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SPECIES DIVERGENCE AND DIVERSITY IN BUCKWHEAT LANDRACES COLLECTED FROM THE WESTERN HIMALAYAN REGION OF PAKISTAN

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Abstract

Attaining food security in subsistence farming system of Himalayan region require novel and original approaches, including improvement of traditional crops, like buckwheat. Little is known about the diversity in local landraces of buckwheat species *Fagopyrum esculentum* (common type) and *F. tataricum* (Tatary type). The present study was thus designed to assess the diversity and divergence in buckwheat germplasm from western Himalayan region of Pakistan. A set of 36 buckwheat landraces collected from the Himalayan ranges of Pakistan were characterized using 20 microsatellite markers, along with the two reference Chinese genotypes. The microsatellite data was analyzed to infer on the divergence between species and diversity in geographically spaced buckwheat landraces. A clear divergence was found between the two buckwheat species (F_{ST} value = 0.331), suggesting their cross incompatibility. The overall diversity was very high, with a genotypic diversity of 1.00 in common type and that of 0.983 in the Tatary type. Buckwheat germplasm showed variable number of alleles per locus, while the dissimilarity in the detected and predicted heterozygosity exposed nonexistence of some inbreeding or clonality in the two species. The higher diversity and information on divergence between species must enable a better crop breeding strategy to select buckwheat genotypes with more production in a subsistence farming system along with further conservation strategy.

Key words: Buckwheat, Traditional crops, Microsatellite, Germplasm resources, Population genetics.

Introduction

Food availability in subsistence farming communities are normally poor and their crop produce is based on diversity in crops and its types (Mathuijs, Noev, 2004). Considering diversification of crops, many underutilized traditional crops enable a better food supply and long term food security in subsistence farming (Prescott-Allen & Prescott-Allen, 1990). Buckwheat is one of these crops, which is least exploited in Pakistan with marvelous health and dietary values (Jiang *et al.*, 2007). Buckwheat (*Fagopyrum* Moench) is the genus of Polygonaceae family, its species are sown as minor, additional or profitable crop in numerous republics, often in subsistence farming (Campbell, 2004). The crop has a high nutritional quality with a potential to fulfill the dietary requirement for proteins, micronutrient minerals (particularly iron), vitamin B, potassium, phosphorus, dietary fiber andvitamin P, that encloses the flavonoid rutin (Eggum *et al.*, 1980). It could be used for human food, animal or poultry feed and to extract rutin for medicinal purposes. Rutin, first discovered in buckwheat in 19th century (Jiang *et al.*, 2007), can effectively reduce thecholesterol level in the blood and strengthen the capillaries and arteries, and elastic (Ihmel *et al.*, 2003; Jiang *et al.*, 2007; Kreft *et al.*, 2006). Ecologically, nectar of buck wheat is utilized by honey bee to produce good quality honey. Thus buckwheat represents an important candidate traditional crop to be exploited both in subsistence and commercialized cropping systems, both worldwide and in Pakistan.

In Pakistan, buckwheat is considered to be a primitive, however, a staple crop in the Himalayan region, particularly Gilgit-Baltistan province. However, its farming and consumption in Pakistan is still partial and scarce. It was thought to be introduced from Tibet via Ladakh through local traders and explorer into Pakistan (Ohnishi, 1993). Two buckwheat species i.e. *F. esculentum* (common type) and *F. tataricum* (Tatary type) are grown for food in the Himalayan regions of Pakistan (Tsuji, Ohnishi, 2000). Despite the vital role of buckwheat in the food security of Himalayan region, the cultivation of buckwheat is on continuous decline, mainly due to lack of improved lines/types to acquire substantial yields (Adachi, 2004; Campbell, 2003). Little efforts have been made for genetic improvement of buckwheat to exploit its actual potential.

The first step of genetic improvement rely on the assessment of genetic diversity in buckwheat, to be exploited in further breeding programs through creation of genetic variation among segregating progeny (Campbell, 2003; Moose & Mumm, 2008; Zamir, 2001). Characterization of diversity in landraces could provide diverse parental sources for a variety of traits (Kump & Javornik, 2002; Zamir, 2001) that would serve as basis for genetic improvement in Buckwheat breeding programs (Facho *et al*., 2016), while their analyses for population genetic structure could shed light on the conservation status and evolution of crop species (Tsuji & Ohnishi, 2000). Several molecular techniques are available for detecting genetic variation of crop plants, out of which simple sequence repeat (SSR)/microsatellites provide an efficient, rapid and cost-effective measure to detect the range of diversity (Adreit *et al.*, 2007; Ali *et al.*, 2011; Ali *et al.*, 2014). Little is known about the diversity in Pakistani Himalayan buckwheat germplasm with less information about its population structure and evolution. Although the Chinese origin has been proposed to the Tatary buckwheat, using Chinese landraces (Tsuji, Ohnishi, 2000), the origin of Pakistani landraces is also unknown. Buckwheat

provides food security to traditional poor resource farmers under subsistence farming but its cultivation and utilization in Pakistan is limited. Both Common (*Fagopyrum esculentum* L.) and Tartary buckwheat (*F. tataricum* L.) are cultivated as a summer crop in valleys of Karakoram and Hindukush, Pakistan. In Baltistan, Common buckwheat is called "jawas", while Tartary buckwheat is called "brow". In Balti language Tartary buckwheat is also known as Kho-brow (bitter), Common buckwheat is known as Sta-Brow / Gevas and wild one is known as Khi-Brow. In the upper Hunza area the word "Bali" is commonly used for buckwheat (Facho *et al.*, 2016).

