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Ethnomedicinal Uses and Inter Specific Diversity Encourage Conservation of *Rhynchosia* species Growing in Hilly Areas of Swat

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Abstract

This work tried to show the medicinal practices of *Rhynchosia*, to assess the consensus factor among people of Swat Valley to measure the potential for novel drugs of herbal origin. There was immeasurable promise among the informers concerning medicinal uses of plants with Informants Consensus Factor (ICF) value extending from 0.913 to 0.992 with an average value of 0.952. The study naked that most of the informants agreed in the application of *R. minima* to use, to treat Urinary infection (ICF 0.992) that unveiled the highest fidelity level (100%). Phylogenetic relationship among the 60 genotypes of *Rhynchosia* Species viz., 20 genotypes of *R. minima*, 20 of *R. capitata*, 20 of *R. rothii* were investigated using morphological and biochemical description. A total of 26 morphological traits were counted for the evaluation of phylogenetic relationship through traits similarity index and cluster analysis. Eight reproducible bands were detected in all of three species with molecular weight ranging from 10KDa to 180KDa. Intra locus contribution toward the genetic disagreement was 62.5% in *R. minima*, 25% in *R. capitata*, 37.5% in *R. rothii*. In the similar way, inter species locus contribution toward genetic diversity was 75%. Out of eight loci L-3, L-6, L-7 were monomorphic in *R. minima* while L-3 L-4, 5, 6, 7 and 8 were monomorphic in *R. capitata*. L-3, 4, 6 were monomorphic in *R. rothii*. Interestingly, Locus 3 (L-3) and locus 6 (L-6) were monomorphic in collected germplasms and marked as generic specific loci for *Rhynchosia* species. .

Keywords: *Rhynchosia* species; Medicinal uses morphology; SDS-PAGE; Phylogenetic relationship; Cluster analysis

Abbreviations: ICF: Informants Consensus Factor; PRA: Participatory Rural Appraisal; LC: Leaf Color; St: Seed Texture; Hc: Hilum Color; SC: Seed Coat Color; SS: Seed Shape; PL: Petiole Length; LL: Leaf Length; LW: Leaf Width; SL: Seed Length; SW: Seed Width; ST: Seed Thickness; SW: Seed Weight; PodL: Pod Length; SP: Seed Per Pod; PP: Pod Per Plant; IL: Inflorescence Length; IW: Inflorescence Width; BPB: Bromo-Phenol Blue

Introduction

The genus *Rhynchosia* belongs to family Fabaceae. This genus is widely dispersed in the hilly areas of the tropics and sub tropics, spreading into temperate regions with roundabout 200 species throughout the world [1]; it is characterized by 7 species in Pakistan Jahan et al. [2]. It is commonly recognized as snout bean. This genus is classified in the Tribe Phaseoleae and Subtribe Cajaninae, a group closely related to beans, pigeon peas and grams (Phaseolus, Cajanus and Vigna spp.). According to the flora of Pakistan this genus is Climbing, prostrate or sometimes erects herbs or subshrubs. The leaves of most of the species are pinnately trifoliolate with axillary or terminal Inflorescence. Its fruits maybe oblong, compressed, 1-2-seeded.

Several examinations has exposed that the decoction of the roots of *Rhynchosia capitata* DC have stomach cleaning action. Various species of *Rhychosia* like *R. minima* roots [3] and *R. nul bilis* seeds Joo Hyuk Yim [4] have been described to be used in Ingestion. Several activities like antibacterial, antifungal and antioxidant have been reported in *R. minima* Gundidza [5]. The anti-inflammatory effect against cotton pellet induced sub-acute inflammation in rats have been reported in the methanolic extracts of flowers of *R. cana* Vimala [6]. In addition, studies have shown the occurrence of C-glycosides, o-glycosides, prenylated flavonoids and aglycones in these species of the plant [5].

It is commonly recognized that morphological description has a key role in the study of genetic diversity in choosing elite variety of medicinal plants but affected by ecological changes harshly and conflicting the analysis of genetic discrepancy Nisar et al. [7]. On the other side genetic diversity evaluation through molecular practices such as biochemical assessment at protein level and DNA based techniques have a number of advantages over the tra-

ditional morphology Ndiaye et al. [8] but matched to biochemical assessment at protein level, molecular investigation of DNA markers is too expensive [9]. Among biochemical techniques, SDS-PAGE process is a simple, reliable, cheap and free of environmental fluxes [10]. SDS-PAGE is now widely used as biochemical procedure to describe the genetic structure of plant species [11]. Massive consideration has been focused on the use of SDS-PAGE over the last two decades for approximation of genetic diversity, reliable judgment and identification of plant varieties. Seed storage protein markers have been effectively used to resolve taxonomic relationships and characterize cultivated varieties in a number of crop plant species [12]; [7] *V. unguiculata* [9]. Proteins are being the end products of gene expression; SDS-PAGE can be employed to identify varities, determine polygenetic relationship in different species, biosystematics analysis and evaluate the passport data [13].

Pakistan is the midpoint of diversity of *Rhynchosia* but yet, there is no scientific report available in the literature on the medicinal uses and their conservation using phylogenetic relationship of *Rhynchosia* species (*R. capitata*, *R. minima*, and *R. rothii*). The objective of the study is to estimate ethnomedicinal uses and phylogenetic relationship in Pakistani *Rhynchosia* species on the basis of morphological and SDS-PAGE investigation.

Material and Methods

Exploration and collection

Various investigative trips were planed to 24 various areas (Ziarat, Rangila, Swegalai, Kohay, Jawand, Amlook Tangay, Kandao, Gharai, Khazana, Gamkot, Saboonkhfa, Nawagai, Barikot, Kandak, Behakan, Khawzakhela, Sangota, Kanju, Derai, Aligrama, Melaga, Dagay and Gadi) of District Swat, Khyber Pakhtunkhwa during years, i.e., 2017-2018. 60 genotypes of three species were recognized and inspected for morphological description and protein profiling.

