GENETIC DIVERSITY WITHIN NATURAL POPULATIONS OF THE MEDICINAL PLANT *RHYNCHOSIA MINIMA* (L.) DC.

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Abstract. Genetic diversity and relationships among 100 genotypes of *Rhynchosia minima* collected from four districts of Malakand Division was assessed using 16 morphological characteristics and total seed protein profile. Based on qualitative characteristics, inter-district trait similarity index among genotypes of Swat-Dir Lower and Swat-Buner was 100%, while for Dir Lower-Buner, Dir Lower-Dir Upper, and Buner-Dir Upper, it was 85.71%, 28.57%, and 42.85%, respectively. Total seed protein profile resulted in 8 reproducible bands/loci, where inter-district locus contribution to the genetic disagreement was 75%. Among the 8 loci, B-6 and B-7 were monomorphic and could be *R. minima* specific, whereas loci B-1, B-4, B-5 and B-8 were observed only in genotypes collected from Buner. All but locus B-1 were present in genotypes of Dir Upper; locus B-8 was missing in genotypes from Dir Lower; loci B-1 and B-4 were absent in genotypes collected from Swat. Two-way cluster analysis resolved genotypes of District Buner and Dir Upper into discrete clusters, highlighting the role of habitat-specific adaptation. Genotypes of Dir Lower and Swat formed mosaic cluster, indicating to the transfer of genes and/or their coevolutionary descent. To the best of our understanding, this is the first ever report addressing genetic variability in *R. minima*.
Keywords: *genetic variability, climate change, SDS-PAGE, two-way cluster analysis, Rynchosia*

Introduction

Grain legumes are important dietary constituents for both humans and livestock and are ranked third in global importance after cereals and oilseeds (Ilyas et al., 2009; Naim-Feil et al., 2017). With the estimated rise in human population, food security is generally uncertain and warrants the need for safeguarding the biodiversity provided by nature and arable land for specific adaptation to stresses as well as ecosystem resilience under changing climatic conditions (Jacobsen et al., 2013; McCouch et al., 2013; Ali et al., 2016). Under these circumstances, demands for grain legume consumption will increase, and biotic and abiotic constraints will push the limits of legume production

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further (Ilyas et al., 2009). Nevertheless, the role of tropical grain legumes is critically important for areas where protein intake from animal sources is low. Population growth and limited land and water resources for agriculture are the major concerns that have gained considerable recent attention (Takeda and Matsuoka, 2008; McCouch et al., 2013). Although there have been consistent increases in crop productivity, these efforts are no longer meet the demands of future populations (Heslop-Harrison and Schwarzacher, 2012). Therefore, exploiting and tapping the wealth of wild and cultivated genetic resources provided by nature and currently warehoused in our seed repositories are vital (Tenskley and McCouch, 1997; Mujeeb-Kazi et al., 2017). However, in spite of all efforts, the world currently faces a greater demand on agricultural output than at any time in history (Tanksley and McCouch, 1997; Naim-Feil et al., 2017).

Indeed, plant breeders have been largely successful in developing high-yielding crop varieties; demands for greater yield potential were previously approached with genetic improvements coupled with increased farming inputs and addition to arable land (Helsop-Harrison and Schwarzacher, 2011). More recently, as the arable land surface has begun to shrink due to population growth, freshwater resources are becoming scarce, and agricultural inputs are associated with increased pollution; thus, major benefits will most likely result from genetic improvement of crops (Lobell et al., 2008; McCouch et al., 2013). However, the last several decades of intense plant breeding and selection are regarded as the most important force that has narrowed the genetic variability of crop species after their domestication some 10,000 years ago (Lu et al., 2009; Schwarzacher et al., 2011; Ali et al., 2012). Today, humanity depends on less than a dozen flowering plant species for 80% of all caloric intake (McCouch et al., 2013). Without sufficient adaptation measures in place, this will not be sufficient to support the feeding demands of 2050 in the face of climate change, habitat fragmentation, and limited water and land resources (Lobell et al., 2008; Mujeeb-Kazi et al., 2017).

Rhynchosia minima (L.) DC., a herbaceous, vining, perennial weed of the family Fabaceae, is found on all five continents (Lopez, 2012). The plant has considerable plasticity and can colonize disturbed areas as well as gaps within natural communities (Shaukat and Burhan, 2000). The plant has tremendous potential to be used in forage, pharmaceutical and other agricultural products, and more importantly, there are no known major threats to this species (Lopez, 2012); however, the plant has negligible uses worldwide, including in Pakistan. Traditionally, *R. minima* is applied to alleviate boils, colds, respiratory infections, diarrhea, dysentery and joint pains, and has been used as an abortifacient and an ecbolic and for general healing. It is also used as a food (in sweets), and its seeds are used as repellents and have antimicrobial potential. Furthermore, a number of important compounds that could fight cancer and may reduce carcinogenesis have been isolated from the seeds (Morris, 2008; Gweru et al., 2009; Jia et al., 2015).

A wide genetic base is critical to adaptation and will determine the future severity of climate change impacts. Genetic bottlenecks jeopardize the potential of crop species for sustainable agriculture and render them vulnerable to stresses (Ali et al., 2012, 2016; McCouch et al., 2013; Mujeeb-Kazi et al., 2017). With the continuous developments in DNA-based technologies and computational tools, more objective measurements of genetic diversity at the genomic level could be made, and important loci could be precisely mapped.

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However, not all of these tools are currently available to plant breeders in developing countries, which house large food-insecure human populations. Plant breeders still rely on traditional approaches to screen entries for a clearly defined character recognizable in the phenotype. Once a line with the desired characteristic is pinpointed, it is crossed with an elite cultivar in order to introduce the genes from the exotic donor into the cultivated type (Tanksley and McCouch, 1997; Mujeeb-Kazi et al., 2017).

Proteins are end products of gene expression, and measuring the diversity of the total seed proteins using the SDS-PAGE method is robust, inexpensive and important in practice to crops such as grain legumes (Nisar et al., 2009, 2016; Zahoor et al., 2015). Furthermore, seed storage protein markers have been successfully used to resolve taxonomic relationships and to identify potential lines in both wild and cultivated crop species (Lioi et al., 1999; Ghafoor et al., 2002; Nisar et al., 2007, 2009; Hameed et al., 2009; Win et al., 20011; Wadood et al., 2016). Overall, a combination of morphology with measurements of SDS-PAGE diversity of the total seed proteins has been largely successful, and the genetic diversity of seed storage proteins has been studied in many plant species of agronomic and commercial importance.

Our understanding of the potential of wild plant species and their objective diversity is still limited and needs enhancement (Payne, 1987; Rogl et al., 1996; Ali et al., 2016; Masood et al., 2017). To date, wild plants have received little attention, and thus, the main objective of the current study was to measure intraspecific genetic variation within the population of *R. minima* naturalized in different geographical regions. The present study was designed to study genetic relationships among natural populations of *R. minima* collected from four districts at random for morphological and seed storage protein evaluation. To the best of our understanding, the present study is the first documented report from Pakistan on the genetic diversity of *R. minima*.

Materials and methods

Climatic conditions of Malakand division

Malakand division in general is a hilly area located within the Northern Pakistan covering an area of nearly 29,872 Km^2 and having a range of climatic conditions (*Fig. 1*). Moving along the foothills of the Hindu Kush, Himalayas and Karakoram ranges, the climate of Malakand division changes from humid subtropical at the foothills to the typical arid climate characteristic of the plains of South Asia (Muhammad et al., 2016). District Swat stretches from 35.2227° North to 72.4258° East longitude and the climatic conditions varies from dry to moist temperate. The climate is influenced by various factors including latitude, altitude, the Indian Ocean Summer, Monsoon and the Western cyclonic currents, coming from the Mediterranean Sea, in the winter. Dir lower is located in the Dry temperate zone (34.9161° N, 71.8097° E; 4411 ft above sea level), Dir upper is a moist temperate region (35.3356° N, 72.0468° E; 3174 ft above sea level) while, District Buner having areas of both Dry and moist temperate regions (34.3943° N, 72.6151° E; 4049 ft above sea level).

