MOLECULAR SYSTEMATICS OF *Cryptocoryne* spp. FROM INDO-MALAYA ARCHIPELAGO

NURUL SHAKINA BINTI MOHD TALKAH

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by

NURUL SHAKINA BINTI MOHD TALKAH

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LIST OF ABBREVIATIONS

bp	Base Pair
CIA	Chloroform-Isoamyl Alcohol
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic Acid
ETS	External Transcribed Spacer
IGS	Intergenic Spacer
IR	Inverted Repeat
ITS	Internal Transcribed Spacer
LSC	Long Single Copy
MW	Molecular Weight
NaOAc	Sodium Acetate Anhydrous
NGS	Next-Generation Sequencing
PCG	Protein Coding Genes
PCR	Polymerase Chain Reaction
rRNA	Ribsomal Ribonucleic Acid
SRA	Sequence Reads Archive
SSC	Short Single Copy
TIS	Transcription Start Site
tRNA	Transfer Ribonucleic Acid
WGD	Whole Genome Duplication
WGS	Whole-Genome Sequencing

SISTEMATIK MOLEKUL SPESIES *Cryptocoryne* DARI KEPULAUAN INDO-MALAYA

ABSTRAK

Beberapa ekspedisi di Sumatera dan pulau-pulau berdekatan telah menghasilkan pengenalpastian spesies *Cryptocoryne* baharu dan hibrid dari spesies yang berlainan dalam genus. Beberapa spesies yang baru ditemui mempunyai campuran struktur morfologi daripada spesies *Cryptocoryne* yang berbeza. Spesies yang baru ditemui ini terbukti mempamerkan pelbagai tahap ploidi walaupun dalam kawasan geografi berdekatan. Keadaan ini telah mengakibatkan kerumitan dalam mengenalpasti taksonomi genus ini. Dalam kajian ini, pembinaan semula genom kloroplas yang lengkap telah dilakukan menggunakan teknologi penjujukan generasi seterusnya (NGS) dan bahasa pengaturcaraan perl. Empat genom kloroplas lengkap telah dikenalpasti dan dibandingkan antara satu sama lain untuk mencari kawasan intergenik homolog dengan variasi yang tinggi. Oleh itu, penanda molekul telah dibangunkan dari kawasan intergenik untuk menjelaskan sejarah evolusi spesis *Cryptocoryne* yang telah ditemui di Sumatera dan pulau berdekatan. Gabungan jujukan intergenik yang baru dibangunkan dan jujukan *trn*K-*mat*K yang digunakan secara meluas telah digunakan untuk menjelaskan sejarah evolusi *Cryptocoryne* berdasarkan warisan dari satu induk. Dua penanda molekul dari kawasan nukleus iaitu penjarak transkripsi dalaman (ITS) dan penjarak transkripsi luaran (ETS) juga digunakan sebagai penanda pewarisan dwi-induk. Analisis filogenetik dari gabungan dua penanda memberikan resolusi yang lebih baik kepada sejarah evolusi spesis berbanding dianalisis secara berasingan. Penanda molekul juga digunakan untuk mendapatkan pandangan awal tentang induk yang dijangkakan dan arah penghibridan

hibrid *Cryptocoryne* yang baru ditemui dan endemik di Sumatera. Spesis hibrid tersebut adalah *C.* ×*jambiensis*, *C.* ×*ardyi* dan *C.* ×*zukalii* nothovar. *sumateraensis*. Bukti molekul menunjukkan bahawa warisan *C.* ×*jambiensis* dan *C.* ×*zukalii* nothovar. sumateraensis berkemungkinan besar adalah dua arah di mana kedua-dua ibu bapanya boleh menjadi penderma debunga dan penerima debunga. Bagi *C.* ×*ardyi*, *C. scurrilis* berkemungkinan besar adalah induk betina manakala *C. cordata* var. *wellyi* menjadi penderma debunga.

MOLECULAR SYSTEMATICS OF *Cryptocoryne* spp. FROM INDO-MALAYA ARCHIPELAGO

ABSTRACT

Several expeditions in Sumatera and adjacent islands have resulted in identifications of new *Cryptocoryne* species and interspecific hybrids within the genus. Some of the newly discovered species possessed a mixture of morphological structure from different *Cryptocoryne* species. These newly described species were proven to exhibit various ploidy level even within nearby geographical area. The situation has resulted in taxonomic complexity of this genus. In this study, complete chloroplast genome reconstruction was done using next-generation sequencing (NGS) technology and perl programming language. Four complete chloroplast genomes were assembled and compared to each other to search homologous intergenic region with high variation. Thus, molecular marker was developed from intergenic region to elucidate the evolutionary history of sampled *Cryptocoryne* species from Sumatera and adjacent islands. The combination of newly developed intergenic region and widely used *trn*K-*mat*K region was utilized to elucidate *Cryptocoryne* evolutionary history based on uniparental inheritance. Two molecular markers from nuclear region, the internal transcribed spacer (ITS) and the external transcribed spacer (ETS) were also utilized to determine biparental inheritance. The combined phylogenetic analysis of two different markers gives better resolution to species evolutionary history compared to being analyzed separately. Molecular markers were also utilized to obtain the preliminary insight on the putative parents and direction of hybridization of newly described *Cryptocoryne* hybrids that are endemic to Sumatera. The hybrid species are *C.* ×*jambiensis*, *C.* ×*ardyi* and *C.* ×*zukalii* nothovar. *sumateraensis*. Molecular

