



Two new *Phragmidium* species identified on *Rosa* plants native to China

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Abstract

Two new *Phragmidium* species, *Phragmidium zhouquensis* and *Ph. longissima*, were identified on two native plants, *Rosa omeiensis* and *R. lichiangensis* respectively, during an investigation of the occurrence of rust fungi in western China. *Phragmidium zhouquensis* is mainly characterized by 3–9-celled teliospores bearing minute verrucae on the surface. *Phragmidium longissima* differs from other *Phragmidium* species in that it possesses echinulate urediniospores with a pore membrane at the germ pore. Phylogenetic analyses based on 28S rRNA partial gene sequences revealed that specimens of *Ph. zhouquensis* and *Ph. longissima* formed two distinct lineages. *Phragmidium longissima* is the first *Phragmidium* species to be identified on *R. lichiangensis*.

Key words: molecular phylogeny, Pucciniales, rose rusts, taxonomy

Introduction

The genus *Rosa* L. (*Rosaceae*) is of worldwide economic importance as the centre of a large ornamental shrub and cut flower industry. *Rosa* species are widely distributed throughout the temperate and subtropical habitats of the northern hemisphere (Matthews 1995). *Rosa omeiensis* Rolfe and *R. lichiangensis* T. T. Yu & T. C. Ku are two species native to central and western China (Lu *et al.* 2003).

The genus *Phragmidium* Link is a common rust fungus restricted to plants belonging to the family *Rosaceae*, especially the genera *Potentilla*, *Rosa* and *Rubus*. *Phragmidium* is characterized by *Caeoma*-type aecia with catenulate aeciospores, *Uredo*-type or *Calodion*-type uredinia with peripheral paraphyses and dark brown teliospores that are typically festooned with several transverse septa along with 2–3 germ pores per teliospore cell (Cummins & Hiratsuka 2003, Yun *et al.* 2011). Most species within this genus produce subcuticular spermogonium, caematoid aecium, uredinium and telium during the autoecious macrocyclic life cycle (Cummins & Hiratsuka 2003, Zhuang *et al.* 2012).

Approximately 60 to 65 species have been recognised as *Phragmidium*, and 30 of these have been reported to infect wild *Rosa* species and ornamental *Rosa* cultivars (Cummins & Hiratsuka 2003). Wahyuno *et al.* (2001) described seven *Phragmidium* species by analysing the morphological characteristics of a maximum of four spore stages from ten previously recorded species. These authors determined that the length, width, degree of tapering toward both ends, and apiculus length were sufficient to determine gross teliospore morphology, and these have been considered as important taxonomic characters. The cell number, wall colour, surface rugosity, and hygroscopicity of the lower part of the pedicel also have been used as taxonomic features at the telial stage. A total of 11 *Phragmidium* species have been reported on *Rosa* in China, including *Ph. butleri* H. Sydow & P. Sydow, *Ph. fusiforme* J. Schröter, *Ph. handelii* Petrak, *Ph. hashiokai* Hiratsuka f., *Ph. kamtschatkae* (F. W. Anderson) Arthur & Cummins, *Ph. montivagum* Arthur, *Ph. rosae-multiflorae* Dietel., *Ph. mucronatum* (Persoon) Schlechtendal, *Ph. robustum* J. Y. Zhuang & S. X. Wei, *Ph. rosae-omeiensis* S. X. Wei, and *Ph. tuberculatum* Jul. Müller. The latter four species have been described on *Rosa omeiensis* (Tai 1979, Wei 1988, Hiratsuka *et al.* 1992, Cao & Li 1996, 1999, Zhuang & Wei 2003, Zhuang 2005, Zhuang & Wang 2006, Zhuang *et al.* 2012, Xu 2013).

During an investigation of rust fungi in western China, two previously unknown *Phragmidium* species were

found on *Rosa omeiensis* and *R. lichiangensis*. Phylogenetic analyses were performed to confirm that the isolates were distinct species. Consequently, they were illustrated and described as the two novel species *Phragmidium zhouquensis* and *Ph. longissima*.

TABLE 1. Sequence data analyzed in this study or obtained from GenBank (new species in bold).

