Assessment Of Genetic Diversity Within And Among Populations Of *Acmella Paniculata* (Wall. ex DC.) R.K Jansen Through RAPD And ISSR Markers

Debmalya Das Gupta, Pallabi Kalita, Hui Tag[1] and A.K Das

Higher Plant Diversity & Validation Unit,Center with Potential for Excellence in Biodiversity

Department of Botany, Rajiv Gandhi University, Doimukh, Arunachal Pradesh, India

[1]**Corresponding author**: huitag2008rgu@gmail.com.

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Abstract: *Acmella paniculata* (Wall. ex DC.) R.K Jansen (syn. *Spilanthes paniculata* DC.) belonging to the family Compositae (Asteraceae) has been reported to be used in traditional system of medicine among different tribal communities of the world as the plant is widely distributed across both tropical and subtropical nations. However in spite of its wide application in traditional medicine healing system, molecular characterization of this particular species has not been explored to date. Here a total of 15 landraces belonging to five populations of *A. paniculata* has been taken in order to establish genetic diversity within and among its populations through RAPD and ISSR marker. In case of RAPD a total of 325 bands were obtained in which 180 bands were monomorphic and 145 bands were polymorphic thereby showing 44.61% polymorphism while in case of ISSR a total of 129 bands were obtained in which 90 bands were monomorphic and 39 bands were polymorphic thereby showing 30.23% polymorphism. The average similarity matrix was used to generate a tree for cluster analysis by UPGMA method which showed higher genetic diversity in some of the populations of *A. paniculata*. ANOVA showed that genetic diversity of *A. paniculata* within population varied at around 74.67% while among population it varied around 25.33%.

Key words: Genetic diversity, *Acmella paniculata*, RAPD, ISSR

Introduction

The genus *Acmella* (*Spilanthes*) belonging to the family Compositae (Asteraceae) has more than 300 species, generally distributed in the tropics (Anonymous, 2013 and Harold *et al*., 1981).This family is known as the aster, daisy, or sunflower family and it is the largest family of flowering plants, in terms of number of species (Paulraj *et al*, 2013) .Commonly known as Toothache plant or Paracress or Eyeball plant, it is native to the tropics of Brazil, and is grown as an ornamental (and as a medicinal) in various parts of the world (Sahu *et al*., 2011). Worldwide around 60 species belonging to the genus *Spilanthes* have been reported (Willis, 1973). The genus *Spilanthes* is represented by six species in India; they are *Spilanthes calva* DC., *S. paniculata* DC., *S. radicans* Jacq., *S. ciliata* Kunth., *S. uliginosa* Sw., and *S. oleracea* L. (Sivarajan and Remesen,1987). *S. calva* DC., *S. mauritiana* L. and *S. paniculata* DC. are found more commonly, whereas *S. acmella* Murr. and *S. acmella* L. *var oleraceae* Clarke are rare in occurrence (Raja,1974). It generally grows on damp areas, near lakes & ponds and near sewage discharge areas. The key identification characteristics of the genera *Acmella* are head solitary, pappus of stiff awns; achenes monomorphic, rhombic, stramineous, cork like margin at maturity, leaves sessile; heads discoid and corollas white to purplish white (Rajan, 1978 and Chandra *et al.,* 2006).The flowers and leaves have a pungent taste, accompanied with tingling and numbness (Chopra *et al*, 1956 and Anonymous, 1989. The plant holds great promise as a commonly available medicinal plant as per as Indian traditional circle is concerned. Some of the *Spilanthes*

species viz. *S. acmella* Murr and *S. oleracea* L. have been reported to be useful in the treatment of malaria (Spelman *et al,* 2011).

A.paniculata (synonym *S. paniculata*) is an annual or short lived perennial herb, erect or prostrate at the base and rooting at the nodes where upwards often strongly branched. The leave blade is broadly ovate to ovate – triangular and measuring (1 - 12) cm X (0.5 – 7) cm. There are (8 – 14) involucral bracts in 2 rows. The peduncle is up to 16 cm long. The flower heads are radiate or conical, and measuring (10 – 15) mm X (8 – 15) mm. The one seeded fruit is (2 – 3) mm long (Verma *et al.,* 1993). It grows in drier places such as upland fields, waste places, roadsides and riversides. It flowers year round and produces plenty of seeds which are dispersed by animals and wind. Young leaves and flower heads are eaten raw, boiled or added to curry. *A. paniculata* has been used as medication for a number of diseases in ancient system of medicine viz. toothache, rheumatism and fever (Agharkar, 1991). The flower heads are chewed to relieve the toothache and other mouth related troubles. Leaves are used externally in treatment of skin diseases (Agharkar, 1991). Root decoction is used as purgative, diuretic and lithotriptic. Whole plant is used in treatment of dysentery (Verma *et al.,* 1993). The plant is also used in the traditional system of medicine for the treatment of various disease complications including toothache, infections of throat and gums, paralysis of tongue, a popular remedy for stammering in children. The raw leaves are used as flavouring for salads, soups and meats in Brazil and India and are grown widely as an ornament because of the attractive colourful heads (Verma *et al.,* 1993).

