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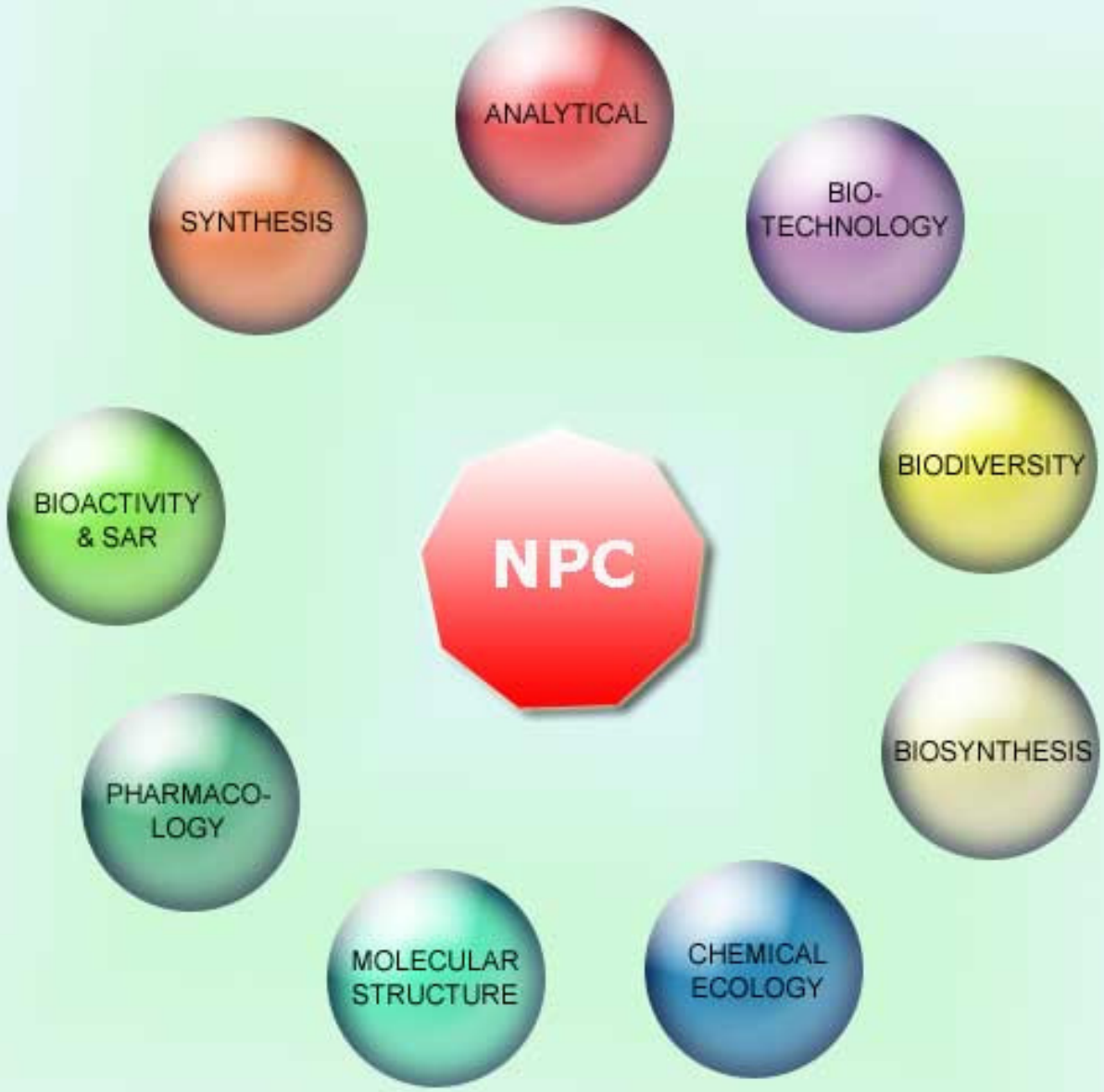

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AFLP-based Detection of Adulterants in Crude Drug Preparations of the 'Safed Musli' Complex

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Safed Musli is an important aphrodisiac herb, which forms an essential ingredient of the preparation of more than one hundred Ayurvedic formulations. It has been found to be an ideal aphrodisiac, with none of the negative side effects associated with the chemical-based products. Various plants belonging to the genera *Chlorophytum* and *Asparagus* have been in use as aphrodisiacs under the common name of 'Safed Musli' because of their white tuberous roots. An AFLP based experiment was carried out to differentiate the members of the 'Safed Musli' complex and resolve the authentication problem prevailing in the herbal drug market.

