



## Phylogenetic position of *Guihailiothamnus* (Rubiaceae): Its evolutionary and ecological implications



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### ABSTRACT

*Guihailiothamnus* (Rubiaceae) is an enigmatic, monotypic genus endemic to southwestern China. Its generic status has never been doubted because it is morphologically unique by having rosette habit, showy, long-corolla-tubed flowers, and multi-seeded indehiscent berry-like fruits. The genus has been postulated to be a relict in the broad-leaved forests of China, and to be related to the genus *Wendlandia*, which was placed in the subfamily Cinchonoideae and recently classified in the tribe Augusteae of the subfamily Dialypetalanthoideae. Using combined evidence from palynology, cytology, and DNA sequences of nuclear *ITS* and four plastid markers (*rps16*, *trnT-F*, *ndhF*, *rbcl*), we assessed the phylogenetic position of *Guihailiothamnus* in Rubiaceae. Our molecular phylogenetic analyses placed the genus deeply nested within *Wendlandia*. This relationship is corroborated by evidence from palynology and cytology. Using a relaxed molecular clock method based on five fossil records, we dated the stem age of *Wendlandia* to be 17.46 my and, the split between *G. acaulis* and related *Wendlandia* species in southwestern China to be 2.11 mya. This young age, coupled with the derived position in *Wendlandia*, suggests an evolutionary derivation rather than an evolutionary relict of *G. acaulis*. Its rosette habit and large showy flowers, which are very distinctive from other *Wendlandias*, are interpreted as a result of recent rapid adaptation to rock and cliff habitats.

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### 1. Introduction

The coffee family, Rubiaceae, is a remarkable group with high diversity in warmer and tropical areas, containing more than 13,000 species in 615 genera (Davis et al., 2009; Govaerts et al., 2007; Ruhsam et al., 2008). It is the fifth-largest family of flowering plants by number of genera, and the fourth largest by number of species (Davis et al., 2009). Besides its richness in genera and species, the most enigmatic phenomena in the diversity of this family may be an unusually high percentage (34.5% of the total) of monotypic genera, which are often morphologically distinctive (Davis et al., 2009). Previous molecular and morphological phylogenetic studies have investigated many of the monotypic genera to reconstruct a phylogenetic frame of the family and/or to assess their generic status, systematic positions, and phylogenetic relationships (Delprete, 1996, 1998a,b; Igersheim, 1993; Nakamura et al.,

2006; Razafimandimbison et al., 2011; Rydin et al., 2008; Thulin and Bremer, 2004). These monotypic genera assessed in previous studies have either been treated as members of other species-rich genera, because of their nested position, or retained as separate genera, when they are phylogenetically isolated from other groups (see a summary by Razafimandimbison et al., 2011). Well-resolved phylogenies, combined with evidence from morphology and reproductive biology, may shed some light on the origin and evolution of these monotypic genera.

Chinese Rubiaceae contains 9 monotypic genera and four of them (i.e. *Dunnia* Tutcher, *Foonchewia* Wang, *Guihailiothamnus* H.S. Lo and *Trilliaedoxa* W.W. Smith and Forrest) are endemic to China (Chen and Taylor, 2011; Wen and Wang, 2012). As such a rare and peculiar plant, however, *Guihailiothamnus* has never been included in any molecular phylogenetic studies of Rubiaceae. During a taxonomic revision and reproductive survey for Rubiaceae in China, we were able to sample several rubiaceaceous species to examine the phylogenetic position and diversification pattern of the monotypic *Guihailiothamnus*. Described by Lo in 1998, *Guihailiothamnus acaulis* H.S. Lo is one of the most endangered angiosperm species in China (Wang and Xie, 2004), and is ranked as critically

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endangered (CR B1ab(ii)) according to the criterion of IUCN (IUCN, 2001). Until now, this species has only been found on the Jiuwan Mountains in northern Guangxi, southwestern China. The increasing human activities and habitat destruction have caused the individuals being dwindling in numbers during the last decades.

*Guihaiiothamnus acaulis* is easily recognized by its rosette habit, terminal inflorescences with 9–25 congested pink long-tubed flowers, and berry-like, indehiscent fruits. When establishing *Guihaiiothamnus*, Lo (1998, 1999) originally placed this genus in tribe Rondeletieae of the subfamily Cinchonoideae, and postulated its close affinity with *Wendlandia* Bartl. ex DC. on the basis of convoluted corolla aestivation, 2-locular ovary with numerous ovules per locule, and reticulate seed testa. However, the circumscription of Rondeletieae has been debated since its establishment (see Delprete (1999) for the detailed classification history). Lo (1998, 1999) adopted Schumann's circumscription of Rondeletieae, which included 15 genera from the pantropic region, when he was studying *Guihaiiothamnus*. However, Rova (1999) and his colleagues (2002, 2009) demonstrated that Rondeletieae sensu Schumann was polyphyletic, and some genera within it were distributed into various other tribes. He thus reduced the delimitation of Rondeletieae to include only closely related genera from the New World tropics, and excluded *Wendlandia* and other unrelated genera from the tribe (Rova et al., 2009). The exclusion of *Wendlandia* out of Rondeletieae has been supported by other molecular phylogenetic studies (Kainulainen et al., 2009, 2013; Razafimandimbison et al., 2011; Robbrecht and Manen, 2006), which consistently suggested that *Wendlandia* is nested within the broadly circumscribed Dialypetalanthoideae Reveal (=Ixoroideae Raf.), and is sister to the genus *Augusta* Pohl from the South Pacific, and Central and South America (Rova et al., 2002; Kirkbride, 1997; Delprete, 1997). Besides molecular evidence, palynological data also supported the transfer of *Wendlandia* from Cinchonoideae to Dialypetalanthoideae (Xie and Zhang, 2010). Lacking sampling of *Guihaiiothamnus* in previous molecular studies, it remains to be assessed regarding its phylogenetic position within Rubiaceae and its relationship with *Wendlandia*.

During recent field works, we found that all individuals of *Guihaiiothamnus* occur only on seasonally dry, barren cliffs, with limited soil under dense forests or near creeks, often growing among moss and ferns, rather than among flowering plants. Morphologically, *Guihaiiothamnus* displays a congruent pattern for an assumed isolated status because both vegetative and flora characters of this genus are distinctive from any other genus of Rubiaceae, and its generic status has not seriously been questioned (Lo, 1998, 1999; Wu et al., 2004, 2005). The proposed sister relationship with *Wendlandia* has been accepted by some authors (Lo, 1999; Wu, 2004; Wu et al., 2005), although Chen and Taylor (2011) carefully stated that its relationships with other Rubiaceae genera are unknown due to lack of detailed information from morphology. If *Guihaiiothamnus* is a separate genus and a sister to *Wendlandia*, it seems reasonable to assume its relict status and its origin from the common ancestor of *Wendlandia*. In a survey of the biogeographical history of flora of China, Wu et al. (2005) considered *Guihaiiothamnus* as one of the many relicts, which were characterized by long evolutionary histories from early Tertiary. Xie et al. (2013), in explaining its unique pollination mechanism, pointed out that, the observed small syrphid flies were unexpected pollinators of *Guihaiiothamnus* because its showy tubular flowers (28–35 mm long, nearly ten times longer than some species of *Wendlandia*) fitted well with a long-tongued butterfly pollination syndrome. The conflict between the expected and the observed pollinators was interpreted as loss of the original pollinators of butterflies, following by a pollinator shift to small syrphid flies during its evolutionary history. However, if its generic status and/or its

sister relationship with *Wendlandia* cannot be supported, the origin and evolution of *Guihaiiothamnus* need to be re-explained.

In the present study, we sampled *Guihaiiothamnus* and representatives from its putative relatives *Wendlandia*, *Augusta* and other taxa either obtained anew in this study or reported in previous molecular studies, and employed DNA sequences from the nuclear ITS and four plastid regions to reconstruct a phylogeny. Using five fossils as calibration points, we estimated the ages of *Guihaiiothamnus* in a broad phylogenetic framework of Rubiaceae. Combining evidence from morphology, cytology, palynology, phylogenetics, divergence time calibration, pollination, and breeding system of *Guihaiiothamnus*, we aim: (1) to pinpoint the phylogenetic position of *Guihaiiothamnus* within the Rubiaceae, which will allow us to assess its generic status and its proposed close relationship with *Wendlandia*, and (2) to examine the hypotheses of its relict origin, and the evolution of its distinctive morphology and pollination mechanism.

