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Molecular marker study in ornamental plant *Euphorbia milii*

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Abstract
The flowering plant family Euphorbiaceae has 300 genera and about 7,500 species, many of these plants are economically and medicinally important. A high range of medicinally important applications is reported for plant *Euphorbia milii* including a cure for cancer and wart. The varieties of this plant have distinct medicinal values. They can morphologically differentiate by its flower color but it is difficult to distinguish at vegetative stage. In the present study, Inter-Simple Sequence Repeat (ISSR) molecular marker study is performed to distinguish the varieties at vegetative stage. DNA was isolated from three varieties of *Euphorbia milii* using standard CTAB protocol. For molecular characterization, 30 different primers of ISSR were tested for all three varieties. The PCR amplification results obtained using ISSR primers for all three varieties showed unique band patterns on agarose gel electrophoresis. From the band patterns, monomorphic and polymorphic bands were statistical analysis. The clear and well-marked ISSR markers were coded in a binary form, 1 or 0 for the presences or absence of the bands, respectively and from these data, the varieties were distinguished. It is observed that ISSR technique is a useful molecular marker to distinguish *Euphorbia milii* varieties for best medicinal formulations and for clonal selection.

Keywords: DNA, *Euphorbia milii*, gel electrophoresis, issr techniques, pcr amplification

Introduction
Medicinal plants play a very important role in human and drug development because it has many resources like alkaloids, phenolic, flavonoids and tannins (Hill 1952) [3]. More than 3.3 billon people make use of medicinal plants; it is a backbone of folk medicine (Latifian *et al.* 2017) [9]. Euphorbiaceace have many plants with its medicinal properties like *Acalyphaindica* L. for cure of bronchitis, asthma, pneumonia, *Acalypha hispsida* Burm. F for diarrhea, ulcer, *Euphorbia hirta* L for inflamed glands, worms, cough, *E. thymifolia* L for eye trouble, breast pain, *E. tiruclli* L. for enlarged spleen, jaundice, stone in the bladder, tumors, *E. helioscopia* L. for hydrogogue, cathartic, cholera.
In this family a high range of medicinally important application is reported for *E. milii* (Sauza *et al.* 1997) [20]. It is native of Madagascar (Yadav *et al.* 2006) [25]; *E. milii* is also known as Crowns of thorns or Christ plant. In ancient time it is suspected that the species was introduced in Middle East, and the legend associated worn by Christ (Zamith *et al.* 1996) [27]. In India it is mainly cultivated as an ornamental plant (Yadav *et al.* 2006) [25]. *Euphorbia* has reported for having antiarthritis, anticancer, anticonvulsant, antidiabetic, anti-eczema, anti-inflammatory, antimicrobial, antioxidant, antispasmodic, antitumor, antitussive properties hormonal and myelopoiesis properties (Bani *et al.* 2007, Milica *et al.* 2011) [1, 11]. Traditionally, few species of *Euphorbia* is used for skin diseases, gonorrhea, parasites and wart cures (Mwine *et al.* 2011) [13]. *E. milii* flower inhibited the growth of *Aspergillus* that causes human disease (Kamurthy *et al.* 2015) [8]. *E. milii* is a species with varieties, like *E. milii* var. *bevilaniensis*, *hislopii*, *imperatae*, *longifolia*, *milii*, *roseana*, *splendens*, *tananarivae*, *tenuispina*, *tulearensis*, *vulcanii* (Huxley, 1992) [4]. At variety levels identification by molecular markers techniques play an important role (Singh and Kumar, 2009) [21]. It is generally used for fingerprinting, diversity analyses, gene mapping, variability, tracking individuals or lines carrying particular genes and for linkage maps (Kalendar *et al.* 2011) [7].
DNA-based molecular marker helps in improvement of medicinal plant species and it is more reliable because the genetic information is unique for each species and is independent of age, physiological conditions and environmental factors (Joshi *et al.* 2004) [5]. The molecular marker technique efficiency is based on the amount of polymorphism it can detect in the given accessions (Leela *et al.* 2009) [10]. ISSR is a technique which is PCR based method, in which DNA sequences amplified at a distance between two identical microsatellite repeat regions oriented in opposite direction (Monpara *et al.* 2017) [12]. It utilizes repeat-anchored primers to amplify DNA sequences between two inverted SSRs.

