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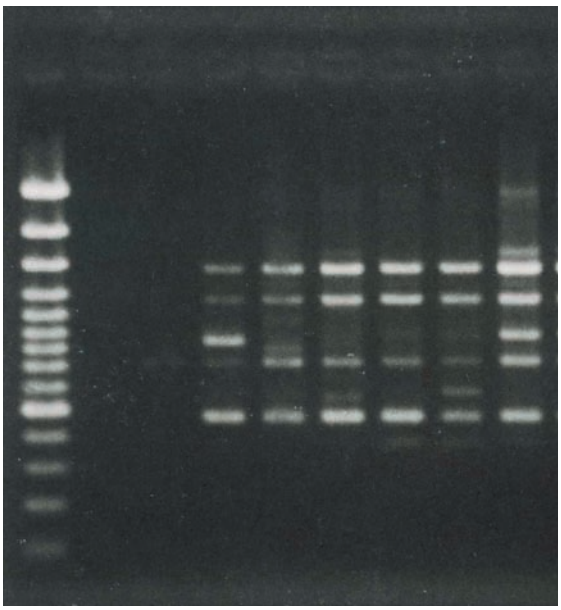

research paper

Optimization of RAPD-PCR conditions for the study of genetic diversity in Nepal's *Swertia chirayita* (Roxb. Ex Fleming) H. Karst

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Optimization of RAPD-PCR conditions for the study of genetic diversity in Nepal's *Swertia chirayita* (Roxb. Ex Fleming) H. Karst

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Of the 30 species (including five varieties) of the genus *Swertia* in Nepal, nine have been reported to possess medicinal properties. Among these, *S. chirayita* is the most valuable species, with high demand in domestic and international markets. Nepal's *S. chirayita* and related species are being recklessly exploited for commercial purposes. Two problems that have emerged with this lucrative market are (a) adulteration and fraudulent labeling of *S. chirayita*, and (b) depletion of *S. chirayita* and allied species from their natural habitats. To address the problem of adulteration and conservation, we studied molecular genetic diversity in *S. chirayita* populations and developed a molecular diagnostic tool for the purposes of authentication. We studied intra-specific genetic diversity in *S. chirayita* using Polymerase Chain Reaction (PCR)-based Random Amplified Polymorphic DNA (RAPD) technique. As a preliminary step, we identified optimal RAPD-PCR reaction and cycling conditions by varying PCR reaction parameters such as concentration of template DNA, $MgCl_2$, dNTPs, primer, Taq DNA polymerase and RAPD-PCR programs. The optimized PCR reaction and cycling conditions were then used in subsequent RAPD profiling experiments for the study of genetic diversity within *S. chirayita* populations from various geographical locations. Genetic diversity characterization of *S. chirayita* populations at the molecular level would furnish information with significant applications in the conservation and sustainable utilization of *S. chirayita* and its allied species in Nepal.

Key words: Polymerase Chain Reaction, Random Amplified Polymorphic DNA, DNA fingerprinting, genetic diversity

Globally, genus *Swertia* is represented by approximately 100 species (Willis 1996). Of the 30 species, including five varieties, that have been reported in Nepal (Press et al. 2000), nine are being traded for their medicinal properties, viz.: (1) *S. chirayita* (Roxb. Ex Fleming) H. Karst, (2) *S. angustifolia* Buch. – Ham. Ex D. Don, (3) *S. tetragona* Edgew, (4) *S. racemosa* (Griseb) C. B. Clark, (5) *S. cilaita* (D. Don ex G. Don) B. L. Burtt, (6) *S. dilatata* C. B. Clarke, (7) *S. multicaulis* D. Don, (8) *S. alata* (Royle ex D. Don) C. B. Clarke, and (9) *S. nervosa* (G. Don) C. B. Clarke (Joshi and Joshi 2001, Rajbhandari 2001). Among these, *S. chirayita* is considered superior to all the others in quality and is in high demand for trade (Pant 2011). *S. chirayita* is distributed in the hills of eastern, central and western Nepal at an altitude of 1200–3000 m in open forests and shady habitats. Bhattarai (1996) reports the species in 40 districts of Nepal; Barakoti et al. (1999) reports distribution in 54 districts.