## 2. Materials and methods

### 2.1. Choice of molecular markers and taxon sampling

The nuclear ribosomal internal transcribed spacer (ITS) and the four plastid regions (*rps16* intron, *trnT-F* region, and *rbcL* and *ndhF* genes) are proved to be informative in numerous molecular phylogenetic studies of Rubiaceae. We thus generated a small data matrix and a large data matrix using combined sequences. The small data matrix contains the nuclear ITS and four plastid regions from 36 samples of *Guihaiiothamnus* and putative related species, and the large data matrix contains exclusively plastid regions from 83 taxa representing the outgroup and all tribes associated with Dialypetalanthoideae (Kainulainen et al., 2013). The sequences of the two data matrices were either generated anew and/or obtained from previous molecular phylogenetic studies. The sequences generated anew are from two individuals of *G. acaulis* (from different populations), 13 species or subspecies of *Wendlandia*, and eight species representing different clades of Rubiaceae. Voucher information and GenBank accession numbers are provided in Table 1.

### 2.2. DNA extraction, amplification and sequencing

The genome DNA was extracted from silica-gel-dried leaf samples with a modified CTAB procedure (Doyle and Doyle, 1987). The primers P17 and 26S-82R (Popp and Oxelman, 2001) were used to amplify and to sequence the ITS region. The four plastid regions were amplified and sequenced using the primers of Razafimandimbison et al. (2008) and Rydin et al. (2008). The PCR and sequencing protocols followed Razafimandimbison et al. (2008).

### 2.3. Phylogenetic analysis

Sequencher 5.1 (Gene Codes Corporation) was used to evaluate chromatograms for base confirmation and to edit contiguous sequences. All the DNA sequences were initially aligned by Clustal X 1.83 (Thompson et al., 1997) and adjusted manually using Se-al 2.0 (Rambaut, 2007).

Parsimony analyses were conducted using PAUP v.4.0b10 (Swofford, 2003) with all characters unordered and equally weighted, gaps treated as missing data. Phylogenies were constructed using heuristic search with 1000 addition-sequence replicates, tree-bisection-reconnection (TBR) branch swapping, steepest descent off, and keeping all most parsimonious trees. Internal branch support (BS) was estimated with 500

**Table 1**

Samples, and GenBank accessions included in the phylogeny reconstruction and in the dating analysis. The superscripts refer to literature or GenBank sources. All the vouchers are deposited in the herbarium of South China Botanical Garden (IBSC).

