Molecularauthentication Of Selected Commerciallysold Medicinal Plants In Quiapo, Manila, Philippines

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Abstract— This study aims to identify and authenticate selected medicinal plants commercially sold in Manila and assess the most effective DNA barcodes using three chloroplast markers (trnH-psbA, matK, rbcL) and a nuclear marker(ITS). Among the four evaluated markers, trnH-psbA was the most easily amplified followed by matK and ITS. The ITSmarker gave 100% sequencing success rate, followed by trnH-psbA and matK at 82% and 78%, respectively. In the BLAST analysis, matK proved to be the most successful and useful marker for identifying all samples up to the species level. For trnH-psbA, 7 out of 10 medicinal plants were identified to species level. Furthermore, the mean interspecific divergence computed using K2P revealed that matK had only 0.7% (0-1.6%),followed byITS and trnH-psbA with 0.11% (0-2.6%) and 0.16% (0-9.7%). In line with the BLAST result, matK can discriminate one species to another due to the minimal intraspecific divergence. In conclusion, matK and trnH-psbA are potential barcodes for identifying commercially sold medicinal plants where details of plant morphology are insufficient.

Index Terms—DNA barcode, medicinal plants, molecular authentication,cpDNA, nrDNA

1. INTRODUCTION

Medicinal plants have been used by mankind for centuries to cure common health problems and continues to be an important source of medicine today.It is estimated that 70% of the population uses traditional and complementary medicines based on Western Pacific Region study under World Health Organization. Of these, 89% do so for particular illnesses, symptoms, cultural needs which biomedicine cannot address as well as for financial reasons.[24] In the Philippines, herbal medicines are widely used and it has been in practice for more than a thousand years. For most Filipinos, medicines sourced from plants have become more than just an alternative to costly commercial drug preparations. They are now acceptable as logical and practical sources of medicines which could provide symptomatic relief for common ailments. [15] Usually, these are being sold in pharmacies as over the counter medicines and various forms in special outlets, stalls in shopping mall and around church areas within Metro Manila. It is utilized by ordinary people and herbal doctors in rural areas and urban cities. In fact, traditional or general practitioners like “albularyo” a Filipino term for traditional healers that used herbal remedies, are accessible, available and affordable, particularly in remote areas.

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This is not surprising since plant extracts have been used for medicinal purposes even before their active ingredients were isolated and used in formal medical practice. [10] At present, medicinal plants are often referred to as alternative medicine, herbal medicine, or natural health products. These terms also include traditional medicines as mentioned under the Philippine Republic Act 8423 known as the “Traditional and Alternative Medicine” Act (TAMA) of 1997. These products are often perceived to be safe because of their natural origin; however, adulterated, counterfeit and low quality products pose serious safety threats to consumers [16],[23] as well as to the existing markets. As an example, there are reported cases of life threatening poisoningdue to toxic adulterant or when substitute is administered.[17], [20],[22] Adulteration of herbal products is an emerging serious problem in the market and it is also growing as a global concern.[14] It has caused a major threat to research of commercial natural products. A recent study conducted in India byRoy et al[18]pointed out that deforestation, extinction of many species and incorrect identification of many plants may have resulted in adulteration and substitution of many drugs. The inaccurate identification of plants genuine resourceshave compromised the therapeutic value of medicinal plants and endangered the safety of the consumers. There is a proliferation of herbal remedies that have been adulterated or substituted with other plant materials, a situation that has stressed the need for quality control.[3] Most of these medicinal products have not been rigorously regulated and they do not guarantee quality and safety for their use. In fact, many cases of toxicity have been reported, like cases due to errors in species identification which is one of the first steps in herbal quality control.[21] It is then important for every herbal product to be botanically identified to make sure that it contains the right plant species with therapeutic value of such plant. Hence, the growing concern on problems of herbal products adulteration has raised the need for the development of analytical tools for species authentication of medicinal plants in the country to prevent the occurrence of similar incidents. Medicinal plants are generally identified based on morphological features but variations among related species are oftentimes limited or vague. Their identities may even bear the same common names. Therefore, efficacies of these

