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Chloroplast gene *matK* holds the barcodes for identification of *Momordica* (Cucurbitaceae) species from Indian subcontinent

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A B S T R A C T

DNA barcoding is a supplementary tool in plant systematics, extensively used to resolve the species level controversies. This paper details the identification of DNA barcodes for seven species of *Momordica*, using the chloroplast gene *matK*. Since the species *M. cymbalaria* has been confused as a member of the genus *Luffa*, 26 accessions of *Momordica* belonging to seven Indian species and two accessions of *Luffa acutangula* were included in this study. Analysis of *matK* sequences has yielded distinct barcodes in *M. charantia* var. *charantia*, M. subangulata subsp. renigera, M. cochinchinensis, M. balsamina, *M. cymbalaria* and also in *Luffa acutangula*. Evolutionary status of each species was reflected as nucleotide polymorphisms in each sequence. The wild species *M. dioica* and *M. sahyadrica* have yielded one barcode but failed to get differentiated. Further, this study provides conclusive proof that *M. cymbalaria* is a member of *Momordica* genus. The phylogram generated was successful to distinguish the monoecious species of this genus, *M. charantia*, *M. balsamina* and *M. cymbalaria*, from the dioecious species *M. dioica*, *M. sahyadrica*, *M. subangulata* subsp. *renigera* and *M. cochinchinensis*. Thus, *matK* locus, by accumulating the evolutionary sequence variations, is proven efficient to differentiate the *Momordica* species and to reveal their relatedness.

Keywords: Cucurbit; DNA barcoding; *Luffa*; Phylogeny; Systematics

1. Introduction

The genus *Momordica* derived from Latin name Mordeo (mordere = to bite) to mention the jagged seeds, is comprised of 59 species (Schaefer and Renner, 2010b). Species *Momordica charantia* (bitter gourd) is a vegetable with many culinary uses especially in Asia and Africa. In India, there are seven well identified species of which four are dioecious and three are monoecious (Joseph, 2005). The monoecious taxa are *M. charantia* L. (2n = 22), *M. cymbalaria* Fenzl ex Naud (2n = 18) and *M. balsamina* L. (2n = 22). The dioecious taxa are *M. dioica* Roxb. ex Willd. (2n = 28), *M. sahyadrica* Joseph et Antony (2n = 28), *M. cochinchinensis* (Lour.) Spreng. (2n = 28) and *M. subangulata* Blume subsp. *renigera* (G. Don) W.J.J de Wilde (2n = 4x = 56). Though the minimal descriptors have been detailed in this genus (Joseph and Antony, 2011), species allocation in few samples remains challenging. Different taxonomic classification approaches have even resulted in controversies about the number of species and their phylogenetic relationships. Further, botanical names and common names are often used incorrectly or interchangeably, making the situation more complicated (Renner and Pandey, 2013). The confusion over the species identification using morphological descriptors alone is such that *M. cymbalaria* (Hook. Fenzl ex Naud.), which is expected to be under *Momordica* has been argued for quite long time as a relative of *Luffa* (Chakravarty, 1982; Bharathi et al., 2011).

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Luffa includes four old world tropical species [*L. echinata* Roxb. (dioecious), *L.* acutangula (L.) Roxb., *L.* aegyptiaca Mill. (syn. *L. cylindrica* (L.) M. J. Roem) and *L. graveolens*] and three Neotropical species (*L. quinquefida*, *L. operculata* and *L. astorii*). Cultivated species *L.* acutangula includes *L.* acutangula var. *acutangula*, *L.* acutangula var. *forskalii* Schwein. ex Harms and *L.* acutangula var. *amara* (Roxb.) C. B. Clarke. *L.* aegyptiaca includes the cultivated var. *aegyptiaca* and wild var. *leiocarpa* (Naud.) (Heiser and Schilling, 1988; Filipowicz et al., 2014). Accessions belonging to *L.* acutangula var. *acutangula* were used in this study to verify the genus status of *M. cymbalaria*.

DNA barcodes enable the rapid and accurate species identification using short, standardized genic regions as internal species tags (Yang et al., 2019). In addition to assigning specimens to known species, DNA barcoding will accelerate the pace of species discovery by allowing taxonomists to rapidly sort specimens and by highlighting divergent taxa that may represent new species (Hebert et al., 2004). Cloroplast loci such as *rbcL*, *matK*, *psbA-trnH, rpoC1, atpF–atpH* spacer and *psbK-psbI* spacer and genomic loci such as ITS have been popularly used as DNA barcodes in plants worldwide (Hollingsworth et al., 2009).

