Research Article

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Diversity Analysis of Common Vetch (*Vicia sativa* L.) Lines and Cultivars Using Pairwise Combinations of Universal Rice Primers

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ABSTRACT

This study has been conducted to determine genetic diversity of the common vetch lines and cultivars by using pairwise combinations of universal rice primers (URPs). A total number of 37 URP marker pairs were tested and twenty of those provided amplicons in the common vetch genome. The pairs of amplified URP markers provided a total of 83 bands and 62 of them were determined as polymorphic and were scattered to the whole genome. The average polymorphism rate of the primers was calculated as 73.5% while the polymorphism information content (PIC) values have ranged from 0.11 to 0.47 with an average of 0.24. The phylogenetic tree constructed based on UPGMA analysis provided three main clades. Two-dimensional plot of PCA and the UPGMA analysis showed that the URP markers successfully distinguished the genetic material based on their genetic origin. In conclusion, this study revealed that the use of pairwise combinations of URP markers could have a better power to reveal the level of polymorphism in plant genome.

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Introduction

Vicia sativa L., common vetch, is considered as one of the most important annual, self-pollinated, diploid forage crop species [1-3]. The common vetch plants have plasticity not only for adaptability to different soil and climate conditions, but it also has diverse use as grain, straw, hay, silage, and green manure along with soil improvement ability with nitrogen fixation [1-3]. Therefore, it is considered one of the most valuable protein and mineral resources for cattle and poultry in Turkey, Australia, New Zealand, China and Eastern Europe [3-7].

PCR based molecular markers are currently one of the best tools to make genetic characterization and to estimate genetic diversity in various organisms including plants [8-11] since they are more reliable than pedigree data to estimate genetic diversity and to

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discriminate individuals from various breeding sources for parental selection in plants [12, 13]. However, the power of a molecular marker is mainly determined by the level of polymorphism detected [14]. As a complex taxon, *V. sativa* represents a diverse phylogenetic relationship [15, 16]. Various type of molecular markers have been previously used to resolve inter- and intra-specific diversity in common vetch by using random amplified polymorphic DNA (RAPD) [15], sequence-related amplified polymorphism (SRAP) and inter-simple sequence repeat (ISSR) [17], amplified fragment length polymorphism (AFLP) [18], simple sequence repeats (SSRs) [19], expressed sequence tag-simple sequence repeat (EST-SSR) [20], cDNA-simple sequence repeat (cDNA-SSR) [21, 22], start codon targeted polymorphism (SCoT) [23] and single nucleotide polymorphisms (SNPs) [24, 25]. The recent advancements in plant biotechnology also resulted in avalanche of information in terms of DNA sequences and functional gene determination in various plant species [20, 21, 23, 26]. Therefore, not only transferability of the current molecular markers among the species has been studied [19] but new and alternative use of those molecular marker techniques have also been developed in diverse plant species [27-29].

The universal rice primers (URPs) were generated based on repetitive DNA sequences of the rice genome [30-32] and their efficiency as well as universal applicability have been previously tested in various prokaryotic [30-32] and eukaryotic [32, 33] genomes for taxonomic and phylogenetic analysis. However, based on the current literature URP markers have never been tested in pairs so far.

The objective of this study was to reveal genetic relationships of some common vetch lines and cultivars obtained from various genetic resources by using pairwise combinations of URP markers.

Materials And Methods

Materials

A total of 24 common vetch lines and cultivars which were obtained from either national or international genetic resources were used in this study (Table 1). The seeds of lines were propagated by selfing under the same field conditions for 2 years, during the plant growing seasons of 2008 and 2009. The taxonomic confirmation of the lines assured that all lines belong to *V. sativa* (Table 1).