Eighty to 90% of the total harvest is exported to India as a crude drug and about 9% to China, Malaysia, Singapore, Germany, Italy, France, Switzerland, Srilanka, Bangladesh, Pakistan and the United States. Nepal consumes only 1% of the total harvest and supplies 45–50% of the world's total volume (Joshi and Dhawan 2005). It has been reported that more than 27.2 tons of *S. chirayita* (valued at more than 2.3 million Indian rupees) was exported from Nepal during 1997–1998 (Joshi and Dhawan 2005).

The medicinal properties of *S. chirayita* are attributed to a number of chemical compounds with therapeutic value; these include chiratanin, gentianine, amarogentin, amaroswerin, and several xanthone, iridoid, triterpenoid and glycoside derivatives (Bajpai et al. 1991, Chakravarty et al. 1991, Benerjee et al. 2000, Joshi and Dhawan 2005). The whole plant is used in crude form, and it is also used to manufacture various ayurvedic, herbal, and allopathic medicines. The importance of *S. chirayita* as a multipurpose drug was perceived already in ancient times (Kiratatikta 2001). In "Ayurvada," *S. chirayita* is said to contain anti-cancerous properties (Chiraito *Swertia chirayita,* www.ansab.org). In Ayurvedic preparations, it is used as antipyretic, hypoglycemic, antifungal and antibacterial agent (Joshi and Dhawan 2005). Although the entire plant has medicinal

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properties, in traditional medicines the root is considered the most powerful part and is used in the treatment of chronic and malarial fever, joint pain, ulcers, cough, cold, asthma, scabies, leucoderma etc. (Chiraito *Swertia chirayita,* www.ansab.org). One of the constituents, Swerchirin, has been found effective in lowering human blood sugar level (Bajpai et al. 1991, Saxena et al. 1993) through the stimulation of insulin release from the islets of Langerhans. Its anti-leishmanial property has been studied in hamster model (Medda et al. 1999), and its anti-inflammatory and hepato-protective effects have been studied in mice model (Islam et al. 1995, Mukherjee et al. 1997).

S. chirayita has been listed as one of the top priority medicinal plants of Nepal as well as of Asia (DPR 2005). The great demand, both in Nepal and abroad, has led to its rapid depletion in the wild. Because of the availability of multiple species of *Swertia* in Nepal, a number of other allied species are also being traded. This trend of adulteration, from human health safety viewpoint is a malpractice. The World Conservation Union (IUCN) has categorized this species as 'Vulnerable' (IUCN 2004). An urgent need has been felt to conserve its existing diversity in different geographical gradients of Nepal while sustainably utilizing it for the economic benefit.

Patterns of genetic diversity in plants can be studied by analyzing morphological, anatomical, embryological, biochemical and molecular traits (Weising et al. 1995). Historically, the method of choice has been to focus on morphological characters as the basis on which different plant species are identified and classified into different taxonomical hierarchical groups. However, the classical tools are being increasingly complemented by advanced biochemical tools (focusing on isozymes, allozymes, seed proteins, and secondary metabolites) as well as molecular tools based on restriction fragment length polymorphism (RFLP) and hybridization, polymerase chain reaction (PCR), and DNA sequencing (Murphy et al. 1996, Judd et al. 2002, Chawla 2003). Various PCR-based molecular marker techniques, have already been widely used for genetic diversity analyses, identification of genotypes in genebank management and molecular phylogenetic studies and development of species diagnostic protocols; these techniques include those based on Random Amplified Polymorphic DNA (RAPD), microsatellites or Simple Sequence Repeats (SSRs), Inter-Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLPs), and Internal Transcribed Spacers (ITS) sequences (Edwards 1998, Matthes et al. 1998, Wetzel et al. 1999, Yasuda et al. 2002, Shrestha et al. 2003, Joshi et al. 2004, Shrestha et al. 2005, Qian et al. 2006, Joshi and Dhawan 2007). RAPD analysis allows detection of polymorphism in closely related organisms (e.g., different populations of single species or individuals within a population) and therefore provides a powerful tool for tasks such as population and pedigree analysis, genetic diversity and molecular diagnostic development (Micheli et al. 1997).

