Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Evaluating the Feasibility of Five Candidate DNA Barcoding Loci for Philippine *Lasianthus* Jack (*Lasiantheae*: *Rubiaceae*)

Muhammad Jefte C. Arshed, Marcos B. Valdez Jr[1], Grecebio Jonathan D. Alejandro[2]

The Graduate School, University of Santo Tomas, España Blvd., 1015 Manila, [1]Department of Biological Sciences, Institute of Arts and Sciences, Far Eastern University, Nicanor Reyes Street, Sampaloc, 1008 Manila, [2]The Graduate School and Research Center for the Natural and Applied Sciences, University of Santo Tomas, España Blvd, 1015 Manila, Philippines

Submitted: 02-01-2017 Revised: 01-02-2017 Published: 13-11-2017

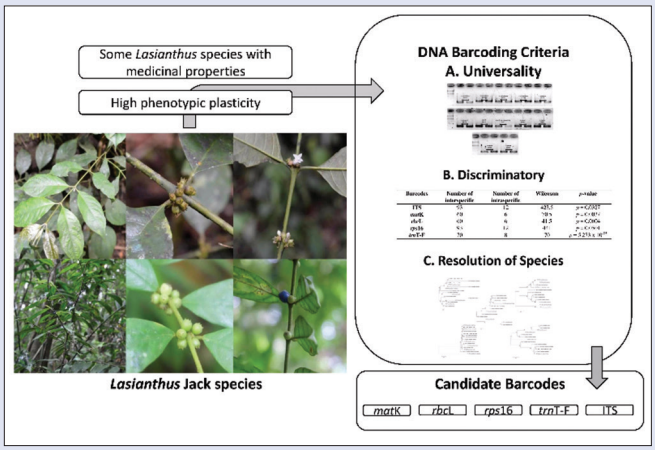
ABSTRACT

Introduction: The pantropical genus *Lasianthus* Jack is identified for high phenotypic plasticity making traditional taxonomic identification difficult. Having some members with important medicinal properties, a precise complimentary identification through DNA barcoding is needed for species delineation. **Materials and Methods:** In this study, 12 samples representing six Philippine *Lasianthus* species were used to determine the most efficient barcoding loci among the cpDNA markers (*mat*K, *rbc*L, *rps*16, and *trn*T-F) and nrDNA (ITS) based on the criteria of universality, discriminatory power, and resolution of species. **Results:** The results revealed that ITS has the recommended primer universality, greatest interspecific divergences, and average resolution of species. Among the cpDNA markers, *mat*K and *rbc*L are recommended but with minimal resolution of species. While *trn*T-F showed moderate interspecific variations and resolution of *Lasianthus* species, *rps*16 has the lowest interspecific divergence and resolution of species. **Conclusion:** Consequently, ITS is the potential ideal DNA barcode for *Lasianthus* species.

Key words: cpDNA, DNA barcoding, *Lasianthus*, nrDNA, Philippines

SUMMARY

- ITS, *mat*K, and *rps*16 markers have the excellent amplification and sequence quality
- ITS marker has the highest interspecific divergence with the maximum values, followed by *mat*K, *rbc*L, *trn*T-F, and *rps*16, respectively
- All markers except *rps*16 yielded average resolution to Lasianthus species
- ITS marker is the most ideal locus in terms of excellent universality, high interspecific discriminatory ability, and average species resolution.



Abbreviations used: ITS: Internal Transcribe Spacer, *mat*K: maturase K, *rbc*L: ribulose-1,5-biphospahte-carboxylase, *rps*16: ribosomal protein 16 small subunit gene.

Correspondence:

Dr. Grecebio Jonathan D. Alejandro,
Rm. 302, Plant Sciences Laboratory,
Research Center for the Natural and Applied Sciences, University of Santo Tomas,
España Blvd., 1015 Manila, Philippines.
E-mail: gdalejandro@ust.edu.ph/ balejan@yahoo.com

DOI: 10.4103/pm.pm_1_17

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Lasianthus Jack is the largest genus of the four genera comprising the tribe *Lasiantheae* of family *Rubiaceae*. The genus consists of about 225 species with the highest diversity in tropical and subtropical Asia.[1] *Lasianthus* is characterized as drupes with thick wall that develop from the ovaries with 3–9 locules.[2] It represents an ecologically important element specifically in its distribution pattern, which is significant in the field of biogeography and speciation patterns in the assemblage of tropical rainforest.[3] Moreover, *Lasianthus* exhibits medicinal uses such as *Lasianthus lucidus* Blume that is used to ease fever, blood loss and has hepatoprotective potential;[4] *Lasianthus verticillatus* (Lour.) Merr. is traditionally used by the Onges tribe as antidote;[5] *Lasianthus oblongus* King and Gamble is applied orally to hasten constriction of the organs for postpartum mothers;[6] and other several species of the genus are with known active chemical constituents such as alkaloids, terpenoids, and glycosides (e.g., *L. attenuatus* Jack, *L. fordii* Hance, and *L. lucidus* Blume).[7,8] Close analysis of literature, protologs, and herbarium specimens reveals uncertainties and difficulties in discriminating *Lasianthus* species based on morphology. The genus is identified for high phenotypic plasticity making traditional taxonomic identification difficult. Knowing some *Lasianthus* species exhibits medicinal and pharmaceutical importance; accurate species identification is necessary.

