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DNA Fingerprinting and Biochemical Characterization of Red Bean (Phaseolus vulgaris L.) Genotypes from Kurram Agency, Khyber Pakhtunkhwa, Pakistan

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Article

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Abstract

Background

Red bean (*Phaseolus vulgaris* L.) is an important grain legume both nutritionally and economically significant, however, the genomic resources of the landraces grown in the combined districts of Khyber Pakhtunkhwa, Pakistan, are poorly documented. The conservation and utilization of the indigenous genotypes require a first step of characterizing their genetic diversity and biochemical composition.

Materials and methods

Nine Start Codon Targeted (SCoT) markers were used to fingerprint 34 red bean genotypes from Kurram Agency. Total phenolic content (TPC) was measured using the Folin-Ciocalteu method, while the antioxidant activity was measured by the DPPH radical-scavenging assay. Genetic relationships were given by UPGMA cluster analysis of Nei and Li's similarity coefficients.

Results

A total of 982 bands were amplified in the nine SCoT primers (mean number of bands/primers 109.1). Polymorphism Information Content (PIC) values were between 0.192 (SCoT 17) and 0.464 (SCoT 15), with an average value of 0.366. The 34 genotypes were separated into three clusters using UPGMA clustering: Cluster I (28 genotypes), Cluster II (the single divergent genotype RBK15) and Cluster III (RBK22, RBK24, RBK25, RBK26, RBK28). Biochemical profiling showed wide variation in TPC, from 10.794 ug/ml (RBK11) to 343.413 ug/ml (RBK13), and in DPPH scavenging activity, from 7.648 ug/ml (RBK3) to 52.462 ug/ml (RBK29), with RBK14 (51.467 ug/ml) and RBK28 (51.829 ug/ml) also ranking among the most active.

Conclusion

The genetic diversity of this germplasm collection was fairly high but well structured with the help of SCoT markers. The significant difference in the phenolics content and antioxidant activity suggests that RBK13, RBK29, RBK14, and RBK28 are potential varieties to be utilized for incorporating these nutrients into the food, and demonstrates the nutritional potential of Pakistan's indigenous red bean landraces for breeding purposes.

1. Introduction

Common bean (*Phaseolus vulgaris* L), one of the most widely used grain legumes in the world [1] is a key leguminous food and nutritional crop. It is a source of protein, complex carbohydrates, fiber, vitamins and minerals that is employed by millions of people in developing countries and sometimes called the

“poor man's meat” [2, 3]. Over 30 million tonnes of dry beans are harvested every year, most of which are grown in Asia, Africa and Latin America [4].

Red bean, a common cultivar of P. vulgaris, is not only a source of macronutrients, but also of phenolic acids, flavonoids, anthocyanins and other antioxidants that give it beneficial properties [5, 6]. They have been associated with lower cardiovascular disease risk, diabetes, and some cancer types [7], and overall, colored seed coats exhibit higher antioxidant activity than white-coated counterparts [8].

Pakistan has significant population of common bean landraces, especially in the northern mountainous areas. Informal farmer-led seed selection is one of the areas that red bean has been traditionally cultivated, such as in Kurram Agency, one of the combined districts in Khyber Pakhtunkhwa [9, 10]. The above “landraces” are the locally adapted genetic resources that have been selected over several generations in response to the agro-climatic environment in which they have evolved, but their genetic diversity and biochemical potential were not previously characterized.

To effectively conserve and breed from plant genetic resources, genetic diversity needs to be assessed [11, 12]. Environmental independence is important for quantifying this variation, and molecular markers can provide such information with an assurance of reliability. The diversity analysis [13] has become a versatile and cost-effective tool among the various marker systems available and is particularly useful for diversity analysis of germplasm. SCoT primers target a short (20 bp) conserved sequence immediately adjacent to the ATG start codon of plant genes, and amplicons are therefore linked to gene-rich areas of the genome and not to anonymous sites of the genome. They are very suitable for the identification of new germplasm not yet studied due to their dominant inheritance and reproducibility, as well as their capacity to show polymorphism [14].

Earlier diversity studies in common bean have relied on RAPD [15, 16], SSR [17–20], and, less commonly, SCoT markers [21]. No studies have been conducted on red bean genotypes of merged districts of Pakistan using SCoT markers so far.

In addition to molecular characterizations, profiling of total phenolics and antioxidant activity can be used to give complementary information for the identification of genotypes with enhanced nutritional value. Total phenolics content (TPC) and DPPH radical-scavenging assay are among the most commonly used and accepted procedures for assessing the bioactive potential of legume seeds [22–24].

The study was thus undertaken to assess: (i) genetic variability of 34 red bean types collected from Kurram Agency by SCoT markers; (ii) the total phenolic contents and their (iii) antioxidant activities.

To assess (i) the antioxidant activity of their seeds; and (ii) combine molecular and biochemical data to select genotypes with special potential for breeding in the future.

2. Materials and Methods

2.1. Collection of samples from a study area and genotype.

The study was carried out at the Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, Pakistan. In Kurram Agency (merged district of Khyber Pakhtunkhwa Province), which has suitable climatic conditions for red bean cultivation, 34 red bean (P. vulgaris) genotypes were collected, labeled as RBK1 to RBK34 (Fig. 1). No formally released cultivars are cultivated in the region and all genotypes were farmer-maintained landraces which were propagated over successive seasons through local seed selection and preservation. There was visible variation in seed color, shape and coat pattern among the collected accessions which reflects the genetic variation.

2.2. Seed Germination and DNA Extraction.

All 34 genotypes were sown into sand and kept in a controlled-environment growth chamber to allow emergence of seedlings. The young leaf tissue was collected and kept at -80 degC for further molecular and biochemical analysis.

The genomic DNA was isolated from leaves using the CTAB method [25]. Fresh leaves were ground to fine powder and incubated in 400 ul of CTAB extraction buffer at 65 degC for 30 min, followed by centrifugation at 13,000 rpm, to obtain the supernatant which was purified using isoamyl alcohol extraction, followed by re-centrifugation and precipitation of DNA with isopropanol, washing and re-suspending in TE buffer for further processing.

2.3. SCoT Marker Analysis

Nine previously published SCoT primers - SCoT 2, SCoT 7, SCoT 9, SCoT 15, SCoT 17, SCoT 22, SCoT 28, SCoT 30, and SCoT 31 - were used to fingerprint the 34 genotypes. These primers amplify the conserved region around the ATG start site; they are used to amplify the gene associated genomic regions.

