Genetic Diversity studies of some ethnobotanical plants belonging to Family Rubiaceae, along the western ghats of India

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Abstract

Genetic diversity helps in long term viability of the plant population by helping them combat stresses and preserving genotypes that are beneficial to the mankind. Geographical factors is one of the main determinant of genetic diversity in plants, as being sessile, they adapt to environmental changes quickly. In this study, ethnobotanical plants, *Meyna spinosa* Roxb. ex. Link from Nasik, *Catunaregam spinosa* (Thunb.) Triveng from Sattari and *Ixora chinensis* Lam from Karwar and all their urban counter- parts from Mumbai were subjected to genetic diversity studies by determining the genetic variation, using RAPD and DNA barcoding. RAPD was performed using five random primers; DNA barcoding was performed using matK and rbcL primers. The analysis culminated into two sets of phylogenetic trees. RAPD generated a phylogenetic tree which revealed that *Catunaregam spinosa* (Thunb.) Triveng from Goa and Mumbai diverged at the ancestral node; DNA barcoding using matK and rbcL primers resulted in 6 matK and 6 rbcL sequences, which were submitted to NCBI and further it generated a phylogenetic tree which revealed that *Meyna spinosa* Roxb. ex Link from Nasik and Mumbai diverged at ancestral node. Nei's gene diversity was found to be 0.4096 ± 0.09, Shannon's diversity index was found to be 0.5943 ± 0.11, percentage of polymorphic loci was found to be 98.67%. We could conclude that genetic diversity could be observed in the same plants from different locations across Western Ghats. Environmental factors such as weather, soil composition and biotic factors could be affecting the genetic make-up of the plants.

INTRODUCTION

Ethnobotanical plants are those which are used by tribal people for various purposes (food/shelter/fodder for animals/medicines) in their day to day life. Ethnobotanical surveys are performed to document such uses of the plants and their identification by authentication. Although many ethnomedicinal plants have been used by tribal people for treating various ailments, validation of the medicinal properties by biological and chemical assays are necessary for scientifically proving their benefits. Foraging of plants for preliminary studies as above can lead to depletion of the same in its original habitat, that is, the tribal settlement area. Alternatively, if the same plants were to be available in different geographical location, from where they could be conveniently sampled, it would reduce the stress on plants present in the original habitat. This would raise the concern of differences in metabolite production, due to geographical variation. Plants being sedentary, experiences the environmental stress much severely compared to other motile organisms. Enzymes play an essential role in the metabolic pathway of secondary metabolite synthesis, regulating the levels of the latter in the plant. On the contrary, enzymes, themselves are regulated at genetic level, due to various cellular and biochemical signals, induced in response various environmental stressors. These environmental factors vary in different geographical locations.

Another reason to study genetic diversity in plants is that it brings about a balance in the ecosystem, since they serve as a repertoire of gene variants, which, can be used for growing better variety plants. Many studies have been carried out to determine the effect of geographical factors on genetic diversity in

certain sets of plants, for example: role of climatic and geographical factors on genetic diversity of *Coffea muritiana* in Reunion island (Garot et al. 2019); effect of environmental factors on genetic diversity of *Caragana microphylla* in northeast China (Huang et al. 2016); Assessing the environmental factors affecting the genetic diversity of *Rhododendron aureum* Georgi (Zhao et al. 2021) and many such studies have been reported. Different approaches to evaluating the genetic diversity include RAPD, AFLP, DNA barcoding etc.

The following plants of ethnobotanical importance, were considered for the current study: *Meyna spinosa* Roxb. ex. Link from Surgana, Nashik- used in treating inflammation and stomach ailments; *Catunaregam spinosa* (Thunb.) Triveng from Sattari, North Goa- used in the treatment of Jaundice & *Ixora chinensis* Lam. from Phaate, Karwar- the roots of the white and red variety was used for treatment of 'Herpes' by the tribal healer. The ethnobotanical uses were recorded in ethnobotanical survey that was conducted between 2016 and 2017.

