

***Lycium amarum* sp. nov. (Solanaceae) from Xizang, supported from morphological characters and phylogenetic analysis**

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Lycium amarum sp. nov. L. Q. Huang is described based on its general morphology and pollen micromorphology, which were compared with closely related *Lycium* species. The phylogenetic relationships among the new species and closely related *Lycium* species belonging to the tribe Lycieae were investigated based on the DNA sequence data of the granule-bound starch synthase (GBSSI) gene. Morphologically, *L. amarum* is similar to *L. chinense* Mill. sharing lanceolate or linear-ob lanceolate leaves, three-lobed calyx, villous ring slightly above the filament base, and adjacent corolla tube, however, *L. amarum* may be diagnosed by its prominent tubercles at the base of spines, thinly leathery leaves, and a two-tine crack on the tip of calyx lobes. According to our results, the new species is close to *L. barbarum* L., *L. chinense*, *L. dasystemum* Pojark, and *L. yunnanense* Kuang & Lu.

The genus *Lycium* L. is one of the largest genera within the family Solanaceae, containing approximately 75 species mainly distributed in South America, southwestern North America, south Africa, with a few species inhabiting temperate Europe and Asia (Miller 2002). Most of the seven species of *Lycium* occurring in China have branches with thorns, perfect flowers, and usually produce red, orange, yellow or black fleshy and juicy berries (Zhang et al. 1994). Mature fruits of *Lycium barbarum* L. have been used in traditional Chinese medicine for thousands of years (Kang 2012).

During fieldwork of the Fourth National Survey on Chinese Material Medical Resources, unusual specimens of *Lycium* were discovered in Qiongjie County, Xizang, China. They could be distinguished from other *Lycium* taxa by the prominent tubercles at the base of their spines, purple pedicels, dense white pubescence on the abaxial side of leaves, and a two-tine crack on the tip of each calyx lobe. This study aimed to present the integrative taxonomy of this new taxon based on its morphological characters and place it in a molecular phylogeny of the genus.

Material and methods

Taxon sampling and morphological studies

Twelve morphological characters of the unusual specimens were obtained and compared to those of *L. barbarum*,

L. dasystemum Pojark., and *L. chinense* Mill. Specimens have been deposited at CMMI (Table 1). Pollen was collected from all specimen flowers, mounted on aluminum stubs, coated with gold-palladium for three to four minutes, and observed under an S-3400N scanning electron microscope at 10–15 kV.

DNA extraction and GBSSI amplification, sequencing and alignment

Total genomic DNA was extracted from the leaf tissues of 3 individuals using the Plant Genomic DNA Kit following the manufacturer protocols. PCR amplification of the granule-bound starch synthase (GBSSI) gene was performed using the primers GBSSI 622-B (5' CACTGCTATAAACGTG GGGTGA 3') and Crmod (5'GGCATAGTATGGGCT CACAGTAA 3') (Peralta and Spooner 2001). The 25 µl PCR reaction contained 2.5 µl of 10 × Ex Taq buffer, 1 µl of dNTPs (10 mM), 0.2 µl of Ex Taq (5 U), 0.25 µl (10 mM) of each primer, 1.0 µl (30–50 ng) of genomic DNA, and 19.8 µl of ddH₂O. Amplification was performed using the following protocol: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 1 min, and extension at 72°C for 2 min; final extension at 72°C for 7 min. PCR products were screened on 1.2% agarose gels and those with the correct size and concentration were sent to Ruibiotech and sequenced in both directions using the amplification primers on an ABI

Table 1. Morphological comparison between *Lycium amarum* and its most closely related congeners.

