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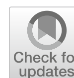
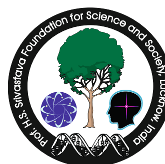

RESEARCH ARTICLE

Isolation and HPLC assisted quantification of two iridoid glycoside compounds and molecular DNA fingerprinting in critically endangered medicinal *Picrorhiza kurroa* Royle ex Benth: implications for conservation

Avinash Kumar[1,4] · Vijay Rani Rajpal[1,2] · Ambika[3,6] · Rachayya Mallikarjun Devarumath[1,7] · Amita Kumari[4] · Rakesh Thakur[5] · Manju Chaudhary[5] · Pradeep Pratap Singh[3,8] · Shiv Murat Singh Chauhan[3] · Soom Nath Raina[1,5]

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Abstract *Picrorhiza kurroa* is a medicinally important, high altitude perennial herb, endemic to the Himalayas. It possesses strong hepato-protective bioactivity that is contributed by two iridoid picroside compounds viz Picroside-I (P-I) and Picroside-II (P-II). Commercially, many *P. kurroa* based hepato-stimulatory Ayurvedic drug brands that use different proportions of P-I and P-II are available in the market. To identify genetically heterozygous and high yielding genotypes for multiplication, sustained use and conservation, it is essential to assess genetic and phytochemical diversity and understand the population structure of *P. kurroa*. In the present study, isolation and HPLC based quantification of picrosides P-I and P-II and molecular DNA fingerprinting using RAPD, AFLP and ISSR markers have been undertaken in 124 and 91 genotypes, respectively. The analyzed samples were collected from 10 natural *P. kurroa* Himalayan populations spread across four states (Jammu & Kashmir, Sikkim, Uttarakhand and Himachal Pradesh) of India. Genotypes used in this study covered around 1000 km geographical area of the total Indian Himalayan habitat range of *P. kurroa*. Significant quantitative variation ranging from 0.01 per cent to 4.15% for P-I, and from 0.01% to 3.18% in P-II picroside was observed in the analyzed samples. Three molecular DNA markers, RAPD (22 primers), ISSR (15 primers) and AFLP (07 primer combinations) also revealed a high level of genetic variation. The percentage polymorphism and effective number of alleles for RAPD, ISSR and AFLP analysis varied from 83.5%, 80.6% and 72.1%; 1.5722, 1.5787 and 1.5665, respectively. Further, the rate of gene flow (Nm) between populations was moderate for RAPD (0.8434), and AFLP (0.9882) and comparatively higher for ISSR (1.6093). Fst values were observed to be 0.56, 0.33, and 0.51 for RAPD, ISSR and AFLP markers, respectively. These values suggest that most of the observed genetic variation resided within populations. Neighbour joining (NJ), principal coordinate analysis (PCoA) and Bayesian based STRUCTURE grouped all the analyzed accessions into largely region-wise clusters and showed some intermixing between the populations, indicating the existence of distinct gene pools with limited gene flow/exchange. The present study has revealed a high level of genetic diversity in the analyzed populations. The analysis has resulted in identification of genetically diverse and high picrosides containing *P. kurroa* genotypes from Sainj, Dayara,

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✉ Vijay Rani Rajpal
vijayrani2@gmail.com

Avinash Kumar
avinashkumar@vbu.ac.in

[1] Department of Botany, University of Delhi, 110007 New Delhi, Delhi, India

[2] Department of Botany, Hansraj College, Delhi University, New Delhi, Delhi 110007, India

[3] Department of Chemistry, University of Delhi, 110007 New Delhi, Delhi, India

[4] Department of Botany, Vinoba Bhave University, Hazaribag, Jharkhand 825319, India

[5] Amity Institute of Biotechnology, Amity University, Sector 125, 201303 Noida, Uttar Pradesh, India

[6] Department of Chemistry, Hansraj College, Delhi University, Delhi 110007, India

[7] Molecular Biology and Genetic Engineering Lab., Vasantdada Sugar Institute, Pune, Maharashtra, India

[8] Department of Chemistry, Swami Shraddhanand College, University of Delhi, New Delhi, Delhi 110036, India

Tungnath, Furkia, Parsuthach, Arampatri, Manvarsar, Kedarnath, Thangu and Temza in the Indian Himalayan region. The inferences generated in this study can be used to devise future resource management and conservation strategies in *P. kurroa.*

Keywords *Picrorhiza kurroa* · Genetic diversity · Molecular DNA markers · Phytochemical diversity · Picrosides · HPLC

Introduction

Plant based compounds offer safer therapeutic options as opposed to many harmful side effects associated with synthetic drugs. Due to this, there has been a tremendous increase in the demand of phyto-pharmaceutical compounds globally. The current needs are largely met by an indiscriminate collection of medicinal plant species from their natural habitats. Harvesting of medicinal plants' germplasm from the wild runs to hundreds of tons of the collected material annually (Kumar et al. 2014; Kumar et al. 2016; Shitiz et al. 2015; Singh and Sharma 2020). This over-exploitation poses a grave threat to many important medicinal plant species, necessitating an urgent development of strategies, for their effective use and conservation.

Picrorhiza kurroa Royle ex Benth (Family Plantaginaceae; Akbar 2020), locally known as 'Kutki' or 'Karu' (Kumar 2019) is a medicinally important, high altitude perennial herb. It is endemic to the Himalayan region and is distributed in India, China, Pakistan, Nepal and Bhutan (Masood et al. 2015). It is widely used in ayurvedic system of medicine to treat the disorders of liver and upper respiratory tract, jaundice, fever, chronic diarrhea and scorpion sting (Krishnamurthy 1969; Vaidya et al. 1996). The species shows hepato-protective, stomachic, anti-inflammatory, anti-tumor, immuno-modulatory, hypolipidemic, hypoglycemic and antispasmodic bioactivities (Tiwari et al. 2012; Bhattacharjee et al. 2013; Sultan et al. 2016; Mahajan et al. 2016). The pharmacological properties of *P. kurroa* are attributed to the presence of various monoterpene-derived Iridoide glycosides known as picrosides that include picroside-I, and picroside-II, metabolites picrosides III, IV and V and other compounds. *P. kurroa* contains as many as 7 such iridoid glycosides namely kutkin, kutkoside, picroside V, pikuroside, mussaenosidic acid, bartsioside and boschnaloside (Bhat et al. 2013; Katoch et al. 2013; Kumar et al. 2016, 2017; Soni and Grover 2019;). Both picrosides P-I and P-II have been found in root and rhizome of the plant, while P-I has also been isolated from the shoots (Kumar et al. 2017; Debnath et al. 2020). Many ayurvedic hepato-protective drug preparations made of *Picrorhiza* extracts, namely Katuki, Picroliv, Livocare, Livomap, Livplus, Livomin etc. that are commercially available in the market contain picroside active principles P-I and P-II in 1:1.5 proportions (Bhat et al. 2013; Kumar 2019; Singh and Sharma 2020). *Picrorhiza* entails an approximate global annual demand of 500 tons against a supply of 375 tons, out of which India contributes 75 tons/year. *P. kurroa* is listed among top 15 most traded medicinal plant species in India based on revenue generation in trade (Thani et al. 2018; Debnath et al. 2020). Due to overexploitation, the existence of *P. kurroa* is threatened and the species is listed as 'endangered' in Appendix II (Aug, 2020 onwards) of CITES (https://cites.org/eng/app/appendices.php) and also in Barik et al. (2018), in India as per IUCN criteria (ver. 3.1).

Although the plant is self-regenerating, but due to reckless collections from its entire wild range of Himalayas, inappropriate methods of extraction of the material, inadequate cultivation practices and low seed viability, the presence of *P. kurroa* in wild, is continuously declining. This has become an important socioeconomic concern among farmers, industry and policy makers (Kumar 2019; Nayar et al. 1990). *P. kurroa* is now listed in the negative lists of exports of Govt. of India (Mehra et al. 2017; Barik et al. 2018; Kumar 2019; Singh and Sharma 2020) with legal restrictions being levied on its collection from the wild (Kumar et al. 2017).This has resulted in illegal collections and adulteration of picrosides P-I and P-II.

To streamline the current demands and supply of Kutki, it is important to devise effective germplasm management and sustainable strategies. One of the important pre-requisites for devising meaningful plant resource management strategies includes partitioning of the available genetic diversity based on characterization of existing gene pools. Such demarcation helps in (i) identifying elite/superior genotypes for multiplication and sustained use, (ii) devising conservation strategies and (iii) planning future breeding programs for genetic improvement of these species. Superior validated germplasm can be directed towards cultivation to make quality raw material available.

Many Molecular DNA markers like RAPD, ISSR and AFLP have been widely used for documentation of genetic diversity and understanding the population structure in many plant species. These multi-locus markers with high multiplex ratio offer the advantages of being free from the developmental and environmental influences, are relatively abundant, and produce sufficient polymorphism to demarcate genotypes in a population (Sarwat et al. 2008; Naik et al. 2010; Singh et al. 2015; Thiyagarajan and Venkatachalam 2015; Costa et al. 2016; Lone et al. 2018; Nazarzadeh et al. 2020). To the best of our knowledge, these markers have not, so far, been used in *P. kurroa* diversity assessment. A few studies have rather used SSR

DNA markers (Katoch et al. 2013; Shitiz et al. 2017; Singh and Sharma 2020) for genetic analysis in *P. kurroa*. Likewise, HPLC-based quantitation of useful phytochemical compounds from roots and rhizomes of *P. kurroa* has been done to identify high yielding elite genotypes (Katoch et al. 2011, 2013; Thapliyal et al. 2012; Shitiz et al. 2015; Sultan et al. 2016; Mehra et al. 2017; Soni and Grover 2019; Singh and Sharma 2020). These studies, though, have reported substantial genetic diversity among populations, but mostly, except Sultan et al (2016) are limited with the use of only a few populations, limited markers and a small sample size. To make meaningful inferences about the overall spectrum of available genetic diversity in this medicinally important species, there is an urgent need to comprehensively characterize its existing wild gene pools using multiple markers on the same set of genotypes.