The following conditions were used during the PCR reactions: 5X PCR master mix 1 ul, genomic DNA template 1 ul and the primer 1 ul. The amplicons were separated in 3% agarose gels with 100–120 V for 60–90 min, using a gel documentation system and a 100 bp DNA ladder.

2.4. Data Scoring and Genetic Diversity Analysis

Since SCoT markers are dominant, each amplified band was considered as a biallelic locus (1 for present, and 0 for absent). Only clear bands were kept and a binary data matrix was compiled. The Polymorphism Information Content (PIC) of each primer was calculated as:

$$PIC = 1 - Sum(pi2)$$

where the proportion of the i-th allele at a particular locus is called the frequency of the i-th allele, or the frequency of the i-th genotype [13].

Genetic similarity between pairs of genotypes (pairwise GS) was calculated by Nei and Li (1979):

$$GS = 2nxy / (nx + ny)$$

Where nxy is the number of bands common to both genotypes x and y and nx and ny are the total number of bands scored in genotypes x and y respectively.

Similarity matrix was used to create a dendrogram using NTSYSpc version 2.02e and MEGA 11, with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering procedure.

2.5. Total Phenolic Content (TPC) Determination

The total phenolic content of the ethanolic and aqueous extracts of the seeds was measured using the Folin-Ciocalteu method with gallic acid as the reference compound [26, 27]. In brief, 20 µl of extract was mixed with 3 ml of distilled water, 90 µl of Folin-Ciocalteu reagent, and 90 µl of 20% Na2CO3. The solution was incubated in dark for 5 min and absorbance was measured at 630 nm. The concentrations were determined from a calibration curve of gallic acid (y = 0.0057x + 0.0665) and reported in micrograms of gallic acid equivalents per milliliter (ug GAE/ml).

2.6. DPPH Radical-Scavenging Assay

The ability of antioxidants was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay (Asadujjaman et al., 2013). An 180 ul aliquot of extract was added to a 20 ul aliquot.

Absorbance was measured at 517 nm and the activity was determined using an ascorbic acid calibration curve (y = 0.0105x + 0.0803) and reported as micrograms of ascorbic acid equivalents per milliliter (ug AAE/ml).

2. Results

2.1. SCoT Marker Polymorphism

982 bands were amplified from the 34 genotypes with the nine SCoT primers, ranging from 46 bands (SCoT 31) to 156 bands (SCoT 17) and an average of 109.1 bands per primer. The range of amplification frequencies were from 0.30 (SCoT 9) to 0.89 (SCoT 17) with an average of 0.60 (Fig. 2A).

The overall discriminatory power was moderate, with values of PIC ranging from 0.19236 (SCoT 17) to 0.4642 (SCoT 15) with a mean of 0.366 (Fig. 2B). SCoT 15 (0.4642) and SCoT 31 (0.4361) were the most informative primers (Fig. 3), and SCoT 17 (0.19236) and SCoT 28 (0.27904) were the least informative primers, although it is interesting to note that the primer giving the maximum number of bands (SCoT 17) was not the most informative, and that allelic evenness and band number were independent properties.

2.2. Allelic Frequencies per Primer

Allelic frequencies were also studied within each primer for each range of amplicon sizes. Frequencies of fragments amplified by Primer SCoT 31 were between 0.23 and 0.38 with the highest being at 400 bp (14 bands, frequency 0.41). SCoT 30 resulted in five band classes (500 to 900 bp) with frequencies ranging from 0.67 to 0.85, with a maximum frequency of 0.85 at 700 bp (29 band classes). SCoT 28 had high frequencies of occurrence throughout all five bp size classes (0.73–0.94), with the most common size band at 600 bp (32 bands, frequency 0.94) (**Table 3**, Fig. 4). It is a pattern that is seen with a few primers yielding a large number of bands which are clustered in a few dominant size classes, as seen with the amplification of conserved, gene-proximal regions, rather than the more even distribution that is seen with anonymous markers.

2.3. Genetic Similarity and Cluster Analysis

The pairwise Nei and Li similarity was between 38.55% (RBK4 vs. RBK9) and 92.31% (RBK11 vs. RBK31) among the 34 genotypes. Within this general range, but outside of it, there were some interesting pairwise comparisons: RBK9 was 64% different from RBK19, 67% different from RBK20, and 76% different from RBK2; RBK2 was relatively similar to RBK13 (83%) (Fig. 5.).

The 34 genotypes were grouped into three major groups by using UPGMA clustering based on the binary matrix of SCoT (Fig. 6).

Cluster I. included 28 of the 34 genotypes, and exhibited moderate intra-cluster similarity, representing the major gene pool.

Cluster II. included only an extremely divergent genotype, RBK15.

Cluster III. Five genotypes (RBK22, RBK24, RBK25, RBK26 and RBK28) were clustered together as a separate sub-population.

2.4. The content of Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used against the gallic acid calibration curve (y = 0.0057x + 0.0665) to determine TP C which had a high variation among the 34 genotypes. RBK13 had the highest concentration (343.413 ug/ml) and RBK11 the lowest (10.794 ug/ml); the collection mean was 181.05 ug/ml (SD = 53.31 ug/ml). Genotypes exceeding 220 ug/ml included RBK7 (224.294 ug/ml), RBK22 (343.413 ug/ml), RBK14 (238.889 ug/ml), RBK26 (236.063 ug/ml), RBK28 (238.143 ug/ml),and RBK29 (220.357 ug/ml) (Fig. 7a).

2.5. DPPH Antioxidant Activity

The DPPH radical-scavenging activity also showed a wide range of values (calibration curve y = 0.0105x + 0.0803). RBK29 showed the highest activity (52.462 ug/ml), followed by RBK28 (51.829 ug/ml) and RBK14 (51.467 ug/ml); RBK3 showed the lowest (7.648 ug/ml). The collection mean was 32.68 ug/ml (SD = 11.54 ug/ml) (Fig. 6b).

(B) DPPH antioxidant activity (ug/ml). Dashed red lines indicate the collection mean.

2.6. Relationship Between TPC and DPPH

The positive correlation coefficient (r = 0.2504) between DPPH and TPC for all of the 34 genotypes was not statistically significant (p = 0.1533) (Fig. 8). The low percentage association indicates that phenolic compounds are involved in, but not entirely responsible for, the antioxidant potential of these red bean seeds; other phytochemicals including flavonoids, anthocyanins and tocopherols may be responsible for some of that antioxidant potential as well.