Table 1. Source of 19 lines and 5 varieties of *Vicia sativa* used in this study

Accession number	Source	Register number
TR41968	Izmir Aegean Agricultural Research Institute	GB-3
TR4471	Izmir Aegean Agricultural Research Institute	GB-5
TR35076	Izmir Aegean Agricultural Research Institute	GB-7
TR12447	Izmir Aegean Agricultural Research Institute	GB-8
TR12474	Izmir Aegean Agricultural Research Institute	GB-13
TR33452	Izmir Aegean Agricultural Research Institute	GB-24
TR4392	Izmir Aegean Agricultural Research Institute	GB-25
TR33253	Izmir Aegean Agricultural Research Institute	GB-29
TR33268	Izmir Aegean Agricultural Research Institute	GB-34
IFVS 490 Sel 2003	International Center for Agricultural Research in the Dry Areas	IC-1
IFVS 1293 Sel 2025	International Center for Agricultural Research in the Dry Areas	IC-2
IFVS 1812 Sel 2083	International Center for Agricultural Research in the Dry Areas	IC-3
IFVS 3026 Sel 2490	International Center for Agricultural Research in the Dry Areas	IC-4
IFVS 715 Sel 2556	International Center for Agricultural Research in the Dry Areas	IC-5
TARM-59998	Ankara Field Crops Central Research Institute	TA-1
TARM-59999	Ankara Field Crops Central Research Institute	TA-2
TARM-60265	Ankara Field Crops Central Research Institute	TA-3
TARM-60279	Ankara Field Crops Central Research Institute	TA-4
TARM-60334	Ankara Field Crops Central Research Institute	TA-5
Alınoğlu-2001	Commercial cultivar	ÇE-1
Bakır-2001	Commercial cultivar	ÇE-2
Cumhuriyet – 99	Commercial cultivar	ÇE-3
Karaelçi	Commercial cultivar	ÇE-6
Kubilay-82	Commercial cultivar	ÇE-7

Methods

DNA extraction and quality control

The young leaves of ten plants of each line or cultivar were bulked and were used for genomic DNA extraction by using plant genomic DNA extraction mini kit (Favorgen, Pingtung, Taiwan) based on the manufacturer's instruction. A known concentration of λ DNA on 0.8% agarose gel electrophoresis was used to determine the sample DNA concentrations and DNAs were diluted to 50 ng/µl by using dH_2O before stored at -22 ºC until used.

Primers and PCR amplification

The primer pairs used in the study were summarized in Table 2. The URP markers were analyzed as described before [32] and was optimized by using 37 URP marker pairs. Amplifications were carried out in a 20 µl reaction mixture containing 1 x PCR buffer

(10 mM Tris-HCl pH 8.3, 50 mM KCl, %0.01 jelatin); 25 mM MgCl2; 50 ng (F+R) primer; 0.5 mM each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP); 0.5 unit of Taq DNA polymerase; and 100 ng of genomic DNA.

The PCR analysis was done in a thermal cycler (Favorgen Gradient PCR, Pingtung, Taiwan). The best annealing temperature of each primer pairs was determined by using a gradient PCR before used in the sample DNA amplifications. To determine the reproducibility of the bands, the PCR reactions were repeated twice. The thermal cycler was programmed to five cycles of 1 min at 94 ºC, 1 min at 35 ºC and 1:30 min at 72 ºC, for denaturing, annealing and extension, respectively. Then main cycles followed by 35 cycles of 1 min at 94 ºC, 1:30 min at 55–58 ºC (depending upon annealing temperature of the primer presented in Table 2, and 1:30 min at 72 ºC, followed by a final incubation for 3 min at 72 ºC. The URP amplicons were separated with a 2% (w/v) agarose gel in 1 X TBE buffer at 90 V for 3 h and were stained with ethidium bromide before photographed under ultraviolet light.

Data analysis

The DNA amplicons on the gel were scored for the presence (1) or absence (0) of the bands. The evaluation of band patters of URP markers in this study revealed that they inherited in dominant pattern although the original research paper did not define their inheritances as dominant or co-dominant [32]. Therefore, the polymorphism information content (PIC) of each primer pairs was calculated as a dominant marker as described previously [34, 35] by applying the formula $PIC= 1-(p^2 + q^2)$ where p and q are the frequencies of presence and absence of bands, respectively. The genetic similarity was calculated by using NTSYSpc-2.1 program based on Dice coefficient [36]. The unweighted pair-group method (UPGMA) with arithmetic averages was used to have the dendrogram. The EIGEN and PROJ modules of NTSYSpc-2.1 program were used for the principal component analysis (PCA).

