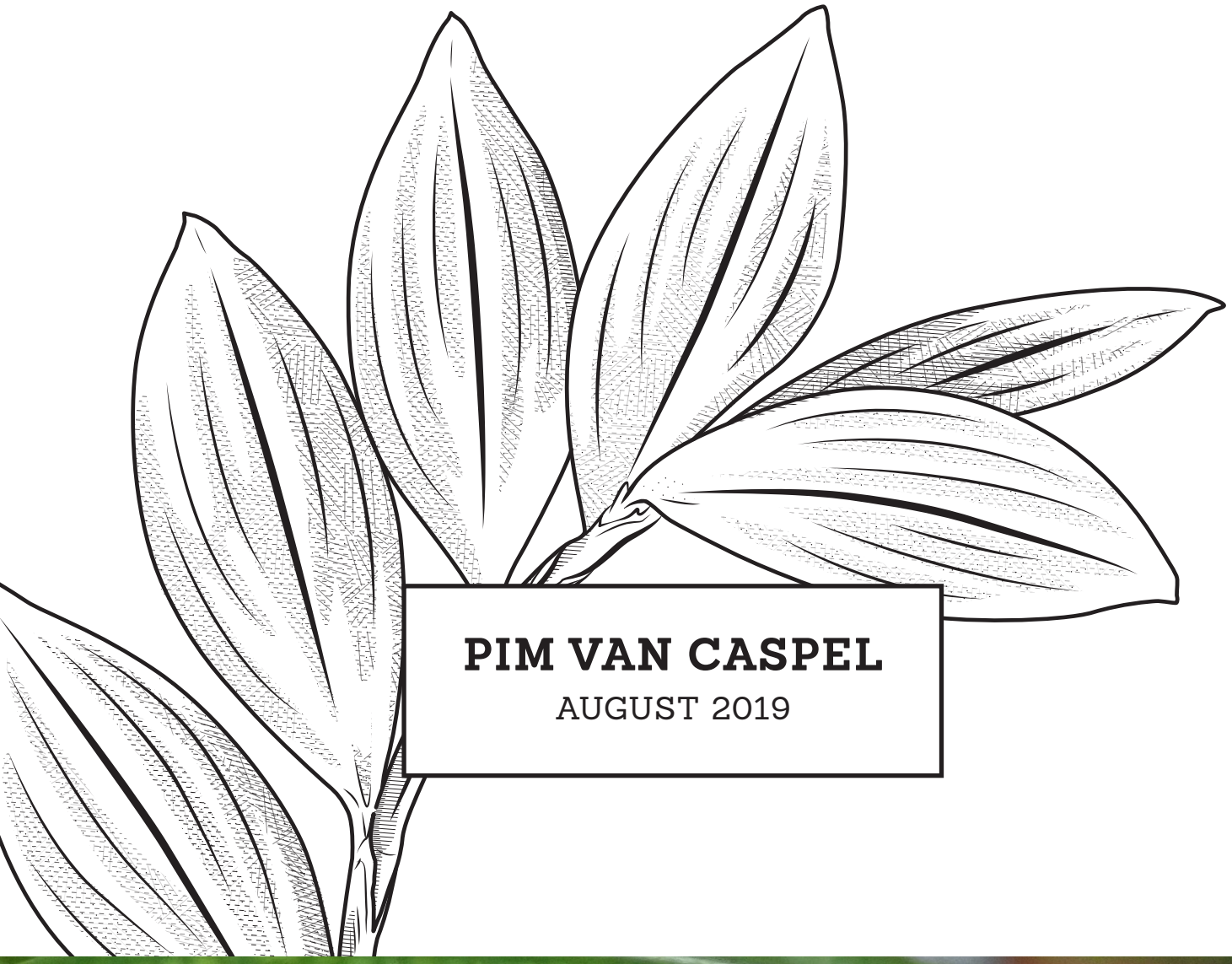
Revision *of* Bornean Costaceae



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AUGUST 2019

THE UNIVERSITY *of* EDINBURGH

Royal Botanic Garden Edinburgh

Thesis submitted in partial fulfilment for the MSc in the Biodiversity and Taxonomy of Plants

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ABSTRACT

This study covers the Bornean Costaceae, a pantropical family sister to the Zingiberaceae are often called the ‘Spiral gingers”. Morphological characters were studied from living specimens in the Royal Botanic Gardens, Edinburgh, and from herbarium and pickled material. A molecular study was done with 24 DNA samples from mostly wild collections, collected by A.D. Poulsen, and some that were collected by the author during the MSc fieldtrip to Colombia in 2019. Morphological characters were examined to find diagnostic characters separating the species and linking the genera together. A Cytological study was done to obtain chromosome numbers, as this was largely unknown for the Asian Costaceae as a whole. These three studies combined has led to a taxonomic treatment revising all currently accepted species of the Bornean Costaceae. Three new combinations were made, and one species was placed in species complex, bringing the species number to seven, in two genera.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisors Axel Poulsen and Greg Kenicer, who gave me a lot of advice and pulled me through in the end, as the stress was starting to get the best of me. This Thesis would not be possible if it wasn't for Laura Forrest, who was a beacon of light during my lab work and provided me with endless advice during the troubleshooting.

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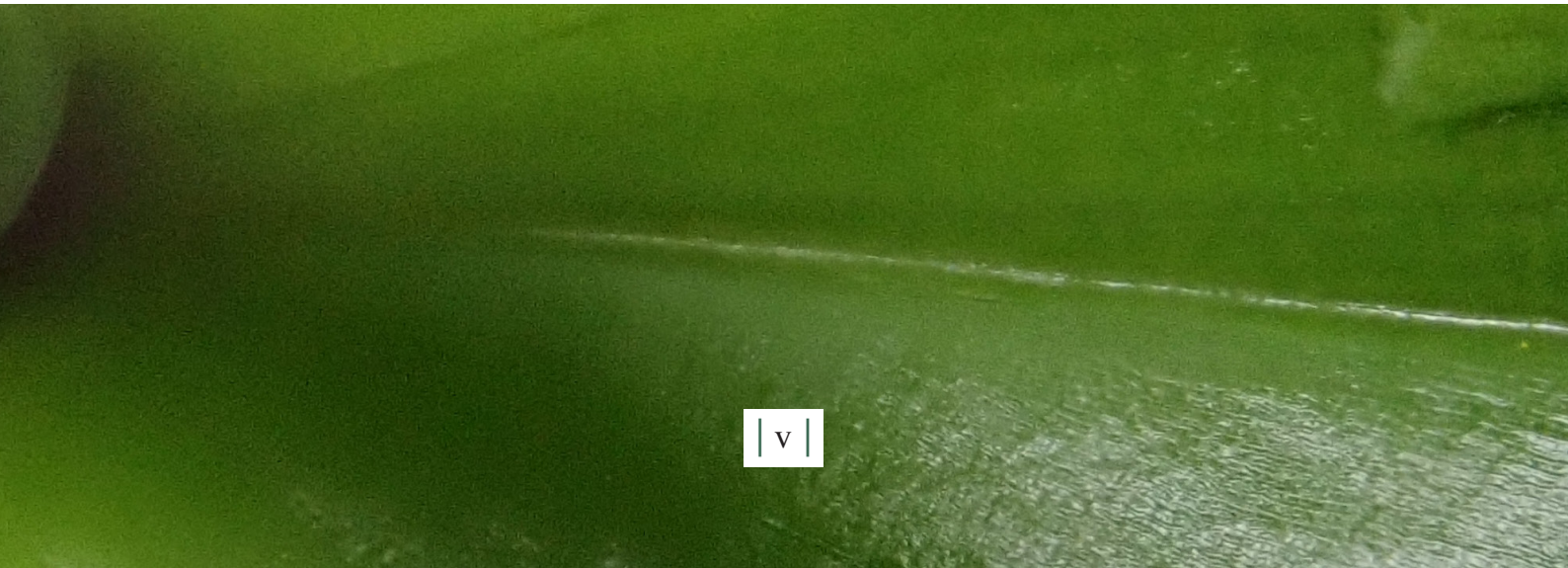
A word of thanks to Simon Milne for trying to induce stress and panic so I would hurry up with my writing in the last week. Last but not least I am indebted to my sister, Inge, who was of great help when I was formatting my thesis and for making the amazing cover drawing.



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1. INTRODUCTION

Costaceae is a relatively small family with ca. 120 herbaceous species distributed over all three major tropical regions (Table 1). They are typically terrestrial or rarely epiphytic and range from 0.5 m to 6.5 m (some *Tapeinochilos* Miq.) in height. The distinctive spiralling leafy shoot of Costaceae has made it a welcome inhabitant of tropical gardens and glasshouses, attracting considerable interest from horticulturists, scientists and the general public. Most of the scientific interest has been focussed on the neotropical Costaceae, where the majority of the species diversity can be found (Figure 1). As mentioned, the core diversity lies in the neotropics, where the genus *Costus* L. accounts for almost half of the species of the family's with ca. 50 species (André *et al*., 2016).

The family is not only distinguished from the other seven families in the Zingiberales by its spiral growth, but also non-aromatic leaves with a closed leafsheath and ligule. In growth form, they range from small to giant understory herbs, including epiphytes, with a terminal inflorescence on a leafy or leafless shoot, or in axillary clusters (*Monocostus* K.Schum., *Paracostus* C.D.Specht).

The most recent review of the family was conducted by Specht in 2006, producing a phylogeny showing that *Costus* only occurs in the Neotropics and Africa, and the Asian species were moved to two new genera; *Cheilocostus* C.D.Specht and *Paracostus* respectively (Specht, 2006; Specht and Stevenson, 2006).

Table 1: Species number and distribution of Costaceae.

Genus	No. of species	Region
Chamaecostus C.D.Specht & D.W.Stev.	8	South America
Costus L.	77	Central & South America, Africa
Dimerocostus Kuntze	3-5	South America
Hellenia Retz.	6-8	South-East Asia, Malaysia, New Guinea
Monocostus K. Schum	1	Peru
Paracostus C.D.Specht	2	Africa, Borneo
Tapeinochilos Miq.	20	Papuasia, tropical Australia

1. INTRODUCTION

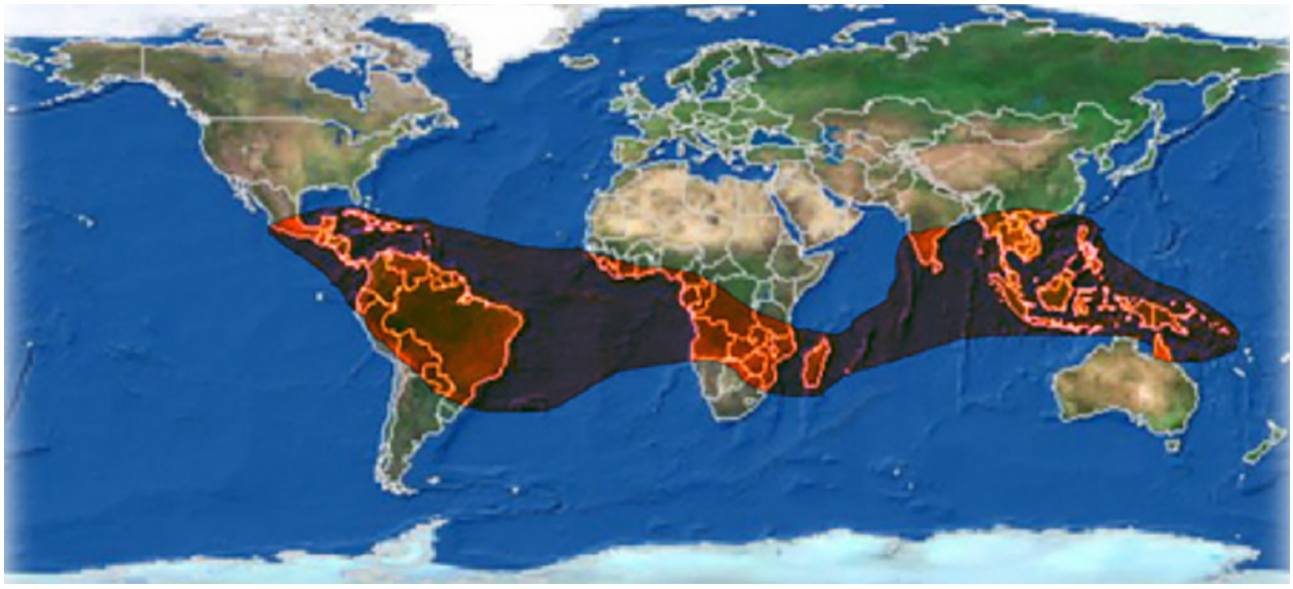


Figure 1: Global distribution map of the Costaceae – species numbers by region are: Neotropics: ca. 70 spp., Africa: ca. 25, Asia: ca. 30. Taken from the Smithsonian website (June 2019)

1.1 Short overview of the(family-level) taxonomy

Costaceae is forming a sister clade with Zingiberaceae, with Cannaceae and Marantaceae as their sisters in turn (Kress, 1990). It can be recognised easily from the other families in the order of the Zingiberales as follows: it is readily distinguished from bananas (Musaceae) and gingers (Zingiberaceae) through the solid, sometimes branched, stems that often have a characteristic spiral (monistichous) phyllotaxy (Kirchhoff and Rutishauser 1990). As its closest relative Zingiberaceae shares ligulate leaves with Costaceae. In Costaceae these emerge from a closed leaf sheath, whereas in Zingiberaceae the leaf sheath is open Costaceae has a unique floral structure within the Zingiberales with a single fertile stamen and five staminodes that have fused forming a petaloid labellum (Troll 1928; Kirchoff, 1988). The labellum accommodates for the pollination syndrome, with either an open or tubular shape, for bee and bird pollination. The labellum in Costaceae differs from the labellum found in Zingiberaceae, as in Costaceae it is made up by the fusion of five stamens, where in Zingiberaceae it forms by the fusion of the two lateral staminodes of the inner stamen whorl (Kress, 1990).

The currently accepted phylogeny shows that of the four old genera in the family, three were monophyletic (*Dimerocostus*, *Monocostus* and *Tapeinochilos*) whereas *Costus* was polyphyletic, with species occurring in three major clades (Specht, 2006). The majority of the species is placed in the *Costus* clade, which consists of Neotropical and African species. The rest is placed in either the Asian group –which consists of the *Cheilocostus* clade (sister to *Tapeinochilos*) and the *Paracostus* clade–

and the new *Chamaecostus clade*, which is sister to *Dimerocostus* and *Monocostus*. The *Cheilocostus* clade has only weak overall support and collapsed with *Tapeinochilos*, only held up by morphological support (Specht, 2006), which could indicate a recent divergence. More recent studies using only molecular results showed *Tapeinochilos* to be embedded within *Hellenia*, whereas *Paracostus* to be basal to the *Costus* clade (Böhmová, 2016, unpublished).

1.2 Nomenclatural controversy

When the genus name *Cheilocostus* was proposed for the Asian clade (Specht and Stevenson, 2006), *Costus speciosus* was chosen as the type specimen, based on *Banksea speciosa* J.Koenig (1783: 75), the only species –and therefore type– of *Banksea* J.Koenig (1783:75). *Banksea* is a later parahomonym of *Banksia* J.R.Forster & G.Forster (1775: 7), to which it should be treated as a homonym for its high similarity (Vienna ICBN Art. 53, 2006). *Cheilocostus* replaced *Banksea*. In 1791 Retzius, however, had already published a replacement name for *Banksea*, namely *Hellenia*. Almeida (2009) tried reinstating the genus name *Pyxa* Noronha, but this genus has never been validly published and lacking a description. Miquel later mentioned *Pyxa* as a synonym but does not change the validity. *Hellenia* is, therefore, the earliest legitimate name. The name has been used for a plethora of species that are now in *Alpinia* Roxb. (Zingiberaceae!), as it was published, albeit illegitimate, for *Hellenia by Wildenow in 1797.*

Because the application of the name *Hellenia* is confusing a proposal for its rejection and conservation of *Cheilocostus* was filed in 2016 (Leong-Škorničková and Šída, 2016). In this thesis, I will consistently use the name *Hellenia* over *Cheilocostus* as this is currently the accepted name until the conservation of another name has been decided.

1.3 Taxonomic history of the Asian species

1828 — Roscoe published his work on Monandrian plants, mainly drawn from specimens living in the botanic garden of Liverpool. He describes one species originating from Asia: *C. nipalenis* Roscoe, now synonymised with *H. speciosa*.

1899 — Schumann formed the subfamily Costoideae within the Zingiberaceae by combining the genera *Costus*, *Dimerocostus* and *Tapeinochilos*. In 1904, he published his monograph on Zingiberaceae in Engler's 'Pflanzenreich'. This study was mainly based on herbarium material and therefore often lacking floral descriptions which limited the taxonomical value.

1924 — Ridley in the Flora of the Malay Peninsula. Ridley lists several species closely related to *Costus globosus.*

1950 — Holttum in Garden's bulletin of Singapore. Three globosa-like species published by Ridley are published here as varieties of *Costus globosus*. These species were mainly based on the indumentum of the inflorescence and the leaves.

1979 — Maas published his notes on Asiatic and Australian Costoideae, after an extensive study on the neotropical members of the family. A key was included for the native and commonly cultivated species, excluding *Tapeinochilos*.

2001 — Specht et al. published a phylogeny based on mainly plastid markers, showing the ancestral floral state and the limited phylogenetic use of pollination syndrome. Until then used for taxonomic and classification purposes.

2006 — Specht published a new phylogeny for Costaceae based on chloroplast and nuclear marker, combined with a morphological dataset. All Asian species are grouped in a distinct clade, with *Paracostus* as a basal clade.

2006 — 2008 Meekiong et al. publishes four new Bornean species in *Costus*, three in *Costus* subgenus *Paracostus*, one supposedly closely related to *H. speciosa*. All descriptions contain serious mistakes, missing essential parts of the description, or have parts of the description cut and pasted in the description of another species. These errors severely impact the overall quality of the paper.

2010 — Poulsen & Specht publish a new species in *Hellenia*, *Cheilocostus borneensis*. A white-flowered species with a radical inflorescence, but without the woolly stamen.

1.4 The island of Borneo: physical features, biogeography and conservation

The island of Borneo is the third-largest island in the world (Meiri *et al.*, 2008) and has been identified as a biodiversity hotspot, with its lowlands containing more vascular plant species than any other ecoregion in the world (Kier *et al.*, 2005; Slik *et al*., 2009). Borneo's forests are different from most forests in the world, as they are mainly covered by Dipterocarpaceae, and have a relatively open canopy due to a higher average tree height and density of emerging trees (Gentry, 1988; Slik *et al.,* 2003, 2009). The island lies on the equator, in the wettest part of Indonesia, getting high temperatures throughout the year (MacKinnon 1996). These conditions combined with Borneo's geological and climate history have led to speciation and high levels of species diversity.

Borneo has a central Mountain range from north to south, with its highest peak Mt. Kinabalu, which extends 4095 meters above sea level (Figure 2).

The island's geological history, in an active and young region, is characterised by its repeated connection –and loss– with the Southeast Asian mainland and the island of Sumatra

and Java during glacial periods (Morley 2000). Together with a high rainfall gradient (nearly 3 m at 1585 to 4566 mm annually), an altitude gradient of more than 4000 metres and its geological history, there are a plethora of ecosystems, ranging from mangroves, kerangas and limestone soils to ultramafic soils, montane and alpine forests (MacKinnon *et al.* 1996)

Borneo is separated to the east from Sulawesi by the Makassar Strait, which even with glacial sea-level fluctuations, never has been less than 75 kilometres (Hall, 2009; Poulsen 2012). This distance has formed a dispersal barrier for both plant and animal life, as observed by Wallace, leading to his infamous Wallace's Line (van Welzen, Parnell and Slik, 2011).

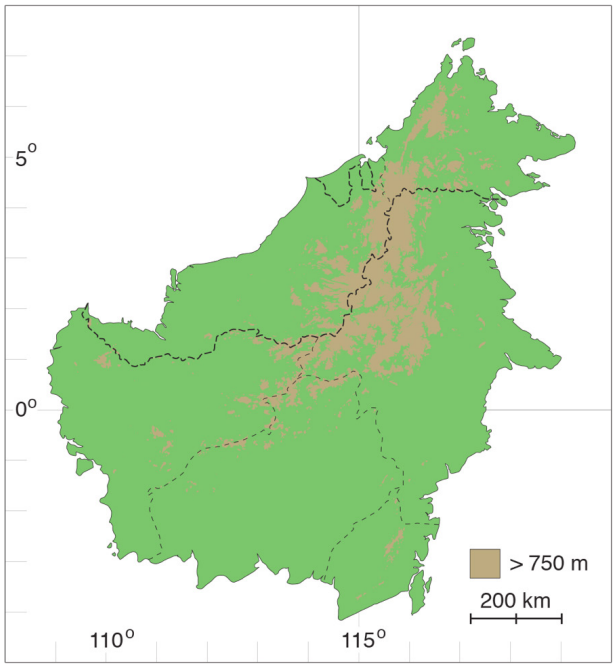


Figure 2: Map of the island of Borneo, showing country and state lines, and the central mountain range extending from north to south.

The biogeography of Borneo is challenging due to the island being on the border of two palaeocontinents that have been separated for a prolonged period (Turner, Hovenkamp and van Welzen, 2001), and Bornean montane areas have served as a forest refugium during glacial periods (Morley & Flenley 1987; Bird, Taylor and Hunt, 2005). Apart from vicariance, two dispersal patterns are to be expected: a pattern of Southeast Asian –possible Laurasian– origin towards Australasian areas, and the opposite pattern of Australian –Gondwanan– flora moving towards the Malay peninsula (Turner, Hovenkamp and van Welzen, 2001). Both Southeast Asian and Australian flora are present in the Pacific, which further complicates the analysis of patterns. Floristic patterns show that diversity is most significant in south-east Borneo, and central Sarawak, and that the central mountain range forms a dispersal barrier, especially for the woody flora (Slik *et al.*, 2003).

