DNA Fingerprint Profiling (RAPD) of superior chemotypes of *'Picrorhiza kurroa'*

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CERTIFICATE

This is to certify that the work entitled, DNA **Fingerprint Profiling (RAPD) of superior chemotypes of '*Picrorhiza kurroa*'** submitted by **Gurpreet Bhatia (071721)** in partial fulfillment for the award of degree of Bachelors of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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DATE -

SUMMARY

P. kurroa Royle ex Benth (Family: Scrophulariaceae) is a medicinal herb mainly found in the North-Western Himalayas at altitudes of 3000-4300 meters. *P. kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, treat dyspepsia, chronic diarrhea, scorpion sting, etc. A commercial formulation named as Picroliv has been prepared from *P. kurroa* extracts containing Picroside-1 as the main component and has been launched as a highly effective hepatoprotective drug. The reckless collection of *P. kurroa* has reduced its populations to a very low levels resulting in its categorization as an endangered species, thereby, warranting the development of alternative strategies for the conservation and production of metabolites of medicinal value. The RAPD technique has been successfully used in a variety of taxonomic and genetic diversity studies. The genetic diversity has been studied in six accessions of *Picrorhiza kurroa* collected from different villages of Himachal Pradesh using four random amplified polymorphic DNA primers. This study revealed rich genetic diversity among *six* accessions of *P. kurroa* from H.P region in India.

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List of abbreviations

2,4-D	2,4,di chloro phenoxy acetic acid
AFLP	Amplified fragment length polymorphism
BA	Benzyl adenine
BAP	Benzyl amino purine
G	Gram
HCl	Hydrogen chloride
HgCl2	Mercuric chloride
IAA	Indole acetic acid
IBA	Indole butyric acid
KN	Kinetin
L	Litre
Lb	Pound
M	Molar
Mg	Milligram
Ml	Millilitre
mM	Millimolar
RAPD	Randomly amplified polymorphic DNA
µM	Micromolar
UV	Ultraviolet