Plant materials

Field trips were arranged during 2016–2017, and 100 genotypes of *R. minima* growing in the wild were collected from four districts of Malakand Division, Pakistan (i.e., 25 genotypes from each of District Swat, Dir lower, Buner and Dir upper) and are

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listed in *Table A1* in the *Appendix*. Plants were identified, the genotypes were labeled [first letter(s) corresponding to District, followed by species abbreviated name and accession number], and voucher specimens were submitted to the Herbarium at Hazara University Mansehra, Pakistan. These genotypes were assessed for estimation of inter- and intra-district variation and genetic diversity of morphological traits and total seed storage protein profile.

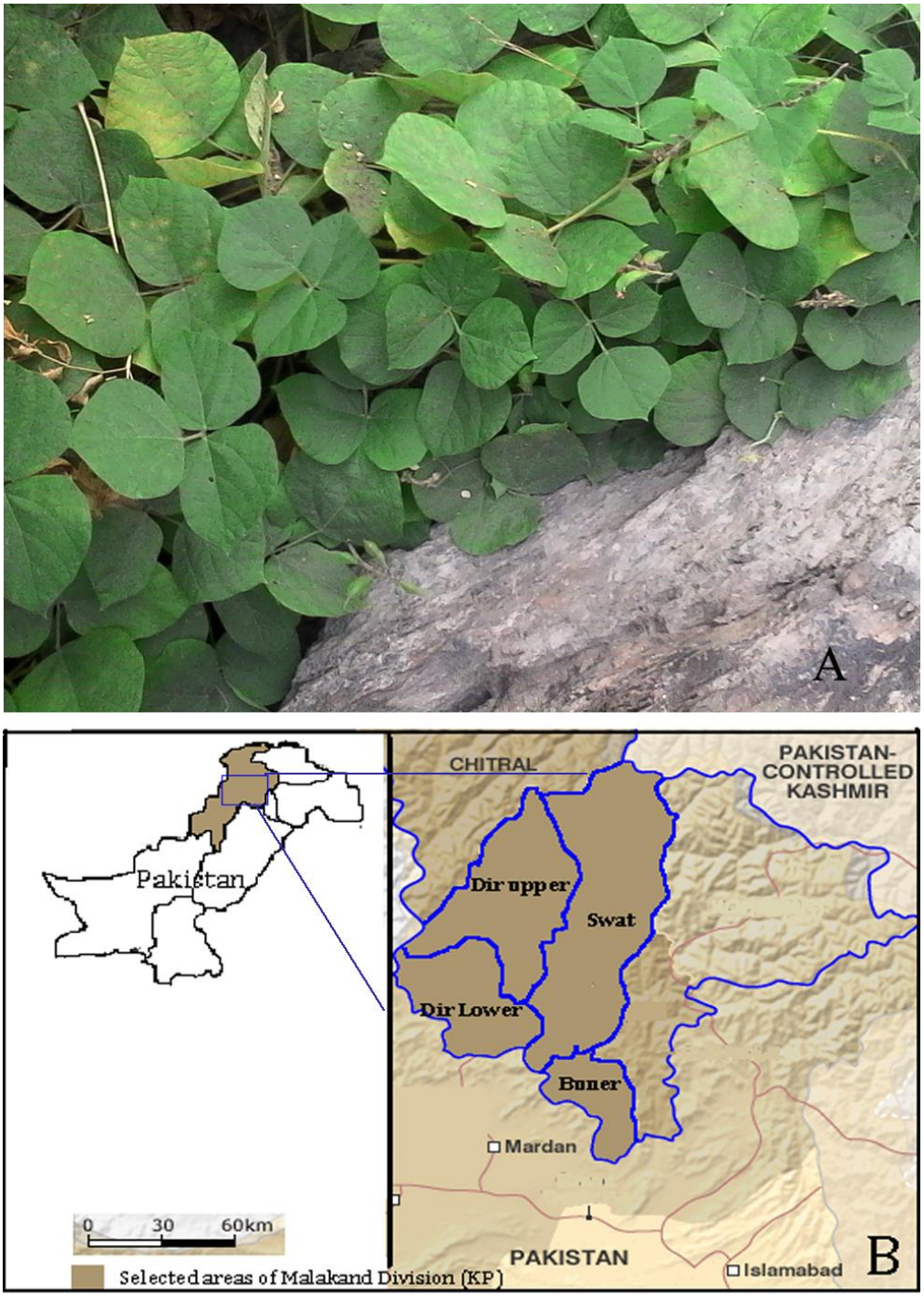

Figure 1. *Rhynchosia minima plants growing on a rocky substrate (A) and map of the study area (B)*

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Morphological characterization

Morphological characterization was registered on site and all randomly selected 25 mature genotypes were accounted (*Fig. 1*). A total of 16 morphological traits, including qualitative and quantitative traits, were scored. Qualitative traits were scored visually and encompassed leaf upper surface color (Luc), leaf lower surface color (Llc), inflorescence color (Ic), seed color (Sc), seed shape (Ss), hilum color (Hc) and testa texture (Tt). Quantitative traits were measured with the help of Vernier calipers or the centimeter scale and encompassed petiole length (PL), leaf length (LL), leaf width (LW), inflorescence length (IL), seed length (SL), seed weight (SW), seed thickness (ST), no. of pods per plant (PP) and 100 seed weight (SWT).

Protein profiling

For a total seed storage protein profile, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method described by Laemmli (1970) and modified by Zahoor et al. (2015). A single seed of each genotype was crushed into a fine flour, and then 400 µl of protein extraction buffer (0.5 M Tris-HCl, 0.2% SDS, 5 M urea, 1% β-mercaptoethanol pH 8.0) was added to 0.01 g of the fine seed powder in an Eppendorf tube and homogenized with a vortex. The mixture was kept for 20 min at room temperature (RT) for protein extraction. Bromophenol blue (BPB) was added to the sample buffer for observing protein sample movement. Samples were centrifuged at 13,000 rpm for 14 min at RT and the supernatant was transferred to a clean 1.5 ml Eppendorf tube and run on a 12% polyacrylamide gel (composition of resolution gel: 3.0 M Tris-HCl pH 9.0, 0.4% SDS and stacking gel 0.4 M Tris-HCl pH 7.0, 0.4% SDS) at 100 V for 2 hrs. The gel was stained with Coomassie brilliant blue for 20 min and destained (5% methanol, 20% acetic acid) until the background color disappeared.

Data analysis

Morphological and SDS-PAGE data were assembled in an MS Excel sheet. Two-way cluster analysis was based on binary data in PC-ORD software (Lepsˇ and Sˇmilauer, 2003). Genetic similarity estimates (F) were designed following Nei and Li (1979), and CANOCO software was used for specific loci/band enrichment in *R. minima* and to find the influence/relationship of the morphological traits and geographical origin on the total seed protein diversity in *R. minima*.

