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ORIGINAL PAPER

Genetic relatedness and population differentiation of Himalayan hulless barley (*Hordeum vulgare* L.) landraces inferred with SSRs

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Abstract A set of 107 hulless barley (*Hordeum vulgare* L. subsp. *vulgare*) landraces originally collected from the highlands of Nepal along the Annapurna and Manaslu Himalaya range were studied for genetic relatedness and population differentiation using simple sequence repeats (SSRs). The 44 genome covering barley SSRs applied in this study revealed a high level of genetic diversity among the landraces (diversity index, DI = 0.536) tested. The genetic similarity (GS) based UPGMA clustering and Bayesian Model-based (MB) structure analysis revealed a complex genetic structure of the landraces. Eight genetically distinct populations were identified, of which seven were further studied for diversity and differentiation. The genetic diversity estimated for all and each population separately revealed a hot spot of genetic diversity at Pisang (DI = 0.559). The populations are fairly differentiated ($\theta = 0.433$, $R_{ST} = 0.445$) accounting for > 40% of the genetic variation among the populations. The pairwise population differentiation test confirmed that many of the geographic populations significantly differ from each other but that the differentiation is independent of the geographic distance ($r = 0.224$, $P > 0.05$). The high level of genetic diversity and complex population structure detected in Himalayan hulless barley landraces and the relevance of the findings are discussed.

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Introduction

Hulless or naked barley (*Hordeum vulgare* L. subsp. *vulgare*) differs from hulled barley by the loose husk cover of caryopses that is easily separable upon threshing in contrast to hulled barley. The hulless grain character is controlled by the single recessive gene '*nud*' located on the long arm of chromosome 7H (Kikuchi et al. 2003). The domestication of naked barley is believed to have occurred after the hulled type around 6500 BC (Zohary and Hopf 2000). Taketa et al. (2004) suggested a monophyletic origin of naked barley as a single mutation event either from wild barley (*H. vulgare* subsp. *spontaneum*) or from domesticated hulled barley (*H. vulgare* subsp. *vulgare*).

Cultivation of naked barley is less common worldwide than that of hulled barley. Its distribution is skewed towards East Asia, namely to the Himalaya range (Nepal, Bhutan and Tibet), China, Korea and Japan where it accounts to as much as 95% of the domesticated barley in some areas (Takahashi 1955; Sun and Wang 1999). Besides East Asia, it is grown in Ethiopia at low frequency (Assefa and Labuschagne 2004). The cultivation is rare in the Western World

(Europe, North America) and in Australia where hulled types are prevalent. Hulless barley is mainly used as animal feed; however, it is an important human food in Himalayas and in Ethiopia. In recent years, its importance is increasing as a human food in non-traditional areas due to its high β-glucan content which acts as an inhibitor of cholesterol synthesis, and due to processing advantages, i.e., the absence of grain husks, for the barley food industry (Bhatty 1999; Atanassov et al. 2001).

The barley landraces from the Himalayas, particularly from the highlands of Nepal, share a significant part of the world barley germplasm resources (Valkoun and Konopka 2004). Due to the fact that naked barley is widely grown in the highlands of Nepal from the East to the West (about 800 km) along the Himalayas, it is frequently represented in Himalayan barley germplasm collections and can be considered as an important genetic resource. The diversity of Himalayan barley is described by various authors. For example, Witcombe and Murphy (1986) assessed the morphological variation of these barleys and Konishi and Matsuura (1991) analyzed isozyme genotypes of Himalayan barley landraces and found that hulless types are highly differentiated from hulled ones. Based on isozyme diversity (Liu et al. 1999) and sequence variation of a DNA marker closely linked to the *nud* locus (Taketa et al. 2004), hulless barley landraces from the highlands of Nepal turned out to be distinct from the Chinese, Korean and Japanese types. Furthermore, it was shown by SSR analyses that these landraces are different from western hulled barley (German cultivars) and Canadian naked barley cultivars (Pandey et al. 2004).

In an extensive survey on Nepalese naked barley germplasm, Sharma et al. (1994) found a vast variation in morphology between and within landrace populations. Besides this, differences in agronomic performance and disease resistance were detected (Baniya et al. 1997). The literatures reviewed here hint for a high level of diversity and genetic uniqueness of Nepalese hulless barley. However, precise and detailed information on genetic diversity is lacking and little is known regarding population structure and geographic differentiation of the Himalayan hulless barley. A precise estimate of genetic diversity is a prerequisite to realize the relative importance of these germplasm in breeding programs, and an in-depth knowledge of population structure and patterns of geographic differentiation allows a more specific use of the genetic resource, e.g., for association studies or to formulate effective management or conservation strategies. We therefore analyzed a large number of gene bank preserved hulless barley landraces originally collected from the Himalayas range in central Nepal with the objectives: (1) to get a precise estimate on genetic diversity of Himalayan hulless barley, (2) to understand the population structure and patterns of geographic differentiation, (3) to estimate the origin of diversity in the region, and (4) to shed light on the relevance of the genetic resources for mainstream barley breeding.

In recent time, SSR markers are widely used in genetic diversity and population studies in different crop species, e.g., rice (Jain et al. 2004), maize (Patto et al. 2004), wheat (Khlestkina et al. 2004), sorghum (Uptmoor et al. 2003; Abu-Assar et al. 2005) and barley (Hamza et al. 2004; Ordon et al. 2005). There are large numbers of barley SSRs available, of which Macaulay et al. (2001) proposed a set for genotyping in barley that is highly informative and provides equidistant genome coverage. In this study, 107 hulless barley landraces were analyzed with 44 well-characterized SSRs out of which 40 are common with Macaulay et al. (2001).

Materials and methods

Nepalese naked barley landraces and molecular genotyping

A total of 107 hulless barley landraces collected from the highlands of Nepal along the Annapurna and Manaslu Himalaya range was studied (Table 1). The samples included in the present study correspond to the set of 115 barley genotypes previously analyzed by Pandey et al. (2004). Seeds were obtained from the Barley Germplasm Center, Okayama University, Kurashiki (Japan) and multiplied for a single generation ensuring self-pollination in a green house at Giessen University. The landraces included in the study were six-rowed spring types, however, some had intermediate growth habit. Detailed descriptions on morphology and important agronomic traits are available in the Catalogue of Barley Germplasm Preserved in Okayama University (1983). The present study covers 85% of the Nepalese hulless barley landraces preserved in the gene bank of Okayama. Using a high resolution (1:25,000) topographical map of the area studied (Survey Department, HMG, Nepal), and information provided on collection sites (Catalogue of Barley Germplasm Preserved in Okayama University, 1983) or the landrace names which in general correspond to the locality of collection, a combined map of the entire region was developed and the origins of the

Table 1 Origin, geographic grouping and sample size of the hulless barley landraces analyzed