Table 2. The pairwise combinations of 20 URP marker pairs, sequences, GC content, annealing temperature, the numbers of amplified and polymorphic bands, polymorphism rate and PIC values for 19 lines and 5 cultivars of *V. sativa*

No.	Primer Name	The base sequences (5'-3')	GC Rate (%)	Ta (ºC)	Amplified bands	Polymorphic bands	Polymorphism Rate (%)	PIC
1	**URP25F**	GATGTGTTCTTGGAGCCTGT	50	56	5	3	60	0.22
	URP2R	CCCAGCAACTGATCGCACAC						
2	**URP30F**	GGACAAGAAGAGGATGTGGA	50	56	2	2	100	0.47
	URP2R	CCCAGCAACTGATCGCACAC						
3	**URP32F**	TACACGTCTCGATCTACAGG	50	55	5	3	60	0.14
	URP2R	CCCAGCAACTGATCGCACAC						
4	**URP38F**	AAGAGGCATTCTACCACCAC	50	55	1	1	100	0.22
	URP2R	CCCAGCAACTGATCGCACAC						
5	**URP2F**	GTGTGCGATCAGTTGCTGGG	50	57	4	3	75	0.35
	URP4R	AGGACTCGATAACAGGCTCC						
6	**URP30F**	GGACAAGAAGAGGATGTGGA	50	56	5	2	40	0.11
	URP4R	AGGACTCGATAACAGGCTCC						
7	**URP32F**	TACACGTCTCGATCTACAGG	50	56	5	4	80	0.23
	URP4R	AGGACTCGATAACAGGCTCC						

8	**URP38F** **URP4R**	AAGAGGCATTCTACCACCAC AGGACTCGATAACAGGCTCC	50	56	4	3	75	0.23
9	**URP1F** **URP6R**	ATCCAAGGTCCGAGACAACC GGCAAGCTGGTGGGAGGTAC	50	57	3	2	67	0.19
10	**URP2F** **URP6R**	GTGTGCGATCAGTTGCTGGG GGCAAGCTGGTGGGAGGTAC	50	57	2	1	50	0.25
11	**URP9F** **URP6R**	ATGTGTGCGATCAGTTGCTG GGCAAGCTGGTGGGAGGTAC	50	55	3	2	67	0.18
12	**URP32F** **URP6R**	TACACGTCTCGATCTACAGG GGCAAGCTGGTGGGAGGTAC	50	55	2	1	50	0.16
13	**URP1F** **URP13R**	ATCCAAGGTCCGAGACAACC TACATCGCAAGTGACACAGG	50	55	3	2	67	0.33
14	**URP2F** **URP13R**	GTGTGCGATCAGTTGCTGGG TACATCGCAAGTGACACAGG	50	55	5	5	100	0.35
15	**URP9F** **URP13R**	ATGTGTGCGATCAGTTGCTG TACATCGCAAGTGACACAGG	50	55	6	6	100	0.28

16	**URP32F**	TACACGTCTCGATCTACAGG	50	55	7	6	86	0.16
	URP13R	TACATCGCAAGTGACACAGG						
17	**URP38F**	AAGAGGCATTCTACCACCAC	50	55	6	6	100	0.33
	URP13R	TACATCGCAAGTGACACAGG						
18	**URP2F**	GTGTGCGATCAGTTGCTGGG	50	55	8	5	63	0.2
	URP17R	AATGTGGGCAAGCTGGTGGT						
19	**URP25F**	GATGTGTTCTTGGAGCCTGT	50	55	5	4	80	0.26
	URP17R	AATGTGGGCAAGCTGGTGGT						
20	**URP30F**	GGACAAGAAGAGGATGTGGA	50	55	2	1	50	0.19
	URP17R	AATGTGGGCAAGCTGGTGGT						
TOTAL					83	62	-	-
MEAN					4.15	3.1	73.5	0.24

