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RESEARCH ARTICLE

DNA Barcoding and Phylogenetic analysis of South Indian *Curcuma* species using chloroplast *mat*K gene.

Santhosh Kumar R. and Yusuf A.*
Interuniversity centre for Plant Biotechnology, Department of Botany, University of Calicut, Kerala-673635, India.

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Abstract

The interspecific relationship between twenty one different *Curcuma* species was studied using MaturaseK gene (*mat*K). Various *Curcuma* species are widely exploited for its medicinal, ornamental and other purpose. Floral plasticity in vegetative characters and developmental portion of inflorescence is widely utilized in morphology based classification which leads to erroneous conclusions makes DNA barcoding an essential tool for deciphering the correct identify and pohylogenetic relationship between the species. The sequence of *mat*K gene of *Curcuma* sp with no barcode gaps were cloned, sequenced and submitted in the Genbank and used for the phylogenetic study. The intra and interspecfic divergence between the species assessed by using K2P of MEGA 6.0.The sequence alignments were performed using Clustal W,transition/transversion rates were predicted and phylogenetic tree was constructed using MEGA 6.Phylogenetic tree was designed to identify the ideal regions of *Curcuma* species for defining inter and intra-species relationships. The phylogenetic analysis using MEGA 6.0 provided two groups with the second group having two subclades. The study revealed the potential of *mat*K gene as a good candidate gene for phylogenetic analysis of the genus *Curcuma*.

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Introduction:-

The genus *Curcuma* L. (Zingiberaceae) comprises of 120 species, widely used is spices, medicines, dyes and as ornamental plants (Skornickova et al., 2007). It is distributed in Asia, Australia and South Pacific (Larsen,et al., 1998; Wu and Larsen, 2000; Ye,et al., 2008; Chen and Xia, 2010). Twenty species and one variety have been reported from South India (Sabu 2006). The taxonomic identification of the genus is difficult and displays certain systematic problems due to morphological variation at the intraspecific level. The flowering season of *Curcuma* species is short and the floral morphology has higher similarity among the species, but differs in colors and inflorescence positions (Bakhuizen van den Brink,Jr 1968; Burtt, 1972). Phenotypic plasticity of the species can lead to wrong taxonomic treatment and identification of individuals. According to Kress et al., (2002), nuclear and plastid (ITS & *mat*K) genes can be used for phylogenetic analysis of *Curcuma* (Zingiberaceae) species.

The constructed phylogenetic trees can be used to study the evolutionary history of living organism and also provide the evidence of climatic and geological history of the earth. A phylogenetic tree displays the relationship of taxonomic groups in a hierarchical order (Futuyma, 2005). Molecular phylogenetic methods are mainly used in the perspectives of biological systematics, and are used for a wide variety of application, such as community ecology

Corresponding Author:- YUSUF A.
Address:- Interuniversity centre for Plant Biotechnology, Department of Botany, University of Calicut, Kerala-673635, India.

(Webb et al., 2002) proteomics, as well as inference of protein–protein interactions (Pazos and Valencia, 2001). Zheng and Xia (2010) used two sequences of ITS and *matK* to study the phylogenetic evolution of the tribe Zingiberaceae. Phylogenetic relationships between *Curcuma* species was studied using the cytological data (Chen et al., 1984)and RAPD fingerprinting (Xiao *et al.,*2000, Rajeshkumar et al.,2016).Ngambriabsakul et al.,(2004) observed that *Curcuma* is a paraphyletic genus in which infrageneric relationships are more complicated.

