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Genetic diversity and phylogenetic relationship of *Nilgirianthus ciliatus* populations using ISSR and RAPD markers: Implications for conservation of an endemic and vulnerable medicinal plant

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ABSTRACT

Nilgirianthus ciliatus is of interest for its extensive applications in Ayurvedic medicine. The study focuses on the assessment of genetic diversity and phylogenetic relationships in *N. ciliatus* populations to aid conservation of this species at risk of extinction. Thirty five inter simple sequence repeats (ISSR) and thirty random amplified polymorphic DNA (RAPD) markers were employed in PCR yielding 298 and 99 bands, respectively. Using ISSR markers, bands of 200–3500 bp were obtained and accounted for 77.0% of polymorphism observed. The polymorphism information content (PIC) and resolving power (R_p) values were 0.59 and 20.8, respectively. RAPD analysis resulted in 99 total bands of which 89 were polymorphic (89.9%). The PIC value for RAPD was 0.61 and R_p value was 18.8. The two different cluster analyses (UPGMA and Neighbor Joining) and principal component analysis showed the genetic variation among the populations. Clustering of *N. ciliatus* populations from different geographical regions in distinct groups can be a good sign for clear separation that is supported by high bootstrap values. Overall, both ISSR and RAPD analyses were valuable for identifying genetic variations and phylogenetic relationships in *N. ciliatus* populations at the DNA level and these differences provides the basic knowledge for better conservation management policies and genetic identification of the different geographical populations of this plant.

1. Introduction

Nilgirianthus ciliatus is an endemic aromatic shrub (Acanthaceae family) of Southwestern Ghats, India with versatile pharmaceutical applications (Ved and Goraya, 2007). In the Ayurvedic system of medicine, the whole plant has been used for the treatment of bronchitis, chest congestion, cough, dropsy, glandular swellings, gum diseases, itching, jaundice, leprosy, lumbago, limping, oedema, paraplegia, leucoderma, rheumatalgia, rheumatism, sciatica, strangury fever, toothache, ulcers, urinogenital tract diseases, general debility, whooping cough, pruritus, scrofula, skin diseases, inflammations, and odontalgia (Warrier et al., 1994; Khare, 2007; Sujatha et al., 2011; Srinivasan et al., 2013; Rameshkumar et al., 2017). *N.ciliatus* is highly traded in India (Ved and Goraya, 2007) and recently many reports were documented for its biological activities viz., hepatoprotective (Usha et al., 2013), radical scavenging (Srinivasan et al., 2013), phytochemical and antimicrobial (Venkatachalapathi and Ravi, 2013; Neethu et al., 2014) and analgesic activity (Jayaraman et al., 2014). Also, this important undershrub possess several pharmacologically important phytochemicals (Reneela, 2011; Venkatachalapathi and Subban, 2012) especially the stigmasterol, lupeol and betulin were accounted for anticancer, anti-inflammatory, antioxidant, hepatoprotective and hypoglycaemic properties (Alakurtti et al., 2006; Nair et al., 2016).

The whole plants of *N. ciliatus* have been uprooted extensively from

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Abbreviations: ISSR, Inter simple sequence repeat; RAPD, Random amplified polymorphic DNA; PIC, Polymorphic information content; UPGMA, Unweighted Pair Group Method Arithmetic average; NJ, Neighbor joining

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the natural habitats due to its extensive curative abilities (Ved and Goraya, 2007; Saha et al., 2015). The International Union for Conservation of Nature (IUCN) has assessed this plant as vulnerable due to its extensive exploitation by pharmaceutical industries, habitat loss and deforestation (Ravikumar and Ved, 2000; Ved and Goraya, 2007; Thomas and Rajeshkumar, 2014; Saha et al., 2015). Moreover, this plant is a monocarp and flowers only once during its six to seven years of the life cycle (Rameshkumar et al., 2017). Inadequate or unavailability of seeds, seed dormancy, and rare seed setting are some of the limitations for its conventional propagation (Chandrashekara and Ramakrishnan, 1993; Rameshkumar et al., 2017). However, it is very important to care about conserve this plant species otherwise the continuous human activities may lead to its total extinction. Previously, our group has conserved this plant through micropropagation (Rameshkumar et al., 2017), however still there is a lacuna regarding genetic diversity and phylogenetic relationship in geographically different *N. ciliatus* populations, which is the base for strong conservation policies and genetics.

