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Integration of Morphological, Protein, and RAPD Markers for Diversity Analysis in Durum Wheat

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Abstract

Triticum durum L. (durum wheat), an allotetraploid species, is widely used for producing noodles, macaroni, and pasta at the commercial level. For qualitative and quantitative improvement, it is essential to estimate the extent of genetic variation within this species. The present study aimed to evaluate the genetic diversity in a world collection of *T. durum* accessions using DNA-based Randomly Amplified Polymorphic DNA (RAPD) markers. A total of 175 accessions were grown in Azad Jammu and Kashmir for morphological characterisation, and 30 genotypes selected randomly were analysed for DNA-based diversity using 40 RAPD primers. RAPD analysis revealed 238 fragments, of which 219 (92.05%) were polymorphic, confirming high genetic variability. Primers GL B-7, B-13, B-17, and D-12 showed the highest polymorphism. Genetic distances among the 30 accessions ranged from 0.00 to 32.0%. The most divergent pair was accession 012967 (population 2) from Azad Kashmir and 013140 (population 21) from Syria, with a genetic distance of 32%. Cluster analysis separated the accessions into two principal groups: the first comprising populations 1 (012966) and 2 (012967), forming a distinct cluster, and the second divided into two subclusters. Overall, the accessions exhibited considerable genetic variability at morphological, seed storage protein, and DNA levels, indicating strong potential for use in breeding programs aimed at developing improved durum wheat varieties to meet the growing industrial demand in Pakistan.

Keywords: Durum wheat, germplasm, genetic diversity, RAPD, DNA-based markers, Cluster analysis, *Triticum durum*.

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1. Introduction

Durum wheat (*Triticum turgidum* subsp. *durum*) is a tetraploid cereal (AABB genome; 2n=4x=28) globally significant for producing pasta, couscous, noodles, and flatbreads. The growing interest in this crop stems from rising demand for nutrient-rich foods and increasing climate variability. Across continents—including West Asia, East Africa, and the Mediterranean—durum yields remain constrained by drought, heat, and soil salinity, underscoring the urgent need to harness its genetic diversity (Robbana et al., 2019; Nigro et al., 2023; Dwivedi et al., 2023).

Genetic variability is the cornerstone of effective breeding programs. Accessing and understanding this variation facilitates trait enhancement for yield, disease resistance, and stress tolerance. Molecular characterisation allows breeders to identify promising parents and monitor genetic gain efficiently (Robbana et al., 2019). In resource-limited regions, cost-effective marker systems like SSRs and RAPDs remain highly practical tools, offering multi-allelic resolution (SSR) or rapid screening capability (RAPD) without extensive genomic infrastructure (Sharma et al., 2020; Kumar et al., 2019; Todorovska et al., 2019). Recent genome-wide studies have elucidated the diversity landscape of durum wheat. Tunisian landraces, profiled via DArTseq, exhibit population structure aligned with geographic origin and adaptive traits (Robbana et al., 2019). In Ethiopia, SSR-based surveys revealed high allelic variation, particularly within local landrace pools (Alemu et al., 2020). A global diversity panel evaluated using SNP arrays highlighted extensive inter- and intra-regional variation, with notable heterozygosity in B-genome loci (Nigro et al., 2023; Dwivedi et al., 2023). Despite these genomic insights, traditional markers like SSR and RAPD continue to deliver practical utility in many breeding scenarios (Kumar et al., 2019; Sharma et al., 2020; Todorovska et al., 2019).

Seed storage proteins, separable via SDS-PAGE, are particularly informative for quality traits in wheat. Polymorphic profiles of glutenins and gliadins correlate with dough strength and end-use quality, and provide a useful complement to DNA-based diversity metrics (Awika et al., 2021). Combining SDS-PAGE, SSRs, and RAPDs can deliver a rich, multi-layered understanding of germplasm variation, useful for breeding decisions in environments like Pakistan's. In Pakistan, durum wheat diversity remains under-characterised at the molecular level. Most local studies focus on agronomic evaluation rather than integrating morphological, biochemical, and molecular markers (Khan et al., 2020). Considering the country's diverse agroecologies and growing climate challenges, systematic characterisation of global and local accessions is crucial for future breeding.

