



# Phylogeny of the Asian *Hedyotis*–*Oldenlandia* complex (Spermacoceae, Rubiaceae): Evidence for high levels of polyphyly and the parallel evolution of diplophragmous capsules

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## ARTICLE INFO

### Article history:

Received 29 May 2012

Revised 5 December 2012

Accepted 8 January 2013

Available online 18 January 2013

### Keywords:

*Dimetia*

*Hedyotis*–*Oldenlandia* complex

Phylogeny

*Scleromitron*

Rubiaceae

*Thecagonum*

## ABSTRACT

Generic delimitation in the *Hedyotis*–*Oldenlandia* complex has a long taxonomically confused history because of the controversy of lumping or splitting these two taxa. Previous morphological and phylogenetic studies with a paucity of Asian taxa suggested that *Hedyotis* should include only Asian species characterized by diplophragmous capsules. In order to test the reliability of this conclusion, assess the phylogenetic value of capsular characters, and evaluate generic circumscriptions in this complex, a phylogenetic study based on expanded inclusion of 63 Asian species was performed using two nuclear regions and eight plastid regions with parsimony and likelihood analyses. The results show that the *Hedyotis*–*Oldenlandia* complex is a highly polyphyletic group. *Hedyotis* should only include most Asian species with erect, robust herbs or shrubs and diplophragmous capsules. *Oldenlandia* s. str. consists primarily of African species, including the type *O. corymbosa*, that are characterized by small herbs, paniculate or corymbose inflorescences, inserted styles and stamens, and loculicidally dehiscent capsules. *Dimetia*, *Scleromitron* and *Thecagonum* are proposed to be resurrected to accommodate three newly resolved clades. Morphological character optimizations indicate that the diplophragmous capsule evolved independently twice within this complex. Plant habit, stipule shape, and capsular dehiscent pattern are of great value in generic circumscriptions.

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## 1. Introduction

*Hedyotis* L. and *Oldenlandia* L. are two of the largest genera within the Rubiaceae. They are very similar and share an herbaceous or shrubby habit, with relatively small, four-merous flowers, bilobed stigmas, and dry, usually two-celled capsular fruits with few to many small seeds (Neupane et al., 2009). The two genera comprise more than 500 species occurring throughout tropical and subtropical regions worldwide (Dutta and Deb, 2004). Over 20 species from the *Hedyotis*–*Oldenlandia* complex, such as *O. biflora*, *O. corymbosa*, and *O. diffusa*, are well-known as folk medicine for treatment of cancers, infections, remittent and bilious fever, and other diseases in many Asian countries (Li et al., 2010). The roots of *O. corymbosa* were used as red dye in India (Dutta and Deb, 2004).

*Hedyotis* and *Oldenlandia* were traditionally placed in tribe Hedyotideae Cham. & Schltdl. ex DC. (Robbrecht, 1988). However, molecular data showed that tribe Hedyotideae is paraphyletic, with Spermacoceae Bercht. & J. Presl deeply nested within it (Bremer, 1996; Andersson and Rova, 1999; Bremer and Manen, 2000). Because of the taxonomic priority of Spermacoceae over Hedyotideae, it was proposed to expand the definition of Spermacoceae to replace and include Hedyotideae (Andersson and Rova, 1999; Robbrecht and Manen, 2006; Groeninckx et al., 2009).

As one of the three main groups of Spermacoceae s. l., taxonomic delimitations within the *Hedyotis*–*Oldenlandia* group have been complicated by highly variable morphological characters (Kårehed et al., 2008). Their circumscriptions and classifications are controversial and have been disputed for many years. Linnaeus (1753) proposed the two generic names simultaneously. Lamarck (1792) considered these two taxa as congeneric because of their similar floral characters and incorporated *Oldenlandia* into *Hedyotis*. Willdenow (1798), however, maintained *Oldenlandia* based on the different type of fruit dehiscence and their seed characters. Thus, the generic delimitation of the *Hedyotis*–*Oldenlandia* complex became the focus of much taxonomic debate and confusion. ‘Lumpers’

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avored a broad genus circumscription by merging most species of the complex into *Hedyotis* (Fosberg and Sachet, 1991; Dutta and Deb, 2004; Chen and Taylor, 2011), while ‘splitters’ preferred recognizing many small genera in addition to narrowly circumscribed *Hedyotis* and *Oldenlandia*, such as *Houstonia* L., *Neanotis* W.H. Lewis, *Arcytophyllum* Roem. & Schult., *Kadua* Cham. & Schltdl., and *Kohautia* Cham. & Schlecht. (Bremekamp, 1952; Terrell et al., 1986; Terrell, 1975, 2001a,b; Terrell and Robinson, 2003).

The formal acceptance of the name *Hedyotis fruticosa* L. as the conserved type for the genus (Jarvis, 1992; McNeill et al., 2006) resolved a long-standing nomenclatural controversy and stimulated a detailed survey of the morphology and taxonomy of the Asian and Pacific species of *Hedyotis* (Terrell and Robinson, 2003). This selection of the type also necessitates redefinition of the generic limits of *Hedyotis* and consequently several other (possibly new) genera around the world (Neupane et al., 2009).

Molecular phylogenetic studies have revealed that *Hedyotis* s. l. is not monophyletic. In contrast, *Hedyotis* s. str., including most Asian and Micronesia species along with the type species (*H. fruticosa*), was proposed as a natural group (Terrell and Robinson, 2003) and has been supported as such (Andersson et al., 2002; Terrell et al., 2005; Kårehed et al., 2008; Groeninckx et al., 2010a).

Although the segregation of several new taxa from the complex received strong morphological and molecular support, it does not mean that the circumscriptions of *Hedyotis* and *Oldenlandia* have been satisfactorily resolved. Taxonomic revisions of ~150 species of *Hedyotis–Oldenlandia* complex that are distributed in Asian countries have yet to be comprehensively conducted because of the high species richness in these areas. On the other hand, molecular studies reported so far were based primarily on non-Asian species. Kårehed et al. (2008) conducted a molecular phylogenetic analysis on the herbaceous tribe Spermaceae, but only 13 Asian *Hedyotis* species and a few globally common *Oldenlandia* taxa were included. The result supported the *Hedyotis* s. str. clade with 12 Asian *Hedyotis* taxa and an isolated lineage consisting only of *H. capitellata*, which left the intrageneric relationships largely unresolved (Groeninckx et al., 2009). Although delimitation of *Hedyotis* s. str. was found to be reasonable (Kårehed et al., 2008; Groeninckx et al., 2009), we did not know if it will be supported with increased sampling of Asian species. In the present study, 63 Asian species were selected from the *Hedyotis–Oldenlandia* complex for a comprehensive molecular phylogenetic analysis on the basis of eight plastid loci (*atpB–rbcL*, *matK*, *petD*, *rbcL*, *rps16*, *trnH–psbA*, *trnL–F*, and *trnL*) and two nuclear gene regions (ITS and ETS). The goals of this study are to: (1) test the monophyly of the observed *Hedyotis* s. str. group that was resolved in previous phylogenetic studies, and whether the diplophragmous capsule represents a unique synapomorphy for *Hedyotis* s. str.; (2) clarify the phylogenetic relationships within the *Hedyotis–Oldenlandia* complex with the broader sampling of Asian samples; (3) determine the generic delimitations of *Hedyotis*, *Oldenlandia*, and their associated genera in Spermaceae; and (4) assess the systematic values of selected morphological characters and identify diagnostic characters to distinguish and highlight the differences among the segregate genera of the *Hedyotis–Oldenlandia* complex.

## 2. Material and methods

### 2.1. Taxon sampling and sequence selecting

We broadened the taxonomic sampling of the *Hedyotis–Oldenlandia* complex presented by Kårehed et al. (2008) and Groeninckx et al. (2009) by adding two additional sets of taxa. First, we sampled 648 sequences, of which 364 sequences came from 76 individuals representing 25 species that we used for barcoding analysis

(Guo et al., 2011). The remaining 284 sequences were obtained from GenBank. Second, we newly generated 381 sequences from 82 individuals (61 *Hedyotis* and 21 *Oldenlandia*) representing 50 Asian species (38 *Hedyotis* and 12 *Oldenlandia*) from the *Hedyotis–Oldenlandia* complex, as well as 27 sequences from two *Neanotis* species. We did not generate any new *atpB–rbcL* or ETS sequences, but we downloaded 81 and 74 of them, respectively, from GenBank to incorporate their phylogenetic signal. A total of 272 individuals from 163 taxa, including 96 individuals of 49 taxa from *Hedyotis* and 80 individuals of 39 taxa from *Oldenlandia*, were sampled in the present phylogenetic analysis (voucher information and GenBank accession numbers are given in Supplementary Table S1).

In preliminary parsimony-based phylogenetic analyses, no *Hedyotis* or *Oldenlandia* taxa were found to be nested within tribe Spermaceae s. str. (*Crusea*, *Diodia*, *Diodella*, *Emmeorhiza*, *Ernodea*, *Galianthe*, *Hydrophylax*, *Mitracarpus*, *Psyllocarpus*, *Richardia*, and *Spermaceae*). Therefore this tribe was excluded from the dataset to improve tree-search efficiency.

