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Integrating morphological and SSR marker analysis to uncover genetic variability in galgal (*Citrus pseudolimon* Tanaka) germplasm

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Abstract

Galgal (hill lemon or Himalayan lemon), an indigenous and drought-tolerant species widely grown across Himalayan states, is renowned for its culinary use in pickles and beverages. Despite its utility, galgal remains underutilized, necessitating comprehensive studies on its genetic and phenotypic diversity. This study investigated the genetic diversity of 13 Indigenous galgal germplasm maintained at the ICAR-Central Citrus Research Institute, Nagpur, using 21 morphological traits and 46 simple sequence repeat (SSR) markers. The study demonstrated the ability to select germplasm for breeding by the highest phenotypic and genotypic coefficients of variation for traits like seed number (61.35 % and 60.57 %), canopy volume (53.33 % and 53.29 %) and rind thickness (45.92 % and 45.49 %). High heritability (>80 %) and genetic advance for characters like canopy volume, fruit weight and rind thickness suggest the predominance of additive gene action, making them ideal targets for improvement. Principal component analysis (PCA) identified seven components explaining 92.3 % of the variability, with fruit and plant traits contributing the most. Cluster analysis grouped the germplasm into three clusters based on morphological characteristics. Molecular characterization using 46 SSR markers showed distinct germplasm with polymorphism information content (PIC) values ranging from 0.260 to 0.698, averaging 0.4851. UPGMA dendrogram and principal coordinate analysis (PCoA) clustered the germplasm into two major groups. The results of the morphological and molecular markers revealed a rich genetic diversity of galgal. The germplasm present in the single cluster should be avoided in breeding. The traits responsible for genetic variation in galgal germplasm should be considered for galgal improvement programmes.

Keywords

galgal; genetic diversity; PCA; PCoA; SSR markers

Introduction

Galgal, or Hill lemon (*Citrus pseudolimon* Tanaka), is indigenous to India (1). It is predominantly used to prepare pickles and squash, particularly in Jammu and Kashmir, Uttarakhand, Punjab and Himachal Pradesh (2). This plant exhibits hardiness and drought tolerance, capable of withstanding high (> 40° C during summer) and low temperatures (< 4°C during winter) without experiencing frost (3). Galgal thrives at elevations up to 3000 meters above mean sea level (4). It is a prolific bearer, producing yields of 30 to 40 t/acre (5)

and its fruits are rich in minerals and vitamin C (5-7). Additionally, the fruits are employed to extract essential oils and process lemonade, juice powder and appetizers (6). The peel is also rich in pectin and flavonoids and the seed is rich in palmitic, linolenic, linoleic and oleic acids (7). Galgal juice, rich in phenols (67.6-77.09 mg GAE/g), flavonoids (34.39-35.31 QE/g) and ascorbic acid (68.25-70.45 mg/100 mL are considered a cost-effective alternative to the more expensive Kagzi lime (*C. aurantifolia* Swingle) juice in the preparation of lime squash (7-8). The tree is venerated as a sacred plant in Himachal Pradesh and the western Himalayas and its unripe fruits are used in worship during the "*Seer*" festival. The leaves are incorporated into "*Grihpoojan"* (the worship of the house or door), a practice referred to as "*patridhalna*" in the local dialect (4). Furthermore, local inhabitants prepare a highly flavoured concentrated fruit juice called "*chukh*" for consumption during the off-season (9).

Galgal grows naturally in wild and semi-wild habitats in the northwestern Himalayas and is widely cultivated in homestead gardens (9, 10). Primarily propagated through seeds, galgal exhibits substantial variability in both cultivated and wild conditions (11, 12). Fruits are often harvested from the wild and sold in local markets, but over-exploitation threatens genetic diversity. These genetic resources are invaluable for galgal improvement. Therefore, systematic collection, conservation, characterization and documentation are crucial. Morphological characterization is a simple and fundamental step in evaluating the genetic diversity revealing the phenotypic variation of crop species, including citrus (3, 9). However, environmental factors such as temperature, rainfall, relative humidity, soil types, etc., often influence the morphological traits, leading to unreliable and biased results (13). Although the environment influences morphological characteristics, they remain crucial for characterization. Studies on galgal genetic diversity are limited but have highlighted significant diversity in quality traits and fruit characteristics (12, 14).

Molecular markers that provide insights at the DNA level are significant tools for characterizing citrus germplasm, as they precisely discriminate closely related germplasm (15). SSR markers are highly polymorphic, co-dominant and reproducible, making them ideal for germplasm characterization. SSR markers have shown high polymorphism in pummelo, mandarin and sweet orange (16, 17, 18). Some studies have utilized molecular markers to identify galgal; however, genetic diversity studies on galgal remain unexplored (19, 20). Integrating morphological traits and molecular markers facilitates a better understanding of trait variability and supports genetic improvement efforts. Therefore, our study aims to characterize the galgal germplasm using integrated morphological and molecular markers to assess genetic diversity.

Materials and Methods

Experimental site

This study was conducted at the ICAR-Central Citrus Research Institute in Nagpur, Maharashtra, India. The site is located at 21°14' N latitude and 79°08' E longitude, 310 m above mean sea level. The average temperature ranges from 13 °C to 42 °C, with May being the hottest month (42 °C) and December the coldest (14 °C). The farm receives an average annual rainfall of 1110 mm, with the wettest month being July (277 mm) and the driest being December (8 mm).

