localized the *S*-adenosyl-L-Met : benzoic acid carboxyl methyltransferase to the cytoplasm of the epidermal cells of the snapdragon flower. The enzyme was located, as we have noticed for the caffeine NMTs in close association with the vacuole surface. Pimenta *et al.*[20] showed that though most of the barely *O*-methyltransferase was cytoplasmic, a portion was adsorbed on the vacuole.

In this study, the sub-cellular targetting of NMTs in coffee was demonstrated using GUS reporter constructs driven by NMT promoter with deletion of the first exon and by immunocytolocalization. We would expect that the different enzymes involved in caffeine production and also that of CGA would co-exist at the same site. Recently, we have reported genetic transformation of coffee using *Agrobacterium rhizogenes* harbouring a binary vector[21]. The findings of this study would be helpful in understanding the regulation of caffeine biosynthesis and also to utilize the NMT promoters to alter the expression of individual NMT genes in transgenic coffee plants in a tissue-specific manner.

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Population structure and genetic relationships among *Delphinium* species – A fast dwindling genus from northwestern Himalaya

Kamal Dev Sharma*, B. M. Singh and Jyoti Saroop

Advanced Centre of Hill Bioresources and Biotechnology,
CSK Himachal Pradesh Agricultural University, Palampur 176 062, India

Delphinium* is a medicinally important genus, several species of which have been reported from the Himalayan region. We estimated population size and possible erosion of *Delphinium* species from Himachal Pradesh, northwestern Himalaya. The study elucidated genetic diversity in *D. denudatum* and studied phylogenetic relationships among *Delphinium* species inhabiting the area. The region was reported to inhabit seven *Delphi-

*For correspondence. (e-mail: kml1967@rediffmail.com)

***nium* species. However, only three, namely *D. brunonianum*, *D. vestitum* and *D. denudatum* were sighted during the present survey. Other species like *D. cashmerianum*, *D. kolzii*, *D. pyramidale* and *D. roylei* were not sighted from the reported and surveyed new locations. Among the three species recorded, two, namely *D. brunonianum* and *D. vestitum* had small populations of 16 and 150 individuals respectively. These species require proper conservation measures. *D. brunonianum* was not found in previously reported locations. However, it was sighted at a new location, i.e. Rohtang. Similarly, *D. vestitum* was also not sighted at one of the previously reported locations. The third species, i.e. *D. denudatum* exhibited relatively larger populations with density of 1.0 to 5.0 individuals/sq. m and did not have immediate threat. *D. denudatum* populations in the study area have evolved from two lineages as revealed by RAPD data. RAPD markers also differentiated the three *Delphinium* species recorded from the region.**

Keywords: *Delphinium*, phylogenetic relationships, population structure.

DELPHINIUM species of the family Ranunculaceae, commonly known as larkspurs, are medicinally important. Pastes, extracts of flowers and roots of many species of this genus have been used traditionally as insecticides, as ingredient of drugs for dysentery and diarrhoea, tonic for toothache, cardiac and respiratory depressant and stimulant. Several alkaloids, which are derivatives of norditerpenoid lycoctonine, have been isolated from roots and leaves of *Delphinium* species[1–3]. Some of these alkaloids possess antifungal activities against human and plant pathogenic fungi, and antifeedant properties to insects *Spodoptera littoralis* and *Leptinotarsa decemlineata*[2,4]. Biomolecules from *D. denudatum* are also being studied for cure of human diseases. Aqueous extracts from roots exhibited properties of antileptic drugs, anticonvulsant activity against seizures[5,6] and antihepatotoxicity[7]. *D. denudatum* extracts have also been implicated in morphine deaddiction[8].

