Genetic variability is one of the most important aspects to find out variation in the nucleotides, genes among the species. *Thottea* is well-defined genus of Sub shrubs or rarely shrubs which mostly occurring in tropical and warm temperate forests of southern-eastern Asian countries, which has totally around 25 species and it, is characterize only eight species in India. This shrub is commonly called as ‘Alpam’ or ‘Apama’. Here we summarize the studies on RAPD outcome that all the eight different species of *Thottea* genus illustrate higher levels of genetic variation along with polymorphism among these species. Cluster analysis based on Unweighted Pair Group Method with Mean Arithmetic (UPGMA) it reveals three clusters comprising *T. abrahami*, *T. dinghoui*, *T. ponmudiana* and *T. barberi* in the first cluster and *T. siliquosa* and *T. sivarajanii* in the second cluster and *T. ducharteri* and *T. idukkiana* in the last cluster. Final observation results the *T. ducharteri* and *T. idukkiana* are similar ones and are separated from each other by small difference.

1. **INTRODUCTION**

Of all the lives on earth, because of its sheer diversity, the incredible multitude of life forms the vast genetic archive they embody, complex ecological associations they form and the fascinating behaviors they exhibit, plant genetic resources are one of the most valuable assets available to humankind. They have proved to be the best friends of man in alleviating his pains and sufferings by providing him with natural drugs. Most of the drugs are obtained especially from the tropical region. Protection and conservation of these resources for future generations, therefore assumes great significance. Recently 28 biodiversity ‘hotspots’ areas with exceptionally high species richness and endemism that are also under threat also been reported. Among these, eight are known to areas of highest diversity and India is privileged to have two of them. An important component for effective management of plant’s genetic resources as well as their utilization and characterization is very essential not only for identification of species, but also for determination of genetic relatedness among them. Genetic diversity is resources for a species own survival and future evolution; it also promotes selective breeding.¹ DNA markers are known to be powerful and reliable tools for discerning variation within the plant germplasm. Polymerase Chain Reaction (PCR)-based approaches using arbitrary primers like RAPD and ISSR are increasingly being used in investigating genetic relatedness and diversity if plant populations owing to their speed, simplicity and accuracy. Studies involving marker techniques in detecting genetic diversity have recently been appeared.²³⁴ Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. RAPD have actually been used for a study of polymorphism and chromosome mapping. There are some new emerging patterns a unique feature to analyze the individual and now a days the term DNA fingerprinting or DNA profiling is used to describe the combined use of several single locus detection systems and is being used as versatile tools for investigating various aspects of plant genomes. Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization –based markers and Polymerase Chain Reaction
(PCR) based markers. Fingerprints generated by these probes are also known as oligonucleotide fingerprints. Specific fingerprints are visualized by hybridization with a labeled micro or mini-satellite probe. Mini satellites are Tandem Repeats with a monomer repeat length of about 11 – 60 bp. These loci contain Tandem Repeats that vary in the number of repeat units between genotypes and are referred to as Variable Number Tandem Repeats (VNTRs) (i.e., a single locus that contains variable number of tandem repeats between individuals) or hyper variable (HVRs). The RAPD assay is a PCR amplification performed on genomic DNA templates using a short, arbitrary oligonucleotide primer and at low annealing temperature conditions that ensure the generation of several discrete DNA products. The genetic relationships of what accessions estimated by polymorphism of RAPD markers, were identical with those inferred by RFLP and ISSR markers, indicating the reliability of RAPD marker for estimation of species. So RAPD was applicable for genomic fingerprinting at the inter species level and inferring genetic relationships among related species.\[6\] Thottea is a well-defined genus of herbs or shrubs occurring mostly in tropical and warm temperate forests of southeastern Asian countries, which has totally 25 species and is represented by 8 species in India. During the floristic explorations along the Western Ghats in Kerala, India, endemic species of Thottea siliquosa (Apama siliquosa), Thottea barberi (Apama barberi), Thottea dinghoui, Thottea duchartrei, Thottea idukkiana, Thottea ponmudiana, Thottea abrahami, Thottea sivarajani were discovered. The Malabaries and Brahmins call the shrub ‘Alpam’ and ‘Apama’. Thottea are used by certain tribes against several diseases and as pain killers. The leaves are used as antidote to stings, while the root crushed with lemon juice is reputed in indigenous systems of medicine for diarrhea, dysentery treatments and against snake poison. The root was found to have antifungal activity against Helminthosporium sativum.\[7\] It was effective for treating itches, very old ulcers etc.
2. MATERIALS AND METHODS