Modern molecular biology tools offer excellent approaches for rapid characterization and precise identification of species. Using short sequences as molecular markers for species-level identification is known

Cite this article as: Arshed MC, Valdez MB, Alejandro GD. Evaluating the feasibility of five candidate DNA Barcoding Loci for Philippine *Lasianthus* Jack (*Lasiantheae*: *Rubiaceae*). Phcog Mag 2017;13:553-8.

as DNA barcoding.[9-12] Applications of DNA barcoding are enormous especially in scenario where morphological approaches cannot resolve identification in species having sexual dimorphism and phenotypic plasticity within species of the same genus.[13] Several genomic regions were proposed for the plant DNA barcoding and the plant working group of the Consortium for the Barcode of Life (CBOL)[14] recommended using *mat*K and *rbc*L as the standard barcodes. Aside from using these two markers, additional three markers were utilized in this study, namely (1) *rps*16, an intron in the single large copy region of the plastid genome[15] that can provide good resolution[16] and has higher divergence than other cpDNA markers;[17] (2) *trn*T-F, a noncoding chloroplast gene that has high variability and useful for species and genus level resolutions for phylogenetic studies (e.g., family *Arecaceae* and *Rhamnaceae*);[18] and (3) ITS, a nuclear locus that has ability to infer closely related genera[19] due to its high repeating units that promote good amplification and sequencing.[20] Moreover, these markers have been utilized in molecular analyses of *Lasianthus* species.[1,17] In this paper, five barcoding loci (*mat*K, *rbc*L, *rps*16, *trn*T-F, and ITS) were evaluated for Philippine *Lasianthus* species to identify the ideal DNA barcode of the genus based on universality, discriminatory ability, and resolution of species.

MATERIALS AND METHODS

Sampling of plant materials

Collections of Philippine *Lasianthus* species [Table 1] from the provinces of Antique, Camiguin, Cebu, Davao, Mindoro, and Quezon, Philippines, by the Thomasian Angiosperm Phylogeny and Barcoding Group (TAPBG) of the University of Santo Tomas (UST), Manila, were used in this study. Field images of *Lasianthus* [Figure 1] and voucher specimens were deposited at the UST Herbarium (USTH) provided with accession numbers [Table 1]. Leaf samples from two different populations were collected and stored in a zip-lock with silica gel.[21] A total of 12 samples representing six Philippine *Lasianthus* species were used in this study. Seven additional sequences of three *Lasianthus* species retrieved in the GenBank were used in the analysis [Table 2].

Table 1: Thomasian Angiosperm Phylogeny and Barcoding Group *Lasianthus* collection used in the study

Codes	USTH accession	Identification	Province
14C-415	USTH 012464	*L. verticillatus*	Cebu
14C-421	USTH 012463	*L. verticillatus*	Cebu
14C-431	USTH 012461	*L. trichophlebus*	Cebu
14-620	USTH 012462	*L. trichophlebus*	Antique
14-814	USTH012458	*L. clementis*	Camiguin
14-513	USTH 012459	*L. clementis*	Davao
14-515	USTH 012457	*L. fordii*	Davao
14-637	USTH 012460	*L. fordii* var. *microphyllus*	Antique
14-541	USTH012466	*L. lucidus*	Davao
14-642	USTH 012465	*L. lucidus*	Antique
14-830	USTH 012470	*L. cyaneus*	Camiguin
14-833	USTH 012472	*L. cyaneus*	Camiguin

L. fordii: *Lasianthus fordii*; *L. lucidus*: *Lasianthus lucidus*; *L. cyaneus*: *Lasianthus cyaneus*; *L. clementis*: *Lasianthus clementis*; *L. trichophlebus*: *Lasianthus trichophlebus*

Table 2: Accession numbers of *Lasianthus* species obtained from National Center for Biotechnology Information-GenBank

Botanical name	NCBI-GenBank accession number		
	*rps*16	*trn*T-F	ITS
L. fordii Hance	KF704883	-	KF704980
L. trichophlebus Hemsl. ex F.B. Forbes and Hemsl.	KF704900	KF704950	KF704999
L. verticillatus (Lour.) Merr.	DQ282640	-	KF705001

NCBI: National Center for Biotechnology Information; *L. trichophlebus*: *Lasianthus trichophlebus*; *L. fordii*: *Lasianthus fordii*; *L. cyaneus*: *Lasianthus cyaneus*

DNA extraction, polymerase chain reaction amplification, and sequencing

Silica gel-dried leaf samples were used for the extraction of genomic DNA using the DNeasy Plant Mini Kit (Qiagen®, Germany) following the manufacturer's protocol. The Biometra T-gradient (Germany) was used for the polymerase chain reaction (PCR) amplification. DNA was amplified using KAPA Taq PCR kit (USA). The universal primers and amplification protocol used are listed in Table 3. The PCR cocktail of 25 μL reaction for the chloroplast markers (*rps*16, *trn*T-F, *mat*K, and *rbc*L) was as follows: 17.35 μL nuclease free water, 2.5 μL × 10 PCR buffer, 1.0 μL 25 $MgCl_2$, 2.0 μL deoxynucleotide triphosphates (dNTP), 1.0 μL of 10 μM forward and reverse primers, 0.15 μL Taq DNA polymerase, and 0.5 μL DNA template. For ITS marker, the PCR cocktail of 25 μL reaction was mixed as follows: 15.3 μL nuclease free water, 2.5 μL × 10 PCR buffer, 2.0 μL $MgCl_2$, 1.5 μL dNTP, 1.0 μL of 10 μM forward and reverse primers, 0.2 μL Taq DNA polymerase, and 1.5 μL DNA. The presence of amplified DNA bands was confirmed using 1% concentration of agarose gel with ×1 tris-borate-ethylenediaminetetraacetic acid buffer [Figure 2]. Amplified DNA was purified using the QIA-quick Purification Kit (Qiagen®, Germany) and were sent to Macrogen, South Korea, for bidirectional sequencing. DNA sequences were assembled and edited using the Codon Code Aligner v. 4.1.1. (CodonCode Co.,USA).

