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Research Article
Assessment of Genetic Diversity and Phytochemical Analysis of *Nigella sativa* Genotypes from Pakistan

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Abstract

Background and Objective: *Nigella sativa* belongs to family Ranunculaceae and is one of the safest plant extracts for human consumption. The present study was conducted to determine the genetic diversity and phytochemical analysis of *N. sativa* from Pakistan. **Methodology:** Study was conducted on 35 *N. sativa* genotypes to assess their genetic diversity using four Random Amplified Polymorphic DNA (RAPD) markers along with six phytochemicals parameters (alkaloids, flavonoids, saponins, phenols, tannins and terpenoids). Qualitative and quantitative analysis were done for phytochemical attributes of all selected genotypes. Phytochemical data were subjected to analysis of variance, correlation and cluster analysis. Molecular data were subjected to cluster analysis based on bivariate data and Unweighted Pair Group Method for Arithmetic averages (UPGMA) using STATISTICA software. **Results:** From molecular analysis 99.29% polymorphism was found in all genotypes. Pk-020990 and Pk-021915 showed diverse phylogenetic relationship (80%). Dendrogram clustered all the genotypes into six groups in molecular study and five groups in phytochemicals. All phytochemicals were present in ethanol extract and were found highly significant ($p \leq 0.05$). Positive correlation was found between solvents; ethanol and n-hexane (0.383*) while negative correlation was found between phenols and tannins (-0.375*). **Conclusion:** Genotypes; Pk-021395 and Pk-020781 exhibited maximum number of phytochemicals (5 times) in frequency analysis based on highest concentration of top ten superior genotypes and are therefore recommended for utilization in future breeding programs.

Key words: *N. sativa*, phytochemicals, RAPD markers, dendrogram, flavonoids, saponins

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nigella sativa L. (black cumin or black seed), the annual flowering member of the family Ranunculaceae is a natural dietary supplement, diploid (2n = 12), containing 20 species and economically important with 2500 years of history, make it one of the safest plant extracts for human consumption[1].

Nigella sativa seeds have been used for thousands of years as folk medicine and some of its active compounds were reported against many ailments. Different pharmacological effects have been reported by different authors of this medicinal plant such as gastric ulcer healing, anti-microbial, anti-viral and anti-cancer activity, cardiovascular disorders, antioxidant activity, anti-inflammatory, anti-asthmatic, anti-anxiety and anti-tumor effects and tumor growth suppression[2-5].

Germplasm is a crucial source to generate new types of plants with desirable traits that increases crop production with quality[6]. The genetic diversity is analyzed by using genetic based tools, advanced molecular methods and DNA techniques etc.[7,8]. Quantitative variances of genetic traits provide an estimate of genetic diversity. For DNA profiling, PCR based method Random Amplified Polymorphic DNA (RAPD) technique is widely used in breeding plans to develop superior cultivars and to find genetic diversity of various plant species because it detects genetic variability in short time which is a pre-requisite for marking genes to increase yields and to stabilize production for environmental fluctuations and disease epidemics[9].

Various plants have been analyzed and cultivated as new oil crops because they have important oil and bioactive compounds which possess unique chemical properties and may enhance edible oils supply. Products derived from plant roots, leaves, fruits and seeds are the part of phytomedicines[10]. For the synthesis of complex chemical compounds understanding of the plants chemical constituents is necessary[11]. *Nigella sativa* has more than 100 diverse phytochemical constituents, containing fiber, sugars, saponins, flavonoids, tannins, alkaloids and organic acids perform activities in different plants and are synthesized by primary or secondary metabolism of living organisms[12,13]. These phytochemicals are widely used in veterinary, human treatment, agriculture and associated studies[14].

Though many studies have been reported on different aspects of *N. sativa* genotypes globally, very few have been reported from Pakistan. So the current research was aimed to assess genetic diversity and phytochemical analysis in the available germplasms of Pakistan. This will help to identify the potential genotypes for exploration of this important medicinal herb as well as to predict some phytochemicals among different genotypes of *N. sativa* which can act as strong inhibitors against wide range of infectious and microbial diseases.

