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The Role of matk Gene in Eucalyptus Species Identification and its Importance in Phylogenetics

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ABSTRACT

There were successful amplifications of DNA for the eight-eucalypt species using gene MatK gene. Its position is can be found within the intron of the chloroplast gene *trn*K. The matK is the longest with 855bp (base pairs). Amplification success rate is almost 100%. For each species there are multiple good quality sequences available for the sequence alignment. The matK is the most useful for species identification and for phylogenetic tree construction. It contains 13 taxonomically useful SNPs. The matK and trnH-psbA are able to meet the barcode criteria well by showing greater interspecific variability. It is found to be best suited for 8 eucalypt cpDNA barcoding. It shows good number of interspecific variable sites. From the multiple sequence alignment of each species, the Single Nucleotide Polymorphism has clearly discriminated 6 species and we can say that each of these taxa has a unique molecular data. Therefore *E. globulus, E. nitens, E. mitchelliana, E. pauciflora* and *E. stellulata* are identified.

Keywords: MatK, SNPs, Variable gene, Phylogenetics, Complementary

INTRODUCTION

In the past few decades, the Genomic research has undergone tremendous changes and there have been several newer technical advances created to better help the structural and functional aspects of genes, chromosomes and sometimes the entire genome. e.g. the sequencing of the entire Human genome, of *Arabidopsis*, Rice, or *Populus* genomes. There are several other researches which taking place. It includes the *Eucalyptus* that has the sequencing of the entire genome. It has a wider application. Genetic information can help us with a good resolution of species boundaries, eventually it may give insights into the patterns and rates of evolutionary diversification among species.

We have seen great explorations on the genetic diversity of different plant species at the phenotypic level for the past century. We can observe a variation of genetic material within the species but what is so astonishing is the genetic variations at the level of conserved regions among distantly related species. We use a short selection DNA (portion of a gene) in order to identify a species. This is called DNA barcoding. The DNA barcoding is a newer system created to provide accurate and automatable species identifications by using short and variable standardized gene regions as species tags or species identity. This has initiated a new method/ technique and eventually led to the formation of Consortium for the Barcode of Life (CBOL, http: barcoding.si.edu) [1]. Our objective of this project is to obtain DNA barcodes from all species all over the world, from different geographical and climatic regions. Though there are great efforts mad by professional taxonomists to collect and preserve, still there a great deal of information about the biological diversity surrounding us is inaccessible. Our objectives of DNA barcoding include facilitating species discoveries based on cluster analysis, Species identification and promoting development of handheld DNA sequencing technology. We have no standard protocol still in for DNA barcoding in land plants. We have low levels of variation in the mitochondrial DNA of land plants as compared to animals where a portion of the mitochondrial gene *Cox1* (*Co1*) is used successfully. There is a standard barcode for animals. Therefore, we look into the plastid DNA for as a suitable region in barcoding. We see there is very slow rate of evolution in plastid genome in plants. Since it shows very low levels of variation in the plastid DNA, therefore, there is need for looking at more than one region for variations. Our challenge is also to find a very suitable region which shows enough variation within it to discriminate among species yet conserved enough to be

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present.

A CRITIQUE OF BARCODING

The advocates of DNA barcoding say that this technology would revitalize biological collections and speed up species identification and inventories. There are only 1.7 million specimens that have been identified by taxonomists and about 10-20 million more which have not been named or explained.

The opponents of barcoding argue that this technology would destroy the traditional systematics and turn it into a service industry [2]. These fears are allayed and in all cases DNA barcoding are applied only in conjunction with classical approach where species are simply unknown or no attempts have been made to delimit them. Therefore, barcoding as originally intended would be limited in its applicability. The DNA barcoding address by matching DNA sequences to 'known' species. As in the words of CBOL "barcoding is neither a substitution for alpha taxonomy nor about interfering phylogenies (Schindler and Miller 2005). Apart from being a diagnostic tool, barcode sequences per se and their ever-increasing taxonomic coverage could become an unprecedented resource for taxonomy and systematic studies. In future in plants multiple markers is likely to be a necessity and it is already being explored [3,4]. It is possible some taxa can be established from the sequence variation alone and re-identified unequivocally while awaiting morphological analysis and formal description, i.e. the 'reverse taxonomy' (Tautz) [5]. Using DNA barcoding the present research is carried out in order to find out the phylogenetic relationship of 6 closely related and 2 distantly related eucalypts.

