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Ardisia crenata Complex (Primulaceae) Studies Using Morphological and Molecular Data

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1. Introduction

Ardisia crenata Sims was a member of Myrsinaceae family in classical taxonomy view, but in the system of APG III (2009), it is included in the expanded family of Primulaceae and the primary Myrsinaceae family does not exist. This evergreen shrub is the most widely distributed species of *Ardisia*, occurring from Japan to Tibet, the Philippine Islands, and southern Asia where it is labelled as medicinal plant (Kobayashi & Mej'ia, 2005) and cultivated as a garden plant (Conover et al., 1989; Lee, 1998). Since *A. crenata* displays a high variability, its identification and species status frequently be confused. This complex includes four species and one variety (*Ardisia crenata* Sims, *A. hanceana* Mez, *A. lindleyana* D. Dietr., *A. linangensis* C. M. Hu, *A. crenata* var. *bicolor* (E. Walker) C. Y. Wu & C. Chen), They all belong to the subgenus *Crispardisia* of *Ardisia*. They have the same characters including inflorescences terminal, with leaf marginal nodules, 5 ovules in one series on the placenta. However, the five taxa also have some characters that could be indentified. There is some controversy between different researchers. Walker (1940) pointed out that *A. hanceana* is closely related to *A. crenata*, from which it may be distinguished by its larger flowers and usually by the lack of raised-punctate glands on the lower surface of the leaves. *A. hanceana*, *A. crenata* and *A. lindleyana* are very similar, the first one differ from *A. crenata* by the larger (6-7mm vs. 4-6mm) flowers, sepal ovate and differ from the last one by the marginal veins near the margin, more lateral veins (12-18 pairs vs. 8-12 pairs) (Chen, 1979). *A. linangensis* was first published by Hu (1992), he noted that this species differs from *A. hanceana* by the black-punctate flowers and by the not scalloped leaves and it is more closely allied to *A. tsangii* Walker, but can be easily distinguished by its glabrous and more corymbose inflorescence and by having more (3-8) leaves on the flowering branches. However, *A. tsangii* was treated as the synonym of *A. lindleyana* and *A. linangensis* was treated as the synonym of *A. crenata* in Flora of China (Chen & Pipoly, 1996). *Ardisia bicolor* was first

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published by Walker (1940), then it was dealt by Wu & Chen (1977) as a variety of *A. crenata* var. *bicolor*, they emphasized that the variety could be distinguished by the purple red of lower surface of leaves, peduncles, sepals and petals. Chen & Pipoly (1996) reduced the variety as the synonym of *A. crenata*. After we checked the specimens deposited in SCBG (Herbarium, Department of Taxonomy, South China Botanical Garden, Chinese Academy of Sciences), PE (Herbarium, Institute of Botany, Chinese Academy of Sciences), KUN (Herbarium, Kunming Institute of Botany, Chinese Academy of Sciences), IBK (Herbarium, Guangxi Institute of Botany), SYS (Herbarium, Life Science College, Sunyatsen University), HITBC (Herbarium, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences) and we found the identification of many specimens of them were incorrect. The five taxa are so similar in morphology, so it is necessary to clarify the relationships among them.

2. Materials and methods

2.1 Plant materials

Eighteen natural populations were sampled for molecular research. These representative populations were from Guangdong, Guangxi, Yunnan, Hainan Provinces. The geographical origins of accessions are given in Table 1. Voucher specimens were deposited in SCBG. Silica-gel dried samples of leaf tissue of each population were prepared for molecular analyses. We used 4 representational species belonging to other subgenera of the genus *Ardisia*, *Ardisia aberrans* (Pimelandra), *A. depressa* (Akosmos), *A. elliptica* (Tinus) and *A. japonica* (Bladhia) as an out-group. As for the morphological materials, we checked more than 2000 specimens in the Herbariums mentioned before and selected more than 570 specimens that have typical characters for morphology research.