MATERIALS AND METHODS

Plant sample: The present research work was carried out in the Department of Biotechnology and Biochemistry, PMAS Arid Agriculture University, Rawalpindi during 2015-2016. Thirty five accessions of *N. sativa* were obtained from PGRI, NARC Islamabad for the study of DNA polymorphism and phytochemical analysis.

DNA isolation and PCR amplification: Genomic DNA was isolated from selected plant materials through standard CTAB method described by Doyle and Doyle[15] with little modifications (i.e., 40 min incubation time at 60°C and three times repeated washing with 70% ethanol). Initially 20 Randomly Amplified Polymorphic DNA (RAPD) markers were tested to find genetic diversity among tested genotypes but finally 4 polymorphic primers (AC-05, OPA-5, GLB-13 and GLB-17) were chosen for PCR amplification of DNA. Polymerase Chain Reaction (PCR) was carried out using the protocols of Roder *et al.*[16]. Initial step of PCR was at 94°C for 3 min, 35 cycles of 94°C for 1 min, annealing temperature 34°C for 1 min and elongation of 72°C for 1 min and final extension of 72°C for 7 min was carried out. The sample tubes were then stored at 4°C. The PCR product was run in 2% agarose gel and visualized under UV light in gel documentation system for checking DNA fragments.

Phytochemical analysis

Extract preparation: Extract of all studied genotypes was prepared in Soxhlet extraction assembly. Four grams seeds were mixed in 250 mL ddH_2O and tested in GC-grade n-hexane and absolute ethanol (99.9 %)solvents. Extract was then filtered, dried, weighed and then kept at 4°C until use[17].

Qualitative analysis: The protocol given by Sheela[18] has been used for the qualitative analysis of alkaloids, flavonoids, saponins, phenols, tannins and terpenoids.

Quantitative analysis

Alkaloids determination: Alkaloid determination in all studied genotypes was done using protocol of Edeoga *et al.*[19].

Tannin determination: Fifty milligrams ground seed sample was added in 5 mL ddH_2O, placed in shaker for 1 h and then filtered in flask. A 5 mL filtrate was taken and mixed in 2 mL of

0.1 M $FeCl_3$, 0.004 M potassium ferrocyanide and 0.1 M HCl. 120 nm wavelength was used to calculate the absorbance in spectrophotometer[20].

Determination of total phenols: Five grams sample was boiled in ether for 10 min, 5 mL extract was taken and mixed in 5 mL ddH_2O, then added 5 mL amyl alcohol and 2 mL NH_4OH. A spectrophotometer at 505 nm wavelength was used to measure the absorbance.

Flavonoids determination: A 5 g sample was mixed in 50 mL ethanol and then filtered, dried in water bath and weighed. Absorbance was measured at 420 nm wavelength for flavonoids determination[21].

Saponin determination: Five grams sample of each genotype was subjected to saponin analysis following the method of Okwu and Nnamdi[22].

Determination of terpenoids: Two grams sample was mixed in 25 mL ethanol and placed for overnight. It was filtered and mixed in ether (50-70°C), then dried and considered as total terpenoids.

Statistical analysis: For molecular analysis bivariate data were subjected to cluster analysis for computing a dendrogram through "STATISTICA" software. Similarity coefficients were generated according to presence and absence of bands. Genetic distance was evaluated using Nei's standard genetic distance[23]. Spectrophotometer (Optima® 3000 plus) was used for quantitative determination of phytochemicals. Correlation and variance analysis (Type 1 ANOVA) were done by SPSS version 19. Cluster analysis was conducted on STATISTICA Version 10 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

During present study 4 RAPD markers produced sum of 269 bands of DNA in 35 genotypes. Out of them 267 (99.29%) bands were found to be polymorphic in one or other variety of all 35 genotypes that is almost in close agreement with the Kapital *et al.*[24] who studied 100% polymorphism using ISSR primers for *N. sativa* genotypes. Average polymorphic bands in each marker were 67.25 (Fig. 1a, b).