In the present study, an attempt has been made to optimize RAPD-PCR reaction and cycling parameters for the study of genetic diversity in *S. chirayita* collections from four districts of Eastern Nepal (Ilam, Terhathum, Dhankuta and Sankhusabha), two districts of Central Nepal (Lalitpur and Kathmandu) and one district from Western Nepal (Kaski). PCR-based molecular genetic diversity studies furnish valuable information regarding genetic diversity in *S. chirayita* populations from various geographical locations and their inherent genetic relationship. A study of molecular genetic diversity in various populations of *S. chirayita* would not only help us understand the evolutionary aspect of the species but also provide valuable insights for its conservation and sustainable utilization. Such a study would also identify a number of taxonomic units for conservation purposes and would enable the linkage of genetic diversity information with data regarding chemical properties (Alam et al. 2008). In addition, since molecular tools hold great promise for diagnostic development (Shrestha et al. 2005, Qian et al. 2006, Shrestha et al. 2010) and medicinal plant authentication (Joshi et al. 2004, Sucher and Carles 2008, Vural and Eri 2009), molecular markers specific to *S. chirayita* could be generated and this in turn could yield a molecular diagnostic tool for authentication purpose. Such a diagnostic tool would be valuable for pharmaceutical applications and for pharmacognosy-based research (Joshi et al. 2004) as well as for intellectual property rights protection (Sharma et al. 2009).

Materials and methods

Plant materials For the RAPD PCR optimization, fresh DNA samples were collected from Godawari, Lalitpur; the rest of the DNA samples were collected in silica gel (**Table 1**). Collected samples from various part of the country were brought to the NAST Biotechnology Laboratory for DNA extraction and analysis.

DNA extraction and DNA estimation Two main DNA extraction techniques (Doyle and Doyle 1990, Graham et al. 1994) were assessed for their usefulness in generating DNA profiles of *S. chirayita* using RAPD-PCR. DNA quantification

Table 1. Details of *S. chirayita* collections for the present study

District/Locality	Region in Nepal*	Number of samples	Altitudinal range
Ilam/ Maipokhari to Maimayuwa	E	15	2100–2600 m
Lalitpur/ Phulchowki	C	15	2150 m
Kathmandu/ Nagarjun	C	15	2000 m
Terhathum/ Tirikhimti to Guphapokhari	E	15	1500–2800 m
Dhankuta/ Pakhribas	E	15	1750 m
Sankhuwasabha/ Lampokhari, Shreemane, Manlabre and Chauki	E	15	2600–2950 m
Kaski/ Sikles	W	15	2000–2500 m

* W = Western, C = Central, E = Eastern

as well as quality assessment was carried out using a Biophotometer (Eppendorf – AG 22331, Germany).

Gel electrophoresis The quality of extracted DNA was also assessed using 1.5% agarose gel electrophoresis (in an EMBI TEC Santiago, CA gel tank) in 1X TBE buffer [10X TBE; 108 gm Tris base, 55 gm Boric Acid and 40 mL of (0.5 M) EDTA pH 8.0] at 50 V (8.47 V/cm) for half an hour. PCR amplification products were analyzed at 25 V (4.2 V/cm) for 1.5 h using the same buffer system.

The gels were stained with ethidium bromide (10mg/ml solution) for 45 minutes and de-stained for 15 minutes in water prior to visualization and photography using UV trans-illuminator (UVITEC, Japan) and Polaroid Gelcam (UK).

RAPD-PCR optimization The RAPD-PCR reaction conditions were optimized by varying key parameters ($MgCl_2$, dNTPs, template DNA, primer and *Taq* polymerase concentration; **Table 2**). Selection of the best RAPD cycling conditions was carried out through the assessment of two randomly selected RAPD-PCR cycling conditions (*viz*. Yu and Pauls 1992, Edwards 1998). All PCR reactions were carried out in the final volume of 25 µL.

Results and discussion

We first compared two DNA extraction protocols in some of our samples. The method developed by Graham et al. (1994) produced multiple sharp bands on electrophoresis gels (**Plate 1**). Doyle and Doyle's (1990) method produced fewer bands. Both techniques produced reasonably pure DNA (A260/280 ratio ranged from 1.8–2.0) for PCR amplification. Graham et al.'s technique was used in all subsequent DNA extractions from multiple collections. The Graham et al. extraction buffer is comprised of 2% CTAB, 1.4 M NaCl, 0.1 M EDTA and 0.1 M Tris HCl pH 8.0. Principal components of this DNA extraction buffer are cetyl trimethyl ammonium bromide (CTAB), sodium chloride (NaCl), ethylene diamine tetra acetic acid (EDTA) and Tris-HCl. CTAB may bind to poly-phenolic compounds during extraction by forming a complex with hydrogen bonds and may help in removing impurities to some extent (Padmalatha and Prasad 2006). RNA that can be co-isolated with DNA can chelate Mg^{2+} and reduce the yield of the PCR. In the present investigation, RNAse has been incorporated in the TE Buffer, which was used to re-suspend DNA pellets at the end of DNA extraction procedure.

