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MOLECULAR CLASSIFICATION OF THE SOME SPECIES OF FAMILY FABACEAE USING RAPD MARKERS

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SUMMARY

The following research comprised the molecular study of cultivars associated with different species of cowpea (*Vigna unguiculata*), broad bean (*Vicia faba*), and peas (*Pisum sativum*) in the family Fabaceae, using random amplified polymorphic DNA (RAPD) markers for genome classification. Overall, the results generated 406 random bands with primers, and some were variant and others had distinct fragment sizes ranging from 100 to 3,000 bp, which distinguished the cultivars of different species. The species *Vigna unguiculata* cultivars showed the highest number of unique bands, while the French cultivar of the species *Pisum sativum* revealed the fewest bands with no unique bands. The genetic distance among the different cultivars ranged from 0.122 to 1.231 cM. The dendrogram revealed three main clusters. The RAPD proves to be a useful tool for evaluating genetic diversity and relationships among different genotypes.

Keywords: Fabaceae, cowpea, broad bean, peas, RAPD markers, molecular identification, genetic distance

Key findings: Based on the studies, the different species cultivars displayed characteristics of unique bands with the highest genetic variation. The determined genetic distance may be effective for breeding programs, as RAPD markers showed the maximum genetic variation, fingerprint, and dimensions in the studied species cultivars.

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INTRODUCTION

Fabaceae is the third largest family of angiosperms, with approximately 20,000 species across 700 genera, including trees, shrubs, vines, and herbs worldwide (Lewis *et al.*, 2005; LPWG, 2013). The said family comprises grain, pasture, and agroforestry species, often serving as forage crops, such as soybeans, peas, alfalfa, cowpeas, and clover. These forage plants have a crucial role in both animal and human nutrition, directly and indirectly, as well as in enhancing the yield and quality of animal products (Reckling *et al.,* 2016; Younis and Saeed, 2023). Recently, using the molecular sections of the DNA has helped elucidate the genetic links among various families and their species. These genetic similarities and variances became the basis in taxonomic and breeding research to acquire the commercially and nutritionally useful crop plants (Mevlude, 2020).

These crops appeared very economical, as their seeds contain the highest levels of protein (23%–37%), in addition to sugars, starches, vitamins, amino acids, carbohydrates, and fatty substances (Matlob *et al*., 1989; Al-Sekmani *et al*., 2018). The genus bean is especially distinct with its lower number of chromosomes (2n = 12) than other species belonging to the same family, followed by peas (2n = 14) and cowpeas (2n = 22) (Hassan, 2002). The leguminous family Fabaceae comprises approximately 20,000 species across 700 genera (Al-Samarrai, 2014).

Knowledge about the genetic variation and differences within the inbred lines of crop species is a prerequisite for the initiation of successful breeding and improvement programs aiming at developing distinct cultivars and new genotypes. Moreover, the assessment of genetic bases that could accrue before and after involvement of breeding programs can bring understanding of genetic variations and kinship among the crop cultivars, developing new strains (Al-Ghamdi, 2009; Al-Sekmani *et al*., 2018).

The efficiency of convenience, accuracy, and availability in identifying the plant genome and cost-effectiveness relative to other methodologies renders random amplified polymorphic DNA markers very appealing in crops' development programs (Omair, 2009). Specific RAPD-PCR markers have proven beneficial for early detection and selection of field crops' enhanced strains and genotypes by exposing superior and stable traits before sowing, offering a less time-consuming and more cost-effective alternative to the traditional methods (Al-Talib, 2021; Alwan *et al.*, 2024). Therefore, based on the above discussion, the presented research will assess the genetic variation and genetic distance across different variants of some Fabaceae species using RAPD-DNA markers.

MATERIALS AND METHODS

Collection of plant samples

The collection of studied genotype samples after one and a half months of planting had 4-6 young and newly developed leaves taken from their developing apex (Al-Jumaily, 2015; Sarhan *et al*., 2015). The leaves, as placed in sterile bags, proceeded to the laboratory directly for isolation and DNA extraction using the specific methodology obtained from the Korean Company, Favorgen and Biotech-CORP.

