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Gymnosporangium przewalskii sp. nov. (Pucciniales, Basidiomycota) from China and its life cycle

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Abstract

In an investigation of rust fungi in Qinghai Province, northwestern China, the novel rust species *Gymnosporangium przewalskii* was identified based on morphology and phylogenetic analyses. Phylogenetic analyses using the internal transcribed spacers (ITS) and the large subunit (LSU) rRNA partial gene revealed that *G. przewalskii* is monophyletic and distinct from other *Gymnosporangium* species. The life cycle of this new taxon was clarified based on molecular data. Its spermogonial and aecial stages occurred on *Sorbus koehneana*, and its telial stage was found on *Juniperus przewalskii*.

Keywords: phylogeny, rust, systematics, taxonomy

Introduction

Approximately 62 species of *Gymnosporangium* R. Hedw. ex DC. have been reported worldwide, most of them in the northern hemisphere (Kern 1973, Azbukina 1997, Zhao & Zhuang 2007, Yun *et al.* 2009, Cao *et al.* 2016). Nineteen species have been recorded from China (Deng 1963, Tai 1979, Wang & Guo 1985, Zhao & Jiang 1986, Cao & Li 1999, Zhuang 2005, 2012, Zhao & Zhuang 2007, Cao *et al.* 2016). Most *Gymnosporangium* species are heteroecious and demicyclic. They usually produce aecia on the leaves and fruits of plants belonging to Maloideae and telia on the needles, stems, and branches of Cupressaceae, causing significant economic losses (Parmelee 1965, 1971, Kern 1973, Sinclair & Lyon 2005).

Juniperus przewalskii Komarov is an evergreen conifer endemic to northwestern China, mainly distributed in the forest regions of the Qilian Mountains (Liu *et al.* 2006). During our investigation of rust fungi in Qinghai Province, northwestern China, we found a telial stage of *Gymnosporangium* species on *J. przewalskii*. Both morphological observations and phylogenetic analyses confirmed that this fungus is distinct from other *Gymnosporangium* species. Upon further investigation in the field, we found an aecial stage of *Gymnosporangium* species on *Sorbus koehneana* C. K. Schneider located in an area near the occurrence of the telial host. The purpose of this study is to clarify the taxonomy of specimens on *J. przewalskii* and *S. koehneana* from China based on morphological and phylogenetic analyses. Based on these analyses, the fungus on these specimens is described as a new species, *G. przewalskii*.

Materials and methods

Specimens

Dry specimens used for this study were collected in Qinghai and Sichuan Provinces and deposited in the Mycological Herbarium, Museum of Beijing Forestry University, Beijing, China (BJFC) and the Mycological Herbarium, the Institute of Microbiology, Academia Sinica, Beijing (HMAS).

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Morphological observation

Spores from specimens were mounted in a drop of lactophenol-cotton blue solution on the slides and were observed under a Leica DM3000 microscope. The length, width, and wall thickness of 30–50 spores from each specimen were measured using a MicroView MVC TWAIN Image Analyser. The surface features of spores were examined by scanning electron microscopy (SEM). For SEM, samples were coated with platinum-palladium using a Hitachi SCD-005 Sputter Coater and examined with an S-4200 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 5 kV.

DNA extraction and PCR amplification

Whole genomic DNA from the spores was extracted following the methods described by Tian *et al.* (2004). DNA concentrations were estimated by electrophoresis in 1.2% agarose gels. The D1/D2 region of nuclear LSU rDNA was amplified with the primer pair No. 4 (5′-ACCCGCTGAAYTTAAGCATAT-3′) and No. 11 (5′-CTCCTTGGTCCGTGTTTCAAGACGC-3′) (Van der Auwera *et al.* 1994). The ITS and the 5.8S region of rDNA were amplified with the primer pair ITS5-u (5′-CAAGGTTTCTGTAGGTG-3′) (Pfunder *et al.* 2001) and ITS4rust (5′-CAGATTACAAATTTGGGCT-3′) (Beenken *et al.* 2012). Amplifications were performed in 25 µl of PCR solution containing 1 µl of DNA template, 1 µl of sense primer (10 µM), 1 µl of antisense primer (10 µM), 12.5 µl of 2×Es Taq MasterMix (Cwbio, Beijing, China), and 9.5 µl of ddH$_2$O. The PCR conditions were as follows: 95°C for 3 min, 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and a final step of 72°C for 10 min. PCR products were purified and cloned for sequencing (Invitrogen, Beijing, China).

