Phylogeny of Celastraceae (spindle-tree family) subfamilies Hippocrateoideae and Salacioideae inferred from chloroplast and nuclear genes

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Picture 1: *Salacia madagascariensis* flower

Picture 2: *Salacia spectabilis* fruits and seeds

Abstract

The phylogeny of Celastraceae (the spindle-tree family) subfamilies Hippocrateoideae and Salacioideae, which include about 360 species of shrubs, trees, and vines in 25 genera, was inferred using plastid (*matK*, *trnL-F*) and nuclear (ITS and 26S rDNA) genes. Together, subfamilies Hippocrateoideae and Salacioideae contain all members of the former Hippocrateaceae, which are now recognized as a derived group within Celastraceae sensu stricto. Based on our results, *Brassiantha*, a monotypic genus endemic to New Guinea, is more closely related to the clade of *Dicarpellum* (New Caledonia) and *Hypsophila* (Queensland, Australia) than it is to the former Hippocrateaceae, in contrast to previous studies. This well supported resolution indicates that having a nectary disk positioned outside the stamens has been convergently derived in these two separate lineages. The clade of *Kokoona* and *Lophopetalum* was resolved as sister to the clade of Hippocrateoideae, *Sarawakodendron*, and Salacioideae. This resolution of *Kokoona* and *Lophopetalum* supports previous assertions that they are a "transitional link" between Celastraceae sensu stricto and the former Hippocrateaceae. *Sarawakodendron*, a monotypic genus endemic to Borneo, was resolved as sister to the clade of Salacioideae, which supports earlier assertions that *Sarawakodendron* is "transitional" between *Kokoona*, *Lophopetalum*, and Salacioideae. Based on our inferred phylogeny, arils as mucilaginous pulp are derived within Salacioideae and winged arils may be primitive within the former Hippocrateaceae as a whole. Finally, the former Hippocrateaceae had an Old World, rather than a New World, origin.

Picture 3: *Brassiantha* (Smith and Bailey, 1941)

Picture 4:*Anthodon panamense* fruits and seeds

Introduction

The phylogeny of the former Hippocrateaceae was inferred using four different loci: two loci from the plastid genome (*matK*, a protein-coding gene, and *trnL-F*, transfer RNA genes) and two loci from the nuclear genome (ITS and 26S ribosomal DNA). There are two subfamilies within the former Hippocrateaceae: Hippocrateoideae [with winged arils (an expansion of the funiculus that arises from the placenta and envelops the seed; Swartz, 1971)] and Salacioideae (with arils as mucilaginous pulp). Genera from both sub-families were sampled, along with four genera that have been associated with the former Hippocrateaceae (*Brassiantha, Kokoona, Lophopetalum*, and *Sarawakodendron*). The methods used to obtain these results include DNA isolation, DNA amplification, DNA purification and sequence alignment.

DNA Isolation

DNA was isolated from dried leaves of all of taxa listed in Table 1 using Qiagen DNeasy Plant Mini Kits. The leaf tissue was pulverized by a Bosch reciprocating saw in tubes containing steel beads. A lysis buffer was then added and heated to break up the cells. The mixture was then centrifuged to separate the DNA in solution from large polysaccharides, proteins and other leaf material. The DNA was then bound to a silica-gel based membrane and washed using ethanol. The DNA was then eluted into a low-salt solution and stored at -20℃ to prevent degradation.

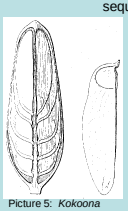
DNA Amplification

The DNA samples were amplified using PCR (polymerase chain reaction) using locus-specific forward and reverse DNA primers, *Taq* DNA polymerase and free nucleotides. The PCR cycle has 3 stages: denaturation, annealing and elongation. Denaturation, run at 94℃, breaks the hydrogen bond s between the two complementary DNA strands, allowing for annealing of the primers. All loci were amplified using a 50℃ annealing temperature, excep t for the 3' end of *trnL-F*, which used a 53℃ annealing temperature. The elongation stage, wherein *Taq* polymerase extends the primers, was run at 72℃. T he entire PCR program consists of 35 cycles, exponentially increasing the number of copies of the targeted locus.