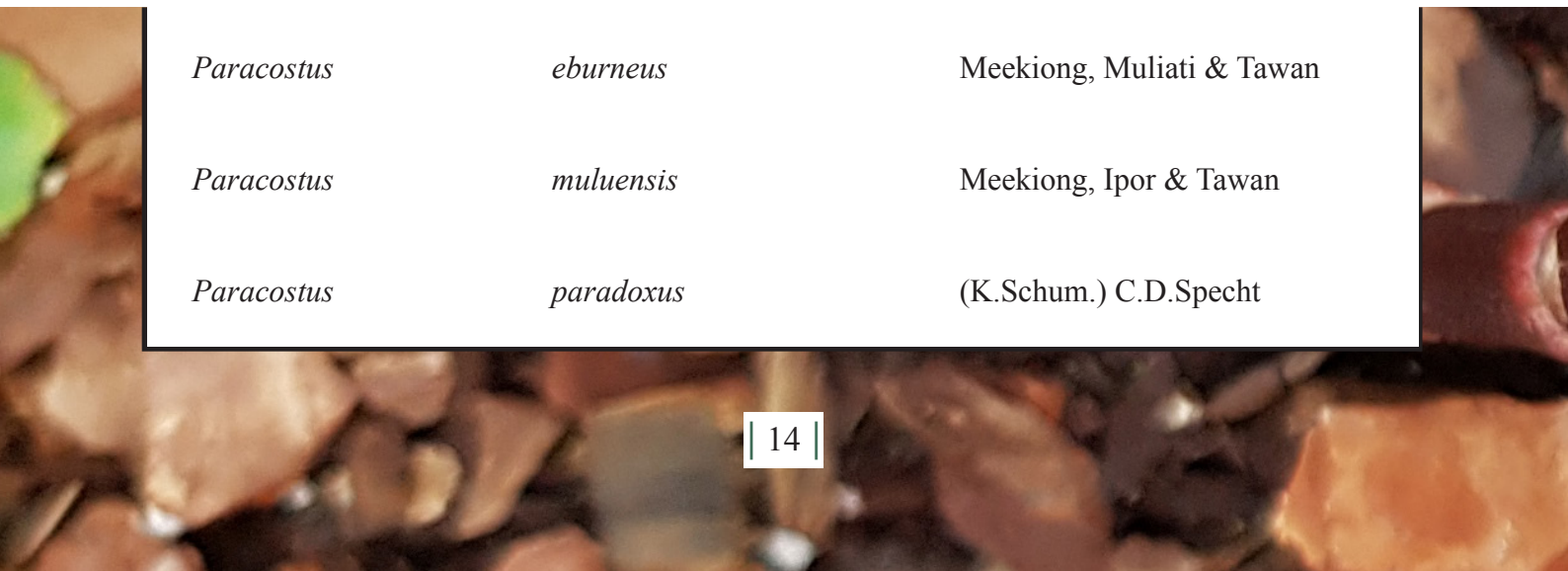
The island is under significant threat from deforestation and the oil palm industry (Hansen *et al.*, 2013), with an estimated loss of fifty per cent of its original forest cover, and 10 per cent of the island taken up by industrial palm and timber plantations (Brookfield and Byron, 1990; LM et al., 2004; Langner, Miettinen and Siegert, 2007; Gaveau *et al.*, 2014, 2016; McAlpine *et al.*, 2018). The effect on forest loss changes the local climate, which could further change the already damaged biodiversity (McAlpine *et al.*, 2018).

1.5 Number of species of Costaceae in Borneo

On the island of Borneo, only two genera naturally occur *Hellenia* Retz. and *Paracostus*. As currently recognised *Hellenia* is represented by a species complex; *H. globosa* and *H. speciosa*. *Paracostus* is represented by only one species, *Paracostus* paradoxus. Meekiong *et al*. (2006) did not accept this classification arguing that the genera had not been sufficiently sampled and therefore placed four new species in *Costus*, three of which were published in the subgenus *Paracostus* (*C. bullatus* Meekiong, Muliati & Ipor, *C. eburneus* Meekiong, Muliati & Tawan and *C. muluensis* Meekiong, Ipor & Tawan, followed two years later by, *Costus mulus* Meekiong, Ipor & Tawan. According to the authors, the latter is related to *Hellenia speciosa* (Meekiong et al., 2008). Including these names, as well as Cheilocostus borneensis A.D.Poulsen (Poulsen *et al.*, 2010) the total number of species in Borneo is currently to seven species, in three genera (Table 2).

Table 2: List of current accepted species in Borneo.

Genus	Species	Authority
Hellenia	*borneensis*	(A.D.Poulsen) Govaerts
Hellenia	*globosa*	(Blume) S.R.Dutta
Hellenia	*speciosa*	(J.Koenig) S.R.Dutta
Paracostus	*bullatus*	Meekiong, Muliati & Ipor
Paracostus	*eburneus*	Meekiong, Muliati & Tawan
Paracostus	*muluensis*	Meekiong, Ipor & Tawan
Paracostus	*paradoxus*	(K.Schum.) C.D.Specht



1.6 Aims and objectives

The main goal of this thesis is to revise the taxonomy of Bornean Costaceae. This taxonomic treatment will be backed up by data from the Morphological, Molecular and Cytological chapters. The Morphological chapter should lay the foundation on which the molecular chapter can by built by providing general characters, and a discussion on specific characters that are of possible taxonomic value and can possibly be used for species delineation.

The molecular chapter will hopefully result in clearly distinct groups on at least genus level. Together with the cytological counts from the cytology chapter this should result in complete species descriptions and a key for identification that can be used in the field. The impact of which reaches further than just Borneo, as it will raise the understanding of the Asian Costaceae for the cytology and clear up some of the taxonomic problems.

2. Morphology

2. MORPHOLOGY

Terms and definitions are used following 'Plant Identification Terminology' by Harris & Harris (2001).

2.1 Material studied

Morphological characters were studied using herbarium specimens and pickled material from RBGE (E), images of herbarium specimens were examined from Aarhus (AAU), Leiden (National herbarium) (L), and the Kepong Herbarium (KEP).

Specimens in the living collections were examined and where possible dissections were made and flowers pickled for later reference. Fresh material was collected from reaches further than just Borneo, as it will raise the understanding of the Asian Costaceae for the cytology and clear up some of the taxonomic problems.

2.2 General morphology

Habit

The plants are herbaceous, terrestrial herbs and generally 0.5 to 3 m tall. The tallest species is *Hellenia globosa*, reaching up to 5 metres. *Paracostus* species do not often extend above 1 m. The rhizomes are often clustered from which the shoots grow vertically, with *Paracostus* species often showing a more prostrate habit.

The shoots can have slightly swollen nodes (*Paracostus*) and often grows in a spiral, showing a monistichous phyllotaxy. The nodes are were branching (*Hellenia*), inflorescences (*Paracostus*) and roots (*Paracostus*) can occur. The leaf sheaths also originate at the node, covering the internode in a closed sheath, with the leave and or ligule arising at the apex. The sheaths are green, hazel (*H. globosa*), (*H. borneensis*), reddish-brown (*P. paradoxus, P. muluensis*) or red (*H. speciosa*). In some species, the sheaths can fall apart or disintegrate into fibres. Both the sheaths and the leaves appear in a monistichous phyllotaxy on the shoots. The shoots either have a terminal inflorescence or are vegetative, with a radical inflorescence. It has not yet been observed in Asian Costaceae that species have multiple types of shoots.

Leaves

When present, the ligule arises at the apical margin from the sheath and is up to 8 mm long. In *Paracostus* the ligule often disintegrates into fibres as the plant reaches maturity, with only small, herbaceous ligules found in young shoots. The ligule is often green, turning yellowish-brown when decaying.

The indumentum of the sheath, ligule and the petiole is most often similar to the indumentum found on the lamina. The petiole is generally short, but can be up to 16 mm long, and slightly swollen. The number of leaves on a shoot varies from two (*P. paradoxus*) to many,

sometimes clustered at the apex (*H. borneensis*). In *C. muluensi*s and *C. bullatus*, the young leaves can be brownish-red. The lamina is generally slightly fleshy, elliptic, but can be narrow or broadly ovate or obovate. It can reach a size of 10 – 42 by 3 – 16 cm. *Costus bullatus* and *C. eburneus* have a bullate leaf surface, whereas a (slightly) plicate surface is more common.

Indumentum on the lamina occurs almost exclusively on *H. globosa* and *H. speciosa*, and is often short, soft, and can be is on the lower side, whereas the upper side is usually glabrous. The base of the lamina is mostly rounded or cuneate. The apex is generally acute to acuminate, in *Paracostus the acumen* can be elongated.

Inflorescence

The inflorescence consists of two parts: the peduncle (scape) and the flowering head (spike) of which the latter is composed of bracts and flowers. The inflorescence can either be terminal (*H. speciosa*), emerging from the base of the shoot as a radical inflorescence (*H. borneensis, H. globosa, H. mulus*), or with flowers from the nodes/rhizome (*Paracostus*). Although the radical inflorescence can become elongated and pendant (*ADP 1801*), it is generally prostrate. *Paracostus* inflorescences are few-flowered, with 2 to 5 flowers per inflorescence, and 2 to 5 inflorescences per plant. In *Paracostus* species, the length of the peduncle is dependent on the origin of the inflorescence. If it arises above ground from the nodes, it is almost absent, whereas if it arises from the rhizome or below-ground it can reach a length of up to 6 cm. The peduncle is often covered in bracts or scales. The length of the inflorescence is skewed mainly by the length of the peduncle, which in some extreme cases from H. globosa can be up to 80 cm (*ADP 1801, ADP 1803*). The length of the scape, however, will not exceed 15 – 20 cm. The bracts are spirally arranged on the spike, with one flower in the axis, which is subtended by a single bracteole. In *H. speciosa* there is a continuous transition from sterile to the lowermost fertile bracts, which may be red, brown, white or green. The bracts are membranous in *Paracostus* and coriaceous in *Hellenia*. An essential character for *H. globosa* is the spiny, pungent, bracts.

Flowers

A Costaceae flower consists of several tubular organs placed within each other (Figure 3). The flowers consist of a 3-lobed calyx, a corolla with three petals, a petaloid labellum (five fused staminodes), one petaloid fertile stamen and a gynoecium that contains an inferior 3-locular ovary. The style is positioned between the thecae of the stamen, with a bilamellated stigma and a dorsal appendage. The colour of the flower (mainly from the corolla and the labellum) ranges from white, to yellow, orange and red. The labellum can have nectar guide, as observed in *H. borneensis, H. globosa* and *P. paradoxus.* The calyx is often the same colour as the bracts and has 3 triangular to deltate lobes. The membranous corolla lobes arise from the apex of the floral tube (extends from the basal part of the ovary to the divergence point of the corolla lobes), and are often elliptic with an acute to rounded apex.

Figure 3: General morphology of a Costaceae flower. **A:** The floral (Ft) and staminal (St) tube and the stamen are indicated. **B:** Detail of the pungent bracts and calyx of *Hellenia globosa*. **C:** Detail of the pilose stamen of *H. globosa.* **D:** Thickened anther cushion in *P. muluensis*. **E:** Detail of the anther crest of *Paracostus muluensis*.

The labellum consists of a joined basal tube that is fused with the petals (floral tube), a staminal tube, and the lobe. The overall shape of the flower is often dictated by the shape of the labellum lobe, which can be either open horizontally flattened (*H. speciosa*) or more funnel-shaped (*H. globosa*) due to a small labellum lobe. The labellum is usually glabrous, although some varieties of *H. globosa* are distinguished by their different types of indumentum on the labellum, calyx and bracts. Glutinose hairs are present in the labellum tube of all Bornean *Paracostus* species.

Nectar guides are found in almost all species, being absent only in *H. speciosa* and some *H. globosa*. It manifests itself as a central, lighter coloured patch or red/purplish lines in the throat of the labellum.

The stamen is generally narrowly elliptic in shape, and bend inwards covering the throat of the labellum. The anther crest is generally reflexed, curling outwards showing the often striped or spotted pattern. The anther is sometimes placed on a thickened punctate cushion (*P. paradoxus*). The apex of the anther crest can be deeply bilobed, trilobed, or fringy with lateral teeth. Although not observed, it has been described to be rounded in some populations.

The gynoecium consists of an inferior ovary style is slender, and lies between the thecae of the stamen wedged in with a dorsal (bilobed) appendage near the apex. The stigma is bilamellate in all Bornean species.

Regarding Costus in Borneo

Based on the general morphology (the inflorescence coming from the nodes, and the small, often prostrate growth form, show that the *Costus* species published by Meekiong et al. (2006) do belong in *Paracostus*, which is not surprising given they were placed in the subgenus *paracostus*. From here onward these species will be referred to as *Paracostus* instead of *Costus*.

Figure 4: Variation in labellum colour in *Hellenia globosa*. Photos courtesy of A.D. Poulsen.

2.3 Character discussion

The undulated, or bullate, leaves, as described by Meekiong (2006) for two species of *Costus*, is a rare character trait in the family. In the whole family, it occurs in two other species: *Costus tappenbeckianus* J.Braun & K.Schum from Africa and *Costus varzearum* Maas from the Neotropics. In these species it occurs frequently, but not always (Maas 2019, pers. comm.). This indicates that this character is not of diagnostic quality. The Bornean species, *Costus bullatus* and *Costus eburneus* being hyper endemic, reinforces this idea, these are localised populations with a bullate leave morphology and may well be distinctive species, but more descriptive characters are needed to separate these on a species level.

There is a remarkable range of colours for the labellum in *Hellenia globosa*. *Paracostus* shows limited variation, with mostly a white labellum with a central yellow patch, but in *H. globosa* it can range from white, with a yellow patch at the apex (*A.D. Poulsen 1801, 1802 – 1805, A.D. Poulsen 2041*), yellow (*A.D. Poulsen 1804*), Red (*JSKOR 74757, JSKOR 74764*), orange (*A.D. Poulsen 2136*) (Figure 4). The overall morphology of these flowers is very similar, with labellum colour and indumentum being the most pronounced differences (Ridley 1924, Holttum 1950). As already posed by Holttum (1950) this alone does not warrant separate species, but more studies will have to be done, especially focussing on the underlying molecular differences.

A character that is less variable is the length of the peduncle. Multiple specimens have been observed with an extremely long peduncle (>50 cm), which in some cases caused the inflorescence to become pendant (*A.D. Poulsen 1801 – 1803, Ambri AA1578*).

he pilose indumentum on the dorsal side of the stamen does not occur in all species in the complex either. Although not occurring not occurring in Borneo, *Costus tonkinensis* Gagnep. does not have a pilose stamen. *Costus mulus* on the otherhand is more similar to *H. globosa* in this aspect.

A character that brings all species together in the *globosa*-complex are the pungent bracts and calyx. This gives the strong suspicion that *Costus mulus* is closely related to *Hellenia globosa* and should be placed in the species complex.

The anther crest shows an exciting variation within *Paracostus*. Maas (1979) and Schumann (1899, 1904) describe the apex as obtuse or bilobed. *Paracostus muluensis* has a fringed apex, with two small lateral teeth, whereas all specimens observed from *Paracostus paradoxus* had a deeply bilobed apex, even though it was described by Meekiong *et al*. (2006) as deeply lobed or acute. In the descriptions of *P. bullatus* and *P. eburneus* they are described as bilobed or mucronate in *P. bullatus* and mucronate in *P. eburneus*. A slide from a specimen with a similar flower morphology as *P. eburneus* (having pinkish lines in the throat) show a deeply bilobed anther crest, which corresponds

to *P. bullatus*. This specimen (*A.D. Poulsen 2081*) does not have bullate leaves however. This could indicate that P. *bullatus* and *P. eburneus* are closely related on possibly a lower level than species level, and it reinforces the earlier mentioned idea that bullate leaves is a population character, and does not delineates on a species level.

In his monograph on neotropical Costaceae Maas covers the two stigma types that occur in the family: 1. Bilamellate, consisting of two appressed, crescent-shaped structures, with a dorsal appendage, to anchor it to the anther. Which occurs in the old *Costus* subgenus *Costus*. 2. Cup-shaped, often ciliate margins and no two-lobed appendage, which occurs in the old *Costus* subgenera *Cadalvena, Monocostus* and *Dimerocostus*.

The species in *Hellenia* and *Paracostus* both have the bilamellate stigma with variable dorsal appendages on the style. Maas only mentions the character for *Paracostus paradoxus* but gives no description. It is a problematic character to see from herbarium study, as flowers, if available, would need to be rehydrated. Specht (2006) published a plate with SEM images of stigma and appendage shape in Costaceae, including both *Hellenia speciosa* and *Paracostus paradoxus*. In Figure 5, the variety of appendages is shown. *Costus muluensis* (5A) does not have a bilobed appendage. Instead, it has a single, rounded bulge. *Paracostus paradoxus* (5B) has a much broader, pronounced bilobed appendage. A horned bilobed appendage was observed in some specimens of *Hellenia globosa* (5C), but this was not always present (5D). This character shows an exciting variety between species; there is, however, insufficient knowledge for its use as a diagnostic character. Further studies, using a scanning electron microscope would be of great interest.

2.4 Problematic description

For some publications it was difficult to extract the right information. In the papers published by Meekiong et al. (2006, 2008), the stamen was consistently described as a corolla lobe.

This resulted in that the description of the stamen was under corolla, for which the number of organs is three, so in in these papers the number of stamens was always three. This was the other way around as well, as description of the actual corolla was under the stamen section.
From the drawings it was clear that the authors had limited understanding of the floral morphology, as the thecae are consistently labelled as the stigma's (Meekiong *et al.*, 2006, 2008). The drawings of combined organs like a flower or inflorescence repeatedly had scalebars that did not match up with the description, or would result in inaccurate sized flowers. It was therefore chosen to not include these drawings into this thesis, but they can be found in appendix 3.

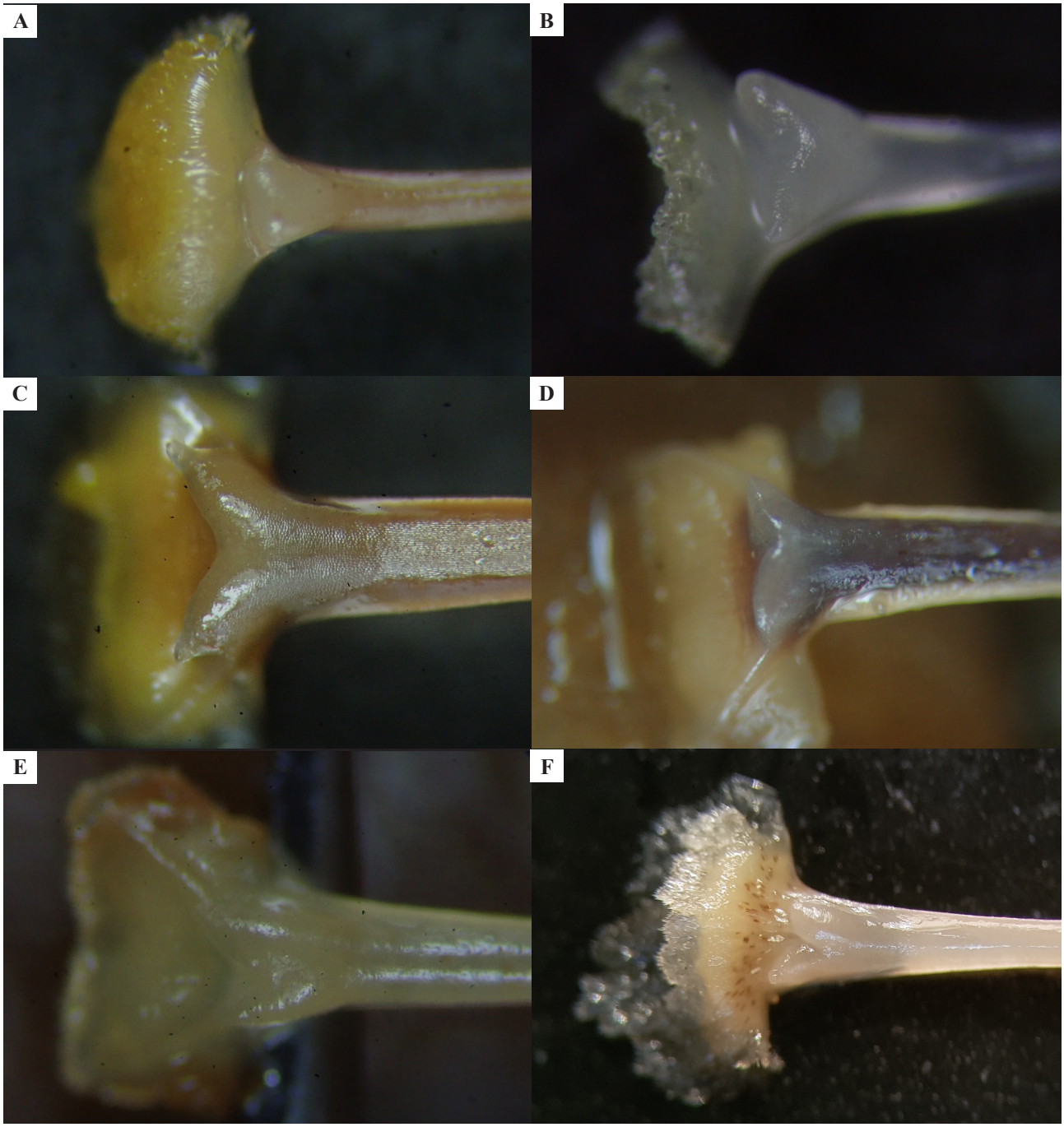
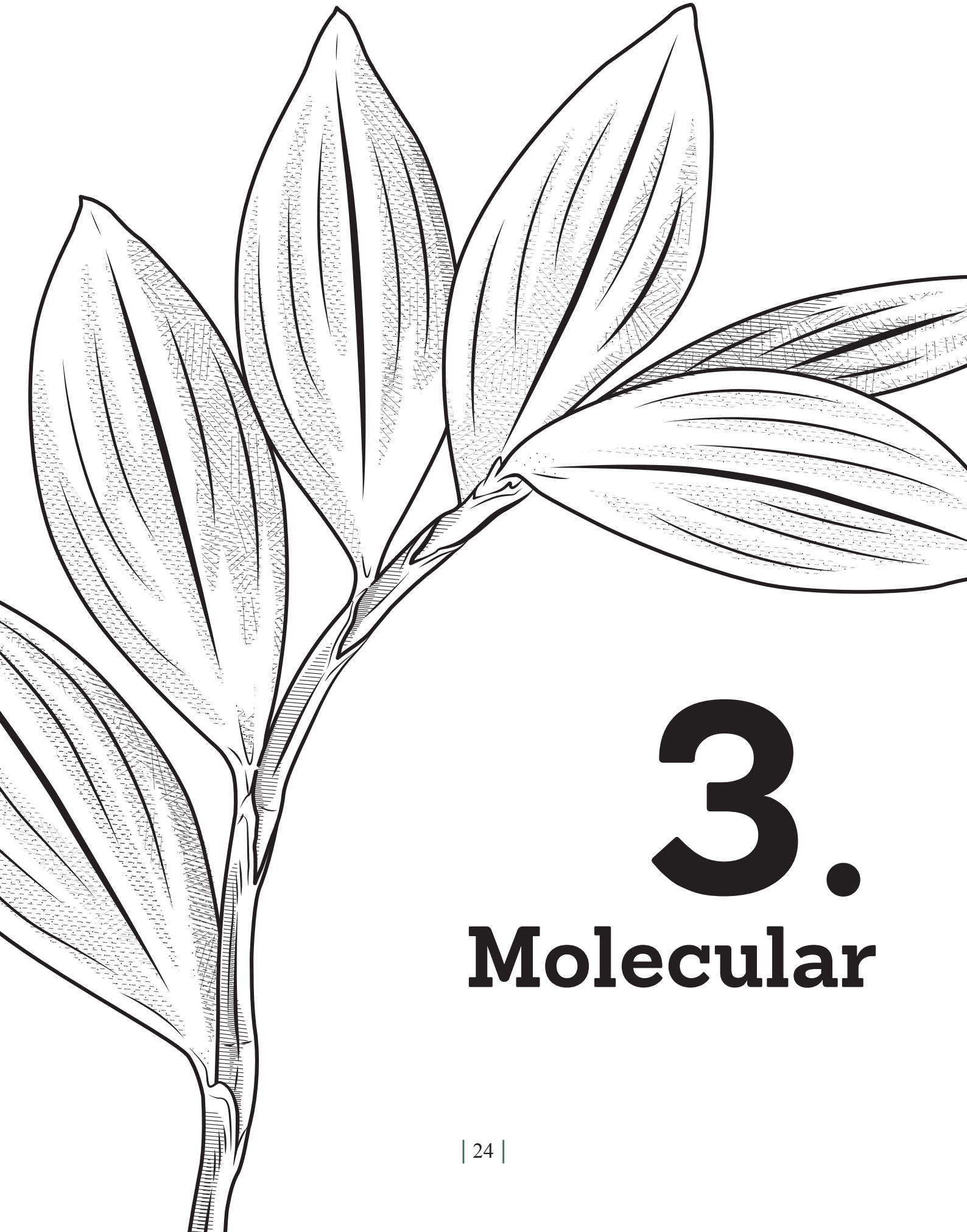


