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Short communication

A universal system for *matK* gene based diagnostic markers to identify the species in Cucurbitaceae

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ABSTRACT

Designing and validation of universal primer combination for *mat*K gene in Cucurbitaceae is described. Using the universal primers already reported, *mat*K gene amplification was attempted in 24 *Momordica* and 4 *Luffa* accessions, which was successful at varying rates. Amplified markers were gel eluted, Sanger sequenced and the sequences along with the *matK* sequences from other Cucurbitaceae genera available at GenBank, were aligned to identify the conserved regions in the locus. Primer combination was designed for the conserved regions and this primer set was found successful for all the *Momordica* and *Luffa* accessions. Subsequently, the primer combination was shown successful for other genera such as *Benincasa*, *Cucumis*, *Cucurbita*, *Citrullus*, *Coccinia*, *Trichosanthes* and *Lagenaria,* suggesting it as a universal system for *mat*K gene in Cucurbitaceae family.

Key words: Cucurbits, genetic diversity, *Momordica*, *Luffa*, systematics, taxonomy.

Genome based markers and techniques such as barcoding are approved as tools to support the phylogenetic results and when it comes to cryptic, primitive or fossil samples, this is the sole strategy in species delineation. In animals, mitochondrial gene cytochrome *c* oxidase subunit 1 (*COI*) will be capable to yield the barcode gaps among the species but in plants, the suitability of multiple loci and their combinations have to be attempted.

The family Cucurbitaceae consists of more than 130 genera representing nearly a thousand species. The tropical and sub-tropical regions of the world carry large diversity for each genus of this family and many new species and sub-species are being frequently described (John *et al*., 2). As of now, to confirm the species status, especially for a new sample, molecular markers and barcodes are extensively recommended. Works on this area are rather rare among the members of Cucurbitaceae and a universal system which could be used for differentiating the entire members of this family is missing. The primers and thermal cycling profiles previously reported for the members of this family were largely genus specific. With higher potential to identify the variation, easy amplification and alignment, a portion of the plastid *matK* gene was proposed as a universal DNA barcode for flowering plants (Lahaye *et al*., 3). Further studies by Seberg and Petersen (7) in *Crocus* have also confirmed this finding. This paper details the development and verification of primer combination for the chloroplast gene *matK* and protocols that could be employed for the development of diagnostic markers to identify the different species in Cucurbitaceae family.

Experiments were started with the attempt to develop barcodes at *matK* chloroplast gene, to differentiate the seven species of *Momordica* available in India (John *et al*., 2 and Mathew, 5). *Momordica charantia* var. *charantia* accessions Preethi, Kurupantara, JNM 7, Vadakara and V53, *M. charantia* var. *muricata* accessions Wild 1 and Wild 2, *M. dioica* accessions Kerala 1, Kerala 2, Kerala 3, Kerala 4, Odisha, *M. sahyadrica* accessions Wild 1, Wild 2, *M. sahyadrica* ssp. *anamalayana* Acc.1, *M. balsamina* Acc.1, *M. cochinchinensis* ssp. *andamanica* Acc.1, *M. cochinchinensis* accession North East, *M. subangulata* ssp. *renigera* Acc.1, Acc.2, Acc.3, Acc.4, Acc.5 and Arka Gaurav along with the *Luffa* accessions Haritham, KAU-MS-1, Deepthi and Arka Sumeet, were employed to identify the *mat*K barcodes. To confirm the universal nature of the primer combination, three accessions of *Benincasa* and two accessions each of *Cucumis*, *Cucurbita*, *Citrullus*, *Coccinia*, *Trichosanthes*, and *Lagenaria* were additionally used. The accessions were obtained from National Bureau of Plant Genetic Resources, Regional Station, Thrissur and Kerala Agricultural University, Thrissur, India.

