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AFLP Markers for Identification of *Aconitum* Species

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ABSTRACT

The genus *Aconitum* is highly complex and its taxonomy has been traditionally difficult due to the high level of variation among the various species. The *Aconitum* species are known for their highly toxic diterpenoid alkaloids but have been described in traditional medicine systems as high-value medicine after proper and prescribed detoxification. In India, *A. heterophyllum*, *A. balfourii* and *A. violaceum* are found mainly in the North-Western Himalayas whereas *A. ferox* is found in the North-Eastern Himalayan region. Among these species, *A. heterophyllum* is the most significant in terms of therapeutic importance and herbal drug market value. It has become critically endangered due to high demand of the herb and indiscriminate overexploitation. There is an existing demand in the bulk herbal drug industry to have an authentic identification system for the *Aconitum* species in order to enable their commercial use as genuine phytoceuticals. In the present study we have used Amplified Fragment Length Polymorphism (AFLP) for developing DNA fingerprints for 4 *Aconitum* species. A total of 10 accessions (4 of *A. heterophyllum*, 3 of *A. violaceum,* 2 of *A. balfourii* and 1 of *A. ferox*) from the 4 species were used in the study, which employed 64 AFLP selective primer pairs. Only 26 selective primer pairs were found to respond with all the accessions and generated a total of 4112 fragments. A number of species-specific markers were identified for all the 4 *Aconitum* species (16 for *A. heterophyllum*, 125 for *A. violaceum*, 79 for *A. balfourii*, and 226 for *A. ferox*). These AFLP fingerprints of the *Aconitum* species could be used in future for authentication of the drug and checking the adulteration-related problems faced by the commercial users of the herb.

Keywords: adulteration, DNA fingerprinting, crude drug, rare plant

INTRODUCTION

Aconitum L. (belonging to the buttercup family Ranunculaceae), which is also known as aconite or monkshood, is a diverse genus with nearly 300 species worldwide, primarily in the temperate regions of the northern hemisphere (Zhang *et al.* 2005). The genus is represented by around 26 species in India, mainly distributed in the sub-alpine and alpine zones of the Himalayas. Interestingly, the *Aconitum* species of North-Western Himalayas are not found in North-Eastern Himalaya and *vice versa* (Chaudhary and Rao 1998). *A. heterophyllum*, *A. balfourii* and *A. violaceum* are found mainly in the North-Western Himalayas whereas *A. ferox* is found in the North-Eastern Himalayan region. Among these species, *A. heterophyllum* is the most significant in terms of therapeutic importance and herbal drug market value. Its importance has further grown due to its critically endangered status and lowest density (1 individual/m^2) among all the threatened plants in the Himalayan region (Singh *et al.* 2008). Illegal and over-exploitation of *Aconitum* species pose a threat to their existence (Nautiyal *et al.* 2002) and the problem has been further complicated by destructive harvesting of root/rhizome of the plants (Pradhan and Badola 2008). Besides, regeneration of *A. heterophyllum* under natural conditions is low due to poor seed germination and low seedling survival and being endemic to the North-Western Himalayas, the species grows only in localized, restricted ecological niches (2500-5000 m above sea level) that have only a few, thin-scattered populations (Beigh *et al.* 2006). The stringent and critical ecological requirements for *A. heterophyllum* have ensured that it neither invades newer areas nor survives at lower altitudes with comparatively higher temperatures. However, *ex situ* conservation of *A. heterophyllum* has been attempted and it has been found that there is some possibility of successful adaptation of the plant in conditions other than its natural habitat (Pandey *et al.* 2005).