evidence showed that the inheritance *C.* ×*jambiensis* and *C.* ×*zukalii* nothovar. *sumateraensis* are most likely to be bidirectional in which both of its parents can become pollen donor and pollen receiver. For *C.* ×*ardyi*, *C. scurrilis* is most likely to be maternal parent with *C. cordata* var. *wellyi* being the pollen donor.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Cryptocoryne species is aquatic plant from family Araceae and have been widely distributed world-wide. Up until recently, more than 60 species were identified with more than 15 varieties and 10 identified interspecific hybrid species. Nevertheless, from time to time, new species was found and identified in various part of Southeast Asia. Recent explorations in Sumatera and adjacent islands resulted in identifications of new species and interspecific hybrids (Wongso et al., 2019). For instance, some of newly described species are the variety of identified species such as *C. cordata* var. *wellyi* and *C. cordata* var. *diderici*. These two *C. cordata* varieties are endemic to Sumatera and slightly identical in their morphological characteristics. Other *C. cordata* varieties were identified from different region such as *C. cordata* var. *cordata* from Peninsular Malaysia, *C. cordata* var. *grabowskiii* from Natuna Island, Borneo, *C. cordata* var. *siamesis* from Thailand. These varieties exhibit different types of ploidy level.

Due to its colorful florets and various leaf shapes, *Cryptocoryne* has been widely popular in aquaspacing. In fact, the species has been exported globally despite some of them such as *C. elliptica* is endemic to Peninsular Malaysia. *Cryptocoryne* natural habitats are usually from low tide to various elevations of river streams depending on the species adaptations capabilities. Although some of the species are proven to be robust to environmental changes, some of them are very sensitive. Thus,

if the natural habitat is destroyed due to developments purposes, chances to discover new species or *Cryptocoryne* populations will also become very slim.

Molecular markers have been proven to become a powerful tool to resolve phylogenetic relationships among different taxa. Countless taxonomic study utilizing molecular markers to understand evolutionary history of *Cryptocoryne* species (Othman et al., 2009; Rusly, 2016; Thirumalai, 2018). These markers are useful to elucidate evolutionary history of *Cryptocoryne* populations. The advances of fast-paced sequencing techniques have made information on genomic data accessible with reasonable cost and short amount of time. Access to genomic information could enabled the development intergenic region with high variations to elucidate low-level taxa.

In this study, comparative analysis of chloroplast genomes was conducted to develop intergenic region with high variation. The molecular marker was combined with universal markers to resolve evolutionary relationships using phylogenetic reconstruction. Two molecular markers from nuclear region were also utilized to give better species resolutions. Putative parents of newly described hybrid species relationships and their evolutionary history were then studied based on molecular perspectives.

1.2 Problem Statement

Several *Cryptocoryne* individuals from Sumatera involved in this study are suspected hybrids due to mixture of complex characteristics in their morphology. Their relationships to other *Cryptocoryne* species are ambiguous due this situation. For newly described species, the generations of molecular data could provide useful insight

to understand their evolutionary history. Utilization of universal primers for intergenic regions in *Cryptocoryne* species were stated to be challenging due to unspecific binding site in chloroplast genome. Current chloroplast marker widely used in phylogenetic studies are from genic regions that show low mutation rate, resulting in poor resolution at lower taxonomical level. Non-coding or intergenic regions show a higher mutation rate however, primers used to amplify these regions are not universally specific across plant families. In many cases, these intergenic primers are not usable in *Cryptocoryne*. Thus, there is a need to develop intergenic specific primers for the genus to increase species resolution especially for closely related species even for species complex. Compared to cpDNA, nuclear DNA namely the ITS region shows higher mutation rate. However, the ITS is not variable enough in *Cryptocroryne* to delineate closely related species. An additional nuclear marker is required to find more variability to increase interspecific resolution.

1.3 Objectives of Research

The objectives of this study are:

1. To develop novel intergenic marker for phylogenetic analysis based on comparative chloroplast genome.
2. To establish phylogenetic relationships of *Cryptocoryne* species based on molecular marker from chloroplast genome (*trn*K-*mat*K and *pet*N-*psb*M).
3. To determine phylogenetic relationship of *Cryptocoryne* species based on two nuclear DNA region (ITS and ETS).
4. To determine the status of naturally occurring *Cryptocoryne* hybrid species and their putative parents using molecular markers.

CHAPTER 2

LITERATURE REVIEW

2.1 The Genus *Cryptocoryne*

Cryptocoryne is an Aroid plant that reside within *Araceae* family. The common name for this genus is 'Water Trumpet' in English and 'Keladi Paya' in Malay. It is characterised by aquatic or amphibious plants that is native to Southeast Asia. *Cryptocoryne* was previously known as *Arum spirale* Retzius. Later on, Wydler established the genus *Cryptocoryne* in 1830 based on species from India, *Cryptocoryne spiralis* (Retzius) Wydler (Othman et al., 2009). More than 65 species have been recognized and many of these species are endemics in certain regions. For instance, *C. cordata* var. *wellyi* is a newly described *C. cordata* variety that endemic to Sumatera region. The endemism pattern in many *Cryptocoryne* species is quite distinctive as the natural habitat of most aquatic plant species is usually known as widely distributed from Sri Lanka to Borneo.

