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Random genomic scans at microsatellite loci for genetic diversity estimation in cold-adapted *Lepidium latifolium*

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Abstract

Lepidium latifolium L. (*Brassicaceae*) grows successfully in a high-altitude cold arid environment. Little molecular data are available for this plant despite its immense ecological importance as a cold- and drought-adapted species. We used a novel approach to identify microsatellite regions using genome walker libraries, called as Random Scans at Microsatellite Regions (RaSMiR), and implemented them on genotypes collected from relatively different topographical conditions within a small geographical area. The success rate of finding a microsatellite sequence using this methodology was 100%, and on developing the RaSMiR technique itself as a molecular marker, 230 electrophoretic bands were obtained using 13 different RaSMiR primers in combination with a microsatellite sequence primer. On an average, 17 bands were obtained for each primer. The electrophoretic profiles generated by RaSMiR markers were distinct from those produced by inter-simple sequence repeat markers. This information has been documented as a dominant marker data, and has been used to construct a neighbour-joining tree that successfully distinguished all genotypes. RaSMiR is an attractive approach for the development of unique and informative microsatellites, or for genome scanning directly as a molecular marker that can potentially be employed for the estimation of genetic diversity or to identify polymorphic loci involved in adaptations particularly in the non-model species, for which sufficient genomic data are not available.

Keywords: cold stress; genome scan; *Lepidium latifolium*; microsatellites; Random Scan at Microsatellite Regions

Introduction

Lepidium latifolium L. (*Brassicaceae*), commonly known as pepperweed or peppergrass, is an invasive plant (Reynolds and Boyer, 2010) native to Western Asia and Southeastern Europe. It is also prominent in Western Himalayas, especially in the high altitude and harsher climate of the cold desert Ladakh (minimally 9800 ft above sea level (asl); 86,904 km^2), with temperature extremes ranging from −35°C in winter to up to 35°C in summer. Similar to native flora and fauna of this region, *L. latifolium* also adapts well to a number of abiotic stresses, namely low temperature, drought, solar radiations, high altitude, etc. High mountain ranges in the north as well as in the south have proven geographical barriers for the biota of the region, thus isolating the species.

Most of the above-ground parts of this plant are edible, and in Ladakh, the spring leaves of this plant are consumed as vegetables. *Lepidium* may be found growing well with green leafy appearance surviving under snow even during winter in Ladakh, when most other plants

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shed their leaves (Gupta *et al.*, 2012). Unfortunately, despite the immense potential of the plant from agronomic and ecological point of view, *Lepidium* has never been in the focus of the scientific community, and absolutely no efforts have been made to understand the molecular biology, genetics or genetic variability of this plant (Gupta *et al.*, 2012). The objective of the present study is to estimate genetic diversity in *Lepidium* based on microsatellites as markers. The benefit of using microsatellites lies in their hypervariable nature that can successfully detect variability within a population distributed in a small geographical area.

Microsatellites or simple sequence repeats are abundant and ubiquitous entities in eukaryotic genomes (Grover *et al.*, 2012). Comparatively higher rates of mutations at these loci compared with other genomic regions constitute the source of variation between individuals of a species, and thus these can be employed as suitable molecular markers. Conventionally, these are isolated from size-selected or enriched genomic libraries of the species under investigation by screening several thousands of clones through hybridization with microsatellite probes (Zane *et al.*, 2002). In recent years, *in silico* mining has been highly advocated for the identification of microsatellites and the development of microsatellite markers (Grover *et al.*, 2012). This is an excellent approach, while working with model species (Sharma *et al.*, 2007); however, its use is limited when working with non-model species (Grover *et al.*, 2010).

As little information is available in the case of *L. latifolium*, we used an innovative strategy, wherein we extracted microsatellite regions from genome walker libraries and used them in DNA fingerprinting studies thereafter, thereby enhancing the success rate of developing microsatellites to 100%. We call this new technique as Random Scans at Microsatellite Regions (RaSMiR). The following sections detail the methodology followed for the development of RaSMiR markers, and their employment for the demonstration of genetic diversity in *L. latifolium*.