Genetic diversity plays a pivotal role in the conservation of rare and endangered species for future generations (Reis and Grattapaglia, 2004). Loss of genetic variation is generally accompanied by decreasing the species capability to survive with the changes in environment, which leads to the species becoming extinct (Ge et al., 1998; Maki and Horie, 1999; Hogbin et al., 2000; Frankham et al., 2002). The knowledge about the genetic diversity patterns will afford insights into the evolutionary and demographic history of a taxon (Milligan et al., 1994). Molecular markers are mainly used in the conservation genetics for the evaluation of genetic variation as well as identification of superior genotypes between the individual. While compared to traditional morphological markers, it directly accesses the hereditary information of the species and helps to understand the relationships between them (Paterson et al., 1991). In addition, molecular marker appears to be more important tool to find the genetic variation rich species from wild conditions for efficient sampling and preservation studies. Random amplified polymorphic DNA (RAPD) technique is a dexterous marker system used in revealing the genetic diversity in population and conservation genetics (Williams et al., 1990; Cruzan, 1998). Inter simple sequence repeat (ISSR) markers amplifies flanking regions of the microsatellite sequences at numerous loci throughout the whole genome (Zietkiewicz et al., 1994). These molecular markers were frequently used to study the genetic variations present among different populations or individuals in plant species (Shilpha et al., 2013).

The aim of the present study was to assess the genetic diversity among the nine *Nilgirianthus ciliatus* wild populations of different places in Eastern and Western Ghats, of India by using ISSR and RAPD markers. The main purpose was to establish the level of genetic variation and identify genetically distinctive populations within the species that belongs to different geographical locations. This is the first report on studying the genetic diversity of *N. ciliatus* populations of different geographical locations of Indian Eastern and Western Ghats. The data obtained from this study will facilitate the development of suitable approach for the conservation of *N. ciliatus* and the approach could be useful for other endangered medicinal plants.

2. Materials and methods

2.1. Plant materials and genomic DNA isolation

In this study, a total of 18 accessions of *Nilgirianthus ciliatus* (two plants from nine populations) were collected from different locations in Indian Eastern and Western Ghats wherein nine were maintained in the shade house, Department of Biotechnology, Alagappa University, Karaikudi, India. Remaining nine plants from each populations were used for genomic DNA isolation and analysis of their genetic diversity through ISSR and RAPD markers (Table 1 and Fig. 1). The genomic DNA was extracted using HiPurA kit (HiMEDIA, Mumbai, India) according to the user manual. The quality and quantity of genomic DNA was confirmed with agarose gel electrophoresis (0.8%) and spectrophotometer analysis (OD ratio 260/280 nm), respectively, according to Satish et al. (2016), and the DNA extracts were diluted to a final concentration of 50 ng/µL.

Table 1
Sampling locations and geographic coordinates.

S. No	Locations	Latitude and Longitude
1	Sirumalai (EG)	10°11′39.28″N, 77°59′48.14″E
2	Aaliyar (E-WG)	10°28′26.04″N, 76°58′22.08″E
3	Kolli Hills (EG)	11°14′54.65″N, 78°20′19.35″E
4	Bangalore (WG)	12°58′17.76″N, 77°35′40.43″E
5	Thirissur (WG)	10°31′39.51″N, 76°12′51.97″E
6	Palakad (WG)	10°47′12.23″N, 76°39′17.26″E
7	Muthanga Forest (WG)	11°40′8.68″N, 76°22′10.26″E
8	Wayanad (WG)	11°42′11.54″N, 76°5′0.24″E
9	Trivandrum (WG)	8°31′26.90″N, 76°56′11.89″E

EG – Eastern Ghats; *E-WG* – Eastern Western Ghats; *WG* – Western Ghats.

2.2. ISSR analysis

For initial screening, thirty five inter simple sequence repeat (ISSR) markers (Sigma, USA) were analyzed (Table 2). Only 17 of these anchored microsatellite primers produced prominent bands and these primers were then chosen for the amplification in all the nine populations of *N. ciliatus*. Polymerase chain reaction (PCR) was set out in 25 µL volumes containing 30 ng each of template DNA, 1X Taq buffer, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 0.4 µM of each primer and 1 unit of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA). The amplification was performed in a thermal cycler (Nexus gradient, Eppendorf, Germany) and the conditions for ISSR marker was programmed as denaturation at 95 °C for 5 min, followed by 42 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The final cycle was allowed to an additional 10 min period of extension at 72 °C.

2.3. RAPD analysis

Initially 30 custom synthesized primers of random amplified polymorphic DNA (RAPD) primers (Sigma, USA) were used for analysis. Only 8 of these primers produced the well resolved banding patterns (Table 2). The PCR mixture was the same as in the ISSR analysis. The PCR amplification conditions using the RAPD primers: pre denaturation at 94 °C for 5 min, then 42 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min.