Figure 5: The diversity of style appendages in the Bornean Costaceae. **A:** The rounded appendage of *Paracostus muluensis*. **B:** *Paracostus paradoxus*. **C:** a horned bilobed appendage in *Hellenia globosa*. **D:** hornless bilobed appendage of *H. globosa*. **E:** The almost appressed appendage of *H. speciosa*. **F:** V-shaped bilobed appendage of *H. borneensis* .



3. Molecular

3. MOLECULAR

3.1 Taxon sampling and Material Collection

The Taxon sampling was set up to cover a full range of taxonomic and morphologic diversity of the Asian Costaceae, with a focus on the species from Borneo. Table 3 lists the taxa examined in this study.

Leaf material for molecular analysis was collected from various sources; from living collections at RBGE, from wild collections by Axel Poulsen from various Asian countries, and my samples collected during the 2019 MSc field trip in Colombia, adding species from *Dimerocostus* and neotropical *Costus*. Clean, healthy leaves were selected for silica drying, torn into small pieces and placed into a tea bag, which was then placed in a container with silica for at least 24 hours.

3.2 DNA region selection

The different genomes in plant evolve at different rates (Wolfe, Li and Sharp, 1987). For the construction of a useful phylogeny, it is essential to choose the appropriate gene regions. Poorly resolved phylogenies can be caused by genome regions evolving too fast, causing long-branch attraction, or too slow due to increased homoplasy (resulting in polytomies). The nuclear genome evolves the fastest, with chloroplast DNA evolving at half the speed of nuclear DNA. Mitochondrial DNA has the slowest evolutionary rate, which is likely caused by a lower mutation rate (Wolfe, Li and Sharp, 1987). The chloroplast genome is frequently used for taxonomic studies on genus and species level, as this generally results in well-resolved phylogenies. Initially, six regions were chosen for this study based on previous studies and expert advice (Specht *et al*., 2001; Salzman *et al*., 2015; André *et al*., 2016, Specht 2019, Pers. comm.). Four chloroplast regions: *matK*, *rps16*, *psbA-trnH* and *trnL-F*, and two nuclear: Calmodulin (*CaM*) and *ITS*. Due to problems with the PCR (which will be discussed later on), only two of these regions were further used for further study: *trnL-F* and *CaM*.

The first region, *trnL-F*, contains the *trnL* intron, and *trnL-F* intergenic spacer (Figure 6). This is a non-coding region of roughly 1200 base pairs, which has been extensively used in phylogenetic studies on angiosperms (Gielly *et al*., 1996) and within the family (Specht *et al*., 2001; André *et al*., 2016).

The second region is Calmodulin, which is a nuclear intron located between two strongly conserved exons, *CaM* exon 1 and 2 (Johansen, 2005). It has been used for phylogenetic studies in Costaceae.

Table 3: List of the taxa examined in this molecular study.

Taxon name	Accession number	EDNA number	Principal collector	Collection number	Country of Origin	Collection date
Paracostus		EDNA19-0053638	Poulsen A.D.	ADP2031	MY	02/07/2003
Hellenia globosa		EDNA19-0053639	Poulsen A.D.	ADP2041	MY	05/07/2003
Hellenia sopuensis		EDNA19-0053640	Poulsen A.D.	ADP2736	ID	13/01/2009
Hellenia borneensis		EDNA19-0053641	Poulsen A.D.	ADP1964	MY	08/12/2002
Hellenia speciosa		EDNA19-0053642	Poulsen A.D.	ADP1806	MY	23/03/2002
Paracostus paradoxus	19622228	EDNA19-0053643	Poulsen A.D.	ADP2465	MY	09/10/2006
Paracostus paradoxus	19773474	EDNA19-0053644	Pim van Caspel	PvCaspel5	MY	16/05/2019
Tapeinochilos		EDNA19-0053645	Poulsen A.D.	ADP2903	PG	05/05/2013
Hellenia speciosa		EDNA19-0053656	Poulsen A.D.	ADP2604	ID	15/02/2008
Costus pulverulentus		EDNA19-0053657	Gutierrez, FF	FFG5359	CO	19/01/2019
Costus lima		EDNA19-0053658	Gutierrez, FF	FFG5360	CO	19/01/2019
Dimerocostus		EDNA19-0053659	Gutierrez, FF	FFG5358	CO	18/01/2019
Paracostus paradoxus		EDNA19-0053660	Poulsen A.D.	ADP2070	MY	17/07/2003
Paracostus paradoxus		EDNA19-0053661	Poulsen A.D.	ADP2081	MY	19/07/2003
Paracostus		EDNA19-0053662	Poulsen A.D.	ADP3027	MY	06/09/2016
Tapeinochilos pubescens		EDNA19-0053663	Poulsen A.D.	ADP3079	MY	06/04/2017
Hellenia speciosa		EDNA19-0053664	Poulsen A.D.	ADP2837	TH	21/09/2010
Hellenia globosa		EDNA19-0053665	Poulsen A.D.	ADP1803	MY	23/03/2002
Paracostus paradoxus	19773484	EDNA19-0053666	Pim van Caspel	PvCaspel6	MY	16/05/2019
Tapeinochilos		EDNA19-0053667	Poulsen A.D.	ADP2999	PG	04/04/2016
Tapeinochilos hollrungii		EDNA19-0053668	Poulsen A.D.	ADP3011	PG	08/04/2016
Hellenia globosa	20070757	EDNA19-0053669	Pim van Caspel	PvCaspel7	ID	28/05/2019
Hellenia globosa	20070755	EDNA19-0053670	Pim van Caspel	PvCaspel8	ID	28/05/2019
Costus oligophyllus		EDNA19-0053671	Sam Y.Y.	FRI69251	MY	19/01/2019

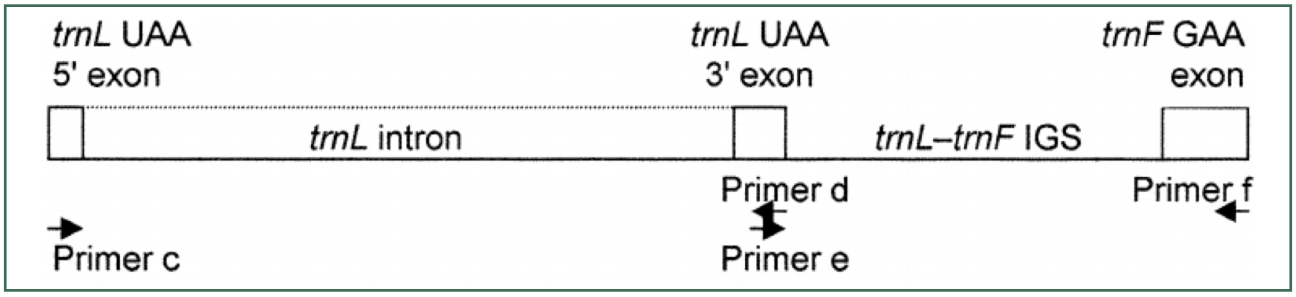


Figure 6: Primer map of *trnL* intron and *trnL-F* intergenic spacer. Primer c to f were all used in this study.

3.3 DNA Extraction

DNA was extracted from silica-dried leaf material using Qiagen DNeasy Plant Mini Kit following the Qiagen DNeasy Plant Mini Kit protocol. To ensure high DNA yields and to avoid DNA degradation, a complete and quick disruption of the starting material is needed. To extract genomic DNA, silica gel dried leaf material was placed in a 2 ml Eppendorf tube with a tungsten cone ball. The leaf material was then disrupted into a fine powder using the TissueLyser II, set to 2 minutes at 20 Hz, with repeats and rotations of the adapters if necessary. The samples were lysed by adding 400 µl AP1 Buffer, vortexed and incubated for 80 minutes at 65ºC in a Thermomixer set at 800 rpm. Following lysis, 130 µl P3 Buffer was added to the lysate and incubated on ice for five minutes.

The samples were then centrifuged to condense the precipitates for five minutes at 13,000 rpm. The clear lysate was then pipetted into the QIAshredder Mini spin column and again centrifuged, for two minutes at 13,000 rpm. The spin-column catches the precipitate while allowing the lysate to flow through.

The flow-through was then pipetted into a new 2 ml tube and 650 µl AW1 Buffer was added to improve the binding of DNA with the DNeasy membrane in the DNeasy spin column. This mixture was then moved into a new DNeasy mini spin column and centrifuged for one minute at 8,000 rpm. After discarding the flow-through this step was repeated using the remainder of the lysate/buffer mixture, afterwards the spin column was placed into a new 2 ml tube.

AW2 Buffer was then added and centrifuged twice, one minute at 8,000 rpm and two minutes at 13,000 rpm, with the flow-through discarded between spins. 75 µl AE Buffer was then pipetted directly into the spin column, which was placed in a new 1.5 ml tube and spun at 8,000 rpm to elute the pure DNA, which was done twice to wash off all the DNA. AE Buffer contains EDTA to prevent DNA degradation by magnesium. The extracted DNA was stored at -20ºC to prevent denaturation.

3.4 Polymerase Chain Reaction

Two regions were amplified for all accessions, and three regions were tested on eight samples, using the primers listed in Table 4. All regions were tested on the first eight extracted samples (Table 3). *ITS*, *rps16* and *psbA-trnH* were not further pursued after this, only *trnL-trnF* and Calmodulin was focussed on due to limited time for troubleshooting.

A general PCR recipe (The primers that were ordered for Calmodulin were shipped dry, so had to be rehydrated. Rehydration is done by adding in sterile deionised water (sigma water). To make a 100 µM stock the nmol (found on the tube is multiplied by 10, which gives the amount of sigma that needs to be added. This needs to be diluted to a working stock, which was done by mixing 20 µL of stock primer, and 180 µl of sigma water.

A general PCR recipe (Table 5) was used for all regions chloroplast regions and *ITS*. Modifications were made where necessary. The PCR recipe for Calmodulin (Table 6) was different, due to the use of Thermo Scientific Phire hot start II DNA polymerase to better replicate current studies.

A master mix was made for each PCR reaction by multiplying the volumes for all ingredients in Table X by the number of samples + 1(the negative control) except for the template DNA. The master mix was prepared in a 0.2 ml reaction tube, vortexed and centrifuged. 19 µl of the master mix was then pipetted in 0.2 ml reaction tubes, with the excess used as a negative control. 1 µl of DNA was added to each reaction tube –except the negative control– adding to a total reaction volume of 20 µl. Additives were used to increase the qualitative output of the PCRs. As additives either CES or TBT-PAR were used, except for Calmodulin, for which DMSO was used.

The primers that were ordered for Calmodulin were shipped dry, so had to be rehydrated. Rehydration is done by adding in sterile deionised water (sigma water). To make a 100 µM stock the nmol (found on the tube is multiplied by 10, which gives the amount of sigma that needs to be added. This needs to be diluted to a working stock, which was done by mixing 20 µL of stock primer, and 180 µl of sigma water.

PCR reactions for *trnL-trnF* were performed using the protocol outlined in Table 7. The protocols for *ITS*, rps16 and *psbA-trnH* were very similar and can be found in Appendix 1. For Calmodulin a gradient PCR was performed to find the optimum annealing temperature, due to extreme differences in the literature found, ranging from 53ºC to 66 ºC (Johansen, 2005; Salzman *et al*., 2015). Multiple different protocols have been used to get the optimal results for the Calmodulin region, which will be covered more in dept in the troubleshooting section below. The protocols, however, revolved around the standard protocol from Thermo Scientific, which is given in Table 8.

Table 4: *Primer pairs and annealing temperatures used in this study. Primers were used for both amplification and sequencing.*

Primer	5'-3'	Annealing temperature (ºC)	Reference
trnL-c	CGA AAT CGG TAG ACG CTA CG	52.5	Taberlet et al. 1991
trnL-d	GGG GAT AGA GGG ACT TGA AC	52.5	
trnF-e	GGT TCA AGT CCC TCT ATC CC	52.5	
trnF-f	ATT TGA ACT GGT GAC ACG AG	52.5	
CaM Costus F	TGC TTC TCT CGA ACG CTA GAT	53	Salzman et al. 2015
CaM Costus R	GAA ACT CGG AAT GCC TCC TT	53	
ITS 7P	GGT GAA GTG TTC GGA TTG C	55	White et al. 1990
ITS 4	TCC TCC GCT TAT TGA TAT GC	55	
ITS 5	GGA AGG AGA AGT CGT AAC AAG	55	Moeller & Cronk 1997
ITS 8	CAC GCT TCT CCA GAC TAC A	55	
rps16	AAA GTG GGT TTT TAT GAT CC	55	Shaw et al. 2007
rps16	GTT GCT TTY TAC CAC ATC GTT T	55	
psbA-trnH	GTT ATG CAT GAA CGT AAT GCT C	50	Shaw et al. 2005
psbA-trnH	CGC GCA TGG TGG ATT CAC AAA TC	50	

Table 5: *Reagent volumes for one sample of PCR.*

Reagent	Volume (µl)
dH_2O	6.1
10X NH4 Buffer	2
MgCl2	0.6
dNTPs	2
Primer forward	2
Primer reverse	2
Additive	4
Biotaq polymerase	0.3
DNA Template	1
Total volume	20

Table 6: *Reagent volumes for one sample of PCR using Phire II hot start DNA polymerase.*

Reagent	Volume (µl)
dH_2O	9.6
5x Phire Buffer	4
dNTPs	0.4
Primer forward	2
Primer reverse	2
DMSO	0.6
Phire Hot Start II polymerase	0.4
DNA Template	1
Total volume	20

Table 7: *PCR protocol for trnL-trnF region.*

Step	Temp. (ºC)	Duration (min:sec)	Process	Repeats
1	94	4:00	Initial denaturation	None
2	94	1:00	Denaturation	40
3	52.5	1:00	Annealing	
4	72	1:45	Extension	
5	72	5:00	Extension	None

Table 8: *PCR protocol for Calmodulin region.*

Step	Temp. (ºC)	Duration (min:sec)	Process	Repeats
1	98	3:00	Initial denaturation	None
2	98	0:05	Denaturation	
3	53	0:15	Annealing	25
4	72	0:20	Extension	
5	72	1:00	Extension	None

3.5 Gel Electrophoresis

Gel electrophoresis was used to visualise the PCR product; through an electric field which moves DNA molecules through the gel. DNA molecules carry a negative charge, when exposed to a current they will migrate to the anode. The migration is determined by the size of the DNA fragment, so the charge it holds. If the amplification of a single region is successful, only a single band should show up after gel electrophoresis.

A 1% agarose gel was prepared by dissolving 1 g of agarose powder into 100ml of 1x TBE Buffer and heating the solution in a microwave until fully dissolved. After cooling, 5 µl of SYBR Safe DNA gel stain was mixed in. The solution was then poured into a 100 ml gel tray with a gel comb and left to set for 30 minutes.

Samples were prepared by loading a plate with 3 µl of PCR product and 2 µl of gel loading dye. The gel was placed in an electrophoresis tank submerged into TBE buffer after which the samples were then loaded into the wells, together with a 1 kb DNA ladder.
The gel was run for 47 minutes at 80 volts. Gel results were visualised using a Syngene Fluorescence Imaging System, under an orange filter panel. Figure 7 is an example of a captured image; images for other runs are included in Appendix 1.

3.6 PCR Product Purification

PCR products were purified using ExoSAP IT, to remove left-over dNTP's and primers, which could affect the sequencing. 2 µl of ExoSAP was mixed with 5 µl PCR product and incubated in a thermal cycler at 37ºC for 15 minutes, followed by 15 minutes at 80ºC to inactivate the enzymes (Bell 2008).

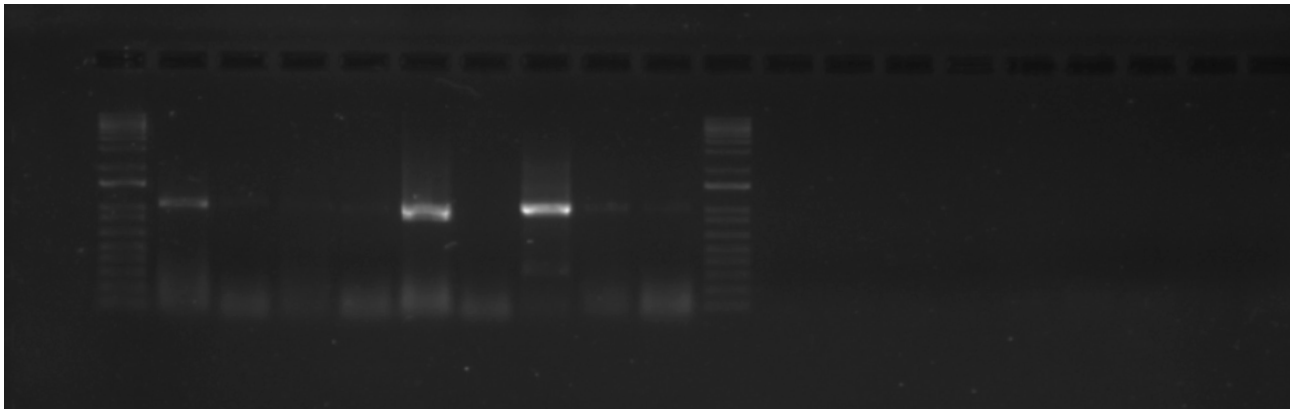


Figure 7: Gel electrophoresis results for *trnL-F* on the left, and *ITS* on the right, which did not amplify during the PCR.

3.7 Sequencing PCR

The same primers from the PCR were used for the sequencing PCR (Table 9). A sequencing was made for both *trnL-F* and *CaM*, with a master mix prepared for each primer. The master mix was prepared by multiplying the numbers found in table 9 with the number of samples to be processed. This was pipetted in an 1.5 ml Eppendorf tube, vortexed and centrifuged, after which 9.5 µl of the master mix was pipetted into each reaction tube, together with 0.5 µl of purified PCR product. The samples were then placed in a thermal cycler and the BigDye protocol was used (Table 10).

Table 9: *Reagent volumes for one reacting for sequencing PCR.*

Reagent	Volume in µl for one sample
Bigdye	0.5
5x Buffer	2
Primer 10 µm	0.32
H_2O	6.58
Template	0.6

Table 10: *Sequencing PCR protocol.*

Step	Temp. (ºC)	Duration (min:sec)	Process	Repeats
1	95	0:30	Denaturation	
2	50	0:20	Annealing	Step 1 – 3
3	60	4:00	Extension	24 times
4	4	0:20	Storage	None

3.8 Sequence Editing

The sequences were edited using Sequencher 5.4.6 (Sequencher). The sequences were trimmed on both the 5' and the '3 end to remove primers and low quality reads. The forward and reverse couplets were aligned using the 'assemble by name' function to form contigs. For some sequences the similarity threshold had to be lowered from 80 to 70, and 65 to get the 'assemble by name' function to work. These contigs were then examined to confirm each sequence ran in the right direction. Ambiguities between sequences were resolved manually. A consensus sequence was produced for all contigs and saved in the fasta format.