The Himalayan region in India is home to several *Delphinium* species that are adapted from subtropical to temperate climatic conditions[9,10] [Herbarium of Forest Research Institute (FRI), Dehradun and Herbarium of Botanical Survey of India (BSI), Dehradun]. Population density estimates on *Delphinium* species in the Himalayan region have not been made so far, except by Kala[11], who reported 11.5–12.0 plants/sq. m of *D. cashmerianum* from Pin Tud and Tarbak eco-climatic zones of Spiti Valley in Lahaul and Spiti. In this context, identification of new locations and confirmation of earlier reported ones for occurrence of *Delphinium* species, and estimates of population density at different locations can reveal the current status of *Delphinium* species in the region. The information can help to devise conservation strategies for species with low population density, if necessary. Further, considering difficulties in identification of the genus *Delphinium*, it would be worthwhile to use molecular markers to differentiate *Delphinium* species. Molecular markers can also be employed for understanding the lineage of the taxon. The present study, therefore, focuses on: (i) estimation of population density of *Delphinium* species in Himachal Pradesh (HP), northwestern Himalaya which spans over 55,000 sq. km, (ii) elucidation of efficacy of RAPD markers in differentiating species inhabiting the study area, and (iii) to study genetic diversity within *D. denudatum*, which is medicinally the most important species.

The study area has nine districts (Hamirpur, Bilaspur, Kullu, Mandi, Shimla, Solan, Sirmaur, Kangra excluding Bara and Chhota Bhangal area, and Lahaul region of Lahaul and Spiti) of HP (30°22′40″–33°12′40″N lat. and 75°47′55″–79°04′20″E long.). The study area lies in four physiographic regions, i.e. Sub Himalaya, Lesser Himalaya, Greater Himalaya and Trans-Himalaya, which represent four climatic conditions – sub-tropical (elevation ≤650 m asl, average rainfall 1110 mm), sub-temperate (elevation 651–1800 m asl, average rainfall 1500 cm), temperate-wet (elevation ≥1800 m asl, average rainfall 1000 mm) and temperate-dry (elevation ≥1800 m asl, average rainfall 250 mm).

The study area was surveyed for three consecutive years (1999–2001). During the first year, the survey was conducted to those locations (20 in number) where *Delphinium* species have previously been reported[9,10] (herbaria of FRI and BSI; Tables 1 and 2). During successive years, previously reported as well as 11 new locations with habitat conditions similar to those of the reported habitats were surveyed. At each location, 99 quadrats (1 m × 1 m) were placed randomly and individuals of *Delphinium* species were counted. Population density, i.e. individuals per unit area was calculated for each location as mean of 99 quadrats. In case of small populations (i.e. *D. vestitum* and *D. brunonianum*), population size was determined as total number of individuals. Species identification was confirmed with the specimens available at the herbaria of FRI and BSI.

Genomic DNA was isolated from young leaves of *Delphinium* plants using the CTAB mini-prep method[12]. Seventeen individuals of *D. denudatum* from three locations (see Figure 1) were used to elucidate genetic diversity within *D. denudatum*. After initial screening of 42 decamer primers (Operon Technologies Inc., CA, USA) using genomic DNA of two plants from two locations, six (OPB-1, OPC-2, OPC-5, OPC-7, OPC-16 and OPC-19) were selected for PCR amplification of genomic DNA of each of the 17 individuals. PCR amplifications were performed in 25 µl volumes containing 2.5 µl reaction buffer (pH 8.8), 0.4 units *Taq* DNA polymerase, dNTP mix containing 0.2 mM each of dCTP, dGTP, dATP and dTTP (Bangalore Genei, Bangalore, India), 5 pm primer and 50 ng genomic DNA. Amplifications were performed in a PCR machine (DNA Engine PTC 200, MJ Research, MA,

Table 1. Locations of occurrence of *Delphinium* species in Himachal Pradesh

Species	Location	District	Altitude (m asl)
D. vestitum and *D. brunonianum*	Rohtang[a,b]	Kullu	3900
D. vestitum	Rahla Falls[a]	Kullu	3000
	Marhi[a]	Kullu	3300
	Jalori Pass	Kullu	3300
	Khoksar	Lahaul and Spiti	3100
	Kukumseri	Lahaul and Spiti	3000
D. denudatum	Dhali	Shimla	2000
	Kotkhai	Shimla	1700
	Rajgarh	Solan	1400
	Kandaghat	Solan	1400
	Solan	Solan	1300
	Nauni	Solan	1300
	Namhol	Bilaspur	450

[a]Sub-populations at Rohtang. [b]New habitat of *D. brunonianum*.

