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Original article

Characterization of indigenous phalsa (*Grewia subinequalis*) genotypes using morphological traits and ISSR markers

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ABSTRACT

Background: Phalsa (*Grewia subinequalis* L.) is a commercial and nutritionally important berry fruit. It is cultivated in Pakistan as a minor fruit crop without any variety characterization. Therefore, the objective of to assess genetic diversity through ISSR markers and morphological features of wild phalsa genotypes collected from different parts of Punjab, Pakistan.

Methods: Morphological characteristics such as plant height, stem circumference, growth habit, leaf length, leaf width, leaf area, leaf color and leaf apex shape showed high variation among genotypes.

Results: Among the twenty inter-simple sequence repeats (ISSR) primers, UBC-812 exhibited the highest PIC values of (0.485) and Dj (0.389) compared to other primers, which considered it better for the identification of phalsa genotypes and prediction of diversity. Moreover, the unweighted pair group method with arithmetic mean cluster analysis divided the sampled genotypes into five clusters (clusters A-E) based on morphological analysis, while molecular data divided the genotypes into eight clusters (clusters A-H).

Conclusion: This study confirmed the high diversity in wild populations especially 'O1P2" and "O7P3" genotypes, with both DNA-based and morphological descriptors that depict their potential use for future phalsa breeding programs.

1. Introduction

Grewia asiatica, commonly known as 'phalsa', is a minor fruit crop native to south Asia and widely cultivated in tropical and subtropical regions (Chundawat and Singh, 1980). Phalsa is an excellent source of antioxidant, antidiabetic, antihyperglycemic, hepatoprotective, radioprotective, antimicrobial, antipyretic, antifungal, analgesic, and antiviral compounds (Zia-ul-haq et al., 2012; Jyoti et al., 2015; Sinha et al., 2015). In particular, the leaves are a source of fodder for cattle and are applied to treat wounds,

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cuts, and painful rashes (Yadav, 1999). The bark and stem are used for sugar refining, and the root bark is used for the treatment of rheumatism (Uddin et al., 2013). Fruit consumption is beneficial for patients with heart, blood pressure, and liver problems (Mukhtar et al., 2012). Additionally, several valuable processed products can be made, such as squash, syrups, beverages, nectars, jams, and chutneys (Mitra et al., 2008; Tiwari et al., 2014).

Despite its high medicinal, nutritional, and economic value, their accessibility for commercial use is limited due to the irregular ripening, perishable nature, and large seed size of the fruits (Wani et al., 2015). These undesirable features also prevent it from attracting attention compared to other grown fruit plants. In addition, there is no suitable characterized cultivar of *G. subinequalis* (Wani et al., 2017). In phalsa breeding sites, some breeders have divided the phalsa genotypes into two groups, local or sharbati and dwarf or tall (Dhawan et al., 1993). It is known and grown as an underutilized native fruit in Pakistan where climatic conditions are suitable for cultivation (Aziz et al., 2018). During the last decade, its area under cultivation is continuously increasing in the country. Currently, it is grown in an area of 1338 ha with annual yield of 4803 tons in 2019–2020 (MNFSR, 2020).

Identification and characterization of the available germplasm are prerequisites for the morphological and genetic development of the crop. In this perspective, germplasm assessment is imperative to understand the genetic background and reproductive value of the existing germplasm (Singh et al., 2004). The use of molecular markers is valuable as an accurate tool to aid plant breeding schemes and effective protection (Koehler-Santos et al., 2003). Some of the most used and readily available applications of DNA fingerprinting are simple sequence repeats (SSRs) or microsatellite DNA markers are ISSR, RFLP, AFLP, and RAPD markers. Among them, ISSR markers are considered highly polymorphic and are very useful in genomic diversity studies, genome mapping, phylogeny, evolutionary biology, and gene tagging (Reddy et al., 2002). In addition, ISSR markers are known as an active marker such as SSR (Ikegami et al., 2009) and RAPD markers (Ruan et al., 2004). On the other hand, Singh et al. (2007) reported that ISSR markers showed great potential for the identification of different genotypes of a particular fruit crop. In the past, ISSR assisted marker technology has been used by many researchers in the characterization of different fruits (Mani et al., 2011; Golein et al., 2011; Santos et al., 2008; Tusa et al., 2002). For traditional morphological characterization, extensive field observations of mature plants are mandatory, which consumes time and effort in addition to their vulnerability to environmental conditions. Despite its disadvantage, the common use of both morphological traits and ISSR markers may provide a more robust framework for exploiting genetic diversity of wild phalsa plants. As the reviewed literature shows, research data on the exploitation and use of the phalsa fruit is very scanty. It is important to consider this fruit, which is currently underutilized to obtain natural bioactive compounds and will expand the fruit market. Considering the importance of this fruit, the present study was designed with the objective to explore the natural genetic diversity of forty-eight phalsa genotypes grown in Punjab (Pakistan) using morphological traits and ISSR markers. Also, statistical calculations were used to clearly define the genotypes and group them into various clusters to establish a relationship between them.