2.2 DNA extraction

Genomic DNA was extracted following a modified 2×CTAB protocol (Doyle & Doyle, 1987) using samples of tissue cut from leaves. The total DNA of each sample was dissolved by 100μL Elution Buffer and diluted ten-fold before using for PCR. Total DNA was deposited at -20°C for long-stem storage. The quality of all DNA preparations was checked by agarose gel electrophoresis (1% w/v) in 0.5×TBE buffer containing 1 μg/mL of ethidium bromide by comparison with a known mass standard.

2.3 PCR amplification and DNA sequencing

ITS can be used for phylogenetic of species, and we also tried to use cpDNA gene, but we found it difficult to solve the relationships among these taxa. The two primers ITS1a (5'-AGAAGTCGTAACAAGGTTTCCGTAGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) used in this study were designed on the basis of the regions of GenBank. The ITS sequences on GenBank are less, such as *Ardisia crenata* (FJ482136, FJ482137, FJ482138, AF547796), *A. japonica* (FJ482143, FJ482144, FJ482145, FJ482146), and we compared the sequence of the former with our sequences and found they were almost the same, for unifying the length of ITS regions, so in this research we just used the sequence we obtained.

For ITS PCR amplifications were performed in a total volume of 30 μl of reaction buffer, 1.5mmol/L $MgCl_2$, 10μmol of each primer, 2.5mmol/L of each dNTP, 5U/μl of Taq DNA

polymerase and 10ng/µl of template DNA. Reactions were performed in a Peltier Thermal Cycler (Bio-RAD DNAEngine) and programmed for an initial denaturation step (3 min at 94 °C) followed by 35 cycles of 45S at 94°C, 50S at 55°C, 1min at 72°C. The last cycle was followed by a final incubation of 10 min at 72°C. Subsequently, 3µl of each amplification mixture was analyzed by agarose gel (1% w/v) electrophoresis in TBE buffer containing 1µg/mL ethidium bromide. The PCR reacions were purified from excess salts and primer using the Qiagen QLAquick PCR purification Kit. Automated DNA sequencing was performed directly from the purified PCR products using ABI 3730 DNA sequencer (Applied Biosystems) by Shanghai Invitrogen biotechnology Co.Ltd. and Shanghai Biosune biotechnology Co.Ltd. All sequences of ITS were bi-directional sequenced and the region was not cloned.

2.4 Sequence alignment and analysis

DNA sequences and overlapping fragments were assembled and edited using SeqMan and checked for orthology to sequences of *Ardisia crenata* complex. The sequence boundaries between the two spacers (ITS1 and ITS2) and coding regions (5.8S) of nrDNA were determined by comparison with the *A. crenata* sequence (Hao et al., 2003).

Multiple alignments were automatically performed using CLUSTAL X 1.83 (Thomson et al., 1997) of DNA Star (DNASTAR Madison, WI), and then further examined and slightly modified manually.

Phylogenetic analyses for each matrix were carried out Bioctrl package using maximum parsimony (MP) and Bayesian inference (BI) methods in PAUP* 4.0b10 (Swofford, 2001) and MrBayes version 3.12 (Ronquist et al., 2003; Huelsenbeck & Ronquist, 2001). For MP analyses, heuristic searches were conducted with 1000 replicates of random addition, one tree held at each step during stepwise addition, tree-bisection-reconnection (TBR) branch swapping, MulTrees options on, and the steepest descent off, Gaps were treated as missing data, characters were equally weighted, and their states were unordered. Relative clade support was evaluated by the bootstrap analyses (Felsenstein, 1985). For Bayesian analyses were accomplished in MrBayes version 3.12 using the best-fit models upon Akaike information criterion (AIC; Akaike, 1974) by using Modeltest 3.7 (Posada & Crandall, 1998; Posada & Buckley, 2004). In Bayesian analyses, trees were generated by running four simultaneous Metropolis-coupled Monte Carlo Markov (MCMC) chains and sampling one tree every 1000 generations for 1,000,000 starting with a random tree. The posterior probability (PP) was used to estimate nodal robustness.

