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Bilirubin: an Animal Pigment in the Zingiberales and Diverse Angiosperm Orders

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

BILIRUBIN: AN ANIMAL PIGMENT IN THE ZINGIBERALES AND DIVERSE
ANGIOSPERM ORDERS

A dissertation submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Cary Lunsford Pirone

2010

To: Dean Kenneth G. Furton
College of Arts and Sciences

This dissertation, written by Cary Lunsford Pirone, and entitled Bilirubin: An Animal Pigment in the Zingiberales and Diverse Angiosperm Orders, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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ABSTRACT OF THE DISSERTATION

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Florida International University, 2010

Miami, Florida

Professor David W. Lee, Major Professor

Strelitziaceae is a tropical monocot family comprising three genera and seven species: *Ravenala* Adans and *Phenakospermum* Endl., which are monotypic, and five species of *Strelitzia* Aiton. All species produce woody capsular fruits that contain vibrantly colored arillate seeds. Arils of the *Strelitzia* species are orange, those of *Phenakospermum* are red, and those of *Ravenala* are blue. Unlike most plant pigments, which degrade after cell death, aril pigments in the family persist for decades. Chemical properties of the compounds are unusual, and do not match those of known pigment classes (carotenoids, flavonoids, betalains, and the chlorophylls). I isolated the orange pigment from the arils of *Strelitzia nicolai*, and performed HPLC-ESMS, UV-visible, ^1H NMR and ^{13}C NMR analyses to determine its chemical structure. These data indicated the pigment was bilirubin-IX, an orange-yellow tetrapyrrole previously known only in mammals and some other vertebrates as the breakdown product of heme. Although related tetrapyrroles are ubiquitous throughout the plant kingdom and include vital biosynthetic products such as chlorophyll and phytochromobilin, this is the first report of bilirubin in a plant, and

evidence of an additional biosynthetic pathway producing orange coloration in flowers and fruits.

Given the unexpected presence of bilirubin, I examined the fruits and flowers of twelve additional angiosperm species in diverse orders for the presence of bilirubin using HPLC and LC-MS. Bilirubin was present in ten species from the orders Zingiberales, Arecales, and Myrtales, indicating its wide distribution in the plant kingdom. Bilirubin was present in low concentrations in all species except those within Strelitziaceae. It was present in particularly high concentrations in *S. nicolai*, *S. reginae* and *P. guyannense*, and is thus responsible for producing color in these species.

No studies have examined the evolutionary relationship among all species in the family. Thus, I also constructed a molecular phylogeny of the family. This information, combined with further studies on the distribution and synthesis of bilirubin in plants, will provide a basis for understanding the evolutionary history of this pigment in the plant kingdom.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
Major Pigment Pathways	1
Minor Pigment Pathways	3
Pigments in the Strelitziaceae	6
Proposed Research	7
Literature Cited	9
II. THE ANIMAL PIGMENT BILIRUBIN DISCOVERED IN PLANTS	14
Supporting Information	16
Acknowledgments	17
Literature Cited	17
III. THE ANIMAL PIGMENT BILIRUBIN IDENTIFIED IN <i>STRELITZIA REGINAE</i> , THE BIRD OF PARADISE FLOWER.	22
Abstract	22
Introduction	22
Materials and Methods	23
Results and Discussion	28
Acknowledgments	29
Literature Cited	30
IV. BILIRUBIN PRESENT IN DIVERSE ANGIOSPERMS	38
Abstract	38
Introduction	39
Discussion	46
Acknowledgments	49
Literature Cited	49
V. PHYLOGENY OF THE STRELITZIACEAE	56
Abstract	56
Introduction	57
Methods	60
Results	65
Discussion	69
Acknowledgments	72
Literature Cited	72

SUMMARY	82
Future Studies	83
Literature Cited	85
VITA	87

LIST OF TABLES

TABLE	PAGE
2.1. ^1H NMR values of bilirubin.....	18
2.2. ^{13}C NMR values of bilirubin	19
3.1. Concentration of bilirubin in <i>S. reginae</i> arils and sepals.....	31
4.1. Plant species sampled.....	52
4.2. Bilirubin detection and concentration.....	53
5.1. Species sampled and taxonomic affiliations.....	77
5.2. Data on individual regions.....	77

LIST OF FIGURES

FIGURE	PAGE
1.1. Pigment structures from each of the four main classes, taken from Lee (2007).....	12
1.2. Pigmented arils of species from three Strelitziaceae genera. From left to right: <i>R. madagascariensis</i> , <i>S. nicolai</i> , <i>P. guyannense</i> . Seeds ~ 4mm in diameter.....	13
2.1. Bilirubin-IX α demonstrating intramolecular H-bonding.....	20
2.2. UV-VIS spectrum of bilirubin from <i>S. nicolai</i> (solvent system: 9:1 0.04 mM sodium acetate in methanol: 1% aqueous ammonium acetate).....	21
3.1. <i>Strelitzia reginae</i> . A. Inflorescence of <i>S. reginae</i> photographed at Fairchild Tropical Botanic Garden. Inflorescence is ~22 cm long. B. Mature and open capsule of <i>S. reginae</i> with arillate seeds exposed, capsules ~5.5 cm across. Photographed in Del Mar, CA by Tom DeFanti. C. Arillate seeds of <i>S. reginae</i> , seeds ~4 mm in diameter, photographed by Tom DeFanti. D. Aril fibers of <i>S. reginae</i> , in longitudinal view. Bilirubin is present in the crystalline granules attached to inner cell wall of these dead cells. Length of image = 300 μ m. E. Paradermal view of epidermal cells of orange sepals of <i>S. reginae</i> . Bilirubin granules are not seen in these cells, the color produced primarily by carotenoids in large chromoplasts. Length of image = 300nm.....	32
3.2. HPLC standard curve of bilirubinIX α	33
3.3. HPLC chromatograms of (A) Bilirubin standard and (B) Aril extract of <i>S. reginae</i>	34
3.4. Bilirubin standard via (+)ESI-Zoom-MS and MS/MS: (A) Zoom-MS of molecular ion region; (B) product spectrum of m/z 585; (C) product spectrum of m/z 583.....	35
3.5. Bilirubin in <i>S. reginae</i> aril via (+) ESI-MS and MS/MS: (A) Zoom-MS of molecular ion region; (B) product spectrum of m/z 585; (C) product spectrum of m/z 583.....	36
3.6. Bilirubin in <i>S. reginae</i> sepal extract via (+) ESI-Zoom-MS and MS/MS: (A) Zoom-MS of molecular ion region; (B) product spectrum of m/z 585; (C) product spectrum of m/z 5.....	37

4.1. Phylogeny of the Zingiberales. Phylogenetic relationships of genera in the Zingiberales (figure adapted from Kress et al., 2001) with photographs of four species sampled in this study.....	54
4.2. Bilirubin production in <i>Strelitzia nicolai</i> during aril development Maturing arils of <i>S. nicolai</i> with increasing amounts of bilirubin. Seeds ~4. mm in diameter.....	55
5.1. Figure 5.1. Strict consensus tree of all analysis types constructed using eleven chloroplast regions. Support values overlaid on nodes: MPBS (above branch, left), MLBS (above branch, right), and PP (below branch).....	78
5.2. Strict consensus tree of all analysis types constructed using <i>ETS</i> and <i>rpb2</i> . Support values overlaid on nodes: MPBS (above branch, left), MLBS (above branch, right), and PP (below branch). “-“ indicates node not present.....	79
5.3. Strict consensus tree of all analysis types using combined nuclear and chloroplast regions. Support values overlaid on nodes: MPBS (above branch, left), MLBS (above branch, right), and PP (below branch). “-“ indicates node not present.....	80
5.4. One of the two most parsimonious trees resulting from the analysis of the combined nuclear and chloroplast regions.....	81

I. INTRODUCTION

Green plants are reliant on light for a multitude of physiological processes including photosynthesis and the regulation of growth and development. The capture and transmission of light energy by plants is achieved using compounds which absorb, transmit, and reflect a broad spectrum of light from the UV-B to far-red. Most of these compounds are pigments, that absorb light in the visible range and thus produce color. Multiple metabolic pathways produce pigments, yet colors observed among flowering plants can be attributed to only four pathways: the terpenoid (carotenoids), the phenylpropanoid (flavonoids), the tetrapyrrole (chlorophyll, phytochromobilin), and the indole (betalains) (Fig. 1.1.).

An unusual orange pigment exists in the arils of several species in the monocot family Strelitziaceae. The pigment is intense, durable, and exhibits chemical properties that differ from those of known plant pigments. The research presented here focuses on the characterization of the pigment, and examines its phylogenetic distribution among flowering plants.

MAJOR PIGMENT PATHWAYS

The carotenoids, flavonoids, and tetrapyrroles are ubiquitous among plants and responsible for the majority of angiosperm coloration. Carotenoids are yellow, orange and red pigments synthesized via the terpenoid pathway. The basic C-40 tetraterpene backbone can be linear or contain cyclic end groups, and, like all pigments, it undergoes modifications such as hydroxylation that cause shifts in wavelength absorption and thus color production (Goodwin and Britton, 1988). Carotenoids play an integral role in

photosynthesis, they are synthesized in the chloroplasts of all photosynthetic organisms, and also accumulate in the plastids of flowers and fruits to produce color. Familiar examples include beta-carotene in carrot roots and sunflowers, lycopene in tomato fruits, and capsaxanthin in red peppers.

Anthocyanins are vacuolar pigments responsible for most magenta, red, and blue colors observed in vascular plants. Anthocyanins are a subclass of the flavonoids, and are thus phenolic compounds produced by the phenylpropanoid pathway. Other flavonoid subclasses include the yellow flavonols and the orange and red aurones and chalcones, which are less common than the anthocyanins (Harborne and Turner, 1984). Flavonoids have a 15-carbon base structure composed of two phenyl rings connected by a three carbon bridge that usually forms a third ring. Structural modifications such as glycosylations are common, and often shift the color of the molecule. Color is also altered by changes in the cellular environment, such as a shift in pH, by self-association in planar stacks, association with other molecules, or by complexation with metal ions (Koes, 1994; Harborne and Williams, 2000). Anthocyanins are often produced in flowers and fruits like blueberries and grapes. Yet they also are produced in leaves and stems, visible as either a red flush or mixed with chlorophyll to produce bronze and brown colors (Lee and Collins, 2001). In addition to facilitating pollination and dispersal, anthocyanin functions include photoprotection and free radical scavenging (Davies, 2004).

Tetrapyrroles include the chlorophylls and phytychromobilin, the photoreceptor of the light sensing protein phytyochrome. All tetrapyrroles are synthesized from eight molecules of δ -aminolevulinic acid (ALA) and are composed of four nitrogenous rings. In the case of chlorophyll, the tetrapyrrole structure is cyclical, and includes a fifth

isocyclic ring and a long phytol chain. The linear tetrapyrrole phytochromobilin is built from the same precursors as chlorophyll and shares the early portion of its biosynthetic pathway. However, its biosynthetic route diverges from that of chlorophyll when the cyclic protoporphyrin-IX is cleaved to form biliverdin-IX, the phytochromobilin precursor (Tanaka et al., 2008). Although tetrapyrroles are of fundamental import for photosynthesis and respiration, they are not typically involved in the coloration of flowers or fruits.

MINOR PIGMENT PATHWAYS

Betalains are a nitrogenous group of water soluble compounds with an indole nucleus. They are subdivided into two groups, the red-violet betacyanins and the yellow-orange betaxanthins (Lee, 2007). Both are conjugates of betalamic acid with cyclo-dihydroxyphenylalanine (cDOPA) glucoside and either an amino acid or an amine. Betalains are produced in all members of the Caryophyllales, with the exception of the Molluginaceae and the Caryophyllaceae, which produce anthocyanins (Strack et al., 2003). Although carotenoids and flavonoids are often produced within the same cells, production of betalains and anthocyanins appears to be mutually exclusive (Stafford, 1994). Betalain compounds contribute to color production in reproductive structures; the flowers of cacti, bougainvillea, and beets are colored by these compounds.

Quinones are widespread in the plant kingdom. The core structure is usually one, two, or three phenolic derived rings modified with carbonyl groups, but larger polycyclic structures also exist. These compounds can be derived from various biosynthetic pathways. Although broadly distributed, quinones do not usually contribute to the color of flowers and fruits for two reasons: they are usually found in roots and wood, and they

are often colorless or weakly pigmented (Davies, 2004).

A rare group of pigments, the phenalenones, are typically yellow to red in color, and occur in Strelitziaceae and some related families (Opitz and Schneider, 2002).

Although they are present in a variety of plant organs including fruits, phenalones are not known to significantly contribute to color. Structures of these compounds are diverse, and their biosynthetic pathways have not yet been completely resolved. Other rare pigments exist, but are excluded from this discussion because of the low frequency of occurrence. For comprehensive coverage of plant pigments and their metabolic pathways, see reviews by Davies (2004), Lee (2007), and Grotewold (2006).

SYSTEMATICS OF STRELITZIACEAE

Strelitziaceae, a small monocot family in the order Zingiberales, includes seven species: *Ravenala madagascariensis* Sonn., *Phenkospermum guyannense* (L.C. Rich.) Endl. ex Miq., *Strelitzia reginae* Banks ex Aiton, *Strelitzia juncea* Link, *Strelitzia alba* Skeels, *Strelitzia nicolai* Regel and Körn and *Strelitzia caudata* R. A. Dyer (APG: Stevens, 2001, Coates Palgrave and Coates Palgrave, 2002; Archer, 2003; Columbus et al., 2006). Like other Zingiberales, Strelitziaceae have an herbaceous habit, large petiolate leaves, and green, boat shaped bracts which subtend colorful inflorescences (Kress, 1990). Synapomorphies of the family include a tree-like growth form, three free sepals, two fused petals that enclose five (or six in *Ravenala*) stamens, and a woody loculicidal capsule (Kress, 1990) containing seeds with brightly colored arils. Three of the members, *S. reginae*, the bird of paradise flower, *S. nicolai*, the white bird of paradise tree, and *R. madagascariensis*, the travelers' palm, are widely cultivated as ornamentals.

Although the monophyly of *Ravenala madagascariensis* and *Phenakospermum guyannense* is widely accepted, *Strelitzia* species have a convoluted taxonomic history. The International Plant Names Index (IPNI) lists 34 names of *Strelitzia* species which have been in use in the past, although today there are just five recognized species. *S. reginae* and *S. nicolai* have been recognized as distinct species throughout their taxonomic history (Wright, 1913). These species can be distinguished on the basis of many morphological traits, perhaps the most obvious difference is that *S. nicolai* is caulescent, having an elongated main shoot, while *S. reginae* is acaulescent. However, the features which distinguish *S. nicolai*, *S. caudata*, and *S. alba* from each other, and *S. reginae* from *S. juncea* are less obvious. *S. alba*, *S. caudata*, and *S. nicolai*, are differentiated only by sepal color, complexity of inflorescence, geographic locale, and habit (Wright, 1913; Dyer, 1945; Goldblatt and Manning, 2000; Coates Palgrave and Coates Palgrave, 2002). They have been classified as distinct species since 1945, when Dyer clarified species boundaries between *S. nicolai* and *S. alba*, and described *S. caudata* (Dyer, 1945). The morphological differences between *S. juncea* and *S. reginae* are also minor; the main distinguishing feature of these taxa is the reduced leaf blade of *S. juncea* (Moore and Hyypio, 1970). Although *S. juncea* was originally described as a species in 1821 by Link, it was reduced to a variety or cultivar of *S. reginae* (Moore and Hyypio, 1970), and then raised again to species status on the basis of developmental differences (Van der Venter, 1975, Dyer, 1975). Because of the minor morphological differences which guide the delineation of some of the *Strelitzia* taxa, molecular data could help clarify species delimitations.

Several large scale phylogenetic studies have sampled a single species from each of the three genera, but results are ambiguous, and no studies have included all *Strelitzia* species. *Ravenala madagascariensis* was found to be basal to the sister taxa *S. nicolai* and *P. guyannense* in the combined three gene (atpB, rbcL, 18s rDNA) analyses of Soltis et al. (2000), Soltis et al. (2007), and Chase (2000), and also in the combined three gene (atpB, rbcL, 18s rDNA) plus morphological dataset of Kress (2001). Using a single gene dataset of ndhF, Givnish et al. (2006) found *S. nicolai* to be basal to *R. madagascariensis* and *P. guyannense*. Thus, although the Strelitziaceae has been confirmed as a monophyletic group, evolutionary relationships within the family remain unclear (Kress et al., 2001, Chase et al., 2000, Givnish 2006, Rudall et al., 1999, Soltis et al., 2000, Soltis 2007).

PIGMENTS IN THE STRELITZIACEAE

Members of Strelitziaceae produce woody tripartite loculicidal capsules, which dehisce at maturity to reveal brightly colored arillate seeds (Kress, 1990). The waxy, funicular arils of *R. madagascariensis* are blue, those of *P. guyannense* are orange-red, and those of the *Strelitzia* species are orange (Fig. 1.2). Remarkably, aril color remains unchanged years after cell death; arils with little or no change in color were observed in herbarium sheets up to 45 years old (personal observation; HUH, Sturrock #93, 1963; and Natal #50,000, 1976). Previous studies of pigments from species within Strelitziaceae have focused on *Strelitzia reginae*. *S. reginae* has striking flowers composed of three vibrant orange sepals and three blue petals. At least nineteen carotenoids are present in the orange sepals (Simpson et al., 1975; Tappi and Menziani, 1955), and the anthocyanin delphinidin-3-rutinoside was found in the blue petals (Harborne, 1967). Phenalenones

were documented in its roots (Holster and Schneider, 2000).