2. Materials and methods

2.1. Sampling area and plant material

Field trials were carried out during 2019–20 in Southern Punjab-Pakistan. A total of elite forty-eight phalsa genotypes were selected in natural ecosystems from sixteen orchards that were selected using snowball methods located in three districts, i.e., Khanewal (30.2864° N, 71.9320° E), Multan (30.1575° N, 71.5249° E) Lodhran (29.5467° N, 71.6276° E)) of Punjab, Pakistan. Selection procedure of these genotypes was based on plant heath with good condition as well as their better morphological traits and high yielding attributes. From each orchard, ten plants were selected using zigzag methods and tagged for sampling. For that purpose, a random sample of twenty fully expanded leaves was collected from each of the selected plants. The detailed information is given in Table 1.

2.2. Morphological characterization and data analysis

For morphological characterization, The Minimal Descriptors of Agri-Horti Crops Part III: Fruit Crops (Mahajan et al., 2002) was utilized. The morphological traits included plant height, and stem girth were measured manually with the help of measuring tape while leaf length and width were determined by using scale. Moreover, growth habit was assessed visually by the attitude of branches. The angle formed by the branches was observed and the average score was registered as erect and drooping. leaf area was calculated by digital leaf area meter. Leaf apex shape was determined using Minimal Descriptors of Agri-Horti Crops. Analysis of variance was done for all the measured morphological characters to check the significance of differences among various genotypes by the help of computer-based software Statistix® 8.1. Moreover, PAST 2000 software was used for PCA and correlation coefficient analysis.

2.3. DNA extraction and quantification

The DNA was extracted from leaves of each genotype by using a modified standard CTAB method described by Doyle and Doyle (1990). Briefly, 3 µg washed leaf sample was grinded in mortar and pestle with 1000 µL of 2X CTAB buffer [100 mM Tris-HCI, 1.4 M NaCl, 30 mM EDTA, PVP and 2 % (w/v) CTAB; pH = 8.0], followed by an addition of 15 µL β- mercaptoethanol. Samples were subjected to separation and purification processes as per the CTAB method. Finally, extracted DNA samples were stored at −20 °C until further use. The isolated DNA was quantified using a spectrophotometer (Implen Nanophotometer, Germany) for downstream application.

2.4. PCR amplification for ISSR analysis

Twenty ISSR markers were selected for the analysis of forty-eight genotypes. A detail of markers is given in Table 2. Individual PCR amplification for each ISSR primer was performed on a programmable thermal cycler (BioRad, California, USA). The PCR protocol involved a total volume of 20 µL reaction mixture containing 40 ng of genomic DNA, 10X PCR buffer (pH 8.3), and 1 unit of Taq DNA polymerase (Fermentas, USA). PCR reactions were carried out with the following temperature program, initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 30 *sec* at 94 °C, 30 *sec* at 55 °C and 1 min at 94 °C, 1 min at 52 or 54 °C and 2 min at 72 °C. The final extension was done at 72 °C for 10 min. Amplified PCR products were visualized using 1 % agarose gel after electrophoresis at 80 voltages for 3 hrs and photographed with a gel documentation system (Photonyx, USA).

2.5. Data analysis for genetic diversity

The binary data were collected as the absence (0) and presence of bands for each locus. Only distinct and unambiguous bands showing polymorphism were used in the analysis. Molecular and

Table 1
Background information on phalsa genotypes collected from different orchards of Punjab, Pakistan.

