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Original Article

Evaluation of cpDNA Barcodes in Selected Medicinal Plants of Mt. Arayat National Park, Pampanga, the Philippines

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ABSTRACT

Objective: One common problem affecting herbal remedies is the incorrect identification of plants due to almost similar morphological characters. A promising technique to ensure correct identification of herbal medicine is the use of DNA barcoding where the gene markers either from Chloroplast (cpDNA) or Nucleus (nrDNA) are commonly used to authenticate plants. In this study, three chloroplast barcodes; *matK, rbcL* and *trnL-F* were evaluated to authenticate 20 selected medicinal plants of Mt. Arayat National Park (MANP), Pampanga, The Philippines. **Methods:** The genetic materials from the 20 selected medicinal plants were isolated, amplified, sequenced and aligned. All generated DNA barcodes were subjected to Kimura two-parameter model to calculate intergeneric and intrageneric variations while discriminatory power was determined using BLAST, neighbour-joining (NJ) and Wilcoxon two-sample test. **Results:** The results show 19 plant samples were successfully amplified. However, *rbcL* gave the highest sequencing success rate and identification rate up to species level over *matK* and *trnL-F*. Though, the three-cpDNA barcodes produced monophyletic clades revealing distinct association among species. The intergeneric divergences of *rbcL, matK* and *trnL-F* are significantly higher than their respective intrageneric divergences. This means the three-cpDNA barcodes can effectively discriminate one species from another. **Conclusion:** The order of efficiency among the DNA barcodes is *rbcL>matK>trnL-F*. Nevertheless, the three-plastid barcodes could be used for practical and accurate authentication of medicinal plants while the baseline data in this study can be used for proper conservation of medicinal plants.

Key words: BLAST, DNA barcoding, Medicinal plants, Monophyletic, Phylogeny.

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INTRODUCTION

Medicinal plants have been identified and used throughout human history. According to the World Health Organization in 2011, about 65% to 80% of the populations in developing countries use medicinal plants as cures for different illnesses and this number is steadily growing.[1] It is estimated that approximately 25% of modern medicines are derived from herbal products. In the Philippines, around 1,500 medicinal plant species are known to be found in the country.[2]

In 1997, the National Biodiversity Strategy and Action Plan (NBSAP) identified several areas in the Philippines as centers of biodiversity including the Mt. Arayat National Park, MANP.[3] This mountain is an isolated and dormant stratovolcano located in the northeastern portion of the province of Pampanga, Luzon which covers an aggregate area of 3,715.28 hectares and with the highest elevation of about 1,030 meters.[4] The richness and the variety of native flora found in the mountain has been used as sources of building materials, food, ornamentals and medicine. However, very limited studies on its biodiversity has been published.

Traditionally, botanists and traditional healers tend to describe medicinal plants based on their appearance and morphology.[5] Unfortunately, this conventional technique can cause confusion, particularly when identifying unstructured plant parts.[6] Thus, the safety of medicinal plants largely depends on the ability to eliminate all forms of misidentification since the efficacy of the drug decreases if it is adulterated and may even be lethal if it is substituted with toxic adulterants.[7]

In pursuit of developing fast and accurate species-level molecular identification methods, DNA barcoding has been known as a powerful tool for ensuring correct identification of herbal medicine. This technique of identifying living organisms uses short DNA sequences from one to several regions.[8-12] Among several candidates are DNA barcodes from chloroplast; *matK, rbcL, rpoB* and *rpoC1*, the intergenic plastidial spacers; *trnH-psbA, atpF-atpH* and *psbK-psbI* and the nuclear internal transcribed spacers that have been used either as one or in combinations.[13-14] In this study, three-cpDNA markers, *rbcL, matK* and *trnL-F* were evaluated to authenticate selected medicinal plants of MANP since these three candidate barcodes have been examined by their universality, discriminatory power and resolution.[15-18]

MATERIALS AND METHODS

Plant material collection and preparation

A total of 20 medicinal plants were selected and initially identified with the help of local herbalists in the area for their rich knowledge and extensive practice on medicinal plants (Table 1).[19] To verify the medicinal values of the collected plants, published books and online sources of ethnobotanical studies in the Philippines were reviewed and consolidated (Table 2). These sources are regarded due to the extensive entries of medicinal plants of the Philippines and accessibility in institution libraries and online sources.[19] The plants were authenticated and deposited at the

Table 1: List of medicinal plants used in this study.

