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RESEARCH ARTICLE

ANALYSIS OF GENETIC VARIATIONS AMONG PONGAMIA PINNATA POPULATIONS IN GINGEE HILLS USING DNA BARCODING TECHNIQUE

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ABSTRACT

The trnL-trnF intergenic spacer of cpDNA has been sequenced from *Pongamia pinnata*. These sequences were used for phylogenetic study to show the variations and relationships found in species *Pongamia pinnata* in its diversity of distribution. The results give sufficient evidence to the morphological and genetic relationships and variations found in *Pongamia pinnata*. The study concludes that no single source of information should be used unequivocally to determine phylogenetic relationships among the closely related but highly diversified taxa associated with the genus *Pongamia*.

INTRODUCTION

In the present era of global warming and climate change, there is an ecological crisis of gigantic proportions looming across the horizons endangering the very survival of the human species together with all other forms within the next millennium. Identifying organisms has grown in importance as one monitors the biological effects of global climate change and attempts to preserve species diversity in the face of accelerating habitat destruction. Less than two million of the estimated 5–50 million plant and animal species have been identified. Scientists agree that the yearly rate of extinction has increased from about one species per million to 100–1,000 species per million. This means that thousands of plants and animals are lost each year. Most of these have not yet been identified (Report of Cold Spring Harbor Laboratory DNA Learning Center on Using DNA Barcodes to Identify and Classify Living Things, 2014). Classical taxonomy, the long standing traditional method used in the identifications of plants, falls short in this race to catalog biological diversity before it disappears. The reason is morphological characteristics or phylogeny of plants alone not sufficient in cataloging newer plants because newer plants exhibit varied diversity in morphological, anatomical and genetical characters due to the erratic climate change. Therefore, there is an urgent need to identify a system that would help the taxonomists to document the species before they disappear from the planet. DNA barcoding has become very instrumental for such study.

DNA barcoding is a novel system designed to provide rapid, accurate, and automatable species identifications by using short, standardized gene regions as internal species tags (Paul *et al.,* 2005). DNA barcoding opens new opportunities for biodiversity research. This technique is now considered to be a powerful tool, both for taxonomical (Hebert *et al.,* 2003) and ecological (Valentini *et al.,* 2009) studies. Taxonomies based solely on morphological analyses are sometimes problematic due to either convergence in phenotypes among distantly related species, or the failure to identify cryptic species where morphologic divergence has not kept pace with genetic divergence (Ahrens *et al.,* 2007). In addition to assigning specimens to known species, DNA barcoding will accelerate the pace of species discovery by allowing taxonomists to rapidly sort specimens and by highlighting divergent taxa that may represent new species. By augmenting their capabilities in these ways, DNA barcoding offers taxonomists the opportunity to greatly expand, and eventually complete, a global inventory of life's diversity. The present study, DNA barcoding and phylogenetic variations, is focused on the aspects of phylogenesis and genomic DNA variations using DNA molecular marker in tender leaves of *Pongamia pinnata* (L.) Pierre collected from four different places in Gingee taluk, Tamilnadu. The advent of fast and low cost DNA sequencing and associated bioinformatics opens the possibility of rapid gene

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discovery as well as genetic variations which in turn help to study the phylogenesis of *Pongamia pinnata* in its natural environment.

Study plant

Pongamia pinnata (L.) Pierre (Papilionoideae) is an Indian Beech, perennial, fast-growing, leguminous tree, widely distributed on the Indian subcontinent, south-east Asia, Oceania, northern Australia, the East-African coast and southern China (Murphy *et al.,* 2012). In addition *Pongamia* has been introduced to other parts of the world, including the United States (Scott *et al.,* 2008). It is used medicinally in India, China, Australia and the Philippines. In the Indian traditional system of medicine Ayurveda, *P. pinnata* has been used in the treatment of bronchitis, whooping cough, rheumatic joints and quench dipsia in diabetes (Bimla *et al.,* 2003). In addition, activities such as anti-plasmodium characteristics (Simonsen *et al.,* 2001), Anti-inflammatory activity (Srinivasan *et al.,* 2001), antidiarrheal (Brijesh *et al.,* 2006), antiulceric (Prabha *et al.,* 2003), hypoglycemic property (Punitha and Manoharan 2006), wound healing property (Ayyanar and Ignacimuthu 2009), like *Jatropa* for oil yielding or bio-fuel source (Naik *et al.,* 2008), anticonvulsant activity (Ashish Manigauha *et al*., 2009) are reported. At a time when society is becoming increasingly aware of the declining reserves of oil for the production of fossil fuels, it has become apparent that bio-fuels are destined to make a substantial contribution to the future energy demands of the domestic and industrial economies. *Pongamia pinnata* will impact most significantly through the extraction of seed oil for use in the manufacture of biodiesel. The potential of *P. pinnata* oil as a source of fuel for the biodiesel industry is well recognized (Azam *et al*., 2005; De and Bhattacharyya 1999; Karmee and Chadha 2005). Moreover, the use of vegetable oils from plants such as *P. pinnata* has the potential to provide an environmentally acceptable fuel, the production of which is greenhouse gas neutral, with reductions in current diesel engine emissions (Raheman and Phadatare 2004). In the study area, *Pongamia pinnata* seeds are used in cultivation as insecticide.