Results

Genetic diversity based on URP marker analysis

A total of 37 URP marker pairs were tested in this study. Twelve of those (URP1F/URP2R, URP9F/URP2R, URP25F/URP2R, URP1F/URP4R, URP25F/URP6R, URP30F/URP6R, URP30F/URP13R, URP1F/URP17R, URP25F/URP17R, URP32F/URP17R, URP38F/URP6R and URP30F/URP17R) did not provide any amplification in the vetch genome. The marker combinations URP38F/URP17R, URP9F/URP4R, URP25F/URP4R and URP25F/URP13R produced monomorphic bands only. The URP13R marker was tested as a single primer and was removed from application of pairwise combination. Twenty UPR marker pairs gave polymorphic bands in different proportions (Table 2).

The URP marker pairs produced a total of 83 bands and 60 of them were determined as polymorphic (Table 2). The average polymorphism rate of the markers used was 73.5%. The URP marker pairs provided 4.15 bands an average. The number of polymorphic bands was calculated as 3.1 per marker pair. Average PIC value was calculated as 0.24. The lowest PIC value was obtained from URP30F / URP4R marker pair with 0.11 while URP30F/URP2R marker pair had the highest (0.47) (Table 2). The highest number of amplicons (8 amplicons) was obtained from the URP2F/URP17R while the URP38F/URP2R marker pair had the lowest (1 amplicon). The polymorphism rate of the maker pairs ranged from 40% to 100% (Table 2). The lowest polymorphism rate (40%) was determined on the URP30F/URP4R while all of the amplicons of URP30F/URP2R, URP38F/URP2R, URP2F/URP13R and URP9F/URP13R marker pairs were polymorphic.

The 24 common vetch lines and cultivars were divided into 3 main clades based on UPGMA analysis presented in Figure 1. The lines came from GB provided clade I (Fig. 1) while the lines encoded as TA provided clade II. The clade III consisted of the cultivar and the lines encoded as IC. The lines IC1 and IC2 were separated from the others in the clade III while the cultivars Bakır-2001 and Kubilay-82 were determined as the most similar cultivars (0.97). The cultivar Alınoğlu-2001 was separated from the rest of the cultivars (Fig. 1).