2.4. Polyacrylamide gel electrophoresis

The PCR amplified products were resolved on 6% polyacrylamide gel using 0.5X TBE (Tris-Borate-EDTA) as running buffer at a constant voltage of 5 V/cm for 3 h. After completion of electrophoresis the gel was stained with ethidium bromide, and then visualized using a gel documentation system (Molecular Imager® Gel Doc™ XR + System, Bio-Rad, USA). For further analyses, the photographs of the gels were stored as digital images in the gel documentation system.

2.5. Scoring and data analyses

For genetic diversity analysis, the well resolved and individual bands produced by the PCR primers were selected and included in the analysis and qualitative binary data matrix was constructed by scoring as 1 for presence or 0 for absence of a band. The ability of each primer to discriminate each accession was analyzed by calculating the

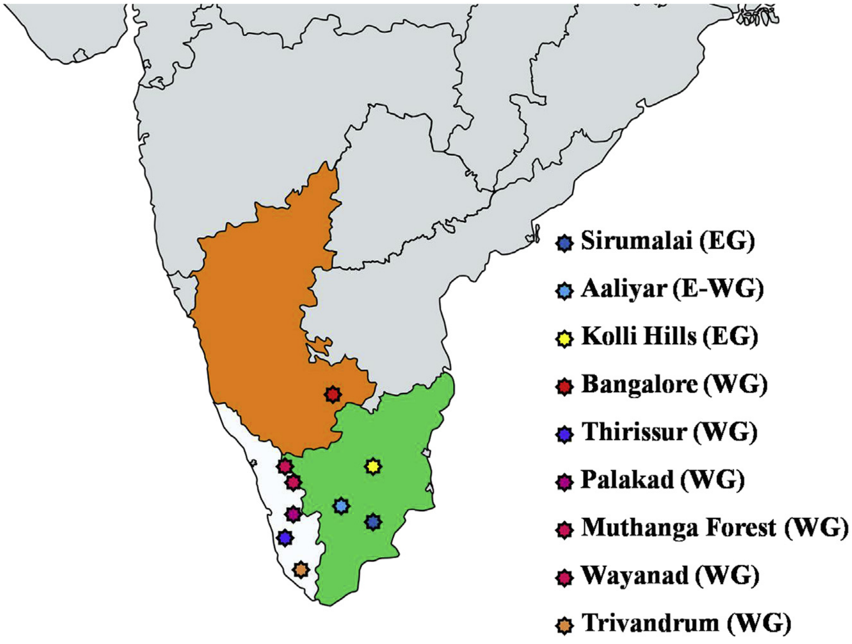

Fig. 1. Collection of *Nilgirianthus ciliatus* populations from Eastern and Western Ghats of India.

Table 2
The extent of genetic polymorphism revealed by different ISSR and RAPD markers.

S. No	Primer name	Primer sequence (5′–3′)	Size range of amplicons (bp)	TNB	NMB	NPB	PPM (%)	R_p	PIC
ISSR									
1	HB 12	CACCACCACGC	350–3500	21	4	17	81%	30	0.73
2	HB 15	GTTGTTGTTGC	400–3500	20	7	13	65%	26.4	0.52
3	ISSR 33	AGAGAGAGAGAGAGAGA	500–1000	23	7	16	69.6%	24.2	0.42
4	ISSR 35	AGAGAGAGAGAGAGAGT	200–2500	26	5	21	80.8%	37.6	0.78
5	ISSR 42	ACACACACACACACACCG	200–900	7	4	3	42.9%	9.2	0.19
6	ISSR 46	ACACACACACACACACGG	200–4000	26	0	26	100%	20	0.63
7	ISSR 67	TCTCTCTCTCTCTCCC	250–2000	24	0	24	100%	22.4	0.71
8	ISSR A1	GAGAGAGAGAGAGAGA	300–1800	14	5	9	64.3%	16.2	0.41
9	ISSR A3	ACACACACACACACACC	750–1200	20	1	19	95%	19.4	0.69
10	ISSR A5	GAGAGAGAGAGAGAGATC	300–1500	15	6	9	60%	20.2	0.47
11	ISSR A6	GACAGACAGACAGACA	500–2000	12	1	11	91.7%	15.8	0.84
12	ISSR A8	ACACACACACACACACT	200–1500	16	3	13	81.3%	21	0.71
13	ISSR A10	AGAGAGAGAGAGAGAGG	500–2500	16	1	15	93.8%	21.4	0.84
14	ISSR A11	GAGAGAGAGAGAGAGAGAT	450–1250	18	3	15	83.3%	20	0.64
15	ISSR A13	CCGCCGCCGCCGCCGCCG	500–3500	12	4	8	66.7%	11.8	0.33
16	ISSR A16	CTCCTCCTCCTCCTCC	450–2000	11	3	8	72.7%	15.4	0.66
17	ISSR Y11	GAGAGAGAGAGAGAGAT	450–1100	17	8	9	52.9%	23.6	0.4
Average				**298**	**62**	**236**	**77.0%**	**20.8**	**0.59**
RAPD									
1	OPA-03	AGTCAGCCAC	500–1000	12	2	10	83.3%	12.8	0.76
2	OPA-10	GTGATCGCAG	450–1100	17	2	15	88.2%	17	0.66
3	OPC-02	GTGAGGCGTC	450–1250	18	2	16	88.9%	13.6	0.48
4	OPC-16	CACACTCCAG	750–1200	9	2	7	77.8%	11.6	0.57
5	OPC-19	GTTGCCAGCC	500–3500	7	1	6	85.7%	16.2	0.75
6	OPD-03	GTCGCCGTCA	200–1500	2	0	2	100%	14	0.63
7	OPD-18	GAGAGCCAAC	200–1500	21	1	20	95.2%	19	0.7
8	OPE-04	GTGACATGCC	500–2500	13	0	13	100%	13	0.75
Average				**99**	**10**	**89**	**89.9%**	**14.6**	**0.66**
Cumulative (25 primers)				**397**	**72**	**325**	**80.8%**	**18.8**	**0.61**