Sr. No.	Genotypes	Orchard location	Geographic locations
1	O1P2	Roshan Ghulam Muhammad Wala, Multan	30° 16′ 5″ N, 72° 6′ 30″ E
2	O1P3	Roshan Ghulam Muhammad Wala, Multan	30° 16′ 5″ N, 72° 6′ 30″ E
3	O1P4	Roshan Ghulam Muhammad Wala, Multan	30° 16′ 5″ N, 72° 6′ 30″ E
4	O2P2	Pul Eesa Shujabad, Multan	30°28′59.99″ N, 72°34′59.99 E
5	O2P3	Pul Eesa Shujabad, Multan	30°28′59.99″ N, 72°34′59.99 E
6	O2P4	Pul Eesa Shujabad, Multan	30°28′59.99″ N, 72°34′59.99 E
7	O2P6	Pul Eesa Shujabad, Multan	30°28′59.99″ N, 72°34′59.99 E
8	O2P7	Pul Eesa Shujabad, Multan	30°28′59.99″ N, 72°34′59.99 E
9	O2P8	Pul Eesa Shujabad, Multan	30°28′59.99″ N, 72°34′59.99 E
10	O4P6	Sarmad Pakipul Shujabad, Multan	30.20402, N 71.45909 E
11	O4P8	Sarmad Pakipul Shujabad, Multan	30.20402, N 71.45909 E
12	O5P7	Haji Zahoor Mohripur, Khanewal	30°24′24″ N, 71°52′00″ E
13	O6P1	Akbar Mohripur, Khanewal	30°24′24″ N, 71°52′00″ E
14	O6P5	Akbar Mohripur, Khanewal	30°24′24″ N, 71°52′00″ E
15	O6P8	Akbar Mohripur, Khanewal	30°24′24″ N, 71°52′00″ E
16	O7P3	Haq Naqaz Qadirpur Rawan, Multan	30°16′ 60.00″ N, 71° 39′ 59.99″ E
17	O7P6	Haq Naqaz Qadirpur Rawan, Multan	30°16′ 60.00″ N, 71° 39′ 59.99″ E
18	O8P6	Muzzaffar Chowk Qadirpur Rawan, Multan	30°16′ 60.00″ N, 71° 39′ 59.99″ E
19	O9P1	Iqbal Aadhi Bagh Shujabad, Multan	30.0510295767, 71.4114599367
20	O10P2	Ishfaq Krari Chowk Shujabad, Multan	29°52′59.9″N 71°18′00.0″E
21	O10P4	Ishfaq Krari Chowk Shujabad, Multan	29°52′59.9″N 71°18′00.0″E
22	O10P5	Ishfaq Krari Chowk Shujabad, Multan	29°52′59.9″N 71°18′00.0″E
23	O10P6	Ishfaq Krari Chowk Shujabad, Multan	29°52′59.9″N 71°18′00.0″E
24	O10P7	Ishfaq Krari Chowk Shujabad, Multan	29°52′59.9″N 71°18′00.0″E
25	O11P2	Adda Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
26	O11P3	Bypass Shujabad, Multan	29°51′56.6″N 71°17′35.1″E
27	O11P4	Bypass Shujabad, Multan	29°51′56.6″N 71°17′35.1″E
28	O12P2	Adda Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
29	O12P3	Adda Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
30	O12P4	Adda Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
31	O3P1	Ghulam Muhammad Wala, Multan	29°51′56.6″N 71°17′35.1″E
32	O13P2	366-WB Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
33	O13P8	366-WB Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
34	O13P9	366-WB Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
35	O14P1	Arshad Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
36	O14P2	Arshad Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
37	O14P3	Arshad Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
38	O14P5	Arshad Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
39	O15P1	363-WB Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
40	O15P2	363-WB Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
41	O15P4	363-WB Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
42	O15P9	363-WB Makhdoom Aali, Lodhran	29.7875° N, 71.5553° E
43	O16P1	Jalla Arain, Lodhran	29°44′8.99″ N 71°35′31.19″ E
44	O16P3	Jalla Arain, Lodhran	29°44′8.99″ N 71°35′31.19″ E
45	O16P6	Jalla Arain, Lodhran	29°44′8.99″ N 71°35′31.19″ E
46	O8P10	Qadirpur Rawan, Multan	30°16′60.00 N, 71°39′59.99E
47	O10P10	Ishfaq Krari Chowk Shujabad, Multan	29°52′59.9″N 71°18′00.0″E
48	O11P10	Bypass Shujabad, Multan	29°51′56.6″N 71°17′35.1″E

Table 2
Different primers and their sequences used to characterize Phalsa genotypes.

Primer Name	Sequence (5′-3′)	Annealing temperature (°C)
UBC-808	AGAGAGAGAGAGAGA GC	52
UBC-809	AGAGAGAGAGAG AGA GG	52
UBC-810	GAGAGAGAGAGAGAG AT	52
UBC-811	GAGAGAGAGAGAGAG AC	52
UBC-812	GAGAGAGAGAGAGAGAA	52
UBC-814	CTCTCTCTCTCTCTCTA	52
UBC-815	CTCTCTCTCTCTCTCTG	52
UBC-816	CACACACACACACACAT	52
UBC-817	CACACACACACACACAA	52
UBC-820	GTGTGTGTGTGTGTGTC	54
UBC-823	TCTCTCTCTCTCTCTCC	50
UBC-825	ACACACACACACACACT	52
UBC-827	ACACACACACACACACG	48
UBC-829	TGTGTGTGTGTGTGTGC	52
UBC-834	AGAGAGAGAGAGAGAGYT	54
UBC-835	AGAGAGAGAGAGAGAGTC	54
UBC-845	CTCTCTCTCTCTCTCTRG	50
UBC-846	CACACACACACACACART	50
UBC-847	CACACACACACACACARC	52
UBC-849	GTGTGTGTGTGTGTGTYA	54

morphological traits were assessed by the Numerical Taxonomy and Multivariate Analysis System (NTSYS) (Rohlf, 2002). A statistical software "STRUCTURE program ver. 2.3.4." was used for evaluation of the genetic structure and the neighbor-joining tree of forty-eight phalsa genotypes.

