

# *Polystichum puteicola*, sp. nov. (sect. *Haplopolystichum*, Dryopteridaceae) from a karst sinkhole in Guizhou, China based on molecular, palynological, and morphological evidence

Li-Bing ZHANG<sup>1,\*</sup>, Hai HE<sup>2</sup>, and Qiang LUO<sup>3</sup>

<sup>1</sup>Chengdu Institute of Biology, Chinese Academy of Sciences, P.O. Box 416, Chengdu, Sichuan 610041, P.R. China and Missouri Botanical Garden, P.O. Box 299, St. Louis, Missouri 63166-0299, USA

<sup>2</sup>Department of Biology, Chongqing Normal University, Shapingba, Chongqing 400047, P.R. China

<sup>3</sup>Department of Environmental & Biological Sciences, Bijie University, Bijie, Guizhou 551700, P.R. China

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**ABSTRACT.** A new fern species, *Polystichum puteicola* (sect. *Haplopolystichum*, Dryopteridaceae), is described from a karst sinkhole in northwestern Guizhou, China. The new species is morphologically similar to *P. obliquum*, a species disjunctly occurring in limestone areas in Taiwan, southwestern China to the western Indo-Himalaya. *Polystichum puteicola* has lamina not contracted towards base (with the largest basal pinnae), petiole scales dark brown adaxially, rachis scales up to  $3.6 \times 0.8$  mm, pinnae dark green, subcoriaceous, lustrous adaxially, and slightly repand or almost entire on margin, and microscales up to  $2 \times 0.5$  mm. In contrast, *P. obliquum* has lamina with basal pinnae contracted, scales of petiole brown adaxially, scales of rachis up to  $2.3 \times 0.5$  mm, pinnae green, chartaceous, matt adaxially, and serrulate on margin, and microscales  $0.2-0.5 \times 0.1-0.2$  mm. Most interestingly, the two species differs in seven positions in the DNA sequences of chloroplast *trnL-F* intergenic spacer. Our phylogenetic analysis shows that the two species are even not closely related. Palynologically, *P. puteicola* has cristate sculpture with numerous spinules on its perispore. Features of the new species in geographical distribution, ecology, spore morphology, and *trnL-F* sequence are given. Conservation assessments are also presented following the IUCN guidelines.

**Keywords:** China; Dryopteridaceae; Guizhou; Karst landscapes; Karst sinkhole; Phylogeny; *Polystichum obliquum*; *Polystichum puteicola*; sect. *Haplopolystichum*; Spore morphology; *trnL-F* sequence.

## INTRODUCTION

Areas of karst topography are characterized by natural features such as sinkholes (Tarbuck and Lutgens, 2002). Sinkholes, also known as dolines or closed depressions, are the diagnostic karst landform, ranging from shallow, bowl-like forms, through steep-sided funnels, to vertical-walled cylinders (Parker, 1984). Southwest China's Guizhou Province offers spectacular karst landscapes including various sinkholes (Zhang et al., 2006).

The Tuntianjing, or the Sky-Swallowing Well,  $27^{\circ}14.42'$  N,  $104^{\circ}59.06'$  E, as the largest karst sinkhole in northwestern Guizhou, is a funnel-shaped karst collapse of carbonate rocks with a dimension of over 400 m long, 150 m wide, and 200 m deep.

As a continuation of our exploration of the diversity and evolution of *Polystichum* Roth in China (Zhang,

1992, 1994, 1996; Kung and Zhang, 1994, 1998; Zhang and Kung, 1994, 1995a, b, 1996a, b, 1998, 1999; Zhang et al., 1995; Kung et al., 2001; Zhang and He, 2009a, b, c, 2010; He and Zhang, 2010), we found in our 2008 field work a species of *Polystichum* sect. *Haplopolystichum* Tagawa sensu Kung et al. (2001) in this sinkhole which is morphologically very similar to but critically distinguishable from the Taiwan-Sino-Himalayan species, *P. obliquum* (Don) Moore. Notably, our molecular work shows that the two species differ in seven positions in DNA sequence of chloroplast *trnL-F* intergenic spacer. Here we describe the new species as *Polystichum puteicola* L. B. Zhang, H. He & Q. Luo.

## MATERIAL AND METHODS

The morphological, palynological, and molecular data are based on the voucher specimens: CHINA. Guizhou: Bijie, L. B. Zhang, H. He, Q. Luo & C. B. Jiang 706 (CDBI, CTC, MO).

\*Corresponding author: E-mail: Libing.Zhang@mobot.org; Tel: +1-314-577-9454; Fax: +1-314-577-9596.

*Morphological Study.* The measurement of roots, petiole, rachis, scales and indusia was conducted with a micrometer under microscope.