Phylogenetic analyses

The new sequences from this study were deposited in GenBank (Table 1). GenBank accession numbers of the downloaded sequences used in the phylogenetic analyses are listed in Table 2. A maximum parsimony (MP) analysis was run using PAUP v.4.0b10 (Swofford 2003). Bayesian inference (BI) and maximum likelihood (ML) were performed using MrBayes v.3.1.2 and PhyML v.7.2.8 (Ronquist & Huelsenbeck 2003, Guindon *et al.* 2010). *Puccinia popowiae* Cooke was selected as the outgroup taxon in the current analysis. Trees were drawn using FigTree v.1.3.1 (Rambaut & Drummond 2010), and the layout was edited in Adobe Illustrator CS v.6.

TABLE 1. The new sequences of *Gymnosporangium przewalskii* used in phylogenetic analyses.

Species	Herbarium accession no.	Locality	Host plant	GenBank accession no.	
				LSU	ITS
G. przewalskii Y.M. Liang & B. Cao	BJFC-R01859	China, Qinghai	*Juniperus przewalskii*	KR814560	KR814563
	BJFC-R01860	China, Qinghai	*Juniperus przewalskii*	KR814561	KR814564
	BJFC-R02083	China, Qinghai	*Sorbus koehneana*	KX528444	KX528446
	BJFC-R02084	China, Qinghai	*Sorbus koehneana*	KX528445	KX528447

MP analysis was inferred using a heuristic search algorithm (1000 random sequence additions) with tree bisection and reconnection (TBR) branch swapping. Maxtrees were set to 5000, branches of zero length were collapsed, and all equally parsimonious trees were saved. Other calculated parsimony scores (Tree Length [TL], Consistency Index [CI], Retention Index [RI] and Rescaled Consistency [RC]) were calculated. ML analysis was performed with a GTR + I + G substitution model selected by MrModeltest v.2.3 (Posada & Crandall 1998). The branch supports of MP and ML analyses were evaluated using a bootstrapping (BS) method of 1000 replicates (Hillis & Bull 1993). Bayesian inference (BI) analysis was performed using a Markov chain Monte Carlo (MCMC) algorithm to construct the topology of the tree (Rannala & Yang 1996). A nucleotide substitution model was also calculated in MrModeltest v.2.3 (Posada & Crandall 1998). The sequence alignment was deposited in TreeBase (http://www.treebase.org/) as accession 19679.

TABLE 2. Sequences of *Gymnosporangium* species used in the phylogenetic analyses.

Species	GenBank accession no.		References
	ITS	LSU	
G. amelanchieris E. Fisch. ex F. Kern	KM486547	KM486546	Fernández *et al.* (unpublished)
	KP261040	KP261041	Fernández *et al.* (unpublished)
G. asiaticum Miyabe ex G. Yamada	KR814568	KT719165	Cao *et al.* (2016)
	KR814569	KT719166	Cao *et al.* (2016)
G. atlanticum Guyot & Malençon	-	KM403111	Fernández *et al.* (2016)
	-	KT160254	Fernández *et al.* (2016)
G. betheli F. Kern	KJ720163	-	Novick *et al.* (unpublished)

...*continued on the next page*

TABLE 2. (Continued)

