Genetic diversity of Melia dubia as revealed by molecular markers

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Abstract

Background

Melia dubia Cav. of the family Meliaceae is a highly valued multipurpose tree species that has a short rotation period and can be harvested for various industrial and domestic wood requirements. However, the availability of genetically improved planting stock is almost non-existent, especially in North East India, and formulating management and conservation strategies are highly in need as it was observed during the survey that the number of Melia trees was very less and the majority of it was found fragmented in home-gardens.

Methods and Results

In the present study, a survey was conducted on the natural population in 11 villages of East Siang District, Arunachal Pradesh and 34 phenotypically superior Melia trees were selected based on the characteristics *viz.* height, clear bole height, girth at breast height, collar girth, crown diameter, straightness and branching. East Siang district of Arunachal Pradesh occupies a geographical area of 3,603 sq. km and a forest cover of 2911.67 sq. km. Genetic diversity of 34 selected trees is estimated with 15 microsatellite markers (SSR). Five out of fifteen SSR primers were found to be polymorphic. The effective number of alleles (Ne = 1.88), Nei's gene diversity (h = 0.47), polymorphism information content (PIC = 0.48) and Shannon's index (I = 0.66).

Conclusion

It is affirmed by the UPGMA dendrogram and the pair-wise genetic dissimilarity that there is no association between the genetic and geographic distance of the genotypes. Understanding the genetic variability of *M. dubia* will help to identify superior seed sources for improvement programs and conservation strategies for the species.

1. Introduction

Melia dubia Cav. (Family Meliaceae) is a highly valued multipurpose tree species commonly known as Malabar Neem or Dreak. The tree is native to India and widely distributed in Khasi Hills, upper Assam, Sikkim Himalayas, North Bengal, Deccan, and Western Ghats (altitude 1,500–1,800 m). It is common in the moist deciduous forests of Kerala [1]. At the end of 20 years, the Melia tree can attain a height of 6–30 m, clear bole up to 8 m and a girth at a breast height of 1.5 m [2]. This tree is usually leafless from December to March and new leaves appear from February to March. It has exceptional wood properties for pulp, paper, plywood and timber industries. As reported, in the world, this is one of the fastest-growing moneymaking tree species under a short rotation agroforestry system [3]. The wood of the species has been reported to be moderately hard, light in weight, easy to saw and durable [4]. Due to the increase in

populations of *M. dubia* in the Indian sub-continent, the genetic diversity of the species has increased. However, very less data is available on intra-specific variation in *Melia* under the Indian subcontinent [5]. As suggested by evolutionary evidence, high levels of genetic diversity are depicted by the species with diverse ecotypes and broad distribution [6]. This necessitated the initiation of a tree improvement program for identifying superior and diverse genotypes for developing breeding strategies. One of the most vital steps is the assessment of genetic diversity, which can be achieved quickly and efficiently using molecular markers. Estimation of genetic diversity can be done with the help of DNA-based molecular. Mostly in molecular studies, RAPDs (randomly amplifies polymorphic DNAs) have been used in genetic analysis, but due to their limitation of reproducibility, SSR markers are preferred more for their formativeness and efficiency in the study of genetic diversity.

Despite the importance of the species and its commercial potential, there is a lack of information on the extent of genetic diversity and knowledge about its genetic improvement is scanty. There is a fragmentation of habitats for various species in the natural forests of tropical and subtropical regions due to anthropogenic activities [7]. Habitat fragmentation is one of the major challenges for biological diversity conservation [8]. Genetic diversity in natural populations occurs due to the interaction of drift, migration and selection which is required for population adaptation, persistence and evolution [9]. The greater the diversity, the greater the adaptability; a species' adaptability to a wide range of climatic conditions is dependent on genetic diversity [10]. Despite the positive outlook on *M. dubia*'s marketable improvement, knowledge about the species' genetic diversity is lacking and widespread misuse of this species leads to further genetic resource depletion. As a result, there is an immediate need to instigate breeding programs for this species to make improved planting stock commercially viable. Recently, 10 populations of *Melia dubia* trees from 8 districts of Karnataka were assessed using SSR markers [11]. Similarly, ISSR markers were used to assess the genetic variability of 232 trees from 7 plantations (147 trees) and 11 natural populations (85 trees) was assessed [12]. There is a lack of information on the genetic diversity of this species through co-dominant markers for the region of Arunachal Pradesh. In light of the foregoing, the current study was undertaken to determine the genetic diversity and differentiation of this species, which could aid in the identification of genetic resources to assist in improvement activities and conservation strategies.

