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Full Paper

Morphology and phylogenetic analysis of *Bucephalandra* from Borneo Island based on matK, ITS and rbcL

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Abstract: *Bucephalandra* is a genus of endemic plants in Borneo. It is highly valued in the aquascape community because of its significant economic potential. This study aims to identify the species type of six *Bucephalandra* sp. accessions from Borneo through morphological characterisation, and evaluate their genetic diversity using maturase K (matK), internal transcribed spacer (ITS) and ribulose-biphosphate carboxylase/oxygenase large subunit (rbcL) markers. The observed characters include the morphology of the leaf, stem and root. Phylogenetic trees were constructed using the unweighted pair group method with arithmetic mean based on each matrix of the respective marker and on the concatenated matrix of all markers. The characteristics of the leaves, stems and roots vary among the six accessions. All accessions exhibit similar leaf colours, except for *B. diabolica*, which has reddish leaves. The accessions are identified as *B. goliath*, *B. forcipula*, *B. diabolica*, *B. kishii*, *B. pubes* and *B. minotaur*. Based on respective ITS and matK sequences, the tree clusters all *Bucephalandra* species into three groups. The tree, based on concatenated sequences, not only clusters them into three groups but also differentiates *B. motleyana* (as reference accession) from other *Bucephalandra* species. The concatenated data set has a haplotype diversity of 1 and a high level of Pi of 0.10917. It indicates that using matK, ITS and rbcL in combination is an optimal and efficient method for differentiating and clustering the *Bucephalandra* species.

Keywords: morphology analysis, phylogenetic analysis, aquatic plant, *Bucephalandra* species, ITS, matK, rbcL

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INTRODUCTION

One of the most popular endemic plant species in Borneo (Kalimantan) is *Bucephalandra* sp. It has unique morphological features. Each species displays distinct colours, leaf shapes and leaf edges that are both attractive and eye-catching. As an aquatic plant, *Bucephalandra* sp. is highly favoured by aquascape enthusiasts at the local, national and international levels. It is used as an elegant decorative element with high value in the market and becomes a focal point for the aquascaping community.

Bucephalandra sp. belongs to the Araceae family. Its natural habitats are on the river beds. It also attaches to rocks, clings to submerged tree roots or grows in areas exposed to water in the tropical rainforests of Borneo Island. It is robust, slow-growing and has a fascinating appearance. Currently, nearly 32 species of *Bucephalandra* originating from Borneo are known [1]. In large-scale trading, the commercial names of *Bucephalandra* sp. are often based on morphological characteristics, leading to uncertainty in species identification [2]. Over the past decade, DNA molecular research has played a significant role in plant taxonomic discoveries, for example using the ribulose-biphosphate carboxylase/oxygenase large subunit (rbcL) and maturase K (matK) in *Lemna* sp. and *Spirodela* sp. [3]. It is also beneficial for morphological investigations, e.g. using internal transcribed spacer (ITS) and matK in *Cryptocoryne* sp. [4], and performing species classification based on similarities and differences in morphological traits in *Amorphophallus* sp. using rbcL, matK and ITS2 [5, 6]. However, DNA molecular studies on *Bucephalandra* sp. remain limited.

DNA analysis can provide insights into the molecular evolutionary processes of species evolution, traits, biogeography and other comparative biological investigations [7]. In this study the diversity of *Bucephalandra* sp. accessions from Borneo Island is analysed using DNA markers, i.e. matK, ITS and rbcL, with *Bucephalandra motleyana*, the first discovered *Bucephalandra* species, serving as a reference sequence, and *Lemna minor* as an outgroup sample [8]. A multi-gene approach combining molecular markers is also utilised to obtain more comprehensive results in the phylogenetic study of the plant species [9]. Molecular markers are beneficial in taxonomic differentiation during plant or gene identification, as they help distinguish between species or organisms. The matK, ITS and rbcL markers can also be employed for DNA barcoding, e.g *Colocasia* sp., using rbcL, matK and ITS [10]. The use of molecular markers in the last decade has significantly facilitated the differentiation of plant species. This research aims to identify species names and reveal the genetic diversity of six *Bucephalandra* sp. accessions from Borneo through morphological characterisation and molecular analysis using matK, ITS and rbcL markers.