Keywords: Drug adulteration, aphrodisiac, DNA fingerprinting.

'Safed Musli' (Liliaceae) is a traditional medicinal plant found in the natural forests of India from the eastern state of Assam to the western state of Gujarat. In spite of the medicinal value of the product and an increase in demand for it, the true identity of the drug is the subject of considerable controversy. Entirely different plant species are referred to as 'Safed Musli' in classical and contemporary texts of the Ayurvedic system of medicine and marketed in different part of the country. The various plant species referred to in classical texts are *Asparagus adscendens, Chlorophytum arundinaceum* and *C. tuberosum*. However, in recent times another *Chlorophytum* species, C. *borivilianum* has become the most acceptable source of 'Safed Musli' in the trade. It is considered as a "wonder drug" in the Indian system of medicine due to its aphrodisiac and natural sex tonic properties, which are responsible for it being referred to as 'Herbal Viagra'. Because of its high therapeutic importance, 'Safed Musli' tubers are the major constituents of more than 100 Ayurvedic preparations [1].

More than 175 species of *Chlorophytum* have been reported worldwide. In other parts of the world, *Chlorophytum* is usually grown as an ornamental plant, but in India it has a reputation as a medicinal plant. A total of 13 species of *Chlorophytum* have been reported from India [2]. All these species are different in their medicinal properties, but due to a lack of correct information, all species are known as 'Safed Musli' in the Indian drug market. The 'Safed Musli' complex is generally supposed to consist of *Chlorophytum borivilianum*, *C. arundinaceum*, *C. tuberosum* and *Asparagus adscendens. C. borivilianum* is believed to have originated in South Africa and been introduced accidentally into India; it propagates through its fleshy roots. Although Indian forests are rich in 'Safed Musli', its demand is increasing rapidly in the Indian and international drug markets. Foreign demand has been estimated as 300-700 tones annually [3]. In India, where 'Safed Musli' is popularly used for medication, its demand is over 35,000 tons per annum, but the supply is only about 5,000 tons a year. Dried roots of *Chlorophytum* contain 42% carbohydrate, 8–9% protein, 3–4% fiber and 2–17% saponin [3].

A lot of confusion prevails in the herbal drug market regarding the identification of true 'Safed Musli' and rampant adulteration of the drug with inferior plant

material is reported. To resolve the 'Safed Musli' complex and to differentiate *Chlorophytum* species in this group and *A. adscendens*, an AFLP-based experiment was carried out with the aim of developing molecular markers for plant authentication purpose.

From a study of the crude drug it is very difficult to identify the species present in the mixture. Although the anatomical features are differentiable between *Cholorophytum* and *Asparagus*, they are not helpful in distinguishing between the various species of *Chlorophytum*. Besides, in the crude drug, it is not possible to study the anatomical features unless intact roots are obtained. So the AFLP based-approach was undertaken to generate a database of unique fragments of DNA. The logic behind this was that the isolated DNA from the crude drug, when subjected to AFLP analysis, would provide an indication of the presence of the different taxa, based on the proximity of fragment-match.