applications are critically dependent on the use of genuine materials. A specific medicinal plant that can treat a particular illness, however, does not follow that its closely related plant species within a genus has also the potential to treat the same illness. Correct identification of medicinal plants is very critical to avoid any adulterants in herbal medicines occurring in the market. Also, it is very important that the morphological differences between species of the same genus or genera of the same family can only be determined through proper plant identification of its parts by professionals and authorities. In Metro Manila, informal markets selling herbal medicine are popular. Quiapo, a busy district of Manila is a well-known area where herbal medicines in various forms can be found, such as herbal decoctions and where consultation with the herbal doctor with their many indigenous treatment tools that is readily available.Thereare many stalls selling herbal medicines around the vicinity of Quiapo church. These come in different forms, such as leaves, twigs, sprigs, seeds, roots, juices or different decoctions of herbal medicinal plants – fresh, dried or powdered, bundled, bottled and assorted decoctions. These medicines might be adulterated or substituted and have low quality imposing a serious threat to the health and well-being of consumers. Problems of misrepresentation in prescribing medicinal plant species are assumed to exist in the area. There are no reported studies yet on substitution and adulteration of plants sold in these location. For instance, packets of medicinal plants named “pito-pito” (7 kinds of leaves in a plastic bag)prepared as a decoction or poultice known for used in applications such as headaches, fever, colds, asthma, abdominal pains, and diarrhea were purchased from different vendors by the researcher. They contained different kinds and forms of leaves. Most of the medicinal plants sold in the area are labeled only by common names with medicinal value. Indeed, there is a need for the authentication of medicinal plants being sold in these area to ensure the authenticity of these plants and assure the safety of consumers. With the advent of molecular technology, DNA sequence data have now become a modern and reliable alternative way for species identification, molecular phylogenetic studies, biodiversity and conservation fields owing to their precise and huge number of characters. [11] DNA barcoding is a new biological tool for accurate,rapid and automated species identificationusing a short fragment of the genomic DNA.The DNA barcoding employs sequence variation within a short, standardized region of the genome, a “barcode” to provide accurate identification at the species-level.[8] In this study, we selected commercially sold plant materials purchased in Quiapo, Manila. The collected samples were sequenced and analyzed to validate their identities and assess the most effective DNA barcode among three cpDNA markers (trnH-psbA, matK, rbcL) and a nrDNA (internal transcribed spacer, ITS). This study provides for the first time the confirmation of the authenticity of these commercially sold medicinal plants in the area using the DNA barcoding analysis. It is part of the on-going project on DNA barcoding of Philippine medicinal plants spearheaded by the University of Santo Tomas (UST).

2. MATERIALS AND METHOD

2.1 Taxon Sampling

A total oftwelve medicinal plants sold in the commercial market along Quiapo, Manila, was included in this study.Due to the limited availability of plant materials, only eight out of twelvemedicinal plants were provided with herbarium vouchers. These vouchers are currently deposited at the University of Santo Tomas Herbarium (USTH) supplied with accession numbers. Table 1 summarizes the scientific names, common names, family affiliation, medicinal uses and the USTH accession numbersof the twelve medicinal plants. Enumerated medicinal uses are based on guidebook on the proper use of medicinal plants,[15]published journals, and data from Health Research Development Information Network (HERDIN), database maintained by Philippine Council for Health Research and Development (PCHRD) as national health research repository of the Philippines.[1],[2],[13] Leaf samples for molecular analysis were stored in resealable plastics with silica gel. [4] Additional 42 Genbank accessionswere included for sequence divergence analyses (Table2). The accession numbers of new sequences generated in this study will be provided in the final revised version of the manuscript.

Table 1. List of medicinal plant specimen collected from Quiapo, Manila.

