Pak. J. Bot., 50(1): 301-305, 2018.

MOLECULAR ANALYSIS IN MEDICINALLY IMPORTANT SPECIES *CARUM CARVI* AND *BUNIUM PERSICUM* (FAMILY APIACEAE) FROM DISTRICT ASTORE

MUNAM ZEHRA, ABDUL RAZAQ* AND IMTIAZ AHMED KHAN

Department of Biological Science, Karakoram International University, Gilgit-Baltistan
**Corresponding author's email: dr.razaq@kiu.edu.pk*

Abstract

Mountain areas of Gilgit Baltistan (GB) is especially rich in medicinal plant species but unfortunately not much genetic analysis work has been conducted on various medicinally important species native to the area. Two plant species of family Apiaceae viz., *Bunium persicum* (black cumin or kala zeera) and *Carum carvi* (cumin or zeera) are medicinally important species of Gilgit Baltistan and have been used by local communities as antioxidant, antimicrobial, antidiabetic, anticarcinogenic / antimutagenic, to treat heart problems, thatching and Roofing, spice, condiments and perfumes / breath freshener / lotions industries etc. Plant material was collected from two locations viz., (i) district Astore (considered as low altitude approximately; 2500 (m.a.s.l.) and (ii) Deosai plateau (considered as high altitude; approximately 4000 (m.a.s.l.). Morphological, biochemical and DNA based markers were used for the first time to estimate genetic diversity in the two species. Two species collected from low and high altitudes showed significant differences for the morphological characters such as plant height, number of branches, canopy area and seed weight.

Total seed protein concentration was estimated using UV spectrophotometry. Average seed protein in the two species ranged from 5.59 to 13.05%. Seed protein banding pattern was studied using SDS-PAGE. Seeds of *Carum carvi* collected from high altitude showed an extra band which could be used as species specific protein band in furhter charactrization and better understanding of txonomic classification of the species. Twenty two Randomly Amplified Polymorphic DNA primers were used for the estimation of genetic distances among the two species. Minimum genetic distance among *Bunium persicum* and *Carum carvi* estimated was 34% (GLD03 and GLK03) while maximum genetic distance among the two species was 100% revealed by 9 RAPD primers viz., GL -A02, -B06, -C01, -D04, -E02, -G07, H09, -K04 and -K09. On an average the two species from the family Apiaceae showed 81% genetic distance.

Key words: *Bunium persicum, Carum carvi*, Morphological characters, Protein concentration, SDS-PAGE, RAPD, PCR, Genetic distance.

Introduction

Pakistan with a wide variety of climates has an abundance of thousands of species of medicinal importance growing widely in the forests, deserts, roadsides and banks of rivers. Over 50% of the population in Pakistan is being cured using traditional medicines by more than 40,000 traditional herbal practitioners (Aslam, 2002; Shinwari, 2010; Shinwari & Qaiser, 2011). Therefore, the rich and varied diversity of medicinal and aromatic plants is one of Pakistan's important strengths and provides the basis for future bio industrial developments in the country (Yousuf *et al*., 2006).

Mountain areas of Gilgit Baltistan (GB) is situated between 710 and 750 east longitude and 320 and 370 north latitude, stretched over an area of 28,000 square miles (Rasool, 1998) and is especially rich in medicinal plant species. Medicinally important genera of GB *include Bunium* and *Carum*. The genera are widely distributed across Asia, Europe and North Africa. *Bunium persicum* also known as "Black Cumin" (black zeera) is one of the economically and medicinally important plant species. Black cumin essential oil is used in pharmaceutical, food sweetening, soft drink, food and hygiene industries. Ripe black cumin fruits contain an essential oil rich in monoterpene aldehydes Cuminaldehyde, p-mentha-1, 3-dien-7-al and p-mentha-1,4-dien-7-al; terpene hydrocarbons are the main components of fruits collected in the wild or harvested unripe. *Carum carvi (Syn.* Caraway, Persian cumin, commonly called "zeera") is a biennial plant in the genus *Carum* of family Apiaceae. It is native to western Asia, Europe, and North Africa. The plant is similar to other members of the carrot family. Main flower stem is 40–60 cm tall, with small white or pink flowers. Caraway fruits/seeds are crescent-shaped 2 mm long. Seeds may be used whole or crushed. Seeds of *Carum carvi* are commonly used as spice, desserts, liquors, casseroles, rice dishes etc. Some time roots of *Carum carvi* are cooked as vegetable. Leaves of *Carum carvi* are used as medicine. Seeds of *Bunium persicum* and *carum carvi* when crushed are highly aromatic. The flavor is similarly pine-like, astringent, and bitter. Average seed yield of *Bunium persicum* and *Carum carvi* ranges from 230-250 Kg/ha (Sofi *et al*., 2009).

