Molecular and morphological evidence for a remarkable new species of Leptopus 1 2 (Phyllanthaceae) from Southeast Yunnan, China 3 Wen-Hua Zhang¹, Xin-Xin Zhu², Bine Xue³, En-De Liu⁴, Yu-Ling Li¹, Gang Yao¹ 4 5 ¹College of Forestry and Landscape Architecture, South China Agricultural University, 6 Guangzhou, 510642, Guangdong, China 7 ²College of Life Sciences, Xinyang Normal University, Xinyang 464000, Henan, 8 9 ³College of Horticulture and Landscape Architecture, Zhongkai University of 10 Agriculture and Engineering, Guangzhou, 510225, Guangdong, China 11 ⁴Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming 12 Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, China 13 14 15 Corresponding author: Gang Yao, 16 483 Wushan Road, Tianhe District, Guangzhou, 510642, China. 17 Email address: gyao@scau.edu.cn 18 19 20 Abstract Leptopus malipoensis, a remarkable new species of Phyllanthaceae from Southeast 21 Deleted: or 22 Yunnan Province, China, is described. The phylogenetic position of the new species 23 within the genus Leptopus was analyzed based on nuclear ribosomal Internal Deleted: the 24 <u>Transcribed Spacer (nrITS)</u> and plastid matK sequence data. The results show that L. Deleted: internal malipoensis is highly supported to be the sister of L. fangdingianus (P. T. Li) Voronts. 25 & Petra Hoff., a species endemic to western Guangxi Province, China. Deleted: transcribed 26 Morphologically, the new species can be distinguished from all known congeneric 27 Deleted: spacer 28 taxa by its long and slim branches usually pendulous or procumbent, large leaves up Deleted: the to 15 cm long and 7 cm wide, and further differs from its sister species by its hirsute stems, leaves and pedicel of female flowers, longer pedicel of male flowers, 3-locular Deleted: DNA region 30 31 ovary and 3 styles. A key to all the Leptopus species is provided. Formatted: Font: Not Italic 32 Deleted: Western 33 Subjects Biodiversity, Molecular Biology, Plant Science, Taxonomy

Keywords: Leptopus, Phyllanthaceae, Poranthereae, taxonomy, Yunnan, China

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Introduction

 Leptopus Decne. is a small genus in the tribe Poranthereae, subfamily Phyllanthoideae of Phyllanthaceae and distributed mainly from the Caucasus to Malesia (Vorontsova & Hoffmann, 2009; Webster, 2014). In the latest taxonomic revision of Leptopus, nine species were accepted and six among them were recorded in China (Vorontsova & Hoffmann, 2009). Results from molecular phylogenetic analyses showed that Leptopus was sister to Actephila Blume with high support (Vorontsova et al., 2007; Vorontsova & Hoffmann, 2008). Morphologically, the genus Leptopus can be distinguished from its sister clade by its leaves membranous to papery (vs. leathery, rarely papery), disk lobed evidently (vs. annular), fruit less than 10 mm in diameter (vs. more than 10 mm in diameter), exocarp adnate to endocarp (vs. free from endocarp) and mature seed with copious endosperm (vs. seed without endosperm) (Li et al., 2008).

During field investigations in Malipo Hsien, southeast Yunnan Province of China, in March 2018, two of the authors (E.D. Liu and X.X. Zhu) collected a Phyllanthaceae specimen belonging to Leptopus, and the same species was collected again in the same locality by another author (G. Yao) in July 2020. The species superficially differs from all the other members of Leptopus in morphology. After detailed morphological investigation and molecular phylogenetic analyses of Leptopus, it was concluded that the specimens represent a species that is new to science, and formally described here.

Materials & methods

Ethics statements

The collection location of the new species reported in this study is outside any natural conservation area and no specific permissions were required for the location. Since this species is currently undescribed, it is not currently included in the China Species Red List (Wang & Xie, 2004). Our field studies did not involve any endangered or protected species. No specific permits were required for the present study.