As per as molecular work of this genus is concerned it is pertinent to mention here that except studying the genetic fidelity of *S. calva* DC using RAPD and ISSR marker (Razzaq and Heikrujam, 2013), no such study has been conducted so far for the molecular authentication of this species. The present study was thus conducted for assessing the genetic diversity within & among populations of *A. paniculata* through RAPD & ISSR markers.

Materials and methods

DNA isolation

DNA was isolated from the landraces using Doyle and Doyle method (Doyle and Doyle, 1990) using 2X CTAB protocol with minor modifications. Approximately 500 mg of fresh leaves of *S. paniculata* were crushed to a powder in the presence of liquid nitrogen, using mortar and pestle. The powder was then transferred to a 25 ml sterile sorvell tube with 1ml of extraction buffer. The Extraction Buffer consisted of 2% (w/v) CTAB (Cetyl-trimethyl ammonium bromide, Sigma), 1.4 M NaCl, 20mM EDTA (Ethylene diamine tetra acetic acid), 100 mM Tris-HCl pH 8.0, and 0.2% (v/v) â-mercaptoethanol. The homogenate was incubated at 65°C for 1 hour with occasional shaking and then mixed with an equal volume of chloroform isoamyl alcohol (24:1), and centrifuged at 10,000 rpm for 20 minutes. DNA was precipitated from the aqueous phase by mixing with 1/10 volume (ml) of 3 M sodium acetate and an equal volume of isopropanol. After centrifugation at 10,000 rpm for 10 minutes, the DNA pellet was washed with 70% ethanol and was air dried using lyophilizer and then resuspended in 40ìl TE (10mM Tris pH 8.0, 0.1 mM EDTA) buffer. The concentration of DNA in the samples was determined by calculation from 260/280 O.D value (UV-2600, Schimadzu wavelength range [300-1100] nm). The DNA samples were subjected to electrophoresis on 0.8% agarose gel with genomic ë DNA (25ng/ìl) as molecular weight standard.

Spectrophotometric analysis of the five populations (viz., SRG, AHV, IR, CH and LV) of *A. paniculata,* collected from Rajiv Gandhi University campus, Nirjuli Directorate office, Itanagar road, Chimpu area and Leki village respectively gave lower absorbance at 230nm, 260/280 value between (1.75 – 2.00) and clear distinct intact band (without smearing) on gel thereby implying the DNA isolated to be ideal for carrying out subsequent PCR steps.

Table 1. Accession number & locality *of Acmella paniculata* (Wall. ex DC.) R.K Jansen.

Sl.No.	Sample code name	Place of collection
1.	SRG1	RGU Campus
2.	SRG2	RGU Campus
3.	SRG3	RGU Campus
4.	AHV1	Nirjuli Directorate Office
5.	AHV2	Nirjuli Directorate Office
6.	AHV3	Nirjuli Directorate Office
7	IR1	Itanagar Road
8	IR2	Itanagar Road
9	IR3	Itanagar Road
10.	CH1	Chimpu
11.	CH2	Chimpu
12.	CH3	Chimpu
13.	LV1	Leki Village
14.	LV2	Leki Village
15.	LV3	Leki Village

RAPD analyis

Four arbitrary 10-base primers (Operon Technologies Inc) were used for RAPD (Random Amplified Polymorphic DNA), following the protocol of (Williams *et al.,* 1990) with minor modifications. Each PCR reaction was performed in a total volume of 25ìl containing 2.5ìl 10X PCR buffer, 0.5mM $MgCl_2$, 0.1mM dNTPs (0.025mM of each dNTP), 1.8 ng/ml decamer primer (Operon), 0.04 U/ml Taq DNA polymerase (New England Biolabs) and a concentration of 40 ng/ìl of genomic DNA. The PCR amplification reaction consisted of an initial denaturation at 94^0 C for 2 minutes followed by 45 cycles, each consisting of denaturation at 94^0 C for 1 minute, annealing at 36^0 C for 1 minute and extension at 72^0 C for 2 minutes. It was followed by a final extension at 72^0 C for 10 minutes. Due to low reproducibility of RAPD, we have repeated each RAPD PCR reaction three times and observed almost similar banding pattern each time. The PCR products were separated by gel electrophoresis in a 1.5% agarose gel containing ethidium bromide in 1X TBE buffer and 1Kb ladder and 100bp ladder (New England Biolabs) were run along with the samples to determine the molecular sizes of the fragments obtained. Gels were photographed in a Gel-documentation system (Bio-Rad).

