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Genetic Diversity Assessment among Native Spinach (*Spinacia oleracea* L.) Cultivars using Morphological Characteristics

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Abstract
Spinach (*Spinacia oleracea* L.) is a nutritious leafy crop worldwide, and the germplasm diversity may influence its performance and application in generating new adaptable cultivars. In this study, some morphological characters were used to assess the genetic variation of 81 local landraces across two years. According to the coefficient of variation, leaf size, dry weight of shoots, number of lateral branches, dry weight of roots, and dry weight of shoots revealed more variations in both years. Based on cluster analysis, spinach landraces were divided into six clusters in both years by verification of the cutoff border via four multivariate ANOVA statistics. Two groups (groups 1 and 6) of the spinach landraces in the first year, along with group 5, had the highest leaf yield performance, while these three clusters (clusters 1, 5, and 6) indicated the highest leaf yield performance in the second year. A comparison of dendrograms from both years indicated that 15 spinach landraces were grouped in the same clusters across the experimental years and did not show any remarkable genotype-by-environment interaction. The current spinach germplasm indicates a good resource of diverse characters that should be managed for future genetic improvement projects aimed at obtaining new cultivars. Finally, the most favorable 15 landraces performed desirably and are recommended for farmers due to their yield performance.
Keywords: Genotype, Environment Interaction, Genetic Diversity, Leaf Yield.

Introduction

Spinacia oleracea L. an annual edible vegetable crop from the family Amaranthaceae, is originated from Iran and identified as a Persian herb (Rafiei et al. 2021). It is one of the desirable leafy vegetables due to its high content of beta-carotene, vitamins, minerals, and other beneficial nutrients. Spinach with a strong nutrient profile such as enzymatic and non- enzymatic antioxidants, boasts active antioxidants that combat cancer and diabetes (Rashid et al. 2022). In the 2022 growing season, China (30 million tons), the United States of America (382 thousand tons), Türkiye (230 thousand tons) and Japan (208 thousand tons) were the top producers of spinach (FAOSTAT, 2022). In Iran, spinach production is relatively modest, with approximately 104,236 tons grown over an area of about 5455 hectares with a mean yield performance of 1.91 tons per hectare which is lower compared to major producing countries. Iranian producers rely on native spinach landraces which exhibit good adaptability to various environmental conditions, but their yield is low (1.91 tons ha^{-1}), compared to the mean global yield (3.53 tons ha^{-1}) and the highest global yields of 5.08 tons ha^{-1} in Jordan and 4.81 tons ha^{-1} in China (FAOSTAT, 2022). Therefore, there is a crucial requirement for spinach breeding aimed at increasing the genetic ability for yield and other target traits.

Being a main center of genetic diversity for several domesticated plants, Iran must manage and preserve these genetic sources. Most Iranian spinach genotypes are landraces, highly adapted to specific and varied environmental conditions, making them useful for genetic variation (Rafiei et al., 2021). Thus, utilizing the current genetic diversity requires knowledge about genetic variations, so

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characterizing and categorizing such genotypes is essential (Arabsalehi et al., 2016). Improvement of yield performance in spinach has been a key objective for breeders, but it is heavily influenced by environmental conditions and thus exhibits low heritability. Consequently, the response to direct selection for yield can be unpredictable. Breeders rarely focus on a single trait, making it essential to examine the associations among various traits, particularly between yield and other traits (Salgotra and Stewart Jr, 2020). Traditional statistical techniques may be insufficient for explaining these associations, necessitating the use of advanced statistical tools for modeling crop yield.