Genomic DNA extraction

The DNA extraction came from young leaves of different species cultivars, which were 45 days old. The purity measurement of the DNA used a NanoDrop device, with the RAPD-DNA markers applied to the DNA of the six different genotypes, employing 13 random primers. The transfer of results on the agarose gel had the samples stained with ethidium bromide, examined using ultraviolet rays, and photographed by digital camera before finally analyzing statistically. The DNA's extraction and isolation from young leaf samples of cultivars of various species used the CTAB method (Table 1) (Haung *et al*., 2013; Shehab, 2020; Al-Talib, 2021; AL-Badrany, 2020). This study had the DNA purification through isolation.

Table 1. Species, cultivars, and the resources used in the study.

No.	Cultivars	Species	Resources
1	Rams harm	*Vigna unguiculata* (cowpea)	Local market, Nenevah, Iraq
2	Local	- do -	
3	LP08-0SSFR	*Vicia faba* (broad bean)	ICARDA
4	LP08-OSS9FR	- do -	
5	Holand	*Pisum sativum* (peas)	Local market, Nenevah, Iraq
6	French	- do -	

Table 2. The primers used in the study.

No.	Primer	Sequence5-3	Reference
1	OPA- 01	CAGGCCCTTC	
2	OPA-03	ACTCAGCCAC	Bukhari *et al*. (2015)
3	OPA-04	AATCGGGCTG	
4	OPA-05	AGGGGTCTTG	
5	OPA-07	GAAACGGGTG	
6	OPA-09	GGGTAACGCC	
7	OPA-10	GTGATCGCAG	
8	OPA-11	CAATCGCCGT	Szilagyi *et al*. (2011)
9	OPA-17	CACCGCTTGC	
10	OPB-10	CTGCTGGGAC	
11	OPC-08	TGGACCGGTC	
12	OPG-14	GGTGAGACC	
13	OPE-06	AAGACCCCTC	

Table 3. Components of the amplification mixture used in the study.

No.	Components	Concentration	Size (ml)
1	Master mix	2x	10
2	Primer	20 Pmol/ml	2
3	$Mgcl_2$	25 mm	1
4	Water		5
5	DNA template		2
Total			20

Reactions RAPD –PCR

Randomly primers prepared and used came from the Korean company, Macrogen (Table 2). Determining the volumes and concentrations of the amplification mix was according to the Macrogen supplier's instructions (Table 3). As the reaction components underwent mixing with a microcentrifuge of the type GLS for 5 s, the genotype samples' placement in a thermocycle device performed the reaction and random amplification. The samples' preparation for electrophoresis followed the amplification process. The mixture of four microliters of volumetric DNA ladder size must be with two microliters of loading dye, added to a specific hole on the one side of the agar at 1.5% concentration.

The 6.5 microliters of polymerase chain reaction (PCR) products, withdrawn and loaded in the agar's pits, excluded the dye and its color, with the power device turned on. Connecting the electrodes had the electric power supply set to 80 volts for a period of 60 min. Following the completion of the relay stage, the gel's transfer and immersion progressed in a water basin containing distilled water to remove the traces of excess dye (ethidium bromide) from the gel. The scrutiny of the gel containing PCR products used a UV transilluminator and its imaging with a high-resolution digital camera.

Table 4: The number of produced, general, and different sites and general, different, unique, and absent bands.

No.	Primers	Produced sites	General sites	Different sites	Total bands	General bands	Different bands	Unique bands	Absent bands	Variance (%)
1	OPA-01	12	0	12	33	0	33	2	0	100
2	OPA-03	12	0	12	28	0	28	1	0	100
3	OPA-04	13	2	11	37	12	25	6	1	84.61
4	OPA-05	11	2	9	31	12	19	2	0	81.81
5	OPA-07	11	0	11	27	0	27	1	1	100
6	OPA-09	14	0	14	30	0	30	6	1	100
7	OPA-10	12	0	12	30	0	30	4	0	100
8	OPA-11	11	0	11	29	0	29	0	1	100
9	OPA-17	10	1	9	21	6	15	4	0	90.0
10	OPB-10	14	0	14	37	0	37	5	2	100
11	OPC-08	15	1	14	49	6	43	0	0	93.33
12	OPG-14	8	1	7	25	6	19	2	0	87.50
13	OPE-06	12	0	12	29	0	29	1	0	100
Total		155	7	148	406	42	364	34	6	