It has been reported that the Chloroplasts are maternally inherited in most Angiosperms and for the eucalypts too this has been demonstrated by Byrne [6] in *E. nitens* of eastern Australia. Schael [7-18] showed a uniparental inheritance of the chloroplast genome and lack of recombination. The variations in the chloroplast genome are ordered accordingly. Since the Chloroplast genome is non-recombining, asexually inherited and evolves slowly, these characters are useful for the estimation of the extension of gene flow between species. Many studies in recent years have been focused on the chloroplast DNA variations in eucalypts because of their economic and ecological significance apart from other important genomic studies. Eucalypts have dominated different land scapes of Australia over the thousands of centuries [7,19-24].

MATERIALS AND METHODS

The Finding of Suitable Regions of the Genome

The DNA barcoding mechanism is a diagnostic tool for species identification- using a short, standard DNA region. Our challenge is to find a suitable genomic region for a wide range of taxa in plants. The sequence variation must be high enough between species so that they can be discriminated from one another and it must be low enough within species that a clear threshold between intra- and inter specific genetic variation can be defined. The Mitochondrial cytochrome C oxidase gene (Co1) segment is used in many animal groups for barcoding. It is said to have been showing necessary universality and variability and more than 95% unambiguous identification in most cases of major animal clades where the studies are done. However, many of the mitochondrial genes are not proved suitable enough for barcoding in plants because of their low mutation rates. This is also the factor of rapidly changing structure of mitochondrial genome in plants. The DNA barcoding has great scientific applications in ecology and evolution. Because of this, a universal barcode is being explored by many scientists and it is yet to be agreed upon. Kress [25] proposed originally a trnH-psbA spacer of plastid region as suitable universal barcode for land plants. Chase [3] has suggested rpocl, rpoB and matK or rpocl, matK and trnH-psbA as a good barcoding region. Taberlet [26] put forward the trnL intron as suitable plant barcode. Kress and Erickson [27] proposed a combination of trnH-psbA with rbcL for plant barcoding (combining from the proposal of Newmaster [12] Lahaye [28] identified a portion of matK gene as a universal DNA barcode for flowering plants. Considering the said reasons from the above proposal the present study uses a multi locus region-*matK,* of the genome for the barcoding of 8 eucalypt species.

The *mat*K gene was formerly called *orf*K. It is 1500 bps in length is a maturase coding gene. Its position is can be found within the intron of the chloroplast gene *trn*K. The *rbcL* is a ribulose-1,5-bisphosphate carboxylase/oxygenase gene.

The Taxa Selection for Barcoding

The group containing *E. moorei, E.affn. moorei, E. dives, E. mitchelliana, E. pauciflora* and *E. stellulata* is considered a highly evolved group in eucalypts. Their mallee form is said to be of recent origin on the evolutionary scale of eucalypts and it is an adaptation to the poor soil and dry climatic conditions (Ian Brooker, 2007 personal communication). The other two species (in the present study) included as a close out- group are *E. globulus* and *E. nitens* and they evolved much before the mallee forms. Of the eight species in this study, *E. moorei*, *E. affn. moorei*, *E. dives*, and *E. pauciflora* came from one population each of Nerega region of New South Wales. The *E. mitchelliana* specimens were collected from one plantation of South Australia. *E. stellulata* collections came from one population of Black Mountain of Canberra, ACT. The *E. globulus* and *E. nitens* collections came from Central Victoria- one population each respectively. There were 30 specimens collected for each of the eight species for DNA barcoding from each population. After extracting the DNA from each specimen, the DNA is pooled for each species.