*Spore Morphology.* The spore samples were taped onto a specimen stub with double-sided tape and sputter-coated with gold-palladium. Observations were conducted using a JSE-5900LV Scanning Electron Microscope (SEM) (Electron Co., Tokyo, Japan) at 20 kV at Sichuan University, Chengdu, China. Measurements were carried out using digital images of 20 spores with the measure tool in Adobe Photoshop (ver. 7.0.1; Adobe Systems Inc., San Jose, California). Descriptive terminology of the spores follows Punt et al. (2007) available online ([www.bio.uu.nl/~palaeo/glossary](http://www.bio.uu.nl/~palaeo/glossary)).

*Molecular Methods.* Total genomic DNA was isolated from silica-dried leaves using Plant Genomic DNA Kits (TIANGEN BioTech., Beijing, China). The plastid *trnL-F* intergenic spacer was amplified with the universal primers e and f of Taberlet et al. (1991). The PCR protocols followed Zhang et al. (2001) and Zhang and Renner (2003). Amplified fragments were purified with TIANquick Midi Purification Kits (TIANGEN). Purified PCR products were sequenced by Invitrogen™ (Shanghai, China). Most DNA sequences were downloaded from GenBank.

Twenty-five species of sect. *Haplopolystichum* s.l. (Zhang and He, 2009a) and seven representatives of the monophyletic *Polystichum* s.s., as resolved in Driscoll and Barrington (2007), Lu et al. (2007), and Li et al. (2007, 2008), constituted the ingroup. Four species of *Cyrtomium* s.s., i.e. *C. caryotideum* (Wall.) Presl, *C. falcatum* (L. f.) Presl, *C. macrophyllum* (Makino) Tagawa, and *C. urophyllum* Ching, were used as outgroups based on Lu et al. (2005) and Li et al. (2008). A few species were represented by more than one accession. In total 42 accessions were included in the analysis. All sequences used in this study together with their GenBank accession numbers and voucher information or source publications are listed in Appendix I. Preliminary alignment of nucleotides was obtained using the default alignment parameters in Clustal X (Thompson et al., 1997) followed by manual adjustments. Gap characters were scored using modified complex indel coding (Simmons and Ochoterena, 2000; Müller, 2006).

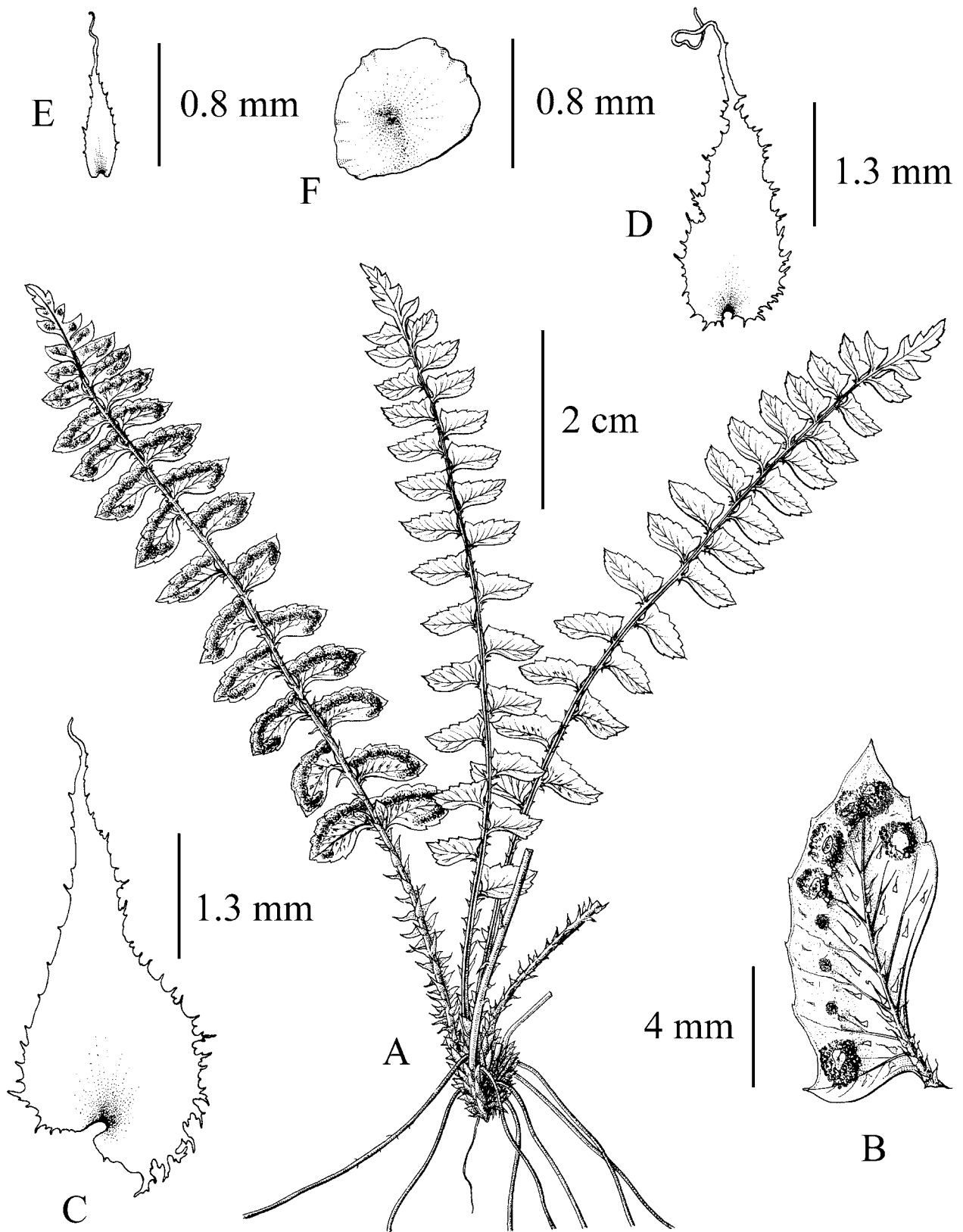
Phylogenetic analysis followed the procedure in Zhang and Simmons (2006) and Zhang and He (2010). Equally weighted parsimony tree searches were conducted using 1,000 tree-bisection-reconnection (TBR) searches in PAUP\* 4.0b10 (Swofford, 2001) with a maximum of 1,000 trees held per TBR search. Parsimony jackknife analyses (Farris et al., 1996) were conducted using PAUP\* with the removal probability set to approximately  $e^{-1}$  (36.946%), and “jac” resampling emulated. One thousand replicates were performed with ten TBR searches per replicate and a maximum of 100 trees held per TBR search.

