Selaginella dianzhongensis (Selaginellaceae), a new spikemoss from China

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Abstract
A new species of spikemoss from Yunnan Province of China, Selaginella dianzhongensis, is described and illustrated based on evidence from gross morphology, micromorphology and molecular phylogeny. S. dianzhongensis is most similar to S. amblyphylla in its habit of creeping stem, leaf size, and obviously dimorphic sporophylls, but is distinct by its ventral leaves ovate-oblong, subcordate at base, basiscopic base entire, axillary leaves ovate and decurrent at base. Molecular phylogeny analysis of three chloroplast gene regions (rbcL, atpI, psbA) shows that S. dianzhongensis forms an independent branch with strong support which is distantly related to S. amblyphylla and S. kurzii, but sister to S. bodinieri which is quite different in habitat of erect or ascending stem and rhizophores restricted to the lower part, and slightly dimorphic sporophyllus.

Keywords
Lycophytes, Selaginella amblyphylla, taxonomy, Yunnan, rbcL, atpI, psbA

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Introduction

The initial critical taxonomic revision of Chinese *Selaginella* was published by Alston (1934), who recognized 41 species from China. In the Flora Reipublicae Popularis Sinicae and Flora of China (Zhang 2004, Zhang et al. 2013) the numbers of recognized species increased to 64 and 72, respectively. Recently, several new species and new records have been reported from China (Zhou et al. 2015a, Zhang and Sun 2015, Wu et al. 2017). Yunnan province is one of the species diversity centers of plants in China, with more than 53 species of *Selaginella* (Chu, 2006).

During field trips in Yunnan, we collected an unknown *Selaginella* species from Yimen County in central Yunnan. Morphology characters show it is similar to *S. amblyphylla* Alston, but phylogenetic analysis based on three chloroplast gene regions show it is close to *S. bodinieri* Hieron. Both molecular and morphology data support the taxon as a new species, which is described and illustrated here.

The new species belongs to subgenus *Heterostachys* in the classification system of Jermy (1986), and sect. *Heterostachys* of subgenus *Heterostachys* in the molecular-phylogeny classification proposed by Zhou and Zhang (2015), but this was rejected in the more robust molecular phylogenetic analysis based on chloroplast and nuclear genes by Weststrand and Korall (2016) that only recognized a broad subgenus *Stachygynandrum*.

Material and methods

Herbarium specimens, silica gel and living materials were collected from Longquan Forest Park of Yimen (Yunnan province), in evergreen forest at 24°40’86”N, 102°08’27.86”E. Herbarium specimens are preserved in PE, and compared with similar species. The morpho-photographs of the plants were taken with a Nikon DXM 1200F camera connected to a stereomicroscope (Nikon SMZ 1000) and computer, measurements were done by D 3.10 (http://www.nikoninstruments.com). The application ImageJ (https://imagej.nih.gov/ij/) was used to measure morphological characteristics (such as axillary, dorsal, ventral leaves, stems and strobili).

For study of spore morphology, scanning electron microscopy (SEM) was used. The spores were taken from mature sporangia and fixed on double line tape, and then covered with gold-palladium mixture. Spores were photographed and measured under different magnifications using a Hitachi S-4800 at 10–20 kV.