After amplification, gel electrophoresis was performed to separate DNA fragments by size. Digital pictures were taken of the gels to ensure that the correct-size fragment was amplified.

DNA Purification

Once amplification was confirmed, the PCR product was purified using Qiagen QIAquick PCR Purification Kits. A high-salt buffer was combined with the product in a tube containing a silica-gel membrane and centrifuged to bind the DNA to the membrane. Once the DNA was bound, the membrane was washed using an ethanol-based buffer and centrifuged. The DNA was then eluted into a low-salt buffer. A small amount of DNA was then run in another gel to quantify the amount of purified DNA. 300 nanograms of DNA were then dried-down and sent, along with the respective primers, to Macrogen, Inc. in South Korea for sequencing.



Picture 5: *Kokoona* (Ding Hou, 1962)

Contiguous Sequences and Alignment

Raw sequences were retrieved from Macrogen, Inc. online and contiguous sequences of the respective loci were created using both forward and reverse sequences for each sample using the program *Aligner* (CodonCode Corporation). Preliminary alignments were performed using *Clustal X* (Thompson et al., 1997) to check for any sequencing errors. Final alignments were performed using the more rigorous program *Muscle* (Edgar, 2004) as the basis for phylogenetic analysis.

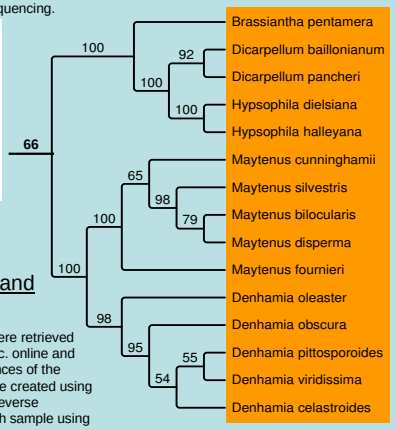


Figure 1. Relationship of *Brassiantha* to an Austral-Pacific clade inferred from the ITS locus.

Picture 6: *Salacia cordata* fruits

Picture 7: fleshy aril (Brock, 1993)

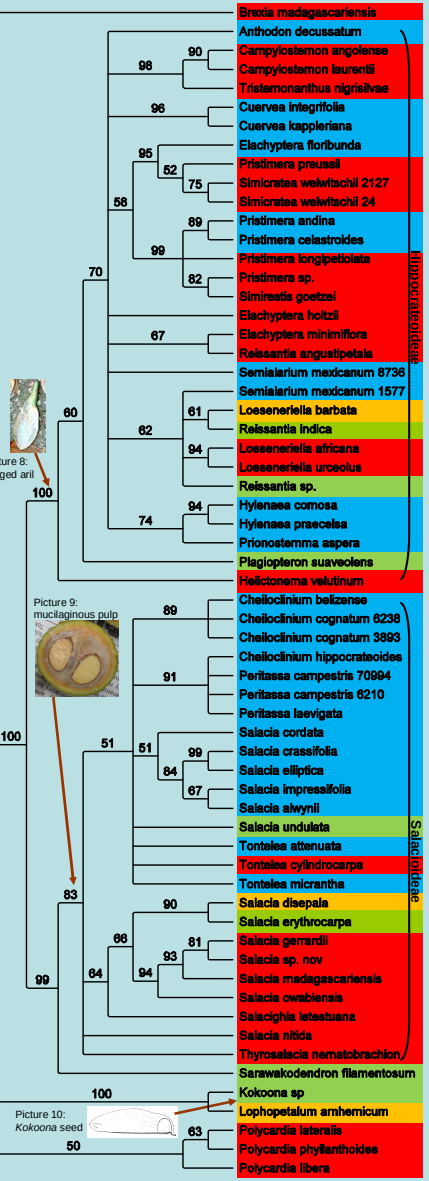


Figure 2. Relationship among lineages inferred using a combined analysis of the four loci. Jackknife are indicated for each clade. The colored backgrounds represent the biogeographical region for each clade: red is for Africa, blue for the Americas, green for Asia and orange for Austral-Pacific.

