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Wax plants disentangled: A phylogeny of *Hoya* (Marsdenieae, Apocynaceae) inferred from nuclear and chloroplast DNA sequences

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Abstract

Hoya (Marsdenieae, Apocynaceae) includes at least 200 species distributed from India to the Pacific Islands. We here infer major species groups in the genus based on combined sequences from the chloroplast *atpB-rbcL* spacer, the *trnL* region, and nuclear ribosomal DNA ITS region for 42 taxa of *Hoya* and close relatives. To assess levels of ITS polymorphism, ITS sequences for a third of the accessions were obtained by cloning. Most ITS clones grouped by species, indicating that speciation in *Hoya* usually predates ITS duplication. One ITS sequence of *H. carnosa*, however, grouped with a sequence of the morphologically similar *H. pubicalyx*, pointing to recent hybridization or the persistence of paralogous copies through a speciation event. The topology resulting from the combined chloroplast and nuclear data recovers some morphology-based sections, such as *Acanthostemma* and *Eriostemma*, as well as a well-supported Australian/New Guinean clade. The combined data also suggest that morphological adaptations for ant-symbiosis evolved at least three times within *Hoya*.

Keywords: *atpB-rbcL* spacer; Bayesian inference; Chloroplast DNA; *Hoya*; Nuclear ribosomal DNA ITS region; Paralogus; Parsimony; *trnL* region

1. Introduction

Hoya is a taxonomically complex genus of flowering plants distributed from India to the Pacific Islands (Fig. 1). Over 500 names have been published (The Plant Names Project, 1999), and at least 200 species are currently recognized (Kleijn and van Donkelaar, 2001), with new ones being added every year. The introduction of about 100 species of *Hoya* into the horticultural trade reflects the plants' large appeal, but the unbridled naming of different forms has led to nomenclatural confusion and heightened the need for a taxonomic revision, also based on a molecular phylogenetic scaffold.

Most species of *Hoya* are herbaceous lianas with succulent leaves, often growing as epiphytes in the rainforest. The genus is generally characterized by persistent inflorescences with flowers having rotate corollas, staminal coronas with revolute margins, pollinia with pellucid margins, and narrow, spindle-shaped seeds without conspicuous wings (Omlor, 1998; this study Fig. 2). Several efforts have been made in the past to subdivide the genus based on morphology (Hooker, 1885; Schumann, 1895; Schlechter, 1913, 1916), but the only recent such attempts come from the horticultural world (Burton, 1985, 1995, 1996; Kloppenburg, 1993, 2001a). Because none of these infrageneric classifications is complete or conclusive, many species of *Hoya* have never been assigned to sections, making appropriate sampling of the genus for phylogenetic work difficult.

The systematic position of *Hoya* in the tribe Marsdenieae of the Asclepiadoideae (Apocynaceae), on the other hand, has been settled by molecular phylogenetic studies (e.g., Meve and Liede, 2004; Potgieter and Albert, 2001; Sennblad and Bremer, 2002). Molecular work has also begun to test the monophyly of *Hoya* (Wanntorp

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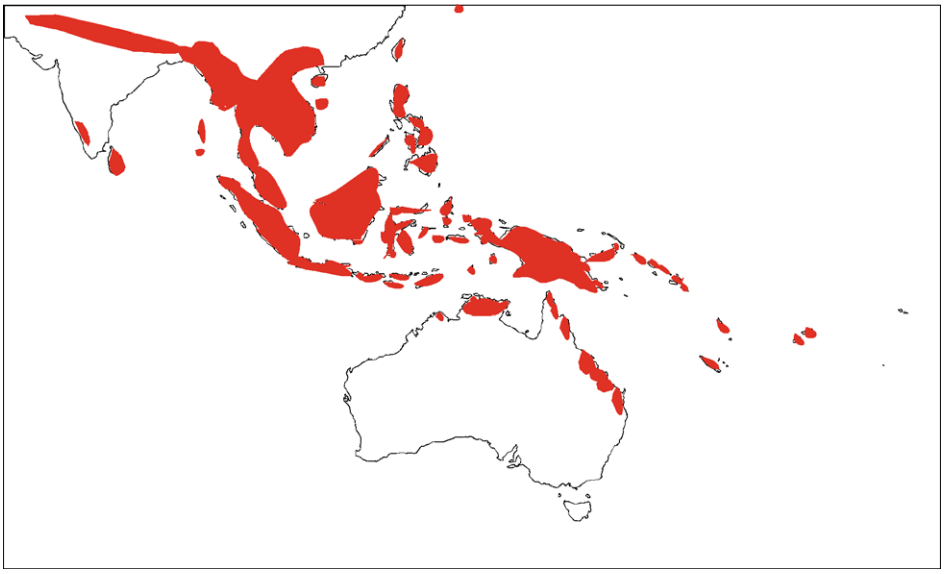

Fig. 1. Geographical distribution of *Hoya*.

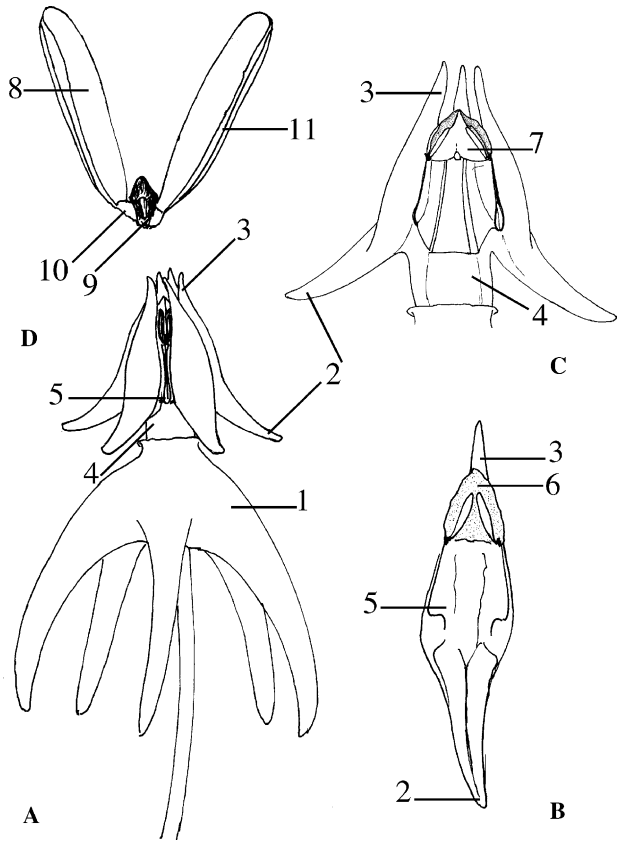

Fig. 2. Schematic drawing of *Hoya multiflora*. (A) Lateral view of flower. (B) Corona lobe and anther from the inside. (C) Lateral view of corona and gynostegium (two anthers removed). (D) Pollinarium. 1, corolla; 2, outer corona lobe; 3, inner corona lobe; 4, column; 5, anther wing; 6, anther appendage; 7, style head; 8, pollinium; 9, Caudicle; 10, Retinaculum; 11, Pellucid margin. Drawn by H-E. Wanntorp.

et al., in press) and *Dischidia*, one of its closest relatives (Livshultz, 2000, 2003). Results for *Hoya* so far show that the genus is paraphyletic unless it includes the monotypic genera *Absolmsia*, *Micholitzia*, and *Madangia* (Wanntorp et al., in press). Although identifying some major clades within *Hoya*, the information from the chloroplast data obtained to date is, insufficient to resolve species relationships within the genus (Fig. 3).