In cucurbits, loci such as *atpB, ndhF, rbcL*, *matK*, trnL, and spacers *trnL-trnF, trnR-atpA, trnS-trnG, rpl20-rps12, psbA-trnH, Ycf9-trnG, Ycf6-PsbM* have been used to study the phylogeny (Zhang et al., 2006; Kocyan et al., 2007; Schaefer, 2007; Schaefer et al., 2008a; H. 2008b, H. 2008c; Volz and Renner, 2009; Schaefer and Renner, 2010b; Sebastian et al., 2010; P. 2012; Holstein and Renner, 2011; Telford et al., 2012; Filipowicz et al., 2014; Chomicki and Renner, 2015; Endl et al., 2018). Genomic locus *ITS* was successful to prove that cucurbits Cucumeropsis manni and Posadaea sphaerocarpa could be treated as one species (Schaefer and Renner, 2010a). Additionally, mitochondrial *nad1 b/c* intron and *matR* gene, the nuclear ribosomal *18S, ITS1-5.8S-ITS2*, and *28S* genes (Schaefer and Renner, 2011) and second intron on nuclear *LFY* gene (Volz and Renner, 2009) were also useful in cucurbit systematics.

The chloroplast gene *matK* has been identified as a leading barcode locus by CBOL Plant Working Group (Hollingsworth et al., 2009) and further it was suggested as a universal barcode locus in land plants (Chase et al., 2007; Pennisi, 2007; Lahaye et al., 2008; Newmaster et al., 2008; Seberg and Petersen, 2009). Subsequently, we have shown that this locus is efficient to differentiate the subspecies within *Momordica cochinchinensis* (Joseph et al., 2018).

However, DNA barcode-based species discrimination is rather rare in cucurbits including *Momordica*. In *Momordica*, even though the morphology and random marker-based species relations are studied (L.K. Bharathi et al., 2012a), DNA barcodes are yet to be developed to exactly differentiate the species and the candidate locus for this purpose is yet to be defined. This study was undertaken with the objective to identify the characteristic barcodes for seven Indian species for *Momordica* using the *matK* chloroplast gene.

2. Materials and methods

2.1. Plant material

Twenty six accessions belonging to seven species of *Momordica* and two accessions of *Luffa acutangula* ('Haritam' and 'Arka Sumit') were used in this study. Details on the accessions used are presented in Table 1.

The accessions were maintained in field under natural conditions and morphological characteristics such as plant growth habit, leaf color, petiole color, leaf margin, leaf shape, leaf size, leaf pubescence, flower color, immature fruit skin color, fruit surface characteristics, fruit shape and fruit size, were recorded following the minimal descriptors (Joseph and Antony, 2011).

2.2. Molecular analyses

Tender leaves, first to third from the tip, were collected on ice, surface wiped with 70% ethanol and used for total genomic DNA isolation (Rogers and Bendich, 1994). The chloroplast gene *matK* was used as the barcode locus. Since the primer combination for PCR amplification of this locus in *Momordica* was not available, universal primers for this gene was initially attempted. Sequences of the universal primer (Saslis-Lagoudakis et al., 2008; van de Wiel et al., 2009; Dunning and Savolainen, 2010; Yu et al., 2011) sets and their combinations attempted are presented in Tables 2 and 3. PCR amplification was performed in a 20 μL reaction mixture consisting 1 μL of genomic DNA (30 ng), 2 μL of 10 × Taq assay buffer A, 1.5 μL of dNTP mix (10 mmol • L^{-1} each), 0.3 μL of Taq DNA polymerase (3 U), and 0.75 μL each of primers (10 pmmol • L^{-1}).

The PCR amplification was carried out with the thermal profile suggested by CBOL (Ivanova et al., 2006) which consisted of initial denaturation at 94 °C for 1 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing for 40 s and extension at 72 °C for 40 s, followed by final extension at 72 °C for 5 min.

PCR products were gel electrophoresed, bands excised and DNA eluted using NucleoSpin® Gel and PCR Clean-up column (Macherey-Nagel, USA) following manufacturer's protocol. Eluted DNA samples were thermal cycled again using their respective primer combinations, electrophoresed and products with single intact thick band were Sanger sequenced. For the samples showing primer dimer or multiple bands on reamplification, thermal cycling conditions were optimized to obtain single band suited for direct sequencing. The PCR amplification and Sanger sequencing were performed two times, independently, and maximum forward and reverse read lengths were obtained for 26 *Momordica* and two *Luffa* accessions.

2.3. Barcode finding and phylogenetic analysis

Forward and reverse reads of each sequence were assembled using CAP3 software. Assembled sequences of 28 lines were aligned along with a reference sequence for *matK* gene from *Momordica*, using Clustal Omega. The characteristic SNPs for each species were identified and their positions were determined based on the reference sequence. SNPs characteristic for the species were considered as barcodes.