Species	Vouchers	GenBank accession				
		<i>ndhF</i>	<i>rbcL</i>	<i>rps16</i>	<i>TrnT-F</i>	<i>ITS</i>
<i>Afrocanthium lactescens</i> (Hiern) Lantz		HM164346 <sup>1</sup>	HM164152 <sup>1</sup>	HM164189 <sup>1</sup>	AJ620127 <sup>2</sup>	–
<i>Airosperma vanuense</i> S.P. Darwin		AM949845 <sup>3</sup>	–	FM204700 <sup>3</sup>	FM207108 <sup>3</sup>	–
<i>Alberta magna</i> E. Mey.		AJ236282 <sup>4</sup>	Y18708 <sup>5</sup>	FM204701 <sup>3</sup>	AJ620118 <sup>2</sup>	–
<i>Aleisanthiopsis distantiflora</i> Tange		HM164350 <sup>1</sup>	HM164154 <sup>1</sup>	AF242903 <sup>6</sup>	HM164306 <sup>1</sup>	–
<i>Anthospermum herbaceum</i> L. f.		AJ236284 <sup>4</sup>	X83623 <sup>7</sup>	EU145496 <sup>8</sup>	EU145544 <sup>8</sup>	–
<i>Appunia guatemalensis</i> Donn. Sm.		AM945252 <sup>9</sup>	AJ288593 <sup>10</sup>	AM945306 <sup>9</sup>	AM945332 <sup>9</sup>	–
<i>Augusta austrocaledonica</i> (Brongn.) J.H. Kirkbr.		HM164352 <sup>1</sup>	EU817412 <sup>11</sup>	HM164193 <sup>1</sup>	EU817454 <sup>11</sup>	–
<i>Augusta longifolia</i> (Spreng.) Rehder		HM164353 <sup>1</sup>	–	AF242913 <sup>6</sup>	HM164309 <sup>1</sup>	–
<i>Augusta rivalis</i> (Benth.) J.H. Kirkbr.		AM949846 <sup>3</sup>	AM949842 <sup>3</sup>	HM164194 <sup>1</sup>	FM207118 <sup>3</sup>	–
<i>Benkara scandens</i> (Thunb.) Ridsdale	XPW160 (IBSC)	KJ680406	KJ680431	KJ680456	KJ680495	–
<i>Bertierra aethiopica</i> Hiern		HM164355 <sup>1</sup>	HM164157 <sup>1</sup>	HM164195 <sup>1</sup>	HM164310 <sup>1</sup>	–
<i>Boholia nematostylis</i> Merr.		AM949848 <sup>3</sup>	AM117210 <sup>12</sup>	AM117286 <sup>12</sup>	HM164312 <sup>1</sup>	–
<i>Burchellia bubalina</i> (L. f.) Sims		HM164358 <sup>1</sup>	Z68833 <sup>13</sup>	HM164198 <sup>1</sup>	HM164314 <sup>1</sup>	–
<i>Caelospermum monticola</i> Baill. ex Guillaumin		AM945255 <sup>9</sup>	AF331644 <sup>14</sup>	AF001438 <sup>15</sup>	AM945334 <sup>9</sup>	–
<i>Captaincookia margaretae</i> N. Hallé		HM164360 <sup>1</sup>	EU817415 <sup>11</sup>	EU817436 <sup>11</sup>	EU817456 <sup>11</sup>	–
<i>Catesbaea spinosa</i> L.		AM117343 <sup>12</sup>	X83628 <sup>7</sup>	AF004032 <sup>15</sup>	AF152706 <sup>16</sup>	–
<i>Cephalanthus occidentalis</i> L.		AJ236288 <sup>4</sup>	X83629 <sup>7</sup>	AF004033 <sup>15</sup>	AJ346955 <sup>17</sup>	–
<i>Chassalia curviflora</i> (Wall.) Thwaites	XPW165 (IBSC)	KJ680400	KJ680425	KJ680450	KJ680489	–
<i>Coffea arabica</i> L.		EF044213 <sup>18</sup>	EF044213 <sup>18</sup>	EF044213 <sup>18</sup>	EF044213 <sup>18</sup>	–
<i>Coussarea hydrangeifolia</i> (Benth.) Benth. & Hook. f. ex Müll. Arg.		EU145422 <sup>8</sup>	EU145460 <sup>8</sup>	EU145501 <sup>8</sup>	EU145549 <sup>8</sup>	–
<i>Cremaspora triflora</i> subsp. Confluens (K. Schum.) Verdc.		AM949850 <sup>3</sup>	Z68856 <sup>13</sup>	FM204715 <sup>3</sup>	FM207121 <sup>3</sup>	–
<i>Crossopteryx febrifuga</i> (Afzel. ex G. Don) Benth.		AM949851 <sup>3</sup>	AM117223 <sup>12</sup>	FM204717 <sup>3</sup>	FM207123 <sup>3</sup>	–
<i>Cruckshanksia hymenodon</i> Hook. & Arn.		–	AJ288599 <sup>10</sup>	EU145502 <sup>8</sup>	EU145550 <sup>8</sup>	–
<i>Doricera trilocularis</i> (Balf. f.) Verdc.		HM164364 <sup>1</sup>	EU817417 <sup>11</sup>	EU817437 <sup>11</sup>	EU817457 <sup>11</sup>	–
<i>Duperrea pavettifolia</i> (Kurz) Pit.	XPW251 (IBSC)	KJ680407	KJ680432	KJ680457	KJ680496	–
<i>Exostema spinosum</i> (La Vavass) Krug & Urb.		GQ852204 <sup>19</sup>	AY205350 <sup>20</sup>	AF242947 <sup>21</sup>	AY763830 <sup>22</sup>	–
<i>Faramea multiflora</i> A. Rich. ex DC.		EU145424 <sup>7</sup>	Z68796 <sup>23</sup>	AF004048 <sup>15</sup>	AF102422 <sup>24</sup>	–
<i>Gardenia hansemannii</i> K. Schum.		AM949852 <sup>3</sup>	AJ318446 <sup>25</sup>	AJ320077 <sup>25</sup>	FM207126 <sup>3</sup>	–
<i>Gelsemium sempervirens</i> (L.) J. St.-Hil.		AJ011984 <sup>26</sup>	L14397 <sup>27</sup>	AJ431033 <sup>28</sup>	AJ430908 <sup>28</sup>	–
<i>Glonnetia sericea</i> (Baker) Tirveng.		HM536207 <sup>29</sup>	HM536223 <sup>29</sup>	HM536229 <sup>29</sup>	HM536235 <sup>29</sup>	–
<i>Guihaiothamnus acaulis</i> H.S. Lo	XPW289 (IBSC)	KJ680408	KJ680433	KJ680458	KJ680497	KJ680475
<i>Guihaiothamnus acaulis</i> H.S. Lo	XPW290 (IBSC)	KJ680409	KJ680434	KJ680459	KJ680498	KJ680476
<i>Heinsia crinita</i> (Afzel.) G. Taylor		HM164372 <sup>1</sup>	Y11849 <sup>30</sup>	HM164210 <sup>1</sup>	HM164323 <sup>1</sup>	–
<i>Henriquezia nitida</i> var. <i>macrophylla</i> (Ducke) Steyererm.		HM164374 <sup>1</sup>	–	HM164211 <sup>1</sup>	HM164325 <sup>1</sup>	–
<i>Ixora coccinea</i> L.		HM164376 <sup>1</sup>	HM164167 <sup>1</sup>	EF205641 <sup>31</sup>	EU817464 <sup>11</sup>	–
<i>Jackiopsis ornata</i> (Wall.) Ridsdale		HM536213 <sup>29</sup>	HM536219 <sup>29</sup>	HM536225 <sup>29</sup>	HM536231 <sup>29</sup>	–
<i>Leptodermis</i> sp.	XPW136 (IBSC)	KJ680404	KJ680429	KJ680454	KJ680493	–
<i>Luculia gratissima</i> (Wall.) R. Sweet		AJ011987 <sup>26</sup>	AM117243 <sup>12</sup>	AJ431036 <sup>28</sup>	AJ430911 <sup>28</sup>	–
<i>Morinda citrifolia</i> L.		AJ236300 <sup>4</sup>	AJ318448 <sup>25</sup>	AJ320078 <sup>25</sup>	AF152616 <sup>16</sup>	–
<i>Morinda longiflora</i> G. Don		–	–	GQ463275 <sup>32</sup>	FJ906986 <sup>33</sup>	–
<i>Morinda lucida</i> Benth.		–	–	GQ463276 <sup>33</sup>	FJ907010 <sup>32</sup>	–
<i>Mussaenda erythrophylla</i> Schumach. & Thonn.		AJ130836 <sup>4</sup>	X83652 <sup>7</sup>	EU145493 <sup>8</sup>	EU145535 <sup>8</sup>	–
<i>Mycetia gracilis</i> Craib	YS134 (IBSC)	KJ680403	KJ680428	KJ680453	KJ680492	–
<i>Mycetia hirta</i> Hutch.	YS79 (IBSC)	KJ680401	KJ680426	KJ680451	KJ680490	–
<i>Myrioneuron tonkinense</i> Pit.	YS68 (IBSC)	KJ680402	KJ680427	KJ680452	KJ680491	–
<i>Nauclea orientalis</i> (L.) L.		EU145410 <sup>8</sup>	X83653 <sup>7</sup>	AJ320080 <sup>25</sup>	AJ346958 <sup>17</sup>	–
<i>Oxyanthus speciosus</i> DC.		HM164384 <sup>1</sup>	AM117252 <sup>12</sup>	AM117330 <sup>12</sup>	AM117375 <sup>12</sup>	–
<i>Pavetta abyssinica</i> Fresen.		AM949854 <sup>3</sup>	Z68863 <sup>13</sup>	FM204726 <sup>3</sup>	FM207133 <sup>3</sup>	–
<i>Pentas lanceolata</i> (Forssk.) Deflers		AJ236304 <sup>4</sup>	L13931 <sup>27</sup>	AM266875 <sup>34</sup>	AM266963 <sup>34</sup>	–
<i>Posoqueria longiflora</i> Aubl.		HM164386 <sup>1</sup>	HM164175 <sup>1</sup>	HM164218 <sup>1</sup>	HM164332 <sup>1</sup>	–
<i>Retiniphyllum pilosum</i> (Spruce ex Benth.) Müll.Arg.		HM164392 <sup>1</sup>	AF331654 <sup>14</sup>	AF004076 <sup>15</sup>	FM207137 <sup>3</sup>	–
<i>Rubia tinctorum</i> L.		DQ359167 <sup>35</sup>	X83666 <sup>7</sup>	–	FJ695421 <sup>36</sup>	–
<i>Saprosma foetens</i> (Wight) K. Schum.		–	DQ662193 <sup>37</sup>	DQ662218 <sup>37</sup>	DQ662168 <sup>37</sup>	–
<i>Scyphiphora Hydrophyllace</i> C.F. Gaertn.		AJ236311 <sup>4</sup>	Y18717 <sup>4</sup>	EU817450 <sup>11</sup>	EU817475 <sup>11</sup>	–
<i>Sipanea hispida</i> Benth. ex Wernham		EU145414 <sup>8</sup>	EU145458 <sup>8</sup>	EU145492 <sup>8</sup>	HM164336 <sup>1</sup>	–
<i>Spermadictyon suaveolens</i> Roxb.		FJ695338 <sup>36</sup>	Z68824 <sup>23</sup>	DQ662219 <sup>37</sup>	DQ662171 <sup>37</sup>	–
<i>Steenisia pleurocarpa</i> (Airy Shaw) Bakh. f.		HM164396 <sup>1</sup>	AM117279 <sup>12</sup>	FM204735 <sup>3</sup>	FM207142 <sup>3</sup>	–
<i>Tarennoidea wallichii</i> (Hook. f.) Tirveng. & Sastre	XPW243 (IBSC)	KJ680405	KJ680430	KJ680455	KJ680494	–
<i>Trailliaedoxa gracilis</i> W.W. Sm. & G. Forrest		HM164400 <sup>1</sup>	HM164183 <sup>1</sup>	HM164227 <sup>1</sup>	HM164339 <sup>1</sup>	–
<i>Uncaria rhynchophylla</i> (Miq.) Miq. ex Havil.		GQ852289 <sup>19</sup>	X83669 <sup>7</sup>	AB178637 <sup>38</sup>	AJ346959 <sup>17</sup>	–
<i>Versteegia cauliflora</i> (K. Schum. & Lauterb.) Valetton		HM164406 <sup>1</sup>	EU817433 <sup>11</sup>	EU817476 <sup>11</sup>	EU817451 <sup>11</sup>	–
<i>Virectaria multiflora</i> (Sm.) Bremek.		HM164407 <sup>1</sup>	Y11861 <sup>30</sup>	HM164233 <sup>1</sup>	HM164345 <sup>1</sup>	–
<i>Wendlandia aberrans</i> F.C. How	YS74 (IBSC)	KJ680410	KJ680435	KJ680460	KJ680499	KJ680477
<i>Wendlandia arabica</i> Deflers		HM164402 <sup>1</sup>	HM164185 <sup>1</sup>	HM164230 <sup>1</sup>	HM164341 <sup>1</sup>	–
<i>Wendlandia cavaleriei</i> H. Lév.	XPW193 (IBSC)	KJ680411	KJ680436	KJ680461	KJ680500	KJ680478
<i>Wendlandia formosana</i> Cowan		HM164403 <sup>1</sup>	HM164186 <sup>1</sup>	HM164231 <sup>1</sup>	HM164342 <sup>1</sup>	–
<i>Wendlandia guangdongensis</i> W.C. Chen	Wang1555 (IBSC)	KJ680412	KJ680437	KJ680462	KJ680501	KJ680479
<i>Wendlandia ligustroides</i> (Boiss. & Hohen.) Blakelock		HM164404 <sup>1</sup>	HM164187 <sup>1</sup>	AF243036 <sup>5</sup>	HM164343 <sup>1</sup>	–
<i>Wendlandia merrilliana</i> Cowan	XPW270 (IBSC)	KJ680413	KJ680438	KJ680463	KJ680502	KJ680480
<i>Wendlandia paniculata</i> (Roxb.) DC.		HM164405 <sup>1</sup>	HM164188 <sup>1</sup>	HM164232 <sup>1</sup>	HM164344 <sup>1</sup>	–
<i>Wendlandia pingpiensis</i> F.C. How	XPW104 (IBSC)	KJ680414	KJ680439	KJ680464	KJ680503	–
<i>Wendlandia scabra</i> Kurz	XPW102 (IBSC)	KJ680415	KJ680440	KJ680465	KJ680504	–

(continued on next page)

Table 1 (continued)