To better resolve the infrageneric structure of *Hoya*, we resorted to the internal transcribed spacer of nuclear ribosomal DNA (nrDNA), which is part of the rDNA cistron comprising 18S, ITS1, 5.8S, ITS2, and 26S. Most eukaryotes have several hundred copies of this cistron, with the copies usually undergoing concerted evolution through unequal crossing over and gene conversion, resulting in their homogenization within individuals, populations, and species (Dover and Tautz, 1986). Studies involving cloning of ITS over the past few years, however, have revealed that incompletely homogenized paralogous copies can persist within species or individuals. Biological phenomena explaining the presence of paralogous copies include polyploid genomes (Muir et al., 2001; Volkov et al., 1999; Wendel et al., 1995), multiple nucleolar organizer regions (Bobola et al., 1992; Karvonen and Savolainen, 1993), and highly divergent ITS pseudogenes no longer capable of undergoing normal concerted evolution (Buckler et al., 1997; Muir et al., 2001; Razafimandimbison et al., 2004). When multiple copies are detected, the ITS region often cannot be used for phylogenetic purposes unless it can be demonstrated that paralogous ITS copies coalesce within species rather than persist through speciation events (e.g., Bellarosa et al., 2005; Buckler et al., 1997; Razafimandimbison et al., 2004; Won and Renner, 2005). When facing evidence of polymorphic ITS in *Hoya*, we decided to assess the level of intra-species polymorphism by cloning the ITS1/5.8S/ITS2 region from about a third of the included accessions. We then analyzed these sequences together with the remaining directly obtained ITS sequences and the available chloroplast DNA sequences using phylogenetic methods.

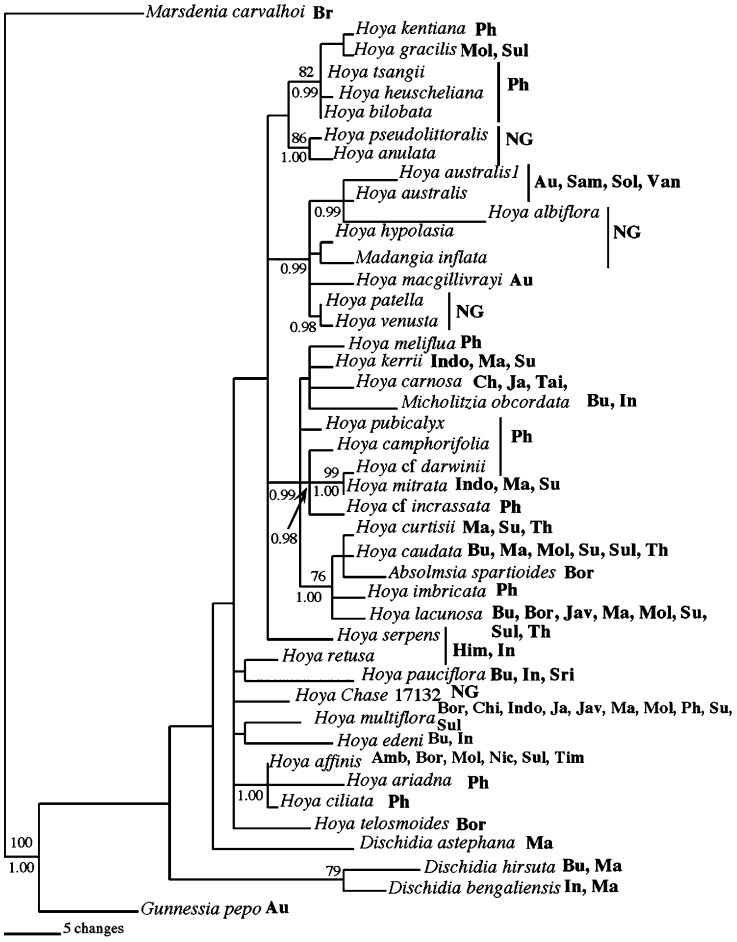

Fig. 3. Phylogram showing a 50% majority rule consensus obtained from the Bayesian analysis, based on the *trnL* region and the *atpB-rbcL* spacer with bootstrap support >75% shown above branches, and Bayesian posterior probabilities >0.97 below branches. Geographic occurrences are abbreviated as follows: Amb, Ambon; Au, Australia; Bor, Borneo; Br, Brazil; Bu, Burma; Ch, China; Him, Himalaya; In, India; Indo, Indochina; Ja, Japan; Jav, Java; Ma, Malaysia; Mol, Moluccas; NG, New Guinea; Nic, Nicobar; Ph, Philippines; Sam, Samoa; Sol, Solomon Islands; Sri, Sri Lanka; Su, Sumatra; Sul, Sulawesi; Tai, Taiwan; Th, Thailand; Tim, Timor; Van, Vanuatu. (Modified from Wanntorp et al., in press.)

Morphological adaptations for ant-symbioses occur in several species of *Hoya* and *Dischidia* (Beccari, 1884; Janzen, 1974; Livshultz et al., 2005; Treseder et al., 1995). In the case of *Dischidia*, Livshultz et al. (2005) has suggested that myrmecophytism may be plesiomorphic, and we were therefore interested in inferring how many times occupation by ants may have arisen in *Hoya*. The principal goals of this study were to further clarify intrageneric relationships in *Hoya*, based on nuclear sequences, and to use the resulting phylogeny to examine the evolution of morphological characters, and of the symbiosis between ants and species of *Hoya*.

2. Materials and methods

2.1. Taxon sampling, DNA isolation, amplification and sequencing

A list of material with voucher information and GenBank Accession Nos. is presented in Table 1. The species sample used here is the same as that of Wanntorp et al. (in press) and covers most of the geographic range and flower morphological variation on which sections have been based (Burton, 1985, 1995, 1996; Hooker, 1885; Kloppenburg, 1993, 2001a; Schlechter, 1913, 1916). In addition to 35 species of *Hoya*, we included *Absolmsia spartioides*, *Dischidia hirsuta*, *D. astephana*, *D. bengalensis*, *Gunnessia pepo*, *Madangia inflata*, and *Micholitzia obcordata* (all Marsdenieae). Trees were rooted with *Marsdenia carvalhoi* based on earlier results (Wanntorp et al., in press).