STUDY AREA: GINGEE HILLS

Gingee is a heritage town bounded by hills of Eastern Ghats. The town falls under the geographical coordinates of 12°.15'N and 79°.25'E., above the Mean Sea Level of 30.45m. The town comprises of a number of small and large hills, rocky outcrops are found here and there. Gingee Forest Range consists of seven Reserve Forests and one Reserve Plains. They are: Gangavaram Reserve Forest (2681.87 ha), Thandavasamudram Reserve Forest (318.49 ha), Poolanjimalai Reserve Forest (236.94 ha), Pakkamalai (2263.81 ha), Siruvadi (1360.16 ha), Muttakadu (1289.72 ha), Padipallam (1457.28 ha) and Karai Reserve Plains (686.75 ha). All these Reserve Forests are located in the south west direction from Gingee towns. There are lots of isolated hillocks present and they are the offshoot of Eastern Ghats. There are 26 villages situated around these Reserve Forest areas. The town has hot, dry climate almost throughout the year. The maximum temperature and the minimum temperature of the town are 36 to 30 C respectively. The town receives rain mainly during the months of October, November through the North East monsoon. On an average the town receives 700mm of rainfall.

MATERIALS AND METHODS

Sample collection and Identification

Fresh tender leaves are collected from four villages in Gingee hills: Gingee Fort (Muttakadu, 1289.72 ha) 12°15N and 79°24E with Mean Sea Level of 112 M; Alampoondi (Poolanjimalai, 236.94 ha) 12°14N and 79°20E with Mean Sea Level of 127 M; Devadanampettai (Pakkamalai, 2263.81 ha) 12°10N and 79°20E with Mean Sea Level of 144 M and Gengavaram (Gengavaram malai, 2681.87 ha) 12°08N and 79°19E with Mean Sea Level of 146 M (Table 1). Taxonomic identification of these plants was carried out by John Britto, Director, The Rapinant Herbarium, St. Joseph's College, Tiruchirappalli. A voucher specimen of each experimental plant was deposited at The Rapinant Herbarium, Tiruchirappalli (RHT), Tamilnadu, India.

Table 1. List and origin of accessions analyzed for cpDNA variation

Sample	Taxa	Voucher Code	Place of Accession	GPS	Date of Accession
1	*Pongamia pinnata*	RHT 65385	Gingee Fort, Villupuram District, Tamilnadu, India	12°15N 79°24E	19.05.2014
2	*Pongamia pinnata*	RHT 65383	Alampoondi, Villupuram District, Tamilnadu, India	12°14N 79°20E	19.05.2014
3	*Pongamia pinnata*	RHT 65487	Devadanampettai, Villupuram District, Tamilnadu, India	12°10N 79°20E	19.05.2014
4	*Pongamia pinnata*	RHT 65488	Gengavaram, Villupuram District, Tamilnadu, India	12°08N 79°19E	19.05.2014

Isolating DNA from plant

2g of fresh leaf samples were taken for the isolation of DNA. Leaves of the samples were sliced into smaller pieces. Care was taken not to choose contaminated specimen. Each sample was carefully labeled with an identification number. 1% β-mercaptoethanol was added to the extraction buffer and warmed at 65ºC for 5-10 min following the protocol of Doyle and Doyle (Doyle and Doyle, 1987). 8-10 ml of warm extraction buffer was added to the ground sample and approximately 0.4 to 2.5 ml CTAB/NaCl solution was added for each gram of fresh leaf tissue and mixed to form slurry. The thoroughly mixed slurry was then transferred to a screw capped 30 ml centrifuge tube. The tubes were incubated at 65ºC for 1-2 hours with occasional mixing. Equal

amount of $CHCl_3$: isoamylalcohol mixture in 24:1 ratio was added to the slurry. The mixture was centrifuged at 10,000 rpm for 5 min. at 4ºC. Clear (greenish yellow) supernatant was transferred to a fresh tube and double the volume of CTAB precipitation buffer was added. Then it was incubated at 37ºC overnight in a water bath. The incubated samples were centrifuged at 8000 rpm for 8-10 min. at 4ºC. The pellet was collected and supernatant was discarded. 1 ml of high salt TE was added to re suspend the pellet. The suspended pellet was transferred 2 ml eppendorff tubes to glass tubes. And 1.0 ml (0.6 volumes) of isopropanol was added and incubated at -20ºC for 30 min. The tubes were centrifuged at 8000 rpm for 10 min. at 4ºC. The pellets were washed with 1 ml of 80% ethanol by spinning at 10,000 rpm for 5 min at 4ºC. The pellets were re suspended in 0.5 ml of 1 X TE. The solution was transferred to 1.5 ml eppendorff tubes. The precipitated samples were centrifuged at 12,000 rpm for 15 min. at 4ºC. The pellet was collected and washed with 0.5 ml of 70% ethanol. Centrifugation was done at 10,000 rpm for 5 min at 4ºC. The pellets were re suspended in 100µl 1X TE. The re suspended pellets were collected and stored at -20ºC.

Spectrophotometric estimation of nucleic acids

The amount of DNA/ RNA present in the pre- diluted sample was quantified using this technique. The absorbance of nucleic acid was read at 260nm while that of protein was read at 280nm. The ratio of the absorbance at 260/280 was used to determine the purity of nucleic acid. Ratio = A260nm/A280nm. The optimum value was taken as 1.8 for pure DNA and 2.0 for RNA.

Sample preparation for UV Spectrophotometry

To 10µl of 1/100 diluted sample in 1X TE buffer 1.90ml of 1X TE Buffer was added. Two ml of the 1X TBE buffer in a quartz cuvette was taken to do baseline correction. The absorbance of the sample both at 260nm and 280nm was read and ratio was calculated. An absorbance (A260) of 1.0 corresponds to 50µg ds DNA / ml of the solution and 40µg of RNA/ ml respectively. From this the concentration of DNA or RNA in the test sample was calculated.

Identifying the regions of Chloroplast DNA (cpDNA)
cpDNA regions analyzed

Chloroplast DNA (cpDNA) sequence variations are now widely used to investigate interspecific relationships among angiosperms and other plants. Total genomic DNA isolation followed the same protocol outlined in Coskun and Parks (2009). Amplifications of *trn*L-F region of the chloroplast DNA were conducted by using the PCR temperature files suggested by Taberlet *et al*. (1991). *trn*L-F molecular marker analyzed in this study belongs to the chloroplast genome (cpDNA). Polymerase Chain Reaction (PCR) amplifications of *trn*L-F cpDNA were performed using the primers designed by Taberlet *et al*. (1991) for all taxa included in this work (Table 2).