Sequence analyses

For determining the most effective barcode marker for the discrimination of *Lasianthus* species, the following conventional barcoding parameters such as mean length of base pair (bp), PCR success rate (%), intra- and inter-specific divergences (%), and the mean sequence divergence in each marker and between the different markers were analyzed using MEGA v. 7.0.14 (Pennsylvania State University), (K2P, Kimura-2-Parameter with pairwise deletion). This was followed by the Wilcoxon Mann–Whitney test to establish if the mean sequence divergence is statistically significant using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). To assess the resolution of species, percentage was calculated base from the neighbor-joining (NJ) tree that was constructed for further evaluation of markers.

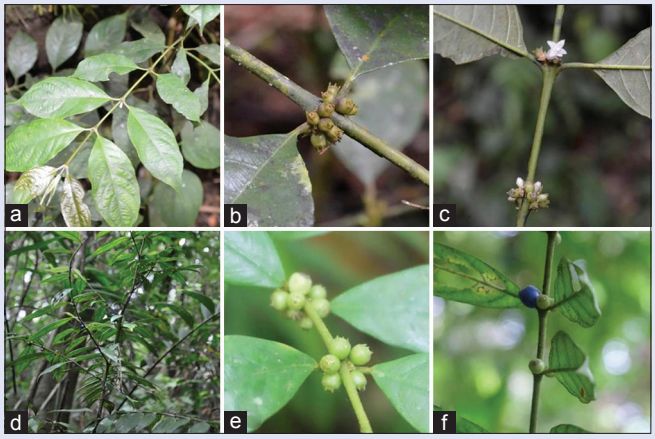


Figure 1: Field images of some *Lasianthus* species. *Lasianthus fordii* Hance: (a) leaves; (b) infructescence; (C) flowers; *Lasianthus clementis* Merr.: (d) habit; (e) infructescence; (f) fruits

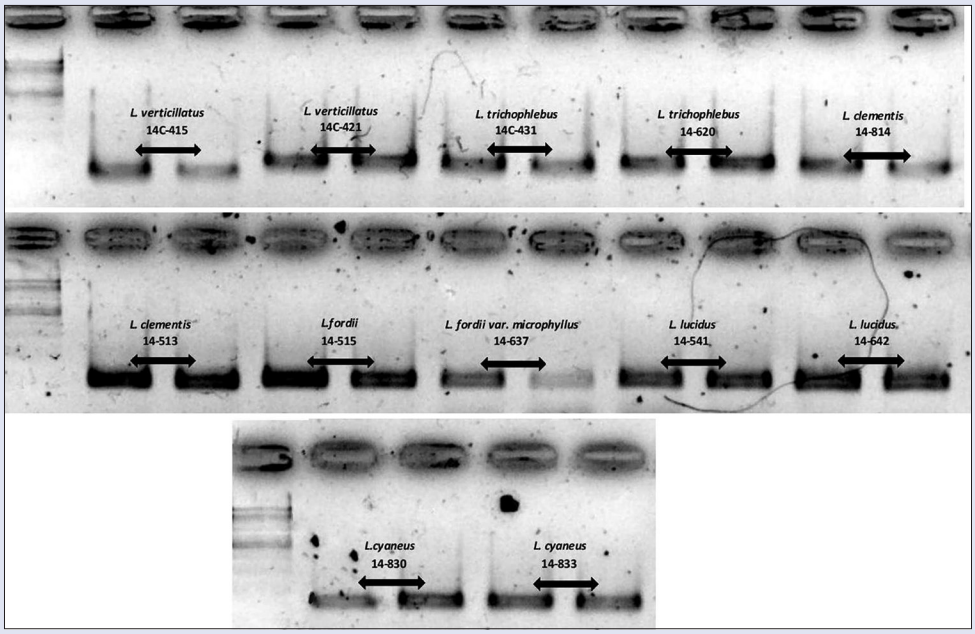


Figure 2: Sample of gel autoradiograph showing polymerase chain reaction products (ITS marker)