The present analysis, in this context, represents the first exhaustive attempt to assess both the genetic diversity in 91 genotypes and phytochemical profiling in 124 genotype of *P. kurroa* representing 10 different populations growing all along its native range (spanning ~ 1000 km) in north east to north west Indian Himalayas. The use of multiple molecular DNA markers like RAPD, AFLP and ISSR fingerprinting will help in scanning different portions of the genome to provide a comprehensive account of genetic diversity. Further analysis of the same set of genotypes for phytochemical quantification of picrosides P-I and P-II will provide a correlation, if any, between genetic heterozygosity and the synthesis of active principles. This study is, by far, the largest genotyping and chemotyping study performed on the same set of genotypes from the wild germplasm of *P. kurroa.*

Material and methods

Plant materials

A list of 91 genotypes, belonging to 10 populations, investigated for their genetic diversity is given in Table 1. Out of 10 populations, 9 populations, represented by 55 genotypes, were collected from major distribution areas of the species from North East to North West Indian Himalayas (Fig. 1). The remaining 36 genotypes, collected initially from 15 regions of Himachal Pradesh, were grown in the experimental farm of Dr. Y. S. Parmar University, Solan. These 36 genotypes have been, therefore, designated as Rahala population. Not more than four to six young leaves were harvested from each plant and were immediately immersed in liquid N_2 to be later stored at – 80 °C.

For the extraction and quantitation of active phytochemical constituents such as picrosides P-I and P-II, 124 genotypes, belonging to 10 populations were collected from North East to North West Himalayas (Table 1). A part of the rhizome was excavated for phytochemical analysis. For preparation of standard and stock solutions ~ 500 g of dried rhizomes procured from the local market in Himachal Pradesh and authenticated at Y.S. Parmar University, Solan, H.P. was used.

Genetic diversity assessment

DNA extraction

The total genomic DNA extracted from young leaves was extracted by a modified DNA extraction protocol as given by Kumar et al. (2014).

RAPD fingerprinting

One hundred arbitrary primers (Operon Technologies, Inc., Alameda, California, USA) were initially tested with three genotypes, out of which 22 primers produced clear amplification products that were easily scorable. These 22 primers were used for comprehensive fingerprinting. The reaction mixture of 25 µl volume contained 2.5 µl 10X assay buffer (Biotools, Spain), 0.24 mM dNTPs (Amersham Pharmacia Biotech, USA), 15 ng primer (Operon Technologies Inc., Alameda, USA), 0.5 U *Taq* DNA polymerase (Biotools), 50 ng template DNA and 1.5 mM $MgCl_2$ (Biotools). DNA amplification was performed in a Perkin Elmer Cetus 480 DNA thermal cycler programmed to 1 cycle of 4 min 30 s at 94 °C (denaturation), 1 min at 40 °C (annealing), and 2 min at 72 °C (extension); followed by 44 cycles of 1 min at 94 °C, 1 min at 40 °C and 2 min at 72 °C ending with 1 cycle of 15 min at 72 °C (final extension).

ISSR fingerprinting

One hundred SSR primers (Biotechnology Laboratory, University of British Columbia, Canada) were tested for amplification in two genotypes. Of these, 15 were found to produce robust amplification products. These 15 SSR primers were finally used for PCR amplification. The 25 µl reaction volume contained 2.5 µl 10X assay buffer (Biotools), 0.24 mM dNTPs (Amersham Pharmacia Biotech), 5 µM primer, 0.75 U *Taq* DNA polymerase (Biotools), 50 ng template DNA, 1.5 mM $MgCl_2$ (Biotools) and 2% formamide. Initial denaturation in Perkin Elmer Cetus 480 DNA thermal cycler was done at 94 °C for 7 min, followed by 44 cycles of 30 s at 94 °C, 45 s at the particular annealing temperature calculated by Wallace rule (Thein and Wallace 1986) and 2 min at 72 °C ending with 1 cycle of 7 min at 72 °C.

Table 1 *Picrorhiza kurroa* germplasm resources collected across the Himalayas and used for genetic and phytochemical diversity assessment in the present study

States/regions	Locations	Population (Pop) number	Number of genotypes	Genotype ID
Jammu and Kashmir	Arampatri	Pop 1	10**	PK01-PK10
	Sonamarg	Pop 2	07***	PK11-PK17
	Manvarsar	Pop 3	04***	PK18-PK21
Sikkim	Temza	Pop 4	4	PK22-PK25
	Thangu	Pop 5	05***	PK26-PK30
Uttarakhand	Kedarnath	Pop 6	10	PK31-PK40
	Dayara	Pop 7	6	PK41-PK46
	Tungnath	Pop 8	4	PK47-PK50
	Jhuni	Pop 9	5	PK51-PK55
Himachal Pradesh	Rahala, Solangnala, Kafnu, Sainj, Pulga, Rohtang, Manimahesh, Holi, Grahan, Saptadhar, Piyyankar, Furkia, Parsuthach	Pop 10*	36	PK56-PK91
Uttarakhand	Tungnath	Pop 8	03****	PK92-PK94
Himachal Pradesh	Kafnu	Pop 10	02****	PK95-PK96
	Sainj		05****	PK97-PK101
	Pulga		02****	PK102-PK103
	Rohtang		04****	PK104-PK107
	Manimahesh		04****	PK108-PK111
	Holi		03****	PK112-PK114
	Grahan		03****	PK115-PK117
	Saptadhar		03****	PK118-PK120
	Piyyankar		03****	PK121-PK123
	Gulaba		08****	PK124-PK131
	Sai Ropa		06****	PK132-PK137
	Rampur		08****	PK138-PK145

*Leaf materials collected from the cultivated genotypes maintained at the experimental farm of Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, designated as Rahala population

**Out of 10 genotypes, only 5 genotypes (PK1-PK5) were used in chemical profiling

***These genotypes were not used in the chemical profiling

****These genotypes were not part of the molecular analysis

The amplification products in both the cases were size separated by standard horizontal electrophoresis in 1.4% agarose (Sigma, USA) gels and stained with ethidium bromide. The reproducibility of DNA profiles was tested by repeating the PCR amplification thrice with each of the analyzed primer. Only the robust bands that were found to be repeatable were scored for data analysis.

AFLP fingerprinting

About 500 ng of genomic DNA was digested with *Eco*RI and *Mse*I at 37 °C for 2 h followed by heat treatment at 70 °C for 10 min to inactivate the enzymes. The digested DNA was ligated to *Eco*RI and *Mse*I adaptors for 2 h at 20 °C. The ligation mixture was then diluted five fold, and selectively pre-amplified (*Eco*RI primer + A, *Mse*I primer + C) during 20 PCR cycles programmed each at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. Twenty-five fold diluted aliquots of pre-amplified fragments were then selectively amplified in the presence of ^{32}P-labelled *Eco*RI + 3 and *Mse*I + 3 (primers with 3 selective nucleotides) primers. The PCR condition for this amplification was one cycle at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s followed by 12 cycles in which the annealing temperature was progressively lowered by 1 °C, and finally 20 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. The amplified fragments were electrophoresed in 6% denaturing polyacrylamide sequencing gel on a Sequi-Gen (BioRad, USA) sequencing cell. Electrophoresis was carried out at 50 W for 3 h in 1 × TBE at 55 °C. Gel was wrapped in Saran wrap and dried for 1 h at 80 °C. Autoradiogram was developed by exposing Konica X-ray film (AX) on the dried gel overnight at − 80 °C with intensifying screens.

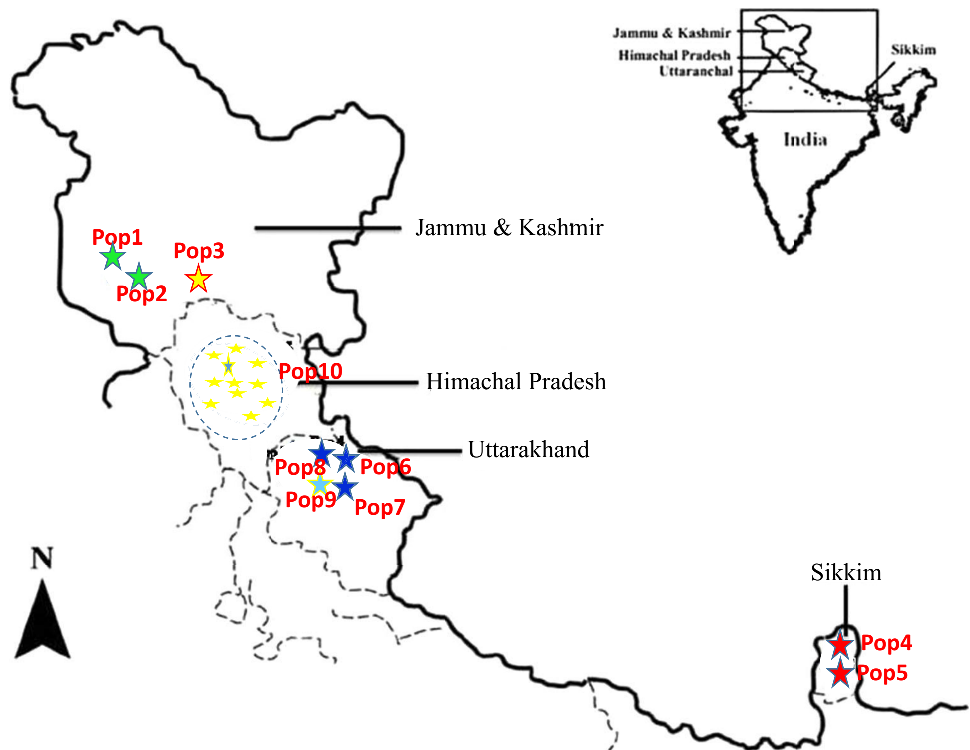

Fig. 1 Germplasm collection sites of *Picrorhiza kurroa* from India

Data analyses

All the amplified bands were scored for the presence (1) or absence (0) and scores were assembled in a rectangular data matrix. The binary matrices were subjected to statistical analysis using the Numerical Taxonomy and Multivariate Analysis System, NTSYS-pc version 2.02 k (Rohlf 1998). Jaccard's similarity co-efficient was employed to compute pairwise genetic similarities. The similarity matrices were constructed for each marker type. Sequential, agglomerative, hierarchical, nested (SAHN) cluster analysis was performed on the data matrix using the unweighted pair group method with the arithmetic averaging (UPGMA) algorithm and 25 iterations. The neighbour joining (NJ) option was also used to construct neighbour joining tree. The validity of the clustering was determined by comparing the similarity and cophenetic value matrices using the matrix comparison module of NTSYS-pc. Principal Component Analysis (PCoA) was done using the PCA function of NTSYS-pc ver 2.02. Bayesian model based clustering method of STRUCTURE ver 2.3.4 (Falush et al. 2007; Pritchard et al. 2000) was employed to estimate the genetic structure. Three independent runs with K values ranging from 3 to 8 and three iterations for each value of K was set. Length of burn-in period and number of Markov Chain Monte Carlo (MCMC) repeats after burn-in were set at 5000 and 50,000, respectively. Results of STRUCTURE were visualized using STRUCTURE HARVESTER (Evanno et al. 2005; Earl 2012) to get the best value of K for the data. Polymorphic information content (PIC) and Marker Index (MI) of each marker was calculated according to Chesnokov and Artem'eva (2015).