When grouped by genetic cluster, Cluster III genotypes (RBK22, RBK24, RBK25, RBK26, RBK28) tended to show higher TPC values than genotypes in the other two clusters (Fig. 9).

3. Discussion

3.1. SCoT Marker Efficacy in Assessing Red Bean Diversity

To our knowledge, this is the first application of SCoT markers to red bean genotypes from the merged districts of Khyber Pakhtunkhwa. The nine primers generated a consistently polymorphic amplification profile, confirming their suitability for characterizing previously unstudied common bean germplasm.

The total number of bands (982) and the per-primer range (46–156) are in line with other SCoT-based diversity studies in legumes. Hromadová, Gálová [14] similarly reported high reproducibility for SCoT markers alongside RAPD markers in common bean, and Osman and Ali [21] used SCoT markers effectively to resolve genetic relationships among Pisum sativum subspecies.

The mean PIC of 0.366 indicates a moderate level of genetic diversity in this collection. SCoT 15 (0.4642) and SCoT 31 (0.4361) were the most discriminating primers and would be reasonable candidates for future, more targeted screening of this germplasm. These values sit below the PIC range typically reported for SSR-based studies of common bean - for example, a mean PIC of

0.63 across 229 Chinese landraces Zhang, Blair and Wang [17] and a range of 0.295–0.831 among 60 red bean landraces from Malakand Division, Pakistan [18] which is expected given that SCoT primers amplify conserved, gene-flanking sequences rather than the hypervariable microsatellite repeats targeted by SSRs. The PIC values obtained here are, however, comparable to or higher than those reported for SCoT markers applied to other grain legumes, including mungbean [28] and blackgram [29], suggesting that the Kurram Agency red bean genotypes retain a useful degree of genetic variation despite the region's relative geographic isolation and reliance on informal seed exchange.

3.2. Genetic Structure of the Collection

UPGMA clustering placed 28 of the 34 genotypes into a single dominant cluster (Cluster I), pointing to a relatively narrow genetic base among most Kurram Agency landraces - a pattern also seen in other regional bean collections.[18] reported genetic similarity values spanning 0.11–0.91 among red bean

landraces from Malakand Division, and Nawaz and Farhatullah [30] similarly resolved 96 red bean genotypes from the Himalayan region of Pakistan into four distinct groups differing considerably in seed characteristics. A comparable pattern of high overall similarity accompanied by a small number of clearly differentiated accessions has also been observed with SSR markers in more distantly related Vigna red beans [31] suggesting that a dominant gene pool punctuated by a few divergent lines is a common feature of regionally maintained bean landraces, irrespective of species or marker system.

The separation of RBK15 into its own cluster (Cluster II), together with the five-genotype Cluster III (RBK22, RBK24, RBK25, RBK26, RBK28), indicates that distinct genetic sub-groups persist within an otherwise narrow gene pool. Such differentiation may reflect separate introduction histories, divergent farmer selection preferences, or adaptation to specific micro-environments within Kurram Agency. At the level of individual genotype pairs, RBK2 and RBK13 were relatively similar (83%), consistent with a shared origin or closely related selection history, whereas the low similarity between RBK4 and RBK9 (38.55%) marks them as strongly

divergent and therefore potentially useful parents for hybridization aimed at maximizing genetic distance.

3.3. Biochemical Variation and Nutritional Potential

The range of TPC values recorded here - from 10.79 to 343.41 ug/ml - is wide relative to several previous surveys of common bean phenolics. Kan et al. (2017) reported TPC of 0.25–3.79 mg GAE/g dry weight across 26 kidney bean cultivars, and Rocchetti et al. (2019) reported approximately 0.90-3.00 mg GAE/g dry weight across several bean types. Because these studies used dry-weight-based units while the present study expresses TPC per milliliter of extract, the two sets of values are not directly comparable in magnitude; nonetheless, the breadth of variation observed among the 34 Kurram Agency genotypes indicates substantial biochemical diversity within this single landrace collection, consistent with Tan [32], who noted that phenolic content differs considerably among Phaseolus species and that greater genetic diversity increases the likelihood of identifying elite genotypes.

DPPH antioxidant activity was likewise highly variable, with RBK29, RBK28, and RBK14 showing the greatest scavenging capacity (52.46, 51.83, and 51.47 ug AAE/ml, respectively). This is consistent with Kan, Nie [24], who linked antioxidant activity in kidney beans to their phenolic and flavonoid content, and with [8] who found that colored bean varieties - particularly red, cream, black, pink, and brown seed coats - generally show the highest antioxidant capacity among common bean types.

3.4. Relationship Between Phenolic Content and Antioxidant Activity

The positive but non-significant correlation between TPC and DPPH activity observed here ($r = 0.2504$, $p = 0.1533$) is weaker than that reported in some earlier studies: Kan, Nie [24],found a strong relationship between FRAP values and TPC ($R2 = 0.8030$), and Mastura, Hasnah and Dang [22] reported a significant positive correlation ($p < 0.001$) between total phenolics and antioxidant capacity across several bean

types. The weaker association in the present collection may reflect the contribution of non-phenolic antioxidants - including flavonoids, anthocyanins, and tocopherols - to overall DPPH scavenging activity [33, 34], as well as the possible influence of anti-nutritional compounds such as phytic acid, which can bind phenolics and reduce their extractability [35, 36]. Taken together, these findings suggest that phenolic content alone is an incomplete predictor of antioxidant capacity in this germplasm and that a broader phytochemical profile would be needed to fully explain the variation observed.

3.5. Implications for Breeding and Conservation

These results have direct implications for the conservation and use of Kurram Agency red bean germplasm. The moderate but structured genetic diversity detected here supports continued in-situ and ex-situ conservation of these landraces as an adaptively valuable resource. The identification of genetically divergent genotypes (notably RBK15) and genotypes with distinctive biochemical profiles (RBK13 for TPC; RBK29, RBK14, and RBK28 for antioxidant activity) provides specific, actionable targets for breeding programs.

Combining molecular diversity data with biochemical profiling allows for more informed parental selection: crossing genotypes from different genetic clusters that also differ in phenolic

content and antioxidant activity could, in principle, combine these complementary traits in progeny while preserving sufficient genetic diversity for future adaptation.