TNB, Total number of bands; *NMB*, Number of monomorphic bands; *NPB*, Number of polymorphic bands; *Rp*, Resolving power; *PIC*, Polymorphic information content.

Resolving Power (Rp) according to Prevost and Wilkinson (1999). To analyze the usefulness of primers, the polymorphic information content (PIC) was calculated according to Botstein et al. (1980). To obtain the genetic relationship between 9 populations, collected from different parts of South India, the genetic similarity matrix was constructed into a dendrogram using the Unweighted Pair Group Method Arithmetic average (UPGMA) and Neighbor joining (NJ) methods. The similarity coefficient (Jaccard's Similarity coefficient) was calculated and applied for constructing UPGMA dendrogram and the principal component analysis were done by using the PAST - PAlaeontological STatistics, ver.

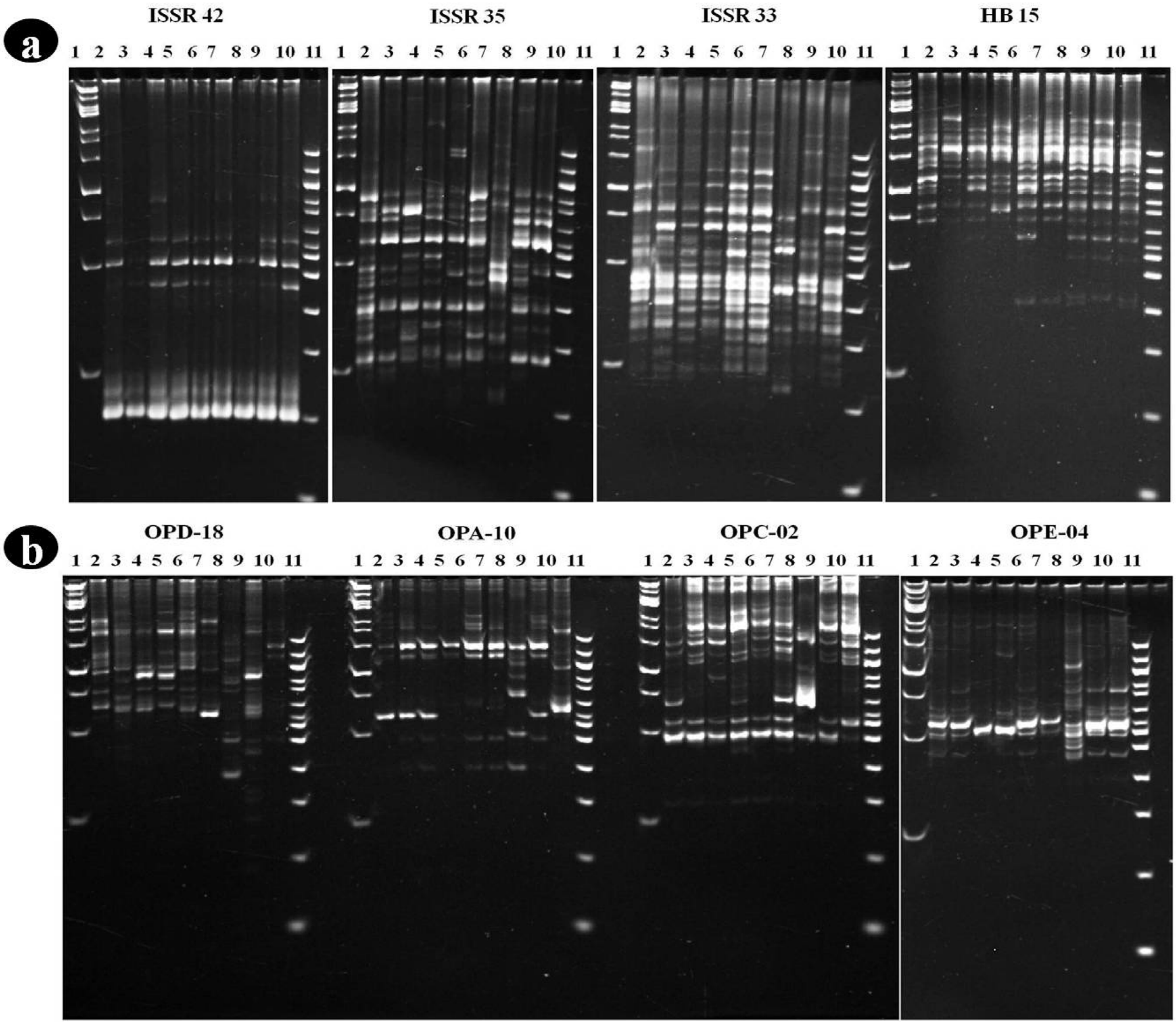

Fig. 2. Genetic diversity analysis using ISSR and RAPD markers.
(a) ISSR analysis; (b) RAPD analysis. Lane 1: 1 Kb ladder; Lane 2: Sirumalai (EG); Lane 3: Aaliyar (E-WG); Lane 4: Kolli Hills (EG); Lane 5: Bangalore (WG); Lane 6: Thirissur (WG); Lane 7: Palakad (WG); Lane 8: Muthanga Forest (WG); Lane 9: Wayanad (WG); Lane 10: Trivandrum (WG); Lane 11: 100 bp ladder.

1.34 (Hammer et al., 2001). Based on the genetic distance the phylogenetic tree, using NJ method was constructed with the DARwin software ver. 6.

3. Results

3.1. ISSR analysis

A total of 35 ISSR primers were tested for amplification, out of which 17 resulted in sharp, clear and highly resolved banding pattern. So, these 17 primers were used to study the genetic variation among the populations of *N. ciliatus* collected from nine different geographical locations. Using these 17 primers, a total of 298 bands were