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Evolutionary Trend in *Passiflora* Species Revealed by RAPD-PCR, Gene Specific ITS and rbcl Markers

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Authors' contributions

This work was carried out in collaboration between both authors. Author BDL designed, experimentation, interpretation and analysis of results also wrote the manuscript. Author ASP contributed suggestion in this study. Both authors read and approved the final manuscript.

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ABSTRACT

Background: The present study represents a preliminary analysis of genetic diversity among *Passiflora* species using amplified genotypic data of specific *ITS* and *rbcL* sequences and non-specific RAPD-PCR markers for investigation of the molecular phylogeny.
Methods: The PCR-RAPD uses ten primers for polymorphic DNA, which are compiled on NTSYS software to construct dendrogram. The gene specific *ITS* and *rbcL* primers are used for specific amplification from genomic. The amplified *ITS* and *rbcL* markers assembled using Maximum Parsimony (MP) and Maximum likelihood (ML) methods. The BLAST, CLUSTAL W, and MEGA 6.0 have been used to conclude final genetic relation tree.
Results: The PCR-RAPD primers translate 133 random amplified polymorphic DNA. NTSYS dendrogram placed *P. vitifolia* from Ramdaspeth and Shankar Nagar Nagpur, India in same clade (similarity coefficient 0.609) confirming same origin Nagpur India. Moreover, *P. foetida* from England is not coming in same clade with Indian *P. foetida* showing geographically intra-specific variation. In addition, the change in a constructed tree was observed with respect to change in

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phylogeny methods MS/ML. The *ITS* MP consensus tree is supported by strong 100 bootstrap value, clusters *P. vitifolia* (HNI and RNI) and *P. foetida* (UAI and UGI) in equivalent clade. However, no single species have been recovered using *rbcL* in MP and ML method. Thus, it is inference that *rbcL* have tendency to differentiate *Passiflora* species not allowing clustering around same species in same clade and the *ITS* region having parsimony informative sites that provide valid resolution and identification at inter-intra species level.

Conclusions: The evaluation of properties of RAPD indicates 100% PCR success, sometimes with low rate of amplification. The *ITS* region found to be best for identification at inter-intra species, On the contrary, *rbcL* region is good to distinguished inter species, making it best as local DNA barcode for marking a *Passiflora* species in phylogenetic community.

Keywords: Passiflora species; PCR-RAPD; ITS4 & rbcL markers; NTSYS; MEGA 6.0 software; maximum parsimony (MP); maximum likelihood (ML); dendrogram; genetic diversity.

1. INTRODUCTION

The classification of plants allows to differentiate among, pharmaceutical important plants that are poisonous. The plant species have characteristic traits helpful for classification. In early times, diversity was purely based on the visual morphological characters [1]. Despite that, some plants have similar external features that made extremely difficult to identify which leads to wrong classification, which is solely biochemical based. Nevertheless, results produced were confusing and had errors, making it unacceptable to scientific community [2].

In the modern taxonomy classification molecular markers are used, which results from deletion, duplication, inversion and insertion are use for assessment of plant genetic diversity. Recently sequence-related amplified polymorphism (SRAP marker) [3]. single-nucleotide polymorphism (SNP) markers [4] are used. Phylogenetic analysis in *P. foetida* from Australia has been investigated using the chloroplast genome data [5].The molecular marker is gene specific and non specific created library using different genes represents diversity among plants. A good review paper by Mondini *et al* [6] and Agrawal *et al.* [7] explained them in detail. The RAPD analysis of *Passiflora* species has been successfully done by Fajardo *et al* [2]; Molinari and Crochemore [8]; Carneiro *et al* [9]; Crochemore *et al* [10]; Loss *et al*. [11]; Junqueira *et al.* [12]; Junqueira *et al.* [13]; Fonseca *et al.* [14]; Viana *et al*. 15]; Castro *et al.* [16].

In animals, COI gene (DNA barcode) is conventionally used to resolve phylogenetic relation [17,18]. Similarly, plastid (chloroplast) ribulose-1,5-bisphosphate carboxylase/oxygenase *(rbcL)* [19-21] and mat K genes are stamped for barcode in plants [22]. However, non coding ITS region i.e internal transcribe spacers of nuclear ribosomal DNA (nr DNA ITS) data compliments the core barcode data [18,23] and used to study evolutionary studies to infer phylogeographic patterns in a wide range of species [24-26]. Therefore, must be incorporated in barcode of plants (Li et al. 2011). The three endangered trees *Coptosperma, Graveolens sub sp. Arabicum (S. Moore) Degreed* has been successfully profiled using PCR-RAPD and *rbcL* gene sequences [27].

The molecular data matrix generated using the sequenced genes and are applied to dendogram construction using software to develop phylogenetic tree by various tree building methods. If sequence similarity is strong, Maximum Parsimony is used: which uses a character-based data matrix (0, 1). Similarly, if sequence similarity is very weak, maximum likelihood is used. Two statistical method tests Bootstrapping (500-1000 time), and Jacknifing with branch point score is around 90%, is best predicated accurate tree analyzed by a) Kishino-Hasegawa b) Bayesian analysis c) Shimodairo- Hasegawa methods [28].

Several plants species have been classified on the basis of modern taxonomy. The *Passiflora* species are valuable as ornamental plants found in the tropical and subtropical region of the globe [29]. They have undergone natural hybridization to form new identities, leading to diversification with beautiful flowers which have been confusing botanists, molecular biologists and taxonomist [2]. Their diversity is also reflected from their inherited pharmacological constituents such as alkaloids, phenols, flavonoids, iso flavonoids, glycosides, esters, volatiles, chrysoeriol, apigenin, luteolin, kaempferol, isoschaftoside, vitexin and isovitexin [30]. These constituents are

used for one of the treatments of various human diseases such as anxiety, asthma, biliousness, sedative, cough, pain, hysteria, headache, insomnia, sexual dysfunction, skin diseases [31,32] digestive problems, including dyspepsia, expectorant nervous conditions, spasms, anti-ulcer, inflammation [32], cancer, diabetes mellitus and rheumatoid arthritis [33].

In the last decade, the phylogenetic studies of *Passiflora* species have been carried out using molecular markers such as *rbcL* and *rps4* genes, the *trnL* intron and *trnL-F* intergenic spacers from the plastid genome, the *nad1* b/c and *nad5* d/e introns from the mitochondrial genome, and a portion of the *26S* gene from the nuclear ribosomal genome organized it into four subgenera: *Astrophaea*, *Decaloba*, *Deidamiodes*, and *Passiflora* [26]. Thus in present work, by using a RAPD marker along with ITS and r*bcL*. Extensive phylogenetic study is conducted to construct evolutionary relation among *Passiflora* species originated from India and England. The phylogenetic tree generated using RAPD data; ITS and *rbcL* sequences were compared and definite classification of *Passiflora* species is presented.

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

The (CTAB- cetyl-trimethyl ammonium bromide detergent), Polyvinylpyrrolidone (PVP) 40000 (1%), liquid nitrogen were used for DNA isolation. Enzymes, Buffers and Instruments: Dream Taq DNA polymerase, Rnase A and Protinase K (Fermentas Inc), Buffer: 10x Dream Taq Green buffer (Fermentas Inc), Nucleotides: dNTPs (G, A, T, C) 2 mM, Gradient thermocycler PCR, Gel electrophoresis in 1X TAE buffer at 50 V, EtBr (ethidium bromide), Gel document system (BioRad). The RAPD primers and *ITS* and *rbcL* marker primer sequence along with references are shown in Table 1. The sequencing of *ITS* and *rbcL* primer done using ABI Big Dye Terminator V3.1 Cycle Sequencing Kit, at Bioresource Biotech Pune, Maharashtra, India.