Fungal taxon	Host plant	Specimen no	Locality and date of collection	GenBank accession no. 28S
<i>Phragmidium zhouquensis</i>	Y.M. Liang & T. Yang			
	<i>Rosa omeiensis</i>	BJFC-R01516	Gansu, China, Aug. 20, 2014	KP407637
		BJFC-R01529	Gansu, China, Aug. 20, 2014	KP407638
<i>Ph. longissima</i>	Y.M. Liang & T. Yang			
	<i>Rosa lichiangensis</i>	BJFC-R00338	Yunnan China, Sep. 18, 2011	KP407633
		BJFC-R00360	Yunnan China, Sep. 19, 2011	KP407634
<i>Ph. biloculare</i>	<i>Potentilla flabellifolia</i>	BPI881121	USA	JF907670 ^a
<i>Ph. fragariae</i>	<i>Potentilla sterilis</i>	—	—	JF907670 ^a
<i>Ph. fusiforme</i>	<i>Rosa hugonis</i>	BJFC-R00942	Gansu China, July. 18, 2013	KP407632
	<i>Rosa pendulina</i>	—	Switzerland	AJ715522 ^a
<i>Ph. handelii</i>	<i>Rosa webbiana</i>	BJFC-R01030	—	KP407631
		BJFC-R01421	Gansu, China, Aug. 15, 2014	KP407628
		BJFC-R01437	Gansu, China, Aug. 15, 2014	KP407629
		BJFC-R01458	Gansu, China, Aug. 17, 2014	KP407630
<i>Ph. ivesiae</i>	<i>Potentilla gracilis</i>	BPI877968	USA	JF907673 ^a
		BPI863637	USA	JF907672 ^a
<i>Ph. mexicanum</i>	<i>Potentilla hebiichigo</i>	BPI881108	South Korea	JF907671 ^a
	<i>P. indica</i>	BPI877884	USA	JF907664 ^a
<i>Ph. montivagum</i>	<i>Rosa cf. woodsii</i>	FO47828	—	AF426213 ^a
<i>Ph. mucronatum</i>	<i>Rosa corymbifera</i>	—	Germany	AJ715520 ^a
	<i>R. rubiginosa</i>	—	Germany	AJ715521 ^a
<i>Ph. potentillae-canadensis</i>	<i>Potentilla canadensis</i>	BPI877885	USA	JF907668 ^a
<i>Ph. rubi-idaei</i>	<i>Rubus idaeus</i>	—	—	AF426215 ^a
<i>Ph. sanguisorbae</i>	<i>Sanguisorba minor</i>	—	—	AF426216 ^a
<i>Ph. tormentillae</i>	<i>Potentilla simplex</i>	BPI877888	USA	JF907669 ^a
<i>Ph. tuberculatum</i>	<i>Rosa rugosa</i>	BJFC-R00936	Gansu, China, July. 18, 2013	KP407635
		BJFC-R00959	Qinghai, China, July. 22, 2013	KP407636
	<i>Rosa sp.</i>	BPI877980	USA	KJ841922 ^a
	<i>Rosa floribunda</i>	BPI877977	USA	KJ841923 ^a
<i>Ph. violaceum</i>	<i>Rubus fruticosus</i>	—	—	AF426214 ^a
<i>Puccinia tanacetii</i>	<i>Artemisia brevifolia</i>	IBA5340	Japan	AB190908 ^{ab}

^a stands for sequences from GenBank.

^b stands for sequences used as outgroup.

Materials and methods

Materials

Fresh specimens used in this study were collected in western China during 2011–2014 and deposited at the Mycological Herbarium, Museum of Beijing Forestry University (BJFC), Beijing, China. This study also included dried specimens on *Rosa*, which were loaned from the Herbarium Mycologicum Academiae Sinicae, Beijing (HMAS) (Tables 1 and 2).

TABLE 2. Comparison of telial characteristics of *Phragmidium* species on *Rosa omeiensis* in China used in this study (new species in bold).

Species	Telia			Teliospores		
	Color	Location on hosts	Number of cells	Size ($\mu\text{m} \times \mu\text{m}$)	Papillae (μm)	Length of Pedicels (μm)
<i>Ph. zhouquensis</i>	dark brown	hypophyllous	(3–)6–8(–9)	67–103 \times 32–39	3.5–6	80–160
<i>Ph. mucronatum</i>	black	hypophyllous	(4–)6–8(–9)	55–104 \times 29–36	5–17	62–184
<i>Ph. robustum</i>	dark brown	hypophyllous	3–7	50–106 \times 35–45(–48)	2–8	75–190
<i>Ph. rosae-omeiensis</i>	black	stem	(4–)7–9(–10)	(67–)80–126 (–160) \times 27–34	2–7(–10)	up to 400
<i>Ph. tuberculatum</i>	black	hypophyllous	(3–)4–6(–7)	52–101(–126) \times 29–36	up to 20	up to 110

Microscopic analysis

For light microscopy (LM) observation, spores and leaf sections were mounted in a drop of lactophenol or lactophenol-cotton blue. For each specimen, approximately 30 spores were randomly selected and measured using a LEICA DM2500 upright microscope (Leica, Germany). To prepare samples for surface structure examination using scanning electron microscopy (SEM), urediniospores and leaf sections with uredinia were adhered onto aluminium stubs covered with double-adhesive tape, coated with gold using the Hitachi SCD-005 Sputter Coater, and then observed with a Hitachi S-3400N scanning electron microscope (Hitachi, Tokyo, Japan) operated at 5 kV.