Three Knoxieae taxa, *Batopedina pulvinellata* Robbr., *Carphalea madagascariensis* Lam., and *Pentanisia parviflora* Stapf ex Verdc., were selected as outgroups following Kårehed et al.’s (2008) and Groeninckx et al.’s (2009) phylogenetic analyses of Spermaceae.

### 2.2. DNA extraction, amplification, and sequencing

DNA was extracted, amplified and sequenced using standard procedures as previously described (Guo et al., 2011). Primer references of seven regions used in this study are given in Table 1. Sequence fragments were assembled using Sequencher ver. 4.5 (Gene Codes Corporation, Ann Arbor, Michigan).

### 2.3. Phylogenetic analyses

Preliminary nucleotide alignments were obtained independently for each gene region using MAFFT ver. 6.5 (Katoh and Toh, 2008a). Q-INS-i, which considers inferred secondary structure of rDNA (Katoh and Toh, 2008b), was used for alignments of ETS and ITS. G-INS-i, the most accurate MAFFT algorithm for aligning loci other than rDNA, was used for all other loci. The two protein-coding loci (*matK* and *rbcL*) were aligned using the 1 PAM nucleotide scoring matrix, whereas the other loci were aligned using the 20 PAM matrix. The default gap opening penalty was applied (1.53) and the gap offset value was set to 0.1 for those loci for which long gaps were not inferred based on precedent from other studies as well as preliminary alignments (ETS, ITS, *matK*, *rbcL*, *trnL* intron), whereas the gap offset value was set to 0 (the default value) for the other loci (*atpB–rbcL*, *petD*, *rps16*, *trnH–psbA*, *trnL–F* spacer).

Manual adjustments to the MAFFT alignments were performed in MacClade ver. 4.03 (Maddison and Maddison, 2001) using the procedure outlined by Simmons (2004) following Zurawski and Clegg (1987). We observed some ambiguously aligned regions where one or more sequences had a duplicate insertion (or the others had a deletion of one of two repeats) and the character-state distribution among the characters in the ambiguously-aligned region was identical for those sequences that have both repeats. In such cases the character-state distribution among the positions in question would be identical for either of the alternative alignments. Therefore, in these cases the ambiguously-aligned regions were kept in the analysis following Davis et al. (1998). A total of 939 ambiguously-aligned positions were excluded from the analyses (ETS: 76 positions from four regions; ITS: 210 positions from seven regions; *petD*: 143 positions from two regions; *rps16*: 151 positions from five regions; *trnH–psbA*: 278 positions from four regions; *trnL–F*: 81 positions from three regions). Ambiguously-aligned

**Table 1**  
Primers used for PCR in the present study.

DNA region	Primer name	Sequence	References
ITS	P17	5'-CTACCGATTGAATGGTCCGGTGAA-3'	Popp and Oxelman (2001)
	26S-82R	5'-TCCCGGTTGCTCGCCGTTACTA-3'	
<i>matK</i>	390F	5'-CGATCTATTTCATTAATATTTTC-3'	Cuenoud et al. (2002)
	1326R	5'-TCTAGCACACGAAAGTCGAAAGT-3'	
<i>petB-petD</i>	PIpetB1365F	5'-TTGACYCGTTTTTATAGTTTAC-3'	Löhne and Borsch (2005)
	PIpetD738R	5'-AATTTAGCYCTTAATACAGG-3'	
<i>rbcl</i>	1F	5'-ATGTCACCACAAACAGAAAC-3'	Olmstead et al. (1992) Fay et al. (1997)
	724R	5'-TCGCATGTACTGCAGTAGC-3'	
<i>rps16</i>	rps16F	5'-GTGGTAGAAAGCAACGTGCGACTT-3'	Oxelman et al. (1997)
	rps16R3	5'-CGATAGACGGCTCATTGGGATA-3'	
<i>trnH-psbA</i>	trnH-05	5'-CGCGCATGGTGGATTCACAATCC-3'	Tate and Simpson (2003) Sang et al. (1997)
	psbA3	5'-GTTATGCATGAACGTAATGCTC-3'	
<i>trnL-F</i>	TabC	5'-CGAAATCGGTAGACGCTACG-3'	Taberlet et al. (1991)
	TabF	5'-ATTTGAAGTGGTACACAG-3'	

nucleotides of individual sequences in regions that could not be unambiguously aligned with the remaining sequences were scored as ambiguous (“?”).

One inversion was inferred in the *petD* intron between positions 259 and 293 in eight terminals. This inversion was reverse complemented in the alignment to reflect the ancestral orientation and a character was added to the analysis to reflect this inversion in an analogous manner to uninode coding (Simmons et al., 2000). Only two terminals are scored for both the *atpB-rbcl* intergenic spacer as well as *rbcl*: *Kohautia caespitosa* and *Phylohydrax carnosus*. These terminals were scored as missing data for the 84 overlapping positions at the 3' end of the *atpB-rbcl* matrix.

Gap characters, whose inclusion often affects the inferred tree topology and increases branch-support values (Simmons et al., 2001), were scored using simple indel coding (Simmons and Ochoterena, 2000) in SeqState ver. 1.4.1 (Müller, 2005). Simple indel coding was applied rather than modified complex indel coding (Simmons and Ochoterena, 2000; Müller, 2006) because of the prohibitive computational cost of analyzing matrices with up to 272 terminals using step matrices (Sankoff and Rousseau, 1975) in PAUP\* ver. 4.0b10 (Swofford, 2001) as well as Simmons et al.'s (2007) simulation results in which the two methods performed similarly to each other. A total of 534 gap characters were scored from the unambiguously aligned regions (*atpB-rbcl*: 39, ITS: 50, *petD*: 136, *rps16*: 95, *trnH-psbA*: 60, *trnL* intron: 59, *trnL-F*: 95) for inclusion in the parsimony analyses.

As a means of data exploration, several alternative potential process partitions (Bull et al., 1993) of the characters were analyzed. Each of the 10 gene regions was analyzed independently of one another to resolve their respective gene trees. Putative coalescent genes (Hudson, 1990; Doyle, 1995) were then analyzed and their trees compared to check for well supported, contradictory signal that may have been caused by lineage sorting, introgression, and/or unrecognized paralogy (Doyle, 1992). As such, gene trees for the two rDNA gene regions and the eight plastid loci were analyzed independently of one another to check for potential introgression of the plastid genome or rDNA (Doyle, 1992; Wendel et al., 1995) or unrecognized paralogy problems with rDNA (Álvarez and Wendel, 2003; Bailey et al., 2003). A simultaneous analysis (Kluge, 1989; Nixon and Carpenter, 1996) of all characters was then performed, which was the primary basis for phylogenetic inference. The simultaneous-analysis data matrix is posted as [Supplementary online data](#).

Equally weighted parsimony tree searches were conducted for each data matrix using 2000 random addition tree-bisection-reconnection (TBR) searches in PAUP\* with a maximum of 10 trees

held per replicate. Parsimony jackknife analyses (JK; Farris et al., 1996) were conducted using PAUP\* with the removal probability set to approximately  $e^{-1}$  (36.7879%), and “jac” resampling emulated. One-thousand JK replicates were performed with 100 random addition TBR searches (each with a maximum of 10 trees held) per replicate. All parsimony analyses were run while collapsing branches with a minimum possible optimized length of zero to increase tree-search efficiency (Davis et al., 2005) and decrease the potential for inflated jackknife values (Simmons and Freudenstein, 2011).

jModeltest ver. 0.1.1 (Posada, 2008) was used to identify the best-fit likelihood model for each locus using the Akaike Information Criterion (Akaike, 1974) without considering invariant-site models following Yang (2006). The models selected all incorporated the gamma distribution (Yang, 1993) except for *trnH-psbA* and *trnL-F*. The Q-matrices selected were all variants of TIM, TPM, TrN, TVM, or GTR.

Likelihood (Felsenstein, 1973) analyses of nucleotide characters from each of the molecular data matrices were performed as (fallible; Gaut and Lewis, 1995; Siddall, 1998; Sanderson and Kim, 2000) tests for long-branch attraction (Felsenstein, 1978). Likelihood analyses were conducted using RAXML ver. 7.2.6 (Stamatakis, 2006) for optimal-tree searches and RAXML ver. 7.0.3 for bootstrap analyses (BS; Felsenstein, 1985) because of insufficient documentation for the bootstrap options that were changed in ver. 7.2.6. Given that RAXML only implements GTR Q-matrices for nucleotide characters, more restrictive variants of the GTR matrix were not used when selected by the AIC. For the plastid, rDNA, and simultaneous analyses, models were partitioned by locus to increase model fit by accommodating locus-specific variation (e.g., Castoe et al., 2004) and potentially decrease the potential for likelihood-based artifacts in both resolution and support caused by non-random distributions of missing data (Simmons, 2012) that varied substantially across loci (Table 2, [Supplementary Table S1](#)). Optimal likelihood trees were searched for using 1000 independent searches starting from randomized parsimony trees with the GTRGAMMA model and four discrete rate categories. Likelihood BS analyses were conducted with 1000 replicates with five searches per replicate using the “-f i” option, which “refine[s] the final BS tree under GAMMA and a more exhaustive algorithm” (Stamatakis, 2008, p. 9).