Species code	Species	Family	Local name	Medicinal use	Medicinal reference
A-15-001	*Albizia lebbeck* (L.) Benth.	Leguminosae	Alalangad	diarrhea, dysentery and hemorrhoids	[35]
M-12-003	*Alocasia macrorrhizos* (L.) G. Don.	Araceae	Biga-biga	joint pains, fevers	35,36,37
M-7-002	*Alpinia elegans* (C. Presl) K. Schum.	Zingiberaceae	Salbak	urticaria, arthritis, diarrhea, stomachache	35,36,37
M-2-004	*Antidesma bunius* (L.) Spreng.	Phyllanthaceae	Isip bundok	diabetes mellitus, diaphoretic	35, 37
M-3-002	*Artocarpus blancoi* Merr.	Moraceae	Tipolo	heart problem, hernia, diarrhea,	36, 37, 38
A-21-005	*Bauhinia integrifolia* Roxb.	Leguminosae	Agkuy	bloody sputum, snake bite	36
M-0-002	*Chromolaena odorata* (L.) R.M. King and H. Rob.	Compositae	Paltuk-paltukan	wounds, fever	35, 36
M-12-008	*Ficus nota* (Blanco) Merr.	Moraceae	Tibig	fever, muscle pain, urinary tract infections, hypertension, diabetes	35, 36
M-0-000	*Gmelina arborea* Roxb. ex Sm.	Lamiaceae	Yemane	arthritis, sprain	35, 36
A-8-001	*Hyptis suaveolens* (L.) Poit.	Lamiaceae	Bunto't pusa	rheumatism, spasm, skin wash	35,36,37,38
M-9-001	*Macaranga grandifolia* (Blanco) Merr.	Euphorbiaceae	Bilua	mouth ulcers	35, 37
A-19-005	*Melanolepis multiglandulosa* (Reinw. ex Blume) Rchb. and Zoll.	Euphorbiaceae	Pakalkal	skin diseases	35,36,37
M-20-004	*Pandanus exaltatus* Blanco	Pandanaceae	Pandan lalake	urinary diseases	35,37, 38
M-12-001	*Parameria laevigata* (Juss.) Moldenke	Apocynaceae	Kuni	wounds	35, 36
A-17-005	*Premna odorata* Blanco	Lamiaceae	Tangle	respiratory diseases, headache	35,36,37,38
M-16-005	*Pongamia pinnata* (L.) Pierre	Leguminosae	Bani	cough, skin diseases, rheumatism	35, 38, 39
A-8-006	*Schefflera odorata* (Blanco) Merr. and Rolfe	Araliaceae	Lima-lima	wounds, arthritis, rheumatism	35,36,37
M-4-001	*Sida acuta* Burm. f.	Malvaceae	Walis-walisan	fever, stomach ache, rheumatism	*
A-19-010	*Streblus asper* Lour.	Moraceae	Kalios	skin diseases	35,36,37
M-13-001	*Wrightia pubescens* R.Br.	Apocynaceae	Lanete	dysentery, arthritis	35

* = all authors and on-line sources

Table 2: Floras used to identify species and as a source of medicinal information about species in the database.

Flora	Reference
Plantas medicinales de Filipinas.	deTavera P, Hermenegildo T. Madrid, Spain. B. Rico. 1892.
Medicinal plants of the Philippines.	Quisumbing, E. 1951. Manila, Philippines. Bureau of Printing.
Philippine medicinal plants in common use: their phytochemistry and pharmacology.	Tan, M.L. 1980. Quezon City, Philippines. Alay Kapwa Kilusang Pangkalusugan. Philippines: The Bookmark, Inc.
Philippine Traditional Knowledge Digital Library on Health.	http://www.tkdlph.com.
Philippine Alternative Medicine.	http://www.stuartxchange.com/AltMedSources.html.