Estimating the genetic dimension

The genetic dimension estimation of the genotypes occurred by converting the results of the obtained RAPD indicators appearing in the gel into characterization tables. Indicating '1' for a band's presence and '0' for a band's absence to find the genetic relationships between the genotypes. The results sustained statistical analysis (Rohlf, 1993) based on the equation mentioned by Nei and Li (1979). Efficiency for each RAPD primer reached estimation using the equation mentioned below (Grudman *et al.*, 1995).

Primer efficiency = (the number of bands per primer/the total number of multiplying bands per primer) × 100

The discriminatory ability, also calculated, relied on the following equation:

Discriminatory power = (the number of differentiated bands per primer/the total number of differentiated bands in all prefixes) × 100

RESULTS AND DISCUSSION

The PCR amplification revealed different patterns of bands (Table 4). The general total of the sites identified by the primers on the sample genome amounted to 155 sites, with an average of 11.9 bands for seven primers. This included a general site, with an average of 0.5 for each primer and 148 divergent sites, with an average of 11.3 bands for each primer. The primer OPC-08 distinguished itself by the highest productive site, amounting to 15, while the primer OPG-14 was the lowest productive site, amounting to eight (Figures 1 and 2). The increased number of divergent bands correlated with the efficacy of the primers in ascertaining the genetic dimension, enhancing the likelihood of acquiring a genetic fingerprint for the examined cultivars. These results were greatly analogous to past findings, wherein 26 examined cultivars of faba beans appeared with 11 RAPD markers (Basheer *et al.*, 2012). The band rates and divergent bands were 8.5 and 5.5 bands per primer, respectively, with variation arising from self-mutation affecting the distance between genetic loci occurring naturally during the evolutionary process of organisms. Variation could be due to the occurrence of deletion and addition at the site to which the primer is linked (Tingey *et al*., 1993).

The total number of bands resulted in those locations (Table 4). The total number was 406, wherein 42 main bands and 364 polymorphic bands existed. The primer OPG-14 produced the fewest bands (8), while the primer OPC-08 recorded with the most bands (15), and the variance percentage was 94%.

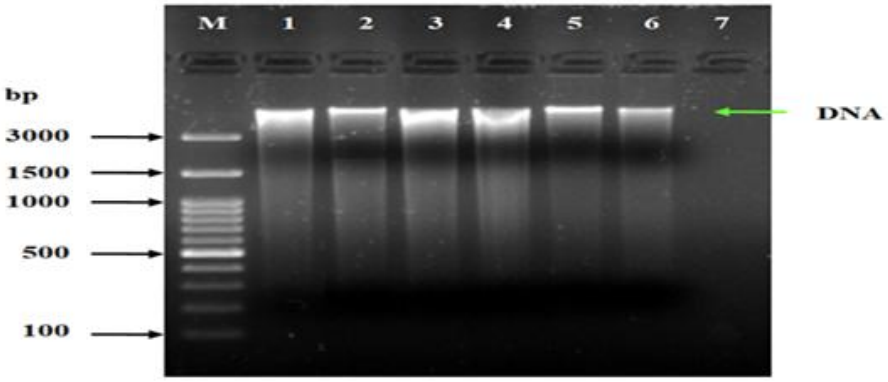

Figure 1. DNA extraction (genome) for species cultivars - Cowpea (1 = Ramsharm, 2 = Local), Faba bean (3 = LPO8-OSSER, 4 = LPOS-OSS9FR), and Peas (5 = Holand, 6 = French).

