A comparative assessment of 16S ribosomal RNA and Cytochrome C Oxidase Subunit I (COI) Primers for Amphibian DNA Barcoding

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Abstract. Amphibians, a diverse and ecologically important group, are facing global declines due to various factors, including habitat loss and climate change. Accurate species identification is crucial for effective conservation efforts, and DNA barcoding has emerged as a powerful tool in this regard. This study compares the efficacy of two DNA barcoding primer sets, targeting the 16S ribosomal RNA gene and the Cytochrome Oxidase I (COI) gene, for identifying 20 amphibian species. While both primer sets successfully amplified sequences, the 16S rRNA gene region identified all 20 samples, whereas the COI region identified 14. The amplified sequences, approximately 550 base pairs for 16S rRNA and 658 base pairs for COI facilitated precise taxonomic placement within amphibian families using Neighbor-Joining phylogenetic trees. These findings enhance DNA barcoding methodology and aid in understanding amphibian diversity, crucial for effective conservation strategies amidst global declines drives by habitat loss, diseases, and climate change.

1 Introduction

Amphibians, a diverse and ecologically significant class of vertebrates, consist of a remarkable array of species exhibiting intricate ecological, morphological, and behavioural adaptations. However, these remarkable amphibian species are increasingly threatened by habitat loss, pollution, and climate change [1-5]. Therefore, there is an urgency to document and conserve amphibian biodiversity. Accurate species identification plays a crucial role in effectively implementing large-scale biodiversity monitoring and conservation [6]. DNA

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barcoding is a widely utilized technique that enables species identification based on specific genetic variations present in short regions of an organism's DNA [7]. This technique also has been widely used by several wildlife species in Malaysia, including the dusky leaf monkey [8], the Malayan tiger [9], the Asian elephant [10], and the amphibian [11]. By employing one or a few regions within the genome, DNA barcoding can effectively distinguish and classify species within a given taxonomic group [12]. This approach has revolutionized DNA-based investigations, encompassing various applications, including phylogenetics and environmental genomics [13-14]. However, one of the critical decisions in DNA barcoding is the selection of genetic markers or primers that reliably and efficiently amplify the target DNA regions for species identification.

In DNA barcoding, a 5′ fragment of the mitochondrial Cytochrome C Oxidase Subunit I (COI) gene is commonly used to classify and identify various animals [7]. Nevertheless, the COI gene in amphibians often encounters challenges due to the notable variability within this fragment. There has been ongoing discussion regarding the effectiveness of COI region as a gene marker for DNA barcoding and this is due to the challenges in amplifying COI gene using Folmer's widely used COI primers and the ambiguity surrounding DNA barcoding gaps [15-16]. Furthermore, the differences in observed divergence often failed to distinguish between variations within a species and differences between species. According to Vences *et al.* [15], universal primers designed for COI often faces challenges in sequencing amphibians because mutations commonly occurred in the priming sites. These complexities complicate the use of COI as a reliable barcoding tool for amphibians [15-17]

Consequently, [16] suggested the use of 16S rRNA gene region has been proposed as a DNA barcoding marker alongside COI, especially for amphibians. 16S exhibits a high degree of conservation, rendering it amenable to the design of universal primers. As a result, many researchers have embraced 16S as the reliable barcoding marker of choice for evaluating biodiversity and identifying species of amphibians [18-20]. The best possible barcoding marker need to show a noticeable difference in genetic variation within species (intraspecific) and between species (interspecific), with an emphasis on accurate species identification [21-22]. However, it is a common challenge that intraspecific and interspecific divergence values for the 16S gene often overlap. This overlap complicates establishing a threshold value that can effectively differentiate between species [16, 18, 20]. In this study, an assessment of the utility of 16S rRNA and COI primers for amphibian DNA barcoding was carried out. Through an analysis of DNA sequences obtained from diverse amphibian species, each marker's advantages and disadvantages were discussed, focusing on their applicability in the context of amphibian conservation, phylogenetics, and ecological research.

