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ORIGINAL ARTICLE

Efficiency of *matK*, *rbcL*, *trnH-psbA*, and *trnL-F* (cpDNA) to Molecularly Authenticate Philippine Ethnomedicinal Apocynaceae Through DNA Barcoding

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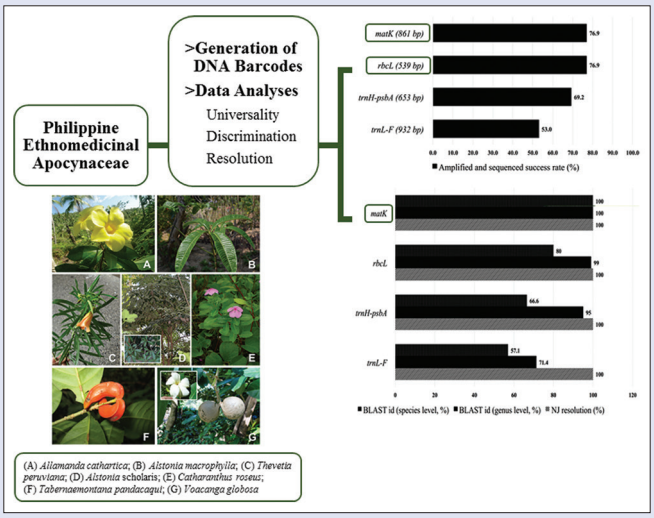
ABSTRACT

Background: The Philippines is home to some ethnomedicinal Apocynaceae that has been used to cure common ailments. They are perceived to be safe, but misidentification can lead to substitution and adulteration. Morphological characters are primarily utilized to identify these species but a new method utilizing molecular characters called DNA barcoding has emerged. In this study, the efficiency of *matK*, *rbcL*, *trnH-psbA*, and *trnL-F* to molecularly authenticate selected Apocynaceae species were tested. **Materials and Methods:** Genomic DNA from silica-dried leaf samples were isolated and used as a template for generating DNA barcodes. Pair-wise sequence divergence using Kimura-2-Parameter was used to analyze inter-specific and intraspecific variations among the barcodes, whereas basic local alignment search tool (BLAST) and neighbor-joining (NJ) analyses were employed to examine discrimination success. **Results:** The results show that *matK* is the best barcode for Apocynaceae as it has the highest amplification and sequencing success together with *rbcL* while having high inter-specific and low intra-specific divergence relative to the other candidate barcodes. Furthermore, *matK* provided the highest discrimination both in BLAST and NJ analyses. **Conclusion:** This study proposes the use of *matK* as the principal barcode for Apocynaceae.

Key words: Apocynaceae, DNA barcoding, ethnomedicinal, molecular authentication

SUMMARY

- Both *matK* and *rbcL* have higher universality compared to *trnH-psbA* and *trnL-F*
- *matK* has relatively high inter-specific divergence and very minimal intra-specific divergence
- *matK* is the best barcode to molecularly authenticate Apocynaceae with either *trnH-psbA* or *trnL-F* as supplements.



Abbreviations used: K2P: Kimura-2-parameter, BLAST: Basic local alignment search tool, NJ: Neighbor-joining.

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INTRODUCTION

The people in Philippine provinces are known to utilize the native plants of their area as logical and practical sources of medicine that provide symptomatic relief for common ailments.[1] Among these plant sources are the members of the family Apocynaceae (sub-family Rauvolfioideae) such as *Alstonia macrophylla* Wall. and G.Don and *Alstonia scholaris* (L.) R.Br. which are traditionally used as an emmenagogue, anti-choleric, and vulnerary and the endemic *Voacanga globosa* (Blanco) Merr. for cancer and tuberculosis.[2,3] Medicinal plants are being marketed today as herbal medicine or natural health products. They are often perceived to be safe but adulterated, counterfeit and low quality products pose serious safety threats to consumers[4,5] as well as to the existing markets. Incorrect identification using primarily morphological characters of many plants has resulted in adulteration and substitution of plant products that compromise their therapeutic value.[6-8] Morphological characterization remains the cornerstone of taxonomic diagnosis in plants. Unfortunately, relying solely on morphology has some considerable limitations.