Few studies have been reported with regard to genetic diversity studies of *Catunaregam spinosa* (Thunb.) Triveng and *Ixora chinensis* Lam., however this is the first study to report barcoding genes in *Meyna spinosa* Roxb. ex. Link. NCBI database lists at least 31 nucleotide results and 17 protein groups belonging to *Catunaregam spinosa* (Thunb.) Triveng. The available data is mostly related to DNA barcoding work, which was carried out for genetic diversity studies (Chao et al. 2019; Kainulainen et al. 2017; Yang et al. 2016). Different studies of *Ixora chinensis* Lam with molecular markers such as nuclear marker gene (nrETS), chloroplast markers (rps16 and trnT-F) by Arnaud et al. (2009); with petD, rps16, rpoB-trnC and trnL by Tosh et al. (2013); with ITS2 by Gong et al. (2018) and lastly a complex plastid and nuclear divergence pattern was studied in Philippine *Ixora* by Banag et al. (2017).

All three plants belong to the family- *Rubiaceae*. The urban counterparts of these plants were sampled from Mumbai, in order to perform the genetic diversity studies.

METHODOLOGY

All reagents used in the experiment were of molecular grade.

Sampling: Tender, fresh leaves of plants under study were collected from the respective location (respective ethnobotanical sites and the urban in zip- lock bags and stored in ice packs. The samples were delivered to the lab within 24- 48 hours of sample collection. The plants are as follows:

1. *Ixora chinensis* Lam from Karwar- ICK

2. *Ixora chinensis* Lam from Mumbai- ICM

3. *Catunaregam spinosa* (Thunb.) Triveng from Goa- CSG

4. *Catunaregam spinosa* (Thunb.) Triveng from Mumbai- CSM

5. *Meyna spinosa* Roxb. ex Link from Nasik- MLN

6. *Meyna spinosa* Roxb. ex Link from Mumbai- MLM

DNA extraction: Genomic DNA extraction was done using CTAB- method with minor modifications. The quantity and quality of DNA isolated was also spectrophotometrically estimated using Nanodrop.

PCR amplification for RAPD and DNA barcoding analysis: The PCR for RAPD was performed using 5 random primers, details of which are listed in **Table 1**. The reaction mixtures set up were different for each random primer. The amplification products were run against a 100bp marker ladder on AGE. The images were captured and subjected to further steps of RAPD. Band scoring and binary data file generation was carried out using PyElph software version 1.4 (Pavel and Vasile 2012) and PopGene Version 1.31 (Yeh and Boyle 1997) and Dendrogram of the data obtained was performed using PopGene program.

Sequencing and In-silico analysis: PCR amplicons obtained with matK and rbcL primers were subjected to sequencing, followed by in-silico analysis for homology match using BLASTn on NCBI (Altschul 1990). They were submitted to Genbank with their respective annotation. The respective sequences (matK/rbcL) were also aligned using CLUSTALW on EMBL, wherein *Vinca major* matK (Acc ID: KX677530) and rbcL (Acc ID: KX678972.1) were used as outgroups. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al. 2013). The alignment was then subjected to MEGA 6 analysis where parameters pertaining to genetic diversity were analyzed- Conserved, Variable and Mark Parism Informative sites information. Genetic distances and phylogenetic tree was constructed which was based on neighbour joining statistical methods, bootstrap method of phylogeny and Kimura 2-parameter model.

Phylogenetic trees resulting from RAPD and DNA barcoding data, along with results of different parameters such as Nei's genetic distance (Nei 1972; Nei 1973 and Nei 1978), Shannon's Diversity Index (Shannon and Weaver 1949), Polymorphic loci, structural similarity of predicted proteins etc. was utilized to understand the evolution and diversity of these ethnobotanical plants across western ghats.