3.9 Troubleshooting

The first PCR, using *ITS* primers 7P and 4, did not result in any DNA amplification. A different primer set was used, 5 and 8, which again did not show any amplification. A PCR for *trnL-F* run at the same time did produce bands for some species after the gel electrophoresis (Figure 7). This showed that the initial DNA extraction was at least partially successful, and the problem lied elsewhere.

A different additive (TBT-PAR) was on the same set of samples (the first eight to be extracted), it did however not make a difference, while also trying two different chloroplast regions: *rps16* and *psbA-trnH*. *rps16* did not show up, like *ITS*, *psbA-trnH* showed a similar pattern as *trnL-F*, where the same samples showed up.

To visualise the quality of the extracted DNA it was directly run on a 2% agarose gel. The results in Figure 8 show that for almost all samples the bands are smeared. This indicates that the DNA was in a degraded state. The samples that performed best were the samples collected from living collections in the glasshouse in the past few weeks (well numbers: 7, 11, 12, 19, 23, 24 in figure 8). To deal with these degraded, shorter segment internal primers were used for *trnL* and *trnF*, in the hope of getter better quality amplification and reads.

This was tested on a different set of samples (samples 17-24), as these showed to be of relatively good quality. The first two PCR results showed only smeared results, as the wrong combination of primers was used. After this mistake was corrected amplification was more successful, with successful amplification for both *trnL* and *trnF*. These regions were then amplified for all 24 samples, with mixed success (Figure 9).

Having established that shorter regions had a reasonable success rate, primers for Calmodulin were ordered together with Thermo Scientific Phire II hot start. With the primers used in this study, Calmodulin reads of around 600 base pairs should be obtained, and therefore a better candidate for the nuclear region than *ITS*. Publications showed a big difference in annealing temperatures, ranging

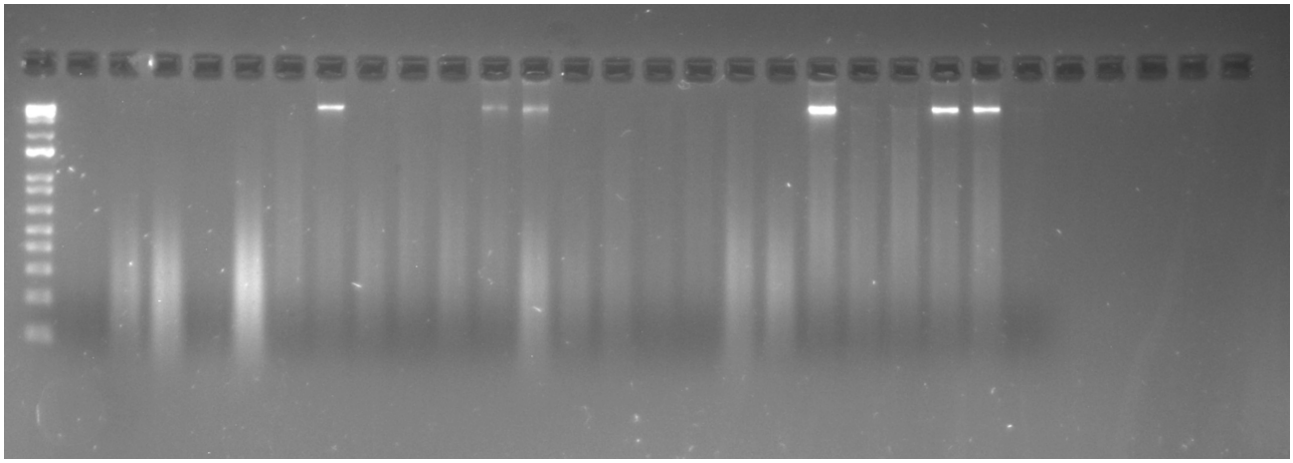


Figure 8: The result of running the extracted DNA on a 2% agarose gel. Most bands are smeared, indicating DNA degradation has taken place.

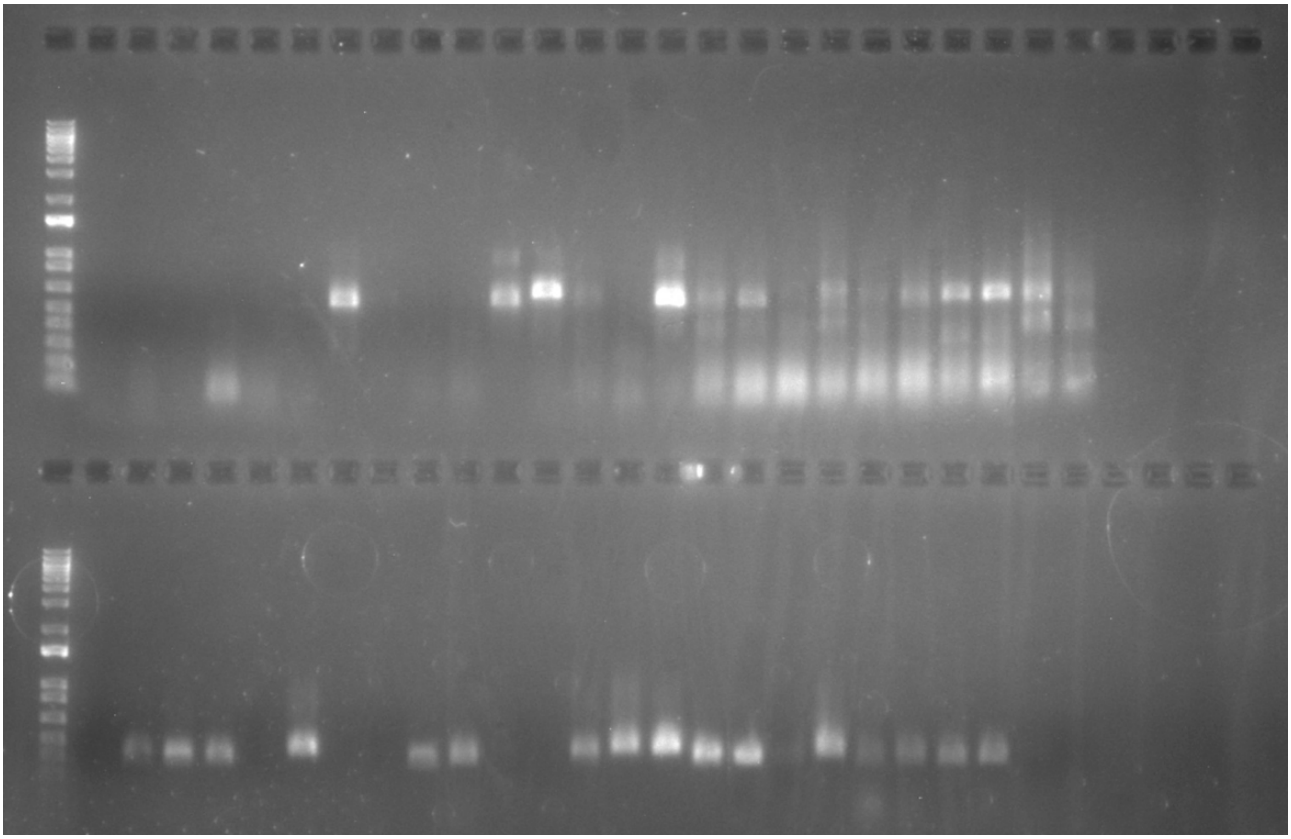


Figure 9: PCR results of amplification of *trnL* (top) and *trnF* (Bottom), for all 24 samples. None of the bands are perfectly bright and clear, selected samples were sent off to sequencing.

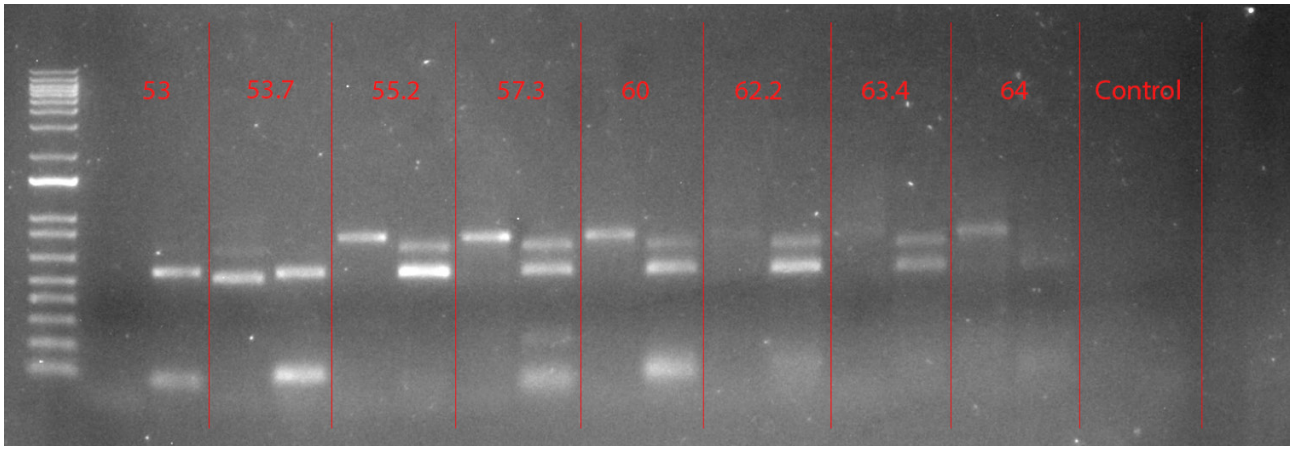


Figure 10: Result of a temperature gradient PCR. Multiple copies are available of this Calmodulin, which amplify at different lengths. The double banding at a higher temperature is unusual, no good explanation was found for this. It was decided to run future PCR's at a temperature of 53ºC.

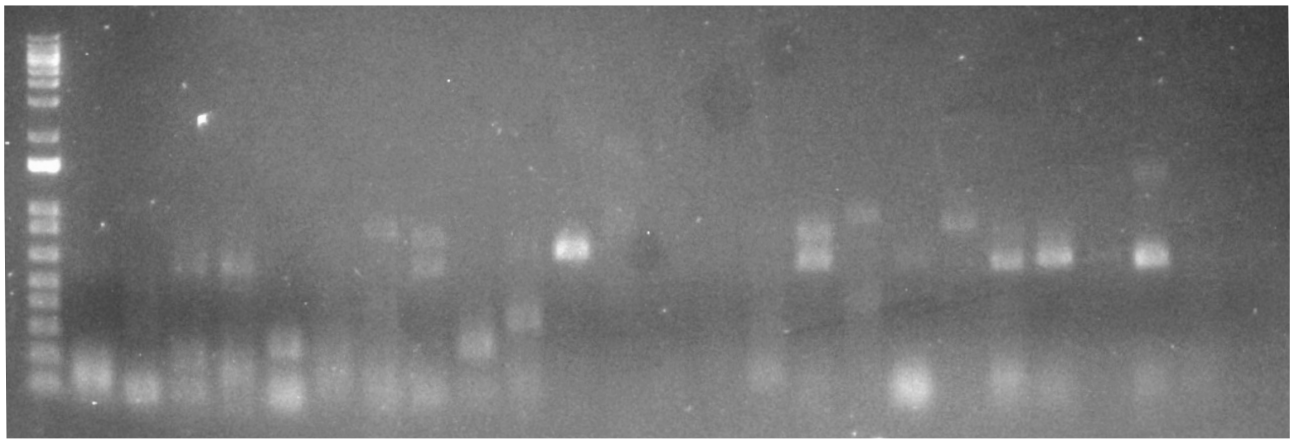


Figure 11: After promising results *CaM* was tried on all 24 samples. This did not result in clear bands, and again many duplicate copies were picked in the samples, resulting in double banding.

from 53ºC to 66ºC (Johanson 2005, Salzman 2015). A gradient PCR was done to find the optimal annealing temperature, which was found the be between 53ºC and 54ºC (figure 10). Initial results showed good amplification across the selected eight samples, after which amplification of all 24 samples was done. This did not however give the desired results, as most regions did not amplify (Figure 11). Instead of DMSO the recipe was changed to use TBT-PAR (recipe in Appendix 1). As this did not make a difference the number of cycles was lowered to 30 instead of 45. This approach also did not lead to better result and in the end the use of Calmodulin had to be abandoned due to time constraints. Only three samples, of two species have been sequenced, the quality of the reads were so low however that they were not used in the final analysis.

3.10 Phylogenetic Methods

Outgroup selection

In order to get sufficient resolution it is important to choose the right outgroup. In this study Siphonochilus J.M.Wood & Franks a basal genus in Zingiberaceae was chosen. Furthermore species were included from neotropical and African *Costus* from genbank. Table 11 shows the genbank accession used in this study. Multiple Asian species were added to give body to the sequences obtained from this study, as there was little overlap between the species for which *trnL* and *trnF* were successfully sequenced. Genbank accession AY041079, a *Hellenia globosa* is labelled as as wrongIDd, as it occurs on genbank twice. The *H. globosa* genbank accession is from Specht's 2001 paper and at the time was identified as a specimen of *Costus globosa*. During the 2006 publication this voucher was again uploaded but with the updated name *Cheilocostus speciosis*. In the analysis here it is labelled as "Costus_globosus_kress945298_wrongIDd".

Parsimony Analysis

The DNA sequences of the two regions were manually compared to the sequences obtained from genbank and manually aligned in a nexus file using MEGA version X (Kumar et al. 2018). Three separate analyses were done; one for *trnL* only, one for *trnF* only and a combined one. The maximum parsimony analyses were run in PAUP 4.0b10 (Swofford, 2003). A heuristic search was done with 10000 replicates using random additions and tree-bisection- reconnection (TBR) branch swapping. A bootstrap analysis was done using 10000 replicates with 1 random taxon addition replicate and TBR branch swapping. The retention and consistency indices were used to estimate the overall degree of homoplasy. Nodes with support values of 85% or higher are considered strongly supported, 75-84% as moderately supported and 50-74% as poorly supported. Mesquite 3.6 (Madison & Madison 2018) was used to display the trees.

Bayesian Analysis

Bayesian analysis was performed in MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001) (Ronquist & Huelsenbeck, 2004) with. Again three analyses were run, one for *trnL*, *trnF* and a combined *trnL-F*. The analyses were run with four Markov chains doing 1000000 generations and a sampling frequency of every 100 trees. The first 25% was removed as burn-in. No evolutionary models were enforced, as not to make any assumption on how it could have evolved.

A majority-rule consensus tree with posterior-probability was created. Nodes with support values of 95% or above was considered as strong support. Figtree 1.4.4 (Rambaut 2018) was used to display the trees.

Table 11: Sequences used from Genbank.

Taxon name	Genbank name	Specimen voucher	Genbank accession number	Comment
Costus afer	*Costus afer*	*Specht02312*	*AY994588*	
Costus amazonicus	*Costus amazonicus*	*Specht02327*	*AY994586*	
Costus gabonensis	*Costus gabonensis*	*Specht02339*	*AY994593*	
Costus pulverulentus	*Costus pulverulentus*	*Kress 94-3680*	*AY041070*	
Hellenia globosa	*Costus globosus*	*Kress 94-5298*	*AY041079*	*Wrong IDd*
Hellenia globosa	*Costus globosus*	*Kress 99-6461*	*AY041089*	
Hellenia lacera	*Cheilocostus lacerus*	*Kress 00-6777*	*AY994578*	
Hellenia speciosa	*Cheilocostus speciosus*	*Kress 94-5298*	*AY994557*	
Hellenia speciosa	*Costus speciosus*	*Specht sn*	*AY994544*	
Hellenia speciosa	*Costus speciosus*	*SSakai2000 Borneo*	*AY994558*	
Paracostus englerianus	*Costus englerianus*	*Kress 94-5279*	*AY994580*	
Siphonochilus decorus	*Siphonochilus decorus*	*00 135*	*AY994539*	
Siphonochilus kirkii	*Siphonochilus kirkii*	*Kress 94-3692*	*AY994538*	
Tapeinochilos ananassae	*Tapeinochilos ananassae*	*NY Cons*	*AY994545*	
Tapeinochilos ananassae	*Tapeinochilos ananassae*	*Specht2001*	*AY041093*	
Tapeinochilos dahlii	*Tapeinochilos dahlii*	*NMNH90012*	*AY994541*	
Tapeinochilos dahlii	*Tapeinochilos dahlii*	*Specht2001*	*AY041094*	
Tapeinochilos queenslandiae	*Tapeinochilos queenslandiae*	*Hay7052*	*AY994542*	
Tapeinochilos queenslandiae	*Tapeinochilos queenslandiae*	*Hay7052A*	*AY041080*	

3.11 Results

In the analysis the original names of the species under which they have been collected has been maintained, therefore some older species names will appear. All *Cheilocostus* are *Hellenia* and all species of *Paracostus paradoxus* are *P. muluensis*. In the parsimony analysis “Paracostus yellow 3662” is the true *P. paradoxus*.

Maximum Parsimony analyses

For the *trnL-F* analysis 1,673 characters were scored for 32 terminals, of which 600 characters were excluded due to poor reads. Of the 1,073 characters used in the analysis 169 (15.7%) were variable and 74 (43.7%) of the variable characters were parsimony informative. 100,000 equally parsimonious trees were produced, with 197 steps (CI: 0.95, RI: 0.92).

For the *trnL* analysis 697 characters were scored for 25 terminals. Of the characters used in the analysis 102 (14.7%) were variable and 46 (45.1%) of the variable characters were parsimony informative. 100,000 equally parsimonious trees were produced, with 113 steps (CI: 0.99, RI: 0.98).

For the *trnF* analysis 376 characters were scored for 31 terminals. Of the 376 characters used in the analysis 67 (17.8%) were variable and 26 (38.8%) of the variable characters were parsimony informative. 100,000 equally parsimonious trees were produced, with 80 steps (CI: 0.93, RI: 0.88).

The analyses of *trnL*, *trnF* and *trnL-F* all resulted in big polytomies. The strict-consensus tree of the *trnL-F* analyses is shown in Figure 12. The strict-consensus trees of the *trnL* and *trnF* analysis can be found in Appendix 1.

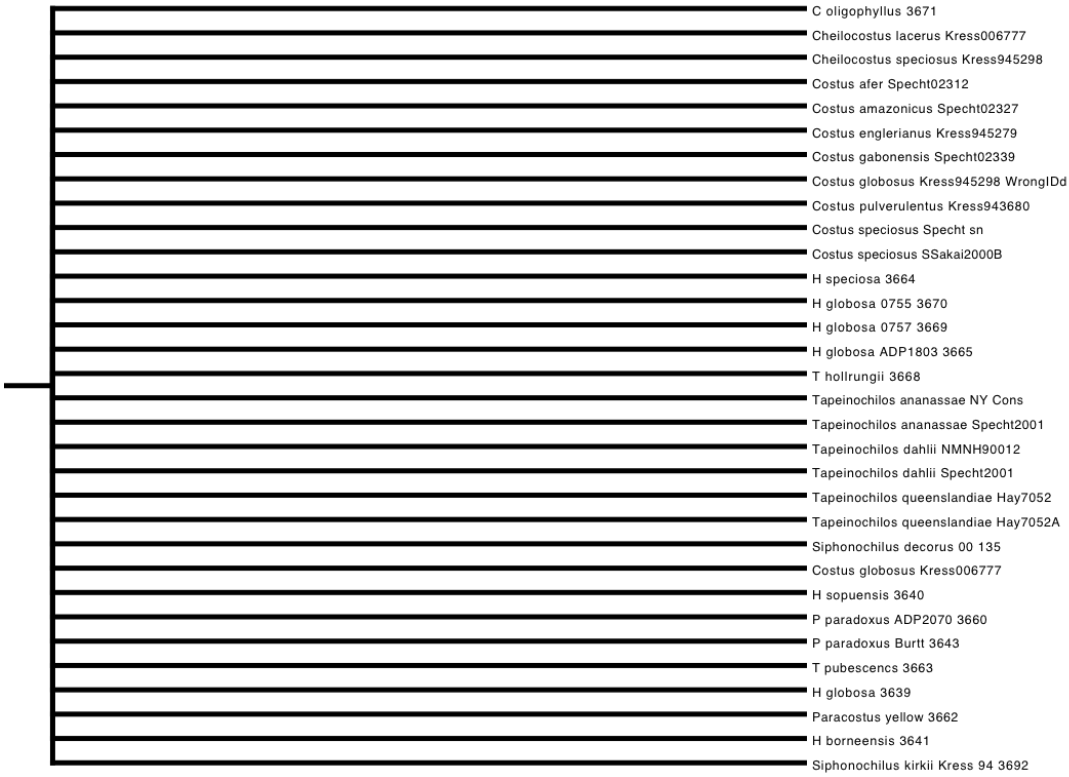


Figure 12: Strict-consensus tree of Maximum Parsimony analysis for *trnL-F*. There is not enough resolution to draw any conclusion on generic or species relations.