Optimization of RAPD reaction conditions was obtained by varying parameters and selection of the best concentrations for each of the constituent of PCR. The optimized RAPD-PCR conditions comprised of 3.0–3.5 mM of $MgCl_2$, 12.5 ng of

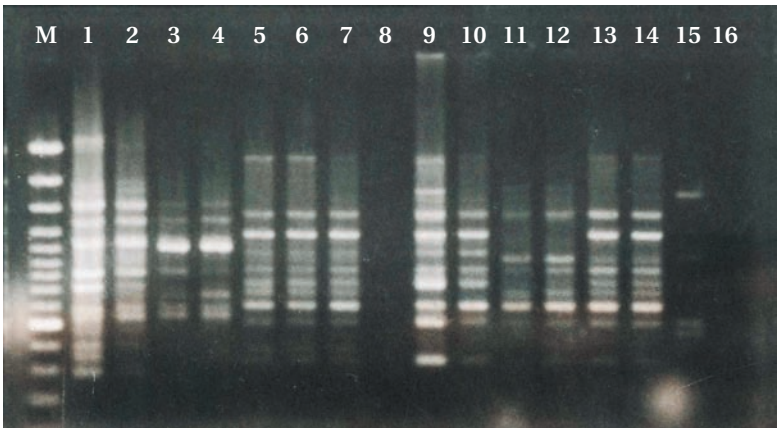

Plate 1. RAPD profile of *S. chirayita* genotypes generated by primer UBC 2 and optimized RAPD reaction parameters. Lane marked M is 100 bp ladder molecular weight marker; lanes 1–2: CTAB extracted DNA (Graham et al. 1994) sample from Godawari, Lalitpur; lanes 3–4: DNA extracted from Godawari, Lalitpur sample using Doyle and Doyle (1990) method; lanes 5–6, 7–8, 9–10, 11–12 and 13–14 are CTAB extracted DNA sample N–1, N–4, N–6, N–8 and N–9 from Pakhribas, Dhankuta respectively. Lane 16 represents negative control.

Table 2. PCR parameters tested and optimized parameters

PCR parameters	Tested range	Optimized conditions found	Remarks
DNA concentration (ng)	12.5, 25, 50, 75, 100, 125, 150, 175	12.5 ng	Highest no. of amplified products observed at 12.5 ng concentration of DNA. Absence of bands from higher concentrations was observed.
$MgCl_2$ concentration (mM)	1.5, 2.0, 2.5, 3.0, 3.5, 4.5, 5.0	3.0–3.5 mM	Lower no. of bands observed at lower (2.0–2.5mM) and higher (4.0–4.5mM) concentrations.
dNTPs concentration (mM)	0.1, 0.2, 0.3, 0.4, 0.5	0.1–0.2 mM	Increased concentration reduced intensity and number of amplified products.
Primer concentration (mM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6	0.2–0.3 mM	Intensity of amplified bands was same at 0.1–0.5mM concentration. Intensity of individual band, number of amplified products and high molecular weight products decreased from 0.6 mM – 1.6 mM concentrations.
Taq polymerase concentration (5U/mL)	0.5, 1.0, 1.5, 2.0, 2.5	2.0U– 2.5U	Faint bands observed at lower concentrations (0.5U–1.5U).

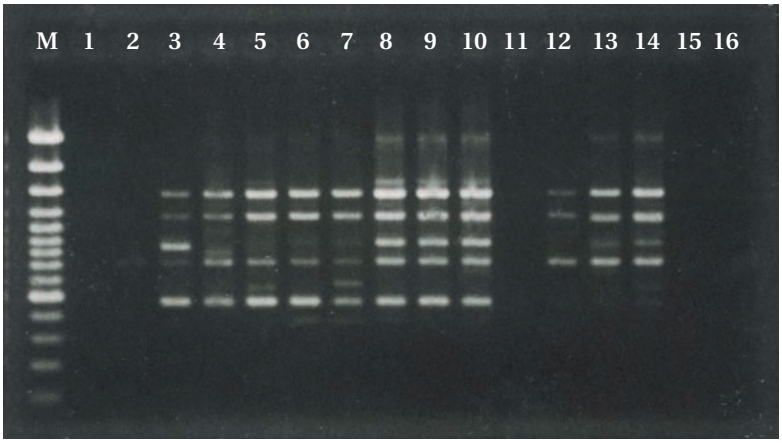

Plate 2. RAPD-PCR optimization for the selection of best $MgCL_2$ concentration for *S. chirayita* using UBC primer 1 and 12.5 ng of DNA. Lane marked M is 100 bp ladder molecular weight marker. Lanes 1–2, 3–4, 5–6, 7–8, 9–10, 11–12, 13–15 represent 1.5 mM, 2.0 mM, 2.5mM, 3.0mM, 3.5mm, 4.0 mM, 4.5 mM concentration of $MgCl_2$, respectively. Lane 16 represents negative control.