Species	Vouchers	GenBank accession				
		<i>ndhF</i>	<i>rbcL</i>	<i>rps16</i>	<i>TrnT-F</i>	<i>ITS</i>
<i>Wendlandia</i> sp.	XPW101 (IBSC)	KJ680424	KJ680449	KJ680474	KJ680513	–
<i>Wendlandia speciosa</i> Cowan	XPW134 (IBSC)	KJ680416	KJ680441	KJ680466	KJ680505	KJ680481
<i>Wendlandia subalpina</i> W.W. Sm.	XPW293 (IBSC)	KJ680417	KJ680442	KJ680467	KJ680506	KJ680482
<i>Wendlandia tinctoria</i> subsp. <i>tinctoria</i>		AM949860 <sup>3</sup>	FM207649 <sup>3</sup>	FM204739 <sup>3</sup>	FM207147 <sup>3</sup>	–
<i>Wendlandia tinctoria</i> (Roxb.) DC.	XPW291 (IBSC)	KJ680419	KJ680444	KJ680469	KJ680508	KJ680484
<i>Wendlandia tinctoria</i> subsp. <i>intermedia</i> (F.C. How) W.C. Chen	XPW109 (IBSC)	KJ680418	KJ680443	KJ680468	KJ680507	KJ680483
<i>Wendlandia uvariifolia</i> subsp. <i>uvariifolia</i>	XPW262 (IBSC)	KJ680420	KJ680445	KJ680470	KJ680509	KJ680485
<i>Wendlandia uvariifolia</i> subsp. <i>uvariifolia</i>	XPW272 (IBSC)	KJ680421	KJ680446	KJ680471	KJ680510	KJ680486
<i>Wendlandia uvariifolia</i> subsp. <i>uvariifolia</i>	XPW284 (IBSC)	KJ680422	KJ680447	KJ680472	KJ680511	KJ680487
<i>Wendlandia uvariifolia</i> subsp. <i>Chinensis</i> (Merr.) Cowan	XPW285 (IBSC)	KJ680423	KJ680448	KJ680473	KJ680512	KJ680488

Published sequences: <sup>1</sup>Kainulainen et al. (2013); <sup>2</sup>Lantz and Bremer (2004); <sup>3</sup>Kainulainen et al. (2009); <sup>4</sup>Bremer et al. (1999); <sup>5</sup>Andreasen et al. (1999); <sup>6</sup>Rova (unpubl. data); <sup>7</sup>Bremer et al. (1995); <sup>8</sup>Rydin et al. (2008); <sup>9</sup>Razafimandimbison et al. (2008); <sup>10</sup>Bremer and Manen (2000); <sup>11</sup>Mouly et al. (2009); <sup>12</sup>Bremer and Eriksson (2009); <sup>13</sup>Andreasen and Bremer (1996); <sup>14</sup>Andersson (unpubl. data); <sup>15</sup>Andersson and Rova (1999); <sup>16</sup>Rova et al. (2002); <sup>17</sup>Razafimandimbison and Bremer (2002); <sup>18</sup>Nalapalli et al. (unpubl. data); <sup>19</sup>Manns and Bremer (2010); <sup>20</sup>McDowell et al. (2003); <sup>21</sup>Rova (unpubl. data); <sup>22</sup>Motley et al. (2005); <sup>23</sup>Bremer (1996); <sup>24</sup>Struwe et al. (1998); <sup>25</sup>Novotny et al. (2002); <sup>26</sup>Oxelman et al. (1999); <sup>27</sup>Olmstead et al. (1993); <sup>28</sup>Bremer et al. (2002); <sup>29</sup>Razafimandimbison et al. (2011); <sup>30</sup>Bremer and Thulin (1998); <sup>31</sup>Mouly et al. (2007); <sup>32</sup>Razafimandimbison et al. (2009); <sup>33</sup>Razafimandimbison et al. (2010); <sup>34</sup>Karehed and Bremer (2007); <sup>35</sup>Manen (unpubl. data); <sup>36</sup>Rydin et al. (2009); <sup>37</sup>Backlund et al. (2007); <sup>38</sup>Aoki et al. (2004).

bootstrap replicates. In each replicate, we performed 10 random addition-sequence replicates.

Bayesian inference (BI) analyses were carried out using MrBayes 3.2.2 (<http://mrbayes.sourceforge.net>). The evolutionary models of the best substitution types and rate distribution models for the combined DNA dataset was determined by the Akaike Information Criterion (AIC) using MrModelTest version 2.3 (Nylander, 2008). The best-fit model of GTR + G + I was identified for all the partitions. Markov chain Monte Carlo (MCMC) analyses were performed with a random starting tree and four chains (one cold and three heated). The chain of the MCMC was sampled every 100th generations from the cold chain. We stopped the MCMC after 5,000,000 generations because the value of average standard deviation was below 0.01, suggesting that the tree samples from the two simultaneous runs became increasingly similar. For the calculation of the Bayesian posterior probabilities (PP), the burn-in period was the first 25% of the sampled generations as determined by the program Tracer version 1.5 (Rambaut and Drummond, 2007).

#### 2.4. Molecular dating

We dated the age of *Guihaiothamnus* and other *Wendlandias* based on a broad phylogenetic framework of Rubiaceae inferred from sequences of the four plastid DNA regions. We constrained ages for five nodes in our phylogeny according to the five reliable fossil records in Rubiaceae followed Nie et al. (2013). We compared likelihood scores for clock and non-clock models using a likelihood ratio (LR) test (Felsenstein, 1981) to determine whether our data conformed to the molecular clock assumption. The LR was calculated as  $2 * (\ln L_{\text{clock}} - \ln L_{\text{nonclock}})$  and assumed to follow a chi-squared distribution with the number of degrees of freedom ( $n$ ) equals to the number of taxa minus two. The assumption of rate constancy was rejected in our study because the constrained and unconstrained analyses differed significantly (LR = 3561.36, d.f. = 81,  $P = 0$ ). We then employed a Bayesian method which allows a relaxed evolutionary model to estimate the divergence time. The Bayesian analysis was conducted using the software BEAST 1.7 (Drummond et al., 2012). The BEAUti was used to set criteria for the analysis. Using the AIC estimated by MrModelTest and the Bayes Factor (BF) calculated by Tracer version 1.5 (Rambaut and Drummond, 2007), we chose the GTR + G + I as the best nucleotide substitution models. In addition, we assumed an uncorrelated lognormal model of rate variation among branches in the

tree and employed a Yule prior on the birth rate of new lineages (Drummond et al., 2006).

The MCMC simulations were run with 10,000,000 iterations to generate trees by sampling every 1000. We discarded the first 10% of the iterations as burn-in and analyzed the log-files in Tracer to evaluate the effective sample sizes (ESS) and the MCMC convergence within chains. We combined the resulting trees using TreeAnnotator version 1.5.2 (part of the BEAST package). The final tree was checked and edited in FigTree version 1.4.1 (Rambaut, 2012). The divergence times are given as the mean and the 95% highest posterior density (HPD) in millions of years (my).

#### 2.5. Palynology

Pollen samples for palynological observation were collected from the two populations. The palynological study was made from fresh flowers of *G. acaulis* fixed in formalin–acetic acid–alcohol (FAA). Before observations, we carefully dissected and squeezed anthers with tweezers to release pollen. After that, we washed the pollen grains with distilled water three times, and then dehydrated them in a progressive ethanol solution from 30% to 70% in an ultrasonic bath. Protocols for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) study followed Xie and Zhang (2010).

Descriptive terminology of pollen follows Punt et al. (2007). For shape classes (P/E), we adopted the wide definitions of Nilsson and Pragowski (1992): oblate (0.50–0.75), suboblate (0.75–0.88), spheroidal (0.88–1.14), subprolate (1.14–1.33), and prolate (1.33–2.00). Pollen size classes were plotted following Dessein et al. (2005): very small, <20  $\mu\text{m}$ ; small, 20–30  $\mu\text{m}$ ; medium, 30–40  $\mu\text{m}$ .

#### 2.6. Cytology

We germinated seeds collected from Sanfang population to get the root tips for cytological observation. Actively growing root tips were pretreated in a saturated santochlor solution for about 3 h at 4 °C, washed with distilled water, fixed in Carnoy's Fluid (95% ethanol:acetic acid = 3:1) for 1 h at 4 °C, then washed three times in distilled water and then hydrolyzed in 1 mol/L hydrochloric acid at 60 °C for 5–6 min. After being washed with distilled water for three times, root tips were stained in Carbol Fuchsin solution for more than 2 h and then squashed on slides for light microscopy observation. The chromosome numbers of at least five metaphase cells were counted.