PCR-based markers have been used extensively for assessing genetic variation within a species to measure its genetic diversity [7, 8]. Amplified fragment length polymorphisms (AFLP) are ideally suited to assess germplasm because of their ability to generate and detect numerous polymorphisms that are largely distributed throughout the genome, and the method is highly reproducible [5, 9, 10]. The AFLP method largely detects single nucleotide polymorphisms [11]. The discriminatory power of AFLP has been used to distinguish between closely related inbred species, cultivars and ecotypes, such as *Lactuca* [12,13] and *Arabidopsis thaliana* [14], and was shown to detect mutations in plants arising from *in vitro* clonal propagation of *Arabidopsis* [15]. So, for achieving the objective to resolve the 'Safed Musli' complex and differentiating *C. borivilianum* from other species of *Chlorophytum* and *A. adscendens*, an AFLP-based experiment was carried out for developing molecular markers for plant authentication purpose. To study the genetic relatedness of *C. borivilianum* with other species of *Chlorophytum* (*C. arundinaceum*, *C. tuberosum* and *A. adscendens*), molecular characterization of these plants was carried out with a set of 64 AFLP selective primer combinations (MseI/EcoRI). The plant material used for isolation of template DNA for AFLP was generated by pooling leaf samples from a diverse population of plants so as to give proper representation to all the genotypes available in a particular case. Of the 64 primer combinations, only 38 primers responded positively to genomic amplification, producing discrete bands with all the samples. These primers produced a total of 1427 bands. Of these, 17 were monomorphic and 1410 were polymorphic. Among the polymorphic bands, 1128 bands were unique. The analysis revealed ~96% polymorphism among the species. In this case, most of the polymorphic bands were unique. This happened because plants taken in the analysis were either from a different genus or different species of the same genus. As small changes in the genetic composition generate a lot of unique bands in AFLP analysis, this may be the reason for obtaining too many unique bands at interspecific and intergeneric level.

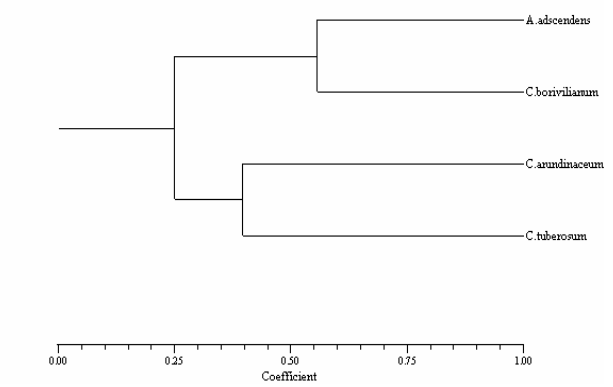

Figure 1: Cluster diagram of *Chlorophytum* species and *Asparagus adscendens* showing relationship among them.

The cluster diagram (Figure 1) generated after cluster analysis showed two major groups. In the first, *A. adscendens* was clustered along with *C. borivilianum*, showing 60% similarity. In the second group, *C. arundinaceum* and *C. tuberosum* were clustered together, showing 40% similarity. The two groups were 25% diverse. Unique bands for all the species were detected and tabulated (Table 1) for identification of adulterants. In the case of *C. borivilianum* 152, of *C. arundinaceum* 431, of *C. tuberosum* 197, and of *A. adscendens* 348 bands were found to be unique. The size of these unique bands ranged between 50 bp and 400 bp. The maximum number of unique bands was detected in the case of *C. arundinaceum*, followed by *A. adscendens*, *C. borivilianum* and *C. tuberosum*. From this analysis we can infer that *C. borivilianum* is closer to *A. adscendens* than to the other species of *Chlorophytum* analyzed in this study. The DNA fingerprint comprised of the unique bands obtained for each of the four pooled populations will provide a reference tool to identify adulterants in the crude

Table 1: Unique/specific AFLP bands identified for differentiating *Chlorophytum* spp. and *Asparagus adscendens* populations in adulterated mixtures.

