

Evaluation of rapid molecular diagnostics for differentiating medicinal *Kaempferia* species from its adulterants



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ARTICLE INFO

Article history:

Received 27 September 2018

Received in revised form

9 April 2019

Accepted 10 April 2019

Available online 16 April 2019

(Editors: Zhekun Zhou and Xuefei Yang)

Keywords:

Ayurveda

Barcoding

Chloroplast

Molecular authentication

Zingiberaceae

Calathea bachemiana

ABSTRACT

Accurate detection of unique herbs is crucial for herbal medicine preparation. Zingiberaceae species, which are important in Ayurvedic medicine of India, are often misidentified in Northeast (NE) Indian herbal markets. *Kaempferia galanga* (Zingiberaceae) is one of the major components of popular Ayurvedic drugs used for rheumatic diseases (i.e., “Gandha Thailam” and “Rasnairandadi Kashayam”), contusions, fractures, and sprains. In NE India, herbal healers often misidentify plants from the Marantaceae family (e.g., *Calathea bachemiana* and *Maranta leuconeura*) as *Kaempferia*, which leads to adulteration of the medicinal herb. This misidentification of herbs occurs in NE India because Zingiberaceae plant barcoding information is inadequate. As a consequence, herbal medicine is not only therapeutically less effective but may also cause adverse reactions that range from mild to life-threatening. In this study, we used eight barcoding loci to develop “fingerprints” for four *Kaempferia* species and two species frequently mistaken for *Kaempferia*. The PCR and sequencing success of the loci *matK*, *rbcl* and *trnH-psbA* were found to be 100%; the combination of *matK*, *rbcl*, and *trnH-psbA* proved to be the ideal locus for discriminating the *Kaempferia* species from their adulterants because the combined loci showed greater variability than individual loci. This reliable tool was therefore developed in the current study for accurate identification of *Kaempferia* plants which can effectively resolve identification issues for herbal healers.

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1. Introduction

Plants have been used since prehistoric times to treat serious ailments. Ayurveda, which is the traditional Hindu system of medicine, utilizes more than 5,000 plant species (Adyanthaya et al., 2016). *Kaempferia galanga* (Zingiberaceae), which is used in 59 different Ayurvedic medicines (Sivarajan and Balachandran, 1994), has become extremely valuable; from 2014 to 2016, the price of this essential herb tripled in India (Preetha et al., 2016). However, the ever-increasing demand for traditional medicine has led to adulteration of Zingiberaceae in the international and national herbal market. For example, in Kerala, India *Kaempferia rotunda*

(Zingiberaceae), the major ingredient of the popular Ayurvedic drug “Hallakam,” which is used to treat stomach aches, wounds, mental disorders, and insomnia, is commonly adulterated by *Lagenandra toxicaria* (Sereena et al., 2011). Consumers are often unaware of this adulteration and buy herbs that have a similar appearance to authentic *Kaempferia* species (Sasikumar et al., 2016).

Previous research has identified common adulterants of Ayurvedic drugs in India (Kumar and Ruba, 2018). During an ethno-medicinal survey in Northeast (NE) India, we found that *Kaempferia* species are adulterated by species belonging to the Marantaceae family (e.g. *Calathea bachemiana* and *Maranta leuconeura*). An additional report also supports our field observation that species from Zingiberaceae (*Curcuma angustifolia* Roxb.) and Marantaceae (*Maranta arundinacea* Linn.) are used interchangeably in the Ayurvedic drug “Tugaksheeree” which aids digestion and metabolism (Rajashekhara and Sharma, 2010). When herbal healers misidentify Zingiberaceae plants, the therapeutic efficiency of *Kaempferia*

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Peer review under responsibility of Editorial Office of Plant Diversity.