Because of high annealing temperature of ISSR primers, this technique is highly reliable, reproducible besides being cost-effective. ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of medicinal plant species (Sarwat *et al.* 2016) [19]. Considering to this, ISSR primers were used in the present study to determine the genetic diversity between three varieties of *E. milii.*

Materials and Methods

Plant material

The three variety of *Euphorbia milii* var. *splendens* (pink), *Euphorbia milii* var. *hislospii* (Red), and *Euphorbia milii* var. *longifolia* (white) were collected from "Women's hostel garden" Saurashtra University Rajkot, Gujarat, India.

DNA isolation from *Euphorbia milii*

The plant material was wash (leaf) with sterile water and leave for air dry. Then leaves of three varieties of were differently crushed in liquid nitrogen. About 300mg of plant material was crushed with 1.5 ml of CTAB extraction buffer and incubated at 65 ºC for 1 hour. After incubation add equal volume of Chloroform: Isoamyl (24:1) and mixed well, centrifuged at 8000 rpm for 20 min. After then take the supernatant and add equal volume of cold isopropanol and incubate at -20 ºC for 1 hour and centrifuged at 3000 rpm for 10min. Then pellet was dissolved in TE (10mMTris, 1mM EDTA, pH 8.0) buffer. Then add RNase (10 mg/ml) for 30 min at 37ºC incubation. 3M (0.1) sodium acetate and absolute cold ethanol is added to the mixture and incubated at -20 ºC for an hour and centrifuged at 8000 rpm for 10 min. After extracting the pellet wash with 70% ethanol and air dried it and dissolve in 100 µl Milli-Q water.

Amplification of ISSR marker

A set of 30 ISSR primers were used for this study (Table 2). DNA was amplified in a total volume of 25 µl. The reaction mixture contained 2.5 µl 10x buffer (10mM Tris-HCL pH 9.0, 50 mM KCL, 0.1% Trion X-100), 2.5 µl 25 mM $MgCl_2$, 1µl 10mM deoxyribonucleotide triphosphate, 10 µM primer and 1 µl of Taq DNA polymerase, 2 µl DNA template. PCR reaction was performed using the Veriti™ Thermal cycler with 40 cycles of denaturation at 92 ºC for 2 min, annealing (36-60 ºC) for 1 min, extension was done at 72 ºC for 7 min. Amplified DNA fragments were analyzed though 2% agarose gel electrophoresis.

Data analysis

ISSR products were scored according to Vafaie-Tabar *et al.* (2004) [23] for each primer and % polymorphism and % monomorphism was calculated. Phylogenetic analysis was performed using unweighted pair-group method with arithmetic averages (UPGMA), as suggested by Sneath and Sokal (1973) [22] and dendrogram was constructed using CLC workbench 7.0.2 Software.

Results and Discussion

DNA markers are very important for genetic diversity, gene mapping and crop breeding studies (Reddy *et al.* 2002) [17]. For the molecular researches good quality of DNA is needed. The successful application of PCR-based downstream applications requires efficient recovery of good quality and quantity of DNA. Plant produces secondary metabolites as well as other substances, that create problems while extraction of DNA. It is difficult to purify and extract high quantity DNA from latex-yielding plants that contain polysaccharide, protein tannins, alkaloids and polyphenols. These compounds are often precipitated with the DNA, and it degrades its quality (Dhakshanamoorthy *et al.* 2013) [2]. In present study three different varieties of *Euphorbia milii* were selected for ISSR marker analysis.