DNA Extraction

About 5-7g of leaves were cut, avoiding the thicker part of the midrib and petiole and any large dead regions. They were immersed in liquid nitrogen. Immediately after liquid N2 evaporates, grind 30-40 sec in coffee grinder. The powder was resuspended in 40 ml of extraction buffer (recipe below) in small plastic beaker. Polytron (homogenise) for 20-30 sec at 3/4 speed. It was then filtered through two layers of muslin (only one layer if it is the very fine muslin) into tube in ice. The tubes were shaken vigorously and then spun at 2000 rpm (750 x g) for 10 min. The supernatant was poured off. The tubes were inverted on paper towel to drain for more than one minute. About 5 ml of wash buffer was added and the tubes were resuspended by vortexing (or with paint brush for species with gummy pellets). About 3 ml of 20% SDS, 20% Triton X-100 were added. They were mixed gently and kept at room temperature for at least five minutes, with occasional gentle mixing by inversion. About 4.0 ml of NaCl/CTAB mix. (34.4g CTAB + 162g NaCl per liter) were added. The tubes were incubated at 65°C for 20-30 min, with occasional mixing. chloroform (24:1) to 45 ml was added to the extract the tubes were inverted for at least 2 min. (Or placed on shaker for 20-60 min. When the aqueous phase retained the green color, it was extracted for longer). It was spun at 5000 rpm (5000 x g) for 10 min. The upper aqueous layer we transferred to a new tube. (If interphase layer is solid enough, the aqueous layer can be poured off-otherwise use a plastic disposable pipette.). About 2/3 volume 100% isopropanol was added. The was hooked (spool) out or spun down if unhook able. It was wash in 1.5 ml of 50% isopropanol/0.3M NH4OAc for several hours or overnight. They were air dried to precipitate for 15-20 min. They were resuspended in a minimal volume of TE (pH 8.0), (usually 200-500 μl).

Preparation of Eucalypt extraction buffer: For 1 L buffer preparation 100 mM Tris,100 mM Tris 12.1 g, 25 mM EDTA 50 ml, 0.35 M Sorbitol 64 g, 100 mM Boric acid 6.2 g 1 M NaCl. 58.4 g were weighed and taken in a 2 L beaker. The pH to 8.0 was added before 2% PVP 40,000 added. Then the following chemicals 10% PEG 8000 100 g, 0.5% BSA 5 g, 0.1 % spermine 1 g, 0.1% spermidine 1 g were added. We prepared the wash buffer with the following chemicals for 1 L. 50 mM Tris50 ml (of 1M, pH8.0), 25 mM EDTA 50 ml (of 0.5 M EDTA, pH 8.0), 0.35 M Sorbitol 64 g. Sodium metabisulphite was added to extraction buffers and wash buffer to 10 mM just prior to use (about 0.2 g per 100 ml-or 10ml per liter of a 1M sol, = 95g/L). PVP 40,000 20 g was added. Then the following chemicals 10% PEG 8000 100 g, 0.5% BSA 5 g, 0.1 % spermine 1 g, 0.1% spermidine 1 g were added. We prepared the wash buffer with the following chemicals for 1 L. 50 mM Tris50 ml (of 1M, pH8.0), 25 mM EDTA 50 ml (of 0.5 M EDTA, pH 8.0), 0.35 M Sorbitol 64 g. Sodium metabisulphite was added to extraction buffers and wash buffer to 10 mM just prior to use (about 0.2 g per 100 ml-or 10ml per liter of a 1M son, = 95g/L).

THE PRIMERS

The primers are selected are universal for the genes matK, (CBOL universal primers. Kress et al., 2005) **(Table1)**. After extracting the DNA individually from all the specimens of each species, the DNA for 30 specimens is pooled together for each is species. The DNA quality is checked using standard protocol before the PCR amplifications are done **(Table 1)**.

Table 1. Primer sequences used in this study (listed 5'- to -3' end).

Gene	Primer sequence
matK	CCTATCCATCTGGAAATCTTAG (1)
	GTTCTAGCACAAGAAAGTCG (2)

Source: Reproduced from COBL Universal primers as Adopted by Kress et al., 2005

RESULTS

There were successful amplifications of DNA for the eight-eucalypt species using DNA barcode gene. namely, matK. The sequence length obtained is quite long with good number of variable sites. Among the genes matK is the longest with 855bp (base pairs).