The study zone was visited four times in different seasons of the year of 2017. Voucher specimens for each species have been gather and treated using standard herbarium procedures [14]. The specimens were recognized referring different Floras, viz., Hooker (1872-1897). Ethnomedicinal data has been collected through Participatory Rural Appraisal (PRA), which is based on communication with indigenous people and direct observation in the field Martin [15]. The data have been noted through semi-structured interviews with people involved in the plants, organization [14]. A total of 130 residents have been interviewed. During the field survey, information on uses of plants to cure various diseases of human being, parts used, of medicine have been collected. Based on the information obtained from the informants in the study area, all the reported diseases have been classified into 12 groups.

The level of similarity among information delivered by various informants was calculated by the Informants' Consensus Factor, ICF Trotter and Logan [16] by applying the following formula:

$$ICF = Nur - Nt / \left(Nur - 1\right)$$

Where, Nur = number of use reports from informants for a specific plant-use category; Nt = number of taxa or species that are used for that plant use category for all informants.

ICF Values range between 0 and 1, where '1' indicates the highest level of informant agreement. The fidelity level (FL), the percentage of informants claiming the use of a certain plant species for the same major purpose, was calculated for the most frequently reported diseases or ailments as:

$$FL\left(\%\right) = \left(Np / N\right) \times 100$$

Where, Np = number of informants that claim a use of a plant species to treat a particular disease; N = number of informants that use the plants as a medicine to treat any given disease [14].

Morphological description

Phenotypic description was performed for evaluation of phylogenetic relationship; 26 morphological traits were documented. Out of 26 characters 9 were qualitative and 17 quantitative. Qualitative characters comprise of leaf color (LC), seed texture (St), Hilum color (Hc), seed coat color (SC), seed shape (SS), leaf pubescent, leaf stipule presence, flower color Whereas, quantitative traits scored were petiole length (PL), leaf length (LL), leaf width (LW), seed length (SL), seed width (SW), seed thickness (ST), and seed weight (SWt), pod length (PodL), No. of seed per pod (SP), No. of pod per plant (PP), inflorescence length (IL), inflorescence width (IW), 100 seed weight, No. of branches per plant, plant height (Ph), stipule length (StL), Biomass. Characters mean was found out after measuring of 3 different samples (small, medium, large) of each quantitative trait. Seven morphological characters were documented, and cluster analysis was performed using software PC-ORD and SPSS.

SDS-PAGE description

For SDS-PAGE examination single seed of each genotype was crushed into a fine powder with the help of mortar and pestle for the extraction of proteins. About 400µl of protein extraction buffer (0.5 M Tris-HCL pH 8.0, 0.2% SDS, 5 M Urea, 1% B-mercaptoethanol) was added to 0.01g of seed flour taken in 1.5ml Effendorf tube. The E-tube was vortexed thoroughly to homogenize the mixture. Bromo-Phenol Blue (BPB) was added to the protein extraction buffer as tracking dye to monitor the movement of protein in the gel. The homogenated samples were centrifuged at 13,000rpm for 13 minutes at 10 ˚C. The electrophoretic procedure was carried out using 12% polyacrylamide gel, separation gel (3.0M Tris-HCl pH9.0, 0.4% SDS) and 4.5% stacking gel (0.4M Tris-HCl pH 7.0, 0.4% SDS). Electrode buffer (0.025 M Tris, 129 M Glycine, 0.125% SDS) was poured into the top pool of the apparatus. A total volume of 8µl of the protein extract solution was loaded in each well of the gel with the help of micropipette. The electrophoresis was run at 100V until the blue line passed through the bottom of gel plates. The gels were then stained in staining solution containing 0.2% BPB dissolved in 10% glacial acetic acid, 40% methanol and water in the ratio of 10:40:50. Gels were de-stained in a solution containing 5% acetic acid and 20% methanol for 15 minutes. The

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data were recorded from the destined gel on the basis of presence and absences of protein bands, i.e., '1' for the presence and '0' for the absence of bands and cluster analysis was carried out using software PC-ORD and SPSS.

Results

In this study 3 plant species of *Rhynchosis* (*R. minima*, *R. capitata* and *R. rothii*) of Fabaceae family in Swat district, have been in listed for curing of 12 categories of diseases. For each species botanical name, family, local name, illnesses to be treated, and part(s) used were recorded (Table 1). Consumption of plant parts as medicine among the informants shows disparities. Seeds are mostly used part for majority, followed by roots, leaves and bark (Table 1). In the current investigation area threat to the species is marginal as seeds are the leading plant part used for medicinal purposes. It was supposed that the collection of part of plant as medicinal part from the wild were not manageable. According to residents, this type of activity is done by the collectors related to illegal activity of medicinal plants. *Rhynchosia* is vulnerable to this type of activity in the study region [5,17,18].

Table 1: Documentation of medicinal plants with scientific name, local name, parts used, and ailments.