Characters	<i>L. amarum</i>	<i>L. barbarum</i>	<i>L. chinense</i>	<i>L. dasystemum</i>
Shrub tall (cm)	100–170	80–200	50–200	ca 150
Thorn (cm)	0.5–1.0, with prominent tubercles at base	0.3–2.0	0.5–2.0	Older growth with thorns 0.6–6.0
Leaves growing	Solitary or in clusters of 2–4	Solitary or fasciculate	Solitary or in clusters of 2–4	Usually fasciculate on short shoots
Leaf blade	Linear-lanceolate or linear-ob lanceolata,	Lanceolate or long elliptic	Ovate, rhombic, lanceolate, or linear-lanceolate	Lanceolate, oblanceolate, or broadly lanceolate
Pedicel (cm)	Purple, 0.5–1.0	Green, 1–2	Green, 1–2	Green, 1.0–1.8
Calyx	3–4 mm long, 3-lobed to halfway, 2-tine at crack tip, lobes marginally pubescent	4–5 mm, 2-lobed, 2- or 3-toothed at lobes apex	3–4 mm, 3–5-divided to halfway, lobes densely ciliate	Ca 4 mm, often 2- or 3-divided halfway
Corolla (mm)	3–5, shorter than or subequaling lobes	8–10, longer than lobes	9–12, subequaling lobes	9–13, tube sparingly villous inside
Lobe	Lobes oblong, apex blunt-ovate	Spreading, margin glabrescent	Pubescent at margin	Half long as corolla tube, ciliolate
Stamen and filament	Stamens longer than corolla, with a villous ring slightly above filament base and adjacent corolla tube	Stamens and style slightly exerted	Stamens shorter or longer than corolla, with a villous ring above filament base and adjacent corolla tube	Stamens exerted from spreading corolla lobes; filaments sparsely villous slightly above base
Berry (mm)	7–12 × 5–7	4–20 × 5–10	10–12 × 5–8	7–15 × 5–8
Flowering	Aug–Sep	May–Aug	May–Sep	Jun–Aug
Fruiting	Sep–Oct	Aug–Nov	Aug–Nov	Aug–Sep

3730. Sequences were assembled with SeqMan, aligned using Clustal X 2.0 (Thompson et al. 1997), and manually adjusted using BioEdit 7.2.0 (Hall 1999) when necessary.

Phylogenetic analyses

GBSSI has been useful to infer the phylogenetic relationships among genera in the tribe *Lycieae* (Levin and Miller 2005, Levin et al. 2007, Wu et al. 2011). In order to understand the phylogenetic position of the new species and the phylogenetic relationships among Chinese *Lycium* species, 76 GBSSI sequences were analyzed: 71 from taxa belonging to the genus *Lycium*, including one from the new species described here; one from *Phrodus microphyllus* (Miers) Miers and one from *Grabowskia boerhavifolia* Schlechl., which belong to tribe *Lycieae*; and one from each of the species constituting the outgroup *Nolana werdermannii* I. M. Johnst., *Nolana coelestis* Lindl., and *Atropa belladonna* L. Sequences were retrieved from GenBank (<www.ncbi.nlm.nih.gov/genbank/>) and their details are provided in Table 2.

Phylogenetic reconstructions using maximum parsimony (MP) and Bayesian inferences (BI) were performed in PAUP 4.0b10 (Swofford 2002) and MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003), respectively. For the MP analyses, a heuristic search was performed considering all characters as independent and of equal weight; gaps were coded as missing data. Topology robustness was evaluated by 1000 bootstrap replicates (Felsenstein 1985) and the most parsimonious trees were summarized as a strict consensus tree.

Before the Bayesian analysis, the Akaike information criterion (AIC) implemented in ModelTest 3.7 (Posada and Crandall 1998) was used to select the best-fit model of molecular evolution for the dataset. Four Markov Chain Monte Carlo (MCMC) simulation chains (three incrementally heated and one cold) were run for 10 million generations and sampled every 1000 generations. When the log-likelihood scores were stabilized, a consensus tree was produced after discarding the first 25% of the sampled trees (burn-in command). The remaining trees were imported into PAUP and a 50% majority-rule consensus tree was produced to obtain the posterior probabilities of the clades.

Results and discussion

Relationships between *L. amarum* and closely related taxa

The new species, *L. amarum*, differ from its most closely related species by its prominent tubercles at the base of thorns, purple pedicels, densely ciliated three-lobed calyx with a two-tine crack on the tip and marginally pubescent lobes, and dense white pubescence on the abaxial side of leaves (Table 1). Moreover, the new species is restricted to high elevation, and its berries are continuously bitter. All these characteristics suggested *L. amarum* as a distinct taxonomic entity.

Table 2. Accession numbers, size, and origin of the 75 GBSSI sequences retrieved from NCBI.