In comparison of Taxonomically useful SNPs (**Table 2**) that might potentially distinguish taxa) for phylogenetic studies **(Figure 1)** the ITS gene is found to be having largest number variable sites in the present study. It shows up 17 taxonomically useful SNPs. The matK is the most useful for species identification and for phylogenetic tree construction. It contains 13 taxonomically useful SNPs. This is followed by the matK with 5.1% variations. The trnH-psbA spacer has 4.41% variability and the rbcL showing only 1.57% variability **(Table 3).**

The matK which shows less divergence (up to 0.038) (**Figure 2**). Among the finding from the study two of the barcodes namely, matK and trnH-psbA are able to meet the barcode criteria well by showing greater interspecific variability.

Table 2 sequence length and percent interspecific sequence divergence for four plastid regions of 2 Subgenera of 8 eucalypts.

Table 2. The Gene, SNP comparison and SNPs percentage.

GENE	No. Bases	No. SNPs	No. Tax useful SNPs	% SNPs
MatK	713	37	13	5.1

The above table is reproduced exactly as it appears in research work, 2009 source.

Note: The barcode gene sequence analysis and SNPS comparison by Jayaraj, 2009. "Evaluation of Genetic diversity of some important Eucalyptus species and construction of Phylogenetic trees. P.72 Copy right. Jayaraj, 2009