Preliminary studies indicated that the aril pigments of *S. nicolai*, *P. guyannense*, and *R. madagascariensis* showed unusual UV-Visible spectra and solubilities that did not match those of the *S. reginae* floral pigments, or any of the aforementioned major pigments. It was not possible to compare aril pigment properties to those of phenalenones because of the diversity of UV-Visible spectra and solubility of these compounds. Thus, the structure of these unusually durable visible pigments remains unknown.

PROPOSED RESEARCH

Given the unusual chemical properties of the persistent orange pigment in *S. nicolai*, it is of interest to determine whether the pigment is restricted to *S. nicolai*, or whether it is present in other flowering plants. Thin layer chromatography of *P. guyannense* aril extracts yielded an orange band with an R_f value identical to that of *S. nicolai* under several different solvent regimes, suggesting that the pigment is not limited to *S. nicolai*.

Using high performance liquid chromatography with UV detection (HPLC/UV) and HPLC/UV/electrospray ionization tandem mass spectrometry (HPLC/UV/ESI-MS/MS), representatives from diverse flowering plant lineages were analyzed for the presence of the pigment. Since structurally related compounds are often found in closely related taxa (Harborne and Turner, 1984), sampling focused on species of phylogenetic proximity to *S. nicolai* within the family, including *P. guyannense* and *R. madagascariensis*. Other members of the Zingiberales were also sampled. Representatives from the “banana group”, a basal clade of the Zingiberales including the

Musaceae, Strelitziaceae, Heliconiaceae, Lowiaceae (Kress et al., 2001) were sampled, with the exception of *Orchidantha*, the sole genus of Lowiaceae. Inclusion of this genus in the analysis is desirable due to its sister relationship with the Strelitziaceae (Kress et al., 2001, Chase et al., 2000, Givnish 2006, Rudall et al., 1999, Soltis et al., 2000, Soltis 2007), but *Orchidantha* is rare and thus difficult to obtain. Species from two of the more derived ginger families, Zingiberaceae, Costaceae, were also sampled. Outside of the Zingiberales, members of the basal dicot order Laurales, two monocot orders, the Arecales and the Pandanales, and the eudicot order Myrtales, a part of the Rosid clade (APG 9: Stevens, 2008) were also sampled to determine the breadth of the orange pigment distribution among angiosperms.

Consideration of these data in a better-resolved phylogenetic framework will provide a basis for understanding the evolutionary history of the orange pigment in plants. Phylogenies are available for the major angiosperm clades (APG 9: Stevens, 2008), yet no phylogeny of Strelitziaceae exists. The research presented here will therefore include a reconstruction of the phylogeny of the Strelitziaceae using eleven chloroplast regions, *rbcL*, *matK* -5' *trnK*, *psbA*- *matK* , *psbB-psbH*, *Ycf6-trnC*, the *rpL16* intron, *rpoB-trnC*, *trnS-trnfM*, *trnY-trnE*, *rpL32-trnL*, *rps16*, and two nuclear regions, *ETS* and *rpb2*.

Research Aims:

1. Identify the orange pigment in the arils of *S. nicolai*.
2. Identify the orange pigment in the arils and sepals of *S. reginae*, and compare this pigment with that of *S. nicolai*.
3. Determine the presence of the orange pigment in diverse angiosperm taxa.

4. Reconstruct the phylogeny of the Strelitziaceae.

Chapters II, III, and IV have been published in the Journal of American Chemical Society, HortScience, and AoB Plants, respectively. Chapter V will be submitted to Systematic Botany.

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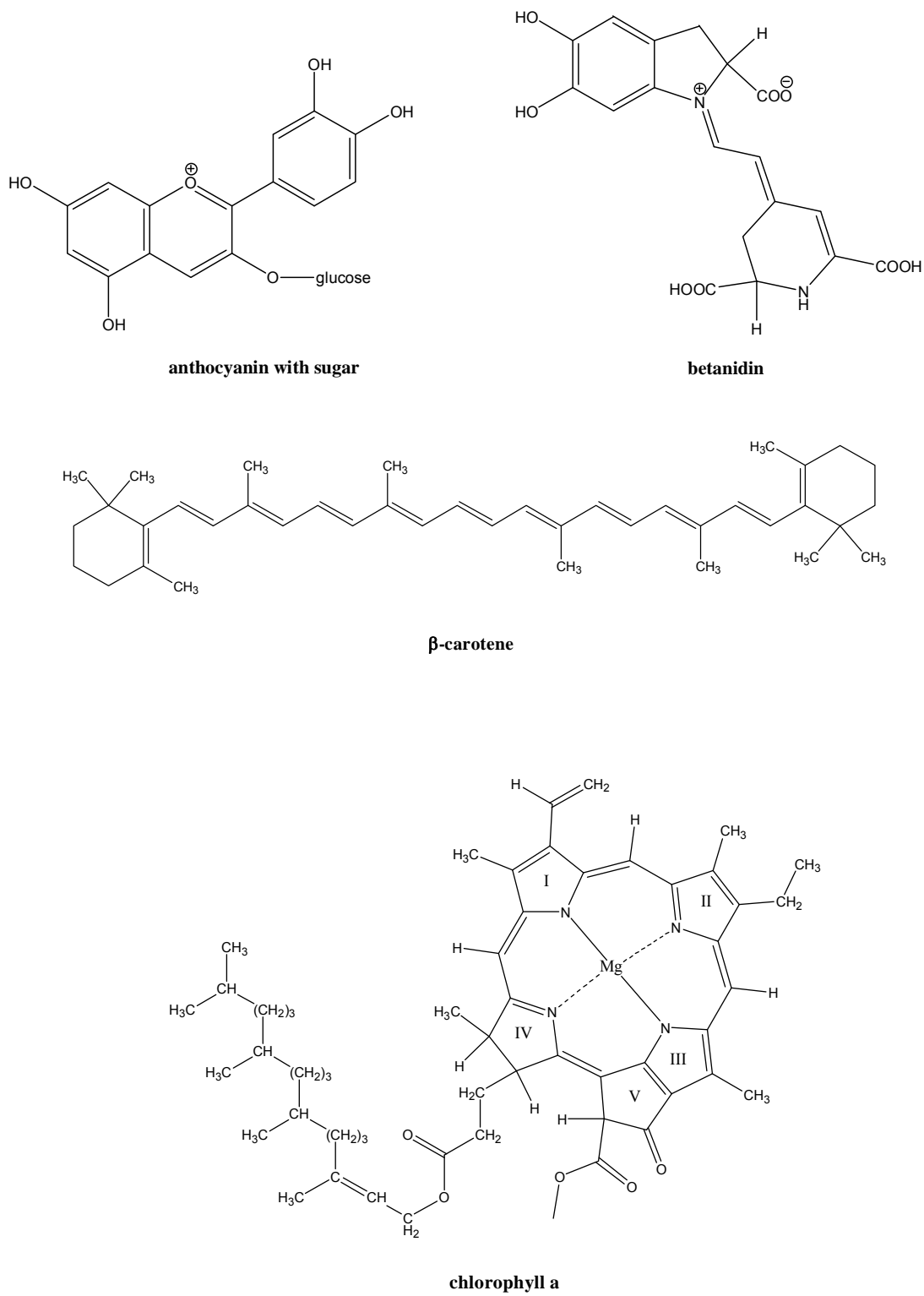


Figure 1.1. Pigment structures from each of the four main classes, taken from Lee (2007).

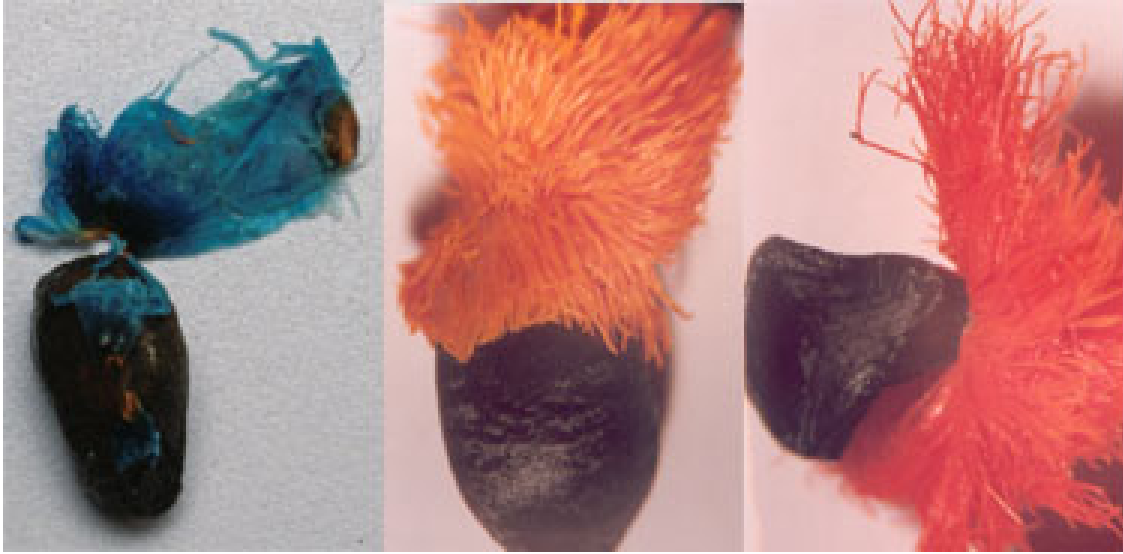


Figure. 1.2. Pigmented arils of species from three Strelitziaceae genera. From left to right: *R. madagascariensis*, *S. nicolai*, *P. guyannense*. Seeds ~ 4mm in diameter.

II. THE ANIMAL PIGMENT BILIRUBIN DISCOVERED IN PLANTS

Reproduced with permission from Pirone, C. L., J.M.E., Quirke, H., Priestap, and D.W. Lee. 2009. Animal pigment bilirubin discovered in plants. J. Amer. Chem. Soc. 131 (8): 2830. Copyright 2009 American Chemical Society."

Cyclic tetrapyrroles occur throughout the plant kingdom and include vital biosynthetic products such as chlorophyll and heme. In plants, oxidative degradation of heme first forms biliverdin IX- α and subsequently phytychromobilin, the precursor of the phytochrome chromophore, an essential light sensing molecule (Tanaka and Tanaka, 2007). In animals, oxidative degradation of heme also leads to the formation of biliverdin-IX α , but it is transformed into the yellow-orange pigment bilirubin-IX α (Fig. 2.1). Here, I present spectroscopic and chromatographic evidence that bilirubin is the major pigment of the orange aril of *Strelitzia nicolai* Regel & Koern. (Strelitziaceae, order Zingiberales), the white bird of paradise tree. This is the first example of bilirubin in a plant (Willows et al., 2004), a finding which likely necessitates the revision of the plant tetrapyrrole pathway since there is currently no known mechanism of bilirubin production in the plant kingdom.

Strelitzia nicolai is native to South Africa and widely cultivated in the tropics. It produces woody capsular fruits that contain orange arillate seeds. Analytical high-performance liquid chromatography with UV detection (HPLC/UV) of the aril extract (Spivak and Yuey, 1986) revealed one major peak, which had a UV-visible spectrum with a maximum absorbance at 444 nm. After purification using preparative scale HPLC/UV, the isolated pigment was analyzed by hydrogen nuclear magnetic resonance spectroscopy (^1H NMR), and carbon NMR (^{13}C NMR) (Brucker, 400 MHz, $(\text{CD}_3)_2\text{S}=\text{O}$),

and HPLC/UV/electrospray ionization tandem mass spectrometry (HPLC/UV/ESI-MS/MS) (Thermo-Finnigan LCQ).

The ^1H NMR and ^{13}C NMR spectra of the isolated pigment matched published values of bilirubin (Kuenzle, 1970; Kaplan and Navon, 1980) (Tables 2.1 and 2.2). Identification was further supported by ^1H NMR analysis of bilirubin standard (Aldrich), which yielded a spectrum which matched that of the *S. nicolai* pigment (Figure 2.2). Both the positive ion electrospray mass spectrum and the product ion spectrum matched those of authentic bilirubin standard and previous published data (Lightner, 1982) (molecular ion, m/z 585 ($\text{M} + \text{H}$)⁺, product ion m/z 299).

Given the unexpected discovery of bilirubin in plants, it was essential to confirm the identity of the pigment as bilirubin-IX α , and not other isomers. Previous chromatographic studies have demonstrated that the ability of bilirubin-IX α to undergo intramolecular hydrogen bonding makes it significantly less polar than bilirubin-IX β , γ or δ (Blanckart et al., 1976; Bonnett et al., 1978). In my HPLC analyses, a single peak was observed when bilirubin-IX α standard was co-injected with the isolated pigment, thereby eliminating the possibility that the pigment was bilirubin-IX β , γ or δ . Furthermore, the visible spectrum of bilirubin-IX α has an intense peak at 458 nm in dimethylsulfoxide (DMSO), which is approximately 50 nm longer than bilirubin-IX β , γ or δ (Blanckart et al., 1976). Other bilirubin isomers, including bilirubin-III α and bilirubin-XIII α , were eliminated because their ^1H NMR spectra are substantially different (McDonagh et al., 1982).

The occurrence of bilirubin is not restricted to *S. nicolai*. Two other species in the Strelitziaceae, *Phenakospermum guyannense* Endl., and *S. reginae* Aiton, the bird of

paradise, contain aril pigments which co-eluted with authentic bilirubin in HPLC and had similar UV-visible spectra. I am currently examining species in related families. This information, in combination with studies on the synthesis of bilirubin IX- α in *S. nicolai*, will provide the basis for a more thorough understanding of the evolutionary origin of this pigment in plants.

SUPPORTING INFORMATION

Isolation of Bilirubin from Plant Aril: Plant arils were removed from the seed and extracted with chloroform until the extracts were colorless. Aril lipids were isolated by re-dissolving the evaporated extract in dimethyl sulfoxide (DMSO) (3 mL per gram of extract) and extracting with hexane in a separatory funnel. The hexane layer was evaporated and the lipid residue stored for future study. The DMSO layer, which contained the pigment, was purified further by preparative HPLC using an XTerra MS C₁₈ OBD column (5 μ m, 50mm, 19mm i.d., Waters), and the binary solvent system developed by Spivak and Yuey (1986). The flow rate was 3 mL per minute. The bilirubin fraction, which eluted from 9 to 13 minutes, was collected from a series of runs, combined, diluted with HPLC grade water (1 mL eluate to 2.5 mL water), and passed through a Waters Sep-Pak C-18 cartridge. Bound pigments were washed with water to remove salts, then acetone, which was a co-solvent for water, and eluted with chloroform until eluates were colorless. The chloroform was evaporated under reduced pressure. Pigments were dried in a vacuum desiccator and stored at -80C. Analytical HPLC analyses were performed on a reversed phase ODS-A column (5 μ m, 4.3 x 150 mm; Waters Corporation, Milford, MA, USA). The solvents and gradient were the same as those used for the preparative HPLC work. The flow rate was 1 mL per minute.

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Table 2.1. ¹HNMR values of bilirubin

Hydrogens	Bilirubin from <i>S. nicolai</i>	Multiplicity	Bilirubin Standard
2-CH ₃	1.94	singlet	1.92
7-CH ₃	2.02 or 2.05	singlet	2.0 or 2.03
13-CH ₃	2.05 or 2.02	singlet	2.03 or 2.0
17-CH ₃	2.11	singlet	2.17
8 & 12-CH ₂ CH ₂ CO ₂ H	2.46	triplet	2.43
8 & 12-CH ₂ CH ₂ CO ₂ H	1.97	triplet (partly obscured)	1.94
10 CH ₂	4	singlet	3.99
18-CH=CH ₂ *	~5.30 (centroid)*	ABX system	~5.31 (centroid)*
18-CH=CH ₂ *	~6.22 (centroid)*	ABX system	~6.21 (centroid)*
18-CH=CH ₂ *	~6.6 (centroid)*	ABX system	~6.59 (centroid)*
3-CH=CH ₂ *	~5.63 (centroid)*	ABX system	~5.62 (centroid)*
3-CH=CH ₂ *	~5.66 (centroid)*	ABX system	~5.66 (centroid)*
3-CH=CH ₂ *	~6.84 (centroid)*	ABX system	~6.83 (centroid)*
5-H	6.1	singlet	6.1
15-H	6.1	singlet	6.1
21-H	10.1	broad singlet	10.04
22-H	10.49 or 10.52	broad singlet	10.45 or 10.48
23-H	10.52 or 10.49	broad singlet	10.48 or 10.45
24-H	9.97	broad singlet	9.92
COOH	12	broad singlet	11.9

*Coupling constants for isolated bilirubin and (bilirubin standard)

C-3: ABX unit: J_{A-B} = 2 Hz (2 Hz); J_{A-X} = 11.8 Hz (11.3 Hz); J_{B-X} = 17.4 Hz (17.1 Hz)

C-18: ABX unit J_{A-B} = 2.8 Hz (2.5 Hz); J_{A-X} = 11.3 Hz (11.5 Hz); J_{B-X} = 17.4 Hz (17.5 Hz)

Table 2.2. ^{13}C NMR values of bilirubin

Carbons	Bilirubin from S. <i>nicolai</i>	Published Bilirubin Values¹⁰
C-1 C=O	171.33	171.5
C-2	123.23	123.4
C-3	140.4	140.5
C-4	127.44	127.6
C-5 methine	98.9	99.2
C-6	122.03	122.1
C-7	119.56	119.6
C-8	123.42	123.4
C-9	130.79	130.6
C-10	23.6	23.7
C-11	131.45	131.4
C-12	124.1	124.0
C-13	119.77	119.8
C-14	122.26	122.3
C-15 methine	100.01	100.1
C-16	128.21	128.4
C-17	141.93	142.0
C-18	122.39	122.6
C-19 C=O	170.39	170.6
C-2'	9.43	9.5
C-3'	127.34	127.5
C-3''	122.08	122.2
C-7'	9.11	9.2
C-8'(C-12')	19.23	19.3
C-8''(C-12'')	34.02, 34.00	34.4
C-8'''(C-12''')	173.95	174.1
C-13'	9.12	9.3
C-17'	9.23	9.4
C-18' CH=CH2	127.06	127.2
C-18'' CH=CH2	117.1	117.2