2.1.1 Morphology and Habitat of *Cryptocoryne*

The name *Cryptocoryne* is a combination from Latin words that described the species structure which are *crypto* means hidden and *coryne* means club (Othman et al., 2009). This refers to the spadix that is totally hidden inside the rounded kettle with no trace of margin fusion. The kettle embraces inflorescence structure which consist of olfactory bodies, spadix, male and female flowers (Plate 2.1). The olfactory bodies main function is to protect the reproductive organs. The kettle is a part of the unique spathe structure which is also consist of the basal tube, an upper tube (long or short),

and a terminal limb formed at the opening upper end of the tube (Wongso et al., 2019). The spathe structure resembles a trumpet; thus, the name water trumpet. Not necessarily flat, the limb can be ovate, extended into tail or even spirally coiled with various sizes and colours. The limb is even the main characteristics to diagnose a species. For example, *Cryptocoryne nurii* has limb that is characterized as cordate, red to black purple sometimes yellow with large, irregular protuberance and narrow collar (Jacobsen et al., 2013). While *Cryptocoryne longicauda* has ovate and rugose limb with distinct collar and a long caudicle (De Wit, 1970).

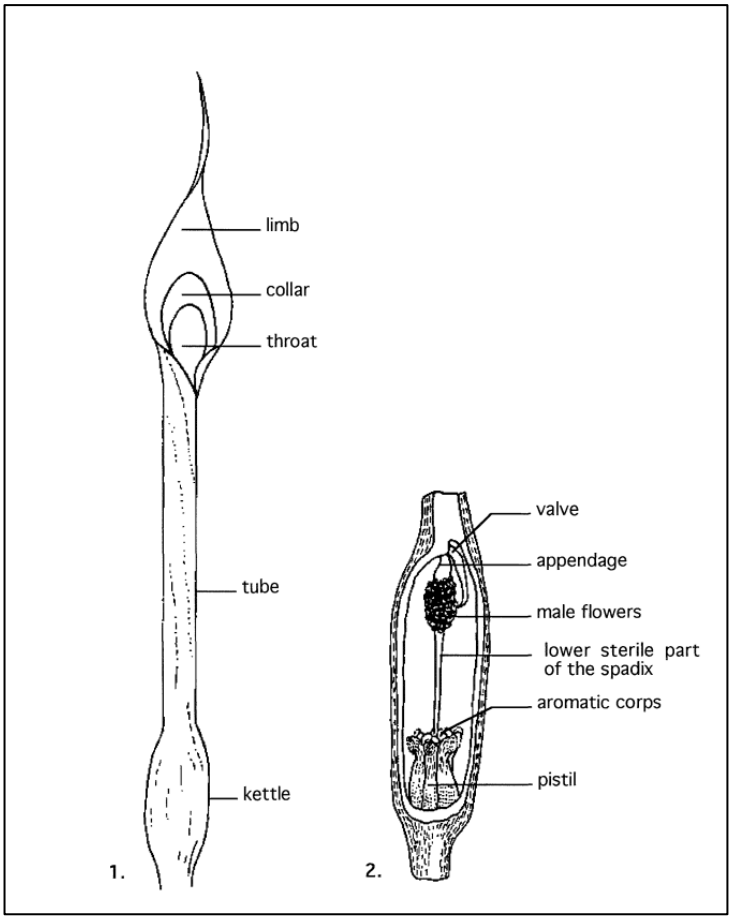

Plate 2.1 Floral morphology of *Cryptocoryne* (Othman et al., 2009)

The leaf structure is another characteristic to be considered to diagnose species of *Cryptocoryne* (Othman et al., 2009; Wongso et al., 2019). Consisting two parts

which are petiole and blade, the leaves grow in a rosette form along the rhizomes. Leaf blades can be varied in shapes, textures and sizes according to species and environment. *Cryptocoryne elliptica* has small, green ovate to obovate leaf blades much like and ellipse structure (Othman et al., 2009) while *Cryptocoryne minima* has longer, green to purple upper leaf ovate to cordate leaf blades (Jacobsen, 1977). These variations can occur even among individuals in the same species, thus making it challenging for taxonomist to identify different species.

In general, *Cryptocoryne* species can be found naturally grow in low tide river with slow running water in the lowland rain forest floor with less than 300 m elevation (Jacobsen, 1986). Though most of them can grow in the similar environment, the position in which part of the river stream may varies due to their aquatic to amphibious characteristics. Some species may only fully submerge in the water when the water is tide which is usually located in the riverbanks, while other species might constantly submerge in the water. The species has been reported to grow on various types of soil from coarse gravel, sand, peat and even mud. *Cryptocoryne affinis* is one of the species that grow on coarse sandy area with muddy bottom. They showed smaller and crumple leaves on when emerged during low water tide. However, the leaves are larger with brownish purple colour when submerged in the water (Othman et al., 2009). Another example that can be seen from Plate 2.2 is *Cryptocoryne elliptica* that emerged on the mudflats is. During heavy rainy season or deeper mud near the small streamlet through the valley it is completely submerge. Rhizomes and runners provide the structure they need to survive any extreme conditions that destroys the leaves completely.

Plate 2. 2 *Cryptocoryne elliptica*; grows on the mudflats in very shallow water in Bukit Panchor, Penang.

2.1.2 Species distribution of *Cryptocoryne* in Sumatera and Adjacent Islands

Cryptocoryne species are widely distributed from West India, Sri Lanka, and Malay-Archipelago (Reumer, 1984). According to The Plant List website from Kew Garden, there are 71 accepted *Cryptocoryne* species published with 8 varieties and two hybrids (The Plant List, 2013). However, the list might not be updated recently with many new taxa identified for the last 5 years. Up until 2019, 65 recognized species, 19 varieties and 10 named interspecific hybrids have been identified only across Southeast Asia (Wongso et al., 2019). The list is expected to be growing in coming years due to ongoing explorations and field studies. As previously mentioned, part of Sumatera and adjacent regions have swampy terrains which are ideal for *Cryptocoryne* species habitat. Several species are known to be endemic in Sumatera and Riau islands while other species can be found naturally in neighbouring region such as Peninsular Malaysia. For example, *Cryptocoryne nurii* can be found naturally in both Peninsular Malaysia and Sumatera (Jacobsen et al., 2013). *Cryptocoryne scurillis* on the other hand are endemic to Sumatera and Riau islands specifically (Reitel et al., 2012). Few

other *Cryptocoryne* species that endemic to Sumatera and Riau islands are *Cryptocoryne bangkensis*, *Cryptocoryne villosa* and *Cryptocoryne wongsoi*. Details on *Cryptocoryne* species within Sumatera and Riau Island regions are being illustrated in Table 2.1.

