 accessions), Portugal (5 accessions), Iraq (2 accessions), Syria (2 accession), Turkey (6 accessions), Iran (2 accessions), Jordan (1 accession), Afghanistan (2 accession), USA (1 accession), China (2 accessions), and India (2 accessions). Population B comprised 19 safflower accessions including; Iran (2 accessions), Turkey (2 accessions), Pakistan (3 accessions), China (5 accessions), France (1 accession), Austria (1 accession), Libya (1 accession), India (2 accessions),

Bangladesh (1 accession), and Russia (1 accession). Clustering of safflower accessions from Mediterranean region with Europe and Asian countries identifies its origin from Mediterranean region and distribution to other parts of the world. The 64 safflower accessions clustered in population C were originated from Syria (1 accession), India (2 accessions), Jordan (4 accessions), Israel (1 accession), Turkey (8 accessions), Afghanistan (3 accessions), China (2 accessions), Canada (2 accession), USA (4 accessions), Iran (6 accession), Bangladesh (3 accessions), Uzbekistan (3 accessions), Australia (1 accession), Austria (1 accession), Pakistan (17 accessions), Hungary (1 accession), Kazakhstan (1 accession), Thailand (1 accession), Argentina (1 accession), Egypt (1 accession), and Portugal (1 accession). Clustering pattern of accessions and their distribution in population C was found similar to populations A and B. Distribution of safflower accessions from Mediterranean region to Asia took place through Turkey, being used as a bridge. According to Nadeem et al. (2018b), Turkey acts as bridge for the diffusion of various crops among the continents. One safflower accession originated from Pakistan (Pakistan-12) made up the unclassified population as its membership coefficient magnitude was less than 50% as proposed by Habyarimana (2016).

Population A included accessions from Asia (29 accessions), Europe (10 accessions), Africa (7 accessions), and American (1 accession) continents. Population B exhibited accessions from Asia (16 accessions), Europe (2 accessions), and Africa (1 accession). Population C revealed accessions from Asia (52 accessions), America (7 accessions), Oceania (1 accession), Europe (3 accessions), and Africa (1 accession). The unclassified population exhibited only one accession that is originated from Asian (Pakistan) continent. Besides sharing common parentage, accessions similarity in the same population during the clustering might also be due to convergent evolution and selection (Golkar et al. 2011). Population C stood the most diverse population as it comprised accessions from all the available continents.

Knowles (1969) suggested the presence of seven similarity centers for safflower throughout the world using various morpho-agronomic traits. Most of the accessions evaluated in this study follow the hypothesis of seven similarity centers at molecular level. But the data obtained from the ISSR markers in this study did not fully support the Knowles's hypothesis of similarity centers. Safflower accessions from different similarity centers clustered together and highlighted the lack of importance of similarity centers at molecular level which was previously reported in the scientific literature (Chapman and Burke 2007). Safflower accessions from Israel, Iraq, Syria, Turkey, Iran, and Jordan were present in population A and can be assigned to the Middle East similarity center. Similarly, accessions from India and Pakistan were also present in population A comprising the India-Pakistan similarity center. Accessions from Pakistan, India, and Bangladesh were clustered in population B and made the India–Pakistan similarity center. Population C revealed the Middle East similarity center as it exhibited safflower accessions from Syria, Jordan, Israel, Turkey, Afghanistan, and Iran. Population C also exhibited safflower accessions from the India-Pakistan (India, Bangladesh, and Pakistan) and Europe (Australia, Austria, Hungary, Argentina, and Portugal) similarity centers. Very recently Ali et al. (2019b) aimed to evaluate the similar centers pattern at molecular level using 13 iPBS-retrotransposon markers and supported the Knowles (1969) hypothesis proposing seven similar centers. Our results are supported by their findings revealing similar safflower similarity centers patterns. Besides obtaining supportive results to the Knowles's hypothesis of seven similarity centers, still there is a need to conduct more research at the molecular level by collecting and testing safflower accessions from the all proposed similarity centers.

The exploration of genetic relationships between the studied 131 safflower accessions using UPGMA resulted in a comparable clustering pattern to that of model-based algorithm with few exceptions as three accessions belonging to population B (Russia-1, India-3, and India-4) clustered with population C (Fig. 2). Seven accessions belonging to population C (Jordan-5, Portugal-5, Egypt-3, Pakistan-7, Iran-2, Uzbekistan-1, and Jordan-2) clustered with population A. Similarly, two accessions from population A (Turkey-3 and Afghanistan-2) clustered with population C. Unclassified safflower accession (Pakistan-12) clustered with population A. Accessions present in the same population revealed full membership coefficients in model-based Structure. The discrepancies displayed in UPGMA clustering might be described by its reduced resolution power relative to the model-based Structure (Bouchet et al. 2012).

Principal coordinate analysis (PCoA) confirmed the clustering based on structure algorithm of 131 safflower accessions into clearly distinguishable three main populations and an unclassified population using 12 ISSR primers (Fig. 3). The occurrence of some light differences between model-based structure and PCoA can derive from its differing clustering resolution, with more resolution revealed by the model-based structure analysis. Existence of the genomic admixture might be the reason for the misclassification in the principal coordinate space of the 131 safflower accessions. Also, same pattern of the similarity centers as obtained through structure based analysis was exhibited by PCoA analysis.

Overall, a reasonable level of genetic diversity was revealed by ISSR markers and the obtained diversity can be used for the improvement of safflower in future breeding programs. As ISSR marker system revealed competitive results in the current and previous studies, it is highly warranted

that ISSR markers should be used as an important tool for the evaluation of the safflower germplasm at DNA level for productivity enhancement. To confirm the uncertainty about the safflower similarity centers, there is a need to include various robust sampling techniques like random sampling without replacement to be implemented in the accessions stored in the various world safflower seed repositories. It will be interesting to characterize those accessions through the various clustering algorithms such as those implemented in this work.

The presence of good level of genome diversity was observed among the studied materials. Clustering algorithms like model-based structure, UPGMA, and PCoA clustered safflower accessions according to collection countries and similarity centers. The current findings greatly support the Knowles hypothesis of seven similarity centers for safflower at DNA level with ISSR markers, but still need to be confirmed by conducting further research work. Analysis of molecular variance (AMOVA) exhibited highly significant differentiation among the identified populations, and population × country combinations. Safflower accessions from Asian continent revealed higher genetic diversity in comparison to other continents. On individual bases, safflower accessions; Pakistan-11, Israel-1, and Pakistan-26 were found most diverse at DNA level and might be suggested as parental germplasm resources for future breeding programs.

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Authors' contributions HJC provided experimental materials. FA, MAN, and AY performed experiment and writing of the manuscript. FSB supervised the experiment. MAN, IHK, SE, GC, and HJC performed editing of the manuscript. EH and MAN supervised the molecular analysis and helped in structuring the manuscript.

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