Materials and methods

Plant material

Seeds of *Lepidium* plants were strategically collected from different parts of the Leh city and its nearby vicinity. *Lepidium* plants growing in relatively different topographical conditions, i.e. in a valley, on a rocky surface, on the roadside, on a sun-facing slope and from the sunshade, were randomly selected. The seeds were germinated in our laboratory on half Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) jellified using agarose and the plants were maintained at 25°C under white light in 16/8 h light and dark cycles. In total, 30 plants from different sites were selected for the analysis (Table 1).

Development of microsatellites for a random genomic scan

DNA was extracted from *Lepidium* plants using the N′, N′, N, N-Cetyl trimethyl ammonium bromide (CTAB) method of Saghai-Maroof *et al.* (1984). Genome walker libraries were prepared using a commercial kit, following the manufacturer's protocol (Clonetech, USA). Polymerase chain reaction (PCR) was carried out with the primer sequence 5′-GCGCGAAATTAACCCTCACTAAAG-3′ (T3), which is complementary to the adaptor used in the kit and either of the microsatellite primers 5′-GAAGAAGAA-GAAGAAGAA-3′ (AG001) or 5′-CTCTCTCTCTCTCTCT-CTCT-3′ (AG002). The PCR was carried out in a total volume of 25 μl, which was constituted of 10 pmol of T3 primer and 30 pmol of either AG001 or AG002 primer, 20 μM of deoxyribonucleotides (dNTP) mix, 1.5 mM $MgCl_2$, 1 × *Taq* polymerase buffer and 1U *Taq* polymerase. Thermal cycling conditions consisted of an initial denaturation of 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, reannealing for 30 s at 43°C when AG001 and T3 primers were used, and at 55°C when AG002 and T3 primers were used, followed by elongation at 72°C for 30 s. A final elongation for 10 min was carried out at 72°C. Amplicons were cloned in a pDrive cloning vector (QIAGEN, Germany) using the QIAGEN PCR Cloning Kit (QIAGEN, Germany), following the manufacturer's protocol. Recombinant vectors were transformed in competent cells of *E. coli* strain DH5α using heat shock at 42°C for 90 s followed by incubation on ice for 5 min. Cells were initially grown in Luria Bertani (LB) medium for 1 h, and later plated on LB Agar plates supplemented with 100 mg/ml of ampicillin, 50 μg/ml 5-Bromo-4-CHloro-3-Indolyl-β-D-galactopyranoside (X-Gal) and 0.5 mM Isopropyl-β-D-thiogalactoside (IPTG). Colony PCR was carried out to determine the size of the insert. Colonies with insert sizes 300–500 bp were sent for sequencing to commercial service providers.

DNA sequencing, preliminary sequence analysis and primer design

Clones and amplicons were sent for sequencing to three different commercial DNA sequencing centres in India: first, at the DNA Sequencing Facility in the Department of Biochemistry, University of Delhi South Campus, New Delhi; second, at Vimta Labs, Hyderabad; third, at

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Table 1. Description of collection sites of the seeds, whose harvested plants were used in the present study

Sample ID	Description of the collection site
1	DIHAR premises; valley
2	DIHAR premises; valley
3	DIHAR premises; valley
4	DIHAR premises; valley
5	DIHAR premises; valley
6	DIHAR premises; valley
7	DIHAR premises; valley
8	DIHAR premises; valley
9	Sun facing slope near DIHAR, Leh
10	Sun-facing slope near DIHAR, Leh
11	Sun-facing slope near DIHAR, Leh
12	Sun-facing slope near DIHAR, Leh
13	Sun-facing slope near DIHAR, Leh
14	Sun-facing slope near DIHAR, Leh
15	Sun-facing slope near DIHAR, Leh
16	2 km from Leh on the Leh–Manali Highway
17	5 km from Leh on the Leh–Manali Highway
18	Growing between Spituk and Nimu on the Leh–Srinagar Highway
19	Growing between Spituk and Nimu on the Leh–Srinagar Highway
20	Growing between Spituk and Nimu on the Leh–Srinagar Highway
21	Rocky surfaces at Nimu
22	Rocky surfaces at Nimu
23	Rocky surfaces at Nimu
24	Rocky surfaces at Spituk
25	Sunshade area near Choglamsar
26	Sunshade area near Choglamsar
27	Sunshade area near Choglamsar
28	Roadside in Leh
29	Rocky surfaces at Spituk
30	Sun-facing slope within DIHAR, Leh