The evolution of molecular biology gave rise to a new approach based on nucleotide sequence diversities among species called DNA barcoding.[9,10]

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Short standardized segment of the genome serving as a pattern "barcode" has been proposed as a technology that offers to expedite accurate species identification.[11,12] The consortium for the barcode of the life (CBOL) plant working group has suggested *matK* and *rbcL* as the core barcode regions for plants.[13]

DNA barcodes are increasingly recognized for their ability to authenticate medicinal plants.[14-17] In this study, we evaluated the efficiency of four candidate DNA barcodes-*matK*, *rbcL*, *trnH-psbA*, and *trnL-F* (cpDNA) for molecular authentication of selected Philippine ethnomedicinal Apocynaceae species namely *A. macrophylla*, *A. scholaris*, *V. globosa*, *Allamanda cathartica* (L.,) *Tabernaemontana pandacaqui* Poir., *Catharanthus roseus* (L.) G.Don., and *Thevetia peruviana* (Pers.) K. Schum. The candidate barcodes were assessed using the criteria set by CBOL stating that an ideal barcode should be routinely retrievable with a single primer pair, be amenable to bidirectional sequencing with little requirement for manual editing of sequence traces, and provide maximal discrimination among species.[13,18,19]

MATERIALS AND METHODS

Sample collection and preservation

Field collections were conducted in different Philippine provinces. Leaf samples for each specimen were stored in re-sealable packs with silica beads. All specimens were provided with herbarium vouchers, currently stored in the University of Santo Tomas Herbarium [Table 1].

Generation of DNA barcodes

Total genomic DNA was extracted from silica gel-dried leaf tissues following the protocols of DNeasy Plant Minikit (Qiagen, Germany). The universal primer pairs for *matK*,[19] *rbcL*,[20] *trnH-psbA*,[18] and *trnL-F*[21] were amplified using Kapa Taq PCR Kit (Kapa, USA) in Biometra® T-Gradient thermocycler [Table 2]. The polymerase chain reaction (PCR) cocktail for all markers contained (25 μL reaction): 17.35 μL water, 2.5 μL ×10 buffer, 1.0 μL 25 mM MgCl2, 2 μL 2 mM dNTP, 1.0 μL of 10 μM forward and reverse primers, 0.15 μL Taq DNA polymerase, and 0.5 μL DNA template. PCR conditions were set as follows: Initial denaturation of 90s at 97°C, followed by 35 cycles of 30s 95°C; 20s 50°C (*matK* and *rbcL*)/55°C (*trnH-psbA*)/20s 72°C (*trnL-F*); 60s 72°C; finishing with 72°C for 10 min.[22]

The amplicons were resolved in agarose gel electrophoresis and specific DNA fragments were purified using the QIA-quick PCR Purification Kit (Qiagen, Germany). Purified DNA was sent to Macrogen Inc. Seoul, South Korea for bidirectional DNA sequencing. All DNA sequences were assembled and edited using CodonCode Aligner v.4.1.1 (CodonCode Co., USA).

Table 1: List of Apocynaceae species used in the study with their University of Santo Tomas Herbarium accession numbers

Species	Code	Location	USTH accession number
Allamanda cathartica L	12-778	Surigao	11302
Alstonia scholaris (L.) R. Br	13-132; 12A-205	Ilocos; Mindoro	011377; 011287
Alstonia macrophylla Wall and G.Don	13-088	Ilocos	011654
Catharanthus roseus (L.) G.Don	B-436; BPI-058; 13-137	Manila; Ilocos	011670; 011465; 011639
Tabernaemontana pandacaqui Poir	12-529; 12A-053	Bicol	011401; 011260
Thevetia peruviana (Pers.) K. Schum	14A-071	Laguna	011338
Voacanga globosa (Blco.) Merr	12A-077; 12A-016; 12A-033	Mindoro; Bicol	011262; 011266; 011268