DNA extraction and sequencing

DNA extraction and amplification of 28S rRNA were modified from the method of Tian *et al.* (2004) using the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGT GTTTC AAGACGG-3') (O'Donnell 1993). The methods for PCR analysis were according to the method of Yang *et al.* (2014). PCR products were examined by electrophoresis on 1% (w/v) agarose gels stained with ethidium bromide in 1 \times TAE buffer. The sequences were deposited in the GenBank database (Table 1).

Phylogenetic analysis

Sequences were aligned using ClustalX 1.83 (Thompson *et al.* 1997) and MEGA 6.0. Partitioned and combined data matrices were analysed by maximum parsimony (MP) and Bayesian analyses (BA) using the *Puccinia tanacetii* (AB190908) sequence obtained from GenBank as the out-group. Sequence alignments were deposited at TreeBase (<http://www.treebase.org/>) under accession number 16998. Parsimony analyses were performed in PAUP* 4.0b10 (Swofford 2002), with all dataset characters treated as equally weighted and gaps treated as missing data. Trees were inferred using the heuristic search option with tree bisection and reconnection (TBR) branch swapping and 1,000 random sequence additions. Clade stability was assessed using a bootstrap (BT) analysis with 1,000 replicates (Felsenstein 1985). The BA was performed using MrBayes 3.1 (Ronquist *et al.* 2005) with Markov chain Monte Carlo (MCMC) and Bayesian posterior probabilities (Larget & Simon 1999). Default parameters were selected, and the

evolutionary model was set to the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites (Ronquist *et al.* 2005). The simultaneous Markov chains were run with 1,000,000 generations, and the tree were sampled every 100th generation.

Results

Morphology

Based on the characteristics of multiple-celled teliospores borne singly on hygroscopic pedicels and 2–3 germ pores in each spore cell, the present two species identified on *Rosa* were assigned to *Phragmidium* and illustrated as *Ph. zhouquensis* and *Ph. longissima* in the taxonomy section (Figs. 1, 2).

Molecular phylogeny

The 28S phylogenetic trees included the 30 samples listed in Table 1. Following alignment, the final dataset contained 620 total characters, with 461 constant characters and 54 parsimony-uninformative variable characters. MP analysis with the remaining 105 parsimony-informative characters resulted in eight equally parsimonious trees with the following parameters: tree length (TL) = 256; consistency index (CI) = 0.742; retention index (RI) = 0.912; and rescaled consistency index (RC) = 0.677. The average standard deviation of split frequencies calculated by BA was 0.008781.

MP and BA gives the same topology that the two new species formed two distinct lineages with a BT value and Bayesian posterior probability of 96/0.97 and 100/1.00, respectively (Fig. 3).

Taxonomy

Phragmidium zhouquensis Y. M. Liang & T. Yang, *sp. nov.* (Fig. 1)

MycoBank no.:—MB811453

Etymology:—*Zhouquensis*, referring to the location of the collection of this species.

Diagnosis:—Telia hypophyllous, dark brown, teliospores 67–103 × 32–39 μm, (3–)6–8(–9)-celled, yellowish brown, apical papilla 3.5–6 μm, verrucose, 2–3 germ pores in each cell, pedicels 80–160 × 14–24 μm.

Type:—CHINA, Gansu Province, Zhouqu County, on *Rosa omeiensis* Rolfe (*Rosaceae*), 20 August 2014, coll. Y. M. Liang & B. Cao (Holotype: BJFC-R01516; Paratype: BJFC-R01529).

Spermogonia, aecia, and uredinia unknown.

