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Research Article

Molecular Identification of Ten Palm Species using DNA Fingerprinting

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ABSTRACT

DNA Fingerprinting is a valuable tool for plant taxonomists. It is an excellent tool for specie identification at molecular level. Several genetic sequences of chloroplast DNA have been proposed as potential fingerprints. But the Scientists have suggested matK and rbcL genes as the universal fingerprints to distinguish most of the plant species. These genetic sequences differ among plant species, but are closely identical in plants of the same species. Following study was conducted for distinguishing various Palm Species through DNA fingerprinting. Ten Palm species were collected from Date Palm Research Station Jhang. DNA was extracted and quantified. Fingerprinting genetic sequences i.e. rbcL and matK were amplified and cloned. The cloned genetic sequences were analyzed for the variation. The sequences were aligned using multiple alignment tool to determine the identity and differences at nucleotide level. It showed 100% similarity of matK and rbcL, amplified from various varieties of P. dactylifera. Variation was observed in the nucleotide sequences of matK and rbcL for seven Palm species. While two species P. dactylifera and P. sylvestris contain no variation in the nucleotides of the fingerprinting genes, although morphologically they were identified as different species. Although both regions have potential to differentiate most of the Palm species but still more genes are required to be found for species distinction and identification.

Key words: *DNA, Fingerprinting, Molecular, Palm, Taxonomy*

INTRODUCTION

Palm family (*Palmae*) is the third most important family after Poaceae and Leguminosae, for human use. This family contains about 200 genera and nearly 2500 species, which are scattered in the tropical and subtropical regions of the world, where their natural biodiversity is concentrated[1]. In Pakistan, there are 16 genera and 18 species of this family[2]. Palms were first appeared in the late Cretaceous period about 80 million years ago and the successful Palm species appeared and spread 60 million years ago. Palms are monocots and characterized by a single leaf in seedling stage. They all are perennial and distinguished by woody stem, large, compound and evergreen leaves.

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They exhibit vast diversity in morphology and therefore can occupy nearly every type of habitat ranges from rainforests to deserts. DNA fingerprinting involves sequencing of short DNA sequences from a particular region of the genome and comparing them between and within species to present a "fingerprint" for species identification[3]. This technique has been proved to be a promising tool for specie identification in animals using the mitochondrial gene cytochrome C oxidase I (COI)[4]. In plants, this is not a straight forward technique because of the lower evolutionary rate of plant mitochondria, resulting in less variation in the COI gene among species. In some plants, the chloroplast genome revealed potential for candidate fingerprinting genes with characteristic features such as conserved gene order, high copy number, and easy amplification by polymerase chain reaction[5]. A multi-locus approach using the chloroplast genome has been successfully used as an effective strategy to barcode various land plants[6,7]. Recently, standard fingerprints for plants have been tested for this purpose[4,5,6,7,8]. The genes maturase K (matK) and ribulose bisphosphate carboxylase (rbcL) have been used as fingerprints by the Consortium for the Barcode of Life (CBOL) because of the easy recovery of rbcL and the high distinction power of matK[7]. These fingerprinting efforts resulted in *in-silico* libraries of DNA fingerprints that could be used as a standard for species identification.

MATERIAL AND METHODS

Leaves of 10 Palm species were collected from Date Palm Research station, Jhang (Table 1).

Table 1: Species of Palm

Species name	Common Name
Chamaedorea elegans	Good luck Palm
Livistona chinensis	Common Palm
P. roebelenii	Pygmy Date Palm
Chaemerops humilis	European fan Palm
Dypsis decaryi	Triangle Palm
Ravenea rivularis	Majesty Palm
Caryota mitis	Fishtail Palm
P. sylvestris	Silver date palm
ArchontoP. Alexandrae	Alexandra
P. dactylifera	Date Palm

Good Quality DNA was isolated from the collected samples using the standard protocol[9]. The genomic DNA concentration of collected germplasm was quantified with a Nano photometer which is used for the quantification of DNA. The optical density of each sample was calculated by measuring the absorption of the ultraviolet light of wavelength λ=260 nm.

PCR Amplifications

Two DNA regions (rbcL and matK) of Chloroplast were used as fingerprints for species identification in palms. Both reverse and forward primers of these two genes were designed at the exon regions of genomic sequence. The primers were designed keeping in view the criteria for specific amplification of the target genes.

The annealing temperature of these primers was optimized and then used in consequent studies. The list of primers along with their sequences is as in (Table 2).