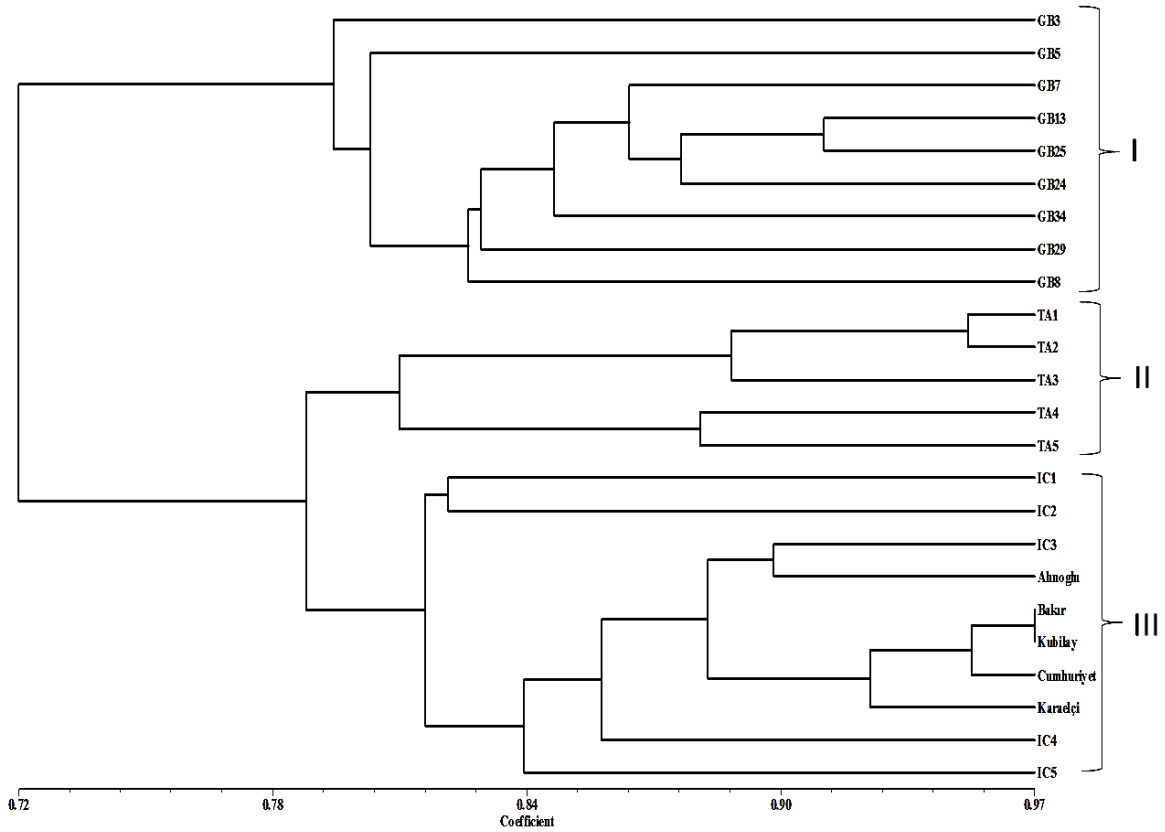

Fig. 1 Genetic similarity dendrogram based given reference [37] for 19 lines and five varieties of *V. sativa* created according to the UPGMA method using 20 pairwise URP markers

The first three components of Eigen values in PCA analysis explained 50.19% of total variation. The PCA analysis revealed that the 20 URP marker pairs as well as their alleles scattered across the vetch genome. For instance, alleles of URP38F/URP13R marker pair provided amplicons came from various loci of different chromosomes (Fig 2). Based on the two-dimensional plot of PCA analysis, the lines GB3 and IC5 were the most distant accessions (Fig. 3).