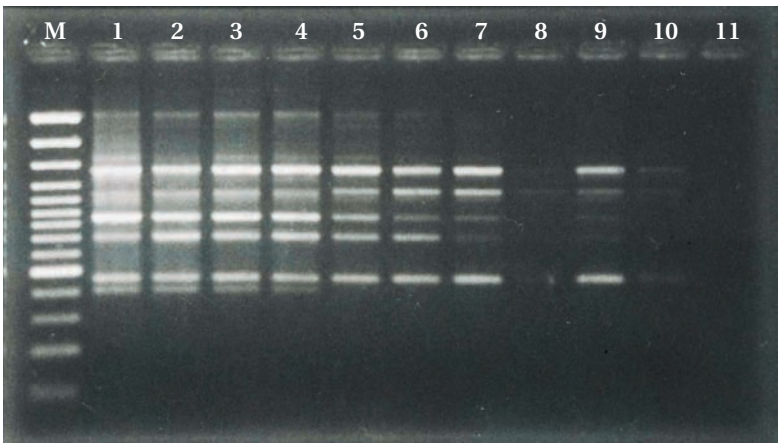

Plate 3. RAPD-PCR optimization for the selection of best dNTPs concentration for *S. chirayita* using UBC primer 1 and 12.5 ng of DNA. Lane marked M is 100 bp ladder molecular weight marker. Lanes 1–2, 3–4, 5–6, 7–8, 9–10 represent 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM concentration of dNTPs, respectively. Lane 11 represents negative control.

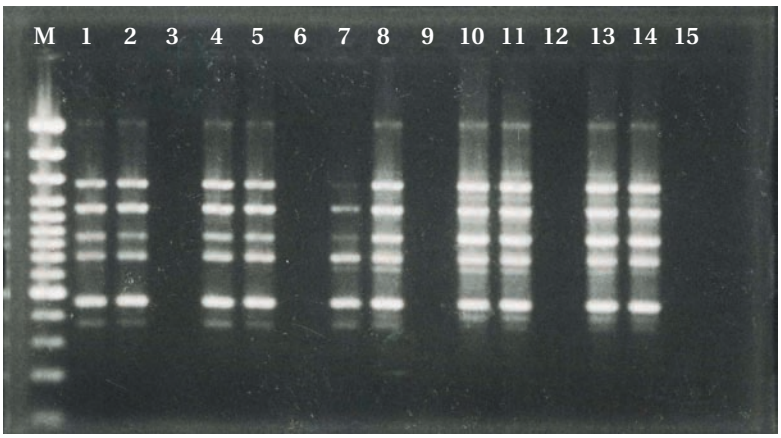

Plate 4. RAPD-PCR optimization for the selection of best *Taq* polymerase concentration for *S. chirayita* using UBC primer 1 and 12.5 ng of DNA. Lane marked M is 100 bp ladder molecular weight marker. Lanes, 1–2, 4–5, 7–8, 10–11, 13–14 represent *Taq* polymerase concentration ranging from 1.0 U, 1.5 U, 2.0 U, 2.5 U and 3.0 U, respectively. Lane 15 represents negative control.