Picture 8: winged aril

Picture 9: mucilaginous pulp

Picture 10: *Kokoona* seed

Picture 11: *Anthodon decussatum* flowers

Phylogenetic Inference

Internal gaps (representing inferred insertions and deletions) in the alignments were coded as separate characters using simple indel coding (Simmons and Ochoterena, 2000) with *SeqState* (Müller, 2005).

Each of the four loci was analyzed separately with previously generated sequences from Islam et al. (2006), Zhang and Simmons (2006), and Simmons et al. (submitted). A simultaneous analysis (Kluge, 1989) of all samples in the former Hippocrateaceae clade (as assessed in the separate analyses) was then performed to increase phylogenetic signal (Figure 2).

For each locus, as well as for the simultaneous analysis, an equally weighted parsimony jackknife analysis (Farris et al., 1996) was performed using *PAUP** (Swofford, 2001) with 2,000 jackknife replicates. Each replicate comprised ten independent random-addition tree searches using tree-bisection-reconnection (TBR; a thorough tree-swapping algorithm).

Results and Discussion

- *Brassiantha*, a monotypic genus endemic to New Guinea, is more closely related to the clade of *Dicarpellum* (New Caledonia) and *Hypsophila* (Queensland, Australia) than it is to the former Hippocrateaceae, in contrast to previous studies (Smith and Bailey, 1941; Simmons and Hedin, 1999). This well supported resolution indicates that having a nectary disk positioned outside the stamens (Picture 3) has been convergently derived in these two separate lineages.
- The clade of *Kokoona* and *Lophopetalum* was resolved as sister to the clade of Hippocrateoideae, *Sarawakodendron*, and Salacioideae. This resolution of *Kokoona* and *Lophopetalum* supports previous assertions that they are a "transitional link" between Celastraceae sensu stricto and the former Hippocrateaceae (Ding Hou, 1964; Görts-van Rijn and Mennega, 1994; Simmons and Hedin, 1999; Simmons et al., 2001).
- *Sarawakodendron*, a monotypic genus endemic to Borneo, was resolved as sister to the clade of Salacioideae, which supports earlier assertions that *Sarawakodendron* is "transitional" between *Kokoona, Lophopetalum*, and Salacioideae (Ding Hou, 1967; Corner, 1976; Simmons and Hedin, 1999).
- Some genera of Hippocrateoideae (*Elachyptera, Loeseneriella, Pristimera*, and *Reissantia)* and Salacioideae *(Cheiloclinium* and *Salacia)* do not appear to be monophyletic groups, but these inferences generally lack strong jackknife support and relationships are poorly resolved. We expect ongoing sequence sampling to improve resolution and support by filling in gaps in our current sampling.
- Based on our inferred phylogeny, arils as mucilaginous pulp (Picture 9) are derived within Salacioideae and winged arils (Pictures 4 & 8) may be primitive within the former Hippocrateaceae as a whole, which would support the inference from Simmons et al. (2001).
- The former Hippocrateaceae had an Old World, rather than a New World, origin based on using Fitch (1971) optimization to map the geographical areas onto the tree (Figure 2).

Picture 12: *Sarawakodendron* (Ding Hou, 1967)