S. No	Botanical Name	Family	Local Name	Voucher No.	Phytochemicals	Habit	Part Used	References
1	*R. minima* (L.) DC	Fabaceae	Zangali Mahe	HUP-7781	isopropyl toluene, O-cymene, camphene, limonene, 2-pinene, _-terpinolene, _-pinene and myrcene	Herb	Bark, seed, Leaves	[5]
2	*R. capitata* (Heyne ex Roth) DC.	Fabaceae	Zangali lobi	HUP-7353	C-glycosides, o-glycosides, prenylated flavonoids and a-glycones	Herb	Leaves	[15]
3	*R. rothii* Benth. ex Aitch	Fabaceae	Panipata	HUP-7254	chymotrypsin and trypsin	Herb	Root , leaves	[16]

ICF values were established to know the settlement among the informants of Swat valley for usage of plants to cure certain illness groups. ICF values are designated in the Table 2. It is known that the ICF values vary from 0.992 to 0.913 with an average value of 0.952. Urinary infection has the highest ICF value 0.992 with 130 use-reports for 2 plant species. The specie liable for this high consensus was *R. minima* with 130 of the defined events, linked by Infertility (ICF = 0.991; 110 use-reports, 3 species), Rheumatism (ICF = 0.988; 87 use-reports, 2 species), Fungal infection (ICF= 0.988, 90 use reports, 2 species) and so on. Medicinal plants thought to be effective in treating specific illness have high ICF values. The high ICF value for Urinary infection possibly unveiled that this ailment is common in the study area High ICF values also designate that the specie predictably used to treat these illnesses are worth searching for bioactive compounds. The least agreement (ICF=0.894) between the informers was detected for plants used to cure Ingestion problems. The low ICF value as noted in our study could be due to a lack of communication among people in various areas (Table 2).

Table 2: Categories of disorders and informant consensus factor (ICF) for each grouping.

S. No	Disease Category	Use Reports	Taxa Used	*ICF
1	Urinary Infection	130	2	0.992
2	Infertility	110	3	0.991
3	Rheumatism	87	2	0.988
4	Fungal Infection	90	2	0.988
5	Liver Protection	85	2	0.988
7	Diarrhea	54	2	0.981
8	Constipation	32	2	0.967
9	wound Healing	28	3	0.925
10	Stomach Cleaning	24	3	0.913
11	Ingestion	20	3	0.894

To discover conventionally significant medicinal species in the society, Fidelity Level (FL) of plants has been predicted based on use reports which have been cited by 50 or more informants for being used against a given disorder. The FL values are shown in Table 3. The examination demonstrated that the highest FL value found in *R. minima* followed by *R. capitata* and *R. rothii* respectively. The least FL value was found in the case of *R. rothii*. FIC and FL studies presented that the most commonly used species in the study area is *R. minima* (ICF = 0.991) with 130 use-reports and FL value (100%). When choosing the most ideal plant species for each ailment category, we took the high-fidelity Level (%) in each category of ailment.

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Table 3: Utmost frequently used plants for various illness groups based on highest FL (%) in each disease category (Total informants = 130).

Scientific Name	Disease Category	Use Report	Fidelity Level (%)
R. minima (L.) DC	Urinary Infection	130	100
R. capitata (Heyne ex Roth) DC.	Infertility	110	84.615
R. rothii Benth. ex Aitch	Wound Healing	28	21.538

Morphological description

For morphological data analysis both the qualitative and quantitative traits were taken. Quantitative traits which were measured with the help of Vernier calipers are: petiole length (PL), leaf length (LL), leaf width (LW), seed length (SL), seed width (SW), seed thickness (ST), and seed weight (SWt), pod length (PodL), No. of seed per pod (SP), No. of pod per plant (PP), inflorescence length (IL), inflorescence width (IW), 100 seed weight, No. of branches per plant, plant height (Ph), stipule length (StL), Biomass. Characters mean was found out after measuring of 3 different samples (small, medium, large) of each quantitative trait. (Table 4).

Table 4: Morphological descriptors used in the characterization of the 60 genotypes *Rhynchosia species*.

Morphological Descriptors	Abbreviations	Morphological Descriptors	Abbreviations
Petiole	PL	Leaf Shape	LS
Leaf Length	LL	Leaf Color	LC
Leaf Width	LW	Leaf Pubescent	LP
Seed Length	SL	Flower Color	FC
Seed Thickness	ST	Seed Texture	St
Seed Width	SW	Hilum Color	HC
Pod Length	PodL	Seed Coat Color	SCc
Seed per Pod	S/P	Seed Shape	SS
Pod per Plant	P/P	Spots on the Seed Coat	SpT
Flower Length	FL	Trait Similarity Index	TSI
Flower Width	FW	Locus	L
Seed Weight	SWT	*Rhynchosia*	R
Branches per Plant	B/P	*R. minima*	RM
Plant Height	PH	*R. capitata*	RC
Biomass	BM	*R. rothii*	RR
Yield per Plant	Y/P		

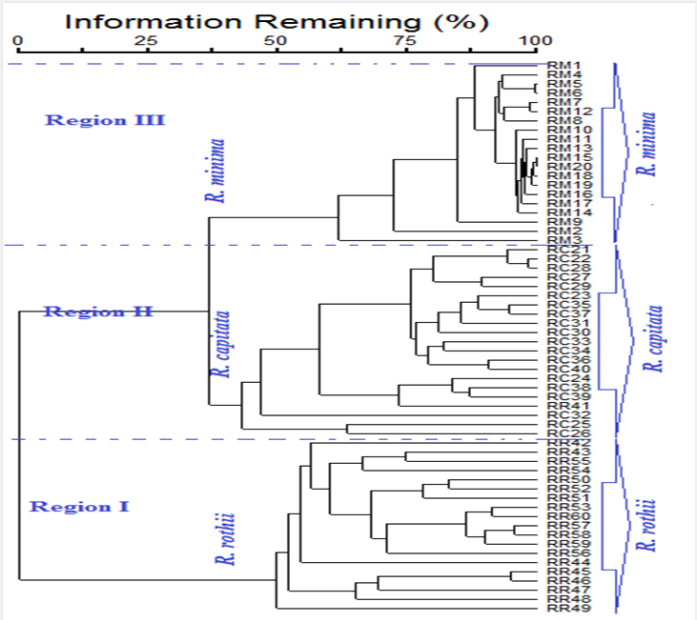

Figure 1: Inter -species phylogenetic relationship identified through morphological traits analysis in 60 different genotypes of Rhynchosia species collected from Swat, Khyber Pakhtunkhwa, Pakistan. RM indicate genotypes of R. minima, and RC represents *R. capitata* and RR represents R. rothii.