Results and discussion

Morphological diversity in R. minima genotypes

For morphological diversity, both the qualitative and quantitative characteristics were assessed, revealing substantial variability among genotypes for most traits: plants collected from different districts harbored higher variability than genotypes from within the same district. Descriptive statistics of the morphological parameters are summarized in *Table 1*. The CV% was calculated for petiole length, leaf length, leaf width, stipule length, inflorescence length, seed length, seed width and seed thickness, No. of pods per plant, 100 seed weight.

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Table 1. *Descriptive statistics of the important morphological parameters*

Area/site of collection	Traits	Minimum	Maximum	Mean	Std. Deviation	CV%
District Swat	**PL**	1.3	1.94	1.5749	0.20889	13.2637
	LL	81.98	101.98	94.7232	5.06394	5.3461
	LW	15.33	33.67	22.58	3.74443	16.582
	IL	1.67	4.33	3.2	0.63828	19.943
	SL	5.67	9	7.684	0.87428	11.377
	SW	2.67	5	3.972	0.52323	13.172
	ST	1.6	3.17	2.516	0.38371	15.251
	P/P	12.67	34	22.2667	6.08428	27.324
	SWT	24	33	28.544	2.44916	8.581
District Dir lower	**PL**	2	2.99	2.654	0.28634	10.789
	LL	107.6	137.8	128	7.04929	5.518
	LW	19.67	112	79.072	32.28988	40.836
	IL	3	7.33	5.0493	1.31576	26.0582
	SL	2.96	8	4.3708	1.22465	28.018
	SW	1.83	4.2	2.9333	0.54815	18.687
	ST	1.23	3.27	2.536	0.57702	22.752
	P/P	23	40.33	33.6933	4.02989	11.961
	SWT	9	33	14.0684	7.74323	55.0398
District Buner	**PL**	6	6.99	6.5473	0.33476	5.112
	LL	63.33	89.78	73.6398	6.29928	8.554
	LW	36	122.67	71.5239	27.31532	38.191
	IL	3.33	6	4.1733	0.90329	21.644
	SL	2.65	8.23	4.8076	1.30897	27.227
	SW	1.67	6.8	3.1561	1.04537	33.122
	ST	1.57	3.63	2.6128	0.57634	22.058
	P/P	35.67	78.67	59.9733	17.37813	28.976
	SWT	9.3	15	12.6412	1.7454	13.809
District Dir upper	**PL**	3.99	5.8	4.3719	0.37533	8.585
	LL	23.25	34	29.223	2.53127	8.661
	LW	17	115	46.78	25.44987	54.401
	IL	1.67	7.67	4.7733	1.55075	32.488
	SL	2.67	8.67	6.1688	1.83278	29.711
	SW	1.57	4.67	3.3835	0.85506	25.271
	ST	1.43	3.37	2.3399	0.51268	21.911
	P/P	13	77.33	45.1867	24.76315	54.801
	SWT	12.14	33	20.5656	7.39849	35.975
	CV% = Std. Deviation/Mean * 100					

PL = petiole length, LL = leaf length, LW = leaf width, SL = seed length, SW = seed width, ST = seed thickness, P/P = no. of pods/plant, SWT = 100 seed weight

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Petiole length in genotypes collected from district Swat was 13.2637%, for district Dir lower was 10.789, for district Buner was 5.112 and for district Dir Upper was 8.585. Significant variation was found for the leaf length among the genotypes of four districts; the highest value was observed for genotypes collected from district Buner (8.554) followed by district Dir Upper (8.661%) and Dir lower (5.518%) while, lowest value was recorded for the genotypes collected from Swat (5.3461%). For leaf width in genotypes collected from Swat was (16.582%), Dir lower (40.836%), for district Buner (38.191%) whereas for district Dir Upper was 54.401%. Furthermore highest variation was observed for seed length and seed width. Number of pods/plant varied for genotypes collected from Swat, Dir lower, Buner and Dir Upper was 27.324%, 11.961%, 28.976% and 54.801% respectively (*Table 1*).

Based on qualitative characteristics, the inter-district trait similarity index among genotypes of Swat-Dir Lower and Swat-Buner was 100%, while for Swat- Dir Upper, Dir Lower-Buner, Dir Lower-Dir Upper, and Buner-Dir Upper, it was 28. 571, 85.71%, 28.57%, and 42.85%, respectively (*Table 2*). Similarly, based on quantitative traits inter-district traits similarity index among genotypes of Swat-Dir Lower, Swat-Dir Upper, Swat-Buner, Dir lower-Buner and Dir upper-Swat was 22.2%; while for Dir Lower-Dir upper was 11.1%; Buner-Dir upper was 33.333% and that of Buner-Dir Upper was 42.85%. Furthermore, the Pearson correlation coefficient revealed a significant positive as well as a negative association ($p = 0.05$ and 0.01) among the studied traits of *R. minima* (*Tables 3* and *4*). Several traits revealed strong interrelationships within phenotype categories, particularly leaf traits with yield contributing traits and a few traits correlating with other categories, such as inherently linked growth and phenology-related traits (*Tables 2* and *3*). Furthermore, quantitative data set of 100 genotypes was used for cluster analysis. The dendrogram resolved all the 100 genotypes of *R. minima* into four discrete clusters (groups), each representing the geographical origin of collection and highlighting the determining role of geography rather than variation within the traits themselves (*Fig. 2*).

Table 2. *Region-wise trait similarity index based on qualitative traits*

Traits	S-RM	DL-RM	B-RM	DU-RM	Traits similarity index				
					S-RM & DL-RM	S-RM & B-RM	DL-RM & B-RM	DL-RM & DU-RM	B-RM & DU-RM
Luc	Green	Green	Green	Moss green	Green	Green	NA	NA	NA
Llc	Green	Green	Green	Yellow green	Green	Green	Green	NA	NA
Ic	White yellow	White yellow	White yellow	Yellow green	White yellow	White yellow	white yell	NA	NA
Sc	Brown	Brown	Brown	Dull yellow	Brown	Brown	Brown	NA	NA
Ss	Oblong	Oblong	Oblong	Oblong	Oblong	Oblong	Oblong	Oblong	Oblong
Hc	White	White	White yellow	White yellow	White	White	White yellow	NA	White yellow
Tt	Smooth	Smooth	Smoth	Smoth	Smoth	Smoth	Smoth	Smoth	Smoth
TSI= homologous traits/Total traits*100 =					100	100	85.71	28.57	42.85

Luc = leaf upper surface colour, Llc = leaf lower surface colour, Ic =Inflorescence color, Sc = Seed color, Ss = Seed shape, Hc = Hilum Color, Tt = Testa texture, S = Swat, DL = Dir lower, DU = Dir upper, B = Buner, RM = R. minima

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Table 3. *Correlation coefficient among nine quantitative traits of DL-RM (italic numbers) and S-RM*

Traits	PL	LL	LW	IL	SL	SW	ST	P/P	SWt
PL	1.00	*-0.501**	*0.01*	*0.39*	*0.884***	*-0.580***	*-.910***	*-0.07*	*-0.743***
LL	-0.08	1.00	*-0.03*	*-0.28*	*-0.450**	*0.20*	*.432**	*0.22*	*0.36*
LW	0.12	0.18	1.00	*0.405**	*0.25*	*-0.540***	*0.15*	*-0.08*	*-0.27*
IL	-0.48	0.34	0.21	1.00	*.659***	*-0.30*	*-0.27*	*-0.01*	*-0.08*
SL	0.39	-0.37	0.06	-0.33	1.00	*-0.543***	*-0.765***	*-0.03*	*-0.07*
SW	0.524**	-0.34	-0.06	-0.34	0.828**	1.00	*.417**	*0.17*	*0.622***
ST	0.402*	-0.36	0.11	-0.09	0.611**	0.707**	1.00	*0.641***	*-0.08*
P/P	0.39	-0.17	-0.16	-0.03	-0.14	0.13	0.18	1.00	*0.14*
SWt	-0.13	-0.31	-0.20	-0.25	0.23	0.32	0.07	0.20	1.00