Taxon	Origin	Accession	Length/bp
<i>Atropa belladonna</i>	China	DQ069253	1671
<i>Crabowskia boerhaviifolia</i>	Mexico	EF137750	958
<i>Lycium acutifolium</i>	South Africa	EF137753	1799
<i>L. afrum</i>	South Africa	EF137754	1805
<i>L. ameghinii</i>	Argentina	DQ124501	1800
<i>L. americanum</i>	Baroza	DQ124502	958
<i>L. amoenum</i>	South Africa	EF137755	1813
<i>L. andersonii</i>	Mexico	DQ124503	1772
<i>L. arenicola</i>	South Africa	JF284375	1709
<i>L. athium</i>	Argentina	EF137756	946
<i>L. australe</i>	Australia	EF137757	872
<i>L. barbarum</i>	China	DQ069268	1672
<i>L. berlandieri</i>	Arizona, USA	DQ124506	1838
<i>L. boscifolium</i>	South Africa	EF137759	1762
<i>L. brevipes</i>	Mexico	DQ124508	1011
<i>L. californicum</i>	Arizona	DQ124509	1762
<i>L. carolinianum</i>	North America	JF284376	1570
<i>L. cestroides</i>	Argentina	DQ124513	1803
<i>L. chanar</i>	Argentina	EF137761	929
<i>L. chilense</i>	Argentina	EF137762	910
<i>L. chinense</i>	China	EF137767	1806
<i>L. ciliatum</i>	Argentina	EF137768	1808
<i>L. cinereum</i>	South Africa	EF137769	1762
<i>L. cooperi</i>	Arizona, USA	DQ124518	1158
<i>L. cuneatum</i>	Argentina	DQ124519	893
<i>L. dasystemum</i>	China	HQ615063	981
<i>L. decumbens</i>	Namibia	EF137770	1805
<i>L. depressum</i>	Israel	EF137771	1806
<i>L. deserti</i>	South America	EU051859	889
<i>L. eenii</i>	Namibia	EF137772	1808
<i>L. elongatum</i>	Argentina	DQ124520	1799
<i>L. exsertum</i>	Arizona, USA	DQ124521	1717
<i>L. ferocissimum</i>	South America	GQ301195	1712
<i>L. fremontii</i>	Mexico	DQ124525	1510
<i>L. fusum</i>	Argentina	EF137774	933
<i>L. gariepense</i>	Namibia	EF137775	1806
<i>L. gilliesianum</i>	Argentina	DQ124526	925
<i>L. grandicalyx</i>	Namibia	EF137776	1793
<i>L. hirsutum</i>	South Africa	EF137777	1791
<i>L. horridum</i>	South Africa	DQ124528	936
<i>L. infaustum</i>	Argentina	DQ124529	954
<i>L. intricatum</i>	Canary island	EF137778	1590
<i>L. leiospermum</i>	Mexico	EF137779	953
<i>L. macrodon</i>	Arizona, USA	DQ124530	1866
<i>L. mescarense</i>	South Africa	EF137780	1805
<i>L. minimum</i>	Ecuador	EF137781	1783
<i>L. minutifolium</i>	Chile	EF137782	861
<i>L. minutifolium</i>	Chile	EF137782	861
<i>L. morongii</i>	Bolivia	DQ124531	851
<i>L. nodosum</i>	Argentina	EF137783	1793
<i>L. oxycarpum</i>	South Africa	EF137784	1806
<i>L. pallidum</i>	Arizona, USA	DQ124534	1763
<i>L. parishii</i>	Arizona, USA	DQ124535	926
<i>L. pilifolium</i>	South Africa	EF137785	1806
<i>L. puberulum</i>	North America	JF284358	1711
<i>L. pumilum</i>	South Africa	EF137786	1805
<i>L. qingshuiheense</i>	China	HQ615060	980
<i>L. rachidocladum</i>	Chile	EF137787	1115
<i>L. ruthenicum</i>	China	HQ615064	983
<i>L. schizocalyx</i>	South Africa	EF137789	1781
<i>L. schweinfurthii</i>	Israel	EF137790	1808
<i>L. shawii</i>	Israel	EF137791	1857
<i>L. shockleyi</i>	Nevada, USA	DQ124540	886
<i>L. stenophyllum</i>	Chile	EF137792	1059