Table 2. Earlier reported and surveyed new habitats where *Delphinium* species were not sighted

District	Location	Altitude (m asl)	*Delphinium* species reported earlier	Reference
Bilaspur	Bilaspur	700	–[a]	
Hamirpur	Bijhari	800	–	
	Hamirpur	800	–	
	Maharal	750	–	
	Barsar	800	–	
Kangra	Palampur	1200	–	
	McLeodganj (area near Bhagsu Nag)	2200	–	
	Kangra	650	*D. pyramidale*	10
Kullu	Great Himalayan National Park	1500–4000	*D. kolzii*, *D. roylei*	10, FRI[b]
Lahaul and Spiti	Keylong (Manglad Nalah)	3300	*D. cashmerianum*	BSI[c], FRI
	Kardang (Bhaga Basin near bridge)	3500	*D. cashmerianum*	BSI, FRI
	Miyar Nalah	2800	–	
	Khoksar	3100	*D. cashmerianum*	BSI
	Chandra Bhaga Basin	3100	–	
	Upper Chanab Area	3200	*D. brunonianum*	FRI
	Kunzum Pass	4590	–	
	Triloki Nath	4000	–	
	Kamring	3500	*D. brunonianum*	FRI
Mandi	Barot	1000	–	
Shimla	Hatu	3000	*D. vestitum*	BSI
	Kullu	1200	*D. roylei*	FRI

[a]Surveyed new habitats. [b]Herbarium of Forest Research Institute, Dehradun. [c]Herbarium of Botanical Survey of India, Dehradun.

USA) with the following temperature profiles: pre-amplification at 94°C for 5 min, 40 cycles of 94°C for 1 min, 37°C for 1 min, 72°C for 2 min and post-amplification cycle of 72°C for 5 min. Amplified products were resolved in 1.4% agarose gel in 1 × Tris-acetate-EDTA buffer at 80 V for 2 h. Gels were stained with ethidium bromide, visualized in UV light and photographed[13]. A Lambda DNA *Eco*RI + *Hin*dIII double digest (Bangalore Genei) was used as DNA molecular-weight marker. Phylogenetic relationships among three *Delphinium* species (*D. denudatum*, *D. vestitum* and *D. brunonianum*) from the study area were elucidated using RAPD markers. Genomic DNA of seven plants of each species from different locations/sites was pooled for PCR in such a way that each of the locations was represented. Fourteen 10-mer primers (OPB-1, OPB-10, OPB-11, OPB-12, OPC-2, OPC-5, OPC-6, OPC-7, OPC-15, OPC-16, OPC-17, OPC-19, OPC-20 and OPX-19) were selected for amplification of genomic DNA of plants after initial testing of 42 primers. DNA fingerprints were scored manually in a binary fashion and data were analysed[14] using the numerical taxonomy system of multivariate statistical program (NTSYS) software package 1.8. Phenograms were constructed by the SAHN clustering using unweighted paired group method of arithmetic averages (UPGMA) option.

During the present survey, only three species, i.e. *D. denudatum*, *D. vestitum* and *D. brunonianum* were sighted at different locations in the study area (Table 1).

Earlier records[9,10] showed the occurrence of seven species in the region. The study indicates that four species (*D. cashmerianum*, *D. kolzii*, *D. pyramidale* and *D. roylei*) might have been lost from all the earlier reported locations, i.e. *D. cashmerianum* from Manglad Nala in Keylong and Bhaba basin near the bridge on the way to Kardang in Lahaul and Spiti District (BSI, FRI); *D. kolzii* from the Great Himalayan National Park, Kullu[10]; *D. pyramidale* from Kangra[10], and *D. roylei* from Kullu (FRI). These species were also not sighted at other surveyed new locations with climatic conditions similar to those of the reported habitats (Table 2). However, *D. cashmerianum* has been reported from Pin Tud and Tarbak eco-climatic zones of Spiti in Lahaul and Spiti (temperate-dry climate)[11], where its population density has been 11.5–12.0 individuals/sq. m.