2.2 Plant Material

The leaves of *Passiflora* species have been used for isolation of genomic DNA. The specific codes have been given to precise *Passiflora* species and their place of origin have been displayed in Table 2. Sampling source: The sampling source from which the experimental plants have been retrieved are shown in Fig. 1. The maps were captured from Google mapsImagery©2015 DigitalGlobe, Getmapping plc, Infoterra Ltd & Bluesky, The Geoinformation Group, Map data ©2015 Google. The maps display distance between the sampling source UK and India. A: Time duration between chiltern seed Willingford UK (CWE) and Ambazari hill top Nagpur (HNI) Maharashtra India is 14 hr 15 min; chiltern seed center location in zoom mode and interval between Ambazari Hill Top (HNI) Nagpur to Ramdaspeth Nagpur (RNI) is 3.7 Km (Approximate 10 min away), B: The gap between Sant Gadge Baba Amravati University campus (AUI) and Ambazari Hill Top (HNI) Nagpur is 150 Km and it takes approximately 2 hrs travel time. The distance between Shankar Nagar (SNI) and Ramdaspeth (RNI) is 2.5 Km while, the stretch between Shankar Nagar (SNI) and Hill Top Nagpur (HNI) is 1.8 Km.

2.3 DNA Extraction

The DNA extraction is performed by improved CTAB method without using liquid nitrogen as described in our previous published work [34].

2.4 PCR Amplification of RAPD

The RAPD reactions were performed using 20 µl of volume containing 20ng of genomic DNA, 2 µl 10X Taq Green Buffer, 0.2 mM of dNTP mix, 0. 20 mM of primers (see Table 1), milli Q water 11-13.5 µl appropriately to make up volume of the reaction, with 0.5 U of Green Taq Polymerase. The gradient thermocycler PCR was programmed for initial denaturation (1) step of 95°C for 4 min followed by 40 cycles of (2) step, 1 min for 94°C, 1 min for 25°C to 43°C, 1 min for 72°C with final extension (3) step of 10 min for 72°C.

NTSYSpc is a Numerical Taxonomy and Multivariate Analysis System Version 2.0 software used to construct dendrogram by molecular matrix data generated in PCR-RAPD analysis. The 12 *Passiflora* species, PCR-RAPD generated fragments used for marking the presence (1) and absence (0) of bands among all species [35]. These 0 and 1 were saved in excel file and used for dendogram construction with UPGMA algorithm [2]. Import excel file from Ntedit program using DDE option was used. Ntsys application version (2. 02i) was opened, and similarity option was selected and further simqual option was carried out. Clustering was performed using SAHN and output file C is

computed to generate dendogram. The compute button takes matrix and generates a Phylogenetic tree and results saved in PDF/ other formats for interpretation of the results.

2.5 PCR Amplification of *ITS4* and *rbcL* Sequence

The primers given in Table 1 were used for PCR amplification of *ITS* and *rbcL* sequence from *Passiflora* species. The final reaction mixture have a final volume of 20 µl, containing 20 ng of genomic DNA, 2 µl 10X Taq Green Buffer, 0.2 mM of dNTP mix, 0. 20 mM of *ITS/ rbcL* primers (forward and reverse) and 0.5 U Green Taq Polymerase. In gradient thermocycler PCR, the programme set as initial denaturation (1) step of 95°C for 3min followed by 30 cycles of (2) step, 30sec for 94°C, 40sec for 55°C, 30sec for 72°C with final extension (3) step of 10 min for 72°C. The 1 µl of amplified DNA from RAPD and *ITS, rbcL* were checked on 1.4 % and 1.2 % agarose gel electrophoresis respectively in 1X TAE buffer at 50 V for three hours stained with 0.5 µg/ml EtBr (ethidium bromide). Finally gel electrophoresis photographed were captured by a gel document system and samples were sent for sequencing.

2.6 Sequencing of *ITS4* and *rbcL* DNA Sequence

In total 52 counts of PCR products were generated among them 26 *ITS* (F/R) and 26 *rbcL* (F/R) were sequenced using ABI Big Dye Terminator V3.1 Cycle Sequencing Kit. The sequence samples applied for BLAST analysis.

2.7 Basic Local Alignment Search Tool (BLAST)

In BLAST search, on NCBI web site, BLAST n selected for nucleotide BLAST (search set), with file FASTA format; optimized for highly similar sequences and clicked BLAST. The several homologous sequences from NCBI database was searched and displayed as result page with the most similar for *ITS* and *rbcL* sequences at the top of the page.

2.8 Molecular Evolutionary Genetics Analysis software (MEGA 6.0)

The sequenced amplified DNA from *ITS* and *rbcL* sequences were assembled for MEGA analysis. It is automatic and manual sequence alignment, building sequence alignments, inferring phylogenetic histories, and conducting evolutionary analysis. In MEGA the two methods maximum parsimony (MP) and maximum likelihood (ML) method were applied as given in Fig. 2 for phylogenetic tree construction.

2.9 Maximum Parsimony (MP) Method

The MP parameters are set automatically in MEGA for *ITS* sequences, nucleotide substitution model with complete deletion of gaps, bootstrap replications set as 1000 and Tree-Bisection-Reconnection (TBR) with 10 number of initial trees, search levels was set 1 and the tree length as 338 parsimony tree 3. Furthermore, same parameters were applied to *rbcL* sequences except, the tree length was 2380, parsimony tree 1 with bootstrap greater than 40% are finalized.

2.10 Maximum Likelihood (MP) Method

Similar to MP method, the ML method considered 1000 bootstrap of replications with deletion of gaps and missing data. The ML characteristic for *ITS* and *rbcL* sequences aligned by clustal W, pair wise and multiple sequence alignments include total 1101 sites with gaps, 781 without gaps and 520 positions respectively. These nucleotide substitution model Kimura 2-parameter was applied and final consensus tree reconstructed by heuristic method with Nearest-Neighbor-Interchange (NNI). The consensus tree, conflicting branching patterns are resolved by selecting the pattern seen in more than 50% of the trees.

2.11 Clustal W

All sequences of (*ITS* and *rbcL)* were considered for pair wise, multiple alignments using CLUSTAL W from MEGA software for phylogenetic tree construction to get number and sequence similarity of nucleotide selected for alignments.

2.12 Bold Search

The sequenced *rbcL* sequence were copied and pasted in a web-based site at plant identification (rbcL and mat K) BOLDSYSTEMS for possible identification of species.

3. RESULTS

3.1 DNA Isolation

The isolated DNA from all the mention species of *Passiflora* produced 168.2 to 1782.5 µg/ml DNA

from 0.5 g of leaf samples; A 260/280 = 1.80 ± 22 and A 260/ 230 = 1.75 ± 20. The optimization of RAPD-PCR protocol and parameters for amplification of genomic DNA fragments are discussed in Lade et al. [34].

3.2 Evaluation of RAPD Marker

The Fig. 3 shows the DNA fingerprinting profile for RAPD-PCR primers (P1-P10). The primer 1-10 turned out to be a good polymorphic marker by producing 133 polymorphic 10-17 bands.

3.3 Tree Description Based on Matrix Data of RAPD

The constructed phenogram using NTSYSpc software is shown in Fig. 4, which consists of two clusters, cluster I and cluster II. Cluster-I consist of two sub clades, which included *P. incarnata* UAI, *P. vitifolia* HNI in 1st clade and *P. foetida* CWE, *P. Lady Margaret* HNI, *P. vitifolia* RNI, *P. vitifolia* SNI, *P. incarnata* SNI in 2nd clade. while, cluster II consist of one clade which contented *P. foetida* UAI, *P. foetida* HNI, *P. caurelia* CWE, *P. incarnate* RNI, *and P. incarnata* CWE. The dendogram confirms high levels of variation within and among *Passiflora* species. A dendrogram placed *P. vitifolia* from Ramdaspeth Nagpur, India and *P. vitifolia* Shankar Nagar Nagpur, India in cluster I and same clade no 2 confirming that they belong to identical geography Nagpur India*. P. foetida* from England (cluster-I) is appearing in a different cluster with *P. foetida* from India (cluster-II) showing geographically intra-specific variation among species.

3.4 Sequencing of Amplified *ITS* and *rbcL* Sequence

The positive PCR product sequenced successfully as *ITS* 680 bp Ramaiya et al [25] and *rbcL* 688 bp Laiou et al [23] and Kress et al. [36] from 13 *Passiflora* species confirmed in gel electrophoresis photographs Fig. 5A & B respectively.