(Continued)

Table 2. (Continued)

Taxon	Origin	Accession	Length/bp
<i>L. strandveldense</i>	South Africa	EF137793	1805
<i>L. tenue</i>	South Africa	EF137794	1806
<i>L. tenuispinosum</i>	Argentina	EF137795	1842
<i>L. tetrandrum</i>	South Africa	DQ124544	933
<i>L. texanum</i>	Arizona, USA	DQ124545	890
<i>L. torreyi</i>	Arizona, USA	DQ124546	883
<i>L. truncatum</i>	China	HM194919	1744
<i>L. villosum</i>	South Africa	DQ124547	935
<i>L. vimineum</i>	Argentina	EF137796	1791
<i>L. yunnanense</i>	China	HM194922	1750
<i>Nolana coelestis</i>	Chile	EF137800	1803
<i>N. werdermannii</i>	Chile	EF137799	1782
<i>Phrodus microphyllus</i>	Chile	EF137801	1760
<i>L. amarum</i>	China	KU745288	852

Pollen morphology can be used for analyzing taxonomic relationships in a wide variety of plant families, as it is a character of considerable systematic significance at the generic and specific levels (Zhang et al. 2009). *Lycium* pollen grains are small, sub-spherical or prolate, three-colporate, with a strip-like exine longitudinally arranged (Fig. 1). Although *L. amarum* pollen grains, with three-colporate apertures, are similar to those of other *Lycium* species, their smaller size compared to that of other species and their irregularly striate exine indicated that *L. amarum* is a different species.

The multiple alignment of the GBSSI gene sequences revealed 696 constant characters, 148 parsimony-uninformative variable characters, and 107 parsimony-informative variable characters among the 951 bp. According to the Bayesian tree, based on the transversional model with invariable sites (TVM + I) selected as the best fit by the AIC, *Lycium* is a monophyletic group (Fig. 2). The Eurasian species (from Europe to China and Japan, 20 spp.) comprised the monophyletic clade I, the Chinese *Lycium* species, i.e. *L. amarum*, *L. barbarum*, *L. chinense*, *L. dasystemum*, *L. qingshuiheense* Jiang et Li, *L. ruthenicum* Murray, and *L. yunnanense* Kuang et Lu, formed another monophyletic group, and *L. amarum*, together with *L. barbarum*, *L. chinense*, *L. dasystemum*, and *L. yunnanense*, constituted a moderately supported clade (PP = 99%, BS = 71%) nested within clade I (Fig. 2).

Thus, morphological characters, pollen micro-morphological characters, and the molecular phylogeny based on GBSSI all indicated *L. amarum* that is closely related to *L. chinense*, *L. barbarum*, and *L. dasystemum*.

Lycium amarum L. Q. Huang sp. nov. (Fig. 3–4)

Although *L. amarum* and *L. chinense* share a three-lobed calyx and a villous ring slightly above the filament base and adjacent corolla tube, these two species are readily distinguished by *L. amarum* having prominent tubercles at the base of spines, purple pedicels, dense white pubescence on the abaxial side of leaves, and a two-tine crack on the tip of calyx lobes.