In this study, we sampled 28 taxa, representing almost all Chinese species with dimorphic strobili. Total genomic DNA was isolated from silica-dried material using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer’s protocols. For each species, we attempted to amplify three chloroplast gene regions (*rbcL*, *atpI*, *psbA*) for the possible new taxa and its putative closely related taxa. These three regions were amplified with newly designed primers *rbcL* 192F (5’CACGTTGGACTACCGTITTGGA3’) and 1324R (TACCCTCAAGAGCGGGATCA3’), *atpI* 119F (5’CYCAGGTTCATGGACAGTAC3’) and *atpI* 540R
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(5'GRGTATYGGGGTTGGTG3'), psbA 169F (5'CACGTGGACTACCGTTTG-GA3') and psbA 1026R (5'ATCTRGWGGGAAGTTGTGAGC3'), respectively. According to recent classification of Selaginella (Zhou and Zhang 2015; Weststrand and Korall 2016), we download 10 rbcL sequences of S. sect. Homostachys (S. laxistrobila, S. helvetica, S. nipponica), S. sect. Auriculate (S. remotifolia, S. kruassiana), S. sect. Oligomacronsporangiatae (S. braunii, S. uncinata, S. delicatula), and S. subg. Selaginoides (S. deflexa, S. selaginoides) from Genbank as outgroups. The 25 μL volume polymerase chain reactions (PCRs) contained 1 μL of plant DNA, 2.5 μL dNTPs, 1 μL each primer, 0.15 μL Taq polymerase (Takara Biotechnology Co., Dalian, China), 16.85 μL ddH₂O. The PCR amplification profiles were identical for the three fragments: one cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 52 °C for 1 min, 72 °C for 1.5 min; and one cycle at 72 °C for 10 min. All PCR products were directly sequenced using ABI 3730XL analyzer (Applied Biosystems, Foster City, California, USA). Newly obtained sequences were assembled with ContigExpress and then aligned with the downloaded sequences using Clustal X v.1.83 (Thompson et al. 1997) followed by manual adjustment in BioEdit v.7.1.11(Hall 1999). We have deposited all sequences into GenBank.

Phylogenetic tree of combined dataset (rbcL+atpI+psbA) was constructed using maximum likelihood (ML) and Bayesian inferences (BI). jModelTest 0.1.1 (Posada 2008) was used to select the appropriate substitution model for ML and BI analyses. The ML analysis was performed on the XSEDE online computing cluster accessed via the CIPRES Science Gateway (http://www.phylo.org) using RAxML-HPC2 v.8.2.8 (Stamatakis 2014), with 1000 bootstrap replicates under the GTRGAT model. Bayesian analyses and posterior probability (PP_{BI}) calculation were conducted in Mr-Bayes 3.2.6 (Ronquist et al. 2012) implemented on the CIPRES Science Gateway Portal (Miller et al. 2010). We ran four chains of the Markov chain Monte Carlo (MCMC), sampling one tree every 100 generations for 1,000,000, starting with a random tree. Bayesian posterior probabilities (PP) were calculated as the majority consensus of all sampled trees with the first 25% discarded as burn-in.

Results

Taxonomic treatment

Selaginella dianzhongensis X.C.Zhang, sp. nov.
urn:lsid:ipni.org:names:60478269-2
Figures 1, 2

Diagnosis. The new species resembles Selaginella amblyphylla in habit and gross morphology, but it is different in stems and branches reddish (vs. stramineous in S. amblyphylla), ventral leaves ovate-oblong, 1.1–2.2 × 0.4–0.8 mm (vs. oblong, 2–3 × 0.6–1.2 mm), base subcordate, basiscopic margin not ciliolate (vs. rounded and margin sparsely ciliolate); dorsal leaves oblique subcordate or cordate at base (vs. obliquely cordate),
Figure 1. *Selaginella dianzhongensis* X.C. Zhang, sp. nov. A habit B adaxial view of strobilus C ventral leaf D axillary leaf E dorsal leaf F adaxial view of lower sporophyll G adaxial view of upper sporophyll H abaxial view of strobilus (Illustration made by Huixia Dong).
Figure 2. *Selaginella dianzhongensis* X.C. Zhang, sp. nov. **A** individual **B** portion of plant **C** habit **D** dorsal leaf **E** ventral leaf **F** strobili **G** axillary leaf **H** proximal surface of megaspore **I** distal surface of megaspores **J** portion of megaspore surface enlarged to show infrastructural detail **K** distal surface of microspore **L** proximal surface of microspore **M** portion of microspore surface enlarged to show infrastructural detail surface (Taken from Yan-Mei Zhu 8158 (PE)).
margin with rather long cilia (vs. denticulate or ciliolate); axillary leaves ovate and decurrent at base (vs. ovate or triangular and obtuse to decurrent at base); strobili 3.2–4.0 × 2.3–3.5 mm (vs. 3.5–10 × 3.2–4.4 mm), ventral sporophylls margin ciliolate, dorsal sporophylls margin denticulate (vs. both sporophylls margin ciliolate).