MATERIALS AND METHODS

Plant Collection

Six aquatic plants were collected from their natural habitats on Borneo Island, Indonesia, in 2022. Based on their morphological characteristics, these plants were identified as endemic *Bucephalandra* species, namely *B. goliath*, *B. forcipula*, *B. diabolica*, *B. kishii*, *B. pubes* and *B. minotaur*. Details of the sampling locations are provided in Table 1 and Figure 1. Fresh samples were used for both morphological analysis and DNA extraction.

Plant Morphology

All dry and clean plant samples were documented. The morphology of the plant samples was characterised using reference books, scientific journals and identification keys to confirm the

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species names and determine their characteristic traits. The lengths and sizes of plant organs (leaves, stems and roots) were measured digitally using ImageJ software.

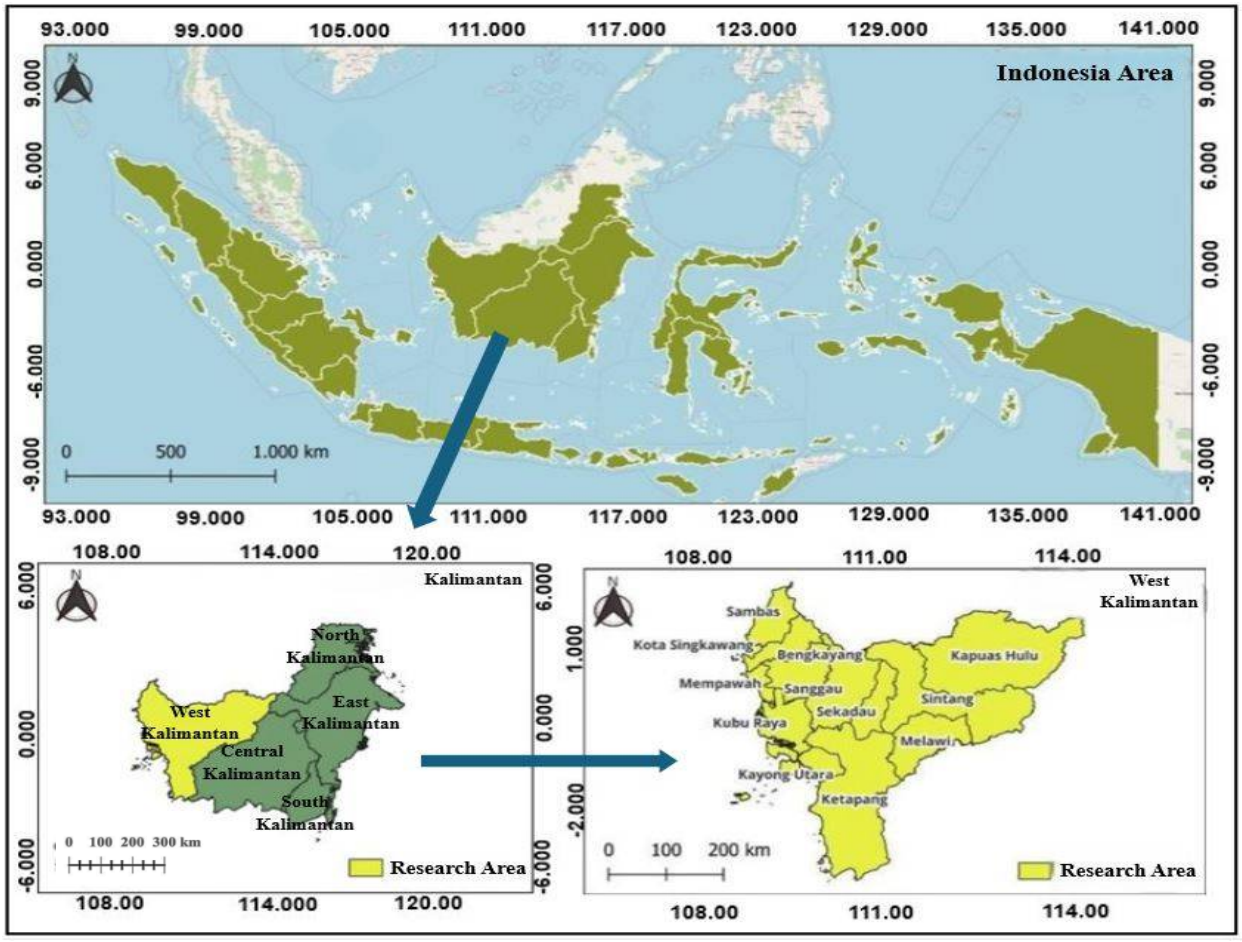

Figure 1. Endemic *Bucephalandra* sp. accessions from West Kalimantan in Borneo Island, Indonesia [11]