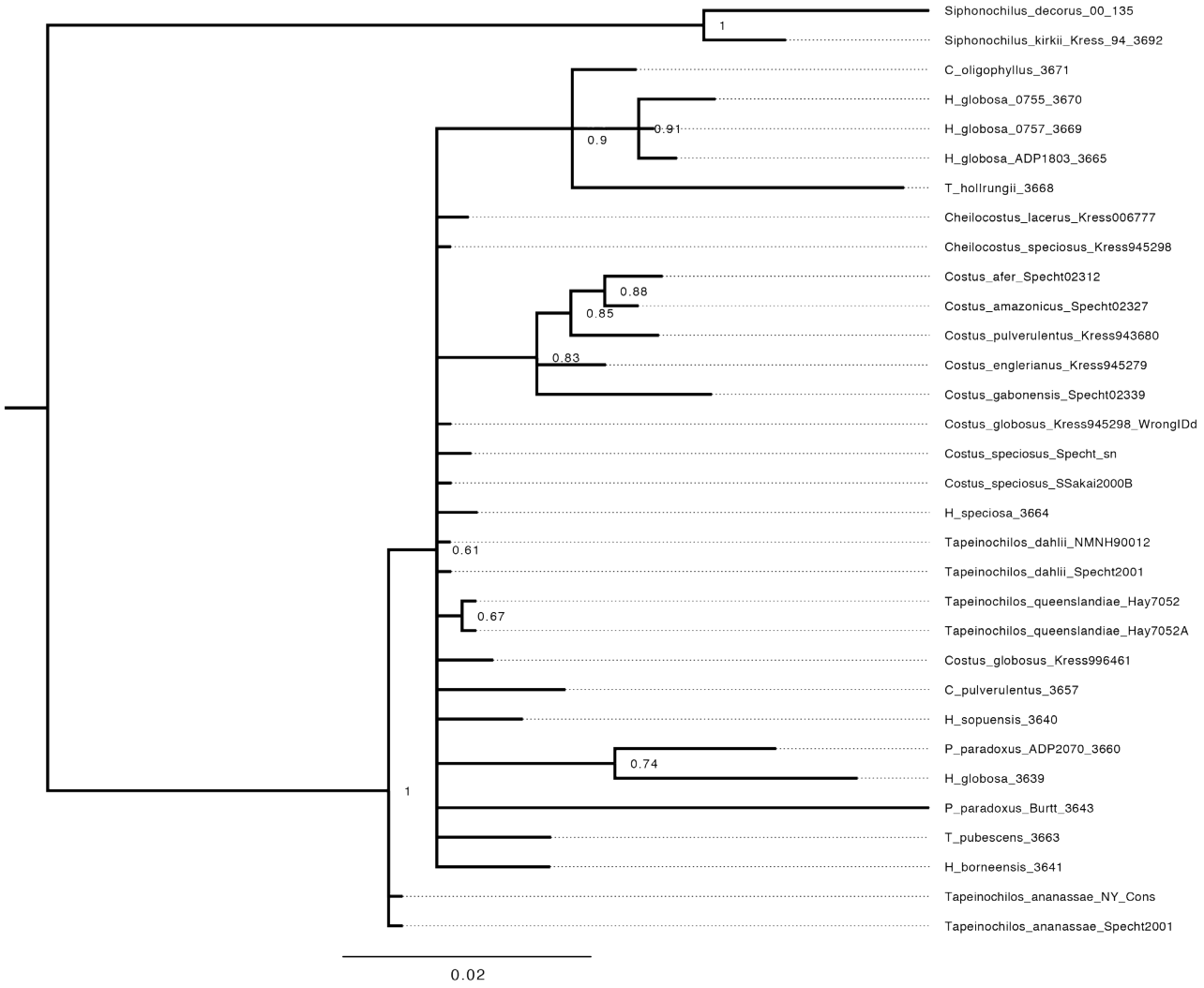


Figure 13: Phylogenetic tree from Bayesian analysis of the whole *trnL-F* region. The posterior probability values show that the neotropical and African Costus clade is moderately supported, with a posterior probability score of 83%.

Bayesian Analyses

The Bayesian analyses showed significantly better resolution than the maximum parsimony analyses. Although most Asian species still form a polytomy (Figure 13). The neotropical and African *Costus* genbank accessions form a moderately supported clade with a posterior probability score of 81%. The tree, however, is not in disagreement with the current phylogeny by Specht, although it does indicate that *Tapeinochilos ananassae* K.Schum is sister to all other Costaceae. The Asian clade holds up, as there are no Asian species that fall within the clade of neotropical and African *Costus*. An interesting find is that *Costus oligophyllus* K.Schum is grouped with *Hellenia globosa*, with a high posterior probability score (90%). This species was recently rediscovered on Peninsular Malaysia and there has been uncertainty in which genus it belongs, *Hellenia* or *Paracostus*. This result could indicate that *Costus oligophyllus* is closely related to *Hellenia* and should be placed in this genus.

4. Cytology

4. CYTOLOGY

The somatic chromosome number for most Costaceae is 2n=18, with *Dimerocostus* deviating with 2n=7 or 2n=14, and *Paracostus* 2n=36 (Maas 1972, Mahanty 1970).
Figure 14 shows the family phylogeny, with chromosome counts plotted onto it. It can be seen that the base number of 9 is constant throughout the whole family, with relatively few recorded exceptions of possible tetraploidy or triploidy.
Up to the present various chromosome, counts have been published for *Hellenia speciosa* (Table 12), but no counts have been reported for other Bornean species. This study will add chromosome counts for *H. borneensis, H. globosa, H. sopuensis* (Maas & H.Maas) Govearts, *P. muluensis* and *P. paradoxus*.

The only available number for the genus *Paracostus* is for *P. englerianus,* which is 2n=36 (Mahanty 1970). It will have to be confirmed if the base number of this species and/or genus is 18, or that in fact, *P. englerianus* is a tetraploid.

Table 12: Previous records of chromosome counts in Bornean Costaceae.

Name	2n	Author
Hellenia speciosa	18	Simmonds (1954)
	18	Satô (1948)
	18	Satô (1960)
	27	Simmonds (1954)
	36	Banerji (1940)
	36	Venkatasubban & Raghavan (1943)
	36	Chakravorti (1948)
	36	Sharma (1959)

All samples are from specimens in the living collection at the Royal Botanic Gardens, Edinburgh and except for one, *Hellenia sopuensis*, all species are from Borneo. Almost all accepted Bornean species are included in this study, except for *Paracostus bullatus* and *Paracostus eburneus* of which no material could be obtained.

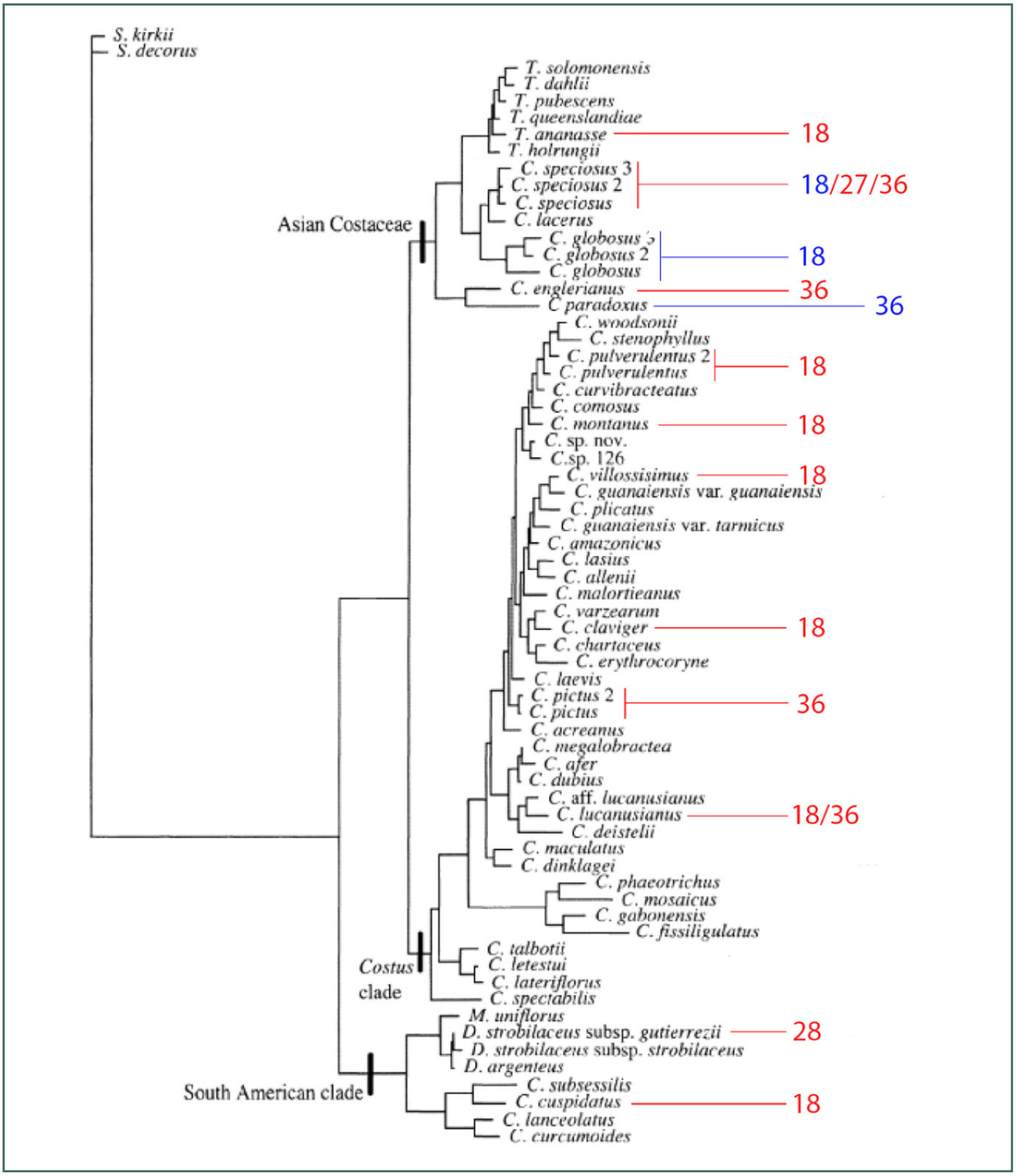


Figure 14: Known chromosome numbers are plotted on the phylogeny from Specht (2006) in red, Numbers found/ confirmed in this study of taxa that appear on this tree are in blue.

4. CYTOLOGY

4.1 Methods

Chromosome numbers have been determined in root tip metaphases of 6 species in 2 genera. To harvest the roots from the selected samples, stem cuttings were made which were grown in a growth chamber for 4 – 6 weeks. For harvesting the roots healthy, young roots were selected as these are most likely to be actively growing. The roots were divided and placed into two different types of pre-treatment: 8-hydroxyquinoline (HQ) and paradichlorobenzene PDB) for five hours at room temperature. The pre-treatment arrests cells in metaphase by inhibiting spindle formation and clarifies chromosome constrictions (Sharma & Sharma, 1980, p.21). For the staining, the Feulgen squash technique was used, with slight alterations (Jong, 1997).

After the fixation in Farmer's fluid and hydrolase step, the roots are placed in Feulgen's reagent for two hours in a dark box. The time is dependent on the age of the Feulgen's reagent, as the reagent loses strength over time. The washing of the roots in sulphur dioxide was skipped; instead, an additional softening step was done in a pre-heated 1:1 mixture of 4% pectinase and 4% cellulose as roots were placed in a 36ºC water bath for 30 minutes.

For slide preparation, the roots were placed on a cleaned slide, and the root tip cut off behind the meristem. The meristem was placed in a drop of acetocarmine for counterstaining and macerated. After a cleaned coverslip was placed on the material, the slide was lightly squashed between two sheets of blotting paper and examined under a microscope.

For *Hellenia globosa* the initial root harvesting did not lead to conclusive results, a second root harvesting was done, with a slightly different pre-treatment: The roots were placed in HQ at room temperature for six hours instead of five.

4.2 Results

A summary of the chromosome numbers established in this study can be found in Table 12.
Paracostus muluensis: The somatic chromosome number is 2n=36 (Figure 15a), 1.2 – 2.5 µm long.
Paracostus paradoxus: The somatic chromosome number is 2n = 18 (Figure 15b), 1.5 – 3.5 µm long.

Hellenia borneensis: has the typical family chromosome number, 2n=18 (Figure 15c), 0.9 – 2.5 µm long.
Hellenia globosa: Multiple counts had to be down, as the first samples were inconclusive. A first number of 2n=20 (Figure 15d was found, which after a second root-harvesting and slightly altered treatment was corrected to 2n=18 (Figure 15e), 1 – 2 µm long.
Hellenia sopuensis: The somatic chromosome number is 2n=18 (Figure 15f), 1.5 –3.1 µm long.
Hellenia speciosa: The somatic chromosome number is 2n=18 (Figure 15g), 1.3 –2.1 µm long.

4. CYTOLOGY

Table 12 summarises the chromosome numbers established in this study. The examined specimens from *Hellenia* all follow the family base number of 9, with 2n = 18. *Paracostus muluensis* has the same number, 36, as *P. englerianus.* Whereas *P. paradoxus* deviates from this number by having 2n=18.

This means that *Paracostus* has the same somatic number as *Costus*, which makes sense when looking a Specht's tree. It will be interesting to know if *P. englerianus* are always tetraploids, and if this is the normal number for this genus. If so it could mean that *P. paradoxus* is basal in this genus. Future cytological studies, and molecular work will have to be done to provide clarity on the matter.
With almost no previous cytological information on Asian Costaceae, these results are an essential addition to understanding the family. A manuscript is in preparation to publish these results. Due to time constraints, this manuscript is not included in this thesis but will be referenced to in chapter 5.

Table 13: Somatic chromosome numbers found in this study.

Spiecies	2n
P. muluensis	*36*
H. borneensis	*18*
H. globosa	*18*
H. sopuensis	*18*
H. speciosa	*18*
P. paradoxus	18

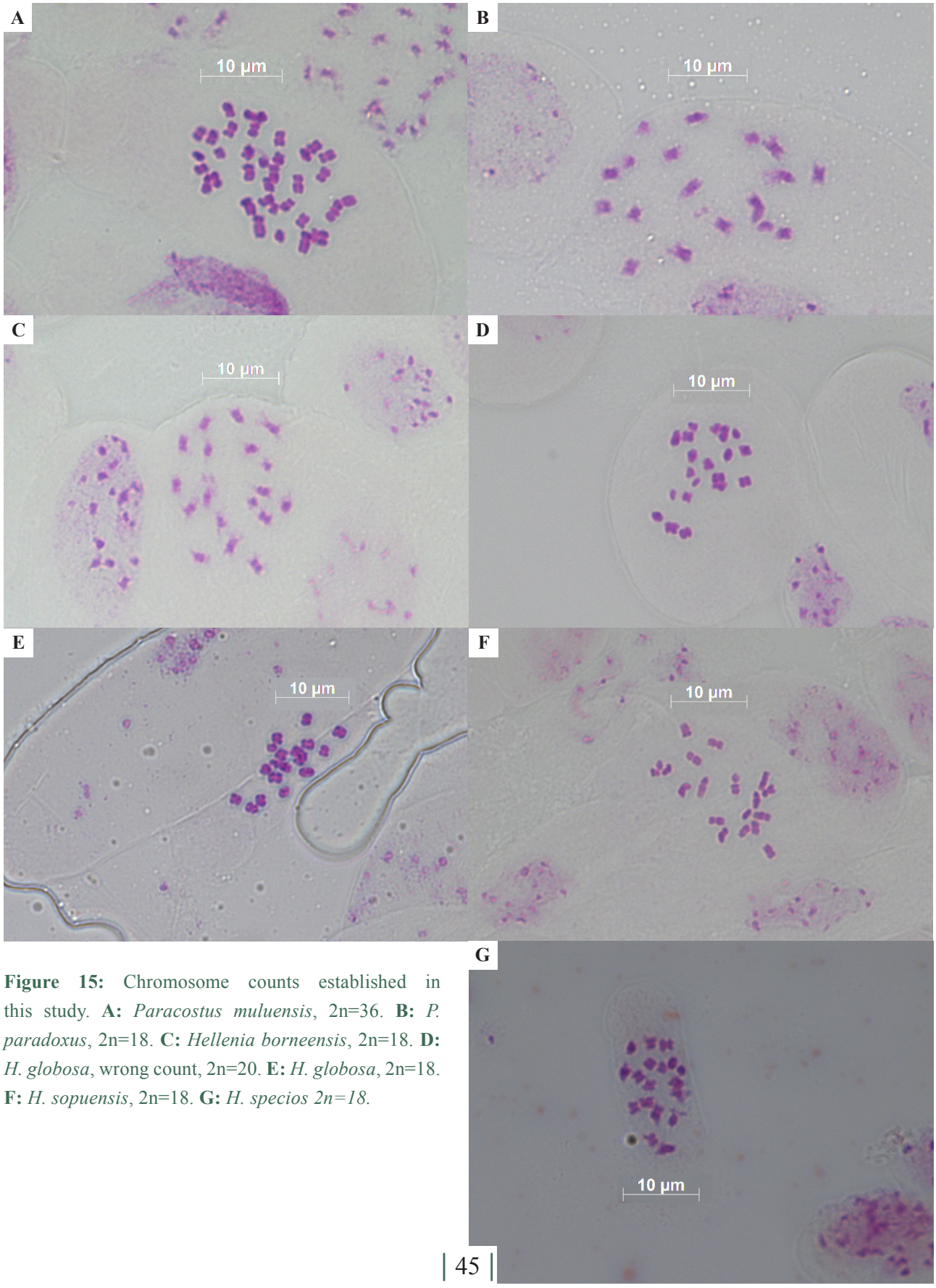


Figure 15: Chromosome counts established in this study. **A:** *Paracostus muluensis*, 2n=36. **B:** *P. paradoxus*, 2n=18. **C:** *Hellenia borneensis*, 2n=18. **D:** *H. globosa*, wrong count, 2n=20. **E:** *H. globosa*, 2n=18. **F:** *H. sopuensis*, 2n=18. **G:** *H. specios 2n=18.*

5. Manuscript

5. MANUSCRIPT FOR PUBLICATION

The following chapter is a manuscript formatted to be published in the journal BLUMEA.

5.1 A revision of the Bornean Costaceae

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Key words:
Hellenia, Paracostus, Sarawak, Sabah, Kalimantan, Zingiberales, Taxonomy

Abstract:
A taxonomic revision of the Bornean Costaceae (*Hellenia* and *Paracostus*) is given.
Within the genus *Paracostus* four species are recognized, of which three are here described as a new combination. In *Hellenia* three species are described, and one is placed within the complex of *Hellenia globosa*.

Acknowledgements:
Pim van Caspel is grateful to Paul and Hiltje Maas for their advice. Chea Yiing Ling for checking recent collections at SAR. Meekiong for answering positively to emails and an intent to collaborate.

Permits for the fieldwork of ADP were processed by the Economic Planning Unit (EPU), Sabah Parks, Danum Valley Field Centre, Universiti Brunei Darussalam, Forestry Department of Brunei, Sarawak Biodiversity Centre, Sarawak Forest Department, National Parks of Sarawak, Indonesian Institute of Sciences (LIPI), The State Ministry of Research and Technology (RISTEK) and other Indonesian authorities. The fieldwork would not have been possible without financial support from HRH Crown Prince Frederik of Denmark, the 'Biodiversity Grant' of WWF Verdensnaturfonden (Denmark) & Novo Nordisk, as well as the Carlsberg Foundation, Frimodt-Heineke, Julie von Müllen, Royal Horticultural Society, Augustinus Foundation, Blaxall Valentine of the Royal Horticultural Society Bursaries, and the Davis Expedition Fund, and the Sibbald Trust. The Royal Botanic Garden Edinburgh (RBGE) is supported by the Scottish Government's Rural and Environmental Science and Analytical Services Division. Logistic support was also facilitated by Julia Sang, Sarawak Forestry Cooperation, the Maliau Project including staff from Yayasan Sabah, Luasong, especially Linus Banggilon and Borneo Adventure, Kuching. The local communities in Borneo, especially in

the Kelabit Highlands and Ng. Sumpa, assisted greatly during the forest exploration. Keepers and staff of the E herbaria allowed us access to herbarium material.5.1 Introduction

In Borneo, the Costaceae consists of two genera: *Hellenia* Retz. and *Paracostus* C.D.Specht. The genus *Hellenia* is widespread in Southeast Asia, extending from India to Papuasia, crossing Wallace's line. *Paracostus,* however, is limited to Borneo, and one species in Africa. A phylogeny published by Specht & Stevenson (2006) sho wed that the, until then pantropical genus *Costus* was paraphyletic, based on phylogenetic analysis from nuclear and chloroplast regions combined with a large morphological matrix. Two new genera were introduced for the Asian species, *Paracostus* and *Cheilocostus* C.D.Specht, to form monophyletic groups. Since then, *Cheilocostus* has been changed to *Hellenia* due to a citation error in the original publication (Govaerts 2013), although a proposal to conserve *Cheilocostus* is currently pending (Leong-Škorničková and Šída, 2016). Until recently *Paracostus* had only one relative in Asia, *Paracostus paradoxus* (K. Schumann) C.D. Specht . Meekiong et al. (2006) published three new species but placed them in *Costus* subgenus *paracostus* K.Schum., awaiting better sampling across Asia before delineating *Costus* L.. A further two species were published in *Hellenia*, *H. mulus* Meekiong, Ipor et. Tawan in 2008 and *H. borneensis* (A.D.Poulsen) Govaerts in 2010 by A.D. Poulsen. Paracostus is characterised by its few leaved prostrate stems, flowering from the nodes and the rhizome in few-flowered clusters. *Hellenia*, on the other hand, is large (>1.5 m) with coriaceous, often spiny bracts, flowering from a radical or terminal inflorescence.

The family of Costaceae is represented by seven species on Borneo. Three new combinations are made in this publication, and one species is placed in the *Hellenia globosa*-complex.

5.2 Materials and methods

Terminology: Definitions are following 'Plant Identification Terminology' by Harris & Harris (2001), especially for indumentum, for which the key proved very useful. Furthermore, the following terms are used to increase comparability for the dichotomous key and the species descriptions. 'Few' means 2 – 7; 'several' means 10 – 18; 'many' means more than 20 leaves, flowers or bracts. Cytological characters are from van Caspel et al. (In prep). Characters are only mentioned when distinctive: e.g. leaf colour is only described when it varies from the typical green, such as 'dark green above' in some species. 'pale green below' was found in almost all species so not mentioned. The ligule ordinarily is green and chartaceous. Leaves are generally herbaceous and thus noted when, for instance, fleshy.

Most measurements were taken from herbarium and pickled material. Where possible, measurements were taken from specimens growing at the Royal Botanic Garden, Edinburgh.