Aconitum species are known for their highly toxic diterpenoid alkaloids (Chan 2009), which, have been used as a source of arrow poisons (Fico *et al.* 2003). The pharmacological effects of preparations of *Aconitum* roots are attributed to these diterpenoid alkaloids. The main alkaloid of these plants is aconitine, which is known to suppress the inactivation of voltage-dependent Na^+ channels by binding to neurotoxin binding site 2 of the alpha-subunit of the channel protein (Ameri 1998). For therapeutic use, *Aconitum* has to be processed and combined with specifically matching herbs to reduce its toxicity (Wang *et al.* 2009). Quantitative structure–activity relationship (QSAR) analyses have been performed to study the mechanism of action of *Aconitum* alkaloids and to provide a rational for their chemical manipulation to reduce their toxicity (Bello-Ramirez and Nava-Ocampo 2004). *A. heterophyllum* finds a mention in Ayurveda for curing stomach ache and fever. It is one of the main ingredients of Ayurvedic medicines, "Ativishadi churna", "Chandraprabha vati" and "Amritarishta" whereas in the Unani system of medicine it is an important ingredient of "Sufuf habib", which is used for curing piles and "Majun jograj guggal" that is used against arthiritis (Uniyal *et al.* 2006). *A. ferox* has traditional use for curing fever, skin diseases, cough and gout (Pradhan and Badola 2008). In recent years, *Aconitum* has been compared with other similar genera like *Delphinium* that produce similar type of alkaloids (Lin *et al.* 2010). Earlier, *Aconitum* was considered to be an antidote of malaria and a substitute of quinine (Chakrabarti 2010).

A high demand for the drug and its endangered status has raised other concerns like adulteration of the authentic

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Table 1 *Aconitum* germplasm collection details.

Name of species and accession number	Place of collection	Voucher specimen number
A. heterophyllum 1	Uttarkashi, Uttarakhand	7781
A. heterophyllum 2	Uttarkashi, Uttarakhand	7780
A. heterophyllum 3	Chamba, Himachal Pradesh	9114
A. heterophyllum 4	Chamba, Himachal Pradesh	8503
A. ferox	Darjeeling, West Bengal	9125
A. balfourii 1	Chamba, Tehri Garhwal, Uttarakhand	7778
A. balfourii 2	Uttarkashi, Uttarakhand	7774
A. violaceum 1	Chamba, Himachal Pradesh	8510
A. violaceum 2	Rohtang Pass, Lahaul-Spiti, Himachal Pradesh	8507
A. violaceum 3	Chamba, Himachal Pradesh	9113

drug with substitutes (that are less effective and often harmful) that could not be identified when the herb is present in the crude drug form. It is therefore an absolute necessity for the herbal drug industry to have stable molecular markers (like DNA markers) for various *Aconitum* species so as to differentiate and authenticate the herbal material when it is present in the form of a crude drug. In the past many molecular marker-based studies have been carried out to analyze *Aconitum* species. Isozyme and random amplification of polymorphic DNA (RAPD) analyses have been quite popular for such studies (Cole and Kuchenreuther 2001). Polymorphic microsatellites (Le Cadre *et al.* 2005) and RAPD (Fico *et al.* 2003) have been used to analyse some European species of *Aconitum* and nuclear intergenic transcribed spacer (ITS) sequences have been used to study the phylogeny of *Aconitum* (Kita and Ito 2000; Luo *et al.* 2005). A Chinese group carried out ISSR-based genetic diversity analysis in *Aconitum carmichaeli* (Luo *et al.* 2006). Since, DNA marker-based studies have not been carried out on the species of *Aconitum* found in India, the present study was undertaken to generate AFLP-based DNA markers for 4 such species (*A. heterophyllum*, *A. balfourii*, *A. violaceum* and *A. ferox*) that are most commonly used in the herbal trade.

MATERIALS AND METHODS

Plant material

The plant material used in this study was collected from the Himalayan region falling in the Indian states of West Bengal, Uttarakhand and Himachal Pradesh and the herbarium was submitted to the National Gene Bank for Medicinal and Aromatic Plants at CIMAP, Lucknow (**Table 1**). Leaf samples from the selected plants were used for DNA isolation. The samples consisted of four accessions of *A. heterophyllum*, three accessions of *A. violaceum*, two accessions of *A. balfourii* and one accession of *A. ferox*.

DNA isolation

DNA was isolated from the plant leaf samples using the protocol described by Khanuja *et al.* (1999) and its quality and quantity were analysed using agarose gel electrophoresis and ND-1000 spectrophotometer (NanoDropTechnologies, USA).

