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## Bilirubin Present in Diverse Angiosperms

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## ABSTRACT

**Background and aims:** 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, we discovered bilirubin in the arils of *Strelitzia nicolai*, the White Bird of Paradise Tree, which was the first example of this molecule in a higher plant. Subsequently, we 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. We were therefore interested in determining whether bilirubin is broadly distributed in the plant kingdom, and whether it contributes to color in other species.

**Methodology:** In this paper, we use we use 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.

**Principal results:** Bilirubin was present in eight species from the orders Zingiberales, Arecales, and Myrtales, but only contributed to color in species within the Strelitziaceae.

**Conclusions:** The wide distribution of bilirubin in angiosperms indicates the need to re-assess 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- $\alpha$ , 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 $\alpha$  is also formed from the degradation of heme, but it is transformed into the yellow-orange pigment bilirubin-IX  $\alpha$ . We have identified bilirubin-IX $\alpha$  (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, we 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., in press).

In *S. nicolai* and *S. reginae*, bilirubin is a novel biosynthetic source of display color. As a rule, the coloration of flowers and fruits is achieved with products from three metabolic pathways: the terpenoid (carotenoids), the phenylpropanoid (flavonoids), and the betalain (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 (Harbourne, 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 our 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 the major groups of the angiosperms. Preliminary high performance liquid chromatography (HPLC/UV) analyses of aril extracts of an additional species in the Strelitziaceae, *Phenakospermum guyanense*, showed a pigment with a retention time and UV-Visible spectra which matched those of bilirubin. Here, we use HPLC/UV and HPLC/UV/electrospray ionization-tandem mass spectrometry (HPLC/UV/ESI-MS/MS) to confirm the presence of bilirubin in *P. guyanense* 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 1). We

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. Tissue for each sample and its replicate were composed of tissue from one or multiple inflorescences or infructescences from a single, sometimes clonal, individual. The replicate aril samples of *Phenakospermum guyanense* came from different individuals (collected by John Kress; Guyana (South America), Demerara-Mahaica region). For the names and taxonomic affiliations of species sampled, see Table 1. 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 analysis. We 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 guyanense* and *Ravenala madagascariensis*. We also sampled species from two of the most derived families in the order, *Costus lucanusianus* (Costaceae) and *Hedychium coronarium* (Zingiberaceae) (Kress,

2001). We 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, we 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 1) was based on tissue availability and fruit color. 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  $\mu\text{m}$  filter, and divided into two equal aliquots. Each aliquot was dried to completion in a rotovap 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. 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

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). Extract was redissolved in DMSO, partitioned with hexane to remove lipids, and chromatographed on a reverse phase ODS-A column (150 mm × 4.3 mm, particle size 5µm; Waters, 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 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 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, we treated non-arillate samples (Table 1) with diazomethane to convert bilirubin to its di-methyl ester, i.e. both carboxylic acids were converted to methyl esters ( $\lambda_{\max} = 453$  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 di-methyl 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 di-methyl 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 di-methyl ester was 31.7 min, thus making it possible to observe the compound without interference from other pigments (Fig. 1). A standard curve for bilirubin di-methyl 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 di-methyl ester was verified by comparison with bilirubin di-methyl 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, we 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 1.

## HPLC/UV/ESI-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<sup>n</sup> scans. HPLC/UV was performed with an Agilent Technologies HPLC with binary pumps (1100 series; Santa Clara, CA, USA), a Symmetry C8 HPLC column (150 mm x 2.1 mm, particle size 5µm; Waters, 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 H<sub>2</sub>O (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 was 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 instrument parameters) 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 due to oxidation during ionization. The  $m/z$  585 and  $m/z$  583 ions underwent 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 due to 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*, *Heliconia collinsiana*, *Costus lucanusianus*, *Ravenala madagascariensis*, *Phenakospermum guyanense*, *Hedychium coronarium*, *Gastrococcus crisper*, and *Eugenia luschnathiana* (Table 1). 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 guyanense*, 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*, *Gastrococcus crisper*, 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 1).

## DISCUSSION

We 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 expected to be found within members of a clade, we concentrated most of our sampling within the Strelitziaceae and the Zingiberales. Kress et al. (2001) divides the order into two major clades, the basal “banana group” which includes the Musaceae, Strelitziaceae, Heliconiaceae, and Lowiaceae, and the more derived “ginger group”, which includes the four remaining families (Fig. 2). Although resolution of intrafamilial relationships varies among other 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 the Lowiaceae as sister to the Strelitziaceae (Rudall et al., 1997; Chase et al., 2000; Soltis et al., 2000; Givnish et al., 2006; Soltis et al., 2007).