4. Conclusions

This study provides the first integrated assessment of genetic diversity and biochemical composition in red bean genotypes from Kurram Agency, Khyber Pakhtunkhwa, Pakistan, combining SCoT molecular markers with phytochemical analysis. The nine SCoT primers detected moderate genetic variation among the 34 genotypes (PIC = 0.192-0.464, mean 0.366). UPGMA clustering resolved three groups, with the majority of genotypes (28 of 34) falling into a single cluster - indicating a comparatively narrow genetic base that nonetheless contains clearly distinct sub-groups (Cluster II: RBK15; Cluster III: RBK22, RBK24, RBK25, RBK26, RBK28).

Biochemical characterization revealed substantial variation in total phenolic content (10.79-343.41 ug/ml) and DPPH antioxidant activity (7.65-52.46 ug/ml). RBK13 (highest TPC) and RBK29, RBK14, and RBK28 (highest antioxidant activity) emerged as the most promising genotypes for nutritional enhancement and further breeding.

Collectively, these findings demonstrate the genetic and biochemical value of locally adapted red bean landraces from Kurram Agency and provide a molecular and phytochemical framework to guide their conservation and use in breeding programs aimed at improving the nutritional quality of red bean in Pakistan.

5. Recommendations

1. Extend molecular characterization of red bean germplasm from other regions of Pakistan, and from other legume crops, using SCoT markers alongside complementary systems such as SSR and SNP markers.

2. Incorporate seed morphological traits together with biochemical markers into selection criteria for breeding stress-resilient, nutritionally enhanced red bean genotypes.

3. Establish an ex-situ core collection comprising the most genetically and biochemically distinctive genotypes identified here - particularly RBK13, RBK29, RBK14, RBK28, and the divergent RBK15 - for evaluation in multi-location field trials.

4. Undertake more detailed biochemical profiling, including individual phenolic acids, flavonoids, and anthocyanins, together with assessment of anti-nutritional factors, to more fully characterize the nutritional potential of this germplasm.

Declarations

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Conflict of Interest

The authors declare no conflict of interest. Data Availability The datasets generated during the current study are available from the corresponding author on reasonable request

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Author Contribution

Omer Khiam (O.K.) conceived and designed the study, performed the experiments and data analysis, prepared the figures and tables, and wrote and organized the manuscript. O.K. also revised and finalized the manuscript and approved the final version.

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The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Data Availability

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Figures



Figure 1

Red beans collected from merged district of Pakistan such as kurram agency

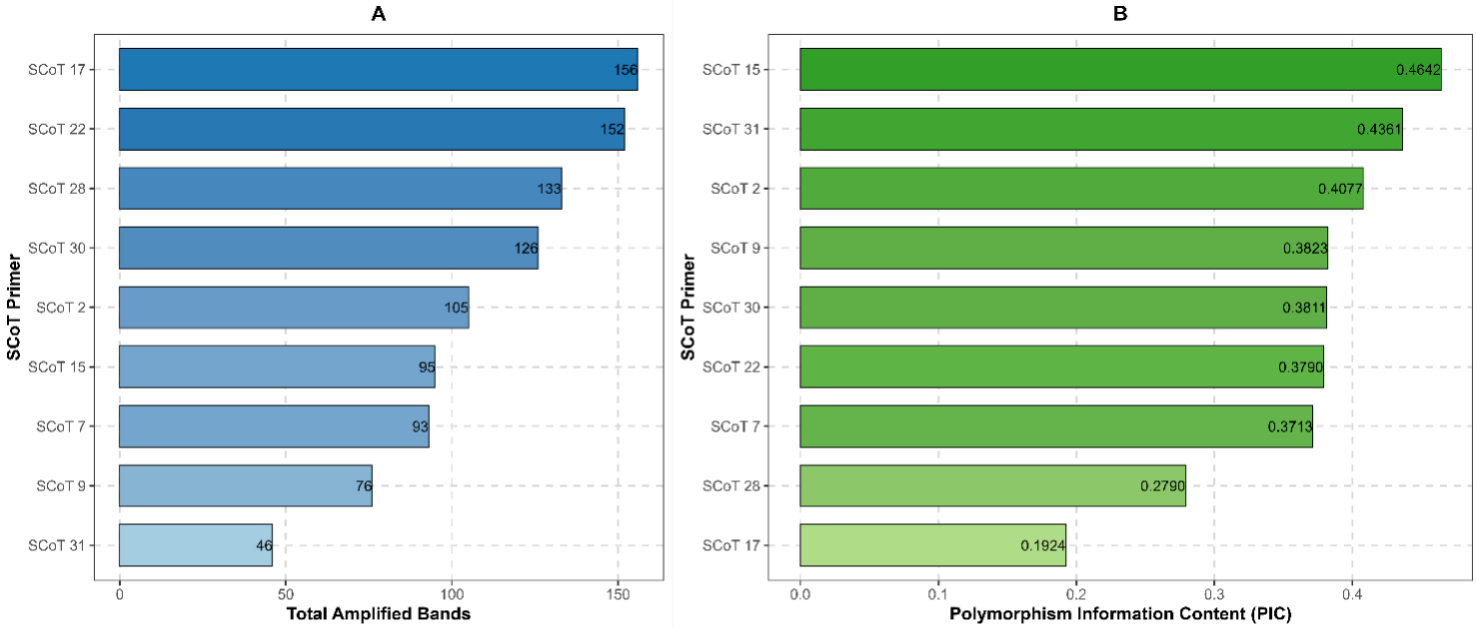


Figure 2

SCoT primer summary across 34 red bean genotypes: (A) total amplified bands per primer; (B) Polymorphism Information Content (PIC) per primer. The highest PIC was recorded for SCoT 15 (0.4642) and the lowest for SCoT 17 (0.19236).