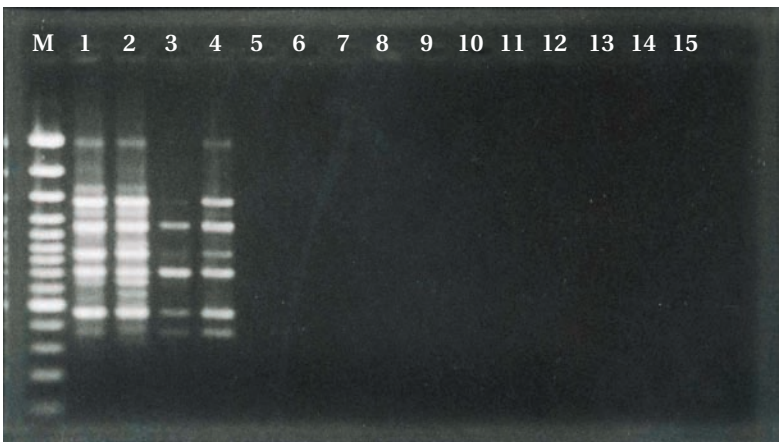

Plate 5. RAPD-PCR optimization for the selection of best DNA concentration for *S. chirayita* using UBC primer 1. Lane marked M is 100 bp ladder molecular weight marker. Lanes 1–2, 3–4, 5–6, 7–8, 9–10, 11–12 represent 12.5ng, 25 ng, 50 ng, 75 ng, 100 ng, 125 ng concentration of template DNA, respectively. Lane 13 represents negative control.

template DNA, 0.1–0.2 mM each of dNTPs, 0.2–0.3 mM each of primers, and 2.0–2.5 units of *Taq* DNA polymerase (MBI Fermentas Company) (**Table 2**). The optimized reaction concentrations were then used in subsequent primer screening and RAPD profiling experiments (**Plates 2, 3, 4 and 5**).

Optimization of reaction parameters in RAPD is crucial in order to maintain reproducibility of RAPD phenotypes among laboratories. In order to generate reproducible RAPD fingerprint profiles, two parameters, *viz.,* quality and quantity of template DNA, have been considered the primary factors affecting reproducibility. Hence controlling these two factors is essential in order to ensure reproducible RAPD patterns (Weeden et al. 1992, Micheli et al. 1997). Apart from DNA, other parameters to be optimized are the concentrations of $MgCl_2$, dNTPs and primers. In the present study, $MgCl_2$ concentration of 3.0–3.5, dNTPs concentration of 0.1–0.2 mM each, primer concentration of 0.2–0.3 mM and *Taq* polymerase concentration of 2.0–2.5 U had no noticeable difference in banding patterns, while template DNA concentration of 12.5 ng was found to be optimal for PCR amplification at all these reagent concentrations.

Of the two randomly selected RAPD-PCR programs (Yu and Pauls 1992, Edwards 1998), the cycling conditions described by Edwards (1998) produced the best profiles for *S. chirayita.* The program consisted of an initial denaturation step at 95°C for 2 min followed by 45 cycles of 95°C for 20 s, 37°C for 60 s and 72°C for 60 s and a final extension step of 72° C for 10 min. The maximum ramp rate available for the PCR machine (Eppendorf, Germany) was used. In some cases, the faster PCR cycle described by Yu and Pauls (1992) would be more applicable. With alfalfa genomic DNA, Yu and Pauls (1992) optimized the denaturing time; they tested 5s, 30s and 60s and found that 5s gave the best PCR products. They also showed that there is a relationship between the time required for primer annealing and the GC content of the primer. For primers having a GC content of 50–80%, a primer annealing time of 30 seconds appeared to be appropriate. It was also shown that strand elongation time affects the size of amplified fragments in the PCR reaction.

For the optimization of RAPD cycling parameters of a number of medicinal and aromatic plants, Padmalatha and Prasad (2006) tested initial denaturation times of 2, 3, 4 and 5 min at 94°C and found 3 min most effective. A range of annealing temperatures (20 to 70° C) and exposure times (30s, 60s, 90s and 120s) was also tested; 37° C for 60 s was found to be the best. In RAPD, random primers should have a minimum of 40% GC content, although 50–80% is generally used (Micheli 1997). While it is generally claimed that RAPD is very sensitive to reaction and cycling parameters, Weeden et al. (1992) concluded that the amplification process is not so sensitive to one or more of the parameters as to seriously affect reproducibility of the technique. Therefore standard reaction conditions and cycling parameters appear to be appropriate for a wide range of plant materials.

Conclusion

Swertia chirayita is one of Nepal's most highly prized medicinal plants both at home and abroad. However, due to the availability of multiple species of *Swertia* in Nepal, not only *S. chirayita* but eight other allied species are also being highly exploited in trade. It is anticipated that the present study aimed at studying genetic diversity within *S. chirayita* populations of Nepal using molecular marker techniques such as RAPDs will furnish valuable information for the sustainable utilization and conservation of these natural resources. Furthermore, DNA samples collected during this investigation and the information thus generated will be highly valuable for planning future molecular projects focusing on *S. chrayita* as well as other *Swertia* species found in Nepal.