This study aims to fill this gap through a comprehensive analysis of 175 durum wheat accessions representing Pakistan, Syria, Egypt, ICARDA, and Cyprus—evaluated under Azad Kashmir conditions across two seasons. In Pakistan, the relevance of durum wheat extends beyond agronomic potential to its growing industrial and nutritional importance. The crop is increasingly demanded by local food industries for pasta, macaroni, noodles, and bakery products, reflecting a shift in consumer preferences toward diversified wheat-based foods. In addition, durum wheat provides superior nutritional quality due to its high protein and gluten strength, making it valuable for addressing dietary needs in a rapidly urbanising population. Despite this potential, molecular-level characterisation of

Pakistani durum wheat resources remains limited, underscoring the need for a system. The integrated approach involves morphological trait characterisation, SDS-PAGE protein profiling, and molecular diversity assessment using RAPD and SSR markers.

The present study was designed to comprehensively evaluate genetic diversity in durum wheat by integrating morphological, biochemical, and molecular approaches. Specifically, phenotypic variation and genotype × environment interactions were examined across two consecutive growing seasons, while seed storage protein profiling and DNA-based markers (RAPD and SSR) were employed to characterise genetic variability at the molecular level. The analysis further aimed to establish genetic relationships and clustering patterns among accessions, ultimately generating insights that could guide breeding programs and support the effective utilisation of durum wheat germplasm under Pakistan's agro-climatic conditions.

2. Materials and Methods

2.1 Plant Material and Experimental Design

A total of 175 *Triticum turgidum* subsp. *Durum* accessions originating from Pakistan, Syria, Egypt, ICARDA, and Cyprus were obtained from the Plant Genetic Resource Institute (PGRI), NARC, Islamabad. The experiment was conducted at the Agricultural Research Station, Garhi Dopatta (Muzaffarabad, Azad Kashmir) during two consecutive winter seasons (2010–2012). A randomised complete block design (RCBD) with three replications was used. Standard agronomic practices were followed, and data on 18 morphological traits were recorded using internationally recognised wheat descriptors (Ali et al., 2021; Eticha et al., 2020).

2.2 Seed Storage Protein Analysis

Seed storage protein polymorphism was analysed in 161 accessions using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Glutenin and gliadin subunits were separated to evaluate protein diversity related to end-use quality. Protein banding patterns were scored as present (1) or absent (0), and a binary matrix was generated for diversity estimation and cluster analysis (Sharma et al., 2019; Chen et al., 2021).

2.3 DNA Extraction and Marker Analysis

Genomic DNA was extracted from young leaves of 30 randomly selected accessions using a modified CTAB method (Al-Khayri et al., 2021). DNA concentration and purity were assessed spectrophotometrically and by agarose gel electrophoresis. Forty RAPD and fifty SSR primers previously validated in wheat diversity studies (Singh et al., 2019; Abou-Elwafa et al., 2022) were used for amplification. PCR products were separated on 1.5% agarose and Metaphor™ gels, stained with ethidium bromide, and visualised under UV illumination.

2.4 Data Scoring and Genetic Diversity Estimation

Amplified fragments were scored as binary data (1 = presence, 0 = absence). The percentage of polymorphism was calculated for each primer. Genetic similarities among accessions were estimated using Nei and Li's (1979) coefficient, and cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in POPGEN v3.5.

2.5 Statistical Analysis

Morphological data were analysed using analysis of variance (ANOVA) under RCBD to test genotypic effects, with means compared using the least significant difference (LSD) test at $p < 0.05$. Coefficients of variation (CV%) and broad-sense heritability (H^2) were calculated

where applicable. Principal component analysis (PCA) identified traits contributing most to variability. Molecular and protein data were subjected to cluster and principal coordinate analyses (PCoA) using NTSYS-pc and POPGEN software. Correlations among morphological, biochemical, and molecular datasets were computed using SPSS v25 and R software (R Core Team, 2023).

3. Results

The evaluation of 175 durum wheat accessions revealed significant phenotypic and molecular variability across all traits. Morphological assessment across two growing seasons demonstrated considerable diversity. Highly significant ($p < 0.01$) differences were recorded among genotypes for traits including days to germination, plant height, spike length, tiller number, peduncle length, and grain yield, confirming the presence of broad genetic variability within the collection. Mean values remained consistent between years, though minor fluctuations suggested an interaction between genotype and environment. For instance, plant height ranged from 95 to 112 cm across years, whereas spike length varied between 8.5 and 10.2 cm. These results highlight the adaptability of durum accessions to local growing conditions, corroborating recent studies that emphasise the value of morphological variability in durum wheat breeding programs.