3. Results

3.1 Sequence characteristics

All the acquired sequences have been submitted to GenBank and can be retrieved using the numbers in Table 1. No evidence of paralogous sequences was found for ITS sequences, because all PCR products were resolved as a single band and no double peaks were encountered in sequencing. The ITS region of nrDNA comprising both ITS sequences (ITS1 and ITS2) and the 5.8S rDNA was amplified by PCR form all 18 taxa of the *A. crenata* complex and 4 samples of outgroup. The ITS aligned sequence data set was 681 bp in length, with 46 positions being variable and 33 parsimony-informative.

Name of species	Subgenus	Locality	Voucher	GB No. ITS
Ardisia crenata Sims	*Crispardisia*	Guangxi, China	J. Wang 200810	JN645183
		Guangxi, China	J. Wang 2007146	JN645181
		Yunnan, China	J. Wang 2007218	JN645182
		Guangdong, China	J. Wang 2007299	JN645180
A. crenata var. *bicolor* (E. Walker) C. Y. Wu & C. Chen	*Crispardisia*	Guangdong, China	J. Wang 2007121	JN645184
		Guangdong, China	J. Wang 2007123	JN645185
		Guangdong, China	J. Wang 2007293	JN645186
		Guangdong, China	J. Wang 200857	JN645190
A. hanceana Mez	*Crispardisia*	Hainan, China	J. Wang 200799	JN645187
		Yunnan, China	J. Wang 2007217	JN645188
		Guangdong, China	J. Wang 2007296	JN645189
		Guangdong, China	J. Wang 200604	JN645191
A. lindleyana D. Dietr.	*Crispardisia*	Guangdong, China	J. Wang 200858	JN645193
		Guangdong, China	J. Wang 2007112	JN645192
		Guangdong, China	J. Wang 2007119	JN645196
A. linangensis C. M. Hu	*Crispardisia*	Guangdong, China	J. Wang 200652	JN645194
		Guangdong, China	J. Wang 200653	JN645195
		Guangdong, China	J. Wang 2007215	JN645197
A. aberrans (E. Walker) C. Y. Wu & C. Chen	*Pimelandra*	Kachin, Myanmar	Xia et al. 381	JN645198
A. depressa C. B. Clarke	*Akosmos*	Guangxi, China	J. Wang 2007169	JN645199
A. elliptica Thunb.	*Tinus*	Guangdong ,China	J. Wang 2007301	JN645200
A. japonica (Thunb.) Bl.	*Bladhia*	Hunan, China	Y. Z. Chen 2007298	JN645201

Table 1. Origin of samples, voucher information, and GenBank database accession numbers of DNA sequences of *Ardisia crenata* complex.

3.2 ITS analysis

The consensus MP phylogenetic tree (L= 95, CI= 0.874, RI= 0.898) and the Bayesian tree derived from ITS/5.8S sequences was shown with bootstrap values in Fig. 1 and Fig. 2. The topology of the strict consensus tree and Bayesian tree are almost identical, just the support values are different, the latter were higher than the former.

All constructed ITS phylogenetic trees congruously suggested that the *A. crenata* complex was divided into three clades, which are strongly supported (MPBPs/BPP: 87/0.98 in clade A; MPBPs/BPP: 62/0.88 in clade B; MPBPs/BPP: 95/1.00 in clade C). Clade A and clade B are composed of all of the samples of *A. hanceana* and *A. lindleyana* separately, *A. crenata*, *A. crenata* var. *bicolor* and *A. linangensis* clustered together with high bootstrap value (MPBPs/BPP: 95/1.00), confirming very close relationships among them. All samples of *A. crenata* and two samples of *A. crenata* var. *bicolor* clustered in Clade D with the bootstrap value (MPBPs/BPP: 93/1.00). All samples of *A. linangensis* and one *A. crenata* var. *bicolor* were in multiple clades.