Table 1. *Bucephalandra* species name and habitat location in West Kalimantan

No.	Species name	Location
1.	*Bucephalandra goliath*	Latitude 0° 34’ 31.90” S, Longitude 111° 56’ 30.80” E.
2.	*Bucephalandra forcipula*	Latitude 0° 27’ 35.40” S, Longitude 111° 2’ 3.20” E.
3.	*Bucephalandra diabolica*	Latitude 0° 24’ 45.20” S, Longitude 111° 59’ 43.90” E.
4.	*Bucephalandra kishii*	Latitude 0° 25’ 24” S, Longitude 111° 17’ 42” E.
5.	*Bucephalandra pubes*	Latitude 0° 16’ 6.60” S, Longitude 111° 08’ 43.91” E.
6.	*Bucephalandra minotaur*	Latitude 0° 58’ 21.80” S Longitude 111° 30’ 54.40” E.

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DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA was extracted from each *Bucephalandra* sp. sample using the Wizard Genomic DNA Purification Kit (Promega A1120, USA). The quality and quantity of the extracted DNA were assessed using 1% gel electrophoresis and a NanoDrop One Spectrophotometer (Thermo Scientific, USA). DNA samples were then amplified using matK, ITS and rbcL primers in a 20-µl reaction volume on a T100 Thermal Cycler (Bio-Rad, USA).

The matK primer sequences are matK-390-Forward-CGA TCT ATT CAT TCA ATA TTT C (22 bases) and matK-1326-Reverse-TCT AGC ACA CGA AAG TCG AAG T (22 bases). The ITS primer sequences are AB101-Forward-ACG AAT TCA TGG TCC GGT GAA GTG TTC G (28 bases) and AB102 -Reverse- TAG AAT TCC CCG GTT CGC TCG CCG TTA C (28 bases). The rbcL primer sequences are rbcLa-Forward- ATG TCA CCA CAA ACA GAG ACT AAA GC (26 bases) and rbcLa-Reverse- GTA AAA TCA AGT CCA CCR CG (20 bases). The polymerase chain reaction (PCR) mixture and cycling instructions followed those in the MyTaq Master Mix manual (Bioline, USA). The PCR conditions used in this study included one pre-denaturation cycle at 95°C for 4 min., followed by 34 cycles of denaturation at 95°C for 30 sec., annealing at 55°C for 30 sec., extension at 72°C for 1 min. and one post-extension cycle at 72°C for 10 min. PCR products were separated on a 1% agarose gel in a 0.5x tris-borate-ethylenediaminetetraacetic acid buffer and visualised with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea) using the FireReader V10 GelDoc System (Uvitec, UK). All PCR products were sequenced at First BASE Laboratories, Taman Serdang Perdana, 43300 Seri Kembangan, Selangor, Malaysia.

Phylogenetic Analysis

Multiple sequence alignment, pairwise genetic distance and phylogenetic analysis were performed on the complete data set from all markers using Mega 11 software [9, 12]. The pairwise genetic distance was estimated using the Kimura 2-parameter model. The phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean [13] based on each matrix of the respective marker and the concatenated matrix of all markers. The method assumes the validity of the molecular clock. All trees were examined using bootstrap methods with 1000 replications [14]. DNA polymorphism characterisation of multiple aligned sequences was analysed using DnaSP version 5 software. The following parameters were calculated, namely haplotype number, variable sites per sequence, parsimony sites, haplotype diversity and nucleotide diversity [15].

