ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization (2013) **11**(1); 90–93
doi:10.1017/S147926211200041X

Short Communication

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

Rahul R. Nair[1], P. S. Udayan[2], S. Thilaga[1], M. Kavitha[1], R. M. Bharathanandhini[3], A. M. Nizzy[3] and D. Ganesh[3]*

[1]*Plant Genetic Improvement Laboratory, Department of Biotechnology, SPK Centre for Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi 627 412 Tirunelveli District, Tamil Nadu, India,* [2]*Department of Botany, Sree Krishna College, Ariyannur 680 102, Thrissur District, Kerala, India and* [3]*Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Palkalai Nagar Madurai 625 021, Tamil Nadu, India*

Received 26 August 2012; Accepted 30 October 2012 – First published online 29 November 2012

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

* Corresponding author. E-mail: ganeshdsneha@yahoo.co.in

online only at http://journals.cambridge.org). The PCR mixture consists of 25 µl PCR mix (contains 10 × Taq buffer, 2 mM $MgCl_2$, 0.4 mM dNTP mix and 2 U 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 µl by adding 22 µl 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 *rbc*L (534 bp) and *mat*K (621 bp) 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 *mat*K (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 *mat*K 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 4000–5000 ft, Kambakam Chingleput, Horsley Konda in Chittoor, southern Western Ghats of Coimbatore, Nilgris and Palakkad at an altitude up to 2000 ft	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
Leaves	Opposite	Opposite
Leaf size	6–12 × 2–3.5 cm	2.5–15 × 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

https://doi.org/10.1017/S147926211200041X Published online by Cambridge University Press