Rashid et al. (2022) assessed the genetic variation of 300 spinach genotypes from 30 countries, via 21 agronomic and morphological characters and found high diversity for height of plant, length of stipule, area of leaves, and according geographic properties reported that the accessions of Türkiye and China had high genetic variation. Rafiei et al. (2021) studied the genetic variation of 22 spinach genotypes via morphological and molecular SSR markers and found more variation for important characters like leaf properties and type of seeds, as well as more polymorphism by SSR markers, and concluded that current spinach genotypes had good variation regarding tolerant genotypes which provides a good chance for breeders for selecting the most favorable individuals. Jahan (2022) evaluated 51 spinach genotypes and reported high diversity for all the traits including leaf yield, attitude of stipule and lateral branches and genotypes were categorized into five clusters based on main characteristics like leaf morphology and yield performance. Characterizing of local landraces can be used in breeding plans for future hybridization projects for yield performance increase and is suitable to categorize of genetic variation of spinach. This study aims to evaluate some Iranian spinach landraces based on multiple traits and to assessment the genetic diversity among them.

Materials and Methods

Spinach genotypes (81 landraces) were collected from a wide geographical range of Iran and cultivated in the field, Maragheh (37°23′N; 46°14′E) using a randomized complete block design with four replications across two years (2014-2015). These genotypes were obtained from local farmers, and the codes and names are given in Table 1. The field altitude was 1477 m, the average annual temperature was 11.2 °C and had a loamy and calcareous soil, with minimum organic mass, shortage of nitrogen, and phosphorus, and sufficient potassium content. Thus, 80 kg ha^{-1} of nitrogen (half at the sowing and half at seedling) was used to support optimal plant growth. In experimental units, six 3-m rows with 25 cm space were generated, so the unit area was 4.5 m² but to ensure accurate yield measurements, only four central rows were harvested after removing marginal effects, so the harvested size of each unit was 2.5 m². Manual weeding was done twice, in the pre- and post-flowering stages when weeds populations were increased.

Some morphological characters were recorded based on the method of Sabaghnia et al. (2014), using ten random samples after five weeks, which were chosen from each unit as: number of leaves at flowering (NLF), length of leaf (LL), the width of leaf (WL), the leaf size (LS), the diameter of stipule (DS), length of stipule (LS), lateral branches number (LBN), dry weight of roots (DWR) and dry weight of shoot (DWS). Also, days numbers to flowering (DF) and female plants percent (FPP) were measured in each plot. Finally, leaf yield performance (LYP) was harvested from central rows and thousand-seed weight (TSW) was recorded based on random samples of seeds from each experimental unit. These measurements and data collection strategies are essential for evaluating the performance and genetic potential of the spinach landraces, providing valuable insights for breeding programs aimed at improving yield and other important traits.

Normal distribution of traits in spinach landraces was assessed via the Shapiro-Wilk procedure with the normality option in Minitab software version 14.0 (Minitab Inc., USA). Prior to clustering, the data were standardized to ensure that all traits contributed equally to the analysis, regardless of their original units of measurement (Salgotra and Stewart, 2020). The standardized data were then subjected to hierarchical cluster analysis using Ward's procedure, which reduced the within-cluster variance. The distance between clusters was measured using the squared Euclidean distance. A dendrogram was generated to visualize the clustering results and to determine the optimal number of clusters. The cutoff point for the dendrogram was chosen based on the height of the vertical lines in the dendrogram plot, which represents the distance or dissimilarity between clusters. By examining the dendrogram, a cutoff point was selected where a significant jump in the distance metric indicated the appropriate number of clusters and balanced between granularity. This was performed by the multivariate analysis of variance through the Wilks' lambda and Hotelling statistic as well as Pillai's trace and Roys' maximum root which were applied with the Multivariate statement in SPSS application version 17.0 (SPSS Inc., USA).

