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RESEARCH ARTICLE

Genetic variation and species relationships in Himalayan buckwheats as revealed by SDS PAGE of endosperm proteins extracted from single seeds and RAPD based DNA fingerprints

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Abstract SDS PAGE of endosperm proteins and RAPD profiles from different accessions of Himalayan buckwheat were studied to determine their genetic variation and phylogenetic relationships. Comparisons based on Jaccard's coefficient and UPGMA clustering revealed interrelationships broadly in conformity with conventional treatments. Cluster analysis of the endosperm protein profiles of the selected accessions revealed three broad clusters. A moderate level of intraspecific variability was detected in the endosperm protein profile of different accessions of *Fagopyrum esculentum*, Moench. Three subgroups were detected in cluster 1. Subgroup 1 included varieties designated as Local, Kamroo local, OC-2 and VL-7 which were collected from VPKAS, Almora. Local, Kamroo local and OC-2 showed a similarity coefficient of 1.0 inspite of their being identified as different accessions. VL-7 emerged out separately from the rest of the three accessions. Accessions having winged grains and those having striations on the seed coat formed a 2nd and 3rd subgroup, respectively. IC-13145 which been identified as ''*F. himalianum*'', showed 100% similarity in endosperm protein profile with IC-13376 (*F. esculentum*) and 85–90% similarity which other accessions of *F. esculentum*. Our results indicate that ''*F. himalianum*'' belongs to the *esculentum* group and should not be regarded as a different species. Cluster 2 included all the accessions of *F. tataricum* (L.) Gaertn. with Sangla 1, 2, 3, 5, 6, 7 and IC-412863 showing 100% similarity. *F. cymosum* emerged as a separate group distinct from both *esculentum* and *tataricum*. Accessions of *F. tataricum* and *F. cymosum* did not show significant intraspecific variation in the SDS PAGE profile of endosperm proteins. Out of the 20 primers used, 3 generated robust, easily interpretable amplified products. While a 1490 bp and a 300 bp RAPD was detected only in *F. tataricum, a* 1154 bp RAPD was detected in all accessions of *F. tataricum* except in Shimla B-1. This variety is early maturing and has high seed yields.

Keywords Buckwheat accessions · *Fagopyrum* spp. · Genetic diversity · RAPD · SDS PAGE · Soluble proteins

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Introduction

Buckwheat is a minor crop in the world but is an indispensable food in the temperate and hill regions of East Asia and Europe. It is a major staple food crop in the Indo-Himalayan region (Jiang and Xing 1992). Buckwheat is a multipurpose crop used for food, feed, medicine and manure. The

tender shoots are used as a leafy vegetable while the flower and green leaves are used for the extraction of rutin (Marshall and Pomeranz 1982). Buckwheat flowers are a good source of honey.

The genus *Fagopyrum* consists of about 19 species, some of which have been recently discovered (Ohnishi 1998a, b; Ohsako and Ohnishi 1998). Of the two cultivated species, *Fagopyrum esculentum* Moench and *Fagopyrum tataricum* (L.) Gaertn., *F. tataricum* is mainly cultivated in the Himalayas. Cultivation of *F. esculentum* extends from temperate Europe to Japan through the Indo-Myanmar region. *Fagopyrum cymosum* (Trevir.) Meissn. (=*F. acutatum* (Lehm.) Mansf. ex K. Hammer; see Hammer 2001), the wild species of buckwheat, occurs mostly in the Himalayan foot hills and China.

Based on molecular data, the genus *Fagopyrum* has been divided into two phylogenetic groups viz. the *cymosum* group comprising of two cultivated species *F. esculentum* (Moench) and *F. tataricum* (L.) Gaertn., and two wild species *F. cymosum* (Meissn), and *F. homotropicum* Ohnishi and the *urophyllum* group comprising *F. urophyllum* (Bur. et Franchet) H. Gross. and other wild species (Ohnishi and Matsuoka 1996; Yasui and Ohnishi 1998a, b; Ohsako and Ohnishi 2000). Baniya (1990, Baniya et al. (1992) have observed significant variations in plant height, number of branches and leaves, clusters per cyme, seeds per cyme, days to maturity, seed weight, grain yield, seed colour/shape/surface in different landraces of buckwheat in Nepal. Joshi and Paroda (1991) have evaluated 408 accessions of Buckwheat from Himalayan region for 31 descriptor parameters including plant height, number of branches and leaves, clusters per cyme, seeds per cyme, days to maturity, seed weight, grain yield, seed colour/shape/surface. They have considered accession no. IC-13145 on the level of a species as ''*Fagopyrum himalianum*''. The accession, housed in the Regional Station, NBPGR, Phagli, Shimla (India), has been listed as ''*F. tataricum* var. *himalianum*'' by IPGRI (http://www.ipgri.cgiar.org/publication). Campbell (1997) has, however, considered it as a race of *F. esculentum*.