## RESULTS AND TAXONOMIC TREATMENT

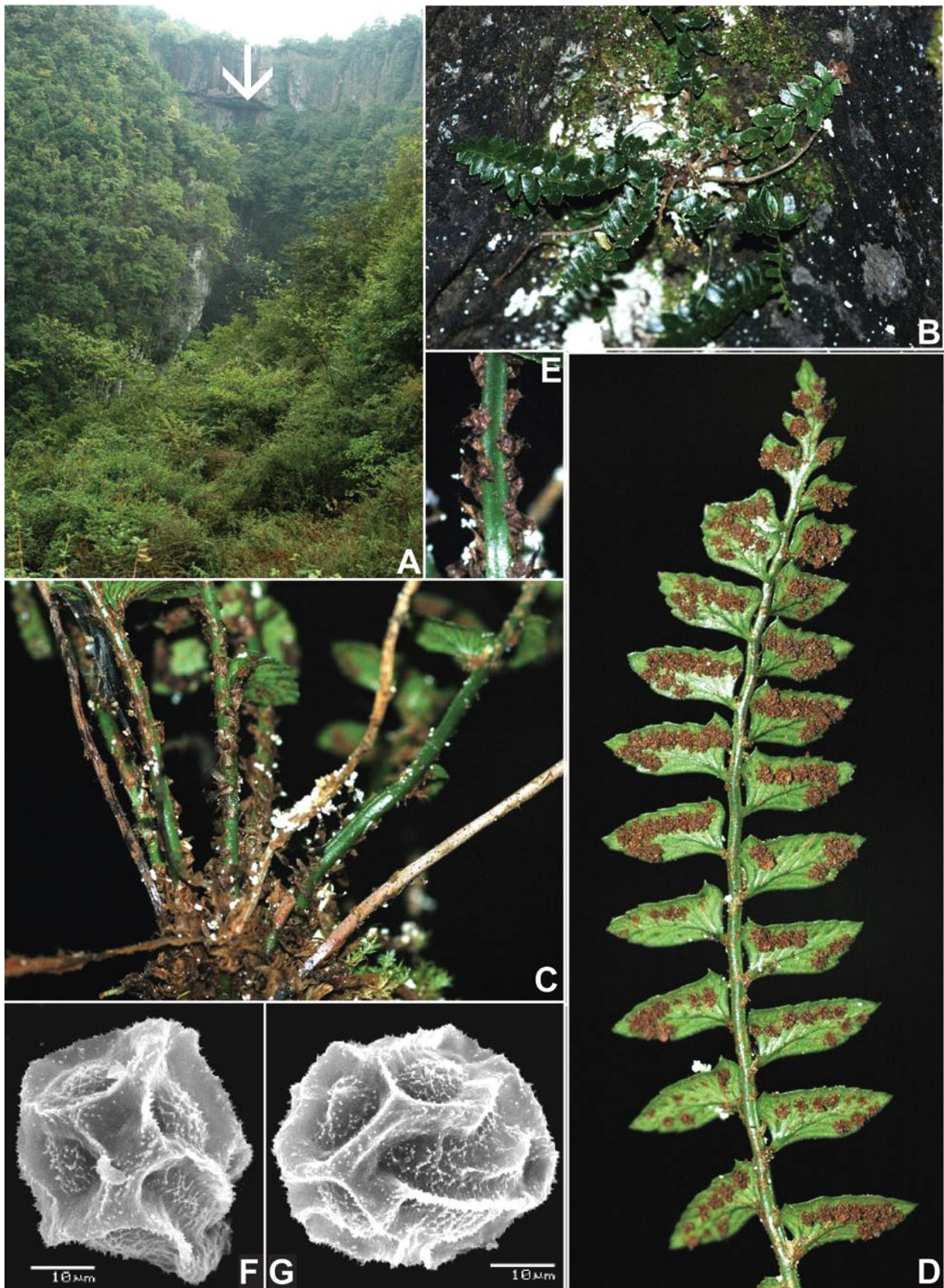
***Polystichum puteicola*** L. B. Zhang, H. He & Q. Luo, sp. nov.—TYPE: CHINA. Guizhou Province: Bijie City, Fangzhu Town, Baini Village, Tuntianjing, 27°14.42' N, 104°59.06' E, on limestone wall, 0.5-1.5 m above the bottom of a sinkhole, c. 200 m under the ground, alt. 1,720 m, 17 Oct. 2008. L. B. Zhang, H. He, Q. Luo & C. B. Jiang 706 (HOLOTYPE: MO; ISOTYPES: CDBI, CTC, HAST, MO). 吞天井耳蕨 Figures 1, 2