### 3. Results

#### 3.1. Populations and habitats

Before this study, *Guihailiothamnus acaulis* was only known from four specimens collected in 1950s from two localities of Rongshui County, Guangxi Province, i.e. Luodong (S. Chen15619 and D. Chen594) and Zhongzhai (S. Chen8783 and W. Rao64009). In this study, we found two additional populations in valleys on the same mountain, which are 30 km apart from each other. The first locality, Huaibao, is 150 m high and hosts ca. 90 individuals of *G. acaulis*. The individuals of this locality are almost stemless and branchless, except for several that have branches up to 10 cm long (Fig. 1B). In the second locality, Sanfang, we recorded ca. 200 individuals on three cliffs, with altitudes of 420 m, 465 m and 480 m, respectively. Unlike the Huaibao population, no individuals with elongated stems or branches were recorded. The microhabitats for *G. acaulis* in both valleys are big cliffs shaded by trees of Fagaceae, Lauraceae, and Theaceae. The companion species of *G. acaulis* are mainly of moss, ferns and Gesneriaceae in both valleys. Comparing to the habitat of the second locality, the first one is richer in soil and less shady.

#### 3.2. Phylogenetic relationships

The phylogenies inferred from any single DNA region are congruent concerning the well-supported clades, we thus combined the five data sets to generate a super data matrix, which consisted of 7331 characters. In the combined most parsimonious (MP) analyses, 1883 characters were variable, of which 993 were potentially parsimony-informative. The MP analyses resulted in 486 equally most parsimonious trees (MPTs) with a tree length of 3048 steps, a consistency index of 0.78, a retention index of 0.77, and a rescaled consistency index of 0.60.

A strict consensus of the MPTs obtained in PAUP\* indicates a backbone phylogenetic pattern congruent to the Bayesian result (Fig. 2). The clade *Augusta-Wendlandia* within the subfamily Dialypetalanthoideae was supported, and *Wendlandia* was strongly supported as monophyletic if including *Guihailiothamnus* within it. Three clades were recovered in the *Wendlandia-Guihailiothamnus* clade (clades I–III in Fig. 2). *Wendlandia ligustroides* (Boiss. & Hohen.) Blakelock, a species from western Turkey to northwestern Iraq, was firstly diverged and was sister to clades II and clades III collectively. Clade II comprised the type species *W. paniculata* (Roxb.) DC. from India, and species from the Arabic Peninsula (*W. arabica* Deflers) and China (*W. speciosa* Cowan, *W. scabra* Kurz, *W. pingpiensis* F.C. How, *W. tinctoria* (Roxb.) DC., *W. cavaleriei* H. Lév., and *W. aberrans* F.C. How). Species in this clade are morphologically variable, including species with either small ovoid or large linear anthers, either triangular, cuspidate, or rounded stipules. The voucher XPW101, as resolved to be a sister of *W. scabra*, bears no flowers and fruits. It resembles *W. scabra* by have rough haired-leaves and rounded stipules, but is more comparable to *W. uvariifolia* Hance vegetatively, of which leaves and stipules larger than *W. scabra*. Clade III consisted of *Guihailiothamnus* and several *Wendlandia* species from Taiwan Island (*W. formosana* Cowan), Hainan Island (*W. merrilliana* Cowan) and the mainland of China (*W. subalpina* W.W. Sm, *W. guangdongensis* C.H. Chen, and *W. uvariifolia*). Two members of Series Montigenae, *W. subalpina* and *W. merrilliana*, belong to clade III, but they were not directly related in our phylogeny. The two samples of *G. acaulis* are identical in all the five sequence regions, and the sister relationship to *W. guangdongensis*–*W. uvariifolia*–*W. formosana* is strongly supported.

#### 3.3. Divergence times

Using a combination of four plastid DNA regions calibrated with five fossil records from Rubiaceae, we estimated the split between *Augusta* and *Wendlandia* to be 17.46 (95% HPD: 12.10–22.70) my. The crown age of *Augusta* is 9.09 (95% HPD: 4.61–14.12) my, and that of *Wendlandia* is 12.57 (95% HPD: 8.03–17.37) my. The split between *Guihailiothamnus* and related *Wendlandias* happened at 2.11 (95% HPD: 0.76–3.79) mya. The divergence times for the nodes in the phylogeny are shown in Fig. 3.

#### 3.4. Palynological characters

Like most *Wendlandia* species (Xie and Zhang, 2010), pollen grains of *G. acaulis* occur in monads and spheroidal in equatorial view and circular in polar view, very small in size ( $P = 17.47 \pm 2.68$  ( $n = 20$ )  $\mu\text{m}$ ,  $E = 18.13 \pm 2.69$  ( $n = 20$ )  $\mu\text{m}$ ), coarsely reticulate exine ornamentation and tricolporate. Each compound aperture comprises an ectocolpus, a mesoporus and an endoaperture. The ectocolpus is long and wide with obtuse or acute ends, and the ectocolpus membrane is slightly granular. The mesoaperture is a circular to oval pore situated in the middle of the ectocolpus at equator (Fig. 1G and H). In fractured pollens, endoapertures are endocolpi perpendicular with ectocolpi. The ends of endocolpi are fishtail-shaped, and the two ends are almost connected with each other to form an endocingulum. The inner surface of pollen is granular (Fig. 1I).

The pollen wall of *G. acaulis* is composed of exine and intine. The exine has four distinct layers: tectum, columellae layer, foot layer and endexine. The tectum is discontinuous because of the exine ornamentation. The columellae layer is the thickest in the pollen wall and constituted by columellae which are irregular in shape and length. The foot layer has little variation in thickness around the pollen except for the aperture region. The endexine is separated from the foot layer by a white line centered lamellae (WLCL) that can be observed in TEM sections (Fig. 1J and K). The endexine is the thinnest layer of exine but it thickens into costa surrounding the aperture, while other layers of exine are thinned into a slim layer at the same region (Fig. 1K). Costa could also be observed from the inner surface (Fig. 1I).

The intine is differentiated into two distinguishable layers (Fig. 1J). Intine 1 is the out layer with low electron density. The inner layer, intine 2, has relatively high electron density and its lower margin being in contact with the protoplast. The intine thickens and protrudes from the aperture forms a protruding oncus (Fig. 1K).

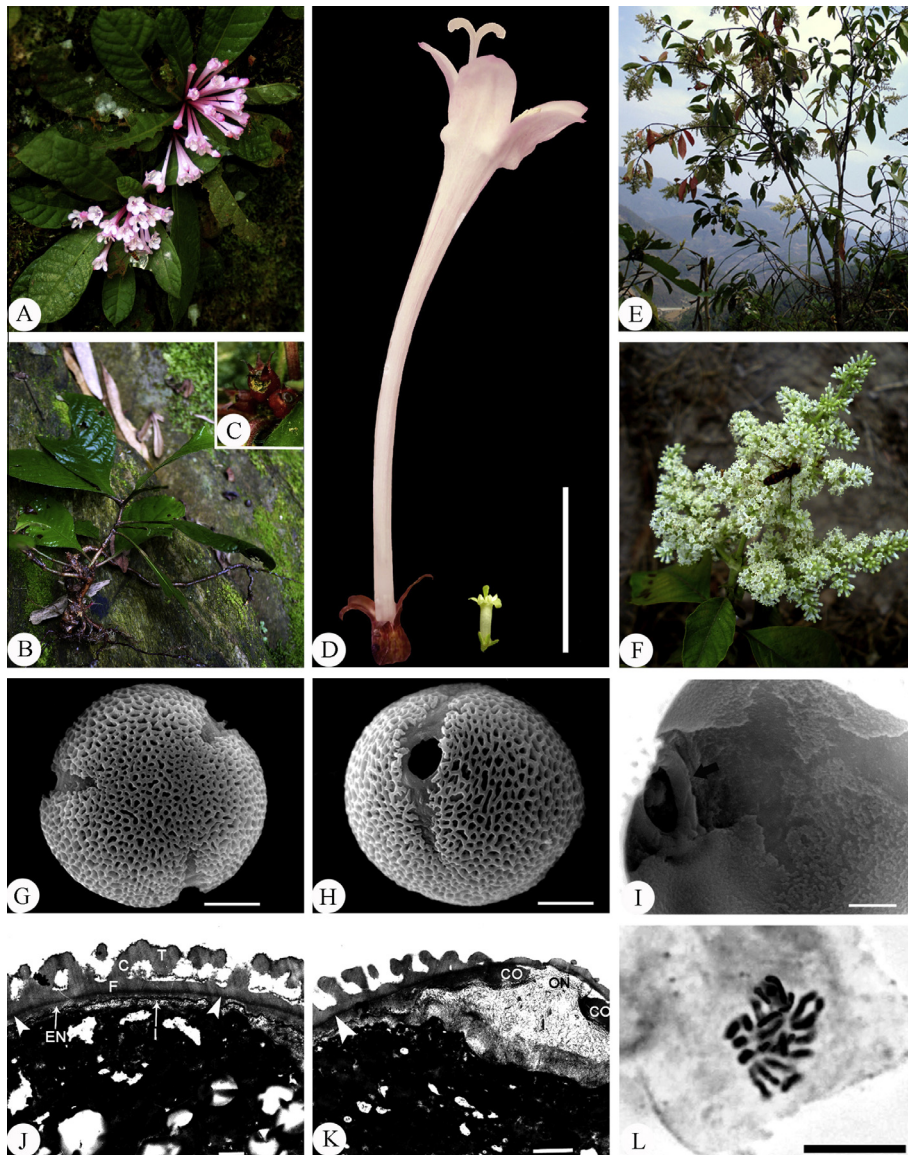
#### 3.5. Cytological characters

The chromosome number of *G. acaulis* in mitotic metaphase cells was counted to be  $2n = 22$ . The karyotype is not available because the chromosomes are too small (Fig. 1L).