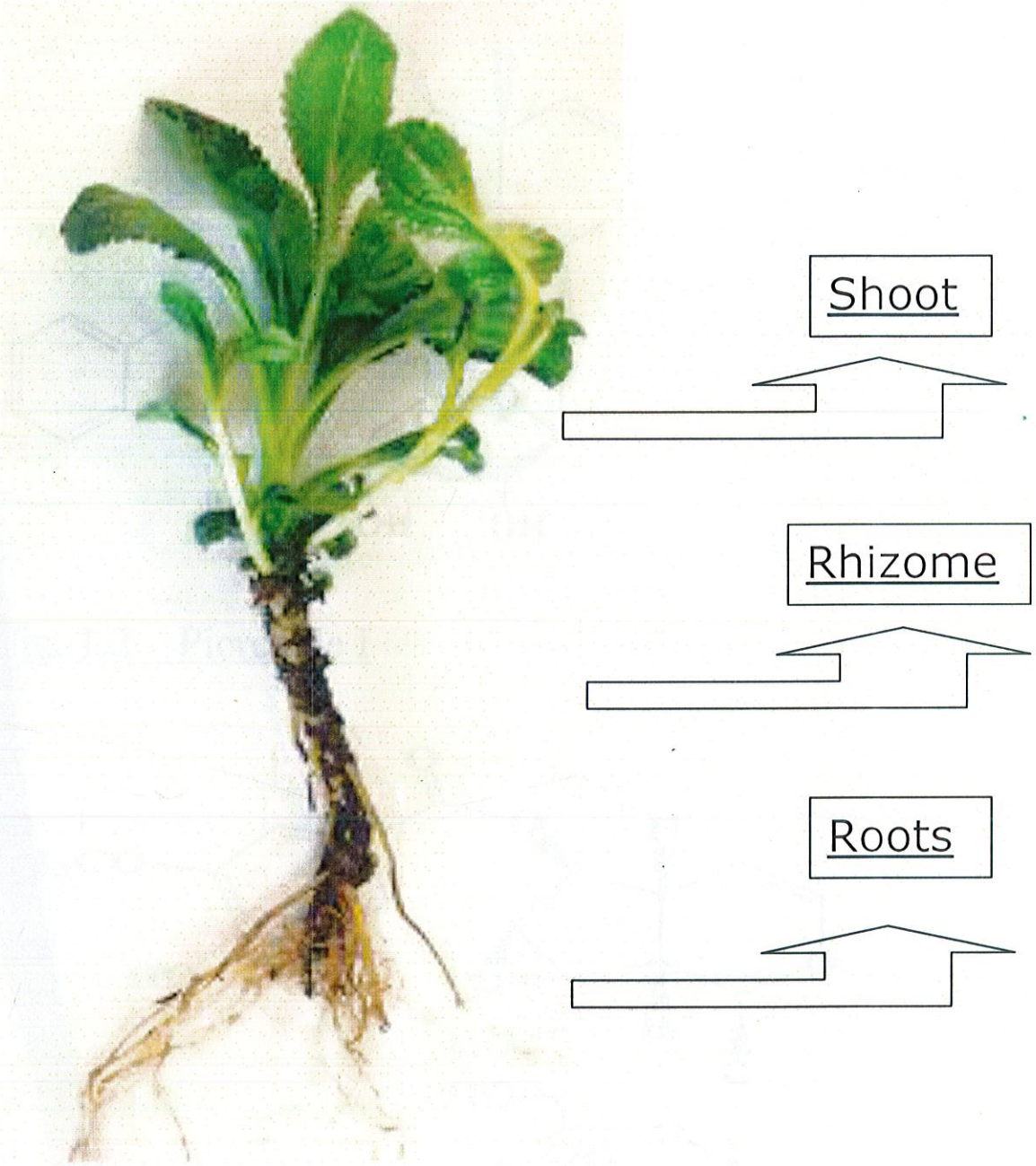


Fig.1.1 Picrorhiza kurroa plant

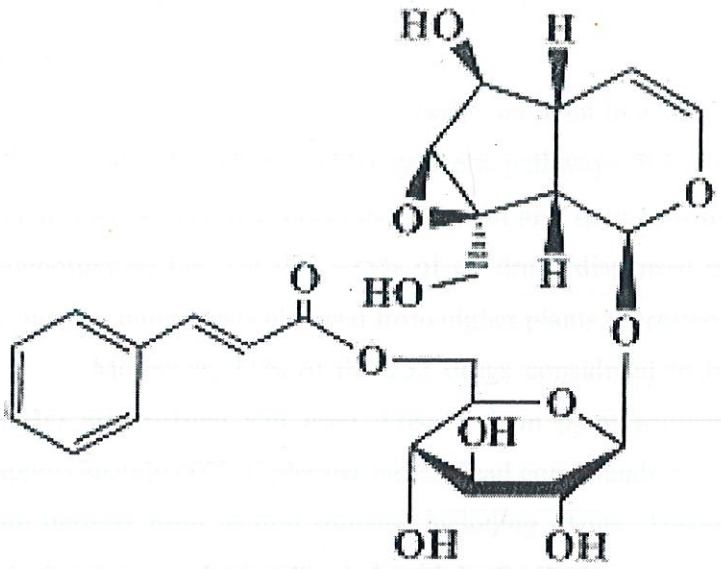


Fig. 1.2 Picroside I

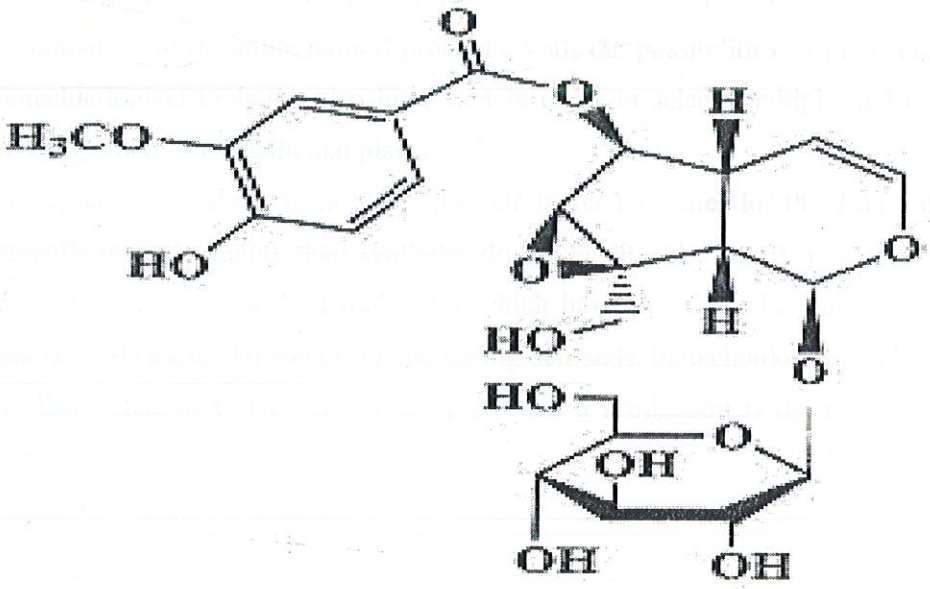


Fig. 1.3 Picroside II

Although current synthetic drugs are in common usage, yet the use of herbal drugs is well accepted, and a continuously high demand for plant material and natural products has been observed.