Phylogenetic analyses on the sequences were performed using the software PhyML 3.0 with 1 000 bootstrap iterations. Substitution model GTR was used with estimated gamma shape parameter and the branch support algorithm was SH-like aLRT (Guindon et al., 2010). The substitution model was automatically determined by PhyML. The Maximum Likelihood tree with boot-

Table 1 Description of Momordica and *Luffa* accessions studied

No.	Species	Description	Accessions	Collection number	Accession number	Collected by	Primer combination and product size/bp
1	*M. charantia* var. *charantia*	Bitter gourd, monoecious, $n = 11$, germination epigeal, annual, non-tuberous, muricate-tubercled, seed sides-rectangular, leaf shape-angular	Preethi	Released variety of Kerala Agricultural University, India			S13 (950)
			Kuruppantara	JDR 01–10	IC321001	Joseph John K., National Bureau of Plant Genetic Resources (NBPGR), India	S13 (950)
			Vadakara	MCC-12	NA	Kerala Agricultural University, India	S4 (1320)
			V53	MCC-07	NA	Kerala Agricultural University, India	S9 (1030)
			JNM7	MCC-18	NA	Kerala Agricultural University	S4 (1320)
	M. charantia var. *muricata*	Wild gathered, $n = 11$, multipurpose, medicinal tuber, germination epigeal, annual, non-tuberous, muricate-tubercled	Muricata 1	SBJ/02–94	IC467682	Joseph John K., NBPGR	S10 (950)
			Muricata 2	SBJ/01–15	IC467645		S1 (1150), S3 (920), S7 (1200)
2	*M. balsamina*	Wild and cultivated, medicinal, monoecious, $n = 11$, germination epigeal, annual, non-tuberous, muricate-tubercled, seed sides-rectangular, leaf shape- angular	Acc. 1	SBJ/03–135	IC467683		S6 (920)
3	var. *M. cymbalaria*	Cultivated, monoecious, $n = 9$, perennial, anthesis late in morning, fruit surface ribbed and seeds were smooth	Periyakulam	PKLM-1	NA	College of Horticulture, Tamil Nadu Agricultural University, Periyakulam, India	S13 (950), S17 (1250)
4	*M. dioica*	Pointed gourd, wild gathered, dioecious, $n = 14$, anthesis in the evening, flower small, pale yellow, intensely musky scented, male calyx whitish yellow, sepals of male flower narrow acute	KL 1	SBJ/01–26	IC467650	Joseph John K., NBPGR	S7 (1200), S9 (1030)
			KL 2	SBJ/01–28	IC467651		S4 (1320), S9 (1030)
			KL 3	SBJ/01–09	IC467670		S1 (1150), S9 (1030)
			KL 4	SBJ/02–62	IC467677		S2 (1290)
			Odisha	CHSG-1			S2 (1290)
5	*M. cochinchinensis*	Under exploited but cultivated vegetable, dioecious, $n = 14$, leaf unlobed or shallowly 3 lobed, margins undulate, male calyx green, broad, tip triangular, fruit with short conical projections, seeds large, smooth on surface	subsp. cochinchinensis var. North-East	JB/11–215	NA		S1 (1150), S6 (920)
			subsp. andamanica	JAS/08–02	IC567226		S1 (1150), S6 (920)
6	*M. sahyadrica*	Wild fruit and leafy vegetable, dioecious, $n = 14$, petals without purple blotch, male calyx hypanthium cup shaped, flower large showy, bright yellow, feeble scented, male calyx blackish purple	Wild 1	SBJ/02–130	IC540802		S1 (1150)
			Wild 2	MS2	NA		S1 (1150), S4 (1320), S7 (1200)
			Wild 3	SBJ/02–127	IC540803		S1 (1150), S4 (1320), S7 (1200)
			subsp. anamalayana	JJK/99–585	IC256223		S6 (920)
7	*M. subangulata* ssp. *renigera*	Teasle gourd, wild and cultivated vegetable, dioecious, $n = 28$, germination hypogeal, perennial, taproot tuberous, nectar of the male flower closed with prominent scales, fruit echinate, petal with black purple blotch, male calyx-hypanthium saucer shaped, leaf cordate, unlobed, margin dentate	*renigera* 1	JS/ 07–61	IC553771		S6 (920)
			renigera 2	JAS/08–12	IC567236		S1 (1150), S4 (1320)
			renigera 3	JAS/08–14	IC567238		S1 (1150), S2 (1290)
			renigera 4	JAS/08–18	IC567242		S2 (1290)
			renigera 5	JAS/08–19	IC567243		S2 (1290)
			Arka Gaurav	Released variety from Indian Institute of Horticultural Research, Bangalore			S4 (1320), S7 (1200)
8	*Luffa acutangula* var. *acutangula*	Ridge gourd or ribbed gourd, monoecious, fruits strongly ridged and not echinate, petals yellow, seeds rugose, without wings, corolla primrose yellow, opening in the evening	Haritam	Released variety from Kerala Agricultural University			S13 (950), S17 (1250)
			Arka Sumit	Released variety from Indian Institute of Horticultural Research, Bangalore			S13 (950), S17 (1250)

Note: Accessions maintained at National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Thrissur and Kerala Agricultural University, India.