A total of 15 young plants of *A. Paniculata* belonging to above mentioned five populations each of RGU Campus (SRG), Nirjuli Directorate office (AHV), Itanagar road (IR), Chimpu area (CH) and Leki village (LV) were taken for PCR studies. RAPD was performed according to the standard protocol taking 4 primers. A total of 325 bands were obtained in which 180 bands were monomorphic and 145 bands were polymorphic thereby showing 44.61% polymorphism.

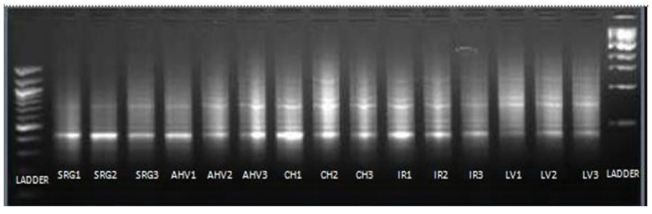

Fig.1. Representative picture of RAPD of 15 *Acmella paniculata* (Wall. ex DC.) R.K Jansen landraces comprising five populations using primer OPAL 02. Some of the bands are more intense showing thereby repetition of the complementary sequence of the primer used and clearly counts for the genetic diversity within and among populations. Ladders each of 100bp (left side) and 1kb (right side) are run along with the samples.

ISSR analysis

Three 5'anchored Simple Sequence Repeats (SSR) (Genetix Labs) were used for ISSR (Inter Simple Sequence Repeats), following the protocol of (Zietkiewicz *et al*., 1994) with minor modifications. Each PCR reaction was performed in a total volume of 25µl containing 2.5µl 10X PCR buffer, 0.5mM $MgCl_2$, 0.1mM dNTPs (0.025mM each dNTP), 1.8 ng/ml decamer primer (Operon),0.04 U/ml Taq DNA polymerase (New England Biolab) and a concentration of 40ng/µl of genomic DNA. The PCR amplification reaction consisted of an initial denaturation at 94^0 C for 2 minutes followed by 45 cycles, each consisting of denaturation at 94^0 C for 1 minute, annealing at 36^0 C for 1 minute and extension at 72^0 C for 2 minutes. It was followed by a final extension at 72^0 C for 10 minutes. The PCR products were separated by gel electrophoresis in a 1.5% agarose gel containing ethidium bromide in 1X TBE buffer and 1Kb ladder and 100bp ladder (New England Biolabs) was run along with the samples to determine the molecular sizes of the fragments. Gels were photographed in a Gel-Documentation System (Bio-Rad)

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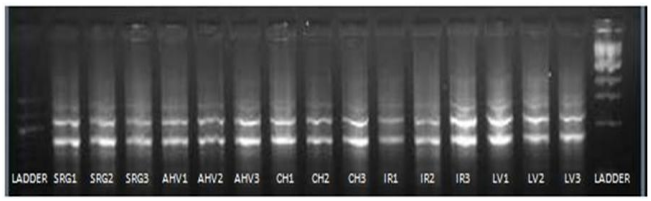

Fig. 2. Representative picture of ISSR of 15 *Acmella paniculata* (Wall. ex DC.) R.K Jansen landraces comprising five populations using primer 5' $C(AG)_8$ 3'. Banding pattern though looks similar but some of the bands are faint as in picture that are quite distinct thereby showing genetic diversity within and among populations. Ladders each of 100bp (left side) and 1kb (right side) are run along with the samples.

Results

Scoring and data analysis

Amplified DNA markers of RAPD and ISSR were scored as present or absent in each plant. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored. Bands were scored as present (1) or absent (0) to form a raw data matrix for diversity analysis. The average similarity matrix was used to generate a tree for cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic mean) method using NTSys v 2.1. (Fig 3) ANOVA (ANalysis Of VAriance) was also done to find out the genetic diversity within and among populations of *A. paniculata* (Wall. ex DC.) R.K Jansen.