2 Materials and Methods

The amphibian sampling was conducted in Gunung Belumut Amenity Forest, Johor, Malaysia. Sampling occurred six times between March and June 2022, with an extra sampling in February 2023. We collected a few individuals for each species and tissue samples for DNA analyses from liver samples, which were then preserved in 95% ethanol [23-25]

The extraction of DNA from amphibian tissues used DNeasy Blood and Tissue Kit (Qiagen, USA). After the extraction process, DNA templates obtained from the tissue specimens were utilized for polymerase chain reaction (PCR) amplification. The PCR reaction mixture consisted of 12.5μL of My Taq Red Mix, 5.5μL of ddH_2O, 4.0μL of extracted DNA, 1.5μL of primers (forward and reverse), both with concentration 10μM. The primers used for the amplification of target genes in amphibians were 16s rRNA [26] and COI gene [27] (Table 1).

Table 1. The sequence for 16S rRNA primer and COI gene primer.

Primer	References	Sequence	Size (bp)
16SrA-L	Palumbi et al. [26]	5'- CGC CTG TTT ATC AAA AAC AT -3'	~ 550 bp
16SrB-H		5'- CCG GTC TGA ACT CAG ATC ACG T -3'	~ 550 bp
Chmf4	Che et al. [27]	5'- TYT CWA CWA AYC AYA AAG AYA TCG G -3'	~ 658 bp
Chmr4		5'- ACY TCR GGR TGR CCR AAR AAT CA -3'	~ 658 bp

The PCR amplification profile cycle for the 16S gene consisted of initial denaturation at 94°C (2 min), 35 cycles of denaturation at 94°C (30 sec), annealing at 52°C (40 sec) and extension at 72°C (1 min). Finally, a final extension step was carried out at 72°C (5 min). For PCR profile cycle for gene COI, the amplification consisted of initial denaturation at 95°C (5 min), 35 cycles of denaturation at 94°C (1 min), annealing at 46°C (1 min) and 72°C (1 min). A final extension was performed at 72°C (10 min). Then, the amplified DNA samples were subjected to gel electrophoresis using a 2.0% agarose gel to assess their quality and integrity. The DNA samples that met the quality criteria were then submitted to the sequencing provider (Apical Scientific Sdn. Bhd.) for DNA sequencing. Each sequence underwent assessment via histogram analysis, with necessary base pair reading corrections made as needed. DNA sequence alignment was carried out using BioEdit Sequence Alignment Editor 7.2.5 software [28]. To analyze the genetic data, MEGA v. 11.0 [29] was utilized to compute sequence divergences and create a Neighbor-Joining (NJ) tree using the Kimura 2-parameter (K2P) model.

3 Results and Discussion

3.1 Results

The sequencing of 16S rRNA was completed for 20 individual samples, representing a success rate of 100%. In contrast, the COI gene was successfully sequenced in 14 out of 20 individual samples, accounting for a success rate of 70%. For 16S, the sequencing process yielded 483 sites comprising 231 conserved sites (47.83%) and 251 variable sites (51.97%). Notably, 224 sites were identified as parsimony-informative (42.38%). For COI gene, a total of 612 sites were analyzed, comprising 296 conserved sites (48.37%), 308 variable sites (50.33%), and 266 parsimony-informative sites (43.46%). The identity of several species, including *Hylarana sundabarat, Limnonectes deinodon,* and *Microhyla mukhlesuri* was confirmed through a comparative analysis with sequences associated with their previous taxonomic using BLAST results.

The DNA genetic divergence across different taxonomic level was summarized in Table 3. In terms of COI intraspecific genetic distances, values varied from 0.001 to 0.132, while interspecific distances ranged from 0.018 to 0.209. Notably, genetic divergences within 16S gene were notably lower. Mean values for within species, within genera, and family genetic distances were 0.011, 0.037, and 0.108, respectively (Table 2). In comparison, the genetic divergence of the COI gene between species within the same genus is one time higher than the genetic difference within species. Furthermore, the average divergence within the 16S gene between species within the same genus was about three times greater than the genetic difference between within species.

The estimation of genetic relationships among the sequences were carried out by analyzing the matrix of sequence divergences using Kimura's two-parameter method [30].

Phylogenetic relationships were determined using the Neighbor-Joining (NJ) method [31]. To construct the phylogenetic trees, the nucleotide sequences of *Trachemys scripta* were utilized as an outgroup, based on the the mitochondrial 16S rRNA and COI genes. MEGA 11 were used to compute bootstraps from 1000 replications for the NJ tree, providing approximate confidence levels for the trees. Fig. 1 shows the phylogenetic tree of NJ for the 16S gene.