Research Findings

According to descriptive statistics (Table 2), the high amounts of coefficient of variation (CV) belonged to leaf size (LS) at 50%, dry weight of shoot (DWS) at 46%, leaf yield performance (LYP) at 46%, lateral branches number (LBN) at 44%, and dry weight of roots (DWR) at 40% in the first year. Similarly, they had high magnitudes of CV in the second year as: LS with 50%, DWS with 31%, LYP with 44%, LBN with 45%, and DWR with 106% (Table 2). The CV of the other remained traits except for days numbers to flowering (DF), was relatively high or moderate which indicated the large variation among studied spinach landraces which collected from different geographical areas of Iran. Thus, this genetic diversity can be utilized to achieve a favorable combination of traits in spinach. Rashid et al. (2020) assessed some spinach genotypes from different regions of the world and reported high variations in the height of plant, length of stipule, leaf area, and leaf morphology and found more genetic diversity in Turkish, Chinese, Afghani and Pakistani genotypes. Identification of the origins helps breeders in using wild species breeding efforts and spinach is supposed originated from the center and southwestern Asia, and was subjected to the domestication process in Iran and immigrated to other areas of the world (Ribera et al., 2021; Gholami et al., 2023). Thus, observation of such huge genetic diversity in the current germplasm of spinach was expected and shows high genetic resources of this vegetable crop in Iran. Also, leaf yield performance (LYP) ranged from 5.5 to 71.8 t ha^{-1} with an average of 23.7 t ha^{-1} in the first year while it ranged from 3.0 to 46.9 t ha^{-1} with an average of 19.2 t ha^{-1} in the second year (Table 2).

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The clustering dendrogram was produced to explore the variation structure among spinach landraces (Fig. 1), and the cutoff border is determined via verification of four statistics of multivariate ANOVA (Table 3). The Wilks' lambada, the maximum root of Roy, traces of Hotelling and Pillai had statistical significance and determined the border of cutoff in the dendrogram across two years. The spinach landraces were grouped into six different groups; the first group (G-1) consisted of 13 landraces, G-2 had 15 spinach landraces and the third group (G-3) contained 12 landraces in the first year (Fig. 1). Also, landraces 3, 16, 32, 45 and 61 were grouped as the fourth group (G-4), 12 spinach landraces fall in the fifth group (G-5), and 24 landraces were categorized as the sixth group (G-6), in the first year (Fig. 1). The clustering method indicated the amount of diversity that could be useful for next genetic improvement projects and some researchers have benefited clustering for the study of morphological traits of spinach showing their intraspecific associations (Sabaghnia et al. 2014; Rafiei et al. 2021). In the second year, the spinach landraces were clustered into six distinct clusters as; landraces 1, 9, 13, 17, 20, 27, 61, 70 and 71 were grouped as the first cluster (C-1), C-2 had 13 landraces and the third cluster (C-3) contained 23 landraces in the second year (Fig. 1). Also, landraces 4, 78, 79, 80 and 81 were categorized as the fourth cluster (C-4), 21 landraces fall in the fifth cluster (C-5), and 10 spinach landraces were located as the sixth cluster (C-6), in the second year (Fig. 1).

Regarding the means of genotypic groups in the first year (Table 4), group G-1 had the highest values for DNF, LL, WL, LS, DS, DWS, TSW and LYP traits. Also, group G-3 indicated the lowest values for all of the measured traits except DNF, DS, LBN and FPP, so group 1 versus group 3 can be assumed as the heterotic groups for cross-breeding programs. The landraces of group G-4 had the highest female plants percent (FPP) and lowest lateral branches number (LBN) in the first year, while the landraces of group G-5 had the biggest leaf size (LS), thinnest diameter of stipule (DS), low FPP and were early maturity, in the first year (Table 4). Finally, group G-6 had the large number of leaves at flowering (NLF), high values of lateral branches number (LBN), and higher dry weight of roots (DWR), while the landraces of group G-2 indicated relatively moderate magnitudes for all of the spinach traits. In the second year, cluster C-1 had the highest values for DNF, LL and LS while cluster C-6 showed the largest amounts for NLF, WL, LBN, DWS and DWR, so both of these clusters were assumed as the best landraces for yield performance and its components (Table 4). The landraces of cluster C-3 had the highest LS and lowest DNF and DS in the second year, while the landraces of group G-5 had the values for DS, TSW and LYP, in this year (Table 4). Finally, cluster G-4 had only high a percent of FPP, and lower values for most of the other remained traits, while similar to the first year, the landraces of cluster C-2 showed relatively moderate values for all of the spinach traits in the second year (Table 4). Two genotypic groups of the first year (G-1 and G-6) following G-5 had the highest leaf yield performance while these three genotypic clusters (G-1, G5 and G-6) indicated the highest leaf yield performance in the second year (Table 4).