2. Materials And Methods

In the present study, a survey was conducted on the natural population in 11 villages of East Siang District, Arunachal Pradesh (Fig. 1), and 34 phenotypically superior Melia trees were selected based on the characteristics *viz.* height, clear bole height, girth at breast height, collar girth, crown diameter, straightness and branching. East Siang district of Arunachal Pradesh occupies a geographical area of 3,603 sq. km and a forest cover of 2911.67 sq. km. [13]. Melia has natural distribution in the region of East Siang district in the form of trees growing in the forests, secluded trees along the roadside, and farmers' fields that have grown naturally. (Table 1). Genetic diversity in selected trees is estimated with microsatellite markers (SSR), which are co-dominant, short tandem repeat motifs, multiallelic and highly polymorphic [14;15].

Table 1
Details of *Melia dubia* trees selected from East Siang District, Arunachal Pradesh

Sl.no.	Genotype	Location	Latitude	Longitude
1	CHF/CAU/MD/1	Medog County	27 56' N	95 20' E
2	CHF/CAU/MD/2	Medog County	27 56' N	95 20' E
3	CHF/CAU/MD/3	Kamlighat	28 03' N	95 30' E
4	CHF/CAU/MD/4	Kamlighat	28 03' N	95 30' E
5	CHF/CAU/MD/5	Gumin Nagar	27 56' N	95 20' E
6	CHF/CAU/MD/6	Mirbuk	27 56' N	95 20' E
7	CHF/CAU/MD/7	Mirbuk	27 53' N	95 18' E
8	CHF/CAU/MD/8	Tebo	27 56' N	95 20' E
9	CHF/CAU/MD/9	Tebo	27 56' N	95 20' E
10	CHF/CAU/MD/10	Mirbuk	28 07' N	95 32' E
11	CHF/CAU/MD/11	Sikatode	28 05' N	95 31' E
12	CHF/CAU/MD/12	Sikatode	28 05' N	95 31' E
13	CHF/CAU/MD/13	Sikatode	28 07' N	95 32' E
14	CHF/CAU/MD/14	Sikatode	27 97' N	95 30' E
15	CHF/CAU/MD/15	Sikatode	27 53' N	95 18' E
16	CHF/CAU/MD/16	Sikatode	27 56' N	95 20' E
17	CHF/CAU/MD/17	Sikatode	27 97' N	95 30' E
18	CHF/CAU/MD/18	Sikatode	28 03' N	95 30' E
19	CHF/CAU/MD/19	Sikatode	27 53' N	95 18' E
20	CHF/CAU/MD/20	Sikatode	28 02' N	95 26' E
21	CHF/CAU/MD/21	Sikatode	28 04' N	95 33' E
22	CHF/CAU/MD/22	Oyan	27 55' N	95 20' E
23	CHF/CAU/MD/23	Oyan	28 04' N	95 33' E
24	CHF/CAU/MD/24	Sille	28 04' N	95 33' E
25	CHF/CAU/MD/25	Sille	28 08' N	95 32' E
26	CHF/CAU/MD/26	Sille	27 56' N	95 20' E