Table 2.1 Distribution of several *Cryptocoryne* species studied Sumatera and adjacent islands region

No	Species name	Chromosome no	Ploidy	Locality / distribution	References
1.	*Cryptocoryne minima* Ridley	2n = 34	Diploid	West Peninsular Malaysia, Sumatera	(Arends et al., 1982; Othman et al., 2009)
2.	*Cryptocoryne scurillis* de Wit.	2n = 34	Diploid	Endemic to Sumatera and Riau islands	(Reitel et al., 2012)
3.	*Cryptocoryne nurii* Furtado	2n = 34	Diploid	Peninsular Malaysia, Sumatera	(Jacobsen et al., 2013)
4.	*Cryptocoryne nurii* Furtado var. *raubensis*	2n = 34	Diploid	Peninsular Malaysia, Sumatera and Riau islands	(Jacobsen et al., 2013)
5.	*Cryptocoryne cordata* Griff var. *diderici* N. Jacobsen	2n = 102	Hexaploid	Endemic to Sumatera and Riau islands	(Wongso et al., 2019)
6.	*Cryptocoryne cordata* Griff var. *wellyi* Wongso	2n = 34	Diploid	Central Sumatera	(Wongso et al., 2019)
7.	*Cryptocoryne cordata* Griff var. *cordata*	2n = 34	Diploid	Thailand and Peninsular Malaysia	(Bastmijer et al., 2010)
8.	*Cryptocoryne bangkaensis* Bastmeijer	2n = 68	Tetraploid	Endemic to Sumatera and Riau islands	(Bastmeijer & Jacobsen, 2007)
9.	*Cryptocoryne schulzei* de Wit.	2n = 34	Diploid	Peninsular Malaysia and Sumatera	(Othman et al., 2009)
10.	*Cryptocoryne griffithi* Schott	2n = 34	Diploid	Peninsular Malaysia, Sumatera and Singapore	(Othman et al., 2009)
11.	*Cryptocoryne villosa* N. Jacobsen	2n = 30	Diploid	Endemic to Sumatera	(Arends et al., 1982)
12.	*Cryptocoryne moehlmannii* De Wit	2n = 30	Diploid	West Sumatera	(Arends et al., 1982)
13.	*Cryptocoryne hudoroii* Bogner and Jacobsen *sp.* nov.	2n = 20	Diploid	Endemic to Borneo	(Jacobsen, 1985)

14.	*Cryptocoryne longicauda* Engler	2n = 30	Diploid	Peninsular Malaysia (Johor), Sumatera, Borneo	(Jacobsen, 1985; Othman et al., 2009)
15.	*Cryptocoryne ciliata* (Roxburgh) Schott var. *ciliata*	2n = 22, 33	Diploid, Triploid	India, Indochina, Indonesia, New Guinea	(Jacobsen, 1985)
16.	*Cryptocoryne pontederiifolia* Schott	2n = 30	Diploid	Sumatera	(Bastmijer et al., 1997)
17.	*Cryptocoryne fusca* De Witt	2n = 34	Diploid	Kalimantan, Sarawak, Sumatera	(I B Ipor et al., 2006; Niels Jacobsen, 1985; Wongso et al., 2019)
18.	*Cryptocoryne wongsoi* I.B. Ipor	2n = 34	Diploid	Endemic to Sumatera	(Jan D Bastmeijer et al., 2016)
19.	*Cryptocoryne griffithi* Schott	2n = 34	Diploid	Malacca, Johor, Singapore, Bintan Island	(Arends et al., 1982)

2.1.3 Key to *Cryptocoryne* species of Sumatera and adjacent islands

Taxonomist from Indonesia described four new *Cryptocoryne* species (Wongso et al., 2019) from his expeditions across Sumatera and adjacent islands. Based on the described species a key is created:

1a. Limb with long cilia at margin…… ***C. ciliata (Roxb.)*** Schott var. ***ciliata***
1b. Limb without cilia at margin……..……. …….. 2
2a. Leaf blade ± covered with hairs on abaxial surface and/or along margins…….3
2b. Leaf blade glabrous …….……..…… 4
3a. Spathe tube short, < half of limb length ……. …… ***C. fusca*** De Wit
3b. Spathe tube long, > 2 × limb length …… ***C. wongsoi*** I. B. Ipor
4a. Tube of spathe > 2 × as long as limb …….……5
4b. Tube of spathe ≤ 2 × as long as limb …….…… 11
5a. Limb surface covered by short black purple hairs ……***C. villosa*** N. Jacobsen
5b. Limb surface not covered by short black purple hairs …….....6
6a. Limb surface with ± distinct protuberances …….. 7
6b. Limb surface without distinct protuberances, but± rough …… 9
7a. Collar with a narrow opening, and with distinct protuberances or conspicuous denticulations along the margins …… 8
7b. Collar with a broad, black-purple, funnel-shaped opening, and smaller protuberances not specifically along the margins…… ***C. schulzei*** De Wit
8a. Limb surface densely covered with many branched protuberances…***C. nurii*** Furt. var. ***nurii***
8b. Limb with distinct protuberances or conspicuous denticulations along the margins …… ***C. bangkaensis*** Bastm.
9a. Limb narrow, brownish to purplish, surface ± smooth, collar zone narrow, brown spotted to evenly purplish …***C. ×zukalii*** Rataj nothovar. ***sumateraensis*** W. Reichert
9b. Limb broad, yellow to brown, surface rough, collar zone broad, yellow ……..10
10a. Leaves green; limb surface yellow, rough ..***C. cordata*** Griff. var. ***wellyi*** Wongso
10b. Leaves purple ± with markings; limb surface ± brownish tinged,±smooth to a little rough ……..……***C. cordata*** Griff. var. ***diderici*** (De Wit) N. Jacobsen
11a. Limb with a tail about as long as the tube ……***C. longicauda*** Engl.
11b. Limb without a long tail ……..12