Telia produced on the abaxial leaf, scattered or loosely grouped, minute, 0.5–2.5 mm, pulverulent, dark brown, leaf colour turns rose-red to aubergine at the position of the sorus (Figs 1A, 1B); teliospores ellipsoid-oblong to cylindrical, 67–103 × 32–39 μm, 3–9-celled, mostly 6–8-celled, the uppermost cell longer than the others, rounded at both ends, often somewhat attenuate at the apex, wall 2–5 μm thick, yellowish brown (Figs 1E, 1F), with coarse and nearly hyaline verrucae on the spore surface (Figs 1C, 1D), apical papilla conical, pale-coloured or hyaline with dense tubercles, 3.5–6 μm long, not constricted at the septa, with 2–3 germ pores in each cell (Fig. 1E); pedicels 80–160 μm long, persistent, upper part colourless or pale brown, lower part with coarse surface and yellowish content, slightly swollen, gradually become lanceolate, approximately 14–24 wide at the broadest diameter (Figs 1D, 1E).

Notes:—*Phragmidium* primarily parasitise *Potentilla*, *Rubus*, and *Rosa*, and rust species do not overlap among these three host genera. Of the 11 *Phragmidium* species reported on *Rosa* in China, four species colonise *Rosa omeiensis*, including *Ph. mucronatum*, *Ph. robustum*, *Ph. rosae-omeiensis*, and *Ph. tuberculatum* (Table 2). *Phragmidium zhouquensis* differed from *Ph. mucronatum* primarily by the dark brown telia (Figs 1A, 1B) and short papilla with lengths up to 6 μm (Figs 1D, E), whereas *Ph. mucronatum* telia were black and teliospores with papillae at the top had lengths up to 17 μm (Wei 1988, Zhuang *et al.* 2012). *Phragmidium robustum* was characterized by wider and more robust teliospores (50–106 × 35–48 μm), mostly 5–6-celled, with longer pedicels of 70–190 μm (Zhuang & Wei 2009, Zhuang *et al.* 2012); these features can be used to distinguish it from the present species. The new species *Ph. zhouquensis* could be distinguished from *Ph. rosae-omeiensis* by its verrucose teliospores (Fig. 1D); by contrast, the surface of *Ph. rosae-omeiensis* teliospores was smooth. The common species, *Ph. tuberculatum*, can be distinguished

from *Ph. zhouquensis* according to its 1–8-celled (mostly 6-celled) teliospores bearing long papillae (7–23 μm) at the spore apices; the papillae of *Ph. zhouquensis* were 3.5–6 μm long (Figs 1D, 1E) (Wei 1988, Zhuang *et al.* 2012).

Phragmidium zhouquensis can be distinguished from other morphologically closely-related *Rosa* species as follows. *Phragmidium fusiforme* is one of the most widespread *Phragmidium* species in the northern hemisphere; it is characterized by multiple-celled (mostly greater than 10-celled) and fusiform teliospore with long papilla up to 15 μm at the spore apex, which is obviously different from that of the present species (Wei 1988, Hiratsuka *et al.* 1992, Wahyuno 2001, Zhuang *et al.* 2012). *Phragmidium zhouquensis* also differed from *Ph. montivagum* by the dark brown telia (Figs 1A, 1B) with ellipsoid-oblong to cylindrical teliospores and lanceolate pedicels (Figs 1D, 1E), whereas the latter species had black telia aggregated by fusiform or subclavate teliospores with hygroscopic and bulbous pedicels.