RESULTS

Genomic DNA extraction of all six samples gave a single, high molecular weight band very close to the well. The concentration of the DNA extracted ranged from 1.8µg/µl to 0.053µg/µl.

RAPD analysis: The PCR amplicons obtained from PCR of genomic DNA of the samples were subjected to agarose gel electrophoresis. For band scoring, 0 was scored for absence of band, while 1 was scored for its presence. A binomial matrix was generated for all the samples with all five primers which were then used to generate "Dominant marker file" in the prescribed format for PopGen16 software. The data of binomial matrix was divided into 6 populations as represented in **Fig 1**.

The Diploid data analysis for dominant marker was chosen for analysis in which “Variable as column” was chosen to be the data format, “Hardy- Weinberg Equilibrium” was the assumption and “Multiple populations” was selected for Hierarchical structure. The following parameters were chosen for estimation- Polymorphic Loci, Gene Diversity, Dendrogram and Shannon Index. Nei’s gene diversity was found to be 0.4096±0.0991 and Shannon’s Information Index was 0.5943±0.1180. The percentage of polymorphic loci was estimated to be 98.67%.

Popgen software analysis as “Groups”: The binary matrix data was re-assessed by categorizing them as Groups, to determine variation between the same plants from different geographical origin. They are generated as follows:

Group 1: *Meyna spinosa* Roxb. ex Link from Nashik and from Mumbai

Group 2: *Catunaregam spinosa* (Thunb.) Triveng from Goa and from Mumbai

Group 3: *Ixora chinensis* Lam from Karwar and from Mumbai

The parameters estimates such as Nei’s genic variation index, Percentage of polymorphic loci and Nei’s genetic distance (by UPGMA method) has been described in **Table 2**. As per this analysis as well, the greatest distance is seen in Group 2, corresponding to *Catunaregam spinosa* (Thunb.) Triveng from Goa and from Mumbai, thereby confirming the former results.

Phylogenetic analysis by DNA barcoding using matK gene:

Amplification products of PCR with matK primers of each sample were observed at around 1000bp. The PCR amplicons were subjected to sequencing and the sequences were submitted to NCBI. They are available on NCBI under the following accession IDs:

Meyna spinosa Roxb. ex Link Nasik- MN108324; *Meyna spinosa* Roxb. ex Link Mumbai- MN096266; *Catunaregam spinosa* (Thunb.) Triveng Goa- MK809391; *Catunaregam spinosa* (Thunb.) Triveng Mumbai- MK697333; *Ixora chinensis* Lam Karwar- MN091839; *Ixora chinensis* Lam Mumbai- MN091840.

Conserved, Variable and Mark Parism Informative sites information: From the aligned data the conserved sequences were found to be 676 out of 842 sequences, which also included gaps. Variable were found to be 155 out of 842 sequences. There were 44 Mark Parism sites found in the aligned sequences. The Distance data matrix was analysed to determine the genetic distances between the plants. Bootstrap consensus tree generated for matK sequences is represented in **Fig 2**.

Phylogenetic analysis by DNA barcoding using rbcL gene:

The amplification products of PCR with rbcL primers of each sample were observed at around 600bp. The amplicons were subjected to sequencing and the sequences were submitted to Genbank. They are available on NCBI under the following Accession IDs:

Meyna spinosa Roxb. ex Link Nasik- MN108323; *Meyna spinosa* Roxb. ex Link Mumbai- MN108322; *Catunaregam spinosa* (Thunb.) Triveng Goa- MN121830; *Catunaregam spinosa* (Thunb.) Triveng Mumbai- MK809390; *Ixora chinensis* Lam Karwar- MN091841; *Ixora chinensis* Lam Mumbai- MN091842.

From the aligned data the conserved sequences were found to be 634 out of 746 sequences, including gaps. Variable were found to be 49 out of 746 sequences. There were 10 Mark Parism sites found in the aligned sequences. The Distance data matrix was analyzed to determine the genetic distances between the plants. The bootstrap consensus phylogenetic tree generated for rbcL sequences, amplified from each plant under study is represented in **Fig 3**.