derived Ayurvedic drugs (e.g., Asaneladi Velichenna, Agasthyar-asayanam, Eladi Velichenna, Gandha Thailam, Triphaladi Thailam, Rasnairandadi Kashayam) is markedly reduced. This taxonomic confusion is related to the rapid radiation in the order Zingiberales (Barrett et al., 2014), which consists of eight families (Marantaceae, Cannaceae, Zingiberaceae, Costaceae, Heliconiaceae, Strelitziaceae, Musaceae, and Lowiaceae) that share similar characteristics. Specifically, plants of these families are rhizomatous herbs, with mid-ribs, ligules, involucre bracts, zygomorphic flowers, which have stamens and filaments with a staminoid structure (Carlsen et al., 2018). Furthermore, the flowering season of *Kaempferia* is short, and inflorescences of some species (e.g., *K. rotunda*) appear before leafy shoots and last only for 1–2 weeks. The absence of floral parts for most of the year and the dormancy throughout the non-rainy season make identifying *Kaempferia* more difficult. Because the preparation of authentic herbal medicine from *Kaempferia* requires accurate discrimination of adulterants from the pure *Kaempferia* complex, investigating effective methods of identifying *Kaempferia* and its adulterant species is crucial.

An accurate marker system that allows non-specialists to scrutinize the purity of the voucher is necessary. When the vouchers for herbal plants are processed into powder form, traditional organoleptic and elemental compositional analysis are often not sufficient to discriminate adulterants. Aside from using high performance chromatography to determine the presence of major compounds (Septyanti et al., 2016), molecular methods to authenticate *Kaempferia*-based herbal products remain scarce. One promising approach in detecting desired plants from the herbal formulation is DNA barcoding (Newmaster et al., 2013). Barcoding has effectively

discriminated adulterants from industrial olive oil (Kumar et al., 2011), tea packets (Stoeckle et al., 2011), and turmeric (Parvathy et al., 2014). Plant barcodes proposed by the Consortium for the Barcode of Life's Plant Working Group include seven loci (*rpoB*, *rpoC1*, *rbcl* and *matK*, *atpF-atpH*, *psbK-psbI* and *trnH-psbA*) (CBOL Plant Working Group, 2009). However, it is unclear whether these barcodes individually or in combination are effective at discriminating individual families of plant species. For example, previous studies on *Kaempferia* phylogeny have attempted to use barcodes that consisted of single loci (*petA-psbI*, *trnH-psbA*), two-locus concatenations combinations (Techaprasan et al., 2010). Efforts to detect adulteration in *Kaempferia*, however, have been scarce. Thus, we aimed to determine which of the DNA barcodes proposed by the CBOL's Plant Working Group are the most effective at discriminating *Kaempferia* from its adulterants.

In this study, we assessed the effectiveness of eight plastid loci (*accD*, *rpoB*, *rpoC1*, *rbcl* and *matK*, *atpF-atpH*, *psbK-psbI* and *trnH-psbA*) in discriminating adulterants from the *Kaempferia* species complex. We also propose the best barcode to authenticate *Kaempferia* species from their adulterants.

2. Materials and methods

2.1. Plant material

Plants belonging to Zingiberaceae and Marantaceae are the dominant food source of elephants in the forests of Assam. Taxonomically, plants of the Marantaceae family are poorly investigated in Asia; previous studies on Marantaceae reported 55 species