Genomic DNA was isolated from fresh or silica gel-dried young leaves using DNeasy Plant kits according to the manufacturer's protocol (QIAGEN, Valencia, CA, USA). Polymerase change reactions (PCRs) were performed with 10 μM primers in 25-μL reactions using "ready to go" PCR beads from Amersham-Pharmacia Biotech (Uppsala, Sweden). PCRs were set at an initial denaturation for 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s for primer annealing, 72 °C for 2 min for primer extension, and 72 °C for 8 min for completion of primer extension, using the primers 18SF (Rydin et al., 2004) and C26A (Yokota et al., 1989). Alternative settings

Table 1
Taxa included in the present study with voucher information and GenBank accession numbers

Taxon	Voucher information	GenBank Accession No ITS region; *trnL-F* spacer; *trnL* intron; *trnL-F* intron and spacer; *atpB-rbcL* spacer
Absolmsia spartioides (Benth.) Kunze (Genus type) = *Hoya spartioides* (Benth.) Kloppenburg	Wanntorp L. 592 (S), Sipitang, Borneo	DQ334484, —; —; DQ334549; DQ334591
Dischidia astephana Scort. ex King & Gamble	Wanntorp L. 562 (S), Cameroon Highland, Pahang, Malaysia	DQ334459; —; —; DQ334534; DQ334576
Dischidia bengalensis Colebr.	920392 (CONN)	—; AF214189; AF214343; —; —
Dischidia hirsuta Decne.,	Wanntorp L. 563 (S), ex hort. Departm. Bot., Stockholm University	DQ334452 (d); DQ334453, DQ334454, DQ334455, DQ334456; —; —; DQ334531; DQ334573
Gunnessia pepo P.I. Forster (Genus type)	P.I.F. Forster PIF6465 (BRI), Queensland, Australia	DQ334446; —; —; DQ334528; DQ334570
Hoya affinis Hemsl.	Chase 17128 (K), RBG-Kew, Liv. Coll. 1983-4478	DQ334481; —; —; DQ334546; DQ334588
Hoya albiflora Zipp. Ex Blume	Wanntorp L. 584 (S), L20000646	DQ334493 (d); DQ334494; DQ334495; DQ334496; DQ334497; —; —; DQ334555; DQ334597
Hoya anulata Schltr.	Wanntorp L. 585 (S), IPPS 8603, L990438	DQ334485; —; —; DQ334550; DQ334592
Hoya ariadna Decne.	Chase 17125 (K), RBG-Kew, Liv. Coll. 1983-4474	DQ334502 (d); DQ334503; DQ334504; DQ334505; DQ334506; —; —; DQ334559; DQ334602
Hoya australis R.Br. ex Traill	Wanntorp L. 564 (S), ex hort Departm. Bot., Stockholm University	DQ334428; —; —; DQ334527; DQ334569
Hoya australis 1 R.Br. ex Traill	Wanntorp L. 565 (S), ex hort. Departm. Bot., Stockholm University	DQ334445; —; —; DQ334524; DQ334566
Hoya bilobata Schltr.	Chase 17129 (K), RBG-Kew, Liv. Coll. 1983-4481	DQ334489; DQ334490; DQ334491; DQ334492; —; —; DQ334554; DQ334596
Hoya camphorifolia Warburg	Wanntorp L. 590 (S), Philippines, Quezon National Park.	DQ334474 (d); DQ334471; DQ334472; DQ334473; DQ334475; DQ334520; —; —; DQ334539; DQ334581
Hoya carnosa R.Br. (Genus type)	Wanntorp L. 566 (S), ex hort. Departm. Bot., Stockholm University	DQ334460 (d); DQ334461; DQ334462; DQ334463; DQ334464; —; —; DQ334535; DQ334577
Hoya caudata Hook. f.	Wanntorp L. 587 (S), ex hort, Departm. Bot., Stockholm University	DQ334483; —; —; DQ334548; DQ334590
Hoya ciliata Elmer ex C.M.Burton	Wanntorp L. 586 (S), IPPS 3071, L920785	DQ334512; DQ334513; DQ334514; DQ334515;—; —; DQ334562; DQ334605
Hoya curtisii King & Gamble	Wanntorp L. 578 (S), 1998-3180, Uppsala Bot. Gar.	DQ334479; —; —; DQ334544; DQ334586
Hoya cf. *darwinii* Loher	Chase 17135 (K), RBG-Kew, Liv. Coll. 1984-2899	DQ334477; —; —; DQ334542; DQ334584
Hoya edeni King ex Hook.f.	Wanntorp L. 579 (S), IPPS 8292	DQ334476; —; —; DQ334540; DQ334582
Hoya gracilis Schltr.	Wanntorp L. 567 (S), ex hort. Departm. Bot., Stockholm University	DQ334439; DQ334440; DQ334441; DQ334442; DQ334443; DQ334444; —; —; DQ334426; DQ334568
Hoya heuschkeliana Kloppenb.	Wanntorp L. 568 (S), ex hort. Departm. Bot., Stockholm University	DQ334416; DQ334417; DQ334418; —; —; DQ334529; DQ334571
Hoya hypolasia Schltr.	Wanntorp L. 588 (S), IPPS 7006, L901824	DQ334470; —; —; DQ334538; DQ334580
Hoya imbricata Decne.	Wanntorp L. 569 (S), ex hort. Departm. Bot., Stockholm University	DQ334480; —; —; DQ334545; DQ334587
Hoya cf. *incrassata* Elmer ex Merr.	Chase 17136 (K), RBG-Kew, Liv. Coll. 1984-3340, Philippines, Palawan	DQ334516 (d); DQ334517; DQ334518; DQ334519; —; —; DQ334561; DQ334604
Hoya kentiana C.M. Burton	Wanntorp L. 570 (S), ex hort. Departm. Bot., Stockholm University	DQ334424; —; —; DQ334522; DQ334564
Hoya kerrii Craib	Chase 17123 (K), RBG-Kew, Liv. Coll. 1982-2786.	DQ334458; —; —; DQ334533; DQ334575
Hoya lacunosa Blume	Wanntorp L. 571 (S), ex hort. Departm. Bot., Stockholm University	DQ334499; —; —; DQ334557; DQ334599
Hoya macgillivrayi F.M.Bailey	Wanntorp L. 572 (S), ex hort. Departm. Bot., Stockholm University	DQ334488; —; —; DQ334553; DQ334595
Hoya meliflua Merr.	Wanntorp L. 591 (S), Philippines, Mindoro Occidental, Puerto Galera	DQ334434 (d); DQ334429; DQ334430; DQ334431; DQ334432; DQ334433; DQ334435; DQ334436; DQ334437; DQ334438; —; —; DQ334525; DQ334567
Hoya mitrata Kerr.	Wanntorp L. 589 (S), IPPS 7684, L914643	DQ334500; —; —; DQ334558; DQ334600
Hoya multiflora Blume	Wanntorp L. 573 (S), ex hort. Departm. Bot., Stockholm University	DQ334487; —; —; DQ334552; DQ334594

(*continued on next page*)

Table 1 (*continued*)