The genomic DNA of all three varieties was isolated (Dhakshanamoorthy *et al.* 2013) [2]. The DNA quality was analyzed by gel electrophoresis and intact bands were observed on 1.0% agarose (Fig. 1). The purity and yield of genomic DNA are presented in Table-1. The A260/A280 ratio was in the range of 1.46% to 1.49% which indicates the purity of the DNA. The *Euphorbia milii* var. *splendens* (pink) concentration was 189 µg/ml, *Euphorbia milii* var. *hislospii* (Red) concentration is 203 µg/ml while the concentration *Euphorbia milii* var. *longifolia* (white) was 154 µg/ml (Table 1).

CTAB buffer was used for the crushing of plant material. It includes detergent cetyltrimethyl ammonium bromide (CTAB) which disrupts the membranes, a reducing agent such as β mercaptoethanol which helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues and for removing the tannins and polyphenols present in the crude extract, a chelating agent such as EDTA which chelates the magnesium ions required for DNase activity. Nucleic acid solution commonly contains undesirable contaminants that are chiefly made of proteins. A classic method of purifying is phenol-chloroform extraction by which the successively washing with a volume of chloroform: Isoamyl alcohol (24:1) was performed. Alcohol precipitation is the most commonly used method for nucleic acid precipitation. This requires diluting the nuclei acid with a monovalent salt adding alcohol to it and mixing gently. The salt used was sodium acetate pH 5.2 (final volume 0.3 M). The nucleic acid precipitated spontaneously and can be pelleted by centrifugation. The salts are removed by washing with 70% alcohol. The DNA is purified by incubating the nucleic acid solution with RNase (10mg/mg) at 37 ºC. RNase treatment is the generally accepted method to remove RNA, however, degradation is often incomplete. The contaminated RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikbart and Villeponteau, 1993) [15] and after that the pellet is washed again with 70% ethanol and it's dissolved in TE buffer or pure MilliQ water.

Many molecular marker techniques are preferentially used for discriminate plants at species and variety levels. However, from 1994, ISSR have become more common and widespread its applications. ISSR have high polymorphism compare to other methods. It has high annealing temperature and longer sequence of ISSR primers but the yield is reliable, bands are reproducible, and the cost of the analysis is relatively lower than other marker, such as AFLP (amplified fragment length polymorphism). A wide range of applications in genetic diversity studies is established by ISSRs for many medicinal plant species (Yao *et al.* 2008) [26].

Nineteen primer set of ISSR successfully distinguished 14 sugarcane (*Saccharum* sp.), for genomic diversity (Rao *et al.* 2016) [16] which is useful in breeding programs for sugarcane varieties. The genetic diversity of *Swertia chirayita* genotypes from the temperate Himalayas of India has analyzed using ISSR primers (Joshi and Dhawan, 2007) [6]. In this study also 30 primer set was used, it have produced total 315 bands resulted in 98.7% polymorphism which indicates high genetic diversity. Similarly, work done in *Psychotria ipecacuanha* (Rubiacea) revealed genetic diversity and geographic

differentiation using ISSR markers (Rossi *et al.* 2009) [18]. Studies on *Vanilla planifolia* (Orchidaceae) (Verma *et al.* 2009) [24] and *Humulus lupulus* (Patzak *et al.* 2001) [14] support the view that ISSR is a reliable technique to distinguished varieties of plant species.

In this study, for ISSR analysis, 30 primers were selected under optimized PCR conditions. The amplified products of ISSR are analyzed by 2% agarose gel electrophoresis. In Table 2 list of primers used for ISSR is given. The annealing temperature of these primer is varying from 30 ºC (UBC803) to 60 ºC (ISSR7). ISSR amplification of DNA polymorphic and monomorphic band pattern observed in electrophoresis (Fig. 2-7). In this work, out of 30 primers, 9 primer were produced 100% polymorphic band with UBC834, UBC876, UBC881, ISSR1, ISSR2, ISSR3, ISSR4, ISSR6, ISSR7 and 2 primer given 100% monomorphic band with UBC803, UBC872.