A second simultaneous-analysis was run after excluding 18 terminals that were resolved as members of large polytomies in the strict consensus of the first simultaneous-analysis parsimony trees ([Supplementary Table S1](#)). Only one or two gene regions were sampled for 13 and five of these terminals, respectively. All 18 of these terminals were represented only by GenBank-sourced sequences.

**Table 2**

Data-matrix and tree statistics for each of the phylogenetic analyses. "CI" = ensemble consistency index (Kluge and Farris, 1969) on the most parsimonious tree(s) for the parsimony-informative characters. "RI" = ensemble retention index (Farris, 1989).

Matrix	# Terminals	# Characters analyzed	# Of parsimony informative characters	% Missing/ inapplicable	Most parsimonious tree length	# Of most parsimonious trees	# Of jackknife/ bootstrap clades $\geq$ 50%	Average jackknife/ bootstrap support (%)	CI	RI
ETS rDNA	74	457	269	9.5	1440	112	55/59	89/85	0.39	0.77
ITS rDNA	246	573	289	11.7	2101	30	144/154	87/84	0.28	0.83
rDNA (ETS, ITS)	247	1030	558	39.0	3554	577	153/161	86/85	0.32	0.81
<i>atpB-rbcL</i>	81	1090	164	31.4	526	5410	37/48	84/78	0.58	0.84
<i>matK</i>	121	862	152	4.7	307	228	44/61	81/80	0.76	0.98
<i>petD</i>	194	1552	384	34.2	1092	10,042	90/105	87/84	0.58	0.92
<i>rbcL</i>	125	611	62	<0.1	141	4646	27/33	79/80	0.64	0.95
<i>rps16</i>	177	990	276	32.0	831	17,200	78/96	84/83	0.59	0.91
<i>trnH-psbA</i>	109	563	135	40.4	251	1218	29/33	77/79	0.78	0.97
<i>trnL</i> intron	103	606	132	17.5	327	5890	44/51	78/79	0.74	0.96
<i>trnL-F</i> spacer	178	898	274	53.9	772	5502	87/101	81/80	0.61	0.92
plastid	242	7172	1577	58.8	4352	1460	134/183	85/85	0.60	0.92
simultaneous – 1st	272	8202	2135	61.0	7998	1850	154/210	87/86	0.53	0.88
simultaneous – 2nd	254	8202	2109	58.9	7806	6830	182/166	88/91	0.53	0.88

### 3. Results

Characteristics of each data matrix and the corresponding tree statistics are presented in Table 2. The simplified 254-terminal simultaneous-analysis tree is presented in Fig. 1 with parsimony JK values  $\geq$ 50% and likelihood BS values  $\geq$ 50%. The entire simultaneous-analysis tree with all terminals represented is presented in Fig. S1 as Supplementary online data. Equivalent trees for each of the 10 individual loci and the plastid and rDNA analyses listed in Table 2 are presented in supplementary Figs. S2–S13. These trees were created using TreeGraph 2 (Stöver and Müller, 2010). Support values were mapped onto the parsimony strict consensus tree so as to help minimize frequency-within-replicates (Davis et al., 1998) and undersampling-within-replicates BS and JK artifacts (Simmons and Freudenstein, 2011).

None of the gene regions were found to exhibit significant nucleotide-frequency heterogeneity when considering only the parsimony informative nucleotide characters among different terminals based on the chi-square test implemented in PAUP\* (which ignores phylogenetic correlations).

#### 3.1. Incongruence

We did not identify any mutually well supported ( $\geq$ 70% JK and BS) topological incongruencies between parsimony and likelihood for any of the individual gene trees or combined analyses, as is typically the case in empirical studies (Rindal and Brower, 2011). In cases of conflict between the parsimony and likelihood results we generally favor the parsimony topology because of the inclusion of gap characters in the parsimony but not likelihood analyses (up to 534 additional characters) and that parsimony is not susceptible to the same artifacts that likelihood is when applied to non-random distributions of missing data that have heterogeneous rates of evolution (Lemmon et al., 2009; Simmons, 2012), for which our data are clearly a severe case in the plastid, rDNA, and simultaneous analyses (e.g., only two terminals were sampled for both the *atpB-rbcL* spacer and the *rbcL* exon; Supplementary Table S1).

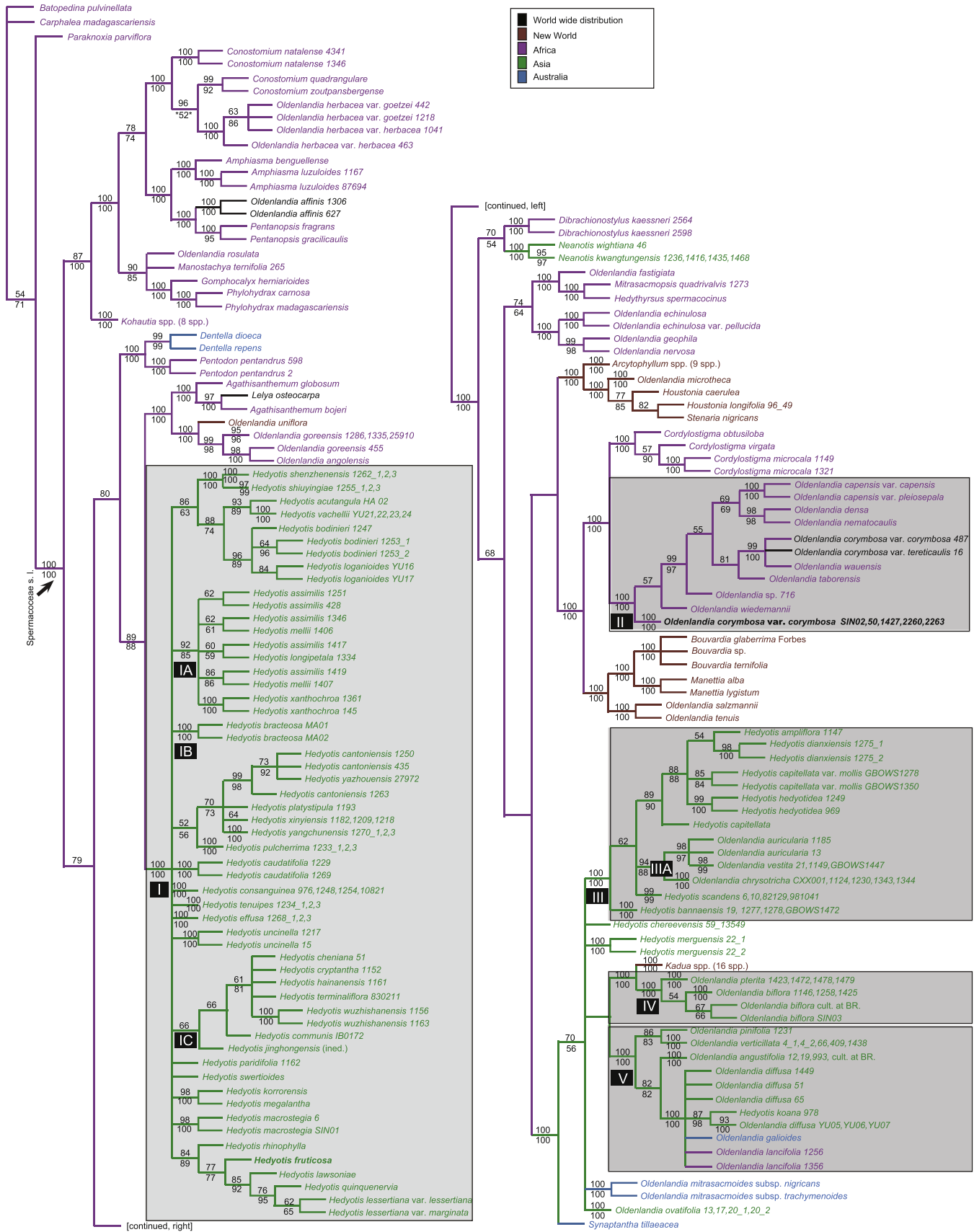
We did not identify any mutually well supported cases of topological incongruence between the plastid gene trees. But we did identify the following three cases of incongruence between the rDNA and plastid trees. First, in the plastid tree (Supplementary Fig. S13), both specimens of *Hedyotis scandens* are resolved as sister to the clade of *Oldenlandia auricularia* + *O. vestita* (74% JK/65% BS), whereas *O. chryso-tricha* is resolved as sister to the clade of *Oldenlandia auricularia* + *O. vestita* (98% JK/94% BS) in the rDNA tree (Supplementary Fig. S4).

Specifically, the incongruence is caused by the alternative resolution of *H. scandens* and only involves one pair of mutually well supported incongruent clades. As such, this is a possible case of differential introgression or lineage sorting of rDNA and the plastid genome relative to each other. We were unable to select among these alternative explanations with the current data.