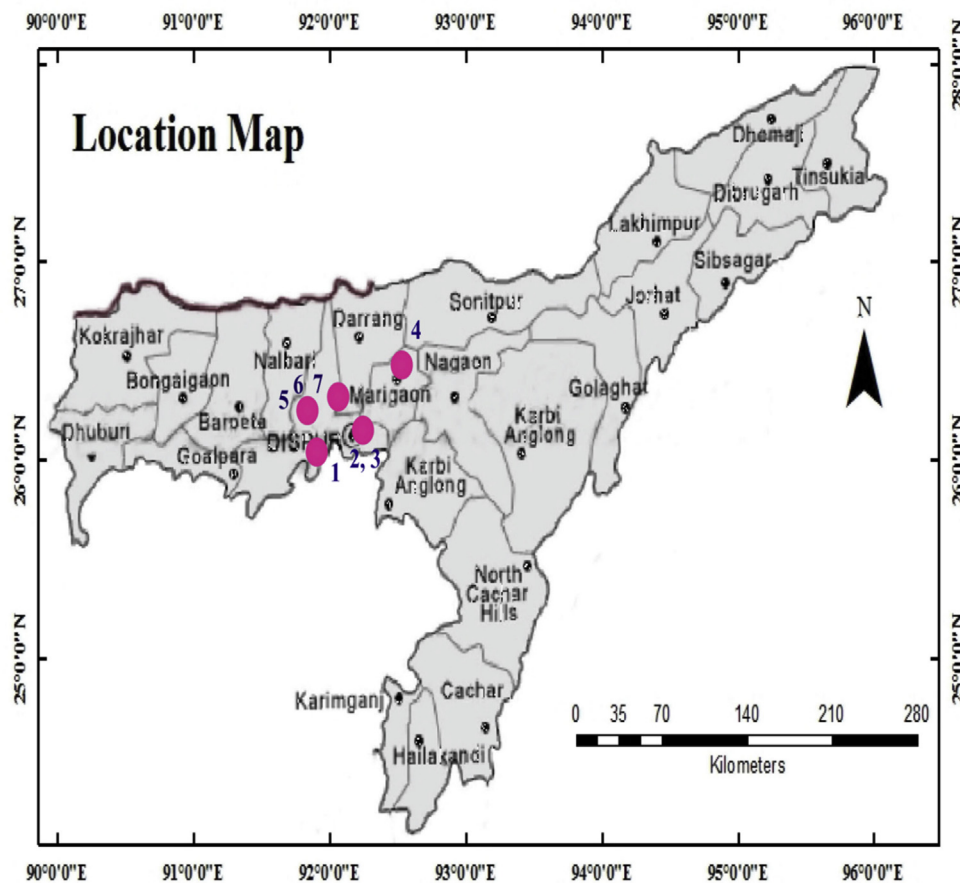


Fig. 1. Collection sites of different *Kaempferia* and their adulterant species used in the current study. Samples have been shown in the figures by their serial number given in Supplementary Table 1. 1. *K. galanga*, 2. *K. angustifolia*, 3. *K. elegans*, 4. *K. rotunda*, 5. *C. bachemiana*, 6. *M. leuconeura* 1, 7. *M. leuconeura* 2.

throughout Asia (Suksathan et al., 2009). The species from the Marantaceae and Zingiberaceae families propagate clonally with rhizomes. To proliferate rapidly, the above ground biomass for species from both families has a very high growth rate. Furthermore, plants of the families Marantaceae and Zingiberaceae become reproductively active during the monsoon. When the flowering season is over, the morphological similarity between the leaves of members of both families make identification at the species level difficult for herbal healers. This difficulty in identification, in turn, increases adulteration of *Kaempferia* by Marantaceae species with similar leaf morphology. To sort out this issue, the species from both families were collected during the monsoon season and identified by a renowned curator of Gauhati University, Dr. Gajen Chandra Sarma. The materials for the present study consisted of four species of *Kaempferia* and two species of family Marantaceae collected from Assam (Supplementary Table 1, Fig. 1).

Two species (*C. bachemiana* E. Morren, *M. leuconeura* E. Morren 1, *M. leuconeura* E. Morren 2) that are commonly used as adulterator were also selected (Fig. 2). The species were maintained in the Departmental green house of IIT Guwahati, Assam.