Plant materials

Thirteen 12-year-old galgal germplasm accessions budded onto Rough lemon rootstock collected from Uttarakhand (IC285430, IC311377, IC285427, IC285440, IC326669 and IC285439), Uttar Pradesh (IC322100 and IC311386), Himachal Pradesh (IC311360), Meghalaya (IC344936, IC285388 and IC346969) and Maharashtra (IC322260) states (Table 1 and Fig. 1) of India were used for study during 2021 and 2022. The trees were planted at 5 x 5 m spacing in a randomized block design (RBD) and replicated thrice with four trees per replication. All the cultural practices were followed uniformly in all the germplasm.

Data collection

Plant characteristics were recorded, including canopy spread (m^2), scion girth (cm), rootstock girth (cm) and plant height (m). The canopy volume (m^3) was calculated as per the Equation 1 formula (21)

$$CV = H (r^2) \qquad \text{(Eqn. 1)}$$

Where,

CV = canopy volume

H = tree height

r^2 = tree radius

The tree height and radius in all the trees in each replication were calculated and the mean was computed. Yield traits viz. fruit weight (g), fruit length (mm), fruit diameter (mm), rind thickness (mm), number of segments, juice content (%) and number of seeds per fruit, seed

Table 1. Geographical location of galgal germplasm collected from different states

Germplasm	State	Location	Longitude (E)	Latitude (N)	Altitude (m)	Status	Frequency
IC-285427	Uttarakhand	Nainital	79^{0}50'	28^{0}91'	1270	Cultivated	Occasional
IC-285440	Uttarakhand	Nainital	79^{0}45'	29^{0}38'	1890	Cultivated	Occasional
IC-326669	Uttarakhand	Pithoragarh	80^{0}12'	29^{0}35'	1570	Cultivated	Abundant
IC-285439	Uttarakhand	Nainital	79^{0}45'	29^{0}38'	420	Wild	Rare
IC-285430	Uttarakhand	Nainital	79^{0}50'	28^{0}91'	2000	Cultivated	Occasional
IC-311377	Uttar Pradesh	Dehradun	78^{0}02'	30^{0}19'	680	Cultivated	Occasional
IC-322100	Uttar Pradesh	Jhansi	78^{0}58'	25^{0}45'	285	Cultivated	Abundant
IC-311386	Uttar Pradesh	Saharanpur	77^{0}33'	29^{0}58'	270	Cultivated	Occasional
IC-311360	Himachal Pradesh	Sirmour	77^{0}50'	30^{0}50'	468	Cultivated	Occasional
IC-344936	Meghalaya	Ribhoi	91^{0}51'	25^{0}30'	850	Wild	Abundant
IC-285388	Meghalaya	South Garo Hills	90^{0}45'	25^{0}30'	250	Cultivated	Abundant
IC-346969	Meghalaya	West Garo Hills	91^{0}23'	25^{0}51'	960	Wild	Abundant
IC-322260	Maharashtra	Ahmednagar	74^{0}81'	19^{0}47'	400	Wild	Occasional

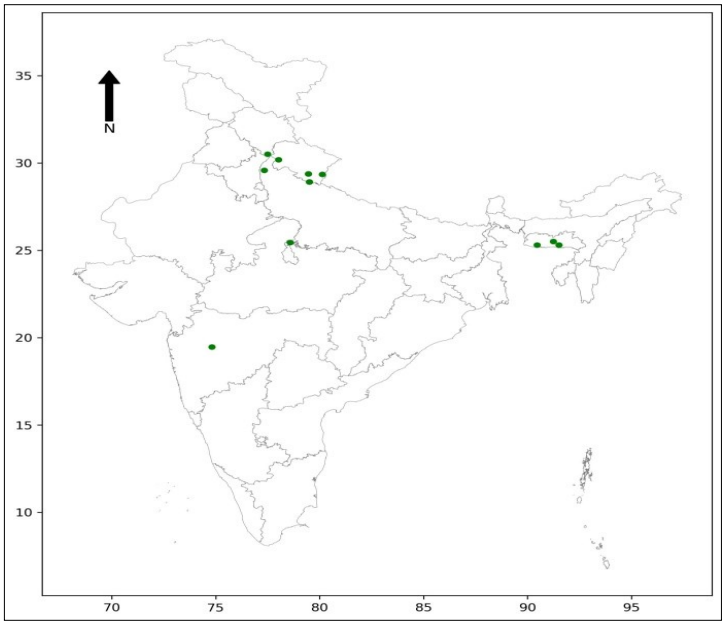

Fig. 1. Germplasm collection site for galgal in India.

weight (10 seeds weight expressed in g), seed length (mm) and seed breadth (mm) were recorded. The TSS (^{0}Brix) was measured using a portable digital refractometer (HANNA Instruments HI96801). The titratable acidity (%) was analyzed per the suggested method (22). These data were collected in each replication from 10 randomly selected fruits and the mean was calculated. The number of fruits per tree and the yield per tree were recorded on four trees in each replication and the mean was computed.

Molecular characterization

46 SSR primers (shortlisted from unpublished data) were screened in galgal (Table 2). The polymerase chain reaction (PCR) amplification was performed in the BIO-RAD T100 thermal cycler. The PCR amplification conditions were as follows: initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 40 sec, and extension at 72 °C for 1 minute followed by a final extension at 72 °C for 10 min (18). Each primer locus allele was measured (bp) with standard (100 bp DNA ladder) (Genexy). The results were then converted to binary codes that indicated whether the corresponding fragment size was present (score 1) or absent (score 0).