A comparison of dendrograms of both years indicated that some spinach landraces were grouped in the same clusters across experimental years and did not show any significant genotype by environment (GE) interaction. Six landraces of the first clusters of both years as 1 (Abhar), 9 (Birjand), 20 (Jahrom), 27 (Kerman), 70 (Tarom) and 71 (Tonkabon), as well as four landraces of the second clusters, cross years as 2 (Amol), 14 (Esfaien), 31 (Lahijan) and 72 (Urmia-A) indicated such ability, so their performance is not affected by GE interaction. Also, seven spinach landraces of the third clusters of both years as 18 (Isfahan-A), 19 (Isfahan-B), 24 (Karaj-C), 38 (Pak-Dasht-B), 53 (Saleh-Aabad), 67 (Tafresh) and 69 (Talesh) as well as six landraces of the fifth clusters cross years as 4 (Ardestan-A), 12 (Brojerd), 15 (Fasa), 28 (Kermanshah), 33 (Mahan) and 52 (Razan) indicated low GE interaction. Finally, the three spinach landraces of the sixth clusters of both years 36 (Nour-Abad), 41 (Qrachak) and 47 (Qum-Saleh) had minimum interaction between genotypes and environments while none of the spinach landraces of the fourth clusters in the first and second years were not similar and their behavior were not predictable due to high GE) interaction occurrence. However, regarding leaf yield performance (LYP) and repeatable results, 15 spinach landraces from three clusters (1, 5 and 6) were suitable for yield production. They were mostly from the center, west and north of Iran as Abhar, Birjand, Jahrom, Kerman, Tarom, Tonkabon, Ardestan-A, Brojerd, Fasa, Kermanshah, Mahan, Razan, Nour-Abad, Qrachak and Qum-Saleh, so they were high widespread and could be advised for cultivation in such areas. Finally, the clustering analysis indicated no significant association between the genetic variation and geographic origin of spinach landraces, as they were from one center origin. Such similar reports were found by Sabaghnia et al. (2014) in the investigation of some local spinach accessions that are cultivated as cultigens.

The coefficient of variations prepared a measure of variability for traits and demonstrated an obvious diversity exist in current spinach landraces, so such genetic diversity for most morphological traits is useful for the next breeding program. Identification of the pattern of this variability in the genetic resources would be significant to planning the perseveration of germplasms, and for exploring the practical usage of gene banks (Peres, 2016). Current findings indicated that there is a good variation among spinach landraces, that can be utilized to get more yield and performance as well as yield components or other targeted characters like thinker diameter of stipule and shorter length of stipule. The grouping of similar spinach landraces depends on the dissimilarity indices among them, which can be explored by morphological markers (Fiore et al., 2019). Our results show a variation among spinach landraces, related to the selection pressures in the breeding programs. The investigation permits a better viewpoint of the spinach landraces via agronomically morphological traits and demonstrates the beneficial aspect of statistical tools like cluster analysis was useful in identifying the most variable traits and can be useful in the future to succeed in genetic improvement projects. We found that, each genotypes have a similar magnitude of genetic variation which may be found and that the distances among genotypes may be restricted. Also, remarkable genetic variation may exist within spinach landraces, so hybridization is possible for obtaining new sources for breeding. The association among traits supports the idea that only a few heritable traits are needed to explain the genetic variation within the spinach germplasm. These traits may engage breeders in the effective management and evaluation of plant materials (Mir-Jalili et al., 2023; Sabaghnia et al. 2023). The 15 desirable landraces are good candidates for commercial release in Iran because they performed well and indicated high amounts of target traits with low GE interaction across two years, so they can be advised for cultivation in semi-arid areas like Iran. In conclusion, the remarkable genetic diversity found in most of the traits showed the genetic factors impact, so the spinach breeding regarding these traits is possible. The spinach landraces used in the present investigation indicated various properties which can be used in different breeding projects. The 15 landraces of current germplasm act in a desirable mood and are recommended for farmers due to their yield performance.