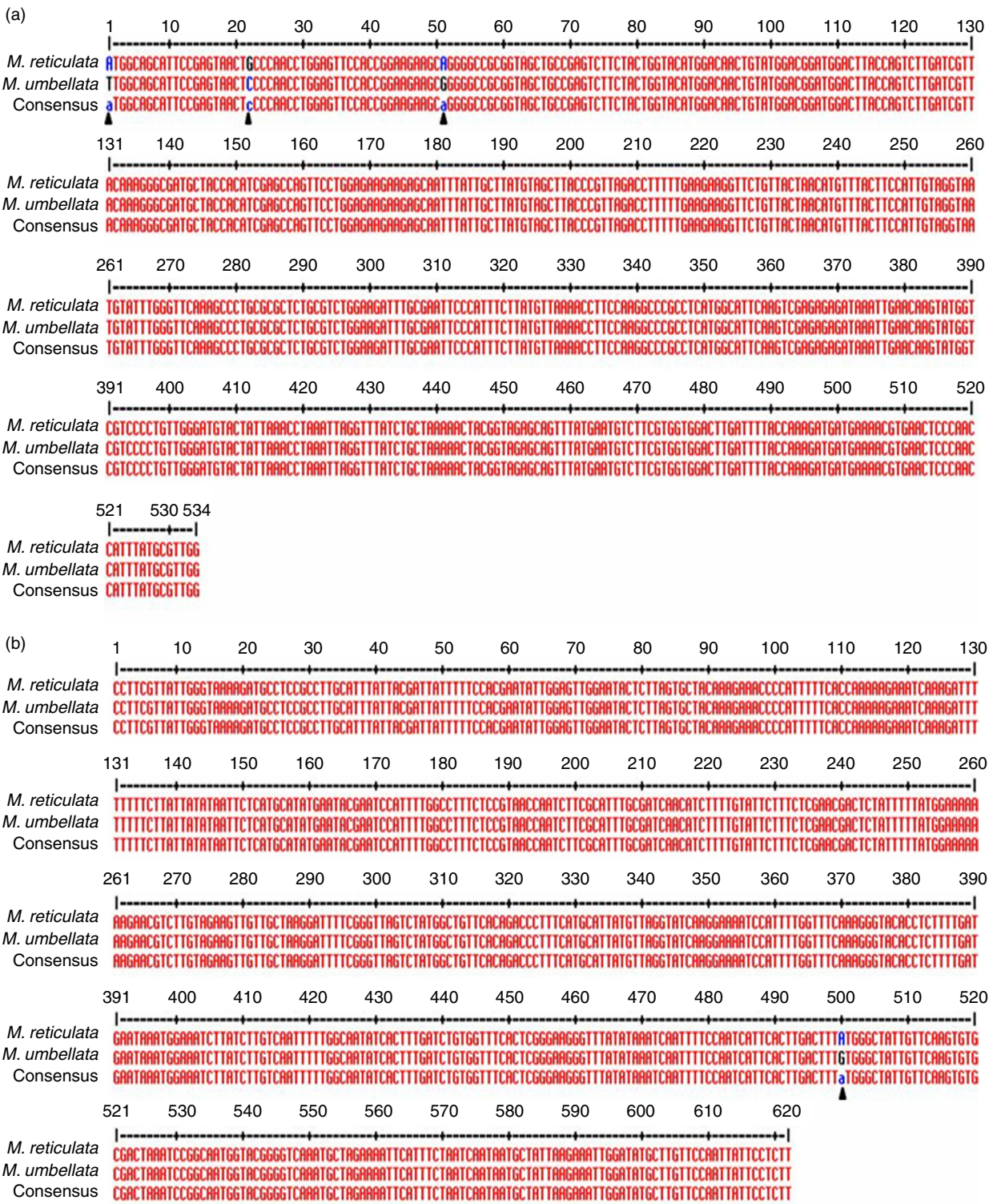

Fig. 1. (a) Nucleotide sequence alignment of a 534 bp fragment spans from position +89 to +622 of the 703 bp *rbc*L open reading frame, using the *Morinda officinalis* (GQ436556) *rbc*L sequence as reference. (b) Nucleotide sequence alignment of a 621 bp fragment spans from position +62 to +682 of the 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

https://doi.org/10.1017/S147926211200041X Published online by Cambridge University Press

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 *rbc*L than in *mat*K, indicating *rbc*L to be more reliable for distinguishing these species. Sequencing of complete *mat*K may reveal more variations since a faster substitution rate has been reported in *mat*K than in *rbc*L (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 *rbc*L and *mat*K genes.

Acknowledgements

The first author would like to thank the Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu for providing facility for the present work. We express our gratitude to Mr Rajagopal, Museum, Trivandrum, Kerala for his assistance in plant collection. We thank Dr N. Sasidharan, Kerala Forest Research Institute, Peechi, Kerala for providing information about *Morinda* spp. This study is part of the PhD work of the first author.

References

Asahina H, Shinozaki J, Masuda K, Morimitsu Y and Satake M (2010) Identification of medicinal *Dendrobium* species by phylogenetic analyses using *mat*K and *rbc*L sequences. *Journal of Natural Medicine* 64: 133–138.

Balasubramani SP and Venkatasubramanian P (2011) Molecular identification and development of nuclear DNA ITS sequence based marker to distinguish *Coscinium fenestratum* Gaertn. (Menispermaceae) from its adulterants. *Current Trends in Biotechnology and Pharmacy* 5: 1163–1172.

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acid Research* 16: 10881–10890.

Dassanayake MD, Clayton WD (eds) (1998) *A Revised Hand Book of the Flora of Ceylon*. New Delhi: Oxford IBH Publishing Corporation Private Limited, p. 321.

Gopalan R, Henry AN (2000) *Endemic Plants of India. (Camp for the Endemics of Agasthiyamalai Hills, SW Ghats)*. Dehra Dun: Bishen Singh Mahendra Pal Singh, pp. 307–310.

Ijinu TP, Anish N, Shiju H, George V and Pushpangadan P (2011) Home gardens for nutritional and primary health security of rural poor of South Kerala. *Indian Journal of Traditional Knowledge* 10: 413–428.

Ismail TS and Sulthana AP (2008) Standardization of *Morinda umbellata* Linn. – an effective crude drug for diarrhoea. *E-Journal of Chemistry* 5: 652–658.

Kaplan J, Chavan P, Warude D and Patwardhan B (2004) Molecular markers in herbal drug technology. *Current Science* 87: 159–165.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.

Mosihuzzaman MM and Choudhary IM (2008) Protocols on safety, efficacy, standardization, and documentation of herbal medicine. *Pure and Applied Chemistry* 80: 2195–2230.

Nair LG and Seeni S (2002) Rapid clonal multiplication of *Morinda umbellata* Linn. (Rubiaceae), a medicinal liana, through cultures of nodes and shoot tips from mature plant. *Phytomorphology* 52: 77–81.

Olmstead RG and Palmer JD (1994) Chloroplast DNA systematics: a review of methods and data analysis. *American Journal of Botany* 81: 1205–1224.

Roonyamarai W, Rungsihirunrat K, Vipunngeun N and Ruangrungsi N (2011) Microscopic and molecular analyses of selected *Morinda* species in Thailand. *Asian Journal of Traditional Medicines* 6: 118–126.

Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406–425.

Singh DR, Srivastava AK, Srivastava A and Srivastava RC (2011) Genetic diversity among the three different *Morinda* species using RAPD and ISSR markers. *Indian Journal of Biotechnology* 10: 285–293.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731–2739.