Table 3: Primers used for the amplification of DNA barcodes

DNA barcode	Primer	Primer sequence (5′→3′)	Amplification protocol
ITS[22]	P17F	5'-CTA CCG ATT GAA TGG TGC GGT GAA-3'	94°C and 5 min, 94°C and 1 min, 50°C and 45 s, 72°C and 1 min, 30 cycles, 72°C and 5 min
	26S-82R	5'-TCC CGG TTC GCT CGC CGT TAC TA-3'	
*trn*T-L[23]	A	5'-CAT TAC AAAA TGC GAT GCT CT-3'	95°C and 1 min, 35 cycles, 95°C and 1 min, 55°C and 1 min 30 s, 72°C and 1 min 30 s, 72°C and 7 min
	B	5'-TCT ACC GAT TTC GCC ATA TC-3'	
*trn*L-F[24]	C	5'-CGA AAT CGG TAG ACG CTA CG-3'	95°C and 1 min, 35 cycles, 95°C and 1 min, 55°C and 1 min 30 s, 72°C and 1 min 30 s, 72°C and 7 min
	F	5'-ATT TGA ACT GGT GAC ACG AG-3'	
*rps*16[25]	*rps*16-1f	5'-GTG GTA GAA AGC AAC GTG CGA CTT-3'	95°C and 1 min, 35 cycles, 95°C and 1 min, 55°C and 1 min 30 s, 72°C and 1 min 30 s, 72°C and 7 min
	*rps*16-2r	5'-TCG GGA TCG AAC ATC AAT TGC AAC-3'	
*mat*K[14]	3F_Kimf	5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3'	94°C and 3 min, 95°C and 30 s, 50°C and 20 s, 72°C and 1 min, 34 cycles, 72°C and 10 min
	1R_Kimr	5'-ACC CAG TCC ATC TGG AAA TCT TGG TTC-3'	
*rbc*L[26]	*rbc*L_aF	5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3'	95°C and 4 min, 94°C and 30, 55°C and 1 min, 72°C and 1 min, 35 cycles, 72°C and 10 min
	*rbc*L_aR	5'-CTT CTG CTA CAA ATA AGA ATC GAT CTC-3'	

Table 4: Characteristics of the different barcoding loci

Characteristics	ITS	*mat*K	*rbc*L	*rps*16	*trn*T-F
Number of taxa	12	12	12	12	12
Number of new sequences	12	12	12	12	12
Number of NCBI sequence	3	0	0	3	1
Total number of nucleotide sequences	15	12	12	15	13
PCR success rate (%)	100	100	100	100	100
Sequence success rate (%)	100	100	92	100	75
Aligned length of base pairs (bp)	841	857	675	882	2101
Number of variable sites	344	101	75	54	270
Number of informative sites	128	48	64	11	164
Resolution of the species (%)	100	50	67	50	67

NCBI: National Center for Biotechnology Information; PCR: Polymerase chain reaction

RESULTS

From the five markers, a total of sixty newly sequences of *Lasianthus* were produced [Appendix 1]. Sequence characteristics for the five barcode loci are presented in Table 4 with their overall results. The longest mean length was from *trn*T-F with 2101 bp followed by *rps*16, *mat*K, ITS, and *rbc*L. As for the most parsimonious informative sites, the *trn*T-F marker was the highest with 164 informative bp from 270 variable sites, followed by ITS, *rbc*L, and *mat*K. Interestingly, *rps*16 with the second highest mean bp still fall short for having the least informative characters of 11 from 54 variable sites. Primer universality was determined using the PCR amplification efficiency and sequence quality. PCR amplification was generally 100% successful to all the candidate barcodes. For the sequence quality, ITS, *mat*K, and *rps*16 exhibited 100% success rates, followed by *rbc*L with 92% sequencing success and *trn*T-F with 75% which was the least efficient.

Pairwise divergence analyses for each candidate barcodes using the two parameters to characterize the inter- and intra-specific divergences are presented in Table 5. The ITS has the highest interspecific divergence (0.1623 ± 0.0810), followed by *mat*K (0.0951 ± 0.0982), *trn*T-F (0.0621 ± 0.0356), *rbc*L (0.0563 ± 0.0232), and *rps*16 (0.0238 ± 0.0376). Results for the intraspecific variations revealed that *trn*T-F (0.0121 ± 0.0122) has the lowest average in all the parameters, followed by *rbc*L (0.0155 ± 0.0161), *mat*K (0.0207 ± 0.0172), *rps*16 (0.0243 ± 0.0469), and ITS (0.0999 ± 0.0613).

NJ tree was used to generate the topology of *Lasianthus* species in each candidate barcodes to determine the species resolution. Using BLAST,

Table 5: Inter- and intra-specific divergences among loci

Parameter	Candidate barcode				
	ITS	*mat*K	*rbc*L	*rps*16	*trn*T-F
Average interspecific distance	0.1623±0.0810	0.0951±0.0982	0.0563±0.0232	0.0238±0.0376	0.0621±0.0356
Average intraspecific distance	0.0999±0.0613	0.0207±0.0172	0.0155±0.0161	0.0243±0.0469	0.0121±0.0122

all of the candidate barcodes were able to classify each species as to genus *Lasianthus*, but the generated tree for each barcodes was unable to categorize some species to its specific resolutions [Figure 3].

None of the markers can completely resolve taxa with closely related species (e.g., *L. lucidus*, *L. fordii*, *L. verticillatus, L. trichophlebus*). Nevertheless, some markers can give better resolution with higher bootstrap (BS) support than other markers used in the study. The *rbc*L marker followed by *mat*K and *trn*T-F can resolve some of the difficult species with greater support value. For ITS, it cannot group same species fully just like *rps*16, but it can generate higher confidence level compared to *rps*16.

