Mycological Progress (2019) 18:529–540
https://doi.org/10.1007/s11557-018-1460-z

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ORIGINAL ARTICLE

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Two new species of *Pucciniastrum* producing dimorphic sori and spores from northeast of China

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Received: 7 September 2018 / Revised: 20 November 2018 / Accepted: 22 November 2018
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Abstract

During field surveys for rust fungi in northeast of China, rust specimens on *Galium* (Rubiaceae), *Aster*, and *Kalimeris* (Compositae) were found in Jilin and Heilongjiang Provinces. Comparative morphology and phylogenetic analyses with 28S and ITS regions of rDNA showed that these specimens belong to *Pucciniastrum*, although no telial stage was found and they were different to *Pucciniastrum* species previously reported from the same host genera. Therefore, the rust fungus on *Galium* and another on *Aster* and *Kalimeris* are described as *P. coronisporum* and *P. verruculosum*, respectively. Both new species are mainly characterized by having two types of uredinia producing morphologically different urediniospores, which are intermingled in most of the uredinia. *Pucciniastrum coronisporum* has echinulate and coronate urediniospores, while those of *P. verruculosum* are echinulate and verrucose to nail-headed.

Keywords *Aster* · *Galium* · *Kalimeris* · Morphology · Phylogeny · *Thekopsora*

Introduction

The genus *Pucciniastrum* G.H. Otth (Pucciniales) was established in 1861 based on *P. epilobii* G.H. Otth on *Epilobium angustifolium* L. and characterized by subcuticular spermogonia (type 3 of Cummins and Hiratsuka 2003), peridermioid acia, uredinia with peridia opening by ostiolar cells, and laterally adherent teliospores in host plants (Hiratsuka 1936, 1958; Cummins and Hiratsuka 2003). Later, *Calyptospora* J.G. Kühn in 1869 and *Thekopsora* Magnus in 1875 were described based on the differences of position of telia in host plants. *Pucciniastrum* produces telia under the epidermal cells, while *Calyptospora* and *Thekopsora* produce them within epidermal cells in stems or leaves (Hiratsuka 1936; Sato et al. 1993; Cummins and Hiratsuka 2003). However, these two genera have been treated as *Pucciniastrum* in broad sense (Arthur 1962; Wilson and Henderson 1966; Cummins and Hiratsuka 2003; Index Fungorum, http://www.indexfungorum.org/) because of similarity of aecial and uredinial morphology. In this study, we followed this taxonomic treatment. About 110 species of *Pucciniastrum s. lat.* have been reported on various plants in the world (Hiratsuka 1936, 1958; Gäumann 1959; Arthur 1962; Wilson and Henderson 1966; Hiratsuka et al. 1992; Azbukina 2015). Among them, 23 species have been reported in China (Tai 1979; Yang et al. 2014, 2015).

Jilin and Heilongjiang Provinces are located in the northeast of China; the east mountainous areas including Changbai mountain ranges are rich in vegetation. However, the inventory and ecology of rust fungi have not been

Section Editor: Meike Piepenbring

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11557-018-1460-z) contains supplementary material, which is available to authorized users.

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sufficiently investigated in these Provinces. Therefore, we surveyed rust fungi in several locations from 2013 to 2017 and collected about 1000 rust specimens. Among these specimens, we found specimens on species of *Galium* (Rubiaceae), and *Aster* and *Kalimeris* (Compositae) producing two morphologically different types of sori and spores on leaves of the same plant. Two morphologically different spores were also observed in the same sorus. One type was similar to uredinial sori and spores of *Pucciniastrum*, and the other was similar to those of *Coleosporium* (Cummins and Hiratsuka 2003). We suspected that two different species of rust fungi were infecting the same host plants, because several rust species belonging to different genera have been recorded on these host plant genera in southeast Asia (Hiratsuka 1936, 1958; Tai 1979; Kaneko 1981; Hiratsuka et al. 1992). For clarification of these rust species, we carried out comparative morphological observations, and molecular analyses with 28S and ITS regions of rDNA. We report here the results of morphological observations and phylogenetic analyses of specimens on *Galium*, *Aster*, and *Kalimeris*, including descriptions of two new species of *Pucciniastrum* with dimorphic sori and spores.