AFLP

For AFLP analysis, DNA was restricted using two restriction endonucleases *Eco*RI and *Tru*9I (an isoschizomer of *Mse*I) and double stranded adapters were ligated to the ends of DNA fragments, generating template for subsequent PCR amplification (preselective followed by selective). Restriction and ligation reactions were carried out simultaneously in a single reaction (Vos *et al.* 1995). To carry out the reaction, an enzyme master mix for 10 reactions was prepared containing 1 µl 10X T4 DNA ligase buffer, 1 µl 0.5 M NaCl, 0.5 µl 1 mg/ml BSA, 1 µl *Tru*9I (10 U/µl), 4.25 µl *Eco*RI (12 U/µl), 0.5 µl T4 DNA ligase (20 U/µl, high concentration) and 1.75 µl water. The restriction ligation reaction consisted of 300 ng of DNA (5.5 µl), 1 µl 10X T4 DNA ligase buffer, 1 µl 0.5 M NaCl, 0.5 µl 1 mg/ml BSA, 1 µl *Mse*I adapter (Applied Biosystems, USA), 1 µl *Eco*RI adapters (Applied Biosystems, USA) and 1 µl enzyme master mix, as described above. The reaction was then incubated overnight at room temperature and subsequently diluted 20-fold with $T_{10}E_{0.1}$ buffer. The ligated adaptors served as primer binding sites for low-level selection in the preselective amplification of restriction fragments. The *Mse*I complementary primer had a 3′-C and the *Eco*RI complementary primer a 3′-A. Only the genomic fragments having an adapter on each end amplified exponentially during the PCR. The preselective amplification mix was prepared by adding 4 µl of 20-fold diluted DNA from the restriction ligation reaction, 0.5 µl AFLP preselective primer (*Eco*RI, Applied Biosystems), 0.5 µl AFLP preselective primer (*Mse*I, Applied Biosystems) and 15 µl AFLP core mix. The preselective amplification was carried out in a thermal cycler programmed as: 72°C for 2 min; 20 cycles of 94°C for 20 sec, 56°C for 30 sec and 72°C for 2 min; 60°C for 30 min and 4°C to infinity.

The preamplified DNA was diluted 20-fold with $T_{10}E_{0.1}$ buffer and selective amplifications were carried out using different *Mse*I and *Eco*RI primer combinations (Applied Biosystems). Primers chosen for the amplification were from 16 available AFLP selective primers (8 fluorescently tagged *Eco*RI and 8 untagged *Mse*I primers). The *Eco*RI primers contained 3 selective nucleotides with the sequence 5′ [Dye-Primer-Axx]-3′, while the *Mse*I primers had the 3 selective nucleotides starting with C i.e. 5′ [Primer-Cxx]-3′. Selective amplification of each sample was done with all 64 (8x8)-primer combinations (*Mse*I/*Eco*RI) using multiplex-PCR reactions. For selective amplification the reaction were set up as follows: 3 µl of 20-fold diluted preselective amplification product, 15 µl AFLP core mix, 1 µl *Mse*I primer 5′-[Primer-Cxx]-3′, 1.5 µl *Eco*RI primers 5′-[Dye-Primer-Axx]-3′ {0.5 µl of 3 *Eco*RI primers each were pooled here}. Selective amplification was carried out in a thermal cycler programmed as 94°C for 2 min; 10 cycles of 94°C for 20 sec, 66°C (-1°C/ cycle) for 30 sec, 72°C for 2 min; 20 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 2 min; 60°C for 30 min; and 4°C to infinity. The samples were loaded onto a 5% (29:1) polyacrylamide gel on an ABI Prism 377 DNA Sequencer (Applied Biosystems, USA). For gel electrophoresis, 3 µl of the selective amplification reaction product was mixed with 4 µl of loading buffer {ROX500 size standard (10%), blue dextran (10%), deionised formamide (80%)}, and 1.5 µl of this mix was finally loaded on the gel. The AFLP amplification modules and the guidelines supplied by Applied Biosystems, USA were used for setting up the reactions as described above.