Assessment of genetic variation in a species is important for initiation of effective breeding programmes because it provides the basis for tailoring desirable genotypes. RAPD and SDS PAGE profiles have been successfully used for analysis of diversity in many crops including buckwheat (Javonik and Kump 1993; Tsuji and Ohnishi 1998; Ohnishi and Asano 1999), *Vigna unguiculata* (L.) Walp. (Mignouna et al. 1998), *Phaseolus vulgaris* L. (Thompson et al. 1998; Duarte et al. 1999), *Brassica juncea* (L.) Czern. (Rabbani et al. 1998), *Vicia sativa* L. (Potokina et al. 2000). Even though much work has been done on the analysis of phylogenetic relationships between different species of the genus *Fagopyrum* using isozyme profiling and RFLP variations in cpDNA (Ohnishi 1998a, b; Ohsako and Ohnishi 1998, 2000), not much information is available on inter- as well as intra-specific variations in molecular fingerprints in this genus. The selection of an appropriate molecular tool for screening of accessions in gene bank collections and elucidation of inter- and intra-specific variations has always been an important consideration. While the endosperm proteins have the advantage of reflecting numerous variations as they belong to very polymorphic multigenic families (Doll and Brown 1979), RAPD markers provide the technical simplicity and speed in elucidation of inter as well as intra-specific variations.

Materials and methods

Accessions of buckwheat used for the present investigation were procured from the National Bureau of Plant Genetic Resources, New Delhi, Vivekananda Laboratory of Hill Agriculture (Indian Council of Agricultural Research), Almora, East Khasi Hills in Meghalaya (India) and the Tawang District of Arunachal Pradesh (India). The accessions used for the present study are listed in Table 1.

Morphology

Germplasm collected from different places was separated into different groups based on their morphological characters. The accessions were grown in the experimental garden to study the qualitative as well as quantitative features

Table 1 Accessions of buckwheat used in the study

No	Accession/Name	Species	Source	Origin
1	IC-188669	*F. esculentum*	NBPGR[a]	Himachal Pradesh (Western Himalaya)
2	IC-18751	*F. esculentum*	NBPGR	Himachal Pradesh (Western Himalaya)
3	IC-13376	*F. esculentum*	NBPGR	Himachal Pradesh (Western Himalaya)
4	IC-13145	*F. esculentum*	NBPGR	Himachal Pradesh (Western Himalaya)
5	IC-13141	*F. esculentum*	NBPGR	Himachal Pradesh (Western Himalaya)
6	IC-13417	*F. esculentum*	NBPGR	Himachal Pradesh (Western Himalaya)
7	Local	*F. esculentum*	VPKAS[b]	Uttaranchal (Central Himalaya)
8	Kamroo Local	*F. esculentum*	VPKAS	Uttaranchal (Central Himalaya)
9	OC-2	*F. esculentum*	VPKAS	Uttaranchal (Central Himalaya)
10	VL-7	*F. esculentum*	VPKAS	Uttaranchal (Central Himalaya)
11	SanglaB-1	*F. tataricum*	VPKAS	Himachal Pradesh (Western Himalaya)
12	SanglaB-2	*F. tataricum*	VPKAS	Himachal Pradesh (Western Himalaya)
13	SanglaB-3	*F. tataricum*	VPKAS	Himachal Pradesh (Western Himalaya)
14	Sangla B-5	*F. tataricum*	VPKAS	Himachal Pradesh (Western Himalaya)
15	Sangla B-6	*F. tataricum*	VPKAS	Himachal Pradesh (Western Himalaya)
16	Sangla B-7	*F. tataricum*	VPKAS	Himachal Pradesh (Western Himalaya)
17	KBB-3	*F. tataricum*	VPKAS	Uttaranchal (Central Himalaya)
18	Himpriya	*F. tataricum*	VPKAS	Uttaranchal (Central Himalaya)
19	Kuppa Local	*F. tataricum*	VPKAS	Uttaranchal (Central Himalaya)
20	Shimla B-1	*F. tataricum*	VPKAS	Himachal Pradesh (Western Himalaya)
21	IC-412863	*F. tataricum*	NBPGR	Arunanchal Pradesh (East Himalaya)
22	EC-323729	*F. esculentum*	NBPGR	Japan
23	*Fagopyrum cymosum*	*F. cymosum*	NBPGR	Meghalaya (East Himalaya)

[a]National Bureau of Plant Genetic Resource, New Delhi, India

[b]Vivekandada Parvatteeya Krishi Anusandhan Sanshan, Almora, India

during their growth span. The seeds were sown in two different seasons i.e. April–July and August–November. The descriptors used for observing variations between different accessions are listed in Table 2. Similarity matrix value was calculated on the basis of the 1 or 0 values for different characters and a dendrogram was developed using NTSYS-PC version-2.1 (Rohlf 1993).