3.3 Morphological and anatomical research

After identifying more than 570 specimens of this complex and dissecting some specimens' flowers, we can easily distinguish *Ardisia lindleyana*, *A. hanceana* from *A. crenata*, *A. crenata* var. *bicolor*, *A. linangensis* in morphology. *A. crenata*, *A. crenata* var. *bicolor* and *A. linangensis* are very similar with each other and many characters of them have different degree of transition, but the typical characters can be easily identified. In this research, we select the typical characters used for summarization, the results are submitted below (Table 2).

Characters/ name of species	*Ardisia crenata*	*A. crenata* var. *bicolor*	*A. linangensis*	*A. hanceana*	*A. lindleyana*
Leaf shape	elliptic to elliptical lanceolate	elliptic to long oblong-elliptical lanceolate	long oblong-elliptical lanceolate or elliptic	elliptic or long oblong-elliptical lanceolate	oblong to elliptical lanceolate
Leaf margin	crisped or crenate	crisped, crenate or dentate	entire or dentate	crenate or entire	entire or dentate
Marginal punctate	obvious, irregular	obvious, uniserial or irregular	obvious, uniserial	without or unconspicuous	unconspicuous or irregular
Leaf margin nodules	between teeth, densely	between or on teeth, densely or sparsely	without or on teeth, sparsely	between teeth, sparsely	without or on teeth sparsely
Flower colour	white	purple to faint red	faint red	Faint red to purple	white
Lobe apex	acute	acute or obtuse	obtuse	acuminate	obtuse
Inflorescences	umbellate or cymose	umbellate or cymose	corymbose or umbellate	compound corymbose cyme	umbel
Flower	4-6mm	4-6mm	5-6mm	6-7mm	ca. 5mm
Flowering branches	4-16cm	2.5-12cm	9-24cm	8-24cm	3-11cm
Leaves of Flowering branches	0-5 pieces	2-5 pieces	3-8 pieces	5-16 pieces	1-3 pieces
Pedicel length	0.5-1.5cm	0.5-1.5cm	1-3cm	1-2.5cm	0.5-1.5cm
Sepal shape	triangular ovate	triangular ovate	triangular or elliptical ovate	ovate	Obtuse triangular ovate
Sepal and pedicel indumentums	without	without	without	without	with
Sepal punctate	yellow-brown	black	black	almost without punctate	black
Fruit diameter	5-6mm	5-6mm	7-8mm	7-9mm	4-6mm