**Type.** CHINA, Yunnan Province, Yimen County, Longquan Forest Park, 9 Feb 2017, Yan-Mei Zhu 8158 (Holotype, PE!; isotype PE).

**Description.** Plants terrestrial, evergreen. Main stems reddish, creeping or suberect, stems 15–25 cm long, 0.6–1.0 mm in diam., branches stramineous. Rhizophores restricted in basal part of main stems or at intervals throughout length of creeping stem and branches, borne on ventral side in axils of branches. Main stems branched from near base, slightly sulcate, primary branches 0.7–1.5 cm apart, secondary branches once or twice pinnately branched, leafy portion of main stem including leaves 6–8 mm wide at middle, ultimate branches 4–7 mm wide including leaves. Axillary leaves on main stems and branches symmetrical, ovate, 1.1–2.2 × 0.4–0.8 mm, at base decurrent, margin sparsely long ciliate at base, ciliolate or denticulate to the apex, apex acute. Dorsal leaves ± symmetrical, on main stems distantly, on branch imbricate, on main stem larger than on branches, ovate to broadly ovate, 1.2–1.9 × 0.5–1.0 mm, slightly carinate on abaxial surface, in base oblique subcordate or cordate, not peltate, margin ciliolate at base, apex long aristate, arista 0.3–0.6 mm long. Ventral leaves asymmetrical, slightly distant, on main stem bit larger than those on branches, ovate-oblong, 1.7–3.9 × 0.7–1.6 mm, in base subcordate; acrosopic base slightly overlapping stems and branch, margin long ciliolate at base, denticulate to the apex, basiscopic bases free from stems, margin entire, apex acute. Strobili compact, solitary, terminal on branch tips, dorsiventrally complanate, 3.2–4.0 × 2.3–3.5 mm. Sporophylls strongly dimorphic: dorsal sporophylls ovate-lanceolate, margin denticulate, apex acuminate, with sporophyll-pteryx slightly incomplete, margin denticulate, ventral sporophylls ovate-lanceolate, ascending, carinate, margin ciliolate, apex cuspidate. Megaspores white-yellow; proximal and distal surfaces verrucate, micro-sculpture densely echinate. Microspores yellowish, proximal and distal surfaces irregularly verrucate, micro-sculpture with interconnected and blunt spinules.

**Etymology.** Dianzhong means central Yunnan in Chinese: the type locality (Yimen) is in the central Yunnan area which is centered on the Provincial capital city Kunming.

**Distribution and habitat.** *Selaginella dianzhongensis* is known only from Yimen county, Yunnan, growing on mossy soils in a mixed evergreen forest, at ca. 1576 m a.s.l. (Fig. 3).

**Conservation status (VU).** *Selaginella dianzhongensis* is known only from one locality inside the Longquan Forest Park in Yimen county, with more than 300 individuals. This park has a heavy recreational load and human pressure, and there are no specific measures to protect the habitats. Considering the restricted distribution and plausible threats, we tentatively assessed *Selaginella dianzhongensis* as vulnerable (VU) according to the IUCN (2018) categories and criteria.

**Specimens examined.** *Selaginella amblyphylla* Alston: THAILAND: Payar, Doi Angka, H. M. Smith 357 – BM [BM000779901, image online!] (holotype), – US [US00134348, image online!], MICH [MICH1191432, image online!], GH...
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Figure 3. Distribution of Selaginella dianzhongensis X.C.Zhang, sp. nov.

[GH00022032, image online!] (isotypes), Doi Cheng Dao, 4800 ft., on rock, Oct–Nov 1922, E. Smith 1262 – SING [!, image] (paratype); Udawn, 900–1400 m, Tagawa c.s. T-1816 [PE01622140]; CHINA: Yunnan, Mengla County, B. G. Li 48926 [PE00405395]; Zhenkang W. M. Chu et al. 15204 [PE00405401]; Yunnan, Gengma, W. M. Chu et al. 15279 [PE00405408]; Guangxi, Lingui, J. X. Zhong 808194 [PE01593730]; Yunnan, Simao (Szemao), ravine, 4000 ft., A. Henry 13529 – NY [NY00127369, image online!] (paratype). Selaginella bodinieri Hieron. Guizhou, Bijie, X. C. Zhang et al. 6842 [PE 01962745]; Guangxi, Fengshan, Alt. 750 m, X. C. Zhang 1272 [PE 00405447]; Selaginella kurzii Baker, Yunnan, Cangyuan, J. C. Zhao 2000-13 [PE 00405796]; Luquan, W. M. Chu 1649 [PE 00405795]; Mengla (Chai-li-Hsien), alt. 950 m, C. W. Wang 77750 [PE 01634093].