3. Results

The plant morphological traits included in the study exhibited considerable variations among forty-eight genotypes (Table 3). In detail, plant height ranged from 5.0 to 13.4 ft whereas stem girth ranged from 1.3 to 4.6 cm. All genotypes showed drooping growth habits, except "O1P3" and "O1P4" which were erect in nature. Leaf length varied from 12.9 to 22.4 cm. Likewise, leaf width ranged from 9.7 to 15.7 cm. The minimum leaf area was noted as 145.20 cm^2 and the maximum was 309.32 cm^2. Leaf color in the studied genotypes was ranged from dark green to whitish green; twenty-five genotypes had light green, twenty-one genotypes had dark green and only two genotypes had whitish-green leaves. Leaf apex was categorized as acute, acuminate, round, and obtuse.

Table 3
Morphological characterization of various phalsa genotypes included in the current study.

Genotype	Growth habit	Plant height (ft)	Stem girth (cm)	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Leaf apex shape	Leaf colour
O1P2	Drooping	8.0	3.8	20.85	15.29	302.84	Obtuse	Whitish green
O1P3	Erect	7.3	3.8	20.65	14.23	286.19	Acute	Light green
O1P4	Erect	5.0	4.7	19.41	15.74	295.94	Obtuse	Whitish green
O2P2	drooping	8.9	2.0	21.17	13.90	287.45	Accuminate	Dark green
O2P3	Drooping	8.2	3.2	17.03	13.51	234.60	Accuminate	Dark green
O2P4	Drooping	7.4	2.8	18.72	12.90	245.41	Accuminate	Dark green
O2P6	Drooping	7.2	3.9	17.50	12.11	213.28	Obtuse	Dark green
O2P7	Drooping	9.8	3.0	18.92	14.24	270.37	Acute	Light green
O2P8	Drooping	7.5	3.2	20.30	13.44	275.37	Accuminate	Light green
O4P6	Drooping	7.8	3.0	16.77	11.98	203.35	Acute	Light green
O4P8	Drooping	5.8	2.3	21.61	13.57	301.21	Acute	Light green
O5P7	Drooping	10.0	3.5	20.32	13.10	260.32	Obtuse	Light green
O6P1	Drooping	7.5	1.5	19.50	15.70	309.32	Obtuse	Light green
O6P5	Drooping	7.2	3.0	15.20	11.50	170.32	Obtuse	Light green
O6P8	Drooping	5.5	2.8	17.90	14.20	260.21	Round	Light green
O7P3	Drooping	6.4	1.5	22.40	9.70	220.32	Round	Light green
O7P6	Drooping	7.0	1.5	21.40	11.70	245.32	Accuminate	Light green
O8P6	Drooping	6.7	1.8	15.20	15.20	229.21	Obtuse	Light green
O9P1	Drooping	9.8	2.7	19.87	14.27	288.86	Acute	Dark green
O10P2	Drooping	13.4	3.4	17.47	12.99	227.81	Acute	Dark green
O10P4	Drooping	12.6	3.1	15.65	14.77	236.87	Obtuse	Dark green
O10P5	Drooping	10.6	2.9	19.55	13.29	260.32	Obtuse	Dark green
O10P6	Drooping	9.8	2.4	18.44	14.26	267.01	Acute	Dark green
O10P7	Drooping	10.3	2.8	13.42	10.76	157.94	Acute	Dark green
O11P2	Drooping	12.4	2.9	15.01	11.15	178.43	Round	Dark green
O11P3	Drooping	8.3	2.2	17.52	13.28	241.49	Accuminate	Dark green
O11P4	Drooping	9.4	2.7	15.58	11.04	173.01	Accuminate	Dark green
O12P2	Drooping	10.0	2.8	17.72	13.36	204.77	Acute	Light green
O12P3	Drooping	9.8	2.2	17.60	11.14	186.43	Obtuse	Dark green
O12P4	Drooping	12.0	3.2	14.24	12.23	173.81	Obtuse	Light green
O3P1	Drooping	7.1	2.5	19.63	11.84	234.91	Accuminate	Light green
O13P2	Drooping	10.3	3.0	14.73	11.15	159.00	Accuminate	Dark green
O13P8	Drooping	8.9	2.4	17.18	11.05	187.36	Obtuse	Dark green
O13P9	Drooping	9.8	2.3	16.74	11.51	190.96	Obtuse	Light green
O14P1	Drooping	7.2	2.3	15.75	10.91	184.81	Round	Light green
O14P2	Drooping	8.1	2.4	14.69	10.79	169.25	Acute	Light green
O14P3	Drooping	10.3	3.2	17.02	13.35	217.72	Accuminate	Light green
O14P5	Drooping	6.5	2.2	15.32	13.37	215.72	Accuminate	Light green
O15P1	Drooping	9.3	2.7	17.96	11.75	206.38	Obtuse	Light green
O15P2	Drooping	8.7	2.8	18.54	13.35	246.25	Obtuse	Dark green
O15P4	Drooping	6.6	2.2	15.60	12.35	184.09	Acute	Dark green
O15P9	Drooping	7.7	2.2	17.52	12.38	206.29	Acute	Light green
O16P1	Drooping	6.8	1.3	16.60	11.98	194.34	Obtuse	Dark green
O16P3	Drooping	9.3	2.7	20.39	13.81	275.81	Acute	Dark green
O16P6	Drooping	8.5	2.8	17.34	12.92	244.27	Acute	Light green
O8P10	Drooping	7.1	1.8	16.00	13.20	217.21	Acute	Light green
O10P10	Drooping	11.8	2.8	12.95	10.92	145.20	Accuminate	Light green
O11P10	Drooping	7.5	2.2	21.17	13.33	276.25	Accuminate	Dark green