Table 2 Sequences of universal matK primers used in this study

No.	Primer	Primer name	Sequence (5′–3′)	Reference
1	matK F1	21.F	CCTATCCATCTGGAAATCTTAG	Yu et al., 2011
	matK R1	5R	GTTCTAGCACAAGAAAGTCG	Dunning and Savolainen, 2010
2	matK F2	Kew matK 2.1F	ATCCATCTGGAAATCTTAGTTC	van de Wiel et al. 2009
	matK R2	3.2R	CTTCCTCTGTAAAGAATTC	Saslis-Lagoudakis et al., 2008
3	matK F3	390F	CGATCTATTCATTCAATATTTC	Dunning and Savolainen, 2010
	matK R3	1326R	TCTAGCACACGAAAGTCGAAGT	
4	matK F4	XF	(T)AATTTACGATCAATTCATTC	
	matK R4	MALV_R1	TAATGAGAAAGATTTCTGCATAT	
5	matK F5	1R_KIM	ACCCAGTCCATCTGGAAATCTTGGTTC	
	matK R5	3F_KIM	CGTACAGTACTTTTGTGTTTACGAG	
6	matK F6	ASP_F	TCAGAATTTACGATCTATTC	
	matK R6	LAM_R	GCACAAGAAAGTCGAAGTATATA	

Table 3 Combinations of forward and reverse primer used for amplifying matK locus in Momordica

No.	Primer set	Annealing temperature/ °C	Primer combination	No.	Primer set	Annealing temperature/ °C	Primer combination
1	S1	51.7	matK F1 matK R1	10	S10	48.4	matK F4 matK R4
2	S2	45.2	matK F2 matK R2	11	S11	49.5	matK F5 matK R5
3	S3	49.5	matK F3 matK R3	12	S12	55.6	matK F6 matK R6
4	S4	45.2	matK F1 matK R2	13	S13	48.4	matK F4 matK R5
5	S5	53.2	matK F1 matK R3	14	S14	48.4	matK F4 matK R6
6	S6	51.7	matK F2 matK R1	15	S15	49.5	matK F5 matK R4
7	S7	52.7	matK F2 matK R3	16	S16	49.5	matK F5 matK R6
8	S8	49.5	matK F3 matK R1	17	S17	57.9	matK F6 matK R4
9	S9	45.2	matK F3 matK R2	18	S18	51.7	matK F6 matK R5

strap values displayed in percentage was viewed in FigTree 1.4.4 (Rambaut, 2018).

3. Results

3.1. PCR amplification and sequencing of matK locus

Twenty six accessions of *Momordica*, representing seven Indian species were initially evaluated using the minimal descriptors. *In situ* maintained fully grown plants at fruiting stage were used for the evaluation. Morphological features and fruit characteristics of these accessions are presented in Fig. S1 and Table S1, respectively. The general fruit characteristics of the different *Momordica* species are presented in Fig. 1.

Of the 18 combinations of universal *matK* primers attempted in *Momordica*, 10 combinations were successful to generate the markers in varying number of accessions, at 900 bp size. In *Luffa*, only two combinations were successful. None of the primer combinations were successful to amplify the markers in all the accessions (Table 1). The *matK* markers from 26 *Momordica* and two *Luffa* accessions were eluted, purified, sequenced and submitted to NCBI GenBank (*Momordica* - KM453229, KP696795, KP696796, KP696797, KP895555, KP895556, KP895557, KP895558, KP895559, KP895560, KP895561, KP895562, KP895563, KP997312, KP997313, KP997314, KP997315, KP997316, KP997317, KT004664, KT004665, KT984124, KT984125, KT984126, MN176105 *Luffa* - KP696798, KP759529). Barcode data generated are also made available at Barcode of Life Data system (BOLD, http://www.boldsystems.org) with Process Ids MCYMB001–15, MCVAD001–15, MCJNM001–15, LAHAR001–15, LAARS001–15.

3.2. Barcode finding

By aligning 28 sequences, contig of 857 bp conserved across all the sequences was identified. Gene *matK* spanned at 155 424:156 932 bp (1 508 bp) in the *Momordica charantia* reference plastome (GenBank: MG022622.1). The contig spanned at 471–1 327 bp in *matK*.

All the five *Momordica charantia* var. *charantia* accessions (Table 1) have shown 17 characteristic barcodes at 566, 568–570, 574–579, 666, 874, 917, 981, 1 002, 1 122 and 1 224 bp positions in the gene (Table 4). The barcodes were definite, establishing the species identity. Interestingly, none of them was shared with the close subspecies *M. charantia* var. *muricata*, showing that evolutionarily, *M. charantia* var. *charantia* and *M. charantia* var. *muricata* are distinct.

Similarly, none of the barcodes in *M. charantia* var. *charantia* were shared with *M. subangulata* subsp. *renigera*, *M. cochinchinensis*, *M. dioica* and *M. sahyadrica*. The *M. charantia* var. *charantia* shared multiple barcodes with *M. balsamina* (566, 568–570, 574–579, 874, 917, 981 and 1 002 bp) and *M. cymbalaria* (566, 568–570, 574–579, 874, 981 and 1 002 bp). In all the dioecious species and *M. charantia* var. *muricata*, there was a characteristic six nucleotide deletion spanning 574–579 bp which generated conserved barcodes in monoecious species *M. charantia* var. *charantia*, *M. balsamina*, *M. cymbalaria* and also in *Luffa acutangula*. Thus, it could be seen that the

Table. 4 Barcodes at *matK* locus in seven species of *Momordica* with their positions in reference sequence

