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MOLECULAR PHYLOGENY STUDY OF *SCHISMATOGLOTTIS* FROM DIFFERENT REGIONS IN SABAH USING INTERNAL TRANSCRIBED SPACER REGION

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ABSTRACT. *The Schismatoglottideae tribe, primarily in Borneo, is notably diverse within the Araceae family. It encompasses various rainforest herbs, adapting to terrestrial, lithophytic, and rheophytic habitats. The Schismatoglottis genus, with over 175 species, mostly exclusive to Borneo due to geological preferences, stands out. A study aimed to understand genetic relationships among Schismatoglottis species from Kadamaian-Kinabalu Park, Kionsom Recreational Area, Imbak Canyon Conservation Area, Mensalong Forest Reserve, Danum Valley Conservation Area, Tawau Hills Park, and Mantailang Crocker Range Park. The Internal Transcribed Spacer (ITS) regions of twelve Schismatoglottis species were sequenced. Genomic DNA was extracted from dried-silica-gel leaf tissue using a commercial kit (Promega, USA). ITS region lengths ranged from 528 bp to 708 bp. BLAST analysis accurately identified species within the Schismatoglottis genus (95% to 98% similarity). Phylogenetic analysis revealed a highly supported sister clade, uniting Schismatoglottis porpax species from Mensalong and Kionsom, despite differing elevational habitats. This pioneering study delves into Schismatoglottis phylogenetics, particularly in Sabah, with seven of the twelve species showing potential for new species classification, pending further research.*

KEYWORDS. Araceae, *Schismatoglottis*, phylogenetic analysis, Malaysia, Sabah.

INTRODUCTION

The Araceae family consists of about seven subfamilies, 32 tribes, and 144 genera with 6,000 estimated species (Mayo *et al.*, 1997). Currently, 4,000 species of Araceae have been formally described and updated in an ongoing list (Boyce & Croat, 2011). The family is defined by having minute sessile either unisexual or bisexual flowers located on the spadix and covered by a modified leaf called a spathe.

The tribe Schismatoglottideae Nakai has more than 120 species, more than 95% of which are endemic, making it the most varied and speciose aroid taxon in Borneo (Low *et al.*, 2018). The tribe includes *Schismatoglottis* Zoll. & Moritz, the biggest genus, in addition, includes four minor genera, or "satellites": *Bucephalandra* Schott, *Aridarum* Ridl, *Piptospatha* N.E. Br. and *Phymatarum* M. Hotta. Although the Schismatoglottideae tribe is prevalent in southeast Asia, it poses many challenges to the current definition of the genus boundaries.

Genus *Schismatoglottis* (included within the Schismatoglottideae tribe) has more than 175 species with a majority endemicity in Borneo with strictly geological obligated (Kartini *et al.*, 2017; Kartini *et al.*, 2020; Low *et al.*, 2018; Wong *et al.*, 2010). Fewer research was done based on aroids especially the genus of *Schismatoglottis* in Sabah resulting in poor understanding of the taxonomy and phylogeny.

The Araceae of Borneo currently stands at 670 species, of which 40% from them is new species and have not been formally described (Wong, 2016). In Sabah, field trips that were conducted often resulted in numerous undeterminable species of aroids which on subsequent flowering in cultivation have proved to be taxonomic novelties (Kartini *et al.*, 2017). In previous molecular phylogenetic studies, mainly examining the Araceae family as a whole, only two or three taxa from the Schismatoglottideae were included. The molecular study indicated that the Schismatoglottideae are not monophyletic (Barabé *et al.*, 2004).

The rapid advancements in molecular techniques, as demonstrated by the polymerase chain reaction (PCR), have had a profound impact on the

application of DNA sequences in molecular phylogenetic studies (Topi & Adi, 2010). DNA-based molecular markers provide a more efficient and reliable method for characterizing germplasm and conducting phylogenetic analysis. An additional advantage of these markers lies in their ability to withstand various environmental variables (Barnajee *et al*., 2016).