3.5 Evaluation of rbcL and ITS4 Markers

In assessment of *rbcL* and *ITS4* markers we successfully sequenced 25 *rbcL* and 21 *ITS* sequences from 13 *Passiflora* species. However, only, *rbcL* 13(F) and 13 *ITS* (R) were considered for phylogenetic analysis. The Table 3 shows evaluation of molecular properties among RAPD, *ITS* and *rbcL* loci in a present study.

3.6 BLAST analysis of ITS and rbcL sequences

The individual sequenced nucleotide was searched against the Gen Bank database using BLAST nucleotide tool and deposited in GENEBANK. The deposited sequence gets a unique identifier as an accession number for each gene sequence (see Table 2 for *ITS* sequences). The BLAST search has identified most similar sequences (search match) for query sequences of *ITS* and *rbcL* that yields 61 % and 15 % of species level respectively (see Table 4).

3.7 Bold Search Analysis

The sequenced *rbcL* query sequences have not returned as an authentic match that may have occurred due to the less numbers of *rbcL* records of *Passiflora*. The BOLD search results for query sequences are shown in Table 5.

3.8 ITS and rbcL Sequences Characteristic

The final data set of aligned 13 *ITS*/*rbcL* sequences characteristic are consider in current phylogenetic investigation. The aligned multiple sequences of ITS site count were 1011 sites with gaps and 924 without gaps. The conserved site were 320, variable sites were 560; Parsimony information sites were 289 and 266 singleton sites respectively. In contrast, for *rbcL* sequences total site used 778 with gaps and 727 without gaps for pair wise and multiple sequence alignments. Then after multiple alignments by clustal W from 764 total sites, the conserved identified as, variable site at 731 parsimony information site were 717 and 12 singleton site respectively.

3.9 Phylogenetic Analysis

The phylogenetic tree is formed based on molecular data of RAPD, sequenced *ITS* and *rbcL* sequences from the *Passiflora* species in MEGA 6.0. The *rbcL* is a coding gene, therefore, the phylogeny construction analysis was performed using the amino acid with Jones-Taylor-Thornton (JTT) model, and tree was inferred by Nearest-Neighbor-Interchange (NNI) ML Heuristic Method. While, *ITS* is non-coding thus nucleotide sequence is considered.

3.10 Tree Description of *ITS4* MP

The results of MP algorithms of *ITS4* sequences show that the *Passiflora* species were distributed into four sub clades, and no specific clade/ cluster was observed (see Fig. 6A). The *P. incarnata* RNI and *P. incarnata* SNI has been a cluster together at a top of a tree with bootstrap score 74. The node joining bootstrap value is 62 to top clade (74) and *P. incarnate* UAI whose descendent is the *P. Lady margrate* (bootstrap score 74). The *P. vitifolia* RNI and HNI are in clade showing similarity at a genetic level as they originate from a nearly equivalent region (bootstrap score 59). The 5 species *P. incarnata* RNI, *P. incarnata* SNI, *P. incarnata* UAI, *P. Lady Margate, P. vitifolia* SNI and *P. vitifolia* RNI*, P. vitifolia* HNI has the same ancestor. The *P. incarnate* CWE and *P. caurelia* CWE both are different species. However, they are coming in the same clade showing genetic similarity due to a same geographical origin. The *P. foetida* is descendent to other species of CWE England that are in sub clades. The 4th well distinguished sub clade includes *P. foetida* UAI and UGI and *P. foetida* HNI is originated from them*. P. foetida* UAI and UGI are grouped together due to their identical growth environment and same region of Amravati based on species similarities. The *P. foetida* and *P. caurelai* are diverse from each other and appeared to a distance group. Similar results were observed by Muschner et al. [37]. The Fig. 6B shows consensus trees prepared by marking multifurcating nodes, in which conflicting branching patterns are resolved by selecting the pattern seen in more than 50 % of a tree consensus tree based on *ITS4* sequence. The bootstrap score of nine nodes is a consensus for 100 scores. The MP tree is in agreement of morphological characters confirming their proper grouping in a constructed.

3.11 Tree Description of *rbcL* MP

The MP tree is divided into two main cluster (see Fig. 7A), cluster-I and cluster-II. Cluster-I (top) is bifurcated into two major branches, branch 1 consists of *P. lady Margaret* HNI, *P. foetida* HNI(bootstrap value 100), *P. incarnata* UAI, *P caurelia* CWE and *P. vitifolia* HNI, *P. incarnata* RNI (bootstrap value 100) and branch 2 consist *of P. vitifolia* RNI and *P. incarnata* SNI (bootstrap value 48%). The -II include *P .foetida* CWE and *P. vitifolia* SNI, *P. foetida* UAI (bootstrap value 100%). Lastly, *P. foetida* UAI and *P. incarnate* CEW separated out from rest of the 2 cluster with 100% Bootstrap value. Fig. 7B shows consensus tree from rbcL sequences.

4. DISCUSSION

The PCR-RAPD markers are successfully used for assessment of genetic diversity in present study [38] and there are reports for population genetics matrix using RAPD [39,40]. The PCR RAPD experiments uses ten primers produces 133 random amplified polymorphic DNA, Nevertheless, Chtourou-ghorbel et al. [40] use10 primers and obtained 127 polymorphous bands. The most important observation for PCR-RAPD study is that *P. vitifolia* (RNI) and *P. vitifolia* (SNI) are grouped side by side in tree showing highly relatedness. This supports the truth and confirms that they originated in around 3 km similar geographical area of Nagpur. These observations correspond with the previous taxonomy and genetic diversity studies suggesting similar species assembled in same clade and diverse species distributed unevenly as suggested by Loss et al. [11]. On comparison of constructed tree from RAPD and ITS sequences, we found some similar results for *P. incarnata* UAI and *P. lady margrate* HNI that displays evolutionary similarity at the different time. The *P. caruelia* and *P. incarnata* from England is having the common ancestors. However, not co-evolved in RAPD tree in contrast, show the co-evolution in ITS tree.

The maximum parsimony (MP) based, and maximum likelihood (ML) based trees were very similar in *ITS4* sequences. For ML for bootstrap higher than 40% using *ITS* (see Fig. 8 A) is slight variation. However, MP and ML show strong bootstrap value for tree, which is in accordance with the species classification. The MP and ML topology, tree using ITS, develops the tree which is up to the mark, with the inter and intra species diversity and is in agreement with the morphological characters. The tree constructed using the ITS sequences in MP method has higher bootstrap value than the tree constructed using ML method as observed by Muschner et al [37]. There is no major difference in a tree except one was *P. foetida* CWE in ML method separate out independently. From MP and ML tree, it is confirmed that, the ancestor *P. caurelia* (C) nucleotide has undergone mutation of A/ T/G for the *P. foetida* [37].

The Muschner et al [37] investigated and found the similar results for MP and ML topology using 61 Passiflora species classified in four genera and 11 subgenera. For phylogenetic tree construction on a basis of ML analysis yields three major clades. In case of *rbcL*, it was observed that both method (MP) based and (ML)

based trees (Fig. 8B) were very dissimilar. The variation in the bootstrap value was observed in both method trees. Even for *rbcL* sequences low branch support was observed in most cases, which urge for more data support. Thus, the MP and ML topology using *rbcL* develops the tree which is not up to the mark for the inter and intra species diversity.