Second, all five specimens of *Oldenlandia verticillata* are resolved as an exclusive lineage in the plastid tree (Supplementary Fig. S13; 99% JK/100% BS), whereas three of these specimens are resolved as more closely related to *O. angustifolia* than they are to the other two specimens (66 and 409) in the rDNA tree (Supplementary Fig. S4; 92% JK/69% BS and 68% JK/<50% BS on successive branches). The identification of all five specimens of *O. verticillata* was re-confirmed. Both specimens 66 and 409 were collected from the same locality. Given that specimens 66 and 409 are resolved consistently between the plastid and rDNA gene trees and that the rDNA-based resolution for specimens 4–1, 4–2, and 1438 is inconsistent with the current taxonomy, we suggest that this may be a case of introgression of rDNA from the *O. angustifolia* lineage to a subset of the *O. verticillata* populations.

Third, there are multiple mutually well supported incongruencies in interspecific relationships between the plastid and rDNA trees within the clade consisting of *Oldenlandia diffusa*, *O. galioides*, *O. herbacea*, *Hedyotis koana*, *O. lancifolia*, and *O. pinifolia*. This clade of six species (except *O. pinifolia* in the plastid tree) is sister to *O. angustifolia* (and three specimens of *O. verticillata* in the rDNA tree), as described above for the second case of incongruence. The only consistent relationship supported by both the plastid and rDNA trees is the clade of three specimens of *O. diffusa* + *O. koana*. Given that there are multiple cases of incongruence within this clade and we are unable to select between the plastid and rDNA topologies using other data, we do not consider the interspecific relationships in this clade that are resolved in the simultaneous analysis to necessarily reflect the predominant phylogenetic relationships.

Finally, an unexpected result on the plastid tree was that two separate clades of *Hedythyrus* + *Mitrasacmopsis* were resolved as distantly related to each other, separated by six branches (Supplementary Fig. S13). In the rDNA tree, one clade of three specimens (*H. spermacoccinus*, *M. quadrialvis* 1226, 1273) was resolved as sister to *Oldenlandia fastigiata* (Supplementary Fig. S4; 84% JK/87% BS). Two of these same specimens (*H. spermacoccinus*, *M. quadrialvis* 1273) were also resolved as sister to *O. fastigiata* on the plastid tree (99% JK/100% BS). In contrast, the third specimen (*M. quadrialvis* 1226) was resolved with *H. thamnoides* and *M. quadrialvis* 11069 on the plastid gene tree (73% JK/78% BS). The latter two specimens were only sampled for *rbcL*. Only two of the five specimens (*H. spermacoccinus* and *M. quadrialvis* 1273) were generated and



**Fig. 1.** The simplified 254-terminal simultaneous-analysis tree with parsimony JK values  $\geq 50\%$  above the branches and likelihood BS values  $\geq 50\%$  below the branches. The one clade that was contradicted by  $\geq 50\%$  BS support is indicated by \*52\*. The type species of *Hedyotis* and *Oldenlandia* are showed in bold. Different colors indicate the region from which samples were collected.

sampled by Kårehed et al. (2008). Based on the congruence between the rDNA and plastid gene trees for the clade resolved as sister to *O. fastigiata*, and incongruent ITS and *rbcL* gene trees for *M. quadrivalvis* 1226, we believe that the three *rbcL* sequences resolved as sister to *O. ovatifolia* in the plastid tree (GenBank accessions AJ616214, AM117232, and AM117248) are not assigned to the correct species.

### 3.2. Simultaneous analysis

With the exception of the three cases of incongruence between the rDNA and plastid trees described above, we consider the simultaneous analysis to be the best estimate of the phylogeny and all of our systematic inferences are based on this tree (Fig. 1).

Our inferred phylogeny is generally consistent with Kårehed et al. (2008) and Groeninckx et al. (2009) with six exceptions, only two of which are mutually well supported. First, Kårehed et al. (2008) resolved *Oldenlandia geophila* and *O. nervosa* as a paraphyletic group [1.0 posterior probability (PP)] whereas both Groeninckx et al. (2009; 1.0 PP) and we resolved them as a clade (99% JK/98% BS). Second, Kårehed et al. (2008) resolved *Conostomium* as a clade sister to *Oldenlandia herbacea* (0.96 PP) whereas *O. herbacea* was resolved as nested within *Conostomium* in our parsimony analysis (96% JK/contradicted by 52% BS).

*Hedyotis* was resolved as a polyphyletic group consisting of at least three lineages. Nested within one lineage are three species of *Oldenlandia* (*O. auricularia*, *O. chrysotricha*, and *O. vestita*; Fig. 1). *Oldenlandia* was resolved as a grossly polyphyletic group consisting of 10 separate lineages, two of which are paraphyletic (with *Kadua* or *Hedythysus* + *Mitrasacmopsis* nested within them). *Neanotis*, which was not sampled by either Kårehed et al. (2008) or Groeninckx et al. (2009), is moderately to weakly supported as sister to *Dibrachionostylus* (70% JK/54% BS; Fig. 1).

## 4. Discussion

The parsimony and likelihood phylogenetic analyses based on two nuclear regions (ETS and ITS) and eight plastid regions (*atpB-rbcL*, *matK*, *petD*, *rbcL*, *rps16*, *trnH-psbA*, *trnL-F* spacer, and *trnL* intron) using a total of 272 individuals resolved the relationships among the taxa of the *Hedyotis*–*Oldenlandia* complex (Fig. 1). In this parsimony-based strict consensus, 11 genera (*Amphisma* Bremek., *Arcytophyllum* Willd. ex Schult. & Schult. f., *Bouvardia* Salisb., *Cordyllostigma* Groeninckx & Dessein, *Dentella* J. R. Forst. & G. Forst., *Kadua* Cham. & Schltdl., *Kohautia* Cham. & Schltdl., *Manettia* Mutis ex L., *Pentanopsis* Rendle, *Pentodon* Hochst., and *Phylohydrax* Puff) that were originally recognized as members of, and segregated as distinct genera from, the *Hedyotis*–*Oldenlandia* complex were supported as monophyletic (Fig. 1). Three other genera (*Agathisanthemum* Klotzsch, *Conostomium* (Stapf) Cufod, and *Houstonia* L.) were resolved as paraphyletic. The phylogeny and taxonomy of these groups were discussed thoroughly by Groeninckx et al. (2009), Thulin and Bremer (2004), and Church (2003), respectively. Therefore, these genera will not be discussed again here because our focus is on the phylogenetic analysis to the *Hedyotis*–*Oldenlandia* complex, especially for the Asian species.

### 4.1. Phylogeny and generic circumscriptions

#### 4.1.1. *Hedyotis* s. str. clade (clade I)

Seventy-six terminals representing 40 Asian species of *Hedyotis* form an unambiguously supported clade (100% JK/100% BS), referred to as *Hedyotis* s. str., including the type species *H. fruticosa*. This clade includes all species that were previously assigned to *Hedyotis* L. sect. *Diplophragma* Wight & Art., as well as some species from *Hedyotis* sect. *Hedyotis* by Ko (1999) and Dutta and Deb

(2004). The members in this clade typically have an erect, robust herbaceous or sometimes shrubby habit, entire stipules with glandular-serrate margins, and diplophragmous capsules with partially apical loculicidal dehiscence followed by complete septicidal dehiscence, resulting in two separate capsular halves (Fig. 3A). Their seeds are dorsiventrally compressed with a ventral hilar ridge topped by a punctiform apical hilum (also referred to as “*fruticosa*-type seeds”; Terrell and Robinson, 2003). The tricolporate pollen has a perforate sexine (Neupane et al., 2009; Guo and Wang, 2011).

The flowers and inflorescence of the species in this clade exhibit diverse patterns. For example, clade IA (Fig. 1) has paniculate inflorescences and homostylous flowers with obviously exerted long styles; clade IB has capitate inflorescences with two large subtending bracts and a long axillary peduncle; and clade IC has terminal cymes with short peduncles and subtending leaves.

Reproductive differences were also observed in clade I. The species in clade IA, including *H. assimilis* (Fig. 3B), *H. longipetala*, *H. mellii*, and *H. xanthochroa* (Fig. 3C), have a cryptic self-incompatibility interbreeding system, whereas other species have both cryptic (in *H. consanguinea*) and a heteromorphic (in most other species, such as *H. cantoniensis* and *H. pulcherrima*; Fig. 3D) self-incompatibility system (Wu et al., 2010, and pers. comm.).

The “diplophragmous capsules” and “*fruticosa*-type seeds” are typical of the *Hedyotis* s. str. clade, but three exceptions, which were previously recognized as members of *Hedyotis* sect. *Hedyotis* by Ko (1999), have either indehiscent (*H. cryptantha*, *H. paridifolia*) or apically dehiscent (*H. platystipula* (Fig. 3E) capsules. In addition, *Hedyotis* sect. *Hedyotis* is not supported and those species are either resolved with members of the *Scleromitron* clade (clade V) or in a separate clade – *Involucrella* (*H. merguensis*), which suggests that this section may be an unnatural group.