University of Santo Tomas Herbarium (USTH) including their accession number and vouchers. Leaf samples were stored in resealable plastic bags with silica gel for desiccation.[20]

DNA extraction, PCR amplification, purification and sequencing

DNA from silica gel-dried leaves was isolated using a modified protocol of Dneasy Plant Mini Kit (Qiagen, Germany), while PCR reactions were performed on a T-gradient thermocycler (Biometra) and conditions were set as follows: Initial denaturation of 90s at 97°C followed by 35 cycles of 95°C for 30s, 50°C (*matK* and *rbcL*) or 55°C (*trnL-F*) for 20s; 60s 72°C and finishing with 72°C for 10 min.[21] These three chloroplast markers were amplified using KapaTaq PCR kit (KapaBiosystems, USA) using universal primers for each marker (Table 3). The PCR products were resolved in agarose gel electrophoresis and specific DNA fragments were purified using the QIA-quick purification kit (Qiagen, Germany). Bidirectional sequencing was done by Macrogen Inc. (Seoul, South Korea). Resulting sequences were assembled and edited using CodonCode Aligner v.4.1.1 (Codoncode Co., USA).

DNA sequence analysis

BLAST was used to initially identify each medicinal plant based on the proportion of individuals assigned to the correct species, genus, or family and the percentage identity of each taxon identified. The generated sequences, including those obtained from NCBI-GenBank were automatically exported to MEGA 7 for sequence characteristic computation. A Neighbor-joining (NJ) method using Kimura-two-parameter distances with 1000 Bootstraps (BS) was performed in MEGA 7.0 to estimate the confidence level of the topology of the consensus tree. To determine if a

DNA marker is more variable within (intra) a genus or between (inter) genera a Wilcoxon two-sample tests by SPSS 15.0 software (SPSS Inc, Chicago, IL, USA) was used.

RESULTS

A total of 19 plant specimens from 11 families were successfully generated new DNA sequences using three candidate barcodes (*rbcL, matK, trnL-F*). Only one plant sample was not amplified, the *Antidesma bunius* (L.) Spreng. The 42 new sequences obtained from all markers together with sequences from NCBI-Gen Bank (Appendix 1) were combined for the succeeding analyses. According to the Consortium for the Barcode of Life (2009), an ideal DNA barcode marker should be easy to amplify, enough variability to be used for species identification and must provide maximal discrimination among species.[16]

PCR and sequencing success rate

The assessment of the primers' universality was based on the comparison in the amplification and quality of the sequences generated from the three regions. Based on the results of the study, both *rbcL* and *matK* produced the highest amplification success rate at 95%, whereas *trnL-F* yielded 70% (Table 4). Interestingly, only *rbcL* gave the highest sequencing success rate at 80%, followed by *matK* and *trnL-F* at 65%. Overall, barcodes were ranked for PCR and sequencing success as *rbcL>matK>trnL-F*.

BLAST and K2P divergence analyses

The BLAST (NCBI-Genbank) was utilized to determine the homology of the plant samples (Table 5). All of which were able to identify from genus to species level. The gene barcode *rbcL* was able to confirm 63% of the initial identity at the species level, followed by *matK* at 46% and *trnL-F* at 38% (Table 6). While other samples, unfortunately, yielded different species although under the same genus or family. The *trnL-F* gave the highest genus identification at 46%, followed by *matK* and *rbcL* at 38% and 31%, respectively. Whereas three other species did not match with expected genus or species but identified under correct family. Like *Ficus nota* (Blanco) Merr. has maximum identity of 98% to *Artocarpus altilis* (Parkinson) Fosberg and *Streblus asper* Lour. has 99% maximum identity to *Castilla elastica* Sesse ex Cerv. using matK but these four genera still belong to same family Moraceae (Table 5). While the *trnL-F* generated maximum identity of 94% to *Abutilon theophrasti* Medik. for *Sida acuta* Burm. f. under same family Malvaceae and *F. nota* has 99% similarity to *Artocarpus fulvicortex* F.M. Jarrett under the family Moraceae. Since taxonomic names change frequently, these changes are not often reflected in GenBank.[22] Thus, two DNA barcodes, *matK* and *trnL-F* were also able to identify its synonym. The plant sample *Pongamia pinnatta* (L.) Pierre was identified as its synonym *Millettia pinnatta* (L.) Panigrahi, but *rbcL* was able to identify the accepted name *P. pinnata*. Therefore, based on BLAST analysis, barcodes were ranked as *rbcL >matK>trnL-F*.