Table 2. ITS primers used in this study with their designers

Primer Name	3'to5' Primer Sequence	Primer designed by	Source
trnL-F			
Forward trnL *e*	GGTTCAAGTCCCTCTATCCC	Taberlet *et al*., 1991	Taberlet
Reverse trnF *f*	ATTTGAACTGGTGACACGAG	Taberlet *et al*., 1991	Taberlet

The chloroplast trnL (UAA) intron may represent a good target region for our purpose. Its sequences have been widely used for reconstructing phylogenies between closely related species or for identifying plant species. Nevertheless, it is widely recognized that it does not represent the most variable non-coding region of chloroplast DNA, but it bears some unique advantages. Universal primers for this region were designed 15 years ago, and subsequently extensively used, mainly in phylogenetic studies among closely related genera and species. The evolution of the trnL (UAA) intron has been thoroughly analyzed and is well understood **(**Downie and Katz-Downie, 1996; Downie and Katz-Downie, 1999**).** Furthermore, this region is the only Group I intron in chloroplast DNA. This means that it has a conserved secondary structure with alternation of conserved and variable regions. As a consequence, the alignment of diverse trnL intron sequences might allow the design of new versatile primers embedded in conserved regions and amplifying the short variable region in between. More specifically, our objective in this paper is to evaluate the power and the limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding, and to assess the possibility for designing a new system allowing species identification with highly degraded DNA.

Primer used

Table 2 presents the sequences of the primers in the chloroplast trnL (UAA) gene. Primers designed by Taberlet *et al*., were used for PCR amplification of the *trnL-trnF* region (5'- GGTTCAAGTCCCTCTATCCC-3' and 5'-ATTTGAACTGGTGACACGAG-3'). This fragment encompasses the entire trnL (UAA) intron plus a few base pairs on each side belonging to the trnL (UAA) gene itself. The primers *e* and *f* were designed for this study on two highly conserved regions after aligning various sequences. The primers are constructed against distal regions of the highly conserved tRNA genes and thus are suitable for amplifying this noncoding region from a broad spectrum of higher plants.

PCR Amplification

To amplify a DNA barcode region, a set of standard primers was selected for each sample. The primers were selected because of the type of DNA studies it is targeted. Double standard DNA amplifications were performed in a volume of 50µl containing 50mM KCl, 10mM Tris-HCL pH 8.3, 2.5mM $MgCl_2$, 1.0µM each primer, 200µM of each dNTP and 1.5U of Taq polymerase (Gen Tec biotech). Amplification reaction was performed in a BIORAD Thermal Cycler. The PCR mixture underwent initial denaturation at 94°C for 5 min, 35 cycles were run. Each cycle consisted of 1 min at 93°C, 1 min at 55°C, and 2 min at 72°C and final extension at 72°C for 10 min. The amplification products were purified by agarose gel electrophoresis and the concentrated DNAs were recovered.

Electrophoresis

The PCR products (5µl) were mixed with 6x gel loading buffer (2µl) and loaded on to an agarose (1.5% w/v) gel electrophoresis in 0.5x TBE (TrisBorate-EDTA) buffer at 100V for 150 min. the gel was stained in ethidium bromide solution, visualized under UV in GEL DOC (UK) Image Analysis system with UViTEC analysis package (Cambridge, UK). (Fig.1)

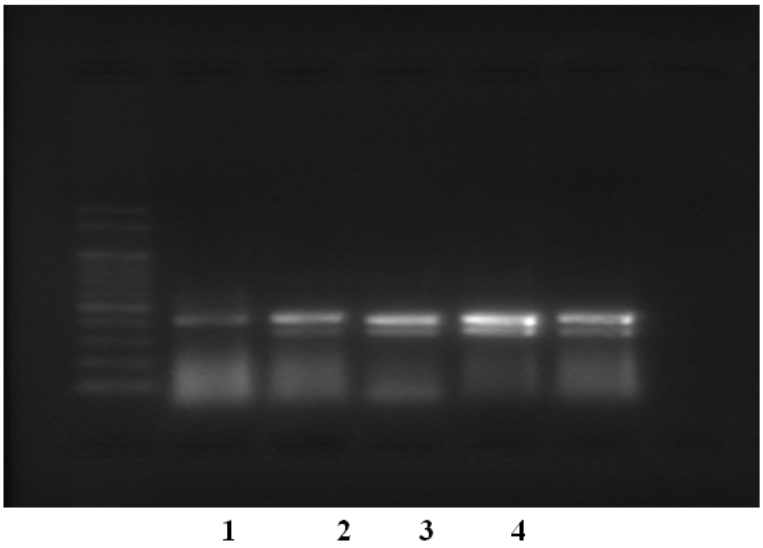


Fig. 1. Electrophorosis of PCR products in a 1.5% agarose gel showing the successful application of the primers over a wide taxonomic range. PCR products of the primers *e* and *f* for the intron of the trnL (UAA) gene. Molecular weight marker, lanes 1 and 4; 2 and 3 show similarities

Sequence Analysis

The remaining PCR products were purified using Purification Kit (Genei, Bangalore) and were sent to Chromous Biotech, Banglore, India for sequencing.