It is essential to take measures for improvement and conservation of these medicinally important species from GB. Prior to make conservation strategy, it is pre requisite to estimate existing genetic diversity in the local material (Yousuf *et al*., 2008). To estimate genetic diversity, previously morphological, cytological and/or biochemical markers were commonly used (Jan *et al*., 2011). But these markers are generally less in number and often influenced by environment so these markers are not considered very suitable for estimation of genetic diversity studies. Recently DNA based markers have been developed which are unlimited in number and are not influenced by environment and hence are considered best available marker system. A number of molecular markers including RFLP (Restriction Fragment Length Polymorphism), PCR (Polymerase Chain Reaction), ASA (Allele Specific Amplification), SSR (Simple Sequence

Repeat), STS (Sequence Tag Site), and SNP (Single Nucleotide Polymorphism) are in common use for the estimation of genetic diversity studies in various plants/animal species (Mago *et al*., 2011; Akbar *et al*., 2012). Among these assays, PCR based assays are easier, cheaper and can be performed in shorter time. Hence PCR based assays are getting more attention for the estimation of genetic diversity all over the world (Sumikova & Heanzalova, 2010; Sultan *et al*., 2013). So far not much work has been documented in Pakistan regarding molecular characterization of *Bunium persicum* and *Carum carvi.* Present research has been formulated for the first time to estimate genetic diversity in *Bunium persicum* and *Carum carvi* in Gilgit Baltistan. Present study was undertaken to characterize *Bunium persicum* and *Carum carvi* collected from low and high altitudes of district Astore, GB using morphological characters. Genetic diversity was estimated using RAPD primers

Materials and Methods

Plants of two species viz., *Bunium persicum* and *Carum carvi* were collected from 2 locations viz., village Hercho, district Astore (considered as low altitude approximately 2500 m.a.s.l.) and Deosai (considered as high altitude, approximately 4000 m.a.s.l.). Five plants per location were collected and average of 5 plants was used for statistical analyses. Morphological data were recorded for plant height (in cm), number of branches, canopy area (in cm) and 1000 seed weight (in g).

Total crude protein was extracted from seeds of two species using protocol described by Yeoh & Wong (1993). Crude seed protein concentration was estimated using UV spectrophotometer (Grimsley & Pace, 2003). Crude seed protein was also separated using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoreses (SDS-PAGE). Modified CTAB based protocol of Doyle & Doyle (1990) was used for isolation of total genomic DNA from seeds of *Bunium persicum* and *Carum carvi*. The quality and quantity of the DNA was checked on 1% agarose/TBE gel. Gel was observed under UV light using "UVitech" Gel Documentation System. Twenty three RAPD primers were used to estimate genetic diversity in *Bunium persicum* and *Carum carvi.*

Basic Statistical analysis (including mean, median, minimum, maximum, standard deviation, standard error and coefficient of variation) were carried out using computer program PAST (PAleontological Statistics, Hammer, 2016) version 3.11. Only reliably scoreable DNA fragments were included in the analyses, faint bands were not included to estimate genetic diversity. Genetic diversities among all the possible comparisons were estimated using unweighted pair group of arithmetic mean (UPGMA) procedure

$$GD=1-dxy/dx+dy-dxy$$

Where GD = Genetic diversity, dxy=number of common DNA fragments in two samples, dx=number of total DNA fragments in sample number 1, dy=number of total DNA fragments in sample number 2 (Nei & Li, 1979).