Table 2. The genetic differences COI and 16S gene across three taxonomic levels using Kimura-2-parameter distance.

Level of comparison	COI				16S			
	Taxa	**Min**	**Max**	**Mean**	**Taxa**	**Min**	**Max**	**Mean**
Species	16	0.001	0.132	0.047	11	0	0.047	0.011
Genus	12	0.018	0.209	0.058	11	0.001	0.124	0.037
Family	5	0.021	0.227	0.158	5	0.006	0.175	0.108

The NJ tree, created from the differences in nucleotide sequence within the 16S gene, showed that the outgroup, *T. scripta* was distinct from the ingroup. Moreover, the ingroup was split into two main clades (Fig. 1). Notably, nearly all amphibian species in the ingroup cluster and their respective reference barcodes display 100% bootstrap value. These species include *Chalcorana labialis, H. sundabarat, Humerana miopus, Odorrana hosii, Occidozyga martensii, Rhacophorus norhayatii, Polypedates macrotis, Polypedates leucomystax, Fejervarya limnocharis, Limononectes blythii, L. plicatellus, Kalophrynus palmatissimus, M. mukhlesuri,* and *Phrynoidis asper*. Notably, *L. deinodon* and *Polypedates discantus* exhibited 99% and 63% bootstrap values, respectively.

Similar to the NJ tree created using 16S rRNA gene, the NJ tree derived from nucleotide sequence divergences within the COI gene also exhibits a distinct separation between the outgroup, *T. scripta* and the ingroup. The ingroup was split into two main clades (Fig. 2). Notably, most amphibian species within the ingroup are observed to form clades with their respective reference barcodes, displaying robust bootstrap support values of 100%. These species comprising *F. limnocharis, P. discantus, H. sundabarat, P. asper, H. miopus, O. hosii, K. palmatissimus, M. mukhlesuri, R. norhayatii, C. labialis*. Notably, *L. blythii* is associated with bootstrap value of 99%. Additionally, sample H54 formed a clade consistent with its respective reference barcode from Genbank, supported by an 84% bootstrap value.

3.2 Discussion

For BLAST identification, several samples did not have reference sequences available in NCBI Genbank. Two samples initially identified as *L. deinodon* were found to correspond to *L. khasianus*, with an identical matching percentage of 94.88%. *L. deinodon*, previously reported under the names *L. laticeps* and *L. khasianus* in [30-33], with taxonomic adjustments proposed by [34]. Hence, *L. deinodon* was identified as the final species for this sample as sequences of this species were not available in NCBI Genbank. Additionally, *M. mukhlesuri* samples exhibited a match with *M. fissipes* at a percentage of 98.16%. *M. mukhlesuri*, formerly recognized as *M. fissipes* in [31], with taxonomic revisions detailed by [35]. *M. mukhlesuri* was identified as the final species of this sample as the distribution of *M. fissipes* was restricted to China, Hong Kong, Macao, Taiwan, and Vietnam [36]. *H. sundabarat* samples were identified as *Hylarana picturata*, also with an identical percentage match of 98.16%. *H. sundabarat* was previously reported as *H. picturata* in [37-38] with taxonomic revisions documented [39]. The quality and completeness of reference databases play a crucial role in species identification accuracy. In this study, reference sequences were

extracted from NCBI Genbank database. Incomplete or outdated reference databases may result in misidentification or underrepresentation of species, particularly for non-model organisms or species with limited genomic data available [40]. The preference for 16S as a dependable marker is because the difficulties in obtaining COI sequencing for amphibians. This leads to a significant difference in the number of available 16S and COI sequences in the database of Genbank [41]. Currently, Genbank repository holds a substantial collection of 16S for amphibians, totalling 7,005 sequences [40]. In contrast, there are only 1,821 accessible COI sequences available [40]. Despite an incomplete database, the 16S gene region, known for its strong phylogenetic signal, continues to be a reliable method for accurate species identification [42]. Thus, sequencing of 16S for amphibians should be continued, along with the development of a comprehensive database of amphibian COI sequences.