Hence, the approaches of medicinal plant biotechnology currently focus more on distinct natural products and biosynthetic pathways. It is not only plants that are of great interest to the pharmaceutical industry, but also defined natural products. This situation is supported by the fact that ~25% of all drugs dispensed during the 1970s in the USA contained compounds obtained from higher plants (Farnsworth et al. 1976).

Moreover, 11% of the 252 drugs considered to be basic and essential by the WHO are isolated and used directly from plant sources (Rates 2001). In addition, approximately 40% of pharmaceutical lead compounds for the synthetic drugs used today are derived from natural sources, including plants. Today, only 10% of all medicinal plant species used are cultivated, with by far the larger majority being obtained from wild collections.

From this background the question arises as to whether there is a need for biotechnology and gene technology for medicinal plants. With regard to medicinal plants, biotechnology could be described as a method for enhancing the formation and accumulation of desirable natural products, with the possibilities of their modifications. Biotechnological tools are also important in order to select, multiply and conserve the critical genotypes of medicinal plants.

Today, natural products from plants provide better templates for the design of potential chemotherapeutic agents than synthetic drugs. Paclitaxel (Taxol), podophyllotoxin and camptothecin are some lead molecules which have proved to be Nature's boon in the treatment of cancer. To meet ever-increasing demands, biotechnological methods offer an excellent alternative, but the economy of such a production is the major hurdle to be overcome.

The successful industrial application of plant cell cultivation for the production of these therapeutic compounds has triggered further research on other promising plant-based chemotherapeutics. The new areas of concern to produce desired products are synergistic product enhancement strategies, along with in-depth knowledge of the biosynthetic pathway. Understanding of biochemistry, enzymology, physiology, bioreactor design and the application of proteomics and genomics are other areas on which to focus.

Several points in a given metabolic pathway can be controlled simultaneously, either by overexpressing and/or suppressing several enzymes, or through the use of transcriptional regulators to control endogenous genes. However, identification of biological pathways and their components remains a challenge for medicinal plants.

It is not an easy task to produce these compounds economically by extraction from intact plants and meet the ever-increasing demand. This may be due to very low concentrations of these active compounds in plants, the slow growth rate of plants, complex accumulation patterns, and high susceptibility to geographical and environmental conditions. Other possible reasons are the non-availability of uniform and unadulterated quality plant material in quantities sufficient for industrial production and uneconomical chemical synthesis, particularly for large complex molecules. Therefore, biotechnological methods offer an excellent alternative for production of such compounds.

P. kurroa Royel ex Benth (Family: Scrophulariaceae) is a perennial herb also known as kutki or karu mainly found in the North-Western Himalayas at altitudes of 3000-4300 meters. ***P.kurroa*** is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, scorpion sting, etc. The active constituents are obtained from the dried roots and rhizomes. The pharmacological importance of ***P.kurroa*** has been demonstrated to be due to rich source of hepatoprotectivepicrosides, **Picroside-I** and **Picroside-II** and other metabolites like Picroside-III, Picroside-IV, Apocynin, Androsin, Catechol, Kutkoside, etc (Weinges et al.1972; Stuppner et al. 1989). Powder, decoction, infusion, confection, and alcoholic extract of the drug are prescribed in Ayurveda and Homeopathy.

P.kurroa has a long, creeping rootstock that is bitter in taste, and grows in rock crevices and moist, sandy soil. The leaves of the plant are flat, oval, and sharply serrated. The flowers, which appear in June through August, are white or pale purple and borne on a tall spike; manual harvesting of the plant takes place October through December.

The pharmacological properties of ***P.kurroa*** have been demonstrated and validated in modern system of medicine like hepatoprotective (Chander et al.1992), antioxidant (particularly in liver) (Ansari et al.1988), antiallergic and antiasthamatic (Dorch et al.1991), anticancerous activity particularly in liver (Joy et al.2000) and immunomodulatory (Gupta et al.2006). A commercial formulation named as Picroliv prepared from *P. kurroa* extracts containing Picroside-1 and kutkoside was launched as a hepatoprotective drug after clinical testing (Ansari et al.1991). Picroliv has also been shown to have immunostimulating effect in hamsters and helps to prevent infections (Puri et al.1992; Gupta et al.2006). The medicinal herb, ***P.kurroa*** is self-regenerating in nature, however, unregulated over-harvesting of plants has reduced its populations in the natural habitat to a level that it has been categorised as a threatened plant species nearing to extinction (Kala 2000). The endangered status of ***P.kurroa*** coupled with its high medicinal value in pharmaceutical and biotechnology industries warrants that alternative means be developed for producing its tissue biomass so that the medicinally important metabolites can be produced under controlled conditions.

OBJECTIVE :-

To study genetic diversity in six accessions of ***Picrorhiza kurroa*** collected from different villages of Himachal Pradesh using four random amplified polymorphic DNA primers.