USTH: University of Santo Tomas Herbarium

Table 2: Universal primers used in this study

Barcode	Primer	Sequence (5'>3')
matK	3F_Kim f	CGTACAGTACTTTTGTGTTTACGAG
	1R_Kim r	ACCCAGTCCATCTGGAAATCTTGGTTC
rbcL	rbcL_aF	ATGTCACCACAAACAGAGACTAAAGC
	rbcL_aR	CTTCTGCTACAAATAAGAATCGATCTC
trnH-psbA	trnHf_05	CGCGCATGGTGGATTCACAATCC
	psbA3'f	GTTATGCATGAACGTAATGCTC
trnL-F	c	CGAAATCGGTAGACGCTACG
	f	ATTTGAACTGGTGACACGAG

Sequence analyses

The sequences of candidate DNA regions were automatically and manually aligned in SeaView v.4.[23] Pair-wise sequence divergence was calculated using Kimura-2-Parameter (K2P)[24] model in MEGA 6.0[25] to evaluate the mean intra-specific divergence, coalescent depth, mean inter-specific distance, and the minimum inter-specific distance.[14,26] The Wilcoxon tests for inter- and intra-specific divergences were conducted using SPSS 15.0 software (SPSS Inc., Chicago, USA). Basic local alignment search tool (BLAST, NCBI-Gen Bank) analysis of acquired sequences was conducted as previously described.[14] Neighbor-joining (NJ) method using K2P distances (1000 bootstrap replicates) were conducted to test phylogenetic relationships in MEGA 6.0.

RESULTS

A total of 13 Apocynaceae specimens representing seven species (*A. macrophylla*, *A. scholaris*, *V. globosa*, *A. cathartica*, *T. pandacaqui*, *C. roseus*, and *T. peruviana*) were obtained. Four candidate barcodes (*matK*, *rbcL*, *trnH-psbA*, *trnL-F*) were used to investigate the feasibility of DNA barcoding for molecular authentication. The 36 new sequences [Appendix 1] obtained from all markers and 14 sequences from Gen Bank were combined for the succeeding analyses. The Gen Bank sequences ensure each species has, at least, a duplicate sequence for intraspecific computation. A previous study by Mahadani *et al.*[15] also looked into the application of DNA barcoding in ethnomedicinal Apocynaceae but they only included two markers for the analyses. This issue is addressed in our study by contrasting the viability of the four candidate barcodes. For amplification and sequencing success, both *matK* and *rbcL* have 76.9% while *trnH-psb A* and *trnL-F* have 69.2% and 53.0%, respectively [Figure 1].

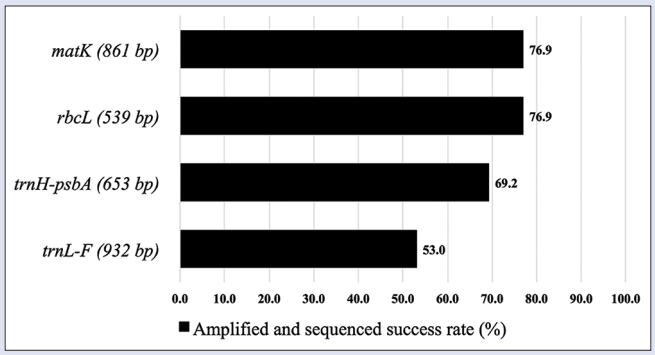

Figure 1: Efficiency of polymerase chain reaction amplification and sequencing success of the four candidate barcodes for the selected Apocynaceae. Numbers in parentheses are aligned lengths

To test the sequence divergence of each barcode, pair wise divergence was calculated using K2P. Table 3 summarizes the results having *trnH-psbA* as the barcode with the highest inter-specific divergence and *rbcL* with the lowest with 0.327 ± 0.090 and 0.028 ± 0.007, respectively. For the intra-specific divergences, *trnH-psbA* also has highest value with and 0.049 ± 0.046 while *matK* has the lowest the lowest with 0.002 ± 0.002. All barcodes were able to identify more than 50% of the specimens at species level using BLAST and gave good resolutions in NJ [Figures 2 and 3].