The chosen molecular markers must be conservative to facilitate PCR amplification and alignment of sequences among distant species, but they must alsobe sufficiently variable to permit the identification of the nearest species. Internal

Transcribed Spacer (ITS) DNA markers are the potential universal marker for plants and animals (Chen *et al.*, 2010). It contains enough variation to identify a wider range of plant taxa and even distinguish between closely related species (Yao *et al.*, 2010), and can be used in conjunction with morphological features analysis (Zhang *et al.*, 2015).

To the best of our knowledge, few molecular investigations on the evolutionary relationships of the tribe Schismatoglottideae were recently investigated utilizing a variety of sequences in Borneo (Ting *et al.*, 2012; Low *et al.*, 2011; Wong *et al.*, 2010; Wong & Boyce, 2007). To date, the vast diversity of *Schismatoglottis* species in Sabah has not been adequately researched, particularly in terms of taxonomy and biosystematics, genetics, and molecular biology. Therefore, this research was done to investigate the evolutionary relationships and provide a basic analysis of a short fragment of the ITS region.

MATERIALS AND METHODS

Plant Materials

In this study, a total of 12 *Schismatoglottis* species were collected from seven different localities in Sabah, Malaysia as presented in Figure 1 and Table 1.

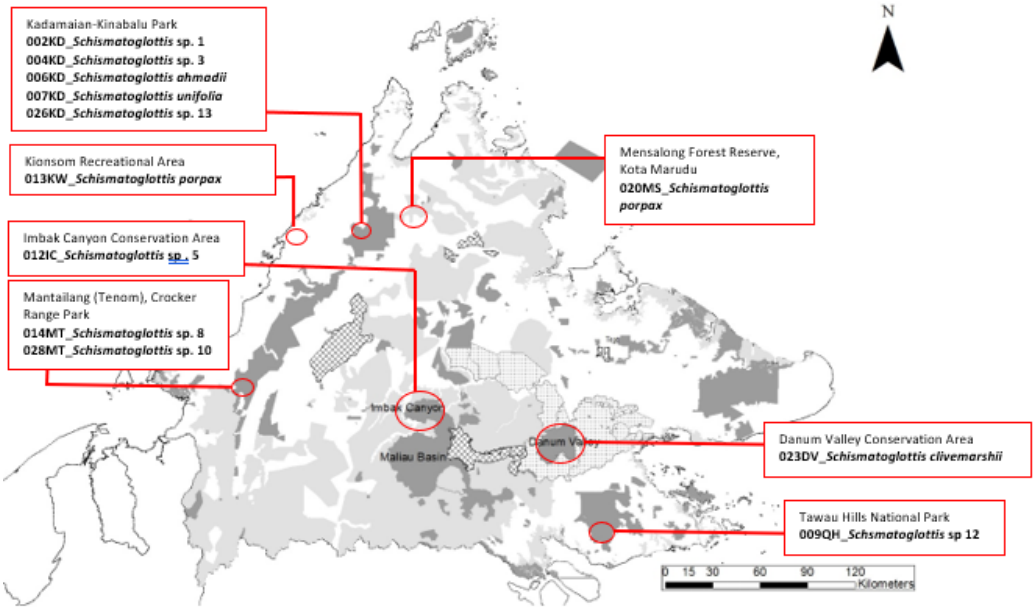

Figure 1. The location of the sampling area.

Table 1: Tabulation of sampling locations and their elevation

No.	Location	Abbreviation	GPS coordinate	Elevation (m a.s.l)
1	*Kadamaian-Kinabalu Park (Kota Belud)*	*K-KP*	*6.105º N, 116.303º E*	*500-1400*
2	*Kionsom Recreational Area*	*KRA*	*5.973° N, 116.206° E*	*200-300*
3	*Imbak Canyon Conservation Area*	*ICCA*	*5.936° N, 116.627° E*	*150-1500*
4	*Mensalong Forest Reserve (Kota Marudu)*	*MFR*	*6.170° N, 116.450º E*	*250-1100*
5	*Danum Valley Conservation Area*	*DVCA*	*4.961° N, 117.689° E*	*500-1100*
6	*Tawau Hills Park*	*THP*	*4.503° N, 117.935° E*	*400-900*
7	*Mantailang (Tenom), Crocker Range Park*	*MCRP*	*5.286° N, 115.978° E*	*500*

Identification of the species is referred to previous references such as Mayo *et al.*, 1997 and Hay & Yuzammi, 2000. In addition, photographs and morphological descriptions of these species were also sent to one of the Araceae experts, Associate Professor Madya Dr. Kartini Saibeh for validation of the species (Table 2). Each species was cut approximately 10 cm with a sterile blade and stored in a tea bag filled with 2 g silica gel (Sigma Aldrich, Germany) to reduce humidity. Once arrived at the laboratory, the samples were then stored at -80 ^{o}C before further use.