For the identification of species variation DNA barcodes are being utilized (Hebert et al., 2003). They are small size DNA of the organelle or nuclear genome with high discriminatory power among the organisms.DNA barcodes have multifarious role in the identification of the plants having problematic taxonomic identity for biodiversity investigation, the identification of cryptic or polymorphic plant species, food adulterants and forensic sample etc .
The *matK* gene is one of the protein coding regions of plastid which is widely used to decipher the coding regions of DNA (Wolfe, 1991). This chloroplast gene is 1500 bp long, located within the intron of the *trn*K and codes for maturase like protein (Wolfe et al., 1992). This gene has high substitution rates within the same species and is a potential candidate to study plant systematics and evolution (Notredame, 2000). The polymorphism of chloroplast DNA regions comprising *trn*K, *mat*K and intergenic *trnL-trnF* have been used to study the phylogenetic evolution of various plants species (Wolfe, 1987). Studies using *mat*K gene are used to resolve intergeneric or interspecific relations among flowering plants, like Malpighiaceae (Cameron et al., 2001), Poaceae (Liang and Hilu, 1996), Cornaceae (Xiang et al., 1998), Nicotiana (Aoki and Ito 2000), Orchidaceae (Goldman et al., 2001; Salazar et al., 2003) and in many other angiosperms (Hiluet al., 2003).

This paper reports the elucidation phylogenetic relationships between 21 different species of *curcuma* by using the chloroplast *mat*K gene sequence.

Materials and Method:-

Collection of Samples:-

Twenty one different *Curcuma* species including one variety were collected from different regions of South India. These plants were maintained in Department of Botany, University of Calicut, and the leaf samples were used for DNA extraction.

DNA Isolation and Purification:-

Genomic DNA was isolated from fresh *Curcuma* leaves using modified CTAB method (Doyle and Doyle, 1987). Extraction buffer contained 2 % (w/v) CTAB, 1MTris–HCl (pH 8), .5M EDTA (pH 8) and 5 M NaCl. The samples were powdered in liquid N_2 and the powder was transferred to pre-heated (65° C) CTAB buffer. 2% (w/v) of PVP was added at the time of homogenization. Proteinase K (10 mg/ml) and 2 % (w/v) β-mercaptoethanol were added to the extract and mixed well. Samples were incubated at 60°C for 60 min with frequent mixing. The extract was centrifuged at 4°C for 12 min at 12,000 rpm and the supernatant was collected in a new Eppendorf tube. Added equal volume of chloroform: isoamyl alcohol (24:1) to the supernatant and centrifuged at 4°C for 12 min at 12,000 rpm. The supernatant was extracted twice with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), followed by extraction with chloroform: isoamylalcohol (24:1), and centrifuged at 12,000 rpm 4°C for 12 min. The supernatant was transferred to a fresh Eppendorf tube and one by third volume of chilled isopropyl alcohol was added, and incubated for one hour at -80°C. The sample was centrifuged at 4°C for 12 min. at 12,000 rpm and the pellet was dissolved in TE. 10µl of RNase was added to dissolve DNA sample. Incubated at 40°C for 30 min and added equal volume of chloroform: isoamyl alcohol (24:1) mixed well and centrifuged at 4°C for 12,000 rpm. The upper layer was transferred to a fresh Eppendorf tube and added chilled isopropyl alcohol and mixed well. The samples were incubated at -80°C for one and half hours and centrifuged at 4° C for 12 min at 12,000 rpm to pellet the DNA. DNA pellet was washed with 70 % (v/v) chilled ethanol and air dried at room temperature and re-dissolved in 20 µl TE buffer. This purified DNA was stored at -80°C.

Quantitative and qualitative assessment of DNA samples:-

Quality and quantity of DNA samples were assessed using a nanodrop spectrophotometer (Thermo, USA). The samples were run on 1 % (w/v) agarose gel and visualized to assess the DNA quality using a gel documentation system.

PCR amplification:-

The PCR reactions were conducted in a final volume of 25 µl containing 10x Buffer 2.5µl, Taq DNA Polymerase (1µl), dNTP mix (10mmol), $MgCl_2$(50mmol), Forward primer (88.4nmol), Reverse primer (72.4nmol),Template

DNA (0.5µl) and the volume was adjusted to 25 µl ddH_2O. The amplification condition consisted of an initial denaturation at 94°C for 2 min, denaturation at 94°C for 15 sec, annealing gradient at 53 to 57°C for 30 sec, extension at 72°C for 1 min. and final extension at 72°C for 10 min. After amplification the PCR product was checked using electrophoresis on a 1% (w/v) agarose gel and the band was eluted and purified by Minigel purification kit (Takara, Japan). Sequencing was done from Scigenom Lab Pvt, Ltd (Cochin, Kerala), on a charge basis.