Phylogenetic Analysis

The combined data matrix included up to 2045 nucleotides for each of the 37 taxa with 374 parsimony informative sites (374/2045 = 18.29%), consistency index (CI) = 0.66, retention index (RI) = 0.80, when the gaps were treated as missing data. The tree recovered from maximum likelihood (ML) and Bayesian inferences (BI), with bootstrap values (BS) of ML and Bayesian posterior probabilities (PP) for each clade is shown in Fig. 4. The new species sampled from Yimen clustered with Selaginella bodinieri with strong support (BS \text{ML} = 99; PP \text{BI} = 1.0), but the new species is quite similar to the S. amblyphylla rather than S. bodinieri in morphological characters.
Figure 4. The 50% majority rule consensus tree derived from maximum likelihood showing the position of *Selaginella dianzhongensis*. Support values (BSML/PPBI) are shown above the main branches; the dash (–) indicates BS < 50%. The new species is shown in bold.

**Discussion**

Morphologically, the shape and margin of ventral and dorsal leaves of *Selaginella dianzhongensis* is most similar to *S. amblyphylla*. But the axillary leaves of the former are ovate, 1.1–2.2 × 0.4–0.8 mm, margin with a few long cilia (vs. ovate or triangular, 2–3 × 0.6–1.2 mm, and denticulate at margin in *S. amblyphylla*). Ventral leaves of the former are ovate-oblong, apex acute (vs. oblong and obtuse or subacute at apex in *S. amblyphylla*), basiscopic margin entire and not ciliolate (vs. basiscopic margin sparsely ciliolate at base in *S. amblyphylla*), acroscopic base of ventral leaf long ciliolate (vs. shortly ciliolate in *S. amblyphylla*).
Molecular data showed that *S. dianzhongensis* clustered with two other species: *S. kurzii* Baker and *S. bodinieri*.

*S. dianzhongensis* is indeed similar to *S. kurzii*, but fertile branches are not erect (vs. erect in *S. kurzii*), dorsal leaves are ovate to broadly ovate with arista at apex (vs. ovate or ovate-elliptic, acuminate or aristate at apex), and ventral leaves are ovate-oblong, basiscopic margin entire and not ciliolate (vs. ovate-triangular, basiscopic margin entire or with 1 or 2 cilia at base).

*Selaginella bodinieri* is widely distributed in the limestone areas from central to southwestern China: main differences between this species (and the other ones mentioned above) and *S. dianzhongensis* are reported in the key below, and in Table 1.

Finally, mega- and microspores of *S. dianzhongensis* are morphologically different from the spores of similar species studied by Zhou et al. (2015b). Megaspores of *S. dianzhongensis* have verrucae on proximal and distal side; micro-sculptures of megaspores are densely echinate on both sides (Fig. 2 H–J). Microspores of *S. dianzhongensis* are verrucate, with blunt spines (Fig. 2 K–M). Morphological comparison of mega- and microspores between *S. dianzhongensis* and closely related species is presented in Table 2.