Qualitative characters are leaf type (LTY), leaf color (LC), seed texture (St), Hilum color (Hc), seed coat color (SC), seed shape (SS), leaf pubescent, leaf stipule presence, flower color. The quantitative and qualitative characters of 60 genotypes (total 26 characters) were documented and data was subjected to computer software the PCORD shown in (Figure 1). The result of the cluster analysis was shown as a phylogenetic tree (Dendrogram) based on the linkage distance (Figure 1).

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Table 5: Correlation coefficient among seventeen quantitative traits of *R. minima*.

Traits	PL	STL	LL	LW	IL	IW	SWT	SL	SW	ST	Pod L	SP	PP	BP	PH	BM	Y/P
PL	1																
STL	-0.04	1															
LL	-0.18	-0.07	1														
LW	-0.07	0.15	0.44	1													
IL	0.09	-0.07	0.21	.489*	1												
IW	0.06	-0.2	0.12	0.44	.873**	1											
SWT	0.17	0.04	-0.04	0	0	0.13	1										
SL	0.02	0.41	0	-0.09	-0.13	-0.31	-0.02	1									
SW	-0.04	-0.09	0.05	-0.08	-0.13	-0.33	-0.2	.527*	1								
ST	-0.13	0.31	-0.25	-0.06	-0.14	-0.14	-0.32	.480*	.581**	1							
Pod L	0.31	-0.07	-0.04	-0.36	-0.09	0.04	-0.26	0.1	-0.08	0.15	1						
SP	-0.17	-0.27	-0.24	-0.41	-0.14	-0.05	-0.19	-0.02	0.35	0.36	0	1					
PP	-0.02	0.32	-0.01	-0.42	-0.18	-0.24	-0.06	0.43	0.12	0.17	0.37	0.2	1				
BP	0.01	-0.15	-0.24	-0.38	0.02	0.16	-0.23	-0.19	0.14	0.27	.487*	.451*	0.03	1			
PH	0	0.27	-0.15	0.19	-0.14	-0.15	.547*	-0.08	-0.17	-0.15	-0.27	-0.38	0.15	-.500*	1		
BM	0.19	0.11	-0.01	0.32	0.42	0.23	-0.15	-0.15	-0.07	0.05	-0.19	0.17	-0.34	0.06	-0.12	1	
Y/P	0.26	0.19	0.1	-0.01	0.43	0.27	-0.03	0.33	-0.14	-0.05	0.09	-0.43	0.17	-0.26	0.01	0.11	1
*. Correlation is significant at the 0.05 level (2-tailed).																	
**. Correlation is significant at the 0.01 level (2-tailed).																	

Table 6: Correlation coefficient among seventeen quantitative traits of *R. capitata*.

Traits	PL	STL	LL	LW	IL	IW	SWT	SL	SW	ST	PodL	SP	PP	BP	PH	BM	Y/P
PL	1																
STL	0.17	1															
LL	0.08	-0.23	1														
LW	-0.04	0.12	.682**	1													
IL	0.24	0.19	-.465*	-0.36	1												
IW	0.17	0.19	-0.31	-0.19	.874**	1											
SWT	0.39	0.21	-0.39	-.556*	0.27	0.11	1										
SL	0.28	.564**	0.16	0.15	0.11	-0.03	0.06	1									
SW	0.11	.450*	0.01	0.06	-0.15	-0.27	0.03	.599**	1								
ST	-0.2	0.09	0.39	0.33	-.578**	-.641**	-0.32	0.4	.490*	1							
Pod L	-0.03	.481*	-0.17	-0.02	-0.03	-0.1	0	0.22	.496*	0.27	1						
SP	0.01	-0.04	0.03	-0.06	-0.18	-0.21	0.01	0.17	0.14	0.25	0.23	1					
PP	-0.14	0.02	-0.03	-0.06	-0.19	-0.15	0.23	-0.11	-0.08	-0.06	-0.28	-0.21	1				
BP	-0.32	-0.13	0.27	0.16	-0.31	0.06	-0.12	-0.4	-0.35	-0.31	-0.25	-0.3	0.4	1			
PH	0.26	-0.13	0.29	0.24	-0.2	-0.19	-0.05	-0.06	0.12	0.29	0.24	0.28	-0.44	-0.22	1		
BM	0.11	0.41	-.444*	-.522*	0.18	0.13	0.26	0.24	0.29	-0.07	0.29	-0.29	0.2	-0.02	-.548*	1	
Y/P	-.581**	-0.07	0.03	0.1	-.609**	-.546*	-0.44	0.03	0.19	.567**	0.11	0.37	0.07	0.05	-0.06	0.11	1
**. Correlation is significant at the 0.01 level (2-tailed).																	
*. Correlation is significant at the 0.05 level (2-tailed).																	

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Table 7: Correlation coefficient among seventeen quantitative traits of *R. rothii*.