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)
PL = petiole length, LL = leaf length, LW = leaf width, IL = inflorescence, SL = seed length, SW = seed weight, ST = seed thickness, P/P = no. of pod/plant, SWT = seed weight. S = swat, DL = Dir lower, RM = R. minima

Table 4. *Correlation coefficient among nine quantitative traits of DU-RM (italic numbers) and B-RM*

Traits	PL	LL	LW	IL	SL	SW	ST	P/P	SWt
PL	1.00	*-.952***	*0.31*	*0.658***	*0.811***	*-0.841***	*-0.888***	*-0.19*	*-0.34*
LL	-0.415*	1.00	*-0.33*	*-0.662***	*-0.828***	*0.817***	*0.878***	*0.11*	*0.32*
LW	-0.21	0.31	1.00	*0.27*	*-0.05*	*-0.18*	*-0.24*	*0.636***	*0.791***
IL	-0.35	0.657**	0.592**	1.00	*0.04*	*-0.15*	*-0.525***	*-0.565***	*0.911***
SL	0.37	-0.32	-0.12	0.11	1.00	*-0.08*	*-0.11*	*-0.707***	*-0.695***
SW	0.15	0.02	0.30	0.434*	0.806**	1.00	*0.19*	*0.519***	*0.862***
ST	-0.03	0.04	0.28	0.444*	0.448*	0.602**	1.00	*0.18*	*0.33*
P/P	0.35	-0.407*	-0.936**	-0.695**	0.11	-0.34	-0.37	1.00	*-0.18*
SWt	0.24	-0.23	-0.747**	-0.552**	-0.20	-0.528**	-0.530**	.800**	1.00

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)
PL = petiole length, LL = leaf length, LW = leaf width, IL = inflorescence, SL = seed length, SW = seed weight, ST = seed thickness, P/P = no. of pod/ plant, SWT = seed weight. DU = Dir upper, B = buner, RM= R. minima

SDS-PAGE analysis

For exploration of genetic polymorphisms, modern tools can provide better understanding of species relationships and heterogeneity. Furthermore, morphometric, biochemical and molecular marker approaches have been used for examining genetic diversity and for inferring the intra- and interspecies relationships in plants (Tomooka et

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al., 2002). Moreover, isozymes (Jaaska and Jaaska, 1990), low-molecular-weight carbohydrates (Yasui et al., 1985), RFLPs (Fatokun et al., 1993), and nuclear as well as chloroplast DNA, (Vaillancourt and Weeden, 1993), RAPDs (Kaga et al., 1996), SSRs, EST-SSRs (Ali et al., 2016) and AFLPs (Tomooka et al., 2002), among others, have been used to study crop species.

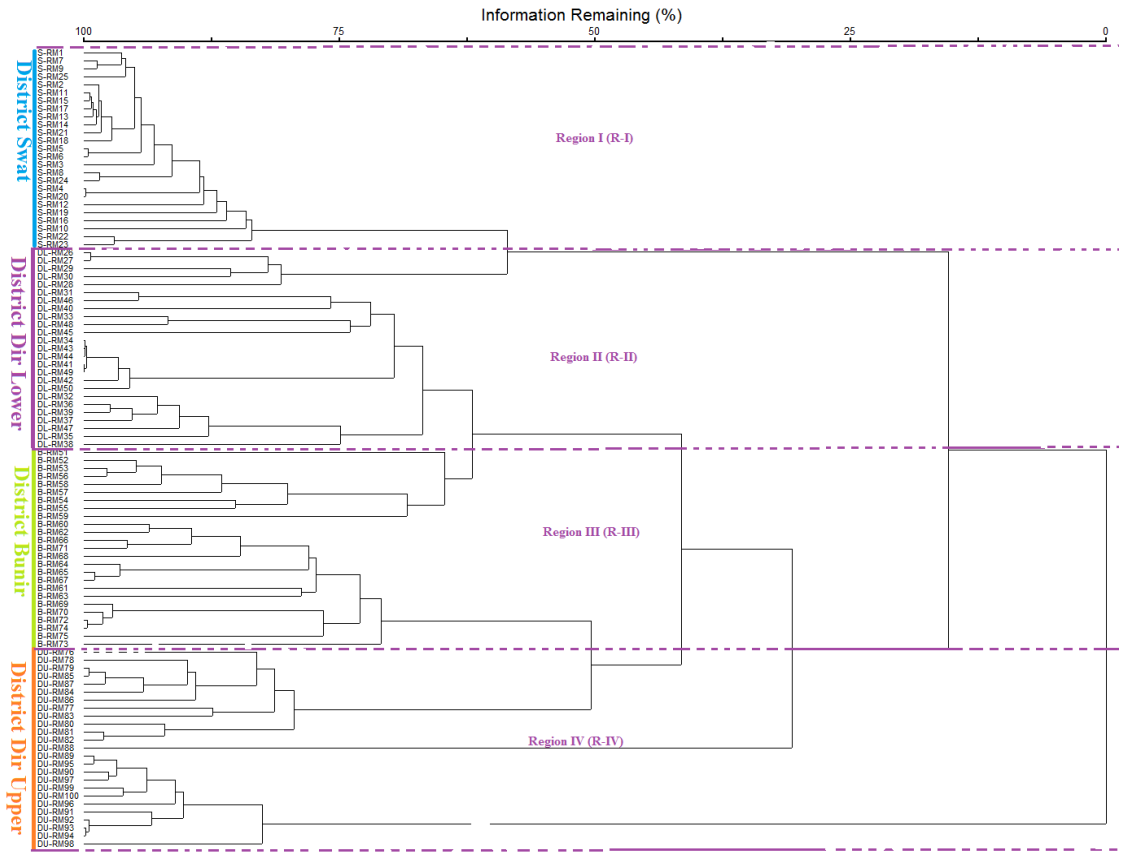

Figure 2. *Intra-species relationship identified through morphological characteristics in different genotypes of R. minima collected from four districts of Malakand Division, Pakistan. RM indicates R. minima, S = Swat, DL = Dir lower, B = Buner and DU = Dir upper*

Eight reproducible bands of ~10–180 kDa were observed in the *R. minima*; a representative PAGE is shown (*Fig. 3*). The binary data of these loci were used to estimate relationships among all genotypes with cluster analysis, and all *R. minima* were divided into six clusters (*Fig. 4*). In cluster-1 (also referred to as Region I, R-I), genotypes collected from District Buner were resolved as having 25% genetic diversity, whereas genotypes of R-II consisted of samples collected from District Dir Upper with 24.07% genetic diversity among genotypes. Genotypes in R-III belonged to District Dir lower with 29.37% genetic variation among genotypes, and R-IV genotypes collected from District Swat had 50% diversity. Similarly, genotypes in R-V and R-VI consisted of genotypes of *R. minima* collected from Dir lower (5 genotypes) and Swat (13 genotypes with 48.07% genetic diversity).