Geographic region	Origin	Nos. of landraces	Landraces
Upper basin of KaliGandaki	Jomson	2	Jomson-1, Jomson-2
	Kagbeni	2	Kagbeni-3, Kagbeni-5
	Tukucha	1	Tukucha
	Dhumpu	1	Dhumpu-2
	Total	6	
Lower basin of KaliGandaki	Sikha	7	Sikha-1, Sikha-2, Sikha-4, Sikha-5, Sikha-6, Sikha-7, Sikha-8
	Ulleri	2	Ulleri-9, Ulleri-21
	Ghara	2	Ghara-1, Ghara-2
	Phalatey	1	Phalatey
	Total	12	
	Annapurna-BC	2	Annapurna BC-1, Annapurna BC-2
Upper basin of Marshyangdi	Chame	8	Chame-2 Chame-3, Chame-8, Chame-9, Chame-11, Chame-12, Chame-13, Chame-14
	Pisang	6	Pisang-4, Pisang-5, Pisang-6, Pisang-7, Pisang-8, Pisang-9
	Thonje	8	Thonje-3, Thonje-4, Thonje-5, Thonje-6, Thonje-16, Thonje-18, Thonje-19, Thonje-21
	Gho	3	Gho-1, Gho-2, Gho-3
	Total	25	
	Bimtakothi	10	Bimtakothi-1, Bimtakothi-2, Bimtakothi-3, Bimtakothi-4, Bimtakothi-5, Bimtakothi-9, Bimtakothi-10, Bimtakothi-11, Bimtakothi-12, Bimtakothi-13
Upper basin of BudhiGandaki	Ngyak	7	Ngyak-1, Ngyak-2, Ngyak-3, Ngyak-4, Ngyak-10, Ngyak-11, Ngyak-12
	Sama	7	Sama-1, Sama-2, Sama-3, Sama-4, Sama-6, Sama-8, Sama-9
	Philem	3	Philem-1, Philem-2, Philem-3
	Pork	2	Pork-1, Pork-2
	Total	19	
East of BudhiGandaki	Sipche	8	Sipche-2, Sipche-3, Sipche-4, Sipche-6, Sipche-7, Sipche-9 Sipche-11, Sipche-12
	Total	8	
	Thomje	5	Thomje-2, Thomje-3, Thomje-4, Thomje-6, Thomje-7
	Thangja	3	Thangja-1, Thangja-2, Thangja-3
	Tilman camp	3	Tilman Camp-1, Tilman Camp-7, Tilman Camp-8
	Lih Dharna Gal	1	Lih Dharna Gal
	Tsumje	2	Tsumje-1, Tsumje-2
	(Unknown)	11	Naked-304, N-6, N-12, Solu Uwa, Nepal-1, Nepal-2, Nepal-3, Nepal-4, Nepal-5, Nepal-6, Nepal-7
	Total	107	

landraces were located (Fig. 1). The geographic location of the study area is between 28°15′ and 28°55′ in the North, and 83°35′ and 85°05′ in the East. This includes the areas both on the South as well as North slope of the main Himalayas crest. The barley samples used are originated from the wider parts of the famous Annapurna and Manaslu conservation areas and represent the highlands, valleys and mountain terraces in the catchments of rivers BudhiGandaki, Marshyangdi and KaliGandaki in central Nepal. The altitude in this region varies greatly from the floor of valleys (500–4,000 m) to the top of the Himalayas (> 8,000 m). Similarly, a strong South-to-North monsoon gradient causes a wide difference in annual precipitation in the region ranging from precipitation peaks at 5,032 mm year^{-1} at about 3,000 (m) altitude on the southern side to ~1,100 mm year^{-1} in the rain shadow to the North of the main Himalayas crest (Putkonen 2004). Because of the vast topographical heterogeneity and impact of the Himalayas on amount and distribution of precipitation, the barley samples used represent highly diverse and isolated eco-geographic locations.

The origins of landraces were divided into five geographic regions following the three river systems (Table 1): (1) upper basin of KaliGandaki, (2) lower basin of KaliGandaki, (3) upper basin of Marshyangdi, (4) upper basin of BudhiGandaki, and (5) East of BudhiGandaki. The accessions derived from Bimtakothi, Annapurna BC, Thomje, Thangja, Tilman camp and Tsumje were not included within the five geographic groups and considered as independent groups according to their origins. Of these, Bimtakothi and Annapurna BC are relatively isolated locations, whereas the positions of Thomje, Thangja, Tilman camps and Tsu-

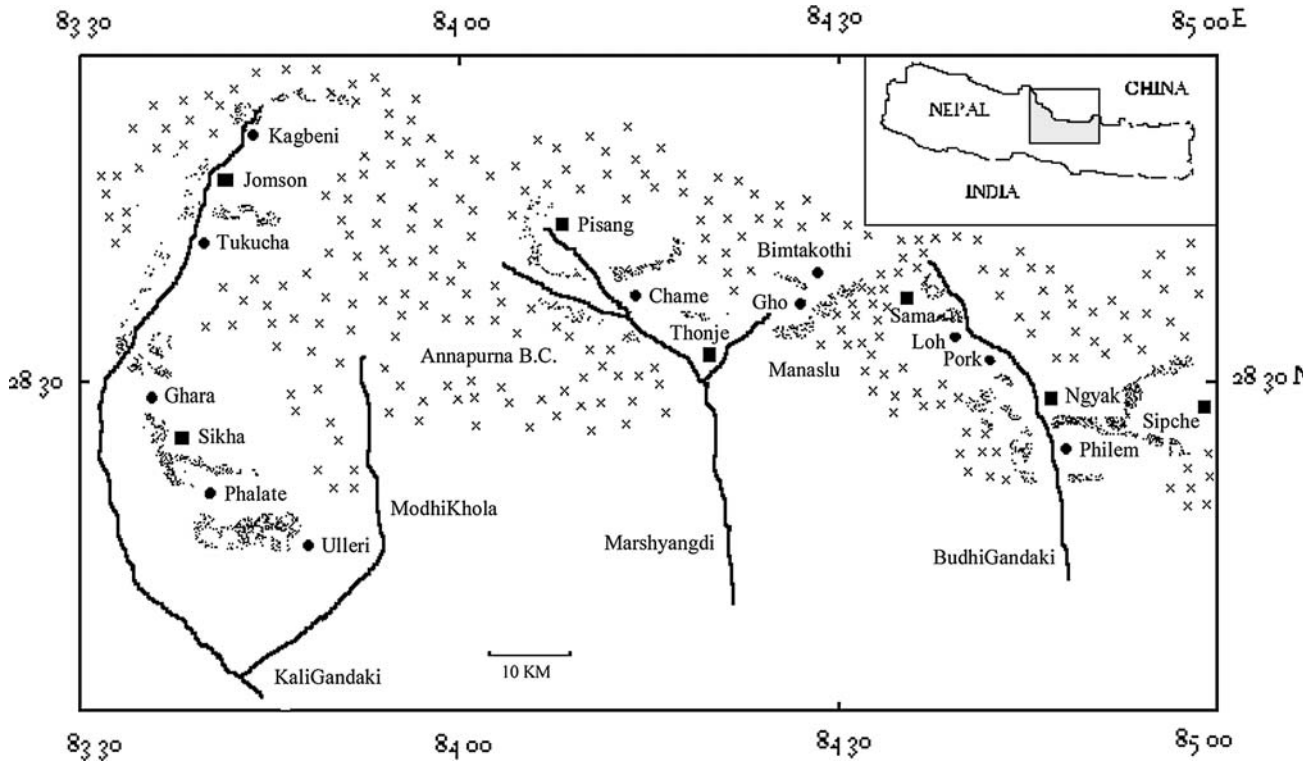

Fig. 1 Combined map of the upper basins of rivers KaliGandaki, Marshyandi and BudhiGandaki extended along the Annapurna and Manaslu Himalaya range in central Nepal. The positions are drawn with an approximate scale. *X* indicates permanently snow covered mountains and *shaded patches* are indicative for dense pine or mixed forest. The locations marked with a *shaded square* comprise distinct barley populations

mje are not indicated in the map because of ambiguity due to the differences in landrace names and the corresponding locality given in the topographic map. The origin of 11 landraces was not known.