Table 2. List of twelve samples collected from different localities

No .	Samples ID	Locality
1	002KD_*Schismatoglottis* sp. 1	Kadamaian
2	004KD_*Schismatoglottis* sp. 3	Kadamaian
3	006KD_*Schismatoglottis ahmadii*	Kadamaian
4	007KD_*Schismatoglotttis unifolia*	Kadamaian
5	026KD_*Schismatoglottis* sp. 13	Kadamaian
6	012IC_*Schismatoglottis* sp. 5	Imbak Canyon
7	009QH_*Schismatoglottis* sp. 12	Imbak Canyon
8	013KW_*Schismatoglottis porpax*	Kionsom
9	020MS_*Schismatoglottis porpax*	Mensalong
10	014MT_*Schismatoglottis* sp. 8	Mentailang
11	028MT_*Schismatoglottis* sp. 10	Mentailang
12	023DV_*Schismatoglottis clivemarshii*	Danum valley

Total genomic extraction

Total genomic DNA was extracted from silica-gel-dried leaf tissue by using the Wizard® Genomic DNA extraction kit (Promega, USA) following the manufacturer's instructions. The quality of DNA was measured using a NanoDrop^{TM} One Microvolume UV-Vis Spectrophotometer, (Thermo Fisher Scientific, USA). The extracted DNA was labeled and stored at -20^{o}C until further use.

PCR amplification and sequence analysis

The PCR fragments were amplified using primer pairs of the Internal Transcribe Spacer (ITS) regions 1 (5' TCCGTAGGTGAACCTGCGG 3') and 4 (5' GCTGCGTTCATCGATGC 3') (White *et al.*, 1990). All PCR reactions were carried out in a 20 µl volume containing 1× GoTaq Flexi PCR buffer (Promega, USA), 3.0 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 2.0 units of Taq DNA polymerase, GoTaq G2 Flexi (Promega, USA), 0.2 mg/ml of BSA (bovine serum albumin, New England Biolabs), 1.5 µl of DNA template (100 ng/µl to 200 ng/µl) and sterile double deionized water.

The expected amplicon size for the Internal Transcribed Spacer is 500-1000 base pairs (bp). The amplification of ITS region was carried out using Bio-Rad T100^{TM} thermal cycler with a thermal profile of primary denaturation for 2 minutes at 95^{o}C, followed by 35 cycles of 30 seconds at 95^{o}C, 1 minute for 57^{o}C, 1 minute and 30 seconds at 72^{o}C and followed by final extension of 72^{o}C at 5 minutes. The PCR was amplified based on Wong *et al.*, (2010) with slight modification.

PCR purification and sequencing

PCR products were checked on 1% TBE agarose gel with 1 Kb DNA ladders. The PCR products are stained using GelRed and visualized under Vilber Lourmat UV scanner (UK) to observe the existing band. The positive PCR products were sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for purification and sequencing.

Sequence search and phylogenetic construction

The sequence of twelve samples was searched for homolog sequence using BLAST analysis against the NCBI database (http://www.ncbi.nlm.nih.gov/blast.cgi). The multiple sequence alignments were performed using the ClustalW program followed by visual adjustment and and then edited using Bioedit version 7.2.5 before constructing the phylogenetic tree. The construction of maximum likelihood (ML) was performed using CIPRES (https://www.phylo.org/portal2/login!input.action) which is an online software. The IQ TREE on XSEDE (Nguyen *et al*., 2015)

was chosen to construct the maximum likelihood phylogenetic tree, whereas, Bayesian phylogenetic analyses were performed using CIPRES (https://www.phylo.org/portal2/login!input.action) which is an online tool and the MrByes 3.2.7a on XSEDE (Ronquist *et al.*, 2011) was chosen to construct the bayesian inference phylogenetic tree.