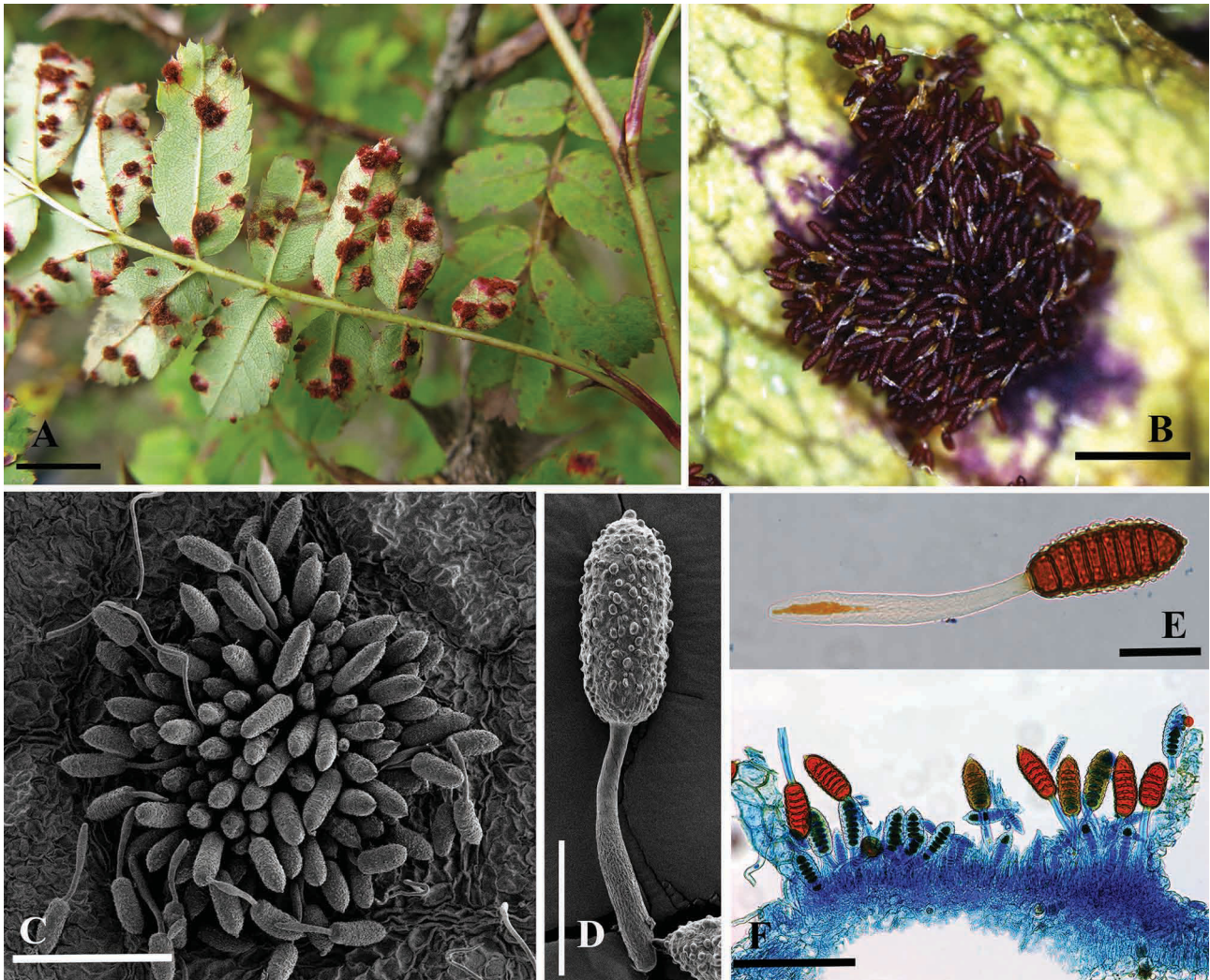


FIGURE 1. *Phragmidium zhouquensis* (BJFC-R01516, holotype). A. Gross features of infected leaves. B and C. Surface view of telium. D. Teliospores with verrucose surface. E. Teliospores with yellow content and hyaline verrucae, 2–3 germ pores in each cell. F. Vertical section of telium. Scale bars: A = 1 cm; B = 500 μm ; C and F = 200 μm ; D and E = 50 μm .

Phragmidium longissima Y. M. Liang & T. Yang, *sp. nov.* (Fig. 2)

Mycobank no.:—MB811452

Etymology:—*Longissima*, referring to the characteristically long teliospores of this species.

Diagnosis:—Urediniospores uniformly echinulate, with pore membrane at the germ pore, telia black, teliospores (8–)9–11(–12)-celled, 85–122 \times 21–30 μm , wall dark brown, verrucose, papilla 2.5–5.5 μm , verrucose, pedicel length 0.5–1 times the spore length.

Holotype:—CHINA, Yunnan Province, Lanping County, Mt. Changyan, on *Rosa lichiangensis* T. T. Yu & T. C. Ku, 18 September 2011, coll. T. Yang, Exsiccate BJFC-R00338.