Taxon	Voucher information	GenBank Accession No ITS region; *trnL-F* spacer; *trnL* intron; *trnL-F* intron and spacer; *atpB-rbcL* spacer
Hoya patella Schltr.	Wanntorp L. 575 (S), ex hort. Departm. Bot., Stockholm University	DQ334498; —; —; DQ334556; DQ334598
Hoya pauciflora Wight	Wanntorp L. 574 (S), ex hort. Departm. Bot., Stockholm University	DQ334465; DQ334466; DQ334467; DQ334468; —; —; DQ334536; DQ334578
Hoya pseudolittoralis C. Norman	Wanntorp L. 582 (S), IPPS 4551	DQ334478; —; —; DQ334543; DQ334585
Hoya pubicalyx Merr.	Wanntorp L. 576 (S), ex hort. Departm. Bot., Stockholm University	DQ334447 (d); DQ334448; DQ334449; DQ334450; DQ334451; —; —; DQ334530; DQ334572
Hoya retusa Dalz.	Wanntorp L. 580 (S), 1998-3127, Rosendal Uppsala, Uppsala Bot. Gar.	DQ334457; —; —; DQ334532; DQ334574
Hoya serpens Hook. f.	Chase 17118 (K), RBG-Kew	DQ334482; —; —; DQ334547; DQ334589
Hoya telosmoides R. Omlor	Wanntorp L. 577 (S), Mount Kinabalu, Sabah, Malaya	DQ334486; —; —; DQ334551; DQ334593
Hoya tsangii C.M. Burton	Wanntorp L. 581 (S), 1998-3136, Uppsala Bot. Gar.	DQ334425; DQ334426; DQ334427; —; —; DQ334523; DQ334565
Hoya venusta Schltr.	Wanntorp L. 583 (S), IPPS 3773	DQ334507; —; —; DQ334560; DQ334603
Hoya–Chase 17132	Chase 17132 (K), RBG-Kew, Liv. Coll. 1983–4484	DQ334469; —; —; DQ334537; DQ334579
Madangia inflata P.I. Forst., D.J. Liddle & I.M. Liddle (Genus type)	I.M. Liddle IML1076 (BRI), Madang Province, New Guinea	DQ334508; DQ334509; DQ334510; DQ334511; —; —; DQ334541; DQ334583
Marsdenia carvalhoi G.Morillo & Carnevali	Chase 17115 (K), RBG-Kew, Liv. Coll. 1982-1949, Brazil, Bahia.	DQ334419 (d); DQ334420; DQ334421; DQ334422; DQ334423; —; —; DQ334521; DQ334563
Micholitzia obcordata N.E.Br. (Genus type)	Seidenfaden s.n. (K) (MWC 733)	DQ334501; AJ431766, AJ431765; —; DQ334601

Accession numbers for the sequences of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (first with the directly sequenced sequences (d), followed by the cloned sequences), the *trnL-F* spacer, *trnL* intron, *trnL-F* intron and spacer, and for the *atpB-rbcL* spacer. Herbarium abbreviations follow Index Herbariorum (Holmgren et al., 1990).

were an initial 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and finally 72 °C for 7 min, using the primers ITS 4 and ITS 5 (White et al., 1990). PCR products were purified with the QIAquick PCR purification kit (QIAGEN) and the Wizard SV genomic DNA purification system (Promega, WI, USA). Sequencing reactions were obtained using the same primers as above and the Big Dye Terminator kit v3.1 (Applied Biosystems, Warrington, UK). After cycle sequencing, products were cleaned by NaOAc/ethanol precipitation or by Sephadex G-50 Superfine gel filtration (Amersham) on MultiScreen TM-HV membrane plates (Millipore, Bedford, USA), according to the manufacturer's protocol to remove unincorporated ddNTPs. Fragments were separated on an ABI Prism 377 DNA sequencer or an ABI 3100 Avant capillary sequencer. Sequences were assembled and edited using the software Sequencher (vs. 4.2; Gene Codes, Ann Arbor, MI, USA) and were then BLAST-searched in GenBank.

The ITS region was cloned for *H. albiflora*, *H. ariadna*, *H. bilobata*, *H. camphorifolia*, *H. carnosa*, *H. ciliata*, *H. gracilis*, *H.* cf. *incrassata*, *H. meliflua*, *H. pauciflora*, *H. pubicalyx*, *H. tsangii*, *Dischidia hirsuta*, *H. heuschkeliana*, *Madangia inflata*, and *Marsdenia carvalhoi*, when detecting a tendency to ITS-polymorphism in these species. For cloning, single PCR bands obtained with the above-mentioned primers 18SF and C26A were purified and cloned into plasmids using the TOPO TA Cloning kit for Sequencing (Invitrogen Life Technologies, Carlsbad, CA) and the Promega pGEM-T Easy Vector system, according to the manufacturers' protocols. One microliter of 10% dimethylsulfoxide (DMSO) was added to the PCRs of *Dischidia hirsuta*, *H. bilobata*, *H. heuschkeliana*, *H. pauciflora*, *H. pubicalyx*, *Madangia inflata*, and *Marsdenia carvalhoi*. Positive plasmid colonies were picked from the agar plates and amplified directly. Ten colonies for *H. gracilis* and *H. tsangii*, and five colonies each for the remaining species were amplified, using the M13F and the M13R primers supplied with the TOPO TA cloning kit. PCR settings were an initial denaturation for 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 45 °C for 30 s, 72 °C for 30 s, and finally, 72 °C for 5 min. The products were purified, and as many clones as possible were sequenced, again using the M13F and M13R primers and the same settings as above.

2.2. Alignment and phylogenetic analyses

Two data sets (available through TreeBASE, study accession number S1420, matrix accession numbers M2559 and M2560) were used in the present study; one including all ITS sequences available (cloned as well as directly sequenced), the other combining chloroplast sequences of the *trnL* region and the *atpB-rbcL* spacer (Wanntorp et al., in press) and one ITS sequence per taxon (see Section 3.3). The *trnL-F* sequence of *Dischidia bengalensis* was downloaded from GenBank. For this spe-

cies, sequences of the ITS and the *atpB-rbcL* spacer regions, respectively, were not available and question marks were therefore used to replace them in the combined analyses. We chose to do this rather than to prune *D. bengalensis* from the analyses, in order to include as many taxa as possible. Alignments were performed using ClustalX (vs. 1.8, Thomson et al., 1997) and adjusted by eye to minimize assumed mutational changes. A 29 bp region of the ITS2, corresponding to bases 424–453 of the nuclear ribosomal DNA ITS region of *Daucus carota* (Lee et al., 2004, direct submission) was excluded from the analyses because it included a homopolymer subregion (poly-C) of variable length. Twenty-three and eleven indels, in the ITS and the combined data sets, respectively, were coded as characters following the simple indel coding method suggested by Simmons and Ochotorena (2000), and in the parsimony analyses, substitutions and indel characters were weighted equally. Parsimony analyses relied on PAUP (vs. 4.0b10; Swofford, 2002) and used heuristic searching with 1000 random taxon addition replicates, saving maximally 10 shortest trees per replicate, tree-bisection–reconnection (TBR) branch swapping, and collapse of zero-length branches. To assess statistical support, we used nonparametric bootstrapping with the same settings as above and, when possible, Bremer support (1994), which was calculated using the program Autodecay (vs. 4.0.2; Eriksson, 1998).

To study the evolution of ant-symbiosis in *H.* cf. *darwinii*, *H. imbricata*, *H. mitrata*, and *Absolmsia spartioides*, a constrained parsimony analysis in PAUP (using the parameters listed above) was conducted on the combined data set, by forcing a single origin for the symbiosis in *Hoya*.

Bayesian analyses relied on MrBayes (vs. 3.0b4; Huelsenback and Ronqvist, 2001; Ronquist and Huelsenback, 2003, on-line manual). Substitution models for Bayesian analyses were selected after exclusion of indels using MrModeltest (vs. 1.1b; Nylander, 2003) in combination with PAUP; MrModeltest is a simplified version of Posada and Crandall's (1998) Modeltest and considers a selection of evolutionary models implemented in MrBayes vs. 3.0. The best-fitting model for the ITS data was the general time reversible (GTR) model (Lanave et al., 1984) with a gamma shape parameter and a parameter accounting for the proportion of invariable sites. The best-fitting model for the chloroplast data had previously been determined to be the GTR + G model without an invariant sites parameter (Wanntorp et al., in press). The combined analysis used the appropriate model for each data partition. Four Markov chains were used, starting from random trees, and 2 million generations were run, saving every 100th tree. The first 15,000 trees sampled were discarded as burn-in (based on inspection of the stationarity plot in MrBayes), and a 50% majority rule consensus tree was calculated for the remaining 5000 trees. Bayesian analyses were repeated three times to assure parameter convergence.