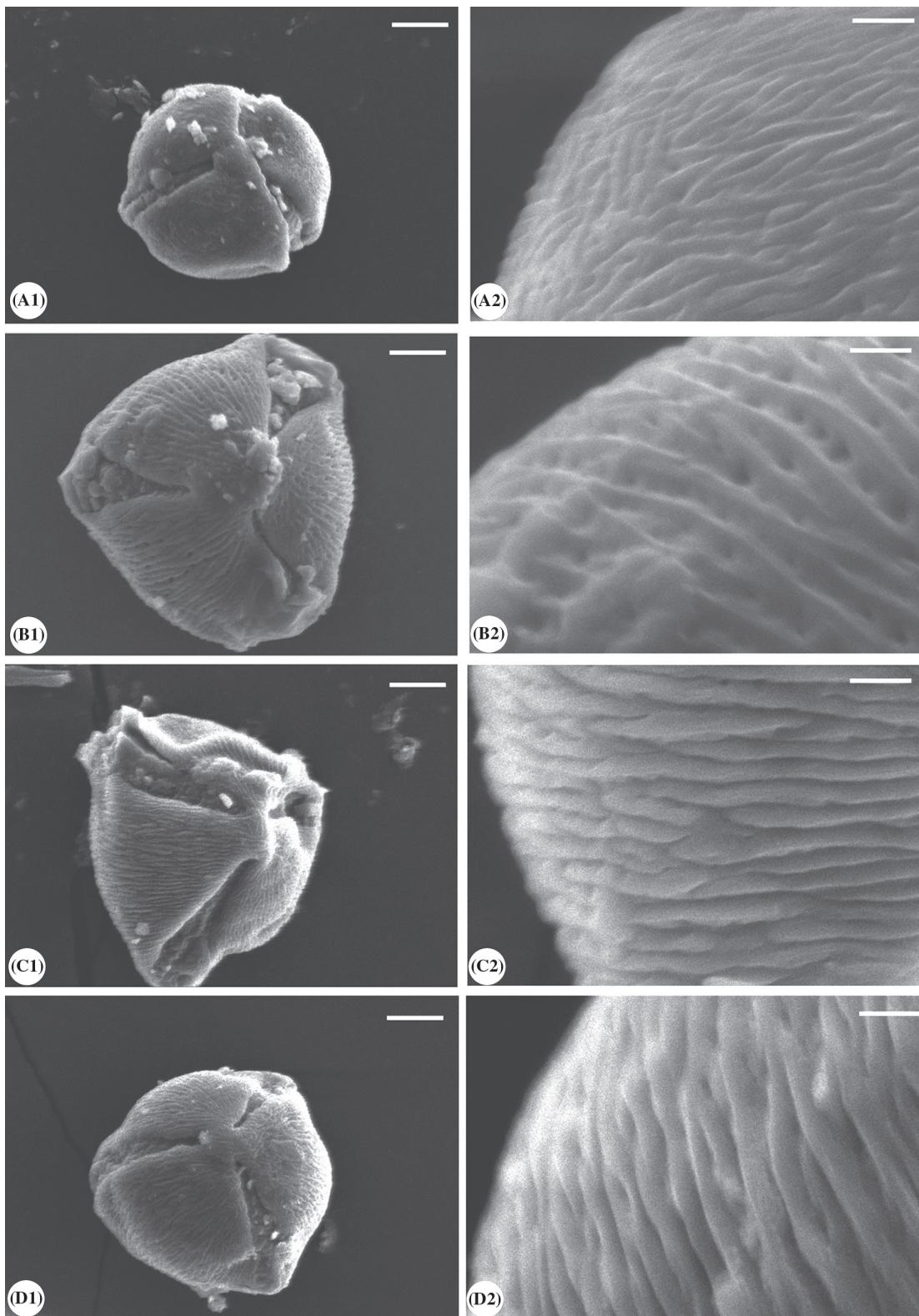


Figure 1. SEM photographs of pollen grains evidencing the morphological differences between *Lycium amarum* and related *Lycium* spp. (A) *L. amarum* (locality: Qiongjie, Xizang, voucher: 542225140917002LY; CMMI), (B) *L. barbarum* L. (locality: Jiuquan, Gansu, voucher: 620921LY0040; CMMI), (C) *L. chinense* (locality: Jiuquan, Gansu, voucher: 620921LY0040; CMMI), (D) *L. dasystemum* (locality: Bazhou, Xinjiang, voucher: 65282612606034; CMMI) (Scale bars: A1–D1 = 5 μ m, A2–D2 = 1 μ m).