Sp. Code.	USTH NO.	Family	Botanical Name	Common Name	Parts used[a]	Medicinal Use
Q-01	11604	Euphorbiaceae	*Euphorbia hirta* L.	tawa-tawa; gatas-gatas	Lf, Sp,Bk,	Dysentery, decreased milk secretion after delivery, ease and regulate urination , prevent bleeding, asthma and bowel complaints
Q-02	11605	Annonaceae	*Annona muricata* L.	guyabano	Bk,Lf,Rt ,Fr,Sp	Sedative, antispasmodic, hypotensive, fever and treatment for worms and parasites
Q-03	11606	Boraginaceae	*Cordia dichotoma* G.Forst.	anonak	Lf, Bk	Ulcers, headaches, boils, mouth ulcers and antidyspeptic
Q-04	11607	Fabaceae	*Tamarindus indica* L.	sampalok	Lf	Fever, cough, wound, and aromatic bath
Q-05	11608	Verbenaceae	*Premna odorata Blanco*	alagaw	Lf	Fever, headache, gaseous distentions, cough and aromatic bath
Q-06	11609	Fabaceae	*Cassia alata* (L.) Roxb.	akapulko	Lf	Scabies, tinea, ringworm and athlete's foot
Q-07	11610	Fabaceae	*Gliricidia sepium* (Jacq.) Kunth ex Walp.	kakawate; madre de cacao	Lf	Scabies, sprains and itching
Q-08	11611	Asteraceae	*Blumea balsamifera* (L.) DC.	sambong	Lf	Gaseous distentions, fever, headache, abscess, diuretic and aromatic bath
Q-09	-	Myrtaceae	*Psidium guava* L.	bayabas	Lf	Aromatic bath, mouth, wound and vaginal washes
Q-10	-	Anacardiaceae	*Mangifera indica* L.	Manga	Lf	Fever,vaginal wash, aromatic bath and for coughs
Q-11	-	Lythraceae	*Legerstroemia speciosa* (L.) Pers.	banaba; melendres	Lf	Diabetes, edema, urinary dysfunctions, fever, jaundice, and dizziness and kidney related diseases
Q-12	-	Rutaceae	*Citrus maxima* Merr.	pomelo; suha	Lf	Fever, dizziness, fainting, hysteria and as aromatic bath

[a] Bk, barks: Fr, fruits: Lf, leaves: Rt, roots: Sp, sap/juice

Table 2. Nucleotide accession numbers of taxa used in this study.

Taxa	NCBI GenBank Accession No.		
	trnH-psbA	*matK*	*ITS*
Euphorbia hirta L.	GQ434946		
	GQ434945		
Annona muricata L.	AY841428	AY743478	
Premna odorata Blanco		HQ384494	
Cassia alata (L.) Roxb.	GU396790		
	HQ161753		
Gliricidia sepium (Jacq.) Kunth ex Walp.		JQ587653	
		JQ587652	
		JQ587651	
Blumea balsamifera (L.) DC.	KF443308	KF443299	KC896714
	KF443297		JF421478
	FM998655		EU195646
Psidium guajava L.	GU135421	AB354958	AY781099
	JQ279707	JQ024987	AY487283
	GQ434986		
Mangifera indica L.	JX856905	AY594472	AB598050
	JX856904	JQ586473	JX856472
		JQ586472	AB071668
Lagerstroemia speciosa (L.) Pers.	JX856902		
Citrus maxima Merr.	JN315365	AB762351	
	GQ267061	JN315361	
	GQ435446	GQ434283	

2.2 Generation of DNA Barcodes.

Total genomic DNA was extracted from silica gel-dried leaf tissues following the protocols of DNeasy Plant Minikit (Qiagen, Germany).ThecpDNA (trnH-psbA, matK, rbcL) and nrDNA(ITS) barcodeswere amplified using KapaTaq PCR Kit (USA), employing the universal primers for each marker (Table 3). The amplicons were resolved in agarose gel electrophoresis and specific DNA fragments were purified using the QIA-quick 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).

2.3 Sequence analysis

The initial species identification of each sample was confirmed using BLAST. The sequences, including those obtained from NCBI-GenBank, were automatically aligned using ClustalX 2.1[12] and manually checked in SeaView v.4 to generate multiple sequence alignment. Pairwise sequence divergence analysis was computed using Kimura-2 Parameter (K2P) implemented in PAUP version 4.0b.[9],[19]

Table 3. Universal primers used for the amplificationand sequencing of DNA barcodes.