DISCUSSION

Ixora chinensis Lam sampled from Karwar, *Catunaregam spinosa* (Thunb.) Triveng, sampled from Goa and *Meyna spinosa* Roxb. ex Link sampled from Nasik and their urban (Mumbai) counterparts yielded good quality genomic DNA which gave reproducible results with RAPD analysis. PCR with all five primers (RP01-RP05) yielded amplification products that ranged from approximately 1,500bp- 300bp. Only distinguishable and genuine bands were scored for binary matrix.

From the results of genetic analysis, it can be observed that there is high level of genetic variation between certain sets of plants. The percentage of polymorphism shows 98.67% which signifies highest level of polymorphism among the alleles in these plants. The genetic distance is highest between *Catunaregam spinosa* (Thunb.) Triveng from Mumbai and *Ixora chinensis* Lam from Karwar. And as per the Dendrogram, three clades were expected to arise from the ancestor, instead only two clades resulted. Although from the dendrogram it may seem that *Catunaregam spinosa* (Thunb.) Triveng, from Mumbai and Goa have joined as a clade with *Ixora chinensis* Lam and *Meyna spinosa* Roxb. ex. Link respectively, they actually are subdivided from the ancestor directly, as opposed to them together forming a different clade.

To support the above analysis, the binary matrix of the bands generated from all these plants were analyzed under “Groups”. The Percentage of polymorphism between *Catunaregam spinosa* (Thunb.) Triveng *from* Goa and Mumbai was high (56%) as compared to *Ixora chinensis* Lam from Karwar and Mumbai (28%) and *Meyna spinosa* Roxb. ex Link from Nasik and Mumbai (29.33%). The Nei’s Genetic distance by UPGMA method showed between *Catunaregam spinosa* (Thunb.) Triveng, from Mumbai and Goa was 41.049.

All these results signify that although morphologically they are the same plants, but *Catunaregam spinosa* (Thunb.) Triveng from Mumbai and Goa have genetically evolved differently from each other. To probably adapt to the environmental conditions of the respective geographical location, the genome may have acquired or lost some genes. The loss or gain of certain portion of the genome depends on their use or function to the plant’s survival. The function of a gene sometimes depends on the climatic/ soil/ temperature/ stress conditions of the respective geographical location. Therefore, from this data we may

conclude that geographical location did affect the gene diversity in *Catunaregam spinosa* (Thunb.) Triveng.

We can also infer that, *Catunaregam spinosa* (Thunb.) Triveng, from Mumbai and Goa, both might harbour functionally or structurally different genes of importance, especially those that take part in metabolic pathways of secondary metabolite production. Micronutrients such as Fe, Mo, Cu etc., are known to be co- factors for most of the enzyme's functioning. As soil nutrients are responsible for the micro and macro nutrients supply to the plants, they in turn may cause certain significant enzymes to be produced. As a part of this study, soil collected from the respective sampling sites was subjected to micro and macro nutrient estimation. Nasik showed least quantities of micronutrients (Fe, Zn, Cu, Mb, Mn & Cl) as compared to other study sites. The Goa sampling site soil revealed less Nitrogen and Phosphorous content as compared to other samples. As opposed to the findings of micronutrients, Nasik sampling site showed highest Magnesium, Nitrogen, Phosphorous, Potassium and Calcium content. Although the concept requires further investigation, the soil composition may be proposed to have an effect on genetic makeup of the plants [**Supplementary soil analysis tables 1–5**]. In one study involving impact of water and soil nutrients on genetic variation and clonal diversity of *Carex nigra* in a central alpine fen, it was found that genetic diversity increased with higher phosphorous contents. In contrast, high genetic variation was also observed in low potassium availability, which maybe due to plant's susceptibility to abiotic stress of this deficiency (Reisch et al. 2020).

