## **Short Communication**

# Molecular distinction of two closely resembling *Morinda* species using *rbc*L and *mat*K loci for quality management of Indian herbal medicines

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### Abstract

*Morinda reticulata* Gamble and *Morinda umbellata* Linn. (Rubiaceae) are medicinally important climbers distributed as a mixed population in southern Western Ghats of India. A close morphological resemblance of these two species misleads the harvester in the identification of plant parts for preparation of herbal medicines. Though both species contain anthraquinone derivatives and share common medicinal properties for treating stomach disorders, each of these species has unique curative properties for treating selective diseases. Conventional methods are not reliable for identification of these species due to similarities in morphology. Thus, misidentification often leads to the deterioration of the quality of medicines. Thus, authentication utilizing conserved gene sequences in the chloroplast genome of these two *Morinda* spp. has been attempted for precise identification. Here we report the use of two barcoding genes (maturase kinase and ribulose 1,5-bisphosphate carboxylase large subunit) to distinguish *M. reticulata* and *M. umbellata* based on single nucleotide polymorphism. The present findings can be used for authenticating leaf samples of *M. reticulata* and *M. umbellata*.

**Keywords:** herbal medicines; *M. reticulata*; *M. umbellata*; quality management; Rubiaceae; single nucleotide polymorphism

### **Experimental**

Morphological parameters of both species are documented (Table 1 and Supplementary Table S1, available online only at http://journals.cambridge.org). About 58 authenticated leaf samples were collected from 16 locations representing eight provinces (Supplementary Table S2, available online only at http://journals.cambridge.org), and genomic DNA was extracted using a DNA isolation kit following the manufacturer's protocol (Helini Biomolecules, Chennai, India).

PCR amplification was performed with 25 ng DNA using universal primers (Supplementary Table S3, available

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online only at http://journals.cambridge.org). The PCR mixture consists of  $25 \,\mu$ l PCR mix (contains  $10 \times$  Taq buffer, 2mM MgCl<sub>2</sub>, 0.4mM dNTP mix and 2U Taq DNA polymerase), forward and reverse primers of 1 µl each (10 pmol/ml) and 1 µl template DNA. The final volume was made up to  $50\,\mu$ l by adding  $22\,\mu$ l nuclease free water. PCR conditions for amplification are detailed in Table S3 (available online only at http://journals. cambridge.org). The amplified PCR products were sequenced using an ABI 373 automated sequencer (Applied Biosystems, Inc., Foster City, CA, USA). These sequences were analysed using the BLAST of NCBI with BLASTX and annotated using the NCBI ORF tool combined with BLASTP (Supplementary Table S4, available online only at http://journals.cambridge.org). Multiple sequence alignment (MSA) was performed using the MultAlin interface (Corpet, 1988). Evolutionary trees were constructed using the neighbour-joining method (Saitou and Nei, 1987) with the Kimura-2 parameter distance correction (Kimura, 1980) implemented in MEGA 5.05 (Tamura et al., 2011).

#### Discussion

The observed morphological parameters of *M. umbellata* and *M. reticulata* were similar to those of published data (Dassanayake and Clayton, 1998; Gopalan and Henry, 2000). It was found that the distinction of these two closely resembling *Morinda* species is difficult in a mixed population distributed at an altitude between 2000 and 5000 ft. The leaves of both species resemble closely (Table 1). *M. umbellata* exhibits variability in leaf size and shape. The observation of *M. umbellata* revealed

eight different shapes of leaves with varying sizes. A similar observation of *M. reticulata* revealed only four different shapes of leaves, resembling to *M. umbellata*. Reproductive parts showed a close resemblance between the two species. Flowering and fruiting seasons were overlapping with each other (Supplementary Table S1, available online only at http://journals.cambridge.org).

The MSA of rbcL (534bp) and matK (621bp) between M. umbellata and M. reticulata revealed three SNPs at sites 1, 22 and 51 in *rbc* L and only one SNP at nucleotide position 499 in matK (Fig. 1(a) and (b); Supplementary Figs S1 and S2, available online only at http://journals. cambridge.org). Genetic divergence between the two species was found to be 0.002 for matK and 0.006 for *rbc*L by taking the number of base substitutions per site into account, and evolutionary dendrograms were constructed (Supplementary Figs S3 and S4, available online only at http://journals.cambridge.org). Sequencing of these genes from both species from 16 samples covering eight provinces did not reveal any intra-specific variations in the sequenced regions. Thus, the SNPs identified for the distinction between *M. reticulata* and *M. umbellata* can be used as a reliable DNA marker.