Genetic structure of population

Matrices based on population genetic data were analyzed using the software Popgene version 1.31 (Yeh et al. 1999) and Arlequin 3.1 (Excoffier et al. 2005). The Shannon index (I), Nei's genetic diversity (*h*), observed numbers of allele (*na*), effective numbers of alleles (*ne*), Nei's genetic identity and distance, number of migrants (N_m) between populations based on Nei's genetic variation (Gst) [$Nm = 0.5(1 - Gst)/Gst$] and the number of polymorphic loci were estimated for each population using POPGENE version 1.31. Analysis of molecular variance (AMOVA) was used to estimate the variation among populations using Arlequin 3.1, providing Fst values which represent the degree of genetic differentiation or population subdivision. The genotypes, populations and the regions were subdivided into small groups on a predetermined criterion so as to test and quantify between and within group variation. In order to confirm the Fst values, AMOVA data were submitted to 1023 independent permutations and P values lower than 0.05 were considered significant.

Chemical prospecting

Isolation and preparation of standard and stock solutions of picrosides

Isolation and extraction procedure

The dried rhizomes (500 g) of *P. kurroa* were extracted with 1L methanol. The methanolic extract was concentrated under reduced pressure and the residue (380 g) was column chromatographed over silica gel (60–120 mesh). The column was eluted with solvents of increasing polarities, starting with petroleum ether, $CHCl_3$, and variable concentrations of $CHCl_3$ and MeOH. Different fractions of 200 ml each were collected and verified by TLC. The fractions obtained by the elution of column using $CHCl_3$:MeOH (96:4) and $CHCl_3$:MeOH (90:10) yielded compound A and compound B. The structure of two isolated compounds was confirmed by the physical and spectroscopic data and they were identified as picroside-I (P-I) and picroside–II (P-II), respectively (Fig. 2). IR, ^{1}H NMR and MS were used to deduce the following spectroscopic details of the two isolated compounds.

Compound A (P-I): Mp: 127-128 °C (lit. mp. 128-129 °C (Mandal and Mukhopadhyay 2004); IR (KBr, umax cm^{-1}): 3200-3420 (broad, OH), 1705 (conjugated ester), 1660 (enol ether); 1H NMR (CD3CN, 300 MHz, d in ppm): H-9 (2.24, dd, *J* = 4.2, 1.6 Hz, 1H), H-5 (2.48, dd, *J* = 9.6, 7.9 Hz, 1H), H-2′, H-3′, H-4’, H-5’ (3.27-3.62, m, 4H), H-7 (3.84, d, J = 8.0 Hz, 1H), H-10a (4.14, d, J = 12.9 Hz, 1H), H-6 (4.38, dd, J = 12.4, 6.0 Hz, 1H) H-10b (4.42, d, *J* = 12.9 Hz, 1H), H-6a’ (4.74, d, *J* = 8.0 Hz, 1H), H-6b′ (4.86, d, J = 9.0 Hz, 1H), H-4 (5.06, m, 1H), H-1 (5.14, m, 1H), H-1’ (5.22, d, *J* = 8.9 Hz, 1H), H-3 (6.24, m, 1H), Ha (6.48, d, *J* = 16.0 Hz, 1H), Ar-H (7.32-7.50, m, 5H), Hb (7.66, d, *J* = 16.0 Hz, 1H); ESI-MS (m/z): 493.1670 (M+1).

Compound B (P-II): Mp: 211–212 °C (lit. mp. 212-213° C (Mandal and Mukhopadhyay 2004); IR (KBr, umax cm^{-1}): 3600-3225 (broad, OH), 1700, 1636 (conjugated ester); 1H NMR (CD3CN, 300 MHz, d in ppm): H-9 (2.60, t, *J* = 8.2 Hz, 1H), H-5 (2.68, m, 1H), H-2’, H-3’, H-4’, H-5’, H-6a’ & H-6b’ (3.20-3.56, m, 6H), H-7 (3.78, d, *J* = 9.2 Hz, 1H), H-10b (3.82, d, J =13.1 Hz, 1H), -OCH3 (3.93, s, 3H), H-10a’ (4.12, d, J =13.1 Hz, 1H), H-4 (4.80, d, *J* = 6.0 Hz, 1H), H-1’ (5.02, d, *J* = 9.7 Hz, 1H), H-6 (5.08, dd, *J* = 9.0, 6.7 Hz, 1H), H-1 (5.12, d, *J* = 9.0 Hz, 1H), H-3 (6.45, d, *J* = 6.0 Hz, 1H), H-5’’ (6.93, d, J = 8.2 Hz, 1H), H-2’’ (7.56, d, J =1.7 Hz, 1H), H-6’’ (7.6, dd, *J* = 8.3, 1.8 Hz, 1H), OH (7.81, s, 1H); ESI-MS (m/z): 513.1457 (M+1).

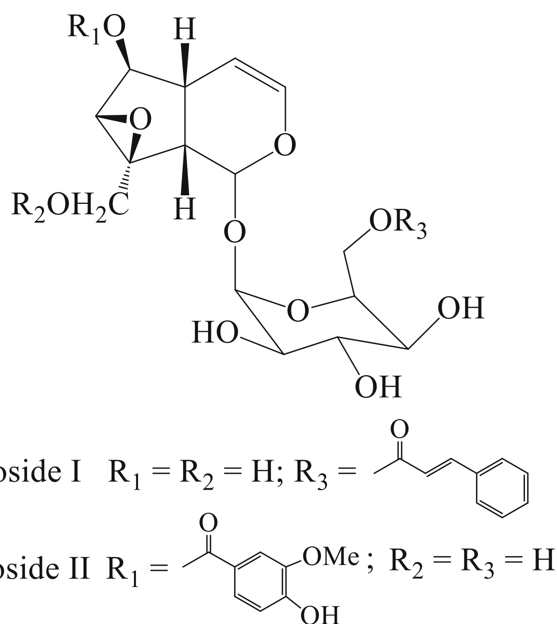

Fig. 2 Chemical structure of picroside I and picroside II

Preparation of standard and stock solutions

Picrosides P-I and P-II were further utilized as standard active compounds for their comparative quantification in 124 collected *P. kurroa* genotypes. Stock solutions of P-I and P-II were prepared by dissolving 1.0 mg of each compound in 1 ml methanol taken in 5 ml volumetric flask. The stock solutions were then filtered through Whatman filter paper No. 41 and different amounts (10, 20, 40 and 60 µl) of the stock solutions were injected into HPLC columns for preparing four point calibration curves.

Preparation of sample solutions

The sample solutions of different genotypes were prepared by dissolving the corresponding dried methanol extract (1.0 mg) in 1 ml methanol using the same procedure (as was done for the standards for preparation of calibration curves) in triplicate.

HPLC analysis

HPLC analysis was carried out using Schimadzu HPLC system with µ-bond pack C-18 column at a wavelength of 274 nm with flow rate of 1 ml/min. The column temperature was maintained at 25 °C. Different compositions of the mobile phase using Water:Methanol (65:35) were tested and desired resolution of P-I and P-II, with symmetrical and reproducible peaks, was achieved. Peaks corresponding to P-I and P-II were observed at retention times of 9.4 and 8.3 min, respectively.

Table 2 Total number of bands (n), number of monomorphic bands (mb), number of polymorphic bands (pb), percentage of polymorphism (pp), polymorphic information content (PIC) and marker index (MI) calculated for RAPD, ISSR primers and AFLP primer combinations in *P. kurroa*

Primer (Sequence)	n	mb	pb	pp	PIC	MI
RAPD						
OPA17 (GACCGCTTGT)	5	1	4	80	0.24	0.77
OPK03 (CCAGCTTAGG)	7	1	6	85.7	0.36	1.85
OPA16 (AGCCAGCGAA)	6	1	5	83.3	0.26	1.08
OPA19 (CAAACGTCGG)	4	2	2	50	0.22	0.22
OPC10 (TGTCTGGGTG)	6	2‘	4	66.6	0.24	0.64
OPH09 (TGTAGCTGGG)	6	1	5	83.3	0.29	1.21
OPH19 (CTGACCAGCC)	5	1	4	80	0.22	0.7
OPJ16 (CTGCTTAGGG)	9	1	8	88.8	0.33	2.35
OPJ08 (CATACCGTGG)	8	1	7	87.5	0.34	2.1
OPT01 (GGGCCACTCA)	7	1	6	85.7	0.33	1.7
OPR17 (CCGTACGTAG)	8	1	7	87.5	0.32	1.96
OPJ09 (TGAGCCTCAC)	7	1	6	85.7	0.33	1.7
OPR06 (GTCTACGGCA)	7	1	6	85.7	0.36	1.85
OPR05 (GACCTAGTGG)	5	1	4	80	0.23	0.74
OPJ18 (TGGTCGCAGA)	7	1	6	85.7	0.28	1.5
OPT02 (GGAGAGACTC)	6	1	5	83.3	0.26	1.08
OPR12 (ACAGGTGCGT)	7	0	7	100	0.41	2.87
OPT18 (GATGCCAGAC)	6	1	5	83.3	0.28	1.17
OPC13 (AAGCCTCGTC)	7	1	6	85.7	0.32	1.65
OPK15 (CTCCTGCCAA)	6	1	5	83.3	0.31	1.29
OPC03 (GGGGGTCTTT)	5	0	5	100	0.43	2.15
OPC16 (CACACTCCAG)	6	2	4	66.6	0.29	0.77
Total	140	23	117	83.5	0.3	1.42
ISSR						
UBC808 (AGA GAG AGA GAG AGA GC)	6	1	5	83.3	0.29	1.21
UBC811 (GAG AGA GAG AGA GAG AC)	7	2	5	71.4	0.26	0.93
UBC815 (CTC TCT CTC TCT CTC TG)	5	1	4	80	0.3	0.96
UBC824 (TCT CTC TCT CTC TCT CG)	6	1	5	83.3	0.32	1.33
UBC826 (ACA CAC ACA CAC ACA CC)	5	1	4	80	0.29	0.93
UBC834 AGA GAG AGA GAG AGA GYT)	6	1	5	83.3	0.31	1.29
UBC836 (AGA GAG AGA GAG AGA GYA)	5	0	5	100	0.37	1.35
UBC840 (GAG AGA GAG AGA GAG AYT)	6	1	5	83.3	0.28	0.88
UBC841 (GAG AGA GAG AGA GAG AYC)	7	2	5	71.4	0.23	0.82
UBC842 (GAG AGA GAG AGA GAG AYG)	8	2	6	75	0.23	0.59
UBC844 (CTC TCT CTC TCT CTC TRA)	6	0	6	100	0.4	1.15
UBC849 (GTG TGT GTG TGT GTG TYA)	6	2	4	66.6	0.24	0.64
UBC850 (GTG TGT GTG TGT GTG TYC)	5	1	4	80	0.36	1.15
UBC854 (TCT CTC TCT CTC TCT CRG)	5	1	4	80	0.33	1.06
UBC858 (TGT GTG TGT GTG TGT GRT)	5	1	4	80	0.31	0.99
Total	88	17	71	80.6	0.3	1.01
AFLP						
E-ACG/ M-CAT	31	11	20	64.5	0.24	3.1
E-AGG/ M-CTT	54	19	35	64.8	0.22	4.5
E-AAG/ M-CTG	52	14	38	73	0.28	7.78
E-AGG/M-CAA	38	10	28	73.6	0.29	5.98
E-AAC/ M-CAT	52	8	44	84.6	0.31	11.54
E-AAC/ M-CTT	54	13	41	75.9	0.33	10.27