generated, of which 62 were monomorphic and 236 were polymorphic or about 77.0% of genetic polymorphism (Table 2). With each ISSR primer, at least 17 distinct scorable bands were produced on an average. Using the ISSR primers 35 and 46 a maximum of 26 scorable bands were produced, while with the primer 42 the least number (only 7 fragments) were amplified (Table 2). The amplicon sizes of the products from using the various primers ranging from 200 bp to 3500 bp were observed across all the populations (Table 2). Among the primers used, ISSR Y11 produced the maximum number (8) of monomorphic bands. With the ISSR primers 46 and 67, in all nine populations of *N. ciliatus* 100% polymorphism of the products were observed. For instance, the amplified products of ISSR markers (ISSR 42, ISSR 35, ISSR 33 and HB 15) has been shown in Fig. 2. The PIC and Rp values were calculated to check the efficiencies of the primers used in analysing the polymorphisms. PIC value ranged from 0.19 (ISSR 42) to 0.84 (ISSR A6 and A10) with the mean of 0.59, and where the Rp value ranged from 9.2 (ISSR 42) to 37.6 (ISSR 35) with the mean of 20.8, respectively.

3.2. RAPD analysis

Thirty arbitrary decamer RAPD primers were used for initial screening. Of these 8 primer sets that provided clear and reproducible amplification products were selected to study genetic diversity of the nine populations of *N. ciliatus*. Using the 8 primers, a total of 99 bands were generated with an average of 12.3 bands per primer. Amplification occurred in all populations and the amplicons varied in size ranging from 200 to 3500 bp (Table 2). Out of the total 99 bands, 89 were polymorphic bands or about 89.9% genetic polymorphism among all the *N. ciliatus* populations which were collected from various