Our results showed that the amplification and BLAST outcomes for the 16S marker exceeded those of the COI marker. The preference for the 16S fragment as a barcoding region in amphibians in primarily attributable to its notably high amplification efficiency [15]. However, this heightened amplification success may come from the conserved region observed in 16S compared to COI, resulting in reduced genetic variation for discriminating closely related species. Furthermore, the PCR process for 16S was straightforward. A standardized protocol was used that yielded consistent success rates. In contrast, initial attempts to amplify COI were successful only for specific species [41]. Given the well-documented historical difficulties associated with their use, this limited success with COI universal primers in amphibians is expected [15]. Notably, the success of amphibian-specific COI primers has improved over the universal primers, where it used to have failure rates from 50% to 70% individual cases [16, 27, 43, 44]. It is likely that the effectiveness of COI when using primers specific for amphibian, could be further enhanced by implementing temperature gradient experiments for each species or family, but this can lead to increased time, cost, and resources used [41]. Despite the clear enhancement in PCR product yield achieved with amphibians-specific COI primers, the question arises whether this improvement is sufficient to justify the uses of COI as the preferred genetic barcode marker for amphibian in comparison to 16S.

The mean genetic distances observed for the COI marker within species, genera, and families are slightly higher than those of 16s marker. Genetic distance reflects the degree of difference between sequences originated from a shared ancestor, as these sequences evolve independently, they gradually diverge from each other over time [45]. The utilization of the COI gene as a universal and widely used DNA barcoding marker for organism is firmly established [7]. Several key attributes for this choice include the gene's universality and a high rate of substitution, particularly in the third codon position [46]. Since the publication of [7], the major growth of public genetic database dedicated to this gene has provided a crucial foundation for its application in specimen identification. Recent assessments have shown the advantages of utilizing the COI gene in comparison to alternative genetic markers [46]. In the context of phylogenetic trees, all species are clearly recovered as monophyletic units. Nevertheless, the phenogram constructed using the 16S marker reveals a more pronounced clustering within genera and family sequences compared to COI. The 16S phenogram yields a greater number of monophyletic taxa. This aligns with findings in a study by Che et al. [27], which observed that COI gene marker did not group individuals accurately within their respective group at the level of genera and higher taxonomic hierarchy.

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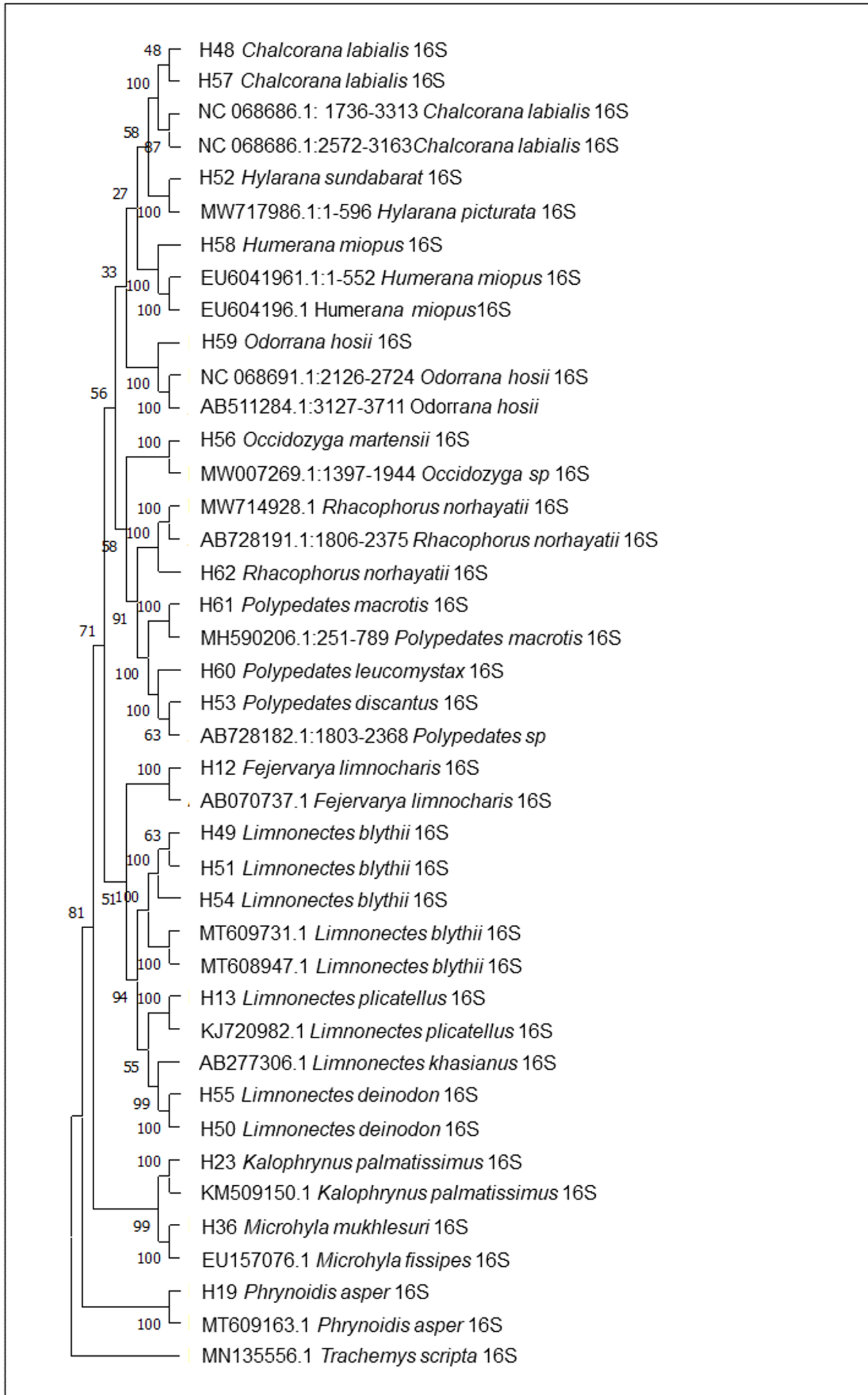