Materials and methods

Specimens

Rust specimens on 4 species of *Galium*: *G. davuricum* Turcz. ex Ledeb., *G. boreale* L., *G. trifidum* L., and *G. bungei* Steud.; 2 species of *Kalimeris*: *K. lautureana* (Debx.) Kitam., and *K. integrifolia* Turcz. ex DC.; and 1 species of *Aster*, *A. tataricus* L. f. were collected at several localities in Jilin and Heilongjiang Provinces in China and were used for morphological observations and molecular analyses (Supplementary Table 1). For molecular analyses, specimens of *Pucciniastrum rubiae* (Kom.) Jørst. on *Rubia cordifolia* L. and *R. chinensis* Regel & Maack, *P. agrimoniae* (Dietel) Tranzschel on *Agrimonia pilosa* Ledeb., *P. tiliae* Miyabe on *Tilia mandshurica* Rupr. & Maxim. and *T. mongolica* Maxim., and *Melampsora laricis-populina* Kleb. on *Populus* sp. were also collected in Jilin or Heilongjiang Provinces and were sequenced (Supplementary Table 1). Specimens of *P. guttatum* (J. Schröt.) Hyl., Jørst. & Nannf. and *P. rubiae* were borrowed from the Mycological Herbarium, Faculty of Life and Environmental Sciences, University of Tsukuba in Japan (TSH) for comparative morphology. All specimens used for morphological observations and molecular analyses are deposited in the Herbarium of Mycology, Engineering Research Center of Chinese Ministry of Education for Edible and Medicinal Fungi, Jilin Agricultural University, China (HMJAU).

Morphological observation

A light microscope (LM) was used to examine morphological characters including the size and shape of sori and spores. Spores or thin sections of sori from dry specimens were mounted in a drop of lactophenol solution on glass slides for LM. The slide preparations were examined and photographed using a Zeiss AXIO imager (Zeiss, Germany) with differential interference contrast (DIC) equipment. Approximately 30 spores from each specimen were randomly chosen and the length, width, and wall thickness of spores were measured using Leica LAS X software attached to a Leica DM2000 microscope (Leica, Germany).

The surface features of spores were examined by a scanning electron microscope (SEM). For SEM, samples obtained from dry specimens were attached to specimen holders by double-sided adhesive tape or carbon paste, coated with platinum-palladium at about 40 nm in thickness using a Hitachi MC1000 Ion Sputter Coater, and examined with a Hitachi SU8010 FE-SEM operated at 5 kV.

DNA extraction and sequencing

The total genomic DNA was directly extracted from about 200 spores containing different types of spores, which were obtained from sori on the leaf, because two types of spores were intermingled in sori and could not be separated clearly. Spores were crushed between two sterilized glass slides and suspended in 30 μL extraction buffer [10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% sodium dodecyl sulfate (SDS), 0.01% Proteinase K], and the suspensions were incubated at 37 °C for 1 h and 95 °C for 10 min, followed by a 4 °C soak (Virtudazo et al. 2001). From the crude extract, 5–7 μL samples were used directly for each polymerase chain reaction (PCR). The rDNA-28S region was amplified using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell 1993), and the internal transcribed spacer (ITS) region (including ITS1, 5.8S, and ITS2) was amplified using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTG ATATGC-3′) (White et al. 1990). The PCR amplifications were performed in 50 μL of mixture containing 5 μL of template DNA, 2 μL of each primer, 25 μL of Premix TaqTM (TaKaRa TaqTM Version 2.0 plus dye) (TaKaRa, Tokyo, Japan), and 18 μL of ddH_2O. Cycling conditions for amplification consisted of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C (ITS) or 55 °C (28S) for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated on 1% agarose gels containing nucleic acid stain (Beijing Dinggou Changsheng Biotechnology Co.) and purified using the TaKaRa MiniBEST Agarose Gel DNA

Exaction Kit Ver.4.0. Purified PCR products were cloned in pEASY®-T1 Cloning Vector (Transgene Biotech, Beijing, China) and then transferred into Trans1-T1 phage, resistant chemically competent cell according to the manufacturer's instructions. The positive clones were sequenced by Sangon Biotech Co., Shanghai, China. All data sequenced in this experiment were deposited at GenBank (Supplementary Table 1).