Species	566	568	569	570	574	575	576	577	578	579	585	666	724	750	787	788	806	826	846	874	913	917	968	981	984	1002	1010	1035	1036	1042	1044	1077	1122	1123	1131	1144	1145	1146	1149	1163	1174	1195	1217	1224	1246	1296	1303	1304
M. balsamina	T	G	A	A	T	C	T	T	A	G	G	C	A	C	G	A	T	G	T	C	C	G	C	T	A	A	C	T	C	T	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	A	G	T
M. cymbalaria	T	G	A	A	T	C	T	T	A	G	T	C	G	C	C	C	T	G	T	C	A	A	A	T	C	A	C	T	C	C	A	T	G	G	T	G	T	A	A	G	T	G	A	A	A	C	T	G
M. sahyadrica 1	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. sahyadrica 2	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. sahyadrica 3	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. sahyadrica ssp. *anamalayana*	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	T	A	T	A	A	C	G	G	C	A	C	T	G
M. dioica KL2	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. dioica KL4	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. dioica KL1	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. dioica KL3	A	T	C	T							G	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. dioica Odisha	A	T	C	T							T	C	G	C	G	C	T	G	A	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	C	G	G	C	A	C	T	G
M. charantia cv. Vadakara	T	G	A	A	T	C	T	T	A	G	T	A	G	C	G	C	T	G	T	C	C	G	C	T	C	A	C	T	C	C	A	G	A	G	T	G	T	A	A	G	T	G	G	T	A	C	T	G
M. charantia cv. V53	T	G	A	A	T	C	T	T	A	G	T	A	G	C	G	C	T	G	T	C	C	G	C	T	C	A	C	T	C	C	A	G	A	G	T	G	T	A	A	G	T	G	G	T	A	C	T	G
M. charantia cv. Kuruppantara	T	G	A	A	T	C	T	T	A	G	T	A	G	C	G	C	T	G	T	C	C	G	C	T	C	A	C	T	C	C	A	G	A	G	T	G	T	A	A	G	T	G	G	T	A	C	T	G
M. charantia cv. JNM7	T	G	A	A	T	C	T	T	A	G	T	A	G	C	G	C	T	G	T	C	C	G	C	T	C	A	C	T	C	C	A	G	A	G	T	G	T	A	A	G	T	G	G	T	A	C	T	G
M. charantia Reference seq.	T	G	A	A	T	C	T	T	A	G	T	A	G	C	G	C	T	G	T	C	C	G	C	T	C	A	C	T	C	C	A	G	A	G	T	G	T	A	A	G	T	G	G	T	A	C	T	G
M. charantia cv. Preethi	T	G	A	A	T	C	T	T	A	G	T	A	G	C	G	C	T	G	T	C	C	G	C	T	C	A	C	T	C	C	A	G	A	G	T	G	T	A	A	G	T	G	G	T	A	C	T	G
M. charantia var. *muricata*1	A	T	C	T							T	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. charantia var. *muricata*2	A	T	C	T							T	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	A	C	T	G
M. subangulata renigera 3	A	T	C	T							T	C	G	A	G	C	T	G	A	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	G	C	T	G
M. subangulata renigera 4	A	T	C	T							T	C	G	A	G	C	T	G	A	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	G	C	T	G
M. subangulata renigera 5	A	T	C	T							T	C	G	A	G	C	T	G	A	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	G	C	T	G
M. subangulata renigera 2	A	T	C	T							T	C	G	A	G	C	T	G	A	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	G	C	T	G
M. subangulata renigera 1	A	T	C	T							T	C	G	A	G	C	T	G	A	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	G	C	T	G
M. subangulata renigera cv. Arka Gaurav	A	T	C	T							T	C	G	A	G	C	T	G	A	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	G	G	C	G	C	T	G
M. cochinchinensis North East	A	T	C	T							T	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	T	G	C	A	C	T	G
M. cochinchinensis ssp. *andamanica*	A	T	C	T							T	C	G	C	G	C	T	G	T	A	C	A	C	G	C	G	C	T	C	C	A	G	G	G	T	G	T	A	A	G	T	T	G	C	A	C	T	G
Luffa acutangula cv. Haritam	T	G	A	A	T	C	T	T	A	G	G	C	G	C	G	C	C	A	T	C	C	G	C	T	C	A	G	C	A	C	C	G	G	T	G	G	T	G	G	G	T	G	G	C	A	C	T	G
Luffa acutangula cv. Arka Sumit	T	G	A	A	T	C	T	T	A	G	G	C	G	C	G	C	C	A	T	C	C	G	C	T	C	A	G	C	A	C	C	G	G	T	G	G	T	G	G	G	T	G	G	C	A	C	T	G

Note: Highlighted bases indicate the nucleotides characteristically conserved among the accessions of accessions of a species and hence were indentified as barcodes.