Fig. 1. The Neighbor-Joining Tree for 16S rRNA gene.

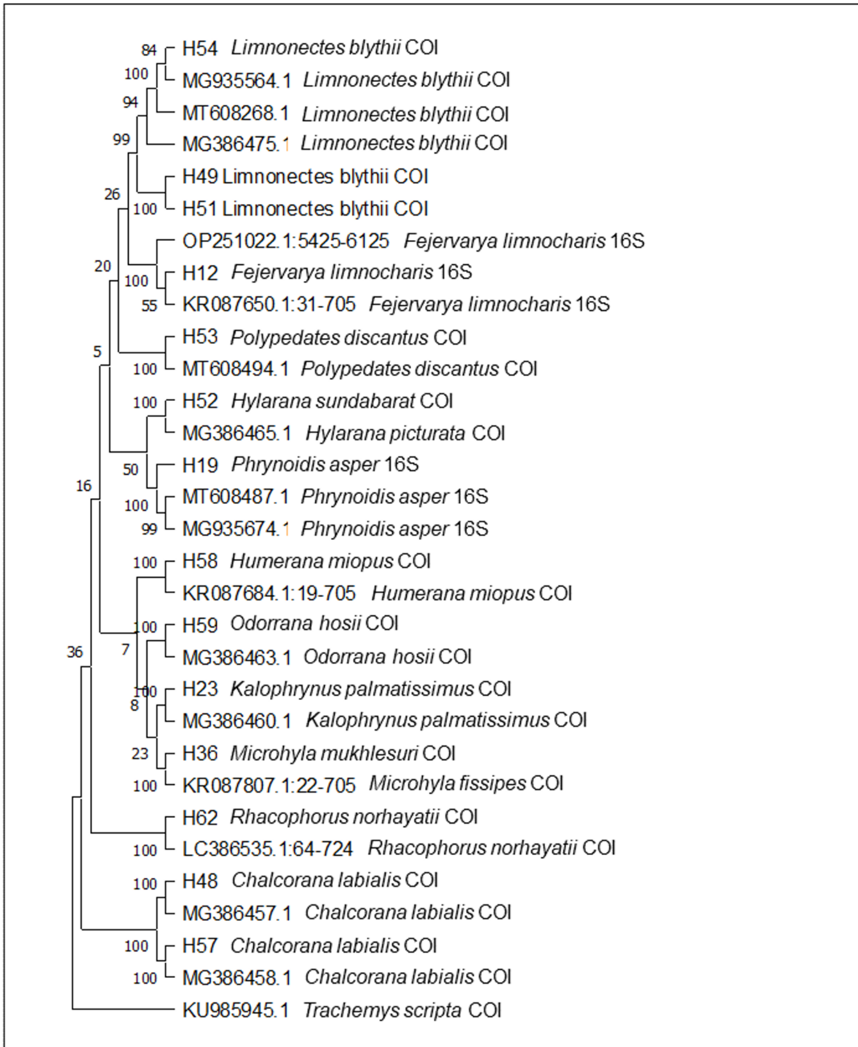

Fig. 2. The Neighbor-Joining Tree for COI gene.