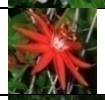
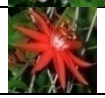
The profile developed using MP & ML for ITS displayed sequences differentiation, and the tree produced shows well resolved inter and intra-species separation in contrast; *rbcL* profiling produced a very different tree, purely inter species-level differentiation. The clades formed were merely the mixture of species, which leads that *rbcL* was not able to resolve any of the experimental species. Except in *rbcL* BLAST search, that identified two species correctly. The *rbcL* does not produce a clade for intra species rather it supports and produce a clade for inter species, suggesting *rbcL* ignoble performance due to low rate of mutation [27]. The sequenced *ITS4* and *rbcL* genes varies greatly in size (bp) and have different GC contents, which reflect the reproductive characteristic, deletion, duplication and excellent rate of evolution of species. The *ITS4* sequences from all the *Passiflora* species show valid conservation and variation in conserved sites (informative sites). Thus, is always the best choice for phylogenetic studies in plants [25]. This region shows better resolution at a species level and useful for genetic diversity study of Passiflora when compared with *rbcL* sequences, *ncpGS* and *trnL-trnF* [24,41,25]. The *ITS* region is considered as the complementing sequence data for DNA barcoding with the advantage of easy amplification. On comparison, it was found that GC content of *rbcL* sequence is 16.4% less than that of *ITS4* sequences.

The other *ITS4* and *rbcL* sequences of top three accession of *Passiflora* species (Table 4) from NCBI based on sequences similarities were also used for establishment of position of trial species in history of an evolution tree. Total 39 accessions were taken from NCBI along with 52 test for both the *ITS4* and *rbcL* gene sequence. A very important evolutionary trend has been observed in the radiate tree, here if we observe the radial tree in clock wise the radial tree concludes a specific pattern of species in order *P. edulis, P. vitifolia, P. cincinnata, P. alata, P. foetida, P. incarnata and P. caurelia.* Though the trial plants grouped in separate clade, the same order of pattern was observed within the experimental species in order *P. vitifolia, P. foetida, P. incarnata and P. caurelai.* The phylogenetic tree resulting from (*ITS4* and *rbcL*) sequence data analysis suggests that the phylogenetic tree constructed has a bootstrap value about 90% (*P. incarnata* CWE, *P. incarnata* RNI), which support strong prediction. The *ITS4* gene provides a valuable set of characters for tree construction and evolutionary studies in Passiflora. The *rbcL* sequences applied to MP and ML method did not identify a species correctly thus establishing its use as a local barcode for development of community phylogeny [19,27].

Table 1. Shows the name of primer fragment (Fw/Rw), respective sequences and references that were used in experimentation of PCR-RAPD and *ITS /rbcL* amplification

Sr. no.	Primer fragment	Sequence (5 ’⟶ 3’)	References
1	Akansha 1	OPA04-AATCGGGCTG	Crochemore et al. [10]
2	Akansha 2	OPB08-GTCCACACGG	Crochemore et al. [10]
3	Akansha 3	OPB18-CCACAGCAGT	Crochemore et al. [10]
4	Akansha 4	OPB19-ACCCCCGAAG	Crochemore et al. [10]
5	Akansha 5	OPB20-GGACCCTTAC	Crochemore et al. [10]
6	Akansha 6	1-CCTGGGCTTC	Aukar et al. 2002
7	Akansha 7	5-CCTGGGTTCC	Aukar et al. 2002
8	Akansha 8	53-CTCCCTGAGC	Aukar et al. 2002
9	Akansha 9	54-GTCCCAGAGC	Aukar et al. 2002
10	Akansha10	CGGGAGACCC	Chalmers et al. [42]
11	*ITS*	TCCGTAGGTGAACCTGCGG	Ramaiya et al. [25]
12	*rbcL*	(Fw)	Ramaiya et al. [25]
		CCTCCGCTTATTGATATGA	Kress et al. [36]
		(Rw)	Kress et al. [36]
		ATGTCACCACAAACAGAAAC	
		(Fw)	
		TCGCATGTACCTGCAGTAGC	
		(Rw)	

Table 2. Represent the sampling source of *Passiflora* species, flower with specific code used for particular species, source code, countries to which they belong and their location on Google's map are summarized

Sr no	Code	Species	Flower	Source	Source code	Gene bank (NCBI) Accession no for *ITS*	Location Map (See Fig. 2)	Country
1	A	*Passiflora incarnata*		Amravati University	UAI	KT003196	B	India
2	B	*Passiflora vitifolia*		Hill top Nagpur	HNI	KT003197	B	India
3	C	*Passiflora foetida*		Chiltern Wallingford	CWE	KT003198	A	England
4	D	*Passiflora foetida*		Amravati University	UAI	KT003199	B	India
5	E	*Passiflora incarnata*		Chiltern Wallingford	CWE	KT003200	A	England
6	F	*Passiflora Lady Margaret*		Hill top Nagpur	HNI	KT003201	B	India
7	G	*Passiflora incarnata*		Ramdaspeth Nagpur	RNI	KT003202	A	India
8	H	*Passiflora vitifolia*		Ramdaspeth Nagpur	RNI	KT003203	A	India
9	I	*Passiflora incarnata*		Shankar nagar Nagpur	SNI	KT003204	B	India
10	J	*Passiflora vitifolia*		Shankar nagar Nagpur	SNI	KT003205	B	India

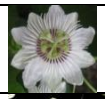
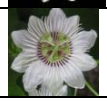
Sr no	Code	Species	Flower	Source	Source code	Gene bank (NCBI) Accession no for *ITS*	Location Map (See Fig. 2)	Country
11	**K**	***Passiflora foetida***		**Hill top Nagpur**	**HNI**	**KT003206**	**B**	**India**
12	**L**	***Passiflora caurelia***		**Chiltern Wallingford**	**CWE**	**KT003207**	**A**	**England**
13	**M**	***Passiflora foetida***		**Amravati University**	**UAI**	**KT003208**	**B**	**India**

Table 3. Shows the evaluation of molecular properties such as PCR, sequencing success, sequence length, overall distance in maximum parsimony and maximum likelihood method, informative, singleton, variable and conserved site, GC %, resolutions of species (%) of RAPD, ITS and *rbcL* loci in a present study

Sr. No.	Parameters	ITS	*rbcL*	RAPD
1	PCR success (%)	100%	100%	100%
2	Sequencing success for 52 PCR(%)	100%	100%	*
3	Sequencing success for 26 PCR(%)	100%	99%	*
4	Sequencing success over all (F)	44.44%	100%	*
5	Sequencing success over all (R)	83.33%	92.30%	*
6	Sequence length	1101	778	*
7	Aligned length (bp)	924	764	*
8	Overall distance in 52 sequences from NCBI (ML method)	0.602	2.741	*
9	Overall distance (MP method)	0.147	3.315.	*
10	No of parsimony informative sites (%)	289	717	133
11	Variable informative sites (%)	560	731	133
12	Singleton site	266	12	*
13	Conserved site	320	3	*
14	Number of sample	13	13	12
15	%GC	59.6	43.5 %.	*
16	Resolution of species (%)	8/13	2/13	2/12

*Note: *: not applicable and not perform, MP: maximum parsimony method, ML: maximum likelihood method, bp: base pair, PCR: polymerase chain reaction*

Table 4. Show's the BLAST database search match for similar and phylogenetic relationship using *ITS* and *rbcL* sequences