The use of adulterated herbal medicines often leads to health hazards (Mosihuzzaman and Choudhary, 2008). DNA markers are the most reliable tool for the precise identification of herbs compared with unreliable conventional methods (Kaplan *et al.*, 2004). Significance in the precise identification of *Morinda* species was emphasized as a high degree of morphological variability was observed within the *Morinda* genus (Roonyamarai *et al.*, 2011). *M. reticulata* and *M. umbellata* are indispensable part of herbal formulations in India. Habitat destruction and large-scale harvesting rendered the status of *M. reticulata* 

Table 1. Resemblance of the vegetative characteristic features between Morinda umbellata and Morinda reticulata

Parameters	M. umbellata	M. reticulata			
Habit	Climbing shrub	Climbing shrub			
Habitat	Deciduous forests and plains	Evergreen forests			
Distribution	Hills of Eastern Bengal, Madgoll Hills of Visakhapatnam at 4000–5000ft, Kambakam Chingleput, Horsley Konda in Chittoor, southern	Strictly endemic to southern Western Ghats of Tamil Nadu (Kanyakumari) and Kerala (Kollam and Travancore mountains) at 1500 ft and Western Ghats up to 5000 ft			
	Western Ghats of Coimbatore, Nilgris and Palakkad at an altitude up to 2000 ft				
Leaves	Opposite	Opposite			
Leaf size	$6-12 \times 2-3.5$ cm	$2.5 - 15 \times 0.5 - 5$ cm			
Leaf shape	Lamina elliptic-lanceolate or oblanceolate obovate abruptly acuminate	Oblanceolate or linear oblong, sometimes abruptly caudate-acuminate			
Leaf texture	Usually pubescent, membranous	Coriaceous and glabrous			
Petiole length	Up to 10 mm	Up to 12 mm			
Venation	6–8 pairs	5–12 pairs			
Reticulation	Fine	Fine			
Stipule	6–7 mm long, connate at the base	Stipules small, connate at the base			