Table 2 Qualitative morphological characters analyzed for determination of variation between the accessions of buckwheat

Sl. No.	Traits	Variable[a]
1.	Seed Shape 1	Winged (1); Non Winged (0)
2.	Seed Shape 2	Non-conical (1); Conical (0)
3.	Seed colour	Brown (1); Dark brown (0)
4.	Seed coat striations	Present (1); Absent (0)
5.	Colour of the stem	Red (1); Green (0)
6.	Blade shape 1	Cordate (1); Sagittate (0)
7.	Blade shape 2	Non Hastate (1); Hastate (0)
8.	Leaf margin colour	Red (1); Green (0)
9.	Flower colour 1	White (1); Green (0)
10.	Flower colour 2	Pink (1); Non Pink (0)

[a]Figures in parenthesis depict the score for the character

Endosperm protein extraction

Endosperm proteins were extracted from grains of different accessions of buckwheat with 50 mM Tris–Cl buffer (pH 6.8) containing 100 mM NaCl, 10 Mm EDTA, 100 mM Glycine, 10% SDS and 1 mM phenyl methyl sulphonyl fluoride (PMSF). The crude homogenate was kept for 45 min at 4°C and centrifuged at 10,000 rpm for 15 min. The extracted proteins were recovered as clear supernatant.

SDS-PAGE of the extracted proteins was carried out on 12% polyacrylamide slab gel under reducing conditions following the method of Laemmli (1970). Samples containing 50 μg protein were loaded on 1.5 mm thick 12% acrylamide gels. Electrophoresis was carried out at a constant voltage of 100 V for 6 h. The gels were stained for 3 h in 0.25% (w/v) coomassie brilliant blue R-250 followed by destaining in methanol : water : glacial acetic acid (4 : 5.3 : 0.7). Protein bands were visualized in a transilluminator under white light.

Random amplified polymorphic DNA

For isolation of total genomic DNA, healthy grains of different accessions were surface sterilized with mercuric chloride (0.01%) and germinated on sterile germinating paper in dark at 27°C in a seed germinator. Total genomic DNA was isolated from 7-day-old etiolated seedlings of different accessions of buckwheat by extraction with CTAB buffer as described by Murray and Thomson (1980). PCR was carried out in 25 µl reaction volumes containing 11.8 µl of sterile distilled water, 0.2 µl (1 unit) of *Taq polymerase*, 2.5 µl of 10× PCR buffer, 2 µl 10 mM dNTP mix, 1.5 µl of 50 mM $MgCl_2$, 100 pmol of primer and 5 µl (50 ng) of template DNA. Amplification was carried out on a Techne thermal cycler programmed for 35 cycles with an initial strand separation at 94°C for 5 min and 94°C for 1 min., followed by annealing at of 37°C for 1 min and extension at 72°C for 3 min. After 35 cycles there was a final extension at 72°C for 10 min. Amplification products were electrophoresed in 1.6% agarose gel and stained with ethidium bromide. Each set of reactions was repeated thrice and only the reproducible ones were included in the analysis.

Statistical analysis

Variation in the morphological characters was determined by visual checking of the features on the plants from each accession. Each character was scored either as (1) or (0). Evaluation of variation in the endosperm proteins and RAPD profile was performed by calculating the individual band frequency for each accession. Polymorphism was scored for the presence (1) or absence (0) of bands. Cluster analysis was performed on the similarity matrix based on Jaccard's coefficient by the UPGMA method. All computations were performed with NTSYS-PC version 2.1 (Rohlf 1993).

Results and discussion

The morphological features analyzed for determination of variations between different accessions of buckwheat are presented in Table 2. While *F. esculentum* and ''*F. himalianum*'' had both winged as well as non-winged grains, *F. tataricum* and *F. cymosum* had exclusively non-winged grains. The grains of *F. esculentum* and ''*F. himalianum*'' had a smooth seed coat with or without striations. On the other hand grains of *F. tataricum* and *F. cymosum* had a rough seed coat without any prominent striations. Variations were also observed in shape of the leaf blade, leaf margin colour and flower colour. While *F esculentum* and ''*F. himalianum*'' showed both cordate and sagittate leaf blade morphology, *F. cymosum* had exclusively sagittate blades. The leaf blade shape in *F. tataricum* was hastate. While *F. esculentum*, *F. cymosum* and ''*F. himalianum*'' had white flowers, *F. tataricum* produced green flowers. Some accessions of *F. esculentum* produced pink flowers.

The SDS-PAGE profile of endosperm seed proteins of different accessions of *F. esculentum*, ''*F. himalianum*'', *F. tataricum* and *F. cymosum* is presented in Fig. 1. The number of polypeptide bands observed in accessions ranged from a minimum of 23 in IC-18751 to a maximum of 29 in accessions of *F. tataricum*. The size of resolved polypeptides ranged between 91 and 14 kDa. Distinct quantitative variations were observed in the SDS-PAGE profile of endosperm proteins of *F. esculentum*, *F. tataricum* and *F. cymosum*. The SDS-PAGE profile of endosperm proteins of ''*F. himalianum*'' showed much similarity with that of *F. esculentum*. Significant intraspecific variations were detected in the endosperm protein profile of different accessions of *F. esculentum*. This could be ascribed to a high degree of grain protein polymorphism in *F. esculentum*. Most of these variations were observed in the protein bands ranging in size between 21 and 74 kDa. Interestingly, most of the protein bands in the 26–54 kDa category belong to the legumin type family of seed proteins (Rout and Chrungoo 1996; Bharali 2002). Accessions of *F. tataricum* and *F. cymosum*, on the other hand, did not show any significant intraspecific variations in the SDS PAGE profile of grain endosperm proteins. These results are consistent with the observations of Nishiyama et al. (1991) and Svetek (1994). One of the most important features of the profile was the presence of a 42 kDa band in accessions of *F. esculentum* having winged grains and a 31 kDa