3. Results

3.1. Analysis of the ITS sequences

In total, we obtained 105 ITS sequences, representing 42 species. ITS sequences (resulted by direct-sequencing and by cloning) from each taxon, generally differed by 1–4 nucleotides (these polymorphic sites were found in different parts of the ITS region and an examination through all the ITS sequences did not reveal any especially variable nucleotide positions/sites in the ITS region of *Hoya* s.l.) except in the three clones of *H. ciliata*, which differed by 6–18 nucleotides from each other and from the directly sequenced ITS. Even so, all *H. ciliata* sequences grouped together as did most other multiple ITS copies from the same species (Fig. 4). Four soft polytomies including cloned sequences of two presumed species each were detected in the ITS tree (Fig. 4). The relationships between the different taxa within each of these clades were unresolved except in one clade that contained a well-supported subclade (marked by an arrow in Fig. 4) in which one accession of *H. pubicalyx* (clone 21) grouped with an accession of *H. carnosa* (clone 3) rather than with other accessions of *H. pubicalyx*.

Clones 17 and 18 of *H. ariadna* had longer branch lengths than any of the other sequences (Fig. 4), their GC contents were lower than that of the other clones of *H. ariadna* (55% and 53% vs. 61%), and they each had five mutations in their 5.8S region, more than found in other sequences. The directly sequenced ITS sequence of *Absolmsia spartioides* also caused a long branch in the tree (Fig. 4) but had a 5.8S region with only three mutations and a sequence length comparable to those of the other ITS sequences here analyzed. The "a priori" exclusion of putative pseudogenes from the data set has recently been criticized by Bailey et al. (2003) who stated that "Disregarding potential pseudogenes may result in under-sampled gene trees, greatly decreasing the potential to fully understand the orthology and paralogy of sequences present in an analysis and the ability to accurately infer species relationships." We chose therefore to include the ITS sequence of *A. spartioides* in the combined analyses. Including or excluding this sequence caused no change in the topology of the combined tree.

3.2. Comparison of ITS and chloroplast trees

With *Marsdenia carvalhoi* as outgroup, 202 of the aligned 809 characters were parsimony-informative (including the 23 informative indel characters). There were no statistically supported contradictions between the Bayesian and the parsimony topologies, and a majority rule consensus tree obtained from the Bayesian analysis is shown in Fig. 4.

The ITS tree contained several well-supported clades, and clones generally grouped by species. However, although the proportion of informative characters of the ITS region (24.9%) was more than twice that of the chloroplast data set (11.28%), the mutual relationships

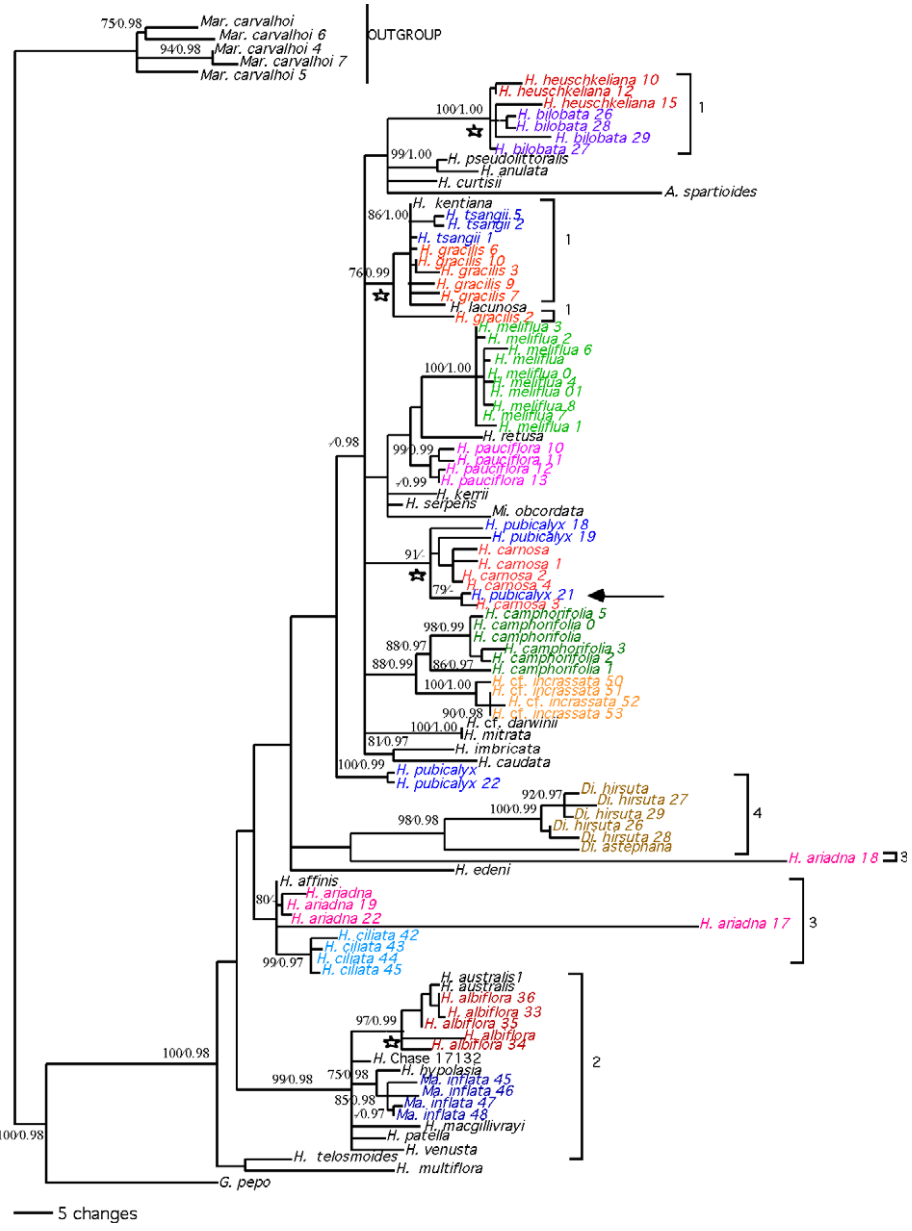

Fig. 4. Phylogram showing a 50% majority rule consensus resulted from the Bayesian analysis based on the internal transcribed spacer (ITS) of nuclear ribosomal DNA. Bootstrap values (>75%) and posterior probabilities (>0.97) are shown above branches. Cloned taxa are shown in color, those sequenced directly in black. Stars indicate four soft polytomies including paralogues of different taxa. The arrow points to the sister relationship between clone 21 of *H. pubicalyx* and clone 3 of *H. carnosa*. Taxon abbreviations: *A.*, *Absolmsia*; *H.*, *Hoya.* Taxon abbreviations: *A.*, *Absolmsia*; *D.*, *Dischidia*; *G. pepo*, *Gunnessia pepo*; *H.*, *Hoya*; *Mar. carvalhoi*, *Marsdenia carvalhoi*; *Ma. inflata*, *Madangia inflata*; *Mi. obcordata*, *Micholitzia obcordata*. Labeled clades according to: (1) the *Acanthostemma* clade; (2) the Australia/New Guinea clade; (3) the *Eriostemma* clade; (4) *Dischidia* clade. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

between most of clades in the ITS tree were not resolved, indicating a higher level of homoplasy in the ITS data than in the chloroplast data. For example, *Hoya bilobata*, *H. kentiana*, *H. tsangii*, *H. gracilis*, and *H. heuschkeliana* formed a well-supported clade in the tree based on chloroplast data (Fig. 2), while in the ITS tree, these species fell into two clades, one consisting of *H. heuschkeliana* and *H. bilobata*, the other of *H. kentiana*, *H. tsangii*, and *H. gracilis*, as well as *H. lacunosa*. This latter species grouped with *H. caudata*, *H. imbricata*, and *Absolmsia spartioides* in the tree obtained by the combined chloroplast data (Wanntorp et al., in press).