2.2. DNA extraction and analysis of partial sequences from potential organelle loci

Total genomic DNA was extracted from fresh tender leaves of the material using a DNeasy Plant mini kit (Qiagen Inc., Venlo, Netherlands). The quality and quantity of the extracted DNA was confirmed by running the extracted DNA on a 0.8% agarose gel, stained with ethidium bromide ($0.5 \mu\text{g } \mu\text{L}^{-1}$). The universal primers of eight plastid loci used to discriminate the *Kaempferia* species from its adulterants were amplified using the coding regions of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcl*), RNA

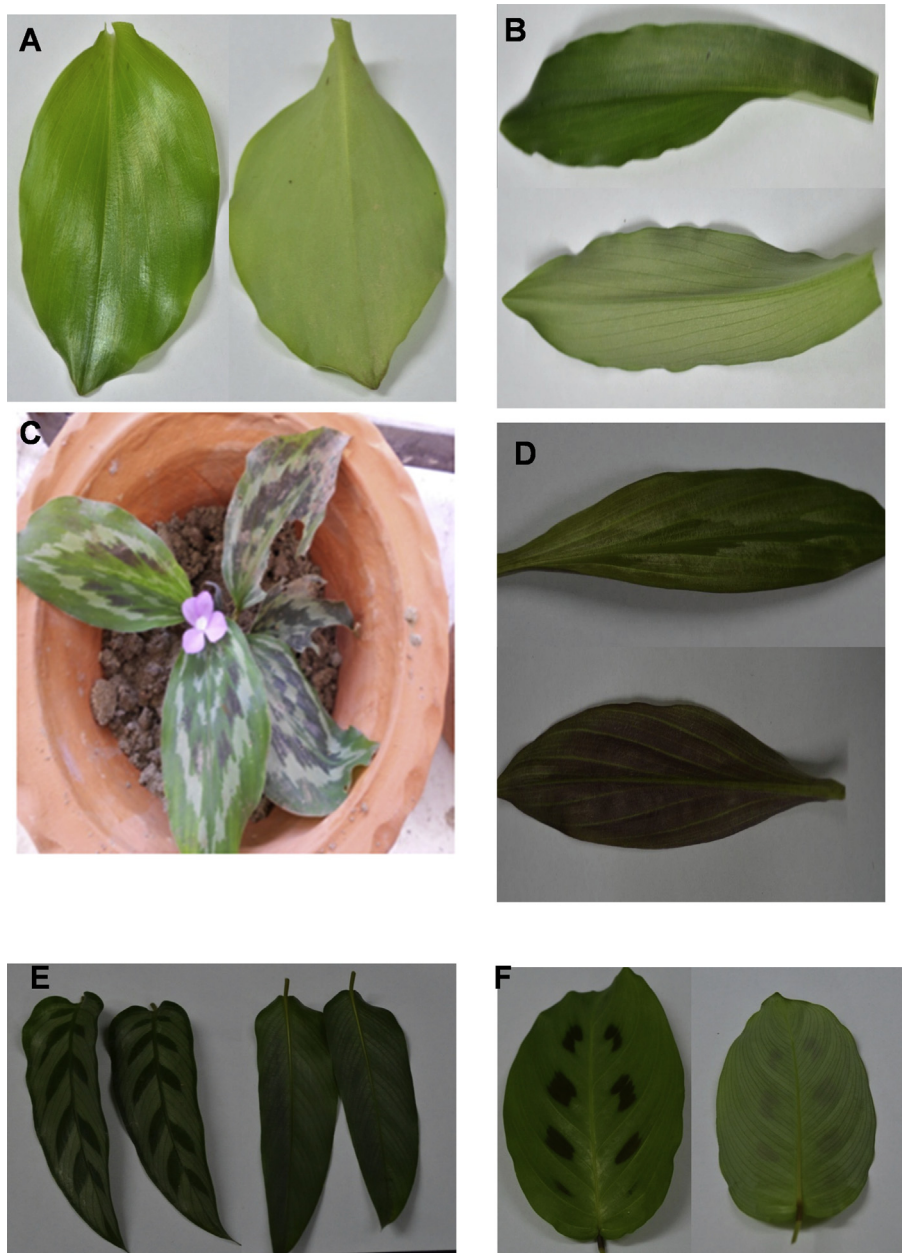


Fig. 2. Morphological variation in leaves (dorsal and ventral side) from *Kaempferia* complex and their adulterants. (a) *K. galanga*, (b) *K. angustifolia* (c) *K. elegans*, (d) *K. rotunda*, (e) *C. bachemiana* (f) *M. leuconeura* 1.