Specimens were examined from the following herbaria; an asterisk indicates if only images were seen. (AAU*, E, KEP*, L* U*).

IUCN conservation status assessments were performed using the IUCN Red List Category Criteria (IUCN 2016), the Extant Of Occupancy (EOO) and Area Of Occupancy (AOO) of each species were calculated using the Geospatial Conservation Assessment Tool (GeoCAT) (Bachman et al. 2011), the AOO with a grid cell size of 2 × 2 km. For species where information on their distribution was available distribution maps were made, which are given below in figure 16.

5.3 Taxonomic treatment

Key to Bornean genera

1. Large herbs (> 1.5 m), erect, shoots sometimes branching. Inflorescence radical or terminal, several to many-flowered, bracts conspicuous, brightly coloured. ……..... *Hellenia*

1. Small herbs (< 1 m), prostrate, shoots never branching. Inflorescence from nodes or rhizome, few-flowered, bracts inconspicuous, light brown. ……..... *Paracostus*

Hellenia Retz.

Protologue: Retzius, Observationes Botanicae 6: 18. 1791.

Large erect herbs. Leaves several to many, large, sometimes plicate; ligule short. Inflorescence terminal on a leafy stem or radical. Bracts brown to red, often pungent, coriaceous. Bracteole tubular. Calyx large and usually exceeding the bracts, often pungent. Corolla white, yellow, orange or red; Labellum large, obovate when spread out, spreading to funnel-shaped, white to yellow, orange and red. Stamen colour same as labellum, dorsal side often pilose. Stigma bilamellate, with a dorsal appendage. Ovary 3-locular, glabrous.

Key to Hellenia

1. Inflorescence terminal on leafy shoot. Lamina hairy beneath. Labellum white. … *H. speciosa*

1. inflorescence radical. Lamina glabrous beneath. Labellum colour variable. ……..... 2.

2. Leafy shoots never branching. Bracts not pungent, dorsal side stamen glabrous....*H. borneensis*

2. Leafy shoots often branching. Bracts pungent, dorsal side stamen pilose. ……. *H. globosa*

5. MANUSCRIPT FOR PUBLICATION

1. *Hellenia borneensis (A.D.Poulsen) Govaerts*
Basionym: —*Cheilocostus borneensis* A.D.Poulsen, gard. Bull. Sing. 62:136 (2010)
Protologue: Govaerts Phytotaxa 151(1): 64. 2013.
Type: Malaysia, Borneo,Sarawak, Batang Ai, Sungai Senkabang, Dec 2002, A.D. Poulsen 1964 (holo, SAR; iso, AAU,Sarawak Biodiversity Centre Flora depository).

Terrestrial herb. Leafy shoot 1.5 – 2 m tall, with 6 – 10 leaves per shoot, clustered at the apex, Base of leafy shoot to c. 3 cm in diameter, pale yellow-green. Sheath reddish-brown (uppermost yellow-green), glabrous. Ligule 1 – 2 mm long, ± truncate. Petiole 5 – 12 mm long, swollen, pale yellowish, lightly canaliculate, glabrous. Lamina 27 – 42 by 9 – 16(-20) cm, narrowly obovate, mid-green above, slightly plicate, glabrous, base narrowly cuneate, apex acuminate. Inflorescence radical, spike 4 – 12 by 5 – 8 cm, lax. Peduncle horizontal to ascending, 6 – 12 cm long, sheaths tubular, brown, glabrous, margin ragged. Fertile bracts 3.2 – 3.5 by 2 – 2.5 cm, elliptic, cucullate, margin membranous, apex softly mucronate (not pungent), dark brown to pale reddish-brown, glabrous. Bracteole 2 – 2.5 cm, split to base adaxially, reddish-brown, glabrous, apex rounded with a small mucro, cucullate. Calyx 3 – 3.2 cm long, dark reddish, glabrous, lobes 7 – 10 mm long, apex acute to mucronate, soft (not pungent). Corolla tube white, tube 15 – 20 mm long, lobes 3.5 by 1.5 cm. Staminal tube 11 – 13 mm long, with yellow hairs inside. Labellum lobe 5 – 5.3 by 5.2 – 6 cm, white, yellow patch in centre, glabrous, margin finely undulated. Stamen 3 – 3.2 cm long, dorsal side glabrous, Anther 7 – 8 mm long, inserted on a cushion-like thickening, anther crest truncate. Ovary to c. 10 mm long, flattened ellipsoid, glabrous. Style 3.5 – 3.7 cm long, white. Stigma bilamellate, with a dorsal bilobed, V-shaped appendage. Fruit 8 – 9 mm in diameter, flattened triangular. 2n=18

Distribution: Endemic to Borneo, known from few populations from Sarawak and Kalimantan.
Ecology: Lowland primary or secondary (logged) mixed dipterocarp forest, along riverbanks, at 130-200 m.
IUCN assessment: Critically endangered.

Notes: In Borneo this species can easily be distinguished on a vegetative level from the other species of *Hellenia* by the hairy underside of the leave.

Specimens examined:
Malaysia
Sarawak
Bernard Lee. S. S54624 (AAU, E), Kapit, Batang Baleh, Sungai Mengiong, Sungai Entulu, 18 July 1987; *Othman S56464 (AAU, E, K),Kapit, Balleh, Ulu Sungai Mengiong, Nanga Sebaning, 1 Nov 1988; MW177, R Ubong, Mulu national Park, 7 Nov 1990.*

Indonesia
Kalimantan
Ambriansyah et al. AA2238 (Bo, L, WAN), Camp Betung Kerihun NP, Putissibau, 28 Feb 2000 .

2. *Hellenia globosa (Blume) S.R. Dutta*
Protologue: Dutta S.R., Pleione 7(1): 228-229 (2013)
Holotype: *Blume s.n.;* Cultivated, introduced from Java to hort. Leiden. (L)
Lectotype: *van Hasselt s.n*.; Nov 1820; Indonesia: Java: Bantam, Tjoeroek Dinding (L-L1480413) designated by J.F.Veldkamp (2018).
Terrestrial herb. Leafy shoot 2 – 5 m tall, sometimes branching at the top, with several to many leaves per shoot, clustered towards the apex. Base of leafy shoot 3 – 10 cm in diameter, pale reddish-brown. Sheath reddish-brown (uppermost yellow-green), velutinous, dehiscing leaving a fibrous cover. Ligule to c. 2 mm long, ± truncate. Petiole to 5 – 10 mm long, swollen, reddish, lightly canaliculate, glabrous. Lamina 13 – 30 by 6.5 – 16 cm, narrowly obovate, slightly plicate, glabrous beneath, base slightly unequal, cuneate, apex acuminate. Inflorescence radical, spike 5 – 9 (30) by 5 – 10 cm. Peduncle horizontal to ascending [pendant], 3 – 15 [80] cm long, sheaths tubular, brown, hairy as bracts, margin ragged. Fertile bracts 2.0 – 3.5 by 2.5 cm, cucullate, apex rounded, with stout acute spine, reddish, yellow towards at the apex, indumentum variable, often stiff. Bracteole 15 – 30 mm by 15 – 25 mm, reddish-brown, glabrous, apex rounded with small spine. Calyx 2 – 3.5 cm long, dark reddish, glabrous, lobes 4 – 8 mm long, apex with spine. Corolla tube white at the base, tube 1.6 – 2.1 cm long, lobes 3.5 by 2 cm, colour white, yellow, orange or red. Staminal tube 11 – 13 mm long, with yellow hairs both inside and outside. Labellum 5 – 5.3 by 5.2 – 6 cm, white, yellow patch in centre or at apex, red, yellow or orange, glabrous to sparsely hairy, margin finely undulated (ciliate). Stamen 2.8 – 3.2 cm long, dorsal side densely pilose Anther 7 – 8 mm long, inserted on a cushion-like thickening, anther crest truncate. Ovary to c. 1.2 cm long, flattened, angular, glabrous. Style 3.4 – 3.6 cm long, white. Stigma bilamellated, with a dorsal (horned) bilobed appendage. Fruit Unknown. 2n=18

Distribution: Endemic to Borneo where it is known from three main areas in Sarawak (one being three collections from the Kapit area) and one in Kalimantan. The furthest localities are about 450 km apart.
Ecology: Lowland primary or secondary (logged) mixed dipterocarp forest, along riverbanks, at 130-200 m.
IUCN assessment: Endangered.

Notes: Within this species complex are the following species and varieties: *C. acanthocephalus* K.Schum. (Sumatra); *C. chrysocephalus* K.Schum. (New Guinea); *C. clemensae* Ridley (Philippines); *C. dhanivatii* K. Larsen (Thailand); *C. globosus* Blume (Java); *C. globosus* var. *kingii* Baker (Malay islands); *C. globosus* var. *ridleyi* K.Schum. (Malay Peninsula and Thailand); *C. globosus* var. *velutinus* Ridley (Malay Peninsula); *C. microcephalus* K.Schum. (Borneo); *C. mulus* Meekiong, Ipor et. Tawan (Borneo); *C. sulfureus* K.Schum. (Celebes); *C.tonkinensis* Gagnepain (Tonkin).

Regarding *Costus mulus*:
The authors state that *C. mulus* is similar to *H. speciosa*, although its morphological characters show an obvious similarity with *H. globosa*. The description by the authors is very incomplete, as many characters are not, or inadequately mentioned. The original drawing from the author can be found in Appendix 3.
The authors describe the importance of field studies and fresh samples, for a better understanding of taxonomic characters and a better description, it was therefore surprising that the description was missing so many essential parts. From the original description, a close relation to *Hellenia globosa* can be inferred. The only character that differs from *H. globosa* is the red spot on the corolla apex, which by itself does not warrant a species-level distinction. No specimens are cited besides the type, of which we have not been able to get any information, or images of. Until further study has been done, this species will be sunken into the *Hellenia globosa*-complex due to its similarities to *Hellenia globosa*.

The collections *1801, 1803 – 1805* by A.D. Poulsen and *Ambri* AA1578 show specimens with an exceptionally long, peduncle, reaching up to 80 cm, with a pendant inflorescence. Further collections and molecular studies are needed to better understand its place within the genus or species.

Specimens examined:
Indonesia
Kalimantan
A.D. Poulsen 3136 (BO, WAN, AAU, L, E), East Kalimantan, Camp Seturan (CIFOR), 28 Aug 2003; *Ambri AA1578* (E), KPC area bengalon, 26 Mar 1996; *Burley 2620* (E), G . Bentuang area, N of Masa village, 13 June 1989.

Malaysia
Sabah
A.D. Poulsen 1801 (SAN, KEP, Universiti Malaysia Sabah, AAU, E) Ula Padas, 22 Mar 2002;

A.D. Poulsen 1803 (SAN, KEP, Universiti Malaysia Sabah, AAU, E) Ula Padas, 22 Mar 2002; *A.D. Poulsen 1804* (SAN, KEP, Universiti Malaysia Sabah, AAU, E) Ula Padas, 22 Mar 2002; *A.D. Poulsen 1805* (SAN, KEP, Universiti Malaysia Sabah, AAU, E) Ula Padas, 22 Mar 2002; *A.D. A Lamb 233 / 86* (E), Lengan, June 1986; *Argent, Ratter et. al,.* 108252 (E), Ulu sg. Segama Lahad datu district, 27 Feb 1985; *Beaman 7274* (E), Tambunan district Crocker range, 19 Oct 1983.

Sarawak

A.D. Poulsen 2041 (Sarawak Biodiversity Centre, SAR, AAU, L) Hose Mountains, SW slope of Buk, 5 Jul 2003; *Burtt & woods 2872* (E), 7th div, Gunong Beumpot, Poi range,16 Aug 1962; *Burtt & Martin B4784* (E), 3rd div, Kaki bukit camp, Bukit Thalong, 4 Aug 1964; *Burtt & Woods B2733* (E), Lundu district, 7 Aug 1962; *Hansen 706* (E), 7th div. Ulu belaga, Sungai, 10 Oct 1981; *J.A.R. Anderson S28692* (E), Bukit Tibang, 13 July 1969.

3. *Hellenia speciosa (J. Koenig) S.R.Dutta*

Cheilocostus speciosus (J.Koenig) C.D.Specht (2006)

Basionym — Banksea speciosa Koenig (1783) 75.

Costus speciosus (J.Koenig) Sm. (1791) 249.

C. lamingtonii J. M. Bailey (1898) 160.

C. formosanus Nakai, J. (1941) 199.

Hellenia grandiflora Retzius 1791, nom. superfl.

Costus spicatus (Jacquin) Swartz var f pubescens risebach (1864) 602.

Costus arabicus auct. non-Linnaeus: Jacquin, 1781;

Protologue: Pleione 7(1): 228 (2013)

Holotype: J. König s.n.; East Indies. (C)

Terrestrial herb. Leafy shoot 1.5 – 3 m tall, sometimes branching at the top, with several to many leaves per shoot, base of leafy shoot to c. 3 – 7 cm in diameter, pale yellow-green. Sheath reddish-brown (uppermost yellow-green), glabrous. Ligule 1 – 2 mm long, ± truncate. Petiole to 5 – 12 mm long, swollen, pale yellowish, lightly canaliculate, glabrous. Lamina 20 – 46 by 6.5 – 15 cm, narrowly elliptic, slightly plicate, puberulous to villose beneath, base rounded to cordate, apex acuminate. Inflorescence terminal, spike 4.5 – 8 by 4 – 10 cm. Peduncle absent. Fertile bracts 2.0 – 5.5 by 1 – 2 cm, ovate, apex mucronate (not pungent), red, glabrous to densely pubescent. Bracteole 9 – 17 mm, reddish-brown, glabrous to densely pubescent. Calyx 2 – 2.9 cm long, red, glabrous to densely pubescent, lobes 9 – 15 mm long, apex obtuse to shortly acuminate. Corolla tube white to pinkish white, tube 15 – 20 mm long, lobes 3.5 by 5 cm. Labellum (including staminal tube) (4–)6 – 7 by (5–)6 – 10 cm, white to pinkish white, tinged in centre, glabrous.

Stamen 3 – 3.5 cm long, dorsal side glabrous, Anther 7 – 13 mm long, anther crest obtuse. Ovary to c. 1 cm long, ellipsoid, densely pubescent. Style 3.5 – 3.7 cm long, white. Stigma bilamellate, with a dorsal bilobed appendage. Fruit 15 – 20 mm in diameter, ellipsoid. 2n=18

Distribution: Common in Borneo.
Ecology: Lowland, often on roadsides, edges or disturbed areas.
IUCN assessment: Endangered

Specimens examined:
Indonesia
Kalimantan
Burley 2655 (E), N of Masa village, 17 June 1989; *Kessler PK 991*(E), East Kalimantan, 29 March 1995; *R Geesink 8923* (E), Timur near Malinau, 2 July 1981.

Malaysia
Sabah
ADP 1806 (E), Ulu Padas, 23 Mar 2002; *A lamb S.n.* (E), Lengan, June 1986; *Amin SAN 69333* (E), Labuk sugut 21 sept 1984; *Beaman 7550* (E), Ranua district, 4 Dec 1983; *Beaman 10230* (E), Labuk/sugut district, 23 June 1984; *Campbell 21/10/12* (E), Lahad Datu, 21 Oct 1987; *M.F. Gardner s.n.* (E), Karamuak river, near Tawa plateau, sept 1977.

Malaysia
Sarawak
Burtt & Woods B2598 (E), Sungei Bena, 28 Jul 1962; *Dyg. Awa & Ilias Paie S47403* (E), Sebako waterfall, Lundu, 3 May 1984; *HJ Othman Ismawi S57186* (E), Gunung Putin, Lundu, 17 Mar 1989; *Ilias & Dami S43947* (E), Niah Forest reserve, 16 Feb 1988; *Mohtar S56110* (E), Serian sri, 17 Jan 1989; *Othman & Munting S54397* (E), Lichok, Roban, 24 June 1987.

***Paracostus* C.D.Specht**
Paracostus C.D.Specht (2006) 162.
Costus L. sect. *Paracostus* K.Schum. (1899) 343.
— *Costus* subg. *Paracostus* (K.Schum.) K.Schum. (1904) 381.

Small prostrate herbs. Leaves few, smooth, plicate to bullate; ligule short to absent. Inflorescence from nodes of lower part of the stem or the rhizome. Bracts light creamy brown, membranous to coriaceous. Bracteole tubular. Calyx without spines. Corolla variable. Labellum yellow, or white with a central yellow spot in the throat; stamen same colour as labellum, anther crest

deeply bilobed or irregularly toothed; stigma bilamellate, with a dorsal bilobed appendage; ovary 3-locular, pubescent.

Key to Paracostus

1. Labellum yellow, with red stripes in throat, leaves never bullate. …..... *P. paradoxus*
1. Labellum white, with a yellow spot in throat, leaves sometimes bullate. ……..... 2.
2. Leaf surface bullate, labellum often with pinkish line in throat, rhizome short-creeping. …3.
2. Leaf surface never bullate, labellum without pinkish lines in throat, rhizome long-creeping. .....*P. muluensis*
3. Young leaves yellowish-green, labellum with pinkish lines in the throat. …….. *P. eburneus*
3. Young leaves pale brownish-red, labellum without pinkish lines. ……..…….. *P. bullatus*

1.*Paracostus bullatus (Meekiong, Muliati & Ipor) Caspel comb. nov.*

Basionym: — *Costus bullatus* Meekiong, Muliati & Ipor Fol. Malay. 7 (1&2) (2006) 65 – 66

Type: K.Meekiong & I.B.Ipor & M.Muliati MK1688 (holo: SAR iso: HUMS)

Terrestrial herb. Rhizomes short-creeping. Leafy shoot 35 – 80 cm tall, prostrate, with 3 – 6 leaves per shoot, base of shoot 2.6 – 5.1 mm in diameter. Sheath greenish-yellow. Ligule 4.5 – 6.8 mm long, light green, glabrous. Petiole to 5.3 – 8.7 mm long. Lamina to 10.5 – 17.5 by 2.6 -– 6.8 cm, lanceolate, bullate, young leaves pale brownish-red, glabrous, base unknown, apex acuminate. Inflorescence from rhizome or nodes of lower part of the stem, 1 – 2 flowers per inflorescence. Peduncle short ascending, 3 – 3.5 mm, brownish. Fertile bracts 5.5 – 7.5 by 13 – 15.5 mm, triangular, brownish-green, membranous, slightly decaying into fibres. Bracteole 7.3 – 8.4 mm long, boat-shaped, green. Calyx 19 – 20 mm long, greenish, indumentum unknown, lobes triangular 2.8 – 4.3 mm long. Corolla whitish to watery green, tube to X cm, glabrous, lobes 2.0 – 2.2 cm long. Staminal tube c. 2.2 cm long. Labellum to c. 3.2 by 2.2 cm, white, yellowish-green stripes in throat, with mucro on individual lobes. Stamen to c. 17.5 mm long, Anther unknown mm long, anther crest deeply bilobed. Ovary 6.5 – 7.5 mm, sub-globose, densely pubescent. Style 3.9 – 4.1 cm long, white. Stigma bilamellated, with a dorsal appendage. Fruit Unknown.

Distribution: Sarawak, so far only known from two collections from the Bau limestone area.

Ecology: Limestone forest, growing in shaded, wet places, flowering recorded in February.

IUCN assessment: Endangered — This species is only known from two collections at the type locality and therefore comes to “Critically endangered”. As the full range of this species is unknown the assessment will be set to “Endangered”.

Notes: The drawing from the original publication (Meekiong et al., 2006) can be found in Appendix 3. The author's description of the petal lobes is found in the stamen section and vice versa, which the authors consistently confuse. Although no specimens were seen the inflorescence and the habit clearly show that this species belongs in *Paracostus*. Although there are strong similarities with *Paracostus eburneus* no specimens could be examined, therefore no other taxonomic decisions can be made at the time.

2. *Paracostus eburneus (Meekiong, Muliati & Tawan) Caspel comb. nov.*

Basionym: — *Costus eburneus* Meekiong, Muliati & Tawan, Fol. Malay. 7 (1&2) (2006) 66-68 — **Type:** K.Meekiong & M.Muliati MK1690 (holo: SAR iso: HUMS)

Terrestrial herb. Rhizomes short-creeping. Leafy shoot 35 – 85 cm tall, prostrate, with 4 – 6 leaves per shoot, base of shoot 4 – 7 mm in diameter. Sheath greenish-yellow. Ligule 3 – 4.5 mm long, light green, glabrous. Petiole 4.5 – 7 mm long. Lamina 12 – 20.5 by 4 –10 cm, lanceolate, dark green above, bullate, glabrous, base cuneate, apex acute. Inflorescence from rhizome or nodes of lower part of the stem, 3 – 4 flowers per cluster. Peduncle short, ascending, 2 – 3 mm, brownish. Fertile bracts to 18 by 12.5 mm, boat-shaped, watery green to pinkish green, membranous, slightly decaying into fibres. Bracteole 9.5 – 14.5 mm long, boat-shaped, green. Calyx 2.4 – 3.3 cm long, watery green, indumentum unknown, teeth triangular 2.8 – 4.3 mm long. Corolla watery green, floral tube length unknown, glabrous, lobes 3.5 – 3.7 cm long. Staminal tube c. 3.5 cm long. Labellum 3.8 – 4.0 by 4.0 – 4.2 cm, white, yellow and pinkish stripes in centre, glabrous, margin irregularly undulated. Stamen 2.0 – 2.1 cm long, Anther c. 3 mm long, anther crest iirregularly toothed to deeply bilobed, cream with pinkish stripes. Ovary 5.5 – 6.5 mm, sub-globose, densely pubescent. Style 4.5 – 4.6 cm long, white. Stigma bilamellated, with a dorsal appendage. Fruit Unknown.

Distribution: Sarawak, known from three collections. Two from the Bau limestone area and one from Long Lellang.

Ecology: (Limestone) forest, growing in shaded areas, flowering recorded in March and July.

IUCN assessment: Endangered —As only one other collection is known apart from the two collections at the type locality the assessment comes to "Critically endangered". The full extent of this species is, however, unknown, so the assessment will be changed to "Endangered".