The molecular genotyping of the landraces was carried out using 44 genome covering barley SSRs (Table 2), out of which 40 correspond to the set proposed by Macaulay et al. (2001) for genotyping in barley. For each accession five seeds were taken from the harvest of several heads of a single selfed plant and genomic DNA was extracted from the composite sample of leaf tissues of 2-week-old plants grown in the greenhouse following the CTAB extraction protocol (Doyle and Doyle 1990). The SSR assay was carried out according to Ramsay et al. (2000) with modifications in a Geneamp 9700 thermal cycler (Applied Biosystems). The respective SSR profiles were detected on an automatic DNA-sequencer (LiCor 4200-S2).

Statistical analyses

The alleles of each SSR locus were scored (bp) with reference to a known size standard followed by transformation to binary codes as presence (1) or absence (0) of the respective fragment size using the software RFLP-scan 2.1 (Scanalytics). Double bands due to residual heterozygosity were not scored and considered as missing values in the statistical analysis.

The polymorphism information content (PIC) was calculated for each SSR according to Weber (1990). Using the 0/1 data matrix, genetic similarity index between the landraces was estimated according to Dice (1945) and UPGMA clustering was performed following the Sequential Agglomerative Hierarchical and Nested (SAHN) method of the software NTSYS-pc (Rohlf 2000). The genetic diversity index (DI) was estimated as the mean gene diversity over the loci and adjusted for the sample size according to Nei (1978):

$$\mathrm{DI} = 2n_a\left(1/n_l\sum_j\left(1-\sum_i x_{ij}^2\right)\right)/(2n_a-1)$$

where x_{ij} is the frequency of the ith allele of locus j, n_l is the number of genetic loci, and n_a is the number of accessions.

Table 2 Repeat motifs, chromosomal locations, number of alleles detected and polymorphism information content (PIC) of 44 SSRs used for genotyping of hulless barley landraces

SSR	Repeat	Chromosome	No. of alleles	PIC	
				Nepalese landraces	Macaulay et al. (2001)
Bmac0399	(AC) 21	1H	7	0.67	0.85
Bmac0032	(AC) 7 T(CA) 15(AT)9	1H	15	0.82	0.75
Bmag0211	(CT) 16	1H	8	0.62	0.75
Bmac0154[a]	(AT) 19 (AC)6	1H	10	0.80	
HvHVA1	(ACC) 5	1H	1	0.00	0.63
WMC1E8	(AC) 24	1H	2	0.07	0.44
Bmac0134	(AC) 28	2H	3	0.44	0.79
HVM36	(GA) 13	2H	4	0.41	0.54
Bmag0378	(AG) 14	2H	3	0.34	0.50
Bmac0093	(AC) 24	2H	5	0.60	0.71
HVM54	(GA) 14	2H	4	0.53	0.76
EBmac0415	(AC) 17	2H	3	0.60	0.66
Bmag0136	(AG) 6-(AG) 10-(AG) 6	3H	3	0.04	0.40
Bmac0067	(AC) 18	3H	7	0.75	0.78
Bmac0209	(AC) 13	3H	4	0.38	0.84
Bmag0225	(AG) 26	3H	4	0.65	0.9
Bmag0013	(CT) 21	3H	8	0.26	0.81
HVM62	(GA) 11	3H	4	0.55	0.56
Bmac0029[a, b]		3H	7	0.71	
HVM40	(GA) 6 (GT) 4 (GA) 7	4H	4	0.40	0.59
Bmag0353	(AG) 21	4H	6	0.66	0.55
Bmag0384	(AG) 18	4H	4	0.70	0.67
EBmac0701	(AC) 23	4H	4	0.47	0.74
HvMLO3	(CTT) 6	4H	2	0.33	0.35
HVM67	(GA) 11	4H	5	0.30	0.50
Bmac0113	(AT) 7 (AC) 18	5H	9	0.83	0.79
EBmac0970	(AC) 8	5H	2	0.07	0.08
EBmac0684	(TA) 7(TG) 11-(TG) 11(TTTG)5	5H	6	0.60	0.67
Bmag0223	(AG) 16	5H	10	0.81	0.90
HVLEU	(ATTT) 4	5H	1	0.00	0.48
Bmag0222	(AC) 9 (AG) 17	5H	3	0.60	0.62
HvLOX	(AG) 9	5H	1	0.00	0.08
Bmac0316	(AC) 19	6H	2	0.02	0.70
Bmag0218	(AG) 6 (AG) 6	6H	2	0.22	0.65
Bmac0018	(AC) 11	6H	3	0.44	0.59
Bmag0009	(AG) 13	6H	5	0.51	0.47
EBmac0806	(AC) 4 (GA) (CA) 8-(CA) 5	6H	4	0.39	0.77
Bmac0040	(AC) 20	6H	2	0.02	0.94
Bmag0007[a]	(AG) 16 (AC) 16	7H	14	0.83	
HVCMA	(AT) 9	7H	2	0.29	0.41
Bmac0273	(AC) 20 (AG) 20	7H	11	0.88	0.59
Bmag0385[a]	(AG) 18 (TG) 10	7H	5	0.54	
Bmag0120	(AG) 15	7H	4	0.53	0.75
Bmac0156	(AC) 22 (AT) 5	7H	17	0.87	0.84
Mean			5.54[c]	0.50[c]	0.64

The SSRs are arranged according to their chromosomal localisation from the telomere of the short arm to the telomere of the long arm of respective chromosomes

[a]Not included in the set of SSRs proposed by Macaulay et al. (2001)

[b]Further information is confidential and subject to commercial license (Scottish Crop Research Institute, Invergowire, Dundee DD2 5DA, Scotland, UK)

[c]Calculated on 41 polymorphic loci

Determination of landrace populations

Due to a limited description provided on the area covered by the landrace samples and a lack of information on the origin of individual landraces within the area (Catalogue of Barley Germplasm Preserved in Okayama University 1983), it was not possible to group the landraces into precise geographic populations.

Therefore, we followed the Bayesian Model-based approach proposed by Pritchard et al. (2000) to assign the landraces into genetically structured groups. The model assumes k number of populations (where k may be unknown) characterized with a set of allele frequencies at each locus that are in Hardy–Weinberg equilibrium. The application tests the presence of a population structure ($k > 1$) and assigns the individuals from the sample population into groups for a given number of populations (k) in a way that Hardy–Weinberg disequilibrium and linkage disequilibrium (LD) is maximally explained. The software package STRUCTURE version 2.0 (Pritchard et al. 2000) was used to perform this analysis. The molecular weight data were used as input file in a haploid format similar to Kraakman et al. (2004). With the knowledge of UPGMA clusters analysis, STRUCTURE software was run for presumed populations (k) from 1 to 12, following the admixture ancestry model. Initially, run length of 10,000-burn-in and 30,000 iterations after burn-in was performed. The run length was increased to 50,000-burn-in and 100,000 iterations after burn-in to achieve consistent results over repeated runs for each value of k and to keep the alpha constant. The run with maximum likelihood was used to assign landraces to groups and to reveal the group membership probability (inferred ancestry) of the landraces. Landraces with ≥ 90% inferred ancestry were considered to constitute a distinct population and those with < 90% were considered as admixtures.