		ITS sequences				rbcL sequence			
Sr no	Species with code	Morphological identification	BLAST search match & similarity (%)	BLAST top 3 search & similarity (%)	Phylogenetic affinity	Morphological identification	BLAST search match & similarity %	BLAST top 3 search & similarity (%)	Phylogenetic affinity
1	*Passiflora incarnata* UAI	*P. incarnata* (781bp)	*P. incarnate* 89% *P. incarnate* 87% *P. edulis* 87%	*P. edulis* 87% *P. edulis* 87% *P. edulis* 87%	*P. incarnata*	*P. incarnate* (734bp)	*p. qabrielliana* 99% *P. incarnate* 98% *P. incarnata*98%	*P. cerasina* 92% *P. qabrielliana* 92% *P. edulis* 93%	*P. incarnata*
2	*Passiflora vitifolia* HNI	*P. vitifolia* (499bp)	*P. vitifolia* 89% *P. jileki* 89% *P. cincinnata* 87%	*P. vitifolia* 89% *P. cincinnata* 87% *P. cincinnata* 87%	*P. vitifolia**	*P. vitifolia* (764bp)	*P. vitifolia* 99% *P. vitifolia* 99% *P. coccinea* 99%	*P. vitifolia* 99% *P.vitifolia* 99% *P. coccinea* 99%	*P. vitifolia**
3	*Passiflora foetida* CWE	*P. foetida* (732bp)	*P. foetida* 83% *P. foetida* 82% *P. foetida* 82%	*P. foetida* 82% *P. foetida* 93% *P. foetida* 82%	*P. foetida**	*P. foetida* (704bp)	*P. foetida* 99% *P. palmeri* 99% *P. qiberti* 98%	*P. cilliata* 98% *P. palmeri* 99% *P. tokuoka* 98%	*P. foetida*
4	*Passiflora foetida* UAI	*P. foetida* (858bp)	*P. foetida* 95% *P. foetida* 94% *P. foetida* 94%	*P. foetida* 94% *P. foetida* 95% *P. foetida* 94%	*P. foetida**	*P. foetida*	*P. foetida* 99% *P. platyloba* 98% *P. foetida* 97%	*P. cilliata* 97% *P. tokuoka* 97% *P. foetida* 97%	*P. foetida*
5	*Passiflora incarnata* CWE	*P. incarnata* (779bp)	*P. edulis* 98% *P. incarnate* 93% *P. incarnata* 93%	*P. edulis* 98% *P. edulis* 98% *P. edulis* 98%	*P. incarnate*	*P. incarnata*	*P. incarnate* 99% *P. incarnate* 98% *P. incarnate* 98%	*P. edulis* 100% *P. odontophylla* 99% *P. edulis* 99%	*P. incarnata*
6	*Passiflora Lady Margaret* HNI	*P. Lady Margaret* (634bp)	*P. villosa* 89% *P. tinuifila* 89% *P.oerstedii* 88%	*P. incarnate* 95% *P. Cincinnata×Incarnata* 94% *P. edulis* 90%	*P. Lady Margaret*	*P. Lady Margaret*	*P. umbilicata* 99% *P. galbana* 99% *P.serratifolia* 99%	*P. incarnate* 99% *P. incarnate* 99% *P. incarnate* 99%	*P. Lady Margaret*
7	*Passiflora incarnata* RNI	*P. incarnata* (647bp)	*P. incarnate* 91% *P. incarnate* 90% *P. edulis* 90%	*P. edulis* 90% *P. edulis* 90% *P. edulis* 90%	*P.incarnata*	*P. incarnata*	*P. incarnate* 98% *P. incarnate* 98% *P. incarnate* 98%	*P. cerasina* 99% *P. qabrielliana* 99% *P. edulis* 99%	*P. incarnata*
8	*Passiflora vitifolia* RNI	*P. vitifolia* (650bp)	*P. vitifolia* 99% *P. cincinnatta* 95% *P. edulis* 95%	*P. vitifolia* 99% *P. cincinnata* 95% *P. edulis* 94%	*P. vitifolia**	*P. vitifolia*	*P. vitifolia* 98% *P. vitifolia* 98% *P. candida* 98%	*P. cilliata* 99% *P. tokuoka* 99% *P. foetida* 99%	*P. vitifolia*
9	*Passiflora incarnata* SNI	*P. incarnata* (654bp)	*P. incarnata* 90% *P. alata* 89% *P. edulia* 89%	*P. vitifolia* 93% *P. alata* 92% *P. cincinnata* 93%	*P. incarnata*	*P. incarnata*	*P. incarnate* 98% *P. incarnate* 98% *P. incarnate* 97%	*P. ambiqua* 98% *P. cerasina* 99% *P. qabrielliana* 99%	*P. incarnata*
10	*Passiflora vitifolia* SNI	*P. vitifolia* (761bp)	*P. cincinnata* 94% *P vitifolia* 93% *P. alata* 92%	*P. alata* 89% *P. incarnate* 90% *P. edulis* 89%	*P. vitifolia**	*P. vitifolia*	*P. vitifolia* 99% *P. vitifolia* 99% *P. riparia* 99%	*P. vitifolia* 99% *P. vitifolia* 99% *P. coccinea* 99%	*P. vitifolia*
11	*Passiflora foetida* HNI	*P. foetida* (856bp)	*P. foetida* 95% *P. foetida* 94% *P. foetida* 94%	*P. foetida* 95% *P.foetida* 95% *P. foetida* 94%	*P. foetida**	*P. foetida*	*P. foetida* 99% *P. clathrata* 995 *P.mathewsii* 99%	*P. ciliata* 99% *P. tokuoka* 99% *P. foeetida* 99%	*P. foetida*
12	*Passiflora caurelia* CWE	*P. caurelia* (744bp)	*P. caurelia* 92% *P. caurelia* 92% *P. caurelia* 92%	*P. caurelia* 92% *P. caurelia* 92% *P. caurelia* 92%	*P. caurelia**	*P. caurelia*	*P. caurelia* 99% *P. caurelia* 99% *P. caurelia* 99%	*P. incarnate* 99% *P. caurelia* 99% *P. caurelia* 99%	*P. caurelia*
13	*Passiflora foetida* UAI	*P. foetida* (924bp)	*P. foetida* 95% *P. foetida* 94% *P. foetida* 94%	*P. foetida* 95% *P. foetida* 95% *P. foetida* 94%	*P. foetida**	*P. foetida*	*P.pilosicorona* 99% *P. speciose* 99% *P. foetida* 98%	*P. vitifolia* 99% *P. coccinea* 99% *P. vitifolia* 99%	*P. foetida*

*Note: The * species have been searched and identified correctly from NCBI BLAST search option*

Table 5. Shows the BOLDSYSTEM database search matches of query *rbcL* sequences for a possible similarity match of *Passiflora* species. The species code; their morphological identifications along bold system search results with similarity % and phylogenetic affinity are displayed

		***rbcL* sequence**			
Sr no	**Species with code**	**Morphological identification**	**BOLD SYSTEM search**	**Similarity (%)**	**Phylogenetic affinity**
1	*Passiflora incarnata* UAI	*P. incarnate*	*P. gabrielliana*	99.12%	*P. incarnata*
2	*Passiflora vitifolia* HNI	*P. vitifolia*	*P. coccinea*	100%	*P. vitifolia*
3	*Passiflora foetida* CWE	*P. foetida*	*P. ciliata*	98.58%	*P. foetida*
4	*Passiflora foetida* UAI	*P. foetida*	*P. ciliata*	95.52%	*P. foetida*
5	*Passiflora incarnata* CWE	*P. incarnata*	*P. odontophylla*	99.71%	*P. incarnata*
6	*Passiflora Lady Margaret* HNI	*P. Lady Margaret*	*P. incarnata*	99.27%	*P. Lady Margaret*
7	*Passiflora incarnata* RNI	*P. incarnata*	*P. gabrielliana*	99.71%	*P. incarnata*
8	*Passiflora vitifolia* RNI	*P. vitifolia*	*P. ciliata*	98.44%	*P. vitifolia*
9	*Passiflora incarnata* SNI	*P. incarnata*	*P. incarnata*	97.06%	*P. incarnata**
10	*Passiflora vitifolia* SNI	*P. vitifolia*	*P. coccinea*	99.85%	*P. vitifolia*
11	*Passiflora foetida* HNI	*P. foetida*	*P. ciliata*	99.42%	*P. foetida*
12	*Passiflora caurelia* CWE	*P. caurelia*	*P. incarnata*	99.56%	*P. caurelia*
13	*Passiflora foetida* UAI	*P. foetida*	*P. coccinea*	98.86%	*P. foetida*

*Note: Only one Passiflora species (P. incarnata) marker as * have been identified correctly using BOLDSYSTEM search database*