The detection of bilirubin in *Gastrococos crispera* (family: Arecaceae, order: Arecales) and *Eugenia luschnathiana* (family: Myrtaceae, order: Myrtales), indicates that bilirubin is not restricted to the Zingiberales and may be broadly distributed throughout the plant kingdom. However, the lack of detection of bilirubin in avocado, *Persea americana* (family: Lauraceae, order: Laurales) and *Pandanus odoratissimus* (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. guyanense* 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*, and the variability of bilirubin concentration within *P. guyanense* may indicate that bilirubin biosynthesis is not constant, but is instead variable and influenced by factors which are currently unknown. For example, we 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. 3), 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 which produces bilirubin in plants remains unknown. In animals, biliverdin-IX  $\alpha$  is reduced by an NAD(P)H-dependent biliverdin-IX  $\alpha$  reductase (BR) to form bilirubin-IX  $\alpha$  (Maines and Trakshel, 1993). Conversely, in plants, biliverdin-IX  $\alpha$  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  $\alpha$  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- $\alpha$  in plants is formed by the reduction of biliverdin IX- $\alpha$  by a BR enzyme or via some other means remains to be determined.

## **CONCLUSIONS and FORWARD LOOK**

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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### **CONTRIBUTIONS BY AUTHORS**

XXX wrote the manuscript, collected plants, prepared extracts, and performed HPLC analyses. XXX performed and interpreted MS/MS analyses. XXX also interpreted MS/MS spectra and oversaw lab work. XXX prepared the *Hedychium* samples and provided general lab advice. XXX was the first to observe the uniqueness of Strelitziaceae pigments, and contributed to the discussion on ecology and color production.

### **CONFLICTS OF INTEREST**

No conflicts of interest

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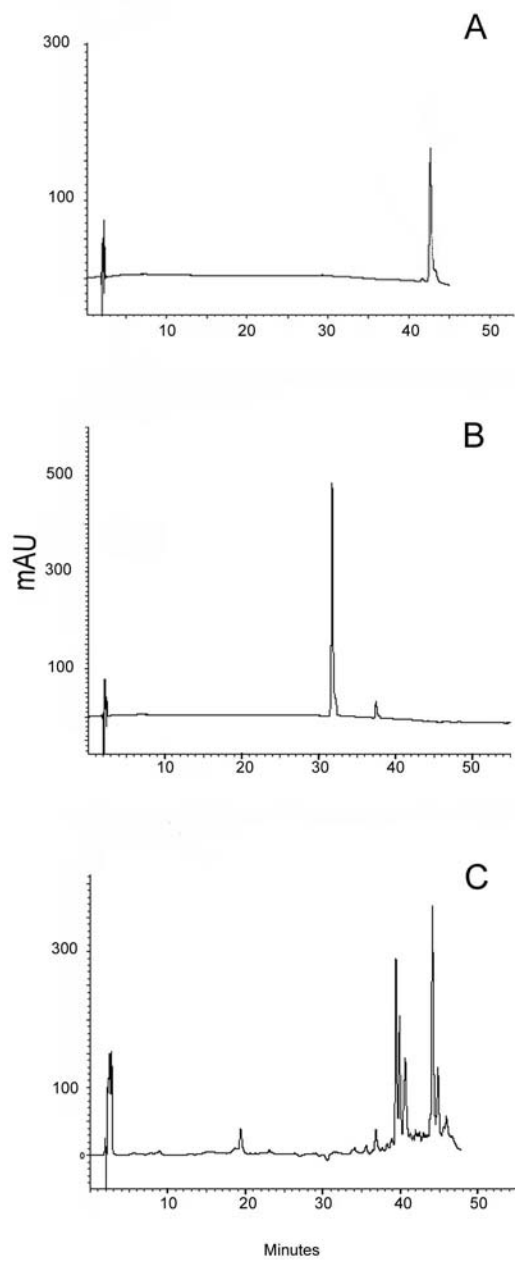
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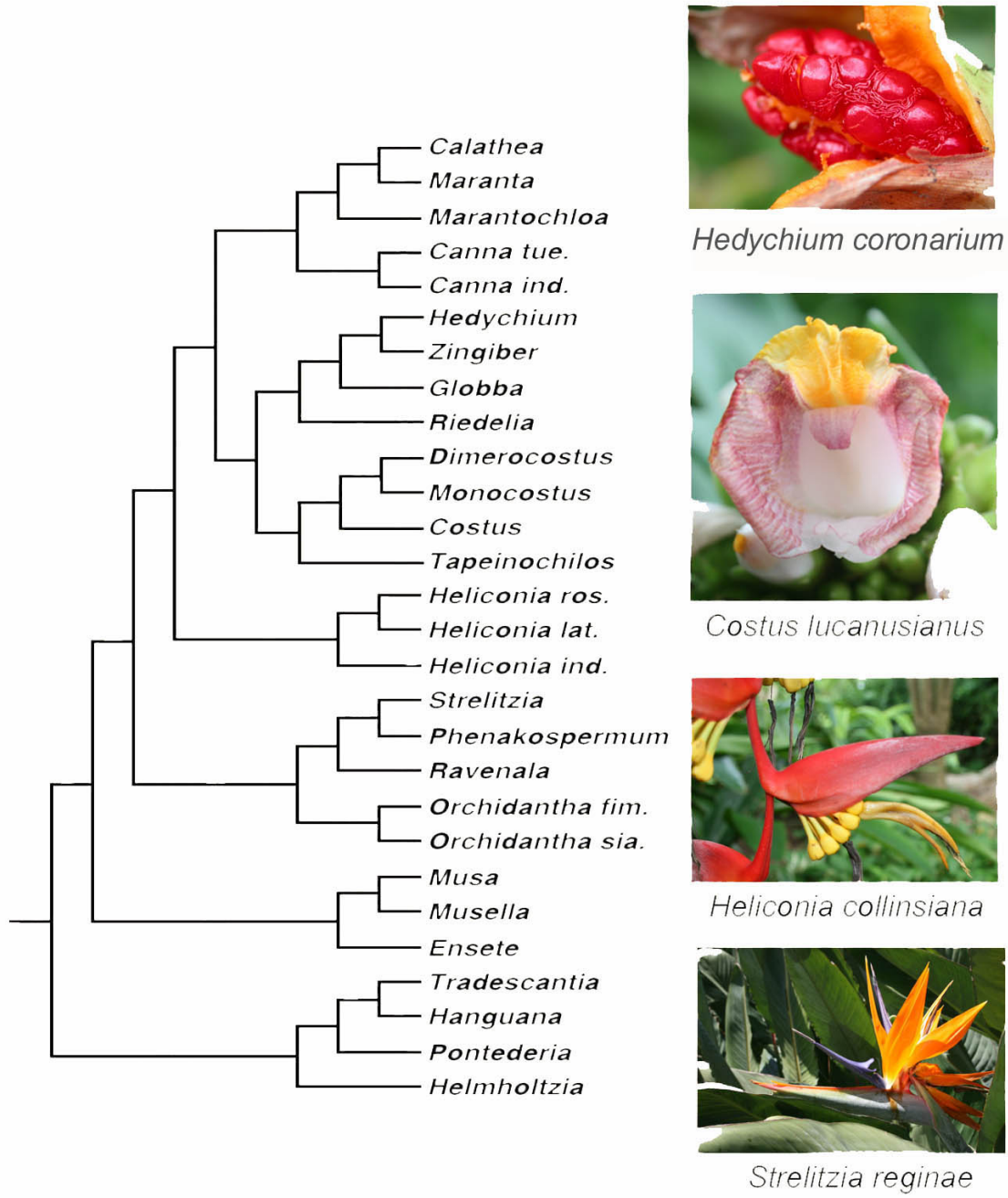
## FIGURE LEGENDS