Among the three recorded species, population size of two (*D. brunonianum* (16 individuals) and *D. vestitum* (150 individuals)) was small. *D. brunonianum* was recorded at a new location, Rohtang, with a single patch of approximately 100 sq. m (Table 3). The species was, however, not sighted at any of the earlier reported or other surveyed new locations (Table 2). *D. vestitum* was recorded from four locations: Rohtang (51 individuals), Jalori Pass (84 individuals), Khoksar (8 individuals) and Kukumseri (7 individuals) (Tables 1 and 3). The species was not sighted at one of the earlier reported locations, i.e. Hatu near Narkanda in Shimla[10] (BSI). The habitats of *D. brunonianum* and *D. vestitum* represented alpine climate (≥3000 m asl). Majority of the plants of the two species were seen growing under bushes. Domestic animals such as sheep, goat, yak and horse belonging to nomads were seen grazing at Rohtang as well as Jalori Pass. Apparently, one of the reasons for small population size might be extensive grazing by animals. *D. denudatum*, the third species, was spread over four districts (Table 1) and has a considerably larger population. The species was sighted in all the earlier reported locations. Plant density varied between 1.0 and 5.0 plants/sq. m and its habitat was spread from Sub Himalaya (elevation as low as 450 m asl) to Lesser Himalaya (up to 2000 m asl). The species was found to inhabit stony slopes with small bushes, except Rajgarh where it was found to grow on slopes under pine (*Pinus roxburghii*) forests.

D. denudatum populations in the study area appear to have originated from two distinct lineages. Amplification of genomic DNA of 17 individuals of this species with six decamer primers (see Figure 2 for photo plates of RAPD profiles obtained with OPC-2 and OPC-7) revealed 42 polymorphic loci. Dendrogram constructed after analysis of PCR amplification data revealed two distinct clusters, each of which was further subdivided into two sub-clusters that were location- or sub-population-specific with few ambiguities (Figure 1). Sub-cluster Ia had individuals from Solan, Ib from site 1 of Rajgarh, IIa from site 2 of Rajgarh and IIb from site 2 of Rajgarh as well as site 1 of Nauni. Among the six individuals from Nauni, two each from sites 1 and 2 were not represented by any of the clusters. In spite of two clusters, overall genetic diversity in *D. denudatum* was low (54% genetic similarity). This suggested that *D. denudatum* lineages from the northwestern Himalayan region might have common ancestry.

Amplification of genomic DNA of *D. brunonianum*, *D. denudatum* and *D. vestitum* with fourteen 10-mer primers revealed 76 polymorphic loci. As against poor morphological differences between *D. brunonianum* and *D. vestitum*, there is low similarity at the genome level, as only 12.5% loci were monomorphic between the two species. NTSYS analysis of RAPD data validated the taxonomical identification of these as distinct species. The resulting dendrogram revealed that both species are phylogenetically

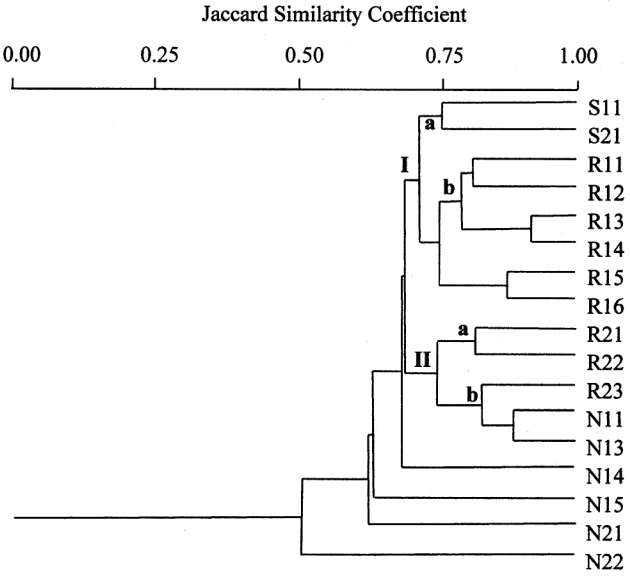

Figure 1. Dendrogram of 17 individuals of *Delphinium denudatum* constructed after NTSYS analysis of 42 polymorhic RAPD loci. Plant numbers are given on the termini of branches where the first letter indicates the location, first number the site and second the plant number. Locations: S, Solan; R, Rajgarh; N, Nauni.