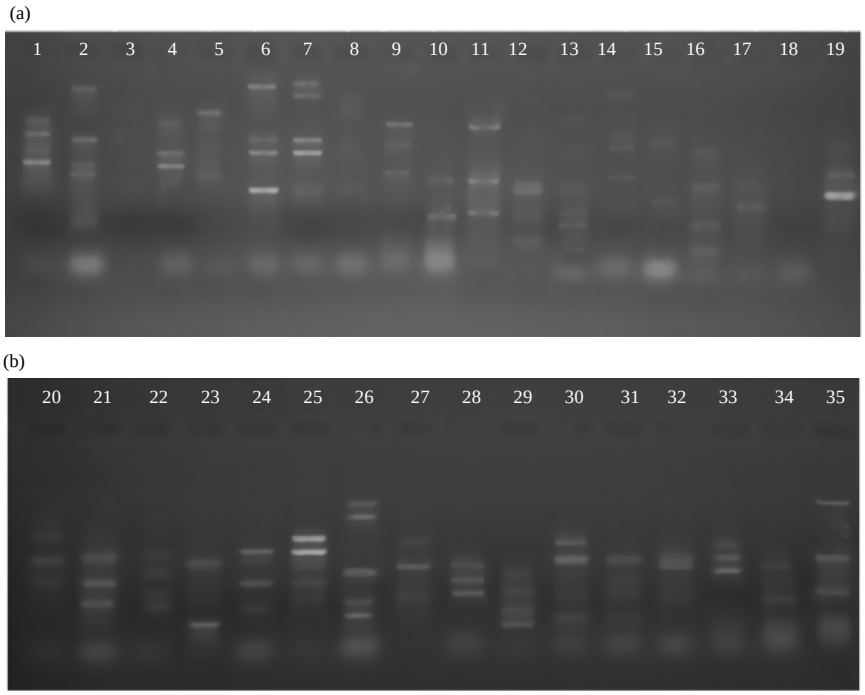

Fig. 1(a-b): PCR amplification profile of *N. sativa* genotypes using primer GLB-17

Lane 1: Pk-020576, Lane 2: Pk-020585, Lane 3: Pk-020609, Lane 4: Pk-020620, Lane 5: Pk-020699, Lane 6: Pk-021395, Lane 7: Pk-021428, Lane 8: Pk-021475, Lane 9: Pk-021753, Lane 10: Pk-021774, Lane 11: Pk-021775, Lane 12: Pk-021776, Lane 13: Pk-021777, Lane 14: Pk-021778, Lane 15: Pk-021805, Lane 16: Pk-021807, Lane 17: Pk-021855, Lane 18: Pk-021913, Lane 19: Pk-021914, Lane 20: Pk-021915, Lane 21: Pk-021916, Lane 22: Pk-021954, Lane 23: Pk-021958, Lane 24: Pk-022053, Lane 25: Pk-022083, Lane 26: Pk-022089, Lane 27: Pk-022115, Lane 28: Pk-022134, Lane 29: Pk-022155 Lane 30: Pk-022301, Lane 31: Pk-020781, Lane 32: Pk-020754 Lane 33: Pk-020904, Lane 34: Pk-020990 and Lane 35: Pk-021090