DISCUSSION

A suitable barcode should exhibit the following criteria: (1) high universality (PCR and sequencing success rates), (2) high discriminatory power based on the inter- and intra-specific divergences, and (3) high species resolution.[14] The results of the study were assessed and vis-a-vis against the criteria.

Universality

PCR amplification efficiency and sequence quality: Amplified and generated sequences of the five barcoding loci were evaluated based on the sequence quality that each barcodes produced. ITS, *mat*K, and *rps*16 markers have the excellent amplification and sequence quality. The *rbc*L and *trn*T-F markers yielded successful amplification but less sequencing success rates. Results show that ITS, *mat*K, and *rps*16 markers are the most universal in terms of quality and coverage of sequences among the barcodes utilized. This corresponds to previous studies[9,27,28] that ITS has high amplification [Figure 2] and sequence capabilities. Likewise, the results confirmed *mat*K exhibiting amplification and sequencing efficiency[29-31] and this was one of the markers recommended by CBOL as a standard barcode in plants. Furthermore, *rps*16 marker also provides high amplification and sequencing success, indicating its universality as it has been used in discriminating taxonomic uncertainties in *Rubiaceae*.[32]

Discriminatory: Inter- versus intra-specific genetic divergence

An ideal barcode should exhibit high interspecific divergences but low intraspecific variation.[9,33,34] Using the Wilcoxon two-sample tests, significant differences between the inter- and intra-specific divergences of the five candidate barcodes were analyzed [Table 6]. Interspecific differences were significantly higher ($P < 0.05$) than their related intraspecific divergences. Thus, settled differences exhibited by both specific divergences give a good lead for the discriminatory efficiency of the markers used.

In comparison of the five barcodes, ITS maker has the highest interspecific divergence with the maximum values, followed by *mat*K, *rbc*L, *trn*T-F, and *rps*16, respectively [Table 5]. The ITS has the second highest number of variable and informative sites. It also yields the highest interspecific mean which corroborates in other studies.[34,35] However, results for intraspecific variations revealed that ITS has the highest value, followed by *rps*16, *mat*K, *rbc*L, and *trn*T-F markers. An ideal barcode should have low intraspecific variations which ITS failed to have. Thus, ITS has high discriminatory power on interspecific level as this marker is useful for identification efficiency of closely related species among numerous genera. Furthermore, ITS region is regarded as more varied than any of the chloroplast genes.[36-38] Results obtained from Wilcoxon signed-rank test [Table 7] support ITS to possess the highest interspecific divergence with almost high significant differences. However, ITS is not a good marker for intraspecific identification of *Lasianthus* species for having the least intraspecific variations among other barcodes. Consequently, *trn*T-F should be the ideal barcode for discriminating species for intraspecific level in genus *Lasianthus*. Furthermore, this marker has the highest number of variable and informative sites. Results obtained using Wilcoxon signed-rank test of intraspecific divergence among loci [Table 8] suggest *rps*16 as the lowest, followed by *trn*T-F and *mat*K with equal rank and then *rbc*L and ITS as the highest. The significant differences were exhibited by *rps*16 and *trn*T-F when compared to ITS alone, making the results inconclusive for the ideal barcode for intraspecific level. There should be a significant difference between all the markers to establish the efficiency of the particular marker to discriminate up to intraspecific level.

Table 6: Wilcoxon two-sample test for inter- versus intra-specific divergences

Barcodes	Number of interspecific	Number of intraspecific	Wilcoxon	*P*
ITS	93	12	423.5	0.0327
*mat*K	60	6	70.5	0.0037
*rbc*L	60	6	41.5	0.0004
*rps*16	93	12	441	0.0501
*trn*T-F	70	8	70	5.273×10^{-5}

Table 7: Wilcoxon signed-rank tests of interspecific divergence among loci

W^+	W^-	Relative ranks		*n*	*P*	Results
		W^+	W^-			
ITS	*mat*K	1450.50	379.50	60	0.000	ITS > *mat*K
ITS	*rbc*L	1777.50	52.50	60	0.000	ITS > *rbc*L
ITS	*rps*16	4368.50	2.50	93	0.000	ITS > *rps*16
ITS	*trn*T-F	2445.50	39.50	70	0.000	ITS > *trn*T-F
*mat*K	*rbc*L	1245.00	585.00	60	0.015	*mat*K > *rbc*L
*mat*K	*rps*16	1708.00	3.00	60	0.000	*mat*K > *rps*16
*mat*K	*trn*T-F	1199.00	631.00	60	0.037	*mat*K > *trn*T-F
*rbc*L	*rps*16	1820.00	10.00	60	0.000	*rbc*L > *rps*16
*rbc*L	*trn*T-F	758.50	1011.50	60	0.340	*rbc*L < *trn*T-F
*rps*16	*trn*T-F	1.00	2484.00	70	0.000	*rps*16 < *trn*T-F

Resolution of species

Alignments for each barcodes were used to generate phylogenetic analysis using NJ tree to evaluate the species resolution if each barcode can generate taxonomic groupings per species and a monophyletic tree. In addition, the BS values were included to give partial tree reliability for each barcodes. All of the markers have insufficient conspecific groupings [Figure 3] where *rbc*L has the highest species resolution of only 67%. The ITS, *mat*K, and *trn*T-F were able to have 50% species resolution and least was from *rps*16 with 33%. Thus, candidate barcodes