Sequencing Reaction: Sanger, 1977

The DNA sequencing method developed by Fred Sanger *et al*. (1977) forms the basis of automated "cycle" sequencing reactions today. In this method, the DNA is used as a template to generate a set of fragments that differ in length from each other by a single base. The fragments are then separated by size, and the bases at the end are identified, recreating the original sequence of the DNA. Sequencing procedure involves complementary ΦX174; restriction enzyme fragments from replicative ΦX174 and buffer were combined and incubated before being diluted. Each sample was then mixed with DNA polymerase, dATP and the appropriate dNTP and ddNTP before being incubated again. After the final incubation, more dATP was added as a 'chase' step to prevent termination at A residue. Each sample was then denatured and applied to acrylamide gel next to one another for electrophoresis.

The Sequencing mix Composition and PCR Conditions are as follows:

10µl Sequencing Reaction

Big Dye Terminator - Ready Reaction Mix: 4µl; Template (100ng/ul):1µl; Primer (10pmol/ ul):2µl and Milli Q Water: 3µl.

PCR Conditions: (25 cycles)

Initial Denaturation : 96°C for 1min; Denaturation : 96°C for 10 sec; Hybridization : 50°C for 5 sec; Elongation : 60°C for 4 min; 1 kb ladder contains 10 DNA fragments of size 1kb, 2kb, 3 kb, 4 kb, 5 kb, 6 kb, 7 kb, 8 kb, 9 kb and 10 kb

Instrument and Chemistry Details

Sequencing Machine: ABI 3130 Genetic Analyzer; Chemistry: Big Dye Terminator version 3.1

Cycle sequencing kit

Polymer & Capillary Array: POP_7 polymer 50 cm Capillary Array; Analysis protocol: BDTv3-KB-Denovo_v 5.2; Data Analysis: Seq Scape_ v 5.2 Software; Reaction Plate: Applied Biosystem Micro Amp Optical 96-Well Reaction plate. Sequences were checked against electropherograms and manually edited, if necessary, using the program chromas lite.

Data analysis

The sequence data were analysed using MEGA (Molecular Evolutinary Genetics Analysis) 6.0 for phylogenetic analysis, DNA Baser 3.4.5 for sequence alignment. The MEGA software is developed for comparative analysis of DNA and protein sequence that are aimed at inferring the molecular evolutionary patterns of genes, genomes and species over time. The following methods were employed in MEGA for the molecular phylogenetic analysis:

Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (1993). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Tamura *et al.,* 2013). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 4 nucleotide sequences. There were a total of 517 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Saitou and Nei, 1987).

Molecular Phylogenetic analysis by Neighbor-Joining method

The evolutionary history was inferred using the Neighbor-Joining method (Tamura *et al.,* 2004). The optimal tree with the sum of branch length = 0.02323176 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2013) and are in the units of the number of base substitutions per site. The analysis involved 4 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 218 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Rzhetsky and Nei, 1992).

Molecular Phylogenetic analysis by Minimum Evolution method

The evolutionary history was inferred using the Minimum Evolution method (Tamura *et al.,* 2004). The optimal tree with the sum of branch length = 0.02323176 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Nei and Kumar, 2000) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Tamura *et al.,* 2013) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. The analysis involved 4 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 218 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

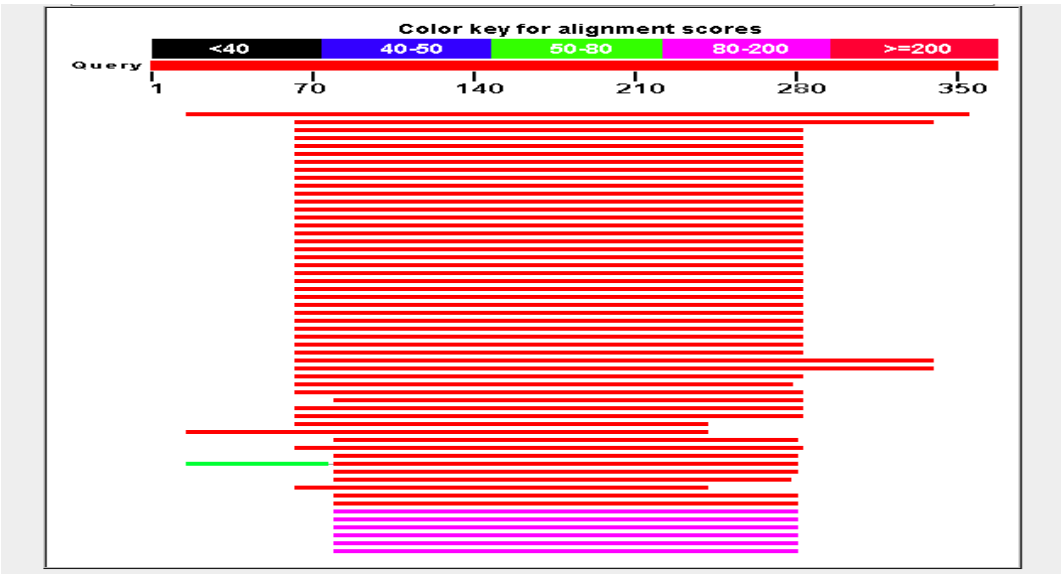
Molecular Phylogenetic analysis by UPGMA method

The evolutionary history was inferred using the UPGMA (Unweighted Pair Group Method Analysis) (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 0.02247522 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura and Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 4 nucleotide sequences. All

positions containing gaps and missing data were eliminated. There were a total of 218 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2004).