Table 2: Primers and their sequences

Primer Name	Primer Sequence
matK-F	5'CGTACAGTACTTTTGTGTTTACGAG3'
matK-R	5'ACCCAGTCCATCTGGAAATCTTGGTTC3'
rbcL-F	5'ATGTCACCACAAACAGAGACTAAAGC3'
rbcL-R	5'GAAACGGTCTCTCCAACGCAT3'

Amplifications were done in 50-µL reactions. Following PCR conditions were observed: denaturation at 95°C for four minutes; ten cycles per minute, Annealing at 50°C for one minute and then final extension at 72°C for ten minutes. The PCR amplifications were separated on a 1% agarose gel with a 1-kb gene ruler (Thermo Fisher Scientific, USA).

Cloning of MatK and rbcL genes in TA cloning vector

The PCR amplified product of MatK and rbcL genes were eluted from 1% agarose gel as individual aliquots in 30 µL elution buffer using Gene jet Gel Elution Kit. The eluted PCR products of MatK and rbcL genes were ligated in TA cloning vector (pTZ57R/T). This ligation reaction was incubated at 16 ℃ and ligation monitoring was done on 1% agarose gel containing 0.05 % Ethidium Bromide along with 1kb standard DNA ladder.

Transformation

E. coli Competent cells were prepared for high transformation efficiency. The ligation mixtures of two genes (rbcL and matK) were transformed separately competent cells by heat shock method.

Plasmid Isolation and Confirmation

White colonies having recombinant vectors were selected and then cultured in LB medium at 37 ˚C overnight. Next day, their plasmids were isolated by using Gene jet Plasmid Isolation Kit. The appropriate clones were selected after restriction digestion of the plasmid based on the insert size. The selected clones were digested with BamH1 & Hind-III and run on 1 % agarose gel containing 0.05% Ethidium Bromide for insert size confirmation. Confirmed cultures were marked and preserved in 50% glycerol for further use.

Sequencing and Data Analysis

Sequencing for forward and reverse primers of both the genes was done by PacBio, California. All these sequences were submitted to BOLD under the sequence submission tool (www.boldsystems.org). All the Sequences were visually checked to clean out the incorrect portion. Reverse compliment was made using complimentor tool (www.justbio.com). Then Forward and reverse primers were marked in the forward sequences and reverse compliment sequences. The overlapped sequence was found between forward and reverse compliment sequences to get a compliment gene sequence. All the sequences were arranged in this way and then aligned by using ClustaIW (www.expasy.org). Then interspecific and intraspecific variations were pointed out. Interspecific and Intraspecific divergence was calculated by Kimura 2 parameter method in BOLD analysis tool. Substitution matrix was estimated by Mega v7.0 software. This substitution matrix gave an estimation of the substitution frequency of four nucleotides in the sequences of both genes. Phylogenetic tree was computed by Neighbor joining method with the help of Mega v7.0.

RESULTS AND DISCUSSION

Success rate of Sequencing

All the primers worked very well for all the samples. The efficiency of sequencing in both rbcL and matK and they yielded the success rate of 95.0% and 80.0% respectively (Table 3). MatK could not be sequenced in some samples. This problem was also reported for *matk* in some previous studies[10,11].

Table 3: Success rate of sequencing of *rbcl* and *matk*

Molecular Marker	rbcL	matK
No. of samples	20	20
Success of Sequencing	19	16
Success rate (%)	95.0 %	80.0%

Estimation of substitution matrix

For the gene rbcL, the analysis involved 9 nucleotide sequences. There were a total of 654 positions in the final dataset. Transition and transversion rates were as in (Table 4).

Table 4: Transition and transversion rate of nucleotides for rbcL

	A	T/U	C	G
A	-	*5.51*	*4.05*	**14.54**
T/U	*5.67*	-	**17.48**	*4.39*
C	*5.67*	**19.61**	-	*4.39*
G	**18.72**	*5.51*	*4.05*	-

Transitions and *Transversions* For simplicity, sum of *r* =100

For the gene matK, the analysis involved 6 nucleotide sequences. There were a total of 874 positions in the final dataset. Transition and transversion rates were as in (Table 5).

Table 5: Transition and transversion rate of nucleotides for matK

	A	T/U	C	G
A	-	*7.68*	*4.09*	**9.51**
T/U	*9.39*	-	**6.65**	*4.42*
C	*9.39*	**12.50**	-	*4.42*
G	**20.19**	*7.68*	*4.09*	-

Transitions and *Transversions* For simplicity, sum of *r* =100

Inter and Intra specific Divergence

Inter and intra specific divergence of each fingerprinting region was computed by calculating kimura-2 parameter distances in BOLD analysis tool. Genetic distances were calculated between all sequences of matK and rbcL within species, Genus and Family.