The present study was designed to characterize Pakistani buckwheat landraces collected across various geographical regions of Himalayas, along with two Chinese Tatary buckwheat reference lines to i). Characterize the level of genetic diversity within two species across geographical regions; ii). Infer on the mode of reproduction in the geographically spaced populations and the two species; and iii). Assess the relationship of Pakistani buckwheat landraces with the previously characterized Tatary buckwheat germplasm.

Materials and Methods

Climatic condition required for Buckwheat: Buckwheat is vulnerable to frost and heat. It has high variation in yield, number of flowers, seed set, lodging and phenological phases. It can ideally be grown in temperature range of 13°C to 26°C with a longer morning period having 18°C to 21°C desirable for the pollination and fertilization. Buckwheat is sensitive to frost, high speed wind and drought. The climatic conditions of GilgitBaltistan are cool and moist which make it suitable for growing buckwheat successfully.

Specimen of buckwheat germplasm: Buckwheat germplasm from the Himalaya and Karakorum, Pakistan was categorized using microsatellite markers. A set of 36 (21 from *F. esculentum*and 15 from *F. tataricum*species) landraces were collected from seven locations of GilgitBaltistan (Fig. 1) genotyped and analyzed for population genetic structure along with two previously characterized "Tatary" genotypes from China (Table 1).

Molecular genotyping of buckwheat: All of these 36 landraces were planted in CIB, Chinese Acad. Sci. China, to extract DNA for molecular studies. Seeds were sown in pots in controlled environment under lab conditions. From each accession, two leaves were harvested. Each leaf was split into two sections, from one section the DNA was extracted, while the remaining half section was stored at -4°C. Genomic DNA was extracted through altered CTAB method (Innan *et al.*, 1995). The extracted DNA was stored at -4°C.A set of 20 SSRs (Konishi *et al.*, 2006) were used for molecular genotyping (Table S1), with calibration for all markers (Table S2). After amplification, the amplified alleles were scored through gel electrophoresis (Chalhoub *et al.*, 1997). The allele scoring was made through visual reading of presence of different alleles in the respective amplicon fragment size region.