DIHAR, Defence Institute of High Altitude Research, Leh.
GPS coordinates of the above-mentioned sites: 1. Choglamsar: 34°06′38.89″N; 77°35′16.08″E; 10,620 ft asl. 2. DIHAR: 34°08′23″N; 77°34′26″E; 11,500 ft asl. 3. Leh: 34°08′43.43″N; 77°34′03.41″E; 11,500 ft asl. 4. Spituk: 34°07′31.8″N; 77°31′34.6″E; 10,852 ft asl. 5. Nimu: 34°11′38.90″N; 77°20′07.80″E; 10,320 ft asl. Based on the collection sites, genotypes were classified into eight groups: I, genotypes 1–8; II, genotypes 9–15 and 30; III, genotypes 16 and 17; IV, genotypes 18–20; V, genotypes 21–23; VI, genotypes 24 and 29; VII, genotypes 25–27; VIII, genotype 28.

Eurofins, Bangaluru. Sequences were obtained in FASTA format. Microsatellites were screened visually in sequences, and 'sense primers' (or reverse primers) were designed using Primer3 (frodo.wi.mit.edu/cgi_bin/primer3/primer3_www.cgi). Primers were designed at least 30 bp away from the microsatellite. An effort was made to keep the annealing temperature ($T_m - 5$)°C of primers as about 55°C and %GC close to 60. T_m was calculated following the formula: (2(A or T) + 4(G or C))°C.

PCR assay and allele detection

Amplification was carried out in a 25 µl reaction mixture containing 200 µM dNTP, 1 × *Taq* polymerase buffer (Bangalore Genei, India), 1.5 mM $MgCl_2$, 50 ng genomic DNA, 1U *Taq* polymerase (Bangalore Genei, India) and 10 pmol each of standard length forward and reverse primers. Reactions were carried out using standard PCR conditions of denaturation, reannealing and extension. The PCR-amplified products were run on 2.0% agarose gels and stained using ethidium bromide. Polymorphic bands were scored based on the presence or absence of bands for each of the genotype. Only clear and unambiguous bands were scored. The sizes of the bands were determined based on their migration relative to molecular weight size markers (1 kb ladder plus; Fermentas, Lithuania).

Data analysis

The frequency of polymorphism was calculated based on the presence or absence of common bands between different genotypes for each type of markers. Popgene

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(version 1.32) was used for the calculation of heterozygosity (*h*; Nei, 1973) and Shannon's diversity index (*I*; Lewontin, 1972), and a neighbour-joining (N-J) tree based on Nei's (1978) genetic distance method and the unweighted pair group method with arithmetic means (UPGMA) (Sneath and Sokal, 1973) was constructed.

Results

Mining microsatellite regions

In the first phase of the experiment, we amplified a region lying between a microsatellite and the ligated anchors (see Supplementary Fig. S1, available online only at http://journals.cambridge.org) using the anchor-specific primers and a microsatellite oligonucleotide primer from the genome walker library template. The amplified region was sequenced and on sequencing, a 100% success rate of isolating a microsatellite was observed. A primer was designed from the region lying between the anchor and the microsatellite (see Supplementary Fig. S1, available online only at http://journals.cambridge.org). In the second phase, this primer was used to amplify the microsatellite region again with the anchor primer from the other end, using the same genome walker library as the template (see Supplementary Fig. S1, available online only at http://journals.cambridge.org). However, as we were interested in the random genome scans at the microsatellite regions, we designed only one primer after sequencing the cloned regions for each of the microsatellite-containing inserts (Table 2). To induce randomness during the genome scan, the microsatellite by itself was used as the second primer. We named the marker system thus developed as RaSMiR. To ensure that the observed amplicons are different from the ones that could have been obtained using only microsatellite primers (inter-simple sequence repeats; ISSR), single primer amplifications were also carried out (Fig. 1).