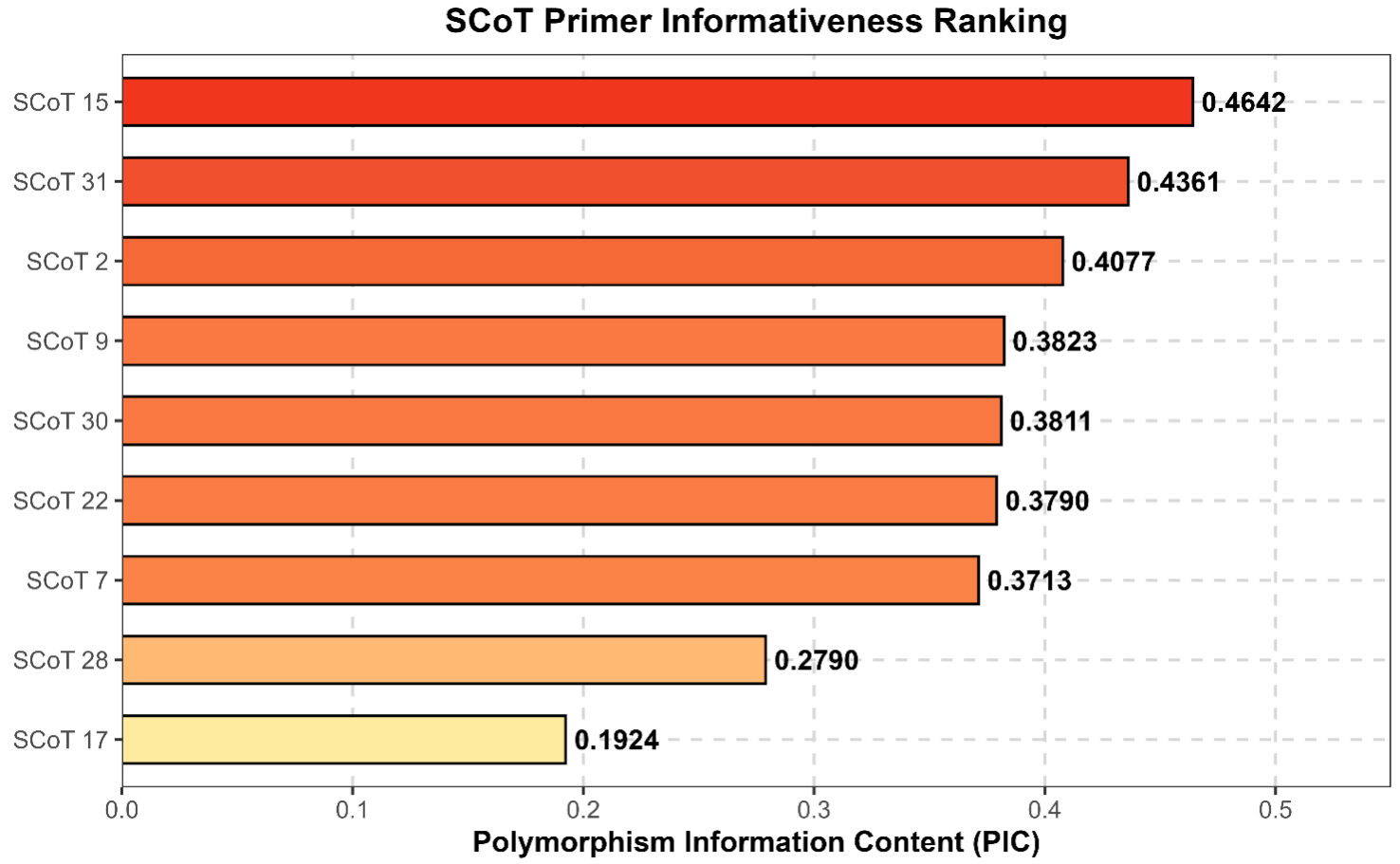
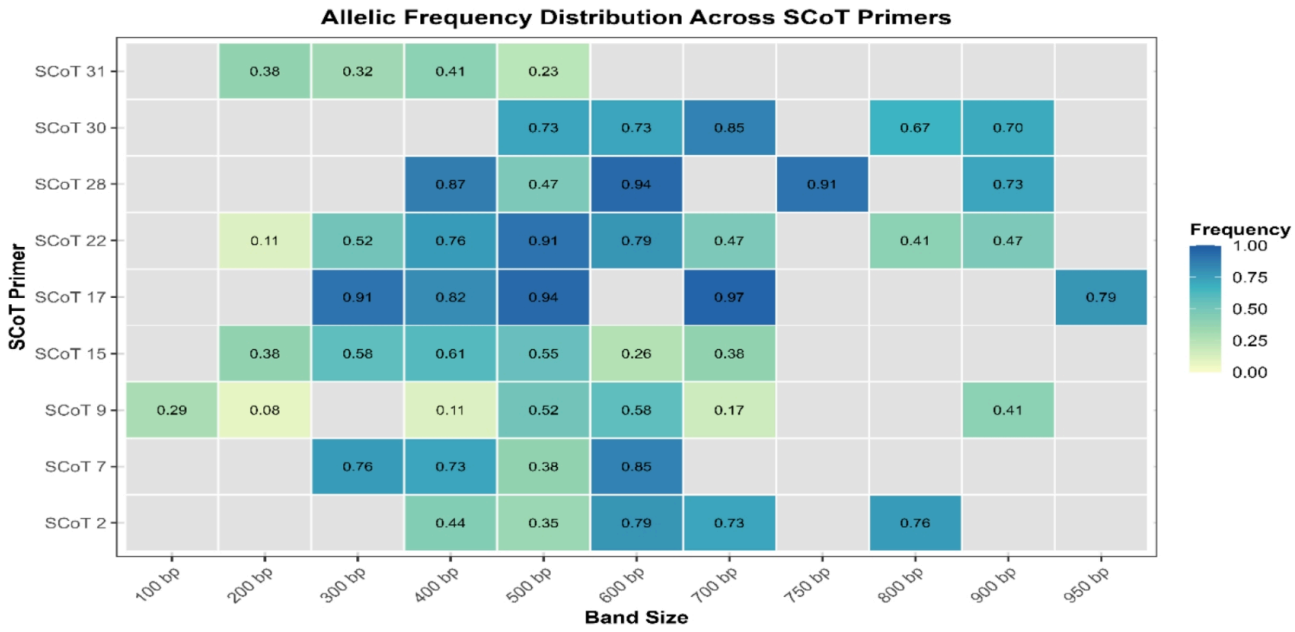


Figure 3

SCoT primer informativeness ranked by Polymorphism Information Content (PIC). SCoT 15 and SCoT 31 were the most informative primers