	1	10	20	30	40	50	60	70	80	90	100	110	120	130
M. reticulata	ATGGCA	GCATTCCGAG	TRACTGCCCA	ACCTEGAET	TCCACCGGAA	GARGC <mark>a</mark> gggg	CCGCGGTAGC	GCCGAGTCT	TCTACTGGTA	CATGGACAAC	TGTATGGACG	GATGGACTT	ACCAGTCTTGA	
M. umbellata Consensus														
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	131	140	150	160	170	180	190	200	210	220	230	240	250	260
M. reticulata														
M. umbellata Consensus														
	261	270	280	290	300	310	320	330	340	350	360	370	380	390 
M. reticulata														
M. umbellata Consensus														
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M. reticulata													0000000000	
M. umbellata														
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	521	530 534												
M. reticulata	100 B	20112020												
M. umbellata														
Consensus	CATTTA	IGCGTTGG												
(b)	1	10	20	30	40	50	60	70	80	90	100	110	120	130
M. reticulata														130
m. renoulate	CCTTC	GTTATTGGGT	AAAAGATGCO	TCCGCCTTG	CATTIATIAC	ATTATTTT	CACGAATATT	+	+	+	+	TTTCACCARA	+	
M. umbellata	CCTTC	GTTATTGGGT	AAAAGATGCO	TCCGCCTTG	CATTTATTAC	ATTATTTT	CACGAATATI	GGAGTTGGA GGAGTTGGA	ATACTETTAG Atactettag	IGCTACAAAG Igctacaaag	ARACCCCATT	TTTCACCARA	iaagaaatcaa iaagaaatcaa	GATTT
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M. umbellata Consensus M. reticulata		GTTATTGGGT GTTATTGGGT 140 	ARAAGATGCC AraagatgCC 150 Artictcate Ratictcate	TCCGCCTTG TCCGCCTTG 160 CATATGAAT	CATTTATTACI CATTTATTACI 170 ACGRATCCATT ACGRATCCATT	ATTATTITI ATTATTITI 180 TTGGCCTTTC	CACGAATATT CACGAATATT 190 TCCGTAACCA	GGAGTTGGA GGAGTTGGA 200 Atcttcgca	ATACTCTTAGI Atactcttagi Atactcttagi 210 TTTGCGATCAI	IGCTACAAAG IGCTACAAAG IGCTACAAAG 220 ICATCTTTTG	ARACCCCATT Araccccatt 230 Tattctttct Tattctttct	240 CGAACGACTC	IAAGAAATCAAA IAAGAAATCAAA IAAGAAATCAAA 250 TATTTTTATGO	260
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M. umbellata Consensus M. reticulata M. umbellata Consensus M. reticulata M. reticulata M. umbellata Consensus M. reticulata M. umbellata	CCTTCC    131    I    131    Zef1    261    I    ARGAR    391    GATA    GATA    521    CGATA	STIATTGGGT    140    CTTATTATAT    CTTATTATAT    270    CGTCTTGTAG    GCGTCTTGTAG    CGTCTTGTAG    400    RATGGARATC    AAGGARATC    530	ARRAGATGCC ARARGATGCC 150 ARTTCTCATC ARTTCTCATC ARTTCTCATC 280 ARGTTGTTGC ARGTTGTTGC ARGTTGTTGC ARGTTGTTGC 410 TTATCTTGTC TTATCTTGTC 540	TCCCCCTTG TCCCCCTTG 160 CATATGRAT CATATGRAT 290 TARGGATTT TARGGATTT 420 RATTITTGG RATTITTGG 550 GTCRAATGC	CATTATTAC CATTATTAC 170 ACGARTCCAT ACGARTCCAT ACGARTCCAT 300 TCGGGTTAGT TCGGGTTAGT CGGGTTAGT CGGGTTAGT CATATCACT CATATCACT 560 TAGARAATTC	ATTATITITI    ATTATITITI    ATTATITITI    ATTATITITI    ATTATITITI    ATTATITITI    ATTATITITI    ATTATITITIC    ATTATITITIC    ATTATITITIC    ATTATITITIC    ATTATITIC	CACCGARTATT CCACGARTATT 190 TCCGTRACCC TCCGTRACCC 320 TCCCGTRACCC 320 TCCCGTRACCC 320 TCCCGGACCCT 450 TCCCGGACCCT 450 TCCCGGACCCT 450 TCCCGGACCCC 580	GGAGTTGGA GGAGTTGGA 200 ATCTTCGCA ATCTTCGCA ATCTTCGCA ATCTTCGCA ATCATCGCA TTCATGCAT TTCATGCAT 460 GGAGGGGTTT GGAGGGGTTT GGAGGGGTTT 590	ATACTCTTAGT ATACTCTTAGT 210 TITGCGATCAT 340 TATGTTAGGTT ATGTTAGGTT 470 ATATARAATCAT ATATARAATCAT 600 GGATATGCTTA	IGCTACARAGE IGCTACARAGE 220 ICATCTITIG ICATCTITIG ICATCTITIG ICATCTITIG ICAAGGAAAA ITCAAGGAAAA 480 ITCAAGGAAAA 480 ITTTCCAATTA 610	ARACCCCATT ARACCCCATT 230 TATTCTTTCT TATTCTTTCT TATTCTTTCT 360 ATCCATTTG ATCCATTTG 490 CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG	TTTCACCARA TTTCACCARA 240 CGAACGACTC CGAACGACTC CGAACGACTC 370 GTTTCAAAGG GTTTCAAAGG 500 ACTTTCAAAGG 500 ACTTTATGGG ACTTTATGGG ACTTTATGGG	IARGARATCARI IARGARATCARI 250 TATTITTATGO TATTITTATGO TATTITTATGO 380 GTACACCTCTT GTACACCTCTT 510 CTATTGTTCRI CTATTGTTCRI	Image: Constraint of the second sec
M. umbellata Consensus M. reticulata M. umbellata Consensus M. reticulata M. umbellata M. reticulata M. umbellata Consensus	CCTTCC    131    I    131    I    131    I    131    I    131    I    261    I    AAGAA    AAGAA    AAGAA    GAATA    521    I    CGACTA    CGACTA    CGACTA	140 CTTATTAGGT 140 CTTATTATAT CTTATTATAT CTTATTATAT 270 CGTCTTGTAG CGTCTTGTAG CGTCTTGTAG CGTCTTGTAG CGTCTTGTAG A00 AATGGAAATC 530 AAATCCGGCA AAATCCGGCA	ARAGATGCC    150    ARATCTCATC    ARATCTCATC    ARATCTCATC    ARATCTCATC    ARATCTCATC    280    ARATCTCATC    ARAGTTGTTGC    ARAGTTGTTGC    ARAGTTGTTGC    ARATCTCATC    410    TTATCTTGTC    540    ATGGTRCGGG	TCCCCCTTG TCCCCCTTG 160 CCATATGAAT CCATATGAAT 290 TAAGGATTT TAAGGATTT 420 RATTTTGG RATTTTGG S50 GCCRAATGC GCCRAATGC	CATTTATTAC CATTTATTAC 170 ACGAATCCAT ACGAATCCAT 300 TCGGGTTAGTT TCGGGTTAGTT 430 CAATATCACT CAATATCACT CAATATCACT 560 TAGAARATTCI TAGAARATTCI TAGAARAATTCI	ATTATTATTATTATTATTATTATTATTATTATTATTATT	CRECEGARTATT 190 TCCGTARCCF TCCGTARCCF TCCGTARCCF 320 TCCGTARCCCT 320 TCCGCAGACCCT 450 TTCCACGACCCT 450 TTCCACGACCCT 450 TTCCACGACCCT 450 TTCCACGACCCT 450	GGAGTTGGA GGAGTTGGA 200 ATCTTCGCA ATCTTCGCA ATCTTCGCA 330 TTCATGCAT TTCATGCAT TTCATGCAT 460 GGAGGGTTT GGAGGGTTT 590 TAAGAAATT TAAGAAATT	ATACTCTTAGE ATACTCTTAGE 210 TTTGCGATCAT TTGCGATCAT 340 TATGTTAGGTT ATGTTAGGTT ATGTTAGGTT 470 ATATARATCAT ATATARATCAT 600	IGCTACARAG IGCTACARAG IGCTACARAG IGCTACARAG IGCTACARAG IGCTACATTIG IGCATCTITTG IGCATCTITTG IGCATCARAG ITCARAGGARA 480 ITTTCCARATA ITTTCCARATA ITTTCCARATA ITTCCCARATA	ARACCCCATT ARACCCCATT 230 TATTCTTTCT TATTCTTTCT TATTCTTTCT AGO ATCCATTTG ATCCATTTG ATCCATTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG CATTCACTTG	TTTCACCARA TTTCACCARA 240 CGAACGACTC CGAACGACTC CGAACGACTC 370 GTTTCAAAGG GTTTCAAAGG 500 ACTTTCAAAGG 500 ACTTTATGGG ACTTTATGGG ACTTTATGGG	IARGARATCARI IARGARATCARI 250 TATTITTATGO TATTITTATGO TATTITTATGO 380 GTACACCTCTT GTACACCTCTT 510 CTATTGTTCRI CTATTGTTCRI	Image: Constraint of the second sec

