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

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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 (matK, rbcL, rps16, and trnT-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, matK and rbcL are recommended but with minimal resolution of species. While trnT-F showed moderate interspecific variations and resolution of Lasianthus species, rps16 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

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.1,3 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

Abbreviations used: ITS: Internal Transcribe Spacer, matK: maturase K, rbcL: ribulose-1,5-biphosphate-carboxylase, rps16: ribosomal protein 16 small subunit gene.

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as DNA barcoding. 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. Several genomic regions were proposed for the plant DNA barcoding and the plant working group of the Consortium for the Barcode of Life (CBOL) recommended using \textit{matK} and \textit{rbcL} as the standard barcodes. Aside from using these two markers, additional three markers were utilized in this study, namely (1) \textit{rps16}, an intron in the single large copy region of the plastid genome that can provide good resolution and has higher divergence than other cpDNA markers; (2) \textit{trnT-F}, a noncoding chloroplast gene that has high variability and useful for species and genus level resolutions for phylogenetic studies (e.g., family \textit{Arecales} and \textit{Rhamnaceae}); and (3) ITS, a nuclear locus that has ability to infer closely related genera due to its high repeating units that promote good amplification and sequencing.

Moreover, these markers have been utilized in molecular analyses of \textit{Lasianthus} species. In this paper, five barcoding loci (\textit{matK}, \textit{rbcL}, \textit{rps16}, \textit{trnT-F}, and ITS) were evaluated for Philippine \textit{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 \textit{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 \textit{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. A total of 12 samples representing six Philippine \textit{Lasianthus} species were used in this study. Seven additional sequences of three \textit{Lasianthus} species retrieved in the GenBank were used in the analysis [Table 2].

**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 (\textit{rps16}, \textit{trnT-F}, \textit{matK}, and \textit{rbcL}) was as follows: 17.35 μL nuclease free water, 2.5 μL × 10 PCR buffer, 1.0 μL 25 MgCl₂, 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 MgCl₂, 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-ethylenediaminetraacetic 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 \textit{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.

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

| Codes   | USTH accession | Identification       | Province    |
|---------|---------------|---------------------|-------------|
| 14C-415 | USTH 012464   | \textit{L. verticillatus} | Cebu        |
| 14C-421 | USTH 012465   | \textit{L. verticillatus} | Cebu        |
| 14C-431 | USTH 012461   | \textit{L. trichophlebus} | Cebu        |
| 14-620  | USTH 012462   | \textit{L. trichophlebus} | Antique     |
| 14-814  | USTH 012458   | \textit{L. clementis}   | Camiguin    |
| 14-513  | USTH 012459   | \textit{L. clementis}   | Davao       |
| 14-515  | USTH 012457   | \textit{L. fordii}     | Davao       |
| 14-637  | USTH 012460   | \textit{L. fordii var. microphyllus} | Davao     |
| 14-541  | USTH 012466   | \textit{L. lucidus}    | Antique     |
| 14-642  | USTH 012465   | \textit{L. lucidus}    | Antique     |
| 14-830  | USTH 012470   | \textit{L. cyanus}     | Camiguin    |
| 14-833  | USTH 012472   | \textit{L. cyanus}     | Camiguin    |

\textit{L. fordii}; \textit{Lasianthus fordii}; \textit{L. lucidus}; \textit{Lasianthus lucidus}; \textit{L. cyanus}; \textit{Lasianthus cyanus}; \textit{L. clementis}; \textit{Lasianthus clementis}; \textit{L. trichophlebus}; \textit{Lasianthus trichophlebus}

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

| Botanical name | NCBI-GenBank accession number |
|---------------|------------------------------|
| \textit{L. fordii} Hance | KS704883, KS704980 |
| \textit{L. trichophlebus} Hemsl. ex F.B. Forbes and Hemsl. | KS704900, KS704950, KS704999 |
| \textit{L. verticillatus} (Lour.) Merr. | DQ282640, KG705001 |

NCBI: National Center for Biotechnology Information; \textit{L. trichophlebus}; \textit{Lasianthus trichophlebus}; \textit{L. fordii}; \textit{Lasianthus fordii}; \textit{L. cyanus}; \textit{Lasianthus cyanus}

**Figure 1:** Field images of some \textit{Lasianthus} species. \textit{Lasianthus fordii} Hance: (a) leaves; (b) infructescence; (C) flowers; \textit{Lasianthus clementis} Merr. (d) habit; (e) infructescence; (f) fruits
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 trnT-F with 2101 bp followed by rps16, matK, ITS, and rbcL. As for the most parsimonious informative sites, the trnT-F marker was the highest with 164 informative bp from 270 variable sites, followed by ITS, matK, and rps16. Interestingly, rps16 with the second highest mean bp still fall short for having the least informative characters of 11 from 54 variable sites.