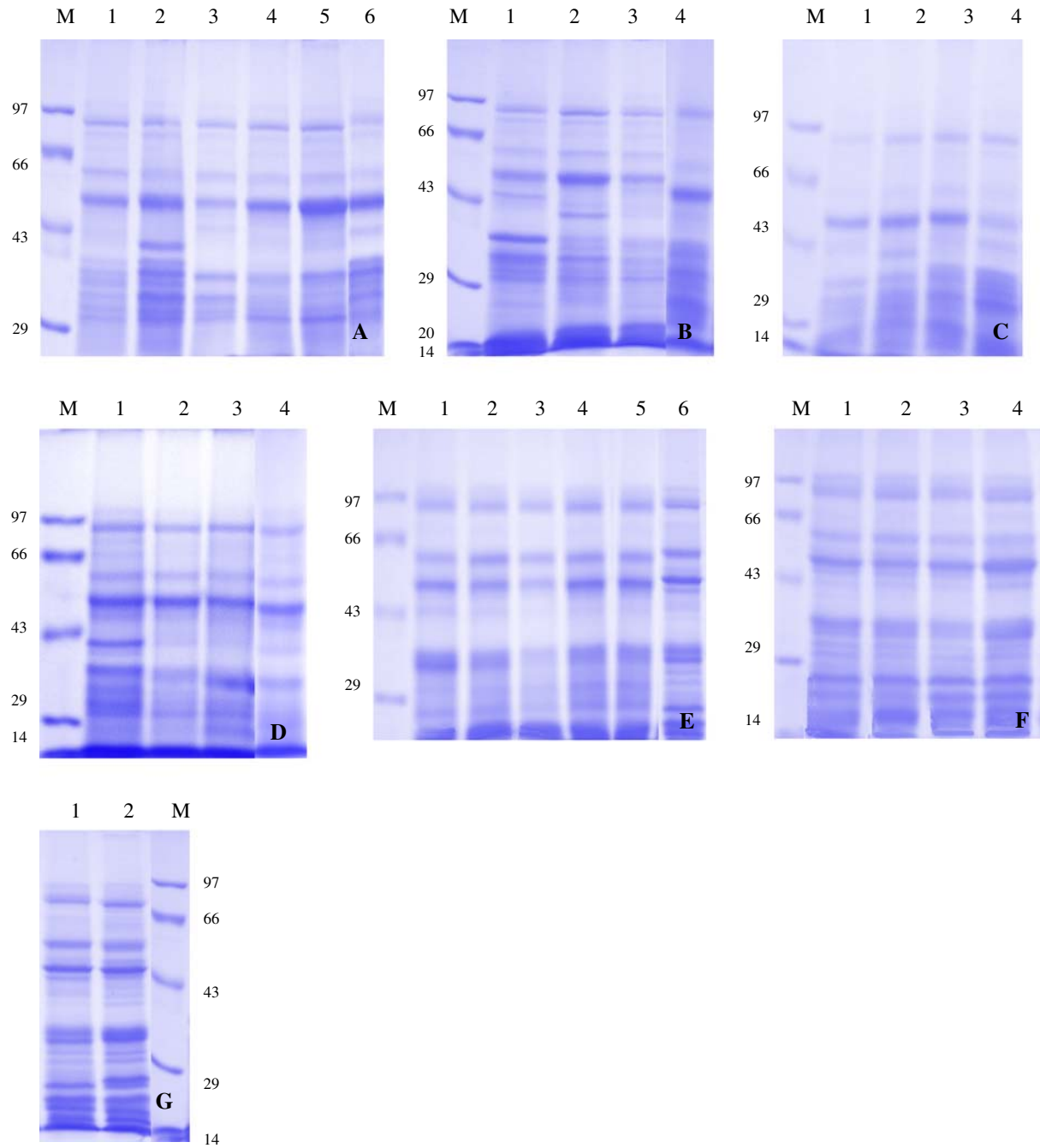

Fig. 1 SDS-PAGE profile of endosperm proteins extracted from single grains of different accessions of buckwheat from Indian Himalayas. **a** Lane1: IC-188669-1, 2: IC-188669-2, 3: IC-18751-2, 4: IC-18751-3, 5: IC-13376-1, 6: IC-13376-4; **b** Lane2: IC-13145-4, 2: IC-13141-4, 3: IC-13141-5, 4: IC-13145-2; **c** Lane1: IC-13417-2, 2: IC-13417-5, 3: EC-323729-1, 4: EC-323729-5; **d** Lane1: VL-7, 2: Local, 3: Kamroo Local, 4: OC-2; **e** Lane1: Sangla B-1, 2: Sangla B-2, 3: Sangla B-3, 4: Sangla B-5, 5: Sangla B-6, 6: Sangla B-7; **f** Lane1: KBB-3 2: Himpriya, 3: Kuppa Local, 4: IC-412863; **g** Lane1: Shimla B-1, 2: *F.cymosum*; M: Molecular Weight Marker (λDNA*Eco*R1 + *Hind*III double digest)