Sl.no.	Genotype	Location	Latitude	Longitude
27	CHF/CAU/MD/27	Sille	28 05' N	95 31' E
28	CHF/CAU/MD/28	Napit	27 55' N	95 20' E
29	CHF/CAU/MD/29	Napit	27 53' N	95 18' E
30	CHF/CAU/MD/30	Napit	28 03' N	95 30' E
31	CHF/CAU/MD/31	Napit	28 02' N	95 26' E
32	CHF/CAU/MD/32	Gumin Nagar	27 56' N	95 20' E
33	CHF/CAU/MD/33	Takilalung	28 04' N	95 33' E
34	CHF/CAU/MD/34	Takilalung	28 08' N	95 32' E

2.1 Genetic Diversity Studies

2.1.1 DNA isolation

Fresh and disease-free juvenile leaves from 34 genotypes sourced from 11 districts were used for DNA isolation. Each leaf sample was packed in polybags, brought to the laboratory and stored in a freezer at -80°C. With the help of the CTAB method by Doyle and Doyle, the total genomic DNA was extracted [16]. The quality of extracted DNA was analyzed on 0.8% of agarose gel (GeNeith, Bangalore, India). Amplification was done using 15 SSR markers (Table 2) that were selected based on the software Primer3web version 4.0.0 and OligoCalc [17]. As per Doyle and Doyle [18] and Rawat et al. [16], the PCR conditions were slightly optimized. The concentration of 3.0Mm $MgCl_2$, 0.20 mM dNTPs mix, 0.20µM of each primer, 0.06U of *Taq*DNA polymerase and 25ng/µl of template DNA were found to be optimal for PCR amplification. PCR amplification for each primer was carried out for 35 cycles. The initial cycle of denaturation was done at 95°C for 5 minutes, followed by 35 cycles of 94ºC for 1 minute, 72°C for 1 minute and a final elongation cycle of 72ºC for 8 minutes. After PCR amplification products were electrophorized on 3% (w/v) metaphor agarose gel with 1×Tris Borate EDTA (TBE) buffer on horizontal gel electrophoresis apparatus (wide Mini-Cell-GI and Sub-Cell GI, Bio-Rad) and stained with ethidium bromide (0.5µg/ml). DNA ladder 100 bp was used as a size standard to ascertain that the amplified products were of the desired product size. DNA fragments (bands) were visualized under UV lights and documented with the gel documentation imaging system (GelDoc-It system, UVP ltd.)

Table 2
List of the primer used for study and to calculate the genetic diversity

Sl.no.	Primer	Sequence (5'-3')	Temp. (°C)
1	Ai11	**F**:GCATCAGTCAGCCATAGTGC **R**:TTGAAAAATCCTGGCGAGTG	55.0
2	Ai13	**F**:CCACAAACAAATGGGAAACC **R**:CCCTTATTACAAAAGAAGAGGGAAG	55.0
3	Ai34	**F**:ATTTGTGTGTGCGTGCTAGG **R**:CGAGGAACTGAGACTCCTGAA	50.4
4	C17	**F**:GAGAAGAGAAGGCTGTGTGC **R**:GAAACCTGATTCGTCGTCGT	59.0
5	C25	**F**:GTGACAGAGCGAGATTCCAT **R**:CCCCTTCAGCTCCAAAATGT	53.2
6	C31	**F**:CTCCTCGTTTGCCACTCATT **R**:AAACAGAGGGTTTTCGGTGC	56.0
7	MAC69	**F**:ATAAGCCAGATGACGGAACG **R**:GCATACGGCTTTCTGGATGT	57.3
8	Md1	**F**:TCAACTCCTTCGCTACTGGG **R**:GCGTATCTTTTGAAGCCAGAATG	59.4
9	Md2	**F**:TGGCTTCAAAAGATACGCCTC **R**:TCGGCTTACTGATGGGATGC	58.9
10	Md3	**F**:ACTTGAAGGGGAAAGGGACA **R**:TACCTTCGCGAGCAAGATCT	54.1
11	Md4	**F**:CCCGTTGCTGGAGAAGAAAA **R**:GGTAAAGTCAAGTCCACCGC	58.9
12	Md5	**F**:GCGGTGGACTTGACTTTACC **R**:ACCCGAAAGTGCATACGATG	55.0
13	Md6	**F**:AAGGCTAAATACGGGCGAGA **R**:GACTCGCACACATGTCAGAC	53.4
14	Md7	**F**:CCGACCGACCTTGATCTTCT **R**:CATCGCCAGTTCTGCTTACC	53.4
15	Sm07	**F**:GATAGCGGAGCCGGTGATT **R**:GGATGGAAGGCTCAAGATTCG	57.2