Results and Discussion

Significance test for the morphological characters studied during present research was performed using F-statistics (Table 1). Differences in plant height between *Carum* and *Bunium* genera (data from 2 altitudes was pooled for this analysis) was highly significantly different (F and p values were 147.5 and 2.1E-10, respectively). Number of branches in *Carum carvi* was significantly different for the samples collected from high and low altitudes. While number of branches in *Bunium persicum* were highly significantly different for the samples collected from high and low altitudes. Statistically, difference between numbers of branches in the two genera (pooled data over altitudes) was also highly significant. Canopy area was highly significantly different in samples of *Carum* and *Bunium* collected from low and high altitudes. When pooled data was used for comparing two genera, statistically f value (1.125) was not significantly different (p value 0.302). 1000 seed weight for *Carum carvi* collected from high and low altitudes were 1.8 g and 1.4 g. Corresponding values for *Bunium persicum* were 1.08 and 1.5 g, respectively. Average 1000 seed weight was subjected to t test for statistical significance. t-value was 10.04 and p value was 0.002 indicating that 1000 seed weight in 2 genera collected from 2 altitudes was highly significantly different. Present findings strengthened previous finding of Majeed *et al*. (2009) who reported that in *Bunium persicum*, highly significant differences for morphological characters exist. They also concluded that morphological data they reported further strengthened genetic diversity estimates using RAPD markers.

Crude protein was extracted from seeds of *Carum carvi* and *Bunium persicum* collected from high and low altitudes. Seed Protein concentration was estimated using UV spectrophotometer. (Grimslay & Pace, 2003). Crude seed protein values for *Carum carvi* and *Bunium persicum* collected from high and low altitudes were 6.85, 6.2, 5.59 and 13.05%, respectively (Table 2). Maximum seed protein (13.05%) was estimated in *Bunium persicum* collected from low altitude. Crude seed protein percentages were highly significantly different (t value=4.58, p value=0.01).

Crude seed protein extracted from *Bunium persicum* and *Carum carvi* were separated using SDS-PAGE. SDS-PAGE banding pattern of the two species showed that seed protein profile of *Bunium persicum* collected from high altitude (lane 1, Fig. 1) and low altitude (lane 3) was same. While in *Carum carvi* collected from high altitude (lane 2) showed an extra protein band as comapred to *Carum carvi* collected from low altitude (lane 4).

Table 1. Statistical significance for morphological characters studied during present research.

	Plant height *Carum* high *Carum* low	**Plant height *Bunium* high vs *Bunium* low**	**Plant ht *Carum* vs *Bunium***	**Branches *Carum* high vs *Carum* low**	**Branches *Bunium* high vs *Bunium* low**	**Branches *Carum* vs *Bunium***	**Canopy area *Carum* high vs *Carum***	**Canopy area *Bunium* high vs *Carum***	**Canopy area *Carum* vs *Bunium***
F value	6.84^{*}	86.6^{**}	147.5^{**}	6.97^{*}	84.7^{**}	36.25^{**}	32.11^{**}	42.21^{**}	1.125^{ns}
P value	0.02	6.5E-06	2.1E-10	0.02	7.1E-6	8.6E-05	0.0003	0.0001	0.302

Table 2. Seed protein concentration in *Bunium persicum* and *Carum carvi* collected from low and high altitudes.