***mat*K data analysis:-**
The Twenty one DNA sequences of the all samples were minimally edited and manually aligned using BioEdit software. The analysis of DNA sequences was conducted by Neighbour-joining to assess topology with MEGA 6.0. All the regions containing gaps and missing data were eliminated. The species identification and homology between the sequences was identified using BLAST method. The phylogenetic tree was developed using Neighbour-joining (NJ) method which was tested with Kimura 2-parameter for evolutionary distances in MEGA6.0 and node support was assessed on 1000 bootstrap replicates.

Results:-
The annealing temperature 55.6°C was found to be ideal to develop a high intensity molecular size band for *mat*K. PCR and sequencing success rate was very high for all the twenty one species in the *mat*K region (99.0% of individuals, 100% species) with a PCR product size of 800 bp (Fig1). The coding region of *mat*K obtained from the 21 species was sequenced and the sequence was submitted to the Genbank of NCBI and the accession numbers were provided by NCBI; KU934093, KU736742, KX170829,KX455852, KX455853,KX455854,KX455855,KX148521,KX418654,KX650813,KX650814,KX650815,KX650816,KX650817 ,KX650818,KX650819,KX650820,KX650811,KX650809,KX650810and KX650812. The sequences were used to construct the phylogenetic tree, having twenty one informative sites and the overall mean distance of all sequences was 0.108.The transition/transversion range between the species was 1.27.Tajima's Neutrality Test was conducted for all twenty one sequences to compare the number of segregating sites per site with the nucleotide diversity(Tajima, 1989)(Table 1).

The phylogenetic tree comprised of two groups in NJ-analysis; **Group 1**contained *Curcuma raktakanta* and *Curcuma aromatica* showed no variation as they was represented with a 100% bootstrap value (Fig2). **Group II** has two branches and two monoclade, branch 1 has two clade A& B and two monoclade *Curcuma kudagensis* and *curcuma neilgherrensis* which comes under clade A with 41% similarity and *curcuma mutabilis* and *Curcuma oligantha var.lutea* comes under the clade B showed similarity with 51% *Curcuma karnatakensis, Curcuma oligantha var.oligantha* and *Curcuma coriacea* exist as a monoclade. Branch II has two clade C and D, Clade C has one subclade and two monoclade *Curcuma haritha* and *Curcuma aeruginosa* comes under same clade and *Curcuma vamana* and *Curcuma zanthorrhiza* which excisted as a monoclade. Clade D has three subcladeD_1,D_2& D_3,*Curcuma longa* and *curcuma pseudomontana* which comes under the same clade. Similarly *Curcuma amada* and *Curucma decipiens* represent the same. *Curcuma Montana* and *Curcuma ecalcarata* which comes under subclade D_1 with 46% similarity. *Curcuma inodora* and *Curcuma bhatii* existed as a monoclade.

Maximum Composite Likelihood Estimate pattern of nucleotide substitution was obtained where each entry showed the probability of substitution (r) from one base (row) to another base (column) (Table 2). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics (Table2). The nucleotide frequency was 32.16% (A), 39.91% (T/U), 15.47% (C), and 12.46% (G). The transition/transversion rate ratios was k_1 = 5.744 (purines) and k_2 = 2.54 (pyrimidines). The overall transition/transversion ratio was R = 1.566, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$.

Table 1:- Results from Tajima's Neutrality Test

m	*S*	p_s	Θ	π	*D*
21	362	0.44691	0.124221	0.071099	-1.767785

m = number of sequences, n = total number of sites, S = Number of segregating sites, $p_s = S/n$, $\Theta = p_s/a_1$, π = nucleotide diversity, and D is the Tajima test statistics

Table 2. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*6.69*	*2.59*	**11.99**
T	*5.39*	-	**6.58**	*2.09*
C	*5.39*	**16.98**	-	*2.09*
G	**30.94**	*6.69*	*2.59*	-