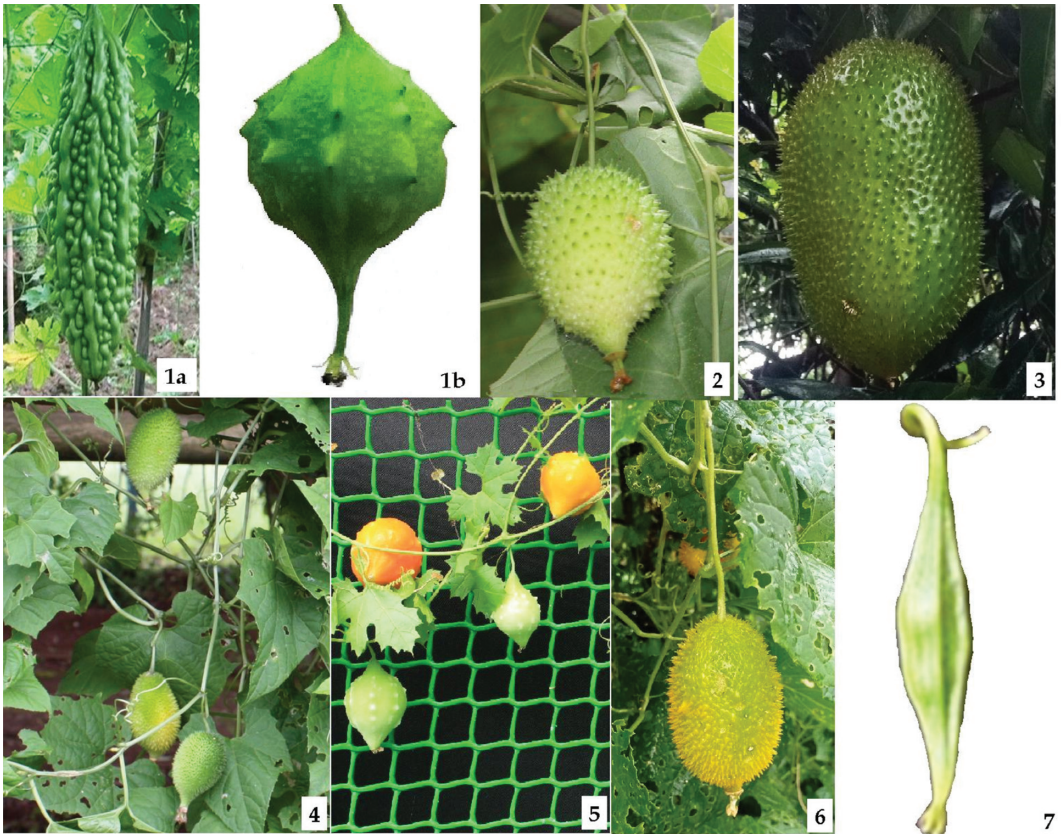

Fig. 1 Fruit characteristics
a: *M. charantia* var. *charantia*; b: *M. charantia* var. *muricata*; 2: *M. dioica*; 3: *M. cochinchinensis*; 4: *M. sahyadrica*; 5: *M. balsamina*; 6: *M. subangulata* ssp. *renigera*; 7: var. *M. cymbalaria*.

cultivated/ monoecious species bear and share more barcodes, pointing to a faster evolution under cultivation compared with the wild types. Though *M. charantia* var. *muricata* and *M. charantia* var. *charantia* belong to the same species, the wild type had no barcodes.

M. subangulata subsp. *renigera* accessions had highly conserved unique barcodes at 750, 846 and 1 246 bp. Among them, barcodes at 846 bp was shared with Odisha accession of *M. dioica*, and others were characteristic to this species. Accessions of *M. cochinchinensis* had one barcode at 1 195 bp, which was unique for this species.

Other wild species *M. dioica* and *M. sahyadrica* shared one barcodes at 585 bp, which was also seen in *M. balsamina*. Among the *M. dioica* accessions, Odisha accession was distinct with the absence of the barcode at 585 bp but two nucleotide polymorphisms were seen at 846 and 1 174 bp. Polymorphism at 1 174 bp was shared with *M. sahyadrica* ssp. anamalayana. The accession *M. sahyadrica* ssp. anamalayana had five additional polymorphisms compared to *M. sahyadrica* ssp. sahyadrica at 1 144–1 146, 1 163 and 1 174 bp, showing a clear distinction among the two subspecies.

M. balsamina had 22 barcodes of which 13 were shared with *M. cymbalaria* and 14 with *M. charantia* var. *charantia*. With 19 barcodes, *M. cymbalaria* was also distinct from the rest of the species. Compared to *Momordica* species, *Luffa* had 25 barcodes. Even though 14 of them were shared with *M. charantia* var. *charantia*, with the locus wide unique barcodes (806, 826, 1 010, 1 035, 1 036, 1 044, 1 123, 1 131 and 1 149 bp), divergence among the genera was evident.

3.3. *Phylogenetic analysis*

The phylogram (*Luffa* rooted) generated using the *matK* sequences (Fig. 2) had distinctly separated *Luffa* and *Momordica* accessions. All the *Momordica charantia* var. *charantia* accessions have been clustered together with high bootstrap values. *Momordica* cymbalaria was found to fall within *Momordica* cluster, clearly showing that even with the fruit shape dissimilarity, this species belongs to *Momordica* genus, only *M. cymbalaria* formed sub-cluster within *Momordica* cluster, showing that *matK* is good enough to establish species delineations in cucurbits.