Estimation of genetic diversity and genetic differentiation

The populations identified with the structure analysis were characterized for genetic diversity and differentiation. The genetic variation within each population was described in terms of number of polymorphic SSRs detected, mean number of alleles per locus, specific alleles and thereafter by DI. Nei's (1978) unbiased genetic distances among the populations were computed and UPGMA cluster analysis was performed. All the calculations described above were carried out using the software POPGENE version 1.32 (Yeh et al. 1999).

Population differentiation was quantified with the parameters θ (Weir and Cockerham 1984) which is analogous to F_{ST} (F-statistics, Wright 1951), and R_{ST} (R-statistics, Slatkin 1995). The θ is calculated on the variances of allele frequencies and defines the relatedness of pairs of alleles within a population relative to the total population. In contrast to this, R_{ST} is an estimator of the genetic differentiation based on the variance of allele size and is designed for genetic markers undergoing a strict stepwise mutation model (SMM). F_{ST} and R_{ST} are the most commonly reported parameters to describe population structure; however, they differ in sensitivity when estimated on SSRs (Balloux and Moulin 2002). The F_{ST} basically assumes the infinite allele model (IAM) of the SSRs and allelic equilibrium at loci thereby underestimating the magnitude of differentiation when populations are highly structured or in a situation when SSRs exhibit a high mutation rate. Contrarily, R_{ST} is independent of the mutation rate, however, it suffers from high associated variance, and any deviation from the assumed mutation model (SMM). For comparison and cautious interpretation of the results, both parameters were estimated (F_{ST} and R_{ST}). The software program FSTAT version 2.9.3.2 (Goudet 2002) was used to compute θ and R_{ST} (unbiased, Goodman 1997) without assuming random mating among the samples. A significance test of population differentiation (pairwise θ) and genotypic disequilibrium was performed by randomizing samples to obtain the log-likelihood G-statistics (Goudet et al. 1996). Significance tests were performed by conducting bootstrapping on loci with a 95% nominal confidence interval and sequential Bonferroni correction was implemented for multiple tests (Rice 1989). The linear association between genetic differentiation (pairwise θ) and the geographic distance (hypothesis: isolation by distance) was tested by correlating the (θ/1–θ) matrix against the log-geographical distance matrix as suggested by Rousset (1997). The calculations and test of significance were performed according to the Mantel matrix correspondence test (Mantel 1967) using the software FSTAT version 2.9.3.2.

Results

Allelic variability and polymorphism information content of the SSR markers

Out of the 44 SSRs analysed on 107 landraces, 41 turned out to be polymorphic and 3 monomorphic (HvHVA1, HvLOX and HVLEU). Residual heterozygosity was observed in a total of six genotypes at five different marker loci (Bmag0223, Bmac0029, Bmac0399, Bmac0093 and HVM62). Accessions N-6 and Nepal-7 had the highest number of heterozygous loci (4), whereas, accessions Pisang-5, Pisang-6, Pisang-7 and Pisang-8 showed double bands only for Bmac0399. The number of alleles detected and PIC values of SSRs are given in Table 2. The 41 poly-

morphic SSRs resulted in 227 alleles averaging 5.54 alleles per locus. The highest number of alleles (17) in polymorphic SSRs was scored for Bmac0156 and the lowest (2) for WMC1E8, HvMLO3, EBmac0970, Bmac0040, Bmac0316, Bmac0218 and HVMCA. The highest PIC value was estimated for Bmac0273 (0.88) and the lowest for Bmac0040, Bmac0316 (0.02) with a mean of 0.50 over the 41 polymorphic loci. The markers Bmac0154, Bmac0032, Bmac0113, Bmag0223, Bmac0273, Bmag0007 and Bmac0156 demonstrated a high level of allelic diversity and were found most informative (PIC > 0.80) on the set of genotypes analyzed. A strong positive correlation ($r = 0.76$) was found between the PIC values and the number of alleles detected at each marker locus. The PIC values of the SSRs averaged on each of the seven chromosomes and compared between Nepalese landraces and data by Macaulay et al. (2001) revealed that these values are similar for chromosomes 4H, 5H and 7H, whereas mean PIC values for the chromosomes 1H, 2H and 3H on Nepalese landraces are slightly lower. A contrasting difference was observed for chromosome 6H in this respect with a mean PIC = 0.266 on Nepalese landraces and a mean PIC = 0.686 on the set of cultivars analysed by Macaulay et al. (2001).

Genetic relatedness among the landraces

Using the 44 SSRs it was possible to uniquely fingerprint the landraces except Sipche-4, Sipche-6, Sipche-7 and Sipche-9. The genetic similarity (GS) between landraces varied from GS = 0.24 to GS = 1.00 with an overall mean of GS = 0.50 (pairwise similarity data not presented). The minimum similarity was observed between the genotypes of Sama *versus* Pisang, and Thomje *versus* Pisang. Comparing the mean genetic similarity between landraces within the geographic region, those derived from Sama exhibited the minimum similarity (mean GS = 0.53, range 0.27–0.93) followed by Ngyak (mean GS = 0.57, range 0.40–0.89) indicating diverse landraces. In contrast to this, a high genetic similarity is observed between the landraces of Sipche (mean GS = 0.87, range 0.61–1.00).

The UPGMA cluster analysis revealed two broad groups of landraces (Fig. 2). A small cluster consisted of 13 landraces from Philem, Sama, Thomje and Sipche. While all the others (94 landraces) were grouped in a big cluster. However, landraces can be seen in ten distinct groups at the sub-cluster level. The largest group (I) consists of 26 landraces that are from Annapurna BC, Bimtakothi, Chame, Pisang, Thangja, Thonje and Tilman camp. In this group, all the genotypes from Bimtakothi except Bimtakothi-13 aggregate in a sub-group; however, landraces of other origins do not show consistency in clustering. The group (II) consists of five genotypes, three from Sama, and landraces Ngyak-11 and Lih-Dharna-Gal. Similarly, group (III) consists of all the landraces of Pisang origin,