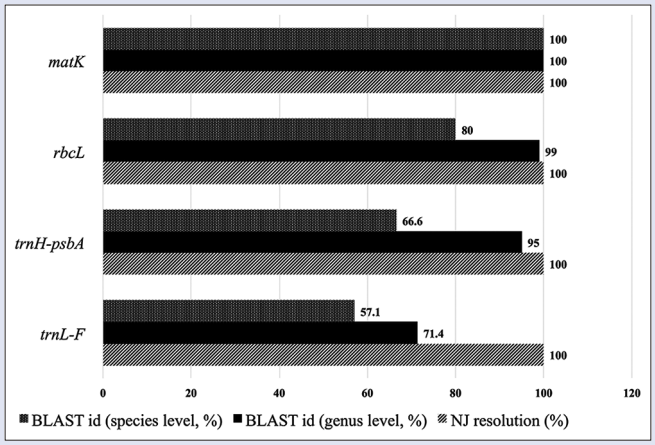

Figure 2: Rate of correct identification of the four barcodes using basic local alignment search tool and neighbor-joining for the selected Apocynaceae

DISCUSSION

Universality of the four candidate barcodes

For a barcode to be considered universal, it must be tractable across a wide range of species. Ideally, it should be relatively short in length to facilitate easy DNA extraction, amplification, and sequencing.[18] Both *matK* and *rbcL* have higher success rate thus, higher universality compared to *trnH-psbA* and *trnL-F*. These are after several attempts to amplify using pure and diluted (1/10 and 1/100) DNA extracts.

Inter-versus intra-specific divergence and barcoding gap analyses

Two parameters (average inter-specific distance and the minimum inter-specific distance) were employed to characterize inter-specific divergence, and two others (average intra-specific distance and maximum

Table 3: Inter- and intra-specific divergences of the four barcodes for selected Apocynaceae inferred using kimura-2-parameter distances

Parameter	Candidate barcode			
	matK	*rbcL*	*trnH-psbA*	*trnL-F*
Average inter-specific distance	0.062±0.021	0.028±0.007	0.327±0.090	0.045±0.011
Minimum inter-specific distance	0.010	0.015	0.142	0.026
Average intra-specific distance	0.002±0.002	0.003±0.005	0.049±0.046	0.008±0.013
Maximum intra-specific distance	0.006	0.013	0.145	0.023