Analysis of diversity at repeat regions

The number of scorable bands for each primer varied from 15 in the case of B28 to 27 in the case of B8 (Table 3). Both ISSR markers produced 16 bands each in the range of 225–3000 bp (Fig. 1). Thirteen specific microsatellite primers in combination with one ISSR primer produced 230 bands (Table 3) with an average 17 bands per primer. The mean *h* or Nei's (1973) gene diversity of RaSMiR markers was computed as 0.2793 and mean Shannon's information index (*I*) (Lewontin,

Table 2. Data on microsatellite discovery and PCR primers for each of the new markers developed

S.No.	Microsatellite ID	Microsatellite motif	Primer used in microsatellite discovery (5′-3′)	Specific primer designed (5′-3′)
1	A4	$(AG)_5$GGGAGG $(GA)_{35}$GG$(GA)_7$	T3 and AG001	TGATGGAAGAAAAGCCAAGG
2	B7	$(TTC)_6$	T3 and AG002	TGAGACTAATGGCCCAAAGG
3	B8	$(GAA)_6$	T3 and AG002	TAACAGCGCTTGAACGTGAT
4	B9	$(TTC)_6$	T3 and AG002	TAACAGCGCTTGAACGTG AC
5	B11	$(GAA)_6$	T3 and AG002	Could not be designed
6	B19	$(GAA)_6$	T3 and AG002	GGTGGACTTGAAGGCAATGT
7	B20	$(TTC)_6$	T3 and AG002	TAACAGCGCTTGAACGTCAC
8	B23	$(GAA)_6$	T3 and AG002	TTTGTGAGCGCTTTGATG TC
9	B24	$(GAA)_6$	T3 and AG002	TTCCTCTCATTTCACTCA CACC
10	B25	$(GAA)_6$ $N_{15}(GA)_4$	T3 and AG002	TATGTTTTGCACTCCCAGCA
11	B28	$(GAA)_6$	T3 and AG002	TAACAGCGCTTGAACGTCAC
12	B32	$(GAA)_7$	T3 and AG002	CCACCACCAAATTTTCTTCC
13	B35	$(GAA)_6$	T3 and AG002	CACCCTCTTCTCCCTCACAT
14	B37	$(GAA)_6$	T3 and AG002	Could not be designed
15	C1	$(GAA)_6$	First PCR using (T3 and AG001, and the product re-amplified with AG001 and AG002	ATGGTGGCTTGTGTCTAGGG
16	C4	$(GAA)_6$	First PCR using (T3 and AG001, and the product re-amplified with AG001 and AG002	CTGGAAGAGGCTTCGTTGTC
17	C8	$(GAA)_6$ CAA(GAA)	First PCR using (T3 and AG001, and the product re-amplified with AG001 and AG002	Could not be designed
18	P2	$(GAA)_4$ $(TC)_4$GA$(TC)_5$	*T3* and *LlaCIPK*MR	Not Applicable

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1972) was 0.4295. These values changed only marginally (0.2786 and 0.4289, respectively), when combined mean of RaSMiR and ISSR markers for the values of *h* and *I* were calculated.

Based on the site of collection, we had tentatively classified all the genotypes into eight different groups, and most of the members in groups I and II were clustered together and these two groups were also placed closer to each other, as expected (Fig. 2).

Discussion

We have demonstrated an innovative method for mining of microsatellites from a non-model plant species. Conventionally, genomic microsatellites are isolated from the size-selected genomic libraries (Zane *et al.*, 2002). This approach requires screening of several thousands of clones through colony hybridization with microsatellite repeat containing probes (Rassmann *et al.*, 1991). However, the portion of the genome screened using this method is very small (<1%), and the success of finding a microsatellite is even lesser (~0.001%) as estimated by Ashkenazi *et al.* (2001) and Grover *et al.* (2009) for potato. To overcome this disadvantage, most workers have switched over to the construction of enriched libraries for the isolation of microsatellites, which improves the success rate by 50% (Grover *et al.*, 2010). There have also been reports of isolating microsatellite sequences using other approaches such as fast