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References

Alam A, PK Naik, P Gulati, AK Gulati and GP Mishra. 2008. Characterization of genetic structure of *Podophyllum hexandrum* populations, an endangered medicinal herb of Northwestern Himalayas, using ISSR-PCR markers and its relatedness with podophyllotoxin content. *African Journal of Biotechnology* **7**(8): 1028–1040

Bajpai MB, RK Asthana, NK Sharma, SK Chatterji and SK Mukherjee. 1991. *Planta Medica* **57**(2): 102–104

Banerjee S, TK Suvra, S Mandal, P Das and S Sikdar. 2000. Hypoglycemic effect of Swerchirin from hexane fraction of *S. chirayita. Indian Journal of Pharmacology* **32**(1): 21–24

Barakoti TP, T Chapagain, Y Thapa and C Bhusal. 1999. Chiraito Conservation and Cultivation Workshop and Achievement, Nepal Agriculture Research Centre, Pakhribas, Dhankuta, Nepal

Bhattarai KR. 1996. Effect of different concentrations of phytohormones on seed germination of *Swertia chirayita. Banko Jankari* **7**(2): 50–51

Chakravarty AK, S Mukhopadhyay and B Das. 1991. Swertane triterpenoids from *S. chirayita. Phytochemistry* **30**(12): 4081–4092

Chawla HS. 2003. *Introduction to Plant Biotechnology,* second ed. Oxford and IBH publishing Co. Pvt. Ltd. New Delhi. 538 p

Doyle JJ and JL Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13–15

DPR. 2005. Prioritized medicinal plants of Nepal for Research and Cultivation. Department of Plant Resources Publication, Ministry of Forests and Soil Conservation, Government of Nepal

Edwards KJ. 1998. Randomly Amplified polymorphic DNAs (RAPDs). In *Molecular Tools for Screening Biodiversity – Plants and Animals*, A Karp, PG Isaac and DS Ingram (eds.). Chapman and Hall, London, Weinheim, New York, Tokyo, Melbourne, Madras, p 171–179

Graham GC, P Mayers and RJ Henry. 1994. A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *Bio Techniques* **15**: 48–50

Islam CN, SN Bandyopady, MD Banerjee and PC Das. 1995. Preliminary studies on the anti-inflammatory effects of *S. chirayita* in albino rats. *Indian Journal of Pharmacology* **27**(1): 37–39

IUCN 2004. National register of medicinal and aromatic plants. IUCN Nepal publication

Joshi KK and SD Joshi. 2001. Genetic heritage of medicinal and aromatic plants of Nepal. Buddha Academic Publishers and distributors Pvt. Ltd., Kathmandu, Nepal

Joshi P and V Dhawan. 2007. Analysis of genetic diversity among *Swertia chirayita* genotypes. *Biologia Plantarum* **51**(4): 764–768

Joshi P and V. Dhawan. 2005. *Swertia chirayita* – an Overview. *Current Science* **89**(4): 635–640

Joshi, K, P Chavan, D Warude and B Patwardhan. 2004. Molecular markers in herbal drug technology. *Current Science* **87**(2): 159–165

Judd WS, CS Campbell, EA Kellog and PF Stevens. 2002. Plant

Systematics, a Phylogenetic Approach. Sinauer, Associates, Inc. Publishers, Sunderland, Massachusetts, USA

Kiratatikta. 2001. The Ayurvedic Pharmacokpoeia of India **1**(1): 71–72

Matthes MC, A Daly and KJ Edwards. 1998. Amplified Fragment Length Polymorphism (AFLP). In *Molecular Tools for Screening Biodiversity- Plants and Animals,* A Karp, PG Isaac and DS Ingram (eds.). Chapman and Hall, London, Weinheim, new York, Tokyo, Melbourne, Madras, p 183–192

Medda S, S Mukhopadyay and MK Basu. 1999. Evaluation of in-vivo activity and toxicity of amarogentin, an antilesmanial agent in both liposomal and niosomal forms. *Journal of Antimicrobial chemotherapy* **44**(6): 791–794

Micheli MR, R Bova and ED Ambrosio. 1997. Random Amplified Polymorphic DNA assay. In' Fingerprinting methods based on arbitrarily primed PCR' Springer Lab manual. Springer-Verlag Berlin, Heidelberg

Mukherjee SA and BR Maiti. 1997. Hepatoprotective effect of *Swertia chirayita* on rat. *Indian Journal of Experimental Biology* **35**(4): 190–194