Statistical analysis

Pooled analysis of variance (ANOVA) (23), genotypic and phenotypic coefficients of variation, heritability and genetic progress as a percentage of the mean were calculated as per standard procedure (24, 25, 26). Principal component analysis (PCA) was performed using SPSS version 16.0 (27). Morphological trait-based clustering was conducted using Wards' method and principal coordinate analysis (PCoA) in R version 4.0 (28). The dendrogram was constructed using the unweighted pair group method with an arithmetic mean (UPGMA) algorithm derived from the dissimilarity matrix computed with DARwin software version 6.0 (29).

Results and Discussion

Analysis of variance

Phenotypic traits provide insights into an individual genetic makeup and environmental influences. The presence of a wide range of specific genes is a crucial indicator of genetic diversity, which serves as the basis for germplasm improvement. The pooled ANOVA analysis in Table 3 revealed significant variations among 13 galgal germplasm for the 21 traits. Traits like canopy volume, fruit weight, fruit length, number of oil glands and number of seeds per fruit showed the highest genetic variations essential for galgal breeding. In contrast, traits like fruit length/diameter, acidity, TSS: acid ratio, seed width and seed thickness showed the lowest genetic variation. These variations may be attributed to differences in germplasm and its interactions with specific agro-climatic conditions. The observed distinctions within galgal germplasm underscore the extensive phenotypic and genotypic variability, offering significant potential for genetic improvement and cultivar development in galgal. The average values are displayed in the Table 4.

Genetic diversity

Assessing genetic diversity within germplasm collections relies heavily on key metrics such as phenotypic and genotypic coefficients of variation (PCV and GCV), heritability and genetic advance as a percentage of the mean (GAM), which are crucial for evaluating genetic variability (30). The genetic analysis of 13 galgal germplasm revealed significant differences across the 21 assessed traits. As shown in Table 4, the PCV was marginally higher than the GCV for all studied traits, indicating minimal environmental influence. The highest percentages of PCV and GCV were observed for the number of seeds (61.35 % and 60.57 %), canopy volume (53.33 % and 53.29 %), rind thickness (45.92 % and 45.49 %), fruit weight (36.27 % and 35.40 %), number of oil glands (32.06 % and 31.47 %), fruit axis diameter (25.88 % and 24 %), seed weight (23.64 % and 21.86 %) and plant spread (23.14 % and 22.90 %). The high PCV and GCV