RESULTS

Molecular Studies of 4 Samples
SAMPLE 1: *Pongamia pinnata* (Collection: Gingee Fort)
>RHT65385 (Lab sample No - 347)
TTCCCACTGTATGTGTGTTCAAGTCCCTCTATCCCCAAAGGGCCGAACAAACAATAGAAGTACTTATCCTATATCTT
CCGTTTTATGAGAAACGTAAAATGAATATCTTATTTTTGAGCAAGAAATCTTTCTATGAATGATTAATAATACAAAA
TCATTACTACTACTGAAACTAACTTACAAAGTCTTATATTTTTTTTCGTCTTTTTGTTTTTTGACTTTATTTTTACTTGA
CATAGACTCATTGACATATACTCCAGTAATCTTTTCAAATAAAAATGAAGAAGACCCGCTAACAACAGTCACCAGA
CAAAAACTGGCAGACCAAAGGACTGAAAATCCTCGTGTCACCAGTTTCACATACAAGT



Millettia pinnata voucher Sirichamorn YSM 2009-25 (L) trnL-trnF intergenic spacer, partial sequence; chloroplast

Sequence ID: gb|JX506503.1|Length: 307Number of Matches: 1

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Sbjct  29   TTATCCTATATCTTCTTTTTTATGATAAACGTAGAAATGAATATCTTATTTTTGAGCAAG  88