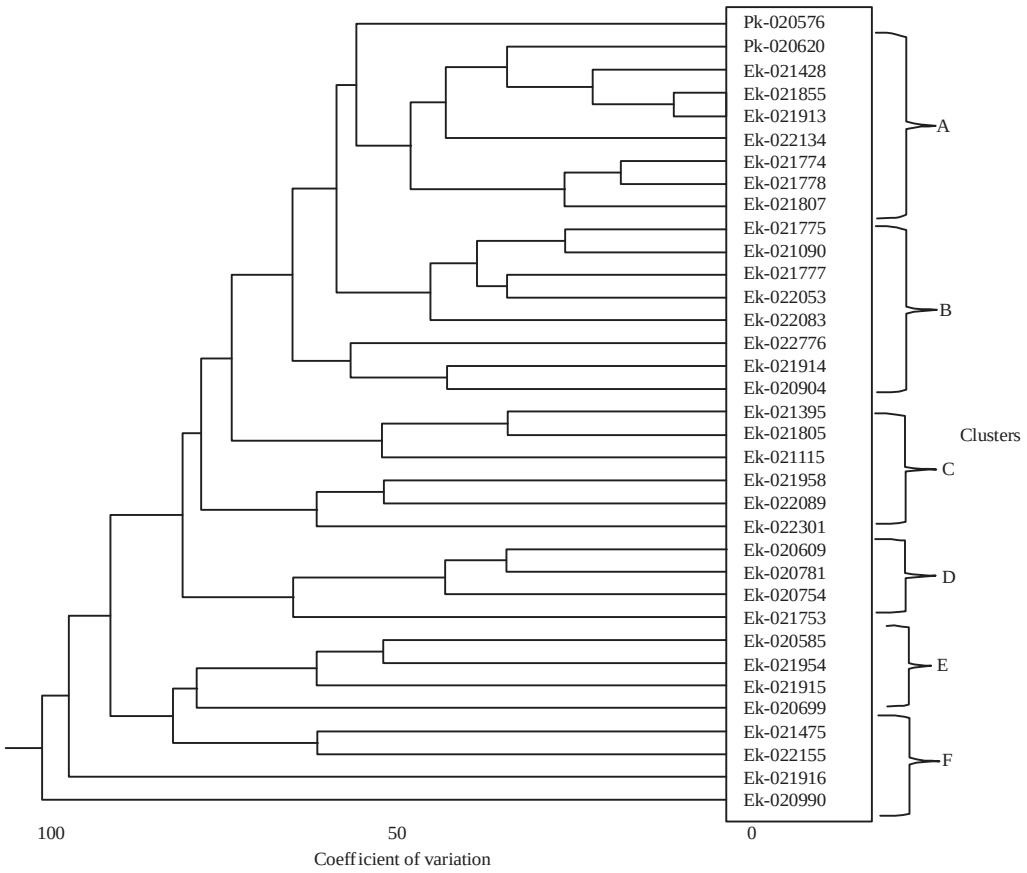

Fig. 2: Dendrogram representing different clusters in 35 genotypes of *N. sativa* for PCR-RAPD markers

Genetic Distance (GD) and cluster analysis: The UPGMA dendrogram constructed using four RAPD primers confirmed that all the 35 *N. sativa* genotypes were sorted into six groups i.e. A, B, C, D, E and F at 50% Euclidean distance by cluster analysis. The group A was sorted into 9 genotypes while group B, C, D, E and F were sorted into 8, 6, 4, 3 and 5 genotypes, respectively (Fig. 2). Different grouping pattern shows that these genotypes are from dissimilar origin and same grouping pattern shows similarities among genotypes.

The overall mean Genetic Distance (GD) was estimated in the range of 0-80% among 35 genotypes of *N. sativa* which is higher than the GD estimated in the range of 25-51% by Kapital *et al.*[24]. The lowest GD (0%) was estimated in three combinations as in Pk-021778 and Pk-021774, Pk-021855 and Pk-021428 and in Pk-021914 and Pk-021913, while the highest GD (0.8) was recorded in between Pk-020990 and Pk-021915 that is 80% (data not shown).