Phylogenetic analysis

Alignments of the sequence data obtained from specimens were performed using MEGA6 (Tamura et al. 2013), and then, it was manually aligned using BioEdit ver. 7.0.9 (Hall 1999). ITS and 28S sequence data were also retrieved from GenBank based on host species and genus and added to phylogenetic analyses (Supplementary Table 2) (Aime 2006; Yang et al. 2014, 2015; Ji et al. 2016; McTaggart and Aime 2017; Aime et al. 2018). The final dataset contained ITS sequences from 64 specimens with a length of about 700 bps, and 43 specimens 28S sequences with a length of about 650 bps. Phylogenetic trees were constructed with the sequence of *Melampsora laricis-populina* as the outgroup. Topologies were constructed based on maximum likelihood (ML) analyses using raxmalGUI1.5b1 (Silvestro and Michalak 2012). The distance matrix values were constructed by MP method. The trees were described based on tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI). Bayesian Markov chain Monte Carlo (MCMC) analyses were performed using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist 2001). In ML and Bayesian analyses, the best-fit substitution models were estimated using Modeltest ver. 3.7 (Posada and Crandall 1998), and K81uf+I+G was selected as the best evolutionary model. The alignment and trees were deposited to TreeBASE under http://purl.org/phylo/treebase/phylows/study/TB2:S23222 (Fig. 1), TB2:S23223 (Fig. 2) and TB2:S23224 (Fig. 3).

Results

Phylogeny

The direct sequencing data obtained from spores mixed with different types were identical among 8 specimens on *Galium*, and those data among 6 specimens on *Kalimeris* and 3 specimens on *Aster* were also identical (Figs. 1, 2). The sequence data obtained from all clones obtained from same specimen are also identical and same as direct sequencing data of same host plant. 28S dataset was finally constructed by 48 sequences of 11 taxa with 72 parsimony-informative characters of 496 total characters. Parsimony analysis yielded one parsimonious tree with tree length (TL = 157), consistency index (CI = 0.771), retention index (RI = 0.946), and rescaled consistency index (RC = 0.729). Bayesian analysis resulted in average standard deviation of split frequencies of 0.008013. ITS dataset was finally constructed by 53 sequences of 11 taxa with 222 parsimony-informative characters of 759 total characters. Parsimony analysis yielded one parsimonious tree with tree length (TL = 538), consistency index (CI = 0.796), retention index (RI = 0.954), and rescaled consistency index (RC = 0.759). Bayesian analysis resulted in average standard deviation of split frequencies of 0.007502. Tree topologies formed by MP, ML, and MCMC methods were identical among trees. The phylogenetic trees by Bayesian analysis are shown in Figs. 1 (28S) and 2 (ITS), respectively. Both 28S and ITS trees showed that specimens on Rubiaceae and Compositae were phylogenetically included into the group of *Pucciniastrum s. lat.* However, as shown in the tree of 28S (Fig. 3), specimens on *Galium*, *Kalimeris*, and *Aster* were phylogenetically distinct from species of *Coleosporium*, which were reported on many plant species of Compositae and had morphologically similar uredinia to one type of sori on these plants.

The specimens on *Galium davuricum*, *G. boreale*, *G. trifidum*, and *G. bungei* collected in Jilin Province were grouped into one clade, which was included into *Pucciniastrum*, but they were distinct from other species on Rubiaceae (*P. guttatum* and *P. rubiae*) with Bayesian posterior probability and bootstrap values of ML and MP, 1/96%/97%, in 28S although they are phylogenetically close to *P. nipponicum* (Hirats. f.) Jørst. reported on species of *Galium* (Fig. 1). The ITS tree of these specimens also showed the same phylogenetic relationships as the 28S tree and they are also phylogenetically distinct from other species on Rubiaceae (*P. guttatum* and *P. rubiae*) with Bayesian posterior probability and bootstrap value of ML and MP, 1/97%/91%.