Sample	Seed crude protein %
Carum carvi high altitude	6.85
Carum carvi low altitude	6.2
Bunium persicum high altitude	5.59
Bunium persicum low altitude	13.05
t value	4.58**
p value	0.01

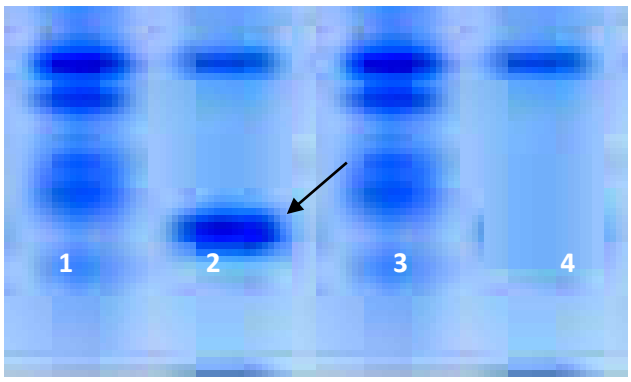

Fig. 1. SDS-PAGE profile of *Bunium persicum* collected from high altitude (lane 1) *Carum carv*i collected from high altitude (lane 2) B*unium persicum* collected from low altitude (lane 3), *Carum carvi* collected from low altitude (lane 4).

Table 3. Genetic distances among *Bunium persicum* and *Carum carvi* estimated using 22 RAPD primers.

S. No.	Name of RAPD	Genetic distance
1.	GLA-02	100
2.	GLA-06	86
3.	GLA-07	67
4.	GLB-02	100
5.	GLB-06	100
6.	GLB-08	50
7.	GLC-09	100
8.	GLC-12	67
9.	GLD-03	34
10.	GLD-04	100
11.	GLD-09	84
12.	GLE-02	100
13.	GLE-10	82
14.	GLG-01	100
15.	GLG-07	100
16.	GLG-09	66
17.	GLH-03	83
18.	GLH-08	57
19.	GLH-09	100
20.	GLI-02	52
21.	GLK-03	34
22.	GLK-04	100
23.	GLK-05	100
Average		**81%**

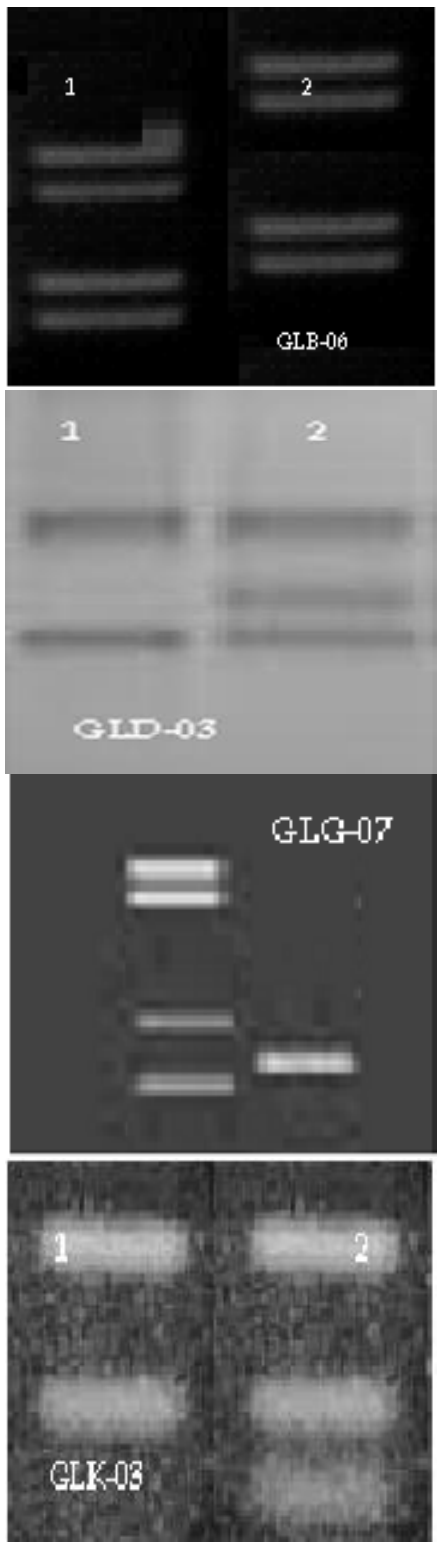

Fig. 2. Few examples of PCR amplification of total genomic DNA isolated from seeds *of Bunium persicum* (lane 1) and *Carum carvi* (lane 2) Using RAPD primers.