### 4. Discussion

#### 4.1. The phylogenetic position of *Guihailiothamnus* and its taxonomic implications

Previous molecular phylogenetic studies have shown that the Asian *Wendlandia* is grouped with the South-Central American and Pacific *Augusta*, and is then sister to a clade of Alberteae–Bertiareae–Coffeae–Gardenieae (Kainulainen et al., 2013; Razafimandimbison et al., 2011; Robbrecht and Manen, 2006; Rova et al., 2002). This phylogenetic relationship led Kainulainen



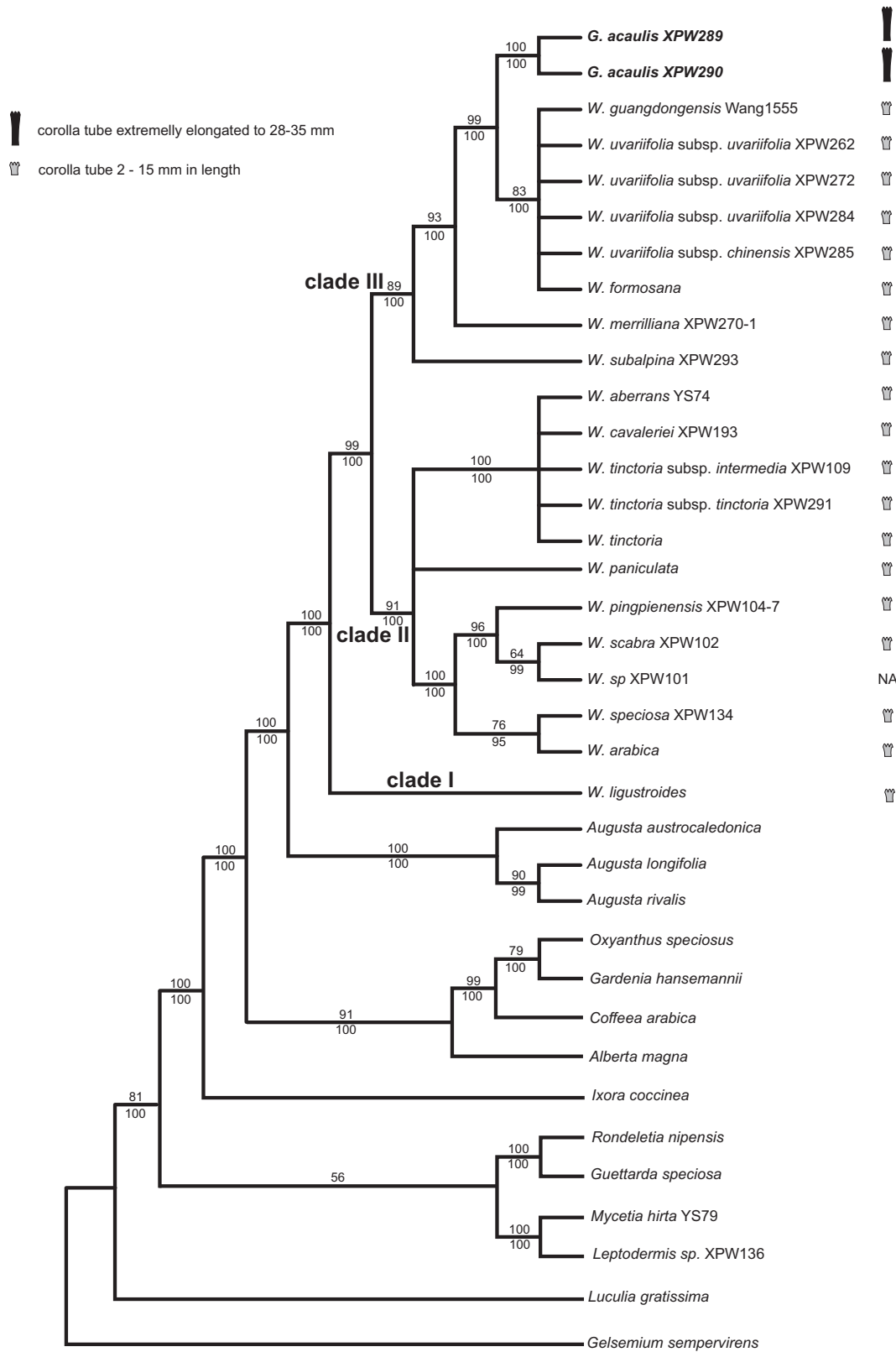
**Fig. 1.** (A–C) *Guihaiiothamnus acaulis*. (A) Showing the rosette shrub habit of *G. acaulis*, with a few but showy and long-tubed flowers clustering on the top of reduced stems. (B) The tallest individual we have found in Huaibao population. (C) The fruit of *G. acaulis* is indehiscent, ca. 3 mm in diameter. (D) The flower of *G. acaulis* is  $28.37 \pm 1.49$  mm in length, almost 10 times than those of *W. uvariifolia* subsp. *chinensis* ( $3.67 \pm 0.48$  mm). Scale bar: 1 cm. (E and F) *W. uvariifolia* subsp. *chinensis*. (E) Showing habit as a big shrub. (F) A single inflorescence is composed of more than one thousand tiny flowers. (G–I) Scanning electron microscopy (SEM) images of *G. acaulis*. (G) Polar view. Scale bar: 5  $\mu$ m. (H) Equatorial view. Scale bar: 5  $\mu$ m. (I) Inner view showing the endexine thickened costa (black arrow) and granular inner surface. Scale bar: 1  $\mu$ m. (J and K) Transmission electron microscopy (TEM) images of the pollen wall of *G. acaulis*. (J) Showing the tectum (T), columellae layer (C), foot layer (F), endexine (EN), intine (I) and white line centered lamellae (white arrow head). Scale bar: 500 nm. (K) Showing the thickened costa (CO) and protruding oncus (ON). Scale bar: 1  $\mu$ m. (L) The chromosome number of *G. acaulis* is  $2n = 22$ . Scale bar: 10  $\mu$ m.

et al. (2013) to describe the new tribe, Augusteae, to accommodate the two genera. Lacking molecular information of *Guihaiiothamnus*, previous authors cautiously suspended it to *Wendlandia* (Lo, 1998, 1999; Wu et al., 2005). By expanding sampling in *Wendlandia*, our analysis corroborates previous studies concerning the close relationship of *Wendlandia* and *Augusta*, and for the first time reveals that *Guihaiiothamnus* is phylogenetically nested within the former genus, being sister to a clade formed by *W. uvariifolia*, *W. formosana* and *W. guangdongensis*.

Palynological survey indicates that the pollen morphology of *G. acaulis*, including pollen size, shape, exine and nexine ornamentation, aperture type and endo-aperture type, are consistent with that of *Wendlandia*. Pollen wall structure of *G. acaulis* and *Wendlandia* are basically the same, except for the white line centered lamellae (WLCL), which is not reported in the studied *Wendlandia*

species (Xie and Zhang, 2010). WLCL is a junction area between foot layer and endexine, it presents in unrelated rubiaceous groups (*Rondeletia odorata* Jacq. of Cinchonoideae (El-Ghazaly et al., 2001); *Tarenna gracilipes* (Hayata) Ohwi of Dialypetalanthoideae (Vinckier and Smets, 2005)) makes it an uninformative character in Rubiaceae classification. Pollen morphology has supported the transfer of *Wendlandia* from Rondeletieae (subfamily Cinchonoideae) to subfamily Dialypetalanthoideae (Xie and Zhang, 2010). So the high uniformity of palynological characters of *Guihaiiothamnus* and *Wendlandia* confirms their close relationship, and in addition, suggests the phylogenetic position of *Guihaiiothamnus* in Dialypetalanthoideae.