The specimens on *Kalimeris lautureana*, *K. integrifolia*, and *Aster tataricus* from Jilin or Heilongjiang Provinces were also grouped into one clade in *Pucciniastrum*, but they were distinct from other species (*P. guttatum*, *P. nipponicum*, and *P. rubiae*), with Bayesian posterior probability and bootstrap values of ML and MP, 1/99%/99%, in 28S. ITS sequences of these specimens also highly supported their distinctness with Bayesian posterior probability and bootstrap values of ML and MP, 1/100%/99%.

Morphology of specimens on *Galium*

Specimens on *Galium davuricum*, *G. boreale*, *G. trifidum*, and *G. bungei* from Jilin Province had two types of sori and spores. One type of sorus was small, yellow, scattered on the leaves, and sometimes gathered in small groups (Fig. 4a, d).

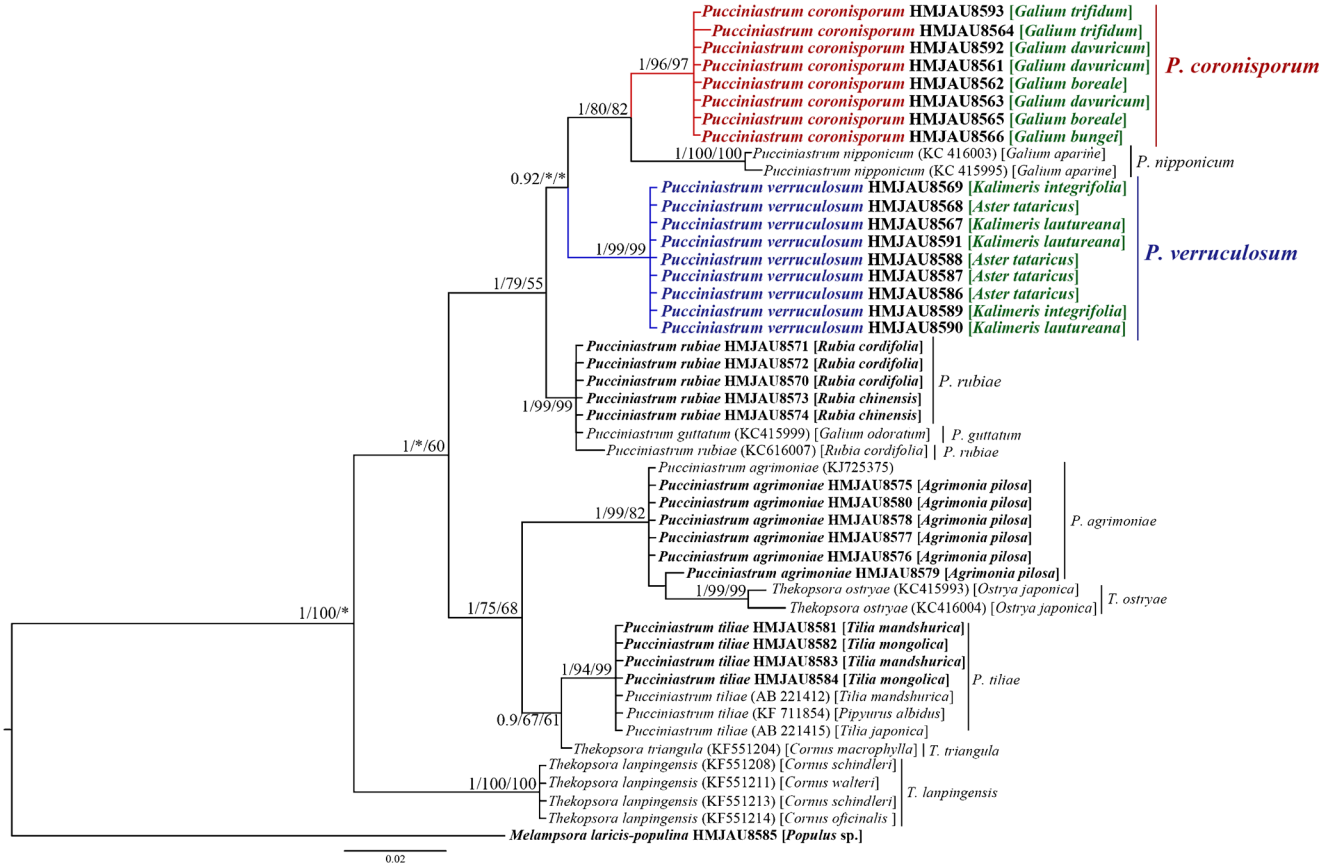

Fig. 1 Phylogenetic tree constructed by Bayesian method based on sequences of 28S regions of rDNA. Bootstrap values of MP and ML are followed by the Bayesian posterior probabilities (Bpp) on the nodes in the topology. Asterisk (*) represents bootstrap values less than 50% or Bpp less than 0.5 in the topology. Sample data are shown with species name, voucher specimen number or GenBank accession number (in parentheses), and host plant. Sequence data determined in this study are shown in bold face

The sori were subepidermal with peridia and erumpent at maturity, but no ostiolar cell was observed (Figs. 4b, 5b). The spores in this sorus were mostly echinulate (Figs. 4f, 5a), but spores with a coronate surface, which were similar to coronate type reported by Sato and Sato (1982) and Lee and Kakishima (1999), were produced and intermingled together (Fig. 4b). The second type of sorus was larger, pale yellow, irregular, and scattered on the leaves (Fig. 4a, d). These sori were subepidermal with hemispherical peridia consisting of polygonal and firmly attached cells, and usually not opened (Figs. 4c, 5d). No