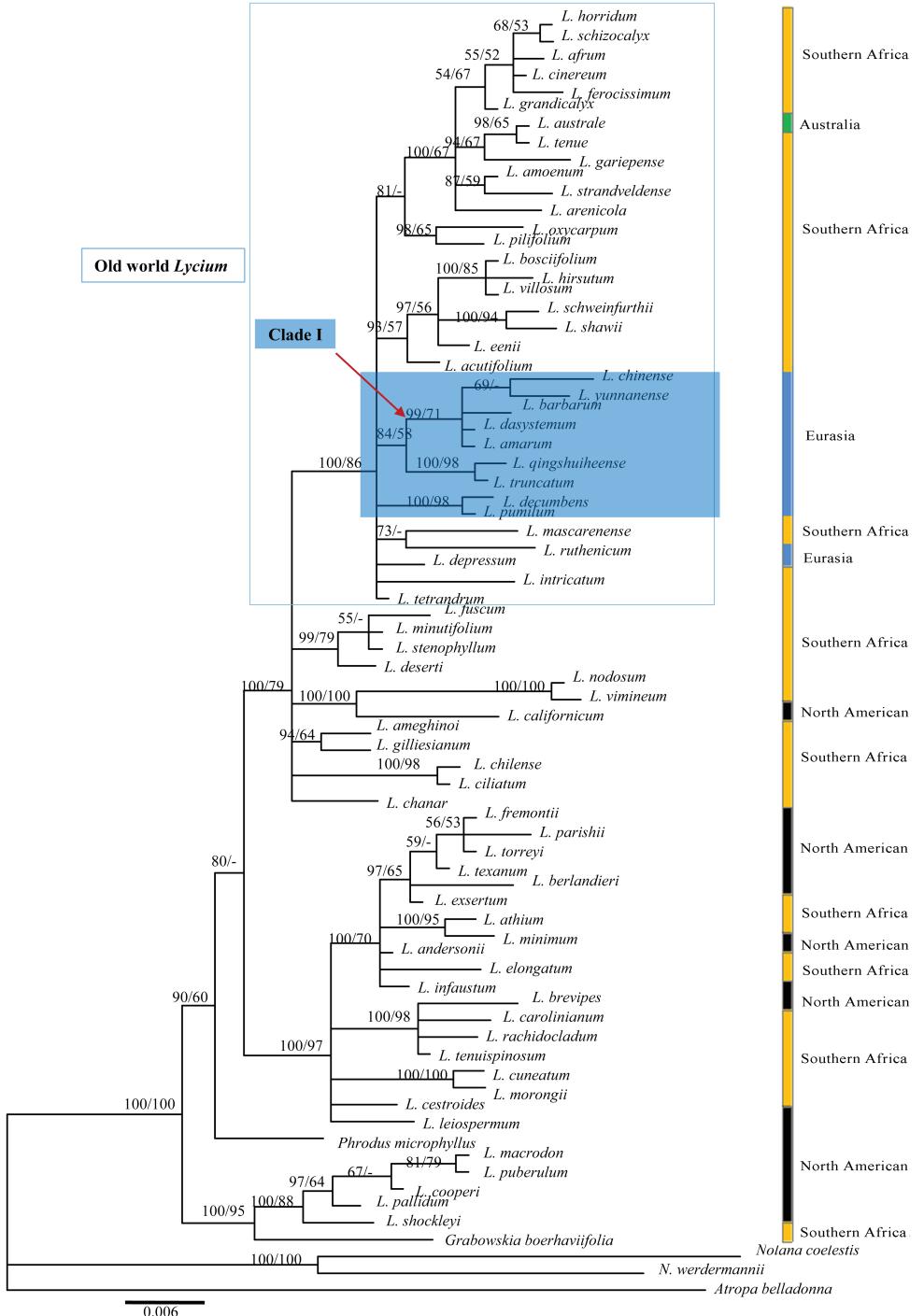


Figure 2. Phylogenetic relationships within the genus *Lycium* resulting from Bayesian analysis of the GBSSI gene using the TVM + I model. Numbers above branches are Bayesian posterior probabilities (PP) and bootstrap values (BS).

Type: China, Xizang, Qiongjie County, Qiongjie Township, on rocks and near roads, 3800–3900 m a.s.l., 17 Apr 2014, L. Q. Huang & X. Zha 542225140917002LY (fl., holotype: CMMI, fr., isotype: CMMI).

Etymology

The specific epithet ‘amarum’ refers to the bitterness of the red berries compared to those of *L. barbarum* and *L. chinense*, which are particularly sweet.