Table 2. Details of the primers used for genetic diversity

S. No	Primer name	Orientation	Primer Sequence (5' to 3')	Bases	Tm (°C)	Ta (°C)
1	Csin.0236	F	AGAAATCCAGTGGTGGTGCT	20	56.6	60
		R	TGAAGTGGGATGGCTTCTTT	20	54.3	58
2	Ma3_72	F	CCAAAACCAAACCTCCACAT	20	54.1	58
		R	CACCTTGCTGGTTGATGATG	20	53.5	60
3	Ma6_10	F	GGAACACAAAGGAGCAAGGA	20	55.2	60
		R	TGGTTTGGCGGTTAAAACTC	20	53.7	58
4	Csin.0551	F	AAAATAGTGAGCAGGGGGCT	20	56.5	60
		R	CGACCACGTTTCCTTCAACT	20	55.3	60
5	Csin.0505	F	AGATTAATTTGAGAATCTTGCCA	23	50.4	60
		R	GATCCATCAAGCAAATGACCT	21	53.0	60
6	Ma3_167	F	CAATGAAGACAGCGACAACG	20	54.4	60
		R	ACAACACAGCTGAACGCAAG	20	56.3	60
7	Ma2_1766	F	TGCCCAAGCATATACCACAA	20	54.3	58
		R	TACTAAATGCACCAACGGCA	20	54.7	58
8	Ma3_1004	F	TCGTTATCCACTTTCTGTGGC	20	55.0	62
		R	AAACGGTGAGGGTGTTGAAG	20	55.4	60
9	Csin.0100	F	TGGGCTTGACCCAATACAGT	20	56.4	60
		R	TGTCCAGTATTCAGTTACGAAGGA	24	55.3	68
10	Ma4_51	F	TCACAAATTTATGCCTTGCG	20	51.8	56
		R	TCGATAGTGCACCACGACAT	20	56.2	60
11	Ma2_1401	F	CCCTCATTTTCCTTCTATCTCTCTC	25	54.3	72
		R	CCCCAGTGGAAAAGAACAAA	20	53.2	58
12	Ma4_178	F	TTTTTCTTTCCCCCTCATCA	20	51.6	56
		R	ATAACTCCCCGACTGCCTCT	20	57.7	62
13	Ma3_44	F	TTTCAGTTTCTGCTGACCCC	20	55.2	60
		R	GGATTGTGACTGTGGCTGTG	20	56.3	62
14	Csin.0167	F	GGATGGCAATTTGAAGGTTG	20	52.4	58
		R	TCGACTTCTCGGGAAAACAT	20	53.8	58
15	Ma2_94	F	GTTATTTGCTTTGGTTCGGC	20	52.8	58
		R	TCAATGATTCCAGAATGCCA	20	52.0	56
16	Ma2_1824	F	GGATAAAGCAGATATTTCCCCA	22	52.3	62
		R	TCCAAATACTTCCTTAAATAGCCC	24	52.7	66
17	Csin.0368	F	TGAAATAACGGTGTCGACCTT	21	54.2	60
		R	GCATATGAGCGCTTTGGAGT	20	55.6	60
18	Ma3_1327	F	CCGTTGCGGTCTACCATTAT	20	54.8	60
		R	CCAGCCACAGAGTTTTCACA	20	55.4	60
19	Ma3_125	F	AACAGTCCTGCCTAGGGCTT	20	58.5	62
		R	AGGGAAATGAAGCTCCGAAT	20	54.1	58
20	Ma2_1162	F	CACTGGCACTGCTCTTTCAA	20	55.7	60
		R	GGCAGGATAGCTTTGGAGTG	20	55.7	62
21	Ma4_83	F	TTATTTTTGAAAGGGATGGGG	21	50.8	58
		R	TCCCTTTCTTTCCCATTTCC	20	52.5	58
22	Ma2_1440	F	CTGTTGAGGGGGTTCCATTA	20	54.3	60
		R	TTGATTCTCTTCACTGCCCC	20	54.8	60
23	Csin.0149	F	CTCACATGGCTTCCAGGTTT	20	55.3	60
		R	AGGCAAAAACCTCAACTCTCC	21	55.3	62
24	Ma3_46	F	AAATGGACACTGCAGGCTTT	20	55.3	58
		R	GCTGAGCAAAATGAAGCAAT	20	52.1	56
25	Ma3_122	F	ACACAAATCTGCCCACATCA	20	54.8	58
		R	GTGTGTGCATGGATGAGGAG	20	55.9	62
26	Ma3_98	F	GATCACCACAAGCAGCACAC	20	56.8	62
		R	TCTCAAGAGCCCAGTTCGAT	20	55.7	60
27	Csin.0514	F	GATGAATCTTTGCCTCTCGC	20	53.8	60
		R	CTCACAGCCCTTGGTTTGAT	20	55.3	60
28	Ma3_1047	F	GCAGCAACAACAACCACATC	20	55.3	60
		R	CCCTTTCCCTCTGATGATGA	20	53.8	60
29	Ma3_153	F	CTGTTGCTGCTCTTGGATCA	20	55.3	60
		R	GTTCCGGATTGAACCATGTC	20	53.8	60
30	Csin.0457	F	TCGTCAAGTTCAAAATCAATGG	22	51.7	60
		R	ATTGCCCTTTTTGACATTGG	20	51.8	56
31	Ma3_5	F	CACGCACGCATAATGAGTTT	20	53.9	58
		R	GGAGATTGCTACCAATAAATCCC	23	53.4	66
32	Ma4_41	F	AATCGAATTCATCCCCATCA	20	51.5	56
		R	CGGTCCAATTCAATCTGCTT	20	53.3	58
33	Ma3_21	F	TCTTCGGATTCGTTGAAAGG	20	52.6	58
		R	CCCGCTCTTTTGTGTATGGT	20	55.3	60
34	Ma3_96	F	ATCATTTCAACAAGGGACGG	20	53.0	58
		R	TCGCCAATCAAACAACAAAA	20	51.3	54
35	Ma2_1201	F	GATCTTCCCGAATGTTGCAT	20	53.1	58
		R	ACTTGGGCTTGCCCTCTTAG	20	57.2	62
36	Ma2_1215	F	GTGGTGGTGATGAAGTGACG	20	55.9	62
		R	TGATTCCATGCAAACTCCAA	20	52.4	56
37	Csin.0287	F	AGAATCCTCATCCCCTCCTAA	21	54.5	62
		R	TCTCTAGCAATTCGTGCCAA	20	51.1	58
38	Csin.0249	F	CGTACTTTAAAATTTCGGACTGA	23	51.2	62
		R	CAGGTGTATATGGCCGCATT	20	55.2	60

39	Ma2_1710	F	TGGAACATTGAAGTGGGTGA	20	54.1	58
		R	ACTTGAGATTAGGGCCGGTT	20	56.1	60
40	Csin.0464	F	TTTGACCAACATGTAAAAGGGA	22	52.5	60
		R	GGTGCATAGAGTACTATGGTGGG	23	56.6	70
41	Csin.0524	F	TTGAACTGCCTGAGTTGGACT	21	56.4	62
		R	CGAACGAAAGTGATGGGAAT	20	52.9	58
42	Csin.0072	F	AGACTTCGTTCAATTAATGTAGATTTT	27	51.5	68
		R	TCTTCTGAGATACCCCTGGA	20	54.2	60
43	Ma2_1856	F	ACCAGAGCTCTTCCTCCTCC	20	58.2	64
		R	AAGCTCACTGACTCCTCCCA	20	57.9	62
44	Csin.0329	F	AAAAGGGCGATTGAAAAGAAA	21	51.2	56
		R	GGTTGTGACGTAGGCTAGGG	20	57.3	64
45	Ma2_1750	F	CTTGCCAACAACCTTTTGCT	20	54.5	58
		R	AGAAGCACAGCCACTGAGGT	20	58.9	62
46	Csin.0373	F	GGACTTGTCGCAAAACCATT	20	53.9	58
		R	CTCCTGCATTATTGGTCGGT	20	54.8	60

Table 3. Pooled analysis of variance (ANOVA) for different traits in galgal germplasm