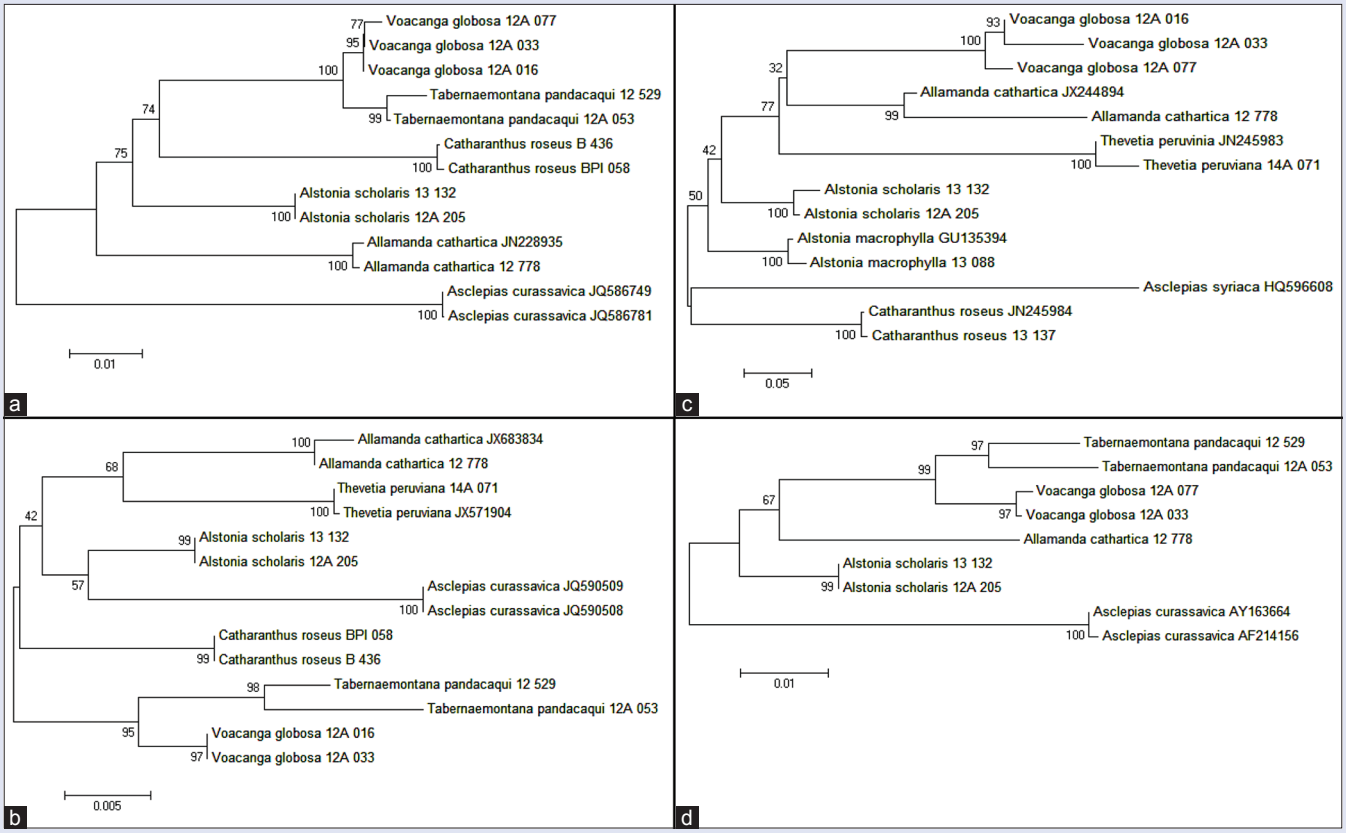

Figure 3: Neighbor-Joining tree of (a) *matK*, (b) *rbcL*, (c) *trnH-psbA*, and (d) *trnL-F* sequences inferred using Kimura-2-Parameter distances. All barcodes have successfully resolved each species but only *matK*and *trnL-F* provided proper polarity with *Asclepia curassavica* as the out group

intra-specific distance) were used to indicate intra-specific variation. By comparison on the inter-specific divergences of four candidate DNA regions (*matK*, *rbcL, trnH-psbA*, and *trnL-F*), *trnH-psbA* has the highest average inter-specific distance followed by *trnL-F* and *rbcL*, respectively. Even with its relatively short length (approximately 450 bp), *trnH-psbA* is considered as the most variable plastid region in angiosperms and is easily amplified across a broad range of land plants with the potential to discriminate among the largest number of plant species for barcoding purposes.[16,18,27]

The Wilcoxon signed rank test among the markers confirms the significant difference between each pair. The same pattern for intra-specific variation is observed. Specifically, intra-specific variation of *trnH-psbA* is higher than those of *matK*, *trnL-F*, and *rbcL*, but there exists no significant difference between those of the latter three candidate DNA regions [Tables 3 and 4]. These results point out that *matK* is better than the rest of the candidate barcodes since it has relatively high inter-specific divergence and very minimal intraspecific divergence compared to *trnH-psbA*. Using the Wilcoxon two-sample test, there are significant differences between the inter- and intra-specific divergences of the candidate DNA regions, with their inter-specific divergences significantly higher than their related intra-specific variations [$P < 0.05$, Table 5]. In consequence, all of them have a potential to discriminate one species from another.

Since the sample size could barely represent a portion of the entire Apocynaceae, DNA barcoding gap analysis is only restricted to the lack of overlap ("gap") between minimum inter- and maximum intra-specific divergences, or presence thereof.[28] Based on this, only *trnH-psbA* has an overlap although the gaps (i.e., difference between minimum inter- and maximum intra-specific divergences) of the other three barcodes are only <0.005 [Table 3].