Table 2 continued

Primer (Sequence)	n	mb	pb	pp	PIC	MI
E-ACG/ M-CTA	46	16	30	65.2	0.31	6.06
Total	327	91	236	72.1	0.28	7.03

Results

RAPD fingerprinting

Twenty two RAPD primers generated a total of 140 amplification products in 91 genotypes with an average of 6.3 bands per primer and showed a high polymorphism of 83.5%. The number of scored markers ranged from 4 with OPA19 to 9 with primer OPJ16. Different primers generated different number of polymorphic bands (Table 2). The order of polymorphism in analyzed populations was found to be Rahala population > Manvarsar > Thangu > Dayara > Arampatri > Jhuni > Tungnath > Kedarnath > Sonamarg > Temza (Table 3).

ISSR fingerprinting

A total of 88 amplification products were generated by 15 ISSR primers with an average frequency of 5.8 bands per primer. The total number of bands produced by an individual primer ranged from 5 in UBC815, UBC826, UBC836, UBC850, UBC854 and UBC858 to 8 in UBC842. Seventy one bands (80.6%) were observed to be polymorphic among analyzed 91 genotypes. (Table 2). The order of percentage polymorphism between populations was found to be Rahala > Manvarsar > Kedarnath > Arampatri > Jhuni > Temza > Sonamarg > Dayara, Tungnath > Thangu.

AFLP fingerprinting

Seven primer combinations resulted in a total of 327 unambiguously scorable bands. The number of bands per primer combination ranged from 31 in E-ACG/ M-CAT to 54 in E-AAC/ M-CTT and E-AGG/ M-CTT, resulting in an average of 46.7 bands per primer combinations. Two hundred thirty-six (72.1%) bands were observed to be polymorphic among the analysed populations (Table 2). However, the number of polymorphic bands within a population ranged from 51 (15.6%) in Sonamarg to 219 (66.9%) in Manvarsar population (Table 3). The order of percentage polymorphism between populations was found to be Manvarsar > Rahala > Thangu > Dayara > Tungnath > Jhuni > Kedarnath > Temza > Arampatri > Sonamarg. The number of polymorphic fragments for each primer pair in the analyzed samples varied from 20 in E-ACG/M-CAT to 44 in E-AAC/M-CAT with an average of 33.8 per primer pair. Based on the number of polymorphic fragments, different levels of polymorphism, ranging from 64.5% in E-ACG/ M-CAT to 84.6% in E-AAC/ M-CAT were observed (Table 2).

To find out the discriminatory power of various primers, indices such as average PIC and MI were calculated (Table 2). The PIC and MI values varied from (0.22 to 0.41 and 0.77 to 2.35) for RAPD, (0.22 to 0.33 and 3.12 to 11.54) for AFLP, and (0.23 to 0.40 and 0.59 to 1.35) for ISSR, respectively.

Cluster analysis

The clustering pattern obtained with RAPD and AFLP marker data showed good congruence with each other. The RAPD and AFLP based dendrograms (Figs. 3 and 4) grouped 91 genotypes into 4 main clusters arranged largely as per their corresponding states/regions. Cluster 5 included individuals from Uttarakhand and Himachal Pradesh while Cluster 6 represented 2 distinctly different samples from Manvarsar (J&K). Individuals of a single population were mostly arranged in population-specific node in their region specific cluster. No difference in the general topology of the main clusters was found between two dendrograms. In contrast to RAPD and AFLP based dendrograms there was no discrete region-wise clustering in ISSR data based dendrogram except for the genotypes collected from Arampatri and Sonamarg. In all, there were 5 clusters (Fig. 5). Two genotypes from Manvarsar were again observed to be most divergent in ISSR based dendrogram as well, clustering at a similarity value of 0.48 with the other groups.

The Mantel's test (Mantel 1967) resulted in a very good fit of co-phenetic values ($0.907 \leq r \leq 0.934$) for all the three marker systems, indicating that the dendrograms obtained with the three marker systems are a proper representation of their respective similarity matrices.

PCoA analysis based on pairwise distant matrix grouped the samples into four region/state specific clusters. All

genotypes from Manvarsar, and few genotypes from Uttarakhand and Himachal Pradesh showed divergence from their region specific cluster (Fig. 6). Bayesian model based STRUCTURE analysis revealed a pattern similar to UPGMA based dendrogram for all the analyzed populations, except for a few admixtures. A peak was produced by STRUCTURE analysis at delta K = 4 (Fig. 7a). The 10 analyzed populations from four states/regions could be classified into four clusters indicating the existence of four distinct gene pools (Fig. 7b).

Partitioning of genetic variation

A hierarchical AMOVA showed that most of the RAPD (43.4%), ISSR (66.2%) and AFLP (48.7%) markers' diversity were due to variation between individuals within populations. The variation due to differences among regions was 35.3%, 23.9% and 32.0%, respectively. A low proportion of genetic variations (21.2%, 9.7% and 19.2%) detected by RAPD, ISSR and AFLP markers respectively, was due to differences among populations within regions/states (Table 4). Average F-statistics indices Fst, Fct and Fsc were 0.56, 0.35 and 0.32 for RAPD markers, 0.33, 0.23 and 0.12 for ISSR markers and 0.51, 0.32 and 0.28 for AFLP markers, respectively. All values were found to be highly significant ($p < 0.001$).

The overall rate of gene flow (N_m) between populations was moderate for RAPD (0·8434) and AFLP (0.9882) to high for ISSR (1.6093), which means the numbers of migrants per generation are less than one for RAPD and AFLP based data and more than one for ISSR marker based estimates.

The observed numbers of alleles at each locus in a population varied from 1.1240 in Temza to 1.6822 in Rahala, 1.1163 in Thangu to 1.7674 in composite population of Rahala, and, 1.1560 in Sonamarg to 1.6636 in Rahala, with RAPD, ISSR and AFLP markers, respectively. The effective number of alleles in the population ranged from 1.0919 in Temza to 1.3596 in Rahala, 1.0836 in Thangu to 1.5195 in Rahala, and 0.3593 in Manvarsar to 1.4069 in Rahala, with RAPD, ISSR and AFLP markers, respectively. Nei's gene diversity within the species was 0.3366 for RAPD, 0.3240 for ISSR and 0.3246 for AFLP based estimates. Shannon's diversity index varied for each population from 0.0742 for Temza to 0.3255 for Rahala, 0.0675 for Thangu to 0.4314 for Rahala, and 0.0848 for Sonamarg to 0.3466 for Rahala, with RAPD, ISSR and AFLP markers, respectively (Table 3).

Pairwise genetic distance were computed for all the populations. The average genetic distance among populations was 0.320, 0.1820 and 0.287, with RAPD, ISSR and AFLP, respectively. The genetic distance among all populations varied from 0.0703 between Dayara and Tungnath

Table 3 Proportion of genetic diversity detected by RAPD, ISSR and AFLP markers for various populations of *P. kurroa*

Population	Sample size	*Na*			*Ne*			H			I			*Number of PL*			*Percentage of PL*		
		RAPD	*ISSR*	*AFLP*	*RAPD*	*ISSR*	*AFLP*	*RAPD*	*ISSR*	*AFLP*	*RAPD*	*ISSR*	*AFLP*	*RAPD*	*ISSR*	*AFLP*	*RAPD*	*ISSR*	*AFLP*
Pop 1 (Arampatri)	10	1.2558	1.3488	1.1682	1.1873	1.2667	1.11	0.1037	0.1495	0.0624	0.1503	0.2158	0.0921	33	31	55	25.58	35.22	16.82
Pop 2 (Sonamarg)	7	1.1628	1.2791	1.156	1.1044	1.1884	1.1026	0.0601	0.1052	0.0575	0.0893	0.1543	0.0848	21	25	51	16.28	28.4	15.6
Pop 3 (Manvarsar)	4	1.5116	1.6279	1.471	1.3077	1.4189	1.3593	0.1848	0.2424	0.2515	0.2773	0.3577	0.2779	66	55	219	51.16	62.5	66.97
Pop 4 (Temza)	4	1.124	1.3023	1.1743	1.0919	1.2222	1.1176	0.0511	0.1247	0.0669	0.0742	0.1812	0.0986	16	27	57	12.4	30.68	17.43
Pop 5 (Thangu)	5	1.3876	1.1163	1.2905	1.2498	1.0836	1.2056	0.1446	0.0463	0.1156	0.2145	0.0675	0.1689	50	15	95	38.76	17.04	29.05
Pop 6 (Kedarnath)	10	1.2093	1.3721	1.1835	1.1455	1.2602	1.1007	0.0809	0.1476	0.0593	0.1189	0.2157	0.0897	27	33	60	20.93	37.5	18.35
Pop 7 (Dayara)	6	1.2791	1.2558	1.2661	1.1718	1.2291	1.1897	0.0985	0.1198	0.1062	0.147	0.1688	0.1549	36	23	87	27.91	26.13	26.61
Pop 8 (Tungnath)	4	1.2326	1.2558	1.263	1.1765	1.1773	1.1886	0.0964	0.099	0.1066	0.1395	0.1454	0.1556	30	23	86	23.26	26.13	28.3
Pop 9 (Jhuni)	5	1.2481	1.3256	1.2202	1.1579	1.2583	1.1497	0.0893	0.1393	0.0851	0.1327	0.1998	0.1253	32	29	72	24.81	32.95	22.02
Pop 10 (Rahala)	36	1.6822	1.7674	1.6636	1.3596	1.5195	1.4069	0.2148	0.2945	0.2336	0.3255	0.4314	0.3466	88	68	217	68.22	77.27	66.36
Overall	91	1.8992	1.8837	1.8899	1.5722	1.5787	1.5665	0.3366	0.324	0.3246	0.4961	0.4765	0.4815	117	71	236	83.5	80.6	72.17

na, observed number of alleles; *ne*, effective numbers of alleles; h, Nei's gene diversity; I, Shannon's diversity index; PL, Polymorphic loci