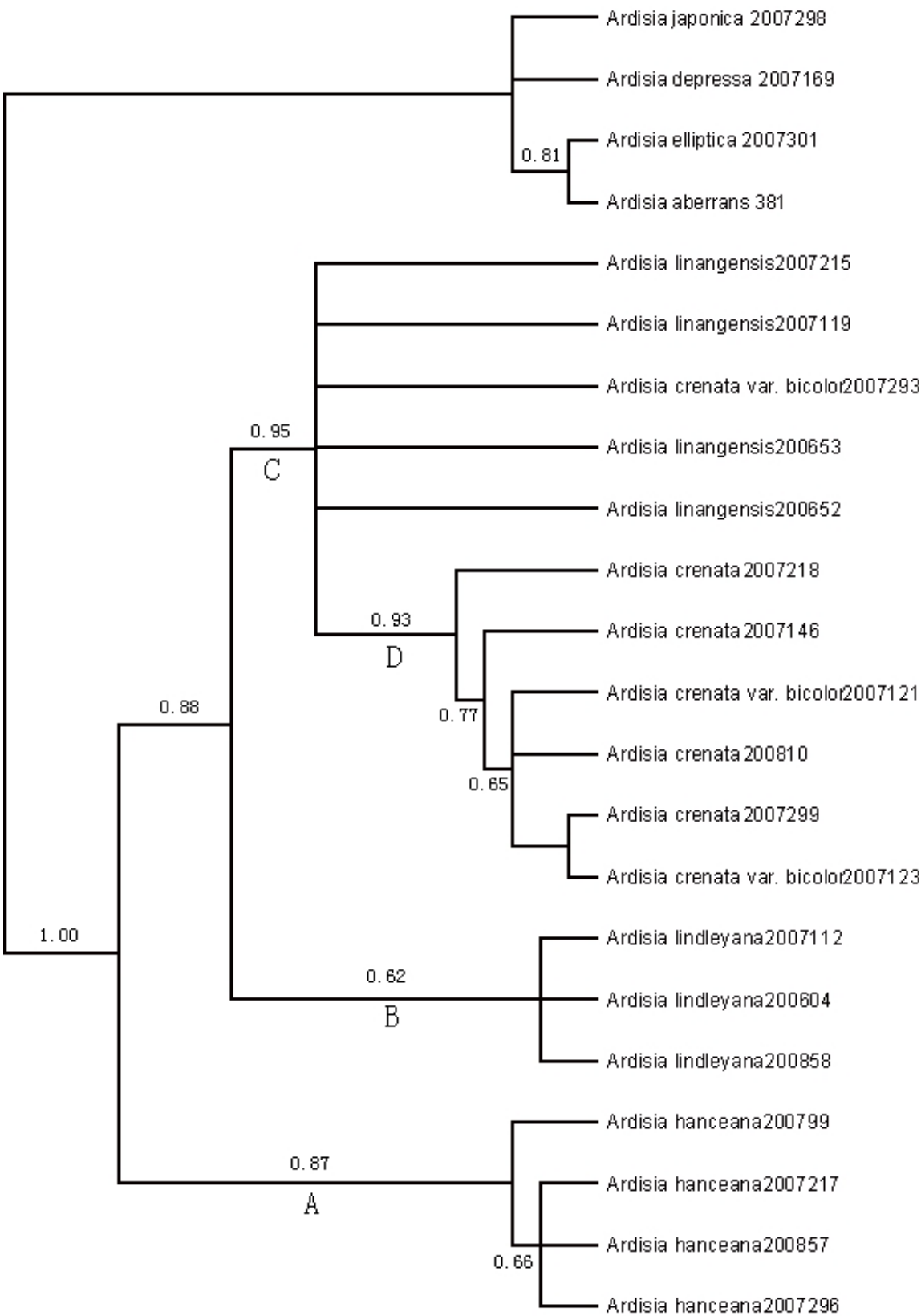Table 2. Morphological and Anatomical comparison of *Ardisia crenata* Complex