RESULTS AND DISCUSSION

PCR amplification

The sequence alignments obtained from Apical Scientific Sdn. Bhd. (Selangor, Malaysia) were successfully generated a total of 12 DNA sequences of *Schismatoglottis* in this study. Figure 2 shows the successful PCR amplification of *Schismatoglottis*.

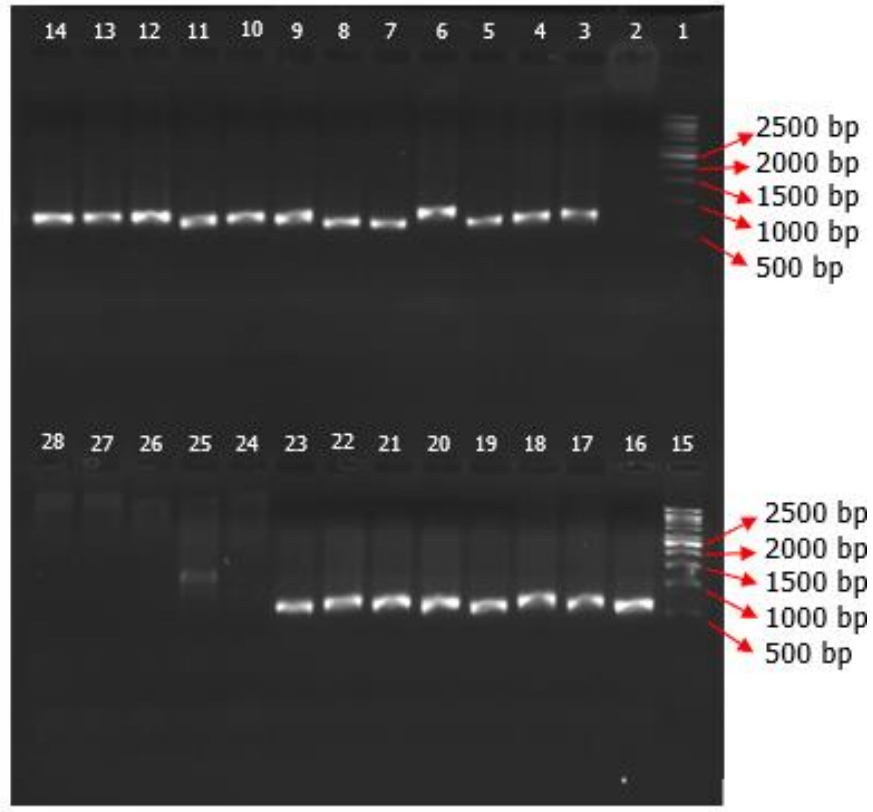

Figure 2. PCR amplification of Schismatoglottis. 1: 1kb ladder, 2: negative control, 3: positive control, 4-5: 002KD_*Schismatoglottis* sp. 1, 6-7: 004KD_*Schismatoglottis* sp. 3, 8-9: 006KD_*Schismatoglottis ahmadii*, 10-11: 007KD_*Schismatoglotttis unifolia* , 12-13: 026KD_*Schismatoglottis* sp. 13. 14-15: 012IC_*Schismatoglottis* sp. 5, 16-17: 009QH_*Schismatoglottis* sp. 12, 18-19: 013KW_*Schismatoglottis porpax*, 20: 020MS_*Schismatoglottis porpax*, 21: 014MT_*Schismatoglottis* sp. 8. 22: 028MT_*Schismatoglottis* sp. 10, 23: 023DV_*Schismatoglottis clivemarshii*, 24-28: unsucessful band.

BLAST analysis

The DNA sequences were verified by comparing them with the sequences of other species by BLAST search in the NCBI. The BLAST analysis was shown in Table 3. The length of the Internal Transcribed Region (ITS) region ranged from 528 bp to 708 (bp).