Efficiency of the four cpDNA markers for resolving identity

One of the main purposes of DNA barcoding is to identify unknown species by matching a particular barcode sequence to available confirmed sequences. This is addressed here using BLAST and NJ method. Correct matches in BLAST and resolutions in NJ (i.e., monophyly of conspecific specimens) are considered as positive results. As shown in Figure 2, *matK* has the highest rates of correct identification in BLAST, both at the genus and species level. It is followed by *rbcL, trnH-psbA*, and *trnL-F*, respectively. The limited available published sequences in the Gen Bank, especially for the endemic species *V. globosa*, played a big factor. It cannot however, deny the fact that *matK* has a superior ability for authentication compared to other candidate barcodes.[13]

This finding corroborates with the NJ resolution results [Figure 3]. All the candidate barcodes are successful in resolving the monophyly of all con-specific specimens. However, only *matK* and *trnL-F* provide the expected polarity in the NJ trees which show *Asclepias curassavica* L. as the out-group. This observation is in agreement with the findings of Mahadani *et al.*[15] Hollingsworth *et al.*[19] stated that for barcoding purposes, *rbcL* meets all the criteria by being easy to amplify, sequence, and align in most land plants. Regrettably, it only has modest discriminatory power, evident in our results. Further, no other 2-marker or multi-marker plastid barcode gave better species resolution than the *rbcL* + *matK* combination. In this study, *matK* is the best barcode to molecularly authenticate Apocynaceae with either *trnH-psbA* or *trnL-F* as supplements.

Table 4: Wilcoxon signed rank tests for inter- and intra-specific divergences among the four barcodes

Divergence	W+	W−	Relative ranks		*n*	*P*	Result
			W+	W−			
Inter-specific	*matK*	trnL-F	161	10	18	0.001	*matK* > *trnL-F*
	rbcL	trnL-F	5	166	18	0.000	*trnL-F* > *rbcL*
	trnH-psbA	trnL-F	171	0	18	0.000	trnH-psbA > *trnL-F*
	rbcL	matK	21	1155	48	0.000	*matK* > *rbcL*
	trnH-psbA	matK	1176	0	48	0.000	*trnH-psbA* > *matK*
	trnH-psbA	rbcL	1830	30.5	60	0.000	*trnH-psbA* > *rbcL*
Intra-specific	*matK*	trnL-F	3	3	3	1.000	*matK*=*trnL-F*
	rbcL	trnL-F	3	3	3	1.000	*rbcL*=*trnL-F*
	trnH-psbA	trnL-F	6	0	3	0.109	*trnH-psbA* > *trnL-F*
	rbcL	matK	3	3	6	1.000	*matK*=*rbcL*
	trnH-psbA	matK	28	0	7	0.018	*trnH-psbA* > *matK*
	trnH-psbA	rbcL	21	0	6	0.028	*trnH-psbA*> *rbcL*

Table 5: Wilcoxon two-sample test for inter- and intra-specific divergences of the four barcodes

Barcode	Number of inter-specific	Number of intra-specific	Wilcoxon W	*P*
matK	48	7	28	2.338×10^{-5}
rbcL	60	6	21	6.238×10^{-5}
trnH-psbA	70	8	37.5	4.687×10^{-6}
trnL-F	18	3	6	0.001504

CONCLUSION

In total, our results support the claim that DNA barcoding in general can provide fast and reliable species identification, especially for the economically important ethnomedicinal plants. In the case of the Apocynaceae species, *matK* is the best barcode for molecular authentication as it gives high universality, discriminatory ability, and can resolve phylogenetic placement accurately. The authors propose the application of *matK* as the primary DNA barcode for Apocynaceae with *trnH-psbA*, and *trnL-F* as supplementary barcodes.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Appendix 1: EMBL accession numbers of the sequences generated in this study

Species	Candidate barcode			
	matK	***rbcL***	***trnH-psbA***	***trnL-F***
Allamanda cathartica	LN883872	LN883882	LN883892	LN883865
Alstonia scholaris	LN883873; LN883874	LN883883; LN883884	LN883893; LN883894	LN883866; LN883867
Alstonia macrophylla			LN883899	
Catharanthus roseus	LN883880; LN883881	LN883889; LN883890	LN883898	
Tabernaemontana pandacaqui	LN883875; LN883876	LN883885; LN883886		LN883868; LN883869
Thevetia peruviana		LN883891	LN883900	
Voacanga globosa	LN883877; LN883878; LN883879	LN883887; LN883888	LN883895; LN883896; LN883897	LN883870; LN883871

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