These efficacy of molecular approaches within the amphibians has been a subject of discussion in numerous manuscripts over the last decades [15, 47, 48]. The DNA barcoding marker, COI gene, as initially proposed by Hebert et al. [7], exhibits a notable level of polymorphism among amphibians, and the successful use of this marker for species identification makes it necessary to use a combination of various primers to ensure accurate amplification across all species. However, the application of the COI gene remains highly questioned, particularly concerning its suitability for identification application. This is probably due to the complexity from the extensive polymorphisms observed in the commonly used COI primer used sites [49]. Challenges could also be observed when attempting to assess population-level diversity among amphibians. Comparisons of mitochondrial and nuclear datasets often yield inconsistent results, and events of occasional hybridization and introgression are known to influence amphibian species frequently and their population substructures [49]. Therefore, it becomes evident that species delimitation among amphibians is not an easy task, especially when relying solely on the COI barcoding approach. Recognizing these challenges, it is widely acknowledged that alternative markers, such as

16S rRNA, should be considered for amphibians. These species are characterized by their relatively ancient lineage and a high degree of genetic mutations [50].

Despite recent efforts to develop more efficient COI primers, a universally accepted method for acquired consistent COI sequences or dependable identification outcomes for amphibians remains elusive. With the declination and threats faced by amphibian species, the window opportunity for establishing a comprehensive COI reference library is limited. Without such a reference database, the utility of COI for accurately identifying unknown specimens is compromised, leading to an increase in time, cost, and resources. This goes against the fundamental idea of rapidly identifying species using DNA barcoding concept. Nevertheless, with the ease of PCR sequencing, the existing reference database, and the historically high success rates for identification, 16S emerges as a more suitable barcoding gene for amphibians. Although the 16S gene has been shown to be more effective than the universal COI barcoding gene for estimation of phylogenetic relationships in amphibians, its advantage over COI in non-phylogenetic inferences like identification of species and delimitation is still uncertain. While a study demonstrated that COI performed better than 16S in identifying species of hynobiid salamanders [43], another study found that 16S did not offer a clear upper hand over COI for the herpetofauna of Korea [44].

The comparison between the 16S and COI gene in identifying amphibian species sheds light on important considerations in molecular taxonomy and phylogenetics, addressing a critical research gap. Multiple studies consistently show that the 16S rRNA gene outperforms COI in this context [16, 49-52]. The significance of 16S gene lies in its evolutionary conservation and utility in determining the evolutionary relationships among different groups of organisms. In amphibians, where molecular evolution rates can vary widely, the 16S gene serves as a more dependable marker for identifying species [16]. In contrast, the COI gene, often utilized in DNA barcoding projects, faces challenges in amphibians due to variations within species and incomplete lineage sorting. This can lead to uncertainties in species delimitation and hinder accurate identification [18]. By favouring the 16S gene, researchers can address crucial gaps in amphibian taxonomy and systematics. Its slower evolutionary rate enables deeper insights into phylogenetic relationships, aiding in both species classification and understanding broader evolutionary patterns within amphibian taxa [18]. Moreover, emphasizing the efficacy of the 16S gene showed the need to reevaluate the suitability of molecular markers for specific taxonomic groups. In doing so, researchers can bridge existing knowledge gaps and enhance the accuracy of amphibian species identification and classification [16].

4 Conclusion

In conclusion, the comparative assessment of genetic divergences between the COI and 16S genes highlights the critical significance of marker selection in molecular studies. The primary objective of DNA barcoding is to offer a reliable, precise, and practical way to identify species, and any potential gene marker must meet these essential criteria. Considering the attributes that a barcoding gene should possess, our analysis indicates that the 16S marker outperforms the COI marker, particularly concerning the ease of obtaining sequences. Additionally, Genbank contains more 16S gene sequences for Malaysian amphibians, resulting in higher success rates in BLAST searches. Hence, the application of DNA barcoding data through standardized markers should progress swiftly for practical purposes, especially considering the growing need for identification of species in the management of amphibian's conservation.

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