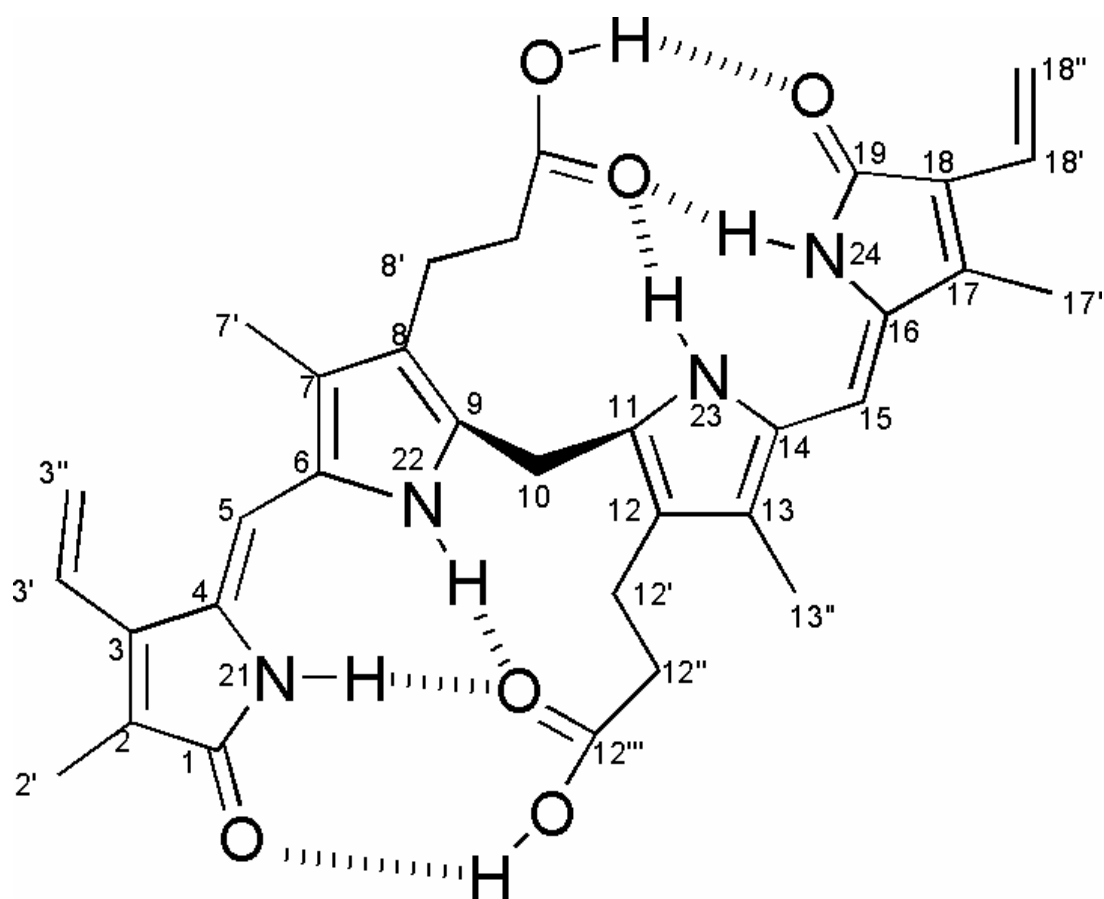


Fig. 2.1. Bilirubin-IX α demonstrating intramolecular H-bonding.

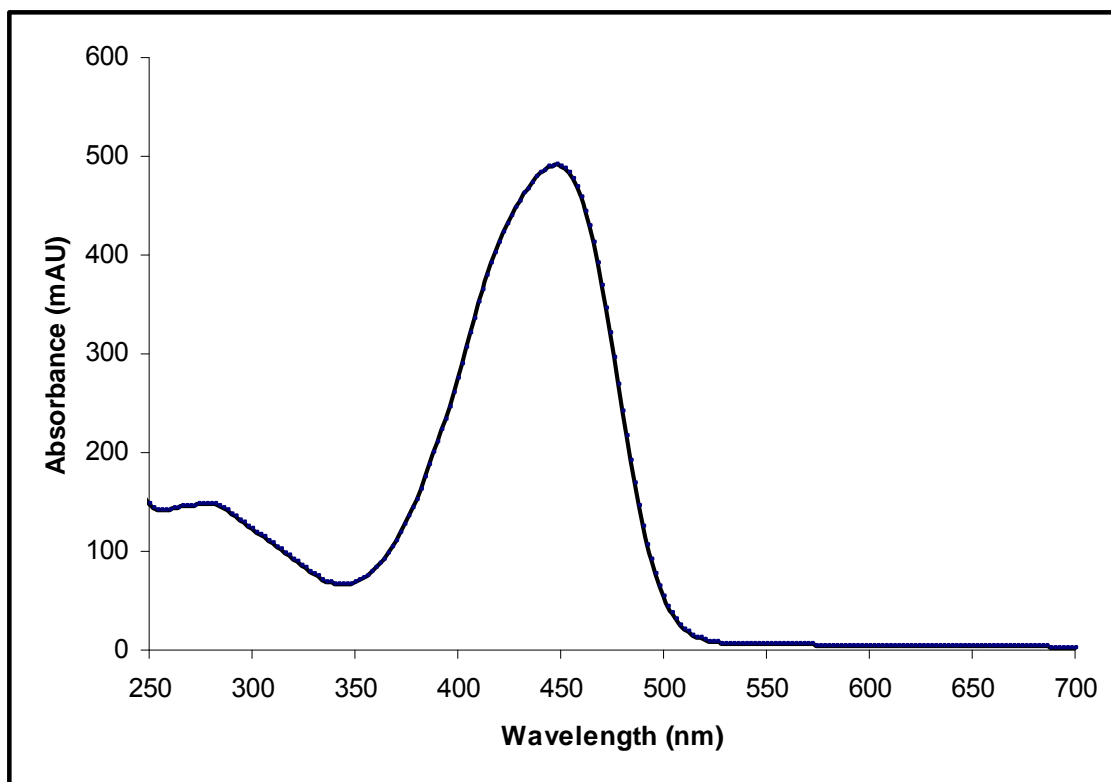


Figure 2.2. UV-VIS spectrum of bilirubin from *S. nicolai* (solvent system: 9:1 0.04 mM sodium acetate in methanol: 1% aqueous ammonium acetate).

III. THE ANIMAL PIGMENT BILIRUBIN IDENTIFIED IN *STRELITZIA REGINAE*, THE BIRD OF PARADISE FLOWER.

ABSTRACT

Strelitzia reginae Aiton is an iconic plant known for its vibrant orange and blue inflorescences. Floral pigments include carotenoids and the anthocyanin delphinidin-3-rutinoside. *Strelitzia reginae* has black seeds with vivid orange arils, yet the basis for the orange color is unknown. I recently discovered bilirubin in the arils of *S. nicolai*. Previously, this pigment was known only to exist in animals as a breakdown product of heme. Using high performance liquid chromatography with UV detection (HPLC/UV) and HPLC/UV/electrospray ionization tandem mass spectrometry (HPLC/UV/ESI-MS/MS), I now show that bilirubin is the primary aril pigment of *S. reginae*, and is also present in low concentrations in its sepals (less than 44.0 ng/g).

INTRODUCTION

Strelitzia reginae (family Strelitziaceae, order: Zingiberales), the Bird of Paradise Plant, is an herbaceous monocot native to South Africa but widely cultivated in warm temperate and tropical regions. Aside from the shape of its inflorescence, which resembles the head of a bird, *S. reginae* is also admired for its brilliant floral coloration. Each flower is composed of three vibrant orange sepals and three blue petals (Fig. 3.1A). At least nineteen carotenoids are present in the sepals (Simpson et al., 1975; Tappi and Menziani, 1955), and the anthocyanin delphinidin-3-rutinoside is found in the petals (Harborne, 1967). Anthocyanins are stored in the vacuole of petal cells (Kronsteadt and Walles, 1986), while carotenoids in mature sepal tissue are stored in spindle shaped chromoplasts

(Simpson et al., 1975). Petal color is enhanced by papillar processes in the epidermis which refract light (Kronsteadt and Walles, 1986).

In contrast to the conspicuous flowers, the capsular fruit of *S. reginae* is pale and partially obscured by the bract during development. However, at maturity the capsule breaks open to reveal intensely colored orange arillate seeds (Fig. 3.1B-C). Remarkably, aril color remains unchanged years after cell death, a quality which likely contributes to the continued attraction of avian frugivores (Frost, 1980). We recently found the orange tetrapyrrole bilirubin to be the primary pigment in the arils of *Strelitzia nicolai*, a close relative of *S. reginae* (Pirone et al., 2009). Bilirubin was previously known in the animal kingdom where it is produced as a breakdown product of heme. Preliminary high performance liquid chromatography (HPLC) and ultraviolet-visible (UV) spectrometry analyses suggested that bilirubin is also present in the arils of *S. reginae*. I was therefore interested in whether this unusual pigment is indeed present in those arils, and whether it is also present in the flowers. I use HPLC/UV and HPLC/UV/electrospray ionization-tandem mass spectrometry (HPLC/UV/ESI-MS/MS) to investigate these questions. I also observe the aril cells using light microscopy to determine the location of pigment within the aril and sepal cells.

MATERIALS AND METHODS

Sepal tissue was collected from a single clonal plant at Fairchild Tropical Botanic Garden in Miami, FL. Aril tissue was obtained from two plants at Ellison Horticulture Pty. Ltd. in Allston, Australia. It was not possible to analyze aril and sepal tissue from the same individuals. *Strelitzia reginae* flowers in Miami, but does not set fruit. Bilirubin is known to decompose in heat, light, and oxygen. Preliminary analyses did not indicate

that bilirubin is stable in sepal tissue, but they did suggest that bilirubin is stable in aril tissue for at least several months, likely a result of the high lipid content of the tissue. Therefore it was necessary to analyze fresh sepal tissue from local plants, but it was possible to analyze imported aril tissue.

For each sepal sample, 20.0 g of fresh tissue was ground in a blender with 100 mL methanol for two minutes, and was then filtered through a Buchner funnel. The residue was re-extracted with 50 mL of chloroform in a mortar and pestle, and this procedure was repeated until sepals were colorless. Methanol and chloroform extracts were pooled, and 100 mL of water was added. The mixture was left in a separatory funnel for five minutes, and then the (lower) chloroform layer was collected, filtered with a PTFE 0.2 μm filter, and divided into two equal aliquots. Each aliquot was dried to completion in a rotary evaporator at 30 C. For each aril sample, 0.05 g tissue from a single aril was ground by a mortar and pestle and extracted with chloroform repeatedly until the chloroform extracts were colorless. As above, the chloroform extract was filtered, divided into two equal aliquots, and dried on a rotovap. Sepal and aril tissue were sampled in duplicate. To determine the presence of bilirubin, one aliquot from each sample was analyzed via HPLC and the second aliquot was analyzed via HPLC/ESI-MS/MS.

HPLC/UV: HPLC/UV analyses were performed on a Thermo-Finnigan SpectraSystem HPLC apparatus with a variable wavelength PDA detector (SMC1000, P4000, AS3000, UV6000LP; Thermo Electro Corporation, San Jose, CA, USA). Extract was redissolved in dimethyl sulfoxide (DMSO), partitioned with hexane to remove lipids, and chromatographed on a reverse phase ODS-A column (5 μm , 4.3 x 150 mm; Waters

Corporation, Milford, MA, USA). Mobile phase A was 0.1% formic acid in methanol, and mobile phase B was 0.1% formic acid in water. The HPLC gradient (at 1.0 mL/min) was started at 40%A and increased linearly to 95%A over 40 min, then held constant at 95%A and 5%B for 10 minutes. Bilirubin was identified by comparing the retention time and UV-Visible spectra of *S. reginae* pigments with bilirubin standard (Sigma-Aldrich; St. Louis, MO, USA), which had a retention time of 42.9 min and a maximum absorbance at 444 nm in the HPLC solvent system described above. Bilirubin concentrations were determined by comparison with a standard curve [$R^2 = .995$] (estimated detection limit = 20 ng injected on column) (Fig. 3.2).

Preliminary analysis of sepal tissue showed compounds which eluted at retention times similar to that of bilirubin. The UV-Visible spectra of these compounds were similar to carotenoids. To avoid the possible overlap of the HPLC/UV spectra of these pigments with that of bilirubin, I treated sepals with diazomethane to convert bilirubin to its dimethyl ester, i.e. both carboxylic acids were converted to methyl esters ($\lambda_{\text{max}} = 453$ nm in HPLC solvents described above) (Kuenzle, 1973). Diazomethane was prepared from diazald according to the method of Vogel et al. (1989). Chloroform extracts of the sepals were treated with an excess of a solution of diazomethane in order to form the bilirubin dimethyl ester. The addition of the diazomethane was deemed to be complete when effervescence was no longer observed. The excess diazomethane was destroyed by the addition of a few drops of acetic acid. Although the diazomethane would also methylate any other carboxylic acid impurities in the extract, such ester byproducts did not interfere in any way with the observance of the bilirubin dimethyl ester peak in the HPLC analyses. Thus, it was unnecessary to carry out additional purification of the of the sepal

extracts. The retention time of bilirubin dimethyl ester was 31.7 min, thus enabling us to observe it without the interference of the other pigments. A standard curve for bilirubin dimethyl ester [$R^2=1$], estimated detection limit = 35.0 ng injected on column] was constructed by treating bilirubin standard with diazomethane. Identification of the peak at 31.7 minutes as bilirubin dimethyl ester was verified by comparison with bilirubin dimethyl ester standard (Frontier Scientific, Logan, UT, USA), which also eluted at 31.7 minutes. For samples in which bilirubin was detected via HPLC/ESI-MS/MS but not HPLC/UV, I assumed the mass of bilirubin to be less than the estimated detection limit of bilirubin or bilirubin treated with diazomethane. Standard deviation values were not calculated because of the low sample size of two. Instead, concentration values for both samples of aril and sepal tissue are presented in Table 3.1.

HPLC/UVESI-MS/MS: The plant extract was redissolved in dimethyl sulfoxide (DMSO) (Certified ACS; Fisher Scientific) and analyzed via reverse phase C8 HPLC/UV/ESI-MS/MS utilizing both positive and negative electrospray ionization ((+) and (-) ESI) and a number of different MS_n scans. The HPLC/UV analyses were performed with an Agilent Technologies HPLC with binary pumps (1100 series; Santa Clara, CA, USA), a Symmetry C8 HPLC column (5 μ m, 2.1 x 150 mm; Waters Corporation, Milford, MA, USA) and an Agilent UV-Visible detector (G1314A). The mobile phase A was 0.2% acetic acid (glacial, biochemical grade (99.8%); ACROS organics, Morris Plains, NJ, USA) in H₂O (HPLC grade, Honeywell Burdick & Jackson, Muskegon, MI, USA) and mobile phase B was 0.2% acetic acid in acetonitrile (LC-MS grade, Honeywell Burdick & Jackson). The HPLC gradient (at 0.2 mL/min) was started at 20% B at time 0 and increased linearly to 85% B over 30 min and then increased linearly to 100%B over 15

min. The column was held at 100% B until monitoring of the UV/MS signal showed no further elution of peaks. For some extracts this was more than 150 min. The UV-Vis response was monitored at 450 nm for bilirubin, which eluted at approximately 39 min.

All mass spectrometry data were obtained with a Finnigan MAT (San Jose, CA, USA) LCQ classic quadrupole ion trap mass spectrometer equipped with an electrospray ionization source (ESI). The ESI was operated with a nitrogen sheath and auxiliary gas flows of 65 and 5, respectively (unitless, software parameter) with a spray voltage of 3.3 kV and a heated capillary temperature of 250 °C. The heated capillary voltage was +15 V and -22 V for (+) and (-)ESI, respectively, while the tube lens was operated at 0 V for both ESI polarities. Collision-induced dissociation (CID) was conducted with a parent ion isolation of 3 u, CID energy of 37.5%, qCID of 0.25 and CID time of 30 ms.

With (+)ESI, bilirubin produced an m/z 585 $[M+H]^+$ ion and an m/z 583 ion because of oxidation during ionization. The m/z 585 and m/z 583 ions underwent collision induced tandem mass spectrometry (CID-MS/MS) to form m/z 299, and 297 ions, respectively, as major product ions. With (-)ESI-MS, bilirubin produced m/z 583 $[M-H]^-$ and m/z 581 ions which were dissociated to form m/z 285 and 537 major product ions, respectively. Bilirubin was identified in the plant extracts by matching of retention time and (+) and (-)ESI-MS and -MS/MS spectra with those of an authentic bilirubin standard.

The DMSO solvent blanks were analyzed before and after analyses of each extract and after analyses of bilirubin standards to check for carryover of bilirubin. These DMSO blanks were repeated if significant levels of bilirubin were carried over.

Microscopy: Sepal and aril tissues were observed in a Leica Model DME brightfield compound microscope with a 40X objective and photographed using a Nikon CoolPix 4500 camera (2272 x 1704 pixels). Tissues were sectioned by hand and mounted in water.

RESULTS AND DISCUSSION

Bilirubin was present in both the aril and sepal extracts of *S. reginae*. Each HPLC/UV analysis of the aril extracts showed a single peak with retention time and UV-Visible spectrum which matched those of the bilirubin standard (Fig. 3.3). Bilirubin identification was confirmed by HPLC/ESI-MS/MS (Figs. 3.4-3.5). Bilirubin was also identified in sepal samples via HPLC/ESI-MS/MS (Fig. 3.6), but was not detected via HPLC/UV, even after treatment with diazomethane. The concentration of bilirubin in the sepals was less than 44.0 ng/g of fresh tissue (n=2), while the average concentration in the aril was 17.4 mg/g fresh tissue (n=2) (see Table 3.1 for concentration values of all samples).