*Species valde affinis* *P. obliquum* (D. Don) T. Moore, sed *microsquamis usque ad*  $2 \times 0.5$  mm [ $(0.5-0.8) \times (0.2-0.3)$  mm in *P. obliquum*], *brunneis* (*brunneolis* in *P. obliquum*), *lamina basi non-contracta* (*leviter contracta* in *P. obliquum*), *paleis stipitis supra atrobunneis* (*brunneis* in *P. obliquum*), *paleis rhachidis usque ad*  $3.6 \times 0.8$  mm (*usque ad*  $2.3 \times 0.5$  mm in *P. obliquum*), *pinnis atrovirentibus, subcoriaceis, supra nitidis, margine leviter repandis vel fere integris* (*pinnis viridibus, supra impolitis, margine serrulatis* in *P. obliquum*) *differt.*

Plants perennial, evergreen, 5-14 cm tall. *Rhizome* 0.5-1 cm long, ascending; scales linear, brown, 0.2-3.6 mm long; roots dark brown when dry, up to 9 cm long, c. 0.6 mm in diam., sparsely or densely covered with scales. *Leaves* caespitose, 4-7 per rhizome; petiole 2-6 cm long, 0.6-1.2 mm in diam. at middle, adaxially canaliculate, green; basal petiole scales ovate-lanceolate,  $3.6-4.5 \times 1.1-2.3$  mm, chartaceous, composed of multiple layers of cells, margin ciliate or erose, apex acuminate or caudate, brown, and matt, adaxially flat, dark brown, and lustrous; distal petiole scales ovate-lanceolate,  $2.7-3.7 \times 1.1-2.1$  mm, differing in size, membranaceous, composed of 1 layer of cells, brown, margin regularly ciliate or with outgrowth, apex caudate, matt. *Lamina* lanceolate, 1-pinnate, 3.5-9.5 cm long, 1.2-2.6 cm wide at middle, 1.3-2.7 cm wide and broadest at base, apex acute; rachis 0.7-1.2 mm in diam. at middle, without proliferous buds, adaxially sulcate; scales of rachis similar to distal petiole scales but smaller, differing in size, margin regularly ciliate, apex caudate, matt. *Pinnae* 6-14 pairs, sparsely arranged, strongly reflexed toward lamina base, basal two pairs 0.7-1.5 cm apart, alternate, oblong, middle pinnae  $7.5-12 \times 3.5-5.5$  mm, basalmost pinnae slightly larger, shortly petiolated, subcoriaceous, acroscopic base slightly auriculate, basiscopic base cuneate, forming 30-90-degree angle with rachis, apex acute, margin slightly repand-serrate ended in tiny tip or nearly entire and without aristate spinules, abaxially scaly, adaxially lustrous and glabrous; microscales on abaxial surface subulate with dilated base (broad-type microscales), 0.5-1.1 mm long, base 0.13-0.26 mm wide, with a few curly outgrowths on margin of base; venation pinnate; midrib slightly raised abaxially, flat adaxially; lateral veins free, 4-5 pairs from midrib per pinna, each lateral vein further dichotomous, indistinct on both surfaces. *Sori* terminal on veins of pinnae, 5-9 (1-3 below midrib, 4-6 above midrib) per fertile pinna, located approximately at middle between midrib and pinna margin and 0.9-1.6 mm distant from pinna margin; all pinnae on