Primer combination MseI/EcoRI	***Asparagus adscendens* (bp)**	***Chlorophytum borivilianum* (bp)**	***Chlorophytum arundinaceum* (bp)**	*Chlorophytum tuberosum* **(bp)**
CAC/AGC	120	180,183,184,185	115,259,309	-
CAC/ACG	234,277,335,356,357	134	130,251,271,311,314	181,182,302
CAG/ACT	239,284,342	325	117,127,138,148,159,169,178,209, 211,222,244,248,251,259,265,288, 374	134,276,336
CAG/AAG	218,248,277,336,367,395	243,245	108,159,162,196,203,211,226,240, 246,256,265,280,333	274
CAG/ACC	114,115,135,162,202,265,273,372	109	107,111,117,142,151,187,194,219, 317,318,324,340,374	108,321
CAG/AGC	127,197,258,306	298	104,246,299,319	199
CAG/ACG	136,141,194,379	131,250,294	108,113,114,118,224,262,279,292, 305,333,338,341,358	151,169,258,336
CAT/ACT	104,120,121,123,124,145,153,155, 178,187,189,195,215,236,256,267, 282,295,302,343,346,359,386,390	133,163	148,151,167,169,186,190,208,223, 238,286,288,301,314,319,320,322, 324,376,377,397	108,129,132,234, 327,331
CAT/AAG	167,175,195,275,331,336,398	136,227,228,229,230,3 99	132,149,209,211,223,315	291,299,300,301
CAT/ACA	124,135,165,173,192,242,255,268, 276,277,295,328,371,372,381,386	147,151,245,282	100,115,133,134,164,175,189,212, 221,261,271,285,323,360	112,287,331
CAT/ACC	102,125,176,177,242,255	107,151,173,174,175,2 95,390	103,111,165,172,189,214,216,232, 233,235,276,277,284,301,335,336, 375,387	109,148,212
CAT/AGC	179	108	130,236,279	400
CAT/ACG	125,188,230,265,371,372,387	132	141,175,177,331,335,336	140,330
CTA/ACT	107,121,151,207,210,232,234,247, 248,254,256,280,301,314,343,359, 360,377,382,394,396,397	110,115,123,134,163, 185,200,201,204,208, 250,255,277,299,353, 378	130,169,180,189,209,239,240,241, 243,249,267,308,318,327,330,336, 346,347,355,357,364,388	102,105,122,125,126, 128,129,139,145,183, 184,233,272,273,274, 294,295,300,309,331, 333,344,371
CTA/AAC	109	147,182	117	-
CTA/AAG	101,107,113,114,115,121,130,134, 136,149,199,220,226,231,238,243, 271,272,291,303,312,320,331,332, 334,363,376,387,395,398	104,185,206,217,218,2 81,283,284,347,348	100,132,133,138,142,160,164,166, 176,186,200,209,210,211,221,222, 237,253,256,260,264,298,315,341, 342,355,361,379,381,382,392	118,127,147,158,171, 172,174,182,213,265, 285,286,294,295,296, 350,368,383
CTA/ACA	105,122,125,129,148,157,199,205, 226,264,313,372	108,133,153,370	106,118,194,213,234	184,220,290,302,366, 383
CTA/ACC	139	103,371	-	365
CTA/AGG	110,179,248,359	105,162,284	144,326,377,382	148,171,172,289,320, 321,368
CTA/AGC	110	-	101	371
CTT/ACG	252,258,275,379	-	308,317	121,173
CTC/ACT	105,112,125,126,137,148,155,156, 175,209,220,222,228,236,243,292, 330,335,339,342,359,370	109,138,140,152,171, 196,208,275,284,285, 286	115,167,176,179,182,183,187,193, 194,199,200,212,214,216,224,269, 272,280,288,295,296,299,301,302, 308,328,340,341,360,361,362,363, 368,374,395	150,151,163,186,217, 219,234,306
CTC/AAC	229,366	140,208	103,107,189	-
CTC/ACA	109,122,134,139,183,204,349	107	120,195,211,304,308,309,334,336	152,165,334
CTC/ACC	113,135,136,139,144,344,371,383	-	-	106,226,227,247,249, 250
CTC/AGG	181,264,275,370	108,109,170,224,320	107,120,123,168,187,222,260,300, 381	106,255
CTC/AGC	218	109,276	127,189,241,300	226,227,250
CTC/ACG	135,290,371,394	108	155,188,203,216,226,257,355,377, 389	146,182,279,318,339, 346,372
CTG/ACT	102,103,178,178,183,188,189,190, 196,204,230,240,369,370	136,182,241,250,353	125,164,165,193,299,322,323,324, 331,334,335	177
CTG/AAC	200,201	-	-	-
CTG/AAG	101,102,104,105,113,116,123,124, 130,152,166,196,200,202,209,211, 214,249,262,296,298,308,310,318, 319,329,333,348,398	233,237,238,268,363	103,107,129,137,143,148,149,160, 162,198,205,212,228,243,244,250, 270,276,287,294,295,344,345,352, 372,373,379,384,387	106,126,173,179,180, 247,337,383

Table 1 (*Continued*)