The low sample size was adequate for ensuring the detection of bilirubin in *S. reginae*, as false positives may have occurred via contamination of the sample or the equipment, but not by chance alone. Contamination was avoided by the washing of glassware multiple times (soap and water, acetone, chloroform), the use of disposable vials when possible, and changing gloves after the analysis of each sample. Blank(s) were run between all chromatographic procedures to avoid the carryover of bilirubin between samples and ensure a flat baseline.

In the arils, bilirubin is not only present in high concentration, but it is also the primary pigment, and is thus responsible for color production. In contrast, it is likely that the low

concentration of bilirubin in the sepals preclude it from contributing significantly to color. Furthermore, high concentrations of sepal carotenoids (Simpson et al., 1975; Tappi and Menziani, 1955) likely mask low levels of bilirubin. Whether bilirubin serves a function other than color production in *S. reginae* remains unclear.

In mature aril tissue, bilirubin was present as granular bodies irregularly distributed throughout the cell (Fig. 3.1D). In mature sepal tissue, I observed elongate structures (Fig. 3.1E) which were previously identified as containing carotenoids (Simpson et al., 1975). I saw no evidence of pigment bodies which might contain bilirubin.

This study provides new insight into color production in an iconic tropical plant.

It also demonstrates the first example of bilirubin in a flower, and verifies the presence of bilirubin in a plant species other than *S. nicolai*. Coupled with further research on the function, distribution, and synthesis of bilirubin in plants, this information may be of use for practical applications such as the manipulation of color through breeding and genetics.

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Table 3.1. Concentration of bilirubin in *S. reginae* arils and sepals.

Sample	Aril	Sepal
1	21.97 mg/g	< 44 ng/g
2	12.82 mg/g	< 44 ng/g



Figure 3.1. *Strelitzia reginae*. A. Inflorescence of *S. reginae* photographed at Fairchild Tropical Botanic Garden. Inflorescence is ~22 cm long. B. Mature and open capsule of *S. reginae* with arillate seeds exposed, capsules ~5.5 cm across. Photographed in Del Mar, CA by Tom DeFanti. C. Arillate seeds of *S. reginae*, seeds ~4 mm in diameter, photographed by Tom DeFanti. D. Aril fibers of *S. reginae*, in longitudinal view. Bilirubin is present in the crystalline granules attached to inner cell wall of these dead cells. Length of image = 300 μ m. E. Paradermal view of epidermal cells of orange sepals of *S. reginae*. Bilirubin granules are not seen in these cells, the color produced primarily by carotenoids in large chromoplasts. Length of image = 300 nm.

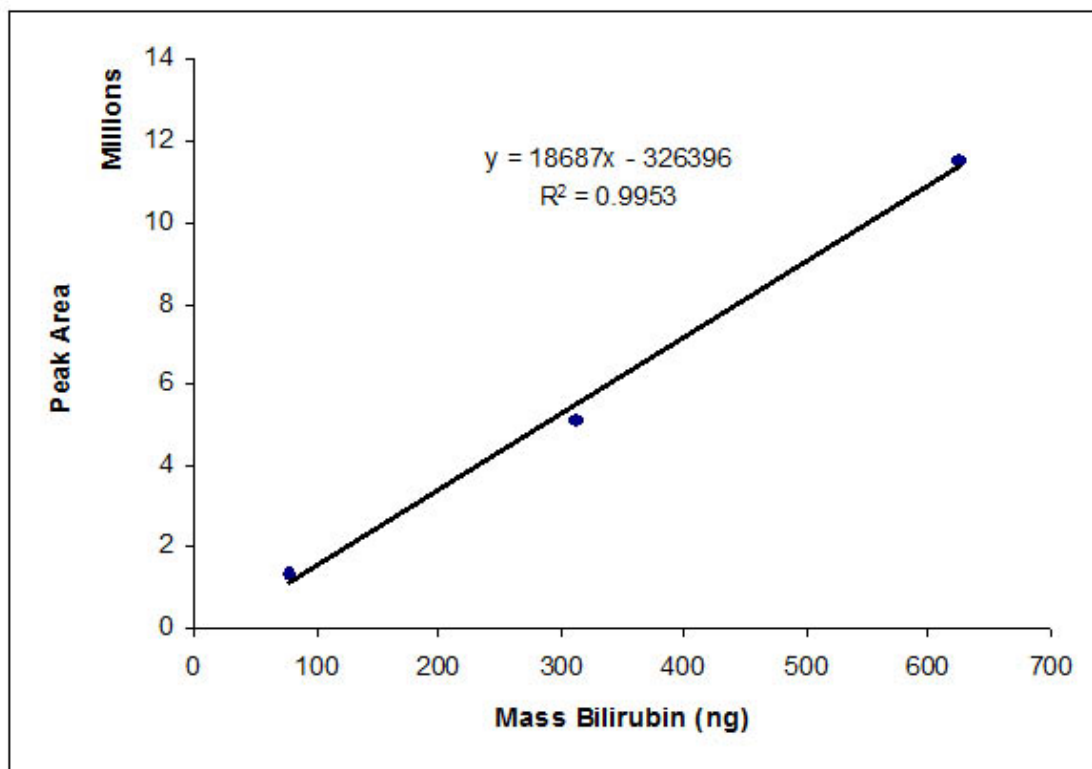


Figure. 3.2. HPLC standard curve of bilirubin-IX α .

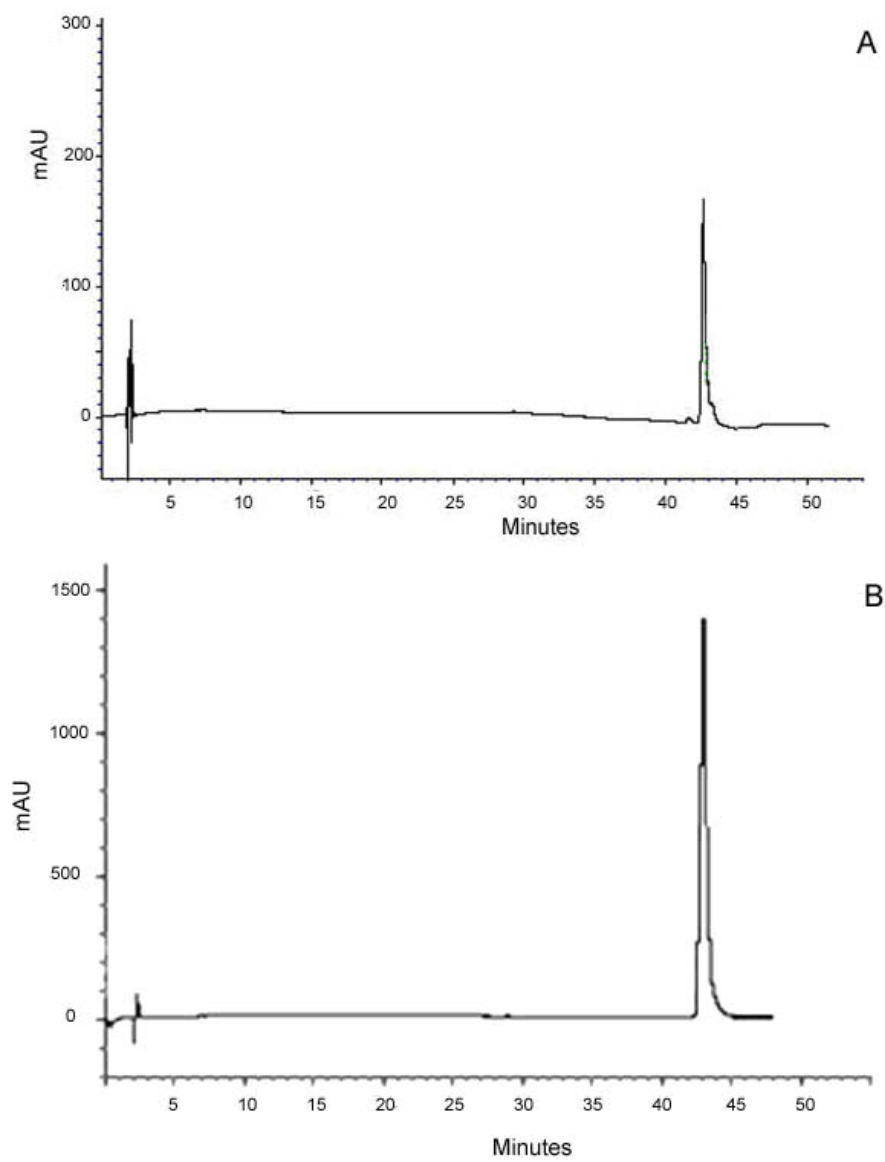


Figure. 3.3. HPLC chromatograms of (A) Bilirubin standard and (B) Aril extract of *S. reginae*.

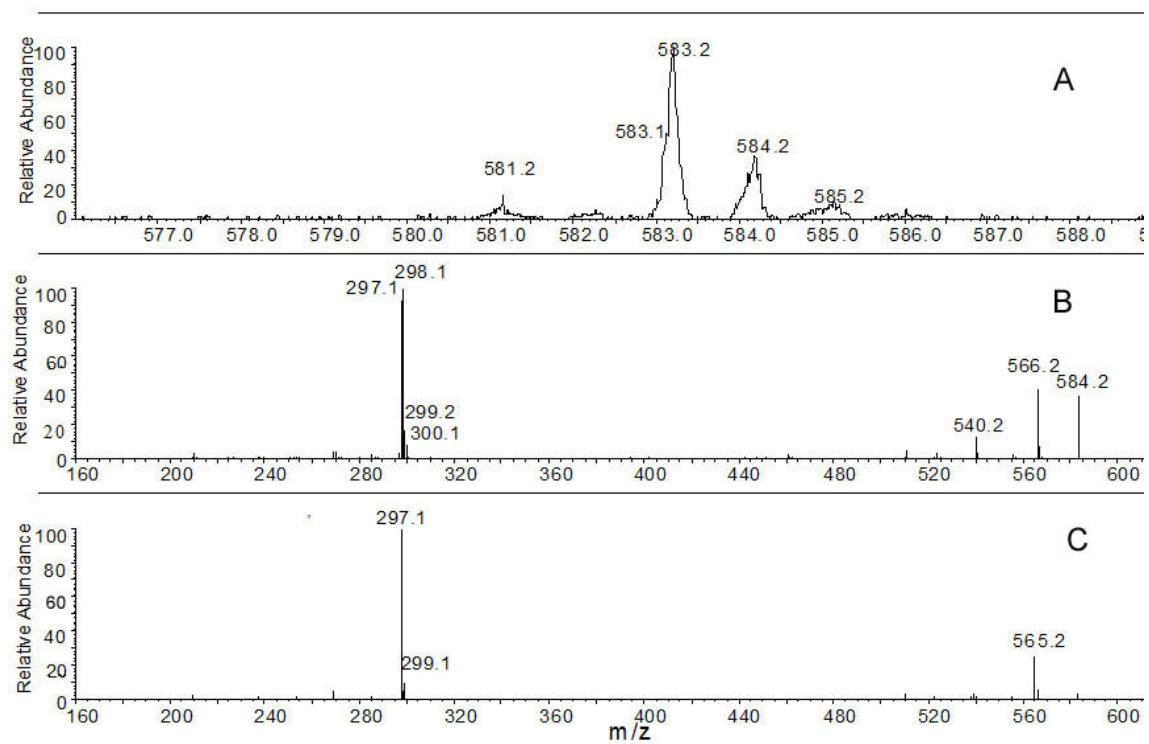


Figure. 3.4. Bilirubin standard via (+)ESI-Zoom-MS and MS/MS: (A) Zoom-MS of molecular ion region; (B) product spectrum of m/z 585; (C) product spectrum of m/z 583.

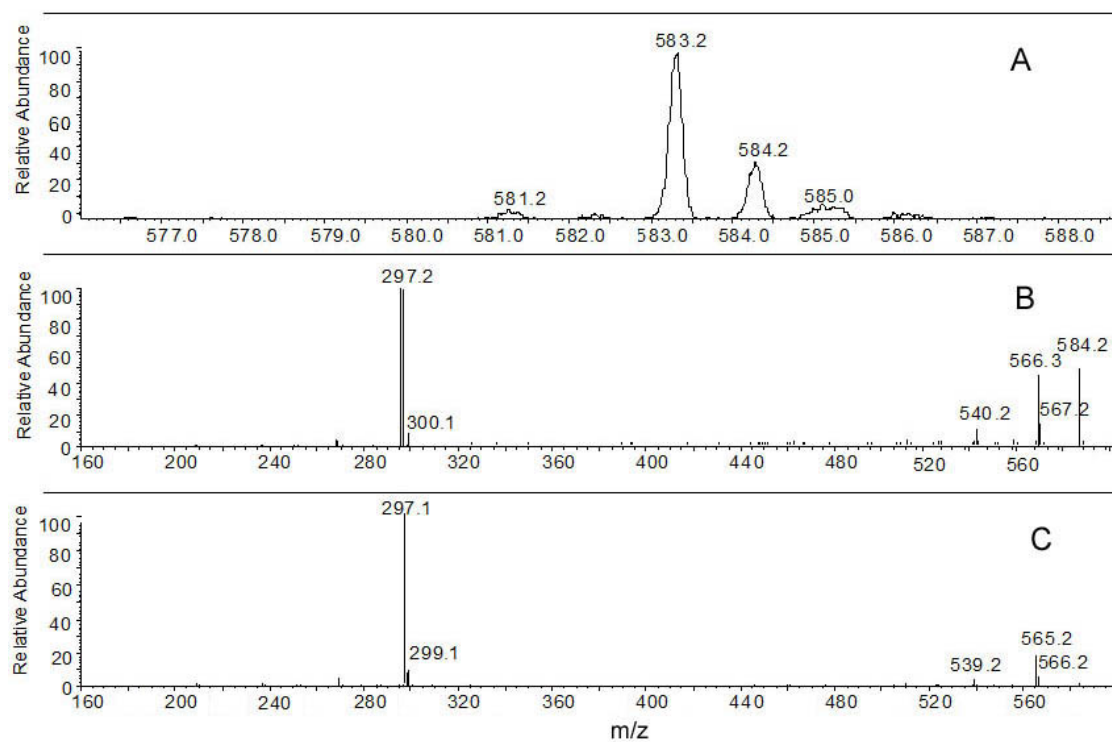


Figure. 3.5. Bilirubin in *S. reginae* aril via (+) ESI-MS and MS/MS: (A) Zoom-MS of molecular ion region; (B) product spectrum of m/z 585; (C) product spectrum of m/z 583.

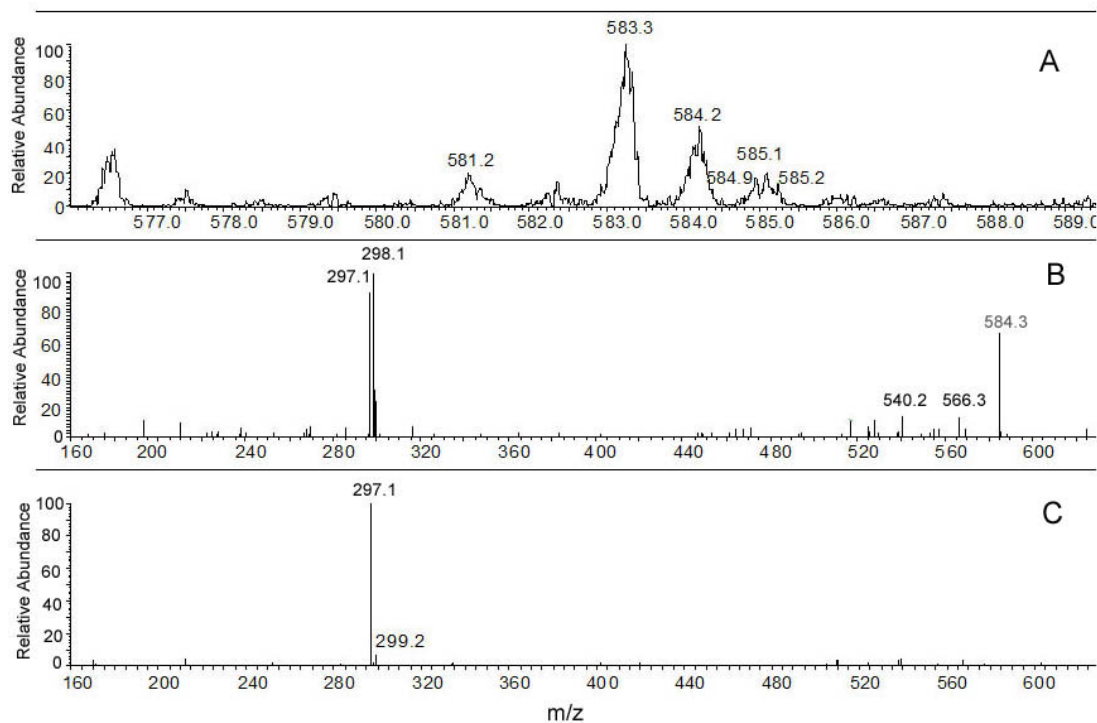


Figure. 3.6. Bilirubin in *S. reginae* sepal extract via (+) ESI-Zoom-MS and MS/MS: (A) Zoom-MS of molecular ion region; (B) product spectrum of m/z 585; (C) product spectrum of m/z 5.