polymerase C (*rpoC1*), RNA polymerase B (*rpoB*), acetyl-CoA carboxylase-D (*accD*), maturase K (*matK*); and the intergenic regions ATP synthase subunit b–ATP synthase subunit c (*atpF-atpH*), Photosystem II reaction center protein K–Photosystem II reaction center protein I (*psbK-psbI*), the intergenic spacer region of *psbA* gene, and *trnH* gene (*trnH-psbA*). PCR was performed in 0.2 µL Eppendorf tubes inside of a Mini Thermal Cycler (Applied Biosystems 9700, USA) as per conditions optimized for each loci (Table 1). PCR products were resolved on agarose gels and purified using a QIAquick Gel Extraction Kit (Qiagen, USA). The purified PCR products were sequenced on an ABI 3730xl instrument (Applied Biosystems, USA) from Macrogen (South Korea) using the same set of primers as defined for PCR amplifications. Both forward and reverse primers were used for sequencing. The consensus sequences were generated in Geneious Pro (5.6.7) (Kearse et al., 2012).

2.3. Sequence analysis

To discriminate *Kaempferia* from Marantaceae species using barcodes, the genuine and adulterated sample were aligned and their sequence variation was quantified. The neighbor-joining (NJ) method was used to test phylogenetic relationships in MEGA 6.0 using K2P distances (2000 bootstrap replicates). These analyses were performed separately for *matK*, *rbcl*, *rpoB*, *rpoC1*, *accD*, *atpF-atpH*, *trnH-psbA*, and *psbK-psbI*; the DNA sequences were also concatenated in a combined analysis. All sequences generated from *Kaempferia* and the adulterant species were deposited in the NCBI database. We also used BLASTn searches to confirm our results.

3. Results and discussion

To test which loci are the most effective barcodes for discriminating *Kaempferia* from contaminants from the Marantaceae family, we assessed eight barcodes on six species (*K. galanga*, *Kaempferia angustifolia*, *Kaempferia elegans*, *K. rotunda*, *C. bachemiana* and *Maranta leuconaria*). These analyses produced 56 DNA sequences (Supplementary Table 1). Sequence characteristics of the eight plastid loci are listed in Table 2. Amplicon size varied from 400 bp in *accD* to 769 bp in *matK*, whereas in *atpF-atpH*, *trnH-psbA* and *psbK-psbI*, it was 750, 775, and 564 bp, respectively. The aligned length varied from 387 bp for *accD* to 769 bp for *matK*. Variable sites ranged from 1.15% for *trnH-psbA* and *rpoC1* to 16.36% for *atpF-atpH*. When sequence length and number of conserved sites were taken into consideration, the most conserved sequences (above 94.00%) among all loci analyzed were *trnH-psbA*, *rpoC1* and *rbcl*. *atpF-atpH* showed the highest nucleotide variation (16.36%), followed by

psbK-psbI (14.18%), *matK* (13.91%) and *accD* (11.63%). Parsimony informative sites varied from 0.53% (*rbcl*) to 13.51% (*atpF-atpH*). The highest number of singleton sites was for *psbK-psbI* (4.79%), whereas *trnH-psbA* showed an absence of singleton sites.