After multiple sequence alignments, the three markers together with the sequences from NCBI-Genbank (Appendix 1), the *trnL-F* produced the highest mean length of 1338 bp, while 899 bp for *matK* and 723 bp for *rbcL*. The *trnL-F* also possessed the highest number of parsimony informative characters with 513 from 670 variable sites (Table 4). The Table 4 also summarizes K2P analysis revealing differences in performance, showing high intergeneric divergence and low intrageneric divergence. The results show *matK* has the highest intergeneric divergence of 0.236±0.005, followed by *trnL-F* and *rbcL* at 0.164±0.004 and 0.118±0.006 respectively. While the highest value of intrageneric divergence was produced by *rbcL* with 0.040±0.009, followed by *matK* at 0.028±0.006 and *trnL-F* at 0.014±0.003.

All generated 42 sequences from the plant samples were subjected to NJ analysis to reveal association among those sequences. The phylogenetic trees produced from three cpDNA markers showed association among close species creating monophyletic clades (Figure 1). The investigated barcodes of species from the same species, genus and family were assigned or grouped properly in the same clades showing the ability of these barcodes for relationship grouping.

Table 3: Universal primers of the three candidate barcodes used in the study.

DNA Barcode	Primer pair	Sequence (5′→ 3′)
matK	3F_Kim f	CGTACAGTACTTTTGTGTTTACGAG
	1R_Kim r	ACCCAGTCCATCTGGAAATCTTGGTTC
rbcL	rbcL_aF	ATGTCACCACAAACAGAGACTAAAGC
	rbcL_aR	CTTCTGCTACAAATAAGAATCGATCTC
trnL-F	c	CGAAATCGGTAGACGCTACG
	f	ATTTGAACTGGTGACACGAG

Table 4: Properties of the three DNA barcoding loci.

Parameter	*trnL-F*	*matK*	*rbcL*
No. of Taxa	20	20	20
PCR Success (%)	70% (14)	95% (19)	95% (19)
Sequencing Success (%)	65% (13)	65% (13)	80% (16)
Conserve Sites	595	318	333
Variable Sites	670	567	388
Informative Sites	513	434	212
Aligned Length (bp)	1338	899	723
Mean intergeneric Distance	0.164±0.004	0.236±0.005	0.118±0.006
Mean intrageneric Distance	0.014±0.003	0.028±0.006	0.040±0.009

Table 5: BLAST analysis with the percentage of maximum identity (Max.Id.) for each sample collected.

Species code	Scientific name	DNA region					
		rbcL	Max. Id.	*matK*	Max. Id.	*trnL-F*	Max. Id.
A-15-001	*Albizia lebbeck*	*Albizia lebbeck*	99%	*Albizia corniculata*	96%	*Albizia berteriana*	96%
M-12-003	*Alocasia macrorrhizos*			*Alocasia scalprum*	99%		
M-7-002	*Alpinia elegans*	*Alpinia elegans*	100%				
M-3-002	*Artocarpus blancoi*	*Artocarpus styracifolius*	99%	*Artocarpus blancoi*	100%	*Artocarpus fulvicortex*	99%
A-21-005	*Bauhinia integrifolia*	*Bauhinia gilva*	99%			*Bauhinia hymenaeifolia*	95%
M-0-002	*Chromolaena odorata*	*Chromolaena odorata*	99%	*Chromolaena odorata*	99%	*Chromolaena stachyophylla*	99%
M-12-008	*Ficus nota*	*Ficus benjamina*	98%	*Artocarpus altilis*	98%	*Artocarpus lanceifolius*	99%
M-0-000	*Gmelina arborea*	*Gmelina arborea*	99%	*Gmelina arborea*	100%		
A-8-001	*Hyptis suaveolens*					*Hyptis suaveolens*	99%
M-9-001	*Macaranga grandifolia*					*Macaranga grandifolia*	99%
A-19-005	*Melanolepis multiglandulosa*	*Melanolepis multiglandulosa*	100%			*Melanolepis multiglandulosa*	98%
M-20-004	*Pandanus exaltatus*	*Pandanus tectorius*	99%				
M-12-001	*Parameria laevigata*	*Parameria laevigata*	99%	*Parameria laevigata*	99%		
A-17-005	*Premna odorata*	*Premna odorata*	100%	*Premna odorata*	87%	*Premna odorata*	99%
M-16-005	*Pongamia pinnata*	*Pongamia pinnata*	99%	*Millettia pinnata*	99%	*Millettia pinnata*	99%
A-8-006	*Schefflera odorata*	*Schefflera minutistellata*	87%	*Schefflera actinophylla*	99%		
M-4-001	*Sida acuta*	*Sida acuta*	100%	*Sida rhombifolia*	99%	*Abutilon theophrasti*	94%
A-19-010	*Streblus asper*	*Streblus asper*	99%	*Castilla elastica*	99%	*Streblus asper*	97%
M-13-001	*Wrightia pubescens*	*Wrightia arborea*	99%	*Wrightia pubescens*	99%	*Wrightia lanceolata*	100%