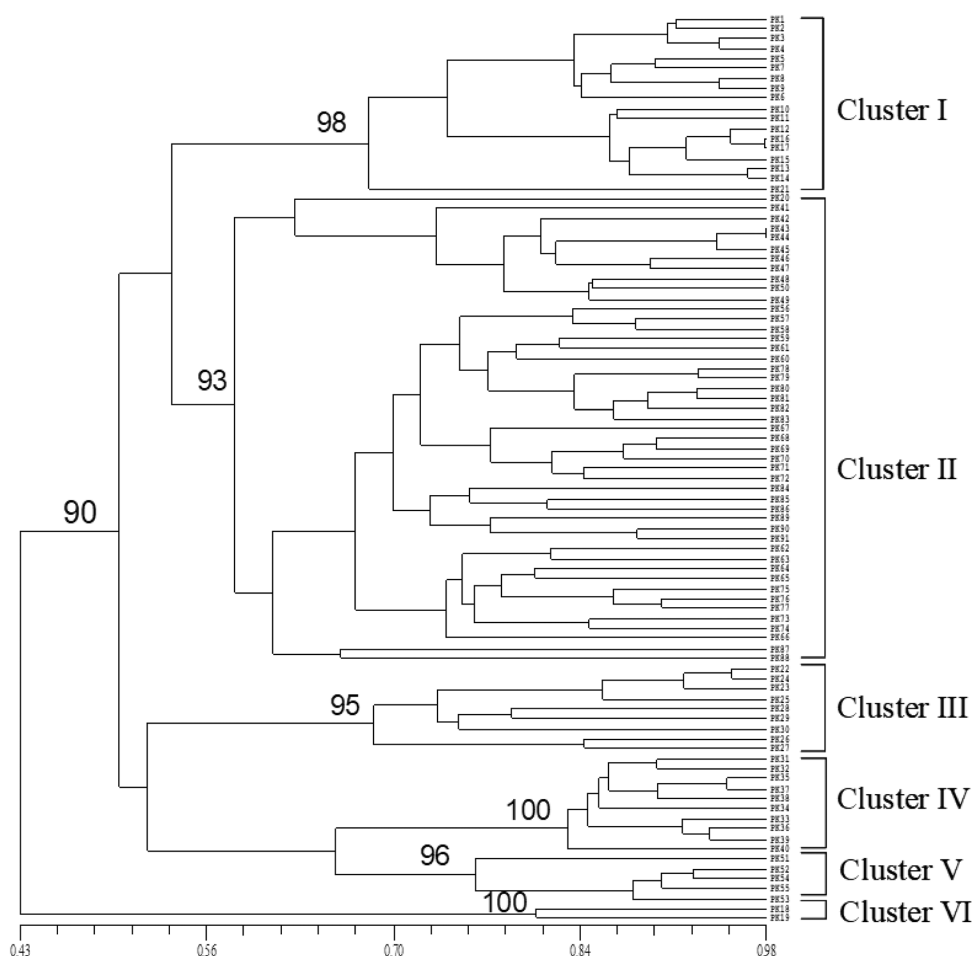

Fig. 3 UPGMA dendrogram of the 91 *P. kurroa* genotypes based on RAPD marker data

to 0.4949 between Arampatri and Temza with RAPD, from 0.0505 between Arampatri and Sandukmore to 0.3740 between Manvarsar and Thangu with ISSR, and from 0.0353 between Arampatri and Sandukmore to 0.4220 between Manvarsar and Temza with AFLP marker data sets. UPGMA dendrograms based on RAPD, ISSR, and AFLP data were constructed to reveal the genetic relationships between populations (Figs. 8, 9 and 10). The RAPD and AFLP based dendrograms were congruent with each other, demarcating 10 populations into region specific four main groups. With a few exceptions, ISSR based dendrogram also segregated 10 populations into their regional groups.

Characterization of isolated compounds

Picrorhiza kurroa dried rhizomes (500 g) were extracted in methanol (1 L). The residue obtained after solvent evaporation was column chromatographed over silica gel (60–120 mesh). Elution of column with $CHCl_3$:MeOH (96:4) gave 1 g of compound A, which gave pink colour with vanillin-sulphuric acid (5%), indicating the presence of iridoid moiety. In ^{1}H NMR spectrum double doublets at δ 2.24 (J = 5.0 & 2.1 Hz) 2.48 (dd, J = 9.6 & 7.9 Hz) and 4.38 (J = 12.4 & 6.0 Hz) ppm were assigned to H-9, H-5 and H-6 protons. Doublets at δ 3.84 (J = 8.07 Hz), 4.14 (J = 12.9 Hz), 4.42 (J = 12.9 Hz), 4.74 (J = 8.0 Hz), 4.86 (J = 9.0 Hz), 5.22 (J = 8.9 Hz) ppm for one proton each were assigned to H-7, H-10_a, H-10_b, H-6_a', H-6_b and H-1′ protons respectively. The multiplets between δ 3.27–3.62, 5.06 and 5.14 ppm were assigned to the sugar protons, H-4 and H-1 protons respectively. Also, doublets at δ 7.66 (J = 16.0 Hz) and 6.48 (J = 16.0 Hz) ppm for one proton each were assigned to H_α and H_β protons of *t*-cinnamoyl ester moiety and a five proton multiplet between δ 7.32–7.50 ppm was assigned to the aromatic protons. Compound A with molecular formula $C_{24}H_{28}O_{11}$ (492.48) showed M + 1 peak at 493.170 in the ESI–MS spectrum. The above spectral data indicated the compound A as picroside-I (P-I).

Further elution of column with $CHCl_3$:MeOH (90:10),1.2 g of another white coloured compound B was obtained, which responded positively for iridoids with vanillin-sulphuric acid (5%). The IR spectrum of the

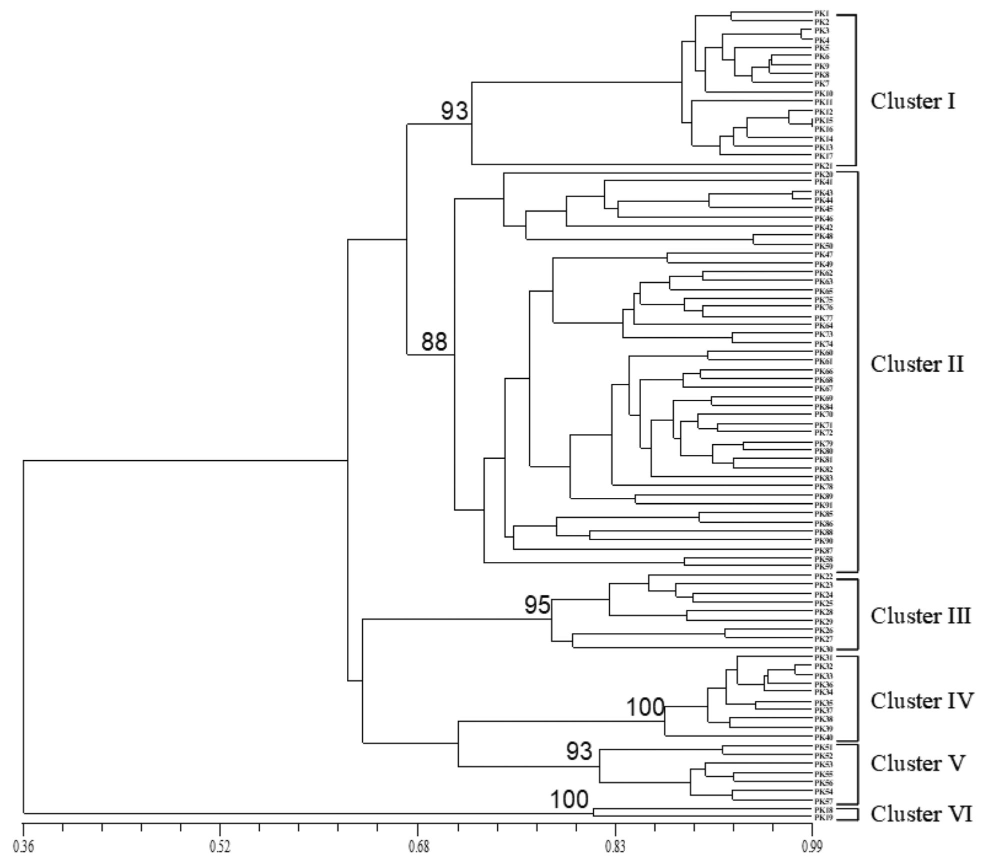

Fig. 4 UPGMA dendrogram of the 91 *P. kurroa* genotypes based on AFLP marker data