band in grains having prominent strips. Further a duplex of 41 and 39 kDa could be detected only in VL-7. This cultivar is high yielding and early maturing, suitable for cultivation in the middle hills (Arora et al. 1995). This protein band could be used as a marker for the identification of this accession.

The dendrogram generated on the basis of SDS-PAGE profile of endosperm proteins revealed the clustering of the accessions into three broad groups (Fig. 2). Cluster 1 included all the accessions of *F. esculentum*. Within this cluster three different subgroups were noticed. Subgroup1 included cultivars designated as Kamroo local, OC-2 and VL-7 which were collected from VPKAS, Almora. Even though Kamroo local and OC-2 have been identified as two different accessions, they showed 100% identity in endosperm protein profile. VL-7 clustered as a separate accession with a Jaccard's coefficient of 0.9 compared to other accessions of the subgroup. Accessions having winged seeds and those having strips on the seed coat formed 2nd and 3rd subgroup, respectively. IC-13145, which had been identified as a separate species viz. ''*F. himalianum*'', showed >90% similarity with IC-13376 (*F. esculentum*). The two accessions clustered together even on the basis of their morphological descriptors. Our results indicate that ''*F. himalianum*'' belongs to the *esculentum* group rather than qualifying as a different species. Cluster 2 included all the accessions belonging to *F. tataricum*. All the accessions/cultivars of *F. tataricum*, except Shimla B-1 clustered into one group with 100% similarity. Shimla B-1 is an early maturing cultivar with higher grain yield compared to other accessions of *F. tataricum*. Cluster 3 included the accessions of *F. cymosum*, which clustered as a separate group distinct from both *esculentum* as well as *tataricum*. *F. cymosum* showed least similarity of 39.4% with *F. esculentum* and a maximum of 57% similarity with *F. tataricum*.

RAPD analysis of buckwheat genomic DNA was carried out under optimized reaction conditions with amplification conditions being identical for all the reactions. All amplification reactions were performed in triplicate and only highly reproducible bands were considered for analysis. The band size of amplified fragments varied from 2.1 to 0.2 kbp (Fig. 3). The primers used in the current study showed high ability to discriminate between accessions from the same as well as different species of the genus. The maximum number of polymorphic bands observed was 14 in accessions of *F. esculentum* and 12 each in accessions of *F. cymosum* and *F. tataricum*. A

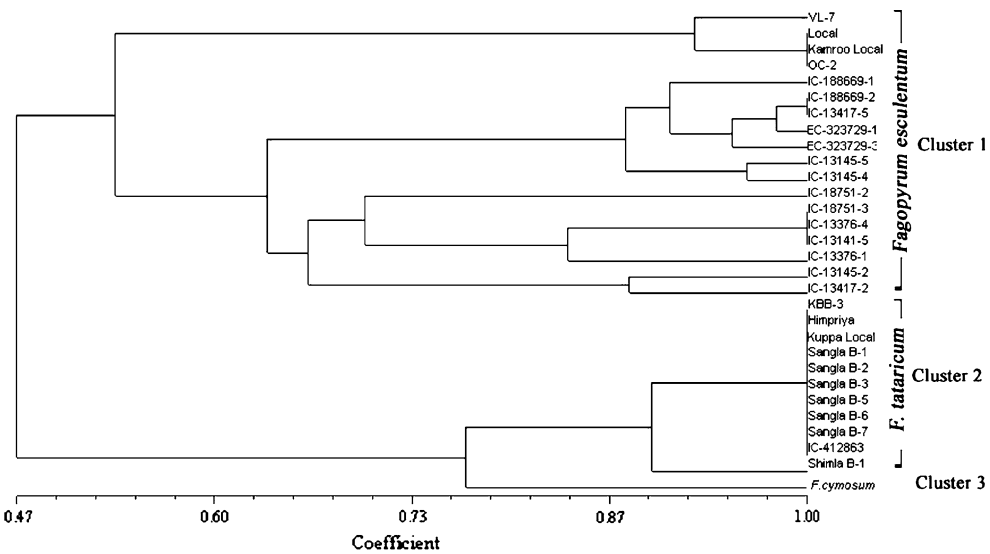

Fig. 2 Dendrogram based on the UPGMA analysis generated from Jaccard's coefficient of the endosperm protein SDS PAGE profiles of different accessions of *Fagopyrum* spp.