Data analysis

Fragment analysis was carried out for bands in the range 35-400 bp. For diversity analysis, bands were scored as present (1) or absent (0) to form a raw data matrix. A square symmetric matrix of similarity was then obtained using Jaccard similarity coefficient (Jaccard 1908) by SPSS v 7.5 software. The average similarity matrix was used to generate a tree for cluster analyses by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method using NTSYSpc version 2.02j (Applied Biostatistics Inc.).

RESULTS AND DISCUSSION

In the AFLP analysis, of the 64 primer pairs used, only 26

Table 2 Unique AFLP marker fragments for the 4 *Aconitum* species.

Primer combination *Mse*I/*Eco*RI	Unique bands of *A. heterophyllum* (size in bp)	Unique bands of *A. ferox* (size in bp)	Unique bands of *A. balfourii* (size in bp)	Unique bands of *A. violaceum* (size in bp)
CAA/ACG	43	67, 124	-	52, 71, 102, 116, 286
CAA/AGC	-	-	-	41, 88
CAC/ACT	122, 205	349	-	133, 149, 199, 220, 234, 243, 283, 318, 353, 392
CAT/ACG	256	77, 87	220	57, 65, 86, 104, 117, 146, 161, 162, 192, 210, 235, 283, 295, 296, 332, 356
CAT/AGC	163, 208	42, 57, 60, 64, 101, 108, 116	72, 209	69, 70, 156, 169, 250
CTG/AGC	-	82, 91, 107, 121, 133, 154, 160, 163, 168, 173, 177, 181, 189, 191, 234, 255, 257, 262, 267, 306, 390, 395	102, 127, 144	54, 97
CTC/ACA	195	45, 48, 53, 58, 87, 98, 105, 110, 126, 131, 140, 151, 170, 185, 202, 205, 220, 227, 242, 248, 266, 285, 316, 325, 326, 329, 335, 345, 348, 359, 366, 381, 389	70, 78	130, 203, 314
CTC/ACG	379	68, 77, 88, 89, 109, 171, 185, 198, 207, 244, 306, 342, 383	49, 92, 142, 150, 182	60, 81, 111
CTG/ACT	190	57, 66, 68, 82, 115, 135, 195, 198, 199, 205, 269, 278, 338, 350, 354, 392, 398	35, 63, 101, 174, 175, 184, 197, 317	48, 122, 222
CTG/AGG	-	50, 65, 85, 89, 218, 237, 239, 250, 272, 285, 291, 308, 313, 349	62, 290	40, 43, 94, 118, 172, 219, 233, 235, 283, 297, 315, 344
CAT/ACC	179, 363	46, 55, 135, 263	61, 78, 137	47, 58, 59, 98, 145, 148, 168, 221, 240, 324, 351
CTA/AAC	-	68, 106, 155	49, 110	82, 329
CTG/ACA	-	67, 69, 71, 74, 90, 91, 101, 125, 134, 140, 150, 153, 176, 186, 196, 199, 207, 210, 213, 223, 225, 230, 233, 240, 261, 262, 265, 267, 283, 285, 288, 304, 328, 329, 344, 349, 359, 374, 384, 398	48, 86, 102, 191, 209	54
CAA/ACA	-	83, 353	45, 46, 47, 58, 60, 68, 74, 128, 129	101, 197, 215, 251
CAA/AGG	-	189	167	-
CAA/ACC	-	276, 289	61, 71, 107, 210, 211	204, 230, 345
CAC/ACA	-	243, 251, 266, 270, 285, 286, 325, 327	49, 217, 278	64, 89, 109, 201, 238, 240, 265, 275, 297
CAG/AGC	-	-	76	47, 80, 128
CAT/ACT	-	63, 65, 73, 78, 94, 95, 105, 117, 128, 159, 181, 219, 269	72	46
CAT/AAC	-	81, 85	-	-
CTA/ACA	-	64, 67, 80, 89, 244	71, 74, 98, 104	177, 209, 317
CTA/AGG	242, 356	111, 124, 141, 351	76, 134, 136	68, 96, 182, 197, 201, 240, 268, 286, 324
CTA/ACC	160	37, 66	64, 68, 152	40, 88, 129, 206, 249, 305, 311, 369
CTA/ACG	-	35, 38, 109, 193, 196	44, 69, 79, 81, 131	55, 165, 229
CTA/AGC	280	35, 85, 169, 313	120, 122, 128, 144, 162, 187, 314	47, 82
CTC/AAG	94	47, 64, 88, 97, 117, 154, 161, 166, 168, 174, 183, 196, 203, 206, 211, 228, 291, 292, 336, 367	72, 120, 124, 189	81, 199, 278, 338, 355
Total	16	226	79	125