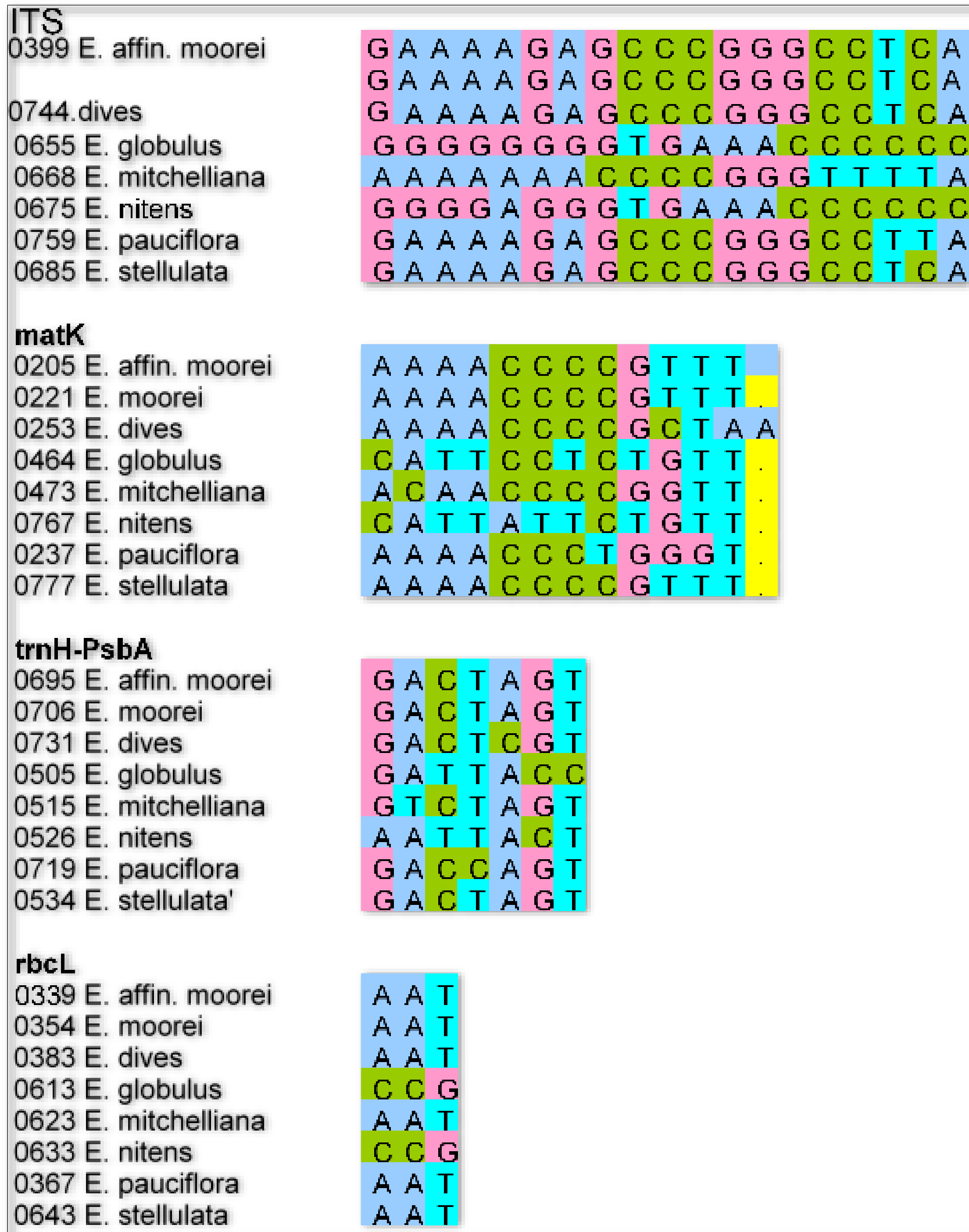

Figure 1. Barcode genes. Taxonomically useful SNPs for species discrimination.

Source: Figure is reproduced exactly as it appears in research work of G. Jayaraj, 2009

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Table 3. Barcode Genes' Sequence Analysis and Snps Comparisons.

Species Name		1	2	3	4	5	6	7	8
Gene length 710bp	Species Name	*E. dives*	*E. globulus*	*E. mitchelliana*	*E. affn. moorei*	*E. moorei*	*E. nitens*	*E. pauciflora*	*E. stellulata*
matK	*E. dives*	-	0.028	0.026	0.028	0.028	0.029	0.029	0.022
	E. globulus	0.028		0.019	0.019	0.022	0.008	0.026	0.015
	E. mitchelliana	0.026	.0.019		0.019	0.021	0.022	0.025	0.015
	E. affn. moorei	0.028	0.019	0.019		0.018	0.021	0.023	0.012
	E. moorei	0.028	0.022	0.021	0.018		0.025	0.028	0.015
	E. nitens	.0.029	0.008	0.022	0.021	0.025		0.038	0.026
	E. pauciflora	0.029	0.026	0.025	0.023	0.028	0.038		0.022
	E. stellulata	0.022	0.015	0.015	0.012	0.015	.0.026	0.022	

Source: The table is reproduced exactly as it appears in research work, 2009

Note: The barcode gene sequence analysis and SNPS comparison by Jayaraj, 2009. "Evaluation of Genetic diversity of some important Eucalyptus species and construction of Phylogenetic trees. P.72 Copy right. Jayaraj, 2009