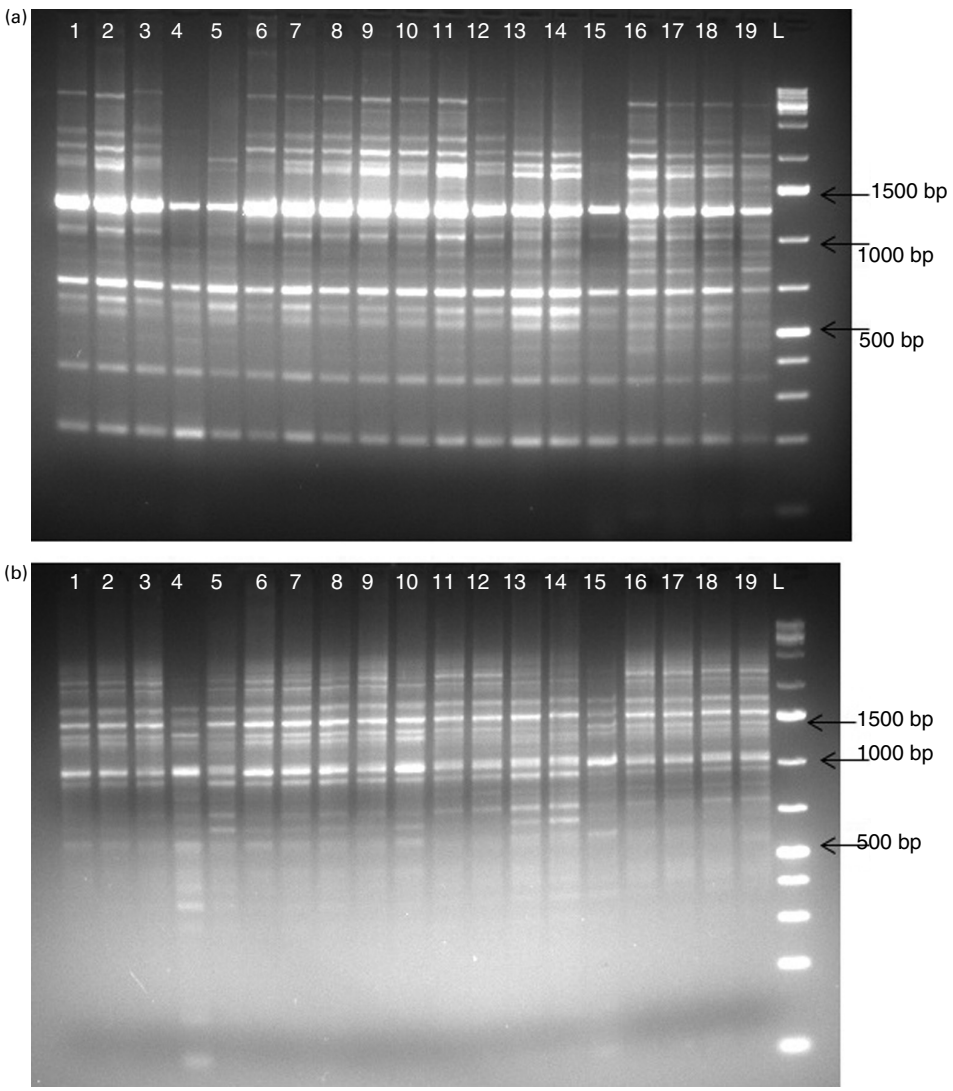

Fig. 1. (a) Representative photograph of electrophoretic mobilities shown by amplicons obtained using primers AG001 and B8. (b) Representative photograph of electrophoretic mobilities shown by amplicons obtained using primer AG001. In both (a) and (b), lanes 1–19 are loaded with amplicons obtained from genotypes 1–19, as listed in Table 1. Lane L is loaded with a 1 kb ladder (Fermentas, USA).