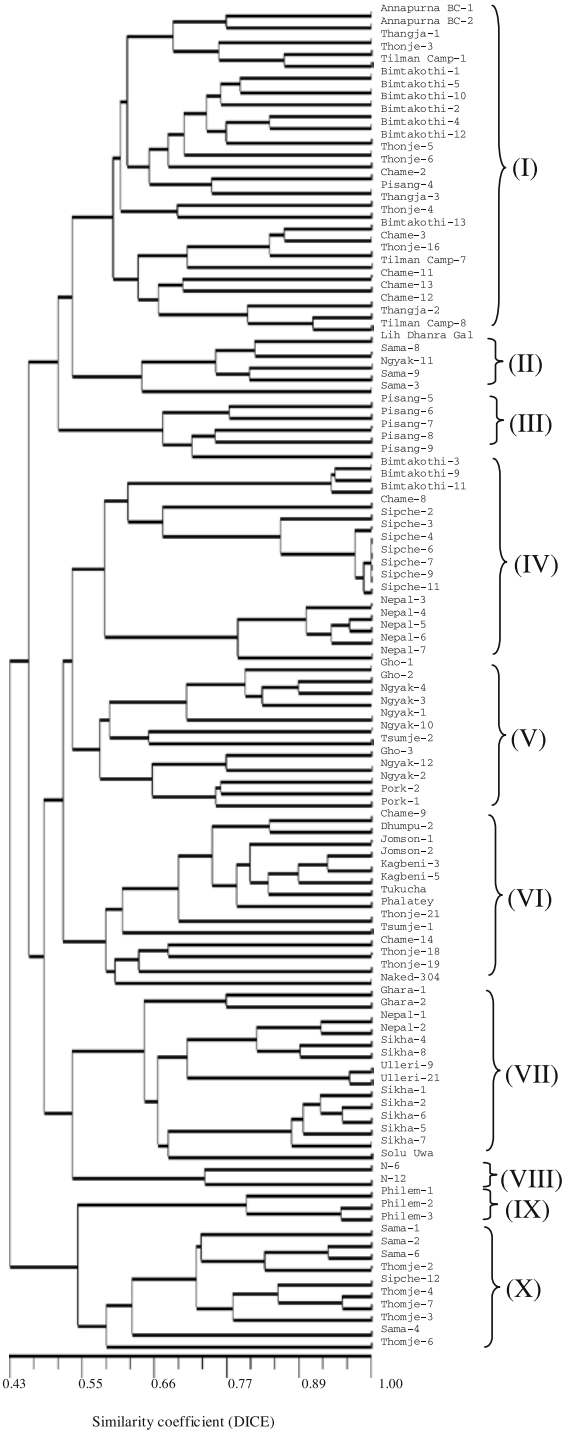

Fig. 2 UPGMA clustering dendrogram of 107 Nepalese hulless barley landraces based on the genetic similarity coefficients. Subclusters are marked with numbers (*I–X*)

except Pisang-4. In contrast to this, group (IV) represents landraces of diverse origin (Bimtakothi, Sipche, Nepal and Chame); however, within this group, landraces are well separated according to their origin. Likewise, group (V) comprised landraces of different origins (Gho, Ngyak, Tsumje and Pork) but unlike in group (IV) the sub-clusters are formed irrespective of the origin. All the landraces of the KaliGandaki (KG) region can be found in group (VI) and (VII). Within group (VI), genotypes from the upper basin of KG (Jomson, Kagbeni and Tukucha) form a distinct sub group, whereas genotypes of different origin, e.g., that of Chame and Thonje, do not converge in clustering. Similarly, group (VII) is comprised of all the landraces of the lower basin of KG (Ghara, Sikha and Ulleri) except the landrace Phalatey which is placed in group (VI) and three other accessions Nepal-1, Nepal-2 and Solu Uwa. The group (VIII) consists of two genotypes, i.e., N-6 and N-12 of unknown origin, and group (IX) consists of three genotypes of Philem origin. Moreover, group (X) is comprised of landraces of diverse origin (Sama, Thomje and Sipche) and none of the sub-clusters in this group represents a specific origin. In general, landraces of common origin are clustered in smaller groups and some of the clusters represent a broad geographic region.

Model-based (MB) groups

The 107 landraces were assigned into nine genetically distinct groups (Fig. 3). Due to large test population and many SSR loci used, the STRUCTURE software required fairly long runs (50,000 burn-in and 100,000 iterations after burn-in) to achieve consistent results. In a range of simulated runs for presumed number of populations (k) = 1–12, the most appropriate number of populations (k) was identified at (k) = 9, where the natural log probability of the data which is proportional to the posterior probability was maximized (–3516.6). Each of the nine groups identified with the structure analysis was named either after the most frequent origin found in the group or the geographic region representative of the landraces in the group. The mean inferred ancestry (%) of nine groups and corresponding geographic origins are given in Table 3. The groups Sikha (1), Nepal (2), Pisang (6), Thonje (7) and Sipche (9) conferred a high mean inferred ancestry (> 90%) and distinct populations were identified from each of these groups. Contrarily, groups Ngyak (3), Sama (4) and Jomson (5) had < 90% mean inferred ancestry, however possessed a number of landraces with ≥ 90% common inferred ancestry and therefore comprised distinct populations.

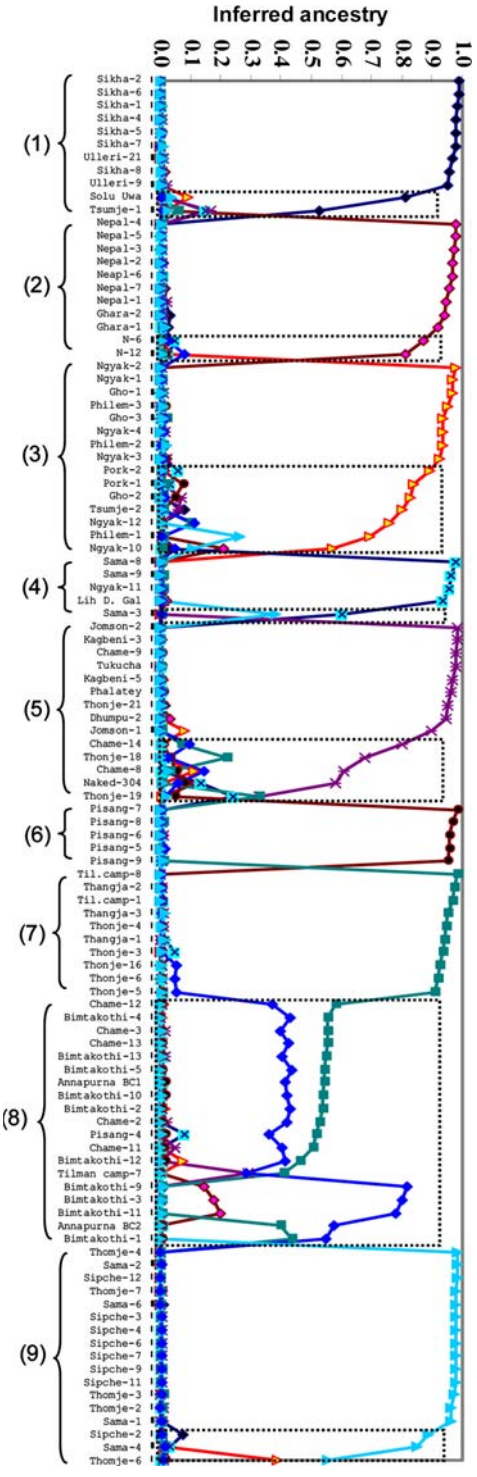

Fig. 3 Inferred ancestry of 107 landraces and the 9 Model-based groups (1–9) identified with the STRUCTURE software (Pritchard et al. 2000). The light areas bounded by the *graphic lines* indicate distinct populations and rectangles denote admixtures

This way, eight distinct populations specific to geographic regions were identified. These populations consisted of landraces from the respective geographic regions with some exceptions (Fig. 3). For instance,

Table 3 Origin and mean inferred ancestry (%) of the nine Model-based groups identified with the STRUCTURE software (Pritchard et al. 2000)