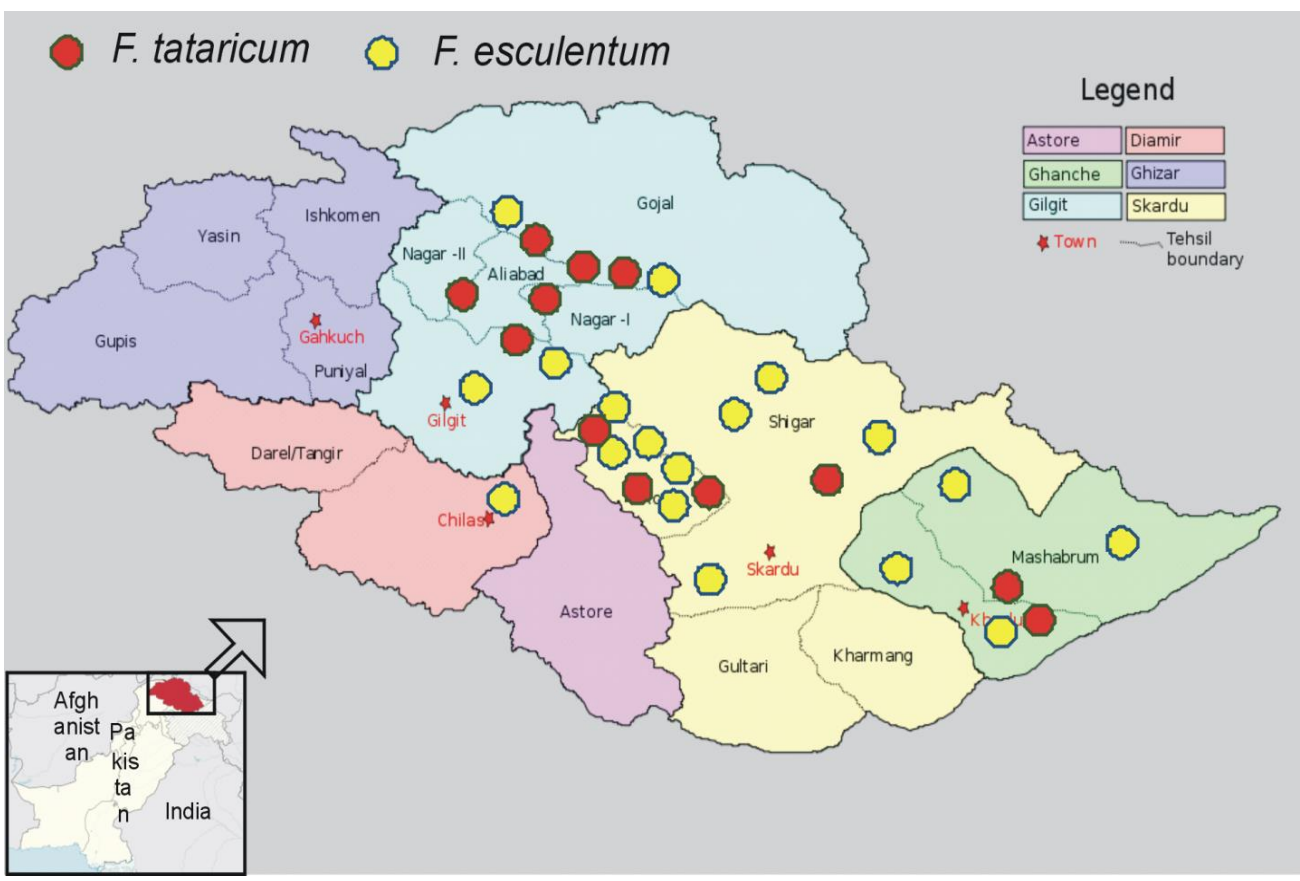

Fig. 1. Sampling locations for the germplasm of buckwheat collected from the Gilgit-Baltistan province of Pakistan.

Table 1. Detail of the germplasm of *F. esculentum and F. tartaricum* collected from different locations of Pakistan, for analysis of genetic diversity based on SSR molecular markers.

Species	District	Collection site	Sampling point	Accession no.*
Common (*Fagopyrum esculentum*)	Ghanche	Balghar	Gh-917	FEG1493
		Haricon	Gh-916	FEG1492
		Tallis	Gh-918	FEG1494
		Thalay	Gh-911	FEG1491
	Gilgit	Gilgit	Gt-589	589
		Chilas	Gt-21081	21081
		Gilgit	Gt-3732	3732
		Skardu	Sk-3723	3723
		Gilgit	Gt-3716	3716
	Skardu	Baghicha	Rd-910	FES1495
		Baha	Rd-919	FEG1498
		Daso C	Rd-920	FEG1499
		Stak	Rd-912	FES1496
		Tormik	Rd-915	FEG1497
		DasoShiger	Sh-908	FEG14910
		Tistey	Sh-914	FEG14912
		Zill	Sh-913	FEG14911
		Manthal	Sh-909	FEG14913
	Reference	KP	Kp-3726	3726
		Islamabad	Pb-3728	3728
Tartary (*Fagopyrum tataricum*)	Ghanche	Ghomo	Gh-905	FTG14915
		Saltoro	Gh-901	FTG14914
	Gilgit	Gilgit	Gt-593	593
		Gilgit	Gt-902	FTGL14916
		Gilgit	Gt-3722	3722
		Gilgit	Gt-1172	1172
		Gilgit	Gt-20727	20727
		Skardu	Sk-3724	3724
	Skardu	Bilamik	Rd-906	FTS14918
		Daso T	Rd-904	FTS14917
		Thowar	Rd-907	FTS14919
		Baha	Sh-903	FTS14920
	Reference	KP	Kp-3725	3725
		Shorkot	Pb-841	841
		China	chinese-1	SWN1
		China	chinese-2	SWN2

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[3] Chengdu Institute of Biology, Chinese Academy of Sciences
* 14 landraces were collected from PGRI, while 22 were collected from the field and submitted to the genebank

Population genetic analyses of the SSR data: Various population genetic analyses softwares were used to evaluate the SSR data, level of diversity and mode of reproduction across the two species and different topographical localities. To test the fitness of SSR loci by plotting the MLGs perceived against the number of loci used, the GENECLONE software (Arnaud-Haond & Belkhir, 2007) was utilized. The Nei genetic distance on SSR data (Nei, 1978) between landraces was calculated, which was used through NJ method to build the un-rooted phylogenetic tree, using POPULATION program (Langella, 2008). Discrepancy amongst populations and species were evaluated by FCA and approximating the F_{ST} data using software GENETIX 4.05.2 as utilized by Belkhir *et al.,* (2004). Discriminate analyses of PC was worked out with software-R utilized by Jombart *et al.,* (2010).

Alleles, linkage imbalance between loci and detected /predicted heterozygosity were assessed using software GENETIX 4.05.2 already reported by Belkhir *et al.,* (2004). Allelic richness and gene diversity was assessed through FSTAT software (Goudet, 2001). Genotypic diversity, frequency of most abundant MLG and linkage disequilibrium (rBarS) was estimated in both the species and across locations using MULTILOCUS software (Agapow & Burt, 2001). The total investigations were deduced succeeding the approach (Ali *et al.,* 2014).

Table S1. Primer sequences of 20 microsatellite markers used to characterize the diversity of *F. esculentum* and *F. tataricum* (Konishi*et al.*, 2006).