Few examples of PCR amplification are presented in Fig. 2. Genetic distances were estimated using UPGMA procedure of Nei & Li (1979). Only reliably scoreable DNA fragments were included in the analyses. DNA fragments were scored as present (1) or absent (0). Genetic distances between *Bunium persicum* and *Carum carvi* are presented in Table 3. Genetic distances among two species

ranged from 34–100% with an average of 81% genetic distance between the two species. Although some common DNA fragments were amplified in the two species (for example DNA fragments 1 and 2 using GLK-03 and DNA fragments 1 and 3 using GLD-03) there was no RAPD prime which showed complete homozygosity between the two species. Eleven RAPD primers viz., GLA-02, GLB-02, GLB-06, GLC-09, GLD-04, GLE-02, GLG-01, GLG-07, GLH-09, GLK-04 and GLK-05 showed 100% genetic distance between *Bunium persicum* and *Carum carvi*.

Recently using DNA sequence analysis, the two species have been reclassified in genera *Bunium* and *Carum* (Zakharova *et al*., 2014). Plants are usually biennial / perennial in nature. Oil extracted from seeds of *Bunium* and *Carum* is rich in monoterpene aldehydes Cuminaldehyde, p-mentha-1,3-dien-7-al and p-mentha-1,4-dien-7-al; terpene hydrocarbons. Plant extracts especially oil extracted from the species is used in pharmaceutical, food sweetening, soft drink, food and hygiene industries, spice, desserts, liquors, casseroles, rice dishes etc (Bashir *et al*., 2014; Shinwari *et al*., 2014). Locally leaves of *Bunium* and *Carum* are also used as herbs by indigenous communities (Sofi, 2009). Worldwide these species are distributed across Asia, Europe and North Africa. In GB these species are found across a wide range of environmental conditions ranging in altitude from approximately 2500 meter above sea level to 4000 meter above sea level (Deosai Plateau which is second highest plateau after Tibetan plateau, Anon., 2016).

Average seed yield of *Bunium persicum* and *Carum carvi* ranges from 230-250 Kg/ha (Sofi *et al*., 2009). Giving due consideration to medicinal / economic importance of *Bunium persicum* and *Carum carvi*, recently lots of research has been conducted from all over the world on utilization of DNA based technology for characterization / improvement of quality and quantity of *Bunium persicum* and *Carum carvi* (Łożykowska *et al*., 2014) . Various researchers have used different DNA based techniques including Restricted Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and Polymerase Chain Reaction (PCR) etc for the estimation of genetic diversity in the local materials of *Bunium persicum* and *Carum carvi* (Plunkett *et al*., 2004; Majeed *et al*., 2009). Unfortunately not much work has been documented on utilization of DNA technology for the characterization / improvement of *Bunium persicum* and *Carum carvi* in Pakistan especially Gilgit-Baltistan. For conservation and management of species like *Bunium persicum* and *Carum carvi*, it is a prerequisite to estimate existing genetic diversity in the material obtained from local resources. Present research is therefore first documented attempt to estimate genetic diversity of *Bunium persicum* and *Carum carvi* using morphological, biochemical and DNA based markers. Plants of two species viz., *Bunium persicum* and *Carum carvi* were collected from 2 locations viz., village Hercho, district Astore (considered as low altitude approximately 2500 m.a.s.l.) and Deosai (considered as high altitude, approximately 4000 m.a.s.l.). The two species *Bunium persicum* and *Carum carvi* collected from low and high altitudes were significantly / highly significantly different for the morphological characters studied viz., plant height, number of branches, canopy area and seed weight except canopy area where species showed non-significant differences. This indicated presence of sufficient genetic differences among the two species collected from various altitudes. Concentration of seed storage protein was estimated using UV spectrophotometry. Maximum crude seed protein (13.05%) was estimated in *Bunium persicum* collected from low altitude while minimum seed protein concentration was observed for *Bunium persicum* collected from high altitude. Crude seed protein percentages were highly significantly different in the 4 samples studied (t value=4.58, p value=0.01). Crude seed protein extracted from *Bunium persicum* and *Carum carvi* were separated using SDS-PAGE. Seed protein profile of *Bunium persicum* collected from low and high altitude were same. While in *Carum carvi* collected from high altitude showed an extra protein band as comapred to *Carum carvi* collected from low altitude. The extra protein band of relatively lower molecular weight (arrowed in Fig. 1) observed in *Carum carvi* colleceted from high altitude strengthens a previous report of Dias *et al*. (2008) who using DNA based markers reported altitudinal variation in clover (genus *Trifolium*). They separated various populations (they studied) collected from low altitude from those populations collected from high altitudes. It is suggested that this extra protein band in *Carum carvi* (collected from high altitude) should be studied in detail for better understanding of proteomics in genus *Carum* and ultimately family Apiaceae.