Groups/origins	Mean inferred ancestry (%)								
	Sikha	Nepal	Ngyak	Sama	Jomson	Pisang	Thonje	Bimtakothi	Sipche
(1) Sikha	91.85	0.75	1.21	1.77	2.00	0.42	0.75	0.37	0.86
(2) Nepal	1.15	93.79	0.84	0.95	0.79	0.47	0.33	1.30	0.41
(3) Ngyak	0.96	2.09	86.50	1.08	1.69	1.59	1.41	1.69	2.93
(4) Sama	0.26	0.82	0.30	88.68	0.48	0.44	0.80	0.44	7.78
(5) Jomson	0.39	1.47	1.86	3.14	83.17	1.96	4.81	2.65	0.56
(6) Pisang	0.28	0.36	0.46	0.46	0.56	96.38	0.56	0.66	0.32
(7)Thonje	0.38	0.31	0.34	1.16	0.44	0.38	94.51	1.92	0.60
(8) Bimtakothi	0.31	3.14	1.01	0.94	2.31	0.66	43.25	48.08	0.29
(9) Sipche	0.96	0.43	2.67	0.42	0.42	0.38	0.39	0.45	93.94

Sama-1, Sama-2 and Sama-6 belong to the Sipche population (9) instead of the Sama population (4). Similarly, Chame-9, Thonje-21 and Phalatey are found in the Jomson group (5) and Ngyak-11 is found in the Sama population (4). However, although the geographic locations of Thomje, Thangja and Tilman camp are not indicated in the map (Fig. 1) because of some doubts, the landraces of Thomje that are found in the Sipche group (9) and landraces originated from Thangja and Tilman camp found in the Thonje group (7) are considered to represent the populations of the Sipche and Thonje regions, respectively.

In total, 36.5% of the landraces are found to be mixtures and all the landraces in group (8) turned out to belong to this category. In this group the mean inferred ancestry corresponding to the origin (Bimtakothi) is only 48.08% (Table 3) and a significant proportion is shared with the Thonje origin (43.25%) which is close to Bimtakothi (Fig. 1). Likewise, groups Sikha, Ngyak, Thonje and Sipche shared the largest proportion of mixed ancestry with Jomson (2.00%), Sipche (2.93%), Bimtakothi (1.92%) and Ngyak (2.67%), respectively. This shows an affinity in shared ancestry between the geographically closest populations. However, an exception exists for Jomson that shared 4.81% mixed ancestry with Thonje and 3.14% with Sama. Similarly, Sama had the largest mixed ancestry (7.78%) with Sipche surpassing the nearest group Ngyak (0.30%). The landraces of Pisang (group 6) shared minimum mixed ancestry ($< 1.00\%$) to any other origin.

Comparing UPGMA clusters and the Model-based groups

The GS based UPGMA clusters (Fig. 2) and the MB groups (Fig. 3) are compared. All the landraces of Bimtakothi are placed in MB group (8) by the structure analysis, whereas three landraces namely, Bimtakothi-3, Bimtakothi-9 and Bimtakothi-11 are separated from the others in the UPGMA clustering (GS groups I and IV). However, in a separate analysis performed for all the landraces of Bimtakothi using the STRUCTURE software, two groups were formed and the three landraces mentioned above were clearly separated from the others (data not shown). The MB approach better elucidates the GS group (I), out of which landraces from the Thonje region formed a distinct group, while all the others are found to be mixtures. The GS groups (II) and (III) are consistent with MB groups (4) and (6), respectively. Similarly, GS groups (V), (VI) and (VII) can be compared to MB groups (3), (5) and (1), respectively. The accessions found in these groups are the same with some exceptions. For example, the MB group (5) consisted of an additional landrace (Chame-8) and lacks Tsumje-1 compared to the corresponding GS group (VI). The accession Tsumje-1 is found in the MB group (1). Moreover, landraces Ghara-1, Ghara-2, Nepal-1 and Nepal-2 that are found in the GS group (VII) are not in the respective MB group (1). The MB group (1) lacking these landraces better represents the distinct population of the Sikha region. The GS groups (VIII), (IX) and (X) converged into MB groups (2), (3) and (9), respectively. The MB groups (3) and (9) having the landraces of GS groups (IX) and (X) represent the broader geographic region of Ngyak and Sipche, respectively.

Genetic diversity and population differentiation

Out of the eight populations determined by structure analysis, seven populations of sample size ≥ 5 were selected for genetic diversity and differentiation studies (Table 4). The Sama population that comprised only four landraces was not included in the analysis, therefore. The total of 64 landraces in the seven populations, representing approx. 60% of the whole set of

Table 4 Sample size, mean inferred ancestry (%), mean number of alleles per locus, % polymorphic loci, number of specific alleles and genetic diversity index (DI) of seven populations studied for diversity and differentiation

Populations	Sample size	Mean inferred ancestry (%)	Polymorphic loci[a] (%)	Mean number of alleles per locus	Number of specific alleles	DI
Sikha	9	97.56	60.0	2.25	10	0.435
Nepal	9	96.11	65.0	2.50	3	0.494
Ngyak	8	95.13	75.0	2.50	5	0.498
Jomson	9	96.11	55.0	2.46	8	0.430
Pisang	5	96.40	52.5	2.43	14	0.559
Thonje	10	94.50	72.5	2.60	17	0.489
Sipche	14	97.71	72.5	2.50	10	0.480
Overall	64			4.46		0.539

[a]With respect to 40 polymorphic loci found in the set of 64 genotypes

107 landraces studied, exhibited 40 polymorphic SSRs (97.56%). Likewise, the overall DI of seven populations was estimated at DI = 0.539 compared to DI = 0.536 for the whole sample (107 landraces). The SSR marker Bmac40 turned out to be monomorphic on the set of 64 landraces.

The populations varied for polymorphic loci, mean number of alleles per locus, number of specific alleles and for genetic diversity (Table 4). The highest variation in terms of polymorphic loci and mean number of alleles per locus was observed for Ngyak (75.0%, 2.5) and Thonje (72.5%, 2.6), whereas Pisang (52.5%, 2.43), Sikha (60.0%, 2.25) and Jomson (55.0%, 2.46) were less variable in this respect. Populations Thonje and Pisang demonstrated the highest numbers of specific alleles (17 and 14, respectively), whereas populations Nepal and Ngyak had the lowest numbers of specific alleles, i.e., 3 and 5, respectively. The numbers of specific alleles detected for Sikha, Jomson and Sipche were comparable (Table 4). The highest genetic diversity was estimated for Pisang (DI = 0.559). The DI estimated for Pisang is higher than that of the sub set of 64 landraces or for the whole sample. A comparable level of genetic diversity was observed between populations Nepal (DI = 0.494) and Ngyak (DI = 0.498), and between Thonje (DI = 0.489) and Sipche (DI = 0.480). The populations Sikha and Jomson revealed minimum genetic diversity comparing all the seven populations, i.e., DI = 0.435 and DI = 0.430, respectively.