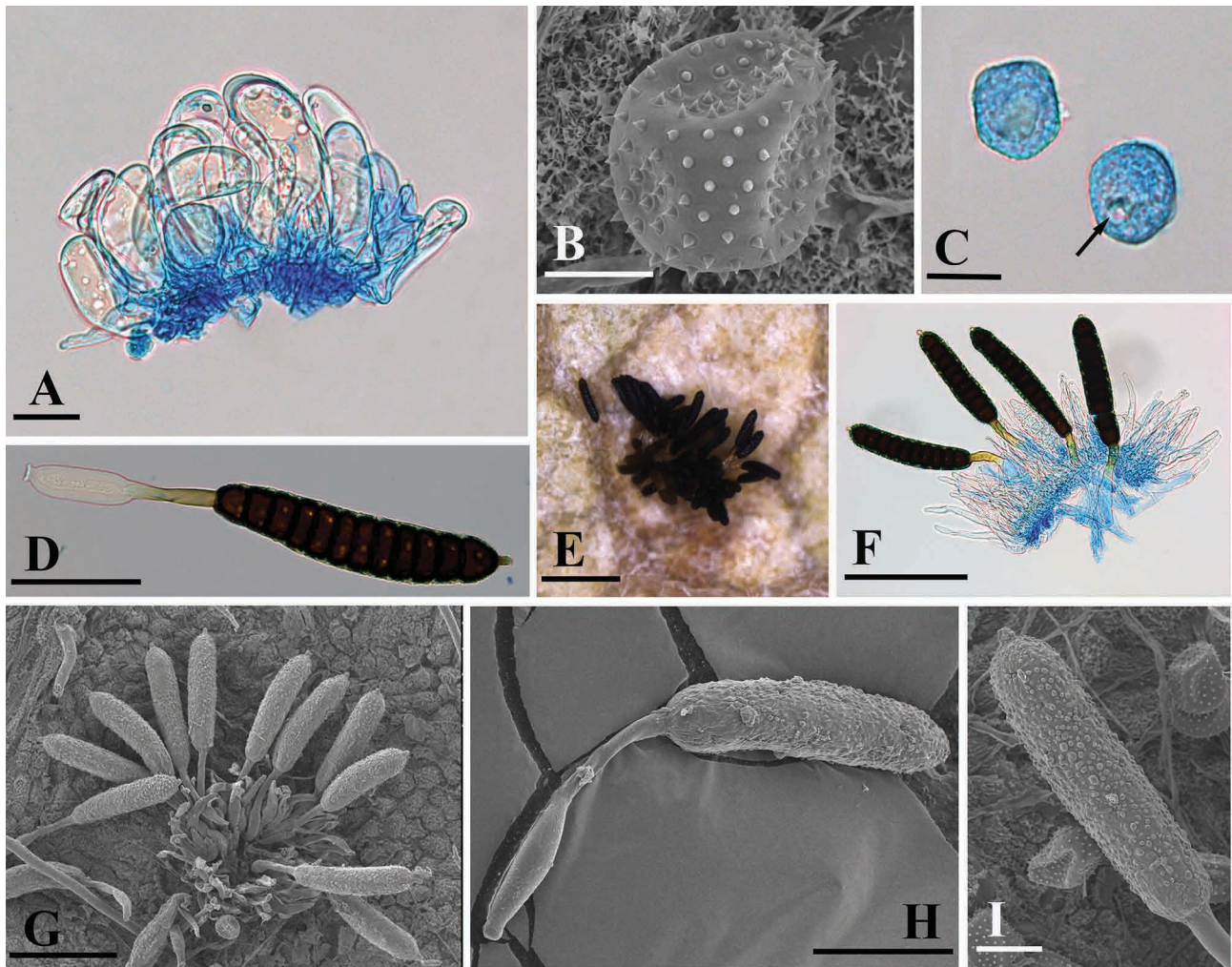


FIGURE 2. *Phragmidium longissima* (BJFC-R00338, holotype). A. Vertical section of paraphyses in uredinium. B. Urediniospore with echinulate surface. C. Globose or sub-globose urediniospores with pore membrane at the germ pore (arrow indicates the position of the pore membrane). D. Teliospore with two germ pores in each cell. E. Black telium on the abaxial leaf. F. Vertical section of telium. G. Surface view of telium. H and I. Teliospores with verrucose surface and smooth pedicel. Scale bars: A and C = 20 μm ; B = 10 μm ; C and I = 20 μm ; D and H = 50 μm ; E = 200 μm ; F and G = 100 μm .

Paratype:—CHINA, Yunnan Province, Lanping County, Mt. Luoguqing, on *Rosa lichiangensis* T. T. Yu & T. C. Ku, 19 September 2011, coll. T. Yang, Exsiccate BJFC-R00360.

Spermogonia and aecia unknown.

Uredinia hypophyllous, scattered or loosely grouped, minute, rounded, 0.05–0.2 mm across, pale yellow; paraphyses numerous, clavate or broadly clavate, 42–75 \times 16–30 μm , sub-erect or incurved, located around the sorus, wall smooth, colourless (Fig. 2A); urediniospores globose or sub-globose, 20–26 \times 18–21 μm , wall approximately 1 μm thick, uniformly echinulate, colourless, wall at germ pore conspicuously intruding in the spore lumen to form a pore membrane (Figs 2B, 2C). Telia produced on the abaxial leaf, scattered or grouped, minute, irregular in shape, 0.1–0.3 mm across, early naked, pulverulent, black (Fig. 2E); teliospores cylindrical, 8–12-celled, generally 9–11-celled, 85–122 \times 21–30 μm , round at both ends, not constricted at the septum, apical papillae obtuse, approximately 2.5–5.5 μm long, brownish-yellow, densely verrucose, usually two germ pores in each cell, wall approximately 2–5 μm thick, dark brown, densely and minutely verrucose, with colourless tubercles (Figs 2D, 2F–2I); pedicles persistent, 65–111 μm long, average length 0.5–1 times the spore length, swelling broadly clavate at the lower half, approximately 11–17 μm at the broadest diameter, brownish-yellow in the upper half, nearly colourless in the lower half, smooth (Figs 2C, 2H).