Individual barcoding loci (except for *matK*) were not effective at discriminating *Kaempferia* species from their adulterants (Supplementary Figs. 1–2). This finding is consistent with previous studies in which single-locus DNA barcodes from plastids were ineffective at discriminating plant species in *Araucaria*, *Solidago*, and *Quercus* (Fazekas et al., 2008; Hollingsworth, 2008; Piredda et al., 2011; Kress and Erickson, 2007). The ineffectiveness of these seven barcode loci (except *matK*) is likely due to hybridization events and the recent divergence of plant species within these families (Ley et al., 2014; Chen et al., 2015; Vinitha et al., 2014). Furthermore, research on molecular phylogeny has indicated that *Kaempferia* is a polyphyletic clade (Techaprasan et al., 2010), whereas species belonging to the Marantaceae family form a monophyletic clade, with Cannaceae as a sister group (Suksathan et al., 2009). In addition, hybridization and polyploidization have been shown to occur in the genus *Kaempferia* (Basak et al., 2018; Nopporncharoenkul et al., 2017), whereas most of Marantaceae species are diploids (Sharma and Mukhopadhyay, 1984). Our previous investigation on genome size of the studied *Kaempferia* species showed that *K. elegans*, *K. angustifolia*, and *K. galanga* are diploid, tetraploid, and pentaploid, respectively (Basak et al., 2018). The Marantaceae species in this study, however, are both diploid (data not shown). The divergence in ancestral relationship, along with difference in the ploidy level between Marantaceae and *Kaempferia* complex, may explain why individual barcodes failed to segregate *Kaempferia* and adulterants.

In phylogeographical studies of land plants, use of multi-locus barcodes is advised in cases where single-locus barcodes fail to discriminate species appropriately. To estimate the discriminatory power of eight barcoding loci and their combinations, we calculated interspecific variability using K2 parameters. The loci *matK* and *psbK-psbI* yielded higher interspecific variability (9.70%). The least variability was shown by *trnH-psbA* (0.70%), followed by *rbcl* and *rpoB* (1.10%) (Fig. 3). For barcoding land plants, previous studies have recommended using a two-locus system that consists of the *matK* + *rbcl* pair (CBOL Plant Working Group, 2009; Kress and Erickson, 2007). In our present investigation, the two-locus systems of *matK* + *rbcl*, *matK* + *psbK-psbI*, *rbcl* + *trnH-psbA* showed 126, 189 and 22 variable sites, respectively (Table 2). Among the two-locus barcode combinations, *matK* + *rbcl* provided the highest resolving power in species discrimination (Supplementary Fig. 3a). In contrast to previous studies (Kress and Erickson, 2007), we found

Table 1

Details of the barcoding primers and PCR conditions optimized in this study for genetic discrimination of *Kaempferia* species from their adulterants.

Primer name	Primer sequence	PCR conditions
<i>rpoC1</i>	F – GGCAAAGAGGGAAGATTTCG R – CCATAAGCATATCTTGAGTTGG	95 °C-5 min; 30 cycles- 94 °C-1 min, 57 °C -30 sec, 72 °C -45 sec; 72 °C -7 min
<i>rpoB</i>	F – ATGCAACGTCAAGCAGTTCC R – CCGTATGTGAAAAGAAGTATA	95 °C-5 min; 30 cycles- 94 °C-1 min, 57 °C -30 sec, 72 °C -45 sec; 72 °C -7 min
<i>matK</i>	F – CGTACAGTACTTTTGTGTTTACGAG R – ACCCAGTCCATCTGGAATCTTGGTTC	95 °C-4 min; 30 cycles- 94 °C-30sec, 50 °C -1min, 72 °C -40 sec; 72 °C -7 min
<i>rbcl</i>	F – ATGTCACCACAAAACAGAGACTAAAGC R – GTAAATCAAGTCCACCRCC	95 °C-4 min; 30 cycles- 94 °C-30 sec, 55 °C -1min, 72 °C -1 min; 72 °C -10 min
<i>accD</i>	F – CTATAGCAATTTGGAGTTATGAATT R – CGGATCAATCAAAAGTTTCGAT TC	95 °C-4 min; 30 cycles- 94 °C-30 sec, 50 °C -1 min, 72 °C -40 sec; 72 °C -7 min
<i>atpF-atpH</i>	F – ACTCGCACACTCCCTTTCC R – GCTTTTATGAAGCTTTAAACAT	95 °C-4 min; 30 cycles- 94 °C-30 sec, 50 °C -1 min, 72 °C -40 sec; 72 °C -7 min
<i>psbK-psbI</i>	F – TTAGCCTTTGTTGGCAAG R – AGAGTTTGAGAGTAAGCAT	95 °C-4 min; 30 cycles- 94 °C-30 sec, 50 °C -1min, 72 °C -40 sec; 72 °C -7 min
<i>trnH-psbA</i>	F – GTTATGCATGAACGTAATGCTC R – CGCCGATGGTGGATTCAATCC	95 °C-4 min; 30 cycles- 94 °C-30 sec, 55 °C -1min, 72 °C -1 min; 72 °C -10 min