Table 6: Interspecific and Intraspecific Divergence by Kimura-2 Parameter method

	N	Taxa	Comparisons	Min. Dist. (%)	Mean Dist (%)	Max. Dist. (%)	SE Dist. (%)
Within Species	22	1	496	0	12.73	75	0.05
Within Genus	24	1	32	0	6.67	75	0.62
Within Family	30	1	252	0.31	16.61	75	0.12

Phylogeny

Neighbor Joining tree was constructed to find out the phylogenetic relationship between species. The tree constructed by using the *rbcl* sequences shows that there is more similarity between *A.alexandrae* and *C.elegance* because they are under the same node. *D. decayri*, *C. humil* and *L. chinensis* are independent entry. While *C. mitis* is more diversed than other species (Fig.1). For *matk*, *C. humilis* and *L. chinensis* are showing more similarity. *P. roebelenii* is representing more similarity with *P. dactylifera* and *P. sylvestris.* These results are in accordance with alignment results (Fig. 2). *P. dactylifera* and *P. sylvestris* are showing a strong relation i.e. 100 % similarity for both the genes. Although they are morphologically different from each other and exist as independent species.

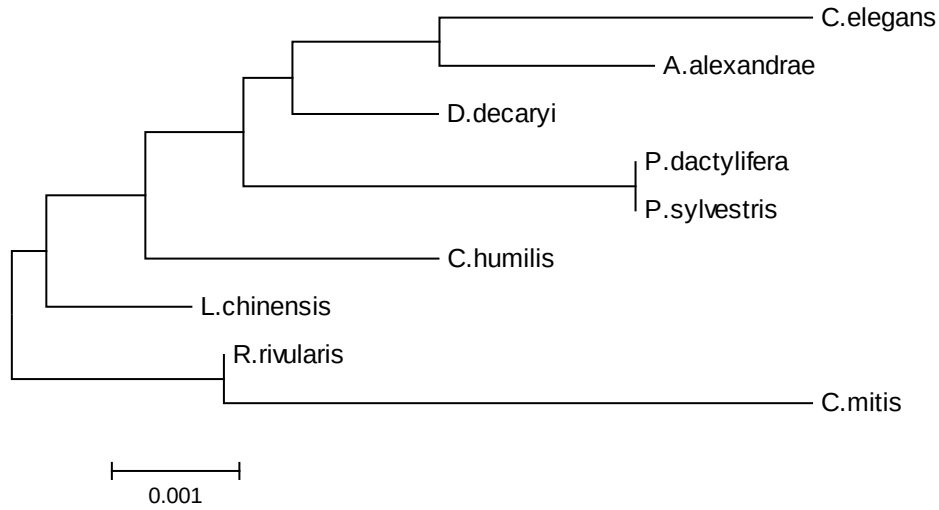

Fig. 1: Neighbor joining tree for rbcL sequences.

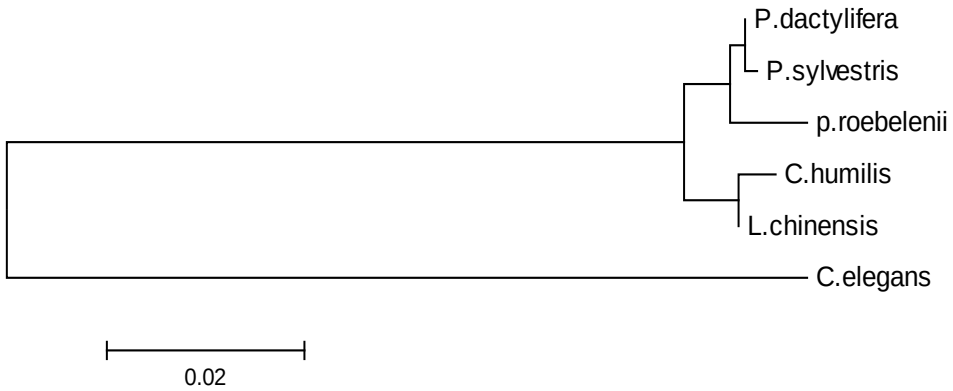

Fig. 2: Neighbor joining tree for matK sequences.

CONCLUSIONS

Such fingerprinting system based on rbcL and matK has the potential to differentiate palm species but when three species of *Phoenix* were focused, It was unable to discriminate between *P .dactylifera* and *P. sylvestris*. Lack in sequence variation of these two species may be due to low rates of sequence evolution and taxonomic misidentification[6]. This shows that these two genes are not sufficient to differentiate among all the Palm species. For complete species identification. It is suggested to include more genes and species to validate this fingerprinting technique.

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