Query  123  AAATCTTTCTATGAATGATTAATAATACAAAATCATTACTACTACTGAAACTAACTTACA  182
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Query  183  AAGTCTTATATTTTTTTTCGTCTTTTTGTTTTTTGACTTTATTTTTACTTGACATAGACT  242
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Query  243  CATTGACATATACTCCAGTAATCTTTTCAAATAAAAATGAAGAAGACCCGCTAACAACAG  302
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Query  303  TCACCAGACAAAAACTGGCAGACCAAAGGACTGAAAATC  341
            ||    | |     |  |||||||  ||  |||||||||||||
Sbjct  269  TCGGGATAGCTCAGCTGGCAGAGCAGAGGACTGAAAATC  307
```

SAMPLE 2: *Pongamia pinnata* (Collection: Alampoondi)

> RHT 65383 (Lab sample No - 348)

CTATATCTTCTTTTTTATGATAAACGTAGAAATGAATATCTTATTTTTGAGCAAGAAATCTTTCTATGAATGATTAATAATACAAAATCATTACTAC
TACTGAAACTAACTTACAAAGTCTTATATTTTTTTTCGTCTTTTTGTTTTTTGACTTTATTTTTACTTGACATAGACTCATTGACATATACTCCAGTAA
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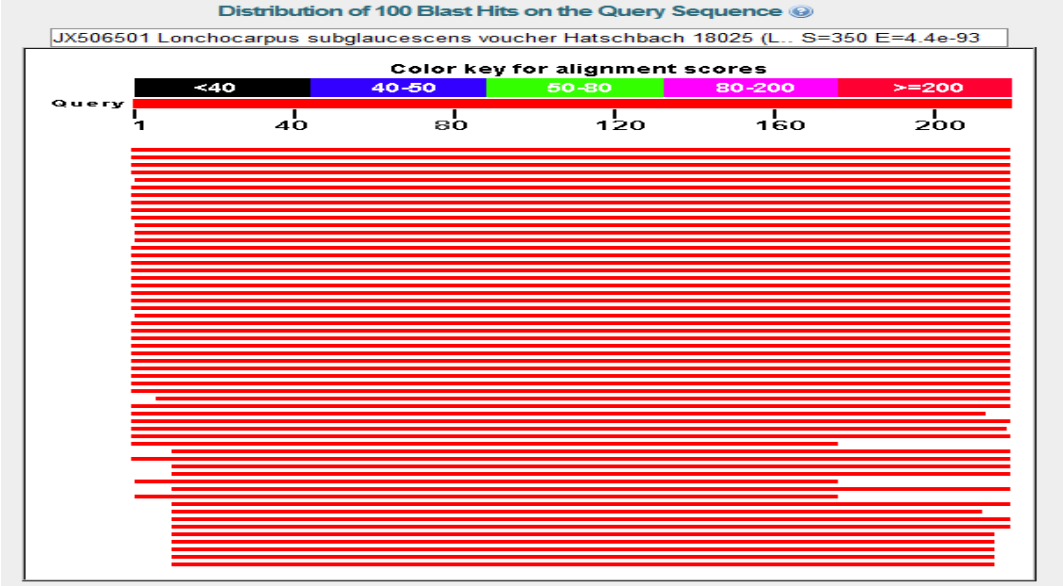

Derris tonkinensis voucher Sirichamorn YSM 2009-11 (L) trnL-trnF intergenic spacer, partial sequence; chloroplast Sequence ID: gb|JX506517.1|Length: 307Number of Matches: 1

Related Information

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Sbjct  214  ACATATACTTCAGTAATCTTTTAAAATAAAAATGAAGCT  252
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SAMPLE 3: *Pongamia pinnata* (Collection: Devadanampettai)

> RHT 65487 (Lab sample No - 349)

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TCCTATATCTTCTTTTTTATGATAAACGTAGAAATGAATATCTTATTTTTGAGCAAGAAATCTTTCTATGAA
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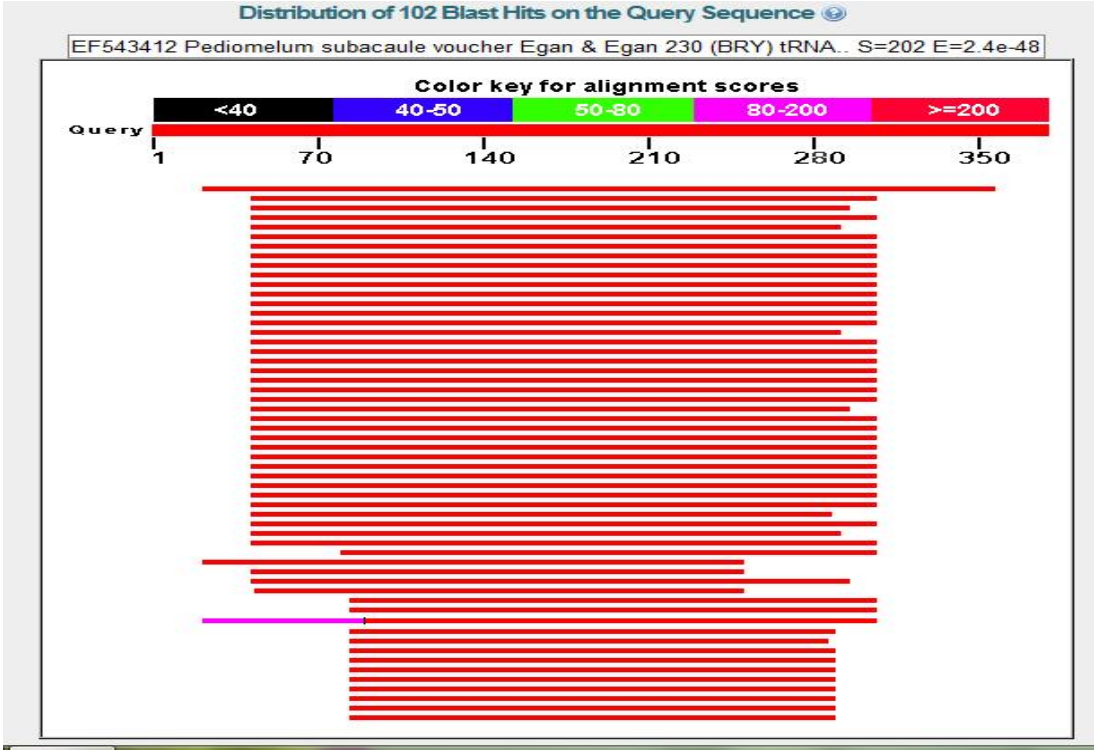

Millettia pinnata chloroplast, complete genome
Sequence ID: gb|JN673818.2| Length: 152968 Number of Matches: 1

Related Information

Score	Expect	Identities	Gaps	Strand
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SAMPLE 4: *Pongamia pinnata* (Collection: Gengavaram)

> RHT 65488 (Lab sample No - 350)

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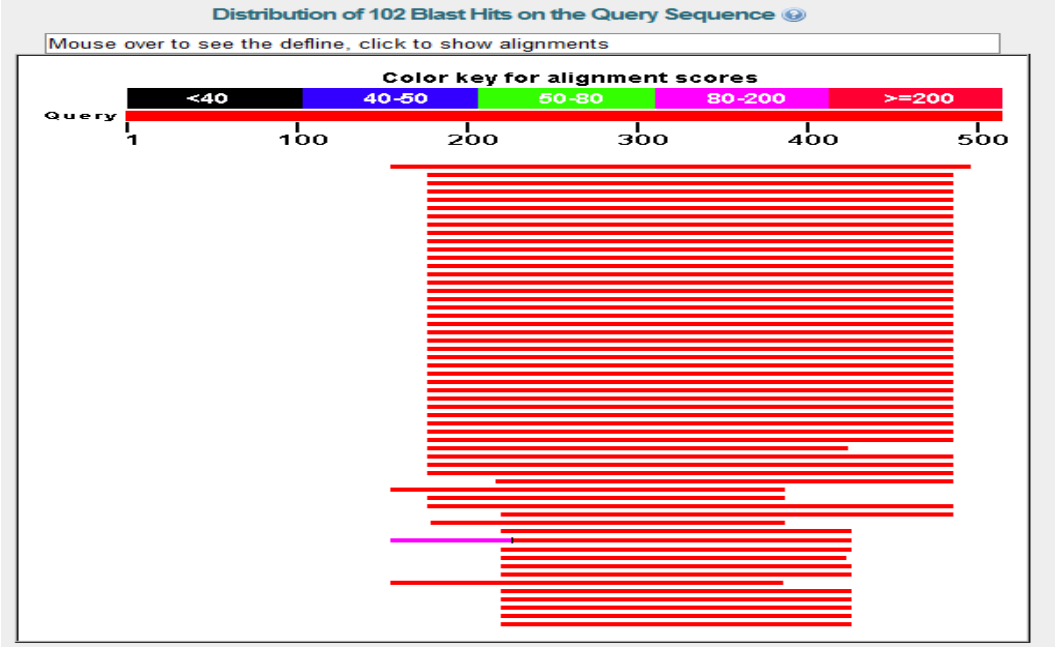

Millettia pinnata chloroplast, complete genome Sequence ID: gb|JN673818.2|Length: 152968Number of Matches: 1

Related Information

Score	Expect	Identities	Gaps	Strand
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```

SAMPLE 4: ***Pongamia pinnata*** *(*Collection: Gengavaram)
> RHT 65488 (Lab sample No - 350)