Species	GenBank accession no.		References
	ITS	LSU	
G. clavariiforme (Wulfen) DC.	KU183498	-	Fernández & Alvarado (unpublished)
	KU183499	-	Fernández & Alvarado (unpublished)
	-	HM114220	Dervis *et al.* (2010)
G. clavipes Cooke & Peck	-	DQ354545	Aime (2006)
	-	HQ317528	Liu *et al.* (unpublished)
G. confusum Plowr.	KP261046	KP261047	Fernández *et al.* (unpublished)
	KT160257	KT160261	Fernández *et al.* (unpublished)
G. cornutum Arthur ex F. Kern	-	AF426210	Maier *et al.* (2003)
	-	FJ848766	Yun *et al.* (2009)
G. cupressi Long & Goodd.	KJ720169	-	Novick *et al.* (unpublished)
G. exiguum F. Kern	KJ720170	-	Novick *et al.* (unpublished)
G. fusisporum E. Fisch.	-	KJ720172	Novick *et al.* (unpublished)
G. globosum (Farl.) Farl.	HQ317506	HQ317506	Liu *et al.* (unpublished)
	HQ317511	-	Liu *et al.* (unpublished)
G. gracile Pat.	-	KM486544	Fernández *et al.* (unpublished)
	-	KM486545	Fernández *et al.* (unpublished)
G. huanglongense Y.M. Liang & B. Cao	KT719167	KT719161	Cao *et al.* (2016)
	KT719168	KT719162	Cao *et al.* (2016)
G. japonicum Dietel & P. Syd.	-	FJ848755	Yun *et al.* (2009)
	-	FJ848756	Yun *et al.* (2009)
G. juniperi-virginianae Schwein.	DQ267127	AY629316	Matheny & Hibbett (unpublished)
	KJ720176	KJ720176	Novick *et al.* (unpublished)
G. kernianum Bethel	KJ720177	-	Novick *et al.* (unpublished)
G. miyabei G. Yamada & I. Miyake	KJ720178	-	Novick *et al.* (unpublished)
G. monticola H.Y. Yun	-	FJ848770	Yun *et al.* (2009)
	-	FJ848771	Yun *et al.* (2009)
G. multiporum F. Kern	KJ720179	-	Novick *et al.* (unpublished)
G. nelsonii Arthur	-	HM591299	Schilder *et al.* (2011)
	KJ720180	KJ720180	Novick *et al.* (unpublished)
G. nidus-avis Thaxt.	KJ720181	KJ720181	Novick *et al.* (unpublished)
G. sabinae (Dicks.) G. Winter	KF925317	-	Filipp & Spornberger (unpublished)
	KF925318	-	Filipp & Spornberger (unpublished)
	-	AF426209	Maier *et al.* (2003)
	-	HM114221	Dervis *et al.* (unpublished)
G. trachysorum F. Kern	KJ720184	-	Novick *et al.* (unpublished)
G. tremelloides R. Hartig	KJ720185	KJ720185	Novick *et al.* (unpublished)
G. unicorne H.Y. Yun	-	FJ848767	Yun *et al.* (2009)
	-	FJ848768	Yun *et al.* (2009)
G. vauqueliniae Long & Goodd.	KJ720186	-	Novick *et al.* (unpublished)
G. yamadae Miyabe ex G. Yamada	KR814566	KT719163	Cao *et al.* (2016)
	KR814567	KT719164	Cao *et al.* (2016)
Puccinia popowiae Cooke	KM217353	KM217353	Beenken & Wood (2015)

Results

The aligned ITS dataset is composed of 781 characters including gaps, of which 351 characters are constant, 73 characters are parsimony-uninformative, and 357 are parsimony-informative. A heuristic search generated three equally parsimonious trees (TL = 1049, CI = 0.737, RI =0.784, and RC =0.578) (Fig. 1). The aligned LSU dataset is composed of 636 characters including gaps, of which 483 characters are constant, 60 characters are parsimony-uninformative, and 93 are parsimony-informative. The MP analysis of sequence data generated 15 equally parsimonious trees (TL = 246, CI = 0.757, RI = 0.836, and RC = 0.633) (Fig. 2). Both ML analysis (GTR + I + G) and BI analysis resulted in the same topology as the presented MP phylogram (Figs 1, 2).

The ITS and LSU sequences obtained from teliospores and aeciospores were identical in an individual clade with high bootstrap values 100/100 (MP/ML) in the ITS phylogenetic tree and 99/100 (MP/ML) in the LSU phylogenetic tree, respectively (Figs 1, 2). Therefore, the rust fungus producing spermogonial and aecial stages on *S. koehneana* was conspecific with the rust fungus producing a telial stage on *J. przewalskii*.