Fig. 1. Strict consensus tree (CI = 0.874, RI = 0.898, RC = 0.126) based on the ITS sequence data for 22 taxa of the *A. crenata* complex, and the number above branches indicated bootstrap values above 50%
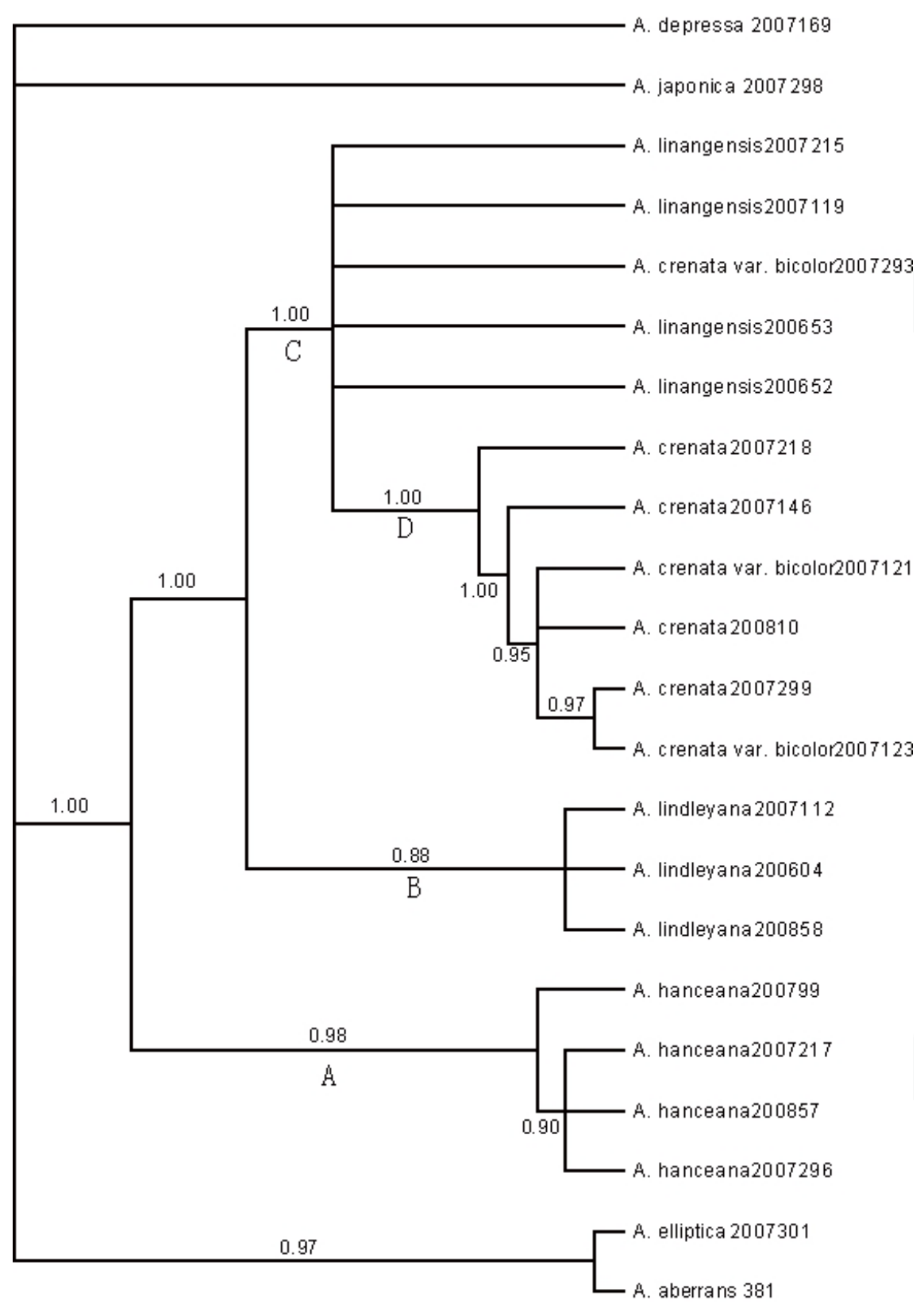