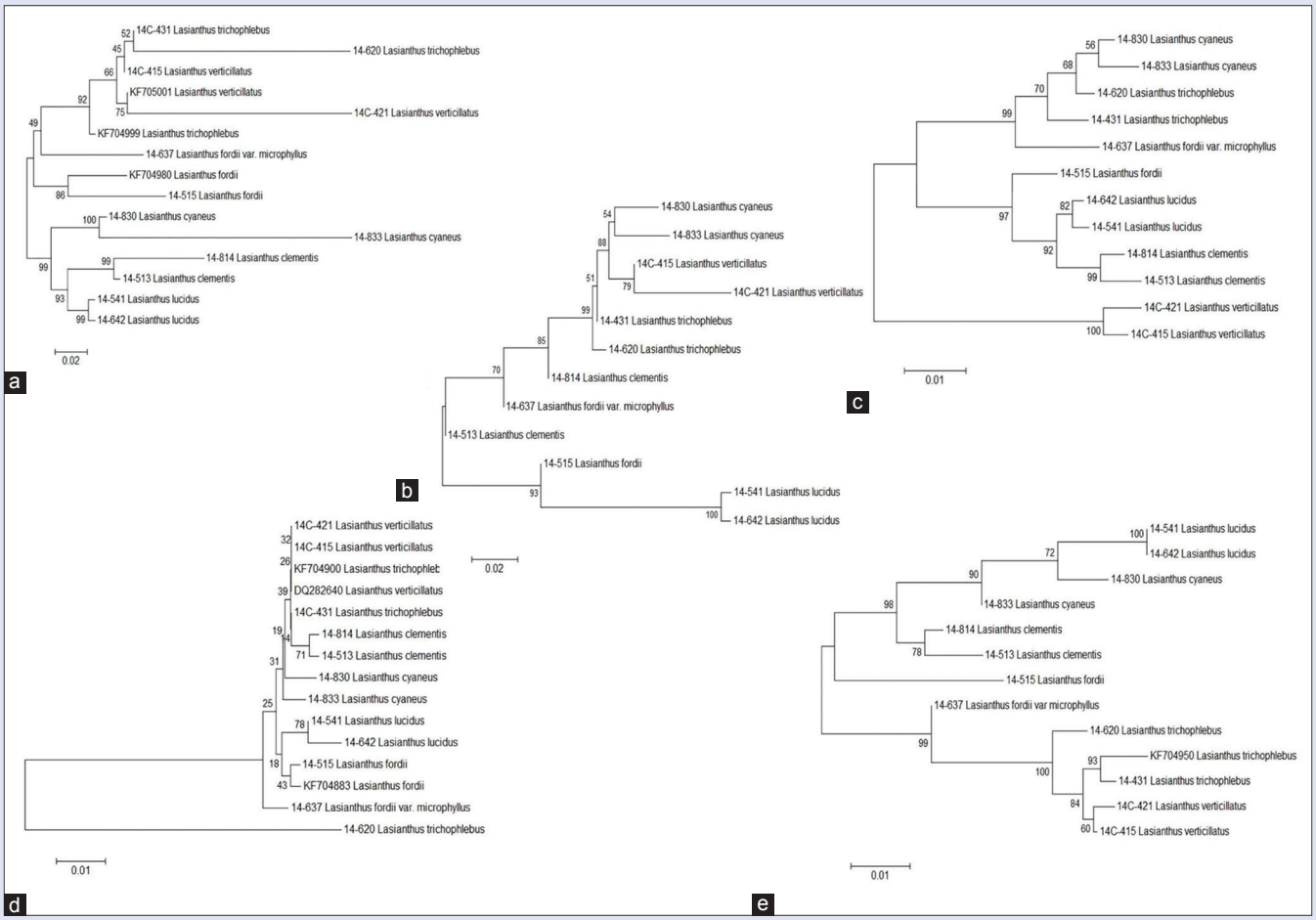


Figure 3: Neighbor-joining bootstrap trees (based on Kimura-2-Parameter) illustrating the resolution of the species for the five barcoding loci: (a) ITS sequences showing 50% species resolution; (b) matK sequences showing 50% species resolution; (c) rbcL sequences showing 67% species resolution; (d) rps16 showing 33% species resolution; (e) trnT-F showing 50% species resolution

used in the study were inadequate for species resolution; nevertheless, inference from this study suggests that most of the barcodes, except for *rps*16, can give average resolution to *Lasianthus* species.

CONCLUSION

This study provides baseline information on the potential barcodes for Philippine *Lasianthus* species. The ITS marker has the most feasible ideal locus for this genus, having excellent universality, high interspecific discriminatory ability, and average species resolution, which can be supplemented by *rbcL* and *mat*K. It would be suitable to increase the sample size of *Lasianthus* species to facilitate more definite results for rapid authentication of Philippine *Lasianthus*.

Acknowledgements

We would like to thank the TAPBG collectors, USTH, for granting access to herbarium collections and the Research Center for Natural and Applied Sciences, UST, for the laboratory facility.

Financial support and sponsorship

The present work is part of the graduate thesis of MJCA in which financial grant has been obtained from DOST-Science Education Institute. GJDA thanks DOST-Philippine Council for Health, Research and Development and CHED-Philippine Higher Education Research Network, for the funding.