Murphy RW, JW Sites, DG Buth and CH Harfler. 1996. Proteins: Isozyme Electrophoresis. In ' Molecular Systematics'. DM Hillis, C. Moritz and BK Mable (Eds.) Sinaur associates Inc. Publishers. Sunderland, Massachusetts, USA

Padmalatha K and MNV Prasad. 2006. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India

Pant S. 2011. Swertia in Nepalese Himalaya (Gentianaceae:Gentianeae:Swertiinae:Swertia). http://gentian.rutgers.edu/genera/genSwerNepal.htm

Pirttila AM, M Hirsikorpi, R Kamarainen, L Jaakola and A Hohtola. 2001. *Plant Molecular Biology Reporter* **19**: 273 *a–f*

Press JR, KK Shrestha and DA.Sutton. 2000. Annotated Checklist of the Flowering Plants of Nepal. Natural History Museum London

Qian YE, Q Ying-Xiong, Q Yan-qi, C Jian-xin, Y Shu-zhen, Z Ming-shui and F Cheng-xin. 2006. Species-specific SCAR markers for authentication of *Sinocalycanthus chinensis. Journal of Zhejiang University of Science* **7**(11): 868–872

Rajbhandari KR. 2001. Ethnobotany of Nepal. Ethnobotanical Society of Nepal. c/o Central Department of Botany, Tribhuvan University, Kirtipur, Nepal

Saxena AM, MB Bajpai, PSR Murthy and SK Mukherjee. 1993. Mechanism of blood sugar lowering by a Swerchirin containing hexane fraction of *Swertia chirayita. Indian Journal of Experimental Biology* **31**(2): 178–181

Sharma KD, BM Singh, TR Sharma, M Katoch and S Guleria. 2009. Molecular analysis of variability in *Podophyllum hexandrum* Royle – an endangered medicinal herb of Northwestern Himalaya. *PGR Newsletter* **124**: 57–61

Shrestha S, SW Adkins, GC Graham and DS Loch. 2003. Phylogeny of the *Sporobolus indicus* complex based on internal transcribed spacers (ITS) sequences. *Australian Systematic Botany* **16**: 165–176

Shrestha S, SW Adkins, GC Graham and DS Loch. 2005. An identification tool for the Australian weedy *Sporobolus* species based on random amplified polymorphic DNA (RAPD) profiles. *Australian Journal of Agricultural Research* **56**(2): 157–167

Shrestha, S, GC Graham, DS Loch and SW Adkins. 2010. Molecular identification of weedy *Sporobolus* species by PCR-RFLP. *Weed Research* **50**: 383–387

Sucher, NJ and MC Carles. 2008. Genome based approaches to the authentication of medicinal plants. *Planta Medica* **74**(6):603–23

Vural HC and AD Eri. 2009. Optimization of DNA isolation for RAPD-PCR analysis of selected (*Echinaceae purpurea* L. Moench) medicinal plants of conservation concern from Turkey. *Journal of Medicinal Plants Research* **3**(1): 016–019

Weeden NF. 1992. Inheritance and reliability of RAPD markers. In '*Application of RAPD technology to Plant Breeding*'. Joint Plant Breeding Symposia Series. Crop Science Society of America, American Society for Horticulture Science, American Genetic Association

Weising K, H Nybom, K Wolff and W Meyer. 1995. DNA Fingerprinting in Plants and Fungi. CRS Press, Boca Raton, USA

Wetzel DK, MJ Horak and DZ Skinner. 1999. Use of PCR-based molecular markers to identify weedy *Amaranthus* species. *Weed Science* **47**(5): 518–523

Willis JC. 1996. Dictionary of flowering plants and ferns (seventh edition), Cambridge, England

Yasuda K, A Yano, Y Nakayama and H Yamaguchi. 2002. Molecular identification of *Echinochloa oryzicola* Vasing. and *E. crus-galli* (L.) Beauv. using a polymerase chain reaction-restriction fragment length polymorphism technique. *Weed Biology and Management* **2**: 11–17

Yu K and KP Pauls. 1992. Optimisation of the PCR program for RAPD analysis. *Nucleic Acids Research* **20**: 2606

Yu, K and Pauls KP. 1994. Optimisation of DNA extraction and PCR procedures for random amplified polymorphic DNA (RAPD) analysis in plants. In *PCR Technology, Current Innovations,* Griffin, HG and AM Griffin (eds.). CRC Press Inc. p 193–200