2.1.2 Scoring of bands and data analysis

The presence or absence of bands was scored 1 or 0 respectively after visualizing the amplified PCR products under the UVP-Gel documentation system. Genetic similarity was measured among sample pairs to genetically similar coefficients. For this purpose, amplified products of 100bp length were taken into consideration. With the help of the SAHN (sequential, agglomerative, hierarchical and nested) technique and UPGMA (unweighted pair group method with arithmetic averages), the binary data produced from 15 SSR primers were put through cluster analysis. Using NTSYS (for Windows, ver. 2.0 e), the final dendrogram was then established [19]. The assessment of discriminatory power for all the markers was done by evaluating PIC (polymorphic information content) and Rp (resolving power).

i. Polymorphic information content was calculated for the co-dominant marker system) using the given formula [20]

$$PIC_i = \{2f_i(1 - f_i)\}$$

where PIC_i is the PIC of marker i, f_i is the frequency of the marker fragments that were present, and $(1 - f_i)$ is the frequency of absent marker fragments; the PIC was averaged over the fragments for each primer combination.

ii. The resolving power is a feature of the primer combination that indicates the discriminatory potential of the primer combination; the Rp of each primer was calculated using the formula, as described by Prevost and Wilkinson [21] as follows:

$$Rp = \sum I_b$$

where I_b is a band in formativeness, which can be represented on a 0–1 scale by adopting the following formula:

$$I_b = 1 - [2 \times |0.5 - p|]$$

Where p is the proportion of all accessions containing bands.

2.2 Data Analysis

Genetic diversity was characterized by estimating an effective number of alleles (Ne) [22], Nei's [23] gene diversity (h), Shannon's Information index(I) [24], Polymorphism Information Content value (PIC) [20] and Resolving Power (RP) [21]. The pair-wise similarity between the genotypes was calculated using NTSYS (PC software, version 2.1) as described by Rohlf [19] and also for cluster analysis using (UPGMA) which would be obtained by (DARWIN for Windows version 1.32 [25].

3. Result

Screening of Melia samples with 15 SSR primers showed polymorphism in 5 primers. The primers which showed polymorphism were C17 (Fig. 3), Ai34 (Fig. 4), C31, MAC69 and Sm07. These five primers were further used for the evaluation of genetic diversity. The genetic diversity of selected genotypes for individual primers and average values concerning all the primers were calculated (Table 3). The SSR primers varied in detecting the genetic diversity of different genotypes. The expected genetic diversity varied from 0.35 to 0.47, averaging 0.41. The highest expected genetic diversity was obtained with primer C31 and the lowest with primer MAC69. The range of the polymorphism information content value (PIC) is from 0.35 to 0.48. The highest was reported with primer C31 (0.48) and the lowest (0.35) with primer MAC69. The Shannon's Information index value varied from 0.52 to 0.66 with an average of 0.59. The highest was obtained with primer C31 and the lowest with primer MAC69. The range of an effective number of alleles (Ne) is from 1.60 to 1.88 with the highest recorded with primer C31 and the lowest with primer MAC69. The average Ne calculated was 1.74. The resolving power had a value ranging from 1.12 to 1.81 with an average recorded value of 1.40. In the estimation of primer-wise genetic diversity, the highest recorded value for h, PIC, I and Ne was obtained with the same primer C31 similarly the lowest

values were recorded with primer MAC69 except in resolving power, the highest value was recorded with Ai34 primer and the lowest with Sm07 primer.