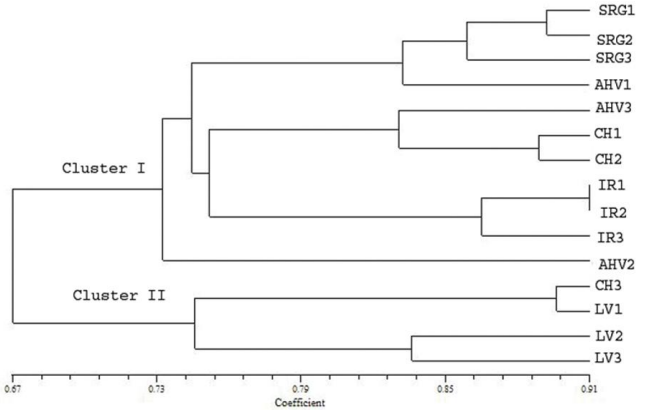

Fig.3. Cluster Diagram showing relationship among *Acmella paniculata* (Wall. ex DC.) R.K Jansen populations. The populations SRG, IR and LV remained together but one of the landrace each of AHV and CH got separated from its group indicating that genetic diversity was noticeably higher in some of the populations of *A. paniculata* (Wall. ex DC.) R.K Jansen within small geographical region. It was also noteworthy that the population of LV got separated from the parent cluster and formed a separate cluster thereby exhibiting higher genetic diversity from the other populations of *A. paniculata* (Wall. ex DC.) R.K Jansen.

The dendogram (Fig. 3) obtained after analysis of data using the NTSys v 2.1 software indicated two major clusters, cluster I and cluster II representing 11 landraces comprising *A. paniculata* (Wall. ex DC.) R.K Jansen populations of RGU Campus, Nirjuli Directorate Office, Itanagar Road and Chimpu area and 4 landraces consisting three of the landraces of Leki Village and one of the landrace of Chimpu Area respectively. The populations of RGU Campus, Naharlagun area and Leki Village remained together but one of the landrace each of Nirjuli Directorate Office (AHV5) and Chimpu area (CH3) got separated from its group indicating that genetic diversity was noticeably higher in some of the populations of *A. paniculata* within small geographical region. It was also interesting to note that the population of Leki Village (LV) as shown in cluster II (Fig 3) got separated from the parent cluster and formed a separate cluster thereby exhibiting higher genetic diversity from the other populations of *A. paniculata* (Wall. ex DC.) R.K Jansen. Within the cluster I it was seen that the genetic diversity among populations of RGU Campus (SRG) and Itanagar Road (IR) were not quite distant as they remained together in the cluster. However one of the landrace of Nirjuli Directorate Office (AHV) was quite genetically diverse from the other two landraces showing distinct sub clustering pattern implying higher genetic diversity.

ANOVA analysis showed that genetic diversity of *A. paniculata* (Wall. ex DC.) R.K Jansen within population varied at around 74.67% while among population it varied around 25.33% (Table 2) as genetic diversity between each populations of *A. Paniculata* were higher than that between each individual plant samples within the population.Thus, it clearly indicates that genetic diversity among populations of *A. paniculata* occurs as per as different populations from different places are taken into account.

Table 2. ANOVA Analysis of *Acmella paniculata* (Wall. ex DC.) R.K Jansen Populations. It indicates that percentage of variation within population (74.67%) was higher than percentage of variation among population (25.33%).

Source of variation	Difference	Sum of squares	Variance components	Percentage of variation
Among population	2	54.971	2.38422va	25.33
Within population	13	105.450	7.03000vb	74.67
Total	15	160.421	9.41422	

Discussion

The preliminary studies of genetic diversity of *A. paniculata* populations sampled from small geographical area of approximately 20 sq.km located within tropical foot hills belt

(Itanagar, Naharlagun, Nirjuli, Rono Hills) of Papum Pare District of Arunachal Pradesh has established the fact that even though the populations collected for this study were not quite distant from each other still it was found that genetically some of the populations of *A.paniculata* (Leki Village LV) was quite distinct from the other populations although they were all collected from tropical zone areas forming altogether a different cluster (Cluster II). As such genetic diversity was seen even though the sample size of each population was small (three landraces for each sample).

Thus from the above study it was found that genetic diversity within and among populations of *A. paniculata* was present within small geographic locations thereby implying the fact that there is further scope of establishing genetic diversity of this genus for large geographical range locations.

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