Table 2
Amplicon size and characteristics of single-locus and multi-locus plastid barcodes in *Kaempferia* and their adulterant species.

	Amplicon size (bp)	Aligned length (bp)	Conserved sites	No of variable sites	Parsimony informative sites	Singleton sites
<i>accD</i>	400	387	342	45	31	14
<i>matK</i>	769	769	662	107	87	20
<i>rbcl</i>	600	566	551	14	3	11
<i>rpoB</i>	425	405	376	29	22	7
<i>rpoC1</i>	525	534	505	29	21	8
<i>atpF-atpH</i>	750	703	585	115	95	20
<i>trnH-psbA</i>	775	695	687	8	8	0
<i>psbK-psbI</i>	564	564	456	80	53	27
<i>matK + rbcl</i>		1335	1209	126	90	36
<i>rbcl + trnH-psbA</i>		1261	1238	22	11	11
<i>matK + psbK-psbI</i>		1334	1109	189	147	42
<i>matK + rbcl + rpoC1</i>		1869	1715	154	111	43
<i>matK + rbcl + rpoB</i>		1740	1582	158	112	46
<i>matK + rbcl + accD</i>		1722	1551	171	121	50
<i>matK + rbcl + atpF-atpH</i>		2038	1794	241	185	56
<i>matK + rbcl + psbK-psbI</i>		1899	1665	206	146	60
<i>matK + rbcl + trnH-psbA</i>		2030	1896	134	98	36
Eight plastid targets		5113	3879	710	316	391