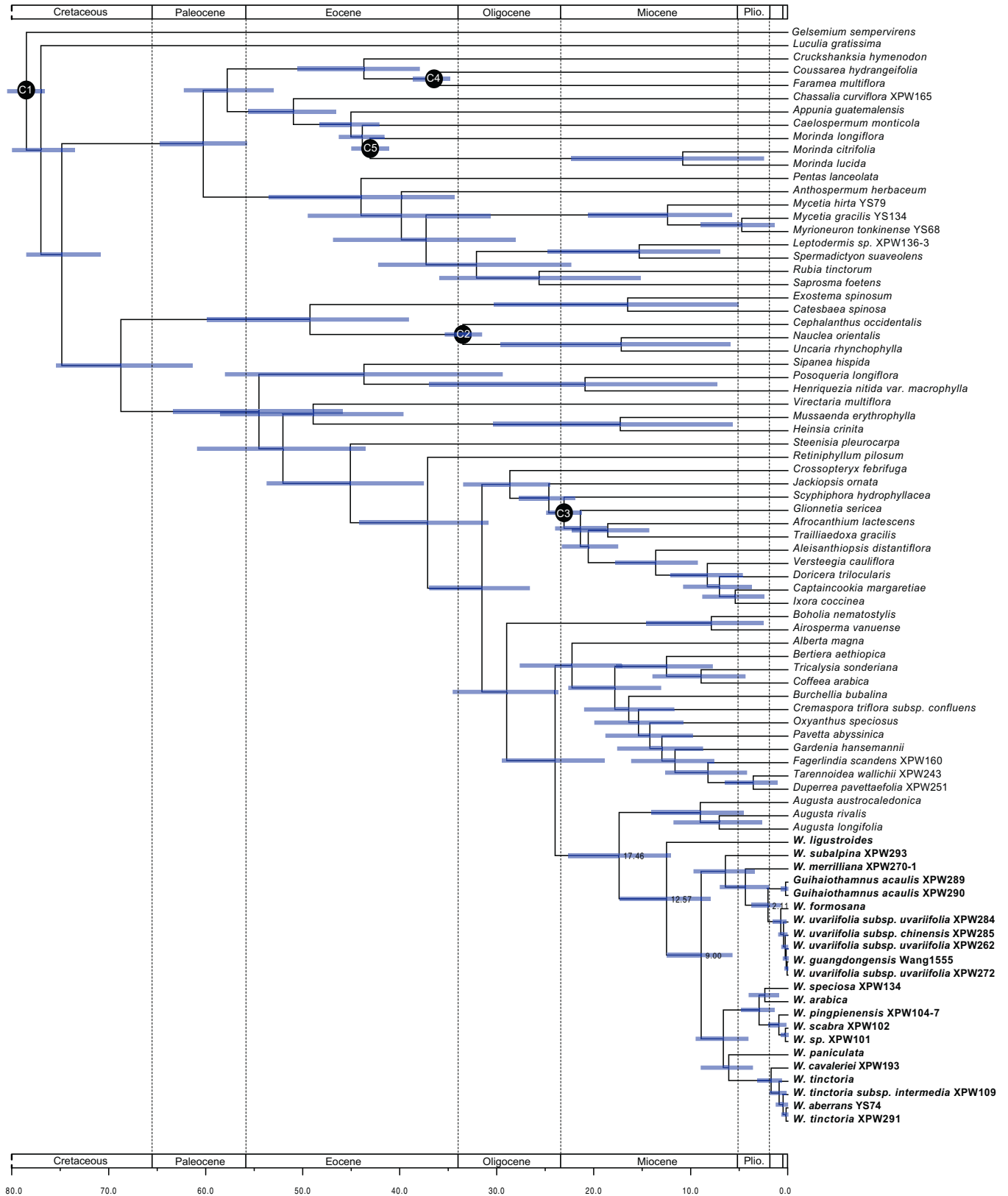
The chromosome number of *G. acaulis* is  $2n = 22$ . The basic number and ploidy level is the same with that of reported *Wendlandia* species (Bedi et al., 1981; Kiehn, 1986; Sandhu and Mann,



**Fig. 2.** Strict consensus of 2383 equally most parsimonious trees based on maximum parsimony analysis of the combined matrix of *ITS* and four plastid regions (*ndhF*, *rbcl*, *rps16*, and *trnT-F*). The shortest trees had a length of 3048 steps, CI = 0.78 and RI = 0.77. Numbers above and below branches are maximum parsimony bootstrap percentages and Bayesian analysis posterior probabilities respectively.

1988; Oginuma et al., 1994). Kiehn (1995) stated that the chromosome number gave few hints for phylogenetic relationships and taxonomies in Rubiaceae without other morphological or karyological information. However, in the same paper, he pointed

out that *Wendlandia* was the only genus with diploid taxa in the Rondeletieae, and its chromosomes were generally smaller than those of other Rondeletieae (Kiehn, 1995). Although cytological data could not help to demonstrate the systematic position of



**Fig. 3.** Bayesian divergence time estimates of *Guihaiothamnus* and *Wendlandia* based on the combined sequence data from four plastid gene markers (*ndhF*, *rbcl*, *rps16*, and *trnT-F*). Five fossil calibration points were marked as C1–C5. The blue bars on the nodes indicate 95% posterior credibility intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*Guihaiothamnus* in Rubiaceae, it confirms its close relationship with *Wendlandia*, and maintains them in the tribe Augusteae.

Combining evidence from molecular phylogeny, palynology and cytology, we hereby propose a new combination for *G. acaulis*,



transfer it to the genus *Wendlandia*, and reduce *Guihaiiothamnus* to synonymy. A description with updated morphological ranges and chromosome number is given here.

*Wendlandia acaulis* (H.S. Lo) P.W. Xie & D.X. Zhang, comb. nov.  $\equiv$  *Guihaiiothamnus acaulis* H.S. Lo in Lo, Bull. Bot. Res. 18: 275–283. 1998. – Holotype: CHINA, Guangxi Province, Rongshui County; on shady rocks near roadside, alt. 180 m; 28 Apr. 1955, S.H. Chen 8783 (IBSC!).

Dwarf subshrubs; stems very short, round, densely villosulous, becoming glabrescent; branches absent or occasionally 2–3, up to 10 cm long, round. Leaves rosettes on stemless and branchless individuals, or arranged sparsely on elongated branches; petiole 5–20 mm, villosulous; leaf blade drying papery, green adaxially and grayish green to deep red abaxially, broadly elliptic-oblong to obovate, 4.5–11  $\times$  2.5–7.5 cm, adaxially sparsely hirtellous or glabrescent, abaxially sparsely hirtellous or moderately to densely tomentulose to strigillose on principal veins, base cuneate to truncate and often shortly decurrent, apex rounded or broadly obtuse and shortly cuspidate; secondary veins 8–12 pairs, slender; stipules 1–2 mm, obtuse and partially reflexed. Inflorescences 1 or 2 on stem terminal or in leaf axils at branchlet apex, 9–25 flowered, peduncle very short; bracts 1.5–1.7 mm. Calyx pilosulous, villosulous, or glabrous; hypanthium portion obovoid-ellipsoid, ca. 1.7 mm; lobes linear-lanceolate to narrowly triangular, 1.7–2 mm, acute to acuminate. Corolla narrow trumpet-shaped, pale red with the throat usually darker than the base, hirsute inside and glabrous outside; tube 25–33 mm, ca. 2.8 mm in diam. at base, 10 mm in diam. at throat, sparsely villous inside; lobes elliptic or ovate-elliptic, ca. 3 mm, rounded; filament short, ca. 1.4 mm in length; anthers linear-lanceolate, 2.6–3.4  $\times$  0.9–1.1 mm, dehiscence longitudinally, partially exerted; Ovary 2-celled, ovules ca. 150 in each cell on peltate axile placentas; style reddish, stigma 2-lobed, ca. 2.0 mm, greenish in bud and white after anthesis, nectar almost undetectable. Fruits berry-like, subglobose, purplish red, 2.5–3.5 mm in diam., 90–140 seeded; seeds 0.2–0.3 mm. Raphides absent. Pollen monads, spheroidal, isopolar and radially symmetrical in shape, very small in size, with tricolporate and coarsely reticulate exine ornamentation. Fl. Apr to Jun, fr. Jul to Sep.  $2n = 22$ .

On shady and moist rocks under forests or on cliffs near creeks; 180–300 m in altitude. Rongshui, Guangxi, China.