From DNA barcoding analysis, the phylogenetic trees generated using matK and rbcL sequences differed from that generated by RAPD. The reason could have been due to targeting a single locus in barcoding as opposed to multiple loci in RAPD. The phylogenetic tree with *Vinca major* as the outgroup showed *Meyna spinosa* Roxb. ex Link Nasik and Mumbai as divergent from the ancestor instead of one clade. A closer observation to the tree reveals that *Meyna spinosa* Roxb. ex Link from Mumbai's landscape and weather is much similar to that of Goa and Karwar; The reason maybe the effect of topography- Mumbai, Goa and Karwar all being hilly and share the similar climatic conditions and soil profile, would most certainly group together. Studies such as Reisch and Rosbakh, 2021, assessed the pattern of genetic variation in European plant species growing on different altitudinal zones; they also observed genetic variation among spatially and environmentally isolated populations, which could have resulted from restricted gene flow. While studying the effect of geographic environment on genetic variation in 46 species of *Erianthus arundinaceus* from natural habitats in China, it was observed that the geographic landforms such as oceans and big mountain and river systems played an important role in determining genetic diversity (Zhang et al. 2017). While comparing rare and endemic species of *Petunia secreta*, the studies showed high genetic diversity. The two species corresponded to two separate sites of species occurrence in different landscapes (Caroline et al. 2016). While assessing microsatellite diversity in 94 individuals of 10 wild barley populations, *Hordeum spontaneum* (C. Koch) Thell, across Israel, it was observed that gene diversity was highest in stressful arid hot environments (Timo et al. 2001).

It can also be observed that *Ixora chinensis* Lam from Karwar is most recently evolved from the matK phylogenetic tree and *Ixora chinensis* Lam from Mumbai being most recently evolved from the rbcL

phylogenetic tree. The transitions and transversion substitutions were calculated for barcoding with both the genes; the transition substitutions from C to T and G to A being highest in both the cases. Polymorphism in the gene sequences between a pair of plants from different geographical location is evident, as can be observed in the phylogenetic tree.

Our study reveals the effect of geographical location on genetic make- up of the plants, at least genomic level. Investigation of the similar effect should be checked at expression level by analyzing the RNA, protein, phytochemical fingerprint and bioactivity to obtain a holistic picture if the differences are significantly large or not. The study also provides a preliminary insight into the molecular evolution of select plants representing Rubiaceae family along western ghats of India.

Declarations

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Competing interests:

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

Author Contributions:

All authors contributed to the study conception and design. Material preparation, data collection were performed by Dr. Sharon D'souza and Dr. Sagarika Damle. The analysis of the data was performed by Dr. Sharon D'souza, Dr. Suruchi Jamkhedkar, Mr. Vikas Jha, Dr. Sagarika Damle and Dr. Anupma Harshal. The first draft of the manuscript was written by Dr. Sharon D'souza and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability:

DNA sequences: Genbank accessions for Matk sequences of the following are deposited in Genbank and available under the accession IDs as mentioned: *Meyna spinosa* Roxb. ex Link Nasik- MN108324; *Meyna spinosa* Roxb. ex Link Mumbai- MN096266; *Catunaregam spinosa* (Thunb.) Triveng Goa- MK809391; *Catunaregam spinosa* (Thunb.) Triveng Mumbai- MK697333; Ixora chinensis Lam Karwar- MN091839; *Ixora chinensis* Lam Mumbai- MN091840.

DNA sequences: Genbank accessions for rbcL sequences of the following are deposited in Genbank and available under the accession IDs as mentioned: *Meyna spinosa* Roxb. ex Link Nasik- MN108323; *Meyna spinosa* Roxb. ex Link Mumbai- MN108322; *Catunaregam spinosa* (Thunb.) Triveng Goa- MN121830; *Catunaregam spinosa* (Thunb.) Triveng Mumbai- MK809390; *Ixora chinensis* Lam Karwar- MN091841; *Ixora chinensis* Lam Mumbai- MN091842.