**Figure 1.** *Polystichum puteicola* L. B. Zhang, H. He & Q. Luo. A, Habit; B, Pinna; C, Scale from base of petiole; D, Rachis scale; E, Microscale; F, Indusium (From the holotype, L. B. Zhang, H. He, Q. Luo & C. B. Jiang 706, MO).



**Figure 2.** *Polystichum puteicola* L. B. Zhang, H. He & Q. Luo. A, Surroundings of karst sinkhole in northwestern Guizhou, China, from where the new species was discovered. Arrow points to the hidden sinkhole; B, Habit with limestone cliff on background; C, Lower portion of plant; D, Lamina; E, Portion of petiole; F, Polar view of spore; G, Equatorial view of spore.

fertile lamina fertile; indusia peltate, 0.8-1.2 mm in diam., membranaceous, brown, margin erose (Figures 1, 2).

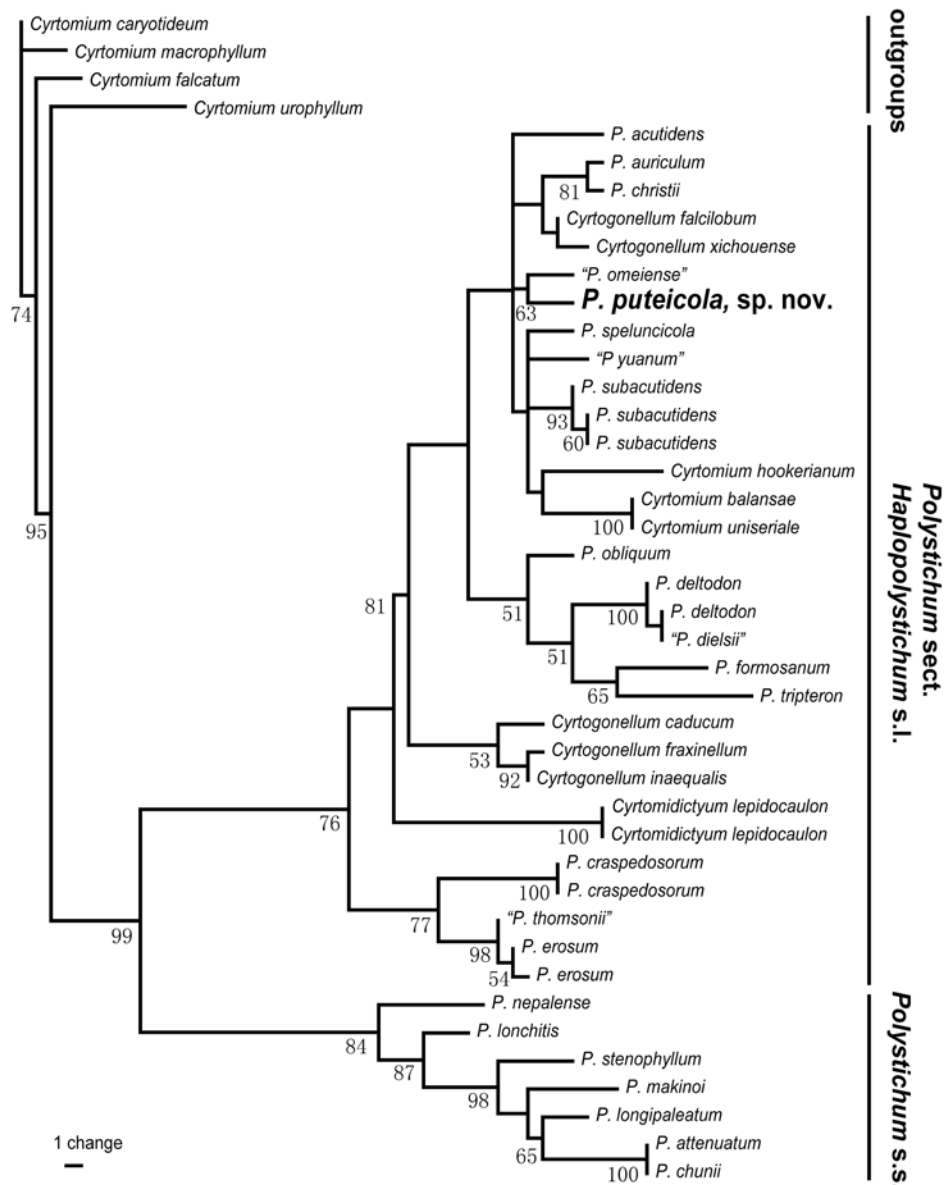
**Spore Morphology.** The spores are subglobose in polar view and elliptic in equatorial view. The spore size is c.  $32.5(28-37) \times 43.5(36-48) \times 34.2(31-38) \mu\text{m}$  (polar axis  $\times$  long equatorial axis  $\times$  short equatorial axis, respectively). The ratio of length of the polar axis to that of the long equatorial axis is c. 0.8. The perispore sculpture is cristate with numerous spinules which are c.  $0.5 \mu\text{m}$  long (Figure 2F, G).

