Summary

Herbal medicines have received a great attention and acceptance worldwide as a primary source of healthcare due to their low cost, promising efficiency and lesser side effects. Medicinal plants are being highlighted as rich sources of natural antioxidants, can effectively reduce the risk of cancer, heart diseases, diabetes, aging and many neurodegenerative disorders. Furthermore, plant based active biomolecules serve as one of the most promising resources of new nutraceutical and dietary supplements due to their varied structures and mode of action, and thus act as a template for drug discovery programs. The increasing demand for many plant-based products has led to the expansion of herbal industry and is accompanied by calls for assurance of quality, efficacy and safety. Generally, the botanical sources of plant-based drugs, medicines and dietary supplements are identified at the species level by their Latin scientific names as the plant species is the basic unit for the preparation of herbal formulations. Misidentification of plant species and advertent adulteration by totally unrelated species or by closely related inferior quality species can hinder the medicinal use, damage the efficacy and the adverse effects of which may even kill the consumer. Therefore, species delimitation is not only a critical step at the beginning of an quality assurance programme but is also of fundamental importance to understand the patterns and mechanisms of speciation, hybridization, and to track the evolutionary history of the organisms by which new biological species evolve and the occurrence of gene flow between closely related species. Furthermore, many medicinally important plant species
are now severely threatened in their natural habitats due to illegal trade and over exploitation for drug preparations in herbal and pharmaceutical industries and therefore need special attention. Rapid depletion of plant genetic resources from their natural habitats demands immediate strategies for their conservation. As plant populations are exposed to a number of factors or forces in their natural habitat, a thorough understanding of the levels and distribution of genetic diversity along with population structure across the geographical landscape is required for the conservation and management of threatened plant species.

The genus *Nardostachys* DC. is a monotypic herbaceous genus of the family Valerianaceae and mostly represented by *N. jatamansi* (D. Don) DC. It is a small, perennial, rhizomatous herb, restricted to some specialized pockets in the alpine Himalayas spanning Himachal Pradesh, Uttarakhand, Sikkim, Arunachal Pradesh in India, Nepal, Bhutan and China at altitudes of 3000-5200 m asl. A lot of confusion exists in different literatures, databases and various herbaria in India regarding the taxonomic identification of the species of *Nardostachys* which has been referred to as the ‘Jatamansi’ or ‘Spikenard’ in the scriptures. The species was first misidentified and described as *Valeriana jatamansi* Jones by William Jones and Roxburgh. The error was detected by D. Don who procured a specimen of true ‘Jatamansi’ and described it again, first as *V. jatamansi* sensu D. Don, non Jones and then as *Patrinia jatamansi*. De Candolle regarded the *Nardostachys* DC as a new genus within the family Valerianaceae and correctly described and illustrated the famous Indian drug ‘Jatamansi’ under the name *N. jatamansi* (D. Don) DC. At the same time, he also described another species as
N. grandiflora DC. However, Weberling reviewed the genus *Nardostachys* over its entire distributional range, covering a wide altitudinal range (3300-5200 m asl) and concluded it to be a single polymorphic species with three main identifiable forms or types. On the other hand, Prakash in his monograph based upon extensive field surveys, reported the existence of two species, namely *N. grandiflora* and *N. jatamansi*. A number of authorities have considered *N. jatamansi* and *N. grandiflora* as two distinct species accounting on the difference in the inflorescences. More than 70 active compounds of medicinal importance have been isolated from *Nardostachys* spp. and many active compounds have still been reported from *N. chinensis* from China. The presence of the active compounds in the genus *Nardostachys* may vary from species to species, and it is critical to identify the species based on the presence of these compounds. Jatamansi has a long history of use as medicine and dietary supplements in Ayurveda, Unani, ethnomedicine since Vedic ages. Due to the habitat degradation, illegal trade and over exploitation for drug preparations in herbal and pharmaceutical industry, *N. jatamansi* has been listed as Critically Endangered (CR) in the IUCN Red list of threatened species and therefore requires an urgent attention for its conservation and sustainable utilization.