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Table 1. Codes and origin of the 81 collected spinach (*Spinacia oleracea*) landraces.

Code	Origin	Code	Origin	Code	Origin
L1	Abhar	L28	Kermanshah	L55	Sanandaj
L2	Amol	L29	Khoram-Abad	L56	Sarasiab
L3	Arak	L30	Kohban	L57	Sari
L4	Ardestan-A	L31	Lahijan	L58	Saveh-A
L5	Ardestan-B	L32	Langrood	L59	Saveh-B
L6	Azna	L33	Mahan	L60	Shiraz-A
L7	Baft	L34	Maragheh	L61	Shiraz-B
L8	Bam	L35	Mobarake	L62	Shirvan-A
L9	Birjand-A	L36	Nour-Abad	L63	Shirvan-B
L10	Birjand-B	L37	Pak-Dasht-A	L64	Sirjan
L11	Bojnord	L38	Pak-Dasht-B	L65	Sirjan
L12	Brojerd	L39	Pishva	L66	Tabriz
L13	Drood	L40	Qazvin	L67	Tafresh
L14	Esfaien	L41	Qrachak	L68	Taft
L15	Fasa	L42	Quchan-A	L69	Talesh
L16	Hamadan	L43	Quchan-B	L70	Tarom
L17	Hamon	L44	Qum	L71	Tonkabon
L18	Isfahan-A	L45	Qum	L72	Urmia-A
L19	Isfahan-B	L46	Qum-Khor	L73	Urmia-B
L20	Jahrom	L47	Qum-Saleh	L74	Varamin-A
L21	Jajarm	L48	Rahim-Abad	L75	Varamin-B
L22	Karaj-A	L49	Rahnan-A	L76	Varamin-C
L23	Karaj-B	L50	Rahnan-B	L77	Yazd
L24	Karaj-C	L51	Ravar	L78	Zabol
L25	Kashan-A	L52	Razan	L79	Zanjan
L26	Kashan-B	L53	Saleh-Aabad	L80	Zarin-Shar-A
L27	Kerman	L54	Salmas	L81	Zarin-Shar-B

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Table 2. description statistics of 13 traits in 81 spinach landraces across two years.

	The first year					The second year				
	Mean	**SD†**	**Min**	**Max**	**CV‡**	**Mean**	**SD†**	**Min**	**Max**	**CV‡**
DNF¶	71.85	8.61	50.00	98.00	11.99	75.17	6.67	52.00	91.00	8.87
NLF	16.36	4.71	6.00	36.00	28.81	14.21	3.87	6.00	29.00	27.21
LL	15.26	4.39	6.61	31.69	28.79	13.25	3.70	5.34	26.82	27.94
WL	9.65	2.68	3.61	17.76	27.73	7.94	2.18	2.86	14.12	27.45
LS	98.68	49.42	14.66	310.30	50.08	81.75	40.97	13.73	254.54	50.12
DS	6.58	1.70	3.21	13.19	25.82	7.73	1.83	3.43	13.64	23.64
LS	9.11	2.40	3.80	17.23	26.34	8.05	1.85	3.76	13.44	23.00
LBN	3.99	1.75	0.00	11.00	43.88	3.07	1.38	0.00	8.00	44.86
FPP	63.67	17.20	15.00	92.00	27.02	58.52	16.47	15.00	93.00	28.15
DWS	12.20	5.64	2.11	66.33	46.21	13.24	4.12	2.47	32.87	31.13
DWR	7.85	3.17	3.99	22.90	40.44	7.13	7.54	0.96	82.88	105.67
TSW	10.62	3.97	2.89	26.18	37.34	8.95	3.54	2.14	21.58	39.49
LYP	23.69	10.87	5.53	71.77	45.90	19.21	8.36	3.04	46.93	43.54

†SD, standard deviation; ‡CV, coefficient of variation.