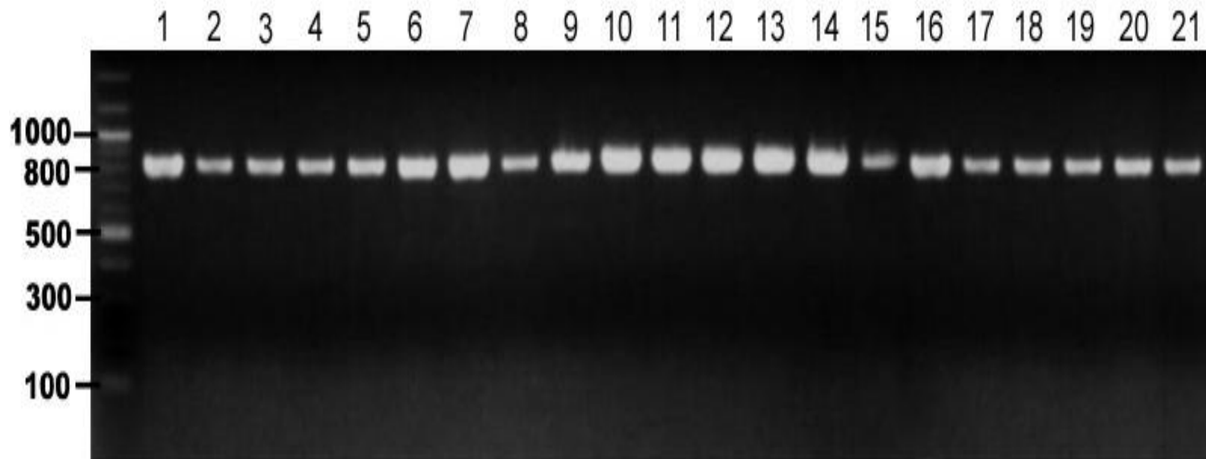

Fig1.Gel-electrophoresis of PCR product of *Curcuma* species shows a band of *mat*K with 800bp.