Fig. 2. Phylogenetic tree obtained from Bayesian inference of ITS Sequence data for 22 taxa of the *A. crenata* complex (Number above branches represcent the values of posterior probability values)
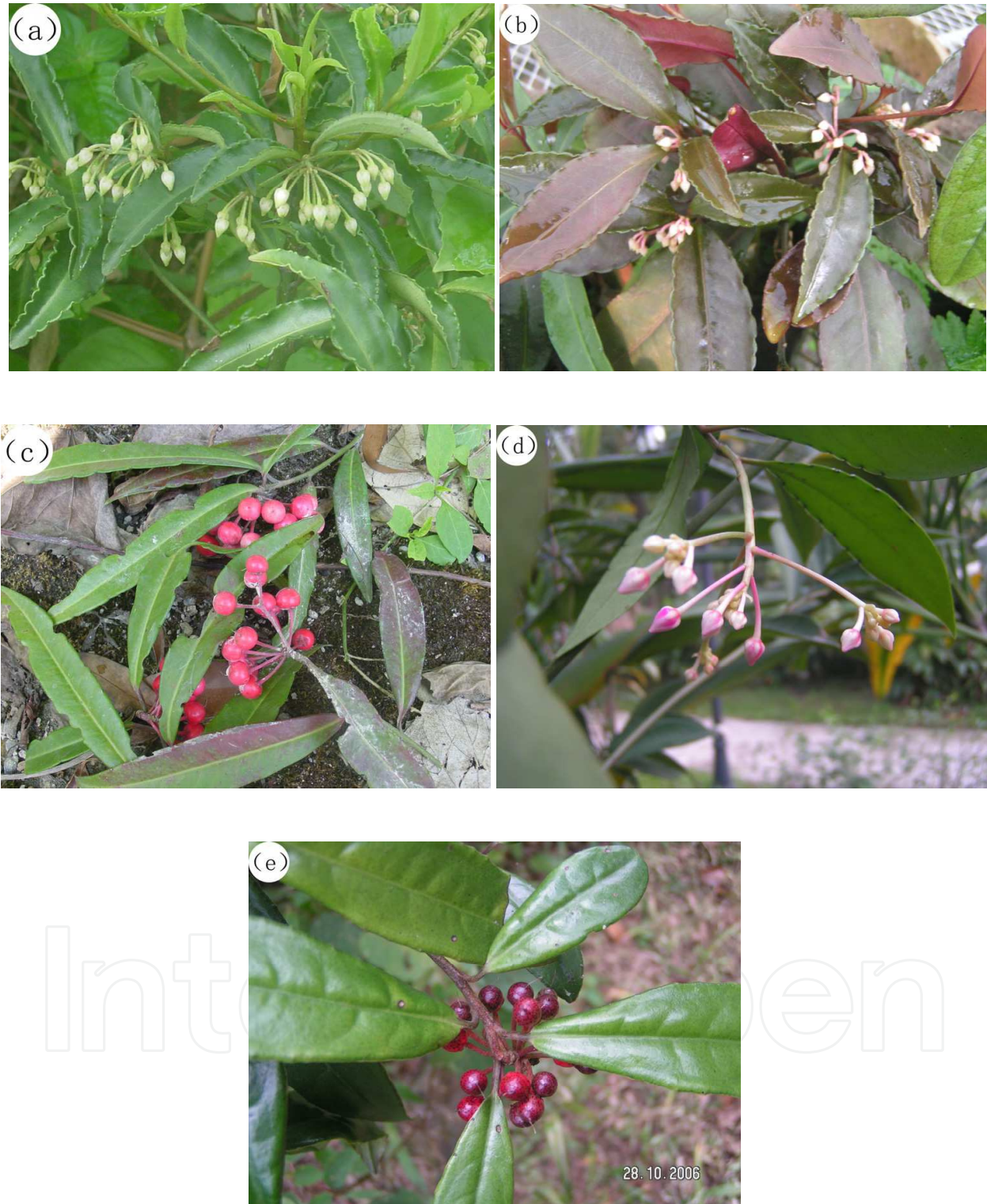
Photo credit: (a)-(d) Jun Wang; (e) Zhong Wang