Wight and Arnott (1834) first proposed an infrageneric classification system of *Hedyotis* and erected sect. *Diplophragma*. Meisner (1838) then treated this section as a distinct genus. Fosberg (1943) adopted the broad sense of the genus while classifying the Polynesian species of *Hedyotis* and treated sect. *Diplophragma* as a subgenus, with typification of *H. fruticosa*. Our phylogenetic analysis supports the segregation of sect. *Diplophragma* from the *Hedyotis*–*Oldenlandia* complex as an isolated genus. The species of this genus are mainly distributed in Asian countries and Pacific islands (several species in Micronesia and only one in Polynesia, but none in Hawaiian islands; Terrell and Robinson, 2003).

#### 4.1.2. *Oldenlandia* s. str. clade (clade II)

*Oldenlandia* s. l. is clearly polyphyletic. As indicated by Kårehed et al. (2008), *Oldenlandia* s. str. should include only the species that are resolved in a clade with the type species, *O. corymbosa*. In the present analysis (Fig. 1), this clade (100% JK/100% BS) is unambiguously supported (100% JK/100% BS), which is congruent with previous studies (Groeninckx et al., 2009, 2010b). Moreover, all species in this clade, other than the pantropically distributed type species, are endemic to Africa and characterized as being small erect or prostrate herbs with terete to quadrangular stems, loculicidally dehiscent capsules and trigonous (3-angled) seeds.

The species in this clade have homostylous [except *O. taborensis* and *O. wiedemannii* and two unclearly described taxa (*O. densa* and *O. sp.* [Dessein et al., 716])] and inserted flowers, which may indicate that there are two different reproductive mechanisms in this clade. *O. corymbosa* is a common weed in moist habitats of tropical America, Africa, Asia, and in the islands of the western Pacific, and its cytogeography was comprehensively studied (Lewis, 1964). It was reported as having originated in Africa and was very probably a post-Columbian introduction to other regions (Bremekamp, 1952, p. 256). Bremekamp's (1952) description showed that stems

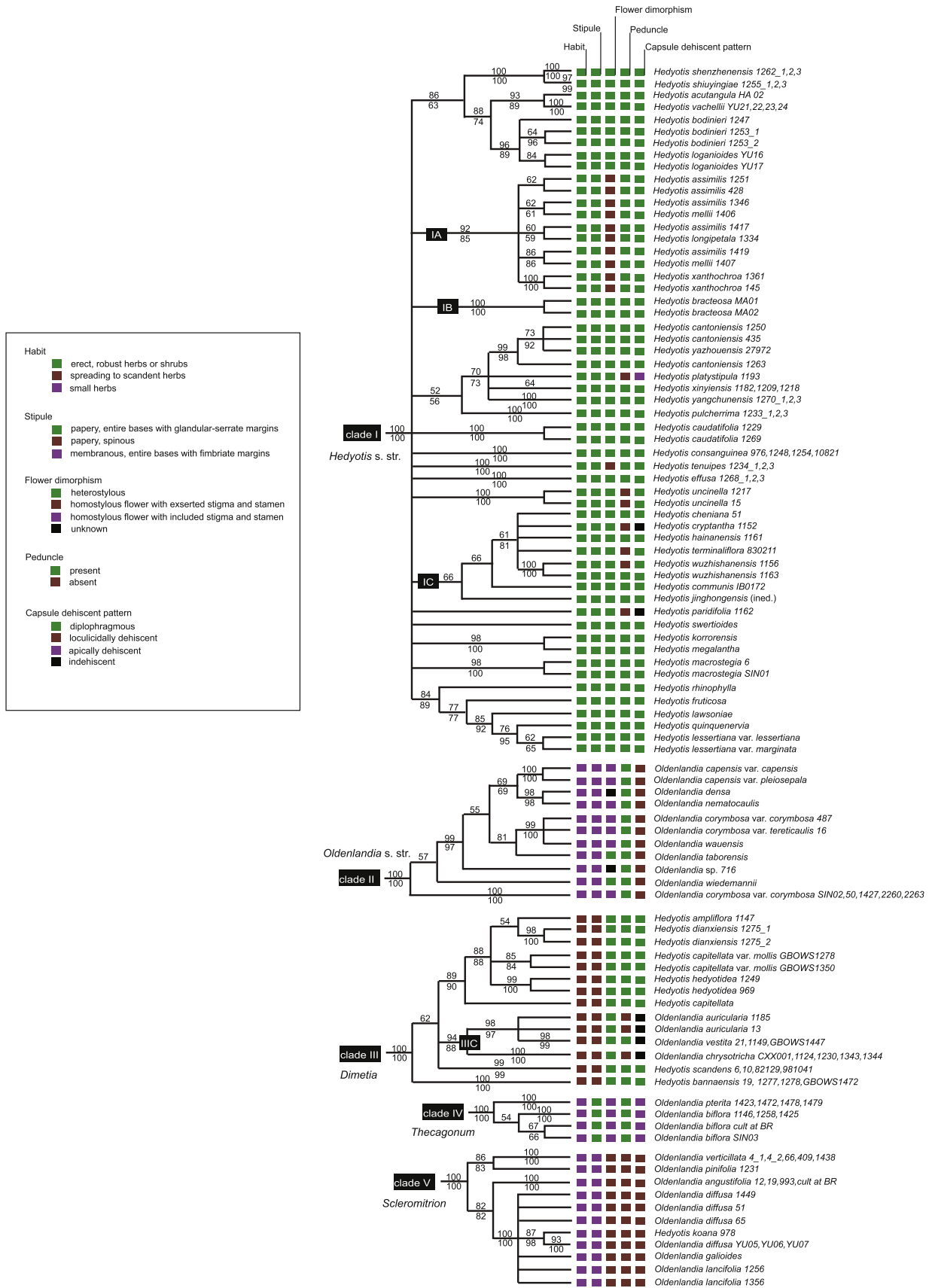
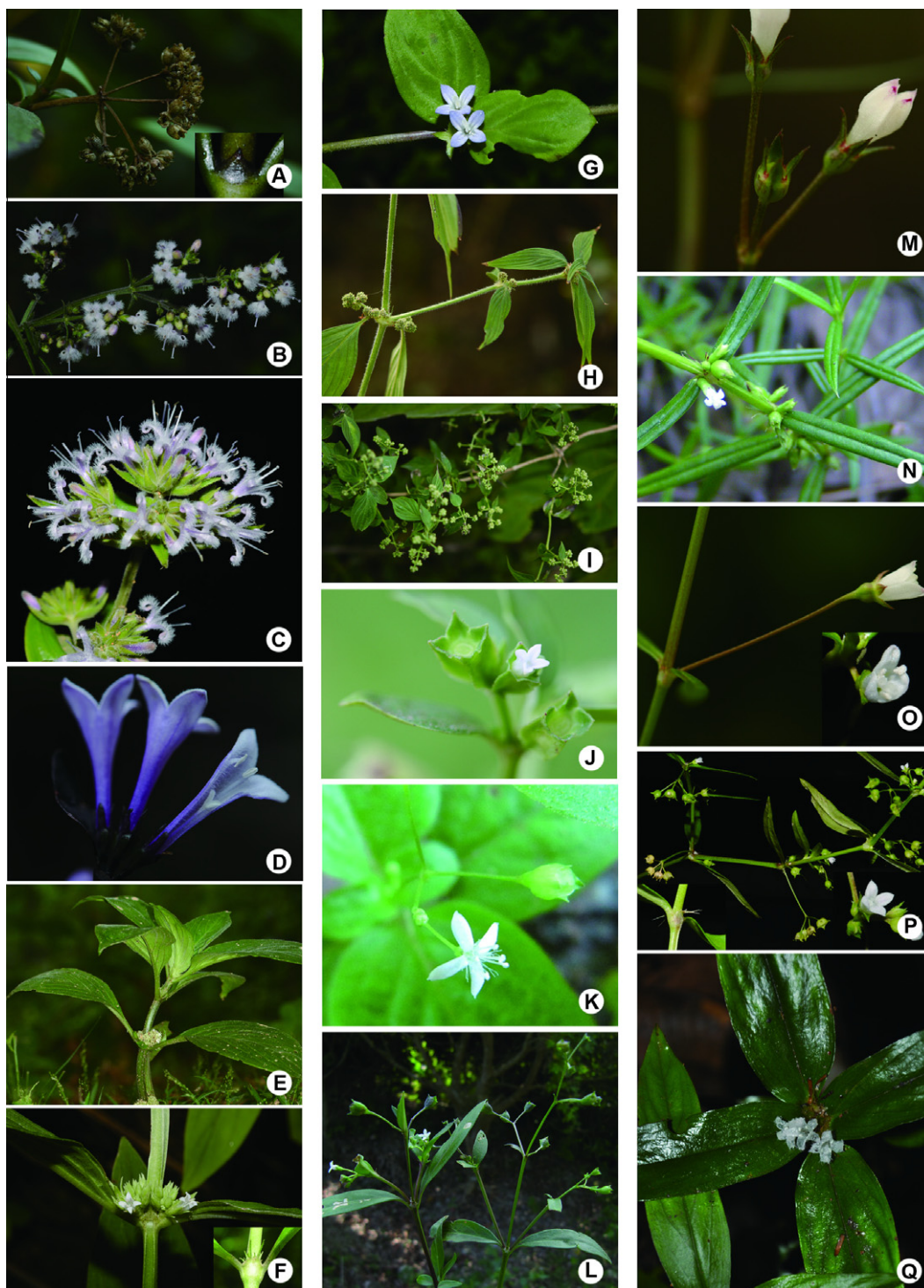