ITS successfully identified 95% to 98% of specimens, respectively at the species level belonging to the genus *Schismatoglottis*. This information could potentially be used in future studies on the phylogeny of *Schismatoglottis* in Sabah

Table 3. Analysis of BLAST X result by using ITS region.

Species	Accession number	E value	Identity	Query cover
Schismatoglottis calyptrata	KU748782.1	0	98.84	99%
***Schismatoglottis* sp. SLL-2016**	KT732169.1	0	98.84	99%
Schismatoglottis ahmadii	KU748781.1	0	98.41	99%
***Schismatoglottis* sp. AR3956**	KP748510.1	0	95.66	99%
***Schismatoglottis* sp. AR3586**	KP748505.1	0	95.64	99%
***Schismatoglottis* sp. AR4331**	KP748501.1	0	95.64	99%
***Schismatoglottis* sp. AR4270**	KP748512.1	0	95.25	99%
***Schismatoglottis* sp. AR4666**	KP748516.1	0	95.36	99%
***Schismatoglottis* sp. AR4096**	KP748511.1	0	95.36	99%
***Schismatoglottis* sp. AR3679**	KP748509.1	0	95.36	99%
***Schismatoglottis* sp. AR3615**	KP748506.1	0	95.24	99%
***Schismatoglottis* sp. AR2549**	KP748503.1	0	95.36	99%
***Schismatoglottis* sp. AR1632**	KP748498.1	0	95.36	99%
***Schismatoglottis* sp. AR1941**	KP748502.1	0	95.11	99%
***Schismatoglottis* sp. AR382**	KP748496.1	0	95.07	99%
***Schismatoglottis* sp. AR1240**	KP748497.1	0	94.95	99%
Schismatoglottis ranchanensis	KP744728.1	0	94.95	99%
***Schismatoglottis* sp. AR4651**	KP748515.1	0	95.18	98%
***Schismatoglottis* sp. AR2588**	KP748504.1	0	94.93	99%
***Schismatoglottis* sp. AR1638**	KP748500.1	0	95.18	98%

Phylogenetic datasets

One dataset was generated, which comprised 12 ITS regions with 22 Bornean samples of *Schismatoglottis* species (Table 4) from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The outgroup species had relatively relevant sequences, with ITS sequences ranging from 640 bp (*Apoballis mutata*).

Table 4. List of species taken from GenBank with accession number

Name	Locality	GenBank (ITS)	Publication
Schismatoglottis nervosa	Sarawak	JX857111	Low *et al.*, 2018
Schismatoglottis nervosa	Sarawak	JX857125	Low *et al.*, 2018
Schismatoglottis sp. AR-2078	Sarawak	JX857121	Low *et al.*, 2018
Schismatoglottis sp. AR-1105	Sarawak	JX857113	Low *et al.*, 2018
Schismatoglottis sp. AR-1930	Sarawak	JX857119	Low *et al.*, 2018
Schismatoglottis asperata	Sarawak	KP744717	Low *et al.*, 2018
Schismatoglottis asperata	Sarawak	KP744712	Low *et al.*, 2018
Schismatoglottis asperata	Sarawak	KP744727	Low *et al.*, 2018
Schismatoglottis asperata	Sarawak	KP744716	Low *et al.*, 2018
Schismatoglottis asperata	Sarawak	KP744715	Low *et al.*, 2018
Schismatoglottis sp. AR1638	Sarawak	KP748500	Low *et al.*, 2018
Schismatoglottis sp. AR3662	Sarawak	KP748507	Low *et al.*, 2018
Schismatoglottis sp. AR3673	Sarawak	KP748508	Low *et al.*, 2018
Schismatoglottis sp. AR382	Sarawak	KP748496	Low *et al.*, 2018
Schismatoglottis sp. AR3679	Sarawak	KP748509	Low *et al.*, 2018
Schismatoglottis sp. AR4096	Sarawak	KP748511	Low *et al.*, 2018
Schismatoglottis sp. AR4023	Sarawak	KP748499	Low *et al.*, 2018
Schismatoglottis sp. AR4651	Sarawak	KP748515	Low *et al.*, 2018
Schismatoglottis sp. AR3615	Sarawak	KP748506	Low *et al.*, 2018
Schismatoglottis patentinervia	Sarawak	KT732134	Low *et al.*, 2018
Schismatoglottis nervosa	Sarawak	JX857116	Low *et al.*, 2018
Schismatoglottis sp. AR3956	Kalimantan	KP748510	Low *et al.*, 2018
Apoballis mutata AR3595	Sarawak	KM433710	Low *et al.*, 2018

Analysis of phylogenetic tree

A combined analysis based on maximum likelihood and Bayesian inference tree based on nuclear Internal Transcribed Spacer (ITS) region sequence data is shown in Figure 3.