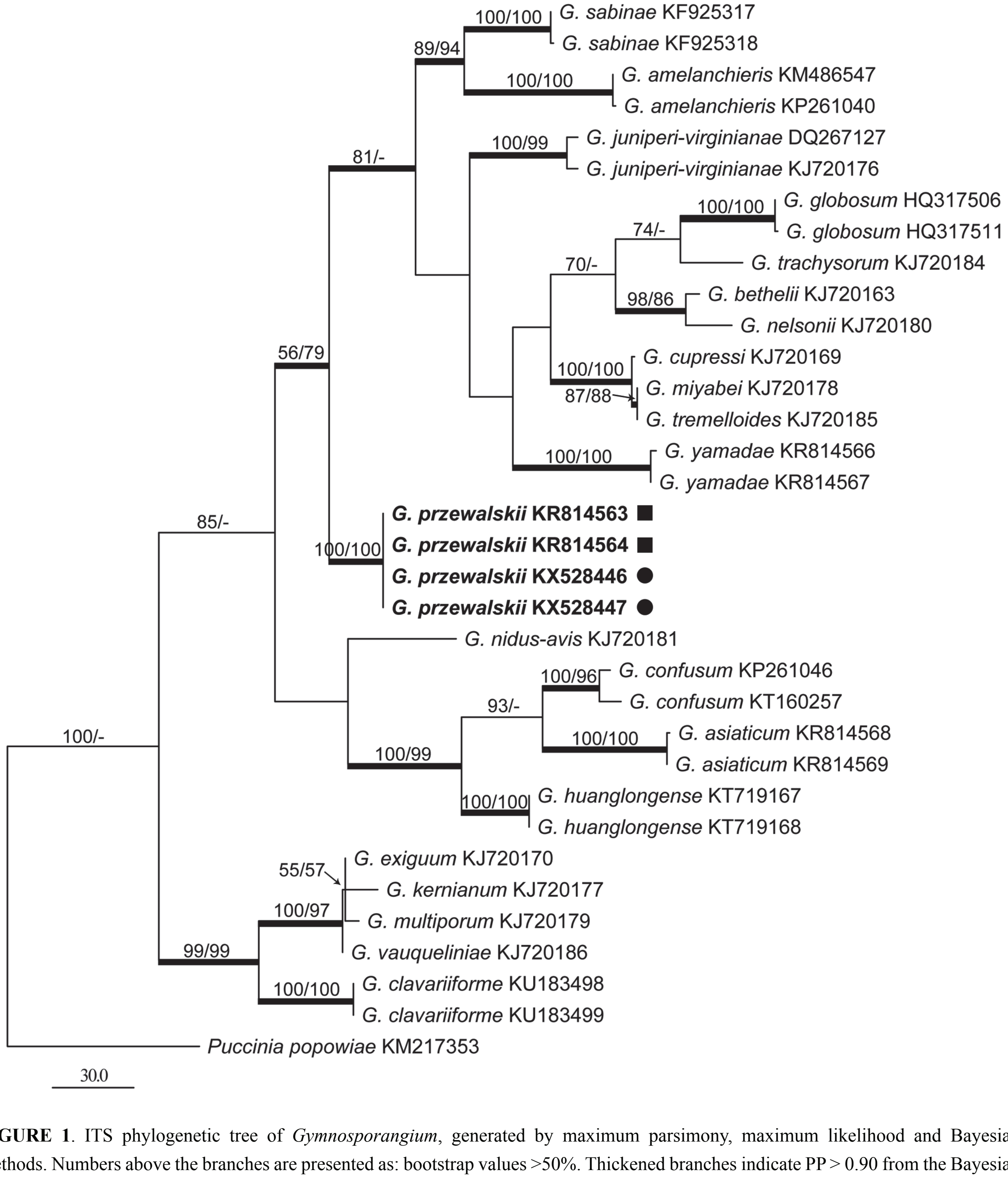

FIGURE 1. ITS phylogenetic tree of *Gymnosporangium*, generated by maximum parsimony, maximum likelihood and Bayesian methods. Numbers above the branches are presented as: bootstrap values >50%. Thickened branches indicate PP > 0.90 from the Bayesian inferences. The new species is shown in bold. Black squares and circles indicate DNA sequences obtained from teliospores and aeciospores, respectively.