SSR Locus	Primer sequence (5_3)	Size (bp)	Ta (°C)
GB-FE-012	F: ACTGCACCCCAGAGGATT R: GCTGTATCCATGCCCGTA	188–209	58
GB-FE-014	F: AGGAGCAGAGGTGGTGGT R: CGGAGCCTCTGCAACC	198–216	59
GB-FE-043	F: TTCAGCACCTGGATGGAC R: TGTCCCCAATGTGAAAGG	186–204	58
GB-FE-169	F: CAACCCTATGCAGCGTTC R: GAGGGGAAGCTGCTTGTT	202–220	58
GB-FE-191	F: AATCAATGACCAGCACGC R: CTGATGGAGGATGCCAAA	141–220	58
GB-FE-001	F: TGAAACCCAACCATCAGG R: CGACAGTGGCTGGAGAAC	268–274	58
GB-FE-035a	F: TGCAATGACTTGGAGGAGA R: ACCACCATTCAACAAGCG	222–404	58
GB-FE-054a	F: TGTTGGACTTCCTAGACCTG R: CATGAAAAGGGGATGCAA	227–247	58
GB-FE-055a	F: CTGCTTGGATCCCATTGA R: AGCCTCTCGATCCCTCTG	256–259	58
GB-FE-080	F: CGAGGTGGGCAGTAGAGA R: GAGGAGGACGAGGAGGTG	231–279	59
Fes 1094	F:GAAGCCTTGGAAGAAGTGAAAT R:TAAAGCTCATCCCAATATGCAA	160	61
Fes 3108	F: GACCTTGACTTCTTTGACCG R: GTAAGCATTCTAGGCATTTG	147	58
Fes 1822	F:ATTGGAACAACTGCGCCTCA R:GGCACGAACTGGGCTCTTAG	156	57
Fes 3214	F: CGTACTCAGCAGGAGATGAC R: TTGACCGCAATTTCCAGAAT	208	59
Fes 2809	F:ATCAACACAAACAATCAAGC R:TAGAATTCCCGTCCTTAGGT	277	58
Fes 1394	F:GCCTTGGCTGCTTGACCTTT R:GATCACACGGGAGGAGAGGA	242	61
Fes 3664	F: TCACGGTCAAAGTGTTCTTCGT R: GAGGTCTTAATCTGTTGCATCC	147	58
Fes 1585	F:GCCTCCTACTAGCCCTTCCA R: AGCTGACCCTTGTGTCTCCC	169	60
Fes 4036	F:ATAACAAGCATCGTCATTGGAG R:CCAAATTGAAACCGAACTTACA	184	60
Fes 1284	F:GAATACACCATTCGGAACAAGA R:ATCATCCTCCTTTGAGTCACCT	114	61

Table S2. Reagents used for DNA extraction and PCR protocol for amplification of SSR markers detailed protocol for PCR amplification of SSR markers.

Reagent	Stock concentration	Reactional concentration	Volume (µL)
ddH_2O	-	-	15.8 µL
PCR buffer	10×	1×	2.5 µL
$MgCl_2$	15mM	1.0mM	1.5 µL
Primer-f	10µM	0.25µM	1 µL
Primer-r	10µM	0.25µM	1 µL
dNTP mix	1mM	100µM	2.0 µL
Taq	5 units	1µL	0.2 µL
Tem DNA	50ng/ µl	50ng	1 µL
Total	---	---	25.0 µL

Detail of PCR cycle

Step	Temperature	Time
Initial denaturation (1st cycle)	94 ºC	2 minutes
Denaturation	94 ºC	30 seconds
Annealing	58-61 ºC	30 seconds
Extension	72 ºC	60 seconds
Final extension (last cycle)	72 ºC	3 minutes
Conservation step	4 ºC	Till further use

Detailed protocol for gel preparation for electrophoresis

Reagents	40ml volume	60 ml volume	80 ml volume	200 ml volume
1) 6% polyacrylamide				
Urea	4.8g	7.2g	9.6g	24g
ddH_2O	6 ml	33.75ml	45ml	112.5ml
10×TBE buffer	4ml	6ml	8ml	20ml
40% Acr-Bis	6ml	9ml	12ml	30ml
2) 10% APS $(NH_4)_2SO_4$	0.4ml	0.6ml	0.8ml	2ml
3) TEMED	27µL	28µL	56µL	110µL

Gel preparation for electrophoresis

Reagents	40ml volume	60 ml volume	80 ml volume	200 ml volume
1) 6% polyacrylamide				
Urea	4.8g	7.2g	9.6g	24g
ddH_2O	6 ml	33.75ml	45 ml	112.5 ml
10×TBE buffer	4 ml	6 ml	8 ml	20 ml
40% Acr-Bis	6 ml	9 ml	12ml	30 ml
2) 10% APS $(NH_4)_2SO_4$	0.4ml	0.6 ml	0.8 ml	2ml
3)TEMED	27µL	28µL	56µL	110µL

Results

The genetic structure, population subsections of the 36 buckwheat landraces representing two species, collected from diverse geographical localities of Himalaya Pakistan, was figured out using 20 microsatellite markers. The general diversity and dissimilarity amongst the landraces was also clarified that could be exploited in future breeding programs.

Summary of SSR markers: Land races of Buckwheat were characterized using 20 microsatellite markers, resulted in a varying no. of alleles $locus^{-1}$ (Table 2), which ranged from two (for SSR loci GB-FE-014, 043, 169, 001, 054a, and GB-FE-080, Fes 1284) to seven (for the locus Fes-1585). The MLGs spotted were plotted against the number of loci re-sampled for detecting the ability of the set of microsatellite loci for identification of the genotype, by calculating the possibility of re-sampling MLG (Fig. S1). The *asymptote* touched the end discovered the adequacy of the markers to describe the landraces (Fig. S1).