Sixteen genotypes had the obtuse apex, fifteen had acute, thirteen had acuminate and four genotypes showed round leaf apex.

The dendrogram obtained with the eight quantitative and qualitative morphological characteristics, considering the forty-eight genotypes, showed the formation of five main clusters (cluster A-E; Fig. 1). Cluster A consisted of seventeen genotypes. Cluster B was comprised of three genotypes. To others, cluster C was the largest cluster with twenty-two genotypes which was further divided into two sub-clusters, i.e., C1 and C2. Cluster D consisted of two genotypes. Cluster E consisted of two genotypes that exhibited the least similarity coefficient. Interestingly, two genotypes (“O1P2” and “O7P3”) remained independent and did not group with any cluster.

A total of 20 UBC (University of British Columbia) ISSRs were applied on collected germplasm to study the genetic diversity (Table 2). Results indicated that all primers are polymorphic (Fig. 2). The range of allele size for ISSRs varied from 300 to 1600 bps (Table 4). The highest value of *PIC* (0.485) and *Dj* (0.389) was obtained through UBC-812, while the lowest value of *PIC* (0.156) and *Dj* (0.126) was obtained through UBC-849 as compared to other markers. Moreover, the highest value of *Cj* (0.779) was calculated in UBC-849, while the lowest value of *Cj* (0.032) was obtained through UBC-812. Further detail regarding the number of loci, the range of allele size, *PIC*, *Cj*, and *Dj* values are given in Table 4.

Dendrogram, based on ISSR markers, grouped forty-eight genotypes into eight main clusters (cluster A-H) while truncated at a similarity coefficient of 0.63 (63 %) (Fig. 3). Cluster A consisted of two genotypes (“O3P1” and “O12P4”) sharing 72 % genetic similarity. Cluster B contained “O15P4” and “O16P3” which were the least similar genotypes (64 %) to others. Cluster C also contained two genotypes (“O11P4” and “O13P9”). O11P4 shared 69 % genetic similarity with 013P9. Cluster D was the second-largest cluster consisting of thirteen genotypes. Cluster E and Cluster G comprised of three genotypes each, whereas cluster F had five genotypes. Cluster H was the largest cluster and compromised of two sub-clusters (H1 and H2). The sub-cluster H1 consisted of six genotypes in which “O2P4” shared 100 % genetic similarity with “O2P7” while there were ten genotypes in sub-cluster H2. Two genotypes, “O12P2” and “O7P3”, remained independent and did not group with any other genotypes.