Seed storage protein analysis using SDS-PAGE further demonstrated high polymorphism. A total of 719 distinct bands (alleles) were identified in 161 accessions, with an average of 4.5 bands per genotype. Genetic distance values ranged widely, from complete similarity (0%) in 127 pairwise comparisons to complete dissimilarity (100%) in 252 comparisons. Cluster analysis based on protein data revealed that accessions originating from Syria and Pakistan frequently grouped, suggesting that geographic origin did not strictly determine genetic relatedness. Similar conclusions have been drawn in recent reports that highlight admixture across wheat populations due to centuries of germplasm exchange.

For DNA-based analysis, thirty randomly selected accessions were screened with 40 RAPD and 50 SSR primers. RAPD markers generated 238 fragments, of which 219 (92.05%) were polymorphic, indicating a high degree of genetic variability. Genetic distances among RAPD profiles ranged from 0.0 to 32%, with cluster analysis grouping accessions into two major clusters. The most distinct grouping was formed by two accessions from Azad Kashmir, while the remaining accessions were subdivided into smaller subgroups. RAPD data thus provided a broad overview of genome-wide variation, supporting their utility in assessing diversity in durum wheat.

SSR markers produced 174 fragments with an average of 3.44 per primer, ranging between two and seven bands per locus. Approximately 48% of fragments were polymorphic, a lower proportion compared to RAPD markers, yet still informative. The genetic distance among SSR profiles ranged from 2 to 38%. Cluster analysis again divided the accessions into two principal groups, consistent with the RAPD results, but with finer resolution in separating closely related accessions. Several primers, such as Xgwm and Xbarc loci, produced highly polymorphic patterns that were particularly useful in distinguishing genotypes. Recent literature also confirms that SSRs remain powerful tools for dissecting genetic structure in durum wheat, although SNP markers are increasingly complementing their role.

Overall, the integration of morphological, protein, and DNA marker data confirmed the presence of substantial genetic variability among durum wheat accessions. The consistency of clustering across different marker systems indicates robust underlying diversity within the germplasm. Such variation provides a valuable foundation for future breeding initiatives aimed at improving durum wheat productivity and adaptability under Pakistan's agro-ecological conditions. These findings are aligned with recent global efforts to utilise both traditional and molecular tools for enhancing genetic gain in durum wheat breeding.

4. Discussion

Understanding the genetic relationships among durum wheat genotypes is crucial for breeding strategies, conservation, and germplasm utilisation. In the present study, morphological characterisation, seed storage protein analysis, and molecular markers (RAPD and SSR) were integrated to evaluate genetic diversity among a global collection of durum wheat accessions grown under Pakistani conditions. The combined evidence confirmed that the durum wheat gene pool harbours substantial variability, which can be exploited in breeding programs.

Morphological traits revealed wide diversity across accessions and years, with highly significant differences in plant height, spike length, grain yield, and maturity parameters. Such phenotypic variation reflects both genetic potential and environmental interactions, highlighting the adaptability of durum wheat to diverse climates. Similar findings have been reported in recent studies from Ethiopia, Turkey, and the Mediterranean, where morphological variability was successfully linked to breeding progress (Eticha et al., 2020; Alemu et al., 2021; Habib et al., 2023). The significance of genotype × environment interaction observed in this study emphasises the need for multi-location trials to identify stable genotypes, a recommendation consistent with recent global breeding initiatives (Maccaferri et al., 2019; Tadesse et al., 2021).

Seed storage protein analysis using SDS-PAGE further confirmed diversity, with 719 alleles identified across 161 accessions. Protein markers are valuable for assessing quality-related traits, especially gluten and storage proteins that influence end-product performance. Interestingly, clustering showed no strict correspondence between geographic origin and genetic distance, suggesting historical germplasm exchange and introgression across regions. This pattern aligns with recent observations where durum populations from the Mediterranean, South Asia, and North Africa showed admixture and lack of geographic clustering (Nadeem et al., 2021; Mangini et al., 2023).