2.1. Sample

Total Genomic DNA was extracted from young fresh leaves of eight *Thottea* species plants like *Thottea siliquosa* *Thottea barberi*, *Thottea dinghoui*, *Thottea duchartrei*, *Thottea idukkiana*, *Thottea ponmudiana*, *Thottea abrahami*, *Thottea sivarajani*.

2.2. DNA Extraction and Amplification: The CTAB (hexadecyltri-methylammonium bromide)

Method of Murray and Thompson method\(^8\) was used to extract total genomic DNA. Leaf material was powdered in liquid nitrogen, mixed with 12 ml extraction buffer [2% CTAB, 1M Tris HCl, 0.5M EDTA, 1.4M NaCl, 1.2% PVP] at 65°C with 1% β – Mercaptoethanol and incubated at 65°C for two hrs. Chloroform extraction was performed by adding equal volume of chloroform to the slurry and centrifuged at 10,000 rpm for 5 min at 4°C. To the supernatant 1/10, volume CTAB/NaCl [10%CTAB/0.2M NaCl] and equal volume of chloroform was added then centrifuged at 10,000 rpm for 5mins. After obtained an equal volume of CTAB precipitation buffer [(1%) CTAB, 50mM Tris HCl, 10mM EDTA] was added and incubated at 37°C for overnight in a water bath. Overnight mixture was centrifuged at 8,000 rpm for 8 – 10 min at 4°C. Pellet was collected then resuspended in 1ml of high salt TE [1M NaCl, 10mM Tris HCl, (pH8.0) 0.1mM EDTA] with that 1ml of isopropanol was added and incubated at -20°C for 30 min, centrifuged at 8,000 rpm for 10min at 4°C. With the Pellet 80% ethanol was added and centrifuged at 10,000 rpm for 5min discard supernatant, pellet was resuspended with 0.5ml 1X TE, RNase (10 µg/ml) added and incubated at 55°C for 10min. After incubation, equal volume of Chloroform was added and centrifuged at 10,000 rpm for 5 min. The upper aqueous portion was collected and double volume 100% ethanol and 1/10 volume of 3M sodium acetate was added ,incubated at -20°C for 1hr. after incubation centrifuged the mixture at 12,000 rpm for 15 min. The sediment was washed twice in 70% ethanol, vacuum-dried and resuspended in 100-μl Tris-EDTA buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 8.0). The quantity and quality of DNA was checked in spectrophotometric method. Readings were taken at wavelengths of λ260 and λ280. The readings at 260nm allow calculation of the concentration of nucleic acid in sample. An OD of one corresponds to approximately 50µg/ml of double –stranded DNA. The ratio at 260nm and 280nm (OD260/OD280) provides to estimate the purity of nucleic acid .The optimum value was taken as 1.8 for pure DNA.

Amplification of genomic DNA were performed in 25-µl reaction volumes containing 1.2 units of *Taq* polymerase (Finnzymes, Finland), 10 mM Tris-HCl (pH 9.0), 25 mM KCl, 2 mM MgCl\(_2\), 0.2 mM of each dNTP, 24 ng each of random primer and 40 ng of template DNA. The cycle program included 3 stages: In stage one 1st cycle (3min at 93°C,1min at 36°C,2min at 72°C) ,In second stage 34 cycles (1min at 92°C,1min at 36°C,2min at 72°C) followed by a final Extension cycle of 15min at 72°C. RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator using a digital camera.

2.3. Estimation of Genetic variation

From ISSR assays genetic variability measures was estimated by WINBOOT AND WINDIST programs. Band profiles generated by PCR were compiled onto a data matrix based on the presence or absence of bands. Nei’s original measures of genetic identity and genetic distance were calculated, generated a phenogram cluster analysis using UPGMA method.