SDS-PAGE has shown promise in understanding the genetic relationships in angiosperms at the generic as well as at the species levels and is reliable for assessing polymorphisms in crops (Payne, 1987; Ghafoor et al., 2002; Nisar et al., 2009; Win et

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al., 2011; Zahoor et al., 2015). The results for the mean square values and response of protein loci/band enrichment within genotypes showed significant variations as indicated (*Fig. 5*). Variability in color and size indicates variation, and the species-specific loci B-6 and B-7 revealed maximum enrichment (present in 100% genotypes), followed by B-8 (72%), B-5 (69%), B-4 (65%), B-3 (63%) and B-2 (50). Furthermore, loci B-2 and B-3 were negatively associated with B-1, B-4 and B-5, while B-8, B-6 and B7 have a neutral association because these bands are evenly distributed among the *R. minima* genotypes collected from these four geographical areas (*Fig. 5*). The overall inter-district locus contribution towards the genetic disagreement was 75%, whereas within the district, it varied from 0.00 to 75%. Among the 8 loci, B-6 and B-7 were monomorphic, present in all genotypes and are likely to be species specific. In contrast, loci B-1, B-4, B-5 and B-8 were observed in genotypes collected from Buner, whereas these genotypes were missing loci B-2 and B-3 and genotypes collected from Dir Upper had all loci except B-1. *R. minima* genotypes collected from Dir Lower and Swat had a mixed protein profile, i.e., showing higher diversity and intermixing within the genotypes. This indicates frequent gene flow within these genotypes and their potential to withstand extreme environmental events (*Fig. 4* and *Table 5*). Details of all of these loci and whether they are monomorphic or polymorphic have been determined and are given in *Table 5*. There was a high inter-district locus contribution towards genetic disagreements among *R. minima* genotypes; SDS-PAGE could be a consistent procedure for documentation of these genotypes, while intra-district locus contributions towards genetic disagreement in genotypes of *R. minima* collected from Swat (S-RM) and Dir lower (DL-RM) were high (100%) compared to those from Buner (B-RM) at 85.71% and from Dir upper (DU-RM) at 28.57% (*Table 2*).

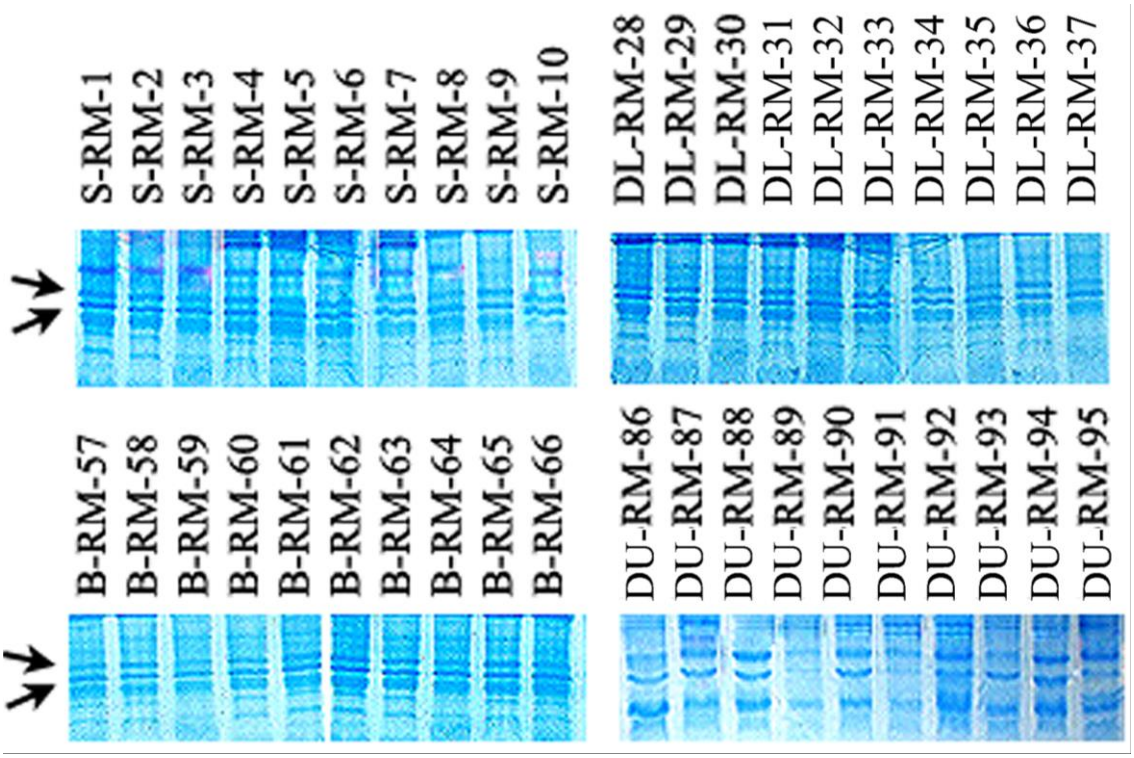

Figure 3. *Representative polyacrylamide gel (12%) electrophoresis showing diversity in total seeds storage proteins among different genotypes of R. minima. Arrow indicates the species-specific loci (B6, B7). S = Swat, DL = Dir lower, B = Buner, DU = Dir upper, RM = R. minima*

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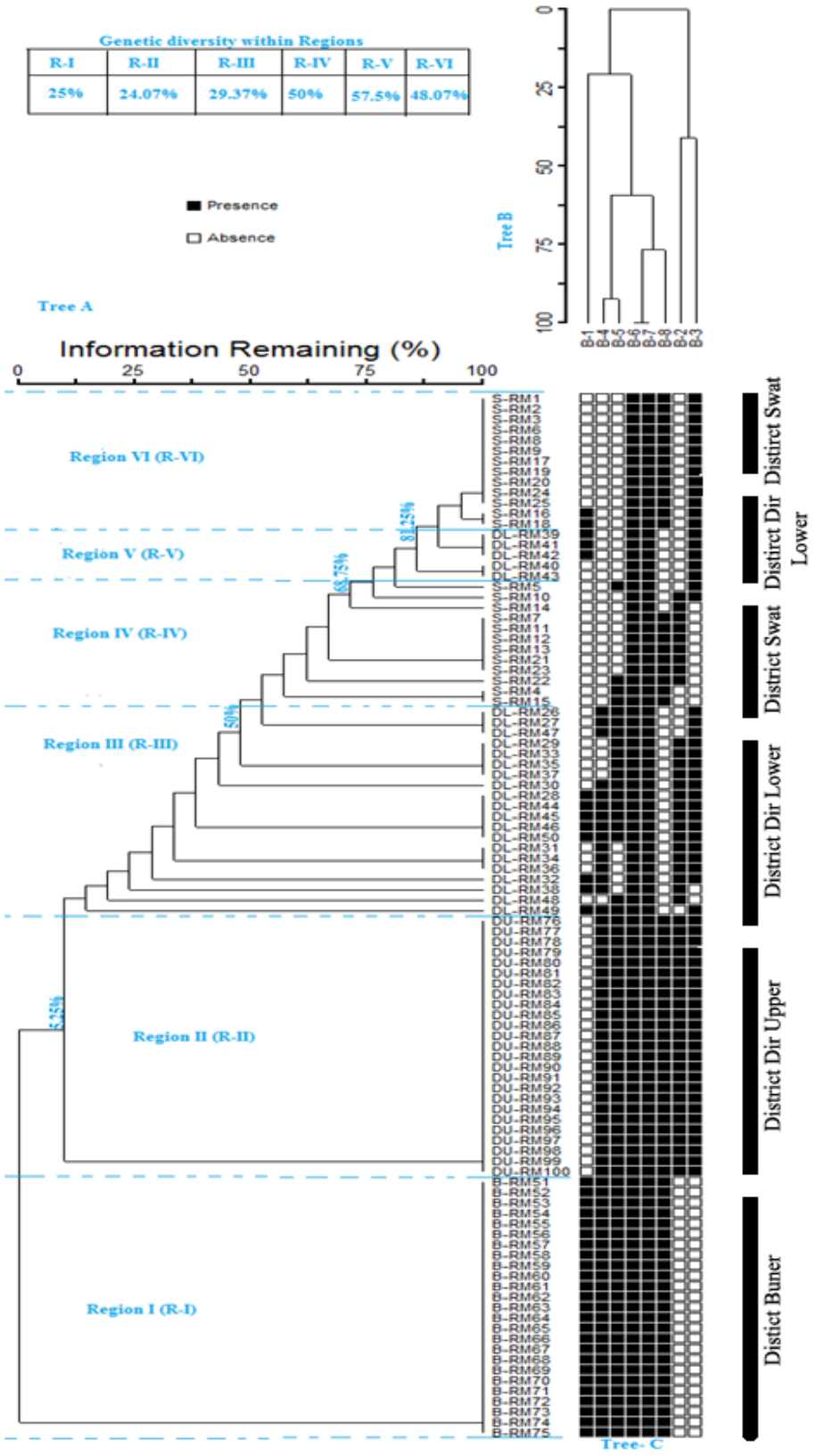