An integrative approach including classical taxonomy, molecular markers-based species delimitation, genome size and secondary metabolite profiling has been taken in this study to resolve the taxonomic identity of the genus *Nardostachys*. In the present study, specimens of *Nardostachys* were collected from 11 localities of 4 different states (Himachal Pradesh, Uttarakhand, Sikkim and Arunachal Pradesh) spanning alpine regions of Western and Eastern Himalayas. Plants were also collected from the ‘Type
localities’ of *N. jatamansi* and *N. grandiflora* following the locations provided by De Candolle and Prakash and compared for any morphological similarities or differences with respect to the other accessions. Specimens of *Valeriana jatamansi* were also collected from natural habitats of Sikkim to assess its synonymy and erroneous misapprehension as famous Indian drug ‘Jatamansi’ in various literatures, databases and herbaria. Critical examinations on the habit, habitat, vegetative as well as reproductive characters of different accessions of *Nardostachys* collected from the entire distributional ranges of alpine Himalayas of India mainly revealed three main identifiable ‘forms’ or ‘types’ viz., jatamansi type, grandiflora type and lineariifolia or chinensis type. *N. grandiflora* or grandiflora type shares several common characters with *N. jatamansi* or jatamansi type, such as habit (perennial herb), long fragmented rootstock covered with fibres, slender stem having simple, undivided leaves (sessile cauline and long petiolate radical leaves), 5-lobed comapanulate corolla tube, 4 stamens, 3-celled ovary with 1 ovule and achene crowned with persistent calyx-lobes. However, *N. jatamansi* (jatamansi type) was found to be typically different from *N. grandiflora* (grandiflora type) in comparative size of inflorescence (bigger bracts, bracteoles and corolla tubes), exerted stamens and achene characters (length of calyx limbs shorter or greater than achene). A significant variation in different vegetative (plant height, stem colour, leaf shape and size, size and fragrance of rhizome) and reproductive characters (simple or compound capitulate inflorescence, flower colour, bract and corolla length, achene length) were observed in different accessions of *Nardostachys* collected from different habitats of Western and Eastern Himalayas. The observed phenotypic variations could be linked with the local
adaptation to that particular habitat. On the other hand, accessions from Thangu and Lachung valley in North Sikkim near the Indo-China border showed resemblance to the lineariifolia or chinensis type (*N. chinensis*). Although the lineariifolia or chinensis type of *Nardostachys* accessions were quite smaller in size with dark pink or purple coloured unicapitulate inflorescence; they showed a great similarities with *N. jatamansi* or jatamansi type in most of the characters (stem, radical and cauline leaves, bracts, calyx, corolla, exerted stamens and achene characters). Plants of this form i.e., lineariifolia or chinensis type were also collected from the same regions in Sikkim and Kumaon as ‘jatamansi type’, but rather from the higher elevations in dry, grassy slopes in alpine Himalayas. Moreover, transitional forms between the jatamansi and lineariifolia (chinensis) (small plants; linear lanceolate leaves; pinkish-white, compound capitulum inflorescence with exerted stamens and larger calyx-lobes than the achene) in Kumaon and Sikkim Himalayas substantially merged this lineariifolia or chinensis type as a morphotype of *N. jatamansi*. On the other hand, it was found that *V. jatamansi* completely differed from *Nardostachys* in its habitat and morphological characters and should not be misidentified with true ‘Jatamansi’. Based on the morphological examination, 2 types or variants, namely, jatamansi type and grandiflora type were recorded while lineariifolia or chinensis type merged with the jatamansi type specimens. However, all *Nardostachys* accessions displayed a great deal of phenotypic variations with respect to elevation and the geographical region, and connected by so many transitional forms, it is not desirable to establish any natural group on the basis of morphology only.