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Table 3. Polymorphism displayed by RaSMiR markers in *L. latifolium* genotypes

Microsatellite ID	Second primer used for genotyping (5′-3′)	Number of Bands	Product size range (bp)	h	I
A4	AG001	16	225–3000	0.3073	0.4661
B7	AG001	17	250–3000	0.3906	0.5713
B8	AG001	27	200–4000	0.2258	0.3615
B9	Primer sequence too similar to that of B8, hence not utilized further				
B11	No amplification obtained				
B19	AG001	16	250–3000	0.2633	0.4138
B20	AG001	16	200–3000	0.2343	0.3832
B23	AG001	18	300–3000	0.2014	0.3224
B24	AG001	16	225–3000	0.2674	0.4157
B25	AG001	17	150–2700	0.2803	0.4307
B28	AG001	15	150–3000	0.2788	0.4290
B32	AG001	18	150–3000	0.3037	0.4569
B35	AG001	15	200–3000	0.2704	0.4197
B37	No amplification obtained				
C1	AG001	23	150–4000	0.2877	0.4345
C4	AG001	16	150–3000	0.3197	0.4794
C8	Primer sequence similar to that of C4; not utilized further				
P2	*LlaCIPK*F and *LlaCIPK*MR	01	661	Not included in analysis	
ISSR1	AG001	16	350–2500	0.2844	0.4357
ISSR2	AG002	16	225–3000	0.2634	0.4131

h- Heterozygosity
I- Shannon diversity Index
*LlaCIPK*F and *LlaCIPK*MR are specific primers for amplification of upstream sequence of CIPK gene from *Lepidium latifolium*, with following sequence-
*LlaCIPK*F- 5′-GTC GCA GCT TTA GCT TTT GC-3′
*LlaCIPK*MR- 5′-GGA GCT CCA CGA TGT TCG-3′

isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) (Zane *et al*., 2002) and a highly successful data mining from sequence databases (Aishwarya *et al*., 2007; Sharma *et al*., 2007). However, FIASCO suffers from the disadvantage that it relies on AFLP, which includes either the use of radioactivity or fluorescence, and data mining from sequence databases, at present, is possible only for a handful of species. Enrichment methodology that we have adopted here interestingly overcomes most of the disadvantages discussed above. While the success rate of following the procedure is 100%, it does not require the use of any labelling technique and can be adopted for any species.

The strategy used in this study may be grouped with PCR amplification-based molecular marker techniques that attempt to scan the genomes. The most popular among these techniques are random amplified polymorphic DNA (RAPD, Williams *et al*., 1990) and AFLP (Vos *et al*., 1995). Several modifications and derivatives of both RAPD and AFLP combining advantages of high polymorphism levels of microsatellites have been demonstrated in the past, and the most popular among them is ISSR (Meyer *et al.,* 1993). In a relatively complicated technique called anchored microsatellite-primed PCR, amplification products are run on polyacrylamide gel in place of agarose gel (Zietkiewicz *et al*., 1994). Siebert *et al.* (1995) cloned ISSR products and sequenced them for designing nested primers from one flanking region, calling the modified technique as dual-suppression PCR. Using the methods similar to ours, a second primer could be designed. Further modifications of RAPD and ISSR include random amplified microsatellite polymorphism (RAMP, Wu *et al*., 1994), double-stringency PCR, a variant of RAMP (Matioli and de Brito, 1995), PCR isolation of microsatellite arrays (PIMA) depending on the cloning of RAPD products in a T-vector and screening as well as the amplification of microsatellites using colony PCR (Lunt *et al*., 1999) and random amplified hybridization microsatellites (RAHM) involving arbitrarily or microsatellite-primed PCR with microsatellite hybridization (Cifaraelli *et al*., 1995). RAHM is also variously known as RAMP (Richardson *et al*., 1995) or randomly amplified microsatellites (Hantula *et al*., 2007).

Selective amplification of microsatellite polymorphic loci (SAMPL), introduced by Morgante and Vogel (1994), is similar to AFLP with one of the primers being compound microsatellite specific in second amplification. Microsatellite–AFLP is a further deviation of SAMPL, in which a 5′-anchored microsatellite primer is used, resulting in about 100 bands on a sequencing gel (Yang *et al*.,