IV. BILIRUBIN PRESENT IN DIVERSE ANGIOSPERMS

ABSTRACT

Bilirubin is an orange-yellow tetrapyrrole produced from the breakdown of heme by mammals and some other vertebrates. Plants, algae, and cyanobacteria synthesize molecules similar to bilirubin, including the protein-bound bilins and phytochromobilin which harvest or sense light. Recently, I discovered bilirubin in the arils of *Strelitzia nicolai*, the white bird of paradise tree, which was the first example of this molecule in a plant. Subsequently, I identified bilirubin in both the arils and flowers of *Strelitzia reginae*, the bird of paradise flower. In the arils of both species, bilirubin is present as the primary pigment, and thus functions to produce color. Previously, no tetrapyrroles were known to generate display color in plants. I was therefore interested in determining whether bilirubin occurs in other plants, and whether it contributes to color in other species. In this paper, I use high performance liquid chromatography with UV detection (HPLC/UV) and HPLC/UV/electrospray ionization tandem mass spectrometry (HPLC/UV/ESI-MS/MS) to search for bilirubin in ten species across diverse angiosperm lineages. Bilirubin was present in eight species from the orders Zingiberales, Arecales, and Myrtales, but only contributed to color in Strelitziaceae species. The wide distribution of bilirubin in angiosperms indicates the need to reassess some metabolic details of an important and universal biosynthetic pathway in plants, and further explore its evolutionary history and function. Although color production was limited to the Strelitziaceae in this study, further sampling may indicate otherwise.

INTRODUCTION

Tetrapyrroles occur throughout the plant kingdom; this class of molecules includes vital biosynthetic products such as chlorophyll and heme. In plants, the degradation of heme forms first biliverdin IX- α , and subsequently phytychromobilin, the precursor of the phytochrome chromophore, an essential light-sensing molecule (Tanaka et al., 2007). In mammals and some vertebrates, biliverdin-IX α is also formed from the degradation of heme, but it is transformed into the yellow-orange pigment bilirubin-IX α . I have identified bilirubin-IX α (henceforth referred to as bilirubin) as the major pigment in the orange arils of *Strelitzia nicolai*, the white bird of paradise tree (Pirone et al., 2009). Although ubiquitous in animals, this is the first example of bilirubin in a plant. Subsequently, I have discovered this pigment in the sepals and arils of *S. reginae*, the bird of paradise flower, indicating the pigment is not unique to *S. nicolai* (Pirone et al., 2010).

In *S. nicolai* and *S. reginae*, bilirubin is a novel biosynthetic source of display color. Most plant pigments are derived from one of three metabolic pathways: terpenoid (carotenoids), phenylpropanoid (flavonoids), or indole (betalains) (Davies, 2004; Grotewold, 2006; Lee, 2007). Betalain synthesis is restricted to families in the order Caryophyllales, while carotenoids and flavonoids (including anthocyanins) are pervasive in the plant kingdom (Harborne, 1967; Goodwin, 1988). A rare group of pigments, the phenalenones, has been documented in several species in the Strelitziaceae and related families (Davies, 2004). However, to my knowledge, neither the phenalenones nor other rare pigments play a significant role in color production. Bilirubin is thus the first product of an additional biosynthetic route, the tetrapyrrole pathway, to produce conspicuous color in a plant reproductive structure. Chlorophylls, which are also

synthesized via the tetrapyrrole pathway, primarily produce color in foliage, thus forming a green background upon which the contrasting colors of flowers and fruits are displayed. While chlorophylls occasionally produce color in reproductive structures, these are fairly inconspicuous.

Given the presence of bilirubin in *Strelitzia*, it is interesting to determine if the pigment is produced by other taxa within the Strelitziaceae, in families closely allied to the Strelitziaceae (as in the Zingiberales), as well as throughout other angiosperms. Preliminary high performance liquid chromatography (HPLC/UV) analyses of aril extracts of an additional species in the Strelitziaceae, *Phenakospermum guyannense*, showed a pigment with a retention time and UV-Visible spectra which matched those of bilirubin. Here, I use HPLC/UV and HPLC/UV/electrospray ionization-tandem mass spectrometry (HPLC/UV/ESI-MS/MS) to confirm the presence of bilirubin in *P. guyannense* and investigate the presence of bilirubin in the mature fruits from nine additional species and the flowers of a single additional species. Six species are within the order Zingiberales, and four are from diverse angiosperm orders (Table 4.1). I discuss our findings within a phylogenetic and biochemical context, and comment on a possible ecological role for bilirubin as a color signal to attract animal dispersers and pollinators.

MATERIALS AND METHODS

Plant material was collected from Fairchild Tropical Botanic Garden in Miami, FL except aril tissue from *Strelitzia reginae*, which was obtained from Ellison Horticulture Pty. Ltd. in Allstonville, New South Wales, Australia, and *P. guyannense*, which was collected by John Kress in the Demerara-Mahaica region of Guyana, South America. Tissue for each sample and its replicate were composed of tissue from one or multiple inflorescences or

infructescences from a single, sometimes clonal, individual. For the names and taxonomic affiliations of species sampled, see Table 4.1. The replicate aril samples of *P. guyannense* came from different individuals. For each sample (except aril samples), 20.0 g of fresh tissue was ground in a blender with 100 mL methanol for two minutes, and was then filtered through a Buchner funnel. The residue was re-extracted with chloroform in a mortar and pestle. Methanol and chloroform extracts were pooled, and 100 mL of water was added. The mixture was left in a separatory funnel for five minutes, and then the (lower) chloroform layer was collected, filtered with a polytetrafluoroethylene (PTFE) 0.2 μm filter, and divided into two equal aliquots. Each aliquot was dried to completion in a rotary evaporator at 30° C. For each aril sample, 0.05 g tissue from a single aril was ground by a mortar and pestle and extracted with chloroform repeatedly until the chloroform extracts were colorless. As above, the chloroform extract was filtered, divided into two equal aliquots, and dried on a rotary evaporator. All tissues were sampled in duplicate. To determine the presence of bilirubin, one aliquot from each sample was analyzed via HPLC and the second aliquot was analyzed via HPLC/ESI-MS/MS.

HPLC/UV: The HPLC/UV analyses were performed on a Thermo-Finnigan SpectraSystem HPLC apparatus with a variable wavelength photodiode array (PDA) detector (SMC1000, P4000, AS3000, UV6000LP; Thermo Electro Corporation, San Jose, CA, USA). Each extract was redissolved in dimethyl sulfoxide DMSO, partitioned with hexane to remove lipids, and chromatographed on a reverse phase ODS-A column (5 μm , 4.3 x 150 mm; Waters Corporation, Milford, MA, USA). The mobile phase A was 0.1% formic acid in methanol, and mobile phase B was 0.1% formic acid in water. The HPLC

gradient (at 1.0 mL/min) was started at 40% A and increased linearly to 95% A over 40 min, then held constant at 95% A and 5% B for 10 minutes. Bilirubin was identified by comparing the retention time and UV-Visible spectra of sample pigments with bilirubin standard (Sigma-Aldrich; St. Louis, MO, USA), which had a retention time of 42.9 min and a maximum absorbance at 444 nm in the above HPLC solvent system. Bilirubin concentrations were determined by comparison with a standard curve [$R^2 = .995$) estimated detection limit = 20 ng injected on column]. Preliminary analysis of some plant extracts showed compounds which eluted at retention times similar to that of bilirubin. The UV-Visible (ultraviolet-visible) spectra of these compounds were similar to carotenoids. To avoid the possible overlap of the HPLC/UV spectra of these pigments with that of bilirubin, I treated non-arillate samples (Table 4.2) with diazomethane to convert bilirubin to its dimethyl ester, i.e., both carboxylic acids were converted to methyl esters ($\lambda_{\text{max}} = 453$ nm in HPLC solvents described above) (Kuenzle, 1973). Diazomethane was prepared from diazald according to Vogel et al. (1989). Chloroform extracts of the sepals were treated with an excess of a solution of diazomethane in order to form the bilirubin dimethyl ester. The addition of the diazomethane was deemed to be complete when effervescence was no longer observed. The excess diazomethane was destroyed by the addition of a few drops of acetic acid. Although the diazomethane would also methylate any other carboxylic acid impurities in the extract, such ester byproducts did not interfere in any way with the observance of the bilirubin dimethyl ester peak in the HPLC analyses. Thus, it was unnecessary to carry out additional purification of the sepal extracts. With the HPLC/UV conditions described above, the retention time of bilirubin dimethyl ester was 31.7 min, thus making it possible to

observe the compound without interference from other pigments. A standard curve for bilirubin dimethyl ester [$R^2=1$], estimated detection limit = 35.0 ng injected on column] was constructed by treating bilirubin standard with diazomethane. Identification of the peak at 31.7 minutes as bilirubin dimethyl ester was verified by comparison with bilirubin dimethyl ester standard (Frontier Scientific, Logan, UT, USA), which also eluted at 31.7 minutes. For samples in which bilirubin was detected via HPLC/ESI-MS/MS but not HPLC/UV, I assumed the mass of bilirubin to be less than the estimated detection limit of bilirubin or bilirubin treated with diazomethane. Standard deviation values were not calculated owing to the low sample size. Instead, concentration values for the replicate samples of each plant are presented in Table 4.2.

HPLC/UVESI-MS/MS: The plant extract was redissolved in DMSO (Certified ACS; Fisher Scientific) and analyzed via reverse phase C8 HPLC/UV/ESI-MS/MS utilizing both positive and negative ESI and a number of different MS_n scans. HPLC/UV was performed with an Agilent Technologies HPLC with binary pumps (1100 series; Santa Clara, CA, USA), a Symmetry C8 HPLC column (5 μ m, 2.1 x 150 mm; Waters Corporation, Milford, MA, USA) and an Agilent UV-Visible detector (G1314A). The mobile phase A was 0.2 % acetic acid (glacial, biochemical grade (99.8%); ACROS organics, Morris Plains, NJ, USA) in H₂O (HPLC grade, Honeywell Burdick & Jackson, Muskegon, MI, USA) and mobile phase B was 0.2% acetic acid in acetonitrile (LC-MS grade, Honeywell Burdick & Jackson). The HPLC gradient (at 0.2 mL/min) was started at 20% B at time 0 and increased linearly to 85% B over 30 min and then increased linearly to 100% B over 15 min. The column was held at 100% B until monitoring of the ultraviolet-visible and mass spectrometry (UV/MS) signal showed no further elution of

peaks. For some extracts this was more than 150 min. The UV-Visible response was monitored at 450 nm for bilirubin, which eluted at approximately 39 min.

All mass spectrometry data were obtained with a Finnigan MAT (San Jose, CA, USA) LCQ classic quadrupole ion trap mass spectrometer equipped with an electrospray ionization source (ESI). The ESI was operated with a nitrogen sheath and auxiliary gas flows of 65 and 5, respectively with a spray voltage of 3.3 kV and a heated capillary temperature of 250 C. The heated capillary voltage was +15 V and -22 V for (+) and (-)ESI, respectively, while the tube lens was operated at 0 V for both ESI polarities. Collision-induced dissociation (CID) was conducted with a parent ion isolation of 3u, CID energy of 37.5%, qCID of 0.25 and CID time of 30 ms.

With (+)ESI, bilirubin produced an m/z 585 $[M+H]^+$ ion and an m/z 583 ion because of oxidation during ionization. The m/z 585 and m/z 583 ions underwent collision induced dissociation tandem mass spectrometry (CID-MS/MS) to form m/z 299, and 297 ions, respectively, as major product ions. With (-)ESI-MS, bilirubin produced m/z 583 $[M-H]^-$ and m/z 581 ions which were dissociated to form m/z 285 and 537 major product ions, respectively. Bilirubin was identified in the plant extracts by matching of retention time and (+) and (-) ESI-MS and –MS/MS spectra with those of the authentic bilirubin standard.

False positives that could result from contamination during handling and analyses were avoided by the washing of glassware multiple times (soap and water, acetone, chloroform), the use of disposable vials when possible, and changing gloves after the preparation of each sample. Dimethyl sulfoxide (DMSO) solvent blanks were analyzed before and after analyses of each extract and after analyses of bilirubin standards to check

for carryover of bilirubin. The DMSO blanks were repeated if significant levels of bilirubin were carried over to subsequent runs.

RESULTS

Bilirubin was present in eight of the ten species tested: *Musa balbisiana* Colla, *Heliconia collinsiana* Griggs, *Costus lucanusianus* J. Braun & K. Schum, *Ravenala madagascariensis* Sonn., *Phenakopsermum guyannense* (L.C. Rich.) Endl. ex Miq., *Hedychium coronarium* J. Koenig, *Gastrococos crispera* (Kunth) H.E. Moore, and *Eugenia luschnathiana* Klotzsch ex O. Berg (Table 4.2). Bilirubin was present in the fruits (including aril and peel) of all species except *Costus lucanusianus*, where it was present in the flowers (fruits were unavailable for sampling). In *Ravenala madagascariensis* and *Phenakospermum guyannense*, HPLC/UV analysis of the aril extracts showed a single peak with retention time and UV-Visible spectrum which matched those of the bilirubin standard. Bilirubin identification was confirmed by HPLC/ESI-MS/MS. Bilirubin was also identified in *Musa balbisiana*, *Heliconia collinsiana*, *Costus lucanusianus*, *Hedychium coronarium*, *Gastrococos crispera*, and *Eugenia luschnathiana* via HPLC/ESI-MS/MS, but was not detected via HPLC/UV, even after treatment with diazomethane. In *Heliconia collinsiana* and *Eugenia luschnathiana*, bilirubin was detected in only one of the two replicate samples, while in all other species bilirubin was detected in both replicates. The concentration of bilirubin was highly variable among species. Concentrations ranged from less than 44 ng/g of fresh tissue (n=2) to 3.73 mg/g of fresh tissue (n=2) (Table 4.2).

DISCUSSION

I initially discovered bilirubin in *S. nicolai* (family: Strelitziaceae, order: Zingiberales), and also in the arils and sepals of *S. reginae*. Since similar secondary metabolites are sometimes expected to be found within members of a clade, I concentrated most of our sampling within the Strelitziaceae and the Zingiberales. Kress et al. (2001) divided the order into two major clades, the basal “banana group” which includes Musaceae, Strelitziaceae, Heliconiaceae, and Lowiaceae, and the more derived “ginger group”, which includes the four remaining families (Fig. 4.1). Although resolution of intrafamilial relationships varies among studies, the ginger clade families are generally well resolved (Rudall et al., 1999; Chase et al., 2000; Givnish, 2006), and there is high support for the position of Lowiaceae as sister to Strelitziaceae (Rudall et al., 1997; Chase et al., 2000; Soltis et al., 2000; Givnish et al., 2006; Soltis et al., 2007). We sampled species from each banana group family, except from the Lowiaceae. This monotypic family consists of fifteen rare species within *Orchidantha*, and we were not able to obtain enough material for analyses. I selected *Musa balbisiana* (Musaceae), one of the wild progenitors of most cultivated bananas (Heslop-Harrison and Schwarzacher, 2007), *Heliconia collinsiana* (Heliconiaceae), and representatives from each of the two Strelitziaceae genera not previously analyzed for bilirubin content, *Phenakospermum guyannense* and *Ravenala madagascariensis*. I also sampled species from two of the most derived families in the order, *Costus lucanusianus* (Costaceae) and *Hedychium coronarium* (Zingiberaceae) (Kress, 2001). I mainly sampled orange fruits to maximize the potential chances of finding bilirubin, but we also included the blue arils of *Ravenala*

madagascariensis, the yellow fruits of *Heliconia collinsiana*, and the multi-colored flowers of *Costus lucanusianus*.

To determine whether bilirubin is present in plants outside of the Zingiberales, I sampled species from the basal dicot order Laurales, two monocot orders, the Arecales and the Pandanales, and the eudicot order Myrtales, which is part of the Rosid clade. Selection of species within those orders (Table 4.1) was based on tissue availability and fruit color. The detection of bilirubin in *Gastrococos crispa* (family: Arecaceae, order: Arecales) and *Eugenia luschnathiana* (family: Myrtaceae, order: Myrtales), indicates that bilirubin is not restricted to Zingiberales and may be broadly distributed throughout the plant kingdom. However, the lack of detection of bilirubin in avocado, *Persea americana* Mill. (family: Lauraceae, order: Laurales) and *Pandanus odorifer* (Forssk.) Kuntze (family: Pandanaceae, order: Pandanales) suggests that bilirubin is not universal in plants at levels detectable by mass spectrometry.

The high concentration of bilirubin in the arils of *P. guyannense* indicates its role in color production. Previous studies indicated bilirubin is also responsible for color production in two other Strelitziaceae species, *S. nicolai* and *S. reginae*. Since brightly colored fruit displays often serve as signals to attract dispersers (van der Pijl, 1982), it is likely that in these species, bilirubin contributes to the attraction of avian frugivores which feed upon the arils (Frost, 1980; Kress, personal communication). Whether bilirubin plays an additional role beyond color production in plants remains to be determined. Bilirubin may function as a potent antioxidant in plants as it does in humans (Stocker, 1987), or serve a different physiological function. Bilirubin may also be a mere metabolic waste product.

The detection of bilirubin in only one replicate of *H. collinsiana* and *E. luschnathiana* likely indicates that bilirubin biosynthesis is not constant, but is instead variable and influenced by factors which are currently unknown. For example, I observed the accumulation of bilirubin in aril cells during the development of *S. nicolai* (personal observation), with a maximum quantity present in mature tissue (Fig. 4.2), suggesting that bilirubin production may be influenced by development. The lack of bilirubin at the time of sampling may also be a function of variable production, and thus may not indicate an absence of bilirubin biosynthesis in the species.