¶Traits are days numbers to flowering (DF), number of leaves at flowering (NLF), length of leaf (LL), width of leaf (WL), leaf size (LS), diameter of stipule (DS), length of stipule (LS), lateral branches number (LBN), dry weight of roots (DWR), dry weight of shoot (DWS); female plants percent (FPP), thousand-seed weight (TSW), and leaf yield performance (LYP).

Table 3. Statistics of Multivariate ANOVA for verification the cutoff point in dendrogram of 81 spinach landraces.

Year	Statistics	Value	F†	Hypothesis df‡	Error df	Sig.
The first year	Pillai's Trace	2.78	6.45	65	335	0.000
	Wilks' Lambda	0.01	10.02	65	301.7	0.000
	Hotelling's Trace	16.06	15.17	65	307	0.000
	Roy's Largest Root	9.43	48.58	13	67	0.000
The second year	Pillai's Trace	2.67	5.93	65	335	0.000
	Wilks' Lambda	0.01	8.36	65	301.7	0.000
	Hotelling's Trace	12.47	11.78	65	307	0.000
	Roy's Largest Root	8.11	41.78	13	67	0.000

†F, statistic of F ratio; ‡df, degrees of freedom.

Table 4. Averages of measured traits for six identified clusters of spinach landraces across two years.

	The first year						The second year					
	G1	**G2**	**G3**	**G4**	**G5**	**G6**	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**
DNF¶	80.50	75.57	74.17	75.81	52.25	78.75	78.92	71.03	54.50	63.17	71.50	74.25
NLF	23.50	14.80	11.10	11.25	11.75	24.25	20.48	15.42	14.75	13.08	22.50	26.25
LL	21.75	13.28	9.97	20.31	15.54	14.63	20.84	14.31	18.57	7.96	17.64	18.40
WL	12.72	8.17	5.52	10.48	7.17	11.03	12.67	9.19	8.73	4.25	11.29	14.38
LS	231.99	82.35	45.44	142.52	71.58	133.96	166.10	87.06	77.91	24.42	126.30	158.80
DS	10.44	8.14	5.94	6.93	4.44	9.35	7.59	6.46	4.00	4.36	8.39	7.82
LS	9.46	8.39	5.97	8.00	11.96	9.58	12.00	8.53	15.38	6.07	9.85	9.64
LBN	4.75	3.20	2.71	1.56	2.50	5.00	4.63	3.88	3.25	3.42	1.25	8.25
FPP	62.00	56.38	60.75	84.31	42.75	69.00	63.77	63.94	63.25	69.75	40.25	51.25
DWS	22.47	14.11	8.30	12.39	14.78	16.59	16.31	11.31	14.10	5.58	15.04	28.91
DWR	7.32	7.79	3.37	3.96	7.22	28.21	8.71	7.60	8.93	6.26	8.55	14.76
TSW	11.71	9.66	5.89	6.88	10.36	9.96	12.79	10.12	11.94	6.80	22.03	11.93
LYP	41.48	20.67	9.65	19.18	22.67	28.95	36.49	21.09	26.04	9.18	47.86	35.32

¶Traits are days numbers to flowering (DF), number of leaves at flowering (NLF), length of leaf (LL), width of leaf (WL), leaf size (LS), diameter of stipule (DS), length of stipule (LS), lateral branches number (LBN), dry weight of roots (DWR), dry weight of shoot (DWS); female plants percent (FPP), thousand-seed weight (TSW), and leaf yield performance (LYP).