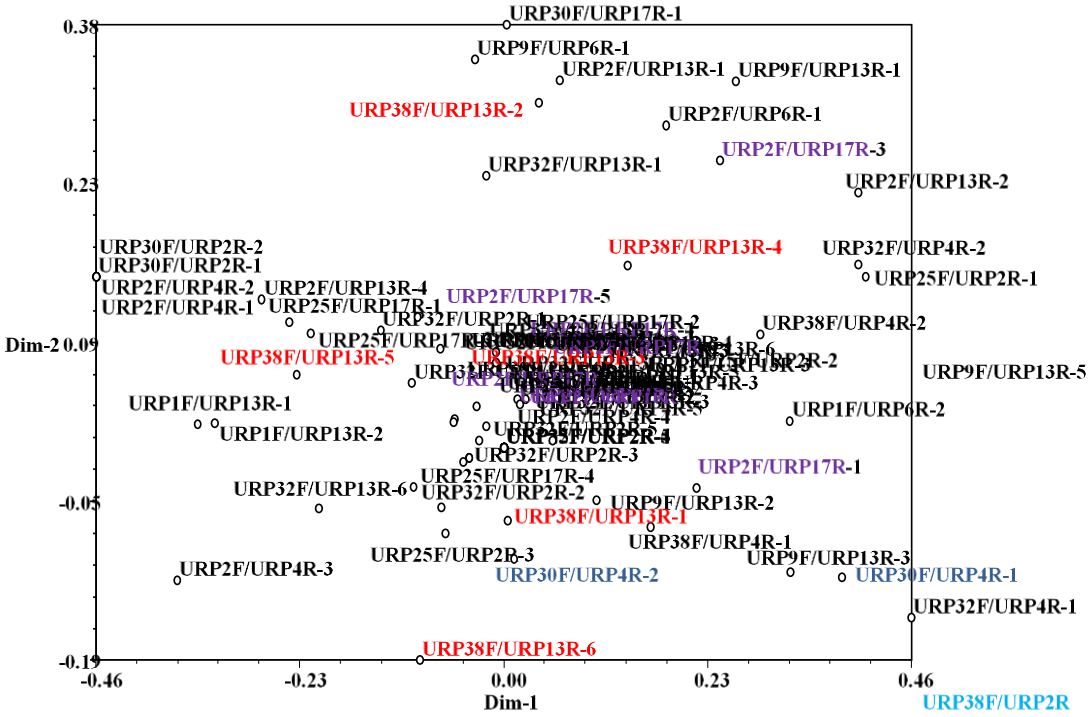

Fig. 2 Two dimensional PCA plot of 20 pair-wise URP markers in the common vetch genome. The 20 pairwise URP markers scattered across the genome. The alleles of URP38F/URP13R, URP2F/URP17R, URP30F/URP4R and URP38F/URP2R markers were given by the same colour along with hyphen and allele numbers

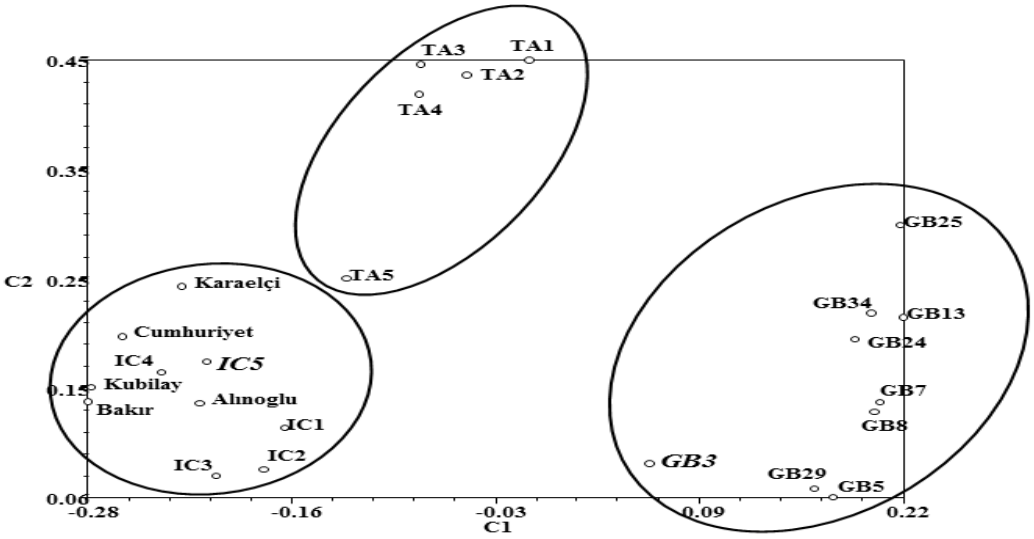

Fig. 3 Two dimensional PCA plot of 19 lines and five varieties of *V. sativa* analyzed by 20 pairwise URP markers. The most distinct lines IC5 and GB3 were indicated in italic

Discussion

The results of the current study revealed that pairwise use of URP markers can successfully be used to determine genetic relationship of common vetch lines and