Traits	PL	STL	LL	LW	IL	IW	SWT	SL	SW	ST	PodL	SP	PP	BP	PH	BM	Y/P
PL	1																
STL	-0.2	1															
LL	.952**	-0.18	1														
LW	.944**	-0.2	.992**	1													
IL	-0.01	-0.16	0.05	0.11	1												
IW	0.13	-0.17	0.2	0.24	.938**	1											
SWT	-.852**	0.11	-.866**	-.857**	-0.22	-0.4	1										
SL	-.481*	0.09	-.563**	-.560*	-0.23	-0.33	0.44	1									
SW	-0.05	-0.17	-0.14	-0.14	-0.28	-0.37	0.11	.804**	1								
ST	-0.01	-0.11	-0.05	-0.05	-0.19	-0.23	0	.767**	.924**	1							
Pod L	.860**	-0.38	.865**	.875**	0.24	0.32	-.818**	-.445*	0.01	0.07	1						
SP	.921**	-0.19	.977**	.967**	0.15	0.3	-.888**	-.639**	-0.23	-0.15	.868**	1					
PP	.556*	-0.31	.549*	.554*	-0.03	0.01	-0.42	-0.07	0.2	0.09	.537*	.561**	1				
BP	.927**	-0.25	.913**	.887**	-0.02	0.14	-.852**	-0.4	0	0.1	.812**	.908**	.588**	1			
PH	.870**	-0.2	.929**	.933**	0.17	0.35	-.923**	-.513*	-0.11	0.02	.872**	.923**	0.41	.859**	1		
BM	.922**	-0.12	.916**	.901**	-0.2	-0.07	-.696**	-0.41	-0.04	0.02	.722**	.842**	.526*	.856**	.755**	1	
Y/P	.649**	-0.24	.567**	.545*	-0.3	-0.3	-0.29	-0.22	0.12	0.09	.452*	.504*	.458*	.624**	0.32	.705**	1
**. Correlation is significant at the 0.01 level (2-tailed).																	
*. Correlation is significant at the 0.05 level (2-tailed).																	

In correlation study of 3 species of *Rhynchosia* (*R. minima*, *R. capitata*, and *R. rothii*) (Table 5-7) the petiole length in the *R. minima* and *R. rothii* is negatively correlated with stipule length positively correlated with stipule length in *R. capitata*. Stipule length is negatively correlated with leaf length in *R. minima* and *R. capitata*. Whereas positively correlated with the leaf length in *R. rothii*. The leaf width is significantly positively correlated with the inflorescence length in *R. rothii*, *R. minima* and *R. capitata*. Seed length seed width, seed thickness, No. of the Pods/Plant, No. of seed/Plant, stipule length and 100 seed weight are negatively correlated with petiole length, leaf length, leaf width, inflorescence length, inflorescence width, pod length and stipule length in all of the three species.

The data of 60 genotypes based on morphology was considered for the construction of phylogenetic tree to represents the similarity of the three species of the *Rhynchosia* and were analyzed for resemblances and the phylogenetic tree was made (Figure 1). The phylogenetic tree separated three species into three regions, the region I was composed of 20 genotypes of *R. rothii* (RR41-RR60) whereas the Region II consisted of 20 genotypes of *R. capitata* (RC21-RC40) at a linkage distance 37.5. While region I included 20 genotypes of *R. minima* (1-20).

Table 8: Intra and interspecific genetic diversity in 26 morphological characters studied in *R. minima*, *R. capitata* and *R. rothii*.

Traits	RM	RC	RR	Traits Similarity Index		
				RM & RC	RR & RM	RC & RR
PL	4.43	7.369	2.218	NA	NA	NA
STL	1.353**	1.69**	1.683	1.521**	1.518**	1.686**
LL	72.863	117.65	35.6	NA	NA	NA
LW	55.376	94.465	28.275	NA	NA	NA
IL	4	5.45	3.15	NA	NA	NA
IW	3.043*	3.55*	2.105	3.296*	NA	NA
SWT	13.702	10.33	27.59	NA	NA	NA
SL	5.0356	3.65	7.756	NA	NA	NA
SW	3.278	2.72	4.26	NA	NA	NA
ST	2.498**	2.353**	2.51**	2.425**	2.504**	2.431**
Pod L	57.9*	57.87*	29.78	57.88*	NA	NA

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SP	3.433	11.216	2.7	NA	NA	NA
PP	72.066	34.716	21.7	NA	NA	NA
BP	12.866	13.1	5.525	NA	NA	NA
PH	293.66	438.05	160.93		NA	NA
BM	112.6	139.885	95.176	NA	NA	NA
Y/P	24.13	39.1	26.6	NA	NA	NA
LS	rhomboid	cordate	rhomboid	NA	rhomboid*	NA
LC	green**	green**	green**	green**	green**	green**
LP	Present**	Present**	Present**	Present**	Present**	Present**
FC	Yellow**	Yellow**	White red	Yellow**	NA	NA
St	Smooth**	Smooth**	Smooth**	Smooth**	Smooth**	Smooth**
HC	white	Yellow*	Yellow*	NA	NA	Yellow*
SCc	brown	green brown	brown	NA	NA	NA
SS	round	rectangular	rhomboid	NA	NA	NA
SpT	Present**	Present**	Present	Present**	Present**	Present**
Total TSI = ((homologous trait/total traits) *100)				34.615	26.92	26.92
* . Traits Similarity Index Within Two Species						
** . Traits Similarity Index Within Three Species						

The similarity indexes were performed for all the genotype of all three species that was 34.615 for *R. minima* and *R. capitata*. Whereas *R. rothii* and *R. minima* were 26.92% similar morphologically. While *R. capitata* and *R. rothii* revealed 26.92% similarity (Table 8).

SDS- PAGE analysis

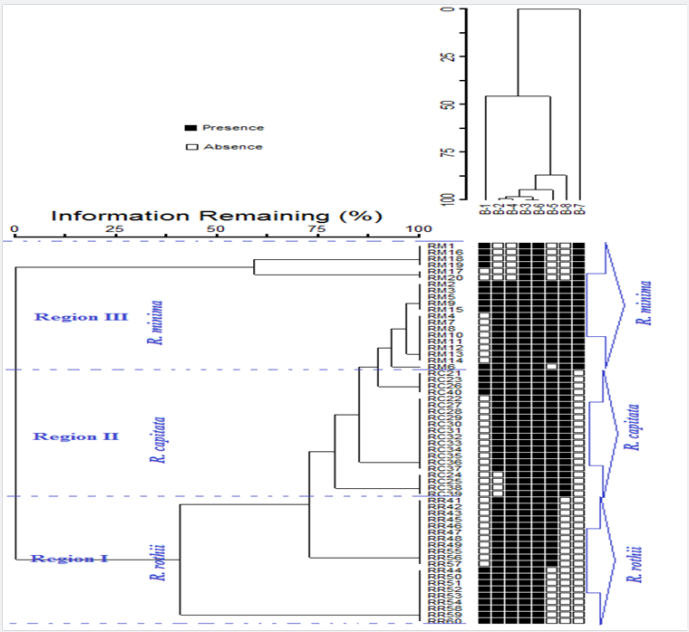

Figure 2: Inter -species phylogenetic relationship identified through SDS-PAGE analysis in 60 different genotypes of Rhynchosia species collected from Swat, Khyber Pakhtunkhwa, Pakistan. RM indicate genotypes of R. minima, and RC represents *R. capitata* and RR represents R. rothii