#### 4.2. Divergence of morphological characters

*Wendlandia* is characterized by trees or shrubs, inflorescence terminal compound cymes or rarely corymbs, corolla lobes (in China) left-convolute, style exerted, ovary bilocular with numerous ovules, and fruit capsular (Cowan, 1932; authors' personal observation) (Fig. 1E and F). We have not observed imbricate corolla aestivation as Chen and Taylor (2011) described for the genus. *Guihaiiothamnus*, with its specially reduced stems and leaves clustering together to form a rosette in habit, looks very different from species of *Wendlandia*. Difference on flower and fruit morphology between *Guihaiiothamnus* and *Wendlandia* is obvious as well: *Guihaiiothamnus* has 9–25 large long-tubed pink flowers per inflorescence clustering on top of the short branches, whereas *Wendlandia* often has more than one thousand small white flowers forming a large panicle; also, *Guihaiiothamnus* has berry-like, indehiscent fruits (Fig. 1C), whereas *Wendlandia* has capsules.

Despite the obvious differences, *Guihaiiothamnus* and *Wendlandia* share characters on left-convoluted corolla, ovary bilocular with numerous ovules, and reticulate seed coat. Thus Lo (1998) hypothesized a relationship for these two genera when establishing *Guihaiiothamnus*. This relationship was then phytochemically supported (Chen et al., 2008). The molecular phylogenetic evidence from the present study reveals a nested position for *Guihaiiothamnus* within *Wendlandia* rather than a sister genus of *Wendlandia*. We

infer that, based on the results, the unique morphology of *Guihaiiothamnus* may represent autoapomorphies, which cannot be used to infer its generic identity but reflect its derived status.

On the other hand, during field observations, significant vegetative variations of *Guihaiiothamnus* among different populations were found. The individuals from Sanfang population are generally stemless and branchless, whereas several individuals from the Huaibao population have somewhat elongated stems and branches, with two extreme examples having branches extending up to 10 cm in length. The significant vegetative variations of *Guihaiiothamnus* from different populations and the morphological difference between *Guihaiiothamnus* and *Wendlandia* suggest a high morphological plasticity for this species.

#### 4.3. Biogeographical origin: evolutionary relict or derivation?

*Wendlandia* species occur mainly in the edges of evergreen broad-leaved forests in tropical and subtropical Asia, India, and the Arabic Peninsula. The populations of *Guihaiiothamnus* known from the Jiuwan Mountains of Guangxi Province are within the northern margin of the distribution range of *Wendlandia*. The Jiuwan Mountains have been stable in tectonics since early Tertiary, and have no records of glaciations in the Quaternary. They have been recognized as a refuge of the Tertiary flora, with numerous relict genera (e.g., *Alsophila* R. Br., *Cephalotaxus* Siebold & Zucc. ex Endl., *Bretschneidera* Hemsl. and *Cyclocarya* Iljinsk., Li and Qiu, 1993). Wu et al. (2005) considered *Guihaiiothamnus* as a typical relict element in this area. The hypothesis, however, is not supported by present results. The age of *Guihaiiothamnus* (2.11 my) is relatively young comparing to the origin of *Wendlandia* in middle Tertiary (17.46 my) and to the long history of the subtropical evergreen broad-leaved forests since early Tertiary (Zhang et al., 1984). Therefore, the origin and evolution of *Guihaiiothamnus* may be better explained by recent derivation coupled with accelerated morphological divergence to adapt to the specific habitat of seasonally dry and barren cliffs in the deep valleys which is very different from typical habitats of other *Wendlandias*.

#### 4.4. Evolution of pollination mechanism

As mentioned above, *Wendlandia* species are erect shrubs or small trees, characterized by panicles with numerous, small, white flowers. Although each single flower on the tree is 2–15 mm long, the high number of flowers makes the inflorescence conspicuous for the pollinators. In contrast to *Wendlandia*, *Guihaiiothamnus* is especially small in plant size, and has less flowers per individual (Fig. 1A). In comparison with those of *Wendlandia*, the flowers of *Guihaiiothamnus* are much larger and showier by having pink, long tubular corollas (28–35 mm long) (Fig. 1D) and exhibit a butterfly pollination syndrome, which is associated with diurnal anthesis, erect, radial flower, with long and narrow corolla tube, well-hidden nectar, and vivid color (Dafni, 1992; Faegri and Van Der Pijl, 1979; Proctor et al., 1996; Willemstein, 1987). However, pollination observations indicated that instead of the expected long-tongued butterflies, pollen-consuming hoverflies and halictid bees with short proboscis were the major pollinators for this self-incompatible species (Xie et al., 2013). The conflict between the observed and expected pollinators was interpreted by Xie et al. (2013) as pollinator shift, that is, *Guihaiiothamnus* was originally pollinated by long-tongued butterflies and, during its evolutionary history, the long-tongued butterflies have been replaced by pollen-consuming hoverflies and halictid bees as the major pollinators. It is noteworthy that, this interpretation was based on the condition that the flower morphology of *Guihaiiothamnus* represents an original rather than derived status comparing to other *Wendlandias*. However, this foundational premise is challenged by the present results, which

suggest the ancestor of *Guihailiothamnus* may have had flowers similar to those of typical *Wendlandias*. Accordingly, we can infer that the ancestor of *Guihailiothamnus* may have pollinators similar to those of typical *Wendlandias*. *Wendlandia* species (*W. uvariifolia*, *W. formosana*, *W. tinctoria*) are pollinated by butterflies, flies, bees, beetles and moths (Kato, 2000; Raju et al., 2011; Xie, unpubl. data), and the plants reward nectar, as well as pollen, to their visitors/pollinators. The ancestor of *Guihailiothamnus* might have also possessed such a large spectrum of pollinators, and supplied nectar and pollen as rewards for visitors/pollinators. Nevertheless, nectar is imperceptible in *Guihailiothamnus* and pollen serve as the only reward for the pollen-consuming pollinators (Xie et al., 2013). Based on the present results, we further support the hypothesis of “pollinator shift” in *Guihailiothamnus*, however, not from long-tongued butterflies to short-tongued flies and bees but from generalized pollinators to pollen-consumed pollinators. In addition, the results suggest the high degree of phenotypic flexibility of *Guihailiothamnus*, which means its phenotypic changes could be achieved in a short time as responses to the pollinator shift (e.g. the expanded corolla throat, abundant pollen and relatively long flower longevity, Xie et al., 2013), and to the shaded habitat shift (e.g. showy flowers and elongated corolla-tubes).

Taking into consideration all the above, we therefore hypothesize that when cloning into the mountains of northern Guangxi, the plant production could be restricted by shortages of water and resources on the cliffs. As a consequence of adaptation to the infertile habitats, *Guihailiothamnus* might have reduced its vegetative biomass. Accordingly, energy and resource saving in blossom by having smaller inflorescences with fewer flowers and less nectar is an optimal selection for the small rosette plants surviving on the rocks and cliffs. The stress-tolerant strategies through plant production decrease might increase the individual's survival capacity under high stress in the specific habitat. The reduction of flower number, however, could make the reproductive organs of *Guihailiothamnus* less conspicuous, which might decrease the flower attractiveness to pollinators and negatively affect the reproductive success for this self-incompatible plant. Comparing with the previous conclusion that the long and pink flowers of *Guihailiothamnus* are the original characters delayed to respond the pollinator shift (Xie et al., 2013), here, we are inclined to believe that the showy flowers are derived as a compensation for its reduction in plant size, inflorescence size and flower number in order to stick out the reproductive organ and thus enhance pollinator attraction and reproductive success. The fruits of *Guihailiothamnus* may have a similar adaptation history like the vegetative and floral organs. The indehiscent, berry-like fruit of *Guihailiothamnus* may have been evolved from dehiscent capsules like those of *Wendlandias*. The dark purple color and the berry-like texture of the fruits suggest that they might be removed and dispersed by fruit-eating birds.

## 5. Conclusions

The present study contributes to the reconstruction of phylogenetic position of *Guihailiothamnus* and to the interpretation for its evolution of morphology and pollination. Comprising a single morphological-distinctive species, *Guihailiothamnus* has never been doubted as an independent genus, whereas the present study resolved a deep nested position for it within *Wendlandia*. Its phylogenetic position and relatively young age dated using five fossil records based on the phylogeny argue a recent adaptive derivation in the unproductive environments. More work in future including extensive sampling from *Wendlandia* is required to clarify the phylogenetic relationships within *Wendlandias* and the details of morphology evolution. Moreover, detailed studies might also be promising to examine whether the recent derivation of

*Guihailiothamnus* has also occurred in other monotypic genera in the forests of southwestern China, including *Clarkella* J.D. Hooker of Rubiaceae, which is comparable to *Guihailiothamnus* with dwarf vegetations, outstanding reproductive organs on the cliffs.

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