ostiolar cell was observed in the peridia (Figs. 4c, 5d). The spores in this sorus had a mostly coronate surface (Figs. 4e, 5c), but spores with echinulate surface were also produced and intermingled together (Figs. 4c, 5d). Two types of spores were clearly recognized inside of the same sorus in observations with SEM (Fig. 5d). No telia and teliospores were found in any specimens. All specimens were morphologically similar to each other, but small sori with echinulate spores were mostly observed in specimens collected in spring and early summer, whereas large sori with coronate spores were produced around the small sori and frequently observed in specimens collected in late summer and autumn.

Morphology of specimens on *Kalimeris* and *Aster*

Specimens on *Kalimeris lautureana*, *K. integrifolia* (Fig. 6a), and *Aster tataricus* collected from Jilin or Heilongjiang Province also had two types of sori and spores. One type of sorus was small, pale yellow or white, scattered on the leaves, or sometimes gathered into small groups. These sori were subepidermal with peridia consisting of irregularly shaped cells, and usually erumpent through stomata, but ostiolar cells were inconspicuous and sometimes not observed (Figs. 6c, 7b). The spores produced in this sorus were mostly echinulate (Figs. 6f, 7a, b), but sometimes spores with verrucose to nail-headed surface, which were similar to verrucose or nail-headed type reported by Sato and Sato (1982) and Lee and Kakishima (1999), were produced and intermingled together (Fig. 6c). The second type of sorus was larger, orange yellow, and scattered on the leaves or gathered into small groups (Fig. 6d). These sori were subepidermal with hemispherical peridia consisting of polygonal and firmly attached cells, and usually not opened (Figs. 6b, 7d). No ostiolar cell was observed in the peridia (Figs. 6b, 7d). Their spores were mostly verrucose to nail-headed (Figs. 6e, 7c), but echinulate spores