RESULTS AND DISCUSSION

Morphological Characterisation

The morphological characterisation of six *Bucephalandra* accessions are performed based on Wong and Boyce [16], who accomplished the detailed characterisation of 19 new species of *Bucephalandra* from Borneo. The shape of leaf tips of six samples varies from spiny (*B. goliath*) to pointed (*B. pubes and B. minotaur*). The leaf edges of those species are flat (*B. goliath* and *B. diabolica*) and wavy (*B. forcipula*, *B. kishii*, *B. pubes* and *B. minotaur*). Their leaf surfaces are generally smooth (*B. goliath*, *B. forcipula* and *B. minotaur*), except for some species with glabrous surfaces (*B. diabolica* and *B. kishii*). The leaf bases vary from blunt (*B. forcipula* and *B. diabolica)* to pointed (*B. pubes* and *B. minotaur*), while leaf shapes range from linear (*B. goliath* and *B. forcipula)* to oblanceolate (*B. pubes* and *B. minotaur*), with curved leaf veins (*B. goliath, B.*

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forcipula, B. kishii and *B. minotaur*) to parallel veins (*B. diabolica* and *B. pubes*) (Figure 2). All identified species have similar leaf colours except *B. diabolica*, which has reddish leaves.

Bucephalandra sp. stem is round and varies in colour, i.e. brown, light green, dark green and reddish-brown. These variations in stem pigmentation might indicate plant adaptations to specific environments. Nodes and lignin are present in all species, indicating that structural properties are required for stem strength and stability (Figure 2). Root colours vary among species, i.e. white, greenish-white, light brown and yellowish-brown (Figure 3).

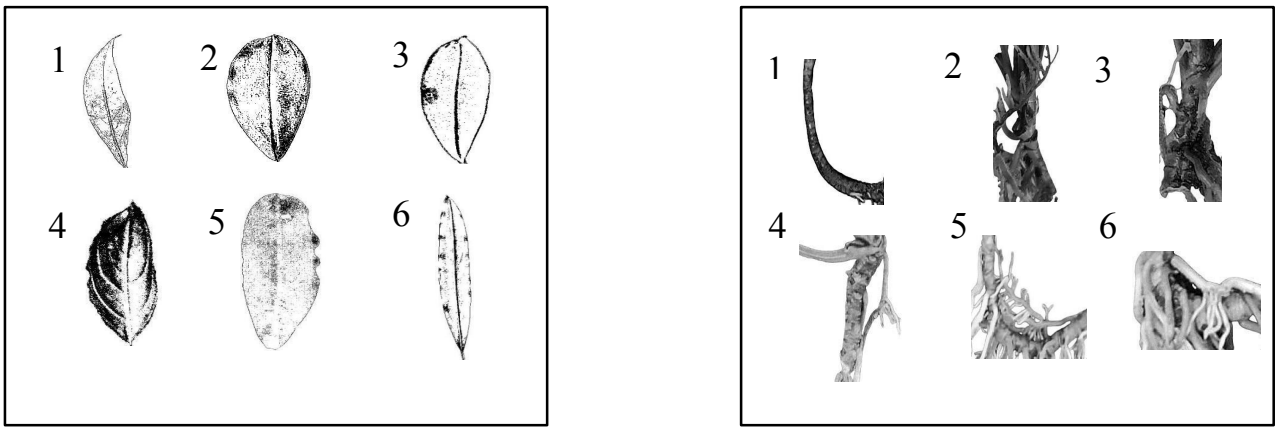

Figure 2. Morphology of leaf and stem of six accessions of *Bucephalandra* sp.: *B. goliath* (1)*, B. forcipula* (2), *B. diabolica* (3), *B. kishii* (4)*, B. pubes* (5) and *B. minotaur* (6)