CHAPTER 2

REVIEW OF LITERATURE

The published literature on various aspects of ***P.kurroa*** has been reviewed under the following headings components :-

2.1. Population status of *P. kurroa* in its natural habitat

The high medicinal value of ***P.kurroa*** coupled with its reckless collection by various pharmaceutical and biotechnology industries has reduced its populations to very low levels, thereby putting it under the category of endangered plant species. The distribution of ***P.kurroa*** was studied by Jain (1996) in the alpine Himalayas from Kashmir to Sikkim giving details about the description, cultural methods, biology, potential value (as a substitute for Indian gentian), and conservation measures. The distribution pattern, population structure and conservation status of rare and endangered medicinal plant species including ***P.kurroa*** were studied in Spiti, Himachal Pradesh, India by Kala (2000) wherein the entire study area was stratified into six zones based on geomorphological and phyto geographical variations. In each zone different habitat types for rare and endangered species were identified and sampled using quadrats. A total of 23 rare and endangered medicinal plants were found, distributed over 10 major habitat types. *P. kurroa* plants were localized and found in patches.

In another study by Rai et al. (2000) suggested that indiscriminate and non-systematic collection of medicinal plants has led to severe pressure on the availability of these plants, many of which are now rare, threatened or endangered. Six species (*Aconitum heterophyllum*, *Podophyllum hexandrum*, *Nardostachys jatamansi*, *Picrorhiza kurroa*, *Swertia chirata* and *Bergenia ciliata*) that are claimed to have therapeutic value and whose survival in the wild is threatened. In a study by Kaul and Handa (2000) discussed in detail the response of two high-altitude medicinal plant species, i.e. *Picrorhiza kurroa* (available in the wild from 3300 to 4000 m) and *Heracleum candicans* (2500-3300 m), at linear altitudes. These species were declared as endangered in view of their overexploitation from wild habitats.

They were successfully domesticated at Srinagar, Kashmir (1750 m) and Kud, Jammu hills (1600 m). Wild populations of these species from three different habitats in the Himalayan region were screened for different phenological details, yield parameters and the presence of active principle at different stages of growth. The analysis of comparative data from wild and cultivated populations depicted that both species show favourable response at lower altitudes. Cultivation was recommended for obtaining better yield of raw material with higher active principles. The study also reported that high-kutkin-yielding genotypes of ***P.kurroa*** showed a tendency to flourish at high-altitude habitats.

Cultivation of ***P.kurroa***, a small creeping, highly medicinal and endangered species of the alpine region was observed at comparatively lower altitude than its natural habitat by Nautiyal et al. (2001). For cultivation of ***P.kurroa***, broadleaf variety, forest litter treatment, levelled ground and intercropping with plants able to retain moisture in the soil for growing plants, and altitude of 2200 m were endorsed as best for higher production. Cost benefit analysis after third year of cultivation indicated benefits of Rs 87, 600/ha based on maximum production. Thus the cultivation of ***P.kurroa*** can provide not only an alternate income-generating resource, but can also provide the opportunity for self-employment to hill farmers.

Another study by Negi and Bhalla (2002) was conducted to systematically document the collection and marketing system of important medicinal and aromatic plants in the tribal areas of Himachal Pradesh, India, for formulation of a suitable policy or modification exercise. It was found that *Jurineama crocephala* and *Picrorhiza kurroa* were the 2 most important species collected in the area. These were followed by *Salvia moorcroftiana*, *Viola serpens* and *Acontium* spp. The collector's net share in consumer's rupee for different products was low and ranges from approximately 14 to 23 per cent. Labour charges were the major component of collectors' cost and account for approximately 17 to 10 per cent of the consumer's price for *J. macrocephala* and *P. kurrooa*.

It was suggested that marketing of medicinal & aromatic plants need to be streamlined and cooperative efforts may be promoted for collectors to get remunerative prices. An interesting study dealt with the indigenous knowledge on 34 medicinal plants of Kumaun Higher Himalaya used by the Bhotia tribes (Satyal et al. 2002). Most of the species studied were native to the Himalayan region. The plant species *Angelica glauca*and *Allium stracheyi* were of narrow range endemic and *Allium stracheyi*, *Picrorhiza kurroa*, and *Nardostachys grandiflora* were recorded in the Red Data Book of Indian Plants. Apart from indigenous uses, the majority of the species were used in the pharmaceutical industries. The study suggested that the annual production of medicinal plants was comparable with the annual production of traditional crops. Hence, development of proper agro-techniques for cultivation, harvesting in the proper season and *in situ* conservation of these species was envisaged.