Characters	Mean sum of square		
	Replication	Genotype	Error
Plant height (m)	0.064	1.852**	0.394
Plant girth (cm)	0.070	1.845**	0.393
Plant spread (m^2)	0.206	5.184**	1.096
Canopy volume (m^3)	8.317	2545.257**	497.813
Fruit weight (g)	5348.999	64793.796	32522.018
Fruit length (cm)	204.258	958.496**	311.800
Fruit diameter (cm)	96.459	627.319*	298.147
Fruit length/diameter	0.023	0.046*	0.019
Number of oil glands	31.814	1837.442**	220.896
Fruit axis diameter (mm)	3.725	137.47**	32.569
Rind thickness (mm)	0.441	18.765*	9.239
Number of segments	0.157	24.555**	0.164
Juice content (%)	9.785	115.952**	30.826
TSS ($^{\circ}$Brix)	0.774	5.157**	0.945
Acidity (%)	0.602	1.386*	0.549
TSS: acid ratio	0.027	0.786**	0.179
Number of seeds	6.783	1669.939**	5.166
Seed length (mm)	1.262	8.945*	3.532
Seed width (mm)	0.691	3.910**	0.884
Seed thickness (mm)	0.649	3.743**	0.608
Seed weight (g)	0.073	1.108**	0.287

Table 4. Estimates of genetic variability, heritability and genetic advance as percentage of mean in galgal germplasm

Traits	Mean	Min	Max	PCV (%)	GCV (%)	Heritability (h^2) (broad sense)	GAM (%)
Plant height (m)	4.024	3.5	5.1	11.49	11.10	0.93	22.09
Plant girth (cm)	80.933	53.0	108.0	19.480	19.405	0.99	39.819
Plant spread (m^2)	4.615	3.65	6.05	23.145	22.901	0.979	46.680
Canopy volume (m^3)	49.945	22.3	93.31	53.330	53.296	0.998	109.722
Fruit weight (g)	483.908	313.0	796.5	36.272	35.404	0.952	71.187
Fruit length (cm)	110.218	87.9	135.7	10.972	10.649	0.941	21.290
Fruit diameter (cm)	94.443	81.1	120.9	11.806	11.517	0.951	23.147
Fruit length/diameter	1.170	1.06	1.26	7.445	6.670	0.802	12.307
Number of oil glands	62.119	29.5	93.0	32.061	31.476	0.963	63.659
Fruit axis diameter (mm)	23.624	11.8	33.3	25.883	24.002	0.860	45.851
Rind thickness (mm)	6.303	3.63	13.5	45.927	45.495	0.981	92.838
Number. of segments	10.590	9.0	13.0	11.997	11.222	0.875	21.626
Juice content (%)	40.344	30.1	47.3	8.927	7.640	0.732	13.470
TSS (^{0}Brix)	7.341	6.6	8.8	10.082	9.718	0.929	19.297
Acidity (%)	3.887	3.11	5.42	17.829	16.828	0.890	32.721
TSS: acid ratio	1.945	1.4	2.6	20.148	19.036	0.892	37.049
Number of seeds	25.791	13.0	72.0	61.358	60.573	0.974	123.186
Seed length (mm)	11.650	8.8	13.0	10.986	9.972	0.823	18.646
Seed width (mm)	6.778	5.4	7.8	12.292	10.212	0.690	17.479
Seed thickness (mm)	4.972	3.1	5.7	18.957	18.088	0.910	35.552
Seed weight (g)	2.341	1.6	3.5	23.648	21.860	0.854	41.627

traits reflect significant genetic variability, identifying them as strong genetic enhancement targets. These higher values suggested that the characteristics are primarily influenced by additive genetic factors, with limited environmental interference, thus improving the selection efficiency and breeding efforts (31, 32). Prioritizing these traits in breeding programs can improve galgal varieties with enhanced yield, quality and adaptability to varying agro-climatic conditions.

Moderate PCV and GCV values were observed for traits such as plant girth, seed thickness, acidity, seed width, number of segments, fruit diameter and plant height, suggesting the influence of both additive and non -additive genetic factors. The lowest PCV and GCV were recorded for the fruit length-to-diameter ratio (7.44 % and 6.67 %, respectively). In this study, traits such as plant spread, canopy volume, fruit weight, rind thickness, fruit axis diameter and the number of seeds per fruit, where PCV was slightly higher than GCV, show significant potential for selecting elite genotypes capable of adapting to environmental changes. Similar findings of high PCV and GCV for traits like fruit weight, number of seeds per fruit, rind thickness, plant girth and seed weight have been reported previously in pummelo, mandarin and rough lemon (17, 33, 34).

Heritability (h^2) reflects the degree to which traits are inherited, with high heritability facilitating the selection of specific characteristics (35). All traits in this study had a high level of heritability (0.690 for seed width & 0.998 for canopy volume). Additionally, a moderate to the high level of genetic advance was recorded as a percentage of the mean for all traits, with maximum GAM recorded for canopy volume (109.7) and number of seeds (123.1). Higher heritability with higher GAM for traits like canopy volume, seed number, fruit weight and rind thickness strongly showed additive gene activity. High heritability with higher GAM also strongly suggested that the environment plays a lesser role (36). Selecting genotypes based on these traits for galgal improvement would be highly effective (37). The germplasm collected from Himalayan states is performing well in Central India. Galgal, predominantly used for pickling in Himalayan states like Jammu, Uttarakhand and Himachal, requires thick rind and large fruits. In this study, these traits have higher PCV, GCV, heritability and GAM, indicating the importance of these traits in selecting superior varieties. Similarly, high heritability and higher genetic advances in fruit traits were reported in mandarin and pummelo (17, 33).