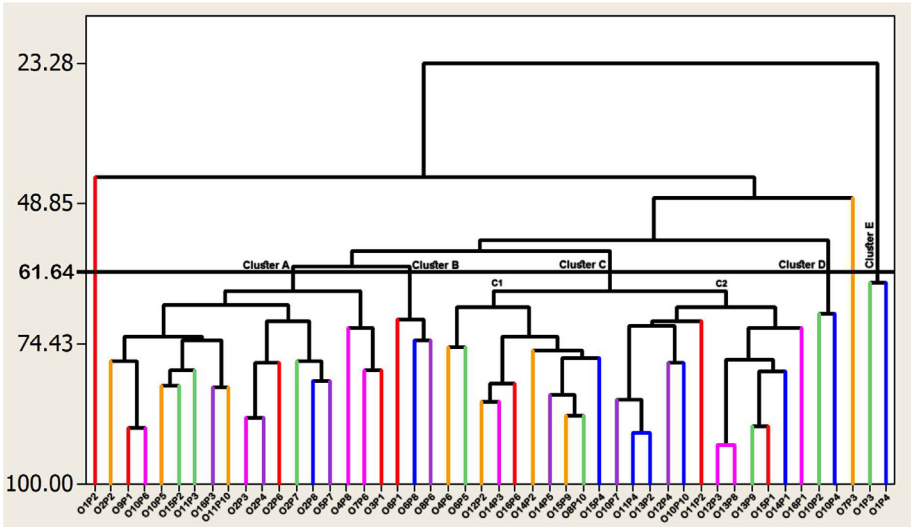

Fig. 1. Dendrogram showing a phenotypic relationship among various phalsa genotypes based on morphological characteristics.

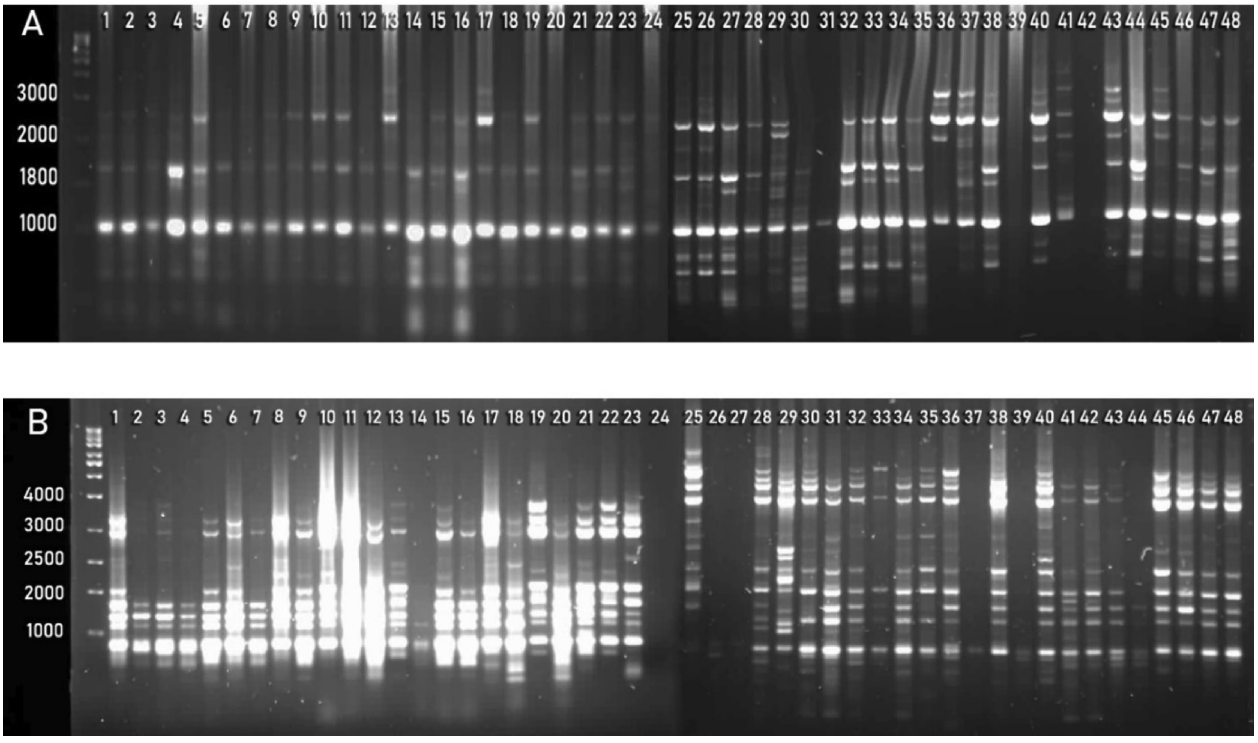

Fig. 2. ISSRs amplification of phalsa genotypes generated with primer UBC-825 (A) and UBC-834 (B).

Moreover, population structure analysis for forty-eight phalsa genotypes was performed based on ISSRs results by adopting an admixed Bayesian model (Fig. 4A-C). An increase in the logarithm of the data likelihood [Ln (PD)] on average was observed with increasing the numbers of assumed sub-populations (K) from 2 to 10. The adhoc quantity based on the second-order rate of change in the log probability (ΔK) exhibited a clear peak at K = 3. Hence, data analysis suggested that the K value of 3 is the most probable prediction for the number of sub-populations for both ISSRs (Fig. 4A). ISSRs based structure analysis depicted that bar plot has been configured into three different colors, i.e., red, blue, and green. The highest contribution was recorded from green color (Fig. 4B and C).