A need for conservation of medicinal plants in Uttaranchal was emphasized by Alam and Kop (2005). The factors responsible for the failure of the policies aiming to promote the cultivation of medicinal plants on a large scale were discussed, including lack of reliable and profitable markets and technical difficulties. The need for interventions to provide the farmers with technical and marketing support so that the risk of cultivating these species is reduced and farmers' income is increased was highlighted. One example of such intervention in Uttaranchal was given, dealing with the cultivation of Kutki (*Picrorhiza kurroa*) for export to a European firm based in The Netherlands.

Information on eight highly traded and locally used medicinal plants (*Aconitum heterophyllum, Bergeniastracheyi, Heracleumcandicans, Jurineamacrocephala, Podophyllum hexandrum, Picrorhiza kurroa, Rheum australe* and *Selinum tenuifolium*) was collected from the alpine zones of Chhota Bhangal by Uniyal et al. (2006). The study aimed to quantify the current status of these plants in terms of density, frequency and biomass, and also document the indigenous use of these plants for traditional healthcare. Informal interviews and discussions were held with local people for recording local uses of the plants. Based on the sampling, it was found that different species had different habitat requirements. Steep slopes of ChhotaBhangal had the highest species richness and

diversity, while rocky areas had the least. Maximum similarity in terms of species distribution was observed between steep slopes and undulating meadows. It was found that these medicinal plants are regularly used by the local people for curing various ailments such as stomach ache, fever and kidney stones. However, illegal extraction of plants for commercial purposes seems to have affected their population in nature. However, in comparison to few other alpine areas of western Himalayas, the present study area supports higher population of medicinal plants

Assessment of population structure on the basis of density, distribution and diversity-dominance pattern was carried out in Kedarnath Wildlife Sanctuary, Uttarakhand, India (Semwal et al. 2007). Besides, distribution pattern, population structure and conservation status of rare and endangered medicinal plants were also evaluated. Different habitat types for these species were identified and sampled using vertical belt transects. Out of ten habitats identified, distribution of most of the species was found to be restricted in 2-3 habitats. However, *Picrorhiza kurrooa* showed wide distribution in six habitats, while *Swertia chirayita* was restricted to a single habitat.

2.2 Medicinal Value of *Picrorhiza kurroa*

The medicinal importance of *P. kurroa* is due to its pharmacological properties like hepatoprotective (Chander et al. 1992), antioxidant (particularly in liver), antiallergic and antiasthamatic (Dorch et al.1991), anticancerous activity particularly in liver (Joy et al.2000) and immunomodulatory (Gupta et al. 2006). A hepatoproctive drug formulation, Picroliv has been prepared from the extracts of P.kurroa (Ansari et al. 1991; Dwivedi et al.1997).Picroliv also provides protection against other ailments such as immunostimulating effect in hamsters and prevention of infections.

Table 2.1 Medicinal Value of *Picrorhiza kurroa*

Medicinal importance	**Reference**
Hepatoprotective	Rangasamy et al. 1999
Nerve growth factor-potentiating	Li et al. 2000
Antitumour and anticarcinogenic	Joy et al. 2000
Antiulcerogenic	Rangansamy et al. 1999
Hypolipemic	Lee et al. 2006
Anti-diabetic	Joy et al.1999
Antiasthmatic	Stuppner et al. 1991
Immunomodulatory	Simons et al. 1989; Sharma et al. 1994
Antiinflammatory	Singh et al. 1993
Cardioprotective	Kumar et al. 2001
Chemopreventive	Sharma et al. 2003

Table-2.2 Pharmacological importance of Picroliv- a commercial drug formulation of *P. kurroa*

Pharmacological importance	**Reference**
Hepatoprotective	Chander et al. 1990 ; Visen et al. 1991,1995
Stimulates liver regeneration	Srivastava et al. 1996; Saksena et al 1995
Antihepatotoxic	Ansari et al. 1991; Diwedi et al.1992
Source of antioxidants	Rastogi et al. 1995
Immunostimulant	Puri et al. 1992
Hypolipidaemic	Khanna et al.1994
Hepatocurative	Rastogi et al. 2000
Protective activity	Singh et al. 2005
Epithelialization and Angiogenesis	Singh et al. 2007

2.3 Intra-specific Genetic Diversity

DNA markers based fingerprinting can distinguish species rapidly using small amounts of DNA and therefore can assist to deduce reliable information on their phylogenetic relationships. Various approaches are available for DNA fingerprinting such as AFLP (Amplified Fragment Length

Polymorphism), SSR (Simple Sequence Repeats) and RAPD (Random Amplified Polymorphic DNA). RAPD is convenient to conduct with good polymorphism and can be used in analyzing genetic diversity and the relation between species. It has been used in analyzing the relationships between strains belonging to same genera and genetic diversity in many plants, especially medicinal plants. Although RAPD is of dominant nature, several strategies have been put forward to minimize the dominance effects on