The clear and well-marked ISSR markers were coded in a binary form, 1 or 0 for the presences or absence of the bands, shown in Table 3 respectively. From total 346 bands, 256 bands were polymorphic and remaining 90 observed as monomorphic (Table 3). The percentage of polymorphic and monomorphic bands showed 73.17 and 26.83, respectively. The total number of band (TNB), number of monomorphic bands (NMB), number of the polymorphic bands (NPB), the percentage of the monomorphic band (M %) and percentage of the polymorphic band (P %), obtained each primer are shown in Table 3.

Jaccard's similarity measure was used to carry out a UPGMA cluster analysis and to generate a dendrogram representing the relationship among three varieties of *E. milii* (Fig. 8). The dendrogram clearly distinguished the three varieties of *E. milii* into two groups whereas band pattern showed clear distinction of all three varieties (Table 3).

In sum, the PCR based ISSR method has been used for detecting genetic relationship among three varieties of *Euphorbia milii.* ISSR- PCR method is easy, fast and cost-effective molecular marker technique which can be widely used to analyzed plant diversity at genetic level.

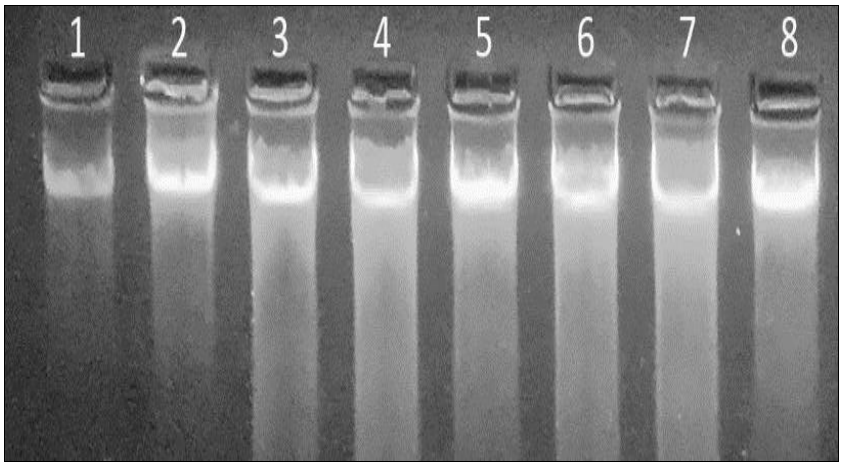

Fig 1: Genomic DNA of three varieties of *E. milii*, lane 1-2 *longifolia*, lane 3-5 *splendens*, lane 6-8 *hislospii*. In 1% gel