cultivars. A total of 37 URP marker pairs were tested and twenty of them were amplified in the common vetch genome. Amplified marker pairs produced a total of 83 bands and 62 of them were determined as polymorphic (Table 2). Similar to many other single primer targeting sites such as single primer amplification reaction (SPAR) [38, 39], directed amplification of minisatellite region DNA (DAMD) [40] and random amplified polymorphic DNA (RAPD) [41, 42], URP-PCR method also uses single primer per PCR reaction [32]. Reliability and reproducibility of URP markers were found relatively high since they were used at higher annealing temperatures because of their longer (20 nucleotides) primer sequences and higher GC contents (50% GC) [32, 43]. Therefore, URP markers become a popular DNA marker system for taxonomic and phylogenetic analysis in various prokaryotic [30-32] and eukaryotic [32, 33, 44] genomes. The pairwise use of URP markers in the present study further improved stringency of PCR amplification in comparison to previous reports [43, 45], suggesting that pairwise use of UPR markers would provide more unique amplicons for taxonomic and phylogenetic analysis. It was recently reported that pairwise use of RAPD primers provided different patterns of PCR amplifications in soybean genome in comparison to single primer target site of RAPD analysis, indicated that RAPD primers could be used as pairs in various combinations and could provide unique amplicons for phylogenetic analysis [46]. This study first time revealed that URP markers could be used as pairs in various combinations to determine genetic relationship of common vetch lines and cultivars, suggesting that pairwise use of URP markers in various combinations could provide an additional marker resource along with their conventional use in both prokaryotic and eukaryotic genomes. It is also possible that the pairwise use of URP markers may provide new unique amplicons which could be converted into other useful markers such as SCAR (sequence characterized amplified region) and CAPS (cleaved amplified polymorphic sequence) for marker assisted selection in plant breeding after their subsequent cloning and sequencing. The results of this study further indicated that the pairwise use of URP markers should be tested in any specific genome since some of the primer combinations did not provide any amplicon in the vetch genome. Unamplified primer pairs URP25F/URP6R and URP30F/URP6R showed a moderate or very weak primer dimer structure. This might be one of the reasons why those URP pairs did not have any amplification although the other unamplified pairs did not have any such structure. Because of their highest polymorphism

rates (100%) (Table 2), the primer pairs URP30F/URP2R, URP38F/URP2R, URP2F/URP13R, URP9F/URP13R and URP38F/URP13R could be the first candidate to test URP markers in pairs for prokaryotic and eukaryotic genomes. Although URP30F/URP2R marker combination had the highest PIC value (0.47), URP2F/URP17R and URP32F/URP13R marker pairs had the highest number of amplicons and gave medium PIC values (0.20 and 0.13, respectively) than the other URP marker pairs tested. Alleles of URP marker pairs scattered to the whole vetch genome (Fig. 2), indicated that pairwise use of URP markers could have a better power to reveal the level of polymorphism than single primer targeting sites in PCR amplifications.

The PCA analysis showed that the PC1 had the maximum variability (29.21%) followed by PC2 (12.29%) and PC3 (8.68%). Two-dimensional plot of PCA and the UPGMA analysis revealed that genetically the most distinct lines (GB3 and IC5) could be used to create an additional genetic diversity in common vetch breeding programs (Figs 1 and 3). The clades I and II successfully distinguished the vetch lines came from the same genetic resource. The clade III included both the cultivars and the lines (IC), suggesting that the cultivars used in this study were originated from IC genetic resource. The results also revealed that the lines grouped in different clades could be used to develop new and alternative common vetch varieties for various purposes. These results confirmed that the pairwise use of URP markers successfully distinguished the genetic material based on their origin. In conclusion, the results of the current study revealed that pairwise use of URP markers could have a better power to reveal the level of polymorphism in plant genome and could be tested in other eukaryotic as well as prokaryotic genomes.

Abbreviations

URPs: Universal Rice Primers; PIC: Polymorphism Information Content; UPGMA: The Unweighted Pair-Group Method; PCA: Principal Component Analysis; G: Guanine; C: Cytosine; RAPD: Random Amplified Polymorphic DNA; SPAR: Single Primer Amplification Reaction; SRAP: Sequence-Related Amplified Polymorphism; AFLP: Amplified Fragment Length Polymorphism; SSRs: Simple Sequence Repeats; EST-SSR: Expressed Sequence Tag-Simple Sequence Repeat; cDNA-SSR: cDNA-Simple Sequence Repeat; SCoT: Start Codon Targeted Polymorphism; SNPs: Single Nucleotide Polymorphisms

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Author Contribution Statements

I.T. conceived the idea, granted financial support, planned the experiments, performed the analysis and all the numerical calculations, interpret the data, designed the figures, M.T. carried out the experiments and drafted the manuscript.

Conflicts of Interest
The authors declare that they have no conflict of interest.

Availability of data and material
Please contact the corresponding author for any data request

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