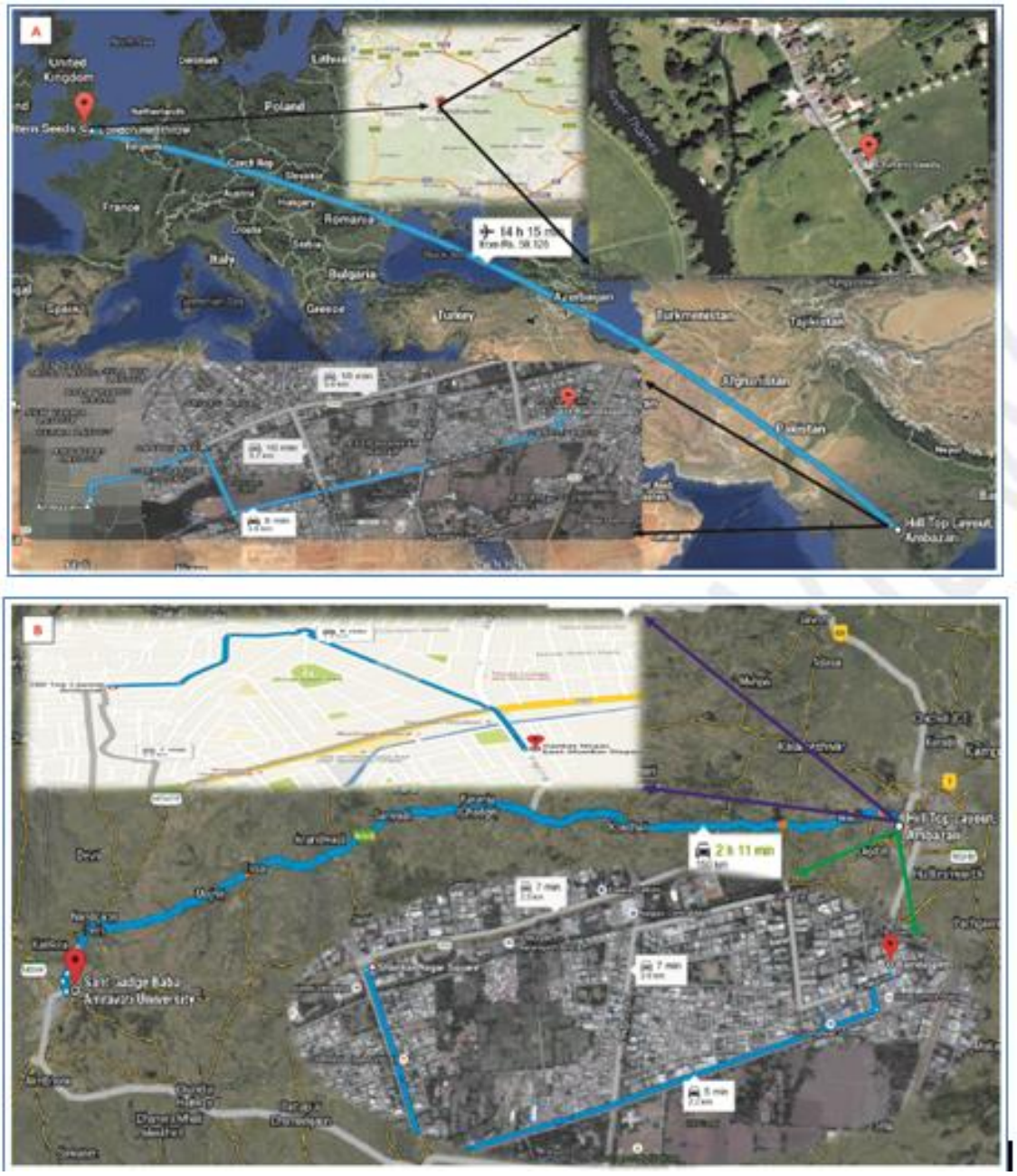


Fig. 1. Displays the google map location of the plant species with their distance in kilometer (Km). The estimated time and kilometer required to cover and distance are showed in A. between UK and India. B. within Indian locations

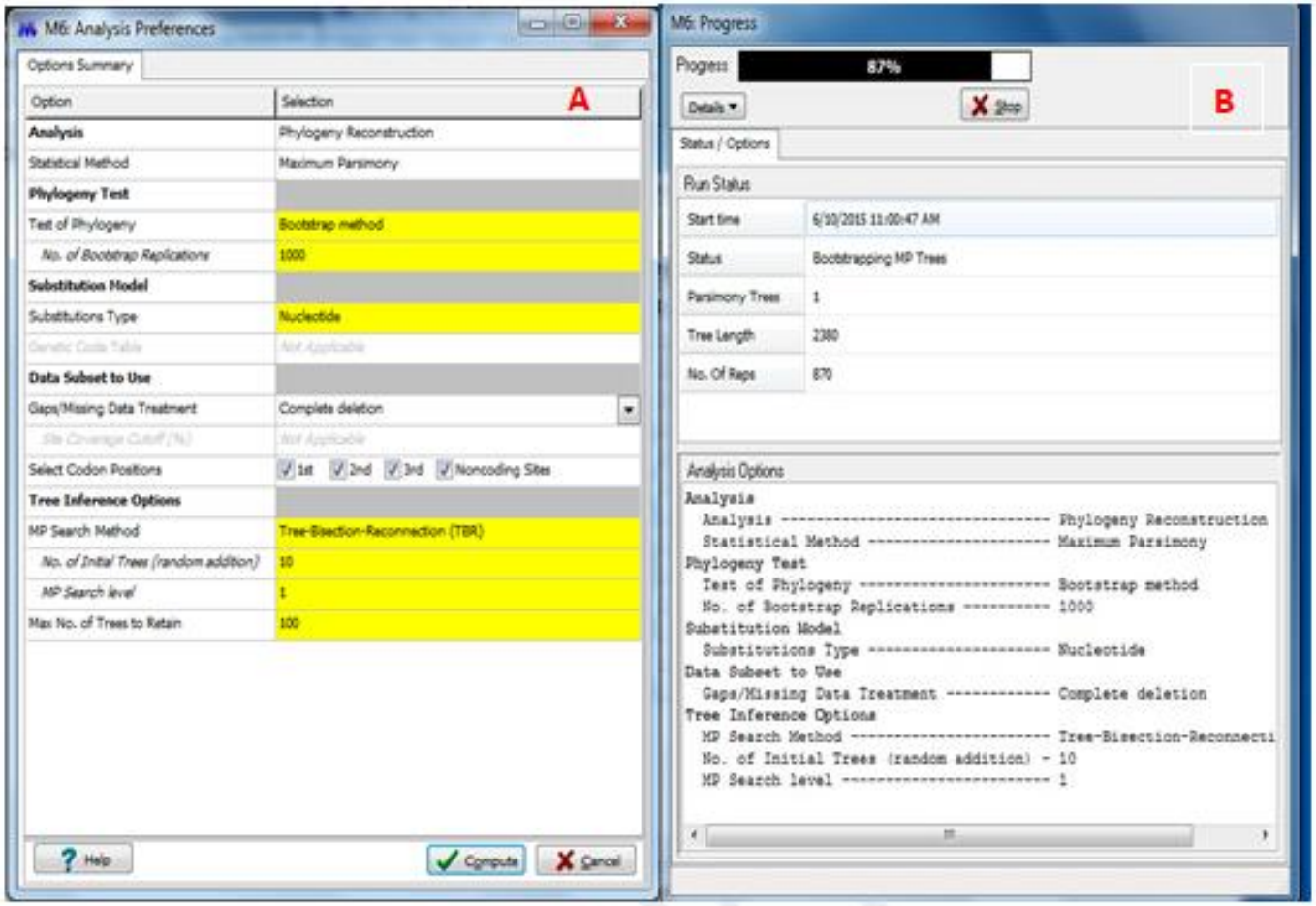


Fig. 2. Shows a snapshot of the phylogeny analysis preferences, statistical maximum parsimony method, A: for *ITS* sequence B: analysis with 1000 bootstrap replications for *rbcL* sequence