4. Discussion

4.1. *Barcode analysis*

The genus *Momordica* includes 59 species (Schaefer and Renner, 2010b) of which about 12 are reported from Southeast Asia and seven from India (Bharathi and Joseph, 2013). Among these seven, *M. charantia* var. charantia is extensively grown and marketed whereas, cultivation of *M. subangulata* subsp. *renigera*, *M. balsamina*, *M. cochinchinensis* and *M. cymbalaria* is restricted to certain pockets in India. For amplification of *matK* locus through

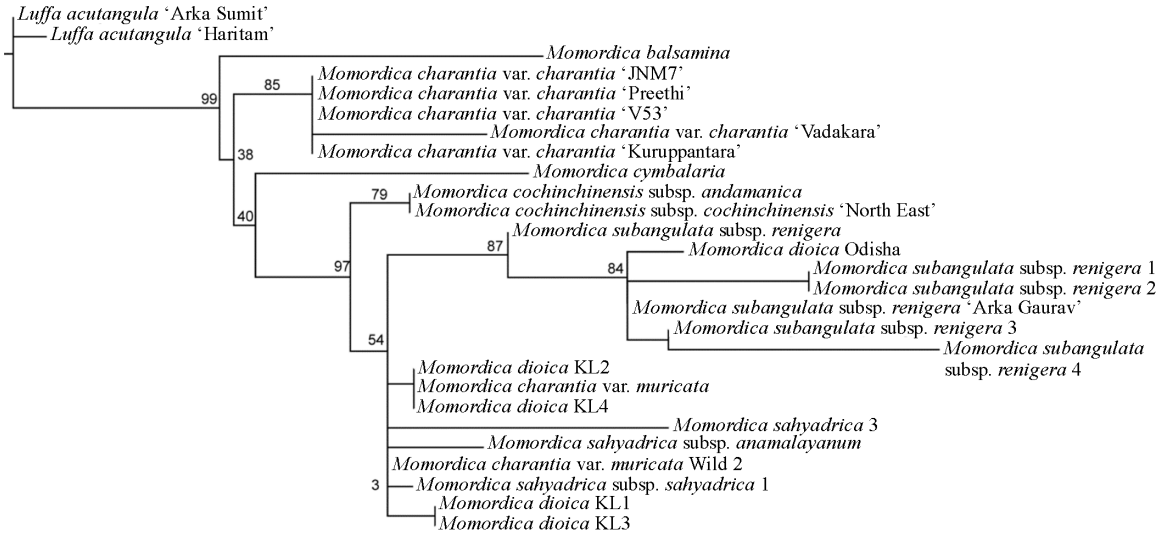

Fig. 2 Maximum likelihood tree of Indian Momordica species derived using the matK gene sequences Numbers indicate the percent (%) bootstrap values.

thermal cycling, primer combination reported by Schaefer and Renner (2010a) was initially attempted in this study. Since this combination has failed to amplify the locus in few *M. charantia* var. *charantia* and *Luffa* accessions, universal primer combinations tried. But those combinations were only partially successful among the accessions. The *matK* locus was thus amplified in all the accessions using different primer combinations, eluted, cleaned, sequenced and analysed.

M. cymbalaria is commercially grown minor vegetable at Periyakulam area in Tamil Nadu state of India. The presence of large number of barcodes obviously points to the extensive cultivation for many years. There has been taxonomic uncertainty on this species, if it belongs to *Momordica* or *Luffa*. Of the 19 barcodes identified in *M. cymbalaria*, 13 were shared with *Momordica* and six were unique.

Species *M. cymbalaria* Fenzl ex Naud. was initially named *Luffa* tuberosa (Roxburgh, 1814, 1832) and subsequently transferred to the genus *Momordica* as *Momordica* cymbalaria (Clarke, 1879). Then it was renamed M. tuberosa (Roxb.) Cogn (Cogniaux, 1881). Still it has been confused to belong to *Luffa* due to long pedicellate distinct shaped flowers with exert anthers, similar to ridge gourd (Bharathi and Joseph, 2013).

Even though the fruit was similar to *Luffa* amara Wall., stopple, one of the generic characters of *Luffa*, was absent in this species and fruits had only eight angles (Roxburgh, 1832). Absence of stopple was a major support for the scientists who argued that it should remain with *Momordica*. But, the absence of true cystoliths of calcium carbonate on the lower surface of the leaf, which is a characteristic feature of *Momordica*, forced *M. cymbalaria* again back to *Luffa* genus (Chakravarty, 1959). Chakravarty (1982) further supported its position in *Luffa* since *Momordica* has either muricate or echinate fruits but never angular.