### Table 1. Morphological characters of *Selaginella amblyphylla*, *S. bodinieri*, *S. dianzhongensis* and *S. kurzii*.

| Characters                  | *S. amblyphylla* | *S. bodinieri* | *S. dianzhongensis* | *S. kurzii*       |
|-----------------------------|------------------|----------------|---------------------|-------------------|
| **Main stems**              | creeping, up to 35 cm | erect or ascending, (15–)30–40(–50) cm | creeping, 15–25 cm | erect or ascending, 10–20 cm |
| **Axillary leaves**         | ovate or triangular, 2–3 × 0.6–1.2 mm | ovate or triangular, 2–3.2 × 0.9–1.6 mm | ovate, 1.1–2.2 × 0.4–0.8 mm | ovate or ovate-lanceolate, 1–2.5 × 0.6–1.6 mm |
| **Base of axillary leaf**   | denticulate      | denticulate or ciliolate | with a few long cilia | long ciliolate   |
| **Dorsal leaves**           | obliquely lanceolate or ovate, 1.4–2.2 × 0.4–0.8 mm | obliquely ovate, 2.4–3.4 × 1.2–1.8 mm | obviate to broadly ovate, 1.2–1.9 × 0.5–1.0 mm | ovate or ovate-elliptic, 1–1.2 × 0.4–0.8 mm |
| **Base of dorsal leaf**     | obliquely cordate, denticulate to ciliolate | obliquely cordate, denticulate to ciliolate | obliquely subcordate or cordate, ciliolate | subcordate or obtuse, ciliolate, |
| **Apex of dorsal leaf**     | aristate, arista ca. 1 mm long | acuminate, arista, or cuspidate | aristate, arista 0.3–0.6 mm long | acuminate or arista, arista 0.3–0.6 mm long |
| **Ventral leaves**          | oblong, 2.2–3.5 × 1.6–2 mm, apex obtuse or subacute | oblong-ovate or oblong, 3.4–4.4 × 1.6–2.2 mm, acute or obtuse | ovate-oblong, 1.7–3.9 × 0.7–1.6, apex acute | ovate-triangular, 1.6–3.8 × 0.6–1.6 mm, apex acute or acuminate |
| **Acroscopic base of ventral leaf** | shortly ciliolate in basal portion, elsewhere entire | denticulate or ciliolate | long ciliolate | rather long ciliolate at base, subentire upward |
| **Basiscopic base of ventral leaf** | sparsely ciliolate at base | entire | slightly auriculate in base, margin entire | entire or with 1 or 2 cilia at base |
| **Strobili**                | 3.5–10 × 3.2–4.4 mm | 4–16 × 1.4–2.4 mm | 3.2–4.0 × 2.3–3.5 mm | 6–8 × 2–3 mm |
Key to the *S. dianzhongensis* and related species in Yunnan

1. Main stems creeping or suberect, fertile stems not erect ..................2
  – Main stems creeping, fertile stems erect .......................................*S. kurzii*

2. Ventral leaves strongly overlapping stems and branches, basiscopic base exau
   riculate and margins ciliolate.......................................................*S. amblyphylla*
  – Ventral leaves not overlapping stems and branches, basiscopic base slightly
   auriculate and margins entire or ciliolate ..................................3

3. Plants 40–50 cm long, main stems unbranched in lower to middle part, with
   stolons at bases, basiscopic base of ventral leaves slightly auriculate, acroscop
   ic base denticulate or ciliolate at margins .................................*S. bodinieri*
  – Plants about 15–25 cm long, main stems branched from near base, rhizo
   phoners restricted to lower part of stem, basiscopic base ventral leaves entire,
   acroscopic base rather long ciliolate at margins ................... *S. dianzhongensis*

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Appendix

Information of the plant materials used in this study is presented in the following order: taxon name, locality (if available), collection number (if available), rbcL GenBank accession number, atpI GenBank accession number, psbA GenBank accession number references (if available). –, sequences not available. *, sequences downloaded from NCBI.