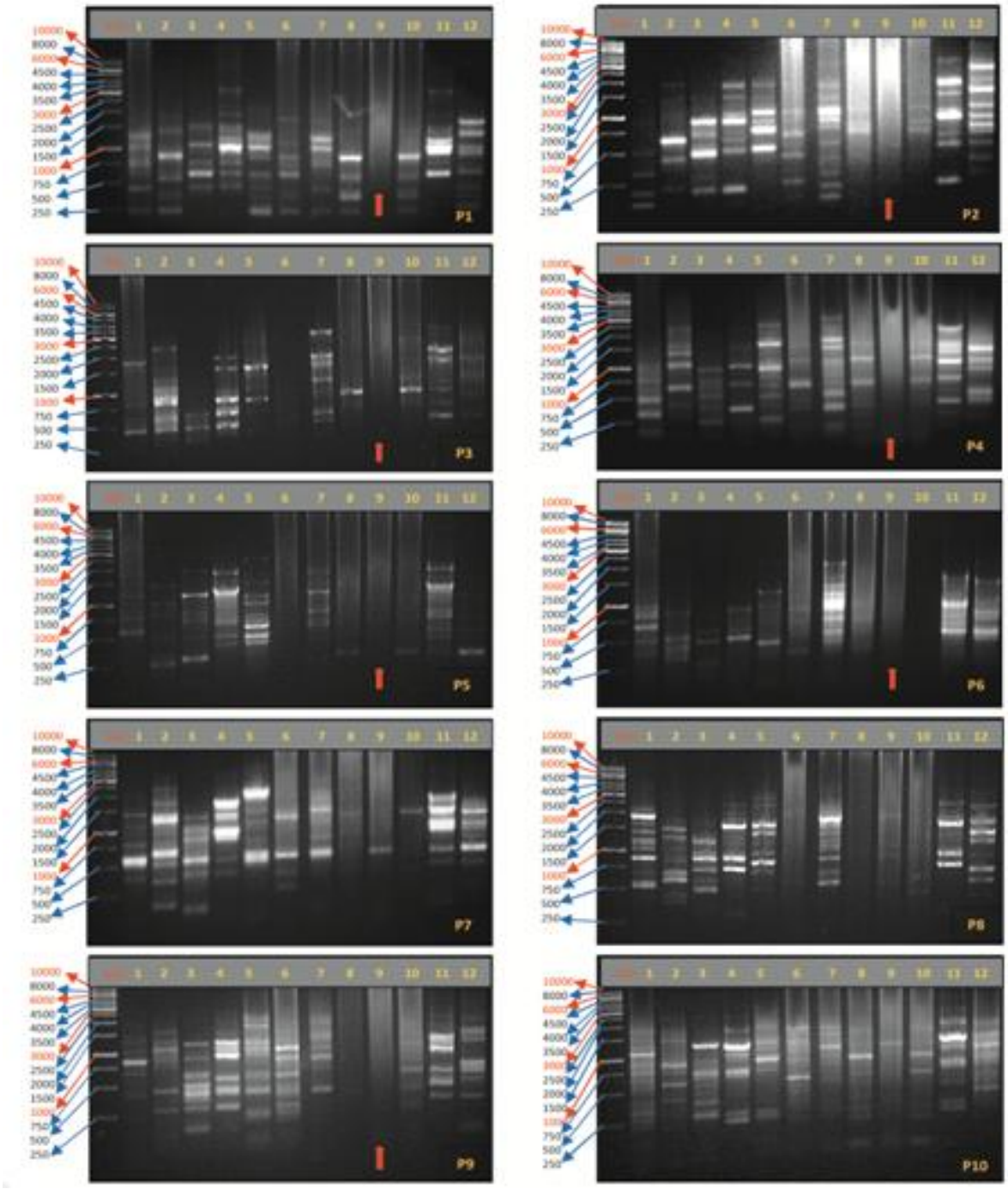


Fig. 3. Shows gel profiled of RAPD-PCR primers (P1-P10), 1 kb: marker ladder and (1-12) are the specific *Passiflora* species (see Table 2) are loaded in 1.2 % agarose gel. In 9th species DNA was not amplified in most cases indicated by (↑) red color upper arrow

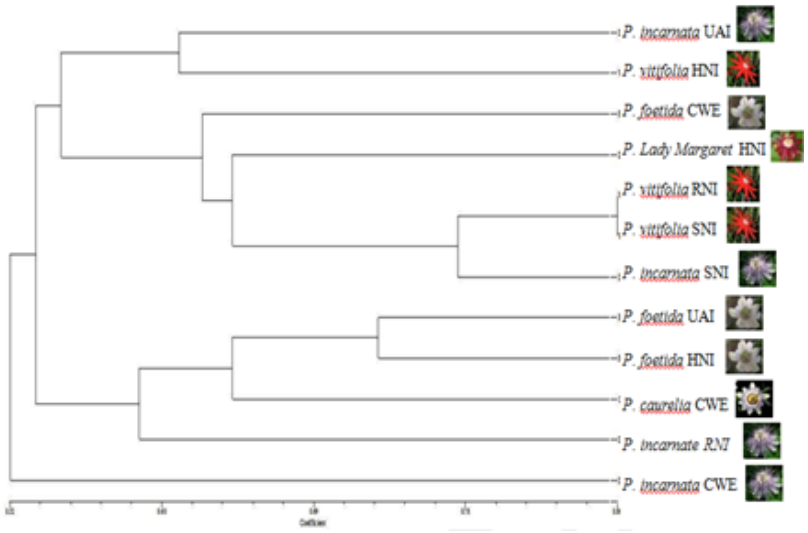


Fig. 4. A dendogram generated using UPGMA analysis showing polymorphism between *Passiflora* species using RAPD PCR markers. The dendogram is generated using NTSYSpc (Numerical Taxonomy and Multivariate Analysis System Version 2.0)

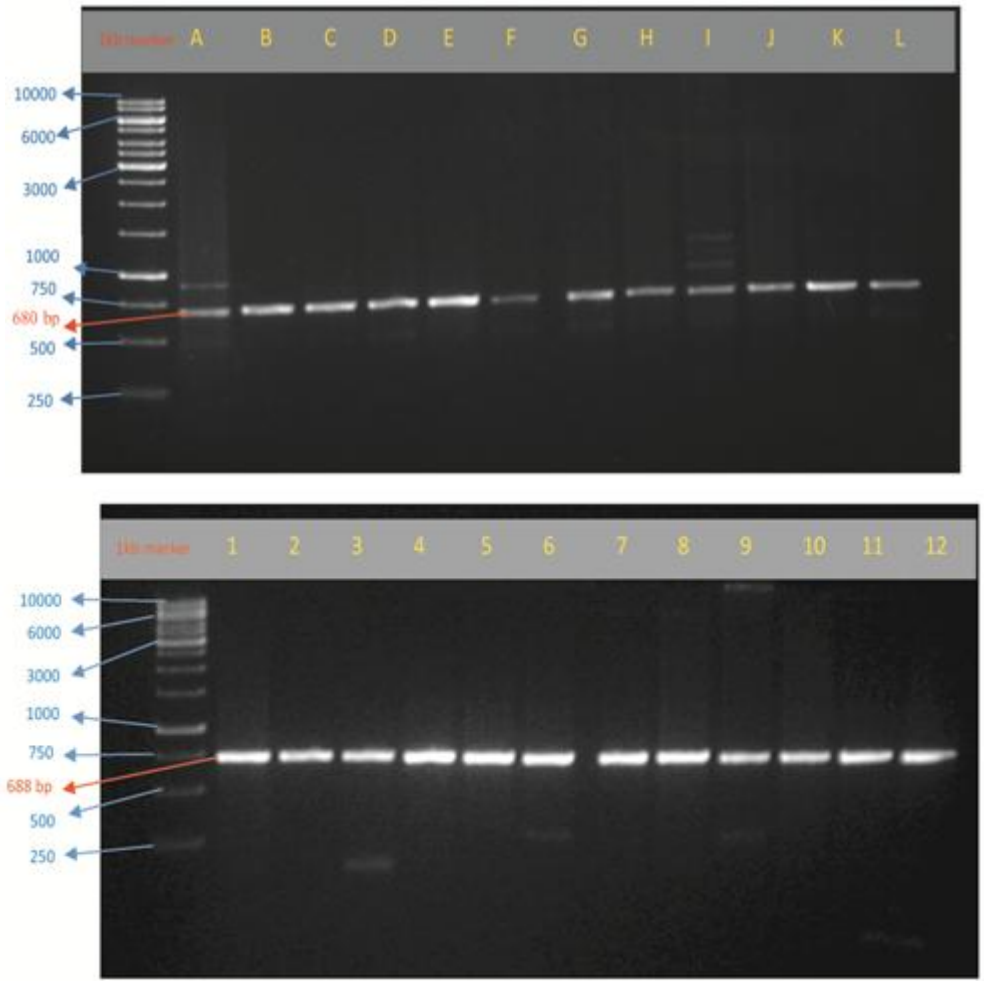


Fig. 5 the PCR amplified products for A: ITS primer (680 bp) and B: *rbcL* primer from 12 *Passiflora* species. 1kb marker, A-L and 1-12 are *Passiflora* species code (see Table 2).