Phytochemical analysis: All phytochemicals were present in ethanol extract while n-hexane extract possessed only saponins (Table 1). This revealed the richness of *Nigella* plant in such chemicals. The presence of these phytochemicals in most of these samples supported the reports of Kamal and Ahmad[25]. They studied the presence of mentioned phytochemicals in different phases of germination of *N. sativa.*

Table 1: Phytochemicals response of studied genotypes in ethanol and n-hexane extracts

Photochemicals	Ethanol extract	n-hexane extract
Alkaloids	++	-
Flavonoids	+++	-
Saponins	+++	+
Phenols	+++	-
Tannins	++	-
Terpenoids	+++	-

+: Present, ++: Moderately present, +++: More present, –: Not found

Alkaloids have significant physiological effects on animals and humans[26]. Flavonoids are present in vegetables, fruits, flowers etc., they possess anti viral, anti fungal and anti bacterial activities. Saponins possess detergent qualities also used in treating injuries, stop bleeding and help in coagulation of RBC's[27]. Phenols can prevent blood clotting and enhance

immunity in some species of plant and can act as anti-oxidant and hormone modulators in plants[26]. Tannins help in fast healing and motivate mucous membranes. Terpenoids have the ability to dissolve oils and other lipids.

Phytochemical analyses were done through Lamda25 UV/VIS Spectrometer. Oil percentage in ethanol extract was 38.29 much higher than n-hexane extract (28.83). Amount of Alkaloid was also recorded as 0.239 mg/100 g (g %) in thirty five genotypes. Flavonoids, saponins, phenols, tannins and terpenoids were recorded as 0.040, 0.37, 0.89, 1.36 and 0.043, respectively (Table 2). Edeoga *et al.*[28] studied the percentage of these phytochemicals in *H. suaveolens* and *O. gratissimum* hybrids from Nigeria. All phytochemicals were present there but with different ratios as compared to current study. The quantitative analysis of these chemicals in this plant will be motivating area in further studies.

The analysis revealed positive correlation between ethanol and n-hexane extracts (0.383). Ethanol extracts showed high number of phytochemicals than that of n-hexane. Analysis of ethanol with alkaloids, flavonoids, saponins and tannins (0.099, 0.032, 0.274 and 0.092), n-hexane with alkaloids, flavonoids, saponins and phenols (0.129, 0.018, 0.085 and 0.072), alkaloids with saponins and tannins (0.075 and 0.061), flavonoids with tannins (0.055), saponins with phenols (0.140), tannins with terpenoids (0.152) was found positively correlated respectively while found negative correlated with the rest of all. Phenol was found highly negative correlated with tannins (-0.375) (Table 3). The correlation matrix of the various phytochemicals was also quantified by Somit *et al.*[29] in leaf of *Croton bonplandianum* which supported the current research in case of tannins with flavonoids and saponin with phenol. But in other cases it doesn't support the present study. The difference came in correlation result between this and Somit *et al.*[29] studies might be due to physiological, morphological and physiochemical differences in plants as well as different habitat and climatic changes in both plants.

From the analysis of variance (ANOVA-1) ($p \leq 0.05$) it was revealed that all the phytochemicals were found to be highly significant (Table 4). It was in contradiction with the study conducted by Somit *et al.*[29] where analysis of variance were non-significant at ($p \leq 0.05$) in many cases. Significant arrangement of these phytochemicals in particular genotypes could be utilized in progress of genotypes in near future as well as could be suggested for herbal researches.

All the 35 studied genotypes were sorted on the bases of phytochemicals into five groups i.e., A, B, C, D and E at 50%

Table 2: Quantitative study of thirty five genotypes of *N. sativa* with values on the bases of phytochemicals

Photochemicals	Quantity (mg /100 g)
Ethanol extract	38.29
n-hexane extract	28.83
Alkaloids	0.239
Flavonoids	0.040
Saponins	0.37
Phenols	0.89
Tannins	1.36
Terpenoids	0.043

Values were taken as average mean of triplicate assessment for all 35 genotypes

Table 3: Correlation for selected phytochemicals in thirty five genotypes of *Nigella sativa*

	Ethanol	n-hexane	Alkaloids	Flavonoids	Saponins	Phenols	Tannins	Terpenoids
Ethanol	1							
n-hexane	0.383*	1						
Alkaloids	0.099	0.129	1					
Flavonoids	0.032	0.018	-0.102	1				
Saponins	0.274	0.085	0.075	-0.019	1			
Phenols	-0.045	0.072	-0.130	-0.204	0.140	1		
Tannins	0.092	-0.041	0.061	0.055	-0.208	-0.375*	1	
Terpenoids	-0.268	-0.286	-0.081	-0.022	-0.148	-0.310	0.152	1