Principal component analysis (PCA)

PCA based on 21 traits resulted in seven principal components contributing 92.3 % of the total variability of the collected 13 galgal germplasm (Table 5). The first three PC accounted for 65.21 % of total variability. The most influential traits in the first PC (28.46 % variability) include fruit weight, length, diameter, acidity, seed length and seed thickness. The second PC accounted for 23.82 % variability and was influenced by plant height, girth, spread, canopy volume, fruit axis diameter and rind thickness. The third PC accounted for 12.92 % variability and was influenced by plant height, spread and canopy volume. The results revealed that the first two components gave the maximum variability and traits influencing the first two components should be considered for galgal improvement programmes. Studies on citrus species consistently highlight fruit traits, including weight, rind thickness and TSS: acid ratio, as key determinants of genetic variability and market success (17, 38-40).

Cluster analysis

Cluster analysis is essential for grouping similar data sets from large data sets based on similarity in their characters. The dendrogram constructed using 21 traits showed variations among thirteen germplasm, which resulted in three clusters (Fig. 2). The first cluster consists of three germplasm (IC322260, IC322100 and IC285440), second cluster consists of three germplasm (IC311360, IC311377 and IC344936) and third cluster consists of seven germplasm (IC285388, IC346969, IC285430, IC285427, IC326669, IC311386 and IC285439) which further sub-divided into three sub-clusters. The results indicate that germplasm within each cluster shares similar morphological traits. Notably, germplasm from hilly states is represented across all three clusters, suggesting that the clustering was primarily based on morphological characteristics rather than geographical origin. Consistent with previous studies on morphological characterization, these findings affirm that grouping in citrus genotypes is driven by morphological similarities (39, 41).

Molecular characterization and principal coordinate analysis (PCoA)

A total of 13 galgal germplasm were analyzed using 46 SSR molecular markers, out of which 9 SSR markers resulted in 23 amplicons (representing gels in Fig. 3). The number of alleles ranged from 1-4 with an average of 3.1 alleles per marker. The SSR marker sizes (Table 6) ranged from 175bp (Ma2_94) to 410bp (Ma4_41). The polymorphic information content (PIC) values ranged from 0.260 to 0.698, with a mean value of 0.4851. Among these, Csin.0329 recorded the highest PIC value and gene diversity. Cluster analysis of the similarity matrix obtained from 23 alleles resulted in a UPGMA dendrogram of genetic relationships, which divided the germplasm into two clusters (Fig. 4). Group I consists of three germplasms, whereas Group II comprises ten germplasms. All the germplasms in Group I were collected from Nainital, Uttarakhand, with IC-285430 and IC-285440 found to be genetically similar. The germplasms in Group II were collected from Maharashtra, Uttarakhand, Uttar Pradesh and Meghalaya. In Group II, IC-344936 (East Khasi Hills, Meghalaya) and IC-322100 (Jhansi, Uttar Pradesh) were found to be genetically similar.

Principal coordinate analysis (PCoA) based on binary data from 13 germplasm was conducted to confirm genetic diversity further. It revealed two clusters similar to the UPGMA dendrogram (Fig. 5). Like the dendrogram, the PCoA plots also showed genetic similarity between IC-285430 and IC-285440. This genetic resemblance could be attributed to the widespread dispersal of galgal across the country, influenced by cultural significance (4), its

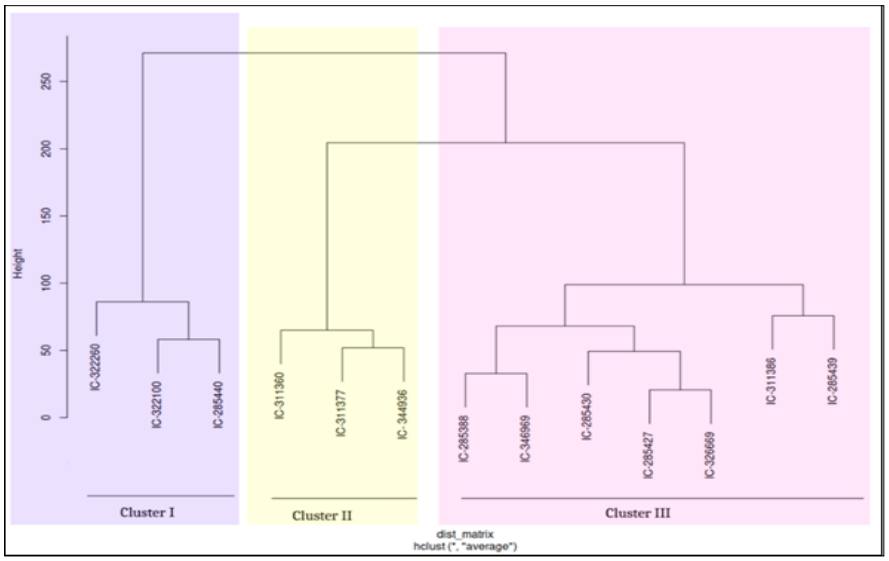

Fig. 2. Dendrogram of 13 galgal accessions based on the 21 morphological traits.