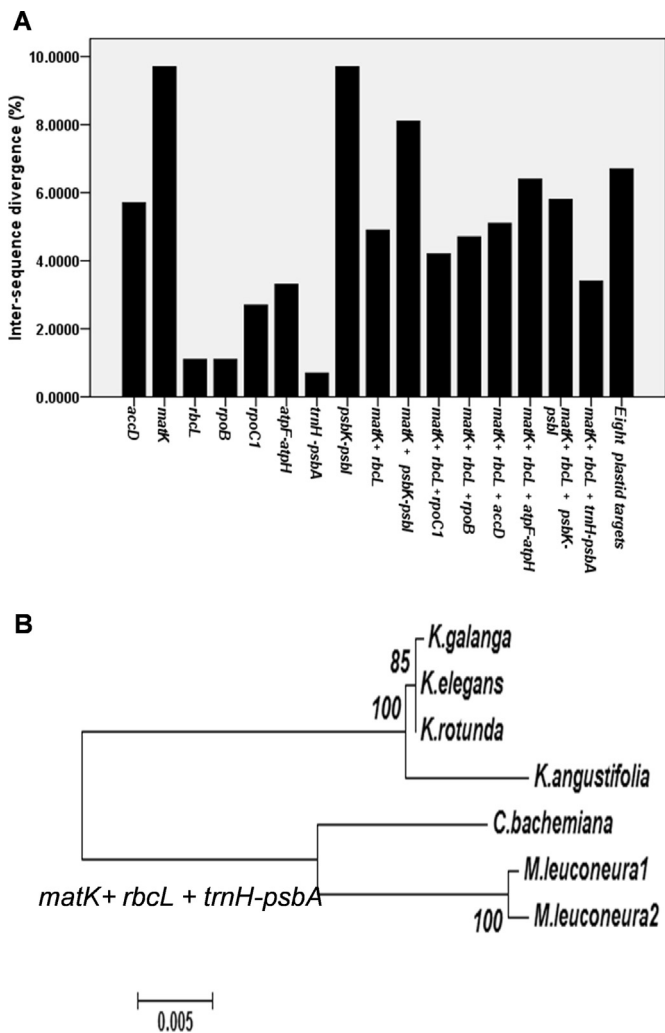


Fig. 3. (a) Overall mean distance calculated using the K2 parameter between species belonging to the *Kaempferia* complex and their adulterants as a measure of the discriminatory power of the analysis. (b) Neighbor joining (NJ) tree for six species based on *matK + rbcl + trnH-psbA* sequence data. *K. galanga* is forming the tri-topological relationship with three other *Kaempferia* species in the dendrogram. The barcoding approach clearly distinguishes the *Kaempferia* complex from its adulterants showing the effectiveness of our study. Bootstrap percentages are shown above the branches which were obtained after 2000 replications.

that *rbcl + trnH-psbA* is not an effective barcode for reliable species discrimination (Supplementary Fig. 3c).

Although the two-locus barcode *matK + rbcl* provided adequate resolution to discriminate between plant species, we assessed multi-locus barcode combinations to increase the resolving power. We analyzed the effectiveness of the following concatenated barcode loci: *matK + rbcl + accD*, *matK + rbcl + rpoB*, *matK + rbcl + rpoC1*, *matK + rbcl + atpF-atpH*, *matK + psbK-psbI*, *rbcl + rpoB + trnH-psbA* (Supplementary Figs. 3a–3c; Supplementary Figs. 4a–4d; Supplementary Figs. 5a–5c). In all cases, the tri-topological relationship among the *Kaempferia* species were either absent or not supported by bootstrap values greater than 70%, making these phylogenetic relationships unsuitable for rapid molecular discrimination. According to bootstrap values and the tri-topological relationship among *Kaempferia*, the loci combinations that were most effective were concatenated *matK*, *rbcl* and *trnH-psbA*. BLASTn analyses confirmed that *matK* was successful at returning the right match. Computer analysis also confirmed that concatenated *matK*, *rbcl* and *trnH-psbA* resulted in higher resolution compared to *matK* alone (Supplementary Table 2). Thus, concatenation of three barcoding genes (*matK*, *rbcl* and *trnH-psbA*) was found to be effective for the discrimination of *Kaempferia* species from their adulterants.

4. Conclusion

This study presents a DNA barcoding method to rapidly identify *Kaempferia* species important in Ayurveda. We found that the *matK* locus can be used as a primary DNA barcode to discriminate between plants in the *Kaempferia* complex and their Marantaceae family adulterants. Furthermore, the *rbcl + trnH-psbA* combination of loci can serve as supplementary barcodes for the same purpose. These results strongly suggest that DNA barcoding is an effective approach to discriminate adulterant species from active species and ensure high quality Ayurvedic medicine.

Conflicts of interest

The authors state that there is no conflict of interest.

Acknowledgements

SB and AMR thank MHRD for fellowship. LR thanks the Department of Biotechnology (DBT), Government of India, for funding the project by way of DBT Twinning Programme for NE (BT/

33/NE/TBP/2010), MS Swaminathan Research Foundation for AFLP facility and Department of Biosciences and Bioengineering, IIT Guwahati, for providing all necessary infrastructural support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pld.2019.04.003>.

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