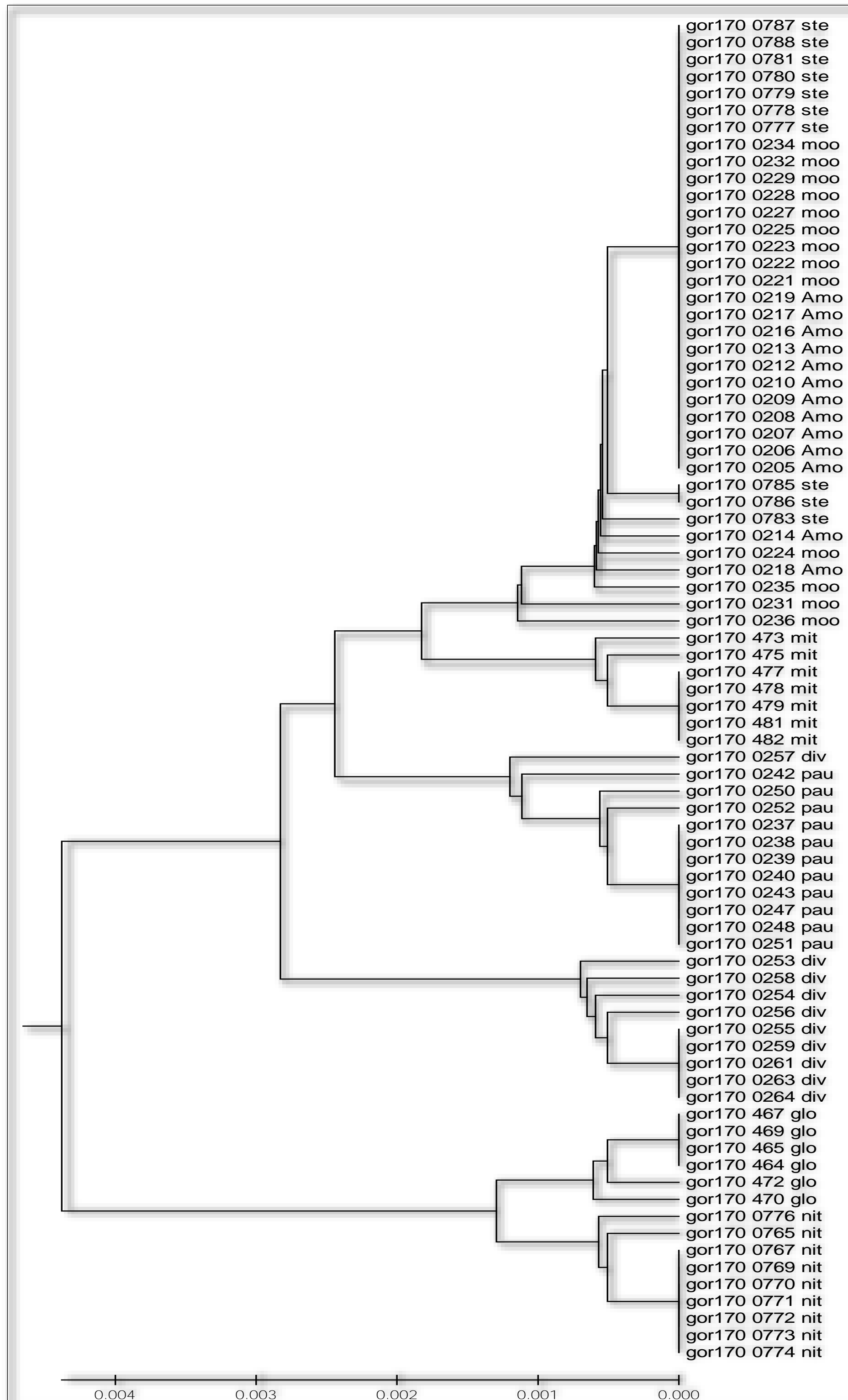

Figure 2. MatK gene. Phylogenetic tree.

Source: UPGMA the Figure is reproduced exactly as it appears in research work, 2009.

Note: The barcode gene sequence analysis and SNPS comparison by Jayaraj, 2009. "Evaluation of Genetic diversity of some important Eucalyptus species and construction of Phylogenetic trees. P.72 Copy right. Jayaraj, 2009

DISCUSSION

Until now there are a few successful findings to give us most suitable DNA barcodes for land plants. These studies helped to focus on the generic level discrimination using barcodes or above this level. One of the prime objectives of this investigation was to compare the cpDNA region of *E. moorei* complex (*E. moorei* and *E. affn. moorei*). The study also included very closely related taxa such as *E. dives, E. stellulata, E. pauciflora* and *E. mitchelliana*. There is a question of the true (disputed) identity of *E. affn. moorei* coming from a disjunct population with diagnostically different morphological features from that of *E. moorei*. The following genes have provided very useful information for the phylogenetic study of eight taxa of eucalypts.

MatK gene: It is found to be best suited for 8 eucalypt cpDNA barcoding cfrom my research work at Yarralumla, NSW (2007). The sequence length is 713 bp long, showing good number of interspecific variable sites. From the multiple sequence alignment of each species, the Single Nucleotide Polymorphism has clearly discriminated 6 species and we can say that each of these taxa has a unique molecular data.Therefore *E. globulus, E. nitens, E. mitchelliana, E. pauciflora* and *E. stellulata* are identified clearly using the matK barcoding. The other two, *E. moorei* and the disputed *E. affn. m*oorei (as part of *E. moorei* complex) have also shown single nucleotide substitutions in some sites in a multiple sequence alignment. The *E. affn. moorei* has two single nucleotide substitutions and the *E. moore*i has 4 substitutions at different site in the gene. The indels are not taken into consideration. Based on these few nucleotide substitutions we cannot give a separate identity for *E. affn. moorei*. This study has shown that there is a small probability of *E. affin moorei* being new species. But there is a greater probability that these are part of one species complex. The explanation can be given by accepting the fact of interspecific hybridization that is very common in closely related species. From the fact that these two are also collected from the same geographical region, it clearly explains why these two have very close sequence homology excepting a few nucleotide substitutions in a few individual sequences of multiple sequences. The chances of gene flow between the two are greater in this context. Moreover, it has been sometimes identified as *E. moorei* var *arborea.*