Figure 4. *Two-way cluster dendrogram based on the presence/absence of seed protein bands in different genotypes of R. minima species collected from four districts of Malakand Division, Khyber Pakhtunkhwa, Pakistan. RM indicates genotypes of R. minima, S = Swat, DL = Dir lower, B = Buner and DU = Dir upper*

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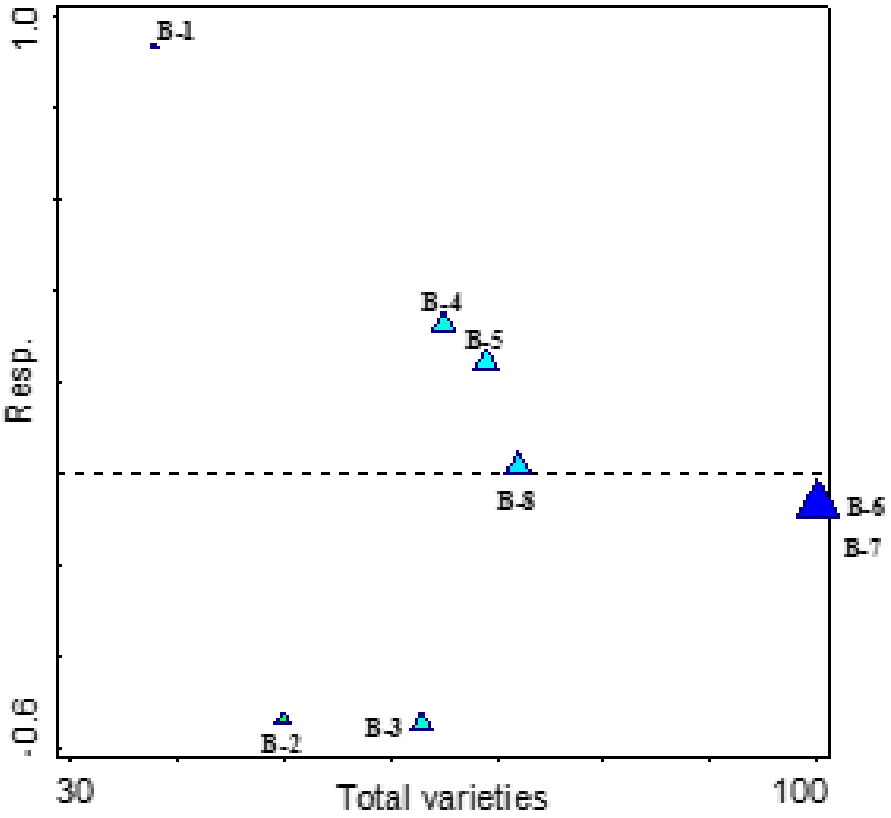

Figure 5. *Different colours showing the R. minima genotype (X-axis) enrichment on the basis of particular loci/band categories*

These four Districts are located adjacent to one another, but altogether, they cover an area of approximately 12,484 km^2, and each District is characterized by distinct environmental conditions and vegetation types. Nonetheless, the wide ecological amplitude and genomic plasticity of *R. minima* allow it to invade new areas and gaps within established plant communities (Shaukat and Burhan, 2000; Jia et al., 2015). This highlights the potential of finding useful genes for adaptation and climatic resilience as well as introgression of genes for improving productivity and resistance in leguminous crops via intergeneric crosses. We have no evidence whether such intergeneric crosses are likely to be successful, but wide crosses (interspecific and intergeneric) have been reported to be tremendously successful in cereals (Ali et al., 2012; Masood et al., 2016; Mujeeb-Kazi et al., 2017). There are numerous reports where not only cultivated plants but also their distant wild relatives have been hybridized successfully, and the size of the alien chromatin associated with linkage drag has then been reduced while still retaining the useful trait (Schwarzacher et al., 2011; Ali et al., 2016; Patokar et al., 2016; Mujeeb-Kazi et al., 2017).

To the best of our knowledge, this is the first study addressing diversity in *R. minima*, although we have no idea whether the seed storage proteins were overlapping as reported in other legumes. Therefore, no attempt was made to assign the recorded polypeptides into respective classes, i.e., legumin and vicilin (Mirali et al., 2007). Instead, for fine resolution, the dendrogram was divided into six regions, and genotypes collected from District Dir lower and Swat segregated with one another, while genotypes from District Dir upper and Buner assorted into discrete clades/groups

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(*Fig. 4*). *R. minima* enrichment on the basis of specific seed protein bands indicated that B-6 and B-7 were species specific and present in all genotypes (*Fig. 5*). The presence of common loci (B-6 and B-7) among these genotypes suggests their close genetic similarity and common ancestry. These loci encoded proteins that have been fixed in different genotypes of *Rhynchosia* over evolutionary time. These findings are in agreement with those of Azeez and Morakinyo (2004), who associated the presence of common protein bands in *Lycopersicum* and *Trichosanthes* species with their common evolutionary origin.

Table 5. *Intra- and inter-district genetic diversity among R. minima genotypes*

Regions	Expressed loci	Status	GD%	Regions	Expressed loci	Status	GD%
S-RM	1	Poly	0.4	B-RM	25	Mono	0.25
	9	Poly	0.36		25	Mono	0.00
	14	Poly	0.56		0.00	Mono	0.00
	0.00	Mono	0.00		0.00	Mono	0.25
	4	Poly	0.16		25	Mono	0.25
	25	Mono	1.00		25	Mono	1.00
	25	Mono	1.00		25	Mono	1.00
	21	Poly	0.84		25	Mono	0.25
GD = poly/Total loci*100 = 62.5				**GD = poly/Total loci*100 = 0.00**			
DL-RM	11	Poly	0.44	DU-RM	0.00	Mono	0.25
	16	**Poly**	0.64		25	Mono	0.00
	23	Poly	0.23		25	Mono	0.00
	15	**Poly**	0.6		25	Mono	0.25
	15	Poly	0.6		25	Mono	0.25
	25	**Mono**	1.00		25	Mono	1.00
	25	Mono	1.00		25	Mono	1.00
	0.00	**Mono**	0.00		25	Mono	0.25
GD = poly/Total loci*100 = 75					**GD = poly/Total loci*100 = 0.00**		

Inter district variation in *R. minima* genotypes

Expressed loci	Status	GD%
37	Poly	0.37
50	Poly	0.5
62	Poly	0.62
65	Poly	0.65
69	Poly	0.16
100	Mono	1.00
100	Mono	1.00
71	Poly	0.71
Intra locus contribution toward GD poly/Total*100 = 75		