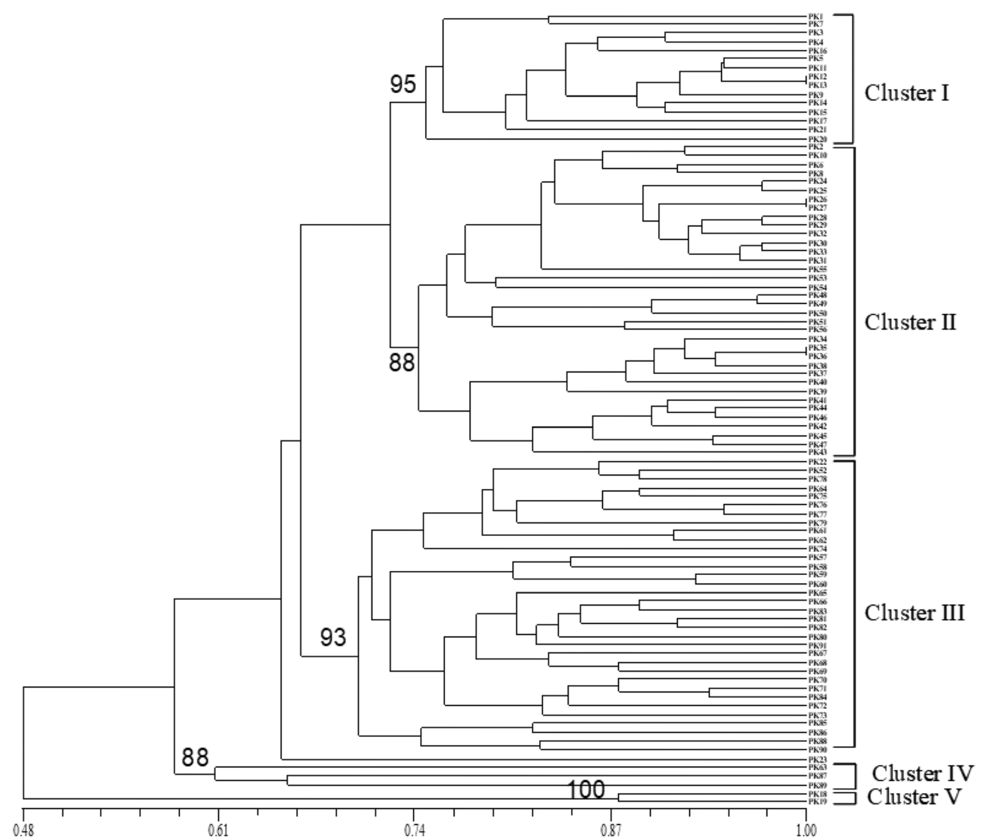

Fig. 5 UPGMA dendrogram of the 91 *P. kurroa* genotypes based on ISSR marker data

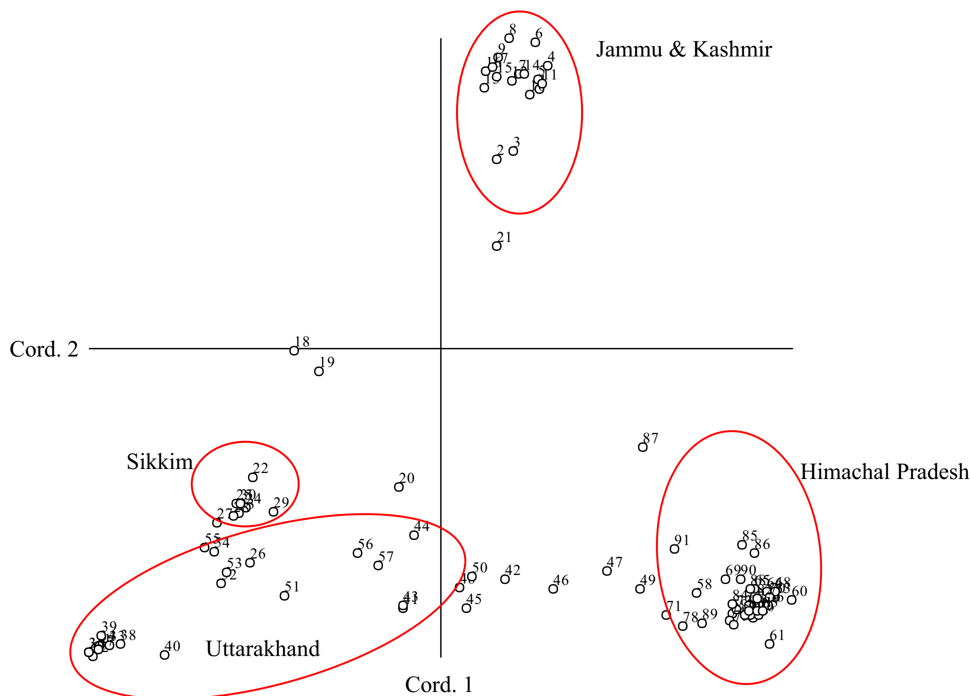

Fig. 6 Principal coordinate analysis (PCoA) of *P. kurroa* genotypes

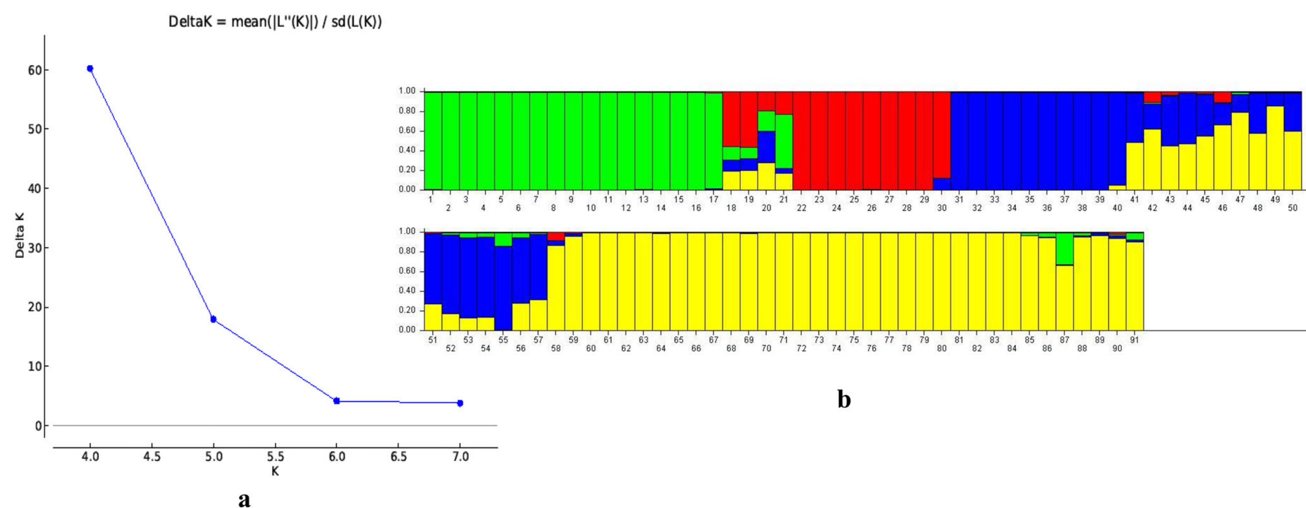

Fig. 7 STRUCTURE analysis based clustering of 91 individuals of *P. kurroa.* **a** Plot showing corresponding value of delta K, **b** Histogram showing distinct genetic pools in population-wise clustering

compound B showed the presence of a peak at 3400–3200 cm^{-1}, while in ^{1}H NMR spectrum a triplet at δ 2.60 (J = 8.2 Hz) and a double doublet at 5.08 (J = 9.0, 6.7 Hz) ppm for one proton each were aggined to H-9 and H-6 protons respectively. A doublet at δ 3.78 (J = 9.2 Hz), 3.82 (J = 13.1 Hz), 4.12 (d, J = 13.1 Hz), 4.80 (d, J = 6.0 Hz), 5.02 (d, J = 9.0 Hz), 5.12 (J = 9.7 Hz) and 6.45 (d, J = 6.0 Hz) ppm were assigned to H-7, H-$10_{a,}$ H-$10'_{b}$, H-4, H-1′, H-1 and H-3 protons. The multiplets between δ 3.20–3.56 and 2.68 ppm were assigned to the sugar protons and H-5 protons respectively. A singlet at δ 3.93 ppm for three protons was assigned to the methoxy group and a singlet at δ 7.81 ppm was assigned to the hydroxyl proton. Two doublets for one proton each at δ 6.90 (J = 8.7 Hz) and 7.56 ppm were assigned to the *ortho* and *meta* coupled protons H-5″ and H-2″ protons, while the double doublet at δ 7.60 ppm was assigned to H-6″ proton, which indicated the presence of vanilloyl group. Compound B with molecular formula $C_{23}H_{28}O_{13}$ (512.46) showed M + 1 peak at 513.157 in the ESI–MS spectrum. The above data indicated that the compound B was picroside-II (P-II).

Table 4 Apportionment of genetic diversity between and within populations of *P. kurroa* genotypes by AMOVA

Source of variation	d.f	Sum of squares	Variance component	Percentage of variation
RAPD				
Among regions	3	723.723	8.59	35.39
Among populations within regions	6	334.430	5.14	21.20
Within populations	81	853.605	10.53	**43.41**
Total	90	1911.758	24.27	
ISSR				
Among regions	3	126.131	1.50	23.93
Among populations within regions	6	57.528	0.61	9.78
Within populations	81	338.362	4.17	**66.29**
Total	90	522.022	6.30	
AFLP				
Among regions	3	1445.334	16.96	32.01
Among populations within regions	6	693.332	10.21	19.28
Within populations	81	2091.422	25.82	**48.72**
Total	90	4230.088	53.00	

Significance tests (1023 permutations)

Bold values indicate that the signify the maximum percentage of variation detected with AMOVA analysis

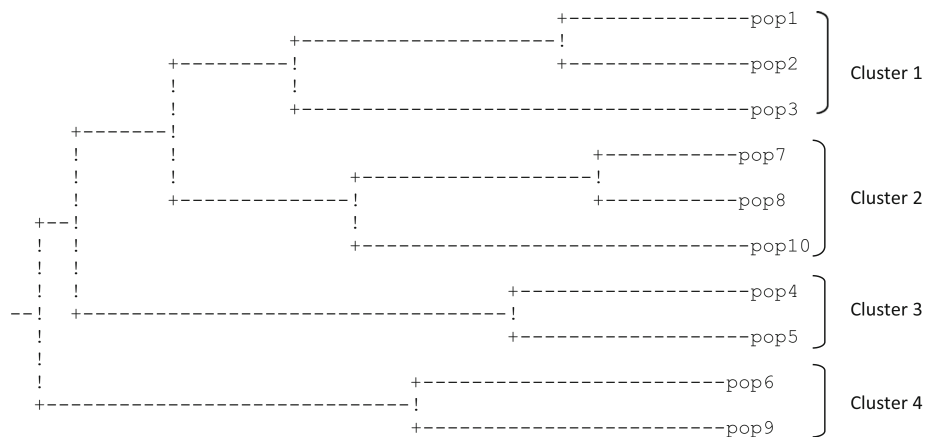

Fig. 8 Nei's genetic distance dendrogram of *P. kurroa* populations based on RAPD marker data