An Australian/New Guinean clade consisting of *H. australis*, *H. albiflora*, *H. hypolasia*, *H. venusta*, *H. patella*, *H. macgillivrayi* and *Madangia inflata* was again retrieved in the ITS tree but with higher resolution than in the tree based on chloroplast data (Fig. 4). The ITS data also added the New Guinean unnamed *Hoya* Chase 17132 to this clade and revealed a sister relationship between *M. inflata* and *H. hypolasia* (Fig. 4).

A previously identified clade, that included *H. meliflua*, *H. kerrii*, *H. carnosa*, *H. pubicalyx*, *H. camphorifolia*, *H.* cf. *incrassata*, *H.* cf. *darwinii*, *H. mitrata*, *H. curtisii*, *H., caudata*, *H. imbricata*, *Absolmsia spartioides*, and *Micholitzia obcordata* (Fig. 2), showed up again in the ITS tree, but now including also *H. pauciflora*, *H. serpens*, and *H. retusa*. *Hoya affinis*, *H. ciliata*, and *H. ariadna* grouped together as in the chloroplast tree (Fig. 2), except for the two long-branched accessions of *H. ariadna*, 17 and 18, discussed above (Section 3.1, Fig. 4).

Dischidia hirsuta and *D. astephana* were sister groups in the ITS tree (Fig. 4) but not in the chloroplast tree, where *D. hirsuta* was instead more closely related to *D. bengalensis* (Fig. 2).

3.3. Combined analysis of ITS and chloroplast sequences

Because most ITS copies grouped by species, we randomly chose one ITS sequence to represent each species (only excluding clones 17 and 18 of *H. ariadna*, see Section 4.1) and combined it with sequences of the *trnL* region and the *atpB-rbcL* spacer, previously obtained from the same DNA aliquots (Wanntorp et al., in press). The within-species polymorphic ITS-sites generally did not contribute information about species relationships, and the particular ITS sequence in the combined analyses did therefore not influence the outcome.

Of 2586 characters in the combined ITS + cpDNA matrix, 216 were parsimony-informative. Heuristic searching yielded 64 equally parsimonious trees of which the strict consensus is shown in Fig. 5. The ant-symbiosis present in *Hoya* appears to have multiple origins as shown in Fig. 5. When forcing these species into a single clade through a constrained parsimony analysis, the tree length increases from 891 steps to 904 steps.

The topology of the 50% majority rule consensus tree from the Bayesian analyses (not shown) was congruent with that resulting from the parsimony analyses. Well-sup-

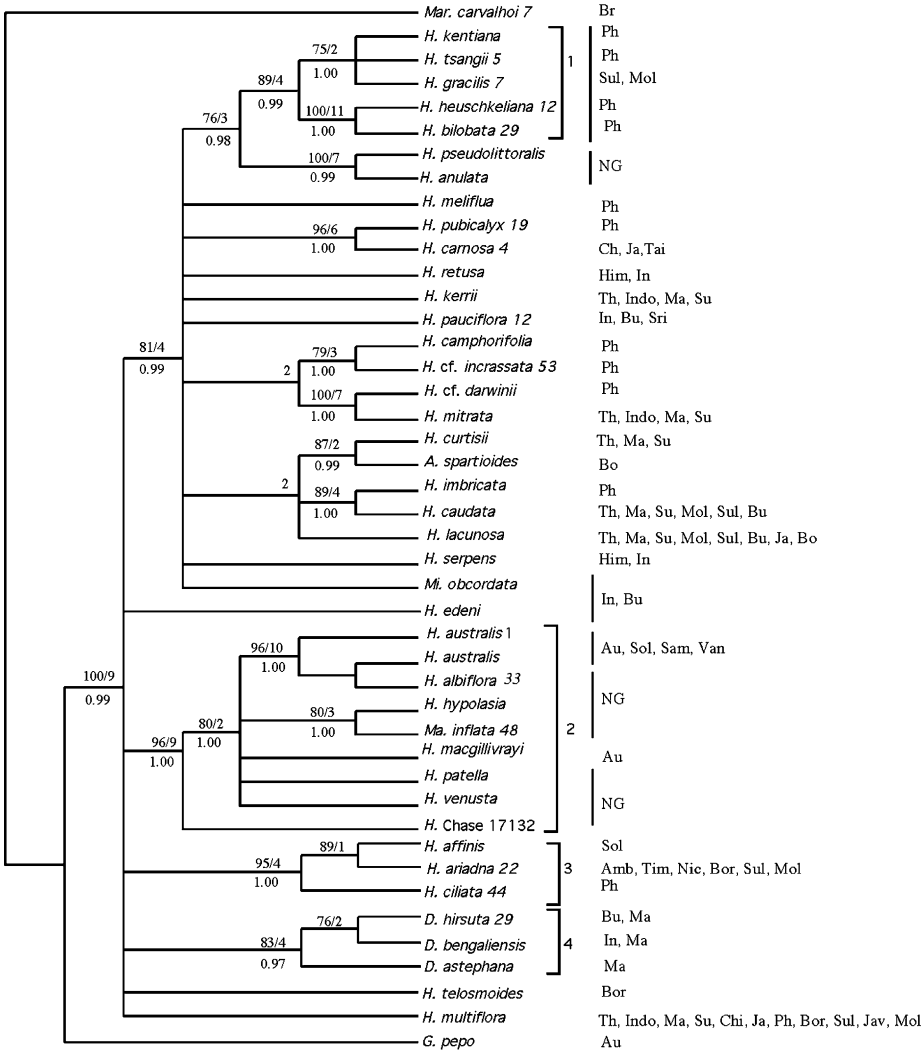

Fig. 5. Strict consensus of the 64 most parsimonious trees obtained from the combined ITS, *atpB-rbcL*, and *trnL* data. Bootstrap values (>75%) and decay indices are shown above branches, posterior probabilities (>0.97) below branches. (1) Geographic occurrences are abbreviated as in Fig. 2. Taxon abbreviations as in Fig. 4.

ported larger clades (numbered 1–4 in Fig. 5) are formed by: (1) species from the Philippines, Sulawesi, and the Moluccas (*H. bilobata*, *H. gracilis*, *H. heuschkeliana*, *H. kentiana*, and *H. tsangii*). These species are members of Schlechter's section *Acanthostemma*, characterized by flowers with hairy corollas, completely revolute petal lobes, and coronas with outer lobes that end in two lateral extensions turning inwards on each other; (2) species from Australia and New Guinea (except *H. anulata* and *H. pseudolittoralis*); (3) *Hoya affinis*, *H. ariadna*, and *H. ciliata*, which are members of Schlechter's (1913) section *Eriostemma*, characterized by large flowers with fleshy corollas, a densely pubescent staminal column formed by the anthers, and club-shaped pollinia; and finally (4) the three species of *Dischidia*.