S = swat, B = buner DL = Dir lower, DU = Dir upper, RM = Rhynchosia minima, GD = genetic disagreement

Detrended correspondence analysis (DCA) revealed grouping and distribution of *R. minima* genotypes into four patches on the basis of their geographical origin coupled

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with the data of morphological as well as total seed protein diversity. Area of collection (origin) played a marked role in the grouping of *R. minima* genotypes as well as a positive/negative association with a particular set of morphological traits and seed protein profile (*Fig. 6*). Furthermore, comparison of the two cluster dendrograms were in general agreement with one another, indicating the stability of the morphological traits and the potential of *R. minima* to adapt to various climatic and soil conditions (compare *Figs. 2* and *4*). The DCA plot revealed important information regarding the distribution of genotypes with morphological traits and total seed protein profile. Here, the role of geographical origin again was overwhelming and leaf-related traits as well as protein loci B-1 had a strong and positive impact (*Fig. 6*). It will be extremely interesting to study specific genes in *R. minima* that allow adaptation to new habitats and to compare them to those that have previously played an important role in plant domestication under human influence (Baudoin and Marechal, 1985; Chen et al., 2006).

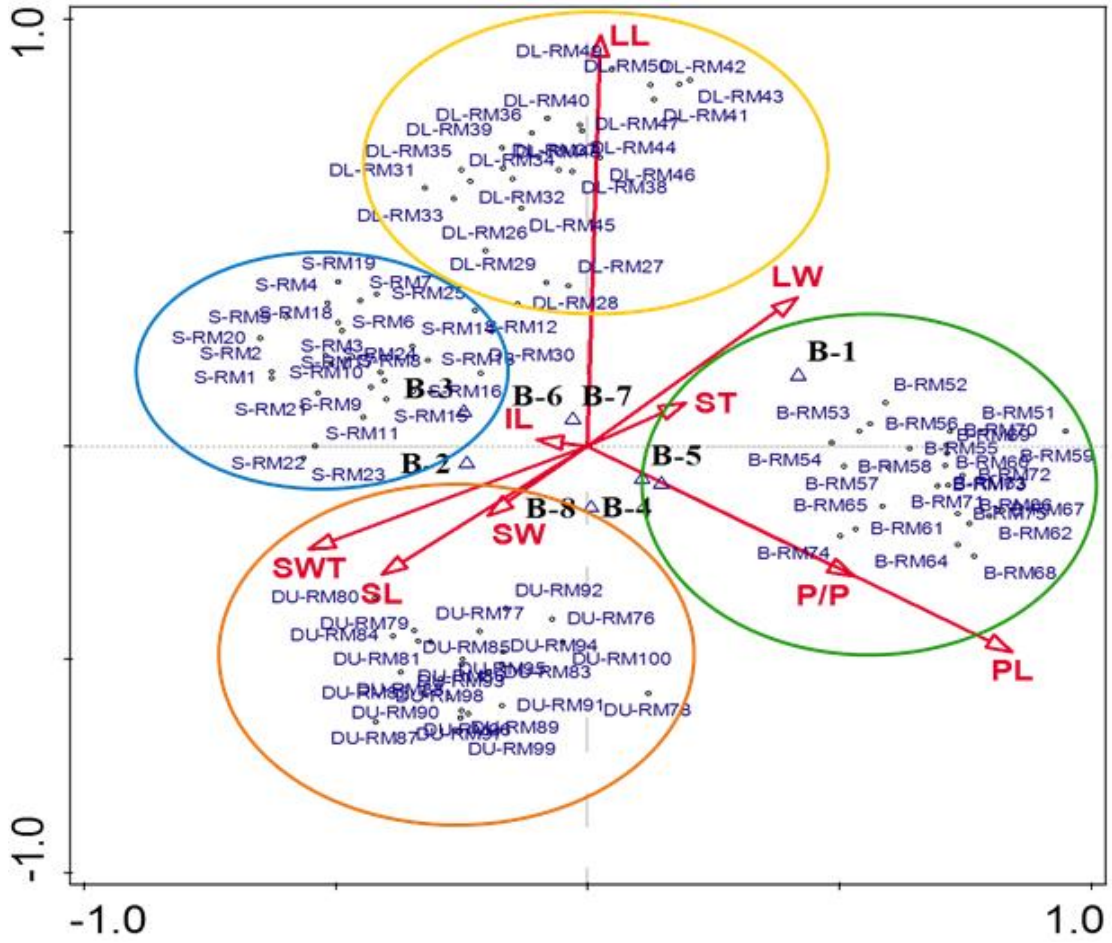

Figure 6. *Detrended correspondence analysis (DCA) diagram showing the distribution of R. minima genotypes and their association/relatedness based on morphological traits and total seed protein profile*

All together, humanity depend on a fraction of the genetic diversity residing in fewer than a dozen of the approximately 300,000 species of angiosperms for 80% of their caloric intake (McCouch et al., 2013). This is not enough to support our food system in

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the future, and unlike conventional breeding that relies on crossing the best with the best, more predictable breeding outcomes are possible with linkages of genes with specific traits. Wild relatives of crops have tremendous genetic potential that can be released by searching for superior genes. In the past, where the increased crop productivity was based on improved agricultural practices and other changes, future gains will rely on improved genetics (Heslop-Harrison and Schwarzacher, 2012).

Conclusion

A total of 16 important morphological traits as well as SDS-PAGE of the total seed protein profile were assessed, and the data revealed the existence of ample diversity. Further, cluster analysis based on seed storage protein analyses demonstrated that the 100 genotypes of *R. minima* collected from four districts had close similarities to each other. The SDS-PAGE electrophoresis results also demonstrated that seed protein profiling provides a powerful tool for genotype discrimination based on geographic differences. Furthermore, the results indicate that high variability exists in *R. minima* and that the differences observed among populations were associated with both genetics and geography. Grain legumes are ranked third after cereals and oilseed crops due to their importance for both humans and livestock (Ilyas et al., 2009; Naim-Feil et al., 2017), and therefore, improvements in leguminous crops are of paramount significance. In the future, where adaptation to stresses is likely to address the severity of climate change impacts as well as worldwide food security concerns (Lobell et al., 2008), minor modifications such as changes in sowing dates are helpful to mitigate negative impacts, but long term benefits will be linked to genetic improvements (Heslop-Harrison and Schwarzacher, 2012). Wild crop relatives offer rich sources of genes for resistance to both biotic and abiotic stresses and therefore could improve agriculture (Vavilov, 1940). Nevertheless, *R. minima*, a distant relatives of crop legumes, has so far undergone only limited utilization in foraging as well as for pharmaceutical purposes (Gundidza et al., 2009; Jia et al., 2015), yet it demands attention for the isolation of important phytochemicals and provides an opportunity for widening the genetic base of important legumes.