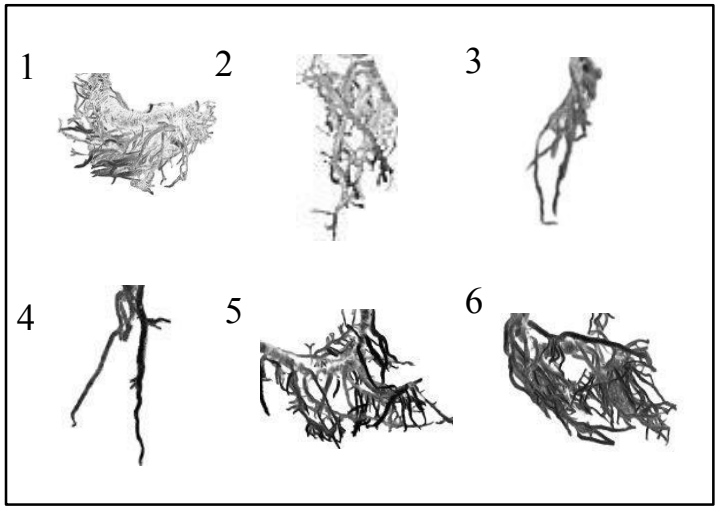

Figure 3. Roots of six accessions of *Bucephalandra* sp.: *B. goliath* (1)*, B. forcipula* (2), *B. diabolica* (3), *B. kishii* (4)*, B. pubes* (5) and *B. minotaur* (6)

The leaf and stem characteristics in Table 2 show that *B. diabolica* has the smallest leaf and stem sizes while *B. goliath* has the largest sizes. *B. forcipula* has the shortest root while *B. minotaur* has the longest one. This result slightly differs from the findings of Wong and Boyce [16], which might be due to some samples being collected at different periods. Thus each sample exists in specific conditions of the environment and plant populations that influence its morphology. Based on the morphology data, we identify six accessions of *Bucephalandra* sp. and conclude that they are namely *B. goliath*, *B. forcipula*, *B. diabolica*, *B. kishii*, *B. pubes* and *B. minotaur*.

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Molecular and Phylogenetic Analysis

The sequences obtained from the amplification of matK, ITS and rbcL loci are used individually and collectively to construct phylogenetic trees using the unweighted pair group method with arithmetic mean (Figure 4). The sequences of *Lemna minor,* an outgroup species, are obtained from GenBank with accession numbers: OR464916.1 (matK), KJ400887.1 (ITS) and GQ436374.1 (rbcL). Based on the estimated pairwise genetic distance matrix, the phylogenetic tree is constructed with the closest species.

Table 2. Results of morphometric identification value of *Bucephalandra* sp.

Parameter	*B. goliath*	*B. forcipula*	*B. diabolica*	*B. kishii*	*B. pubes*	*B. minotaur*
Leaf length (cm)	9.28	7.64	2.59	3.6	5.20	7.37
Leaf width (cm)	1.71	2.27	0.69	1.8	1.18	1.40
Stem diameter (cm)	0.84	0.45	0.39	0.57	0.45	0.84
Stem length (cm)	15.32	2.90	1.91	3.16	6.49	3.05
Internode distance (cm)	0.82	0.42	0.19	0.40	0.63	0.27
Root diameter (cm)	0.16	0.09	0.11	0.13	0.15	0.21
Root length (cm)	2.47	1.78	2.49	3.08	2.95	4.47