Eight bands were noticed in three species of *Rhynchosia* (*R. minima, R. capitata* and *R. rothii*) Figure 3. The phylogenetic relationship among all the three species through phylogenetic tree has been shown in the (Figure 2). The phylogenetic tree divided all the sample of three species into three regions. The Region I included 20 genotypes of *R. rothii* RR41-RR60); these were collected from

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(RR41 Ziarat, RR42 Rangila, RR43 Swegalai, RR44 Kohay, RR45 Jawand, RR46 Amlook Tangay, RR47 Kandao, RR48 Gharai, RR49 Khazana, RR50 Gamkot, RR51 Saboonkhfa, RR52 Nawagai, RR53 Barikot, RR54 Kandak, RR55 Behakan, RR56 Khawzakhela, RR57 Sangota, RR58 Kanju, 59 Derai, RR60 Aligrama while the Region II consisted of 20 genotypes of *R. capitata* ; RC21 Melaga, RC22 Dagay , RC23 Gadi, RC24 Jawand, RC25 Amlook Tangay, RC26 Kandao,RC27 Gharai, RC28 Khazana, RC29 Gamkot, RC30 Saboonkhfa, RC31 Nawagai, RC32 Barikot, RC33 Kandak, RC34 Behakan, RC35 Khawzakhela, RC36 Sangota, RC37 Kanju, RC 38 Derai, RC39 Aligrama, RC40 Swegalai. Whereas the Region III was composed of the 20 genotypes *R. minima* these were collected from; RM1 Gharai, RM2 Khazana, RM3 Gamkot, RM4 Saboonkhfa, RM5 Nawagai, RM6 Barikot, RM7 Kandak, RM8 Behakan, RM9 Khawzakhela, RM10 Sangota, RM11 Kanju, RM12 Derai, RM13 Aligrama, RM14 Melaga, RM15 Dagay, RM16 Ziarat, RM17 Rangila, RM18 Swegalai, RM19 Kohay and RM20 Jawand. Moreover, dendrogram based on SDS-PAGE showed that the genotypes of *R. rothii* were 37.5% similar with the genotypes of *R. capitata* whereas the genotypes of *R. minima* have 87.5 similarities with the genotypes of *R. capitata* (Figure 2).

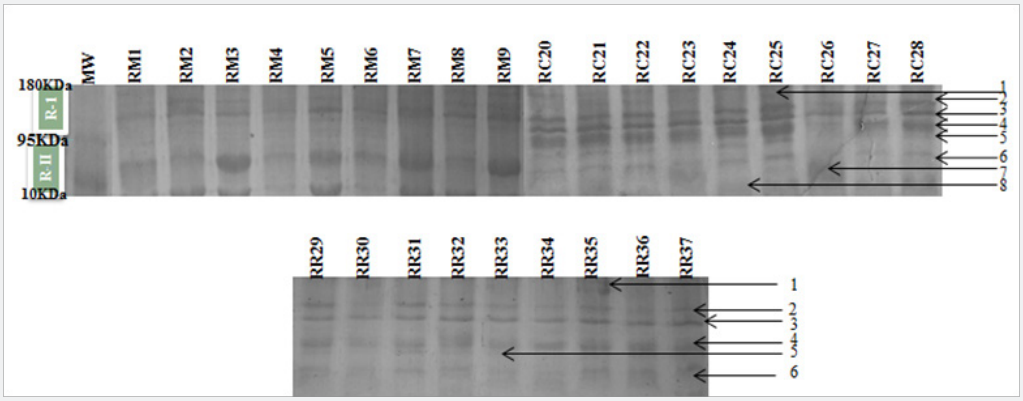

Figure 3: Electrophoregram of 12% polyacrylamide gel banding pattern showing diversity in total seeds storage protein of three species of Rhynchosia (R. minima, *R. capitata*, and R. rothii). Arrow indicates the location of protein bands in the electropherogram. MW=molecular weight marker, RM= R. minima, RC= *R. capitata*, RR= R. rothii.

Decisively, the Region I grouped 20 genotypes of *R. rothii*. The Region II comprised of 20 genotypes *R. capitata*. Whereas the Region III consisted of 20 genotypes *R. minima* revealed a clear-cut evidence for species identification on the basis of seed storage protein.

Locus dissimilarity

Strangely, table 9 displays interspecific variation among 60 genotypes of the *Rhynchosia* species. Among all the genotypes, eight loci (L1-L8) were noted, out of these L3 and L6 were monomorphic and were marked as generic specific which is used to distinguish the *Rhynchosia* species. Moreover, the loci L-1, L-2, L-4, L-6 and L-8 were marked as polymorphic with 60, 45, 90, 75, 28.33 and 60 percent genetic diversity, respectively. The inter species comparative locus contribution toward genetic disagreement was 75% in the three species of 60 *Rhynchosia* genotypes (Table 9).