The biochemical pathway that produces bilirubin in plants remains unknown. In animals, biliverdin-IX α is reduced by a nicotinamide adenine dinucleotide phosphate (NAD(P)H) dependent biliverdin-IX α reductase (BR) to form bilirubin-IX α (Maines and Trakshel, 1993). Conversely, in plants, biliverdin-IX α is converted to a structural isomer of bilirubin, 3Z-phytychromobilin, the precursor of the phytyochrome chromophore (Tanaka and Tanaka, 2007), by a ferredoxin-dependent bilin reductase, phytychromobilin synthase (Terry et al., 1995; Kohchi et al., 2001). In cyanobacteria and some algae, biliverdin-IX α is also reduced by ferredoxin-dependent enzymes, the bilin reductases, to form the phycobilin chromophores (Beale, 1993; Frankenburg et al., 2001). A BR enzyme was identified and cloned in the cyanobacterium *Synechocystis* (Schluchter and Glazer, 1997), and BR enzymes have also been found in a variety of other bacteria. Whether bilirubin IX- α in plants is formed by the reduction of biliverdin IX- α by a BR enzyme or via some other means remains to be determined.

Bilirubin was present in eight species from three diverse angiosperm orders, and contributed to aril color in species within the Strelitziaceae. Further sampling of bilirubin

in plants, both across species, under variable conditions, and across different time scales, will be necessary to gain a comprehensive understanding of the distribution of bilirubin in plants, and to determine whether color production is limited to the Strelitziaceae.

These studies, combined with work on the biosynthesis of bilirubin in plants, will provide a more comprehensive understanding of the evolution of this unusual molecule in the plant kingdom.

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Table 4.1. Plant species sampled. All plants were collected from Fairchild Tropical Botanic Garden, except *Persea americana*, which was collected from the Kampong, a branch of the National Tropical Botanic Garden.

Species	Accession Number	Family	Order	Organ
<i>Musa balbisiana</i> Colla	6222B	Musaceae	Zingiberales	peel
<i>Heliconia collinsiana</i> Griggs	58523 A	Heliconiaceae	Zingiberales	fruit
<i>Costus lucanusianus</i> J. Braun & K. Schum	95815 A	Costaceae	Zingiberales	flower
<i>Ravenala madagascariensis</i> Sonn.	P.1395 H	Strelitziaceae	Zingiberales	aril
<i>Phenakopsermum guyannense</i> (L.C. Rich.) Endl. ex Miq.	N/A	Strelitziaceae	Zingiberales	aril
<i>Hedychium coronarium</i> J. Koenig	78-333	Zingiberaceae	Zingiberales	aril
<i>Gastrococos crispa</i> (Kunth) H.E.Moore	661025A	Arecaceae	Arecales	fruit
<i>Pandanus odorifer</i> (Forssk.) Kuntze	68222A	Pandanaceae	Pandanales	fruit
<i>Persea americana</i> Mill.	20052309	Lauraceae	Lurales	fruit
<i>Eugenia luschnathiana</i> Klotzch ex O.Berg	X.3-48B	Myrtaceae	Myrtales	fruit

Table 4.2. Bilirubin detection and concentration.

Species	BR detection via diazo- methane derivative	BR detection via HPLC-UV	BR detection via HPLC- MS/MS	BR conc. Sample 1	BR conc. Sample 2	Mean BR conc. (n=2)
<i>Musa balbisiana</i>	n	n	y	< 44 ng/g	< 44 ng/g	—
<i>Heliconia collinsiana</i>	n	n	y	—	< 44 ng/g	—
<i>Costus lucanusianus</i>	n	n	y	< 44 ng/g	< 44 ng/g	—
<i>Ravenala madagascariensis</i>	n/a	y	y	0.001 mg/g	0.001 mg/g	0.001 mg/g
<i>Phenakopsermum guyannense</i>	n/a	y	y	3.041 mg/g	5.787 mg/g	3.725 mg/g
<i>Hedychium coronarium</i>	n	n	y	< 44 ng/g	< 44 ng/g	—
<i>Gastrococos crispa</i>	n	n	y	< 44 ng/g	< 44 ng/g	—
<i>Pandanus odorifer</i>	n	n	n	—	—	—
<i>Persea americana</i>	n	n	n	—	—	—
<i>Eugenia luschnathiana</i>	n	n	y	< 44 ng/g	—	—

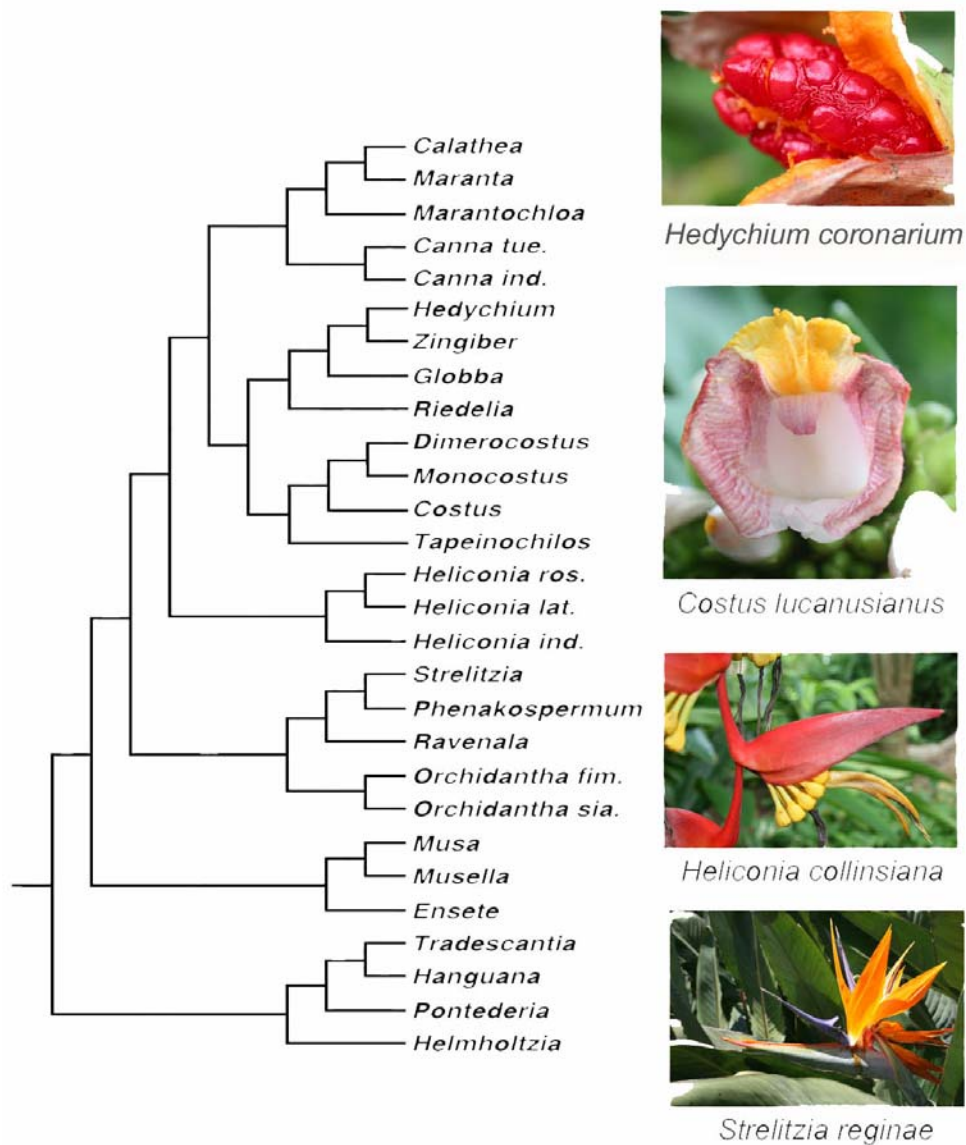


Figure 4.1. Phylogeny of the Zingiberales. Phylogenetic relationships of genera in the Zingiberales (figure adapted from Kress et al., 2001) with photographs of four species sampled in this study.



Figure 4.2. Bilirubin production in *Strelitzia nicolai* during aril development. Maturing arils of *S. nicolai* with increasing amounts of bilirubin. Seeds ~4 mm in diameter.

V. PHYLOGENY OF THE STRELITZIACEAE

ABSTRACT

Strelitziaceae, a small monocot family in the order Zingiberales, consists of seven species: *Ravenala madagascariensis* Sonn., *Phenkospermum guyannense* (L.C. Rich.) Endl. ex Miq., *Strelitzia reginae* Banks ex Aiton, *Strelitzia juncea* Link, *Strelitzia alba* Skeels, *Strelitzia nicolai* Regel and Koern and *Strelitzia caudata* R. A. Dyer. Two of the members, *S. reginae*, the bird of paradise flower, and *R. madagascariensis*, the travelers' palm, are widely cultivated as ornamentals. Despite this horticultural importance, and despite the close relationship of the Strelitziaceae with well known families including the bananas (Musaceae) and the gingers (Zingiberaceae), evolutionary relationships among the seven taxa have not been explored. Here, I reconstruct a phylogeny of the family using eleven chloroplast genes and two nuclear regions using parsimony, maximum likelihood, and Bayesian analyses. Trees resulting from all analyses of data from different compartments (chloroplast, nuclear) and from the combined regions are in good agreement. Strelitziaceae was found to be monophyletic and sister to the *Orchidantha* species. In most trees, *P. guyannense* is sister to the remaining Strelitziaceae taxa, and in all trees, the *Strelitzia* species form a monophyletic clade. Most relationships within *Strelitzia* are well established, except for that of *S. reginae* and *S. juncea*, which requires further study.

INTRODUCTION

The Strelitziaceae Hutch. is one of eight families in the order Zingiberales. It includes three genera and seven species: *Ravenala* Adans and *Phenakospermum* Endl., which are both monotypic, and five species of *Strelitzia* Aiton. (Coates Palgrave and Coates Palgrave 2002; Archer 2003; Simpson, M.G. 2006). Taxa in the family share an herbaceous habit, large petiolate leaves with transverse venation, and colorful, bracteate inflorescences with other members of the order, but can be differentiated by an arborescent growth form (lost in two species of *Strelitzia*), three free sepals, two fused petals that enclose five (or six in *Ravenala*) fertile stamens, a woody loculicidal capsule (Kress 1990) and seeds with brightly colored arils. Two of the genera, *Strelitzia* and *Ravenala*, are widely cultivated as ornamentals. *Strelitzia reginae*, the bird of paradise flower, is particularly well known for its showy inflorescence which resembles the head of a bird. *Phenakospermum*, *Ravenala*, and *Strelitzia* are distributed across French Guyana, Madagascar, and southern Africa, respectively. The family is estimated to have diverged from other Zingiberales 55- 49 million years ago, likely via dispersal events (Kress and Specht 2006).

Strelitziaceae was formally included in Musaceae *sensu lato* (Bentham and Hooker 1883), but was later recognized as a distinct family by Hutchinson (1934), who included *Heliconia*, *Ravenala*, *Phenakospermum*, and *Strelitzia* in his circumscription. A monophyletic Strelitziaceae without *Heliconia* was recognized by Nakai (1941), and this classification was supported by most subsequent authors (see Kress et al. 1990, for a review of taxonomic history of the Zingiberales). The monotypic nature of *Ravenala madagascariensis* Sonn. and *Phenakospermum guyannense* (L.C. Rich.) Endl. ex Miq.

has been little disputed, with the exception of the temporary recognition of a second species of *Phenakospermum*, *P. amazonicum* (Mart.), now recognized as conspecific with *P. guyannense* after temporary classification as a distinct genus, *Musidendron* Nakai (Kress and Stone, 1993). In contrast, the classification of *Strelitzia* species has undergone multiple revisions, with five species currently recognized: *Strelitzia reginae* Banks ex Aiton, *Strelitzia juncea* Link, *Strelitzia alba* Skeels, *Strelitzia nicolai* Regel & Koern and *Strelitzia caudata* R.A.Dyer (Coates Palgrave and Coates Palgrave 2002; Archer 2003; Simpson 2006). *Strelitzia reginae* and *S. juncea* differ from *S. alba*, *S. caudata*, and *S. nicolai* by a shrub-like growth form, orange (to yellow) sepals, and dark blue petals. In contrast, *S. alba*, *S. caudata* and *S. nicolai* have a tree-like habit reaching heights of up to 15 m, white sepals, and blue (white in *S. alba*) petals (Coates Palgrave and Coates Palgrave 2002; Dyer, 1945; Goldblatt and Manning 2000; Moore and Hyypio 1970).

Strelitzia nicolai can be differentiated from *S. alba* and *S. caudata* by a compound inflorescence (i.e., a tiered structure with multiple spathes), including up to five spathes (boat-like bracts) (Wright 1913; Dyer 1945; Coates Palgrave and Coates Palgrave 2002). In contrast, *S. caudata* and *S. alba* have simple inflorescences comprising a single spathe with a cincinnus of between six and ten flowers. *Strelitzia caudata* has a similar coloration of sepals and petals to that of *S. nicolai* (white and blue, respectively), while *S. alba* has completely white flowers (Dyer 1945; Coates Palgrave and Coates Palgrave 2002). Identification of species is sometimes supported by geographic location and habit. *S. nicolai* occurs in coastal dune vegetation of the Eastern Cape and KwaZulu-Natal in South Africa, and in Mozambique, *S. caudata* is the only montane species, occurring on

steep rocky slopes in Mpumalanga and Limpopo provinces of South Africa, and in Swaziland and the eastern highlands of Zimbabwe, and *S. alba* has a limited distribution in the coastal forests near Knysna and Plettenberg Bay in the Western Cape province of South Africa (Wright 1913; Dyer 1945; Goldblatt and Manning 2000; Coates Palgrave and Coates Palgrave 2002).

Strelitzia reginae and *S. juncea* are difficult to distinguish; the principal morphological difference between the two is a variable lamina width and shape. The lamina of *S. reginae* is large and ovate (Wright 1913; Dyer 1975; Goldblatt and Manning 2000), while that of *S. juncea* is highly reduced, usually with just the midrib remaining (Dyer 1975). Geographic locale is therefore useful in distinguishing among the taxa. *Strelitzia juncea* has a very limited distribution in the Eastern Cape, and occurs in bushveld as opposed to the riverine and coastal bush habitat of *S. reginae*. Dyer (1975) suggested that *S. juncea* may have arisen from *S. reginae*.

Phylogenetic analyses place the Strelitziaceae as one of the more basal lineages in Zingiberales, along with the Lowiaceae, Musaceae, and Heliconiaceae (Kress 1990; Smith et al. 1993; Kress 1995; Stevenson and Loconte 1995; Chase et al. 2000; Kress 2001). Most studies strongly support the sister relationship of Strelitziaceae and Lowiaceae (Rudall et al. 1999; Chase et al. 2000; Soltis et al. 2000; Kress et al. 2001; Givnish 2006; Soltis 2007), with some support for Musaceae as sister to the rest of the order (Kress 1990; Kress, 1995; Chase et al. 2000; Kress et al. 2001). In contrast, Johansen (2005) positioned Lowiaceae as sister to all remaining Zingiberales taxa, but sampling outside of *Orchidantha* was sparse. Other intrafamilial relationships among the Strelitziaceae, Lowiaceae, Musaceae, and Heliconiaceae have not been well established.

Relationships within the family Strelitziaceae are also unclear. *Ravenala madagascariensis* was found to be sister to the clade comprising *S. nicolai* and *P. guyannense* in the combined three gene (*atpB*, *rbcL*, *18s* rDNA) analyses of Soltis et al. (2000), Soltis et al. (2007), and Chase (2000), and also in the combined three gene (*atpB*, *rbcL*, *18s* rDNA) plus morphological dataset of Kress (2001). In contrast, Givnish et al. (2006) found *S. nicolai* to be sister to the clade comprising *R. madagascariensis* and *P. guyannense* based on analysis of the single gene data set of *ndhF*.

In the present study, I reconstruct the phylogeny of the Strelitziaceae using eleven chloroplast regions, *rbcL*, *matK* -5' *trnK*, *psbA*- *matk* , *psbB-psbH*, *Ycf6-trnC*, *rpL16* *intron*, *rpoB-trnC*, *trnS-trnfM* , *trnY-trnE*, *rp132-trnL*, *rps16*, and two nuclear regions, the nuclear ribosomal external transcribed spacer (ETS) and the 23rd intron of RNA polymerase beta subunit II (*rpb2*). I sample each of the seven species in the family as well as selected outgroups from within Musaceae, Heliconiaceae, and Lowiaceae. My objectives include the elucidation of relationships within the Strelitziaceae and comparison of phylogenetic signals between the nuclear and chloroplast regions, and the evaluation of current species delimitation in *Strelitzia*.

METHODS

Taxon Sampling: All seven currently recognized species of Strelitziaceae, *S. reginae*, *S. nicolai*, *S. caudata*, *S. alba*, and *S. juncea*, and four outgroup species, *Orchidantha fimbriata*, *Orchidantha siamensis*, *Musa acuminata*, and *Heliconia pendula* were represented in this study (see Table 5.1 for a list of species with accession numbers). Outgroup species were chosen from each of the four most basal families in the Zingiberales (Kress 2001).

Molecular Characters:

Rapidly evolving intron and intergenic spacers were selected from chloroplast and nuclear genomes that have previously been shown to resolve species level phylogenies (Shaw 2005; Shaw 2007), and the *rbcL* coding region was included to help resolve phylogenetic relationships at higher taxonomic levels (Small 2004).

A single accession was sequenced for the chloroplast and nuclear regions of both ingroup and outgroup taxa. A second accession for all ingroup species was sampled for all nuclear regions, and for three chloroplast regions: *matK* -5'*trnK*, *rpS16*, and the *rpL16* intron.