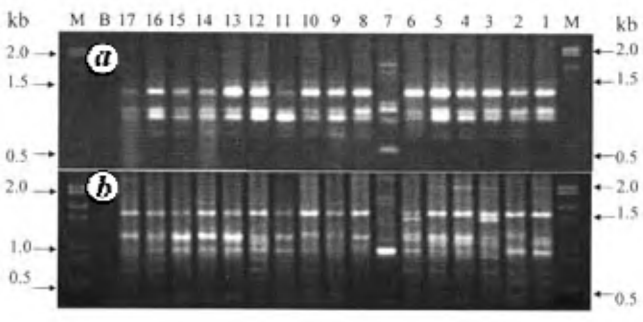

Figure 2. RAPD profiles of 17 individuals of *D. denudatum* obtained with decamer primers (*a*) OPC-2 and (*b*) OPC-7. Lanes 1–17, *D. denudatum* plants; Lane M, Molecular weight marker, and Lane B, Control.

Table 3. Population size of *D. brunonianum* and *D. vestitum* at different locations in Himachal Pradesh

Species	Location	Site	Number of patches	Population size (no.)	Number of individuals/sq. m
D. brunonianum	Rohtang	Rohtang	One	16	0.16
D. vestitum	Rohtang	Rohtang	One	10	0.10
	Rohtang	Marhi	One	33	0.21
	Rohtang	Rahla Falls	Two	8	0.08
	Khoksar	Khoksar	One	8	0.08
	Kukumseri	Kukumseri	One	7	0.07
	Jalori Pass	Jalori Pass	One	84	0.28

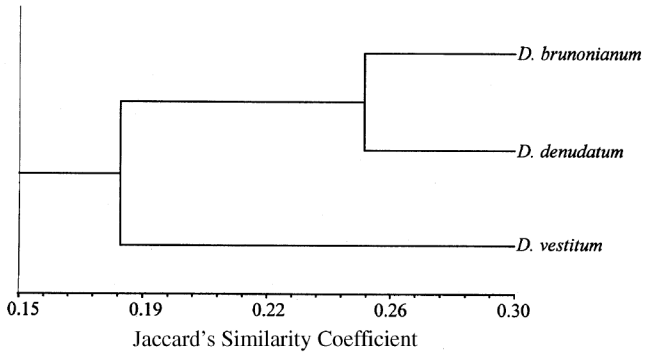

Figure 3. UPGMA dendrogram of relationships among *Delphinium* species from north-western Himalayan region based on Jaccard's coefficient of similarity obtained from 76 polymorphic RAPD loci. Names of the species are given on the termini of branches.

different from each other with as low as 19% genetic similarity (Figure 3). Likewise, *D. denudatum* could also be differentiated as it shared only 25% genetic similarity with *D. brunonianum* and 19% with *D. vestitum*. Thus RAPD could differentiate these three species precisely.

Absence of four species (*D. cashmerianum*, *D. kolzii*, *D. pyramidale* and *D. roylei*) from the previously reported locations and small population size of *D. brunonianum* and *D. vestitum* indicated that *Delphinium* species in this region are under threat. Small population size and low genetic variation reinforce the endangered status of a species[15]. Small population size of *D. brunonianum* and *D. vestitum* and their absence in several previously reported locations is indicative of their critically endangered status. This calls for proper conservation measures to avoid their erosion from the northwestern Himalaya. As such, erosion of diversity in natural plant populations in the Himalayan region during the recent past is not uncommon and has been attributed to reckless exploitation for extraction of drugs[11,16], socio-economic factors, various environmental perturbations, loss of habitat and overgrazing by animals[17–19]. Heavy grazing appears to be one of the major causes of diminished/diminishing populations of *Delphinium* in HP. Scientific management of heavy grazing and creation of botanical reserves would be required essentially to conserve *Delphinium* in this region. In addition, seed banks and field gene banks in areas having climate similar to natural habitat might also prove beneficial for conservation of this genus.