Fig. 3. Images of the members of *Ardisia* complex. (a)*Ardisia crenata* Sims; (b) *A. crenata* var. *bicolor* (E. Walker) C. Y. Wu & C. Chen; (c) *A. linangensis* C. M. Hu; (d) *A. hanceana* Mez; (e) *A. lindleyana* D. Dietr.

4. Discussion

The topology in the analysis of two methods (MP, Bayesian) above was consistent based on molecular data, the differences were the bootstrap support values. These results showed that the *A. crenata* complex could be divided into three major groups, which were strongly supported by two phylogenetic methods (MP, Bayesian). The 4 samples of *A. hanceana* were clustered into Clade A and The Clade B was composed of 3 samples of *A. lindleyana*, each one became a true clade in the ITS phylogenetic trees, Clade A was at the base of the trees and the Clade B following closely, which suggested that *A. hanceana* and *A. lindleyana* were divergent earlier than the other three taxa of this complex. From the morphological analysis, *A. hanceana* is different from *A. crenata* in the compound corymbose cyme (vs. umbellate or cymose) inflorescences, 5-16 pieces leaves of flowering branches (vs. 0-5 pieces), sepal almost without punctuate (vs. yellow-brown), fruit diameter 7-9 mm (vs. 5-6mm). *A. lindleyana* also can be distinguished from *A. crenata* with the umbel inflorescences (vs. umbellate or cymose), entire or dentate leaf margin (vs. crisped or crenate), obtuse lobe apex (vs. acute), obtuse triangular ovate sepal (vs. triangular ovate), with sepal and pedicel indumentums (vs. without), so we could separate *A. hanceana* and *A. lindleyana* from the other three taxa of this complex. 4 samples of *A. crenata*, 3 samples of *A. crenata* var. *bicolor* and 4 samples of *A. linangensis* clustered into Clade C. The phylogenic analysis based on ITS with two methods indicated that these three taxa differentiated later than *A. hanceana* and *A. lindleyana*, they might be the same ancestor yet subsequently divergent in different evolutionary patterns almost at the same time. Although all samples of *A. linangensis* not clustered into one Clade, we should pay attention to the Clade D, which all samples of *A. crenata* fell into and it was well supported with (MPBPs/BPP: 95/1.00). This indicated that there has difference between *A. crenata* and *A. linangensis* from the molecular data. From morphological research, we could know there are many characters different between them, *A. linangensis* differ from *A. crenata* in leaf margin (entire or dentate vs. crisped or crenate), marginal punctuate (uniserial vs. irregular), leaf margin nodules (without or on teeth, sparsely vs. between teeth, densely), inflorescences (corymbose or umbellate vs. umbellate or cymose), lobe apex (obtuse vs. acute) and its distribution area is so narrow, can only be seen in south area of Nanling Mountain, which is the nomenclature origin of the species. 2 samples of *A. crenata* var. *bicolor* clustered in Clade D with all samples of *A. crenata*, this indicated that they have very close phylogenetic relationship. The morphological characters of *A. linangensis* and *A. crenata* var. *bicolor* are also very close, they are composed of a multiple branches in the two phylogenetic tree, but *A. linangensis* differ from *A. crenata* var. *bicolor* in many characters such as inflorescences, leaf margin nodules, leaf margin, fruit diameter (Tab. 2) and they are also have different distribution. As for *A. crenata* var. *bicolor*, it was very close to *A. crenata* except for the colour of the leaf. In conclusion, we agree with the opinion of allocating *A. crenata* var. *bicolor* in *A. crenata* and do not support the idea of making *A. linangensis* as the synonym of *A. crenata*. If possible, we want to further research on the population of *A. crenata* complex and find more information used for taxonomy study.

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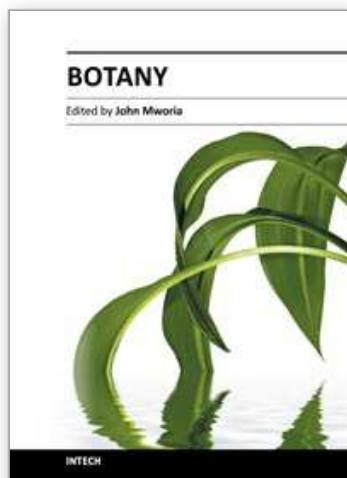
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This book is devoted to botany and covers topical issues in this diverse area of study. The contributions are designed for researchers, graduate students and professionals. The book also presents reviews of current issues in plant-environment interactions making it useful to environmental scientists as well. The book is organized in three sections. The first section includes contributions on responses to flood stress, tolerance to drought and desiccation, phytotoxicity to Chromium and Lead; the second has aspects of economic botany including a review of Smut disease in sugarcane and properties of plant extract used Tassaboount date juice; the last covers topical issues on morphogenesis and genetics on cotton fiber special cell, secretory glands Asphodelus aestivus flower ,pollen tube growth in Leucojum aestivum , morphological studies of Ardisia crenata complex, and hybrid lethality in the Genus Nicotiana.

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