**Molecular Phylogenetics.** The chloroplast *trnL-F* intergenic spacer of *P. puteicola* was sequenced. The sequenced region is 381 basepairs long (including a few basepairs of *trnL* and *trnF* genes at ends) and the

GC content is c. 39.1%. The newly generated sequence has been deposited in GenBank with accession number GQ244335.

Heuristic searches yielded 194 most parsimonious trees with tree length = 212, consistency index = 0.6934, and retention index = 0.8298. One of the most parsimonious trees is shown in Figure 3. Species with doubtful identity, whose *trnL-F* sequence were downloaded from GenBank, were indicated with quotation marks in Figure 3.

**Geographical Distribution.** So far *Polystichum puteicola* is known only from the type locality in Fangzhu Town, Bijie City, northwestern Guizhou, China (Figure 4). Comparable habitats may be difficult to find in the neighborhood with high altitudes, moist, and twilight



**Figure 3.** One of the 194 most parsimonious trees based on DNA sequences of chloroplast *trnL-F* intergenic spacer. Tree length = 212, consistency index = 0.6934, and retention index = 0.8298. The numbers below or next to the branches are jackknife values. The bar indicates one change. The species in bold face is the new one described in this study.

conditions, and limestone substrate. *Polystichum puteicola* might be endemic to this sinkhole.

**Ecology.** *Polystichum puteicola* grows on moist limestone walls, 0.5-1.5 m above the sinkhole bottom, c. 200 m below the above ground, alt. 1,720 m.

Associated plants on limestone walls occurring at similar positions as the new species include moss species of *Hymenostylium* and *Zygodon* (Pottiaceae) and fern species *Polystichum cyclolobum* C. Chr. (Dryopteridaceae). Other plants found at the bottom of the sinkhole include fern species *Allantodia laxifrons* (Rosenst.) Ching, *Athyriopsis japonica* (Thunb.) Ching (Athyriaceae), *Coniogramme caudiformis* Ching et Shing (Hemionitidaceae), *Cyrtomium caryotideum* C. Presl, *Polystichum acutidens* Christ, *P. craspedosorum* (Maxim.) Diels, *P. pseudoxiphophyllum* Ching ex H. S. Kung (Dryopteridaceae), and *Struthiopteris eburnea* (Christ) Ching (Blechnaceae), and seed plants *Boehmeria clidemioides* Miq., *Geranium* sp., *Gynostemma pentaphyllum* (Thunb.) Makino, *Impatiens* sp., *Kalopanax septemlobus* Koidz., *Lindera megaphylla* Hemsl., *Paraprenanthes sororia* (Miq.) C. Shih, *Rubus* sp., *Sambucus chinensis* Lindl., *Strobilanthes* sp., and *Toxicodendron* sp.

**Conservation assessments.** Only one population with c. 50 individuals was found so far. The status of the new species clearly should be classified as CE - Critically Endangered category based on current information and following the IUCN (The International Union for Conservation of Nature and Natural Resources) guidelines (IUCN, 2008), but more extensive searches are needed to accurately assess the conservation status. The maintenance of the cool, moist, and twilight habitat of the new species in the sinkhole relies on the continuous flowing of water from above ground and the vegetations in the sinkhole and around the mouth of the sinkhole. Fortunately, the bottom

of the sinkhole is not easy to access and thus the habitat is less susceptible to human disturbance. Notably, Bijie National Forest Park including the sinkhole area has been established to protect the local natural vegetations and habitats.