CTG/ACA	157,158,325	108,187,353	104,111,118,129,150,153,163,356, 372,392	189
CTG/AGG	324,325	-	114,134,152,190,211,245,267,361	268
CTG/ACG	106,158,268,302,337	398	287	321,393
CTT/ACA	130,189,388	119	235,263,341	223,224,330,331
CTT/ACC	-	328	299,303	335
CTT/AGG	120,	175,347,385	266,317	146,330,331
CTT/ACG	189	367	147,148,149,150	329,333,335

drug supposedly consisting of a particular population. The frequency of 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 population in it. This could help in quantification of the adulteration of the crude drug of different species. This will go a long way in establishing the authenticity and credibility of the ayurvedic drug 'Safed Musli', which presently suffers from the problem of adulteration. The principal component of this drug, *A. adscendens*, is mainly responsible for the aforementioned therapeutic properties. However, the presence of other *Chlorophytum* species in the crude drug may alter its efficacy and therapeutic value. Although there has been no study on the deleterious effects of the adulterants on human beings, there is a distinct possibility that they may be harmful for human usage. The importance of the present study stems from the fact that it provides an authentic tool to detect adulterants in the crude drug 'Safed Musli' and validates the scientific basis of this drug in Ayurveda.

Experimental

Plant material: The plant material used in this study consisted of diverse collections of *Asparagus adscendens*, *Chlorophytum borivilianum*, *C. arundinaceum* and *C. tuberosum* taken from the CIMAP gene bank. For each of the four samples, leaf material pooled from the respective populations was used for DNA isolation (20 plants in each species).

DNA isolation: DNA was isolated using the protocol described by Khanuja *et al.* [4] and quantified by fluorimetry using a DyNa Quant 200 fluorimeter.

AFLP analyses: For AFLP analysis, DNA was restricted using two restriction endonucleases, EcoRI and Tru9I (an isoschizomer of MseI), and double-stranded adapters were ligated to the ends of DNA fragments, generating templates for subsequent PCR amplifications (preselective followed by selective). Restriction and ligation reactions were carried out simultaneously in a single reaction [5]. To carry out the reaction, an enzyme master mix for 10 reactions was prepared containing 1µL 10X T4 DNA ligase buffer, 1µL 0.5 M NaCl, 0.5µL 1 mg/mL BSA, 1µL Tru9I (10 U/µL), 4.25µL EcoRI (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 MseI adaptors (Applied Biosystems), 1 µL EcoRI adaptors (Applied Biosystems) and 1 µL enzyme master, as described above. The reaction mix was incubated overnight at room temperature and subsequently diluted 20-fold with $T_{10}E_{0.1}$ (10 mM Tris and 0.1 mM EDTA) buffer. The ligated adaptors served as primer binding sites for a low-level selection in the preselective amplification of restriction fragments. The MseI complementary primer had a 3'-C and the EcoRI complementary primer a 3'-A. Only the genomic fragments having an adaptor 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 (EcoRI, Applied Biosystems), 0.5 µL AFLP preselective primer (MseI, 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 for infinity.

The preamplified DNA was diluted 20-fold with $T_{10}E_{0.1}$ buffer and selective amplifications were carried out using different MseI and EcoRI primer combinations (Applied Biosystems). Primers chosen for the amplification were from 16 available AFLP selective primers (8 fluorescently tagged EcoRI and 8 untagged MseI primers). The EcoRI primers contain 3 selective nucleotides with the sequence 5' [Dye-Primer-Axx]-3', while the MseI primers had the 3 selective nucleotides starting with C with the sequence 5' [Primer-Cxx]-3'. Selective amplification of each sample was done with all the 64 (8x8)-primer combinations (MseI/EcoRI) using multiplex-PCR

reactions. For selective amplification the reactions were set up as follows: 3 µL of 20-fold diluted preselective amplification product, 15 µL AFLP core mix, 1 µL MseI primer 5'-[Primer-Cxx]-3', 1.5 µL EcoRI primers 5'-[Dye-Primer-Axx]-3'{0.5 µL of 3 EcoRI 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 for infinity. The samples were loaded onto a 5% polyacrylamide gel on an ABI Prism 377 DNA Sequencer. 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. For AFLP reactions the AFLP amplification modules and the guidelines supplied by Applied Biosystems, USA were used.

Data analysis: 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 [6] 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 average) method using NTSys *v*2.1.

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