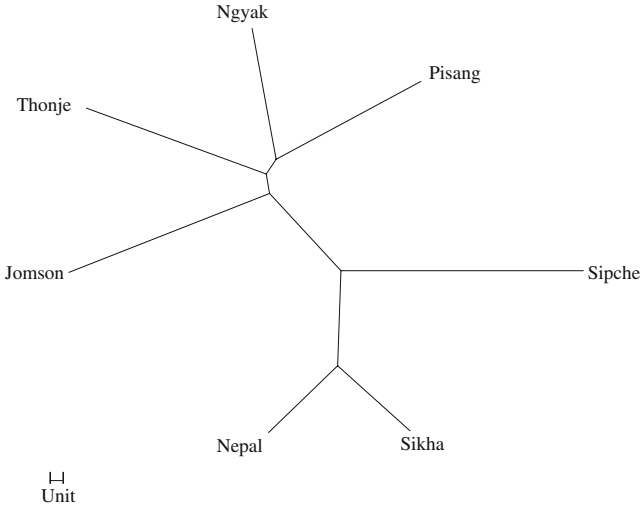

Fig. 4 Genetic relationship among the seven geographic populations revealed with UPGMA clustering on genetic distances (Nei 1978) and displayed as an un-rooted tree diagrams

The genetic relationship among the seven populations is displayed in an unrooted tree diagram, forming two broad groups (Fig. 4). The populations Pisang, Ngyak, Thonje and Jomson are in one group while Sikha, Nepal and Sipche are found in the other. The population Jomson in the first and Sipche in the latter group are, however, more distinct from the others. In a more specific interpretation, landraces from the upper basin of rivers Marshyangdi and BudhiGandaki corresponding to the populations Pisang, Thonje and Ngyak are genetically closer. Similarly, landraces from the lower basin of KaliGandaki (population Sikha) and that of population Nepal (location not known) are also closely related, whereas landraces from the upper basin of the river KaliGandaki, i.e., population Jomson and BudhiGandaki (population Sipche) derived from east of the river are more independent. The population differentiation parameters θ and R_{ST} are estimated at 0.433 and 0.445, respectively. The pairwise population differentiation and significance tests are shown in Table 5.

Out of 21 pairwise combinations among the seven populations, 15 conferred a significant differentiation. Thonje differed from all the other populations, whereas Sipche, Jomson and Nepal differed from all the others except population Pisang. Similarly, Sikha was differentiated from Nepal, Jomson, Thonje and Sipche, and Ngyak was differentiated from Nepal,

Table 5 The population differentiation measured by pairwise θ

Populations	Sikha	Nepal	Ngyak	Jomson	Pisang	Thonje
Nepal	0.310*					
Ngyak	0.473^{ns}	0.335*				
Jomson	0.542*	0.435*	0.387*			
Pisang	0.588^{ns}	0.491^{ns}	0.321^{ns}	0.507^{ns}		
Thonje	0.520*	0.443*	0.335*	0.455*	0.345*	
Sipche	0.462*	0.348*	0.349*	0.476*	0.509^{ns}	0.420*

The lower triangle matrix is the θ calculated for all the population-pairwise combination and significance test performed by permuting genotypes among populations (1,000 randomizations). Multiple significance tests were performed after sequential Bonferroni corrections

*Significant at $P \leq 0.05$; *ns* non-significant

Jomson, Thonje and Sipche. The population Pisang however, differentiated only from Thonje. The linear relationship test between coefficient of genetic differentiation (pairwise θ) and geographic distance revealed a non-significant correlation coefficient ($r = 0.224$, $P > 0.05$) rejecting the hypothesis of isolation by distance.

Discussion

The SSRs used in this study selected from the SSR set proposed by Macaulay et al. (2001) for genotyping in barley proved to be highly informative. In comparison to the results of Macaulay et al. (2001), differences in polymorphism information content (PIC) values were observed (Table 2). The SSRs Bmac0032, Bmac0113, Bmag0223, Bmac0273 and Bmac0156 were highly informative in our experiment with a PIC value of > 0.80, while e.g., Bmac0273 had a low PIC value (0.59) in the work of Macaulay et al. (2001). The mean PIC-value of 41 polymorphic loci (0.50) in our study is comparable to that of Hamza et al. (2004, PIC = 0.45) estimated on 26 Tunisian winter barley accessions and cultivars with 17 SSRs. The PIC of an SSR marker, which is also defined as its capacity to discriminate genotypes, largely depends on the allelic diversity. In this respect, we estimated a correlation coefficient of $r = 0.76$ between the PIC-values and the number of alleles detected. A strong positive correlation ($r = 0.624$) between genic diversity of an SSR locus (~PIC) and the number of alleles detected is also reported by Yu et al. (2003).

The DI on the set of 107 landraces is estimated at DI = 0.536. Russell et al. (2003) reported DI = 0.652 on a set of 125 barley lines collected from various regions of Syria and Jordan, and assessed with 20 SSRs of which 15 are common to our experiment. In a more recent experiment, we have estimated a DI-value of 0.595 on a set of 52 barley cultivars covering a wide range concerning the time of release and representative for the major barley growing areas of the world (East and central Europe, East Asia and North America) using a subset of 30 SSRs analyzed in the present study (unpublished data). Therefore, it may be concluded that a DI estimated at 0.536 on 107 hulless Nepalese barley landraces is moderate to high indicating that the highlands of Nepal are rich in barley diversity. This is in agreement with the findings of Witcombe and Murphy (1986), and Murphy and Witcombe (1986) based on the morphological variation and with results by Konishi and Matsuura (1991) based on isozyme diversity.

The landraces studied are divided into different groups using genetic similarity (GS) based UPGMA clustering and a Bayesian Model-based (MB) structure analysis (Pritchard et al. 2000). The MB method is widely used in population structure analysis for association studies in human and animal genetics. Recently, the method that was developed by Pritchard et al. (2000) and provided as software program STRUCTURE, is frequently being used for population structure analysis or to define genetically distinct groups in crop plants (Remington et al. 2001; Liu et al. 2003; Jain et al. 2004; Lu et al. 2005; Stich et al. 2005). The structure analysis revealed nine groups of Nepalese hulless barley landraces out of which eight distinct populations are identified (Fig. 3). Using a similar approach, Remington et al. (2001) and Liu et al. (2003) were able to differentiate a large set of maize inbred lines into genetically distinct groups with high pedigree conformity.

In our work seven populations out of eight identified are localized in the map and represent specific geographic regions (Fig. 1). The landraces named as 'Nepal' along with the landraces from Ghara constituted a population (MB group 2) which, however, could not be located in the map because the geographic origin of landraces named 'Nepal' was not known. All the landraces of Bimtakothi and many from Chame con-

ferred a highly mixed ancestry (MB group 8). The complex genetic makeup of the landraces of Chame and Bimtakothi detected with the structure analysis can also be seen in the UPGMA clustering as the landraces are widely dispersed in the dendrogram (Fig. 2). The landraces with an inferred ancestry corresponding to a different origin, for example Chame-9 and Thonje-21 found in Jomson (MB group 5), represent freshly introduced populations in the region that can result from seed exchange among the farmers.

Comparing GS based UPGMA clusters and the MB groups, the UPGMA clusters are in accordance to origin only among the landraces sharing high genetic similarity, and the clusters rarely represent geographic groups. On the other hand, the MB approach was able to cluster landraces representing most appropriate geographic groups that can be explained by the information available on landrace origin. Jain et al. (2004) reported highly comparable results of GS based UPGMA clustering and MB structure analysis. Similarly, Lu et al. (2005) found both approaches equally effective to define genetically distinct groups of 145 US rice cultivars, however, concluded that UPGMA clusters have a greater conformity with pedigree data. In this study, a difference existed between GS groups and MB groups, although results are comparable to a larger extent. Based on our results, it can be suggested that structure analysis can define more informative groups than GS based UPGMA cluster analysis when genotypes are of complex origin or the pedigree is not known, e.g., admixture populations and/or gene bank accessions.