Divergence and genetic diversity in two buckwheat species: A very clear and strong divergence between the two species was revealed by all the analyses used viz. through FCA, creation of phylogenetic tree and approximation of F_{ST} values. In FCA, the first axis symbolized 100% genetic inconsistency, established on the basis of species. Genotypes from two species were undoubtedly divergent from each other (Fig. 2). This divergence (amongst species) was further validated by assessing an F_{ST} value of 0.331, which was significant ($p \geq 0.000$; Table 3). The phylogenetic tree and DAPC analyses further established the difference amongst the landraces from two species (Figs. 3 & 4). The overall investigations clarified the genetic deviation among two species by splitting landraces into two clusters, clearly explained by the tree.

Table 2. Details of the 20 microsatellites markers used to genotype the buckwheat DNA samples (Konishi *et al.*, 2006).

Marker/Primer	Motif type	Total number of alleles	Size range (pb)
GB-FE-012	$(CAG)_5(CT)(CAG)$ & $(GAK)_8$	6	280-260-280
GB-FE-014	$(GA)_{10}C(GA)$	2	240-220
GB-FE-043	$(CCA)_5$	2	175-100
GB-FE-169	$(ACA)_6$	2	260-240
GB-FE-191	$(CAT)_5$	3	240-26—280
GB-FE-001	$(CAA)_7$	2	290-260
GB-FE-035a	$(GAY)_{14}$	3	180-140-180
GB-FE-054a	$(TR)_{12}$	2	140-120
GB-FE-055a	$(GAK)_6$&& $(GAT)_3$& $(GAT)_2$	3	300-350-190
GB-FE-080	$(CST)_7$	2	250-140
Fes 1094	$(AG)_{37}$	3	500-400-250
Fes 3108	$(AT)_7(TG)_{11}CT(GT)_{11}$	3	220-150
Fes 1822	$(GA)_{22}$	0	--
Fes 3214	$(GA)_{19}$	3	700-600
Fes 2809	$(CA)_{13}$	5	260-210
Fes 1394	$(CT)_{18}$	4	240-160-100
Fes 3664	$(CT)_{14}$	0	--
Fes 1585	$(GA)_7$	7	400-300-240220
Fes 4036	$(GA)_{40}GGA(G)_{10}$	3	250-180
Fes 1284	$(ATG)_4$	2	140-120

Table 3. Estimates of genetic diversity measures for the two species of buckwheat based on landraces collected from Pakistan and two reference Chinese genotypes.

	Common buckwheat	Tartary buckwheat
Number of samples	20	16
Number of MLGs	20	14
Frequency of most abundant MLG	1	2
Genotypic diversity	1.000	0.983
Gene diversity	0.491	0.374
No. of alleles per locus	2.722	2.500
Allelic richness	2.695	2.500
rBarS (probability)	0.022 (0.634)	-0.012 (0.932)
F_{ST} between the two species (significance)	0.331 (0.000)	

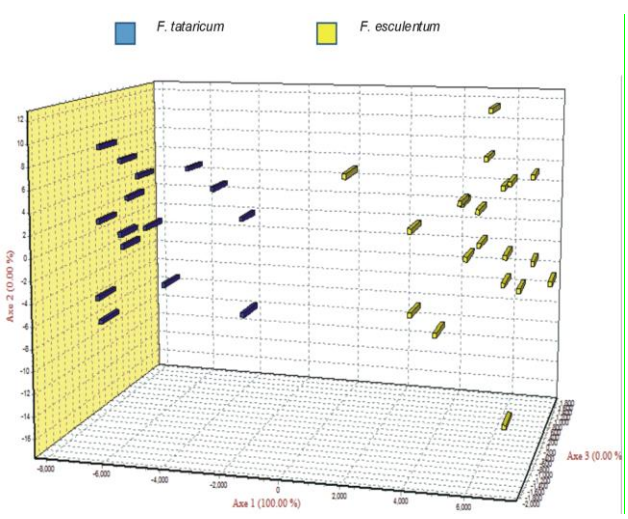

Fig. 2. Distribution of 36 Buckwheat landraces on the basis of Factorial Correspondence Analysis (FCA) of the SSR data, separating clearly the two species.

The overall high gene and genotypic diversity was revealed in common species (0.491 and 1.00, respectively) than the tartary species (0.870 and 0.374, respectively). A non-significant change in the detected / predictableheterozygosity discovered absence of any inbreeding or clonality in both species (Fig. 5; Table 3). This was further confirmed by the non-significant linkage disequilibrium observed in both common (rBarS = 0.022; p-value = 0.634) and tartaricum species (rBarS = -0.012: p-value = 0.932). Furthermore, in the overall population, 34 MLGs were detected out of the 36 samples examined, revealed a general high genotypic variation (Table 3).

Spatial structure and diversity in geographically spaced buckwheat landraces: Divergence among the geographically spaced populations was analyzed using the distribution of landraces in FCA analyses, phylogenetic tree and the discriminate analysis of principal components (DAPC) along with estimation of F_{ST}. In FCA, three axes clarified 72 % of the entire dissimilarity. Partition of

populations was not clear in the geographically spread out populaces, excluding that a very clear grouping due to species was evident (Fig. S2).