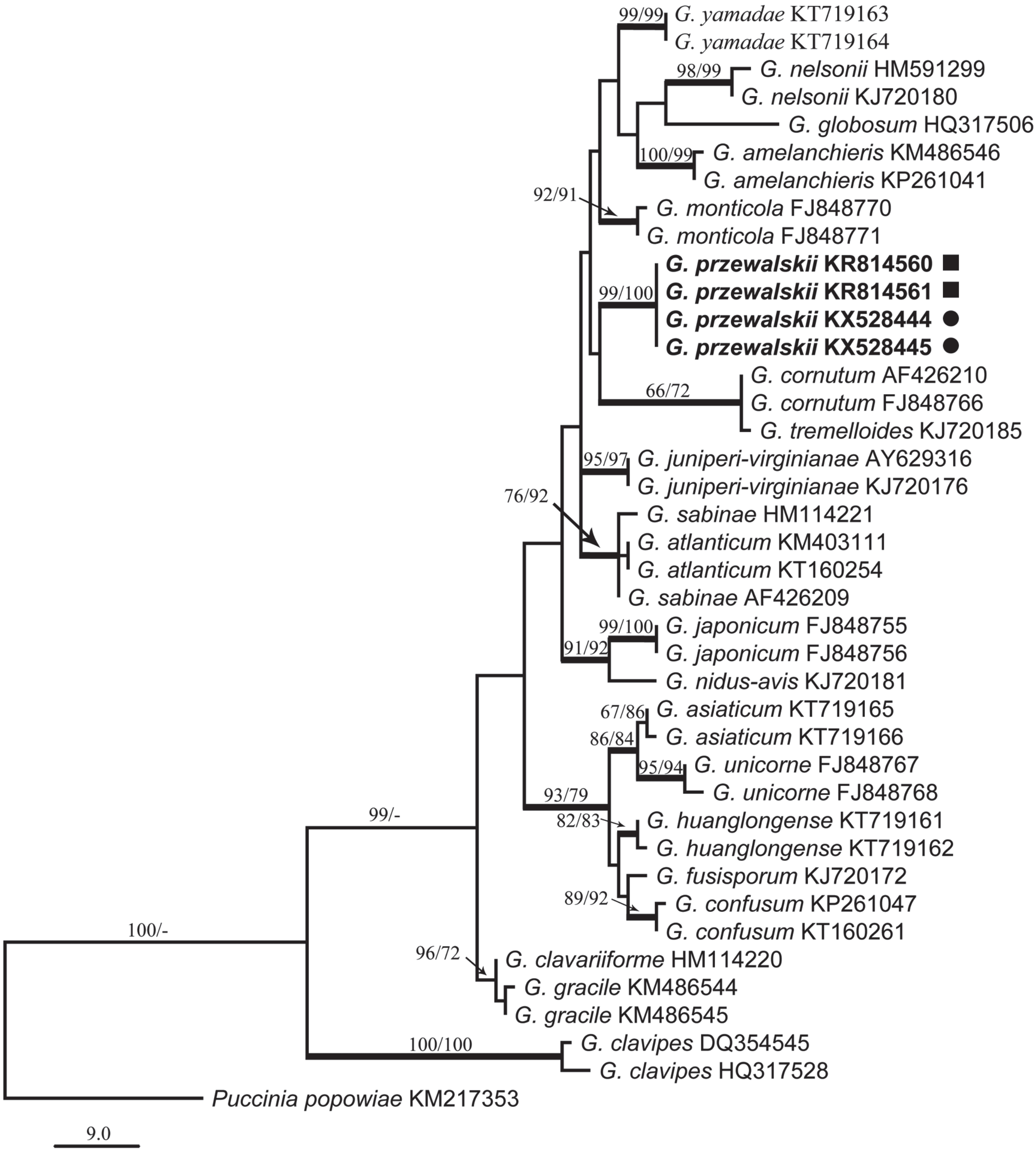

FIGURE 2. LSU phylogenetic tree of *Gymnosporangium*, generated by maximum parsimony, maximum likelihood and Bayesian methods. Numbers above the branches are presented as: bootstrap values >50%. Thickened branches indicate PP > 0.90 from the Bayesian inferences. The new species is shown in bold. Black squares and circles indicate DNA sequences obtained from teliospores and aeciospores, respectively.

Taxonomy

Both morphological and molecular phylogenetic results revealed that the rust fungus on *J. przewalskii* and *S. koehneana* from China is a distinct species, and it is described as a new species.

Gymnosporangium przewalskii Y. M. Liang and B. Cao, *sp. nov.* (Figs 3, 4)

MycoBank no.:—MB817891.