The number of bands that a genome showed was one of the bases the RAPD depends on, which, in turn, represents the number of sites the primer finds and connects with. The numbers of those sites become affected by two basic factors, i.e., the sequences of the primers and the size of the studied genome. Previous research revealed random primers emerged with 2.10 sites on the DNA genome of the plants (Borovkova *et al.*, 1997; Al-Talib, 2021).

From Table 4, the presence of unique bands ranged from 166 to 1,946 bp, with the highest molecular weight (1,964 bp) recorded in the PCR amplification produced when using the primer OPA-01. Meanwhile, the lightest molecular weight (166 bp) was evident when using the primer OPA-04. The primers' efficiency differed among the studied species cultivars, where the primer OPC-08 recorded with the highest efficiency (12.07), while the lowest efficiency resulted in the primer OPA-17 (5.17) (Figure 2). A varied discriminatory ability of the primers existed, where OPA-17 has characteristics of the lowest efficiency (4.12), while the primer OPA-08 has an absence of bands. The total distinct bands produced by different primers were 40, with the absent bands (6) and unique bands (34). The French cultivar of the pea had the lowest number of unique bands and did not show any unique band. Several reports stated the same results emerging in previous studies (Al-Assi, 2002; Al-Badrany, 2020; Al-Talib *et al.*, 2021).

Concerning the absent bands, the cowpea (*Vigna unguiculata*) cultivar exhibited the highest number of absent bands (4), while the pea cultivar Ramsharm did not have any absent bands. These bands were seemingly a discriminatory and diagnostic characteristic of the studied cultivars. It is because the appearance of these bands in certain cultivars revealed the mutation occurred at a specific site, leading to the identification of the primer and the emergence of the unique bundle. Study results corroborated past findings of Al-Asi (2002) and Al-Qaisi (2013). These bands play an important role in shortening breeders' efforts to reach their goals (Tingey *et al*., 1993; Borovkova *et al.*, 1997). This opens horizons of the future to find the distinctive bands of other cultivars, as well as link such bands with other analytical characteristics (Smith and Smith, 1992; Al-Samarrai, 2014).

The primers used considerably differed in their molecular weights, and the recorded highest discriminative ability was 11.81 (Table 5). The RAPD results showed the primers recorded with varying efficiency; however, most of the primers possessed an appropriate efficiency. The study concludes the efficiency of the primers enhanced with the increase in the number of bands produced. The assessment of genetic distance based on the RAPD technology among the examined species cultivars utilized the genetic program NTSYS-pc version 2.10. It relied on the presence of shared bands among