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Tables

Table 1: The RAPD primers used for the amplification of Plant genomic DNA are as follows:

Primer Name	Sequence 5'-3' Direction	Tm ^{o}C
RP01	AAAGCTGCGG	32
RP02	AACGCGTCGG	34
RP03	AAGCGACCTG	32
RP04	AATCGCGCTG	32
RP05	AATCGGGCTG	32

Table 2: The different parameters estimation for each “Group”

	Nei’s genic variation index	Percentage of polymorphic loci	Nei’s genetic distance (by UPGMA method)
Group 1	0.14±0.22	29.33	17.359
Group 2	0.28±0.24	56	41.049
Group 3	0.14±0.22	28	16.425

Figures

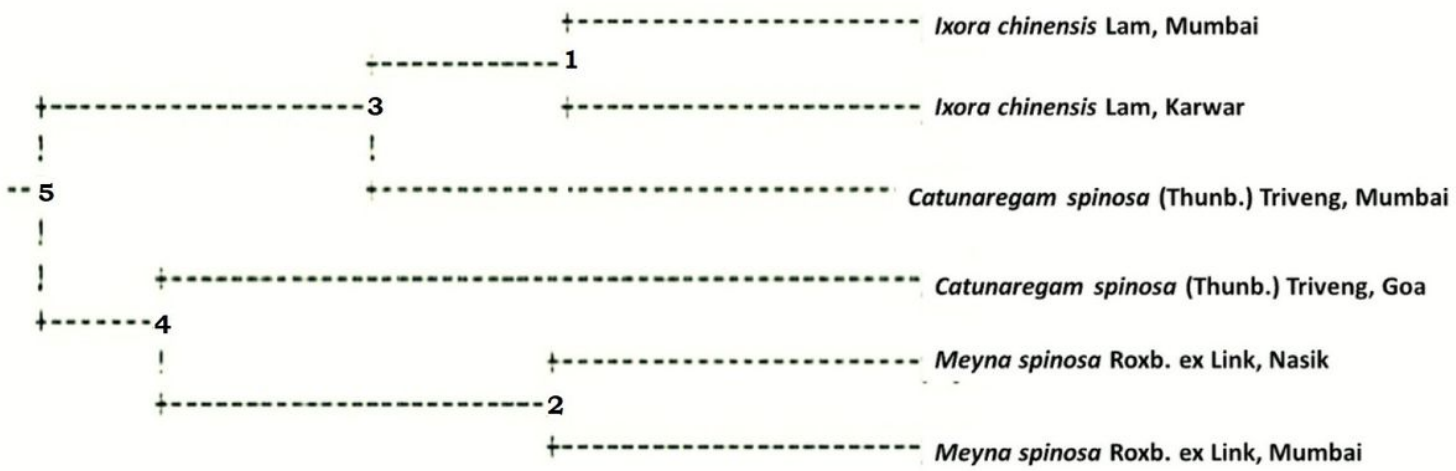

Figure 1

The Dendrogram obtained by UPGMA method based on Nei’s genetic distance.