Fig. 6. Shows the maximum parsimony analysis of *Passsiflora* species using amplified *ITS* nucleotide sequences. A: The parsimonious trees with length = 338, the maximum node score is 97 for *P. foetida* CWE and rest of above species. B: Reconstructed consensus tree, all the nodes of branches show 100 bootstrap score except bottom 3rd, which shows 33 scores for *P. foetida* UAI and rest above branch

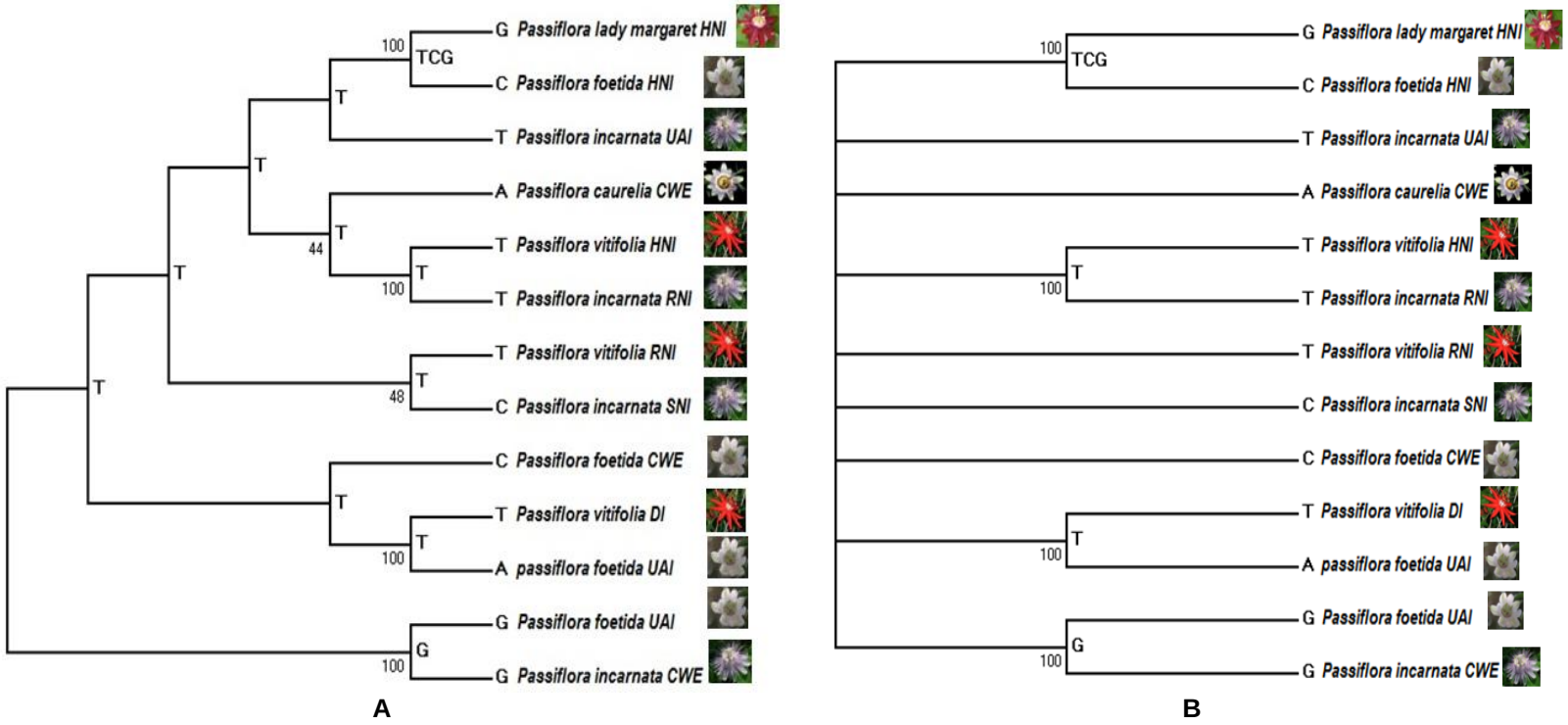


Fig. 7. Shows the A: evolutionary history using maximum parsimony analysis of *rbcL* nucleotide sequences from *Passiflora* species. The bootstrap 100 is obtained for four clades, except 44 for fouthr branch from top. B: Shows consensus tree of *rbcL* nucleotide sequences with the bootstrap 100 is obtained for four clades

A B

Fig. 8. Shown the molecular phylogenetic analysis by maximum likelihood method using A: ITS sequences from *Passiflora* species with bootstrap value greater than 50% are displayed with maximum 93 bootstrap was noted. B: *rbcL* based on the Kimura 2-parameter model were a total of 505 positions taken in the final dataset with bootstrap value higher than 40% with maximum 90 was displayed

To sum up, the established PCR-RAPD results would be convenient for specific classification of species according to genetic similarity, and it will help for breeding program and hybridization for crop improvement [42] and *ITS4* is a very applicable for evaluation of intra species diversity in *Passiflora*. However, *rbcL* results were unpredicted and very different from *ITS* tree. The intra species variation is not distributed evenly among species and its complex evolutionary history. As a result, in fewer cases led to classify the same plant species in a different group example (*P. edulis, P. caerulea*). Thus, combination pack of various genetic markers and large number of samples needs to consider together for inference of perfect robust phylogenetic studies. Moreover, the Passiflora species is highly diversified, which have been confirmed not only by morphological, secondary metabolites and phytochemical composition of leaves; fruit and flower. However, the information and resource for molecular studies are lacking in NCBI. The BOLD search has not retrieved the authenticate species may be due to fewer numbers of species *rbcL* sequence database except (*P. incarnata*) have been identified correctly using BOLD.

5. CONCLUSION

In conclusion, the phenotypic character for morphological characterization and genotypic molecular data of PCR-RAPD, *ITS4* and *rbcL* sequences have a good applicability to dissect genetic diversity among *Passiflora* species from Indian and England.

This work successfully used ten RAPD primers that are polymorphic in nature, rapid, easy techniques, which avoid the use of radio labeled isotopes [40] which produced 133 random amplified polymorphic DNA among 12 *Passiflora* species. The NTSYS dendrogram placed *P. vitifolia* from Ramdaspeth Nagpur, India and *P. vitifolia* Shankar Nagar Nagpur, India in same clade with a similarity coefficient of 0.609 confirming that they belong to identical geography Nagpur India. In contrast, P. *foetida* from England is not coming in same clade with *P. foetida* from India showing geographically intra-specific variation among species. *ITS* MP consensus tree is supported by strong 100 bootstrap value clusters same species in equivalent clade as *P. vitifolia* (HNI and RNI) and *P. foetida* (UAI and UGI) belong to same place Amravati University. The MP & ML methods works independently based on similarity of sequences and suggest that *rbcL* is useful in genetic diversity studies. Our results suggest that *ITS* regions provide valid resolution at species level and are best for molecular identification and inter-intra species differentiation of *Passiflora* species. Nevertheless, *rbcL* region is good to distinguished inter species, yet unsuitable for intra-species differentiation. The *ITS* marker having parsimony informative sites may be used for identification at a species level, and *rbcL* can be used as local DNA barcode. However, further studies may be undertaken to increase the sequencing data and including the large number of species and sub genera of *Passiflora* that will assist conservation as well as to get perfect resolution of hidden evolutionary history of *Passiflora.*

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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