Table 3
List of the primer used for study and to calculate the genetic diversity

S.No.	Primer	Sequence	Temp (°C)	Ne	h*	I	PIC value	Rp
1	**C17**	**F**:GAGAAGAGAAGGCTGTGTGC	59.0	1.61	0.36	0.53	0.37	1.41
		R:GAAACCTGATTCGTCGTCGT						
2	**Ai34**	**F**:ATTTGTGTGTGCGTGCTAGG	50.4	1.81	0.44	0.63	0.44	1.81
		R:CGAGGAACTGAGACTCCTGAA						
3	**C31**	**F**:CTCCTCGTTTGCCACTCATT	56.0	1.88	0.47	0.66	0.48	1.24
		R:AAACAGAGGGTTTTCGGTGC						
4	**MAC 69**	**F**:ATAAGCCAGATGACGGAACG	57.3	1.60	0.35	0.52	0.48	1.41
		R:GCATACGGCTTTCTGGATGT						
5	**Sm07**	**F**:GATAGCGGAGCCGGTGATT	57.2	1.78	0.42	0.60	0.35	1.12
		R:GGATGGAAGGCTCAAGATTCG						
Average				1.74	0.41	0.59	0.43	1.40

*ne = Effective number of alleles, Kimura and Crow (1964), * h = Nei's (1973) gene diversity, * I = Shannon's Information index, Lewontin (1972), PIC vaue = Polymorphism information content, Rp = Resolving power.

The UPGMA-based cluster analysis was performed to obtain a dendrogram (DARWIN version 1.32 Yeh and Boyle [25]. The 34 genotypes were grouped into 8 different clusters with cluster 1 as the major cluster with 15 genotypes and cluster 7 with 5 genotypes (Fig. 2). The clustering pattern showed that some genotypes of different geographical locations were clustered together and *vice-versa*. The genetic dissimilarity between pair-wise comparisons of the genotypes was obtained using NTSYS (Table 4). The overall dissimilarity values among the selected plus trees ranged from 0.001 to 0.845. The highest dissimilarity coefficient (0.845) was observed between genotypes, CHF/CAU/MD/19 with CHF/CAU/MD/12 & CHF/CAU/MD/30. Whereas the lowest dissimilarity coefficient (0.001) was observed between CHF/CAU/MD/4 & CHF/CAU/MD/27 genotypes.

4. Discussion

In the current study, 15 SSR primers were selected for genetic diversity studies, of which five primers were found to be polymorphic. Similarly, Rawat et al. [11] reported the 6 highest polymorphisms out of 40 SSR primers in genetic diversity studies of *Melia dubia*. In earlier studies of Melia dubia's genetic diversity

studies, a similar number of markers has been used by Rawat et al. [12], *Toona ciliata* [26], *Azadirachta indica* [27;28] and *Swietenia macrophylla* [29;30] in Meliaceae family.

The SSR primers varied in detecting the genetic diversity of different genotypes. As revealed by microsatellite markers, several alleles and the effective number of alleles show diversity and polymorphism [31]. In the present study, an effective number of alleles (Ne = 1.88), Nei's gene diversity (h = 0.47), polymorphism information content (PIC = 0.48) and Shannon's index (I = 0.66) are shown by the same primer (C31) were found to be the highest which indicates diverse and polymorphic nature of the primer. Rawat et al. [12] reported Shannon's index value of 0.51 and Nei's gene diversity value of 0.34 in *Melia dubia*. Rawat et al. [11], reported an effective number of alleles (Ne = 4.6), observed heterozygosity, (H_o=0.64), and Shannon's index (I = 1.58) in 10 natural populations of *Melia dubia*. However lower levels of H (0.29 and 0.28) and I (0.44 and 0.43) were reported in other tropical species such as *Gmelina arborea* and *Pongamia pinnata* [32;33]. The level of polymorphism detected (information content) and the extent to which an assay could identify multiple polymorphisms was balanced by the utility of a given marker system. Resolving power (RP) however, did not show any considerable variation in its range. Among all the SSR primer pairs tested in *Melia dubia*, C31, Ai34 and Sm07 were the most informative with high PIC values and RP values