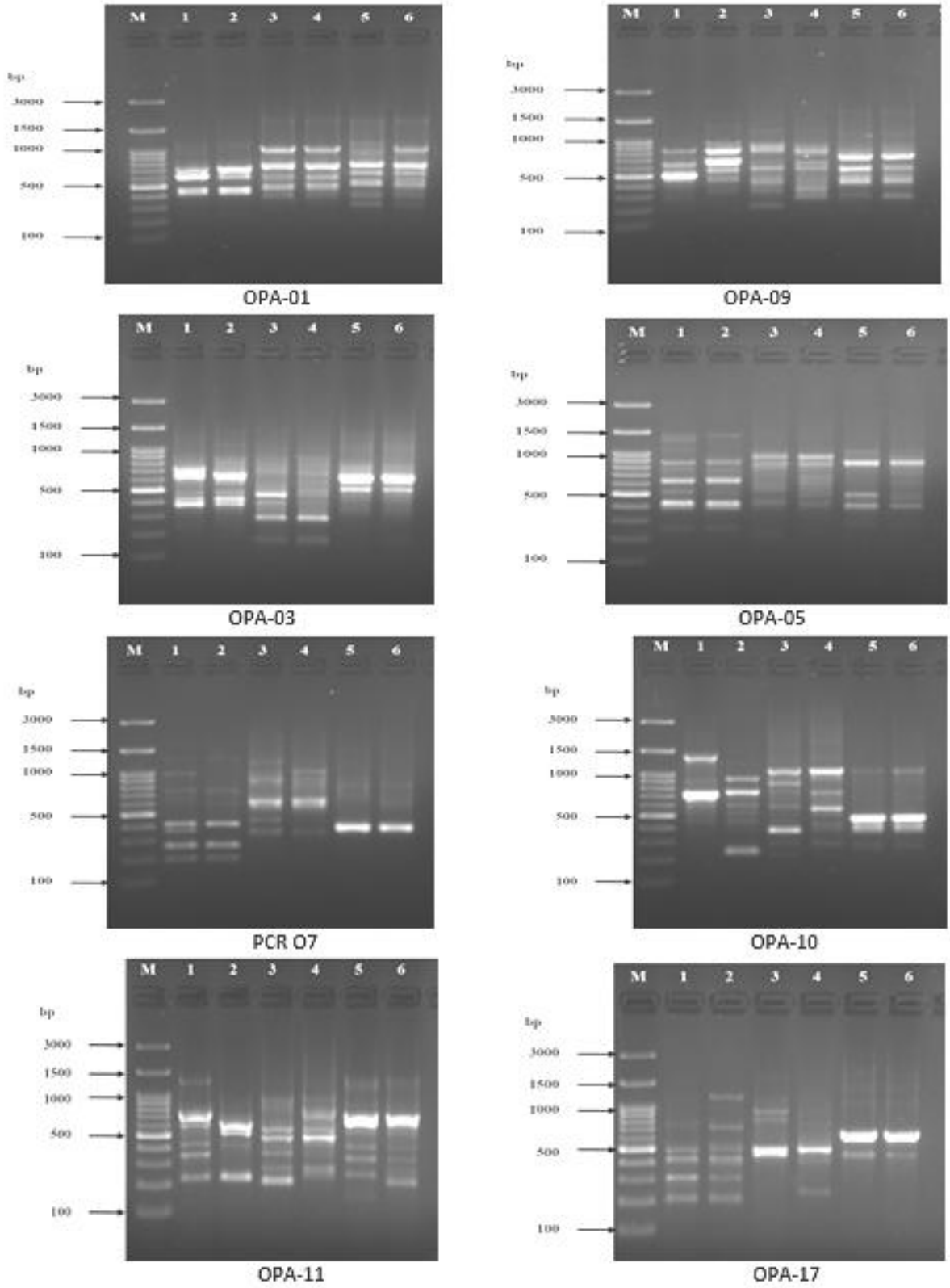

Figure 2. Profiles of the species cultivars - Cowpea (1 = Ramsharm, 2 = Local), Faba bean (3 = LPO8-OSSER, 4 = LPOS-OSS9FR), and Peas (5 = Holand, 6 = French).

Table 5. Genetic distance centimorgan (cM) among the species cultivars.

Cultivars	P1	P2	P3	P4	P5	P6
P1	0					
P2	0.358	0				
P3	1.220	1.002	0			
P4	0.916	0.858	0.217	0		
P5	1.016	1.201	0.975	0.909	0	
P6	0.991	1.231	0.719	0.781	0.122	0