Five cpDNA (rbcLa, matK, rpoC1, psbA-trnH, trnL-F) and one nDNA (nrITS2) were taken for phylogenetic and species delimitation analyses. Comparisons of the 6 marker candidates (rbcLa, matK, rpoC1, psbA-trnH, trnL-F and ITS2) by TaxonGap revealed the overall separability was found higher in psbA-trnH, ITS2 and trnL-F followed by the coding regions. However, lower heterogeneity values of each marker region suggested a combined marker datasets for the species delimitation and phylogenetic analyses. Phylogenetic analysis with concatenated 6 marker sets with 3 different approaches (maximum parsimony, maximum likelihood and Bayesian) showed two clear groupings- Nardostachys clade and Valeriana clade with strong bootstrap support values. All Nardostachys accessions were recovered as a monophyletic group with considerably higher bootstrap and branch length values in the concatenated species tree. Within the Nardostachys clade, several accessions clustered into small sub-branches, then intermixed each other. In most of the cases, Nardostachys accessions clustered together based on their geographical distributions, forming separated sub-clades. Plant samples of Eastern Himalayan and Western Himalayan regions were found to group together regardless of the method of phylogenetic reconstruction used. NJAP01 samples from Arunachal Pradesh remained isolated from the other Nardostachys accessions from the respective geographical regions which could be linked to the higher population genetic differentiation and reduced gene flow. More interestingly, in the present study, lineariifolia or chinensis type plants collected from Thangu and Lachung valleys (NJSK01 and NJSK02) near Indo-China border were also found to be grouped with the Eastern Himalayan accessions. In the present study, all the 3 methods of
phylogenetic analyses revealed \textit{N_jatamansi}_GB as completely separated from the \textit{Nardostachys} clade. In the \textit{Valeriana} clade, \textit{V. jatamansi} was found well separated from \textit{V. hardwickii} with significantly higher bootstrap values with \textit{L. japonica} as outgroup. Profile neighbour joining (PNJ) analysis with aligned sequence-secondary structure of ITS2 also revealed 4 distinct groups where all \textit{Nardostachys} accessions grouped together, and \textit{V. jatamansi} and \textit{V. hardwickii} as 2 other separate clades with \textit{L. japonica} as outgroup. However, no formation of two well defined separated bigger clades corresponding to \textit{N. grandiflora} and \textit{N. jatamansi} were observed which clearly indicated \textit{Nardostachys} as a conspecific. In addition 3 species delimitation methods were used to determine putative species boundaries in \textit{Nardostachys-Valeriana} species complex. Distance-based-Automatic Barcode Gap Discovery (ABGD) identified \textit{N. jatamansi/grandiflora/chinensis} group as conspecific or a single species but \textit{V. jatamansi} and \textit{V. hardwickii} as 2 other distinct species. Non-ultrametric tree-based Poisson Tree Process model (PTP and bPTP) species delimitation approaches suggested the existence of 3 distinct species in terms of higher support value in resulted tree topology and partitioning of putative species. All \textit{Nardostachys} accessions were grouped into a single partition (Support value= 1) clearly indicating \textit{Nardostachys} as a single monotypic genus with different morphological variants. In all \textit{Nardostachys} accessions, either sampled from the field or retrieved from the NCBI-GenBank, no Compensatory Base Changes (CBCs) or hemi-CBCs were observed while cumulative 3 CBCs+hemi-CBCs were found to be present between \textit{Nardostachys} and \textit{V. jatamansi} indicating \textit{V. jatamansi} as a distinct species despite its identical species name with \textit{Nardostachys}. Furthermore,
consensus secondary structures of all *Nardostachys* accessions did not reveal any changes in nucleotides in all the 4 helices.

Intact nuclei were isolated from the leaves of *N. jatamansi* (jatamansi type), *N. grandiflora* (grandiflora type), *N. jatamansi* (lineariifolia or chinensis type), *V. jatamansi* and *V. hardwickii* to compare the nuclear DNA content following FCM analysis. Nuclear DNA contents of different accessions of *N. jatamansi* (jatamansi type) from different locations of Western and Eastern Himalayas were also evaluated for the intra-specific variations in genome size. Using *Pisum sativum* Citrad nuclei (9.09 pg/2C) as an internal standard, the nuclear DNA content of *N. jatamansi* (jatamansi type) (2n) was calculated to be 1.40±0.1 pg/2C. The 1C genome size of *N. jatamansi* was found to be 684.6 Mbp (1 pg=978×10^9 Mbp). Nuclear DNA content of *N. grandiflora* (grandiflora type) and *N. jatamansi* (lineariifolia or chinensis type) were 1.43±0.2 pg/2C (699.27 Mbp) and 1.44±0.1 pg/2C (702.40 Mbp), respectively. Genome size of *V. jatamansi* was found to be 4.7 times larger than *N. jatamansi* and genome size of *V. hardwickii* 1.57 times smaller than *V. jatamansi*, and thus FCM approach provided a clear species discrimination boundary. Based on observed intra-specific variations of different accessions of *N. jatamansi* (jatamansi type), it was found that the genome size of grandiflora and lineariifolia types was within the range of variability of *N. jatamansi*. Furthermore, the comparative genome size analysis did not also reveal any sign of hybridization events among these ‘Types’ of *Nardostachys*.