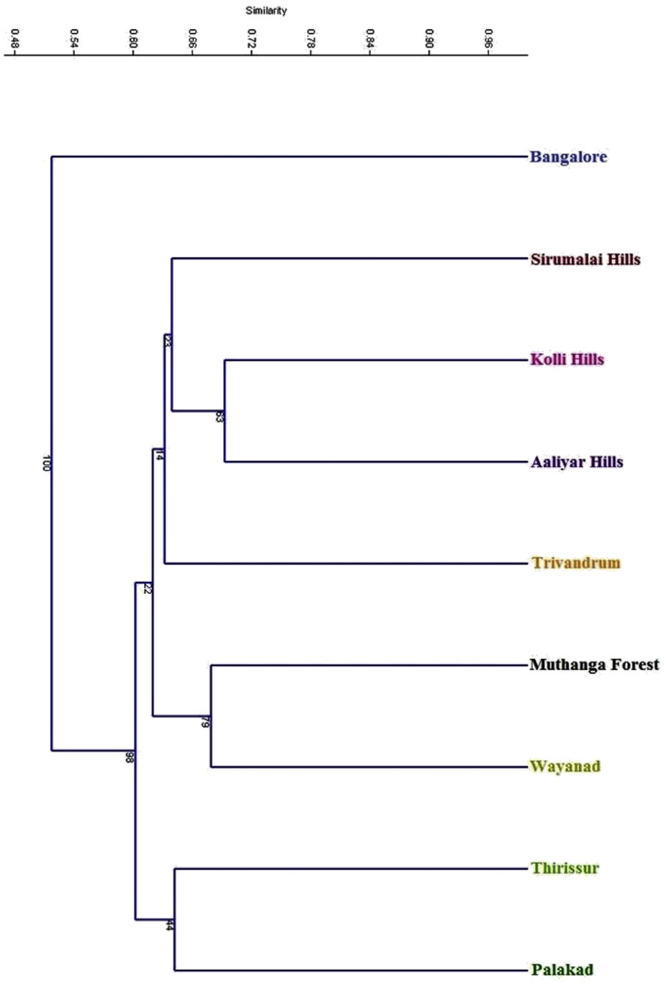

Fig. 3. UPGMA phenogram illustrating the genetic relationships among nine natural populations of *Nilgirianthus ciliatus*, based on Jaccard's similarity coefficient calculated from 25 (17 ISSR and 8 RAPD) markers.

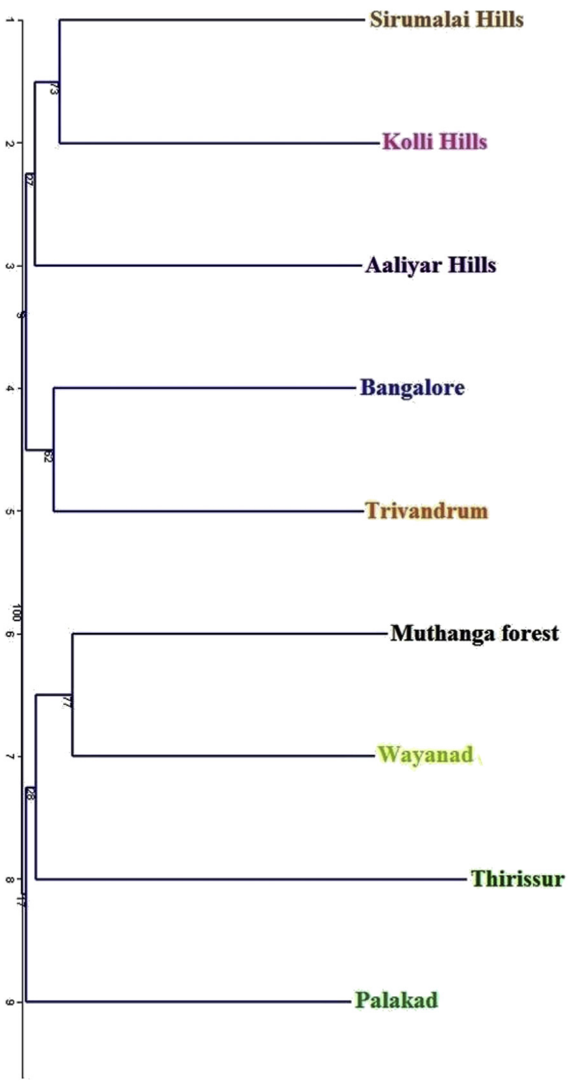

Fig. 4. Neighbor-joining (NJ) dendrogram illustrating the genetic relationships among nine natural populations of *Nilgirianthus ciliatus*, based on Euclidean genetic distance measure calculated from 25 (17 ISSR and 8 RAPD) markers.

parts of South India. The number of amplified fragments ranged from 2 (OPD-03) to 21 (OPD-18) and the percentage of polymorphism also varied for each RAPD primer used. A maximum of 100% polymorphism was obtained using OPD-03 and OPE-4 while a minimum of 77.8% was exhibited by OPC-16 (Table 2). For instance, the amplified products of RAPD markers (OPD 18, OPA 10, OPC 02 and OPE 04) has been shown in Fig. 2. The Rp value ranged from 11.6 (OPC-16) to 19 (OPD-18), and the average was 14.6. The calculated average PIC value was 0.66. The average PIC for the primer OPC-02 was low (0.48) and that for the primer OPA-03 was of a relatively higher value of 0.76.