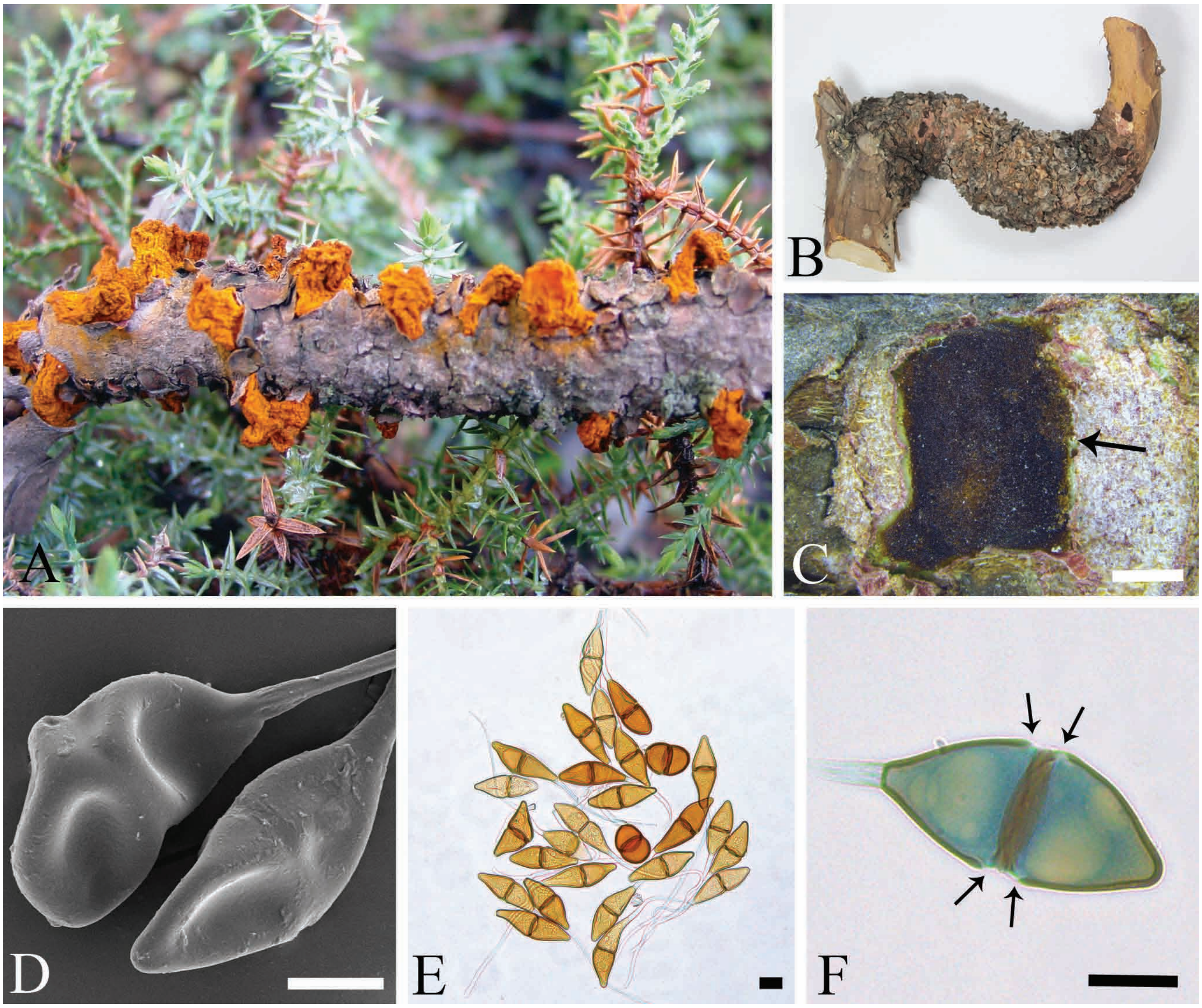

FIGURE 3. Telial stage of *Gymnosporangium przewalskii* on *Juniperus przewalskii* (holotype, BJFC-R01859). A. Telia emerge from the branches in saffron yellow gelatinous form under moist conditions. B. Telia formed on a branch. C. An enlarged view of a telium. D, E. Teliospores. F. Teliospore with two septal germ pores in each cell (arrows). Scale bars: C = 2 mm; D, E, F = 20 µm.

Holotype:—China, Qinghai Prov., 35°13′47″N, 101°56′48″E, 3020 m asl, on *Juniperus przewalskii* Komarov, Apr 4, 2014, F. Z. Han, BJFC-R01859. (Isotype HMAS 247194).

Etymology:—The epithet "przewalskii", referring to the telial host "*Juniperus przewalskii*".

Distribution:—China (Qinghai and Sichuan Prov.).

Hosts:—0, I on *Sorbus koehneana* C. K. Schneider; III on *Juniperus przewalskii*.

Original description:—Spermogonia epiphyllous and globoid, subepidermal and in groups on discoloured spots, type 4 of Cummins and Hiratsuka (2003). Aecia hypophyllous and fructicolous, 1–3 mm high, roestelioid. Peridium rupturing at apex but retaining tubular form. Peridial cells rhomboid, 39–96 × 13–27 µm ($\bar{x}$ = 64 × 21 µm, n = 50), yellowish brown; outer walls smooth, inner walls moderately rugose with irregular blunt papillae, and side walls moderately rugose. Aeciospores globoid, minutely coronate, 18–30 × 15–27 µm ($\bar{x}$ = 25 × 21 µm, n = 50), yellow to yellowish-brown, wall 1–3 µm.

Telia caulicolous, on fusiform or irregular swelling with knotty rough surface, irregularly pulvinate, brown to black-brown, 3–10 mm across; teliospores two-celled, rarely one-celled, fusiform, more or less constricted at the septum, yellowish brown, 42–80 × 15–28 µm ($\bar{x}$ = 59 × 22 µm, n = 50), walls 0.5–2 µm; two pores near the septum in each cell; pedicels ≥ 200 µm long.