Description

Erect shrub, 100–170 cm tall. Stems highly branched; branches with spines; young branches pale gray; spines 0.5–1.0 cm long, with prominent tubercles at the base. Leaves alternate on long branches, in clusters of 2–4 on short branches, linear-lanceolate or linear-ob lanceolate, 1–4 × 0.5–1.0 cm, thinly-leathery, with dense white pubescence on the abaxial side; base almost wedged; apex blunt-acute; petiole not distinct. Flowers solitary or paired on

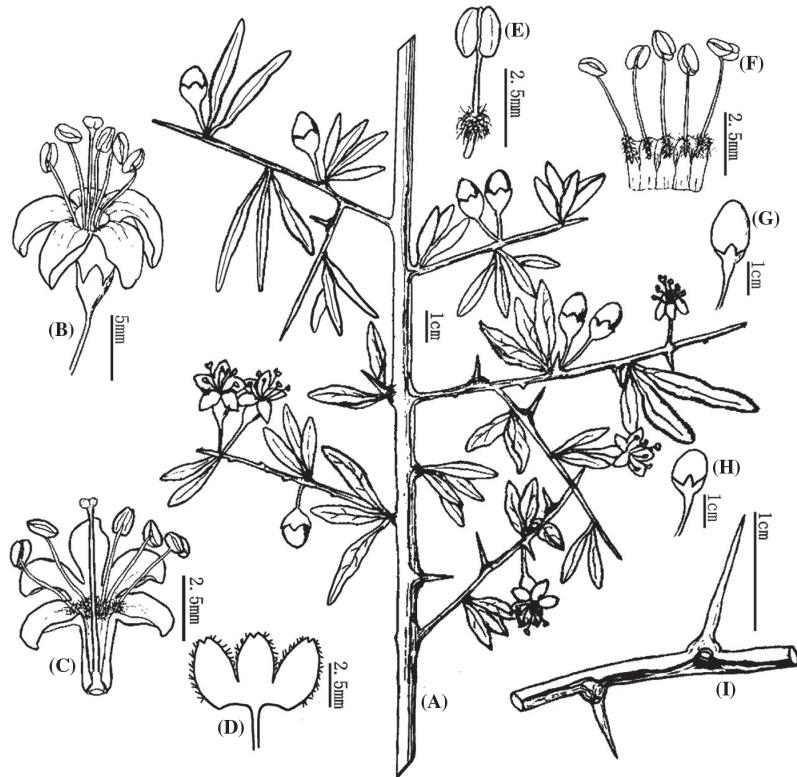


Figure 3. *Lycium amarum* sp. nov. (A) flowering and fruiting branches, (B) flower, (C) open corolla showing the stamens, (D) calyx, (E)–(F) stamen and filament, (G)–(H) fruits, and (I) thorn with tubercles at the base. Drawn by Hai-Yan Cao from the holotype in ACM.



Figure 4. *Lycium amarum* sp. nov. (A) habit, (B) berry, (C) inflorescence, (D) two tines on the tip of calyx lobe with pubescent margins, (E) flower, and (F) thorn with tubercles at the base.

long shoots; pedicels 0.5–1.0 cm long, purple; calyx campanulate, 3–4 mm long, three-lobed to halfway, with a two-tine crack on the tip of calyx and pubescent margins; corolla pale purple, funnel-shaped, 3–5 mm long, usually 5-fid, shorter than or sub-equal to lobes; lobes oblong, blunt-ovate at apex, glabrescent. Stamens slightly longer than corolla, with a villosus ring slightly above the filament base and adjacent to the corolla tube. Berry red, ovoid, or oblong, 7–12 × 5–7 mm. Seeds numerous, yellow, about 2 mm long. Flowering in Aug–Sep and fruiting in Sep–Oct (Fig. 3–4).

Ecology, distribution, and importance

This new species is only known from the type locality, nearby the Tombs of the Tibetan Kings, Qiongjie County, Shannan Prefecture of Xizang, southwestern China. Annual rainfall in the area is about 345 mm, belonging to the a region with temperate semi-arid monsoon climate. *Lycium amarum* grows on rocks and along road banks at an elevation between 3800 and 3900 m a.s.l.

Acknowledgements – This work was supported by the special fund of the Fourth National Survey on Chinese Material Medical Resources, by the Foundation of Special Protection of Biological Diversity of the Dept of Environmental Protection of China (2013), and by National Funds for Distinguished Young Scientists (81325023). We are also grateful to Hai-Yan Cao for providing the drawings.

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