**Figure 1. HPLC/UV Chromatograms of A. Bilirubin standard, B. Bilirubin standard treated with diazomethane, and C. Carotenoid-like pigments from the flowers of *Costus lucanusianus*, which elute around the same retention time as bilirubin.** Bilirubin standard and flower extract from *C. lucanusianus* monitored at 444 nm, bilirubin standard treated with diazomethane monitored at 453 nm.

**Figure 2. 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 3. Bilirubin production in *Strelitzia nicolai* during aril development.** Maturing arils of *S. nicolai* with increasing amounts of bilirubin. Seeds ~4 mm in diameter.

**Fig. 1**



**Fig. 2**



**Fig. 3**

**Table 1. Summary of HPLC/UV and MS/MS results of the analysis of bilirubin (BR) in ten angiosperm species. N/A indicates that samples were not treated with diazomethane.**

Species	Family	Order	Organ	BR detection via Diazomethane Derivative	BR detection via HPLC/UV	BR detection via HPLC-MS/MS	BR conc. Samp. 1	BR conc. Samp. 2	Mean BR conc. (n=2)
<i>Musa balbisiana</i>	Musaceae	Zingiberales	peel	N	N	Y	< 44 ng/g	< 44 ng/g	—
<i>Heliconia collinsiana</i>	Heliconiaceae	Zingiberales	fruit	N	N	Y	—	< 44 ng/g	—
<i>Costus lucanusianus</i>	Costaceae	Zingiberales	flower	N	N	Y	< 44 ng/g	< 44 ng/g	—
<i>Ravenala madagascariensis</i>	Strelitziaceae	Zingiberales	aril	N/A	Y	Y	0.001 mg/g	0.001 mg/g	0.001 mg/g
<i>Phenakospermum guyanense</i>	Strelitziaceae	Zingiberales	aril	N/A	Y	Y	3.041 mg/g	5.787 mg/g	3.725 mg/g
<i>Hedychium coronarum</i>	Zingiberaceae	Zingiberales	aril	N	N	Y	< 44 ng/g	< 44 ng/g	—
<i>Gastrococos crispera</i>	Arecaceae	Arecales	fruit	N	N	Y	< 44 ng/g	< 44 ng/g	—
<i>Pandanus odoratissimus</i>	Pandanaceae	Pandanales	fruit	N	N	N	—	—	—
<i>Persea americana</i>	Lauraceae	Laurales	fruit	N	N	N	—	—	—
<i>Eugenia luschnathiana</i>	Myrtaceae	Myrtales	fruit	N	N	Y	< 44 ng/g	—	—