1490 and 300 bp RAPD was detected only in *F. tataricum*. While a 1150 bp RAPD was detected in all cultivars/accessions of *F. tataricum*, it could not be detected in Shimla B-1. Shimla B-1 is an early maturing cultivar and has been shown to give the highest grain yield. Similarly a 250 bp RAPD was detected only in KBB-3 (*F. tataricum*). Such diagnostic markers can be of importance in cultivar identification and can be used to detect instances of natural interspecific gene introgression.

The dendrogram generated on the basis of RAPD profile revealed clustering of the accessions into three groups (Fig. 4). All the accessions of *F. esculentum* clustered as one group. Since the similarity value within the accessions of *F. esculentum* varied from 47–100% numerous subgroups were formed within the group. Even though IC-13145 had been identified as ''*F. himalianum*'' it showed 100% similarity of its RAPD profile with IC-13376 (*F. esculentum*). Accessions belonging to *F. tataricum* clustered as a separate group with two sub-groups. While one of the groups comprised KBB-3, Sangla 1, 2, 3, 5, 6, 7 and DRLT-1247 (IC-412863) the other comprised Himpriya, Kuppa local and Shimla B-1. The percentage of similarity varied from 69 to 100% within the accessions belonging

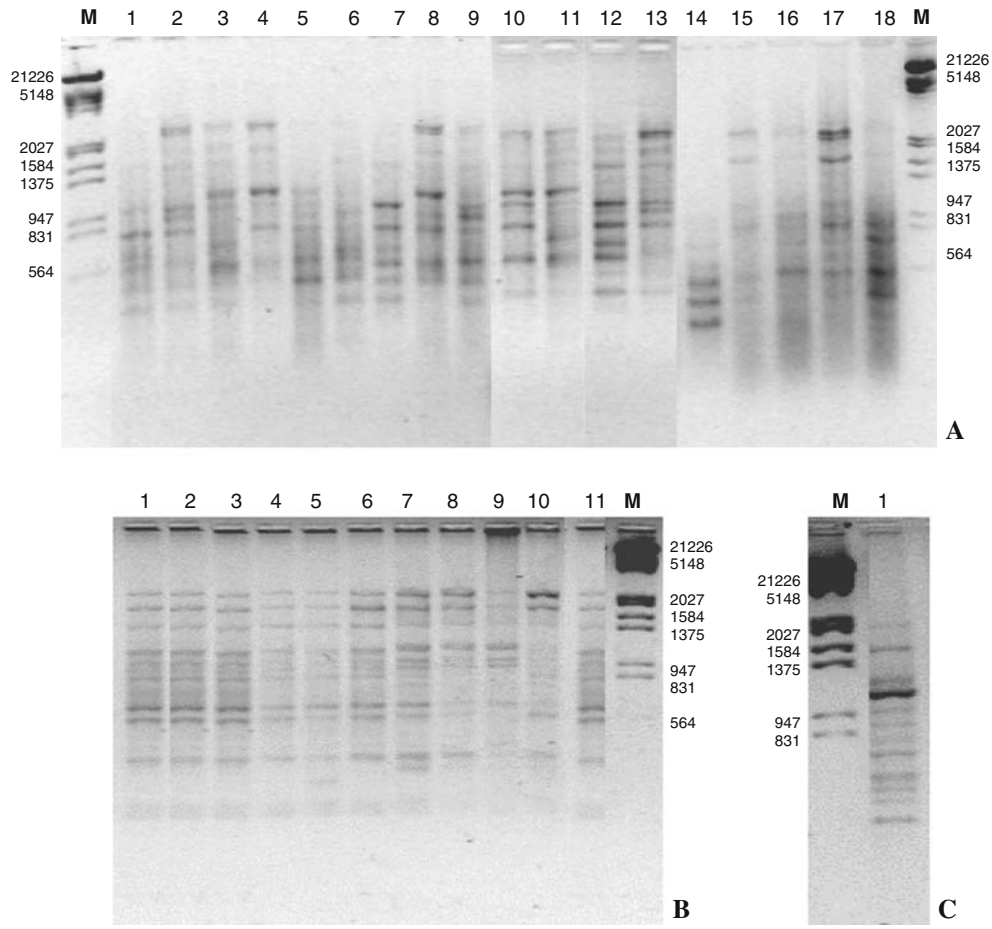

Fig. 3 RAPD profile of genomic DNA extracted from single etiolated seedlings of different accessions of buckwheats from Indian Himalayas. **a** Lane1: IC-18751-2, 2: IC-13376-1, 3: IC-13376-4, 4: IC-13141-1, 5: IC-13141-5, 6: IC-188669-1, 7: IC-188669-2, 8: IC-13145-4, 9: IC-13145-2, 10: IC-13417-5, 11: EC-323729-1, 12: EC-323729-3, 13: IC-18751-3, 14: IC-13417-2, 15: OC-2, 16: VL-7, 17: Local, 18: Kamroo Local; **b** Lane1: Sangla B-1, 2: Sangla B-2, 3: Sangla B-3, 4: Sangla B-5, 5: Sangla B-6, 6: Sangla B-7, 7: KBB-3, 8: Himpriya, 9: Kuppa Local, 10: Shimla B-1, 11: IC-412863; **c** Lane1: *F. cymosum*; M: Molecular Weight Marker (λDNA*Eco*R1 + *Hind*III double digest)