DNA Barcode	Primer		Sequence (5'-3')	Author
ITS	ITS4	Forward	5'-GGAAGTAAAAGTCGTAACAAGG-3'	White *et al.*, 1990
	ITS5	Reverse	5'-TCCTCCGCTTATTGATATC-3'	
matK	3F_KIM f	Forward	5'-CGTACAGTACTTTTGTGTTTACGAG-3'	CBOL, 2009
	1R_KIM r	Reverse	5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3'	
rbcL	a_f	Forward	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	Kress & Erickson, 2007
	a_r	Reverse	5'-CTTCTGCTACAAATAAGAATCGATCTC-3'	
trnH-psbA	trnHf_05	Forward	5'-CGCGCATGGTGGATTCACAATCC-3'	Kress *et al.*, 2005
	psbA3'f	Reverse	5'-GTTATGCATGAACGTAATGCTC-3'	

3. RESULTS AND DISCUSSION

A total of twenty-two new sequences was generated in this study. Additional 42 sequences from the NCBI-GenBank (Table 2)were used for sequence divergence analysesand interspecific divergence. According to the Consortium for the Barcode of Life (2009)[5] an ideal DNA barcode should be (1) routinely retrievable with a single primer pair, (2) responsive to bidirectional sequencing with sequences that are easily edited manually, and (3) provide maximal discrimination among species. Each of these criteria is covered in the succeeding discussion.

3.1PCR and Sequencing Success Rate

Based on the results of the study, the success rate of generating unambiguous DNA sequences by routine PCR with universal primers varied among the DNA barcodes. The universal primers for trnH-psbA, matK, andITSwere successful in amplifying their respective target sequences for at least 9 out of 12 samples (Fig. 1). Among the four evaluated markers, trnH-psbA was the most easily amplifiedat 92% (11/12), followed by matK and ITSwith 75% (9/12), 50% (6/12), respectively.Although rbcL is one of the recommended barcodes by CBOL (2009), only 17% (2/12) of the samples produced amplicons. This is after several attempts to amplify using pure and diluted (1/10 and 1/100) DNA extracts. Moreover, both of the two rbcL sequences did not yield reliable consensus sequences after the manual editing.Since two of the criteria of being a good barcode are the ability to be amplified routinely using a universal primer pair andyielding reliable sequences, rbcLwas subsequently excluded in the succeeding analyses.

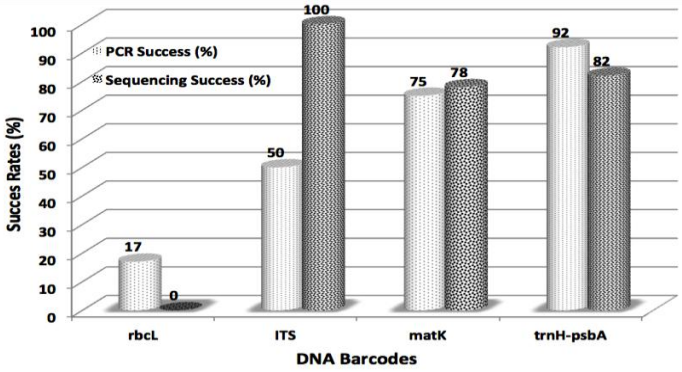

Fig. 1. PCR and Sequencing Success Rates of the four DNA Barcodes for Quiapo samples.

In comparison of the three barcodes, ITS gave 100% sequencing success rate, followed by trnH-psbA and matK at 82% and 78%, respectively.These three markers with relatively higher PCR and sequencing success rates are the ones left for sequence divergence analysis. Together with the sequences from NCBI-Genbank, the mean length of these DNA barcodes were 380.83base pairs (bp) (179-559bp) for trnH-psbA, 789bp (720-851bp) for matK, and 580.55bp (306-744bp) for ITS.

3.2 BLAST and K2P Divergence Analyses

In the BLAST (NCBI-Genbank) homology search results (Table 4),matK was able to confirm the initial identity among the available samples (7/7). It proved to be the most successful and useful marker for identifying all samples of up to the species level. For trnH-psbA, 7 out of 10 medicinal plants were identified to species level or its synonym. For example, the medicinal plant Euphorbia hirta is identified as its synonymChaemescyle hirta. Theremaining samples unfortunately yielded different species although under the same genus or family.In the ITSBLAST results, two samples were contaminated by funginamely Aspergillus gracilis and Eurotium tonophilum. The fungal contamination may have been caused by molds on the final dried medicinal leaves and were sequenced. This is similar to some reported studies on difficulties of using ITS as a marker for barcoding due to problems such as polymorphism and fungal contamination.[6],[7]

Table 4. BLAST homology search results for Quiapo samples.