3.3. Analysis of pooled data with ISSR and RAPD

For further analysis, the data of both ISSR and RAPD markers were pooled together. Using a total of 25 primers (17 ISSR and 8 RAPD), 397 bands were produced, of which 72 were monomorphic and the remaining 325 bands were polymorphic. Overall, an average 80.8% of genetic polymorphism in the nine populations of the *N. ciliatus* collected from different zones of South India were found. The mean values of PIC and Rp were 0.61 and 18.8, respectively (Table 2).

Then, from the combined dataset, UPGMA cluster analysis was done by using Jaccard's similarity coefficient value and the NJ dendrogram was constructed with Euclidean genetic distance which showed the phylogenetic relationships and the amount of genetic variation in these nine populations. The UPGMA cluster (Fig. 3) and NJ (Fig. 4) analyses were done using the combined data of both RAPD and ISSR markers which separated the nine populations of *N. ciliatus* into the four different clusters and were grouped based on their geographical locations. The factorial analysis (Fig. 5) also supports the cluster analyses, which shows the genetic distribution based on the genetic variations among the nine populations.

4. Discussion

Based on the geographical conditions the plant species may differ in their genetic content (Shilpha et al., 2013). In here, the IUCN red listed medicinal plant, *N. ciliatus* have been assessed for genetic diversity through molecular markers. Analysis of that genetic diversity is important measure to conserve the plant genetic resources especially threatened medicinal plants. Estimating the level and distribution of genetic variation in endangered species is a primary objective of conservation genetics (Fritsch and Rieseberg, 1996). Precise estimates of genetic variability are very useful for enhancing sampling strategies of conserving and managing the genetic resources of valuable plants (Hamrick et al., 1991). Molecular markers offer an effective methodology to reveal genetic diversity based on DNA polymorphisms in plant species. In medicinal plants, it is very important to study the genetic base of a species to conserve the plants from extinction. In this