RAPD markers delineated *D. denudatum*, *D. vestitum* and *D. brunonianum*, validating their taxonomical classification as distinct species and at the same time indicating the importance of these markers in differentiating the species. Similar to our findings, RAPDs have already been found suitable to differentiate among three tall larkspurs (*D. glaucum*, *D. occidentale* and *D. barbeyi*)[20]. Knowledge of the existence of genetic diversity in *D. denudatum* will help in selecting populations for *in situ* and *ex situ* conservation. Since it has two distinct phylogenetic lineages, its diversity cannot be conserved until populations representing both the lineages are included.

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Seasonal variation in the litter chemical quality of a wet evergreen forest in the Western Ghats

Anto Anu and Thomas K. Sabu*

Litter Entomology Research Unit, P.G. & Research Department of Zoology, St. Joseph's College, Devagiri, Calicut 673 008, India

Seasonal pattern in the resource quality of freshly fallen leaves in a wet evergreen forest floor of Western Ghats was assessed. High resource quality litter was shed during monsoon seasons and low quality litter during the summer season. We propose that the observed variations are related to nutrient source–sink interactions during summer periods and pulse of increased soil nutrient availability and uptake during rainy season, resulting from wind-mediated green leaf fall. Significant seasonal variations in the nutrient content of fresh litter suggest complex decomposition patterns during different seasons of a year.

Keywords: Evergreen rain forest, litterfall, nutrient levels, seasonal variation, Western Ghats.

*For correspondence. (e-mail: sabukthomas@gmail.com)

THE view that tropical evergreen rainforests are static communities which function under consistently optimal moist and warm climatic conditions[1], has changed with the recent recognition of a strong seasonality of leaf litterfall, with a peak at the end of the dry season followed by a surge in nutrient availability in the forest floor[2]. The following potential mechanisms lead to seasonal variation in leaf litter quality in tropical wet forests. (i) Rainfall-mediated leaching of nutrients from live and senescent leaves and decomposing litter in forests[3], known as low nutrient and high rainfall hypothesis. (ii) Stronger winds during high precipitation and consequent rise in nutrient-rich green litterfall[4]. (iii) Persistent cloud cover and reduced insolation reaching the canopy during rainy seasons leading to lower photosynthetic rates and lower litter nutrient levels in wet tropical forests[5]. (iv) Fall in litter nutrient concentration arising from mobile nutrients from old tissue (source) to new tissue (sink) during flushing of new leaves and fruit production[6,7] in summer. (v) Premature senescence of leaves due to water stress during non-rainy periods[8].

Despite these evidences indicating the influence of seasonality on patterns of nutrient cycling in wet evergreen tropical rainforests, litterfall nutrient measurements beyond a single season are never considered in the tropical moist evergreen forests of the Western Ghats. In the present study, we report the seasonal variation in leaf litter nutrient levels of freshly fallen litter present in a wet evergreen forest in the western windward region of the Western Ghats.

The study was conducted in a wet evergreen forest at Chanthanathode, North Wayanad, the Western Ghats ecoregion (11°50′N lat. and 75°49′E long.), 800 m asl and having an area of 85.12 sq. km. An annual precipitation of 3752 mm was received during the study period (2002–03), of which 81% was received during the southwest monsoon (June–August), 10% during the northeast monsoon (September–November), 8% during summer season (March–May) and 1% during presummer season (December–February; KSEB Rain Gauge Station, Periya).

Two samples of freshly fallen litter were collected using 20 × 20 cm wooden frame from randomly located plots of 20 m × 80 m, three times during a season and covering all ffour seasons in 2002–03. Samples were dried at 30°C to constant weight, ground and passed through a 1 mm mesh screen. Thereafter, required amount of sub-samples of litter were taken for nutrient analysis. Total phenols were quantified by Folin Ciocalteu method[9], carbon by Walkley and Black method[10], nitrogen levels by micro-Kjeldahl digestion[11] followed by distillation and titration; Na, Ca and K levels by acid digestion and flame photometery[12] and lignin by acetyl-bromide extraction procedure[13]. All analyses were carried out in triplicate and mean values were taken. Significance levels of the seasonal variation in chemical variables were analysed using non-parametric statistics (Mann–Whitney *U* test) after multivariate com-