*Significance of correlation was at the level of 0.05% (2-tailed)

Table 4: Comparative analysis for selected phytochemicals in thirty five genotypes of *N. sativa* on the bases of analysis of variance

Photochemicals	SS	Df	Mean square	F	Significance
Ethanol	91.08060	34	2.67884	281.28	0.000
n-Hexane	88.01270	34	2.58861	250.64	0.000
Alkaloids	7.51456	34	0.22102	17686.6	0.000
Flavonoids	0.07979	34	0.00235	7.34	0.000
Saponins	7.18596	34	0.21135	53.84	0.000
Phenols	46.01660	34	1.35343	60.20	0.000
Tannins	477.35300	34	14.03980	1537106	0.000
Terpenoids	0.30217	34	0.00889	599.53	0.000

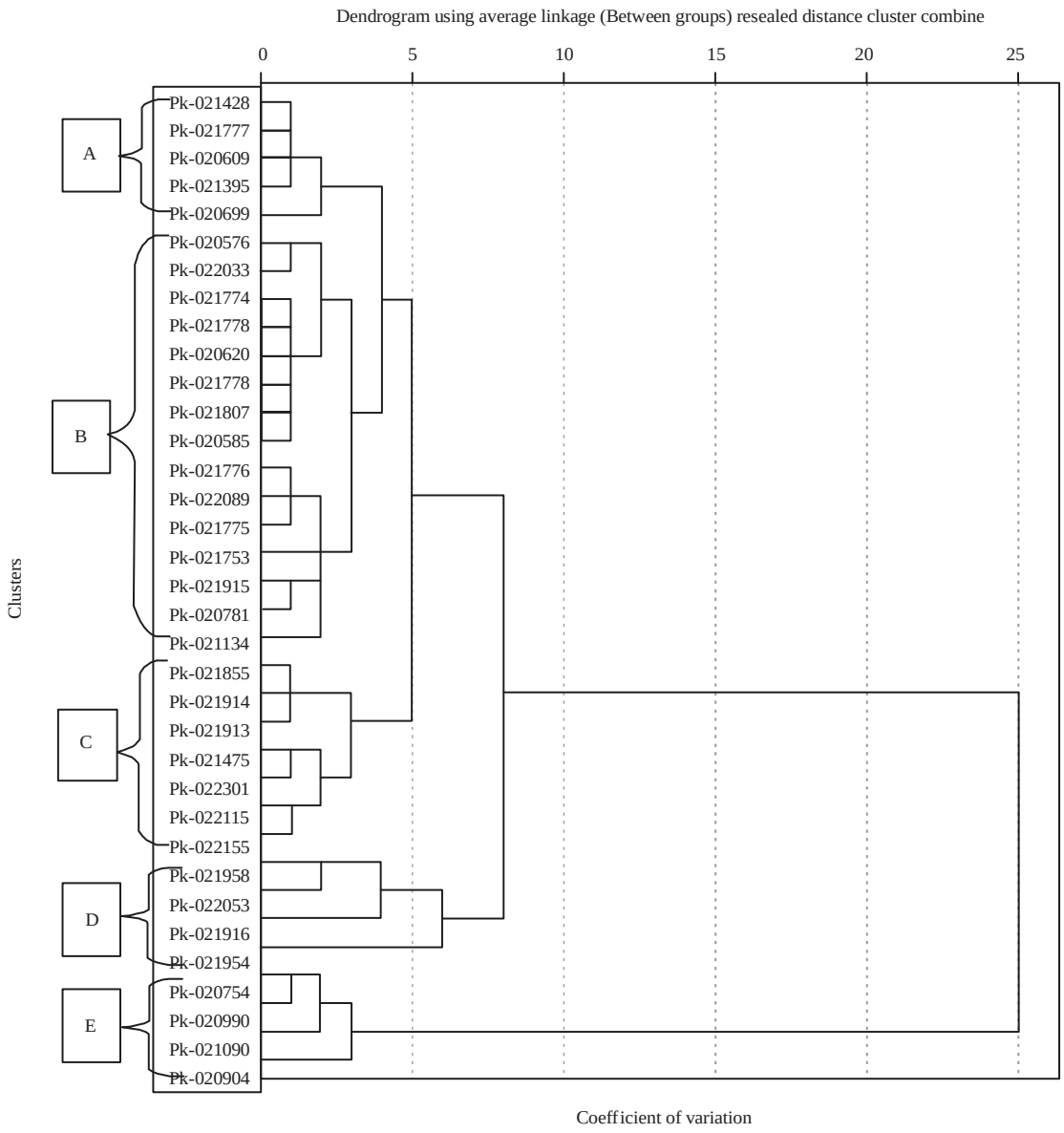

Fig. 3: Dendrogram representing different clusters for selected phytochemicals in 35 genotypes of *N. sativa*

Euclidean distance by cluster analysis. Group A was comprised of 5 genotypes, group B was the largest group having 15 genotypes. Similarly group C, D and E were consisting of 7, 4 and 4 genotypes, respectively. These genotypes showed similarity to each other while different from the rest of genotypes (Fig. 3).

Top ten superior genotypes: On the bases of analysis of top ten superior genotypes for frequency analysis Pk-021395 and Pk-020781 revealed maximum number of phytochemicals (5 times) followed by Pk-021777 and Pk-020609 recorded 4 times for frequency (Table 5). These genotypes could be utilized by farmers for local use in the country as well as could be used to get specific phytochemicals in higher amount for herbal research. The information on genetic diversity can further be linked to active medicinal compounds of the *N. sativa* seed. This could be very useful for the selection of germplasm resource for breeding and commercial sourcing. In spite of low reproducibility and dominant nature RAPD technique proved to be useful tool in identifying *N. sativa* germplasm and determining genetic diversity among the available accessions. This study can be further extended by using advanced molecular marker investigations and their association with valuable attributes of this species.