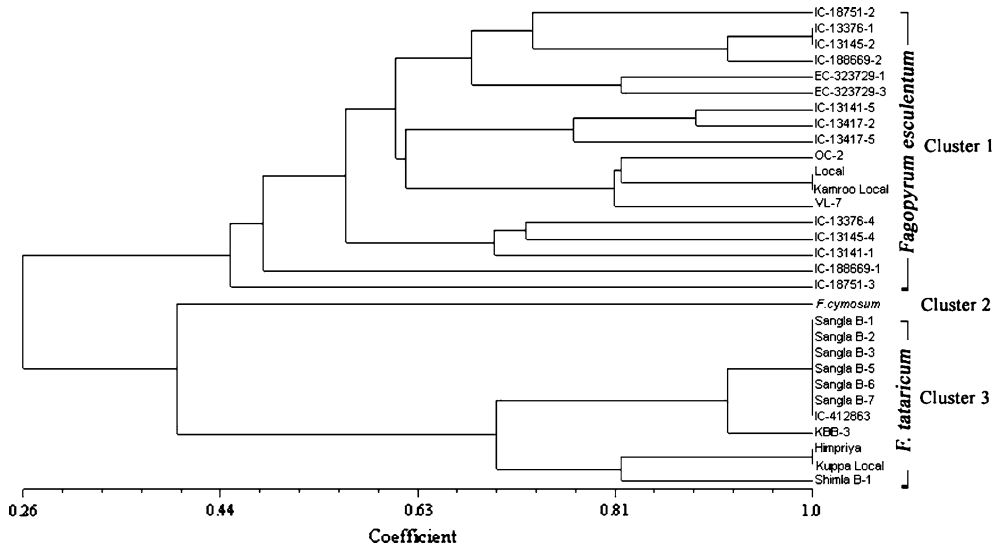

Fig. 4 Dendrogram based on the UPGMA analysis generated from Jaccard's coefficient of the RAPD profiles of different accessions of *Fagopyrum* spp.

to *F. tataricum*. The third cluster consisted of *F. cymosum*.

No variability was observed in qualitative morphological features (Fig. 5), endosperm protein SDS- PAGE (Fig. 2) and RAPD (Fig. 4) profiles in the Sangla group of accessions and IC-412863. A similarity coefficient of 1.0 for these accessions indicates that these varieties/accessions may be genetically similar. While Sangla is an accession from the western Himalayas, IC-412663 is an accession from eastern Himalaya (Tawang valley of Arunachal Pradesh). *F. tataricum* is self compatible and is cultivated extensively in the Indian Himalayan foot hills. The loss of variability in tatary buckwheat could have occurred during the process of domestication. Ohnishi (1998b, 2004) has suggested that tartary buckwheat acquired four variants through mutations during the process of domestication. Each of the variants got fixed in the local populations during diffusion of buckwheat cultivation in the Indian Himalayas.

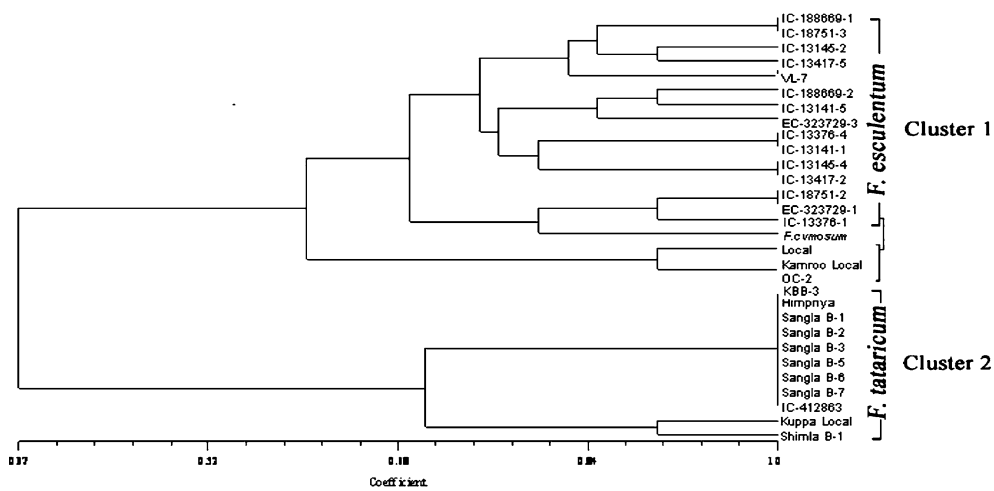

Fig. 5 Dendrogram based on the UPGMA analysis generated from Jaccard's coefficient of the morphological characters of different accessions of *Fagopyrum* spp.