Nomenclature

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants (ICN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. In addition, new names contained in this work which have been issued with identifiers by IPNI will eventually be made available to the Global Names Index. The IPNI can be accessed and the associated information contained in this publication viewed through any standard web browser by using the web address "http://ipni.org/". The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central, and CLOCKSS.

Material collection

Flowering and fruiting specimens of the new species were collected in the mountain

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area, in Nandong to Bajiaoping, Laoshan, Malipo Hsien of Yunnan Province, China, for morphological study. Leaf materials for DNA extraction were collected and dried using silica gel in the field.

Morphological study

Specimens of *Leptopus* deposited in the herbaria GXMG, IBK, IBSC, KUN and PE were carefully examined for the present study. Field investigation of Chinese *Leptopus* species were also conducted in recent years. Morphological characters of stems, leaves, flowers and fruits of relevant species were photographed and measured. In addition, morphological comparisons between the new species and all the nine *Leptopus* species accepted by Vorontsova & Hoffmann (2009) were also conducted.

Phylogenetic study

Phylogenetic relationships among species of Leptopus were previously investigated by Vorontsova et al. (2007) based on analysis of two DNA regions, viz. the nuclear ribosomal Internal Transcribed Spacer (ITS) and plastid matk. To study the phylogenetic position of the new species within the genus Leptopus, the two above mentioned DNA regions of the new species were sequenced. Relevant DNA sequences of Leptopus species included in Vorontsova et al. (2007) were obtained from GenBank (https://www.ncbi.nlm.nih.gov/) and used in the present phylogenetic analyses. Additionally, outgroups were selected from the other seven genera of the tribe Poranthereae [Actephila Blume, Andrachne L., Meineckia Baill., Notoleptopus Voronts. & Petra Hoffm., Phyllanthopsis (Scheele) Voronts. & Petra Hoffm., Pseudophyllanthus (Müll. Arg.) Voronts. & Petra Hoffm. and Poranthera Rudge] and the genus Heywoodia Sim of the tribe Wielandieae, based on previously published phylogenetic frameworks (Kathriarachchi et al., 2005; Vorontsova et al., 2007). DNA sequences of outgroups were also downloaded from GenBank. Detailed information about the species sampled and DNA sequences are provided in Table 1.

Total DNA of the new species was extracted and then sequenced by genome skimming following the protocol of Zeng et al. (2018). Plastid and the nrITS sequence reads were assembled using the software GetOrganelle (Jin et al., 2020), with the reference plastid genome of *Glochidion chodoense* C.S. Lee & Im (GenBank accession number: NC_042906) and nrITS sequence of *Leptopus chinensis* (Bunge) Pojark. (MH710764), respectively. Genes in the plastid genome obtained were annotated in the software PGA (Qu et al., 2019). The *matK* sequence was then extracted from the assembled whole plastid genome.

Sequences were aligned using MAFFT v. 7.221 (Katoh & Standley, 2013) and then three data sets were constructed: the *matK* dataset, the nrITS dataset and the combined dataset (including *matK* and nrITS). All the three datasets were analyzed using two approaches: Bayesian Inference (BI) and Maximum Likelihood (ML). Detailed information about the parameter settings in the BI and ML analyses referred the phylogenetic analyses conducted in Yao et al. (2020). The models of nucleotide substitution of the two DNA regions used were selected under the Akaike Information Criterion (AIC) using jModeTest v. 3.7 (Posada, 2008): TVM+G for *matK* and

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Results & discussion

Phylogenetic analysis

The *matK* dataset, nrITS dataset and combined dataset <u>alignments</u> contained 2000 bp, 812 bp and 2812 bp, respectively. Conflicted topologies were found between the *matK* and nrITS frameworks (Fig. 1), but relevant conflicted phylogenetic nodes were all <u>poorly</u> supported in analyses of the nrITS dataset (Fig. 1B). Phylogenetic relationships derived from the combined dataset were much better resolved compared with those obtained from analyses based on the other two datasets, and phylogenetic relationships among *Leptopus* species sampled here were all resolved with high <u>support values</u> (Fig. 2). Thus we focus on describing phylogenetic relationships based on the result derived from the combined dataset.