4. Discussion

4.1. ITS pseudogenes in Hoya

Among the 105 ITS sequences (from 42 species) analyzed here, only two (clones 17 and 18 of *H. ariadna*) may be pseudogenes as judged by low overall GC content, mutations in the 5.8S, and/or high mutation rates (i.e., exceptionally long branch lengths). Divergent pseudogenic ITS copies can coexist with functional copies—at least for some time—if concerted evolution is relatively slow. This is the case where unusually different parental genomes are interacting, for example, after hybridization, followed by polyploidy that lead to heterogeneous karyotypes (Álvarez and Wendel, 2003; Buckler et al., 1997; Muir et al., 2001; Wendel et al., 1995). The 36 species of *Hoya* whose chromosome numbers have been counted, however, all have $2n = 22$, with no indication of recent polyploidy or other forms of karyotype divergence (Nakamura, 1991, 1992a,b, 1993, 1996; Navaneetham and Sampathkumar, 1984; Sarkar, 1988; *Absolmsia*, *Micholitzia*, and *Madangia* have not been counted). We are unaware of published accounts of natural hybridization in *Hoya*. Our results, showing that in *Hoya* the paralogous ITS copies mostly sort by species, make *Hoya* another example of species-level phylogenetic signal from paralogous genes (Bellarosa et al., 2005; Razafimandimbison et al., 2004; Won and Renner, 2005). Based on the available data, except for one suspected case of lineage sorting (that of *H. carnosa–H. pubicalyx*), normal concerted evolution in *Hoya* appears fast enough to homogenize copies within individuals and species.

4.2. Major groups in Hoya and traditional sectional classifications

The first infrageneric classification of *Hoya* is that of J. D. Hooker (1885) in his *Flora of British India*. Hooker recognized four sections: *Crytoceras* Hook. f. (= *Cyrtoceras* J.J. Bennett in Schumann 1895), *Pterostelma* (Wight), *Ancistrostemma* Hook. f. and *Euhoya* Miq. (by today's nomenclatural rules, the last becomes section *Hoya*, with the generic type species *H. carnosa* (L.) R. Br.). While Schumann (1895) still followed Hooker's classification in his contribution on the Asclepiadaceae, Schlechter (1913) created five additional sections, *Eriostemma* Schltr., *Oreostemma* Schltr., *Otostemma* (Bl.) Schltr., *Physostelma* (Wight) Schltr., and *Plocostemma* (Bl.) Schltr. and considered *Cyrtoceras* as a separate genus. In 1916, Schlechter added one more section, *Peltostemma*, to this classification. Since then, no comprehensive revision has been attempted, but modified classifications have been proposed by horticulturists, who today recognize 14 or 16 sections (Burton, 1985, 1995, 1996; Kloppenburg, 1993, 2001a). These classifications, mainly based on flower morphology like the earlier ones, have nomenclatural problems and provide few explicit arguments for particular decisions.

Those of Schlechter's sections that in the present study were sampled for at least two species so far are monophyletic or unresolved (*Eriostemma*, *Pterostelma*, and *Physostelma*, all discussed below), indicating that he and earlier workers identified key morphological traits that contain phylogenetic signal.

The following sections discuss the groups seen in the trees based on the combined chloroplast data, the single nuclear ITS region, and the combined chloroplast and nuclear DNA data, respectively (Figs. 2, 4, and 5).

4.2.1. Basal polytomy of Dischidia, Eriostemma, the oddballs Hoya multiflora and H. telosmoides, and core hoyas

The first-branching clades in Fig. 4 (compare also Fig. 5) are formed by species that have variously been included in, or excluded from *Hoya*. The first of them consists of *H. telosmoides* Omlor (1996) and the widely cultivated *H. multiflora* (Fig. 3). The latter was sometimes considered a separate genus (e.g., Schlechter, 1913), *Centrostemma* Decne, or placed in *Hoya* section *Cyrtoceras* together with *C. reflexum* (Schumann, 1895). *Hoya telosmoides* is endemic to northern Borneo and resembles *Dischidia* in having urceolate corollas, otherwise known only in *H. heuschkeliana* and *Micholitzia*. However, its corona lobes with revolute margins and pollinia with a pellucid germination margin argue for a placement in *Hoya* (Omlor, 1996). In the ITS tree (Fig. 4), *H. telosmoides* weakly clusters with *H. multiflora* (Fig. 3) with which it shares relatively long pointed corolla lobes, a trait found also in the rarely collected monotypic genus *Oreosparte* Schltr. from Sulawesi. The latter has a stalked gynostegium and a corona similar to that of *H. multiflora* (Omlor, 1996, 1998; Fig. 3).

Another major branch found in the combined analyses of this study ('clade 3' in Fig. 5), consists of *H. affinis*, *H. ariadna*, and *H. ciliata*. All are members of Schlechter's (1913) section *Eriostemma*, which he considered possibly a subgenus rather than a section. Following Schlechter's suggestion, Kloppenburg and Gilding (2001) raised Schlechter's section *Eriostemma* to generic rank in a nomenclaturally problematic publication. *Eriostemma* contains mostly New Guinean species, namely *H. ariadna*, *H. coronaria*, *H. gigas*, *H. hollrungii*, *H. lauterbachii*, *H. pur-*

purea, and *H. neoguineensis* as well as *H. guppyi* and *H. affinis* (both from the Solomon Islands), plus two species from the Philippines and Sulawesi, the first of them later described as *H. ciliata*. The most distinctive character of the *Eriostemma* clade is the terrestrial, not epiphytic habitat of its species. The relatively basal position of this clade in the phylogeny suggests that a terrestrial offshoot evolved early in *Hoya* or that the terrestrial habit is plesiomorphic and shared with *Marsdenia*. Besides being terrestrial, species of *Eriostemma* have densely pubescent, large flowers on deciduous peduncles, with fleshy corollas, corona scales standing upon a densely hairy column, and club-shaped pollinia lacking a pellucid margin and with twisted translators, all characters generally not present in other species of *Hoya*.

Clade 4 in Fig. 5 is formed by three species of *Dischidia*. *Dischidia*, which contains about 80 species of succulent epiphytic vines, has always been considered closely related to *Hoya* (Omlor, 1998; Schlechter, 1913; Schumann, 1895). Molecular data support this relationship (Livshultz, 2003; Meve and Liede, 2004; Potgieter and Albert, 2001; Wanntorp et al., in press; this study). A phylogeny based on the second intron of the nuclear gene *LEAFY* and which encompasses the morphological diversity of *Dischidia*, plus *Micholitzia* and four species of *Hoya* (all also sampled here), also supports the monophyly of *Dischidia* plus *Hoya*/*Micholitzia*, but does not resolve *Dischidia* and *Hoya* as mutually monophyletic (Livshultz, 2003).