Fig. 2. Character states for five morphological characters are mapped onto five clades from the simultaneous-analysis phylogenetic tree.

of *O. corymbosa* in Africa are subterete and glabrous, whereas the Asian species have acutely angular and glabrous or scabridulous

stems with prominent ridges (Ko, 1999; Dutta and Deb, 2004). Likewise, three chromosomal races (a diploid race [2x=18] from



**Fig. 3.** Morphological characters of selected species in the *Hedyotis*–*Oldenlandia* complex. (A) *Hedyotis cantoniensis*, showing the diplophragmous capsules and stipule with glandular-serrate margins; (B) *Hedyotis assimilis*, showing the exserted long styles in the homostylous flowers; (C) *Hedyotis xanthochroa*, showing the exserted styles and stamens; (D) *Hedyotis pulcherrima*, showing the dimorphism of the flowers; (E) *Hedyotis platystipula*, showing the sessile inflorescence; (F) *Oldenlandia auricularia*, showing the sessile inflorescence, indehiscent capsules, and spinous stipe; (G) *Oldenlandia chrysotricha*, showing its scandent habit; (H) *Oldenlandia vestita*, showing its scandent habit and capitate inflorescence; (I) *Hedyotis bannaensis* (ined.), showing its scandent habit and capitate inflorescence; (J) *Oldenlandia pterita*, showing its winged capsules; (K) *Oldenlandia ovatifolia*, showing the appendages in the flowers and the somewhat winged capsules; (L) *Oldenlandia biflora*, showing its winged capsules; (M) *Hedyotis koana*, showing the long and gracile pedicel for a solitary flower; (N) *Hedyotis angustifolia*, showing the sessile and axillary inflorescence; (O) *Oldenlandia diffusa*, showing the solitary flower with a gracile pedicel and exserted style and stamens; (P) *Oldenlandia corymbosa*, showing the paniculate inflorescence with obvious peduncles and pedicels, flowers with inserted style and stamens, and stipules with entire base with fimbriate margins; (Q) *Hedyotis merguensis*, showing its terminal inflorescences that are half embraced at their bases by involucre-like uppermost leaves.

America, Asia, and the western Pacific, a tetraploid race [ $4x=36$ ] from Africa and India, and a hexaploid race [ $6x=54$ ] known only in western Africa) have been reported (Lewis, 1964).

Five samples identified as *O. corymbosa* from Australia, China, Gabon, and Singapore form an exclusive lineage that does not include the Zambian *H. corymbosa* (Fig. 1); these separate lineages

may represent two independent races or might be a composite of two distinct species. But before making a definite conclusion, further examination and analyses of more samples are necessary.

Bremekamp (1952) segregated *O. wauensis* as a new monotypic genus (*Thecorchus* Bremek.) based on its pseudo-axillary flowers or flower triads, included anthers and stigma, and distinctly elongated capsules. But this segregation was not supported in Kårehed et al. (2008), Groeninckx et al. (2009), and our study.

Africa is the center of diversification of *Oldenlandia*. Bremekamp (1952) narrowly circumscribed *Oldenlandia* for his treatment of about 60 African species. But our understanding of this lineage is still limited because of insufficient data. As an important component of the *Oldenlandia*–*Hedyotis* complex, the poorly known African *Oldenlandia* species should be prioritized for future systematic analyses.

#### 4.1.3. *Dimetia* clade (clade III)

This unambiguously supported clade (100% JK/100% BS), which includes 26 terminals, is distantly related to *Hedyotis* s. str. (Fig. 1). The three *Oldenlandia* species [*O. auricularia* (Fig. 3F), *O. chrysotricha* (Fig. 3G), and *O. vestita* (Fig. 3H)] formed a well supported subclade (Fig. 1, IIIA; 94% JK/88% BS) in the simultaneous analysis, but this clade is contradicted in the plastid gene tree (Supplementary Fig. S13). These three species were previously either treated as members of *Hedyotis* sect. *Hedyotis* (Ko, 1999; Dutta and Deb, 2004) or as a separate genus *Exallage* by Bremekamp (1952), with designation of *O. auricularia* as the type in both treatments. *Oldenlandia auricularia* is distinct from other members of *Oldenlandia* by having a prostrate or subprostrate habit, axillary inflorescences, indehiscent and globose capsules, and obconical seeds with concave lateral faces. Bremekamp (1952, p. 140) established the genus *Exallage* but did not provide a distinctive description of the morphological differences relative to *Oldenlandia* because “it would be premature... and require a study of the numerous Asiatic species”. Ridsdale (1998, p. 237) commented that *Exallage* was “an ill-defined genus” because many species in *Exallage* should belong to “*Hedyotis* s. str.” Terrell and Robinson (2003) compared the morphological similarities and differences of *O. auricularia*, *O. lapeyrousii*, and *O. vestita* with “true” *Oldenlandia* and supported recognizing them as “*Oldenlandia* subg. *Exallage* (Bremek.) Terrell & H. Robinson”, whose main distinctive trait is the presence of indehiscent capsules.

In addition to the *Oldenlandia* taxa in this clade, other terminals representing six species of *Hedyotis* have a scandent or climbing habit, diplophragmous capsules usually with a protruded crown, angular seeds with narrow wings, and 4-(rarely 3-)colporate pollens with a double reticulum sexine (Neupane et al., 2009). Wight and Arnott (1834) assigned these species to *Hedyotis* sect. *Dimetia* Wight & Arnott (Ko, 1999), and *H. capitellata* and *H. scandens* were also included in their protologue for describing the new section. *Hedyotis bannaensis* (ined.; Fig. 3I) is very similar to *H. capitellata* and *H. scandens* in habit, but the character combination of its pubescent stems, inflorescences, calyx tubes, and spinous stipules, together with small capitate inflorescences and sessile flowers distinguish this species from the others. The outstanding trait of these scandent plants is, as documented by Wight and Arnott (1834, p. 406), that their mature capsules are “compressed at top and protruded beyond to the tube of calyx, splitting at apex transversely to the dissepiment”. Dutta and Deb (2004) did not recognize *Hedyotis* sect. *Dimetia* but combined it with sect. *Hedyotis* for the Indian species, as with *H. fulva* Hook. f. Neupane et al. (2009) thought *H. scandens* has diplophragmous capsules and “*fruticosa*-type” seeds (dorsiventrally flattened, lenticular with an irregular narrow wing-like margin) and treated it as a member of *Hedyotis* sect. *Diplophragma*. These treatments indicate the confusion about these two sections and their close relationship, as also indicated in our analysis (Fig. 1).

Although species in the *Dimetia* clade vary in the capsule-dehiscence type and seed morphology, they all have sub-ligneous stems; a prostrate, subprostrate to scandent habit; spinous stipules; sessile or sub-sessile pedicels; small heterostylous flowers; and 4-(rarely 3-)colporate pollen with a double reticulum sexine. Given the morphological distinctiveness and robust support for this clade, it should be recognized as a distinct genus that needs to be resurrected.

***Dimetia*** (Wight & Arnott) Meisner, Pl. Vasc. Gen. [Meisner] 5 (Tab. Diagn.): 160. 1838. – Type: *Dimetia scandens* (Roxb.) R. J. Wang, **comb. nov.** Basionym: *Hedyotis scandens* Roxb., Fl. Ind., ed. Carey, 1: 369. 1820.

*Exallage* Bremek., The African species of *Oldenlandia* L. sense Hiern et K. Schumann, 1952, 140. **syn. nov.** – Type: *Exallage auricularia* (L.) Bremek.

Herbs or scandent shrubs. Stems prostrate, subprostrate to climbing, herbaceous or sub-ligneous. Spinous stipules; inflorescences axillary or terminal, flowers heterostylous, pedicels sessile or sub-sessile, pollen 4-(rarely 3-)colporate, with a double reticulum sexine. Capsules globose to ovoid, septicidally dehiscent or indehiscent. Seeds lenticular or obconical, with irregularly narrow wing-like margin or concave lateral faces. Distributed mainly in Asia, but also in Africa, Micronesia, and Australia.

#### 4.1.4. *Thecagonum* clade (clade IV)

Babu (1969) proposed the genus *Thecagonum* and designated *T. pteritum* (syn. *Oldenlandia pterita*, Fig. 3J) as the type species. It was unambiguously supported (100% JK/100% BS) as sister to the genus *Kadua* in our analysis (Fig. 1). *Thecagonum* is distinguished from *Oldenlandia* by having 4-winged loculicidally dehiscent capsules and ovoid or subglobose seeds with relatively narrow and shallow surface depressions and conspicuously sinuous borders (Terrell and Robinson, 2007; our obs.). But the capsules of *O. ovatifolia* (Fig. 3K) are not obviously winged and the seeds are conoidal or irregularly conoidal with reticulate and polygonal areoles, which is different from the typical obvious depressions in other *Thecagonum* species. Moreover, *O. ovatifolia* was not resolved within the clade of *O. biflora* (Fig. 3L) + *O. pterita*.