Phylogenetic results showed that the genus *Leptopus* was sister to *Actephila* with high support values (bootstrap, BS, = 100%, posterior probability, PP = 1.00), and the monophyly of *Leptopus* was strongly supported (MLBS = 100%, PP = 1.00). Within *Leptopus*, three major clades were recovered. *Leptopus australis* (Zoll. & Moritzi) Pojark. represents the earliest divergent clade within this genus. The new species is strongly supported as the sister of *L. fangdingianus* (P.T. Li) Voronts. & Petra Hoffm. (MLBS = 100%, PP = 1.00) and this pair in turn sister to *L. clarkei* (Hook. f.) Pojark. (MLBS = 100%, PP = 1.00). *Leptopus chinensis* (Bunge) Pojark. and *L. cordifolius* Decne. formed the third clade and sister to the (new species-*L. fangdingianus*)-*L. clarkei* clade with strong support (MLBS = 100%, PP = 1.00). Furthermore, the sister relationship between the new species and *L. fangdingianus* was highly supported in both of the *matK* (Fig. 1A) and nrITS (Fig. 1B) analyses.

Morphological comparisons

Morphologically, the new species has pendulous or procumbent stems (Fig. 3A-B) and large leaves up to 15 cm long and 7 cm wide. These characters distinguish it easily from all the other Leptopus members, which usually have ascendant or erect stems and smaller leaves less than 10 cm long and 5 cm wide. The procumbent habit is also recorded in L. clarkei, a species widely distributed from southern China, extending west to Assam and Burma, and south to northern Vietnam (Vorontsova & Hoffmann, 2009). It is also closely related to the pair of the new species and L. fangdingianus in the phylogenetic analyses (Figs. 1 and 2). However, the new species differs from L. clarkei by having straight and not ribbed stems (Fig. 3C-F) (vs. flexuous and longitudinally strongly ribbed stems), larger leaves up to 15 cm long and 7 cm wide (vs. less than 10 cm long and 3 cm wide), leaves hirsute on both surfaces (Figs. 3C–E & 4D) (vs. glabrous adaxially and glabrous to sparsely hirsute abaxially), margin of leaves densely hirsute (Figs. 3C-E & 4D) (vs. glabrous to hirsute). While the new species differs from its sister L. fangdingianus by its leaves hirsute on both surfaces (Figs. 3C-E & 4D) (vs. glabrous to sparsely hirsute on both surfaces), pedicel of male flowers 10-25 mm long (vs. usually less than 10 mm long), pedicel of female flowers hirsute (Fig. 4D) (vs. glabrous), ovary 3-locular (vs. 4-5) and styles 3

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Four species of *Leptopus* [viz., *L. micans* (Dunn) Pojark., *L. hainanensis* (Merr. & Chun) Pojark., *L. pachyphyllus* X.X. Chen and *L. robinsonii* Airy Shaw] were not sampled in the present phylogenetic analyses. Morphologically, the new species also can be easily distinguished from these four congeneric species based on its above-mentioned pendulous or procumbent stems and large leaves. Additionally, the new species has hirsute indumentum, not ribbed branches, chartaceous leaves with 4 (or rarely 5) pairs of secondary veins. In contrast, *L. micans* has glabrous branches longitudinally ribbed and 8–10 pairs of secondary veins in leaves. *Leptopus hainanensis* also has glabrous branches and leaves, as well as 2–3 pairs of secondary veins in leaves. While the other two species *L. pachyphyllus* and *L. robinsonii* both have glabrous and bilaterally flattened branches, and the leaves of *L. pachyphyllus* are glabrous on both sides and almost succulent.