The remaining three branches in the basal polytomy (Fig. 5), namely *H. edeni*, the Australia/New Guinea clade ('clade 2' in Fig. 5), and a large clade comprising all other species of *Hoya* (including *H. carnosa*, the type species of the genus) can be considered 'core *Hoya*.' Core *Hoya* clearly includes species generally referred to the monotypic genera *Madangia*, *Micholitzia*, and *Absolmsia* (Forster et al., 1997; Goyder and Kent, 1994; Kloppenburg, 2001b).

4.2.2. The Australia/New Guinea clade: "New Guinean Whites" and Australian Physostelma

Except for *H. pseudolittoralis* and *H. anulata* (sometimes considered as belonging to a single species, Forster and Liddle, 1992), all Australian and New Guinean species sampled, formed a clade (Figs. 2, 4, and 5, clade 2). Australia and New Guinea, being part of the same continental plate, were joined by a series of island-arc terranes during Pleistocene glacial maxima (McLoughlin, 2001). The two areas are considered a single biogeographic region, and numerous studies have found sister clade pairs in Australia and New Guinea (e.g., Linder and Crisp, 1995; Raven and Axelrod, 1972; Wanntorp and Wanntorp, 2003). A well-supported Australian/New Guinean sister species pair in our analysis consists of *H. hypolasia* and *Madangia inflata* (Fig. 5). *Hoya hypolasia* has flowers that have recurved corollas and laterally compressed corona lobes with erect apices. *Madangia inflata* has flowers with globose corollas and inflated coronas with contiguous lobes (Forster et al., 1997), but shares similar flat style heads with *H. hypolasia* (Wanntorp, pers. obs.).

Other members of the Australia/New Guinea clade, such as the Australian *H. macgillivrayi* and the New Guinean *H. patella* and *H. venusta*, have large flowers on long peduncles with rotate or bell-shaped corollas and shortly apiculate style heads. Schlechter (1913) grouped these species in his section *Physostelma* of which *H. venusta* is the type. Another Australia/New Guinea section, *Pterostelma*, of which we sampled *H. albiflora* and *H. australis*, comprises species known as the "New Guinean Whites" because of their showy, white flowers (Forster and Liddle, 1992). There are problems with the delimitation of these species, mostly deriving from the many cultivated forms of *H. australis*. In the ITS tree (Fig. 4), copies of *H. albiflora* and *H. australis* do not sort by species, but our data are insufficient to decide whether *H. albiflora* and *H. australis* are the result of recent hybridization or if what we observe is the persistence of paralogous copies through a recent speciation event and insufficient time for completion of concerted evolution.

4.2.3. The Acanthostemma clade and other core hoyas

The *Acanthostemma* clade (Fig. 2 and 'clade 1' in Fig. 5) comprises species with small flowers clustered into umbels that have a concave shape. The densely pubescent corollas have revolute lobes, and the coronas have outer lobes that end in two lateral extensions turning inwards on each other. With the exception of *H. heuschkeliana*, these are generally placed in section *Acanthostemma* Blume (Kloppenburg, 1993). *Hoya heuschkeliana* has urceolate or pseudourceolate corollas that are papillose (not hairy) on the inside and that resemble corollas of *Dischidia*. Light and scanning electron microscopic studies of floral details, however, support the relationship of *H. heuschkeliana* to *Acanthostemma* species: All have similar winged pollinia and coronas with lateral extensions more or less turned inwards (Wanntorp, pers. obs.).

Two taxa from New Guinea, *H. anulata* and *H. pseudolittoralis*, appear closely related to the *Acanthostemma* clade (Fig. 5). However, their flowers differ from those of species of *Acanthostemma* in the flatter shape of the corolla and the oblong-linear corona lobes that lack lateral extensions. Forster and Liddle (1990) suggested that *H. pseudolittoralis* may instead be "closely allied to *H. eitapensis* Schltr. and *H. microstemma* Schltr.," both members of Schlechter's huge section *Hoya* (with 100 species). They continued, "further studies of variation in this group [i.e., *H. anulata* and *H. pseudolittoralis*, *H. eitapensis*, and *H. microstemma*] may show all to be part of one variable taxon."

The type species of section *Hoya* (and thus of the genus), *H. carnosa*, occurs wild in China, Taiwan, and Japan, but is now extensively cultivated because of the sweetly perfumed scent of its showy flowers and its tolerance to dry conditions that makes it a hardy indoor plant. It is sister to another horticulturally popular species, *H. pubicalyx* (Figs. 4, 5). *Hoya pubicalyx* is from the Philippines, and its flowers are very similar to those of *H. carnosa*. Our finding that two

of the cloned ITS sequences of these species are interspersed with each other suggest that *H. pubicalyx* and *H. carnosa* may actually be one species.

Other well-supported subclades (Fig. 5) consist of *H. curtisii* plus *Absolmsia spartioides* and *H. imbricata* plus *H. caudata*. Flowers of *H. curtisii* differ from those of *A. spartioides* in the shape of the corolla and the corona, and in a conspicuous columnar structure only present under the corona of *H. curtisii* (Wanntorp, pers. obs.), not supporting a close relationship between these species. By contrast, the sister relationship between *H. imbricata* and *H. caudata* (Fig. 5) fits with their similar flowers which in both species have revolute corolla lobes and extremely long caudate anther appendages. The latter apparently were the main reason why Schlechter (1916) placed *H. imbricata* in its own section *Peltostemma*.

4.3. Multiple evolution of ant symbiosis in Hoya

Several species of *Dischidia* are regularly occupied by ants (Beccari, 1884; Janzen, 1974; Livshultz et al., 2005; Treseder et al., 1995) and the same is true of *Hoya* species such as *H. darwinii*, *H. lambii* (not sampled here), *H. mitrata*, and *Absolmsia* (= *Hoya*) *spartioides* (Nyhuus, 2004). Both, *Hoya* and *Dischidia*, also include ant-garden epiphytes, meaning that ants plant seeds of these plants in their nests (Kaufmann et al., 2001). The ant-occupied hoyas have a range of adaptations to ants. *Hoya darwinii* has dimorphic leaves somewhat resembling those of *Dischidia*, with one leaf forming a pouch for ants, the other being a regular leaf. *Hoya imbricata* has concave leaves appressed to the substrate, under which ants are sheltered. Two other species, *H. mitrata* and *H. lambii* have very short internodes, resulting in tightly stacked leaves that provide nesting space for ants. Lastly, in *A. spartioides* ants live between the tangled roots of the plants. Our combined data (Fig. 5) suggest that *H. mitrata* and *H. darwinii* are sister species and that their different adaptations, stacked leaves versus leaf pouches, evolved from some common ancestral form. However, the remaining ant-housing species sampled so far, *A. spartioides* and *H. imbricata*, do not fall in a clade with *H. darwinii* and *H. mitrata*, and it therefore seems that ant-symbiosis evolved at least three times within *Hoya*. A single origin for the ant-symbiosis in *Hoya* based on the topology found in the combined analyses (Fig. 5), would imply an increase of 15 steps in tree length.

This study has improved the resolution of the available phylogeny within *Hoya* by adding information from the nuclear ribosomal DNA ITS region to an available chloroplast data set (Wanntorp et al., in press). Preliminary analyses of additional nuclear gene regions, e.g., the second intron of the *LEAFY* gene and the *G3pdh* region (Wanntorp, unpubl.), indicate that molecular markers suitable for further resolving phylogenetic relationship within *Hoya* may be difficult to find and underline a need for developing detailed morphological and ontogenetical data to be added to the molecular data.

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