The molecular analysis added a higher-resolution picture of genetic variation. RAPD markers exhibited 92.05% polymorphism, underscoring their efficiency in revealing genome-wide diversity despite being dominant markers. The genetic distances obtained (0–32%) indicate that some accessions are genetically very close, while others are highly divergent. SSR markers provided codominant, highly reproducible data, generating 174 fragments with 48% polymorphism. The genetic distance from SSRs ranged from 2–38%, broadly consistent with RAPD findings but offering better discrimination among closely related accessions. These results corroborate earlier reports that RAPD and SSR markers complement each other in resolving diversity in durum wheat (Singh et al., 2019; Abou-Elwafa et al., 2022; Javid et al., 2023).

Molecular tools such as SSRs have become increasingly important in breeding programs because of their high allelic diversity, reproducibility, and genome-wide coverage. Recent advances in durum wheat genomics, including high-density SNP arrays and whole-genome resequencing, are gradually replacing older markers. However, SSRs remain relevant in resource-limited breeding programs due to their cost-effectiveness and ease of use (Said et al., 2021; Al-Saghir et al., 2024). Moreover, the integration of SSRs with next-generation sequencing data is enhancing the precision of diversity assessments and marker-assisted selection in durum wheat (Mangi et al., 2022; Bassi et al., 2023).

The clustering of accessions into two major groups across both RAPD and SSR datasets further validates the robustness of the observed genetic structure. The agreement between different marker systems suggests that the underlying genetic variation is strong and reliable. Such findings highlight the potential of combining multiple marker systems for accurate germplasm characterisation. This integrated approach has been advocated in recent studies aiming to accelerate durum wheat improvement under climate stress (Kabbaj et al., 2022; Habib et al., 2023).

Looking forward, the integration of SNP-based high-throughput markers and next-generation sequencing technologies could further enhance the resolution of genetic diversity analyses in durum wheat. While RAPD and SSR markers provide a solid foundation, SNP platforms allow genome-wide association mapping and genomic selection, which will be particularly valuable for accelerating breeding progress in Pakistan's variable agro-climatic conditions. Future studies should therefore consider combining traditional markers with SNP arrays and whole-genome approaches to maximise both cost-effectiveness and genomic coverage.

Overall, the study demonstrates that Pakistani-grown durum wheat accessions possess wide genetic variability at the morphological, protein, and molecular levels. This diversity can be harnessed for breeding programs focused on yield stability, stress tolerance, and quality improvement. The findings are consistent with recent global efforts to broaden the genetic base of durum wheat by exploiting diverse germplasm collections and applying modern molecular breeding approaches (Tadesse et al., 2021; Mangini et al., 2023; Al-Saghir et al., 2024).

5. Conclusion

This study demonstrated substantial genetic diversity among 175 durum wheat accessions evaluated under Pakistani conditions using morphological traits, seed storage protein profiles, and molecular markers. RAPD and SSR analyses revealed complementary insights into genetic structure, while SDS-PAGE highlighted protein-based variation relevant to quality traits. The clustering patterns confirmed that genetic relationships were not strictly linked to geographic origin, reflecting historical germplasm exchange. Overall, the identified variability provides a strong foundation for breeding programs aimed at yield stability, stress tolerance, and quality improvement. These findings reinforce the importance of integrating traditional and molecular approaches for effective durum wheat improvement.

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8. Author Contribution Statement

Shabnam Niaz designed and conducted the study; Muhammad Waleed analysed data and drafted the manuscript; Muhammad Akhlaq contributed to writing and correspondence; Nasir Ahmad Khan assisted in writing.

9. Conflict of Interest

There is no conflict of interest.

10. SDGs Addressed

This study aligns with UN Sustainable Development Goals 2 (Zero Hunger), 12 (Responsible Consumption and Production), and 13 (Climate Action) by promoting sustainable wheat improvement and food security.