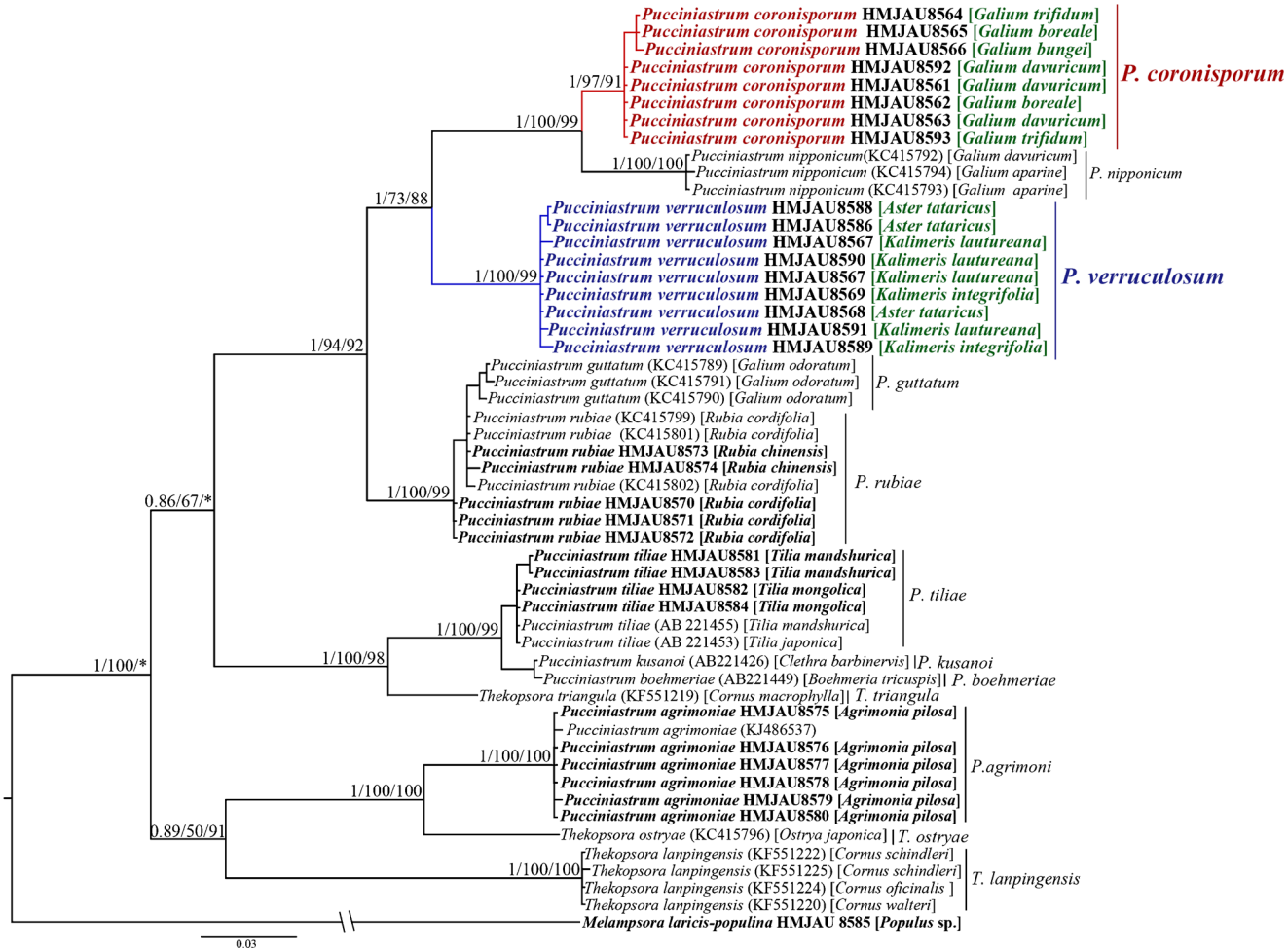

Fig. 2 Phylogenetic tree constructed by Bayesian method based on ITS regions of rDNA. Bootstrap values of MP and ML are followed by the Bayesian posterior probabilities (Bpp) on the nodes in the topology. Asterisk (*) represents bootstrap values less than 50% or Bpp less than 0.5 in the topology. Sample data are shown with species name, voucher specimen number or GenBank accession number (in parentheses), and host plant. Sequence data determined in this study are shown in bold face

were also produced and intermingled (Figs. 6c, 7d). Two types of spores were clearly recognized inside of the same sorus in observations with SEM (Fig. 7d). No telia and teliospores were found in the specimens. All specimens observed were morphologically similar to each other.