**Fig. 1.** (a) Nucleotide sequence alignment of a 534 bp fragment spans from position +89 to +622 of the 703 bp *rbc*L open reading frame, using the *Morinda officinalis* (GQ436556) *rbc*L sequence as reference. (b) Nucleotide sequence alignment of a 621 bp fragment spans from position +62 to +682 of the 809 bp *mat*K open reading frame, using the *M. officinalis* (GQ434175) *mat*K sequence as reference. A colour version of this figure can be found online at journals.cambridge.org/pgr

(a)

as endangered. The roots of *M. umbellata* possess high medicinal properties and the root bark contains colouring constituents (Nair and Seeni, 2002). Thus, the misidentification of *M. reticulata* as *M. umbellata* poses threats to the existing population. Both species share common medicinal properties. However, M. reticulata was precisely used for back pain, blood purification and postnatal care (Ijinu et al., 2011), whereas M. umbellata is used for leukaemia, gonorrhoea and syphilis (Nair and Seeni, 2002; Ismail and Sulthana, 2008). Thus, misidentification leads to the deterioration of the quality of herbal drugs as they exhibit unique medicinal properties. The mixed population of these species with variations in morphology due to environmental conditions (Singh et al., 2011) confounds the harvesters. Thus, the development of a DNA marker is essential for monitoring the population besides maintaining the quality, as M. umbellata has been extensively supplied as an adulterant/substitute for Coscinium fenestratum Gaertn. (Menispermaceae) (Balasubramani and Venkatasubramanian, 2011). DNA barcodes have been utilized for detecting adulterants in Chinese herbal medicines (Asahina et al., 2010); however, no serious attempt has been made in the context of Indian herbal medicines. This work describes the utilization of DNA barcodes for the two closely related Morinda spp. of medicinal importance. The frequency of SNPs was higher in rbcL than in matK, indicating rbcL to be more reliable for distinguishing these species. Sequencing of complete matK may reveal more variations since a faster substitution rate has been reported in matK than in rbcL (Olmstead and Palmer, 1994). This result is expected to be useful for authentication of these species either in the state of raw material or with other herbal medicines. This result is reliable over phytochemical methods due to reproducibility. Moreover, minute quantities of samples are sufficient for analysis. A similar study can be applied to distinguish other closely related medicinal plants in addition to monitoring adulterants in commercial herbal formulations by utilizing the rbcL and *mat* K genes.

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