Additional specimens examined:—China, Qinghai Prov., 35°13′47″N, 101°56′48″E, 3020 m asl, on *Juniperus przewalskii*, Apr 4, 2014, F. Z. Han, BJFC-R01860 and BJFC-R01861; 35°12′32″N, 102°02′13″E, 2699 m asl, on *Sorbus koehneana*, Aug 15, 2015, B. Cao, BJFC-R02083, BJFC-R02084 and BJFC-R02085. Sichuan Prov., 32°54′33″N, 103°40′59″E, 3225 m asl, on *Juniperus przewalskii*, Apr 23, 2015, B. Cao, BJFC-R01911 and BJFC-R01912.

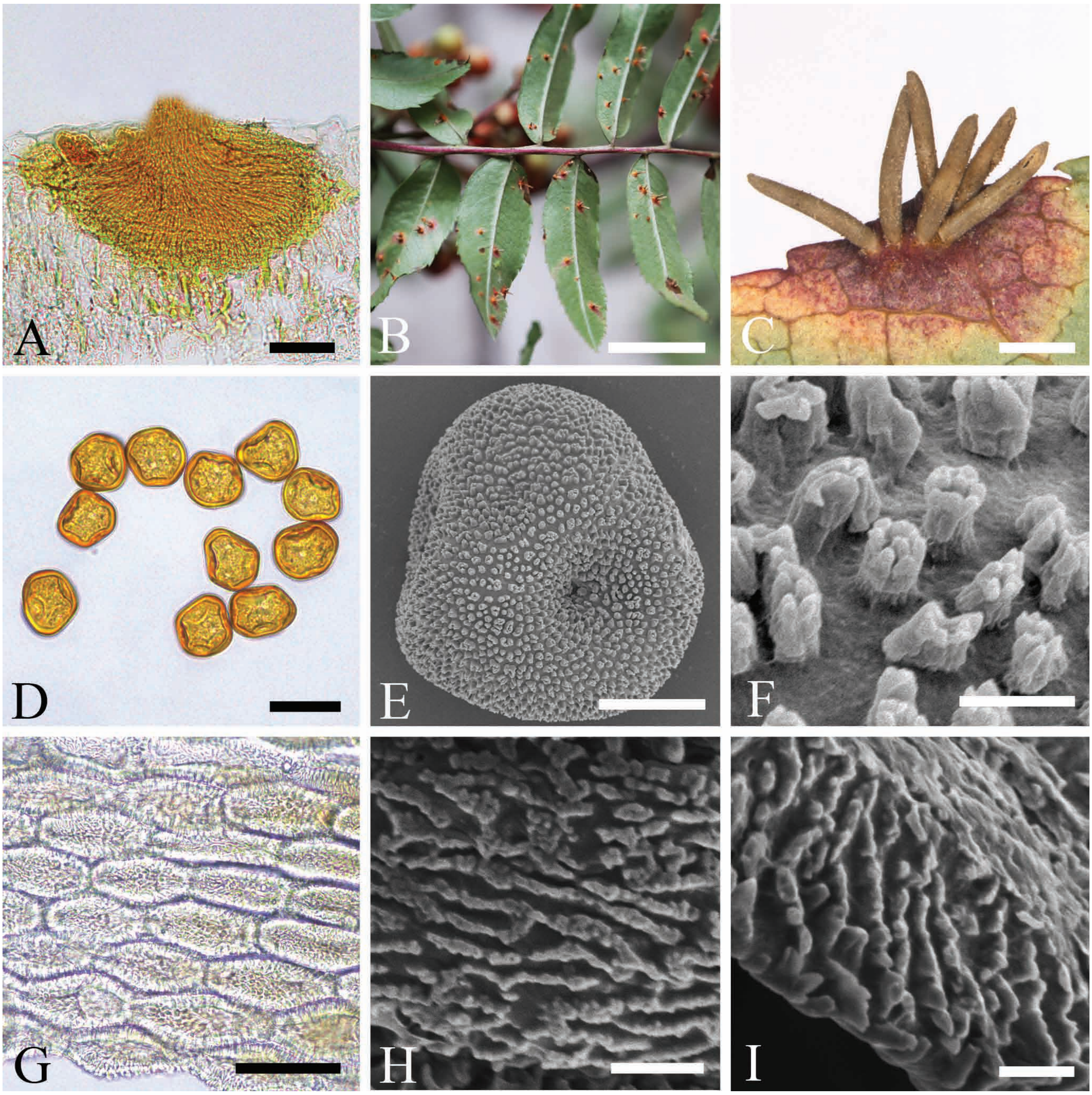

FIGURE 4. Spermogonial and aecial stages of *Gymnosporangium przewalskii* on *Sorbus koehneana* (BJFC-R02083). A. Vertical section of a spermogonium. B. Aecia on the abaxial leaf surface. C. An enlarged view of aecia. D. Aeciospores. E. Surface structure of aeciospores. F. Minutely coronate processes on the aeciospores surface. G. Peridial cells. H. Moderately rugose processes on the inner wall of the peridial cells. I. Moderately rugose processes on the side wall of the peridial cells. Scale bars: A = 30 µm; B = 3 cm; C = 1 mm; D = 30 µm; E = 10 µm; F = 1 µm; G = 60 µm; H, I = 3 µm.

Discussion

In this study, the sequences obtained from teliospores and aeciospores were identical in a distinct clade with high support values (Figs 1, 2). Thus, the phylogenetic analyses confirmed the host alternation between *J. przewalskii* and *S. koehneana* of *G. przewalskii*. According to previous studies, teliospore length and telia shape are useful in discriminating among species of *Gymnosporangium* (Kern 1973, Yun *et al.* 2009). Based on these two characteristics, *G. przewalskii* is similar to four *Gymnosporangium* species, namely *G. formosanum* Hirats. f. & Hashioka, *G. inconspicuum* F. Kern, *G. miyabei* G. Yamada & I. Miyake and *G. tremelloides* R. Hartig (Table 3); however, it can be distinguished based on the following morphological characteristics. The septum in *G. formosanum* is 6–12 µm thick (Kern 1973, Zhuang

2012), which is considered the main characteristic for differentiating it from the other species. *G. przewalskii* differs from *G. inconspicuum* and *G. miyabei* mainly in having two pores near the septum in each cell of the teliospores (Fig. 3F), while the latter two species have only one pore per cell (Kern 1973). The new species and *G. tremelloides* are similar in the telial stage, but they can be distinguished in the aecial stage. The surface of *G. tremelloides* aeciospores is type E (Lee & Kakishima 1999a), while the surface of aeciospores of the new species is type MC (Lee & Kakishima 1999a). In addition, *G. tremelloides* typically has larger aeciospores (30–45 × 28–35 μm) (Parmelee 1971, Kern 1973) than those of *G. przewalskii* (18–30 × 15–27 μm). Further, the phylogenetic analyses showed that the new species is distinct from *G. tremelloides* (Figs 1, 2). The LSU rDNA phylogenetic analyses indicated that two species, *G. cornutum* Arthur ex F. Kern and *G. tremelloides*, were closely related to *G. przewalskii*. As previously discussed, *G. tremelloides* differed morphologically from *G. przewalskii* in terms of the surface structures of its aeciospores. *G. cornutum* could be distinguished from *G. przewalskii* by its teliospore pores, which are covered with hyaline papillae, and its shorter teliospores (30–58 × 15–24 μm) (Parmelee 1965, Kern 1973) than those of *G. przewalskii* (42–80 × 15–28 μm).

In a previous study, only *G. huanglongense* was reported on *Juniperus przewalskii* (Cao *et al.* 2016). In our research, the new taxon was collected from the same host. However, it can be distinguished from *G. huanglongense* by its irregularly pulvinate telia on fusiform or irregular swelling; in contrast, the telia of *G. huanglongense* are often conic with no hypertrophy on the smaller branches (Cao *et al.* 2016). In addition, the phylogenetic analyses showed that the new species is distinct from *G. huanglongense*. Only the telial stage has been found for *G. huanglongense*. However, in this study, we confirmed the host alternation of *G. przewalskii* through phylogenetic analyses (Figs 1, 2).

TABLE 3. Comparison of telium habit and teliospore morphology between *Gymnosporangium przewalskii* and its morphological allies.

Species	Telia		Teliospore	
	Position	Shape	Size (μm)	Germ pores
G. przewalskii	caulicolous	pulvinate	42–80 × 15–27	2/cell, near septum
G. formosanum[ab]	caulicolous	pulvinate or applanate	57–75 × 27–39	2/cell, near septum
G. inconspicuum[a]	caulicolous or foliicolous	pulvinate	55–80 × 25–29	1/cell, near septum
G. miyabei[a]	caulicolous	pulvinate or applanate	40–80 × 11–20	1/cell, near septum
G. tremelloides[ac]	caulicolous	applanate	37–73 × 18–29	2/cell, near septum (or 1 apical + 2 near septum in distal cell)

References: [a] Kern (1973); [b] Zhuang (2012); [c] Parmelee (1971).

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