Gas chromatography-mass spectroscopy (GC-MS)-based metabolic profiling revealed presence of mainly terpenoid compounds (mono-, di-, sesquiterpenes and
coumarins) in all *Nardostachys* accessions whereas valepatriates-iridoids were identified in *Valeriana* spp. A total of 20 metabolites (VIPs>1) were identified which contributed significantly to the species separation, generated from orthogonal partial least squares discriminant analysis (OPLS-DA). Jatamansone or valeranone, the principal sesquiterpene of *N. jatamansi* was found to be present in the highest amount in all three types of *Nardostachys* accessions. Spirojatamol, aristolone, valencene, ledol, α-vatirenene, (-)-a-Panasinsen, stigmast-4-en-3-one and an alkaloid, actinidine were some of the key marker compounds that were also present in all *Nardostachys* accessions (jatamansi, grandiflora and linearifolia type). However, the presence of secondary metabolites varied quantitatively depending on the different collection sites and geographical regions.

The combined approaches including classical taxonomy, molecular phylogeny and species delimitation, genome size and comparative secondary metabolite profiling identified *Nardostachys* as a monotypic genus with significant morphological variations which might be due to its local adaptive evolution to the various micro and macro environmental conditions in the respective habitats in alpine Himalayas.

Genetic diversity and spatial population genetic structure of 94 wild individuals of *N. jatamansi* collected from 7 naturally distributed populations from alpine Himalayas of India were carried out using cumulative marker-based genome scan approach comprising 4 molecular markers *viz.*, Start Codon Targeted Polymorphism (SCoT), Intron Splice Junction (ISJ), Inter-Retrotransposon Amplified Polymorphism (IRAP) and Intron Targeted Amplified Polymorphism (ITAP). A total of 406 fragments were
produced cumulatively by 49 primers using 4 markers, of which 350 fragments were polymorphic (85.7%) with an average 7.05 polymorphic fragments per primer, and genetic distance recorded using Jaccard’s coefficients of similarity ranged from 0.56-0.96. The observed number of alleles (Na) and effective number of alleles (Ne) ranged from 1.35 (NJUKG, Tungnath, Uttarakhand) to 1.75 (NJAP, Tawang valley, Arunachal Pradesh), and 1.22 (NJUKG, Tungnath, Uttarakhand) to 1.46 (NJAP, Tawang valley, Arunachal Pradesh), respectively. The highest number of polymorphic loci (Pp= 75.49%, h= 0.269±0.240, I= 0.401±0.168) was exhibited in NJAP population (Tawang valley, Arunachal Pradesh) and the lowest (Pp= 35.57%, h= 0.125±0.214, I= 0.187±0.303) in NJUKG (Tungnath, Uttarakhand) population. In the present study, unexpectedly high level of genetic variation (Na= 1.8814, Ne= 1.5342, h= 0.312, I = 0.466) was observed with 88.14% polymorphic loci despite the rarity and severely fragmented populations of *N. jatamansi* in the natural habitats.. The genetic differentiation among population (Gst) value was calculated to be 0.4108 and estimate of gene flow (Nm) was 0.717. The fixation index or F statistics (F<sub>ST</sub>) ranged from 0.4108 (NJSKNa, Nathula, East Sikkim) to 0.4558 (GHNP, Himachal Pradesh). Populations of *N. jatamansi* across the alpine Himalayas were found to be significantly differentiated as revealed by overall F<sub>ST</sub> (0.4331). An AMOVA analysis of pair wise distances revealed that 43.32% of the genetic variation was partitioned between populations, and 56.68% within populations based on four cumulative markers. The gene flow values and high genetic diversity found in this study suggest a migratory pattern with colonists recruited from random individuals of previously existing populations which should not be interpreted as the
Bayesian cluster analysis using the STRUCTURE programme suggested that the populations used in this study could be divided into 6 clusters where populations from Arunachal Pradesh (NJAP) and Himachal Pradesh (NJHP) were found as 2 distinct clusters with a little or insignificant admixture. Populations from Garhwal (NJUKG) and Kumaon (NJUKK) Himalayas in Uttarakhand appeared as a single group whereas 2 populations from East Sikkim (NJSKZ, NJSKNa) and one population from North Sikkim (NJSKL) showed a greater admixing amongst themselves. The observed clustering pattern indicated that geographically proximate populations were more efficiently connected by gene flow than populations separated by greater distance. An UPGMA dendrogram constructed based on the Nei’s genetic distances revealed 4 main clusters and 6 sub-clusters and the genetic distance recorded using Jacard’s coefficient of similarity ranged from 0.57-0.96. The result of UPGMA cluster analysis was further supported by the principle component analysis (PCA). Mantel test with spatial autocorrelation analyses revealed a significant correlation between genetic and geographical distance (r= 0.5425, P<0.05), which indicated presence of one or several barriers among populations that restricted population intermingling, resulting in geographically distinct populations. In addition, the Genetic Landscape Shape interpolation analysis produced 4 major surface peaks or ridges in the landscapes, indicating potential barriers of gene flow that caused increased population differentiation of *N. jatamansi* through the entire Himalayan range. BOTTLENECK analysis was carried out to model a population bottleneck based on the stepwise mutation model (SMM), the two-phase model (TPM), and the infinite alleles model (IAM). A
significant (P<0.05) heterozygosity excess and shifted mode of allele frequency distributions were detected in 4 out of 7 populations which suggested the occurrence of recent population bottlenecks in those populations. This population reduction event indicated the higher genetic diversity in the existing populations which in turn reflected the historical gene flow and populations admixing during glaciations events. Population bottlenecks reduced both allele frequency and heterozygosity via enhanced genetic drift leading to the loss of some alleles within a population. This might suggest that populations of *N. jatamansi* which have undergone contraction would face a greater extinction risk in near future via enhanced inbreeding and genetic drift.