Taxonomy

The morphological observations showed that the two types of sori and two types of spores were produced on the same host plant, and two types of spores were also produced in the same sorus. Two same types of spores were produced in different types of sori on the same host plant. Two types of sori were also produced closely each other on the same host plant. The molecular analyses also supported that two types were genetically same because the direct sequencing data obtained from all samples on the same host plants including two types of spores and sequence data of all clones obtained from same specimen were identical. Therefore, it was concluded that they were not caused by intermingling of two species, but were produced by a single species of rust fungi, even though the sorus structures of these types differed. The phylogenetic analyses also showed that these specimens belonged to *Puccinistarum*, but specimens on *Galium* and those on *Aster* and *Kalimeris* separated into two distinct clades, which were different from other species of *Pucciniastrum* reported on the same host genera, or on any other genera. Therefore, we describe them as new species of *Pucciniastrum* producing dimorphic sori and spores.

Pucciniastrum coronisporum Jing X. Ji & Kakish., **sp. nov.** Figs. 4, 5.

MycoBank no. MB 824851

This species differs from other species of *Pucciniastrum* by the dimorphic sori with peridia, and spores with echinulate and coronate surfaces. The echinulate spores (12.0–20.5 × 9.0–14.0 μm) are smaller than those of *P. guttatum* and *P. rubiae* reported on species of Rubiaceae. The coronate

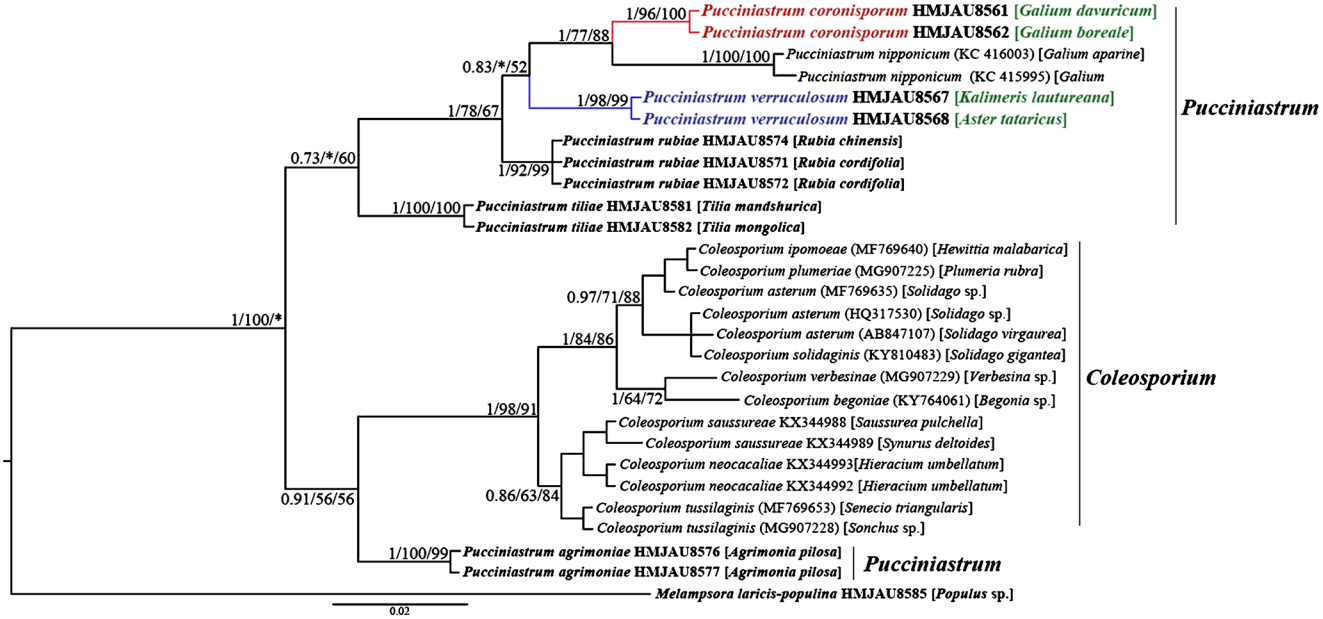

Fig. 3 Phylogenetic tree constructed by Bayesian method based on sequences of 28S regions of rDNA. Bootstrap values of MP and ML are followed by the Bayesian posterior probabilities (Bpp) on the nodes in the topology. Asterisk (*) represents bootstrap values less than 50% or Bpp less than 0.5 in the topology. Sample data are shown with species name, voucher specimen number or GenBank accession number (in parentheses), and host plant. Sequence data determined in this study are shown in bold face