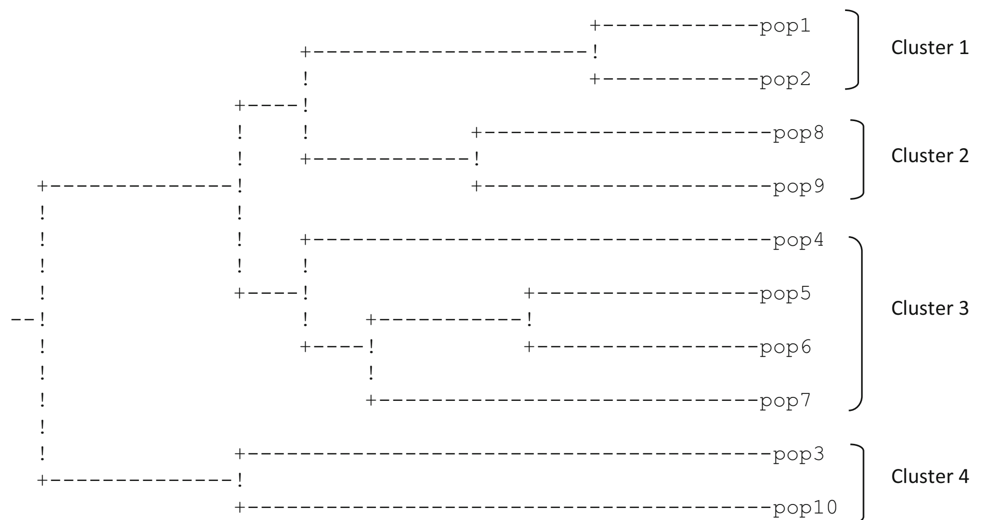

Fig. 9 Nei's genetic distance dendrogram of *P. kurroa* populations based on ISSR marker data

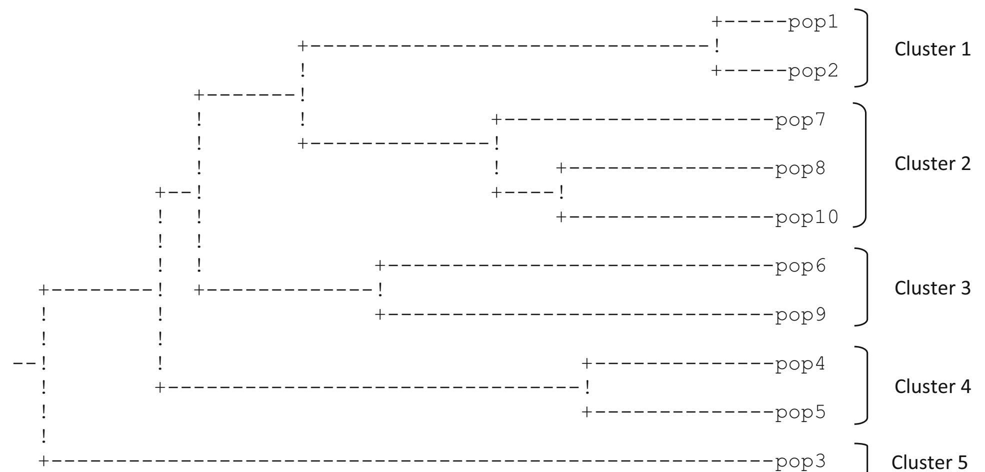

Fig. 10 Nei's genetic distance dendrogram of *P. kurroa* populations based on AFLP marker data

Quantification of picrosides P-I and P-II

Well defined peaks were observed for picrosides P-I and P-II in the analyzed samples (Fig. 11a–c). Peak purity tests were done by comparing UV–Visible spectra of P-I and P-II in standard and sample track. The peaks corresponding to P-I and P-II were symmetrical and well separated from other peaks. The quantified range of P-I and P-II content in the analyzed samples collected from different locations is detailed in Table 5**.**

The levels of P-I and P-II for individual genotypes varied substantially and for a representative sample have been shown in Fig. 12. The P-I concentration in individual samples varied from 0.01 percent in genotype 'PK116' from Grahan to 4.15% in a genotype 'PK92' from Tungnath. The P-II concentration in individual samples varied from 0.01% in individual genotypes from Temza and Kafnu to 3.18% in genotype 'PK68' from Sainj.

The P-II concentration (> 1.0%) was detected in the genotypes of Sainj, Temza, Dayara, Furkia and Rahala. Similarly, more than 1.0% P-I concentration was detected in the genotypes of Tungnath, Sainj, Dayara, Parsuthach, Mani Mahesh, Pulga, Kedarnath, Temza, Rohtang, Holi and Gulaba. On a closer observation, it was found that low P-I concentration in a genotype was associated with largely a high P-II concentration as evident in Fig. 12 and Table 5. Accordingly, most of the genotypes were either P-I or P-II rich, and a few only showed almost similar amount of both P-I and P-II (Fig. 12).

Discussion

This study provides comprehensive insights into the genetic and phytochemical diversity across 91 and 124 *P. kurroa* genotypes belonging to 10 geographically distinct Indian Himalayan populations, respectively. Molecular DNA marker analysis revealed a sufficient level of polymorphism by ISSR (80.6%), RAPD (83.5%) and AFLP (72.1%) analysis. The data generated was found to be useful in estimating the level of genetic diversity and understanding the population structure in *P. kurroa*. The average PIC and MI values for RAPD (0.30, 1.42), ISSR (0.30, 1.01) and AFLP markers (0.28, 7.03), even with the limited no. of genotypes available in some analyzed population(s) indicated that these markers are capable of providing useful information on genetic diversity.

Revelation of a higher level of polymorphism by RAPD as compared to AFLP markers as seen in the present study have also been reported in *Antirrhinum microphyllum* (Torres et al. 2003), *Digitalis minor* (Sales et al. 2001), *Hordeum vulgare* (Russell et al. 1997), Olive (Belaj et al. 2003) and *Valeriana jatamansi* (Kumar et al. 2014). Further, the incongruence of ISSR data based dendrogram with both RAPD and AFLP based dendrograms, indicates that each of the analyzed markers spans different regions of the genome and genetic relationships and distances are dependent on genome coverage and/or the type of sequence variation recognized by each marker system (Powell et al. 1996; Pejic et al. 1998; Degani et al. 2001). Further, the inherent biasedness in the information generated by dominant RAPD, ISSR and AFLP markers was compensated in the present analysis by analyzing a large number of loci with all the three markers system, across many genotypes. The unbiased analysis is further supported by high levels of significance, which suggests that results are robust (Lynch and Milligan 1994).

AMOVA values estimates within and among populations for RAPD (43.4%, 35.3%), ISSR (66.2%, 23.9%) and AFLP (48.7%, 32.0%), respectively indicated that populations are not genetically divergent, although the genotypes within the populations show a high level of genetic diversity. F-statistics was used to estimate the proportion of genetic variability among populations (F_{ST}), among

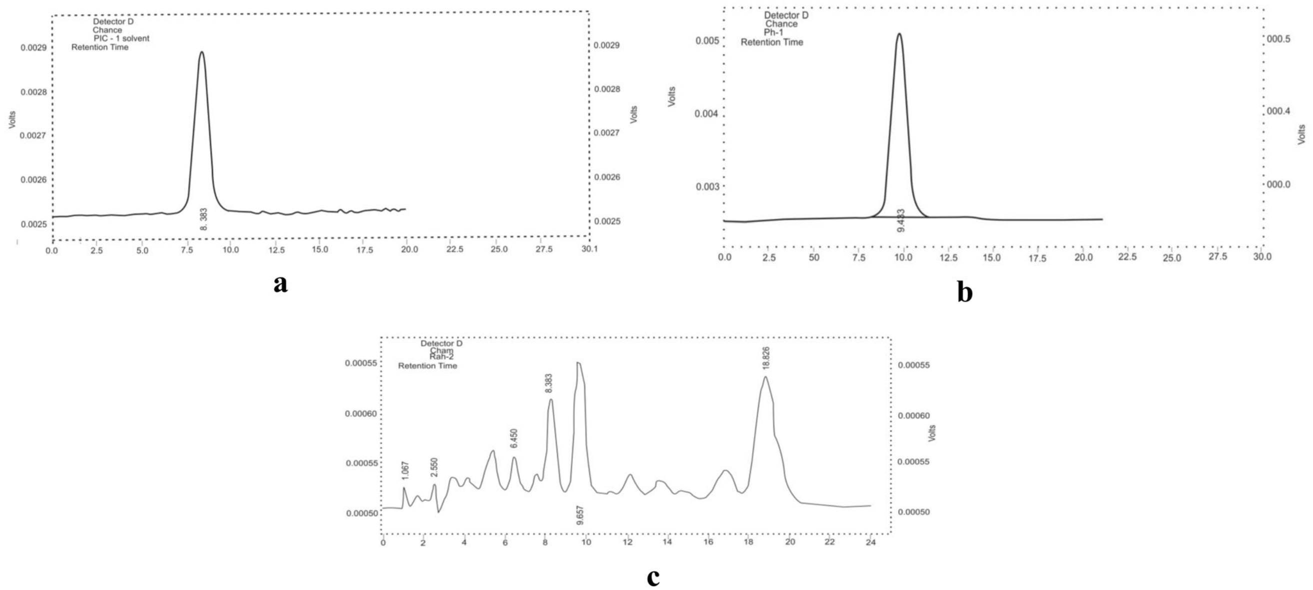

Fig. 11 Chromatogram of standards picroside-1 (**a**), picroside-II (**b**) and Tungnath genotype showing major peaks for two isolates (**c**)

Table 5 Range of P-I and P-II detected from *P. kurroa* collected from different Himalayan Locations

S. no.	Region	Location	Altitude (mt.)	Picroside I (%)	Picroside II (%)
1	Jammu & Kashmir	Arampatri	3000	0.02–0.68	0.10–0.56
2	Sikkim	Temza	3000	**0.32–1.66**	0.01–1.41
3	Uttarakhand	Jhuni	2008	0.19–0.55	0.0–0.87
4		Kedarnath	3553	**0.0–2.0**	0.0–0.63
5		Dayara	**3580**	**0.12–4.04**	**0.06–1.3**
6		Tungnath	**3680**	**0.13–4.15**	**0.0–0.54**
7	Himachal Pradesh	Sairopa	1800	0.10–0.29	0.03–0.81
8		Rampur	2020	0.02–0.46	0.0–0.83
9		Holi	2200	0.22–1.03	0.04–0.92
10		Saptadhar	2200	0.21–0.87	0.05–0.69
11		Grahan	2298	0.01–0.75	0.03–0.49
12		Kafnu	2496	0.63–0.86	0.01–0.69
13		Sainj	**2733**	**0.16–4.07**	**0.04–3.18**
14		Parsuthach	**2750**	**1.24–3.54**	**0.12–0.16**
15		Rahala	2800	0.20–0.59	0.43–1.0
16		Pulga	2895	**0.13–2.56**	0.04–0.24
17		Solang nallah	3060	0.02–0.29	0.002–0.76
18		Furkia	3250	0.1–0.13	**1.0–1.1**
19		Piyyankar	3613	0.24–0.65	0.002–0.32
20		Rohtang	**3980**	**0.18–1.26**	**0.10–0.79**
21		Gulaba	**4000**	**0.22–1.03**	0.02–0.43
22		Mani Mahesh	**4080**	**0.37–2.75**	0.04–0.57

Bold values indicate that the signify the maximum concentration (at different altitudes) of either P-I and P-II detected in the analyzed genotypes

populations within groups (F_{SC}) and among groups (F_{CT}). Average F indices F_{ST}, F_{CT} and F_{SC} values were 0.56, 0.35 and 0.32 for RAPD markers, 0.33, 0.23 and 0.12 for ISSR markers and 0.51, 0.32 and 0.28 for AFLP markers, respectively, indicating thereby that most of the observed variability is due to variation among genotypes within population followed by among populations.