The overall DI of seven populations (64 landraces) and that of the whole sample (107 accessions) is estimated at the same level, 0.539 and 0.536, respectively. Likewise, the number of polymorphic SSRs detected among the seven populations remained at 97.6% of the whole sample even after the population size was reduced to 59.81%. This indicates that structure analysis was quite effective to define genetically distinct populations among the 107 landraces. The DI estimations for each of the seven populations varied, and populations from the Marshyangdi and the BudhiGandaki region in the East are more diverse compared to those of the KaliGandaki region in the West (Table 4). The highest genetic diversity existed in the Pisang population (DI = 0.559) which is larger than that estimated for the whole sample or within the sub-set of 64 landraces. This population consisted of five landraces, all are of Pisang origin and the landraces are highly consistent to the origin having < 1.00% mixed ancestry of any other origin (Table 3). Furthermore, considerable numbers of specific alleles were detected within the Pisang population which ranked second after the Thonje compared among all the seven populations (Table 4). Geographically, Pisang is fairly isolated and represents the uppermost basin of the river Marshyangdi (Fig. 1). The region can be considered a hot spot of hulless barley diversity. Schoen and Brown (1991) have emphasized the existence of such hot spots of genetic variation in self-pollinated crops which are of high relevance to the conservation of genetic resources.

The population differentiation parameters estimated, $\theta = 0.433$ and $R_{ST} = 0.445$, indicated a high level of differentiation, and over 40% of the total genetic variation resided among the seven populations. The R_{ST} value is estimated slightly higher than the θ-value. Similar results are reported by Zhou et al. (2003) for different rice populations ($\theta = 0.491$; $R_{ST} = 0.519$). In our study, θ and R_{ST} are comparable suggesting SSRs do not fit strictly to either of the mutation models (SMM or IAM). The population-pairwise differentiation test revealed that not all of the seven populations are significantly different from each other (Table 5). Indicated by the estimated high DI value and not being differentiated from all the populations except Thonje, landraces from the Pisang region possess a broad genetic base and can be considered as a founding population in the highlands of central Nepal. However, highly differentiated landraces found in Thonje which is geographically close to Pisang makes it difficult to explain whether the Thonje population is a descendent of Pisang or evolved independently. The highly differentiated landraces and many conserved alleles found in Pisang and Thonje affirmed the upper valley of river Marshyangdi as origin of hulless barley diversity within the Himalayas range in central Nepal.

When the genetic relationship among the populations is compared to geographic distances, striking results are found. For example, the population Ngyak is found genetically closer to Thonje and Pisang than to Sipche which is geographically closer to Ngyak. Similarly, the populations Sipche and Sikha are genetically close, however, are geographically most distant. These findings are also verified by the test of isolation by distance hypothesis resulting in a non-significant correlation ($r = 0.224$, $P > 0.05$) between pairwise θ and the geographic distance. However, the influence of geographic distance on the genetic relationship can be deduced from the mean inferred ancestry (%) of the MB groups (Table 3). Most of the groups comprised the largest proportion of mixed ancestry from the nearest group indicating adjacent populations shared common parentage to some extent.

The patterns of diversity and genetic relationship among the populations are largely related to the altitude that varies sharply from the South to the North creating a range of agro-ecological environments, e.g., warm temperate climate with high monsoon rain in the South (Sikha) and cool temperate or sub-alpine climate affected by the rain shadow of the Himalayas in the North (upper valleys of KaliGandaki and Marshyangdi) (Fig. 1). The distribution of hulless barley is more frequent in higher altitudes towards the North (Baniya et al. 1997), and its value as the sole food crop increases with the increasing altitude where other cereals cannot be grown successfully (Sharma et al. 1994). In this study, we found the most diverse landraces at the highest altitude in the North, i.e., Pisang (~ 3,500 m). The populations from Thonje, Ngyak and Sipche (altitude ≤ 2,800 m) showed a comparable level of diversity, however, less than that estimated for Pisang (Table 4). The patterns of diversity detected on populations from the upper basins of Marshyangdi, BudhiGandaki and the East of BudhiGandaki are in accordance with the trends of hulless barley distribution in relation to altitude. However, the diversity estimated on populations derived from the KaliGandaki region in the West did not concur with latitudinal variation, i.e., populations from the North (Jomson, altitude ~ 3,000 m) and the South (Sikha, altitude ~ 2,000 m) revealed the same level of diversity, which is significantly lower than the populations from the East (Table 4). The less diverse hulless barley populations observed in the KaliGandaki valley may be due to a high preference for hulled types in this region. The genetic relatedness among the populations also reflected a North–South differentiation pattern with some exceptions (Fig. 4). For example, landraces from the upper basin of KaliGandaki are genetically close to the landraces from the upper basins of Marshyangdi and BudhiGandaki, and are distinct from those originated from the South (Sikha). However, a closer association between the populations Sipche and Sikha, one derived from a higher altitude in the East and the other from a lower altitude in the West, respectively, needs some further explanation. Factors other than agronomic and eco-geographic, e.g., historical and/or ethnicity, may have a role which must be considered in future studies. Moreover, information on the origin of the landraces named 'Nepal' would help to further elucidate the genetic and eco-geographic relationship of the barley populations.

The hulless barley landraces analyzed here correspond to the set of 115 barley genotypes previously analyzed by Pandey et al. (2004) with some SSRs that are common to the present study. In this preliminary study we showed that Nepalese hulless barley landraces are genetically different from western cultivars, i.e., Canadian naked cultivars or German hulled cultivars. Besides this, a contrasting difference in polymorphism on chromosome 6H has been detected in the present study between the Nepalese landraces and the set of western barley cultivars and accessions analyzed by Macaulay et al. (2001). Furthermore, Nepalese hulless barley landraces revealed a considerable number of specific alleles (13%) when analyzed together with a set of 52 barley cultivars originated from the major barley growing areas of the world covering East Asia, Europe and North America (unpublished data). Therefore, these landraces can be considered as an important genetic resource for mainstream barley breeding in the West, particularly for spring types. This will broaden the genetic base of the present barley gene pool and provide novel alleles for the western cultivars.

The Himalayas are well known to harbour a tremendous diversity in cultivated barley and therefore are considered a region of domesticated barley diversification (Badr et al. 2000; Li et al. 2004). The present study also revealed considerable genetic diversity and highly complex genetic structure of the Himalayan barley populations supporting this statement. Genetic differentiation results from the joint effects of mutation, migration, selection and drift, which in turn must operate within the historical and biological context of each plant species. In crop plants, human selection plays a major role in shaping population structure. However, in case of landrace populations which are found in more natural environments, natural selection in response to environmental heterogeneity (biotic and abiotic) is the major cause of population differentiation (Linhart and Grant 1996). The diverse and highly differentiated barley landraces found in the Himalayas can be primarily attributed to the vast eco-geographical diversity prevailing in the region. Furthermore, frequent seed exchange among the farming communities seen in the highland agriculture definitely accelerates the process of diversification and contributes to complicate population structures.

In summary, the highlands of central Nepal, particularly the valleys along the upper basin of the river Marshyangdi (Pisang and Thonje) are rich in hulless barley diversity and landraces in this region are highly differentiated. These landraces have shown resistance reaction against soil borne mosaic viruses (Pandey et al. 2004) and can be used for barley improvement therefore, or for genetic studies to complement existing linkage maps. The hulless barley populations can be used for association studies to localize complex traits of

agronomic importance. Such small, isolated local populations can be more effective for an association study than large cosmopolitan populations (Nordborg et al. 2002).

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