12a. Limb surface with ± distinct protuberances ……...13
12b. Limb surface without ± distinct protuberances ……..19
13a. Limb surface with uniformly rounded protuberances ……..***C. griffithii*** Schott
13b. Limb surface with irregular protuberances ……14
14a. Limb surface with ± branched protuberances ……15
14b. Limb surface without ± branched protuberances ……..……18
15a. Collar thick, asymmetrical collar opening, protuberances irregular, only slightly branched ……***C. scurrilis*** De Wit
15b. Collar thin, regular in opening, protuberances branched …….....16
16a. Limb distinctly twisted ……..…… ***C. ×jambiensis*** Bastm.
16b. Limb flat, ± recurved ……..……17
17a. Limb surface densely covered with many branched protuberances.. ***C. nurii*** Furt. var. ***nurii***
17b. Limb surface with fewer branched protuberances ……***C. ×timahensis*** Bastm.
18a. Limb narrow, < 1 cm broad …… ***C. minima*** Ridl.
18b. Limb broad, > 1 cm broad ……..…….……..***C. ×ardyi*** Wongso
19a. Limb distinctly recurved and narrowed, with a broad, vertical, funnel-shaped purple collar zone ……..……***C. schulzei*** De Wit
19b. Limb not distinctly recurved, yellow or purple, upright or forward obliquely twisted without a vertical funnel-shaped purple collar ……..…..……20
20a. Limb ± purplish, upright to forward obliquely twisted ..***C. moehlmannii*** De Wit
20b. Limb ± yellow to reddish, upright to somewhat twisted …….***C. pontederiifolia*** Schott

2.1.4 Species Evolution of *Cryptocoryne* Species

One of the earlier evolution study of *Cryptocoryne* genus was done by utilizing data from morphological characteristics and chromosome numbers (Arends et al., 1982; Reumer, 1984). The chromosome numbers recorded can be seen previously in Table 2.1. Arends et al (1982) concluded the most primitive number of chromosome in *Cryptocoryne* is 2n = 36 based on closely related species chromosome number *Lagenandra*. Arends et al. (1982) also hypothesized that the evolution of *Cryptocoryne*

genus is going towards reduction in chromosome numbers. Although he agreed with the conclusion, Reumer (1984) combined the chromosome number data with biogeographical data to form hypothetical phylogeny of the subtribe *Cryptocoryninae*, *Araceae*. According to Reumer (1984), relationship between different chromosome numbers, is to divide species chromosome number based on their distribution across specific continent. For example, haploid x = 10 is found exclusively on the island of Borneo and considered to be descended from x = 11. The phylogeny interpretation is *Cryptocoryne* have a diphyletic characters whereby two primary base $x_1 = 11$ and $x_2 = 18$ are descended from $x_1 = 9$. The primary base $x_1 = 11$ is a result from aneuploidy $x_1 = (9+2)$ and $x_2 = 18$ from chromosome doubling. It can be seen from these findings that topological changes in the land surfaces have made big impact on species dispersal and chromosomal data could give an overview to inter-species relatedness.

Another efficient method of studying species evolution is from molecular data. A phylogenetic tree from molecular data was constructed from nuclear and chloroplast region of *Cryptocoryne* DNA (Othman, 1997; Thirumalai, 2018). For nuclear region, Internal Transcribed Spacer (ITS) has been used due to their high substitution rate. Similarly, for chloroplast region, intergenic region has been used to resolve intra-species relationship also due to their higher rate of polymorphism compared to coding region. More detailed discussion on ITS and chloroplast intergenic region will be discussed in the later section. Returning to the molecular studies for *Cryptocoryne*, the phylogeny based on ITS data by Othman (1997) showed 3 different clades (Figure 2.2). These clades represent species from different geographical regions; Mainland Asia, Malay-Archipelago and Sri Lanka. The first clade revealed that *Cryptocoryne spiralis* which distributes from India to Bangladesh is the most primitive member of *Cryptocoryne* genus. This finding supports Reumer's (1984) and Arends et al., (1982)

inference based on similarities in chromosome number and morphological characteristics of *Cryptocoryne spiralis* with *Lagenandra*. Following that, three other taxa; *C. albida* Parker, *C. crispatula* Engler var. *balansae* and *C. retrospiralis* (Roxburgh) Kunth are also placed in the same clade which is consistent with phylogeny results from Arends et al. (1982). Based on the positions of these species in the phylogenetic tree and their distributions, Othman (1997) suggests that the ancestors of *C. crispatula* and *C. albida* have evolved in western India, dispersed through the river systems throughout Bangladesh, Myanmar, Thailand and South China and resettled. The situation which inferred as allopatric speciation in *Cryptocoryne* evolution has been described earlier by Jacobsen (1977) from chromosome number and morphological characteristics data.