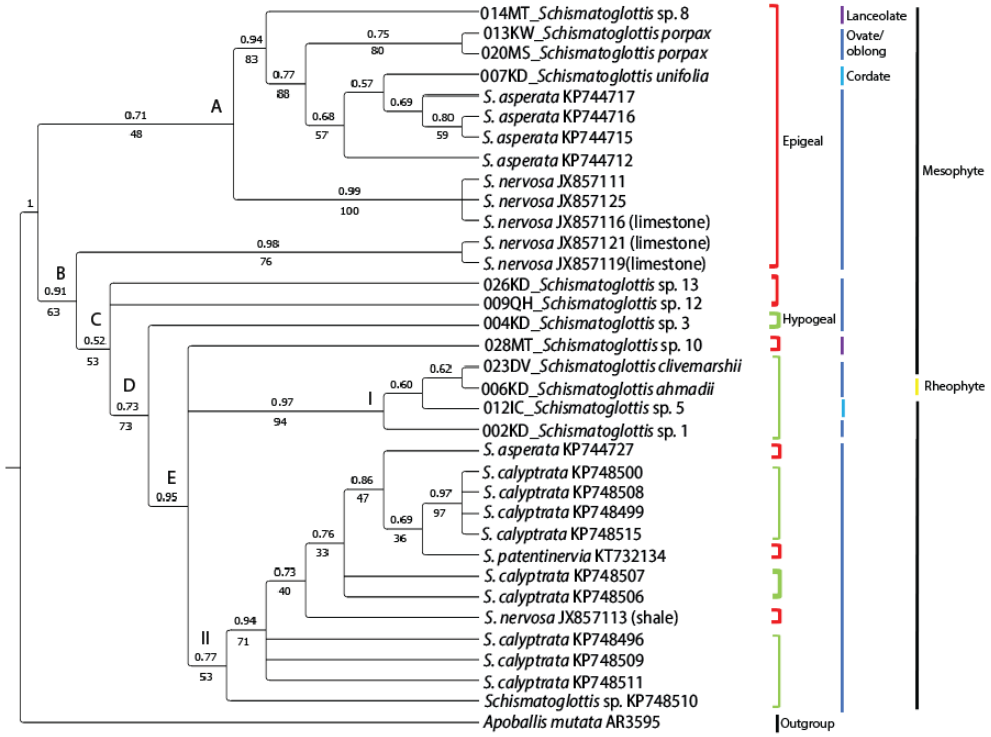

Figure 3. The combined consensus tree results from Maximum Likelihood analysis (lower branch) and optimal tree results from Bayesian analysis (upper branch) generated from the Internal Transcribed Spacer (ITS) region.

Based on the consensus tree, five major clades were formed. Clade A moderately supported with 0.71 posterior probability and 48% bootstrap. *Schismatoglottis porpax* taken from Mensalong Forest Reserve and Kionsom Recreational Area was grouped together with moderately high support value (PP: 0.75, BS: 80%). *Schismatoglottis unifolia* formed a basal root to *Schismatoglottis asperata* (KP744717, KP744716, and KP744715) with a moderately bootstrap support value of 57%. *Schismatoglottis unifolia* is a unique single leaf of the *Schismatoglottis* genus. This species is endemic to Sabah and usually found on steep slopes (Figure 4).

Figure 4. A colony of *Schismatoglottis unifolia* in its habitat at Kadamaian-Kinabalu Park.

In Clade B, *Schismatoglottis nervosa* from the limestone area formed a sister clade with a strongly supported posterior probability of 0.98 and bootstrap of 76%. *S. nervosa* JX857121 and JX857119 belong to clade B, form a separate clade from *S. nervosa* JX857116, which belongs to clade A. These species are thought to have originated in the limestone areas of Sarawak but have not been identified yet.