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 matK (0.0951 ± 0.0982), trnT-F (0.0621 ± 0.0356), rbcL (0.0563 ± 0.0232), and rps16 (0.0238 ± 0.0376). Results for the intraspecific variations revealed that trnT-F (0.0121 ± 0.0122) has the lowest average in all the parameters, followed by rbcL (0.0155 ± 0.0161), matK (0.0207 ± 0.0172), rps16 (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,
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 rbcL marker followed by *matK* and *trnT-F* can resolve some of the difficult species with greater support value. For ITS, it cannot group same species fully just like *rps16*, but it can generate higher confidence level compared to *rps16*.

**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. 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, *matK*, and *rps16* markers have the excellent amplification and sequence quality. The *rbcL* and *trnT-F* markers yielded successful amplification but less sequencing success rates. Results show that ITS, *matK*, and *rps16* markers are the most universal in terms of quality and coverage of sequences among the barcodes utilized. This corresponds to previous studies that ITS has high amplification [Figure 2] and sequence capabilities. Likewise, the results confirmed *matK* exhibiting amplification and sequencing efficiency and this was one of the markers recommended by CBOL as a standard barcode in plants. Furthermore, *rps16* marker also provides high amplification and sequencing success, indicating its universality as it has been used in discriminating taxonomic uncertainties in *Rubiaceae*.

**Discriminatory: Inter- versus intra-specific genetic divergence**

An ideal barcode should exhibit high interspecific divergences but low intraspecific variation. 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 *matK*, *rbcL*, *trnT-F*, and *rps16*, 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. However, results for intraspecific variations revealed that ITS has the highest value, followed by *rps16*, *matK*, *rbcL*, and *trnT-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. 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, *trnT-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 interspecific divergence among loci [Table 8] suggest *rps16* as the lowest, followed by *trnT-F* and *matK* with equal rank and then *rbcL* and ITS as the highest. The significant differences were exhibited by *rps16* and *trnT-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 interspecific level.

**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 *rbcL* has the highest species resolution of only 67%. The ITS, *matK*, and *trnT-F* were able to have 50% species resolution and least was from *rps16* with 33%. Thus, candidate barcodes

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

| Parameter                        | Candidate barcode | ITS       | matK      | rbcL     | rps16    | trnT-F   |
|----------------------------------|-------------------|-----------|-----------|----------|----------|----------|
| Average interspecific distance   |                   | 0.1623±0.081 | 0.0951±0.0982 | 0.0563±0.0232 | 0.2328±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 |

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**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|
| matK     | 60                      | 6                       | 70.5     | 0.0037|
| rbcL     | 60                      | 6                       | 41.5     | 0.0004|
| rps16    | 93                      | 12                      | 441.1    | 0.0501|
| trnT-F   | 70                      | 8                       | 70       | 5.273×10^-3 |

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

| W+   | W-   | Relative ranks | n   | P     | Results   |
|------|------|----------------|-----|-------|-----------|
| ITS  | matK| 1450.50        | 379.50 | 60   | 0.0000 | ITS > matK |
| ITS  | rbcL| 1777.50        | 52.50  | 60   | 0.0000 | ITS > rbcL|
| ITS  | rps16| 4368.50       | 2.50   | 93   | 0.0000 | ITS > rps16|
| ITS  | trnT-F | 2445.50    | 39.50  | 70   | 0.0000 | ITS > trnT-F|
| matK | rbcL| 1245.00        | 585.00 | 60   | 0.0150 | matK > rbcL|
| matK | rps16| 1708.00       | 3.00   | 60   | 0.0000 | matK > rps16|
| matK | trnT-F | 1199.00    | 631.00 | 60   | 0.0370 | matK > trnT-F|
| rbcL | rps16| 1820.00        | 10.00  | 60   | 0.0000 | rbcL > rps16|
| rbcL | trnT-F | 758.50       | 1011.50| 60   | 0.3400 | rbcL < trnT-F|
| rps16| trnT-F | 1.00          | 2484.00| 70   | 0.0000 | rps16 < trnT-F|
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 \( matK \). It would be suitable to increase the sample size of \( Lasianthus \) species to facilitate more definite results for rapid authentication of Philippine \( Lasianthus \).

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**Conflict of interest**

There are no conflicts of interest.

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APPENDIX

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

| Species          | ITS   | matK  | rbcL  | rps16 | trnT-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. fordi         | LT717431     | LT717467     | LT717479     | LT717443     | LT717455     |
| L. fordi 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; LT717477; LT717448 | LT717459; LT717460 |

L. fordi: Lasianthus fordi; L. lucidus: Lasianthus lucidus; L. cyaneus: Lasianthus cyaneus; L. clementis: Lasianthus clementis; L. trichophlebus: Lasianthus trichophlebus