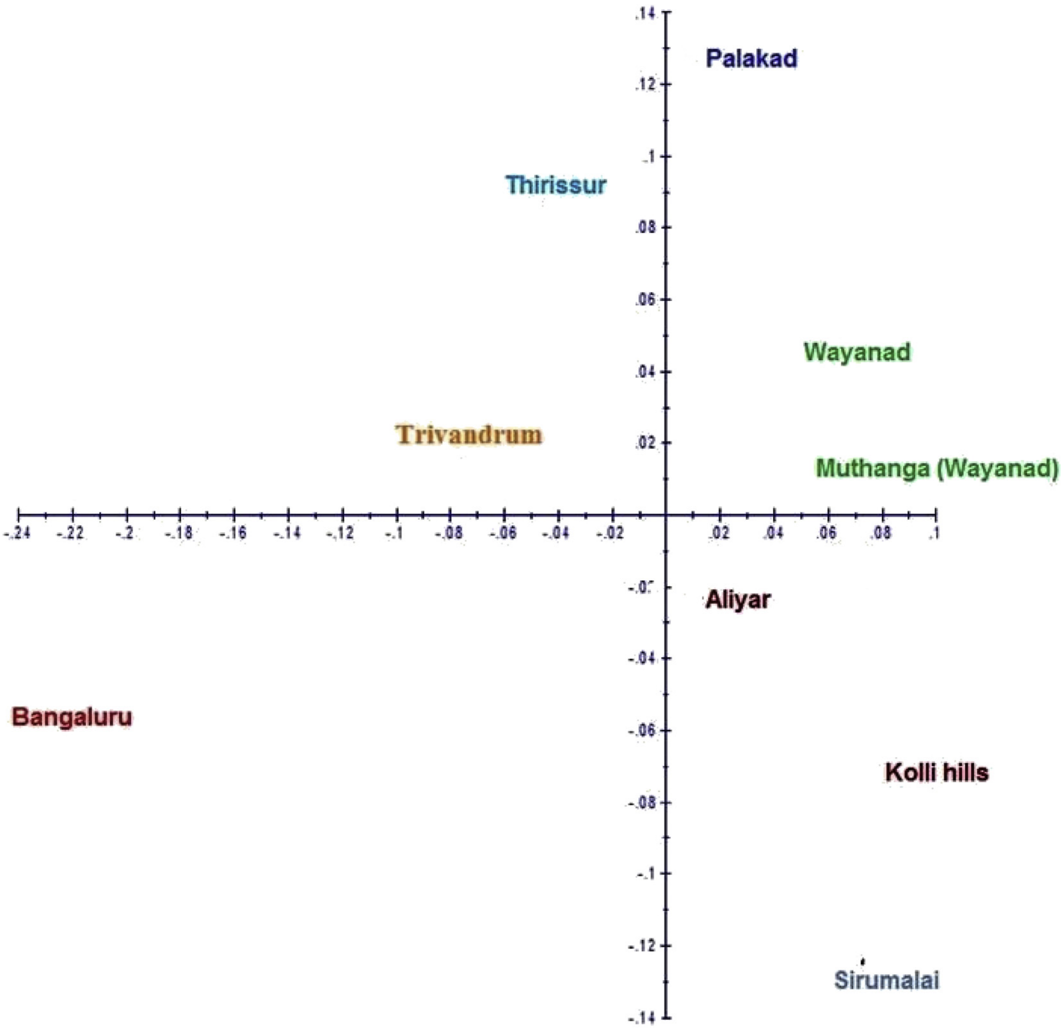

Fig. 5. Principal Component Analysis (PCA) illustrating the geographical distribution and genetic relationships among nine natural populations of *Nilgirianthus ciliatus*.

study, two important dominant markers such as ISSR and RAPD were used to study the genetic variations between nine populations of *N. ciliatus* collected from different geographical locations of South India. Previously, there is no report on studying the genetic polymorphism and diversity of this vulnerable medicinal plant. Here, the molecular marker analysis results were depicting that, all nine populations from different geographical locations have higher level of genetic variations probably due to the different environmental conditions (Hamrick and Godt, 1989). The various climatic conditions such as temperature, humidity and soil are may be the reason for the greater percentage of genetic diversity among the *N.ciliatus* population. The similar response was previously observed in the medicinal plant, *Caragana microphylla* wherein environmental conditions influence the genetic diversity (Huang el al. 2016).

The combination of different marker systems will be always helpful in significant analysis and to produce useful genetic resources about a particular plant species (Satish et al., 2016). Previously, several studies have been done using the combination of ISSR and RAPD primers in *Moringa oleifera* (Saini et al., 2013), *Solanum trilobatum* (Shilpha et al., 2013), *Andrographis paniculata* (Tiwari et al., 2016), *Withania somnifera* (Khan and Shah, 2016). Diversity analysis revealed that the genetic variation of *Nilgirianthus ciliatus* populations was very abundant, and the nine populations were divided into four groups in the UPGMA and NJ dendrograms. The populations belong to the close locations were placed in a single group. This is due to the plants are growing in the same or moderately similar environmental conditions (close locations) compared to different places (Huang et al. 2016). However, further research work is needed for the assessment of other environmental factors which impact the genetic diversity in plant population. The populations from Western Ghats region of Kerala such as Thirissur, Palakad, Wayanad and Muthanga forest of Wayanad were very closely located in two different clades of the dendrogram. Next to that, the populations belong to Eastern and Western Ghats of Tamil Nadu such as Sirumalai, Kolli hills and Aaliyar were placed in a single cluster. Finally, the Karnataka populations collected from Western Ghats region of Bangalore were separately placed in the dendrogram. A wide range of geographical distribution in *N. ciliatus* populations was observed in the present study which is due to the higher level of variations at the DNA level (Liu et al., 2012). Based on the cumulative percentage of polymorphism, each accession of nine populations is having the high genetic variation and also having the similarity with nearby places growing plants. The factorial analysis that is PCA also supports the results obtained by the cluster analysis, which shows the distribution of the nine populations based on their geographical locations. In wild condition, the genetic diversity of each *N. ciliatus* is more and it was successfully revealed by ISSR and RAPD genetic markers. In future, this is base to develop sequence characterized amplified region (SCAR) marker for plant identification and effective preservation.

5. Conclusions

This is the first report on the genetic diversity analysis of *Nilgirianthus ciliatus*, which will be highly needed to protect the vulnerable and pharmacologically important medicinal plants. Indiscriminate collections of medicinal plants from wild conditions are caused major threat to the plant existence. A genetic variation within/among the populations helps to sustain the plants which are under threatened condition for long term survival. Also, the findings of this study suggest that it seems possible to protect the natural populations by maintaining the genetic variations of the populations. Assessment of that genetic diversity could be fruitful to concentrate on its threat status and means and give an idea to frame the strong conservation schemes.

Compliance with ethical standards

Conflict of interest

The authors have no conflicts of interest to declare.

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