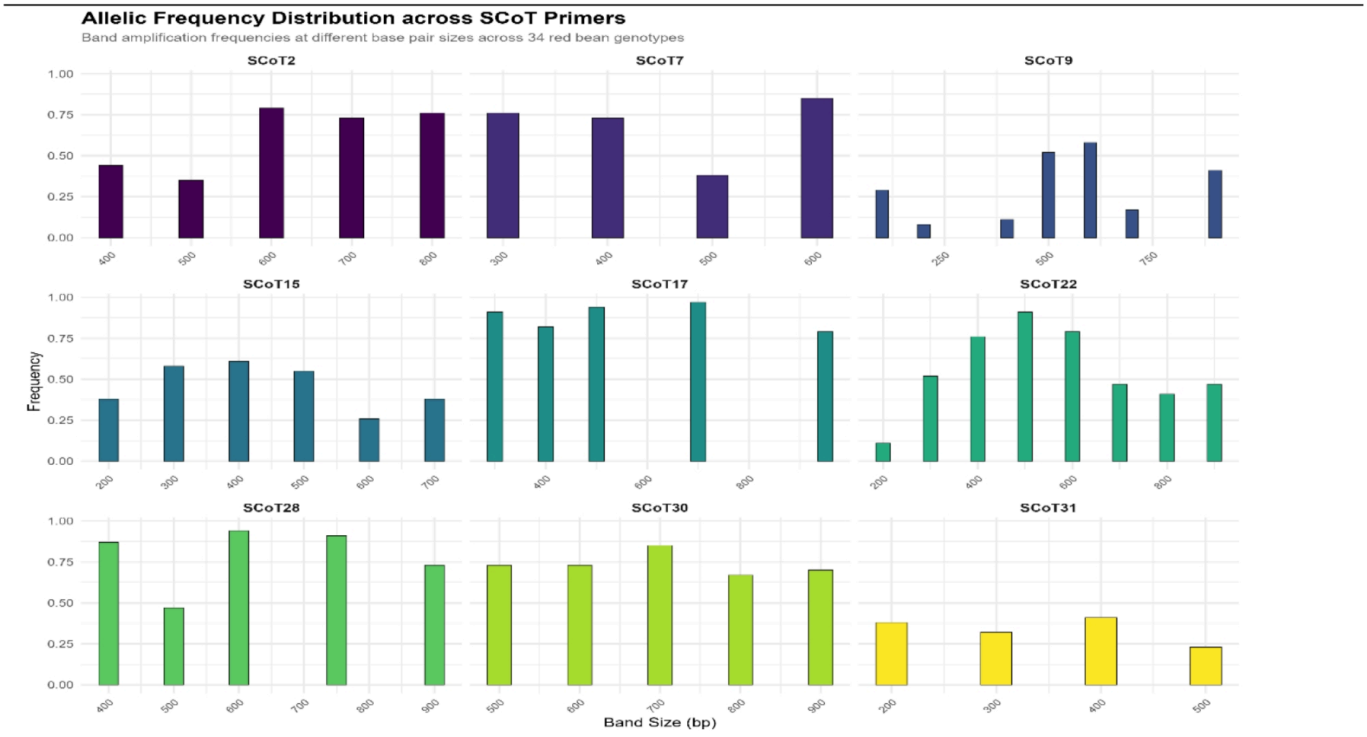


Figure 4

Allelic frequency distribution across nine SCoT primers in 34 red bean genotypes. Darker shading indicates higher amplification frequency.

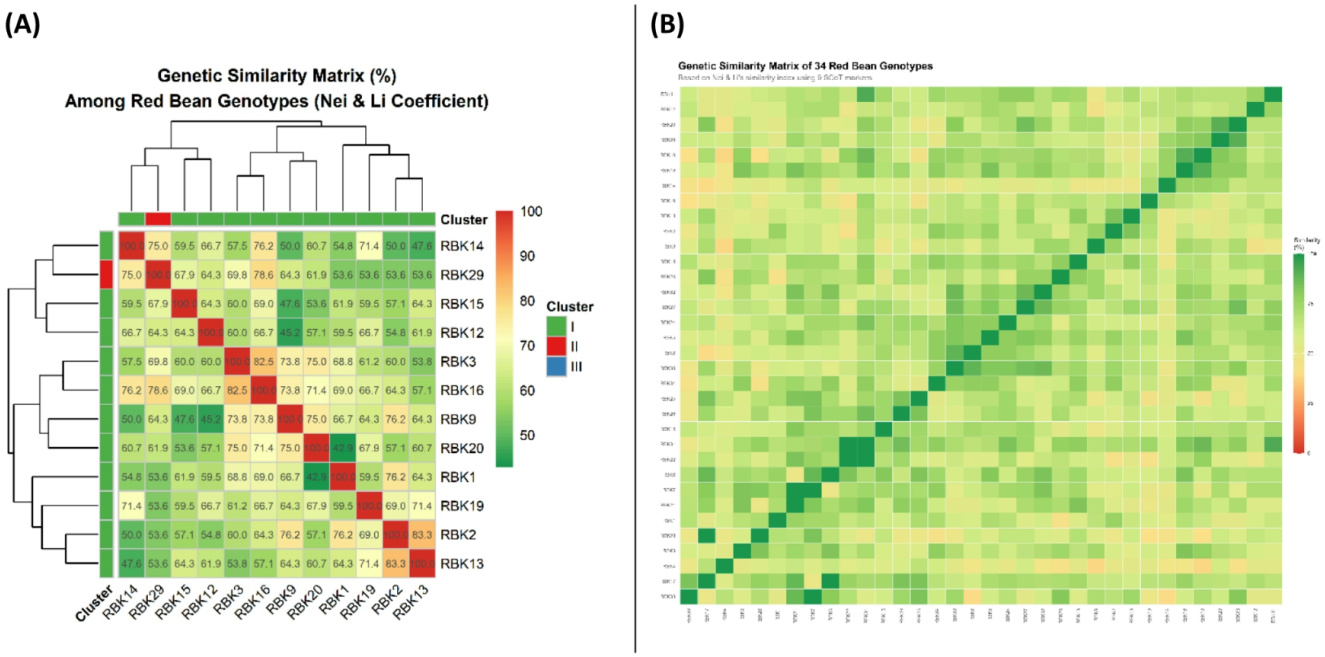


Figure 5

Nei and Li coefficients similarity heatmap of 12 red bean genotypes. Values are percentage similarity, with darker green colours signifying greater similarity. (b) Genetic similarity heatmap of 34 red bean genotypes based on Nei and Li's similarity index using 9 SCoT markers. Values range from ~38% (green) to 100% (dark green). Genotypes ordered by hierarchical clustering