The 36 Buckwheat landraces belonging to 2 species and sampled from three localities were distributed into 5 groups i.e. G1, G2, G3, G4 and G5 (Fig. 4). The G1 enclosed eight landraces; Group 2 included 5 landraces; Group 3 also had 8 landraces; Group 4 included 7 landraces; and the Group 5 had also 7 landraces. This grouping was comparable with the results obtained on FCA (Fig. S2) and phylogenetic tree (Fig. 3), where despite the major 2 groupings due to 2 buckwheat species, sub-groups were observed, at least within *F. tartaricum* (Fig. 3). Inspection of this grouping in relation to geographical locations revealed lack of a clear spatial structure. This was further strengthened through estimation of pair wise F_{ST} values between the 3 geographical locations within 2 species (Table 4). The F_{ST} values among the 3 locations ranged from 0.000 to 0.066 in *F. esculentum*and from 0.000 to 0.151, all non-significant (Table 4).

A high gene and genotype diversity was revealed in the observed buckwheat germplasm when analyzed according to their geographical location within the 2 species (Table 5). A non-difference in observed and expected heterozygosity and a non-significant linkage disequilibrium (rBarS) was evident across all locations (Table 5 and Fig. 5), showing the absence of any clonal or inbreeding reproduction in these populations. The genotypic diversity was the maximum at all locations (1.00) with none of the MLG repeated more than once (Table 5). The allelic richness was the maximum at Gilgit for *F. esculentum* (2.015), while it was the minimum at Ganche for *F. tartaricum* (1.500).

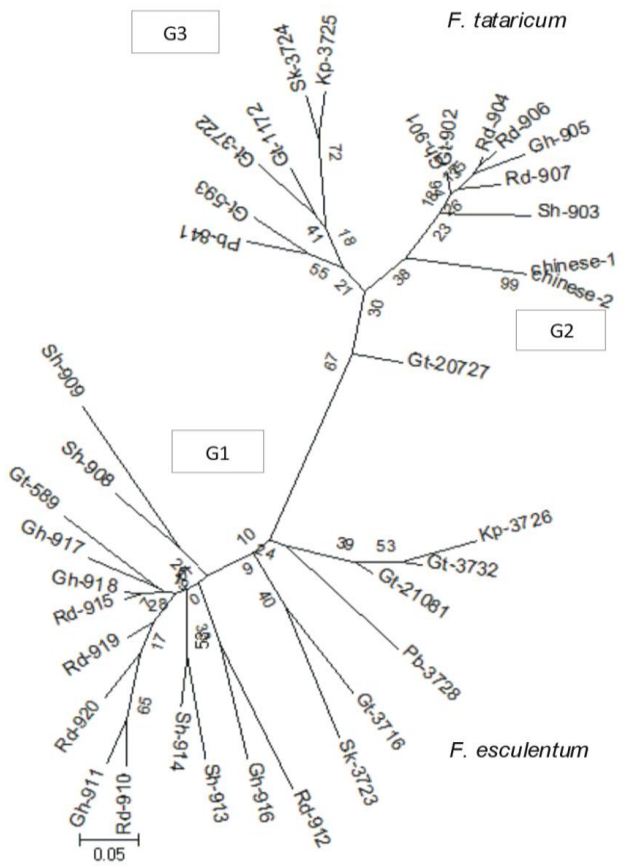

Fig. 3. Phylogenetic tree constructed for 36 landraces (comprising of 33 multilocus genotypes) of buckwheat germplasm from the two species, *Fagopyrum esculentum* and *F. tataricum.*

Table 4. Genetic differentiation (*F_{ST}*; in the upper diagonal) and its significance (in the lower diagonal) among different geographically spaced buckwheat populations representing the two species.

Species		Common type				Tartary type			
	Location	Ghanche	Gilgit	Skardu	Reference	Ghanche	Gilgit	Skardu	Reference
Common type	Ghanche	-	0.066	0.000	0.124	0.427	0.352	0.452	0.396
	Gilgit	0.071	-	0.061	0.000	0.329	0.263	0.380	0.298
	Skardu	0.571	0.023	-	0.088	0.384	0.334	0.411	0.373
	Reference	0.000	0.409	0.023	-	0.347	0.272	0.431	0.290
Tartary type	Ghanche	0.000	0.000	0.000	0.000	-	0.091	0.000	0.078
	Gilgit	0.000	0.000	0.000	0.000	0.103	-	0.144	0.000
	Skardu	0.000	0.000	0.000	0.000	0.807	0.046	-	0.151
	Reference	0.000	0.000	0.000	0.000	0.136	0.758	0.024	-

Table 5. Estimates of genetic diversity measures for the two species of buckwheat based on landraces collected from Pakistan and two reference Chinese genotypes.