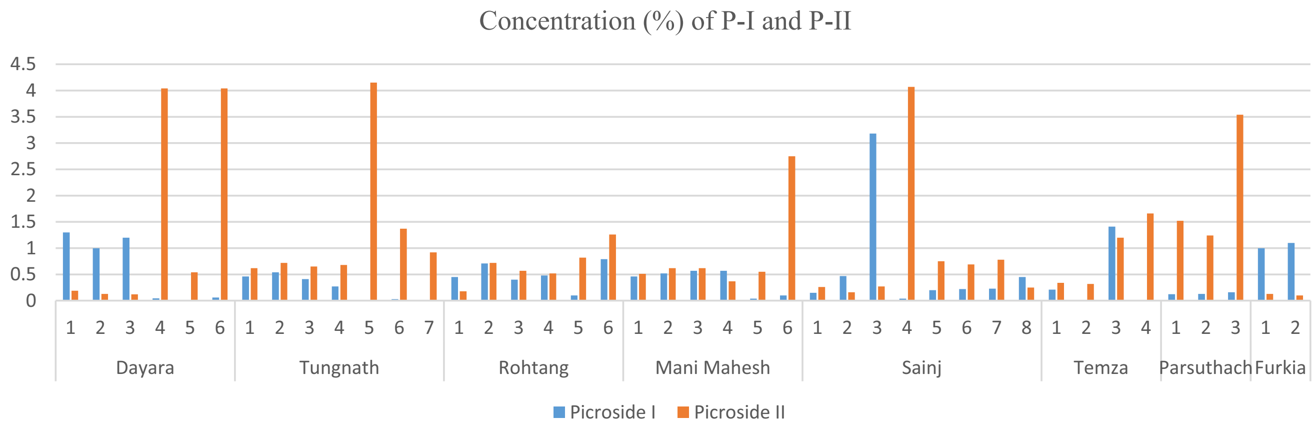

Fig. 12 Representative comparative histogram of P-I and P-II extracted from rhizome of *P. kurroa* genotypes. X-axis represents number of genotypes from the respective location while Y-axis represents concentration (%) of picrosides

The calculated estimates of Nei's gene diversity for RAPD (0.33), ISSR (0.32) and AFLP (0.32) data sets for *P. kurroa* fell in between a high value of, for example 0.49 reported in wild populations of outcrossing *Brassica oleracea* (Lannér-Herrera et al. 1996) and 0.274 and 0.278 reported for narrow endemic species of *Allium aaseae* and *A. simillium* (Smith and Pham 1996), respectively. Analysis of gene flow (*Nm*) values for RAPDs (0.8434) and AFLPs (0.9882) to comparatively higher levels for ISSRs (1.6093) indicated moderate levels of gene flow. The analyzed samples were collected from a distance spanning 1000 km separated by geographical barriers that might have allowed little pollination and seed dispersal. The estimated levels of gene flow and Nei's gene diversity values indicate that *P. kurroa*, which is largely an outcrossing species, has had a continuous habitat across the Indian Himalayas running from western to eastern fronts that has now been fragmented due to over-exploitation and/or habitat loss. The fragmentation reduces populations to smaller isolates which are subjected to increasing genetic drift, inbreeding and reduced gene flow (Carvalho et al. 2019).

Co-phenetic correlation values of Mantel's test are 0.907 to more than 0.934 for the three marker systems. This validates the trees developed on the basis binary matrices are a true representation of their similarities. Region specific clusters with NJ, PCoA and Bayesian model based STRUCTURE analysis were observed with all the three analyzed markers with small intermixing in some groups indicating limited gene flow between them. The admixtures might reflect past genetic exchange events as STRUCTURE only estimates global ancestory by implementing different models of population structure to the data.

Although, a fairly high overall genetic variation was reflected within the analyzed populations, the level of polymorphism detected among populations of *P. kurroa* is low and might prove detrimental in the evolutionary and ecological context of the species (Barrett and Kohn 1991).

A high level of genetic diversity revealed in the present investigations in *P. kurroa* populations fall in line with the earlier reports by Katoch et al. (2013) and Singh and Sharma (2020) in the species. Outcrossing perennials have generally been reported to exhibit higher levels of genetic diversity and lower levels of population differentiation (Hamrick and Godt 1990, 1996). As observed in many outcrossing species, populations isolated by distance can set forth independent genetic differentiation in them to accumulate divergent alleles (Prentice et al. 2003) and bring in evolutionary differences. This can in turn affect the population structure.

HPLC analysis has been used as a preferred method for isolation, chemical characterization, and quantification of phytochemicals in many medicinal plant species (Han et al. 2008; Sultan et al. 2008; Thapliyal et al. 2012; Song et al. 2015; Mehra et al. 2017; Thakur et al. 2020).The concentration of picrosides in dry rhizomes of different populations of *P. kurroa*, has been found to vary with altitude in different ecogeographical regions in Himalayas. In Himachal Pradesh, the highest concentration of P-I and P-II was observed between an altitude of 2733 m (Sainj) to 2750 m (Parsuthach), in Uttarakhand it was observed between 3580 m (Dayara) to 3680 m (Tungnath), while in Kashmir and Sikkim regions, it was observed at 3000 m altitude in Arampatri and Temza, respectively. In general, more picrosides content was observed in genotypes growing at higher altitudes. With an increase in altitude, cold weather period increases resulting in slow growth and consequently increasing the content of picrosides per gram of the tissue. Similar observations have previously been made by Sultan et al (2016) who showed higher P-I (2.78–5.18%) and P-II (2.53–5.39%) variation in *P. kurroa* collections from 2799 to 3750 m altitudes. In the present study, we quantified

slightly lower ranges of P-II (0.01–4.15%) and P-II (0.01–3.18%) in genotypes collected from altitudes 1800–4080 m which might be due to effect of changes in growth conditions, season of collection and local geographical and climatic condition on accumulation of picrosides.

Further, it was interesting to observe that low P-I content in a given genotype was generally observed to be associated with high P-II content suggesting an interconversion of PI and PII. The present analysis allowed identification of P-I rich, P-II rich or both P-I and P-II rich genotypes. Similar observations were made by Kumar et al. (2017), who identified genotypes PKST-3 and PKST-5 as maximum P-II and minimum P-I genotypes, while PKST-16 and PKST-18 were identified as minimum P-II and maximum P-I genotypes. They proposed that metabolic network of picrosides I and II biosynthesis is very complex. An increase in P-II upon reduction in P-I indicated that P-I and P-II skewed from a common metabolic node and P-I and P-II biosynthesis is regulated by metabolic modulations (Kumar et al. 2017). Based on present analysis, the genotypes of Sainj, Dayara, Parsuthach, Tungnath, Furkia and Temza may be considered as superior genotypes with higher quantity of P-I and P-II.

Upon correlation of data generated by genetic and phytochemical markers, it was observed that some genotypes with higher concentration of either P-I or P-II, like from Temza and Tungnath populations did not exhibit much genetic polymorphism. Similar negative correlation between genotypic and phytochemical diversity has been observed in *Ocimum basilicum* (De Masi et al. 2006), *Thymus caespititius* (Trindade et al. 2008), *Cymbopogon* sp. (Kumar et al. 2009) and *Zataria multiflora* (Hadian et al. 2011). On the other hand, both a high level of genetic polymorphism as well as picrosides P-I or P-II in Rahala population, especially in genotypes from Grahan, Rohtang, Holi, Mani Mahesh and particularly Sainj clearly displayed a positive correlation between the two marker systems as seen previously in *Podophyllum hexandrum* (Sultan et al. 2008).

To sum up, although the present comprehensive molecular and phytochemical analysis in *P. kurroa* revealed a high genetic diversity and various statistical analysis indicated that populations did not show much genetic divergence, and the observed genetic diversity resides largely among the genotypes within the populations. Nevertheless, on the basis of molecular and phytochemical markers, the genotypes from Sainj, Parsuthach, and Furkia (Himachal Pradesh), Arampatri and Manvarsar (Jammu and Kashmir), Dayara, Kedarnath and Tungnath (Uttarakhand) and Temza and Thangu (Sikkim) with high genetic heterozygosity and picrosides content can contribute towards a probable core collection of most variable genotypes in *P. kurroa* which can be further characterized and used for multiplication, conservation and genetic improvement purposes.

Conclusions and conservation implications

In the present study, both molecular DNA and phytochemical markers efficiently partitioned the genetic variation in different populations of *P. kurroa*. Diversity and clustering analysis grouped the populations distinctly providing a clear spatial population structure depicting limited gene flow between them. Most of the genetic variability was reflected at the intra-population level with low inter-population variation. The present study has helped in the demarcation of most divergent *P. kurroa* genotypes with high percentage of genetic polymorphism and picrosides content. These elite genotypes may potentially be used for further characterization, multiplication, industrial utilization and conservation of *P. kurroa* germplasm. An imperative conservation strategy in *P. kurroa* may include integrating in situ and ex situ management processes, setting up of protected areas and cultivation practices of divergent *P. kurroa* genotypes. Intensive botanical surveys followed by characterization of genetic diversity by various approaches can be really helpful in identification of elite *P. kurroa* genotypes. Additionally, involvement of all the stakeholders, i.e. local communities, academicians and industries at all levels of planning, execution, monitoring and assessment of conservation processes can result in better resource management. Further, biotechnological interventions including molecular markers and phytochemical quantification analysis as done in the present analysis followed by micropropagation of identified elites can help support the conservation program in the species. The present information on genetic and phytochemical diversity, gene flow and population structure of *P. kurroa* might be useful to understand the evolutionary pathways, prioritize suitable sampling strategies for resource management, sustained use and conservation of this important medicinal species.

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