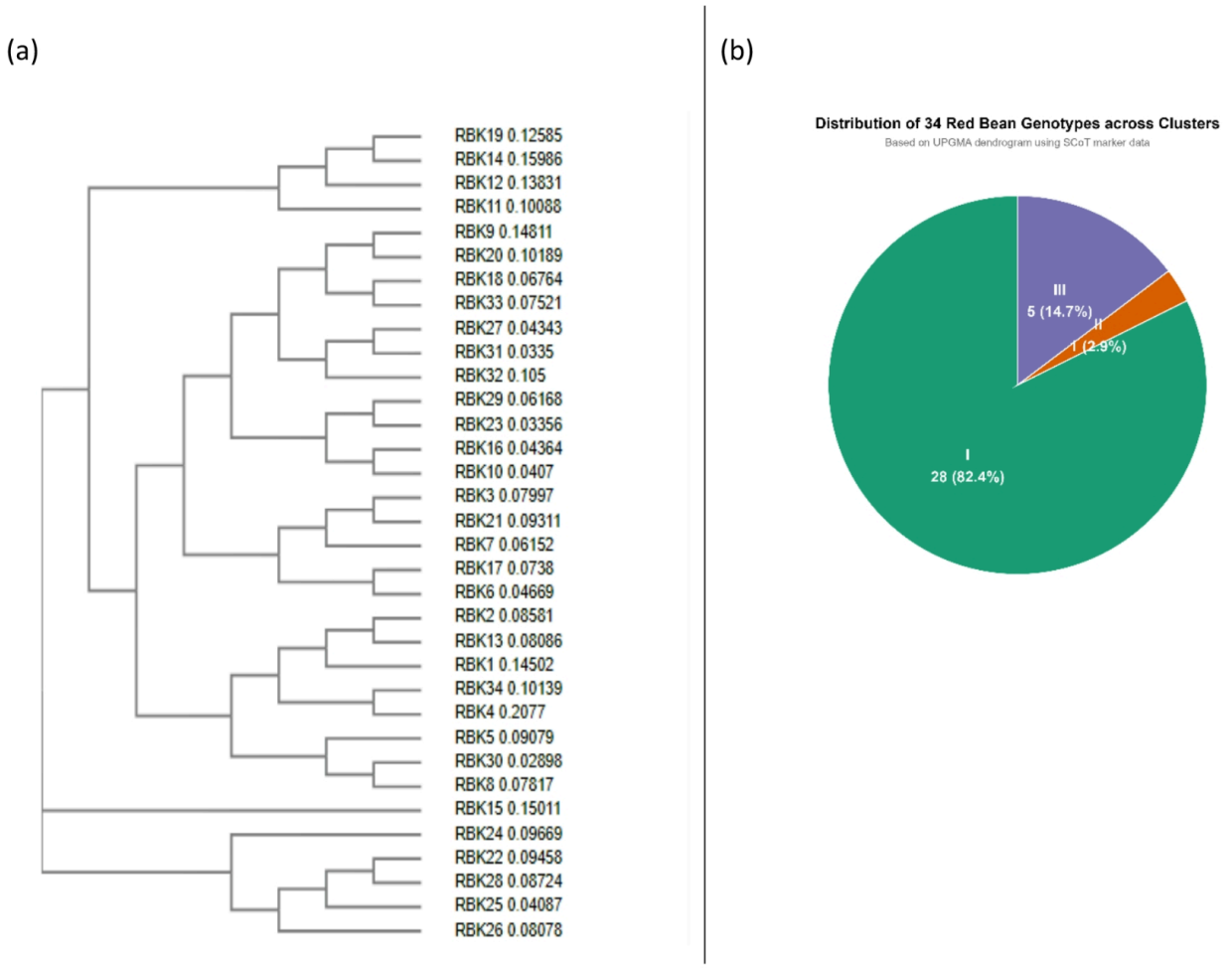


Figure 6

UPGMA dendrogram of genetic relationships among 34 red bean genotypes based on SCoT markers and the Dice coefficient. Branches are colored by cluster assignment. (B) Cluster composition of 34 red bean genotypes based on UPGMA dendrogram analysis. Cluster I: 28 genotypes (82.4%), Cluster II: 1 genotype (2.9%, RBK15), Cluster III: 5 genotypes (14.7%).