responded positively and generated discrete bands with all the plant samples. From a total of 4112 bands, 4 were monomorphic and 4108 were polymorphic. A polymorphism of 99.9% was detected among the species tested. A total of 446 bands were found to be unique for various *Aconitum* species. In this analysis, species-specific markers were identified for the 4 *Aconitum* species (16 for *A. heterophyllum*, 125 for *A. violaceum*, 79 for *A. balfourii*, and 226 for *A. ferox*) (**Table 2**). In the cluster diagram obtained after analysis (**Fig. 1**) accessions of each of the 4 *Aconitum* species grouped separately. The single accession of *A. ferox* was found to be closest to *A. balfourii*. The four *A. heterophyllum* accessions grouped together with a similarity of 28%. Similarly, the two *A. balfourii* accessions had 49% similarity and the three *A. violaceum* had 74% similarity.

Possibly many ecotypes and/or chemotypes of even a single species like *A. heterophyllum* exist in nature. While using the plant commercially as a herbal drug it is very important to identify the correct chemotype having the maximal content of the therapeutically useful secondary metabolites. Besides, correct identification and quality assurance of the starting plant material is an essential prerequisite for ensuring reproducible quality of herbal medicine and also contributes to its safety and efficacy (Joshi *et al.* 2004). This identification requires the use of molecular markers that are unique to the relevant plant and are stable under different conditions (plant age, environment, etc.). Although, chromatographic fingerprinting combined with similarity and hierarchical clustering analysis has been recently applied to distinguish closely related *Aconitum* species (Zhao *et al.* 2009), DNA markers are best suited in terms of stability to serve this purpose.

In an earlier study, for clarification of the circumscription and relationships among the six species within the *Aconitum delavayi* complex that is distributed mainly in the Hengduan Mountains of China, RAPD markers were employed to examine the differentiation of the populations representing the species (Zhang *et al.* 2005). The AFLP markers (unique bands) for *Aconitum* species generated in the present study (**Table 2**) would provide a useful reference tool to identify the herbal material when present in the form of crude drug and circumvent the problems associated with morphological, chemotypic and isozyme markers. The occurrence of these unique bands in the analysis of the DNA isolated from the crude drug preparation could be used as an assay for the presence of a specific species popu-