Notes:—*Phragmidium longissima* differed from *Ph. rosae-multiflorae* in that it had uniformly echinulate urediniospores (Fig. 2B) and generally 9–11-celled teliospores (Figs 2D, 2F), whereas the latter was characterized by verrucose urediniospores and mostly 7–8-celled teliospores. *Phragmidium. rosae-multiflorae* pedicels were

obviously wider (up to 30 μm) than those of *Ph. longissima* (Wei 1988, Hiratsuka *et al.* 1992, Zhuang *et al.* 2012). *Phragmidium americanum* was similar to *Ph. longissima* with respect to teliospore size and cell number, but was distinct in that teliospores were sometimes slightly narrowed above and the pedicel length averaged 1–1.5 times the spore length (Cummins 1931, Wahyuno 2001). *Phragmidium longissima* resembled *Ph. rosae-californicae* in the size of teliospores and pedicels, but was distinguished by the rounded cells at both teliospore ends (Fig. 2D). *Phragmidium rosae-californicae* was characterized by teliospores strikingly acuminate above and with a typically longer apical cell, which graded directly into the apiculus (Cummins 1931). *Phragmidium americanum* and *Ph. rosae-californicae* are distributed primarily in North America and have never been recorded in China (Cummins 1931). *Phragmidium longissima* was the first *Phragmidium* species reported on *Rosa lichiangensis*, which obviously differed from all the previously described *Phragmidium* species by the uredinial and telial host range (Wei 1988, Hiratsuka *et al.* 1992, Wahyuno 2001, Tykhonenko 2007, Zhuang & Wei 2009, Zhuang *et al.* 2012).