3. RESULTS AND DISCUSSION

Analysis of RAPD data of all the eight different species of genus *Thottea* shows higher levels of genetic variation. 15 primers studied amplified a total of 353 DNA fragments .Out of them 3 were found to be polymorphic (0.849%) in one or other of the 8 species. The levels of polymorphism were different with different primers among these species. Primers BCG 04 & BCG 06 produced maximum numbers of amplified products and BCG08 & BCG 14 produced the least (Figure. 1.).
Figure 1. Amplification of Thottea species by RAPD primer from *Thottea abrahamii*, *Thottea barberi*, *Thottea duchartrei*, *Thottea dinghoui*, *Thottea idukkiana*, *Thottae ponmudiana*, *Thottea siliquosa*, *Thottea sivarajanii*. (a) RAPD profile of eight thottea species amplified with BCG 04 (b) RAPD profile of eight Thottea species amplified with BCG 06 (c) RAPD profile of eight thottea species amplified with BCG 14 (d) RAPD profile of eight Thottea species amplified with BCG 08. The number of amplified products from each species varies significantly for all the 15 primers. The number of amplified products from rest of the species varied from 2 to 30. Genomic DNA concentration for all *Thottea* Species were checked by spectrophotometric in 260nm and 280nm (Table 2.).

Table 2. Spectrophotometric readings of *Thottea* Species DNA ranging from 1.71 to 1.89.

<table>
<thead>
<tr>
<th>Species</th>
<th>260nm</th>
<th>280nm</th>
<th>260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thottea abrahamii</em></td>
<td>0.223</td>
<td>0.120</td>
<td>1.86</td>
</tr>
<tr>
<td><em>Thottea barberi</em></td>
<td>0.085</td>
<td>0.047</td>
<td>1.80</td>
</tr>
<tr>
<td><em>Thottea duchartrei</em></td>
<td>0.426</td>
<td>0.228</td>
<td>1.87</td>
</tr>
<tr>
<td><em>Thottea idukkiana</em></td>
<td>0.312</td>
<td>0.182</td>
<td>1.71</td>
</tr>
<tr>
<td><em>Thottae ponmudiana</em></td>
<td>0.450</td>
<td>0.244</td>
<td>1.84</td>
</tr>
<tr>
<td><em>Thottea siliquosa</em></td>
<td>0.343</td>
<td>0.184</td>
<td>1.86</td>
</tr>
<tr>
<td><em>Thottea sivarajanii</em></td>
<td>0.489</td>
<td>0.261</td>
<td>1.87</td>
</tr>
</tbody>
</table>

The similarity matrix based on Jaccards method shows the coefficient of similarity value ranging from 0.87 to 0.99 with a mean value of 0.93. The observed value signifies the extent of genetic variation in these species. Cluster analysis based on UPGMA reveals three clusters comprising *T. abrahami*, *T. duchartrei*, *T. ponmudiana* and *T. barberi* the first cluster and *T. siliquosa* and *T. sivarajanii* in the second cluster, *T. duchartrei*, and *T. idukkiana* in the last cluster (Figure 3.).
The moderate level of polymorphism detected in the plant is attributed to the vegetative mode of propagation prevailing in the plants though the major mode of propagation in through seeds. Though higher level of polymorphism in bands is useful for easy and rapid resolution of interspecific variation, lower to moderate levels of polymorphism contributes towards accurate and reliable estimates of genetic variability and thereby help in defining the nature of existing gene pools. The high number of polymorphic products generated by certain primers as discussed above might attributed to the fact that in RAPD even small divergence between two cultivars can result in distinct patterns as polymorphism. May be any one of the various reasons such as (a) single nucleotide change within the primer binding site, (b) insertion or deletion with the amplified region (c) complete absence of complementary sites, (d) the region between the binding sites on opposite strands is beyond the normal amplifiable length (Table 3.). The reproducibility of RAPD profiles for all the primers with respect to the samples was observed in the present study indicate genetic stability of the plant.

Some RAPD values is sometimes limited for rare endangered medicinal plants because of their limited genetic variation although they have been widely and effectively use to analyze genetic diversity within and among population. The damage to their habitats is a main reason they are so rare. It is good strategy to protect more of their habitats. The result of RAPD and UPGMA also show that we need to take individuals from more populations that are different if we are to construct an artificial conservation area to preserve their diversity for the future.

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4. REFERENCES


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