Though the diagnostic morphological features in the disputed taxon of *E. affn. moore*i are notable, taxonomically this taxa still needs to be well defined or explained in order to ascertain its identity. The present study has not clearly distinguished the two based on molecular data with 100% certainty. At the same time there are indications from the data that there is 30% probability of identifying these individual species based on DNA barcodes. As suggested in the beginning of this study a further taxonomic study is awaited from the Blackheath in Blue Mountain population (New South Wales). Therefore, we can suggest to carry out a matK barcoding from the population of Blackheath in order to ascertain the identity of *E. affin mooorei* as new species. We can recommend that matK gene as a good candidate for DNA barcoding for diversity studies at interspecific level.

Implications of the Present Study and Future Prospects

The present research findings high lights the importance of matK (coding region). The matK which has been recommended as an important barcoding gene recently Lahaye & Kress [28] proved again its great resolving power at interspecific discrimination. Contrary to the earlier findings this gene matK is amplified easily with the universal primers and showed potentially important variable sites for species discrimination and they useful for phylogenetic construction **(Figure 3).** In spite of large number of indels, this gene has yielded relatively significant PIC values. This work has focused on a small group consisting of five Blue ashes, one Peppermint and two symohyomyrts (eight closely related) *Eucalyptus* taxa. These are said to be one of the most rapidly evolving group of individuals in the genus *Eucalyptus.* There are many reports of interspecific hybridizations and introgressions. It is not always easy to distinguish the hybrids because the hybrids share the maternal plastid DNA. Therefore, the choice of this study actually enters into a problem-group area. In a sense this might be a good start with a problem group and apply the DNA barcodes for the 8 closely related taxa is challenging. Sometimes we also know the fact that discrimination of some taxa might be lost with greater taxonomic and geographic sampling. This gives us a grasp of things on a minor scale before launching a largescale study. The use of the matK gene helped us not only to identify successfully six taxa out of eight but it also it has indicated once again the successful universal amplification of matK gene and its application in eucalypts.

We conclude from this study that one gene (matK) may be better suited for phylogenetic study than the other three. This could be further tested by including many individuals from the same subgenus to test the utility of this barcode. However, a study like this one on a small group (8 taxa) can be useful to explain their taxonomic relationships in detail. This might be a good tool for predicting the overall levels of variations that are likely to be found in a large scale.

CONCLUSION

In the Phylip tree construction based on Nucleic Acid sequence Maximum likelihood method has given us comparative confidence limits of interspecific divergence of eight closely related Eucalyptus species. Among the eight taxa, there are five species with significant confidence levels **(Table 3**). They are as following. *E. dives* 0.17 confidence, *E. pauciflora* 0.18 confidence, *E. mitchelliana* 0.37 confidence, *E. globulus* 010 confidence, and *E. nitens* 0.24 confidence. These levels of confidence are positively significant. In other three cases of *E. stellulata*, *E. moorei*

and *E. affn. moorei* the confidence limits are insignificant. These form a one taxonomic complex. We can also call this an aggregated species based on molecular data. But the taxonomic identity of *E. steullulata* is well defined based on morphological characters. There is an ambiguity about the identity of the disputed *E. affn. moorei*.