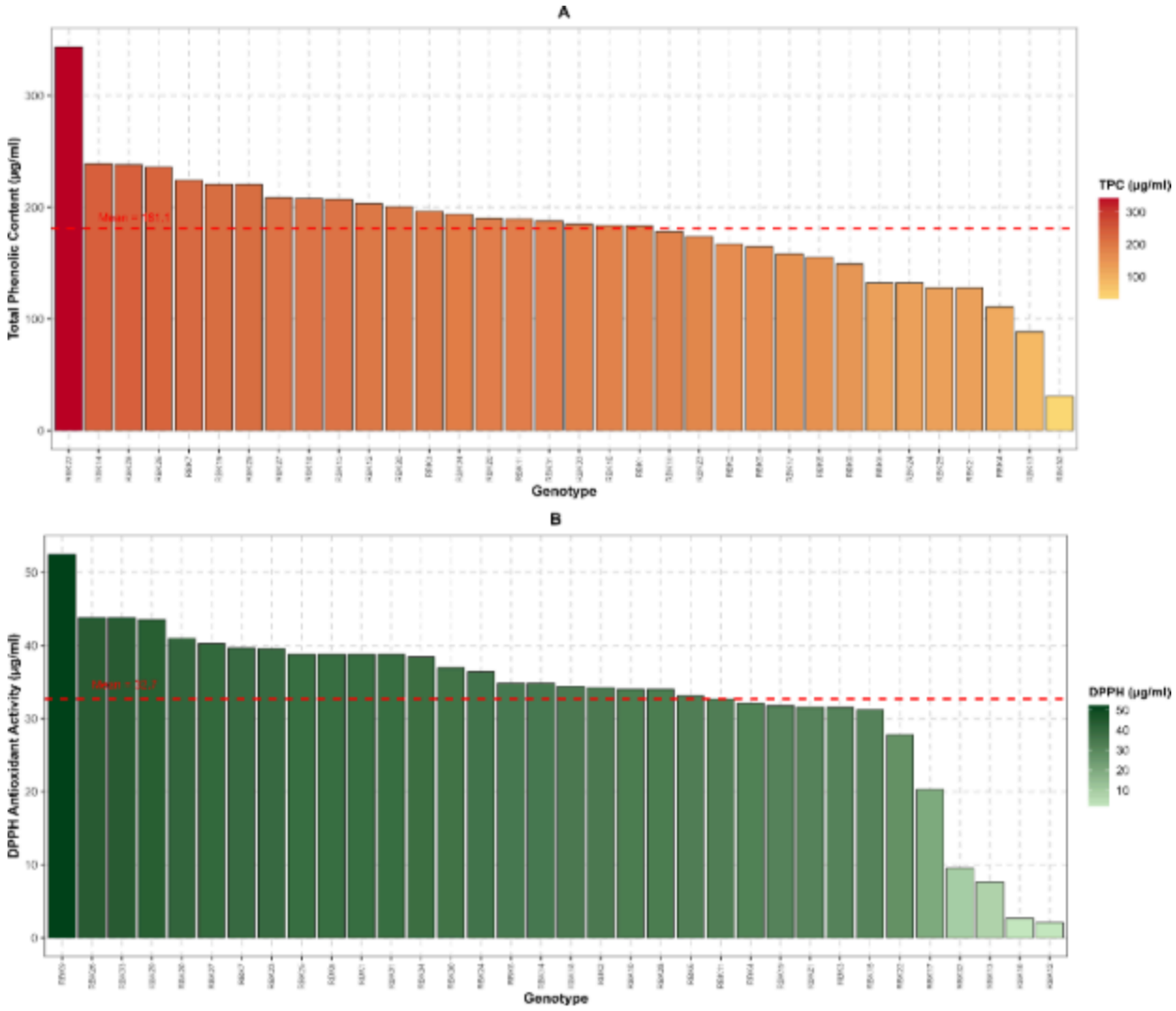


Figure 7

Biochemical parameters of 34 red bean genotypes: (A) total phenolic content (ug/ml);

(B) DPPH antioxidant activity (ug/ml). Dashed red lines indicate the collection mean.

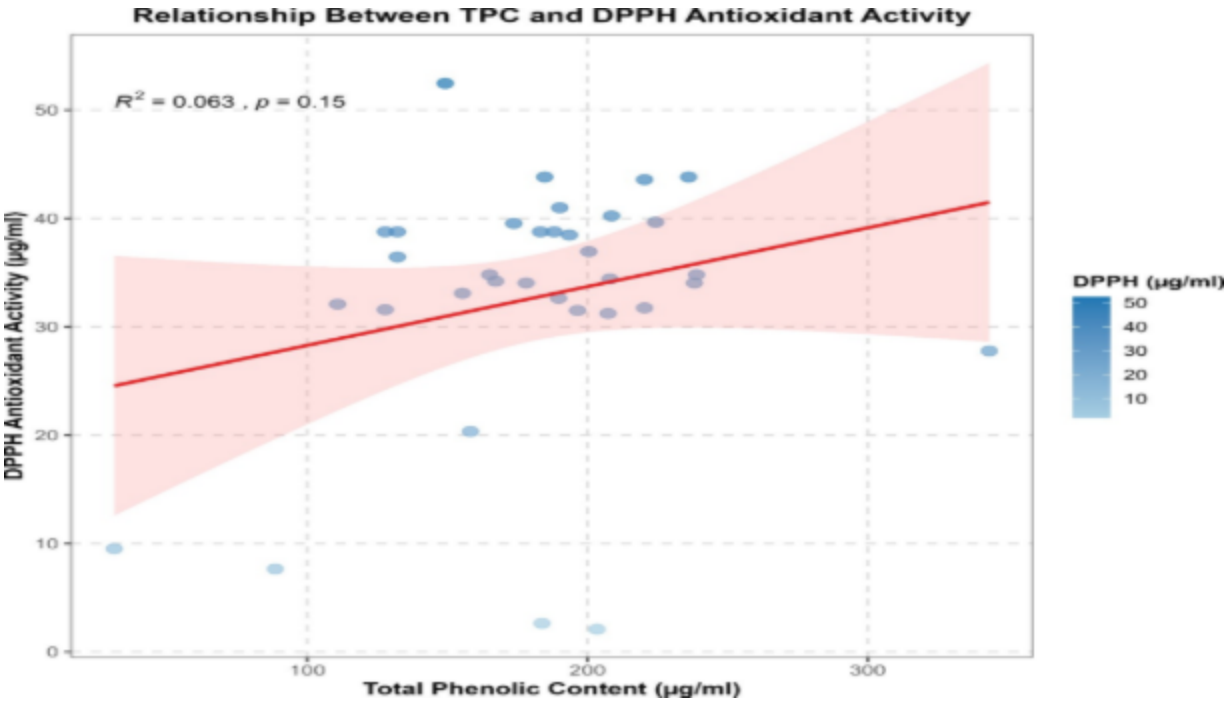


Figure 8

Relationship between total phenolic content and DPPH antioxidant activity across 34 red bean genotypes, with linear regression line and 95% confidence band (r = 0.2504, p = 0.1533)

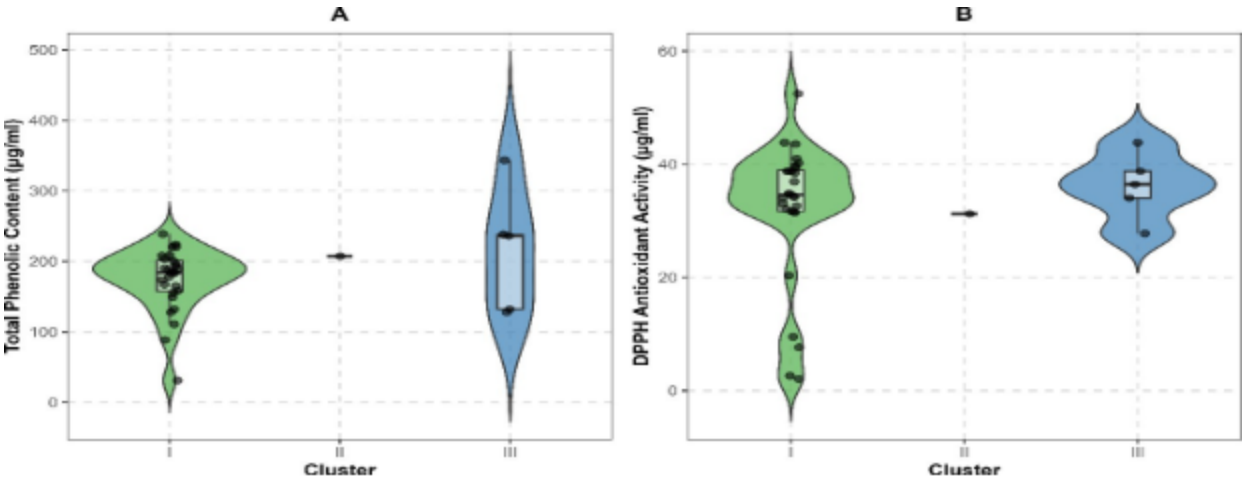


Figure 9

Biochemical parameters grouped by genetic cluster: (A) total phenolic content; (B) DPPH antioxidant activity. Clusters are as defined by UPGMA analysis