However, similarity of *M. cymbalaria*'s seed coat (Singh and Dathan, 2001) and seed fat (Azeemoddin and Rao, 1967) characteristics with other members of *Momordica* genus has forced its retention with *Momordica*. More recent studies on pollination biology and comparative morphology made scientists to place it under *Luffa* (Joseph and Antony, 2010) and differences of this species from other Indian species was detailed (Bharathi et al., 2011; L.K. 2012a). Still it is closer to African species such as M. sessilifolia, M. kirkii, M. humilis, M. boivinii (Schaefer and Renner, 2010b) and M. cabraei (Ali et al., 2010). Recently, based on ITS sequences of nuclear ribosomal DNA (Ali et al., 2010) and genomic phylogeny (Schaefer and Renner, 2010b), the status of this species in *Momordica* was established. Till today, *M. cymbalaria*'s position is unclear especially since multiple attempts to cross it with *Momordica* species available in India are reported to have failed (Pandey et al., 2006; Bharathi et al., 2011). In the phylogram generated from the present study, it is evident that *M. cymbalaria* falls within the genus *Momordica* and the *Luffa* lines have clustered outside the *Momordica* cluster.

4.2. *Phylogenetic analysis*

The phylogram has clustered monoecious species *M. charantia* var. *charantia* and *M. balsamina* close to each other. Both these species form the sect *Momordica*. Interestingly, these two species lie close to the third monoecious species *M. cymbalaria* which forms the sect Raphanocarpus. Thus, *matK* locus is successful to differentiate the monoecious species of *Momordica* from dioecious species.

Even though the varieties charantia and muricata are accommodated within *M. charantia*, barcode analysis at *matK* locus suggests their independent evolution. Their limited crossability (Bharathi et al., 2012b; Asna et al., 2018) also points to the evolutionary distinctiveness. Accessions of *M. charantia* var. *muricata* had no barcode, suggesting that from the base sequence in *M. charantia* var. *muricata*, other species with sequences having multiple SNPs might have been evolved. Lack of evolutionary variations at this maternally inherited locus shows that this is the least evolved and progenitor species in *Momordica* genus. The present results provide the molecular level proof for the previous reports that the wild variety *M. charantia* var. *muricata* is the progenitor of cultivated *M. charantia* var. *charantia* (Degner,

1947; Walters and Decker-Walters, 1988). Similarly, extensive sequence level variations in cultivated species in comparison with the progenitor species are reported in many plants (Olsen, 2004; Hand et al., 2008; Cheung et al., 2009). Abundant single nucleotide polymorphisms at *matK* locus in extensively cultivated species *M. charantia* var. *charantia* shows that commercial cultivation and human selection in any species leads to rapid generation advancements and evolution (Tang et al., 2010). Thus, commercially grown species are more likely to have barcodes in these candidate loci. Similarly, one accession each in *M. dioica* and *M. sahyadrica* had SNPs at this locus, supporting the primitive status of these species (Ali et al., 1991).

4.3. *Additional information from the barcode pattern*

Accessions of Asiatic dioecious wild species *M. dioica* and *M. sahyadrica* used in this study were diverse, including a distinct one from Odisha state (India) and an accession belonging to the subspecies anamalayana from Anamalai hills (Kerala state, India), respectively. The species *M. sahyadrica* was identified from *M. dioica*, mainly based on the time of flower anthesis (Joseph and Antony, 2004). In both these species, no definite barcode was identified, but the variability observed at *matK* in Odisha and *M. sahyadrica* ssp. anamalayana points to their distinct status, demanding further investigations.

In the phylogram generated, the dioecious species *M. cochinchinensis*, *M. dioica*, *M. sahyadrica* and *M. subangulata* subsp. *renigera* belonging to the sect Cochinchinensis have clustered together. *M. dioica* with *M. cochinchinensis* and *M. dioica* with *M. subangulata* subsp. *renigera* are reported to be completely cross compatible. *M. cochinchinensis* with *M. subangulata* subsp. *renigera* and *M. sahyadrica* with *M. cochinchinensis* are partially cross compatible (Bharathi et al., 2012b). Similarly, *M. dioica* and *M. sahyadrica* fall within the same cluster. It is well shown that *M. dioica* and *M. sahyadrica* are genetically closer and cross compatible (Bharathi et al., 2012b). These results clearly show that *matK* is a candidate locus to differentiate the *Momordica* species and to understand their relations.

5. Conclusions

Confusions over species identity have been a concern in *Momordica* genus. Very often, *M. subangulata* subsp. *renigera* is confused as *M. dioica* (Hossain et al., 1996) or *Momordica* cochinchinensis (Sanwal et al., 2011) and similarly, *M. cymbalaria* has been debated to be included in the genus *Luffa*. DNA barcoding assists plant taxonomy by studying the characteristic variations for each species in the widely recognised genomic loci. The present study using *matK* chloroplast gene sequences in 26 accessions of *Momordica* belonging to seven species and 2 accessions of *Luffa* has established characteristic barcodes for each species except the wild *M. dioica* and *M. sahyadrica*. Additionally, the analysis indicated that *M. charantia* var. *muricata* is the progenitor for genus *Momordica*.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hpj.2021.04.001.

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