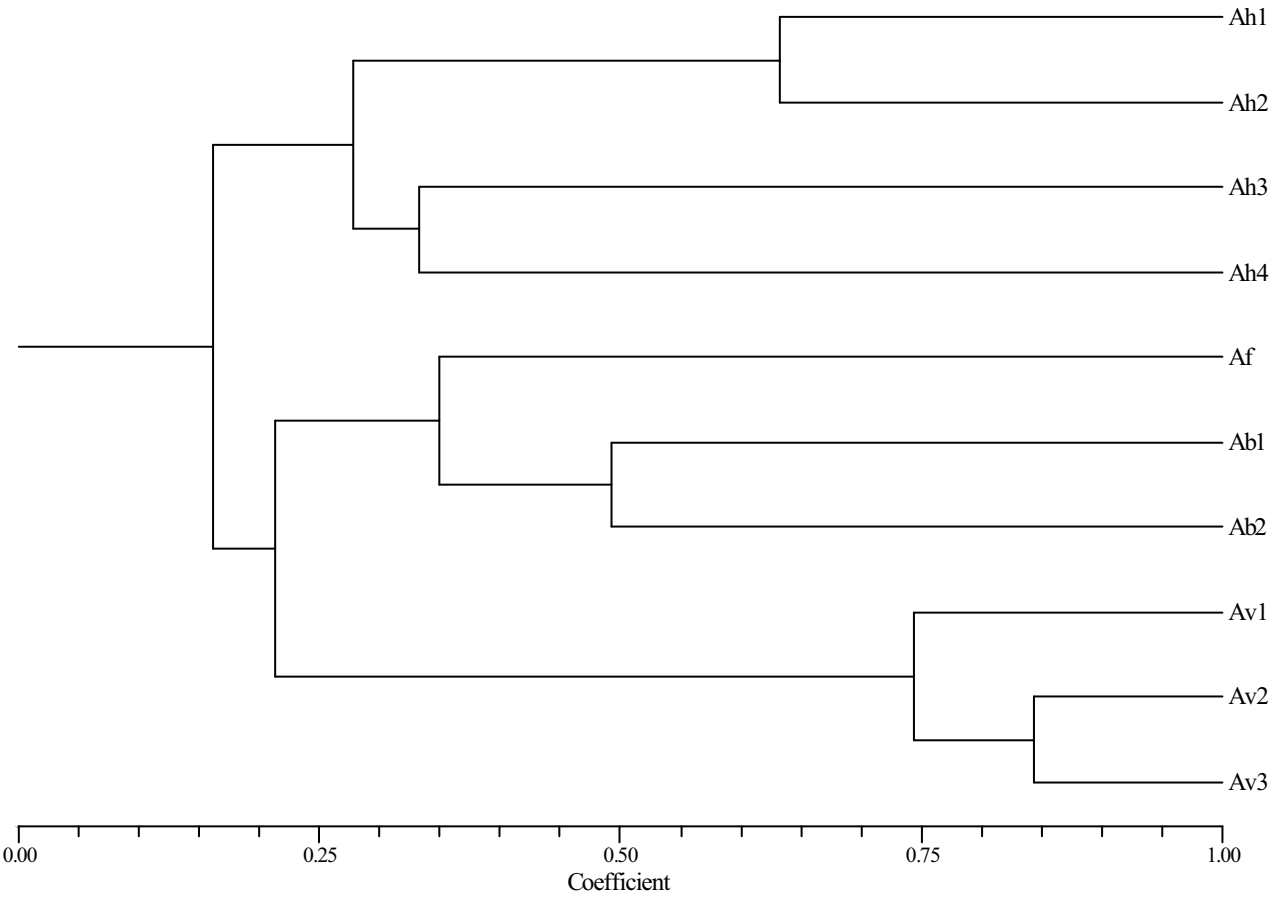

Fig. 1 Cluster diagram showing the relationship among various accessions of the 4 *Aconitum* species. *A. heterophyllum* (Ah), *A. ferox* (Af), *A. balfourii* (Ab) and *A. violaceum* (Av).

lation in it. Previously also AFLP and other DNA markers have been used to resolve complex polyherbal mixtures and identify specific species present therein. In a previous study, AFLP markers have been used to resolve the "Safed Musli" complex and detect the presence of adulterants in crude drug preparations of the herb that is commonly known to contain *Chlorophytum* species along with *Asparagus adscendens* (Misra *et al.* 2007). Species-specific sequence characterized amplified region (SCAR) markers have been used to tag *Phyllanthus* species that are used in herbal drug trade (Jain *et al.* 2008). AFLP has been particularly useful for discriminating closely related species and authentication of herbs as exemplified in an earlier study for *Plectranthus* genus (Passinho-Soares *et al.* 2006). AFLP has also been used for determining the levels of genetic diversity of other critically endangered herbs like *Dendrobium officinale* (Li *et al.* 2008) and *Primulina tabacum* (Ni *et al.* 2006). The present study also provides a well defined grouping pattern for all the 4 *Aconitum* species analysed. The significance of this study lies in the fact that it has provided an authentic tool to detect adulterants in the crude drug preparations of *Aconitum* and help the herbal drug industry in maintaining the quality standards.

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