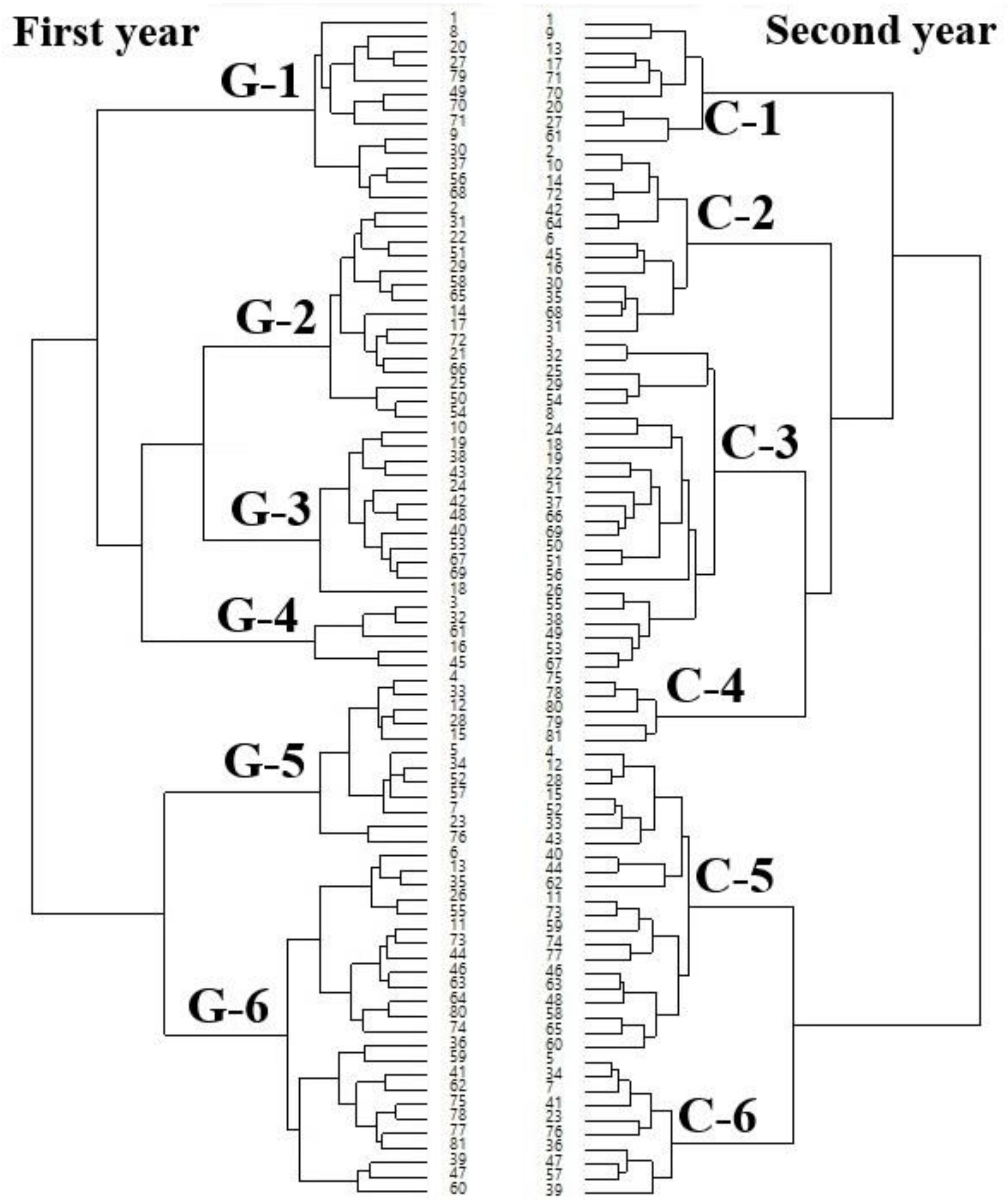

Fig. 1. Dendrograms of cluster analyses for 81 spinach landraces across two years.

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