For estimation of genetic diversity, total genomic DNA isolated from seeds of *Bunium persicum* and *Carum carvi* were amplified using 23 RAPD primers. Genetic distances among two species ranged from 34–100% with an average of 81% genetic distance between the two species. Although some common DNA fragments were amplified in the two species (for example DNA fragments 1 and 2 using GLK-03 and DNA fragments 1 and 3 using GLD-03) there was no RAPD primer which showed complete homozygosity between the two species. Eleven RAPD primers viz., GLA-02, GLB-02, GLB-06, GLC-09, GLD-04, GLE-02, GLG-01, GLG-07, GLH-09, GLK-04 and GLK-05 showed 100% genetic distance between *Bunium persicum* and *Carum carvi*. Present research demonstrated that RAPD markers can successfully be used for the estimation of genetic diversity in *Bunium persicum* and *Carum carvi*. Therefore it is suggested that cheaper, easier and user friendly technique of PCR using RAPD primers should be utilized at larger scale for the estimation of important plant species especially those endangered in Gilgit-Baltistan due to uncontrolled harvesting for medicinal purposes. This will help in formulating better strategies for the conservation / management /improvement of various economically important plant species of the area and local communities.

References

Akbar, F., N. Yousaf, M.A. Rabbani, Z.K. Shinwari and M.S. Masood. 2012. Study of total seed proteins pattern of sesame (*Sesamum indicum* L.) landraces via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *Pak. J. Bot*., 44(6): 2009-2014.

Anonymous. 2016. *Deosai, National Park. "Deosai National Park 2nd Highest Plateau in the World". www.skardu.pk. Skardu.pk. Retrieved 23 Nov*.

Aslam, A. 2002. Conservation, cultivation and trade of medicinal herbs and spices in Pakistan. Paper presented in International workshop on Health Challenges of 21st Century and Traditional Medicines in SAARC Region November 4-6. 2002 Islamabad, Pakistan.

Bashir, I., Z.A. Kaloo, S. Singh and S. Rashid. 2014. Chemical composition & medicinal importance of *Bunium persicum* (Boiss.) B.fedtsch. – A review . *International Journal of Advanced Research*, 2(7): 244-247.

Dias, P.M.B., V.F. Pretz, M.D. Agnol, M.T. Schifino-Wittmann and J.A. Zuanazzi. 2008. Analysis of genetic diversity in the core collection of red clover (*Trifolium pratense*) with isozyme and RAPD markers. *Crop Breed. & Appl. Biotech*., 8: 202-211.

Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus*, 12: 13-15.

Grimsley, G.R. and C.K. Pace. 2003. Spectrophotometric determination of protein concentration. Current protocols in protein science. 3.1.1-3.1.9. John Wiley and Sons, Inc, USA. http://onlinelibrary.wiley.com/book/10.1002/0471140864/homepage/Archive.html

Hammer, Q. 2016. Natural History Museum. University of Oslo. http://folk.uio.no/ohammer/past/. Retrieved on March, 17, 2016.