Notes: The drawing from the original publication (Meekiong *et al*., 2006) can be found in Appendix 3. The author's description of the petal lobes is found in the stamen section and vice versa, which the authors consistently confuse. Based on the inflorescence and the habit it is clear that this species belongs in *Paracostus* and will therefore be moved over to this genus.

Specimens examined:
Malaysia
Sarawak
Long Lellang, ADP 2081 (AAU, E, SAR), 19 Jul 2003.

3. *Paracostus muluensis (Meekiong, Ipor & Tawan) Caspel comb. nov*
Basionym: — C*ostus muluensis* Meekiong, Muliati & Tawan, Fol. Malay. 7 (1&2) (2006) 60-65 — **Type:** K.Meekiong & I.B.Ipor MK1190 (holo: SAR iso: HUMS)

Terrestrial herb. Rhizomes long-creeping. Leafy shoot 30 – 80 cm tall, prostrate, with 4 – 8 leaves per shoot, Base of shoot 6.2 – 15.0 mm in diameter. Sheath green turning hazel or reddish-brown. Ligule 4.2 – 4.8 mm long, light green, glabrous. Petiole 7.0 – 16.0 mm long, swollen, yellowish. Lamina 11 – 17.5 (–21) by 6.5 – 10.8 cm, ovate to lanceolate, dark to mid-green above, slightly plicate, fleshy, young leaves pale brownish-red, glabrous, base cuneate to rounded, apex acuminate, acumen often elongated. Inflorescence from rhizome or nodes of lower part of the stem, 2 – 4 flowers per inflorescence. Peduncle short ascending, 2 – 8.2 mm, brownish. Fertile bracts 7.9 – 14.5 by 10.5 – 12.5 mm, triangular, creamish brown, membranous, slightly decaying into fibres. Bracteole 7 – 10 mm long, boat-shaped, green. Calyx 20 – 25 mm long, watery green, glabrous, lobes triangular 2.8 – 3.5 mm long. Corolla whitish to watery green, tube to 1.2 cm long, glabrous, lobes 2.5 – 2.7 cm long. Staminal tube c. 1.2 cm long. Labellum to c. 2.8 by 3.2 cm, white, yellowish patch in throat. Stamen to c. 20 mm long, Anther 3 – 4 mm long, inserted on a cushion-like thickening, anther crest irregularly toothed to trilobed. Ovary 3 – 6 mm, angled, densely pubescent. Style 2.9 – 3.2 cm long, white. Stigma bilamellated, with a dorsal rounded appendage. Fruit unknown. 2n=36.

Distribution: Endemic to Borneo
Ecology: Understory forest herb, growing in shaded, moist places.
IUCN assessment: Endangered

Notes: The drawing from the original publication (Meekiong et al., 2006) can be found in Appendix 3. The author's description of the petal lobes is found in the stamen section and vice versa, which the authors consistently confuse. Based on the inflorescence and the habit it is clear that this species belongs in the genus *Paracostu*s.

Specimens examined:
Brunei.
ADP 400, Temburong: Amo, Batu Apoi Forest Reserve.

Malaysia
Sarawak
A.D.Poulsen 2070 (SAR Biodiv Cent. SAR, AAU, E), Guunung Mulu, 17 Jul 2003; Bernard Lee S54663 (E), Sg. entulu Sg Mengiong, Btg Beleh, Kapit 19 July 1987; *Burtt & woods B2063* (E), Gunong Mulu, 13 June 1962; Kerby 216, Gunong Mulu, 1977; Kerby 226, Gunong Mulu, 1977; Kerby 818, Gunong Mulu, 25 Nov 1977.

4. *Paracostus paradoxus (K. Schum) C.D.Specht*
Basionym: — *Costus paradoxus* K.Schum (1899) 345
Protologue: C.D.Specht in Taxon 55(1): 162, f. 1. 2006. 162.
Type: Beccari 3791 (Fl), Bellaga, prov. Redjang, Sarawak, Borneo.

Terrestrial herb. Rhizomes long-creeping. Leafy shoot 20 – 70 cm tall, prostrate, with 2 – 5 leaves per shoot, Base 5 – 15 mm in diameter. Sheath yellowish to reddish-brown. Ligule to c. 1 mm long, decaying into fibres at early stage. Petiole to 7 mm long. Lamina 12 – 17 by 5.5 – 9 cm, elliptic to ovate, fleshy, glabrous, base cuneate, apex acute to acuminate. Inflorescence composed of 1 – 2 clusters, from rhizome or nodes of lower part of the stem, 3 – 5 flowers per cluster. Peduncle subterranean to 6 cm long, on stem almost absent, ascending. Fertile bracts 10 – 15 by 8 – 12 mm, triangular, creamish, membranous. Bracteole 9 – 12 mm, membranous, light creamy brown, Calyx 17 – 24 mm long, green, glabrous, teeth deltoid. Corolla whitish, turning yellow towards apex, tube 2 – 2.5 cm, lobes 2.5 – 2.8 cm long. Staminal tube c. 20 mm long. Labellum 2.5 – 3.0 by 2.0 – 3.0 cm, yellow, red stripes in centre, glabrous, margin irregularly undulated. Stamen 15 – 25 mm long, Anther 4 – 4.5 mm long, inserted on a cushion-like thickening, anther crest deeply bilobed. Ovary 3.0 – 5.0 mm long, sub-globose densely pubescent. Style 2.5 – 3.0 cm long, white. Stigma bilamellated, with a dorsal rounded to slightly bilobed appendage. Fruit 12 – 15 mm in diameter, sub-globose. 2n=18.

Distribution: Endemic to Borneo.
Ecology: Mixed Dipterocarp forest, shady areas.
IUCN assessment: Endangered

Notes: The description of *Costus paradoxu*s from Meekiong et al. (2006) contains contradicting information for the calyx, corolla and the labellum. It seems a part of the description of *C. muluensis* was pasted into the description of *C. paradoxus*, as this part is missing the description of *C. muluensis.*

Specimens examined:

Malaysia

Sarawak

ADP 2031 (AAU, SAR) Sungau Rayu, 2 Jul 2003; *Burtt 11293* (E), Punan Lusong, 24 Aug 1978;

Chai S33579 (E, KEP, L), Ulu sg. Kaup, Bukit Uba riba, 10 Mar 1974 ; *Julaihi S83569* (K, Kep), Sabal, Balai Ringin forest reserve, 23 Apr 2000 ; *Carlo Hansen 622* (E), 7th division, Ula belaga, Sungai semawat, 15 Oct1981; *Purseglove 5359* (L), Sungei Mayeng, Tau Range, 4 Jun 1956; *Teck S68611* (E, KEP) Lubok Antu, 3 Feb 1995;

Indonesia

Kalimantan

Winkler 1274 (E), Sungei Malang, 28 Jan 1925; *Amdjah 303* (L), 13 Jul 1912.

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Figure 16: Distribution maps of most Bornean Costaceae. **A:** *Hellenia borneensis*, **B:** *H. globosa*, **C:** *H. speciosa*, **D:** *Paracostus eburneus*, **E:** *P. muluensis*, **F:** *P. paradoxus.*

6. Conclusions

6. CONCLUSIONS AND SUGGESTED FURTHER WORK

Even though molecular studies were not successful this study has made a significant contribution to the understanding of Costaceae in Borneo, and the Asian Costaceae in general. The addition of chromosome counts has resulted in uncovering previous unknown information to science, and the taxonomical treatment has cleared up some taxonomical difficulties. This is not just a benefit to the science but can aid any enthusiast in better understanding this wonderful family.

As with all studies that are largely based on herbarium vouchers, there are many characters that can't be studied from dried material. Fieldwork will have to be conducted on Borneo to collect samples on the *Paracostus* species in particular, for they show a remarkable variation, but with the very limited amount of information currently available it is difficult to explains these differences.

Although herbarium investigations inevitably lead to deficiencies in information (Burtt & Smith 1972), I am confident that this thesis provides clarity on the taxonomic situation, and will be of benefit to everyone who is looking for more information on the Bornean Costaceae.

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Appendix 1

Table 1: PCR recipe used for *CaM, ITS, rps16, psbA-trnH* and *trnL-F.*

Reagent	Volume in µl for one sample
dNTPs	2
10xBuffer	2
$MgCl_2$	0,6
primer A	2
primer B	2
Taq	0,3
CES	4
Template	1
H_2O	6,1

Table 2: PCR recipe, used in *trnL* and *trnF.*

Reagent	Volume in µl for one sample
dNTPs	2
10xBuffer	2
$MgCl_2$	0,6
primer A	2
primer B	2
Taq	0,3
TBTPAR	4
Template	1
H_2O	6,1

Table 3: Additive free PCR recipe used for *trnL.*

Reagent	Volume in µl for one sample
dNTPs	2,5
10xBuffer	2,5
$MgCl_2$	1,25
primer A	0,75
primer B	0,75
Taq	0,125
Template	1
H_2O	16,125

Table 4: Alternative Phire Hot Start recipe.

Reagent	Volume in µl for one sample
dNTPs	0,4
5x Phire buffer	4
TBT-PAR	4
primer A	2
primer B	2
Phire Hot Start II	0,4
Template	1
H_2O	6,2

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Table 5: SPCR protocol *ITS*.

Temp. (ºC)	Duration (min:sec)	Repeats
95	4 min	1
94	1 min	
55	1 min	30
72	45 sec	
72	5 min	1
10	forever	1

Table 6: SPCR protocol *rps16*.

Temp. (ºC)	Duration (min:sec)	Repeats
94	3 min	1
94	45 sec	
55	45 sec	
72	1 min	35
72	5 min	1
10	forever	1

Table 7: SPCR protocol *psbA-trnH*.

Temp. (ºC)	Duration (min:sec)	Repeats
94	3 min	1
94	45 sec	
50	45 sec	2
72	1 min	
94	45 sec	
45	45 sec	30
72	1 min	
72	5 min	1
10	forever	1

Table 8: Additve free PCR protocol *trnL*.

Temp. (ºC)	Duration (min:sec)	Repeats
94	3 min	1
94	1 min	
55	1 min	30
72	90 sec	
72	5 min	1
10	forever	1

Table 9: *CaM* PCR, without Phire Hot Start.

Temp. (ºC)	Duration (min:sec)	Repeats
94	1 min	
52	1 min	30
72	90 sec	
72	5 min	1
10	forever	1

Table 10: *Cam* PCR protocol, less cycles.

Temp. (ºC)	Duration (min:sec)	Repeats
98	3 min	1
98	5 sec	
53	15 sec	30
72	20 sec	
72	1 min	1
10	forever	1

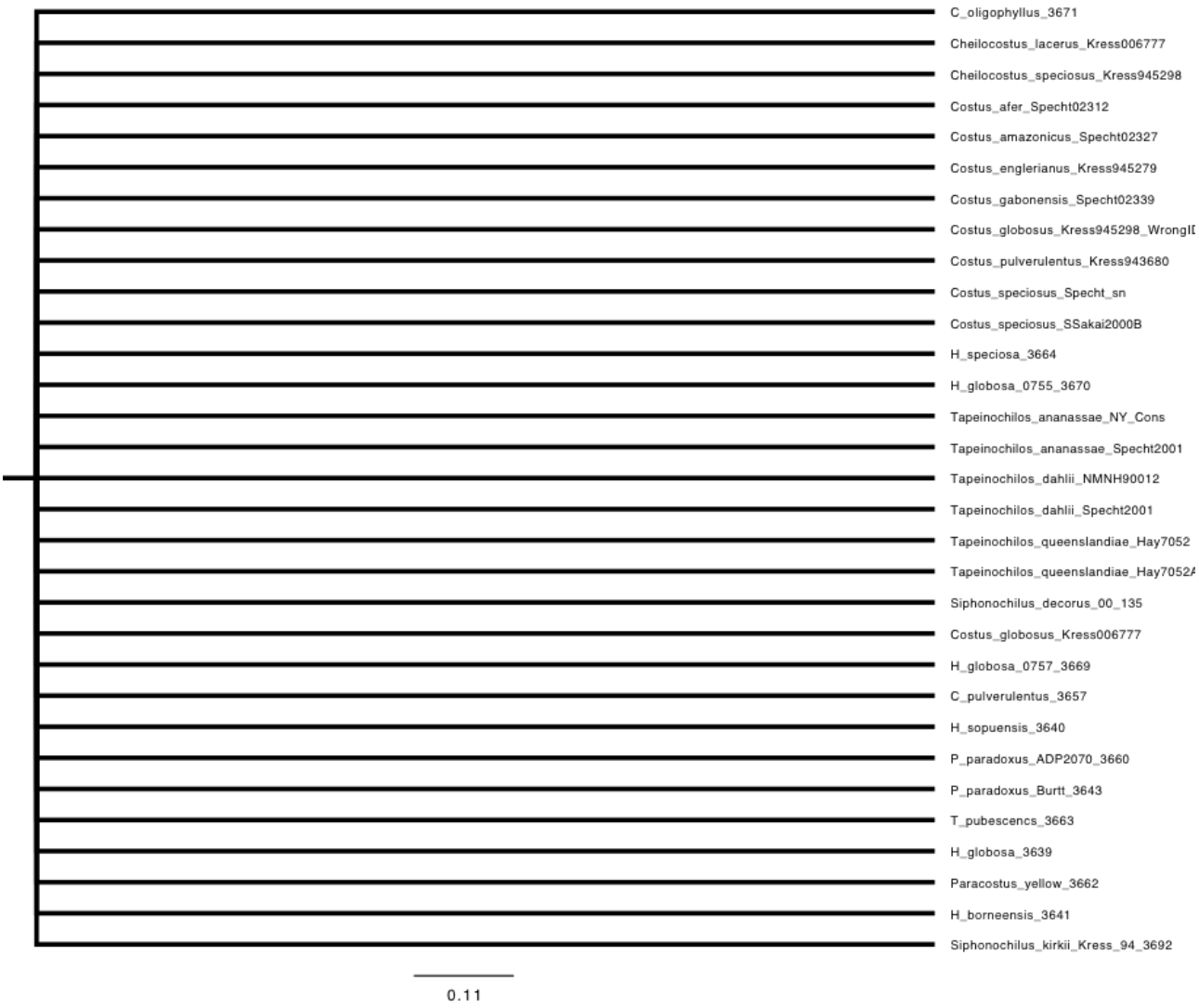

Figure 1: Maximum Parsimony Strict consensus tree of *trnF*.

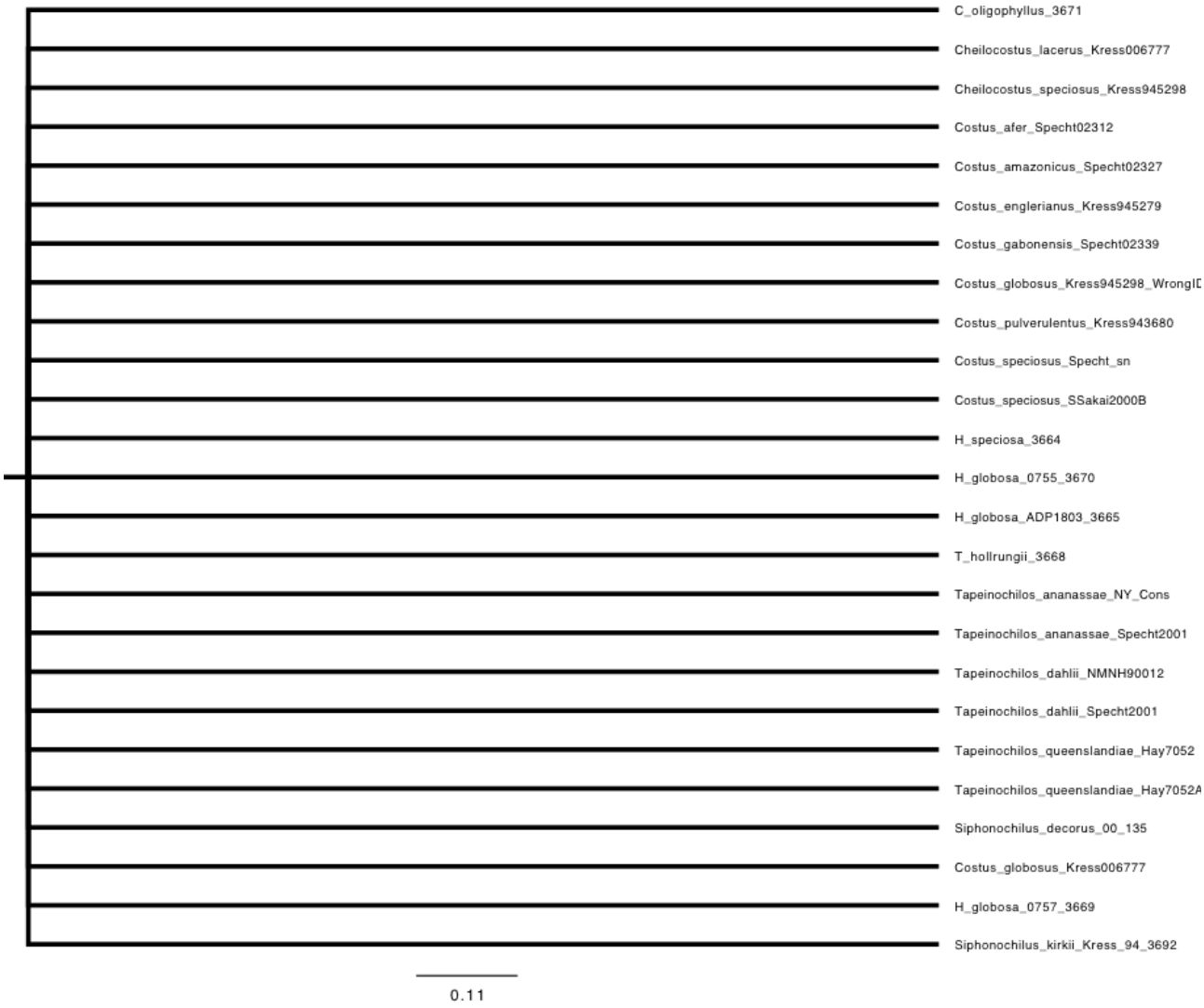

Figure 2: Maximum Parsimony Strict consensus tree of *trnL*.

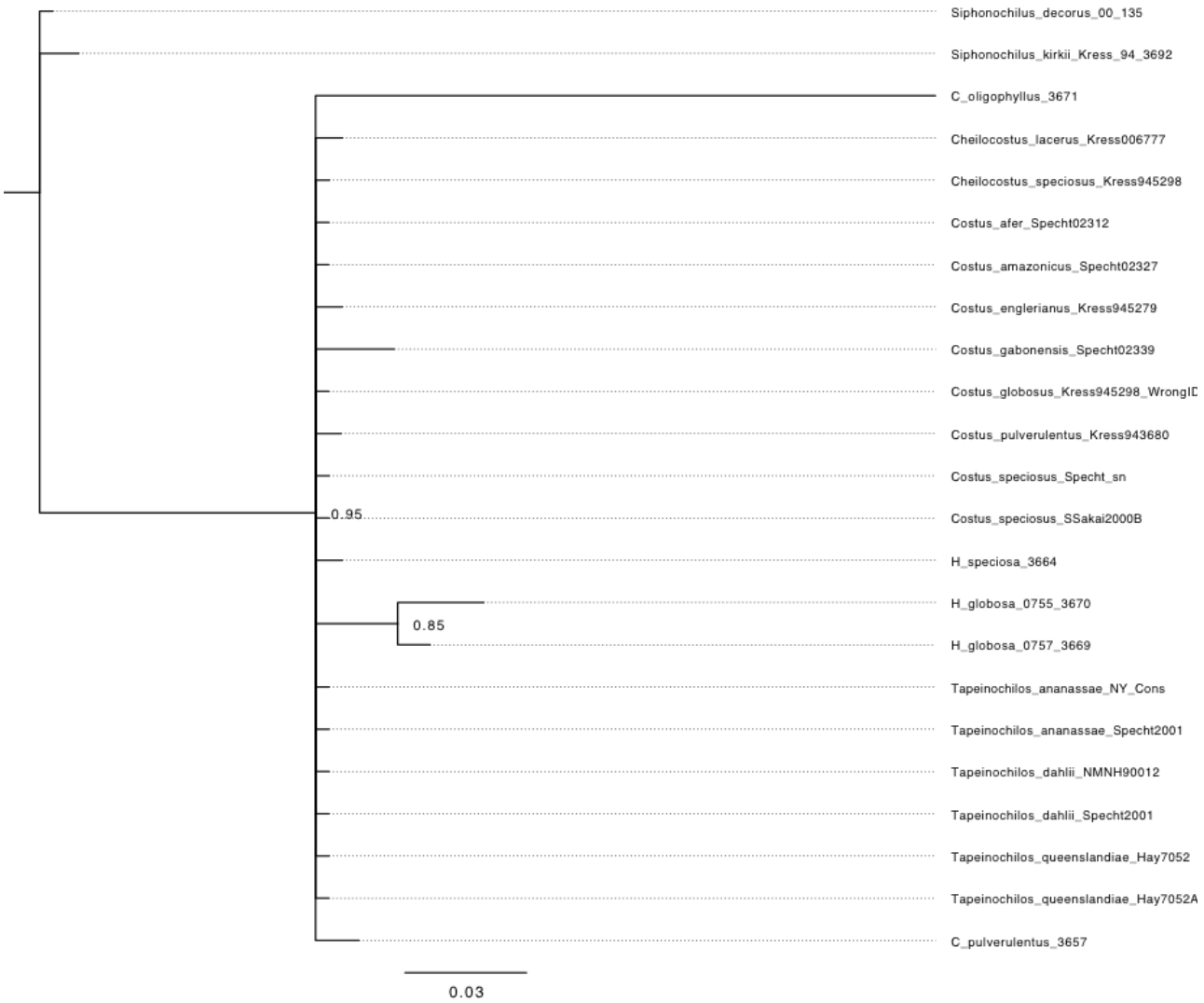

Figure 3: Result of Bayesian analysis for *trnL*.