Universal *mat*K primers reported by Dunning and Savolainen (1), Saslis-Lagoudakis *et al*. (6), Van De Wiel *et al*. (8) and Yu *et al*. (9) were attempted since no primer sets for this locus in *Momordica* or its sequences have been reported. The primers got synthesised (Sigma, India) and all possible combinations of forward and reverse primers from the five primer sets (Table 1) were tried to amplify the locus from 24 *Momordica* accessions belonging to seven species and 4 *Luffa* accessions. The PCR

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reaction mixture (20 µL) consisted of 30 ng DNA (1 µL), 10X Taq buffer A (2 µL), dNTP mix (10 mM each) (1.5 µL), Taq DNA polymerase (3U) (0.3 µL), primers (10 pM) (0.75 µL each) and water (MB grade, 13.7 µL). Conditions for thermal cycling were optimized to initial denaturation at 94 °C for 1 minute followed by 40 cycles with denaturation at 94 °C for 30 seconds, primer annealing at temperature optimised for each primer combination for 40 seconds and primer extension at 72 °C for 40 seconds, followed by final extension at 72 °C for 4 minutes. PCR products were electrophoresed on 2 % agarose gel and documented.

The bands generated in 24 *Momordica* and 4 *Luffa* accessions using different universal primers were eluted from the gel and sequenced independently using the specific forward and reverse primers. The forward and reverse sequences of individual accessions were aligned using BioEdit, annotated using MEGA v.6.0 as detailed by Mathew (4,5) and deposited in GenBank.

Subsequently, three *matK* sequences each of *Cucumis, Citrullus, Trichosanthes, Cucurbita* and *Coccinia*, were retrieved from GenBank and aligned along with these *Momordica* and *Luffa* sequences using MEGA v.6.0. The conserved sequence regions were identified and using Primer3, the forward and reverse primers were designed in the conserved regions across all the sequences, for a product size of 900 bp.

The already reported universal primer combinations were differentially successful to amplify the locus from different accessions. Only nine combinations were successful to amplify the locus from at least one *Momordica* accession whereas the primer combination 2 (*matK* F2 and *matK* R2) was most successful by amplifying the locus from seven accessions (Fig. 1).

Annotated *Momordica* sequences are available at GenBank with accession numbers KP696795, KP696796, KP696797, KP895555, KP895556, KP895557, KP895558, KP895559, KP895560, KP895561, KP895562, KP895563, KP997312, KP997313, KP997314, KP997315, KP997316, KP997317, KT004664, KT004665, KT984124, KT984125, KT984126 and MN176105 and *Luffa acutangula* accessions with accession numbers KP696798, KP759527, KP759528 and KP759529.

Table 1. The *matK* primer sets used to amplify the *Momordica* accessions

Sl. No.	Primer	Primer name	Sequence (5'-3')	References
1	*matK* F1	2.1F	CCTATCCATCTGGAAATCTTAG	Yu *et al*. (9)
	matK R1	5R	GTTCTAGCACAAGAAAGTCG	Dunning and Savolainen (1)
2	*matK* F2	Kew *mat*K 2.1F	ATCCATCTGGAAATCTTAGTTC	Van De Wiel *et al*. (8)
	matK R2	3.2R	CTTCCTCTGTAAAGAATTC	Saslis-Lagoudakis *et al*. (6)
3	*matK* F3	390F	CGATCTATTCATTCAATATTTC	Dunning and Savolainen (1)
	matK R3	1326R	TCTAGCACACGAAAGTCGAAGT	
4	*matK* F4	XF	(T)AATTTACGATCAATTCATTC	
5	*matK* F5	3F_KIM	CGTACAGTACTTTTGTGTTTACGAG	
	matK R4	1R_KIM	ACCCAGTCCATCTGGAAATCTTGGTTC	