**Etymology.** From the Latin *puteus*, well, pit or hole, and the Latin suffix *-cola*, dweller, referring to the sinkhole-dwelling habit of the species.

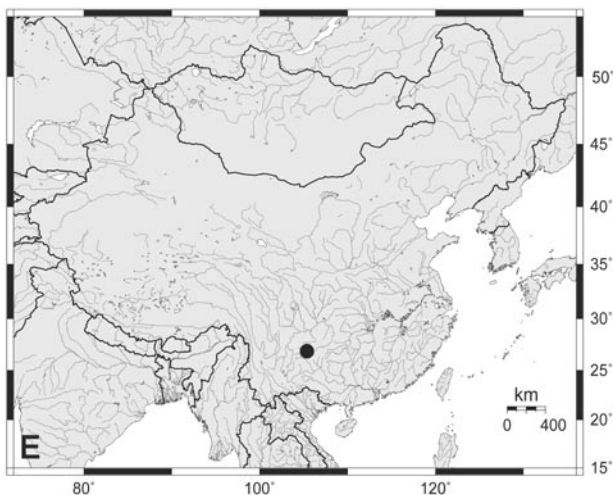
**Additional specimen examined.** CHINA. Guizhou Province: Bijie City, Fangzhu Town, Baini Village, Tuntianjing, 27°14.42' N, 104°59.06' E, on limestone wall, 0.5-1.5 m above the bottom of a sinkhole, c. 200 m under the ground, alt. 1,720 m, 17 Oct. 2008, Q. Luo 08363 (Bijie University Herbarium).

## DISCUSSION

*Polystichum puteicola* is most similar to *P. obliquum* in the stature, pinna shape, and pinna size, but *P. puteicola* has the lamina widest at the base, petiole scales blackish brown adaxially, rachis scales up to  $3.6 \times 0.8$  mm, pinnae oblong, dark green, subcoriaceous, lustrous adaxially, and slightly repand-serrate or almost entire on margin, and microscales brown and up to  $2 \times 0.5$  mm. In contrast, *P. obliquum* has slightly oblanceolate lamina with the basalmost pair of pinnae contracted, petiole scales brown adaxially, rachis scales up to  $2.3 \times 0.5$  mm, pinnae rhombic-ovate or rarely oblong, green, chartaceous, matt adaxially, and serrulate on margin, and microscales brownish and of  $0.5-0.8 \times 0.2-0.3$  mm. As pointed out by C. R. Fraser-Jenkins (pers. comm.), there is some overlap in lamina shape and glossiness between *P. puteicola* and material of *P. obliquum* from the Indo-Himalaya, from where only a few specimens were examined for this study.

*Polystichum puteicola* is also similar to *P. paradeltodon* L. L. Xiang in the stature and pinna size. In comparison with *P. paradeltodon*, *P. puteicola* has leaves thicker and dark green (vs. thinner and green or yellowish green in *P. paradeltodon*), pinnae oblong (vs. falcate-triangular in *P. paradeltodon*), more or less reflexed and with basicopic base attached at 30-90-degree angles to rachis (vs. upwards at 100-110-degree in *P. paradeltodon*), and perispore with cristate sculpture (vs. filamentous-retiform sculpture in *P. paradeltodon*; Xiang, 1994; Kung et al., 2001).

Interestingly, *P. puteicola* differs from the morphologically most similar species, *P. obliquum*, in seven positions (six nucleotide substitutions and one 4-basepair long gap) in the *trnL-F* sequences (*trnL-F* sequence of *P. obliquum*: GenBank accession no. EF177284, submitted by Driscoll and Barrington, 2007), suggesting that the two species are impossible to be conspecific. Our phylogenetic analysis shows that the two species are even not closely related (Figure 3). This to some extent also suggests decoupling of morphological and molecular evolution rates contrary to that suggested in *Aquilegia* (Hodges and Arnold, 1994) and Hawaiian silverswords (Baldwin and Robichaux, 1995) where



**Figure 4.** Distribution (●) of *Polystichum puteicola* L. B. Zhang, H. He & Q. Luo in northwestern Guizhou Province, China.

remarkably low molecular variation was observed in contrast with considerable morphological divergence. We do not have *trnL-F* sequence of *P. paradelton* to compare with.

In addition to the limestone substrate and the cool and twilight conditions inside the sinkhole, a small stream flowing down from the above ground keeps the sinkhole moist. This ideal habitat for many lime-loving ferns such as species of *Polystichum* sect. *Haplopolystichum* s.l. (Zhang and He, 2009b) and the absence of gene flow between the species inside and outside of the sinkhole caused by the physical barrier of the karst collapse may have played an important role in the speciation of *P. puteicola*.