Taxonomic treatment

Leptopus malipoensis W.H. Zhang & Gang Yao, sp. nov. (Figs. 3-4)

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Type. CHINA. Yunnan Province, Wenshan State, Malipo Hsien, Laoshan, Nandong to Bajiaoping, on stone slopes near the roadsides of the semi-shady forests, at the elevation of ca. 1200 m, 15 July 2020, *G. Yao YGYN2020071501* (holotype: IBSC; isotypes: KUN, CANT).

Diagnosis. The species is similar to *L. fangdingianus* (P.T. Li) Voronts. & Petra Hoffm. in morphology, but differs from the latter by its procumbent habit with long and slim branches usually pendulous, larger leaves up to 15 cm long and 7 cm wide, hirsute stems, leaves and pedicel of female flowers, longer pedicel of male flowers, 3-locular ovary and 3 styles.

Description. Shrub, monoecious. Stems straight, terete, hirsute; branchlets long and slim, sometimes up to 1.5 m long, usually pendulous or procumbent, hirsute. Leaves alternate, chartaceous, elliptic to ovate, 2-15 cm long and 1.2-7 cm wide, both surfaces hirsute, densely hirsute when young, margin densely hirsute, base cuneate to round, apex acuminate; midvein adaxially impressed, abaxially raised; secondary veins usually 4 pairs, rarely 3 or 5 pairs, adaxially slightly impressed, abaxially raised, obliquely ascending, sometimes arcuately anastomosing near margins. Petiole 4-12 mm long, hirsute. Stipules narrowly triangular, apically acuminate. Inflorescences unisexual or bisexual, axillary, fasciculate. Male flowers 1–3 per fascicle, ca. 3 mm in diameter, light yellow to slightly green; pedicel 10-25 mm long, glabrous; sepals 5, oblong, apically round, adaxially glabrous, abaxially sparsely hirsute, 0-3-veined; petals 5, clavate to slightly linear, alternating with sepals; disc extrastaminal with 5 contiguous regular segments deeply billobed, apices of lobes truncate to rounded; stamens 5, opposite sepals; filaments 5, free; anthers 5, longitudinally dehiscent. Female flowers usually 1 per fascicle, 3.5–4 mm in diameter; pedicels usually 15–25 mm long, sparsely hirsute, apically dilated evidently; sepals 5, oblong to ovate-triangular, apically acute to round, adaxially glabrous, abaxially glabrous or sparsely hirsute, usually 0-5-veined; petals 5, Jinear, alternating with sepals; disc with