	Common buckwheat			Tartary buckwheat		
	Ghanche	Gilgit	Skardu	Ghanche	Gilgit	Skardu
Number of samples	4	5	9	2	6	4
Number of MLGs	4	5	9	2	6	4
Frequency of most abundant MLG	1	1	1	1	1	1
Genotypic diversity	1	1	1	1	1	1
Gene diversity	0.449	0.508	0.454	0.306	0.396	0.289
No. of alleles per locus	2.111	2.389	2.278	1.500	2.389	1.667
Allelic richness	1.884	2.015	1.860	1.500	1.818	1.551
rBarS (*p*-value)	0.297 (0.072)	-0.023 (0.870)	0.044 (0.618)	-1 (0.980)	0.028 (0.760)	0.121 (0.580)

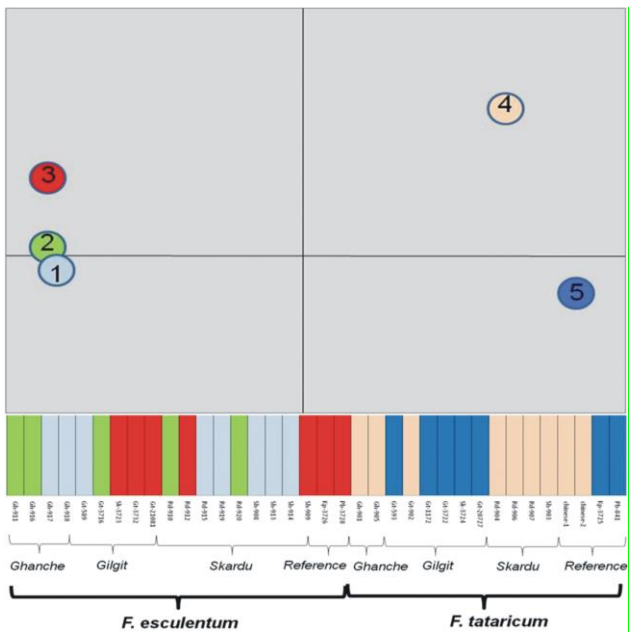

Fig. 4. Discriminate Analyses of Principle Components (DAPC) graph made with ADEGENET (R-environment), presenting the five populations for the Buckwheat samples taken from 7 locations (above) and assignment of 36 buckwheat individuals into five genetic groups following the DAPC analyses (below).

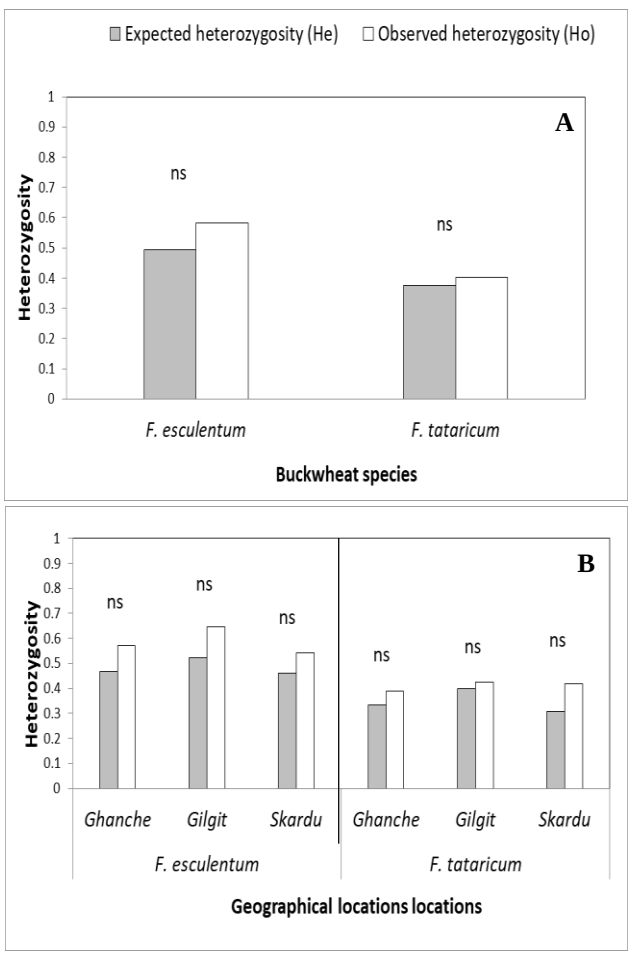

Fig. 5. Expected (He) and observed (Ho) heterozygosity for two Buckwheat species, *F. esculentum*and *F. tartaricum*, (A) and for three geographically spaced populations (B) sampled from Himalayan region of Pakistan.

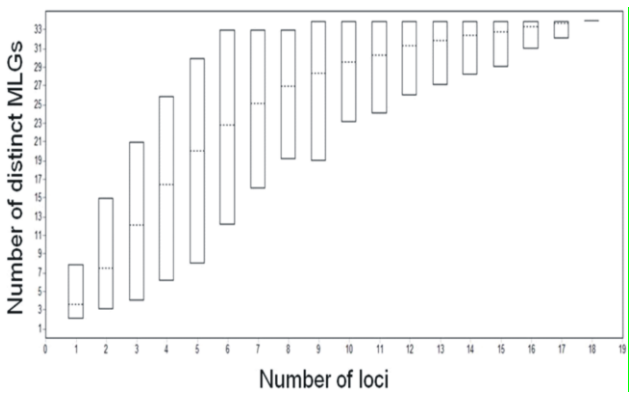

Fig S1. Number of multilocus genotypes (MLGs) detected as a function of the number of loci re-sampled within the 18 microsatellite markers.