Jan, H.U., M.A. Rabbani and Z.K. Shinwari. 2011. Assessment of genetic diversity of indigenous turmeric (*Curcuma longa* L.) germplasm from Pakistan using RAPD markers. *J. Med. Pl. Res*., 5(5): 823-830.

Łożykowska, K.S., A. Kuczyńska, K. Mikołajczyk, J. Nowakowska and J. Bocianowski. 2014. *Acta Sci. Agron*., 36(2):

Mago, R., H. Simkova, G. Brown-Guedira, S. Dreisigacker, J. Breen, Y. Jin, R. Singh, R. Appels, E.S. Lagudah, J. Ellis, J. Dolezel. and W. Spielmeyer. 2011. An accurate DNA .marker assay for stem rust resistance gene *Sr2* in wheat. *Theor. Appl. Genet.*, 122(4): 735-744.

Majeed, S., M. Sayyed, R. Kaur, M. Gupta, A. Kashyap and D.R. Sharma. 2009. Molecular analysis of genetic diversity in wild accessions of *Bunium persicum*: A critically endangered medicinal plant of temperate Himalaya. *The Asian and Aust. J. Pl. Sci. & Biotech*., 3(1): 7-10.

Nei, M. and W.H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proc. of the Nat. Acad. of Sci. USA*, 76(10): 5269-5273.

Plunkett, G.M., G.T. Chandler, P.P. Lowry, S.M. Pinney and T.S. Sprenkle. 2004. Recent advances in understanding Apiales and a revised classification. *South Afr. J. Bot*., 70(3): 371-381.

Rasool, G. 1998. Medicinal Plants of the Northern Areas of Pakistan: Saving the plants that save us. Gilgit, Pakistan.

Shinwari, Z.K. 2010. Medicinal Plants Research in Pakistan. *J. Med. Pl. Res*., 4(3): 161-176.

Shinwari, Z.K. and M. Qaisar. 2011. Efforts on conservation and sustainable use of medicinal plants of Pakistan. *Pak. J. Bot*., 43(SI): 5-10.

Shinwari, Z.K., H. Rehman and M.A. Rabbani. 2014. SDS-Page based genetic divergence in safflower (*Carthamus tinctorius* L.). *Pak. J. Bot*., 46(3): 811-815.

Sofi, P.A., N.A. Zeerak and P. Singh. 2009. Kala zeera (*Bunium persicum* Bioss.): A Kashmirian high value crop. *Turk J. Biol*., 33: 249-258.

Sultan, M., N. Zakir, M.A. Rabbani, Z.K. Shinwari and M.S. Masood. 2013. Genetic diversity of guar (*Cyamopsis tetragonoloba* L.) landraces from Pakistan based on RAPD markers. *Pak. J. Bot*., 45(3): 865-870.

Sumikova, T. and A. Heanzalova. 2010. Multiplex PCR assay to detect rust resistance genes *Lr26* and *Lr37* in wheat. *Czech J. Genet. Pl. Breed*., 46(2): 85-89.

Yeoh, H.H. and P.F.M. Wong. 1993. Nutritative value of leaf protein concentrates from some tropical plants. *J. Singapore Academy. Sci*.,

Yousaf, Z., S. Masood, Z.K. Shinwari, M.A. Khan and A. Rabani. 2008. Evaluation of taxonomic status of medicinal species of the genus *Hyoscyamous, Withania, Atropa* and *Datura* based on poly acrylamide gel electrophoresis. *Pak. J. Bot*., 40(6): 2289-2297.

Yousuf, Z., S. Masood, Z.K. Shinwari, M.A. Khan and A. Rabbani. 2006. Evaluation of taxonomic status of medicinal species of the genus *Solanum* and *Capsicum* based on poly acrylamide gel electrophoresis. *Pak. J. Bot*., 38(1): 99-106.

Zakharova, E.A., G.V. Degtjareva, E.V. Kljuykov and P.M. Tilney. 2014. The taxonomic affinity of *Carum piovanii* Chiov. and some *Bunium* species (Apiaceae). *South Afric. J. Bot*., 94: 122-128.

(Received for publication 15 December 2016)