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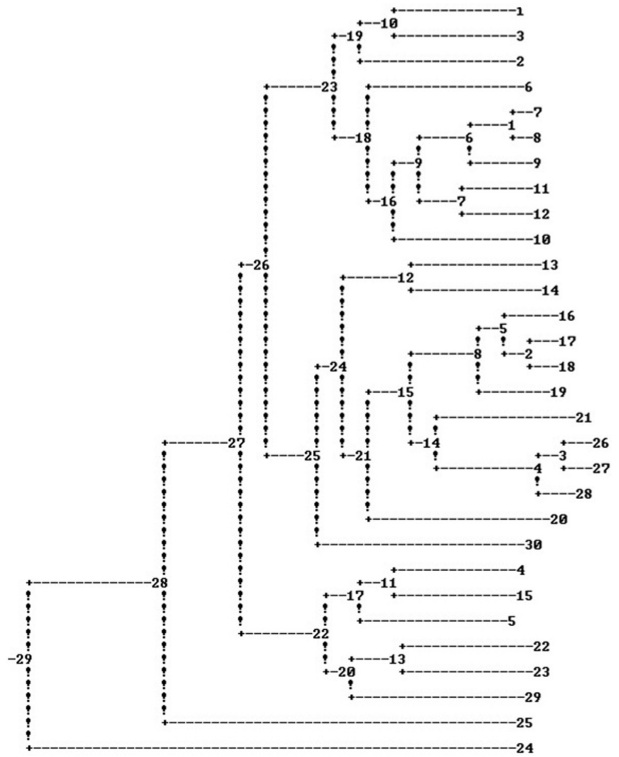

Fig. 2. N-J tree constructed based on Nei′ (1973) genetic distance and UPGMA algorithm. The digits at the end of the branches correspond to genotypes mentioned in Table 1.

2001). Selectively amplified microsatellite (SAM) analysis, described by Hayden and Sharp (2001), is comparable to the methods employed by us in terms of success of mining microsatellite sequences, and the reliance on a single locus-specific primer. However, our protocol is shorter than the SAM, for the development of microsatellite markers. Acquadro *et al.* (2005) modified the SAM protocol (called the microsatellite amplified library) to generate both the primers for the development of microsatellites, which is similar to our method that is open for exploitation for the development of the second primer.

Microsatellites thus developed have been implemented for the demonstration of genetic diversity in a representative sample of the *Lepidium* population distributed in a small geographical area (Table 1). The plants were collected from topographically distinct places in the Leh region. *Lepidium* easily grows in all kinds of terrains including on the rocky surfaces. We expected the genotypes collected from nearby places to be clustered together (null hypothesis), i.e. we expected groups I, II and VII to be clustered together, and groups IV, V and VI to be clustered together. However, on drawing the N-J tree, we found that the clustering of genotypes deviated from our expectations, and thus the null hypothesis was proved to be wrong (Fig. 2). As trees are the outcome of mapping and clustering the microsatellite alleles together, first in the form of a matrix and then in a phylogenetic tree (carried out using some software), the distribution of microsatellite alleles thus appeared random and did not show any association with the genotypes collected from a particular site. However, it is very difficult to ascertain whether microsatellite alleles provide any selective advantage, or microsatellite mutations are simply following a neutral mode of evolution. Proving the evolutionary and ecological significance of microsatellites is a difficult task. If a microsatellite occurs within a gene sequence or is conserved in its regulatory region, its functionality can be studied (Vasemagi *et al.*, 2005; Edelist *et al.*, 2006; Olafsdottir *et al.*, 2007; Makinen *et al.*, 2008).

Generally, microsatellite loci showing no polymorphism are considered to be of recent origin. From the evolutionary point of view, this could be the result of several bottlenecks, which might be further regulated either by the environment or by the vitality of the molecular or meiotic function of the locus involved (Grover and Sharma, 2011). Such loci apparently do not play any role in the evolution of the species (Grover and Sharma, 2011). However, such conclusions must be drawn with caution, as a microsatellite associated with an important region of the genome (from the evolutionary point of view) can significantly display lower variability during genetic drift and selective sweeps (Sclotterer, 2003), leading to allele excess.

In the present study, both hypervariable and nonmutable loci were observed (Fig. 2(a)). Functional vitality might be the determinant of their differential mutabilities. Genome scan methods, as studied here, have a high potential to identify the loci involved in adaptations as well as the ones that drive genome divergence. However, an experimental validation using selection experiments is recommended.

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