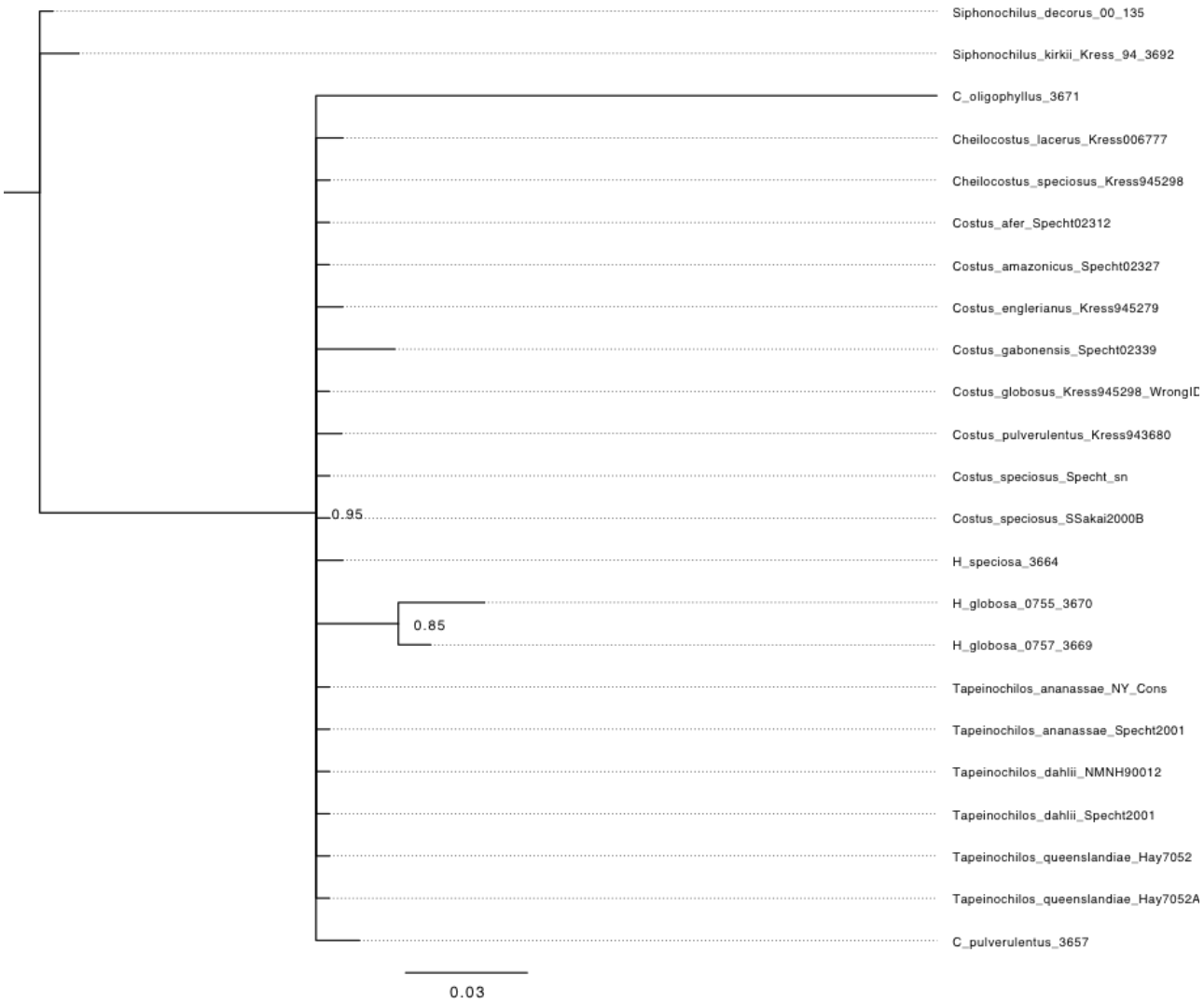

Figure 4: Result of Bayesian analysis for *trnF.*

Appendix 2

Table 1: Accessions used for cytological study.

Taxon name	Accession number
Paracostus muluensis	19773484
Paracostus paradoxus	20040971
Hellenia speciosa	19751812
Hellenia borneensis	20040728
Hellenia globosa	20070757
Paracostus muluensis	19773474
Hellenia sopuensis	20090617
Hellenia globosa	20070755

Appendix 3

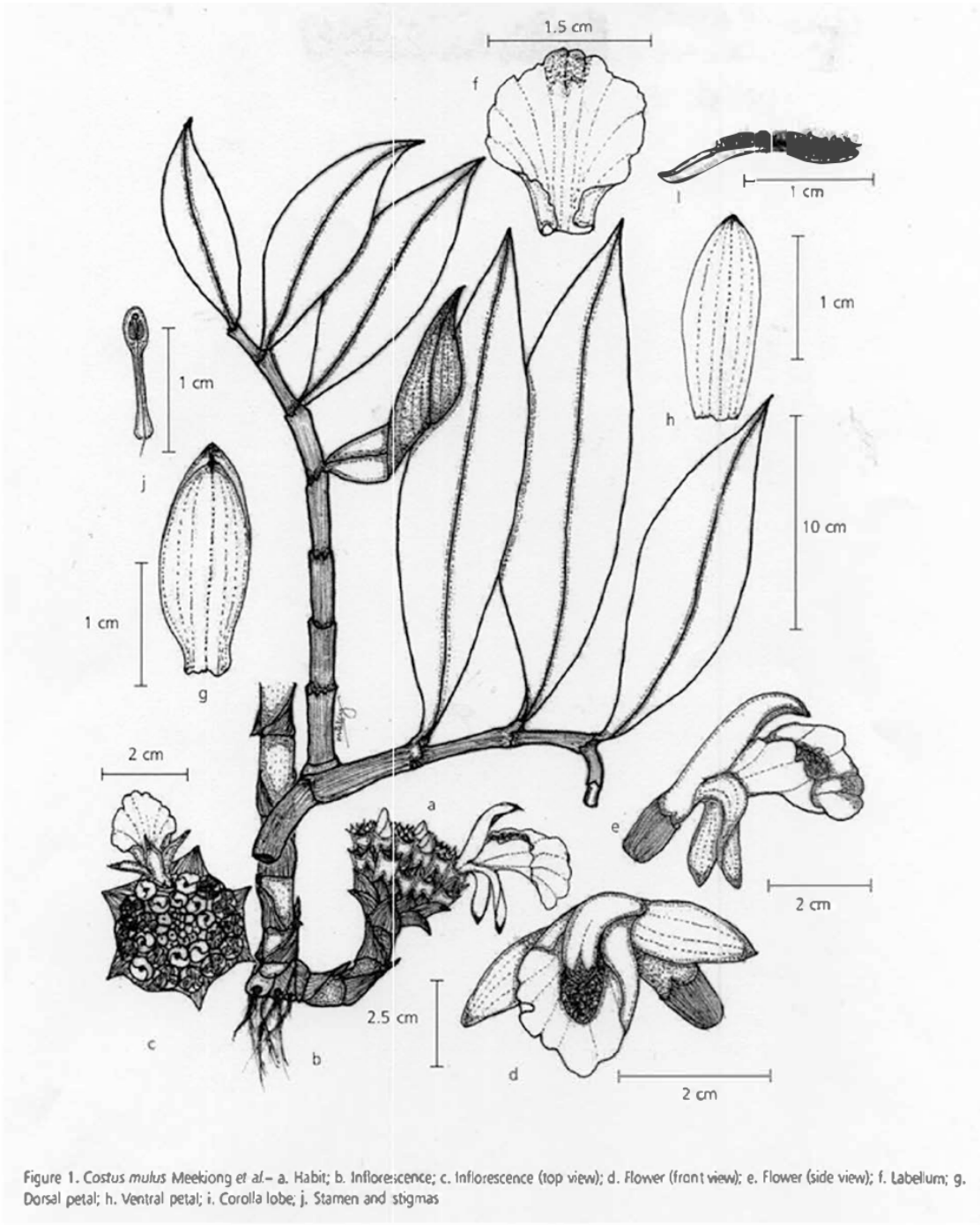

Figure 1. *Costus mulus* Meekiong *et al.*– a. Habit; b. Inflorescence; c. Inflorescence (top view); d. Flower (front view); e. Flower (side view); f. Labellum; g. Dorsal petal; h. Ventral petal; i. Corolla lobe; j. Stamen and stigmas

Figure 1: Drawing from publication of *Costus mulus*.

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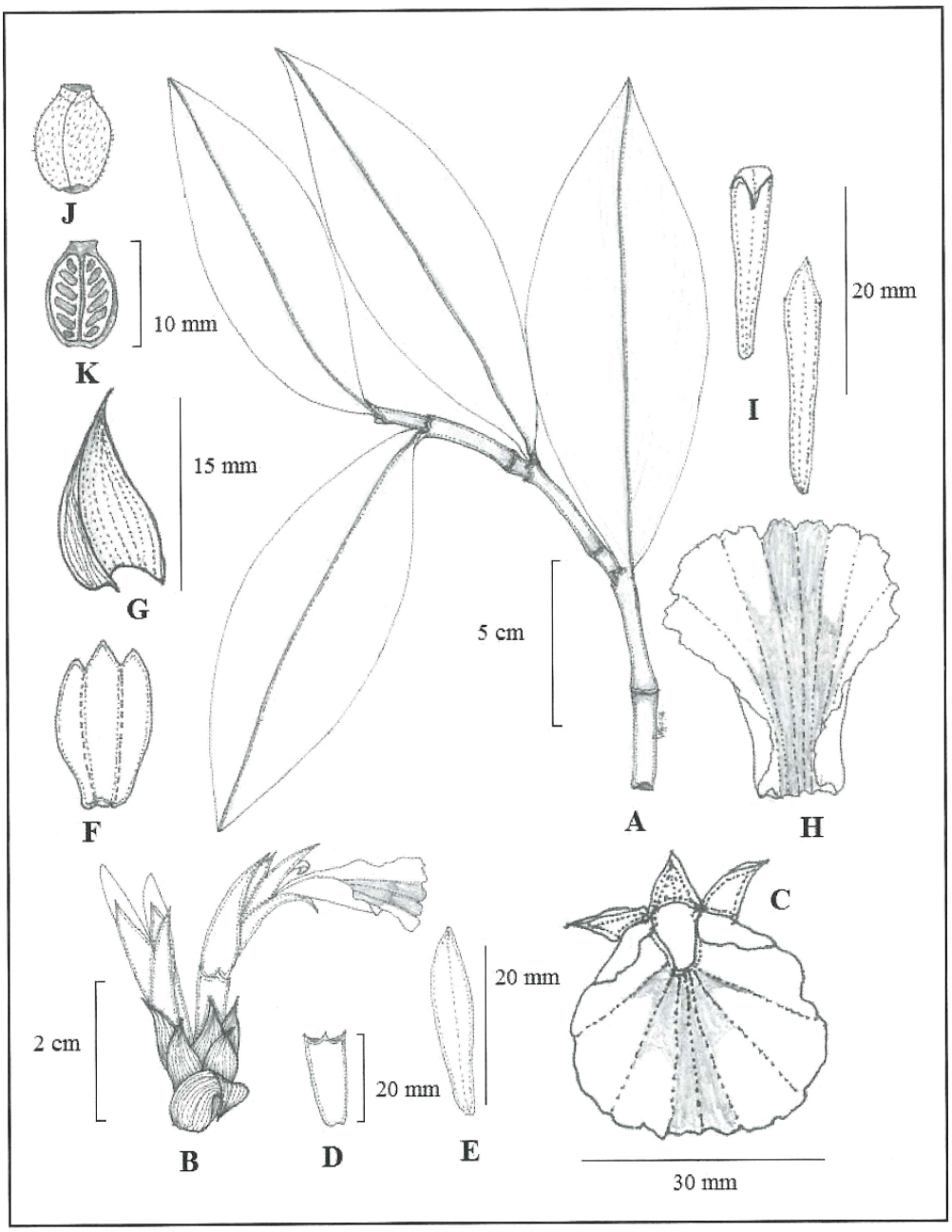

Plate 2: ***Costus muluensis*** Meekiong, Ipor & Tawan **sp. nov. A**: leaves; **B**: inflorescence; **C**: flower; **D**: calyx; **E**: lateral lobe; **F**: dorsal lobe; **G**: bracteole; **H**: labellum; **I**: corolla lobe; **J**: fruit; **K**: longitudinal section of fruit. Specimen no.: *MK1190* (**HUMS**). Type locality: Mulu National Park, Miri, Sarawak. {Drawing by K. Meekiong]

Figure 2: Drawing from publication of *Paracostus muluensis.*

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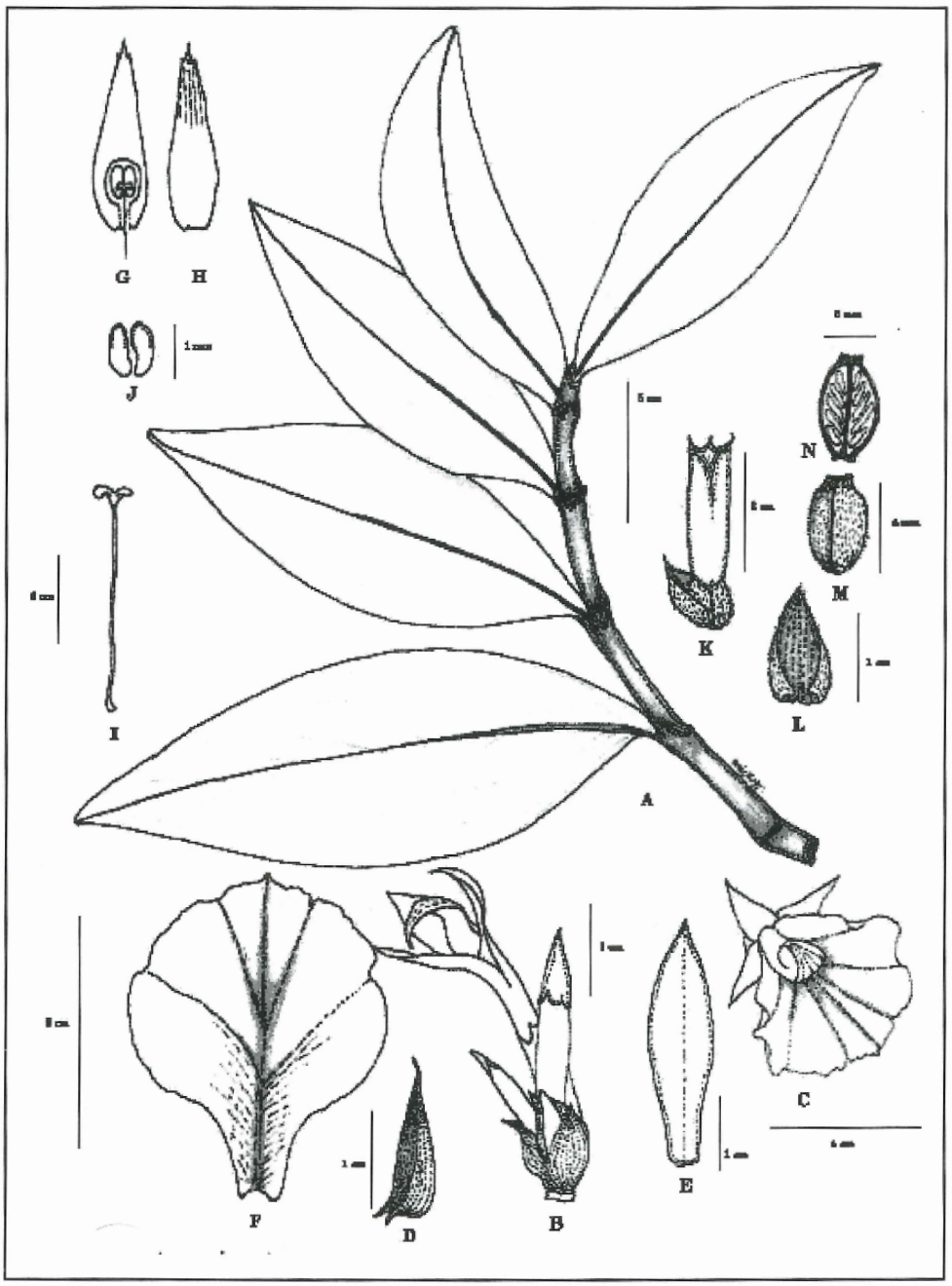


Plate 10: ***Costus eburneus*** Meekiong, Muliati & Tawan **sp. nov. A:** leaves; **B:** inflorescence (labellum fallen); **C:** flower; **D:** bract; E: sepal; **F:** labellum; **G, H:** Corolla lobe; **I:** style with stigmas; **J:** stigmas; **K:** ovary with corolla tube; **L:** bracteole; **M:** ovary; **N:** ovary longitudinal section. Specimen no.: *MK1690* (HUMS).

Figure 3: Drawing from publication of *Paracostus eburneus.*

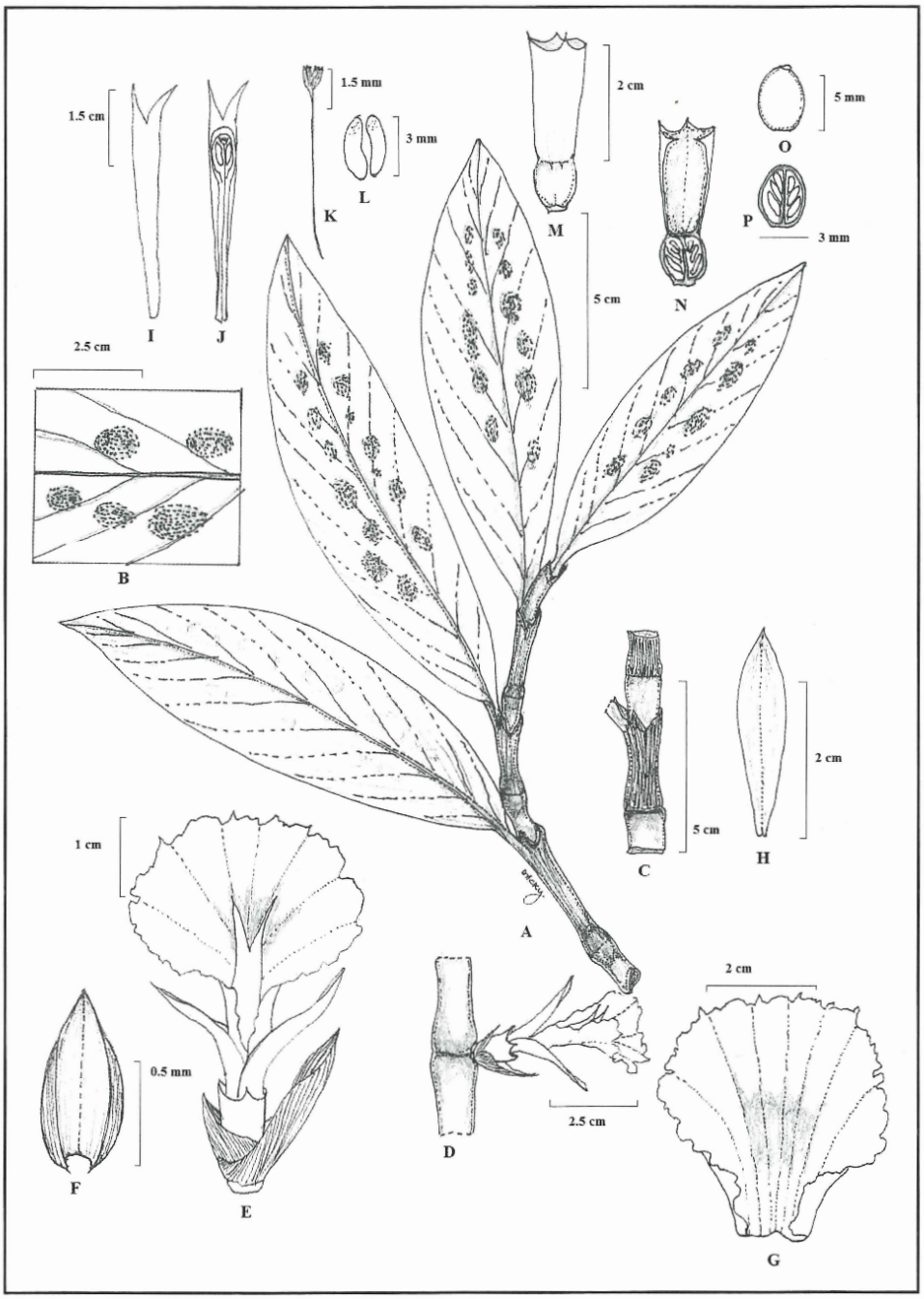
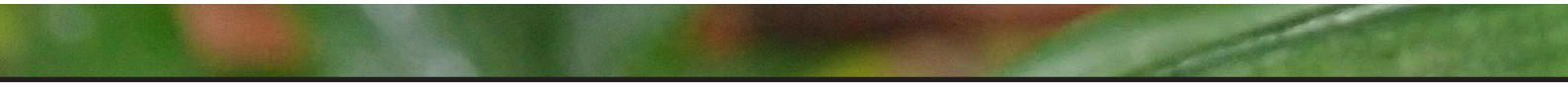


Plate 3: ***Costus bullatus*** **Meekiong, Muliati & Ipor sp. nov. A:** leaves**; B:** leaf surface with bullate; **C**: inter node; **D**: inflorescence from node at upper part of stem; **E**: flower; **F**: bract;**G**: labellum; **H**: petal; **I**: corolla lobe; **J**: corolla lobe inner; **K**: style; **L**: stigmas; **M**: corolla tube; N: longitudinal section of corolla tube with ovary; **O**: ovary; **P**: longitudinal section of ovary. Specimen no.: *MK1688* (**HUMS**). [Drawing by K. Meekiong]

Figure 4: Drawing from publication of *Paracostus bullatus.*

Revision *of* Bornean Costaceae



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