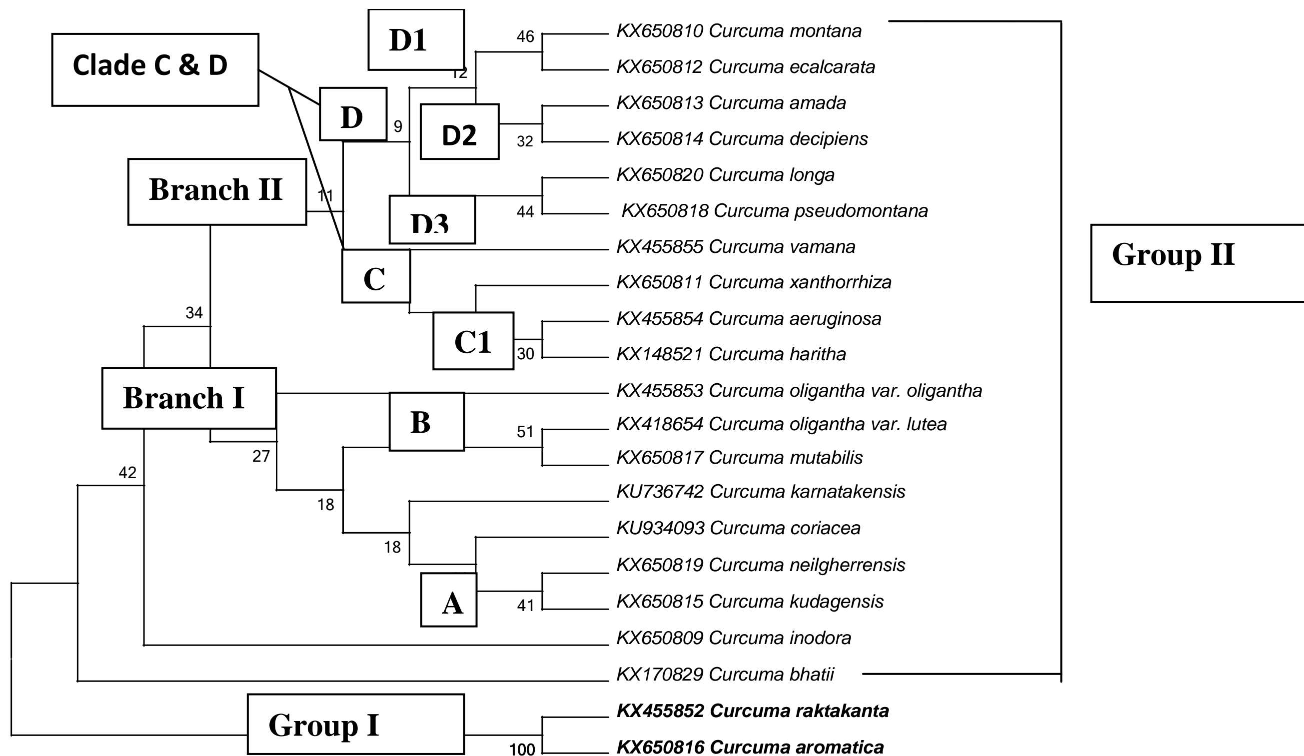

Fig 2:- Phylogenetic tree showing Evolutionary relationship between 21 Curcuma species inferred using the Neighbour joining method

Discussion:-

Chloroplast *mat*K gene is considered as one of the most rapidly evolving gene (Vijayan and Tsou, 2010).PCR and sequencing problems have been reported for *mat*K gene (Sass, et al., 2007; Fazekas,et al., 2008; Ford,et al., 2009;

Hollingsworth et al., 2009). PCR and sequence amplification success rate are important criteria for developing a DNA barcode (Kress and Erickson, 2007; Ford,et al., 2009; Hollingsworth et al., 2009).However, in the present study the results showed PCR and sequencing success rate of *mat*K as high as 99%.Transversions are considered illegitimate mutations in constructing phylogenies (Lake, 1987; Quicke, 1993). In recently diverged sequences the observed Transition/ transversion ratios were high. Transitions are higher than transversions for highly diverged sequences and low for less diverged sequences due to rate of higher transversions than transitions (Holmquist, 1983).Here, the observed low transition to transversion (Ts/Tv) ratio (1.27) indicates comparatively high proportion of transversions in the gene. The evolutionary rate of *mat*K gene makes phylogenetically resolving intergeneric and interspecific relationships of many angiosperms (Johnson and Soltis, 1995; Soltis & Soltis, 1998).According to Savolainenet al.,(2000a)the relationships revealed using *matK* data are more robust than those derived from combining *rbcL* and *atpB* sequences.

Earlier intrageneric classifications of *Curcuma* were based on morphological characters using the position of the inflorescence that are confusing for many species (Škorničková, 2007). Molecular phylogenetic studies showed that *Curcuma* is a paraphyletic genus (Kress,et al., 2002; Ngamriabsakul, et al., 2004; Záveskáet al.,2012).Based on the morphological classification of the species *C. haritha* showed close relationship with *C.aromatica* and share a few characters with *C.raktakanta,* however *C.raktakanta* closely resembles *C.aeruginosa* in morphological character (Sabu,2006).Based on the molecular character related phylogenetic analysis, *C.aromatica* and *C.raktakanta* are close to each other. The present study showed the paraphyletic origin of these two species is moderate with (100 %) bootstrap value; however *C.raktakanta* did not show any relationship with *C.aeruginosa.* On morphological character, close relationship was established between *C.karnatakensis* and *C.oligantha* (Amalrajet al., 1999) that is incongruent with our work where these two species are grouped with different clade based on *mat*K sequence data. However based on *mat*K sequence both were placed in different clades.

Conclusion:-

The genus *Curcuma* of the family Zingiberaceae is complicated to differentiate using traditional morphological parameters because the morphological differences developed due to wide species hybridization. Then a Molecular marker based identification and phylogenetic analysis is required for these medicinal and economically important species. The current study suggests the effectiveness of *matK* gene sequences data to resolve phylogenetic relationship in the genus *Curcuma*. This study reveals the sequence variation, mean evolutionary rates, patterns and transition/transversion rate in nucleotide sequence, nucleotide diversity of *mat*K gene can be used for the interpretation of evolutionary relationship within inter species level of genus *Curcuma*. The *matK* sequence successfully discriminated the closely related *Curcuma* species. So these sequences can be used as a DNA barcode for the genus *Curcuma*.

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