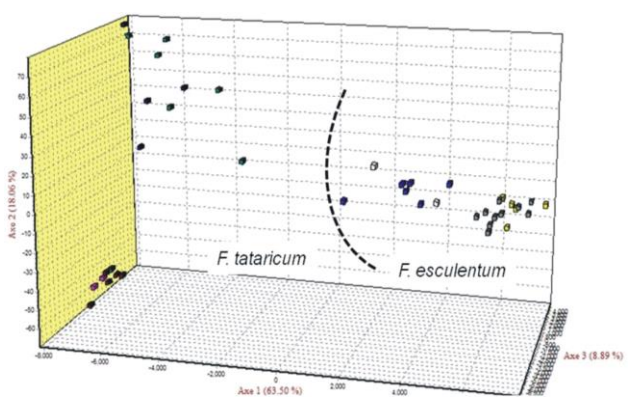

Fig. S2. Distribution of 36 Buckwheat landraces from three districts of Khyber Pakhtunkhwa and two species based on Factorial Correspondence Analysis (FCA) on 18 SSR data.

Discussion

The buckwheat germplasm was characterized for the first time from Pakistan for their species divergence and genetic diversity. Genotyping with 20 SSR markers discovered a great diversity in buckwheat germplasm collected from Himalaya with clear divergence among the two genetic groups established on the basis of 2 species of Buck wheat.

SSR markers resolution: Characterization of 36 genotypes exposed that 18 out of 20 SSRs formed perfect bands and distinguished both species using PSA, where eight or nine markers already perceived the distinct MLGs and the diversity of buckwheat landraces. These SSRs must provide enough information to assess the genetic multiplicity and PS among and within population of common and tartary buckwheat (Garima *et al.*, 2011; Iwata *et al.*, 2005).

Divergence and gene flow at the species and spatial population level: The most frequently grown buckwheat is *F. esculentum*, thus called common buckwheat, while *F. tataricum* is less common and is known as Tartary buckwheat (Adachi, 2004). A robust separation was evidenced among both species, *F. esculentum* and *F. tataricum*. The divergence between both species was

examined using thelevel of diversity showing the low gene flow between the two species. Although both species reside in sympatric at dissimilar sites, the low gene movement and robust population subdivision would suggest a free preservation of the two populations. Some cross-incompatibility may present at the origin of this low gene flow amongst members of both species (Marshall, 1969). Buck wheat is grown in small valleys in Gilgit-Baltistan with variable climatic conditions and cultural practices. Lack of specific aim for a homogenous crop in this subsistence farming region (Baiphethi & Jacobs, 2009), could contribute to high diversity in the buckwheat germplasm. In such subsistence farming, crop diversity is maintained naturally in response to biotic and abiotic stresses. In contrast to modern crops like wheat or maize with more homogeneity, diverse subsistence farming crops has less vulnerability to emergence of new crop diseases, insects and pests (Prescott-Allen & Prescott-Allen, 1990).

The population division and gene flow across ecological localities exposed absence of a clear distinction, as evaluated by FCA, DAPC, phylogenetic tree and F_{ST} approximation. Interestingly the reference isolates were not very much divergent from the Himalayan landraces. The investigation revealed that although there was less gene flow between two species, the level of gene flow across locations was the minimum. Indeed, the buckwheat pollen have been reported to spread over long distance (Adhikari, Campbell, 1998). However further study involving a larger sample size must be carried out to investigate the level of population subdivision.

Mode of reproduction and genetic diversity: Both species are very distinct and across different geographic localities. Similarly, a sexual reproduction arrangement was witnessed devoid of any proof of clonal reproduction or inbreeding in both species of buckwheat from the Himalayan region of Pakistan. *F. esculentum* is self-incompatible due to its heteromorphic behavior (Marshall, 1969) with dimorphic white, pink and red color flowers. *F. tataricum* has homomorphic, self-fertile and cleistogamous flowers that are pollinated prior to flower opening, having light green sepals (Samimy, 1991). Due to the flower structure, inbreeding within *F. esculentum*is not possible (Woo *et al.*, 1999). Due to lack of inbreeding or clonal reproduction was also evident across locations, through the sample size needed to be increased for more authentic results.

The diversity could be exploited for future crop improvement. Presently, there are no improved cultivars to be recommended to farmers, but they select their own grown seed for year to year cultivation (Adachi, 2004). Exploitation of this germplasm and others for enhancement yield, disease resistance will also boost yield as well as its acceptance by the growers. This would increase diversity in our food and nutrition.

Conclusions

This is the first report on the divergence and diversity in buckwheat landraces from two species collected from the Himalayan region of Pakistan. Our work clearly identified the divergence among both buckwheat species *F. esculentum* and *F. tartaricum*, revealing cross incompatibility between the two species. Considering, the importance of buckwheat in the Northern part of Pakistan, the observed diversity in buckwheat could be used for genetic improvement of the crop and to develop resistant varieties. The observed divergence between the species must further be explored using landraces and wild species in the region and their association with wild species in Hushe and Indus valleys of Gilgit Baltistan for their exploitation and conservation.

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