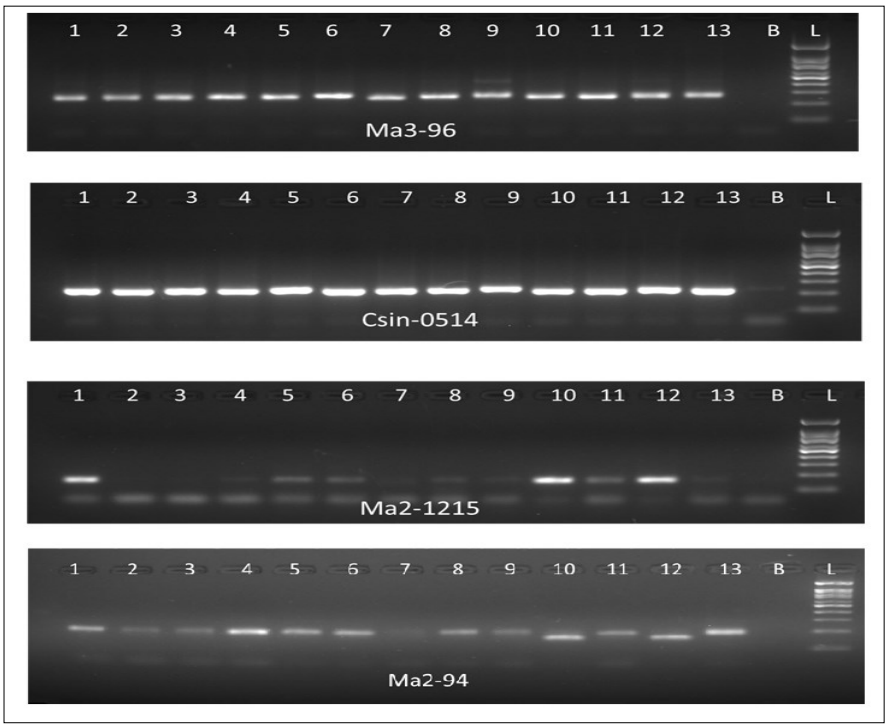

Fig. 3. Polymorphic SSR markers observed in galgal accessions**.** 1)IC-322100, 2) IC-311360, 3) IC-285430, 4) IC-311377, 5) IC-322260, 6) IC-285427, 7)IC-285440, 8) IC- 344936 9) IC-326669, 10)IC-285388, 11) IC-346969, 12)IC-311386, 13) IC-285439, B- Blank, L – 100 bp Ladder.

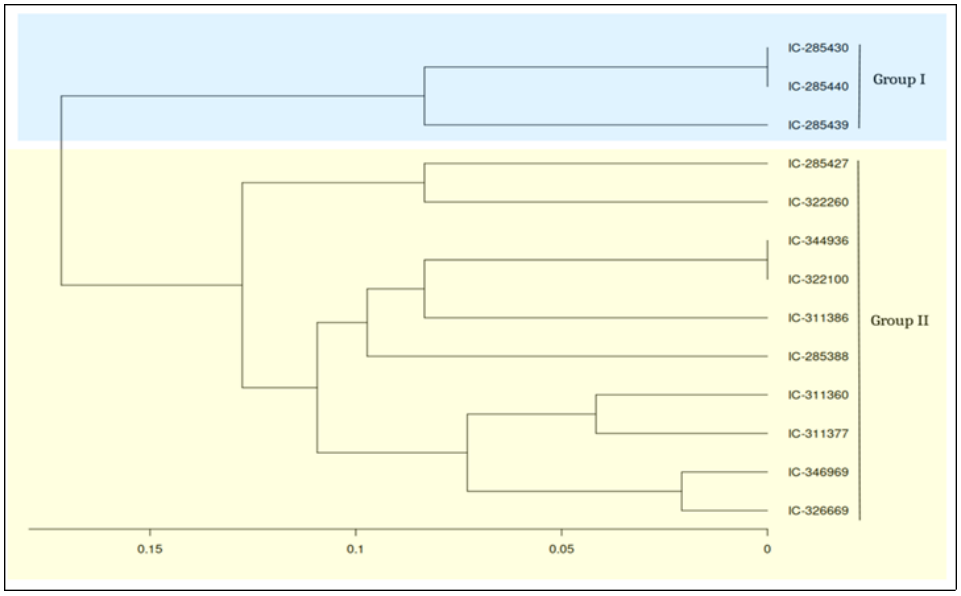

Fig. 4. UPGMA dendrogram demonstrating the genetic diversity and relationships among 13 galgal germplasm**.**

Table 5. Principal component analysis for 21 traits in galgal germplasm

Traits	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Plant height (m)	-.605	.530	.502	.218	-.046	-.107	.073
Plant girth (cm)	-.712	.572	.017	-.001	-.081	.049	-.033
Plant spread (m^2)	-.247	.546	.762	-.038	.154	.044	-.010
Canopy volume (m^3)	-.420	.542	.717	-.027	.076	-.019	.042
Fruit weight (g)	.631	.728	-.129	-.111	.022	.045	.021
Fruit length (cm)	.821	.503	.103	.038	.105	-.027	-.006
Fruit diameter (cm)	.634	.699	-.092	-.082	-.014	-.119	.122
Fruit length/diameter	.353	-.564	.419	.263	.264	.237	-.346
No.of oil glands	-.357	.440	-.593	.020	-.160	.134	.369
Fruit axis diameter (mm)	.406	.672	.113	.405	.117	.039	.118
Rind thickness (mm)	.089	.746	-.366	-.361	.094	-.200	-.203
Number. of segments	.114	.406	-.257	.653	-.356	-.277	-.220
Juice content (%)	-.307	-.002	-.230	.496	.241	-.660	-.166
TSS (^{0}Brix)	-.645	.170	-.081	.337	-.197	.307	.366
Acidity (%)	.751	-.314	.107	-.030	-.400	-.085	.312
TSS: acid ratio	-.830	.277	-.158	.185	.257	.204	-.113
Number of seeds	-.190	.398	-.184	-.818	.247	-.074	-.091
Seed length (mm)	.567	.598	.318	.029	-.327	.152	-.150
Seed width (mm)	.227	.122	-.368	.320	.707	.161	.288
Seed thickness (mm)	.805	-.107	.247	.149	.341	-.069	.278
Seed weight (g)	.364	.318	-.406	.246	-.042	.515	-.452
Plant height (m)	5.978	5.004	2.714	2.11.	1.403	1.129	1.049
% total variance	28.465	23.829	12.922	10.049	6.679	5.374	4.998
% cumulative variance	28.465	52.294	65.216	75.265	81.944	87.318	92.315