*Polystichum puteicola* is the second species in the genus described from and so far only known from a single karst sinkhole (the first one is *P. nayongense* P. S. Wang & X. Y. Wang; Wang and Wang, 1997).

In addition to *P. obliquum* and *P. paradelton*, two more species in sect. *Haplopolystichum*, *P. peishanii* L. B. Zhang & H. He and *P. delton* (Baker) Diels var. *henryi* Christ, share with *P. puteicola* in pinnae not aristate on margin, acute apically, and with the length to width ratio  $\leq 2$ . These five species can be distinguished from one another with the following key:

#### Key to *Polystichum puteicola* and its allies

1. Margin of pinnae repand or slightly toothed; pinnae less than  $2 \times 1$  cm.
  2. Pinnae green or yellowish green, chartaceous, with basiscopic base attached at 90-110-degree angles to rachis.
    3. Pinnae oblong or falcate-triangular, 16-40 pairs; sori between midrib and margin or close to margin of pinnae.
      4. Sori close to margin of pinnae; pinnae up to 40 pairs. .... *P. delton* var. *henryi*
      4. Sori between midrib and margin or slightly closer to margin of pinnae; pinnae fewer than 20 pairs. .... *P. paradelton*
    3. Pinnae ovate, rhombic-ovate or rarely oblong, 7-15 pairs; sori between midrib and margin of pinnae. .... *P. obliquum*
  2. Pinnae dark green, subcoriaceous, with basiscopic base attached at 30-90-degree angles to rachis. .... *P. puteicola*
1. Margin of pinnae entire or slightly repand distally; pinnae  $2.4-3.3 \times 1.1-1.3$  cm. .... *P. peishanii*

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work. Three anonymous villagers from Baini Village helped access the bottom of the sinkhole. Yu Wang helped with the molecular work and Bo Xu with the SEM work. Si He identified the two associated moss species. Yingbao Sun prepared the basic line drawing. Helpful comments were received from C. R. Fraser-Jenkins, Peishan Wang, and George Yatskievych. Special thanks go to Ning Wu and Xinfen Gao for their support. The curators of the herbaria CDBI, CTC, and MO provided access to the specimens in their care.

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# 中國貴州喀斯特天坑耳蕨屬一新種：吞天井耳蕨（鱗毛蕨科半開羽耳蕨組）——基於分子、孢子周壁紋飾和形態學證據

張麗兵<sup>1</sup> 何海<sup>2</sup> 駱強<sup>3</sup>

<sup>1</sup> 中國科學院成都生物研究所 (CDBI); 密蘇里植物園 (MO)

<sup>2</sup> 重慶師範大學 生物系 (CTC)

<sup>3</sup> 貴州畢節學院環境與生命科學系

本文描述了在中國貴州西北部一喀斯特天坑中發現的耳蕨屬半開羽耳蕨組 (*Polystichum* sect. *Haplopolystichum*) 一新種：吞天井耳蕨 (*P. puteicola*)，並提供線繪圖與彩色照片以資辨識。吞天井耳蕨形態上與間斷分佈於臺灣、中國—喜馬拉雅石灰岩地區的斜羽耳蕨 (*P. obliquum*) 相似，但吞天井耳蕨的葉片基部最寬，向基部不縮短，葉柄鱗片近軸面深棕色，葉軸鱗片達  $3.6 \times 0.8$  mm，羽片深綠色，近革質，向軸面有光澤，邊緣具略波狀微齒或近全緣，小鱗片達  $2 \times 0.5$  mm；而斜羽耳蕨的葉片的基部縮短，葉柄鱗片向軸面棕色，葉軸鱗片達  $2.3 \times 0.5$  mm，羽片綠色，草質，向軸面無光澤，邊緣有鋸齒，小鱗片達  $0.5-0.8 \times 0.2-0.3$  mm。最有趣的是，兩者在葉綠體 *trnL-F* 基因間區的 DNA 序列上有七個位點的差異。系統學分析表明，兩種耳蕨並不是最近緣的。吞天井耳蕨的孢子周壁紋飾為冠狀並具眾多微刺。本文給出了吞天井耳蕨在地理分佈、生態學、孢子形態和 *trnL-F* 基因間區的 DNA 序列特徵，並對該種的瀕危狀況做出了評價。

**關鍵詞：** 中國；鱗毛蕨科；貴州；喀斯特地貌；喀斯特天坑；斜羽耳蕨；吞天井耳蕨；系統發育；半開羽耳蕨組；孢子形態；*trnL-F* 基因間區序列。

**Appendix 1.** Voucher information, GenBank accession numbers, and source publications.

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