4. Discussion

According to the current findings, significant levels of diversity were observed in the wild phalsa population of Southern Punjab (Pakistan), both at the morphological and molecular levels. For example, variation in leaf color ranged from dark green to whitish green. Interestingly, the growth habit of two genotypes ("O1P3" and "O1P4") was upright while the rest of the genotypes showed drooping growth habit. Similarly, variations were observed for other characters of the leaf and plant. Overall, the two genotypes ("O1P2" and "O7P3") showed significant variability among all genotypes. These differences may be due to environmental changes, as the samples were collected from different locations

Table 4
Marker discriminating indices of ISSRs used to characterize different Phalsa genotypes included in the current study.

Primer Name	Number of loci	Range of allele size (bp)	*PIC*	*Cj*	*Dj*
UBC-808	8	500–3000	0.284	0.7157	0.355
UBC-809	9	500–3000	0.415	0.601	0.300
UBC-810	9	500–3200	0.467	0.493	0.246
UBC-811	8	500–2500	0.381	0.637	0.318
UBC-812	5	500–1500	0.485	0.032	0.389
UBC-814	7	500–2500	0.413	0.603	0.301
UBC-815	10	500–3000	0.471	0.542	0.271
UBC-816	5	500–1500	0.459	0.510	0.255
UBC-817	5	500–1200	0.276	0.748	0.374
UBC-820	8	500–3000	0.357	0.663	0.330
UBC-823	7	500–3000	0.475	0.537	0.268
UBC-825	6	500–3000	0.430	0.479	0.239
UBC-827	8	500–3000	0.448	0.489	0.244
UBC-829	4	600–1200	0.419	0.597	0.298
UBC-834	6	700–2500	0.248	0.163	0.263
UBC-835	8	500–2200	0.271	0.699	0.349
UBC-845	8	500–3000	0.400	0.560	0.280
UBC-846	8	500–3000	0.166	0.526	0.164
UBC-847	6	500–1200	0.334	0.579	0.370
UBC-849	9	500–3000	0.156	0.779	0.126

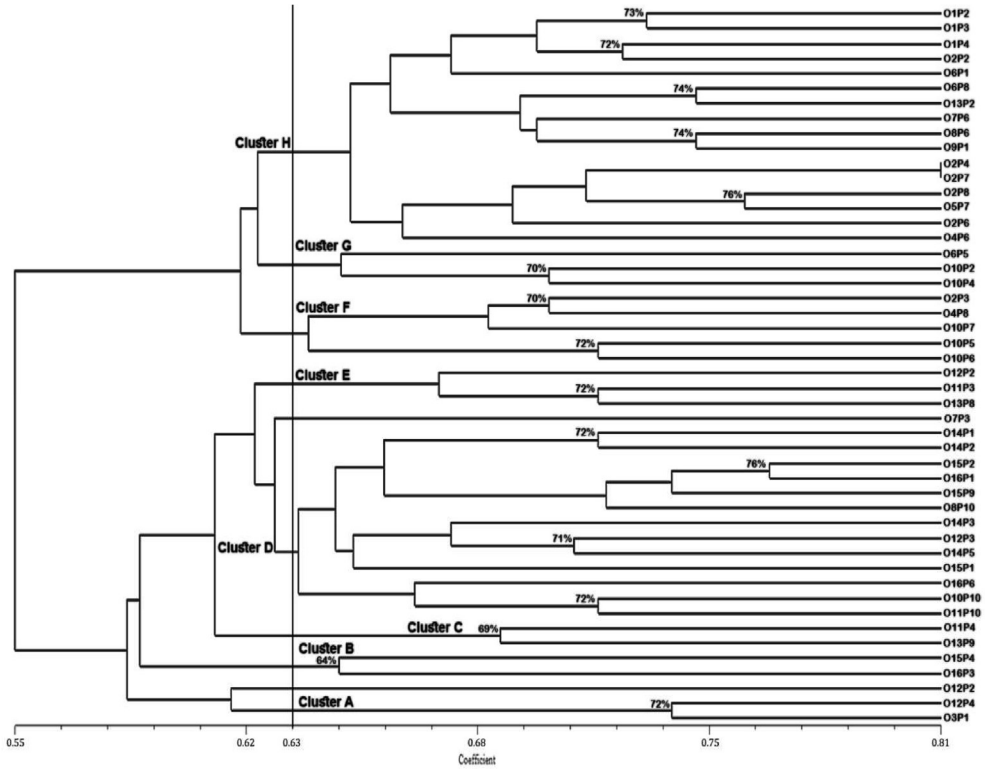

Fig. 3. Dendrogram showing a genotypic relationship among phalsa genotypes based on ISSR markers.

with varying climatic conditions. It is well known that the interaction of a given genotype with different environments produces possible variations in phenotypic characters (Teng et al., 2002). Pettersen et al. (2006) stated that the differences in essential oil content in the rose plant may be caused by different environmental conditions such as temperature, humidity, and precipitation. Therefore, morphological characterization alone has limitations in providing precise information due to environmental influence (Belaj et al., 2003).