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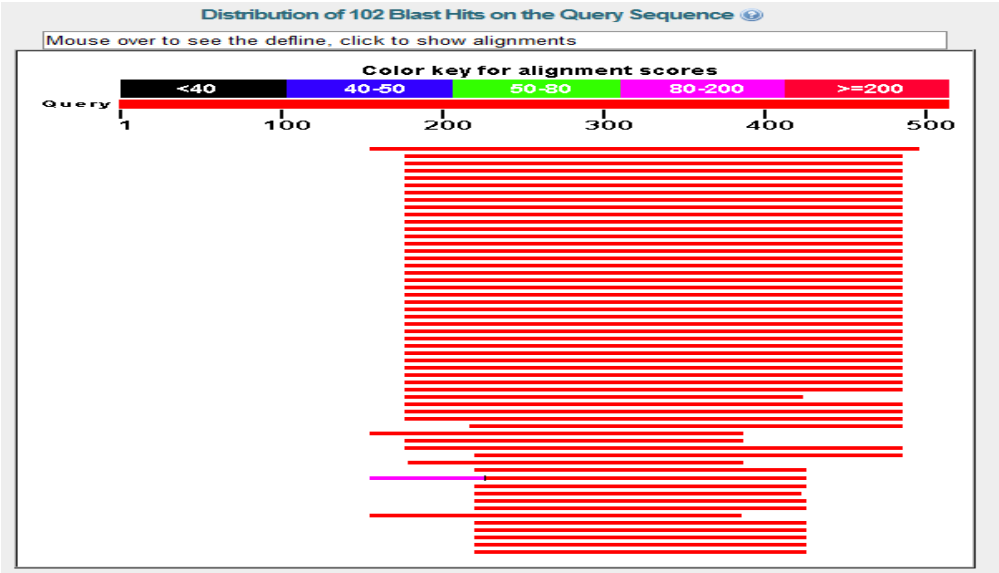

Millettia pinnata chloroplast, complete genome Sequence ID: gb|JN673818.2|Length: 152968Number of Matches: 1

Related Information

Score	Expect	Identities	Gaps	Strand
507 bits(274)	6e-140	318/340(94%)	0/340(0%)	Plus/Minus

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Query  157    AGGGTTCAAGTCCCTCTATCCCCAAAGGGCCTGTTAAACTCTAGAAGTACGTATCCTATA  216
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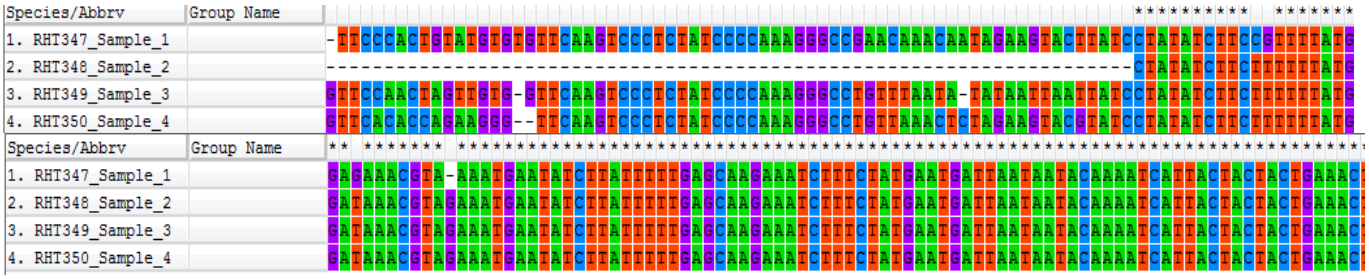
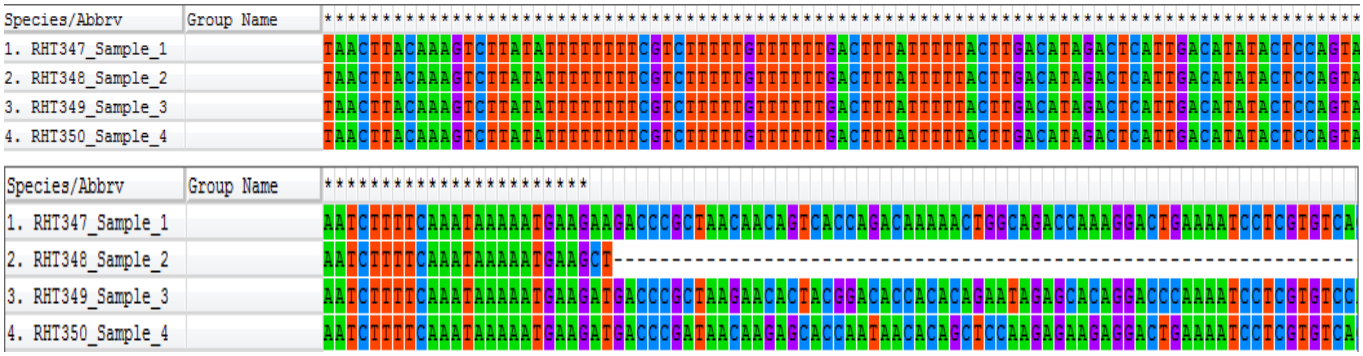
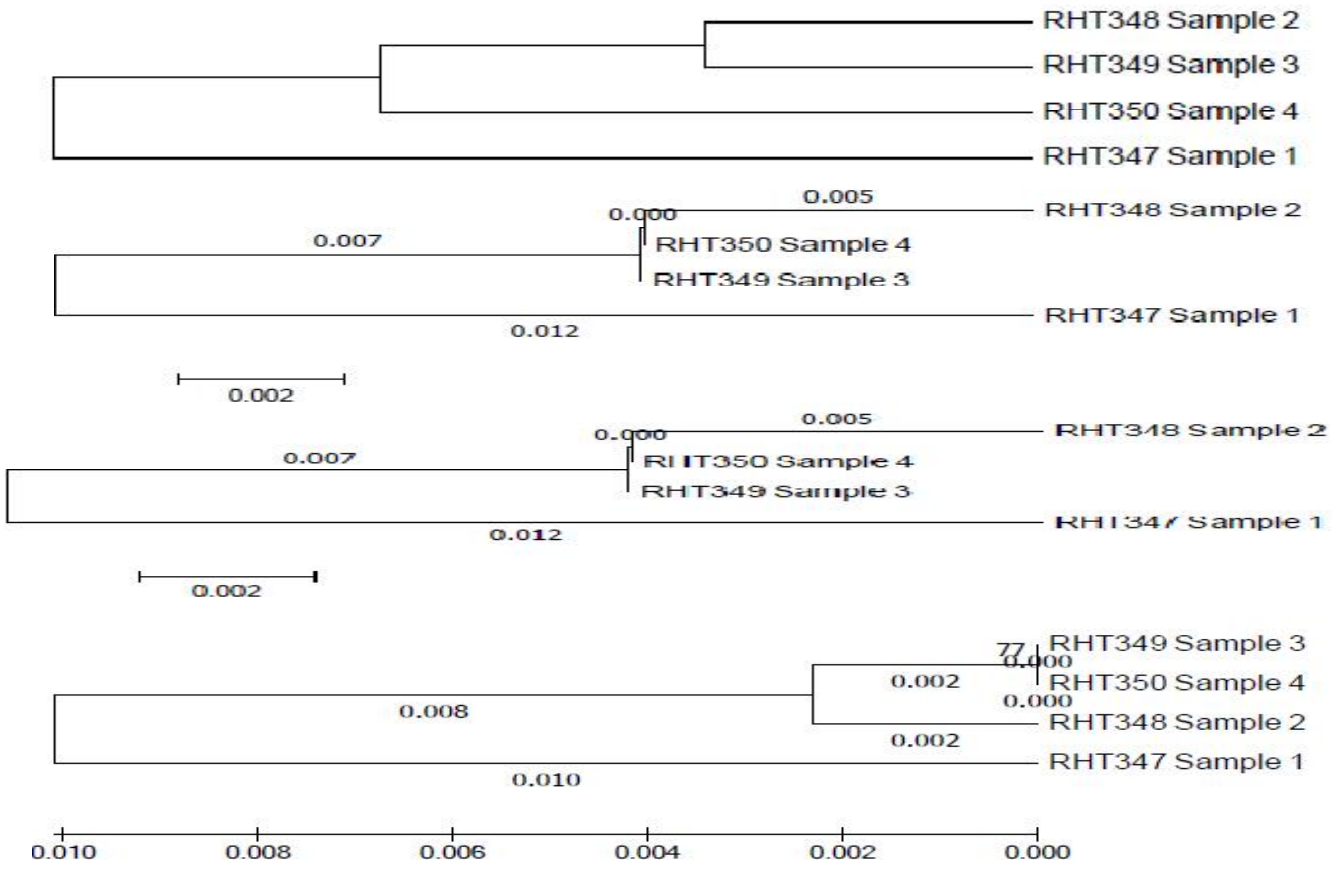
Multiple sequence alignment of 4 samples





Evolutionary relationships of taxa



SAMPLE 1: ***Pongamia pinnata*** **(**Collection: Gingee Fort**)**

The MEGA analysis of trnL –trnF sequence data involving 4 Pongamia pinnata accessions show

DISCUSSION AND CONCLUSION

There were four accessions of *Pongamia pinnata* surveyed, collected and subjected to DNA barcoding for the phylogenetic analysis from Gingee hills. Morphological variations such as tree/shrub along with leaf variations and time of flowering. Sample collection from Gingee Fort (RHT 65385; Lab sample No 347) showed good variations. Some variations were identical to the sample collection from Devadanampettai (RHT 65487; Lab sample No 349). While other two sample collections from Alampoondi (RHT 65383; Lab sample No 348) and Gengavaram (RHT 65488; Lab sample No 350) were found to be identical and showed not much variations. The results of cpDNA with the help of DNA barcoding showed the following results.

Sample 1: RHT 65385 (Lab sample No 347) exhibits a total of 279 nucleotide base pair coding regions in the cpDNA. The length of the base pair region is 307. About 257 nucleotide base pairs are found to be identical which about 92% is. It is reported that 8% shows non-identical in the nucleotide base pair and while there is only one non-coding region found in nucleotide.

Sample 2: RHT 65383 (Lab sample No 348) exhibits a total of 219 nucleotide base pair coding regions in the cpDNA. The length of the base pair region is 307. About 214 nucleotide base pairs are found to be identical which about 98% is. It is reported that 2% shows non-identical in the nucleotide base pair and while no non-coding region in nucleotide has been found.