Selaginella albociliata P.S. Wang, Guizhou, China, Zhang X.-C. 7242.(PE), MH814882, MH814826, MH814854. Selaginella amblyphylla Alston, Yunnan, China, Zhang X.-C. 2924.(PE), MH814883, MH814827, MH814855. Selaginella amblyphylla Alston, Yunnan, China, Zhang X.-C. 7951.(PE), MH814884, MH814828, MH814856. Selaginella bodinieri Hieron., Chongqing, China, Zhang X.-C. 5.(PE), MH814885, MH814829, MH814857. Selaginella bodinieri Hieron., Sichuan, China, Zhang X.-C. 526.(PE), MH814886, MH814830, MH814858. Selaginella bodinieri Hieron., Guizhou, China, Zhang X.-C. 7069.(PE), MH814887, MH814831, MH814859. Selaginella chaetoloma Alston, Guizhou, China, Guo Z.-Y. 2016014.(PE), MH814888, MH814832, MH814860. Selaginella chaetoloma Alston, Guizhou, China, Zhang X.-C. 7347.(PE), MH814889, MH814833, MH814861. Selaginella chingii Alston, Guangxi, China, Zhang X.-C. 7904.(PE), MH814890, MH814834, MH814862. Selaginella ciliaris (Retz.) Spring, Yunnan, China, Zhang X.-C. 86.(PE), MH814891, MH814835, MH814863. Selaginella ciliaris (Retz.) Spring, Yunnan, China, Zhang X.-C. 86.(PE), MH814892, MH814836, MH814864. Selaginella decipiens Warb., Guangxi, China, Zhang X.-C. 7253.(PE), MH814893, MH814837, MH814865. Selaginella dianzhongensis X.C.Zhang, Yunnan, China, Zhu Y.-M. 8158.(PE), MH814894, MH814838, MH814866. Selaginella chrysoacaulos (Hook. & Grev.) Spring, Sichuan, China, Zhang X.-C. 86.(PE), MH814895, MH814839, MH814867. Selaginella heterostachys Baker, Guizhou, China, Zhang X.-C. 7088.(PE), MH814896, MH814840, MH814868. Selaginella heterostachys Baker, Guizhou, China, Zhang X.-C. 7268.(PE), MH814897, MH814841, MH814869. Selaginella kurzii Baker, Yunnan, China, Zhang X.-C. 1934.(PE), MH814898, MH814842, MH814870. Selaginella labordei Hieron. ex Christ, Hubei, China, Zhang X.-C. 3356.(PE), MH814899, MH814843, MH814871. Selaginella megaphylla Baker, Tibet, China, Jin X.-H. 19301.(PE), MH814901, MH814845, MH814873. Selaginella monospora Spring, Guangxi, China, Zhang X.-C. 7889.(PE), MH814902, MH814846, MH814874. Selaginella ornata (Hook. & Grev.) Spring, Yunnan, China, Zhang X.-C. 8520.(PE), MH814903, MH814847, MH814875. Selaginella repanda (Desv. ex Poir.) Spring, Yunnan, China, Zhang X.-C. 5655.(PE), MH814904, MH814848, MH814876. Selaginella repanda (Desv. ex Poir.) Spring, Yunnan, China, Zhang X.-C. 9273.(PE), MH814905, MH814849, MH814877. Selaginella repanda (Desv. ex Poir.) Spring, Yunnan, China, Li B.-G. sn_20.(PE), MH814906, MH814850, MH814878. Selaginella vaginata Spring, Shaanxi, China, Zhang Z.-S. 161.(PE), MH814907, MH814851, MH814879. Selaginella xipholepis Baker, Guizhou,
China, Zhang X.-C. 7422. (PE), MH814908, MH814852, MH814880. *Selaginella dianzhongensis* (Selaginellaceae), a new spikemoss from China. *Selaginella braunii* Baker, voucher Zhang 1332 (PYU, CDBI), KT161420.1 *, -,-. *Selaginella delicatula* (Desv. ex Poir.) Alston, voucher Gao & al. HGX10734 (CDBI), KT161441.1*, -,-. *Selaginella helvetica* (L.) Link, voucher Zhou 093 (CDBI), KT161472.1*, -,-. *Selaginella kraussiana* (Kunze) A. Braun, voucher Zhou 062 (CDBI), KT161498.1*, -,-. *Selaginella laxistrobila* K.H. Shing, voucher Chu & al. 24449 (PYU), KT161509.1*, -,-. *Selaginella nipponica* Franch. & Sav., voucher Zhou & al. DJY07479 (CDBI), KT161542.1*, -,-. *Selaginella remotifolia* Spring, voucher Zhou 005 (PYU, CDBI), KT161580.1*, -,-. *Selaginella uncinata* (Desv. ex Poir.) Spring, voucher Zhang & Zhou DJY04101 (CDBI), KT161626.1*, -,-. *Selaginella deflexa* Brack., AF093253.1*, -,-. *Selaginella selaginoides* (L.) P. Beauv. ex Schrank & Mart., voucher S. Weststrand 104 (UPS), KY023148.1*, -,-.