DISCUSSION

Universality

According to Kress *et al*., in 2005 for a barcode to be considered universal, it should have a relatively short length to facilitate easy DNA extraction, amplification and sequencing. Moreover, it must be tractable across a wide range of species.[18] The results show that both *rbcL* and *matK* produced higher amplification rate thus, higher universality compared to *trnL-F*. These are based after several attempts to amplify DNA extract using pure and diluted (1/10 and 1/100). However, only *rbcL* gave the highest sequencing success with minimum editing which indicates it is the most universal among the barcodes used. This is comparable to the finding of Huang *et al*. in 2015. wherein *rbcL* obtained higher sequencing success rate at 90.80% while *matK* obtained relative low at 79.50% suggesting *rbcL* is the best barcode for PCR, sequencing and aligning in most tropical trees at Xishuangbanna Nature Reserve, Southwest China.[23] As for *matK*, Hollingsworth *el al*., in 2011 . suggested further work is needed on development to enable routine and efficient PCR and sequencing. Since the best currently available 'universal' primer pair of matK (3F/1R) which produces 70% PCR and sequencing success in angiosperms on diverse sample sets, it was recommended to use a secondary primer pair (390F/1326R) which can increase amplification and sequencing success by another 10%.[15] While in the study of Wang *et al*. (2016) in the DNA barcoding of ferns, Chinese *Adiantum* L., the amplification of *trnL-F* was discovered to be relatively difficult because of the universal presence of mononucleotide repeats in the intergenic spacer that affected sequence quality and reduced the universality of this marker.[24.]

Discrimination

To characterize the pairwise divergences of the samples per barcode, the mean intergeneric and intrageneric divergences were analyzed. It is vital to compare the pairwise divergence of the DNA barcodes to significantly distinguish one species from another but not individuals of the same species.[25-26] Based on the Wilcoxon two-sample test, there are significant differences between the intergeneric and intrageneric divergences of the candidate DNA barcodes. The intergeneric divergences of *rbcL, matK* and *trnL-F* are significantly higher than their respective intrageneric variations ($p < 0.05$, Table 7). However, *rbcL* is better than the two candidate barcodes since it has relatively high intergeneric divergence at 482 and very minimal intrageneric divergence at 14 even both *matK* and *trnL-F* have relatively higher mean intergeneric distances against *rbcL*.

Though *trnL-F* is not considered as a core barcode by CBOL-PWG (2009), the marker has been used for the identification not only for angiosperms but also of trees and leguminous crops.[27-28] In fact, *trnL-F* has been recommended as an efficient tool to identify field gametophytes[29] and as a good barcode for the identification of wild, ethnomedicinal plants.[30] While the choice of *rbcL and matK* as core barcodes by CBOL-PWG (2009) are based on its recoverability, sequence quality and level of species discrimination.[16] In consequence, all of them have a potential to effectively discriminate one species from another.