Table 5: Top ten superior genotypes on the basis of phytochemical analysis

Accession No.	F	Ethanol	n-hexane	Alkaloid	Flavonoid	Saponin	Phenol	Tannin	Terpenoid
pk-021395	5	+	+	+	+	+	-	-	-
Pk-020781	5	+	-	+	+	-	-	+	+
Pk-021777	4	+	+	-	-	+	+	-	-
Pk-020609	4	+	+	+	-	-	+	-	-
Pk-021775	4	-	+	+	-	+	-	-	+
Pk-022053	4	-	+	+	-	+	-	+	-
Pk-021954	4	-	-	+	-	+	-	+	+
Pk-020699	3	+	+	+	-	-	-	-	-
Pk-021428	3	+	+	-	-	-	+	-	-
Pk-021916	3	+	-	-	+	-	+	+	-

+: Present, -: Absent

CONCLUSION

In the current study, RAPD analysis of thirty five genotypes of *N. sativa* revealed maximum amount of polymorphism (99.29%). The Pk-020990 and Pk-021915 genotypes exhibited the highest diverse phylogenetic relationship. Genotypes Pk-021395 and Pk-020781 recorded maximum number of phytochemicals (5 times) and thus are recommended for maximum cultivation across the country. Genotypes with great potential of yielding maximum number of phytochemical and having maximum DNA based genetic differentiation could be proved promising candidates in future breeding programs.

SIGNIFICANCE STATEMENTS

This study discovers the genetic affiliation and phytochemical analysis profile of available *N. sativa* genotypes from Pakistan that can be beneficial for future breeding programs of this miracle herbal specie. The phytochemicals screened in this study play a vital role in economic as well as therapeutic advancement in countries like Pakistan that have great potential for mass production of *N. sativa*. This study will provide a basic knowledge about N. *sativa* future research and management to get both genetically and phytochemically promising varieties.

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