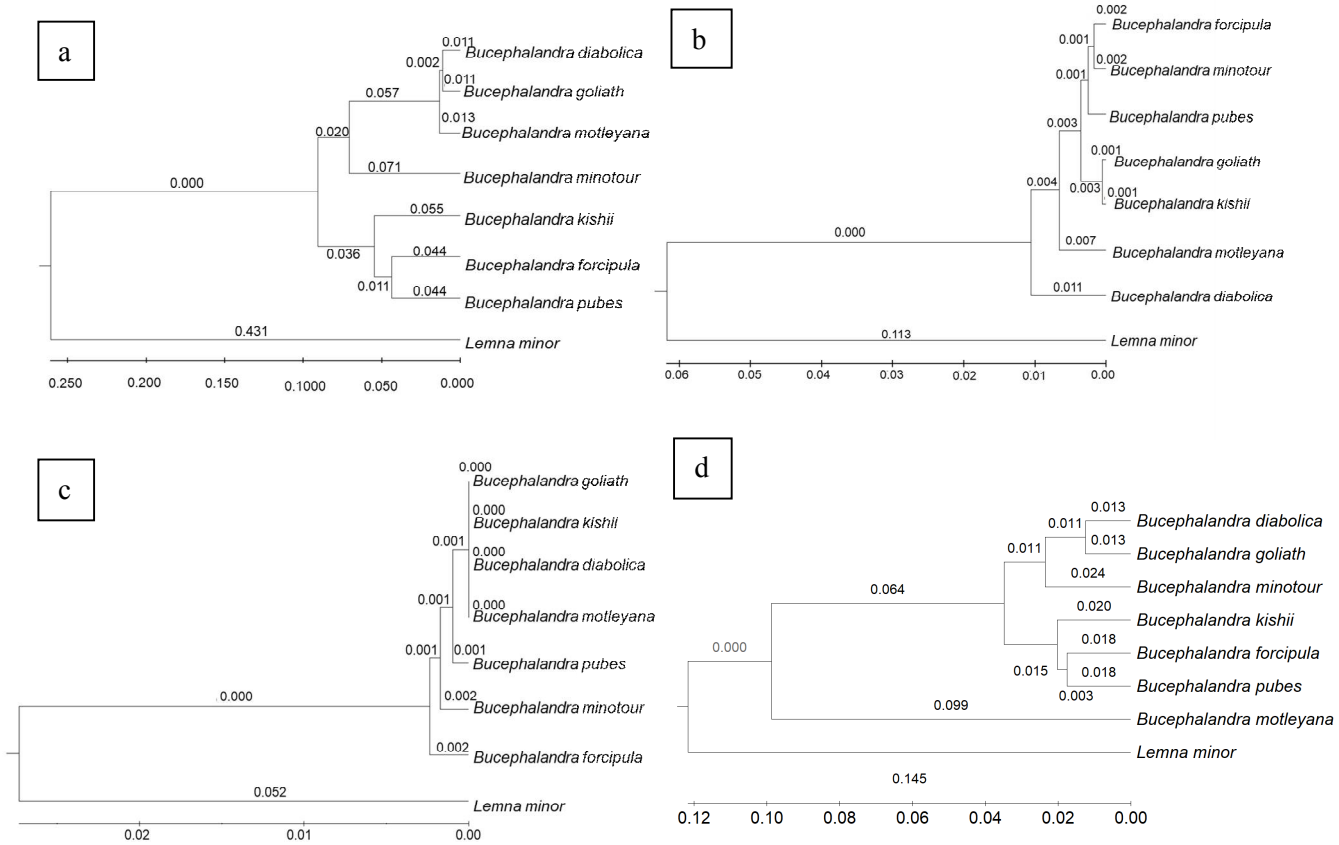

Figure 4. Phylogeny tree based on matK (a), ITS (b), rbcL (c) and concatenated (matK, ITS and rbcL) (d) sequences

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Phylogenetic analysis using respective and concatenated markers reveals distinct phylogenetic trees. Among the four phylogenetic trees, those constructed using matK (Figure 4a), ITS (Figure 4b) and concatenated sequences (Figure 4d) clearly differentiate the six *Bucephalandra* sp. into three groups. The three groups are a result of the matK sequence differing from the ITS sequence. The matK sequence generates Group 1 (*B. forcipula*, *B. minotaur* and *B. pubes*), Group 2 (*B. goliath* and *B. kishii*) and Group 3 (only *B. diabolica*) (Figure 4a), while the ITS sequence generates Group 1 (*B. diabolica*, *B. goliath* and *B. motleyana*), Group 2 (*B. minotaur*) and Group 3 (*B. kishii*, *B. forcipula* and *B. pubes*) (Figure 4b). Interestingly, the tree from concatenated sequences successfully differentiates six species of *Bucephalandra* from *B. motleyana*. The six species of *Bucephalandra* are divided into three groups, i.e. Group 1 (*B. diabolica*, *B. goliath* and *B. minotaur*), Group 2 (*B. kishii*, *B. forcipule* and *B. pubes*) and Group 3 (*B. motleyana*). In contrast, the phylogenetic tree by the rbcL marker consists of only one large group (Figure 4c).

However, there is a slight difference in the genetic distance values of the respective and concatenated markers. The genetic distance values of the matK marker range from 0.001 to 0.011 (Figure 4a). while those of the ITS marker range from 0.002 to 0.027 (Figure 4b). The concatenated markers have the highest genetic distance values, ranging from 0.2173 to 0.0251 (Table 3), which indicates that they are more powerful in detecting differences between species. The rbcL marker produces the lowest genetic distance values ranging from 0.000 to 0.002.