Authentication

The ability of DNA barcoding as a powerful tool for correct identification of medicinal plants rely on BLAST identification of species up to the specific epithet. It is primarily dependent on comprehensiveness of the taxon representation in the NCBI-GenBank.[31] As shown in Table 6, *rbcL* has the highest rate of correct identification in BLAST up to species level. It is followed by *matK* and *trnL-F,* respectively. While the rest of the

plant samples were identified up to genus level. This means that BLAST is highly reliable for genus identification but low in species identification.[32] The limited available published sequences in the NCBI-GenBank, especially for the endemic species restricts the efficiency of gene barcodes.[33]

According to Cabelin *et al* in 2015 BLAST alone will not suffice as the basis for molecular authentication. Thus, a phylogenetic study using NJ analysis must come in handy to test the reliability of phylogenies generated from a single gene analysis.[33] Based on the phylogenetic analyses, all candidate barcodes are successful to generate well-resolved monophyletic clades of all taxonomic groupings which are strongly supported by high bootstrap values (Figure 1).

The multilocus combination of barcodes have been suggested to increase the success rate of species resolution for land plants when no single locus is satisfactory.[34] Although the three candidate barcodes in this study, *rbcL*, *matK* and *trnL-F* gave better species resolution individually, the *rbcL* has still emerged as the best barcode to molecularly authenticate selected medicinal plants of MANP with either *matK* or *trnL-F* as supplements.

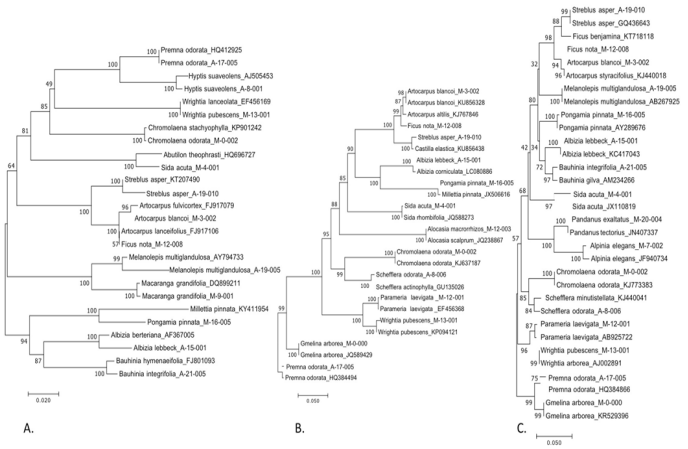

Figure 1: Neighbour-joining tree of (A) trnL-F, (B) matK and (C) rbcL sequences inferred using Kimura two-parameter distances. Numbers at the nodes are Bootstrap (BS) support values. A BS>70% is considered strongly supported.

Table 6: Comparison of the identification efficiency of the three candidate sequences using BLAST.

Marker	No. of sequences	Species-level identification	Genus-level identification
trnL-F	13	(5) 38%	(6) 46%
matK	13	(6) 46%	(5) 38%
rbcL	16	(10) 63%	(6) 38%

Table 7: Wilcoxon two-sample test for inter- and intra-generic divergences among the three barcodes.

Barcode	Number of intergeneric	Number of intrageneric	Wilcoxon W	P value
rbcL	482	14	990	$p <= 0$
matK	314	12	348	$p <= 0$
trnL-F	317	8	75	$p <= 0$

CONCLUSION

This study serves as good baseline information regarding the application of DNA barcoding in selected medicinal plants of MANP to differentiate the genuine species of medicinal plants from other plants. In conclusion, the order of efficiency among the DNA barcodes evaluated is *rbcL*> *matK*>*trnL-F*. These cpDNA barcodes could be used for practical and accurate authentication of medicinal plants that may and can prevent the utilization of adulterants and substitutes. The data gathered from this study can also be used to ensure proper conservation and management options for monitored harvesting and trade of important medicinal plants of MANP. While the nucleotide sequences generated can be a part of the local database to be used as a reference data of unidentified species for other future barcoding studies in the country.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool; **cpDNA:** Chloroplast DNA; **K2P:** Kimura-2-Parameter; **matK:** Maturase K; **NJ:** Neighbor-Joining; **nrDNA:** Nuclear Ribosomal DNA; **rbcL:** Ribulose Bisphosphate Carboxylase Large

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