DNA Extraction and Manipulation:

cpDNA

DNA was extracted from silica dried or fresh tissue using the DNeasy Plant Mini Kit (Qiagen). Protocol for cpDNA polymerase chain reactions (PCR) followed (Green et al. *in revision*). Primers for the regions *rbcL*, *matK* -5'*trnK*, *psbA*- *matk* , *psbB-psbH*, *Ycf6-trnC*, *rpoB-trnC*, *trnS-trnfM* ,*trnY-trnE*, and *rpL16* were taken from Shaw (2005), *rp132-trnL* from Shaw (2007), *rps16* from Levin et al. (2004), and *rbcL* from Kress and Erickson (2007). Polymerase chain reactions were carried out in 25µl volume using 21.4 µl of 1X PCR buffer from New England BioLabs: (Taq PCR Kit # E5000S), 0.125 µl each of forward and reverse primer at 100mM, 0.5 µl dNTP at 10mM, 0.1 µl Taq (5,000 units/ml), 0.25 µl DNA (diluted in 5 of 11 taxa in 1:3 volume, others at full concentration). For several of the taxa, a 2X Diamond Reaction Mix from Bioline was substituted for the buffer, dNTP, and Taq. Amplifications were performed in a BioRad MyCycler, according to the following protocol: One cycle of 3 min. at 95°C, 10 cycles

beginning at 95°C for 45 sec., followed by a variation in temperature per cycle at a 1°C gradient beginning at 55°C and ending at 45°C, then 72°C for 45 sec. Then 20 cycles of 95°C for 45 sec, 50°C for 45 sec, and 72°C for 45 sec. Cycling was finished with a final extension time of 72°C for 5 min. The PCR products were held at 4°C for until they were checked for purity in a 1% agarose gel. The PCR products were purified using a MultiScreen PCR_{p96} Plate from Millipore. Cycle sequencing reactions were performed using Big Dye version 3.1 ABI (Applied Biosystems International) and sequenced on an ABI 3730 capillary sequencer. Sequencing of the *rbcL* region followed Kress et al. (2009).

Continuous stretches of DNA, contigs, were assembled using the commercially available software Sequencher v. 4.1 (GeneCodes) and preliminary sequence alignments were performed using the Clustal W algorithm (Thompson et al. 1994) accessed via Geneious Pro 5.0.2 (Drummond et al. 2010). For chloroplast regions, manual adjustments were made using MacClade v. 4.08 (Maddison and Maddison 2005) or Geneious Pro 5.0.2 (Drummond et al. 2010). Evolutionary events were minimized (Zurawski and Clegg 1987) and alignment of insertion-deletion (indel) events followed recommendations of Kelchner (2000). Areas of ambiguous alignment were excluded from analyses.

Nuclear

Nuclear DNA was extracted from silica-dried leaf material using a modified sodium dodecyl sulfate (SDS) extraction protocol (Edwards et al. 1991; Konieczny and Ausubel 1993). Previously published primers and protocols were used for PCR amplification in both cases (Baldwin and Markos 1998; Kay et al. 2005; Sass and Specht 2010).

Phylogenetic Analyses:

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed on the combined chloroplast regions, the combined nuclear regions, and the combined chloroplast and nuclear regions. For Bayesian analyses, the data matrix was partitioned according to three partitioning schemes: (1) unpartitioned, (2) a separate partition for each sequenced gene, and (3) separate partitions for markers within each compartment (chloroplast, nuclear) when relevant. All analyses (MP, ML, and Bayesian) were performed on two datasets, one which included all taxa, and one which excluded taxa which were incompletely sequenced for the chloroplast region (i.e., the second accession of each *Strelitziaceae* species). Tree topologies and support values from the analyses of the two datasets were compared to evaluate the effects of the missing data.

Gaps were treated as missing data. In unambiguously aligned regions, parsimony-informative gap characters were scored according to the simple indel coding method of Simmons & Ochoterena (2000) and added as binary characters to the end of the matrix. All regions were analyzed both with and without indels. The ML analyses were only performed on datasets with indels excluded.

MP and ML analyses were conducted with PAUP* (Swofford 2003). Parsimony analyses of individual and combined datasets were performed with characters equally weighted and unordered. Branch and bound searches were performed using the furthest addition sequence. Topological support was evaluated by performing the bootstrap analysis (Felsenstein 1985). A heuristic search strategy was used to analyze 1000 bootstrap replicates, each with 10 random addition replicates. To measure the fit of characters to the trees, the consistency index (Kluge and Farris 1969) and the retention index (Farris 1989) were calculated using PAUP*.

For ML analyses, models were chosen for the chloroplast data (GTR+I+G), the nuclear data (HKY+G), and the combined chloroplast and nuclear data (GTR+I+G) on the basis of the Akaike Information Criteria (AIC) criteria of ModelTest v. 3.7 (Posada and Crandall, 1998). Models identical to those suggested under the AIC were identified under hierarchical likelihood ratio test (hLRT) of Modeltest for the nuclear and the combined chloroplast-nuclear datasets, but for the chloroplast region, the model (TVM+G) was identified. Chloroplast data were analyzed under both suggested models. Heuristic searches with a random order of sequence addition and tree bisection and reconnection (TBR) branch-swapping were repeated 100 times. Bootstrap tests were performed using 100 replicates, each with 10 random addition sequence addition replicates.

Strict consensus trees were computed for MP and ML analyses. All MP and ML trees were rooted for output using *Musa acuminata*. Congruence of data from the chloroplast and nucleus was assessed by the comparison of MP and ML consensus tree topologies and bootstrap values. In addition, strict consensus trees were computed within each partition (chloroplast, nuclear, and combined nuclear and chloroplast) across analysis types (MP, ML, and Bayesian).

For Bayesian analyses, model selection for each partition was performed using MrModeltest v2 (Nylander 2004). The coded indels were included as a separate data partition, under a modified F81 model (Ronquist and Huelsenbeck 2003). Bayesian inference of phylogeny was performed on the data matrix under each partitioning scheme, with and without indels, using MrBayes v3.2.1 (Huelsenbeck and Ronquist 2001). In each analysis, two analyses of 10 million generations sampled every 1,000

generations were run to completion. In all cases, the two parallel analyses converged (average standard deviation of split frequencies < 0.005). The first using 10% of the trees were discarded as burnin and a 50% majority rule tree was constructed from the remaining trees.

RESULTS

The inclusion of a second accession, albeit incompletely sequenced, did not appear to negatively affect the outcome of the study in any way. The *Strelitzia* clade in the parsimony consensus tree of the chloroplast region was slightly less resolved when incomplete taxa were included than when they were excluded. However, ML and Bayesian consensus trees were dichotomously resolved and placement of taxa was constant, regardless of whether incomplete taxa were included. Support values for all trees were comparable (trees not shown). Thus, in this paper we present only the results of analyses which include all taxa.

Chloroplast data from each region was assumed to undergo similar evolutionary histories, therefore these regions were combined. Nuclear regions (*ETS*, *rpb2*) were also combined based on the comparison of tree topologies and MP and ML bootstrap values from consensus trees for each region, which differed only where bootstrap support was less than 70%. Comparison of tree topologies and MP and ML bootstrap values from consensus trees for each data compartment (combined chloroplast, combined nuclear) showed differences only where bootstrap support was less than 70%, therefore data from these regions were also combined.

Bayesian analyses of data under all partitioning schemes produced trees with identical topologies and comparable support values. Thus, only the results of the unpartitioned analyses are presented here.

Chloroplast DNA

The length of the tree for the eleven combined chloroplast regions was 6738 bp, with 623 variable characters and 355 parsimony informative characters. A total of 53 informative and unambiguous indels were coded as binary characters. A single accession was sequenced for all eleven regions, and a second accession sequenced for three regions, *matK* -5'*trnK*, *rpS16*, and the *rpL16* intron. Thus, the remaining seven regions of the second accession were coded as missing data. It was not possible to sequence the second accession of *R. madagascariensis* and *S. alba* for the *rpL2* intron, thus these regions were also coded as missing data. Total missing data was 30.8% (25.3 % due to the incomplete taxa).

Information regarding individual chloroplast regions is presented in Table 5.2. For the combined chloroplast region, there was a total of 132 equally most parsimonious trees (steps = 730 CI = 0.8973, RI= 0.8654). For ML analyses, the TVM+G model and the GTR+I+G yielded five most likely ML trees which were identical under each model with -log-likelihood (-Ln) = 13333.702.

The outgroups *M. acuminata* and *H. pendula* are sister taxa. *Orchidantha* species form a well supported clade which is sister to the Strelitziaceae (maximum parsimony bootstrap support (MPBS)=100, maximum likelihood bootstrap support (MLBS) =100, posterior probability (PP)=1.0), a monophyletic group (MPBS = 100, MLBS = 100, PP = 1.0). Placement of *R. madagascariensis* and *P. guyannense* differ among the three

analyses. They are unresolved in the parsimony consensus trees, but are sister to each other in a clade which is sister to the *Strelitzia* clade in the Bayesian consensus tree (PP = .92) (Fig. 5.1). In the ML consensus tree, *P. guyannense* is sister to remaining Strelitziaceae taxa (MLBS=100) (Fig. 2). *Strelitzia* species form a monophyletic clade in all analyses (MPBS = 95, MLBS = 100, PP= 0.99), but differ in the degree of species resolution. In the parsimony analyses, the *Strelitzia* clade is unresolved at the base. Both accessions of *S. reginae* and *S. juncea* are ungrouped, as is *S. nicolai*, and *S. caudata* forms a polytomous clade with *S. alba* (MPBS = 72) (tree not shown). In the ML consensus tree, *S. alba* accessions group together in a clade with *S. caudata* (MLBS=97.8) which is sister to a dichotomous clade containing the remaining *Strelitzia* taxa (MLBS=100). This clade consists of *S. nicolai* in one clade, and the poorly resolved accessions of *S. reginae* and *S. juncea* in the other. In the Bayesian consensus tree, *S. nicolai* is sister to the remaining *Strelitzia* species (PP=.99), which are split into two clades, one with *S. alba* paraphyletic with respect to *S. caudata*, and one clade containing *S. reginae* and *S. juncea*. *Strelitzia juncea* is paraphyletic with respect to *S. reginae*.

In the parsimony analyses, no changes in tree topology occurred when indels were included. In the Bayesian analyses including indels, *P. guyannense* is sister to other Strelitziaceae taxa, and *R. madagascariensis* is sister to a poorly resolved *Strelitzia* clade (trees not shown).

Nuclear

It was not possible to unambiguously align sequence information for rpb2 outgroup species (*M. acuminata*, *H. pendula*, *O. fimbriata*, and *O. siamensis*), thus this data was excluded from analyses. Missing data was 21% (16.3% due to missing rpb2

sequence for outgroup taxa). A total of 23 informative and unambiguous indels were coded as binary characters. Information regarding individual regions is shown in Table 5.2. Two most parsimonious trees were found (tree length = 535, CI= 0.8953, RI= 0.8519), and a single most likely tree was found ($-\text{Ln}= 4918.81777$).

Outgroup relationships in all analyses are similar to those described in the chloroplast trees. In the parsimony and Bayesian consensus trees, *P. guyannense* is sister to all other Strelitziaceae taxa (MPBS = 97, PP = 0.84), and *R. madagascariensis* is sister to the *Strelitzia* clade (MPBS = 75, PP = 0.76). *Strelitzia nicolai* is sister to remaining *Strelitzia* species (MPBS = 97, PP = 0.88), which is split into two clades (MPBS = 98, PP = 0.96) (Fig. 5.2). One clade contains *S. alba* and *S. caudata*, and one contains *S. reginae* and *S. juncea*. In the ML and Bayesian consensus trees, *S. juncea* is nested within a paraphyletic *S. reginae* (MLBS= 67, PP=.99), while in the parsimony consensus tree, the *S. reginae* accessions are unresolved. Outside of the clade containing *S. reginae* and *S. juncea*, the nuclear ML consensus tree is poorly resolved and is thus not further discussed. When indels were included in the data matrix, no changes in tree topology occurred in either parsimony or Bayesian analyses.

Combined Chloroplast and Nuclear

A total of 76 informative and unambiguous indels were coded as binary characters. Missing data was 29%. Two most parsimonious trees were found (steps = 1266, CI = .8957, RI = 0.8588), and a single most likely tree was found ($-\text{Ln}= 18773.771$).

Topologies of the combined nuclear-chloroplast consensus trees (parsimony, ML, and Bayesian) are identical to each other, with one exception (Fig. 5.3). The two *S.*

reginae accessions are paraphyletic in the trees produced via Bayesian and ML analyses, but have variable positions in the two most parsimonious trees. MP and Bayesian trees are identical to the corresponding parsimony and Bayesian trees produced from nuclear data. Overall, support values are comparable among consensus trees from each analysis, but are slightly lower in the most likely tree at the node which divides *S. alba*, *S. caudata*, *S. reginae*, and *S. juncea* into two separate clades. MLBS values are also slightly lower in the node which defines the relationship between *S. reginae* and *S. juncea*.

The relationship between *P. guyannense* and *R. madagascariensis* changes when indels are included in parsimony and Bayesian analyses. *Phenakospermum guyannense* is sister to *R. madagascariensis* in a clade which is sister to the *Strelitzia* clade when indels are included, whereas *P. guyannense* is sister to other Strelitziaceae taxa when indels are excluded. Positions of other taxa remain unchanged.

DISCUSSION

The inclusion of incomplete taxa in analyses has been shown to reduce phylogenetic accuracy under some circumstances (Huelsenbeck 1991; Wiens and Reeder, 1995; Lemmon et al., 2009). However, the inclusion of incomplete taxa in our study did not appear to create ambiguity in our datasets or result in low support values. This observation is supported by other studies which have shown that the presence of missing data does not necessarily lead to erroneous results (see Kearney 2002; Wiens 2006; Wiens 2008 for reviews). In the chloroplast trees, species relationships within *Strelitzia* are poorly resolved in the parsimony consensus tree. However, ML and Bayesian consensus trees are dichotomously resolved, with the exception of *S. reginae* and *S. juncea*. In the nuclear region, ML analysis yielded a consensus tree which lacked

resolution at its base, but the parsimony and Bayesian consensus trees are dichotomously resolved for all taxa with good support values. Combined chloroplast-nuclear trees are also dichotomously resolved.

Trees resulting from all analyses of data from different compartments (chloroplast, nuclear) and from the combined regions are in good agreement. Thus, relationships discussed here are common to all trees, unless otherwise specified. Strelitziaceae was found to be monophyletic and sister to the *Orchidantha* species, a position which agrees with previous studies (Rudall et al. 1997; Chase et al. 2000; Soltis et al. 2000; Kress et al. 2001; Givnish 2006; Soltis 2007). In all trees, *P. guyannense* is sister to the remaining Strelitziaceae taxa, except in the parsimony and Bayesian consensus trees from the chloroplast region which either lacked resolution of the genera (MP), or yielded *P. guyannense* and *R. madagascariensis* as sister taxa in a clade which is sister to the *Strelitzia* species (Bayesian). The latter relationship appears in the chloroplast-nuclear trees when indels are included, but the placement of genera in all other trees remains constant. In contrast, most previous studies place *R. madagascariensis* as sister to a clade containing the remaining genera (Kress, 2001, Soltis et al., 2000, Soltis et al., 2007, and Chase, 2000). However, the single loci study of Givnish et al. (2006) also found *S. nicolai* to be sister to the remaining taxa.

In all analyses, the *Strelitzia* species form a monophyletic clade. Despite few morphological differences between *S. nicolai*, *S. alba*, and *S. caudata*, I found high molecular support for the resolution of these taxa. In most trees, *S. nicolai* is sister to a dichotomous clade containing the other *Strelitzia* species; *S. alba* and *S. caudata* are resolved as sister taxa in one of the two clades. The other clade consists of *S. reginae* and

S. juncea, but relationships among these accessions vary among trees, and overall we did not find support for the classification of these taxa as distinct species. In the consensus chloroplast trees, accessions of *S. reginae* and *S. juncea* form a polytomy, while in the nuclear and chloroplast-nuclear consensus trees, *S. reginae* is paraphyletic with respect to *S. juncea* with moderate to high support. The addition of indels into the analyses did not alter the relationships between the taxa.

Given the debated taxonomic history of *S. reginae* and *S. juncea*, these ambiguous results are not surprising. *Strelitzia juncea* was first described by Link (1821), although *S. parvifolia* Aiton var. *juncea* Ker Gawl. was described and illustrated by Ker-Gawler earlier that same year (Wright 1913; Dyer 1975). It is not absolutely certain that these are the same plants (Dyer 1975). Considering that leaf blade shape is the principal distinguishing feature among the taxa, Moore and Hyypio (1970) proposed that *S. juncea* should be classified as a variant or cultivar of *S. reginae*. However, Van der Venter (1975) observed variable leaf blade shapes in plants grown from two distinct seed stocks in a controlled environment, and concluded that genetic differences govern leaf development. Based on these observations, as well as geographic information, he supported the classification of the taxon as a distinct species. This position was supported by Dyer (1975) and is currently accepted by most authors today.

This study is the first to examine the evolutionary relationships among all species within the Strelitziaceae. With the exception of *S. reginae* and *S. juncea*, relationships within the family are well supported. In light of the sparse morphological evidence supporting the taxonomic division of *S. reginae* and *S. juncea*, the paraphyletic or ambiguous relationships shown here in the molecular data, and the short branch lengths

differentiating the species (Fig. 5.4), further investigations are needed. Population level studies which sample additional accessions, both within and among populations of both taxa will help to clarify these species boundaries. This information, combined with morphological data, will help to achieve a robust understanding of the evolutionary history of the family.

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Table 5.1. Species sampled and taxonomic affiliations.