Genetic variation analysis in occasional cases, RAPD is poor in reproducibility but this can usually be solved by optimization of reaction conditions. RAPD analysis requires only a small amount of genomic DNA and can produce high level of polymorphism and may facilitate more effective diversity analysis in plants. It provides information that can help to define the distinctiveness of species and phylogenetic relationships at molecular level. Use of such techniques for germplasm characterization may facilitate the conservation and utilization of plant genetic resources, permitting the identification of unique genotypes or sources of genetically diverse genotype. As *P. kurroa* plant is having very high medicinal value there is a need for genetic diversity analysis of this plant in Himalayas of Himachal Pradesh region to explore the hidden role of genes according to different factors. In the present study, genetic diversity of different accessions of *P. kurroa* was studied by random RAPD markers.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant material

A total of 6 plant samples (one from each location) were collected from six different places of Himachal pradesh as shown in (Figure 1) Plant were collected from six different places of Himachal pradesh: Rohtang, Malana, Bhrigu, Dalau pathar, Bhagi and Chitkul. The average rainfall, latitude and longitude of these places are given in Table 1.

These areas have different climatic and soil conditions. The nutrient composition, water holding capacity, nature and type of soil is variable in these districts.

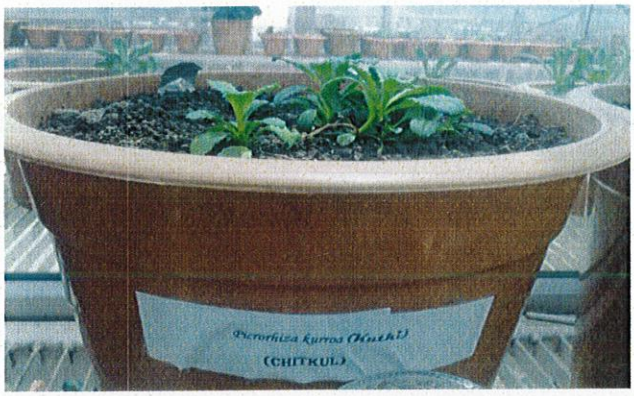


Fig. 3.1 CHITKUL

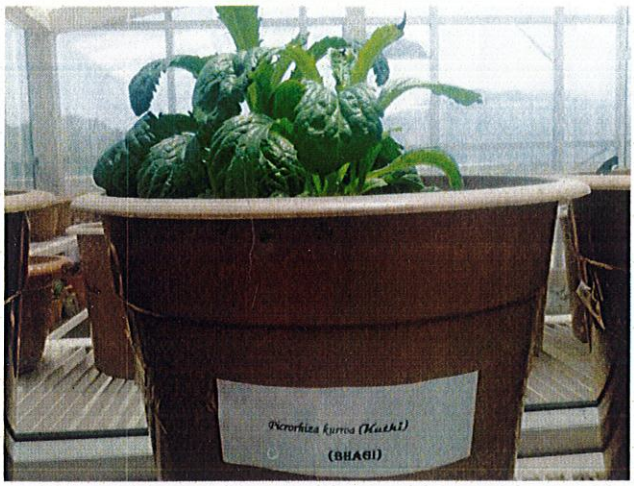


Fig. 3.2 BHAGI

Fig. 3.3 DALAU PATHAR

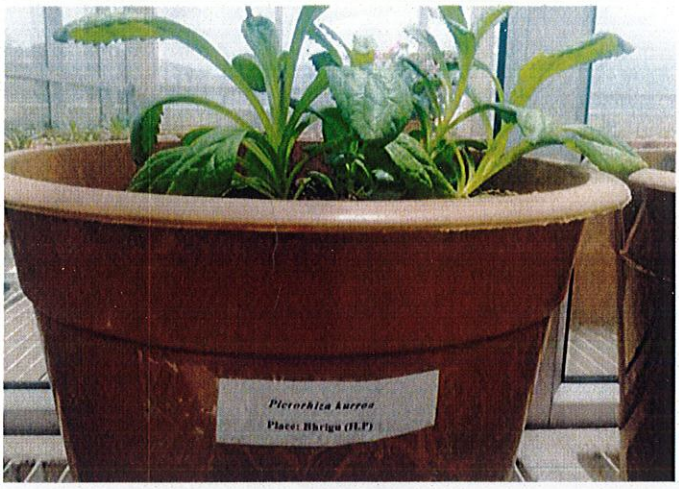


Fig. 3.4 BHRIGU

Fig. 3.5 MALANA JYOT

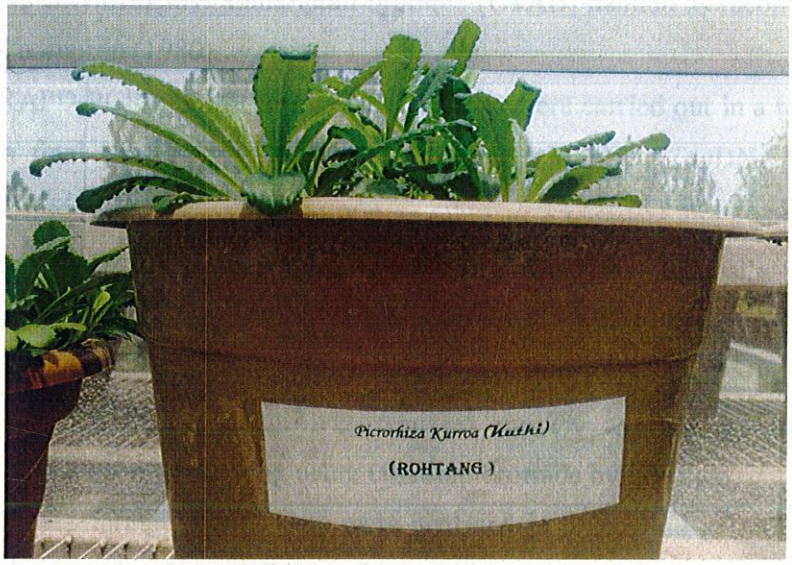


Fig. 3.6 ROHTANG

3.2 Genomic DNA isolation of Samples collected from six different locations and RAPD fingerprinting