Table 8: Wilcoxon signed-rank tests of intraspecific divergence among loci

W^+	W^-	Relative ranks		*n*	*P*	Results
		W^+	W^-			
ITS	*mat*K	18	3	6	0.116	ITS > *mat*K
ITS	*rbc*L	18	3	6	0.115	ITS > *rbc*L
ITS	*rps*16	69.50	8.50	12	0.017	ITS > *rps*16
ITS	*trn*T-F	34	2	8	0.025	ITS > *trn*T-F
*mat*K	*rbc*L	11	10	6	0.917	*mat*K < *rbc*L
*mat*K	*rps*16	12	3	6	0.225	*mat*K > *rps*16
*mat*K	*trn*T-F	13	8	6	0.599	*mat*K = *trn*T-F
*rbc*L	*rps*16	17	4	6	0.172	*rbc*L > *rps*16
*rbc*L	*trn*T-F	9	6	6	0.686	*rbc*L > *trn*T-F
*rps*16	*trn*T-F	8	28	8	0.161	*rps*16 < *trn*T-F

Conflict of interest

There are no conflicts of interest.

REFERENCES

1. Smedmark JE, Razafimandimbison SG, Wikström N, Bremer B. Inferring geographic range evolution of a pantropical tribe in the coffee family (Lasiantheae, *Rubiaceae*) in the face of topological uncertainty. Mol Phylogenet Evol 2014;70:182-94.
2. Zhu H. *Paralasianthus* (*Rubiaceae*), a new genus from Southeast Asia. Phytotaxa 2015;202:273-8.
3. Zhu H, Roos MC, Ridsdale CE. A taxonomic revision of the Malesian species of *Lasianthus* (*Rubiaceae*). Blumea Biodivers Evol Biogeogr Plants 2012;57:1-102.