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APPENDIX

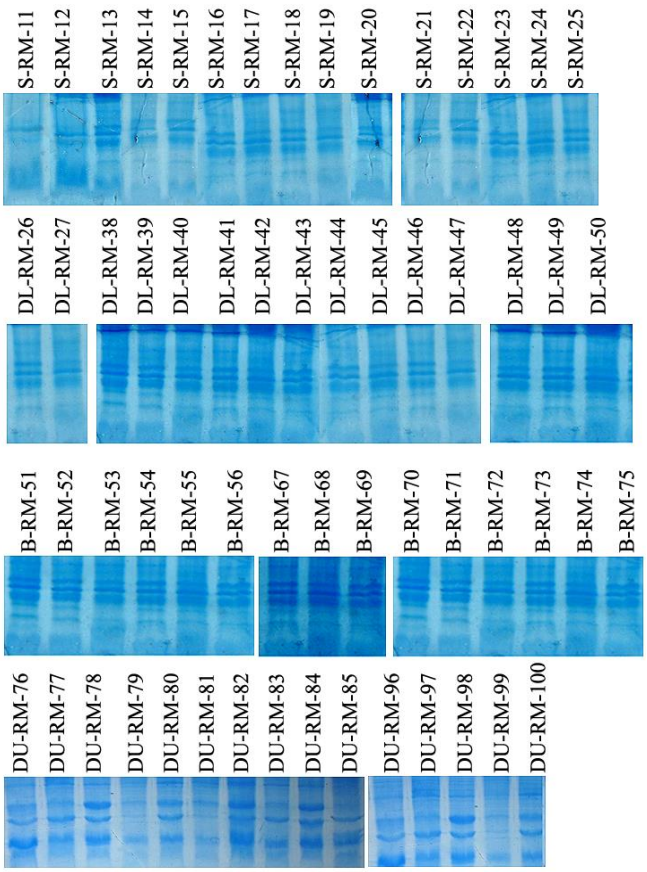

Figure A1. *Polyacrylamide gel (12%) electrophoresis showing diversity in total seed storage protein among different genotypes of R. minima collected from four districts of Malakand Division, Pakistan. S = swat, DL = Dir lower, B = buner, DU = Dir upper, RM = R. minima*

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Table A1. *List of R. minima genotypes collected from four districts of Malakand Division, Pakistan*

S. No.	Species name	District of collection	Specific area of collection	Accession No.	S. No.	District of collection	Specific area of collection	Accession No.
1.	*R. minima*	Swat	Ziarat	S-RM1	51.	Buner	Karakar	B-RM51
2.	*R. minima*	Swat	Swegalai	S-RM2	52.	Buner	Jowar	B-RM52
3.	*R. minima*	Swat	Dadahara	S-RM3	53.	Buner	Toorwarsk	B-RM53
4.	*R. minima*	Swat	Kohay	S-RM4	54.	Buner	Sawarai	B-RM54
5.	*R. minima*	Swat	Gadi	S-RM5	55.	Buner	Chamtalai	B-RM55
6.	*R. minima*	Swat	Sharif Abad	S-RM6	56.	Buner	Matwanai	B-RM56
7.	*R. minima*	Swat	Zarakhela	S-RM7	57.	Buner	Bara	B-RM57
8.	*R. minima*	Swat	Gora Gat	S-RM8	58.	Buner	Nawagai	B-RM58
9.	*R. minima*	Swat	Chongai	S-RM9	59.	Buner	KozaNawagai	B-RM59
10.	*R. minima*	Swat	Qabar Shah	S-RM10	60.	Buner	Narbatawa	B-RM60
11.	*R. minima*	Swat	Landakay	S-RM11	61.	Buner	Naway Kalay	B-RM61
12.	*R. minima*	Swat	Kota	S-RM12	62.	Buner	Anghapur	B-RM62
13.	*R. minima*	Swat	Aboha	S-RM13	63.	Buner	Derai	B-RM63
14.	*R. minima*	Swat	Terang	S-RM14	64.	Buner	Amnawar	B-RM64
15.	*R. minima*	Swat	Dool	S-RM15	65.	Buner	Sangara	B-RM65
16.	*R. minima*	Swat	Chargo Tangay	S-RM16	66.	Buner	Agarai	B-RM66
17.	*R. minima*	Swat	Amlook Garai	S-RM17	67.	Buner	Ashezomaira	B-RM67
18.	*R. minima*	Swat	Shamra	S-RM18	68.	Buner	Bampokha	B-RM68
19.	*R. minima*	Swat	Mula Hassan Baba	S-RM19	69.	Buner	Balo Khan	B-RM69
20.	*R. minima*	Swat	Malak Abad	S-RM20	70.	Buner	Bahi Kaly	B-RM70
21.	*R. minima*	Swat	Soray	S-RM21	71.	Buner	Barjokaney	B-RM71
22.	*R. minima*	Swat	Tangai Chena	S-RM22	72.	Buner	Bar Kaley	B-RM72
23.	*R. minima*	Swat	Qalagay	S-RM23	73.	Buner	Beshonai	B-RM73
24.	*R. minima*	Swat	Sarkhanai	S-RM24	74.	Buner	Bazargey	B-RM74
25.	*R. minima*	Swat	Yakhtangay	S-RM25	75.	Buner	Mula Banda	B-RM75
26.	*R. minima*	Dir lower	Faqir Abad	DL-RM26	76.	Dir upper	Batal	DU-RM76
27.	*R. minima*	Dir lower	Ramora	DL-RM27	77.	Dir upper	Khwage-Oba	DU-RM77
28.	*R. minima*	Dir lower	Gul Abad	DL-RM28	78.	Dir upper	Bekari	DU-RM78
29.	*R. minima*	Dir lower	Shah lam Baba	DL-RM29	79.	Dir upper	Patrok	DU-RM79
30.	*R. minima*	Dir lower	Ouch	DL-RM30	80.	Dir upper	Shoor	DU-RM80
31.	*R. minima*	Dir lower	Asbanr	DL-RM31	81.	Dir upper	Balkot	DU-RM81
32.	*R. minima*	Dir lower	Nawagai	DL-RM32	82.	Dir upper	Jandrai	DU-RM82
33.	*R. minima*	Dir lower	Khwas	DL-RM33	83.	Dir upper	Islam-Gat	DU-RM83
34.	*R. minima*	Dir lower	Totano Bandai	DL-RM34	84.	Dir upper	Jelar	DU-RM84
35.	*R. minima*	Dir lower	Bambolai	DL-RM35	85.	Dir upper	Kakad	DU-RM85
36.	*R. minima*	Dir lower	Kityari	DL-RM36	86.	Dir upper	Haji Shai	DU-RM86
37.	*R. minima*	Dir lower	Shwa	DL-RM37	87.	Dir upper	Kharkani	DU-RM87
38.	*R. minima*	Dir lower	Badwan	DL-RM38	88.	Dir upper	Thal	DU-RM88
39.	*R. minima*	Dir lower	Dalbar	DL-RM39	89.	Dir upper	Kalkot	DU-RM89
40.	*R. minima*	Dir lower	Laaram	DL-RM40	90.	Dir upper	Lamotai	DU-RM90
41.	*R. minima*	Dir lower	Sarkhanai	DL-RM41	91.	Dir upper	Jagram	DU-RM91
42.	*R. minima*	Dir lower	Jawaro	DL-RM42	92.	Dir upper	Bandagai	DU-RM92
43.	*R. minima*	Dir lower	Babar Ghakhey	DL-RM43	93.	Dir upper	Karodara	DU-RM93
44.	*R. minima*	Dir lower	Medan	DL-RM44	94.	Dir upper	Warai	DU-RM94
45.	*R. minima*	Dir lower	Talash	DL-RM45	95.	Dir upper	Laspoor	DU-RM95
46.	*R. minima*	Dir lower	Fingal	DL-RM46	96.	Dir upper	Mastooj	DU-RM96
47.	*R. minima*	Dir lower	Bagh	DL-RM47	97.	Dir upper	Drosh	DU-RM97
48.	*R. minima*	Dir lower	Khaal	DL-RM48	98.	Dir upper	Ashreet	DU-RM98
49.	*R. minima*	Dir lower	Rabaat	DL-RM49	99.	Dir upper	Domail	DU-RM99
50.	*R. minima*	Dir lower	Kotigram	DL-RM50	100.	Dir upper	Qashqar	DU-RM100

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