Determination of plant genotype by evaluating genetic differences is an effective tool that strengthens and confirms morphological characterization results. With the use of molecular markers and DNA fingerprinting, genotypes can be accurately identified and the actual relationship between the genotype affected by environmental conditions and geographic location in a population group can be measured (Ahmad et al., 2019). Because the genetic structure is not affected by climatic conditions. Thus, the variability observed by ISSR markers in this study can distinguish and identify different genotypes of different genetic makeup. This study revealed a wide variety of genetic variations in the phalsa germplasm.

In this study, the dendrogram based on ISSRs shows noticeable differences in the number of clusters as well as the position of genotypes within the clusters. The reason for this difference was

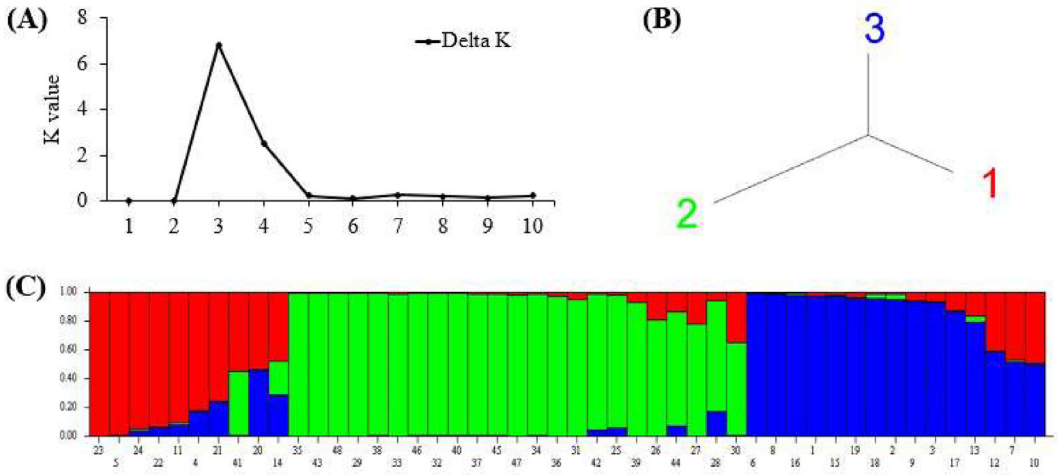

Fig. 4. Population structure analysis showing a genetic relationship among forty-eight phalsa genotypes based on ISSR markers: A = best K value graph, B = neighbour-joining tree and C = bar plot.

that molecular markers recognized distinctive regions of DNA variation within the genome (Dongre et al., 2004). The two genotypes ("O2P4" and "O2P7") showed maximum genetic similarity (100 %) and therefore they were very close to each other in the dendrogram among all genotypes. The fact that "O12P2" and "O7P3" do not cluster with any other genotype indicates that these genotypes have different genetic makeup, distinctive background, and a high degree of polymorphism. Maximum genetic variation in these genotypes requires protection of these genotypes from natural disasters and human activities. Also, collecting and then maintaining various populations is better than collecting a few samples from each population (Kaundun and Park, 2002).

The effectiveness of primers is an important factor in scientific studies, especially when genetic diversity is estimated. PIC is considered equivalent to genetic diversity as it determines the frequency of alleles per locus and the number of alleles expressed. The PIC value also indicates the dominant or co-dominant nature of the marker. ISSR markers PIC values range from 0.0 to 0.5 as they are dominant markers, the higher the PIC value, the higher the genetic diversity. PIC is directly proportional to Dj and inversely proportional to Cj. Primers with a minimum Cj value and a maximum PIC and Dj value are excellent for detecting allelic variation in genotypes (Riek et al., 2001). Minimal genetic diversity was revealed by primer UBC-849 due to low PIC (0.156), Dj (0.126), but highest Cj (0.779). Therefore, the UBC 849 primer would not be more efficient for assessing genetic diversity. Overall, the UBC-812 primer gave maximum PIC (0.485), Dj (0.389) but minimum Cj (0.032). Therefore, it is considered a more efficient primer for the estimation of genetic diversity among plant populations.

5. Conclusion

In the present study, forty-eight genotypes were investigated for identification and characterization. A wide variation was found among genotypes based on morphological and molecular characters. Among twenty ISSR markers, UBC-812 was found to be the most effective for the estimation of genetic variability of phalsa genotypes. Particularly, two genotypes ("O1P2"and "O7P3") showed distinct variability in morphological and molecular characters among all the genotypes and this variability would be interesting to broaden the genetic base of phalsa breeding programs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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