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256	5 contiguous regular segments deeply billobed, apices of lobes truncate to rounded;	Deleted: apical
257	ovary 3-locular, globose, glabrous; styles 3, free, deeply bifid, lobes usually recurved.	
258	Fruiting pedicel 2–3 cm long, hirsute; capsules depressed globose, smooth, glabrous	
259	or sparsely hirsute when young, 4-6 mm in diameter, 2.5-3 mm high, persistent	
260	sepals oblong; seeds 6, brown to dark-brown, hemispheric or laterally compressed, ca.	
261	2.5 mm long and 2 mm wide, lacking appendages.	
262	Etymology. Leptopus malipoensis is named after its type locality, Malipo Hsien.	
263	Malipo Hsien is a hotspot for biodiversity research in Yunnan Province, China, and	
264	many new species have been described recently from there, including Bredia	Deleted: were
265	malipoensis D. H. Peng, S. Jin Zeng & Z.Y. Wen (Wen et al., 2019), Habenaria	
266	malipoensis Q. Liu & W.L. Zhang (Zhang et al., 2017), Primulina malipoensis L.H.	Deleted: such as
267	Yang & M. Kang (Yang et al., 2018), and Salacia malipoensis X.D. Ma & J.Y. Shen	
268	(Ma et al., 2020).	
269	Phenology: Flowering in April to August, and fruiting in May to October.	
270	Paratype: CHINA. Yunnan Province, Wenshan State, Malipo Hsien, Laoshan,	
271	Nandong to Bajiaoping, under the semi-shady forests, at the elevation of 1171 m, 5	
272	March 2018, Z.D. Wei, F.Z. Shangguan, X.X. Zhu, et al. LiuED8755 (KUN).	
273	Distribution and habitat: The species is known only from its type locality, Malipo	
274	Hsien in <u>southeast</u> Yunnan Province, China (Fig. 5).	Deleted: Southeast
275	Habitat. The species grows on stone slopes near the roadsides of the semi-shady	G + I DYOYG 2 1 2
276	forests or under the semi-shady forests, in limestone environments, at an elevation of	Commented [MOU6]: Rocky?
277	1171–1200 m.	Deleted: the
278	Chinese name. Ma Li Po Que She Mu (麻栗坡雀舌木)	
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280	Key to species of <i>Leptopus</i> , modified from Vorontsova & Hoffmann (2009)	
281	1a. Leaf blades coriaceous, almost succulent; endemic to Guangxi Province,	
282	China	
283	1b. Leaf blades membranaceous to thick chartaceous, never	
284	succulent	
285	2a. Leaf blades with 8-10 visible pairs of secondary veins; fruit strongly reticulate;	
286	seed with orange micropylar appendage	
287	2b. Leaf blades with 0–6 (7) visible pairs of secondary veins; fruit smooth to faintly	
288	reticulate; seed without appendage	
289	3a. Ascendant herb to subshrub up to 0.5 m high; female pedicels 2–5 mm in flower,	
290	5–9 mm in mature fruit	
291	3b. Erect to procumbent herb or shrub 0.5-4 m; female pedicels 5-30 mm in flower,	
292	7–36 mm in mature fruit	
293	4a. Male pedicels less than 3 mm in length	
294	4b. Male pedicels no less than 3 in length, and usually up to 10 mm or longer 6	
295	5a. Branches white to light brown; male petals, filaments and styles mostly	
296	glabrous; seeds smooth; endemic to Hainan Province,	

5b. Branches reddish; male petals, filaments and styles hirsute; seeds transversely to irregularly ridged, sometimes pitted; endemic to Khanh Hoa Province, 7a. Leaves usually less than 3 cm in length, and never longer than 5 7b. Leaves usually longer than 5 cm in length, sometimes even longer than 10 cm in length8 8a. Leaves less than 10 cm in length and less than 3.5 cm in width; leaf blades with 8b. Leaves sometimes longer than 10 cm and wider than 3.5 cm; leaf blades 4 or 5 9a. Stems and branchlets usually pendulous or procumbent; ovary 3-locular; styles 9b. Stems and branchlets ascendant or erect; ovary 4–5-locular; styles 4–5; endemic

Adhikari et al. (2010) described the new species *Leptopus nepalensis* B. Adhikari, R.P. Chaudhary & S.K. Ghimire from Nepal, the holotype (*B. Adhikari 224*, TUCH) of which had been <u>originally</u> identified as *Phyllanthus griffithii* Müll.Arg. On the basis of the morphological description provided by Adhikari et al. (2010), the new species is characterized by its 6 petals in two whorls, 6 stamens (or 3 as observed from the <u>illustration</u> drawn based on the holotype), and 3 styles connate into a column up to about halfway. However, all of these characters are very different from those of the genus *Leptopus* as currently circumscribed (Vorontsova & Hoffmann, 2009; Webster, 2014). Thus the species *L. nepalensis* is not included in the above key, and the taxonomic status of the species needs to be clarified in further study, especially in molecular phylogenetic analysis.

Acknowledgements

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345 346 The authors are grateful to the curators and staff of the herbaria GXMG, IBK, IBSC, KUN and PE <u>for</u> hosting our visits or providing images of specimens, <u>and</u> to Mr. Long in Malipo Hsien, Laoshan, Yaowang Valley for help in field investigations.

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