Table 6. Genetic diversity parameters of galgal accessions

Locus	No of alleles	Allele size	Ho	He	PIC
Ma2_94	2.000	175-200bp	0.000	0.260	0.260
Ma3_96	3.000	280-300bp	0.000	0.615	0.615
Csin.0514	4.000	220-240bp	0.000	0.663	0.663
Ma2_1215	1.000	260bp	0.000	0.284	0.260
Ma3_98	3.000	270-280bp	0.000	0.917	0.627
Csin.0329	3.000	200-210bp	0.077	0.660	0.698
Ma4_41	2.000	280-410bp	0.000	0.278	0.272
Csin.0524	2.000	260-280bp	0.000	0.970	0.379
Ma3_153	3.000	190-200bp	0.154	0.521	0.592

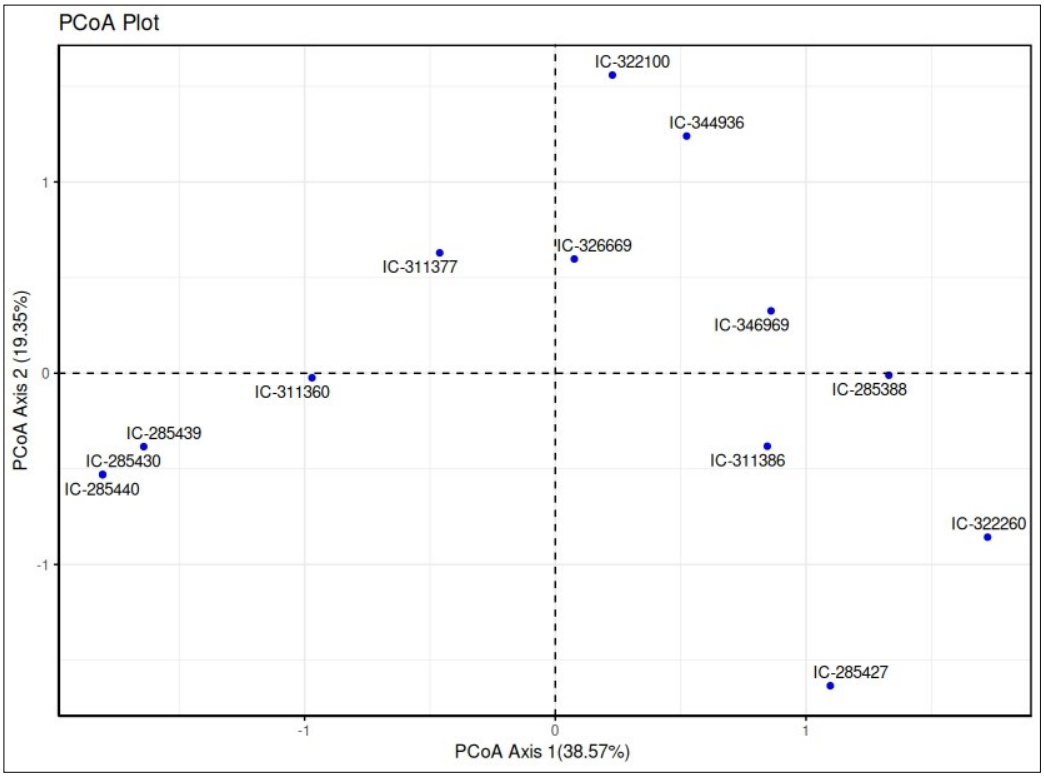

Fig. 5. Principal coordinate analysis of galgal germplasm in India.

nutritional and medicinal properties, its wider adaptability and its use in culinary purposes such as pickles, squash and more (3, 4, 6). The differences in clustering patterns based on morphological traits and SSR markers highlight the complex factors influencing genetic variation, including geographical location, soil type and climate. In citrus, genetic variability is enriched by diverse hybrids, taxonomic species and occurrences of limb sports and bud mutations. The Himalayan region, recognized as one of the primary centres of origin for citrus species, significantly contributes to the genetic pool (42).

Conclusion

Despite its rich diversity and utilisation in the Himalayan tract, Galgal remains one of the underutilized crops within the citrus group. The integration of morphological and molecular analyses in the current study showed substantial genetic variability among the 13 galgal germplasm. While morphological clustering did not show a geographical influence, molecular characterization identified genetic similarities shaped by environmental and evolutionary factors. These findings emphasize the necessity for comprehensive approaches to understanding genetic diversity. Furthermore, the current study identified canopy volume, fruit weight, seed number and rind thickness as essential traits to be considered in galgal improvement programmes.

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Authors' contributions

TA, PT and AA conceived the study, experimented and participated in sequence alignment, statistical analysis, writing, JU, PR and CP collected morphological data and fruit quality analysis. SA carried out isolation DNA, PCR and gel electrophoresis.

Compliance with ethical standards

Conflict of interest: No conflict of interest among the authors

Ethical issues: None

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