Picture 13: *Salacia madagascariensis*

Picture 14: *Salacia madagascariensis*

Table 1. Species Sampled

Taxon	Source
Anthodon decussatum Ruiz & Pav.	*G. Montgomery 19*, Panama (MO)
Campylostemon laurentii De Wild.	*W.J.J.O. de Wilde et al. 3754*, Cameroun (MO)
Cheiloclinium belizense (Standl.) A.C. Sm.	*J. Meave & A. Howe 1435*, Belize (MO)
Cheiloclinium cognatum (Miers) A.C. Sm.	*J.A. Lombardi 6238*, Brazil (HRCB)
Cheiloclinium hippocrateoides (Peyr.) A.C. Sm.	*J.A. Lombardi 6532*, Brazil (HRCB)
Cuervea kappleriana (Miq.) A.C. Sm.	*G.L. Sobel et al. 4864*, (NY)
Elachyptera floribunda (Benth.) A.C. Sm.	*E.M. Martinez et al. 23410*, Guatemala (MO)
Elachyptera holtzii (Loes. ex Harms) R. Wilczek	*O.A. Kibure 228*, Tanzania (MO)
Elachyptera minimiflora (H. Perrier) N. Hallé	*R.H. Archer et al. 2934*, Madagascar (CS)
Helictonema velutinum (Afzel.) Pierre ex N. Hallé	*T.B. Hart 1580*, Zaire (MO)
Hylenaea comosa Miers	*J.A. Lombardi 6400*, Brazil (HRCB)
Kokoona sp.	*M.W. Chase 2092*, cult. Bogor, Indonesia (K)
Loeseneriella barbata (F. Muell.) C. T. White	*A. Ford 4475*, Australia
Loeseneriella urceolus (Tul.) N. Hallé	*R.H. Archer et al. 3002*, Madagascar (CS)
Lophopetalum arnhemicum Byrnes	*W. Price s.n.*, Australia
Peritassa campestris (Cambess.) A.C. Sm.	*J.A. Lombardi 6210*, Brazil (HRCB)
Peritassa laevigata (Hoffmanns.) A.C. Sm.	*J.A. Lombardi 6399*, Brazil (HRCB)
Plagiopteron suaveolens Griff.	*M.W. Chase 1335*, cult. Bogor, Indonesia (K)
Pristimera celastroides (Kunth) A. C. Sm.	*Nee & Taylor 26588*, (NY)
Pristimera longipetiolata (Oliv.) N. Hallé	*R.H. Archer 2174*, South Africa (PRE)
Pristimera preussii (Loes.) N. Hallé	*Harris 4969*, Central African Republic (K)
Pristimera sp.	*R.H. Archer et al. 2948*, Madagascar (CS)
Prionostemma aspera Miers	*Pires 1399*, Brazil (NY)
Reissantia angustipetala (H. Perrier) N. Hallé	*R.H. Archer et al. 2939*, Madagascar (CS)
Reissantia sp.	*M.W. Chase 2095*, cult. Bogor, Indonesia (K)
Salacia alwynii Mennega	*J.A. Lombardi 6333*, Brazil (HRCB)
Salacia cordata (Miers) Mennega	*J.A. Lombardi 6352*, Brazil (HRCB)
Salacia crassifolia (Mart.) G. Don	*J.A. Lombardi 6206*, Brazil (HRCB)
Salacia disepala (C.T. White) Ding Hou	*W.W.C. 1896*, Australia
Salacia elliptica G. Don	*J.A. Lombardi 6121*, Brazil (HRCB)
Salacia gerrardii Larv. & Sprague	*R.H. Archer 2121*, South Africa (PRE)
Salacia madagascariensis DC.	*R.H. Archer et al. 2919*, Madagascar (CS)
Salacia nitida N. E. Br.	*J. Munzinger & Karamoko 14*, Ivory Coast (BH)
Salacia owabiensis Hoyle	*J. Munzinger & Karamoko 17*, Ivory Coast (BH)
Salacia sp. nov.?	*R.H. Archer et al. 3040*, Madagascar (CS)
Salacia undulata Cambess.	*M.W. Chase 2096*, cult. Bogor, Indonesia (K)
Salacighia letestuana (Pellegr.) Blakelock	*M. Fay & Harris 8430*, Central African Republic C46(K)
Semialarium mexicanum (Miers) Mennega	*E. Cabrera & H. de Cabrera 8736*, Mexico (F)
Semialarium mexicanum (Miers) Mennega.	*J.F. Morales et al. 1577*, Costa Rica (F)
Simicratea welwitschii (Oliver) N. Hallé	*C.C.H. Jongkind & D.K. Abbiw 2127*, Ghana (MO)
Simirestis goetzei (Loes.) N. Hallé ex R. Wilczek	*G. Simon & I.L. Mollel 82*, Tanzania (MO)
Thyrosalacia nematobrachion (Loes.)	*O.S. Invido & N. de Makokoa 1294*, Gabon (MO)
Tontelea cylindrocarpa A.C. Sm.	*C. Feuillet et al. 10191*, French Guiana (F)
Tontelea micrantha Spreng.	*J.A. Lombardi 6442*, Brazil (HRCB)

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