We found that the corolla lobes of *O. ovatifolia* are much longer than the corolla tube and are horizontally extended during anthesis. In addition, hairs in the corolla throat are as long as the stamens and styles, and exerted upward. This unique and interesting trait has not been previously reported in other species of the *Hedyotis*–*Oldenlandia* complex. We expect that this trait may play an important role for pollination. These morphological distinctions, together with our unambiguously supported resolution of the *Thecagonum* s. str. clade as sister to *Kadua*, strongly support the exclusion of *O. ovatifolia* from *Thecagonum*. *Oldenlandia ovatifolia* might be recognized as a distinct genus in the future.

We support Babu's (1969) proposal to reinstate the genus *Thecagonum* and the generic characters are summarized as: small herbs, inflorescences terminal, flowers heterostylous, 4-winged fruits, loculicidally dehiscent, ovoid or subglobose seeds with relatively narrow and shallow surface depressions and conspicuously sinuous borders. *Thecagonum* is distributed in Asia and Micronesia.

#### 4.1.5. *Scleromitron* clade (clade V)

This unexpected and unambiguously supported (100% JK/100% BS) clade includes taxa from Africa, northern Australia, and tropical and subtropical Asia. All species within this clade have an herbaceous habit; sessile or subsessile, linear to narrowly lanceolate leaves; homostylous flowers that are sessile or have a long and gracile pedicel (Fig. 3M); exerted stamens and styles; deeply divided corolla lobes; oblong anthers; subglobose capsules that are loculicidally dehiscent at the apex; and many obconic seeds.

Morphological comparison showed that *Scleromitrium* is very similar to *Oldenlandia* with respect to habit, stipule shape, and capsule dehiscent type (Fig. 2). Our detailed observation and comparison revealed that these two genera can be distinguished by their inflorescence and flower traits. *Oldenlandia* s. str. usually has terminal or axillary panicles with obvious or very short peduncles and 2–5 pedicelled flowers in each peduncle. In contrast, *Scleromitrium* has either axillary clusters of 2–5 sessile flowers or a single flower with a long and slim pedicel that is borne terminally or axillarily. Moreover, the homostylous androecia and gynoecia are usually inserted within the corolla tube in *Oldenlandia*, but exerted in *Scleromitrium*. *Scleromitrium* is distributed in Asia, Africa, and Australia, and *Oldenlandia* s. str. is mainly limited to Africa, except for the pantropical species, *O. corymbosa*.

Following Schumann (1891)'s system, Ko (1999, p. 69) employed “*Hedyotis* sect. *Euoldenlandia* K. Schum.”, which should be *Hedyotis* sect. *Oldenlandia* (L.) Wight & Arn., rather than sect. *Scleromitrium*, to accommodate these plants with “sessile or pedicelled solitary flowers, and exerted stamens and style”. Dutta and Deb (2004) adopted sect. *Scleromitrium* Wight & Arn. and included three species (*H. angustifolia* [Fig. 3N], *H. pinifolia*, and *H. verticillata*), which were also sampled in our analysis (Fig. 1).

Bremekamp (1952) described the flowers of African *O. verticillata* as heterostylous, but the individuals sampled from China in this study have homostylous flowers based on our own observations and the description in the *Flora of China* (Chen and Taylor, 2011; likewise by Dutta and Deb, 2004 for Indian species). Given that both of these samples of *O. verticillata* from two continents were correctly identified, there may have been a functional change from hetero- to homostylous flowers during the species' dispersal and adaptive history (Barrett and Shore, 2008).

*Oldenlandia diffusa* (Fig. 3O) and *O. corymbosa* (Fig. 3P) are two morphologically similar species that were proposed as the “*Hedyotis corymbosa-diffusa* complex” by Sivarajan and Biju (1990) based on the Indian collections. Dutta and Deb (2004) then clarified this confusion and provided a proper treatment. In China, *O. diffusa* is a traditional herbal medicine for the treatment of hepatitis and malignant tumors of the liver, lung, and stomach (Liang et al., 2008); it is frequently adulterated by *O. corymbosa* (Li et al., 2010). Besides the morphological method to distinguish the two species, Lau et al. (2012) also developed a simple chromatographic method to check the presence of the chemical constitution of hedyotiscone A, which is only found in *O. corymbosa* and 6-*O*-(*E*)-*p*-coumaroyl scandoside methyl ester, which is only found in *H. diffusa*. Thus we confidently conclude that the “*Hedyotis corymbosa-diffusa* complex” is not natural and that these are two clearly distinct species within two separate genera.

Halford (1992) reported that the Australian *O. galioides* and *O. tenelliflora* (synonym of *O. angustifolia*) have obconic seeds that are slightly laterally compressed and obtriangular in outline. Dessein (1998) reported similar seeds in the African species *O. lancifolia*. Neupane et al. (2009) examined the Nepalese species *O. brachypoda* (a synonym of *O. diffusa*), *O. diffusa*, and *O. erecta* (a synonym of *O. corymbosa* var. *linearis*) and noticed that the seed coat of *O. diffusa* and *O. brachypoda* have distinct punctations, which are less obvious in *O. erecta*. Molecular phylogenetic analyses also confirmed that these two species are in separate clades (Guo et al., 2011; Li et al., 2010).

Kårehed et al. (2008, p. 855) discussed the relationships among the species within their clade H and suggested that “one or several new genera should better be recognized to acknowledge the early branching members”. Our increased sampling uncovered some doubts and threw light on the complicated relationships of this lineage. We reinstate the genus *Scleromitrium* to include these diversely distributed but phenotypically distinctive species in clade V.

***Scleromitrium*** (Wight & Arnott) Meisner, Pl. Vasc. Gen. [Meisner] 5 (Tab. Diagn.): 160. 1838. – Type: *Scleromitrium angustifolium* (Cham. & Schlecht.) Benth., J. Bot. Kew Gard. Misc. 4: 172. 1852.

Herbs annual or perennial, diffusely branched at base erect or decumbent. Stem 5–20 cm long. Leaves sessile or subsessile, elliptic lanceolate, linear-lanceolate or linear, usually 1-nerved. Stipule membranous, with 3–7 bristles. Flowers homostylous, terminal or axillary panicles with obvious or very short peduncles and 2–5 pedicelled flowers in each peduncle, stamens and styles both exerted. Corolla divided equally or more than half way down. Anthers oblong. Capsule subglobose, loculicidal dehiscent on the top. Seeds many, obconic. Distributed in Africa, Australia, Asia and Micronesia.

#### 4.1.6. *Hedyotis merguensis*

The Asian species *Hedyotis merguensis* is distributed in China, India, Malaysia, Myanmar, Philippines, Thailand, and Vietnam. It is distinctive based on its terminal or upper axillary inflorescences that are half embraced at their bases by involucre-like uppermost leaves (Fig. 3Q). The phylogenetic relationship of *Hedyotis merguensis* was not resolved because it is part of a polytomy (Fig. 1), but its unique inflorescence character states highlighted that this Asian species may represent a distinct evolutionary lineage that is not nested within the other lineages that are part of this polytomy. *Hedyotis cheniana*, *H. terminaliflora*, and *H. wuzhishanensis*, etc. in *Hedyotis* s. str. also have terminal inflorescences subtended by four leaves, which seems to be similar to that of *Hedyotis merguensis*, but these leaves do not enclose the inflorescences. Also, their pedicels and peduncles are present, though short in most cases.

*Hedyotis* sect. *Involucrella* was first proposed by Bentham and Hooker (1873) on the basis of the monotypic species *H. merguensis*. This treatment was accepted by Schumann (1891). Bakhuizen van den Brink (1965) synonymized the widely used name *H. coronaria* with *H. merguensis*, but Ko (1999) seemed to overlook this treatment and ascribed *H. coronaria* to sect. *Hedyotis* because of its indehiscent capsule. Dutta and Deb (2004) recognized this infrageneric section with this monotypic species. Our phylogenetic analysis only included two Chinese samples from one population. We cannot draw a robust conclusion to establish a monotypic genus at this stage and many samples from different populations will help enable an objective future decision on whether to erect a new genus.

#### 4.1.7. *Neanotis*

The genus *Neanotis* Lewis was segregated from *Hedyotis* sect. *Anotis* Wight et Arn. because of its notable plurizonocolporate pollen grains; in other *Hedyotis*–*Oldenlandia* complex members the aperture number rarely exceeds five (Lewis, 1966; Groeninckx et al., 2009). *Neanotis* has an herbaceous habit, septicidally dehiscent capsules, and houstonioid (cymbiform) seeds with a hilar ridge in a ventral depression (Terrell and Robinson, 2007). It is distributed in Southeast Asia and Melanesia (Ridsdale, 1998).