To further test the extent and pattern of genetic relatedness of the 34 selected genotypes of *Melia dubia*, subjected to an unweighted pair group method with arithmetic mean (UPGMA) based cluster analysis. Different genotypes were found to be clustered together though they belong to different geographic locations and *vice-versa*. It was evident that the clustering of genotypes was random and was not as per the geographical location. A similar study has been reported for variation amongst progenies of *Melia dubia* by Rawat et al. [11;12;34]. Similarly, no association between genetic and geographic distance is documented in many tree species selected from natural populations *viz*. *C. latifolia* and *Theobroma speciosum* [35;36].

The pair-wise comparisons of each genotype were generated based on NTSYS which gives the percentage of dissimilarity between each pair of genotypes analyzed. The dissimilarity coefficient ranged from 0.001 to 0.845. Zhang et al. [37] reported a similarity coefficient ranging from 0.620 to 1 in Chinese jujube (*Ziziphus jujuba* Mill.). Subramanyam et al. [38] also reported the similarity coefficient varying from 0.001 to 1.00 *Jatropha curcas* (L.). The highest dissimilarity coefficient (0.85) was observed between genotype CHF/CAU/MD/19 with CHF/CAU/MD/12 and CHF/CAU/MD/30 which implies that the genotypes belonging to the different geographic locations are the most genetically distant. Whereas the lowest dissimilarity coefficient (0.00) was observed between the genotypes CHF/CAU/MD/4 & CHF/CAU/MD/27 indicating genetic similarity although they belong to different geographic locations. Johar et al. [34] and Dhillon et al. [39] reported similar findings in *Melia dubia* and *Jatropha curcas* respectively.

5. Conclusion

As per the findings of the study, the overall genetic diversity of the *Melia dubia* population in Arunachal Pradesh is moderate level. The SSR primers varied in detecting the genetic diversity of different genotypes and the result of the cluster analysis and dissimilarity coefficient suggest distinction was not as per the geographical location of the genotypes. To avoid inbreeding and alterations to the genetic composition of the species, plantations from highly diverse superior seed sources should be raised. This will not only help in maintaining genetic diversity but also assist in the conservation of the species.

Abbreviations

Syn.	Synonymous
SSR	Simple Sequence Repeats
PIC	Polymorphism information content
I	Shannon's diversity index
Ne	Effective number of alleles
h	Nei's gene diversity
Rp	Resolving power
°C	Celsius
%	Percent
µl	Microliter
Bp	Base pair(s)
CTAB	Cetyltrimethylammonium bromide
TE	Tris. EDTA
Tris.	Trisaminomethane
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase chain reaction
dNTPs	Deoxynucleoside triphosphates
UPGMA	Unweighted Pair Group Method With Arithmetic Averages
SAHN	Sequential, Agglomerative, Hierarchical and Nested)
NTSYS	Numerical Taxonomy and Multivariate Analysis System
via.	By means of
viz.	Namely

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Author 1 & 2 contributed to the study conception, design and material preparation. Author 3 & 4 contributed to the laboratory work. Analysis was performed by Shivani Dobhal. Data collection and first draft of the manuscript was written by Unshani Daryal and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Ethical approval: This article does not contain any studies with human or animal participants performed by any of the authors.

Data availability

The data used to assist the findings of this research are obtainable from the corresponding author upon request.

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Table 4

Table 4 is available in the Supplementary Files section.