Table 9: Inter-locus variations among *R. minima, R. capitata* and *R. rothii.*

Locus	Present (%)	Absent (%)	Variation (%)	Status	GD
L-1	24(40%)	36(60)	60	poly	0.4
L-2	23(55)	27(45)	45	poly	0.55
L-3* Generic Specific Locus	60(100)	0	Nil	mono	1
L-4	6(10)	54(90)	90	poly	0.1
L-5	15(25%)	45(75)	75	poly	0.15
L-6* Generic Specific Locus	60(100)	0	Nil	mono	1
L-7	43(71.66)	17(28.33)	28.33	poly	0.43
L-8	24(40)	36(60)	60	poly	0.6
Locus Contribution Toward Genetic Disagreement GD =75 (Poly loci/total loci*100)					

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Intraspecific locus variation among 20 genotypes of *R. minima* unveiled high intra-specific locus variation, denoted in Table 10. Notably, L-3, L-6 and L-7, were monomorphic in *R. minima* L-1, L-2, L-5 and L-8 shows 50, 30, 35 and 30 percent variation and the locus contribution toward genetic disagreement of *R. minima* was 62.5% Table 10.

Table 10: Intra-locus variations among genotypes of *R. minima*.

Locus	Present (%)	Absent (%)	Variation (%)	Status	GD
L-1	10(50%)	10(50)	50	poly	0.5
L-2	14(70)	6(30)	30	poly	0.7
L-3* Generic Specific Locus	20(100)	0	Nil	mono	1
L-4	14(70)	6(30)	Nil	poly	0.7
L-5	13(65)	7(35)	35	poly	0.1
L-6* Generic Specific Locus	20(100)	0	Nil	mono	1
L-7	20(100)	0	Nil	mono	1
L-8	14(70)	6(30)	30	poly	0.7
Locus Contribution Toward Genetic Disagreement GD =62.5 (Poly loci/total loci*100)					

Table 11: Intra-locus variations among genotypes of *R. capitata*.

Locus	Present (%)	Absent (%)	Variation (%)	Status	GD
L-1	3(15%	17(85%	85	poly	0.15
L-2	16(80)	4(20)	20	poly	0.8
L-3* Generic Specific Locus	20(100%)	0	Nil	mono	1
L-4	20(100%)	0	Nil	mono	1
L-5	20(100%)	0	Nil	mono	1
L-6* Generic Specific Locus	20(100%)	0	Nil	mono	1
L-7	20(100%)	0	Nil	mono	1
L-8	20(100%)	0	Nil	mono	1
Locus Contribution Toward Genetic Disagreement GD =25 (Poly loci/total loci*100)					

The Table 11 represents the intraspecific variation among the 20 genotypes of *R. capitata*. Among eight loci, out of which L-3, L-4, L-5, L-6 L-7 and L-8 were monomorphic, while L-1 and L-2 were polymorphic. L-2 and L-3 represent 85 and 20 percent variation. The locus contribution toward genetic disagreement of *R. capitata* was 25% Table 11.

Table 12: Inter-locus variations among genotypes of *R. rothii*.

Locus	Present (%)	Absent (%)	Variation (%)	Status	GD
L-1	9(45%)	11(55%)	55	poly	0.45
L-2	20(100)	0	Nil	mono	1
L-3* Generic Specific Locus	20(100)	0	Nil	mono	1
L-4	20(100)	0	Nil	mono	1
L-5	11(55)	9(45)	45	poly	0.55
L-6* Generic Specific Locus	20(100)	0	Nil	mono	1
L-7	0	20(100)	Nil	mono	0
L-8	18(90)	10(35.714)	35.714	poly	0.9
Locus Contribution Toward Genetic Disagreement GD =37.5 (Poly loci/total loci*100)					

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The Table 12 represents the intraspecific variation among the 20 genotypes of *R. rothii*. Among eight loci, out of which L-2, L-3, L-5 and L-6 L-7 were monomorphic, while L-1 and L-8 were polymorphic. The L-7 was missing in 20 *R. rothii* genotypes. Hence this missing band in this specie can be helpful to identify this specie. L-1 and L-8 represent 55 and 35.714 percent variation respectively. The locus contribution toward genetic disagreement of *R. rothii* was 37.5 % table 12.

Discussion

Most of the public's living in the region depends on direct medicinal plant to treat a wide range of illnesses. However, the vanishing of these plant species is steadily reported chiefly due to fluctuations in the environment, land degradation and unsustainable use of these plants; moreover, the expansion of invasive species has donated a lot to their disappearance Mohammed et al. [19]. Conservation of medicinal plant genetic diversity has freshly created a lot of attention in the tropics as a result of many years of misconduct, adverse environment as well as socio-economic changes. Population genetic theory expects that the reduction in the genetic diversity limits a species ability to keep pace with the changing selection pressure Young and Merriam [20]. Plant species mainly the medicinal plants rely on the existing genetic diversity for constancy and survival under the ever-fluctuating environments [21]. Understanding medicinal plants species population genetic structure is vital for their conservation, planning and justifiable organization Sun et al. [22]. Therefore, a common goal line of conservation is to preserve genetic diversity in "red listed" species, which is crucial for long-term survival and evolutionary response to the altering environment [23]. One main implication of this method, from the viewpoint of conservation genetics, is that it could help us set sampling intervals of areas within populations to optimize the genetic diversity in collections from local populations of rare, endangered, or endemic plant species Chung et al. [24]. For the purpose of conservation of plant species, most of the investigations deal only with determination of genetic diversity in individual populations [12].

This work has been consequently initiated with an objective to file the knowledge and practices on the *Rhynchosia* medicinal practices by the local residents and its genetic diversity. The present research is supposed to add up the publics' knowledge in the country's database of traditional knowledge and will offer a baseline data for future pharmacological and phytochemical investigation. Genetic diversity suits more vital in view of climatic change and allied unexpected events as it may serve as the source of novel traits considering tolerance to different biotic and abiotic stresses Muhammad et al. [12].