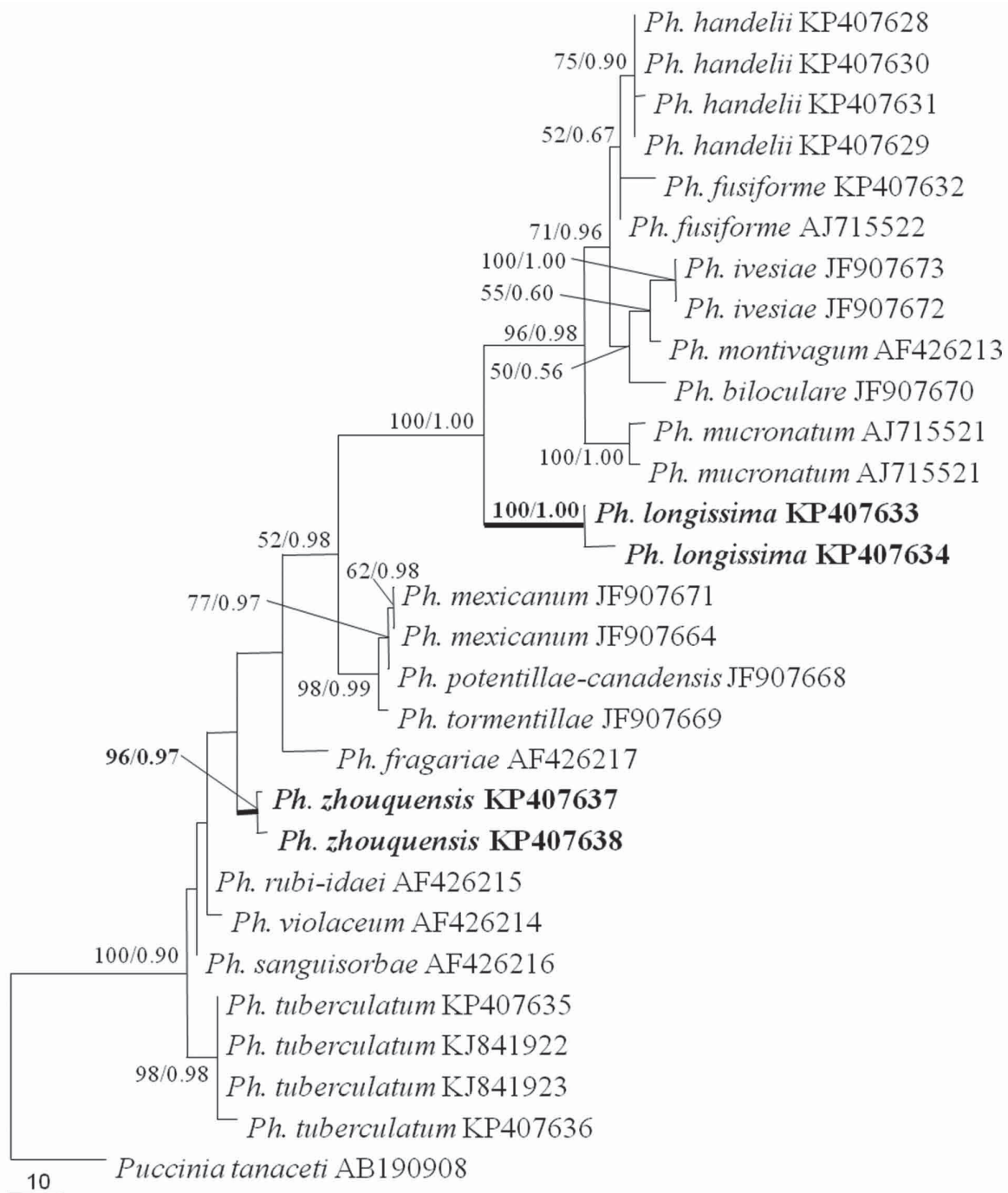


FIGURE 3. Phylogram constructed by maximum parsimony and Bayesian analyses based on 28S sequences. Bootstrap values were calculated from 1,000 replications. Parsimony bootstrap (before the slash marks) and Bayesian posterior probabilities (after the slash marks) greater than 50% are shown. Bars: 10 nucleotide substitutions. New species are shown in bold.

Discussion

The new species *Ph. zhouquensis* was characterized by dark brown telia and 3–9-celled teliospores (Fig. 1E) with minute verrucae on the surface and a 3.5–6 µm long papilla at the spore apex (Fig. 1D). The other new species, *Ph. Longissima*, was characterized by echinulate urediniospores (Fig. 2B), with a pore membrane at the germ pore (Fig. 2C), 9–11-celled teliospores with typically two germ pores per cell, and hygroscopic pedicels of about 0.5–1 times the spore length (Fig. 2D).

The phylogenetic results indicate that *Ph. zhouquensis* and *Ph. longissima* are two distinct lineages with high BT and Bayesian posterior probability (96/0.97 and 100/1.00, respectively) (Fig. 3). The two new species are phylogenetically distinct from other *Phragmidium* species. *Phragmidium zhouquensis* is more closely related to *Ph. fragariae*, which is parasitic to *Potentilla* plants; however, they are clearly different in terms of telial characteristics. The telia of *Ph. fragariae* are often present on the petioles of *Potentilla*, while the telia of *Ph. zhouquensis* are only found on the leaf surfaces of *Rosa*. Except for the difference of host range and infect different portions of the plants. *Ph. zhouquensis* is characterized by larger teliospores (67–103 × 32–39 µm) with mostly 6–8-celled and conical papillae at the top of spores, while *Ph. fragariae* has 2–5-celled teliospores (46.5–77.5 × 24–34.5 µm) without papillae. Furthermore, *Ph. fragariae* also differs from *Ph. zhouquensis* in that it has shorter pedicels (24.5–57 µm in length vs. about 80–160 µm in length) (Petrova & Denchev, 2004). The high supported cluster formed by two *Ph. longissima* specimens is treated as a sister clade of *Ph. mucronatum*, which is also found on *Rosa* plants. However, they are morphologically different in many respects. *Phragmidium longissima* is characterized by long, mostly 9–11-celled teliospores (85–122 × 21–30 µm), while the teliospores of *Ph. mucronatum* are commonly 5–9-celled and 67.5–103.5 µm long. In addition, *Ph. mucronatum* has papilla at the top of the teliospore (up to 13.5 µm long), which are clearly longer than those of *Ph. longissima* (Wei 1988, Hiratsuka *et al.* 1992, Wahyuno 2001, Zhuang *et al.* 2012). According to the consensus results of morphological and phylogenetic analyses, *Ph. zhouquensis* and *Ph. longissima*, which were collected from two native *Rosa* species (*R. omeiensis* and *R. lichiangensis*, respectively), are two distinct taxa.

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