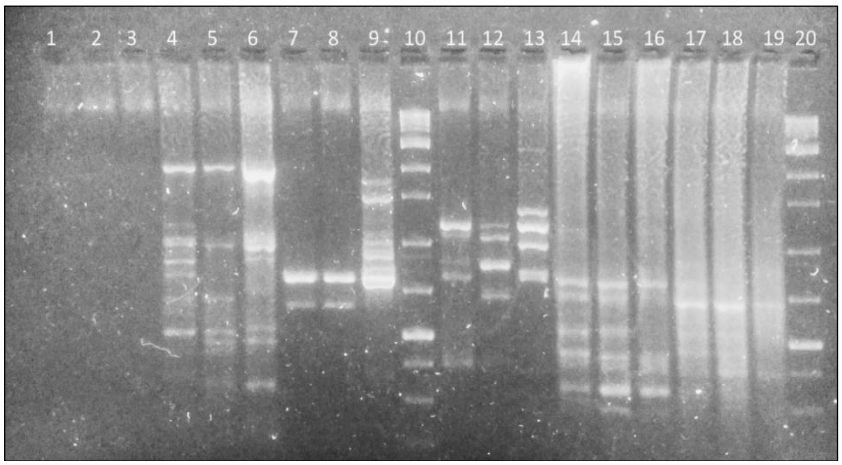

Fig 2: ISSR amplification pattern in three *Euphorbia milii* varieties. ISSR primers in these lanes are: Lane 1-3 primer UBC803, Lane 4-6 primer UBC808, Lane 7-9 primer UBC814, Lane 11-13 primer UBC822, Lane 14-16 primer UBC826 and 17-19 primer UBC862. Each three successive lane from 1-20 contains three varieties of *Euphorbia milii* which are *E. milii* var. *longifoli*, *E. milii* var. *splendens* and *E. milii* var. *hislospii* respectively, while lane 10 and 20 are of BioLit ProxiO 1kb DNA Ladder

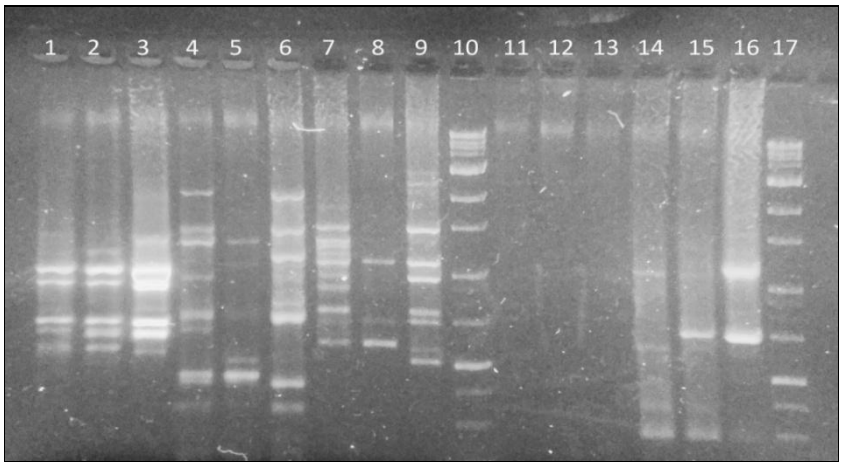

Fig 3: ISSR amplification pattern in three *Euphorbia milii* varieties. ISSR primers in these lanes are: Lane 1-3 primer UBC894, Lane 4-6 primer UBC867, Lane 7-9 primer UBC868, Lane 11-13 primer UBC872 and Lane 14-16 primer UBC873. Each three successive lane from 1-20 contains three varieties of *Euphorbia milii* which are *E. milii* var. *longifoli*, *E. milii* var. *splendens* and *E. milii* var. *hislospii* respectively, while lane 10 and 17 are of BioLit ProxiO 1kb DNA Ladder