Though, the indigenous knowledge that has been recognized was previously eroded, if not in all, previously in most of the parts studied Mohammed et al. [19]. Contributions concerning conservation of traditional knowledge still remain scarce [25]. Therefore, the study focused on recognizing local factors that promote the knowledge on introduced and native medicinal plant species, and to designate medicinal uses of plant species.

This work is one of the first efforts to count the ethnomedicinal information and interspecies genetic diversity in *Rhynchosia* species (*R. minima*, *R. capitata* and *R. rothii* from Swat which offer better choice for the selection of broadly used medicinal plants looking for bioactive compounds to cure illnesses. The study described 3 medicinal plants with their uses from the Swat. The effectiveness and safety of all the reported ethnomedicinal plants need to be assessed by phytochemical and pharmacological studies. Plants with high informant consensus factor use report and fidelity level should be given priority to carry out bioassay and toxicity studies. From this study we suggest *R. minima* for further ethno-pharmacological studies, since this species has the high ICF and FL values. The results presented that this species may be used for the development of new, cheap, effective, and eco-friendly herbal formulations for healthcare organization. Additional use of these herbal formulations for healthcare management will need safety and effectiveness testing. According to occupants and our observations in the field, *R. minima* are now a very infrequent plant in the area. Illegal and unsustainable collection of leaves and seeds from this plant by the local crude drug traders is one of the major causes of depletion of this species from nature. There is crucial want to formulate suitable conservation strategies for naturally growing ethnomedicinal plants to overcome their depletion from natural resources and to make these practices more ecofriendly.

The examination of genetic diversity and phylogenetic relationship in medicinal plant species is helpful in their conservation. Molecular markers are the most effective markers for genetic polymorphism studies in many species [26]. However, the use of biochemical markers based on total seed protein and enzyme by SDSPAGE method has confirmed to be a consistent, yet inexpensive technique of developing countries. Genetic markers for identification and genetic analyses of several plant species, as they reveal differences between storage proteins or enzymes encoded by different alleles at a single (allozymes) or more gene loci (isozymes) Oppong-Konadu et al. [27].

The SDS-PAGE of seed storage protein is a technique to examine genetic variation and to categorize plant varieties [28]. The arrangement between various subgenera, species and subspecies are based primarily on morphological attributes. However, these morphological traits may be unstable and influenced by environmental instabilities [29]. Plant identification is often masked by high species dissimilarity. Re-evaluation of the morphological variation within taxa and populations is therefore essential [30]. Among several biochemical techniques, SDS-PAGE (Sodium Dodecyl Sulphate) has been used successfully to resolve taxonomic and evolutionary problems of several plants [31]; Lioi et al. [32]. In this technique protein is separated according to their molecular weights. Resolution of this technique is very high and therefore it could be used as a reliable tool for taxonomic purposes Bartke et al. [33]. Its banding pattern is very stable which encouraged for

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identification purpose in medicinal plants. It has been broadly recommended that such banding patterns could be used as important supplemental method for medicinal plant documentation Tanksley et al. [34]; Thanh et al. [35]. Analyses of SDS-PAGE are simple and inexpensive, which are added advantages for its use in practical plant breeding practices [36].

SDS- PAGE has been considered as useful tool for the estimation of genetic and systematic relationships in flowering plants at generic and specific levels Crawford [37] because the seed storage protein electrophoretic pattern shows genetic affinities within taxon and even between different biological taxa [38]. Hence, in this work three different species of *Rhynchosia* were selected and their relationship was investigated using SDS-PAGE.

The three plants species under the genus *Rhynchosia* study exposed that no two plants have similar protein banding patterns which shows, the presence of genetic diversity among these species. The occurrence of common bands/loci (L-3 and L-6) among these three *Rhynchosia* species suggests their close genetic similarity and common ancestry. These loci coded for by a gene that has become fixed in different species under genus *Rhynchosia* over evolutionary time that is an agreement with finding of Nisar et al. [7] that the appearance of common bands in *Lycopersicum* and *Trichosanthes* species labels their common evolutionary origin. Also, Alkinwusi and Illoh [39] accredited the appearance of a common band in all individual in a population to the fact that the gene coding for the enzyme or protein does not differ.

Due to High inter-species locus contribution toward genetic disagreement SDS-PAGE could be a reliable technique for identification of these three species, while intra-specie locus contribution toward genetic disagreement was high in genotypes of *R. minima* (62.5%) as compare to *R. rothii* (37.5%) and *R. capitata* (25%) respectively.

In our present study, phylogenetic tree based on seed storage protein analyses of selected species of *Rhynchosia* showed that the three species of *Rhynchosia* had close similarity to one another. The result showed that the *R. rothii* was clustered adjacent to *R. capitata* showed 37.5% genetic similarity. Whereas the genotypes of *R. minima* have 87.5 similarities with the genotypes of *R. capitata*. The results obtained after SDS-PAGE electrophoresis disclosed that the method provided a powerful tool for reliable germplasm discrimination based on genetic differences in seed storage protein. Thus, the present study explores the existing polymorphism of total proteins through SDS PAGE to facilitate description of selected germplasm of *Rhynchosia*.

Conclusion

The current work delivers indication that medicinal plants have an important role in the healthcare system of Swat urban public. They still endure to depend on medicinal plants for the treatment of healthcare problems. The existing paper denotes significant ethno-botanical information on medical plants which provides baseline data for future pharmacological studies and genetic diversity is helpful in selection elite genotypes for future conservation.

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Conflict of Interest

The authors declare that they have no conflict of interests.

Author's Contribution

NM collected plants and carried out experimental work analysed data and wrote paper, NA conceived the overall project, and helped in interpretation of the results, NA and NU wrote and critically reviewed the manuscript. All authors have read and approved the final manuscript.

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