For genomic DNA isolation, fresh leaf tissue was harvested from ***P.kurroa*** collected from six different locations of Himachal Pradesh having different altitudes which showed lower or higher and no change in the picroside content, dipped in liquid nitrogen and stored at -80^{0}C till further use.

For DNA extraction, approximately 0.5g of leaf tissue was ground into fine powder using a hand held grinder with liquid nitrogen and then total genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) method as recommended by Murray and Thompson (1980).

For RAPD-PCR amplification, PCR reactions were carried out in a total volume of 20 µl at a final concentration of 1 mMMgCl$_2$, 2 mMdNTP, *Taq*DNA polymerase enzyme (1u /20 u), with approximately 200 ng DNA as a template and a single random primer (0.2 mM). Thermal cycle conditions were 94°C for 2 min, one cycle, 94°C for 15 sec, 35°C for 15 sec, 72°C for 30 sec which were repeated in 40 cycles followed by 5 min-extension at 72°C.

Then 8 µl of each PCR product was revealed on 2% agarose gel subjected to electrophoresis at 80 V after staining using ethidium bromide by UV light.

The RAPD fingerprinting patteren data were analysed in a Bio-Rad gel documentation system wherein size of each amplified band was determined.

3.3. Random primer sequences

In this work total of 20 random primers were tested for amplification on ***P.kurroa*** six genomic DNA out of which 4 random primers showed polymorphism.

The 4 primers with their corresponding sequences are given in the Table 3.1.
DNA sequences of RAPD primers.

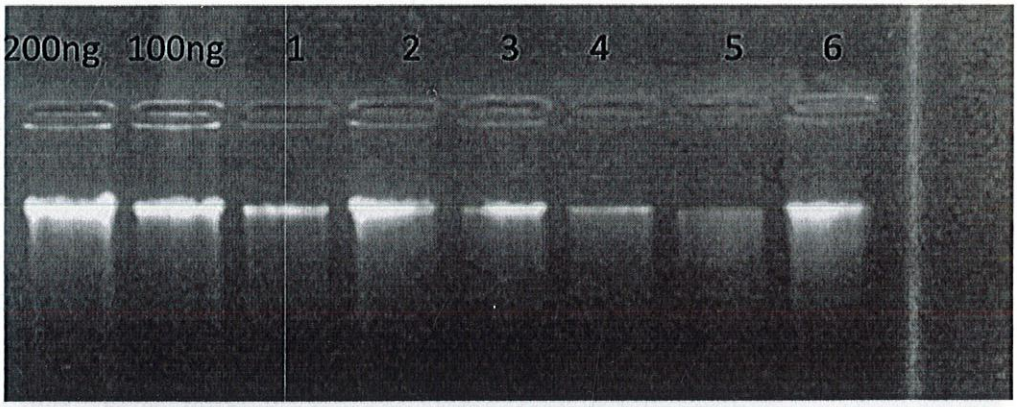
Primer Name	**Sequence (5`- 3`)**
OPA-02	TGCCGAGCTG
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPC-06	GAACGGACTC

Table 3.1. Primer showed a wide range of RAPD polymorphism among six *P. kurroa* accesions collected from different villages of Himachal Pradesh ranging from presence and absence of amplification, number of amplified bands from one to ten among different accesions.