4. Choudhury KD, Choudhury MD, Baruah MK. Hepatoprotective potential of *Lasianthus lucidus* leaf extracts against carbon tetrachloride induced liver damage in Swiss albino mice. World J Pharma Pharm Sci 2014;3:1536-47.
5. Sharief MU. Plants folk medicine of Negrito tribes of Bay Islands. Indian J Tradit Knowl 2007;6:468-76.
6. Ong HC, Faezah AW, Milow P. Medicinal plants used by the Jah Hut Orang Asli at Kampung Pos Penderas, Pahang, Malaysia. Ethno Med 2012;1:11-5.
7. Takeda Y, Shimidzu H, Mizuno K, Inouchi S, Masuda T, Hirata E, *et al*. An iridoid glucoside dimer and a non-glycosidic iridoid from the leaves of *Lasianthus wallichii*. Chem Pharm Bull (Tokyo) 2002;50:1395-7.
8. Choudhury KD, Dutta C, Anupam DT. Chemical constituents and biological activities of the genus *Lasianthus* Jack: A review. Assam Univ J Sci Technol 2010;6:129-38.
9. Gao T, Yao H, Song J, Zhu Y, Liu C, Chen S. Evaluating the feasibility of using candidate DNA barcodes in discriminating species of the large Asteraceae family. BMC Evol Biol 2010;10:324.
10. Yu X, Xie Z, Wu J, Tao J, Xu X. DNA barcoding identification of *Kadsurae caulis* and *Spatholobi caulis* based on internal transcribed spacer 2 region and secondary structure prediction. Pharmacogn Mag 2016;12 Suppl 2:S165-9.
11. Zhou J, Wang W, Liu M, Liu Z. Molecular authentication of the traditional medicinal plant *Peucedanum praeruptorum* and its substitutes and adulterants by DNA-barcoding technique. Pharmacogn Mag 2014;10:385-90.
12. Mahadani P, Sharma GD, Ghosh SK. Identification of ethnomedicinal plants (Rauvolfioideae: *Apocynaceae*) through DNA barcoding from northeast India. Pharmacogn Mag 2013;9:255-63.
13. Chapple DG, Ritchie PA. A retrospective approach to testing the DNA barcoding method. PLoS One 2013;8:e77882.
14. CBOL Plant Working Group. A DNA barcode for land plants. Proc Natl Acad Sci U S A 2009;106:12794-7.
15. Lee J, Hymowitz T. A molecular phylogenetic study of the subtribe *Glycininae* (Leguminosae) derived from the chloroplast DNA rps16 intron sequences. Am J Bot 2001;88:2064-73.
16. Marazzi B, Endress PK, Queiroz LP, Conti E. Phylogenetic relationships within *Senna* (Leguminosae, Cassiinae) based on three chloroplast DNA regions: Patterns in the evolution of floral symmetry and extrafloral nectaries. Am J Bot 2006;93:288-303.
17. Xiao LQ, Zhu H. Paraphyly and phylogenetic relationship in *Lasianthus* (*Rubiaceae*) inferred from chloroplast rps16 data. Bot Stud 2007;48:227-32.
18. Borsch T, Hilu KW, Quand, D, Wilde V, Neihuis C, Barthlott W. Noncoding plastid trnT-F sequences reveal a well resolve phylogeny of basal *Angiosperms*. J Evol Biol 2003;16:558-76.
19. Soltis DE, Soltis PS. Choosing an approach and an appropriate gene for phylogenetic analysis. In: Soltis PS, Doyle JJ, editors. Molecular Systematics of Plants. Boston, Massachusetts: Kluwer; 1998. p. 1-42.
20. Baldwin B, Sanderson MJ, Porter JM, Wojciechowski MF, Donoughe MJ. The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. Ann Mo Bot Gard 1995;82:247-77.
21. Chase MW, Hills HH. Silica gel: An ideal material for preservation of leaf samples for DNA studies. Taxon 1991;40:215-20.
22. Popp M, Oxelman B. Inferring the history of the polyploid *Silene aegaea* (*Caryophyllaceae*) using plastid and homoeologous nuclear DNA sequences. Mol Phylogenet Evol 2001;20:474-81.
23. Razafimandimbison SG, Bremer B. Phylogeny and classification of Naucleeae s.l. (*Rubiaceae*) inferred from molecular (ITS, rBCL, and tRNT-F) and morphological data. Am J Bot 2002;89:1027-41.
24. Taberlet P, Gielly L, Pautou G, Bouvet J. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 1991;17:1105-9.
25. Oxelman B, Lidén M, Berglund D. Chloroplast rps16 intron phylogeny of the tribe *Sileneae* (*Caryophyllaceae*). Plant Syst Evol 1997;206:393-410.
26. Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: The coding rbcL gene complements the non-coding trnH-psbA spacer region. PLoS One 2007;2:e508.
27. Yao H, Song J, Liu C, Luo K, Han J, Li Y, *et al*. Use of ITS2 region as the universal DNA barcode for plants and animals. PLoS One 2010;5. pii: e13102.
28. Gu W, Song J, Cao Y, Sun Q, Yao H, Wu Q, *et al*. Application of the ITS2 region for barcoding medicinal plants of *Selaginellaceae* in *Pteridophyta*. PLoS One 2013;8:E67818.
29. Yu H, Wu K, Song J, Zhu Y, Luo K, Lin Y. Expedient identification of magnoliaceae species by DNA barcoding. Plant Omics 2014;7:47-53.
30. Cabelin VL, Santor PJ, Alejandro GJ. Evaluation of DNA barcoding efficiency of cpDNA barcodes in selected Philippine *Leea* L. (Vitaceae). Acta Bot Gallica 2015;162:317-3.
31. Cabelin VL, Alejandro GJ. Efficiency of matK, rbcL, trnH-psbA, and trnL-F (cpDNA) to molecularly authenticate Philippine ethnomedicinal *Apocynaceae* through DNA barcoding. Pharmacogn Mag 2016;12 Suppl 3:S384-8.
32. Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, *et al*. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am J Bot 2005;92:142-66.
33. Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F, Gigot G, *et al*. DNA barcoding the floras of biodiversity hotspots. Proc Natl Acad Sci U S A 2008;105:2923-8.
34. Li HQ, Chen JY, Wang S, Xiong SZ. Evaluation of six candidate DNA barcoding loci in Ficus (Moraceae) of China. Mol Ecol Resour 2012;12:783-90.
35. Chase MW, Salamin N, Wilkinson M, Dunwell J, Kesanakurthi RP, Haidar N, *et al*. Land plants and DNA barcodes: Short-term and long-term goals. Philos Trans R Soc Biol Sci 2005;360:1889-95.
36. Chase MW, Cowan RS, Hollingsworth PM, van den Berg C, Madriñán S, Petersen G, *et al*. A proposal for a standardized protocol to barcode all land plants. Taxon 2007;56:295-9.
37. Roy S, Tyagi A, Shukla V, Kumar A, Singh UM, Chaudhary LB, *et al*. Universal plant DNA barcode loci may not work in complex groups: A case study with Indian berberis species. PLoS One 2010;5:e13674.
38. Liu Y, Zhang L, Liu Z, Luo K, Chen S, Chen K. Species identification of *Rhododendron* (*Ericaceae*) using the chloroplast deoxyribonucleic acid PsbA-trnH genetic marker. Pharmacogn Mag 2012;8:29-36.

APPENDIX

Appendix 1: European Molecular Biology Laboratory accession numbers of the sequences generated in this study

Species	*Candidate* barcodes				
	ITS	*mat*K	*rbc*L	*rps*16	*trn*T-F
L. verticillatus	LT717425; LT717426	LT717461; LT717462	LT717473; LT717474	LT717437; LT717438	LT717449; LT717450
L. trichophlebus	LT717427; LT717428	LT717463; LT717464	LT717475; LT717476	LT717439; LT717440	LT717451; LT717452
L. clementis	LT717429; LT717430	LT717465; LT717466	LT717477; LT717478	LT717441; LT717442	LT717453; LT717454
L. fordii	LT717431	LT717467	LT717479	LT717443	LT717455
L. fordii var. *microphyllus*	LT717432	LT717468	LT717480	LT717444	LT717456
L. lucidus	LT717433; LT717434	LT717469; LT717470	LT717481; LT717482	LT717445; LT717446	LT717457; LT717458
L. cyaneus	LT717435; LT717436	LT717471; LT717472	LT717483; LT717484	LT717447; LT717448	LT717459; LT717460

L. fordii: *Lasianthus fordii*; *L. lucidus*: *Lasianthus lucidus*; *L. cyaneus*: *Lasianthus cyaneus*; *L. clementis*: *Lasianthus clementis*; *L. trichophlebus*: *Lasianthus trichophlebus*