On the second clade of ITS phylogenetic tree by Othman (1997), the list of species comes from Sri Lanka. Two taxa can be seen from this clade which from base number x = 18 (*C. alba*) and base number x = 14 (*C. backettii*, *C. undulata*, *C.* ×*willisii*, *C. walkeri* and *C. wendtii*). This groupings are tally with analysis based on cytological and morphological data by Jacobsen (1977) and Arends et al. (1982). On the other hand, two of the species which are *C. backettii* and *C. undulata* branched out from other with other species on their clade was not well resolved using ITS sequences. Moving on to the third clade, the clade representing species from Malesia or Malay-Archipelago region. Overall species relationship for Malay-Archipelago region was not satisfactory. However, Othman (1997) concluded that there are closed relationship between two Sumatran species (*C. pontederifollia* and *C. moehlmannii*) and two Peninsular Malaysia species (*C. elliptica* and *C. schulzei*) as they emerged in the same monophyletic groups. Another notable finding is *C. pygmea* which from Philippines are more closely related to *C. elliptica* and *C. schulzei* than another species from

Philippines which is *C. aponogetifollia*. Instead, *C. aponogetifollia* is positioned in the same monophyletic group with a species from Vietnam which is *C. annamica*. It has been stated that ancestor of these two distinct geographically isolated species dispersed to both areas (Othman et al., 2009).

Utilization of chloroplast gene also has been proven to be useful in resolving phylogenetic relationship among interspecific individuals. Othman (1997) finds that even though the resolution level is lower compared to ITS region, chloroplast sequence can complement ITS phylogeny especially when separating the species into three clades representing their geographical origin.

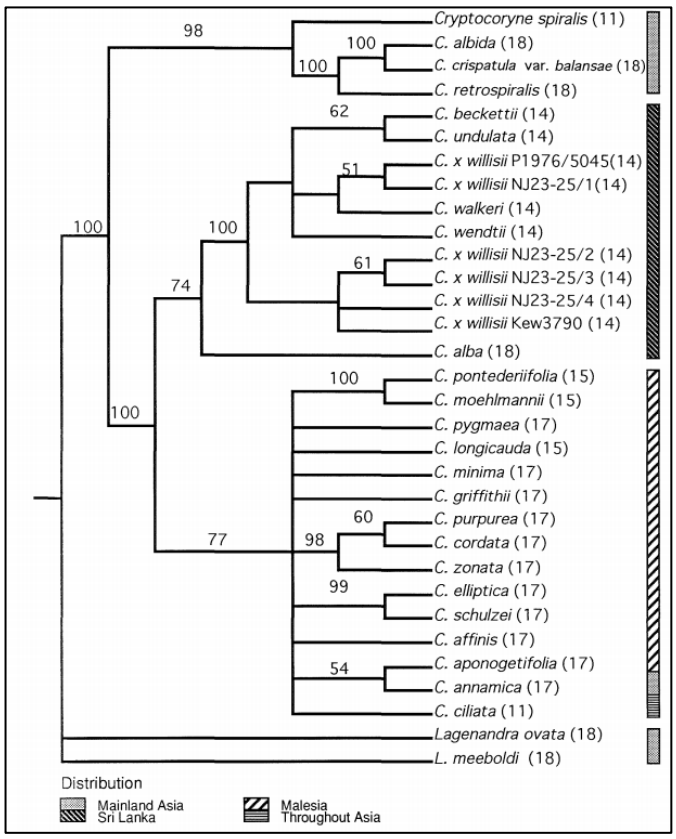

Figure 2.1 Phylogeny tree (strict consensus) of 25 *Cryptocoryne* species from ITS region (Othman, 1997).

2.1.5 Human Uses and Vulnerability to the Environment

Cryptocoryne species such as *C. wendtii* are widely known to be use in aquascaping. Aquascaping is basically an art of arranging aquatic plants along with the rocks, stones, cave work or driftwood in aesthetically satisfying manner to their enthusiasts (Kumari & Kumar, 2021). The art can come with animals (fish, amphibians, or reptiles) or without animals in the aquarium. It is not easy to achieve the level of success in aquascape because many factors need to be in balance in the closed system environment. Not all *Cryptocoryne* species are being used for aquascaping, most of the species chosen because they are easier to grow and have a beautiful rosette. Nevertheless, for some species such as *C. nurii* and *C. cordata*, even though it can be tedious for the species to thrive after habitat relocation, the growing demand resulted them to be sold for RM30-RM40 per individuals. The species even available in e-commerce site such as 'Shopee'. *C. nurii* for instance required acidic environment which can be achieved by putting Ketapang leaves litter (*Terminalia catappa*) on the bottom of plain brown soil and creating submerged environment for the plant individuals. The resulting blackwater setup are great for the plants to thrive. Air-conditioned environment in timely manner with minimal sunlight can also be helpful with the long-term plant survival. Unfortunately, *Cryptocoryne* species has been collected in high numbers and exported to different parts of the world leaving alarming rate of declined quantities in their natural habitat.

Another threat to the existence of *Cryptocoryne* species in natural environment is the loss of habitat. The reason for this matter are due to massive land clearance, logging, and other human activities (Mansor & Masnadi, 1994). Practically, the development process is usually inevitable and beneficiary to human being. Even though, some *Cryptocoryne* species able to adapted with changing environment in

order to survive (Jacobsen et al., 2013; Saibeh & Mansor, 1996), it is necessary to provide a suitable habitat. Thus, smart utilization in the landscape is required to successfully delivered our agricultural needs while reserving a reasonable amount of area for natural species to thrive.