CHAPTER 4

RESULTS AND ANALYSIS

Twenty random decamer primers purchased were screened taking DNA of two *P. Kurroa* samples before performing RAPD analysis in all the genotypes. Out of Twenty primers used for screening, 16 did not amplify any fragment. While, other 4 primers generated amplicons ranging from 3 to 13. The reproducibility of the bands generated by these 4 primers was confirmed by replicating the amplification twice and if needed thrice.



λ 1 – 200ng
λ 2- 100ng
1 - Malana Jyot
2 - Bhagi
3 - Dalahu Pathar
4 - Chitkul
5 - Rohtang
6 - Bhrigu

Fig.4.1 Genomic DNA of *P. kurroa* strains.

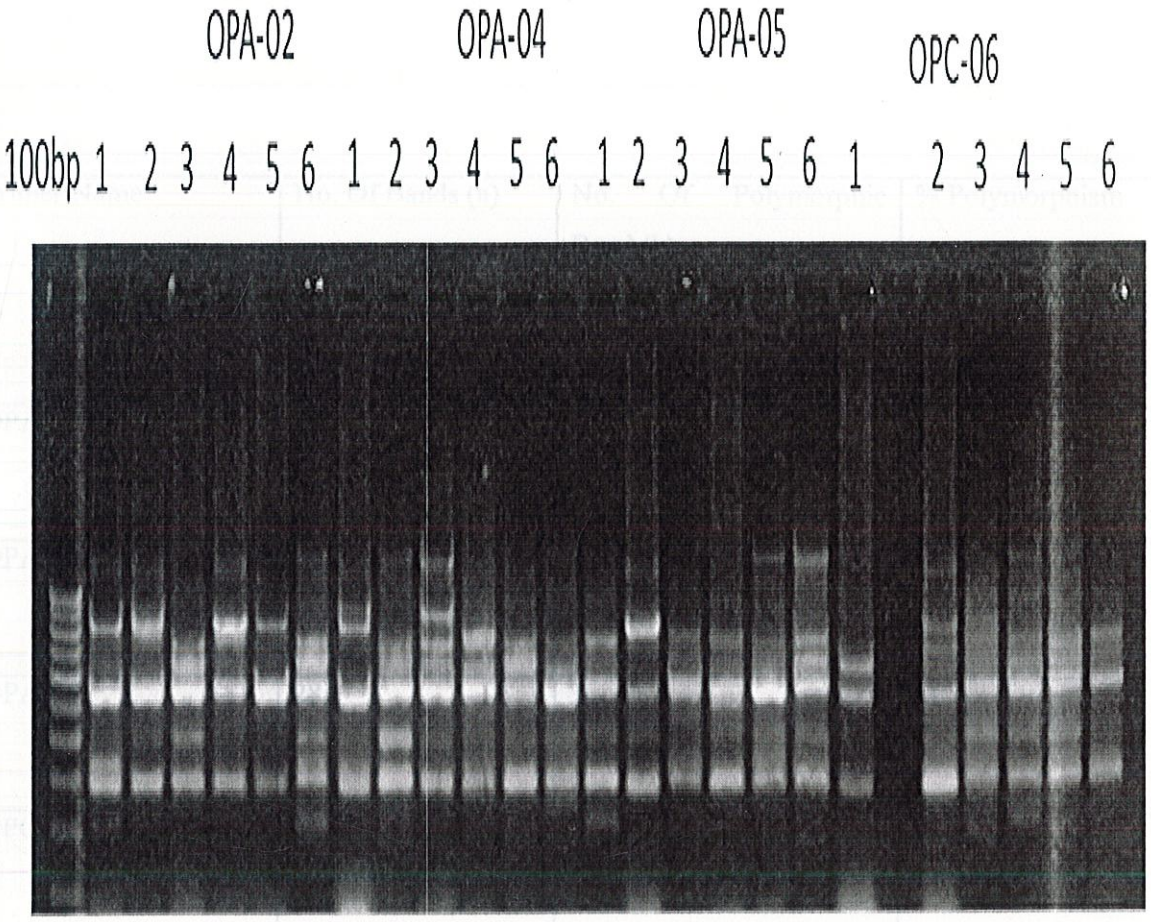


Fig. 4.2 RAPD fingerprinting profile of *P. kurroa*

Table 4.1 Analysis of the RAPD Fingerprint Profile of *P.Kurroa*

Primer Name	No. Of Bands (a)	No. Of Polymorphic Band (b)	% Polymorphism
OPA-02	34	1	3
OPA-04	34	2	6
OPA-05	28	3	11
OPC-06	26	2	8

CHAPTER 5

CONCLUSION

Conclusion :-

Results confirm that the variation in ***P.kurroa*** samples collected from six different locations may reflect strong variations on genomic level. In addition, different responses may be related to specific individual strains. Therefore, RAPD markers successfully proved their ability to evaluate the genetic variations in ***P.kurroa*** samples collected from six different locations and grown under same conditions for one year.

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