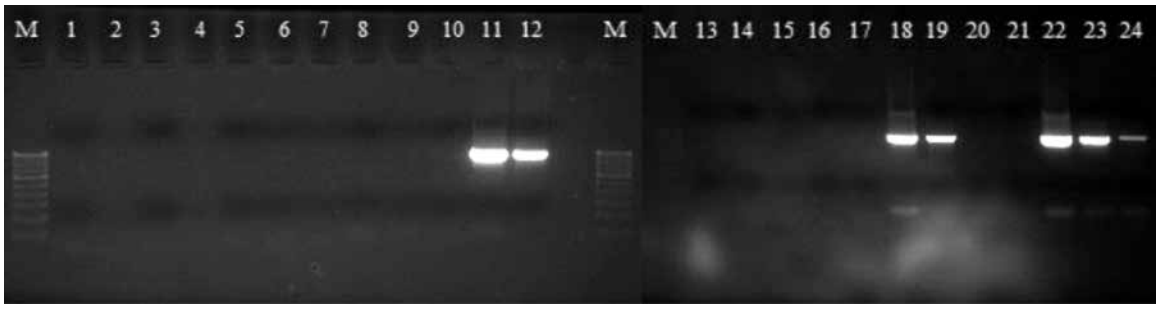

Fig. 1. Amplification of *matK* locus using the primer combination F2-R2. (Lanes – M: 1000 bp marker, 1: Preethi, 2: Kurupantara, 3: JNM 7, 4: Vadakara, 5: V53, (all *Momordica charantia* var. *charantia*), 6: *M. charantia* var. *muricata* Wild 1, 7: *M. charantia* var. *muricata* Wild 2, 8: *M. dioica* Kerala 1, 9: *M. dioica* Kerala 2, 10: *M. dioica* Kerala 3, 11: *M. dioica* Kerala 4, 12: *M. dioica* Odisa, 13: *M. sahyadrica* Wild 1, 14: *M. sahyadrica* Wild 2, 15: *M. sahyadrica* ssp. *anamalayana*, 16: *M. balsamina*, 17: *M. cochinchinensis* ssp. *andamanica,*18: *M. cochinchinensis* ssp. *cochinchinensis* North East, 19: *M. subangulata* 1, 20: *M. subangulata* 2, 21: *M. subangulata* 3, 22: *M. subangulata* 4, 23: *M. subangulata* 5 24: Arka Gaurav.

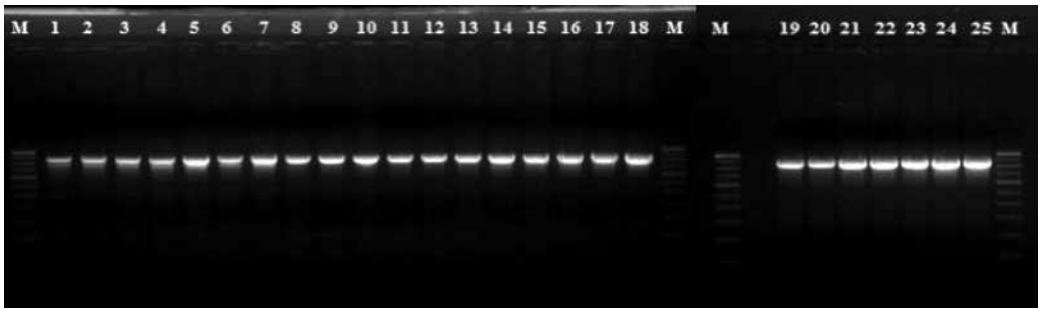

Fig. 2. Amplification of *matK* locus (900 bp) under the universal system (Lanes 1-7: Seven *Momordica* species, 8-10: *Luffa*, 11-13: *Benincasa*, 14-15: *Cucumis*, 16-17: *Cucurbita*, 18-19: *Citrullus*, 20-21-*Coccinia*, 22-23: *Trichosanthes*, 24-25: *Lagenaria.*

The primers designed by the multiple sequence alignment were F- 5'AGGGTTTGGAGTCATTGTGG3' and R- 5'GAATCGATCCAGGTCGTCTT3' with annealing temperature 59.82 °C and 59.09 °C, respectively. Under the PCR conditions detailed above, except for the annealing temperature, this combination was successful to amplify 900 bp markers of *matK* locus in all the samples including seven species of *Momordica*, three accessions each of *Luffa* and *Benincasa*, and 2 each of *Cucumis*, *Cucurbita*, *Citrullus*, *Coccinia*, *Trichosanthes* and *Lagenaria*. (Fig. 2). Currently, this primer combination and protocols are widely used to develop markers in different genera under Cucurbitaceae and hence recommended as a universal system for the *matK* based markers for species identification.

DECLARATION

The authors declare no conflict of interest.

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