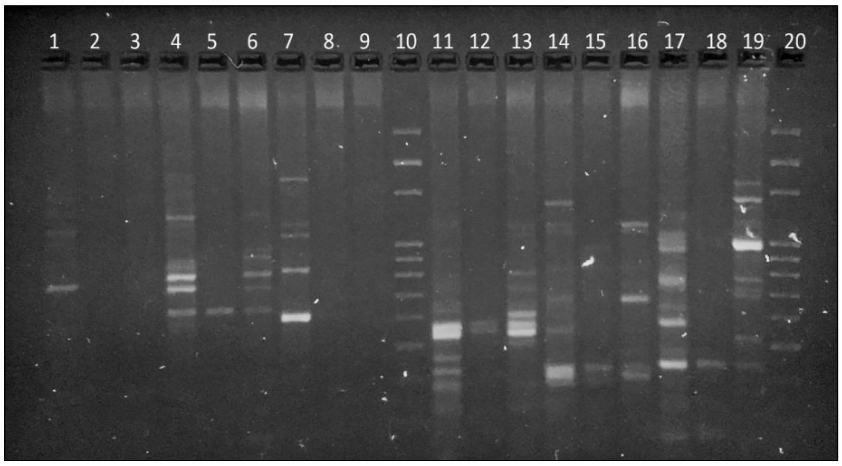

Fig 4: ISSR amplification pattern in three *Euphorbia milii* varieties. ISSR primers in these lanes are: Lane 1-3 primer UBC876, Lane 4-6 primer UBC880, Lane 7-9 primer UBC881, Lane 11-13 primer UBC884, Lane 14-16 primer UBC885 and 17-19 primer UBC886. Each three successive lane from 1-20 contains three varieties of *Euphorbia milii* which are *E. milii* var. *longifoli*, *E. milii* var. *splendens* and *E. milii* var. *hislospii* respectively, while lane 10 and 20 are of BioLit ProxiO 1kb DNA Ladder

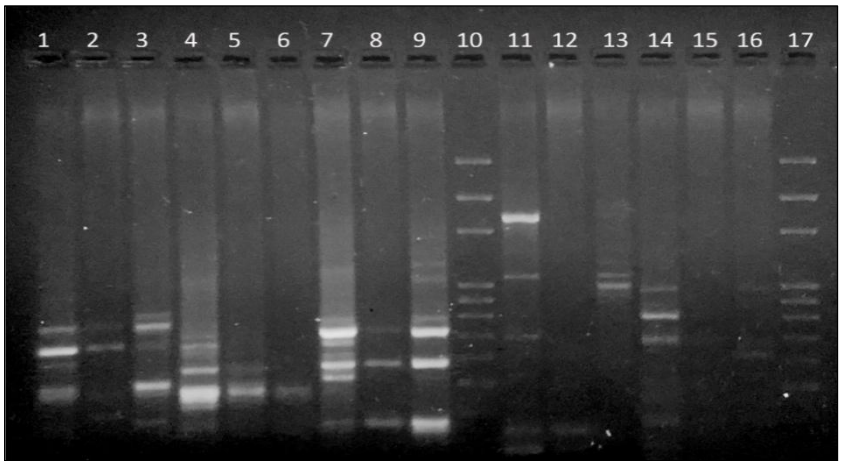

Fig 5: ISSR amplification pattern in three *Euphorbia milii* varieties. ISSR primers in these lanes are: Lane 1-3 primer UBC888, Lane 4-6 primer UBC889, Lane 7-9 primer UBC889, Lane 11-13 primer ISSR6 and Lane 14-16 primer ISSR5. Each three successive lane from 1-20 contains three varieties of *Euphorbia milii* which are *E. milii* var. *longifoli*, *E. milii* var. *splendens* and *E. milii* var. *hislospii* respectively, while lane 10 and 17 are of BioLit ProxiO 1kb DNA Ladder