Figures

Figure 1

Distribution map of *M. dubia* in East Siang district, Arunachal Pradesh

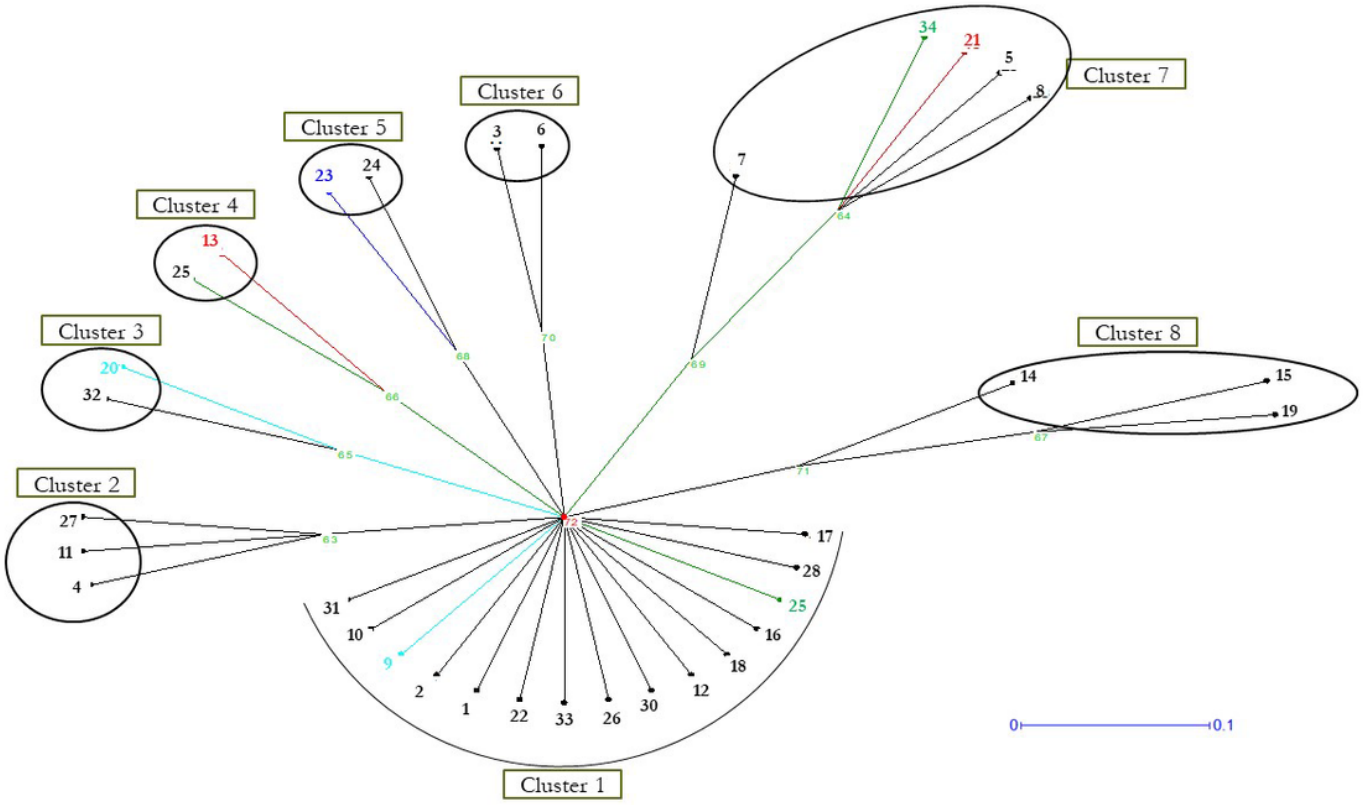

Figure 2

UPGMA-based dendrogram generated by SSR molecular data

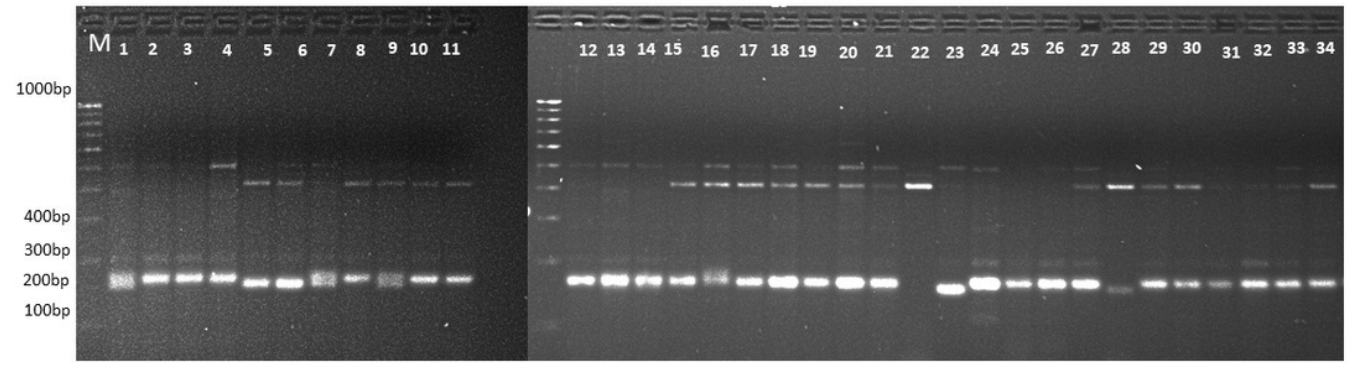

Figure 3