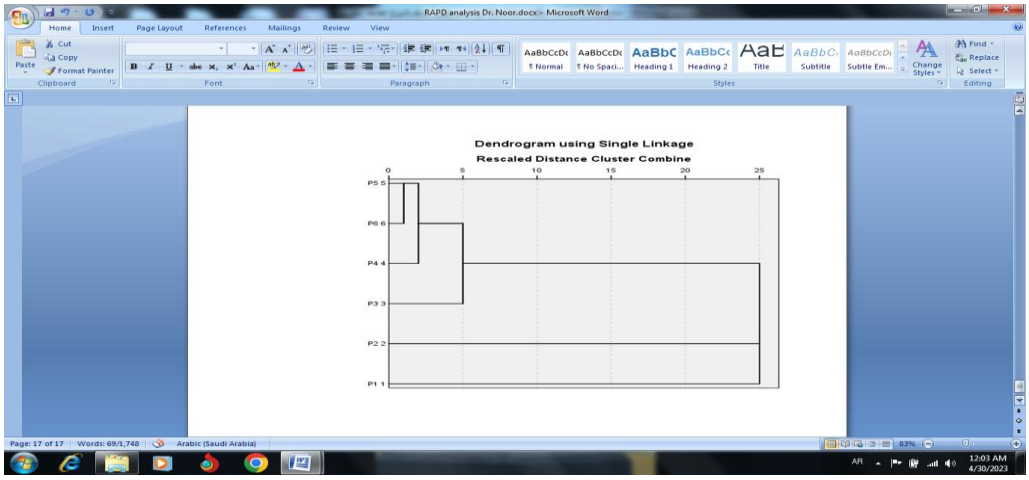

Figure 3. Dendrogram showing the relationships among the studied species cultivars.

the studied genotypes analyzed according to the equation proposed by Nei and Li (1979). These results were consistent with past studies on the use of several primers, which can distinguish the genotypes through the resulting different bands (Borovkova *et al.,* 1997; Hormaza *et al*., 1998; Al-Talib, 2022).

The genetic dimension indicated the genetic distance values among the examined cultivars, utilizing 13 RAPD primers, revealed an identical genetic material among two cultivars corresponding to a genetic distance of zero. Conversely, the percentage of genetic similarity quantifies the degree of similarity among the crop genotypes (Khierallah *et al.,* 2014; Rasheed *et al*., 2024). The genetically diverse types were those possessing the fewest shared bands, attributable to variations in their nucleotide sequences within the genome (Liu *et al.,* 2005; Yassin, 2011). The lowest genetic distance (0.885) was evident between the Dutch and French cultivars of the *Pisum sativum*, whereas the genetic distance of the other genotypes varied in ranges. Based on the genetic dimensions, the studied cultivars' classification gave a genetic kinship tree through a meta-analysis dendrogram, as founded on the genetic spectrum connecting the primary groups. Consequently, the inclusion of a subset of cultivars within a specific group signifies a comparable genetic dimension among the group cultivars (Elkichaoui *et al.,* 2013; Al-Badrany, 2020).

The family tree illustrated the meta-analysis in Figure 3. The dendrogram of the examined species cultivars indicated their genetic relationship based on RAPD technology, revealing a division into three primary groups. The first primary group comprised the cultivar Ramsharm of *Vigna unguiculata*, while the second principal group consisted of the cowpea cultivars belonging to the same species. The third main group, as subdivided into two subgroups, includes the first, represented by the cultivar Lpo8-055FR of *Vicia faba*, with the second further dividing

into two segments. The first segment included the cultivar Lpo8-059FR of *Vicia faba*, and the second segment encompassed the Dutch and French cultivars of *Pisum sativum*. The presented findings implied a degree of genetic similarity and variance among the examined genotypes. These results aligned with the phenotype owing to the resemblance in the non-coding areas of the genes (Abboud *et al.*, 2014). The findings align with numerous researchers employing RAPD markers to investigate genetic variation among various plant cultivars (Al-Samarrai, 2014; Taher and Saeed, 2023). Similar genetic relationships were prevalent in the bean genotypes through the application of RAPD primers (Basheer *et al.,* 2012; Al-Talib, 2022; Taher and Saeed, 2022).

The augmented quantity of used primers rises, with some connection zones indicated by the sequence of the employed primers. The genetic divergence among the genotypes was indicative of the quantity of common bands. An increasing number of such bands denotes a diminished genetic distance, whereas a decreased number implies an augmented genetic distance. The common bands signify the genetic similarity at specific genomic loci among examined species cultivars, which may correlate with phenotypic traits relevant to productivity, reproduction, disease resistance, and the genetic adaptation to environmental conditions favorable to particular subspecies' growth. In past studies, the broad bean genotypes bore scrutiny with random primers, indicating the genetic distance across these genotypes linking the current study to previous studies, such as Al-Ghamdi (2009).

CONCLUSIONS

The results revealed the used primers produced 406 bands, including different bands (364) and the general bands (42). The most crucial result obtained for genetic distance was 0.122 to 1.231, with genetic structures determined by a number of distinctive bands amounting to 40, wherein 34 were unique bands and six were absent bands.

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