Despite having medicinal properties, religious importance and critically endangered status, *N. jatamansi* has not received much attention with respect to conservation and sustainable utilization. Therefore, an efficient, genetically stable protocol using both indirect and direct shoot organogenesis has been optimized for *N. jatamansi* in the present study. Among different explants tested, leaf, petiole and rhizome explants were selected based on their response for callus induction frequency and callus growth. Temperature had a profound effect on callus induction, growth, shoot regeneration and maintenance of this alpine plant. It was found that culturing the explants in medium supplemented with different auxins and cytokinins resulted in better callus induction frequency (%), further proliferation (callus fresh weight) and shoot regeneration preferably at low temperature (13±1 °C) under 14 h photoperiod. Murashige and Skoog (MS) medium containing 1.5 mg/l α-naphthaleneacetic acid (NAA) along with 1.0 mg/l meta-Topolin (mT) was found to be beneficial for optimum callusing
response (88%) and growth (0.446±0.06 gm FW) from leaf and petiole explants at low temperature (13±1 °C). Callus cultured at 22±1 °C turned brown within 15 days of incubation which might be due to temperature stress. It was also observed that amount of calli at 13±1 °C were almost two times higher in amount which remained healthy for more than 45 days without subculture. Similarly, optimum callusing from rhizome explants was achieved in MS medium fortified with 1.5 mg/l each of NAA and mT with 81.5% callus induction frequency and 0.469±0.04 gm FW callus growth at 13±1 °C. The highest regeneration frequency (92%), maximum number of shoots (25.2±0.4) with 6.1±0.2 leaves per shoot were achieved in medium supplemented with combination of 1.0 mg/l mT and 0.5 mg/l NAA. Shoot primodia appeared from both friable as well as compact nodular calli after 2 weeks of culture in the regeneration medium when calli were cultured only at 13±1 °C temperature for 14 h photoperiod. Calli cultured at 22±1 °C either did not respond or resulted in rhizogenesis. Although in thidiazuron (TDZ) containing medium better response of the explants was observed at lower concentrations, the use of its higher concentrations resulted in increased hyperhydricity (60% at 1.5 mg/l, and 75% at 2 mg/l). In the present findings, mT was nearly twice more effective than conventional cytokinins [6-benzylaminopurine (BAP), Kinetin (KN)] in shoot regeneration. No hyperhydric shoots were observed even at higher concentrations of mT in the medium. Also, direct organogenesis from shoot tip and petiole explants was achieved in MS medium containing 1.0 mg/l mT. Optimum rooting was achieved in the same medium supplemented with 1.0 mg/l indole acetic acid (IAA) wherein average of 4.51 roots/shoot was induced. Genetic stability of in vitro-derived plantlets was assessed
and compared to mother plant using molecular markers and flow cytometry. ISJ and SCoT marker-based profiling revealed uniform banding profile in case of direct shoot organogenesis-derived plants while callus mediated organogenesis-derived plants showed slight variations as compared to mother plant. Although organogenic calli showed mixoploidy but no major phenotypic and genetic rearrangements were detected by flow cytometry in callus-derived plants. Cytogenetically normal (diploid) cells present in the callus usually maintain totipotency and regenerate well. However, polyploid and aneuploid cells may not regenerate at the same efficiency. Therefore, the population of regenerated plants from a mixoploid callus is usually biased towards normal karyotype. The developed protocol could be utilized for the improvement of bioactive constituents by genetic manipulations, and commercial propagation of this highly valued critically endangered, medicinal plant of alpine Himalayas.