Amplification of *M.dubia* using SSR primer C17; 100 bp ladder

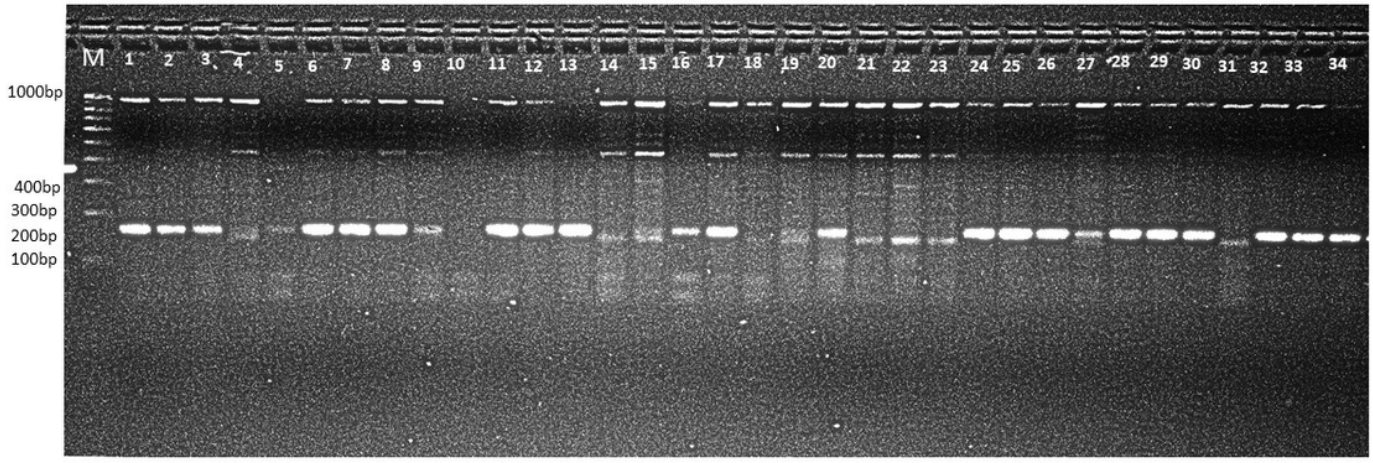

Figure 4

Amplification of *M.dubia* using SSR primer Ai34; 100 bp ladder

Supplementary Files

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- Table4.doc