2.2 Natural Hybridization in Plants

Evolutionary biologist define hybridization in different meanings (Rieseberg & Carney, 1998). A broad meaning for this term is the cross between individuals from different populations producing progenies 'which are distinguishable on the basis of one or more heritable character' (Harrison, 1990). A similar term to hybridization but with narrower definition is introgression which is focusing on interspecies movement of genes mediated by backcrossing or between genetically distinguishable populations. Another meaning which is more straight forward and simplified is cross-fertilization of different species. This definition however will require us to look back for the definition of species itself. According to Mayr (1963), the species are groups of interbreeding natural populations which are reproductively isolated from all other such groups (Mayr, 1963). Rieseberg and Carney (1998) agreed to this definition as it is applicable for studies of hybridization and speciation with small changes in the 'reproductively isolated' to 'genetically isolated' from all other such groups.

In 1766, Joseph Gottlieb Kölreuter initiated the systematic study of plant hybridization (Goulet et al., 2017). The experiment which involved the crossing between *Nicotiana paniculata* and *N. rustica* has resulted in two important conclusions (López-Caamal & Tovar-Sánchez, 2014). The first one is the first generations (F1) of interspecific crosses are likely to become sterile or 'botanical mules'. Secondly, hybrids will not emerge without difficulty such that human intervention or disturbance

of the habitat. This can be related to plant adaptability to environmental changes to survive. Following the experiment, the hybrids produced by Kölreuter tend to revert to their parental forms when being backcrossed with the parental species. The statement shows that the genetic materials of hybrids is not stable or constant to keep their parental intermediate morphology. Prior to this hypothesis, Kölreuter also demonstrated that hybrids are usually intermediate morphologically relative to their parents. Initially, Kölreuter considered the conclusions as nature's way to conserve the preexisting process. Nevertheless, Kölreuter efforts has caused the development of various hybridization hypotheses among botanists. For instance, Abbott and Rieseberg (2012) claimed that hybrids were constant or true-breeding for several generations will eventually form new species.

2.2.1 Polyploidy in Plants

Polyploidy is a term described to an organism or cells that possess more than a single sets of basic chromosomes (Chen, 2010). The formation of hybrids is usually due to polyploidy ability in the plants. Polyploidy plays big role in plant evolution on retaining favoured hybrid combinations during sexual reproduction (Breese et al., 1981). In nature, favoured hybrid combinations occur to extend adaptation, however, humans manipulate this condition in the crops to improve crops quality such as in rice and maize. In the early years of plant polyploidy research, scientists speculated about half of all angiosperms were polyploid (Tate et al., 2005). Although few follow ups have been done on estimated frequency of plant polyploidy, Otto and Whitton (2000) suggested that "polyploidization may be the single most common mechanism of sympatric speciation in plants". The statement infers that hybrid generations that come from interspecies parent may eventually fail to breed with their respectful parental

species. Despite the theory, Tate et al. (2005) in their review states that polyploidy seems to appear less in plant lineage other than angiosperms and ferns. However, it is worth to note there are scarce research focusing on the other plant lineage. On contrary, Tate et al. (2005) also mentioned about how genomic investigations reveals whole genome duplications (WGD) could be the result of gene redundancy across species. This has been proved by the study from complete genome of *Arabidopsis thaliana* by Bennett et al., (2003). The study coupled with recent advancements in genomic sequencing technology has led to revelations of possibilities of polyploidy occurrence in other plant generations.

Two types of widely known polyploidy are allopolyploidy which is genome doubling through hybridization of two or more different but related species and autopolyploidy which is genome doubling of single species (Qiu et al., 2020). Following previous discussion, WGD could be the results from either allopolyploidy or autopolyploidy. However, both polyploidies have the prevalence in such a way that conferred heterozygosity which has major advantage to their diploid progenies (Mason & Wendel, 2020). The statement is proven by vast researches and theories that shows plant adapted this mechanism as part of evolution process in order to survive global change (Van de Peer et al., 2017). An interesting example from Cai et al. (2019) that utilized transcriptomics and complete genomes data of 42 species in plant order *Malpighiales*. The study identified 22 ancient WGDs that widely distributed across the order and clustered around Eocene-Paleocene transition.

2.2.2 Hybrid Zones

In response to climate change, hybrid zones may formed prior to achievement of reproductively or genetically (as in previous discussion) isolated plant species

(Abbott, 2017). The formation of hybrid zone can be either through primary intergradation along an environmental gradient (Gompert et al., 2017) and secondary contact that previously differentiated in allopatry (Mullen, 2017). Studies focusing on hybrid zones is very limited however many researchers concluded most hybrid zones are formed through secondary contact which is usually caused by environment disturbance. If the hybrid zone is stable, it may exist for hundreds and thousands of generations otherwise hybrid zone will only exist temporarily and produce speciation reinforcement, co-exist with parental populations or extinction of one of parental species (Mullen, 2017). Hybrid fitness played a massive role in the hybrid zones stability. In theory, new hybrid generations that is fitter than their parents' generations in adapting environment changes will eventually dominate their shared habitat. Nevertheless, most studies of hybrid taxa shown habitat divergent is the limitation factor for hybrids generations to dominate the habitats even they have higher fitness than their parents. Thus, these hybrids co-exist with their parental species in the habitat (Abbott & Rieseberg, 2012).