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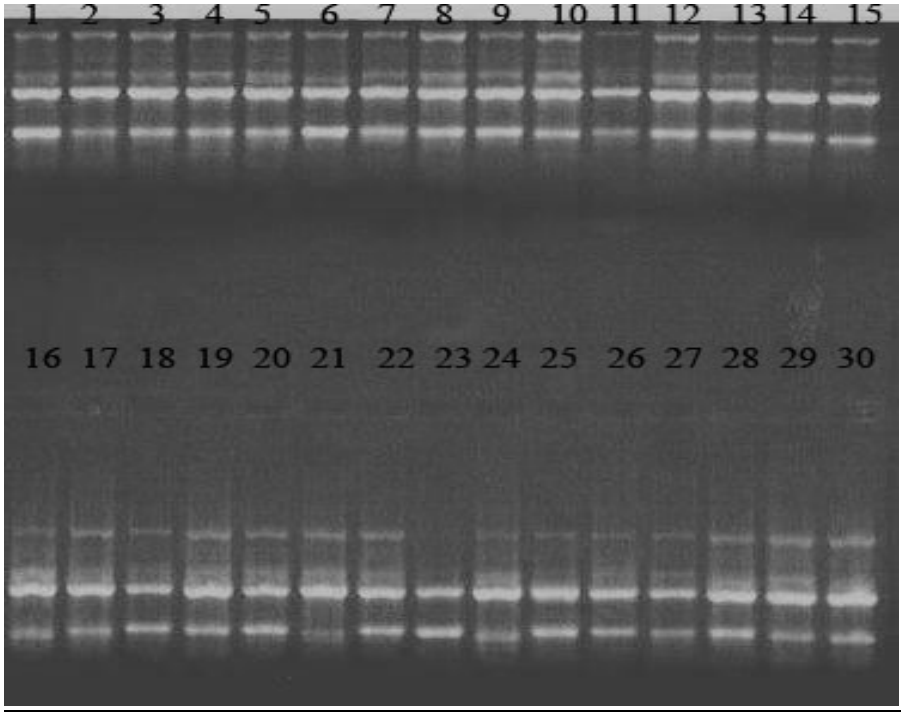

Figure 1. Representative gel of the amplification of 30 durum wheat accessions using RAPD primer GLI-17