Accessions of *F. esculentum* showed greater closeness with accessions of *F. tataricum* than with *F. cymosum*. Similar observations have also been made by Yasui and Ohnishi (1998a, b) who compared the nucleotide sequence of the *rbcl–accD* region of cpDNA in the genus. These results are in agreement with the molecular systematic studies of Ohnishi and Matsuoka (1996), Ohsako and Ohnishi (1998, 2000) on *Fagopyrum*.

Our results indicate that *F. cymosum*, which to a greater extent resembles *F. esculentum* morphologically (Fig. 5) is closer to *F. tataricum*. These observations are also brought out by a combined dendrogram generated on the basis variations in qualitative morphological characters, SDS PAGE and RAPD profiles (Fig. 6). These observations are in conformity with earlier findings reported on species relationships in *Fagopyrum* using different marker approaches (Kishima et al. 1995; Sharma and Jana 2002).

Principal component analysis of the endosperm protein SDS PAGE and RAPD profiles (Fig. 7) supports the taxonomic treatment of the three species and the relationship within accessions within the genus *Fagopyrum*. One of the significant features revealed by PCA was the emergence of VL-7 and EC-323729 as accessions distinct from other accessions of the same species. While VL-7 is a cultivar released by VPKAS, Almora EC-323729 is an East European accession. These results indicate that SDS PAGE profile of endosperm proteins and RAPD can be used to determine intraspecific variability in *Fagopyrum* spp.

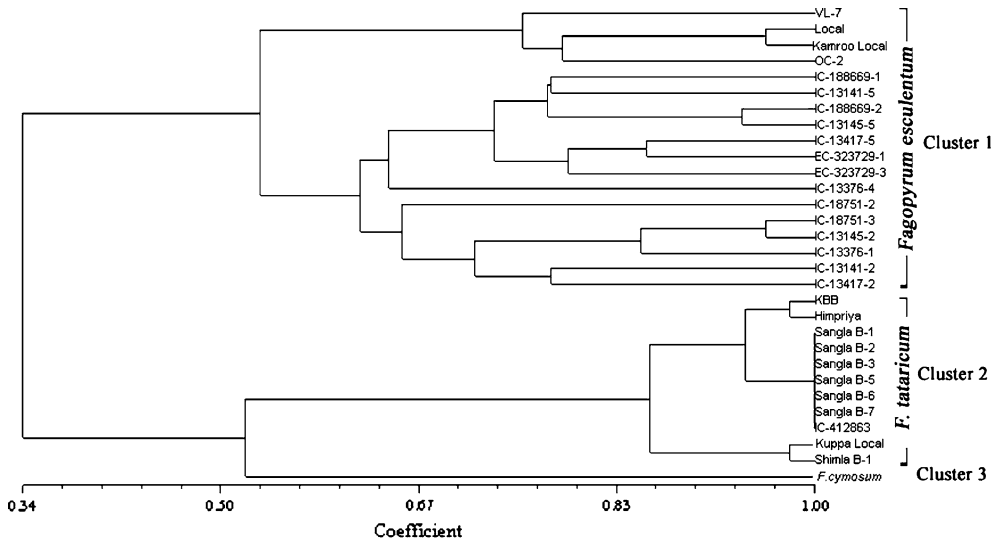

Fig. 6 Dendrogram based on the combined UPGMA analysis generated from Jaccard's coefficient of the morphological characters, SDS PAGE and RAPD profile of different accessions of *Fagopyrum* spp.

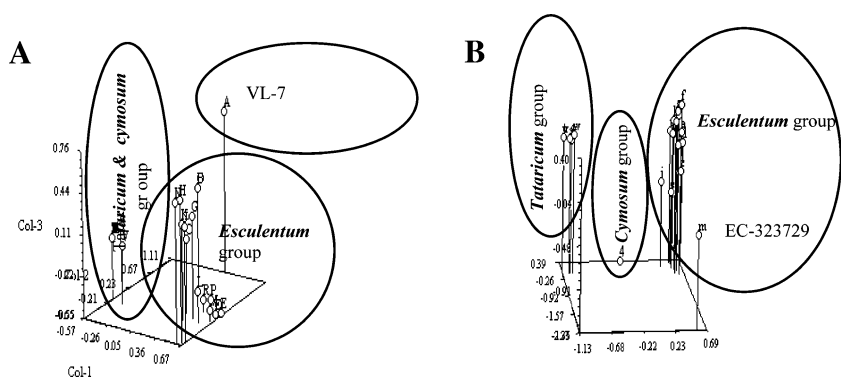

Fig. 7 Plot of principal component analysis based on SDS PAGE profile of endosperm proteins extracted from single grains (**A**) and RAPD profile of genomic DNA extracted from single etiolated seedlings (**B**) of different accessions of buckwheats from Indian Himalayas

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