Table 3. Matrix pairwise genetic distance based on concatenated sequence (matK, ITS and rbcL) markers between *Bucephalandra* species

	1	2	3	4	5	6	7	8
1	0.000							
2	0.1603	0.000						
3	0.2149	0.0572	0.000					
4	0.1743	0.0251	0.0628	0.000				
5	0.2173	0.0617	0.0401	0.0609	0.000			
6	0.2064	0.0558	0.0926	0.0384	0.0893	0.000		
7	0.2110	0.0567	0.0351	0.0597	0.0411	0.0858	0.000	
8	0.3715	0.2046	0.2334	0.2016	0.2345	0.2240	0.2338	0.000

Note: 1) *B. motleyana*, 2) *B. diabolica*, 3) *B. forcipula*, 4) *B. goliath*, 5) *B. kishii*, 6) *B.minotaur*, 7) *B. pubes* and 8) *Lemna minor*

Our study shows that the concatenated markers (rbcL, ITS and matK) are optimal and efficient in differentiating and clustering the *Bucephalandra* family based on molecular genetic differences. These findings hold promising implication for future research as these markers will be beneficial in performing phylogenetic analysis, species classification and DNA barcoding of the abundant *Bucephalandra* genetic resources in Borneo.

Haplotype Diversity

The ITS and concatenated data sets exhibit a haplotype diversity of 1.000 and the number of haplotypes is 8 (Table 4), which confirms that each individual is a unique haplotype. In contrast the matK data set shows slightly lower haplotype diversity (0.964), indicating minor redundancy or genetic similarity between some sequences. The rbcL data set shows the lowest haplotype diversity at 0.786. The ITS and concatenated data sets exhibit the highest nucleotide diversity of 0.16568 and 0.10917 respectively, which highlights greater overall genetic variability at the nucleotide level. The

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matK data set follows with a nucleotide diversity of 0.03369 while the rbcL data set has the lowest value (0.01508), which indicates that the rbcL marker is less effective in detecting phylogenetic differences.

Table 4. Comparison result of DNA sequence polymorphism version 5.0 for each parameter

Parameter	matK sequence	ITS sequence	rbcL sequence	Concatenated sequences
Number of haplotypes	7	8	5	8
Number of variable sites	99	240	23	656
Parsimony informative sites	6	57	3	107
Haplotype diversity	0.964	1.000	0.786	1.000
Nucleotide diversity	0.03369	0.16568	0.01508	0.10917

The number of haplotypes generated by each marker is different. The ITS and concatenated markers deliver eight haplotypes, indicating that the markers can identify the high diversity of samples. On the other hand, matK marker gains seven haplotypes, indicating relatively high diversity. However, the rbcL marker only has five haplotypes, which means it recognises lower genetic diversity than the other markers. The number of sites in the concatenated data set shows the highest number of variable sites (656), followed by the ITS data set (240), matK data set (99) and finally rbcL data set with only 23 variable sites. This shows that using the concatenated markers provides greater variability, thus providing a more comprehensive view of genetic diversity. The parsimony sites of the three markers are significantly different. Those achieved by the concatenated (107) and ITS (57) markers provide more substantial phylogenetic information. In contrast the matK and rbcL markers gain six and three parsimony sites respectively, which shows the limitation of their phylogenetic resolution. In the genetic analysis of *Bucephalandra* sp. the matK and rbcL markers should be used in conjunction with other markers to provide better capture of the genetic diversity.

CONCLUSIONS

For the morphologically identified six accessions of *Bucephalandra* sp., namely *B. goliath*, *B. forcipula*, *B. diabolica*, *B. kishii*, *B. pubes* and *B. minotaur*, phylogenetic analysis using the concatenated markers (matK, ITS and rbcL) generates three groups with the highest genetic distance values, ranging from 0.2173 to 0.0251. This follows the results of DNA polymorphism analysis. The concatenated data set has the highest values of haplotype diversity, number of variable sites and number of informative parsimony sites. Thus, it is the most suitable data set for detailed *Bucephalandra* sp. phylogenetic analysis.

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