2.2.3 Plant Hybrids Identification

There are few techniques utilized by researchers to identify hybrids in certain species. López-Caamal and Tovar-Sánchez (2014) highlighted four different types of hybrid identification method which are morphological characteristics, chemical characteristics, DNA fingerprinting and chromosome number. In the field, taxonomists will adapt the first insight on potential species hybrids that are usually morphological intermediary of parental species. However, identification based on species morphology alone have several weaknesses that can potentially lead to erroneous results. Firstly, morphology is highly influenced by their environment (López-Caamal & Tovar-Sánchez, 2014). For example, there is a study proved that

gene expression is highly regulated by their physical environment such as soil water stress (Ni et al., 2009). Secondly, morphological characters are usually correlated (the presence of several morphological characteristics are influenced by each other), therefore several characters are reduced greatly. Correlation of characters can originate from intra-organisms that shares the same phylogenetic history or even forced by character-state coding scheme (i.e pleitropy) (Guillerme & Brazeau, 2018). For this reason, it might impact the identification process. Thirdly, morphological intermediary is not only caused by hybridization. According to example from Rieseberg (1995), plesiomorphic characteristics could be shown by plants with the same ancestral population.

Other than morphological intermediary, chemical markers or also known as secondary metabolites are being used to identify hybrid in plants. Theoretically, secondary metabolites were used as a marker due to their simple inheritance mechanisms. Few secondary metabolites studied by researchers are phenolic, terpenoid, alkaloid, isothiozyanates and flavonoid compounds. Flavonoids however is the most studied secondary metabolites in hybrids identification due to its stability and high variability (Rieseberg et al., 1993). Nevertheless, it has been discovered later that even secondary metabolites have complex patterns of inheritance (qualitatively and quantitatively) in hybrids (Cheng et al., 2011).

Genetic data are very useful in plant hybrids identification. Two types of genetic data approach are chromosome number study and DNA fingerprinting. Based on the assumption of hybrid individuals always undergo an instant duplication of the chromosome complement as discussed earlier, chromosomal number analysis are being adapted to indicate hybrid organism (Lawton-Rauh et al., 2003; Roose &

Gottlieb, 1976; Strong & Ayres, 2013). The analysis are relatively easy to conduct (López-Caamal & Tovar-Sánchez, 2014), but it is really important to confirm the chromosome duplication is due to alloploidy not autoploidy. In order to differentiate that, Soltis et al. (2010) proposed to observe the absent or presence of multivalents during meiosis and explore the segregation ratios and many loci. This is time consuming and costly making it less effective to determine potential hybrids individuals. Even though allopolyploidy are often related to hybrids output, some hybrids species also recorded a number of homoploid hybrid speciation (Abbott & Rieseberg, 2012). Homoploid hybrid speciation is formed when there is no duplication of chromosome occur during the hybridization process. Due to this occurrence, it has been proposed that chromosome number alone does not reliable to determine the status of putative hybrids individuals. The hypothesis for more robust method to utilize chromosomal data is being combined with morphological or DNA fingerprinting data (Newaskar et al., 2013). In the case of *Cryptocoryne* species, natural hybrids are identified via pollen stainability whereby species with pollens that show low fertility or completely sterile are usually enticed as a hybrid species (Bastmeijer & Kiew, 2001; Jacobsen et al., 2016; Wongso et al., 2019). This procedure has been used from 1970s among taxonomist that studied *Cryptocoryne* systematics and then realized that some species in their collections are interspecific hybrids. The procedure requires placing a drop of 'cotton blue' which is a mixtures of Aniline Blue, phenol, glycerol, lactic acid and distilled water (Wongso et al., 2019). Deep blue stained pollen indicates, fully fertile pollen while transparent light blue indicates less fertile pollen (Jacobsen et al., 2016). Certainly, this observation needs to be combined with the knowledge of other morphological characteristics, chromosome numbers and their potential putative parents' population within their natural habitat.

The statement led us to the final method of hybrids identification which is via DNA fingerprinting. This method is usually focused on utilization of various markers present in the DNA region. These markers are usually present in the large number within the genome, not really influenced by other factors, inherited strictly under Mendelian ratios and can be useful for detecting different hybrid class (López-Caamal & Tovar-Sánchez, 2014). Few classical markers that has been used to identify hybrids are Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Simple Sequence Repeats (SSR) (Shasany et al., 2005; Snow et al., 2010). Although, the techniques mentioned proven to have many advantages and widely used among scientists for quite some times (Othman, 1997; Tovar-Sánchez et al., 2012), limitation occur in the availability of markers that can differentiate between two closely related species which usually haver lower resolution and identify of later hybrid generation (A D Twyford & Ennos, 2012). The limitation highlighted by Twyford & Ennos (2012) was then followed with the proposal of utilizing Next-Generation Sequencing (NGS) data to find the best multiple markers to further analyse hybrid identification. This emerge from the basic principle of NGS data that usually produce a bulk of genomic data that can be manipulated in various ways where with the addition of latest software that could assist the process. For instance, there is a report of *Mangifera casturi* Kosterm utilizing NGS data to study cross-hybridizations of multiple ancestors (Matra et al., 2021).

2.2.4 Occurrence of Hybrids among *Cryptocoryne* Species

Cryptocoryne species has been proven to have natural hybrids occurrence within their habitats. Wongso et al. (2019) identified three new interspecific natural hybrids among *Cryptocoryne* species, hence there are currently 10 interspecific

hybrids and more than 15 unnamed hybrids known. Wongso demonstrated that hybridization among *Cryptocoryne* species with more distant relationship will produce sterile progenies by observing their pollens. Thus these hybrids thrive with their vegetative progenies utilizing their subterranean stolons and dormant buds (Othman et al., 2009). As previously mentioned, some plant species will be adapted to the environment changes to survive. This is evidenced by Jacobsen & Ørgaard (2019) regarding *Cryptocoryne* hybrids across Southeast Asia. Based on their observation, hybridization is a major drive for speciation and populations are the units and medium in evolution. This is because in the hybrid zone, different species population have the potential to produce different types of hybrids depending on the changes that influence selection pressures in this case for *Cryptocoryne* species.