Sample 3: RHT 65487(Lab sample No 349) exhibits a total of 336 nucleotide base pair coding regions in the cpDNA. The length of the base pair region is 152968. About 315 nucleotide base pairs are found to be identical which about 94% is. It is reported that 6% shows non-identical in the nucleotide base pair and while there are three non-coding region found in nucleotide.

Sample 4: RHT 65488(Lab sample No 350) exhibits a total of 340 nucleotide base pair coding regions in the cpDNA. The length of the base pair region is 152968. About 318 nucleotide base pairs are found to be identical which about 94% is. It is reported that 6% shows non-identical in the nucleotide base pair and while there is no non-coding region found in nucleotide. The evolutionary distances were computed using the Maximum Composite Likelihood method. The phylogenetic tree shows the evolutionary relationships of taxa where sample 1 and sample 4 have much similarities and are identical in characters which is also recorded at the time of collection of the sample. Sample 2 and sample 3 show very close relationships between them. It is observed sample 1, 2, 3 are very similar to *Millettia pinnata* and while sample 2 gives genetic resemblance to Derris tonkinensis only.

The phylogenetic analysis of cpDNA in addition to biparentally inherited nuclear genes provide a means of evaluating phylogenetic relationships among taxa. Sequence analysis of the intergenic spacer between ther trnL (UAA) 3' exon and the trnF (GAA) gene provides valuable additional data on the cpDNA variation in *Pongamia pinnata* plant species. As expected from the non-coding regions and non-identical regions, the divergence values calculated in *Pongamia* species which gives is very necessary for the phylogenetic study of a species. A general conclusion emerging from the present study is that no single source of information should be used unequivocally to determine phylogenetic relationships among the closely related but highly diversified taxa associated with the genus *Pongamia*. The combined use of chloroplast and nuclear molecular marker is invaluable for assessing the occurrence of genetic exchange among related forms. Nevertheless, further analysis using additional multiple nuclear and cytoplasmic markers would be required to determine the actual extent and evolutionary significance of introgression in *Pongamia* species.

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