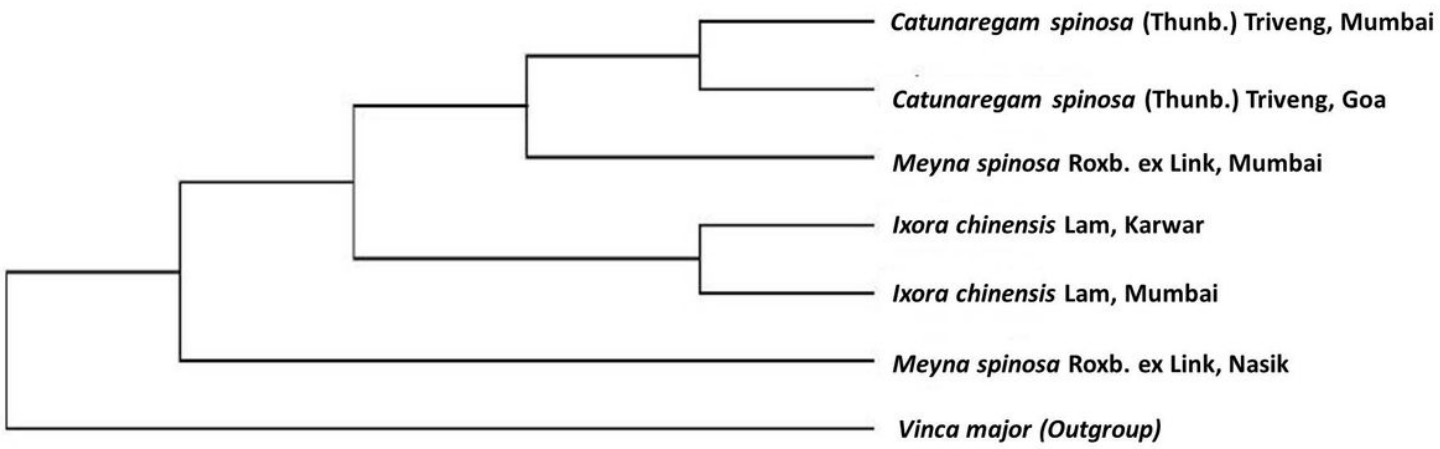

Figure 2

Bootstrap consensus tree generated for matK sequences

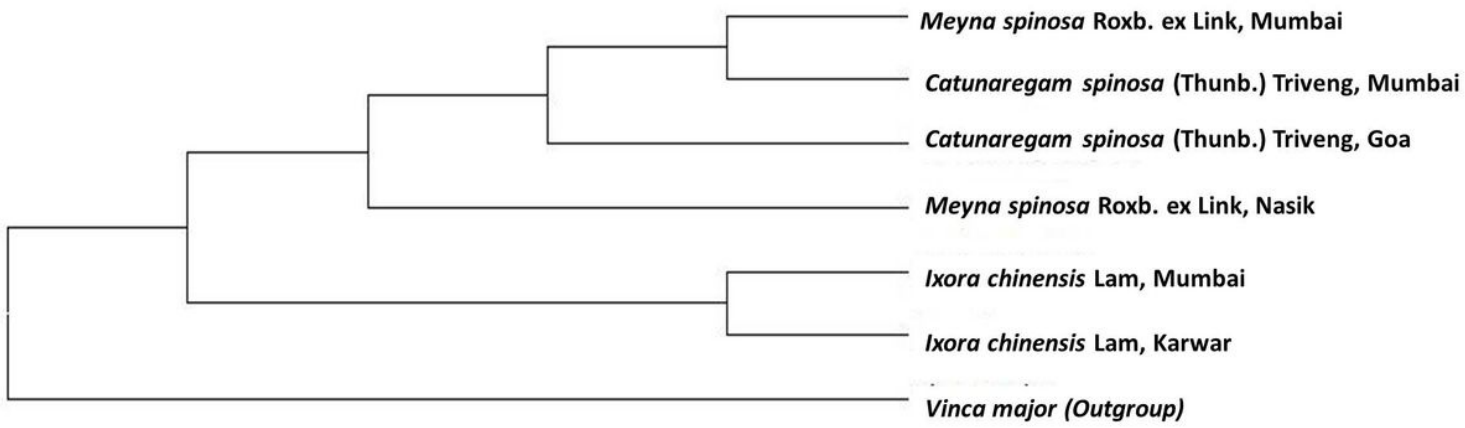

Figure 3

Bootstrap consensus phylogenetic tree generated for rbcL sequences amplified from each plant under study.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables14.pdf