The presence of aromatic vegetative tissues (terpenoids), longitudinally ribbed petioles, and leaf blades with tessellate tertiary venation led to the placement of these species in the Nervosa Grade.

Schismatoglottis nervosa is an intriguing plant species that exemplifies the complex process of evolutionary divergence among populations. Schismatoglottis nervosa, which has a common ancestor, has endured significant changes over time. The species initially flourished as a single population, but environmental changes led to the separation of the population into two distinct groups. In isolation, these communities may have **Molecular**

accumulated genetic variations through mutations, a hallmark of evolution. Over the course of generations, these genetic differences manifested as unique traits and adaptations in each group, such as variations in leaf morphology and growth patterns. As a result, the two groups progressively evolved into distinct clades within the larger *Schismatoglottis nervosa* species, each with a unique evolutionary history. Phylogenetic analysis, which entails the study of the genetic material of individuals from each clade, reveals the complex branching pattern of their evolution. This analysis provides not only insights into the intriguing history of *Schismatoglottis nervosa*, but also a broader comprehension of how species can diversify in response to their environments.

In Clade C, *Schismatoglottis* sp. 13 from Kadamaian-Kinabalu Park and *Schismatoglottis* sp. 12 form sister taxa with a posterior probability of 0.52 and bootstrap of 53%. Both of these species share similar morphology traits which they have an ovate to oblong shape of leaf (Figure 5).

Figure 5. A) *Schismatoglottis* sp. 12; B) *Schismatoglottis* sp. 13, both of these species have an ovate to oblong shape of leaf (red circles).

For Clade D, this clade is supported with posterior probability of 0.73 and bootstrap of 73%. Moving to Clade E, this clade is divided into two clades which are Clade E-I and Clade E-II. In Clade E-I, *Schismatoglottis* sp. 1 formed basal root to *Schismatoglottis* sp. 5, *Schismatoglottis ahmadii* and

Schismatoglottis clivemarshii with posterior probability of 0.97 and bootstrap of 94%.

Schismatoglottis genus had a lack of identification based on ITS rDNA sequence data however they have a significantly moderate level of nucleotide variation between species that are closely related species.

CONCLUSION

To conclude, the phylogenetic tree of combined analysis for ITS gene in this study produced five clades. For Clade A, there are 11 species were included that is *Schismatoglottis* sp. 8, *Schismatoglottis porpax*, *Schismatoglottis porpax*, *Schismatoglottis unifolia*, *Schismatoglottis asperata* (KP744717, KP744716, KP744715, KP744712) and *Schismatoglottis nervosa* (JX857111, JX857125, JX857116). In Clade B, there are two species included that is *Schismatoglottis nervosa* (JX857121 and JX857119). In Clade C, there are two species also that is *Schismatoglottis* sp. 13 and *Schismatoglottis* sp. 12. Whereas in Clade E, is separated into two subclades: Clade E-I includes four species that is *Schismatoglottis clivemarshii, Schismatoglottis ahmadii, Schismatoglottis* sp.5 and *Schismatoglottis* sp.1. Lastly, in Clade E-II, 13 species included that is *Schismtoglottis asperata* (KP744727), *Schismatoglottis calyptrata* (KP748500, KP748508, KP748999, KP748515, KP748507, KP748506, KP748496, KP748509, KP748511), *Schismatoglottis patentinervia* (KT732134), *Schismatoglottis nervosa* (JX857113) and *Schismatoglottis* sp. (KP748510). The conclusions drawn from the phylogenetic tree lead to the formation of one highly supported sister clade. The sister clades are the *Schismatolgottis porpax* (from Mensalong) and *Schismatolgottis porpax* (from Kionsom). Even though they lived at different elevations, the two species of *Schismatoglottis porpax* were closely related to one another. The results of this study are the pioneers of the phylogenetic study of *Schismatoglottis* focusing on species in Sabah. A total of seven (7) out of 12 species would have a high potential to be described as a new species, however, further confirmation needs to be made in future research.

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