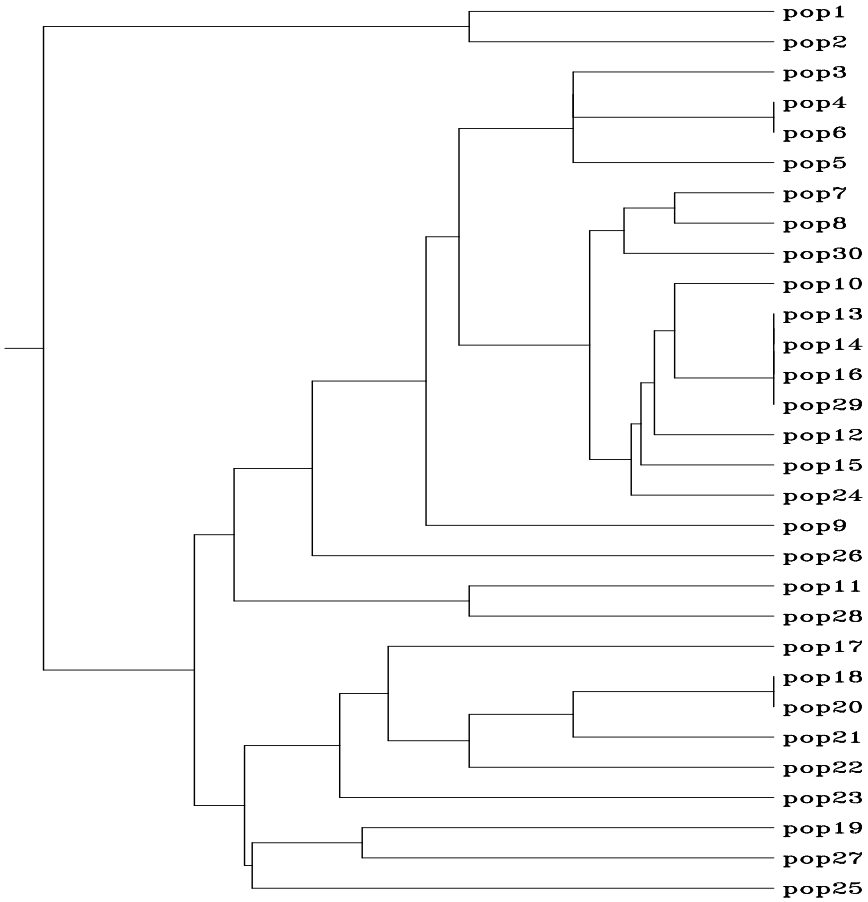

Figure. 2. Clustering pattern of durum wheat genotypes using RAPD data

Sr. No	Primer Name	Sequence
1	GL DecamerB-07	GGTGACGCAG
2	GL DecamerB-10	CTGCTGGGAC
3	GL DecamerB-11	GTAGACCCGT
4	GL DecamerB-13	TTCCCCCGCT
5	GL DecamerB-17	AGGGAACGAG
6	GL DecamerD-06	ACCTGAACGG
7	GL DecamerD-12	CACCGTATCC
8	GL DecamerD-13	GGGGTGACGA
9	GL DecamerD-19	CTGGGGACTT
10	GL DecamerD-20	ACCCGGTCAC
11	GL Decamer I-02	GGAGGAGAGG
12	GL Decamer I-05	TGTTCCACGG
13	GL Decamer I-06	AAGGCGGCAG
14	GL Decamer I-07	CAGCGACAAG
15	GL Decamer I-09	TGGAGAGCAG
16	GL Decamer I-10	ACAACGCGAG
17	GL Decamer I-11	ACATGCCGTG
18	GL Decamer I-15	TCATCCGAGG
19	GL Decamer I-17	GGTGGTGATG
20	GL Decamer I-20	AAAGTGCGGG
21	GL Decamer J-04	CCGAACACGG
22	GL Decamer J-05	CTCCATGGGG
23	GL Decamer J-06	TCGTTCCGCA
24	GL Decamer J-13	CCACACTACC
25	GL Decamer J-14	CACCCGGATG
26	GL Decamer J-19	GGACACCACT
27	GL Decamer J-20	AAGCGGCCTC
28	GL Decamer K-07	AGCGAGCAAG
29	GL Decamer K-11	AATGCCCCAG
30	GL Decamer K-13	GGTTGTACCC
31	GL Decamer K-15	CTCCTGCCAA
32	GL Decamer K-17	CCCAGCTGTG
33	GL Decamer K-18	CCTAGTCGAG
34	GL Decamer K-19	CACAGGCGGA
35	GL Decamer K-20	GTGTCGCGAG
36	GL Decamer L-07	AGGCGGGAAC
37	GL Decamer L-09	TGCGAGAGTC
38	GL Decamer L-10	TGGGAGATGG
39	GL Decamer L-16	AGGTTGCAGG
40	GL Decamer L-19	GAGTGGTGAC

Table 1. RAPD primer sequences used for the amplification of genomic DNA

Table 2. Estimates of genetic divergence among 30 durum wheat accessions using RAPD primers

Pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
2	0.0780																													
3	0.1335	0.1625																												
4	0.1335	0.1625	0.0513																											
5	0.1335	0.1625	0.0513	0.0513																										
6	0.1335	0.1625	0.0513	0.0000	0.0513																									
7	0.2231	0.1924	0.0780	0.0780	0.0780	0.0780																								
8	0.1924	0.2231	0.0513	0.0513	0.0513	0.0513	0.0253																							
9	0.1924	0.1625	0.1054	0.1054	0.1054	0.1054	0.0780	0.1054																						
10	0.1335	0.1625	0.1054	0.1054	0.1054	0.1054	0.0780	0.0513	0.1054																					
11	0.1924	0.1625	0.1625	0.1625	0.1625	0.1625	0.1924	0.1625	0.1625	0.1054																				
12	0.1335	0.1625	0.0513	0.0513	0.0513	0.0513	0.0780	0.0513	0.0513	0.0513	0.1054																			
13	0.1625	0.1924	0.0780	0.0780	0.0780	0.0780	0.0513	0.0253	0.0780	0.0253	0.1335	0.0253																		
14	0.1625	0.1924	0.0780	0.0780	0.0780	0.0780	0.0513	0.0253	0.0780	0.0253	0.1335	0.0253	0.0000																	
15	0.1924	0.2231	0.1054	0.1054	0.1054	0.1054	0.0780	0.0513	0.1054	0.0513	0.1625	0.0513	0.0253	0.0253																
16	0.1625	0.1924	0.0780	0.0780	0.0780	0.0780	0.0513	0.0253	0.0780	0.0253	0.1335	0.0253	0.0000	0.0000	0.0253															
17	0.0780	0.1625	0.1625	0.1054	0.1625	0.1054	0.1335	0.1054	0.1625	0.0513	0.1625	0.1054	0.0780	0.0780	0.1054	0.0780														
18	0.1625	0.2549	0.1335	0.1335	0.1335	0.1335	0.1054	0.0780	0.1335	0.0780	0.1924	0.0780	0.0513	0.0513	0.0780	0.0513	0.0780													
19	0.1924	0.2231	0.1625	0.1625	0.1054	0.1625	0.1335	0.1054	0.1625	0.1054	0.1625	0.1054	0.0780	0.0780	0.1054	0.0780	0.1625	0.1335												
20	0.1625	0.2549	0.1335	0.1335	0.1335	0.1335	0.1054	0.0780	0.1335	0.0780	0.1924	0.0780	0.0513	0.0513	0.0780	0.0513	0.0780	0.0000	0.1335											
21	0.2231	0.3216	0.1924	0.1924	0.1924	0.1924	0.1625	0.1335	0.1924	0.1335	0.1924	0.1335	0.1054	0.1054	0.1335	0.1054	0.1335	0.0513	0.0780	0.0513										
22	0.1924	0.2231	0.2231	0.1625	0.2231	0.1625	0.1924	0.1625	0.2231	0.1625	0.1625	0.1625	0.1335	0.1335	0.1625	0.1335	0.1054	0.0780	0.1625	0.0780	0.0780									
23	0.1924	0.2877	0.2877	0.2231	0.2877	0.2231	0.2549	0.2231	0.2877	0.1625	0.1625	0.2231	0.1924	0.1924	0.2231	0.1924	0.1054	0.1335	0.1625	0.1335	0.0780	0.1054								
24	0.1335	0.1625	0.1054	0.0513	0.1054	0.0513	0.0780	0.0513	0.1054	0.0513	0.1625	0.0513	0.0253	0.0253	0.0513	0.0253	0.0513	0.0780	0.1054	0.0780	0.1335	0.1054	0.1625							
25	0.1625	0.2549	0.1924	0.1924	0.2549	0.1924	0.2231	0.1924	0.2549	0.1924	0.2549	0.1924	0.1625	0.1625	0.1924	0.1625	0.1335	0.1625	0.1335	0.1625	0.1054	0.1335	0.1335	0.1335						
26	0.1924	0.2877	0.1625	0.1625	0.1625	0.1625	0.1335	0.1054	0.1625	0.1054	0.2231	0.1054	0.0780	0.0780	0.1054	0.0780	0.1054	0.0780	0.1625	0.0780	0.1335	0.1625	0.2231	0.1054	0.1335					
27	0.2549	0.2877	0.2231	0.2231	0.2231	0.2231	0.1924	0.1625	0.2231	0.1625	0.1625	0.1625	0.1335	0.1335	0.1625	0.1335	0.1625	0.1335	0.1054	0.1335	0.0780	0.1054	0.1625	0.1625	0.1335	0.1054				
28	0.1625	0.1335	0.1335	0.1335	0.1335	0.1335	0.1625	0.1335	0.1335	0.0780	0.0780	0.0780	0.1054	0.1054	0.1335	0.1054	0.1335	0.1625	0.1924	0.1625	0.2231	0.1924	0.2549	0.1335	0.2231	0.1335	0.1924			
29	0.1625	0.1924	0.0780	0.0780	0.0780	0.0780	0.0513	0.0253	0.0780	0.0253	0.1335	0.0253	0.0000	0.0000	0.0253	0.0000	0.0780	0.0513	0.0780	0.0513	0.1054	0.1335	0.1924	0.0253	0.1625	0.0780	0.1335	0.1054		
30	0.1924	0.1625	0.1054	0.1054	0.1054	0.1054	0.0253	0.0513	0.0513	0.0513	0.1625	0.0513	0.0253	0.0253	0.0513	0.0253	0.1054	0.0780	0.1054	0.0780	0.1335	0.1625	0.2231	0.0513	0.1924	0.1054	0.1625	0.1335	0.0253	