DNA parsimony algorithm for ITS, rbcL, trnH-PsbA and matK genes (version 3.67 using Phylip)

TREE -2

```
+---E. nitens
 |
 1--E. globulus
 |
 |                 +---E. pauciflora
 |              +--4
 |              | +---------E. mitchelliana
 +------------------------------2
                | +E. stellulata
                | |
                +--3-E. moorei
                  |
                  +E. affin. moorei
                  |
                  +-----E. dives
```

requires a total of 43.000

between	and	length
1	*E. nitens*	0.071429
1	*E. globulus*	0.047619
1	2	0.523810
2	4	0.023810
4	*E. pauciflora*	0.071429
4	*E. mitchelliana*	0.166667
2	3	0.023810
3	*E. stellulata*	0.000000
3	*E. moorei*	0.000000
3	*E. affin. moorei*	0.000000
3	*E. dives*	0.095238

Figure 3. DNA Parismony algorithm for ITS,rbcl, trnH-PsbA and matk genes(version 3.67 using Phylip).
Source: The Figure is reproduced as it appears in the Ph.D. thesis, G. Jayaraj, 2009
Note: The DNA Parsimony algorythm ITS, rbcL, trnH-psbA spacer and matK genes using 3.67 Phylip.
The comparison by G. Jayaraj, 2009. "Evaluation of genetic diversity of some important Eucalypus species and construction of phylogenetic trees.

Our present study using 4 different cp DNA and nrDNA-ITS barcodes have not resolved the issue fully although it has indicated us some haplotypes of *E. moorei/ E. affn. moorei*. It is recommended (Ian Brooker personal communication, 2008) to make a collection from a population of Blackheath, New South Wales, for this species complex in order to do a comparative study based on morphological characters as well as molecular data This might either clarify or brings out

useful information for identifying *E. affn. moorei* as a different species or consider it still part *E. moorei* species complex. We can also use some low-copy nuclear genes to resolve the identity and position of *E. affn. moorei*.

There are many success stories so far reported using either a single cp DNA barcode or in combination two or three in different individual groups of land plants. But there is no consensus as yet if an individual or a multilocus barcodes that would work very well in plants belonging to different families coming from various geographical locations of the planet. Some taxonomists view (Chase) [3]. that DNA barcodes based on uniparentaly inherited markers can never reflect the complexity that exists in nature.

There are ambiguities created as far as species limits are concerned by barcodes in some cases. Therefore, some taxonomists have a suspicion or skepticism of barcodes. They are critical of this work. For most taxonomists it is important to have a reasonable barcode based on a multiple low copy nuclear DNA locus, a multi-locus barcode system (MBC). This would mean looking for identification of conserved flanking regions containing variable sites. These sites may be introns of appropriate size. These conserved sites would serve as universal PCR priming locations. The reasons for MBC are because of detection of hybridization/ introgression cannot be reliably done by examining a single cp DNA region.

The starting presupposition was to identify all the 8 species of this group using 4 cp DNA barcodes coding and noncoding and expected them to be taxonomically discriminated based on molecular data. The findings are not far from the objectives. Though there is no 100% perfection of the results, these have clarified and illumined the understanding that matK gene and trnH-psbA spacer are better suited for low-level molecular phylogenetic studies in eucalypts. Therefore, it is not a question of mathematical precision of usefulness of data but the value and the significance of the information it has provided us in order to enhance the ongoing search for the most suited barcode regions for plant identification in a wider concern for recording and preserving the biodiversity on our planet.

Since 1995 we have seen a great deal of increase in using the noncoding cp DNA and the coding matK data for molecular systematic investigations. The regions included for this kind of study are trnH-psbA spacer, trnL-trnL-trnLF, trnK-trnK or matK, ITS and rbcL. There are many other non-coding regions of cpDNA investigated but they are not recorded or not explored yet. Because of these reasons we have little information about relative rate of evolution among different non-coding regions. Each research group designed its own experiments to test different barcodes on different group of plants. Some of the works are on a large scale. However, there is no consensus as yet. Apart from various recommendations, some of the latest such as Lahaye [25] which correctly classified 90% of the species by using matK and trnH-psbA (either alone or in combination). The final agreements seem to be in the direction of using multiple regions than one. The latest CBOL' conference in Taipei proposal is for using matK, trnH-psbA and atpF-H. The present findings are in the direction of above research work [26-33].

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