Initial Identification	DNA Barcodes			
	trnH-psbA	*matK*	*rbcL*	*ITS*
Euphorbia hirta L.	*Chaemescyle hirta*	-	-	***
Annona muricata L.	*Annona muricata*	*Annona muricata*	-	-
Cordia dichotoma G.Forst.	-	-	-	-
Tamarindus indica L.	-	-	-	-
Premna odorata Blanco	*Premna microphylla*	*Premna odorata*	***	-
Cassia alata (L.) Roxb.	*Pandanus dubius*	-	-	-
Gliricidia sepium (Jacq.) Kunth ex Walp.	*Robinia pseudoacacia*	*Gliricidia sepium*	***	***
Blumea balsamifera (L.) DC.	*Blumea balsamifera*	*Blumea balsamifera*	-	*Blumea balsamifera*
Psidium guajava L.	*Psidium guajava*	*Psidium guajava*	-	*Psidium guajava*
Mangifera indica L.	*Mangifera indica*	*Mangifera indica*	-	*Mangifera indica*
Lagerstroemia speciosa (L.) Pers.	*Lagerstroemia speciosa*	-	-	*Aspergillus gracilis* (uncultured fungus)
Citrus maxima Merr.	-	*Citrus maxima*	-	*Eurotium tonophilum* (uncultured fungus)

*** Amplified; Unsuccessful sequencing

After evaluation and identification were done on the medicinal plants using the sequences generated from the three barcoding regions, a total of 10 out of 12 medicinal plants with confirmed identification. Using the Kimura 2-Parameter(K2P) analysis, the mean sequence divergence revealed that trnH-psbA and ITS are the most variable among the markers included with 53.9% and 48.8%, respectively.Although matKhad the least mean difference with 20.9%, this marker yielded likewise the least value of mean interspecific divergencewith only 0.7% (0-1.6%). It is followed by ITS and trnH-psbA with 0.11% (0-2.6%) and 0.16% (0-9.7), respectively. In line with the BLAST result, matK clearly can better discriminate one species to another because the intraspecific divergence is very minimal. In other words, individuals of the same species have very minimal sequence variation. This study generated the standard DNA barcodes of nine commonly used medicinal plants which are potential molecular markers to differentiate the genuine species of medicinal plants from other plants that may and can be used as adulterants and substitutes. It demonstrated potential use of DNA barcodes to identify a specific medicinal plant species that are commercially sold in Quiapo, Manila. The following species of medicinal plants were molecularly authenticated in the study such as Euphorbia hirta with synonym of Chaemescyle hirta; Senna alata with synonym of Cassia alata; Premna odorata from its close relative adulterant Premna microphylla. Interestingly, the rest of the samples have confirmed identity such as Annona muricata, Gliricidia sepium, Blumea balsamifera, Psidium guajava, Mangifera indica, Lagerstroemia speciosa, Citrus maxima. It is regrettablethough that no data was generated for the molecular authentication of Cordia dichotoma and Tamarindus indica.

4. CONCLUSION

The order of efficiency among the DNA barcodes evaluated is matK,trnH-psbA,and ITS. AlthoughtrnH-psbA is the most easily amplified and had a relatively high sequencing success, it did not give the best discriminatory power. In contrast, even if matK was not as successful in amplification, it gave the best identity confirmation and had the least intraspecific divergence. Furthermore, due to the nuclear nature of ITS, it is prone to fungal contamination and is therefore the least efficient barcode among the evaluated markers. The authentication results are reliable and not affected by the physical form or physiological conditions of the plant samples. Therefore, the method derived from the cpDNA trnH-psbA and matK, nrDNA ITS sequences in this study could be used for practical and accurate authentication of medicinal plants and other adulterants. Further, molecular identification using DNA barcode is expected to be a routine practice in the future and can be use by regulatory bodies in the Philippines to monitor the presence and/or absence of adulterants and substitutes for herbal medicines in the market. The sequences generated in this study, particularly thematK andtrnH-psbA are potential markers for identifying medicinal plants from the market that lack morphological features for species identification. It is therefore recommended that a reference barcoding database for medicinal plants from the market be included in future research studies to gather large number of sequences for monitoring the commercially sold medicinal plants in the area,and in the country in general.

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