Species	Accession Number	Source
<i>Musa acuminata</i>	02 075	Smithsonian
<i>Heliconia pendula</i>	94 650	Smithsonian
<i>Orchidantha fimbriata</i>	2003-1178 A	Fairchild
<i>Orchidantha siamensis</i>	94 843	Smithsonian
<i>Ravenala madagascariensis</i>	P.1395 H	Fairchild
<i>Phenakospermum guyannense</i>	1994-768	Smithsonian
<i>Strelitzia nicolai</i>	77117 A	Fairchild
<i>Strelitzia reginae</i>	74529 A	Fairchild
<i>Strelitzia juncea</i>	2002-0688-A	Fairchild
<i>Strelitzia alba</i>	K19A	Chetty
<i>Strelitzia caudata</i>	K01A	Chetty

Table 5.2. Data on individual regions. * = parsimony informative characters.

locus	# accessions sequenced	compartment	length (bp)	var. sites	% var.	PICs*
<i>matK</i> -5' <i>trnK</i> intron	2	chloroplast	767	79	10.30%	45
<i>matK-psbA</i>	1	chloroplast	844	73	8.65%	45
<i>rps16</i> intron	2	chloroplast	708	70	9.89%	38
<i>psbB-psbH</i>	1	chloroplast	560	30	5.36%	19
<i>rp132-trnL</i>	1	chloroplast	1045	148	14.16%	83
<i>rpL16</i> intron	2	chloroplast	662	75	11.33%	47
<i>rpoB-trnC</i>	1	chloroplast	604	53	8.77%	23
<i>trnC-ycf</i>	1	chloroplast	670	52	7.76%	27
<i>trnS-trnfM</i>	1	chloroplast	184	10	5.43%	7
<i>trnY-trnE</i>	1	chloroplast	157	7	4.46%	4
<i>rbcL</i>	1	chloroplast	537	26	4.84%	17
<i>ETS</i>	2	nuclear	574	307	53.48%	141
<i>rpb2</i>	2	nuclear	1294	87	6.72%	55

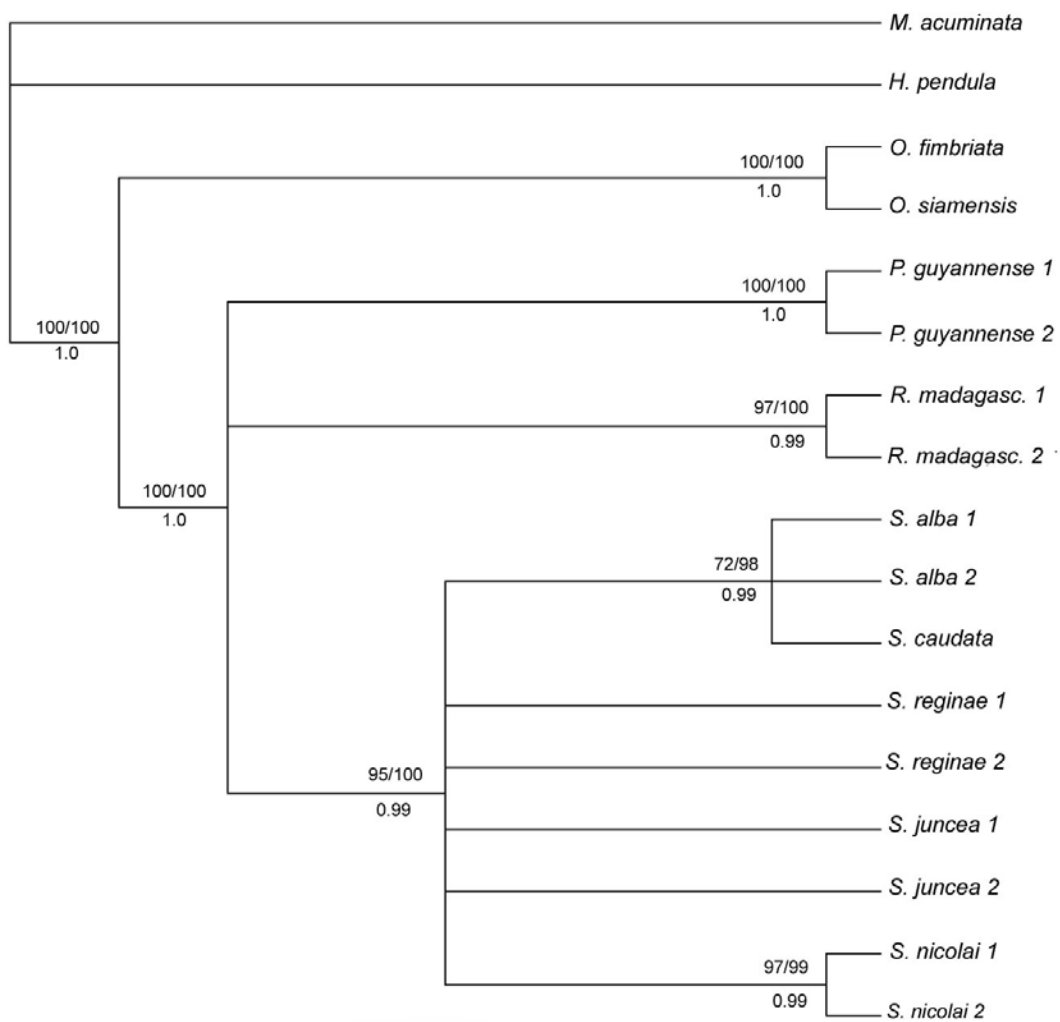


Figure 5.1. Strict consensus tree of all analysis types constructed using eleven chloroplast regions. Support values overlaid on nodes: MPBS (above branch, left), MLBS (above branch, right), and PP (below branch).

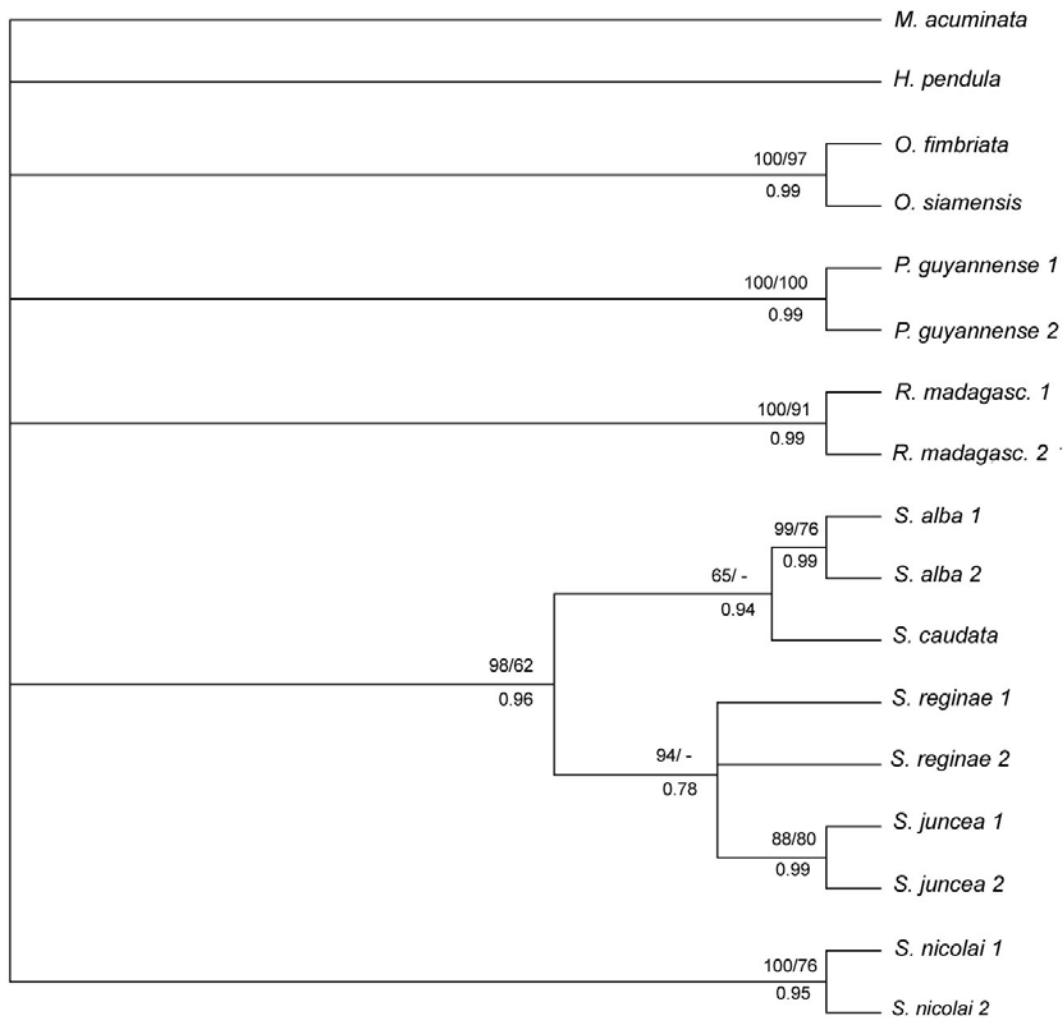


Figure 5.2. Strict consensus tree of all analysis types constructed using *ETS* and *rpb2*. Support values overlaid on nodes: MPBS (above branch, left), MLBS (above branch, right), and PP (below branch). “-” indicates node not present.

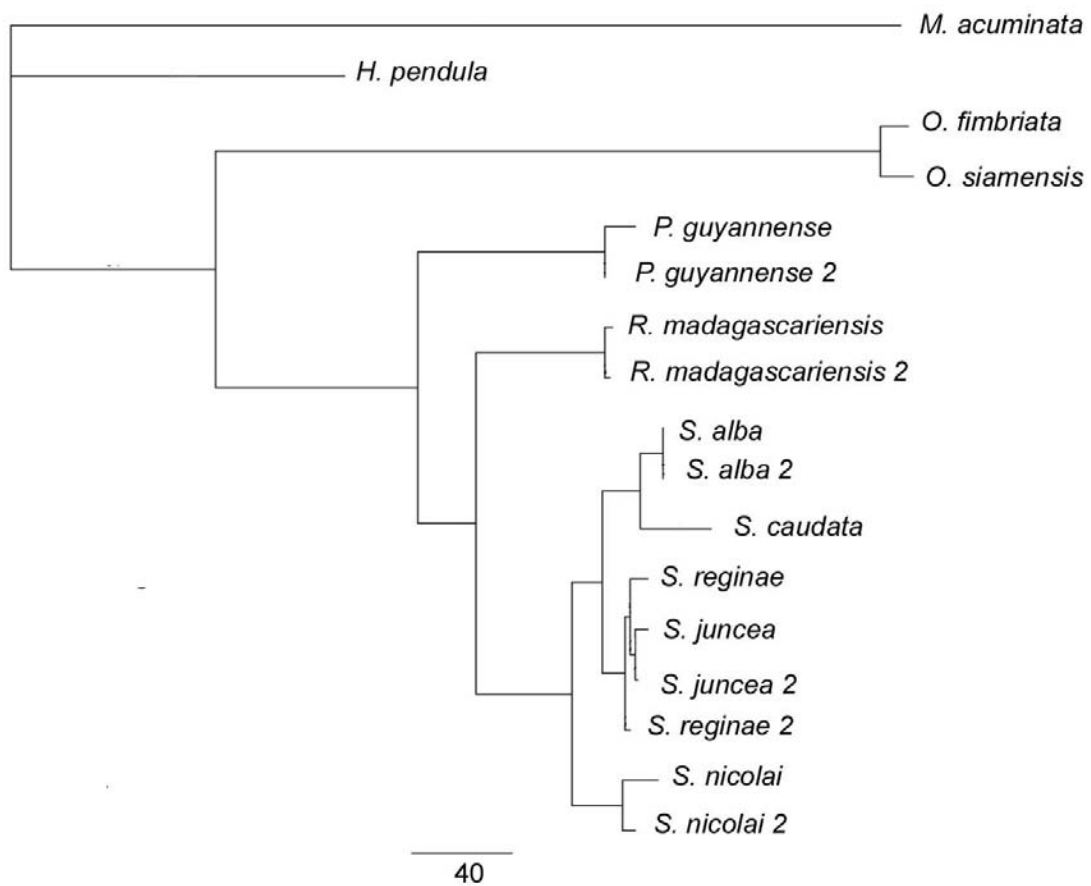


Figure 5.4. One of the two most parsimonious trees resulting from the analysis of the combined nuclear and chloroplast regions.

SUMMARY

Although tetrapyrroles are ubiquitous in plants and include vital molecules such as chlorophyll, heme, and phytochromobilin, the presence of bilirubin- IX α in *Strelitzia nicolai* was the first example of this compound in a plant (Pirone et al., 2009). Remarkably, the pigment was also present in a diverse sampling of angiosperm species, from the monocot orders Zingiberales and Arecales to the more derived eudicot Myrtales. In most species sampled, bilirubin was present in low levels detectable only by mass spectrometry (Chapter 3). However, in the family Strelitziaceae, pigment concentrations were high in the orange arils of *Phenakospermum guyannense*, *Strelitzia nicolai*, and *S. reginae*, and thus contribute to color. Although bilirubin concentrations in *Ravenala madagascariensis* were high enough to be detected via high performance liquid chromatography with UV detection (HPLC/UV), the arils of this species are blue, and thus bilirubin does not make a significant contribution to color, at least not one which is observable by the human eye. Our preliminary analyses indicate an unusual blue protein is responsible for the vibrant blue aril color in this species (Pirone, unpublished observations).

Investigation of phylogenetic relationships within the Strelitziaceae indicates that *Phenakospermum guyannense* is sister to a clade containing *R. madagascariensis* and the *Strelitzia* species. However, when indels were included in the combined chloroplast-nuclear analyses, *R. madagascariensis* and *P. guyannense* are sister taxa in a clade which is, in turn, sister to the *Strelitzia* clade. In neither case is the accumulation of bilirubin to produce color a synapomorphy.

FUTURE STUDIES

The unexpected discovery of bilirubin in plants provides a basis for additional research in diverse fields. Of high priority will be the determination of the biochemical pathway by which bilirubin- IX α is produced in plants. In animals, biliverdin-IX α is reduced by biliverdin-IX α reductase (BR) to form bilirubin-IX α (Maines and Trakshel, 1993). Conversely, in plants, biliverdin-IX α is converted to a structural isomer of bilirubin, 3Z-phytochromobilin, the precursor of the phytochrome chromophore (Tanaka and Tanaka, 2007), by phytochromobilin synthase (Terry et al., 1995; Kohchi et al., 2001). In cyanobacteria and some algae, biliverdin-IX α is reduced by the bilin reductases, to form the phycobilin chromophores (Beale, 1993; Frankenburg et al., 2001). A BR enzyme was identified and cloned in the cyanobacterium *Synechocystis* (Schluchter and Glazer, 1997), and BR enzymes have also been found in a variety of other bacteria. Whether bilirubin IX- α in plants is formed via a BR enzyme or some other means remains to be determined.

It is also of great interest to establish the botanical function of bilirubin. As a novel source of plant color, bilirubin likely attracts pollinators and dispersers when present in high concentrations. Aril color is preserved for decades under mild conditions; we observed brilliantly colored arils in herbarium sheets up to 45 years old (HUH, Sturrock #93, 1963; and Natal #50,000, 1976). Thus, persistence of aril color may contribute to a prolonged period of dispersal. Bilirubin may also provide other benefits to the avian dispersers. Perhaps they use its antioxidant properties (Stocker, 1987), or they may sequester it as feather color. Within the plant, bilirubin may also function as a potent lipid soluble antioxidant, or serve other functions which are unknown

at this time. Cellular and molecular studies which explore the possible physiological role(s) of bilirubin in plants will be useful.

Further sampling of bilirubin-IX α in plants will be necessary to gain a more complete view of its phylogenetic distribution, and determine whether its role in color production is limited to the Strelitziaceae. The research presented here focused on sampling within the Zingiberales, and included some representatives from the Arecales, Pandanales, Laurales, and Myrtales. Future sampling should include additional members of these and related orders, as well as basal angiosperm taxa from the Magnoliid clade, and derived representatives from the Asterid clade. To determine whether bilirubin-IX α is unique to flowering plants, sampling should also include ferns and gymnosperms (Cycadales, Ginkgoales, Pinales, and Gnetales). Dense sampling of species within selected clades will elucidate fine scale phylogenetic patterns of bilirubin production. In addition to fruits and flowers, stems, leaves, and roots should also be sampled. Like many secondary compounds, it is likely that bilirubin production varies with time, developmental stage, or environment. Thus, species should be sampled under diverse conditions to accurately assess the presence or absence of bilirubin within a species. Observations of bilirubin under different conditions may also elucidate production trends which could provide clues regarding its function.

Further sampling should include species which were previously analyzed for pigment (carotenoid or flavonoid) content, as bilirubin-IX α may have been present but undetected due to its unique biochemical properties. Bilirubin is insoluble or minutely soluble in solvents routinely used for the extractions of carotenoids and flavonoids (acetone and hexane, and alcohols, respectively) (Goodwin and Britton, 1998; Harborne

1998) and thus may have remained in plant tissues after extraction. Also, routine chromatographic procedures may have been insufficient to detect bilirubin. I observed the retention of bilirubin in both C-18 and silica columns under various solvent regimes which would have otherwise eluted molecules with a wide range of polarities. Furthermore, the chromatographic peaks or bands corresponding to bilirubin may have been obscured by those of other plant pigments (personal observation). Bilirubin-IX α decomposes in heat, light, and change in pH (Lightner, 1982); therefore degradation may also occur during handling and chromatography and further preclude its detection.

The unique biochemical and chromatographic properties of bilirubin-IX α are likely attributable, at least in part, to conformational changes in the molecule. Bilirubin-IX α is a linear tetrapyrrole, but under most conditions folds in on itself to create a “ridge-tile” conformation. Specifically, two sets of intramolecular hydrogen bonding occur, each involving a carboxylic acid, a pyrrole-imino hydrogen, and a terminal lactam system (Bonnett et al., 1978). This conformation renders the molecule more nonpolar than its linear form and thus interacts differently with its environment.

Future analyses which follow protocols designed specifically for the detection of bilirubin-IX α will result in the successful acquisition of data and lead to a more complete understanding of the evolutionary history of this unusual molecule in plants.

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PUBLICATIONS AND PRESENTATIONS

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