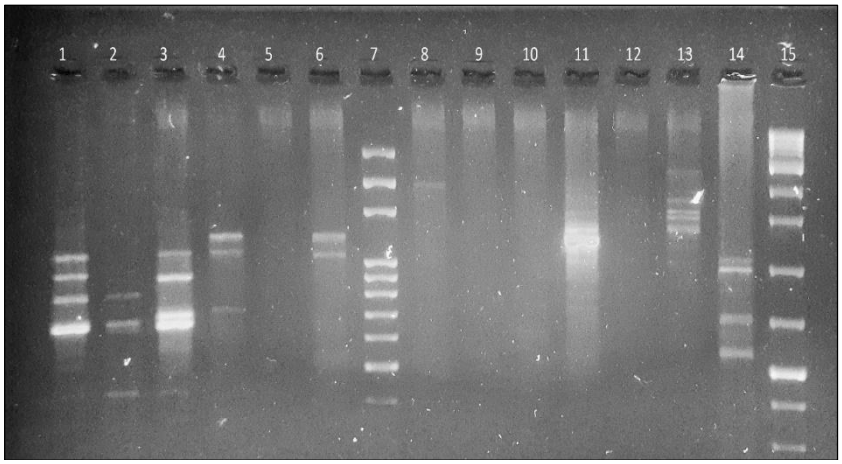

Fig 6: ISSR amplification pattern in three *Euphorbia milii* varieties. ISSR primers in these lanes are: Lane 1-3 primer UBC817, Lane 4-6 primer UBC824, Lane 8-10 primer UBC840 and Lane 11-13 primer ISSR1. Each three successive lane from 1-20 contains three varieties of *Euphorbia milii* which are *E. milii* var. *longifoli, E. milii* var. *splendens* and *E. milii* var. *hislospii* respectively, while lane 7 and 15 are of BioLit ProxiO 1kb DNA Ladder

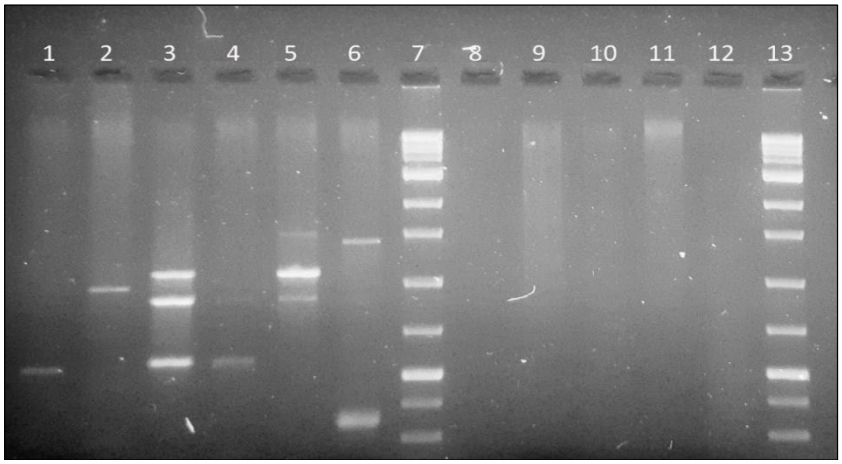

Fig 7: Amplification pattern of selected ISSR primer for three variety of *Euphorbia milii.* Lane 1-2 primer ISSR2, Lane 1 var. *splendens, Lane* 2 var. *hislospii.* Lane 3-5 primer ISSR3 Lane 3 var. *longifolia,* Lane 4 var. *splendens, Lane* 6 primer ISSR4- *var. splendens,* Lane 8-9 primer ISSR4 Lane 8– *var. splendens, Lane* 9- *var. hislospii.*, Lane 10-12 primer ISSR7 Lane 10- *var. longifolia,* Lane 11– *var. splendens, Lane* 12- *var. hislospii.*, Lane 7 and 13 are of BioLit ProxiO 1kb DNA Ladder

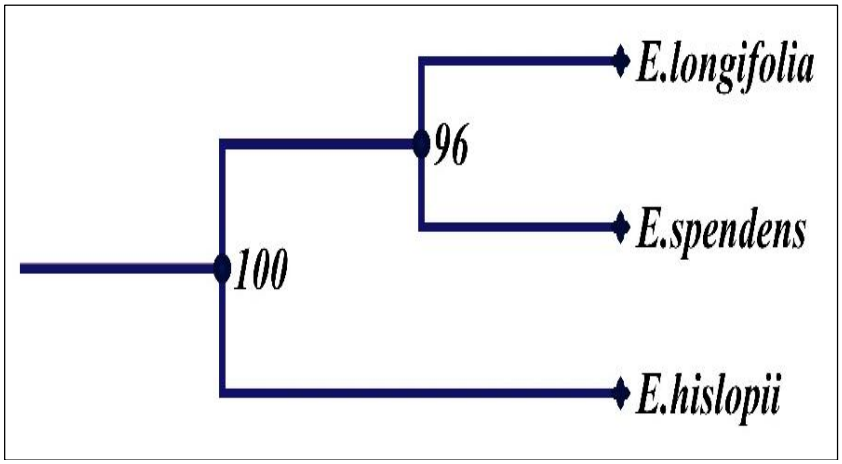

Fig 8: A dendrogram constructed from ISSR data in three variety of *Euphorbia milii*, based on Jaccard's similarity coefficient and UPGMA clustering