Jovet (1941) suggested a close relationship between the Madagascar genus *Astiella* Jovet and the Asian *Neanotis* because of their common character states of fimbriate stipules and 4-merous corollas, but he also noted the difference between the capsule dehiscence (loculicidal and septicidal in *Astiella*). Without sequences of *Neanotis* for analysis and considering its geographical distribution, Groeninckx et al. (2009) also tentatively proposed that *Neanotis* is closely related to the *Pentanopsis* clade, including *Amphasma* Bremek., *Conostomium* (Stapf) Cufod., *Manostachya* Bremek., *Pentanopsis* Rendle, and *Phylohydrax* Puff.

Five accessions from two *Neanotis* species were sequenced for the first time in this study. Our results provide moderate to weak support (70% JK/54% BS) for *Neanotis* as sister to the Kenyan monotypic genus *Dibrachionostylus* Bremek., which was separated from *Oldenlandia* largely on the basis of its both loculicidally and

septicidally dehiscent capsules. *Dibrachionostylus* has tricolporate pollen (Lewis, 1965), which is clearly distinct from the plurizonocolporate pollen of *Neanotis*.

#### 4.2. Morphological character evolution

The *Hedyotis*–*Oldenlandia* complex includes over 500 species and is distributed in both tropical and subtropical areas. The criteria for generic delimitation within this group are complicated due to the highly diverse morphological variation. Verdcourt (1958) presented a list of characters and briefly discussed their reliability for subfamilial classification of Rubiaceae. Robbrecht (1988) described and evaluated the vegetative and reproductive characters for the tropical woody Rubiaceae. But for characters of the *Hedyotis*–*Oldenlandia* complex, Dutta and Deb (2004) first provided a very detailed and valuable description and illustration mainly for Asian *Hedyotis* s. l., although Bremekamp (1952) gave a concise summary previously for the African *Oldenlandia*. In order to assess the reliability of the morphological characters traditionally used in the taxonomy of *Hedyotis*–*Oldenlandia* complex, five characters were selected and mapped onto the simultaneous-analysis phylogenetic tree (Fig. 2).

Habit—Habit is a valuable character for generic delimitation (Verdcourt, 1958). Species of the *Hedyotis* clade (clade I) are usually erect and robust herbs or (sub)shrubs; the *Dimetia* clade includes spreading herbaceous or ligneous scandent plants, and the small and gracile herbs are commonly in the *Thecagonum*, *Scleromitron*, and *Oldenlandia* clades. This effective characterization of the different clades was overlooked in many previous studies about Asian species. However, this character shows limited correlation to the worldwide (clade V) and African species (clade II).

Stipules—The presence of stipules is one of the most important vegetative characters to distinguish Rubiaceae from other angiosperms. The size and shape of the stipules and the way how they are connected with the leaves may be used for the characterization of define genera (Bremekamp, 1952, p. 24). In the *Hedyotis*–*Oldenlandia* complex, stipule morphology varies in size, shape, and margin, but the characters do not vary among members of individual clades (see Fig. 2) and can be applied for generic delimitation. The margin of stipules is glandular-serrate in *Hedyotis* and *Thecagonum* clades, spinous in the *Dimetia* clade, and fimbriate in the *Scleromitron* and *Oldenlandia* clades.

Inflorescences—Inflorescence type is usually constant within a genus but varies among some tribes and groups of genera (Verdcourt, 1958). This character can be used for delimiting the *Scleromitron* and *Oldenlandia* clades. *Scleromitron* has sessile or single flowers with long and slim pedicels, whereas *Oldenlandia* has paniculate or corymbose inflorescences with obvious peduncles.

Flower dimorphism—In herterostylous flowers anthers are included and styles are exerted in one form and exactly the opposite in the other form. Homostyly is characterized by styles and anthers being of the same height and is generally regarded as derived from the normal heterostylous state as a result of reversal of the stamens and style height (Dowrick, 1956). The evolutionary patterns of flower dimorphism in our study vary in clade I (*Hedyotis* s. str.) and clade II (*Oldenlandia* s. str.), in which herterostylous and homostylous flowers co-exist (Fig. 2). The homostylous flowers having both stamens and styles included are present in clade II (*Oldenlandia* clade) and the exerted homostylous flowers are found in the *Scleromitron* clade. This character shows limited phylogenetic signal but may represent an adaptation of the pollination syndrome. For example, the species in clade IA, including *H. assimilis* (Fig. 3B), *H. longipetala*, *H. mellii*, and *H. xanthochroa* (Fig. 3C), have homostylous flowers with a cryptic self-incompatibility interbreeding system, while other species are heterostylous but have both cryptic (in *H. consanguinea*) and a heteromorphic (in most other species, such

as *H. cantoniensis* and *H. pulcherrima*) self-incompatibility system (Wu et al., 2010, and pers. comm.).

Capsule and its dehiscence pattern—Shape, size and texture of capsules are of diagnostic significance in different clades. The *Hedyotis* s. str. clade (clade I) usually has globose, ellipsoid or oblong capsules with flat or slightly protruded apices. The *Dimetia* clade (clade III) is characterized by having a protruded top in mature fruits, and the *Thecagonum* clade (clade IV) has obvious winged capsules. The pattern of capsule dehiscence (septicidal, loculicidal, or indehiscent) is an important character that is extensively used for generic delimitation within the *Hedyotis*–*Oldenlandia* complex (Bremekamp, 1952; Terrell, 1975; Ko, 1999; Terrell and Robinson, 2003, 2007; Dutta and Deb, 2004; Neupane et al., 2009). *Hedyotis* s. str. was established on the basis of its diplophragmous capsules, which are similar to those in the type species *H. fruticosa* (Terrell and Robinson, 2003). This name was generally accepted for Asian and Micronesia taxa (Terrell and Robinson, 2003, 2007). Groeninckx et al. (2009) also supported this delimitation of *Hedyotis* s. str. based on their molecular phylogenetic analysis that included 10 Asian species. But with our increased sampling of Asian samples, the evolutionary patterns observed indicate that the diplophragmous capsule does not represent a unique synapomorphy of *Hedyotis* s. str. and therefore this trait alone is not diagnostic for *Hedyotis* s. str. As shown in Figure 1, the clades of *Hedyotis* s. str. and *Dimetia* are two distinct lineages that are not closely related. Both lineages have diplophragmous capsules, which implies that this fruit syndrome evolved independently at least twice – once in clade I and once in clade III. This phylogenetic distribution of indehiscent capsules indicates that capsule dehiscence is homoplasious and should not be the only basis for generic delimitation.

Seeds and pollen—Seed morphology (shape, surface) and pollen characters (aperture number and position, exine sculpturing, stratification) are very helpful for generic classification in this complex. The *Thecagonum* clade (clade IV) is characterized by having ovoid or subglobose seeds with relatively narrow and shallow surface depressions that have conspicuously sinuous borders. The *Dimetia* clade (clade III) can be distinguished from the *Hedyotis* clade (clade I) by having 4-(rarely 3)-colporate pollen with a double reticulum sexine. The potential phylogenetic value of pollen and seed indicate that thorough micromorphological study would be helpful for generic delimitation within this complex.

According to the above discussion, uniquely derived morphological synapomorphies with which to define genera are rare within the *Hedyotis*–*Oldenlandia* complex and in most cases character-state combinations must be applied to distinguish genera. Therefore, many other characters, such as the habit, leaf micromorphology and stipule morphology should be explored more broadly rather than relying strictly on reproductive characters. Studying the chemical compounds should also be pursued as an additional source of characters.

#### 4.3. Center of origin

Phytogeography may contribute to plant classification. Fossils usually play a key role in understanding biogeographical patterns, but the summarization of the accepted fossils of the Rubiaceae, especially of subfamily Rubioideae and Spermaceae (only *Borreria* fossils), showed that they are too young (Neogene) to provide an informative indication about the origin of the family (Graham, 2009; Shi et al., 2012).

On the basis of cytoplasmic analysis, Lewis (1964) strongly suggested an origin of *O. corymbosa* in eastern Africa, which was also in agreement with Bremekamp's (1952) deduction about the continental origin of the species. Wang and Zhao (2001) analyzed the available data from systematics, paleogeography, and

cytogeography of *Hedyotis* s. l. and proposed that this group originated in northeastern Gondwana before the late Jurassic or even in the early Triassic. Based on mapping geographical distribution of taxa onto our inferred phylogeny (Fig. 1) we infer that the *Oldenlandia*–*Hedyotis* complex originated in Africa. This African lineage later dispersed to Asia at least three times and to the New World at least four times.

## Acknowledgments

We are grateful to the curators of CDBI, CUHK, HITBC, IBSC, KUN, and SZ for permission to examine specimens; Professor D.X. Zhang and Dr. X.Q. Wu (IBSC) for sharing their Chinese-*Hedyotis* reproductive results; Mr. X.X. Huang (IBSC) for collecting plant materials; and suggestions from two anonymous reviewers. The project was funded by the National Natural Science Foundation of China (Grant No.: 30770156, 31070177) and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-EW-J-28).

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2013.01.006>.

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