Table 1: Purity and concentration (µg/ml) of genomic DNA of three variety of *Euphorbia milii*

Varieties	Purity (%)	Concentration µg/ml
E. milii var. longifolia	1.46	154
E. milii vars. splendens	1.49	189
E. milii vars. hislospii	1.48	203

Table 2: Primers used for ISSR amplification and their conditions

Sr. No.	Primer name	Sequence	Ta (°C)
I-1	UBC803	(AT)8C	36
I-2	UBC808	(AG)8C	48
I-3	UBC814	(CT)8A	48
I-4	UBC817	(CA)8A	44.2
I-5	UBC822	(TC)8A	48
I-6	UBC826	(AC)8C	48
I-7	UBC834	(AG)8TT	44.2
I-8	UBC840	(GA)8TT	44.2
I-9	UBC862	(AGC)6	48
I-10	UBC864	(ATG)6	48
I-11	UBC867	(GGC)6	48
I-12	UBC868	(GAA)6	48
I-13	UBC872	(GATA)4	48
I-14	UBC873	(GACA)6	48
I-15	UBC876	(GATA)2(GACA)2	48
I-16	UBC880	(GGAGA)3	48
I-17	UBC881	(GGGGT)3G	48
I-18	UBC884	HBH(AG)7	48
I-19	UBC885	BHB(GA)7	48
I-20	UBC886	VDV(CT)7	48
I-21	UBC888	BDB(CA)7	48
I-22	UBC889	DBD(AC)7	48
I-23	UBC891	HVH(TG)7	48
I-24	ISSR1	(AGC)5GC	55
I-25	ISSR2	(CA)7AC	44.2
I-26	ISSR3	(GT)7AC	44.2
I-27	ISSR4	GCA(GA)7	47.2
I-28	ISSR5	(GA)9C	51.7
I-29	ISSR6	(GA)9A	49.5
I-30	ISSR7	(CG)8C	60

Table 3: ISSR amplicon profile, % monomorphism and % polymorphism of three variety of *Euphorbia milii*

Primer	Total band	Monomorphic band	Polymorphic band	% Monomorphism	% Polymorphism
I-1	3	3	0	100.00	0.00
I-2	24	3	21	12.50	87.50
I-3	12	3	9	25.00	75.00
I-4	13	3	10	23.08	76.92
I-5	18	6	12	33.33	66.67
I-6	22	12	10	54.55	45.45
I-7	6	0	6	0.00	100.00
I-8	4	3	1	75.00	25.00
I-9	13	6	7	46.15	53.85
I-10	22	9	13	40.91	59.09
I-11	23	3	20	13.04	86.96
I-12	26	3	23	11.54	88.46
I-13	3	3	0	100.00	0.00
I-14	13	3	10	23.08	76.92
I-15	2	0	2	0.00	100.00
I-16	10	3	7	30.00	70.00
I-17	5	0	5	0.00	100.00
I-18	14	3	11	21.43	78.57
I-19	14	3	11	21.43	78.57
I-20	16	3	13	18.75	81.25
I-21	12	3	9	25.00	75.00
I-22	10	3	7	30.00	70.00
I-23	18	9	9	50.00	50.00
I-24	12	0	12	0.00	100.00
I-25	7	0	7	0.00	100.00
I-26	7	0	7	0.00	100.00
I-27	2	0	2	0.00	100.00
I-28	6	3	3	50.00	50.00
I-29	8	0	8	0.00	100.00
I-30	1	0	1	0.00	100.00

Total	346	90	256	738.05	2194.18
Average	11.53	3	8.53	26.83	73.17

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