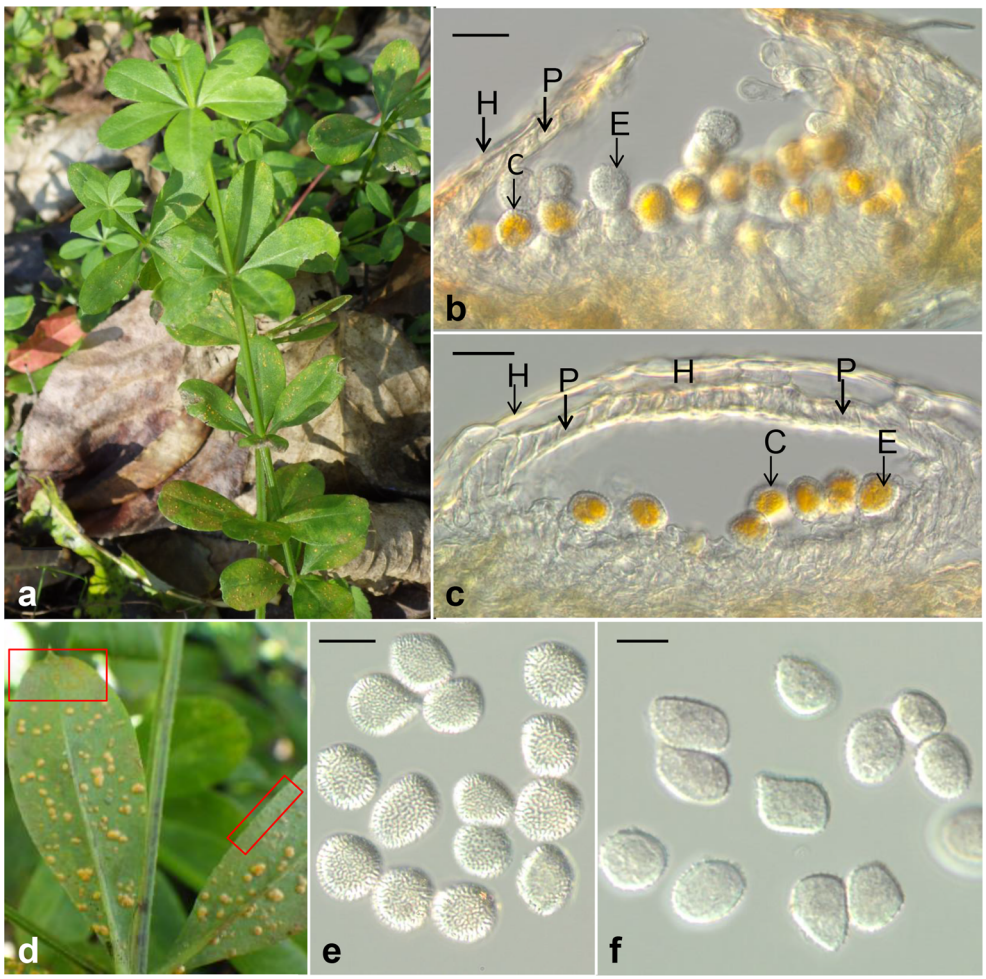

Fig. 4 *Pucciniastrum coronisporum*. **a** *Galium davuricum* producing two types of uredinia. **b** Vertical section of small type uredinium with peridium (P) produced under the epidermis of plant (H) and opening by an apical pore. Echinulate (E) and coronate urediniospores (C) produced in the uredinia. **c** Vertical section of large type uredinium with peridium (P) consisting of firmly attached cells, produced under the epidermis of plant. Coronate (C) and echinulate urediniospores (E) produced in the uredinium. **d** Two types of uredinia produced on the same leaves of *G. davuricum*. Small type (inside of red rectangles) and large types (outside of red rectangles). **e** Coronate urediniospores mostly produced in large type uredinia. **f** Echinulate urediniospores mostly produced in small type uredinia. Bars **b**, **c** 25 μm; **e** 15 μm; **f** 10 μm

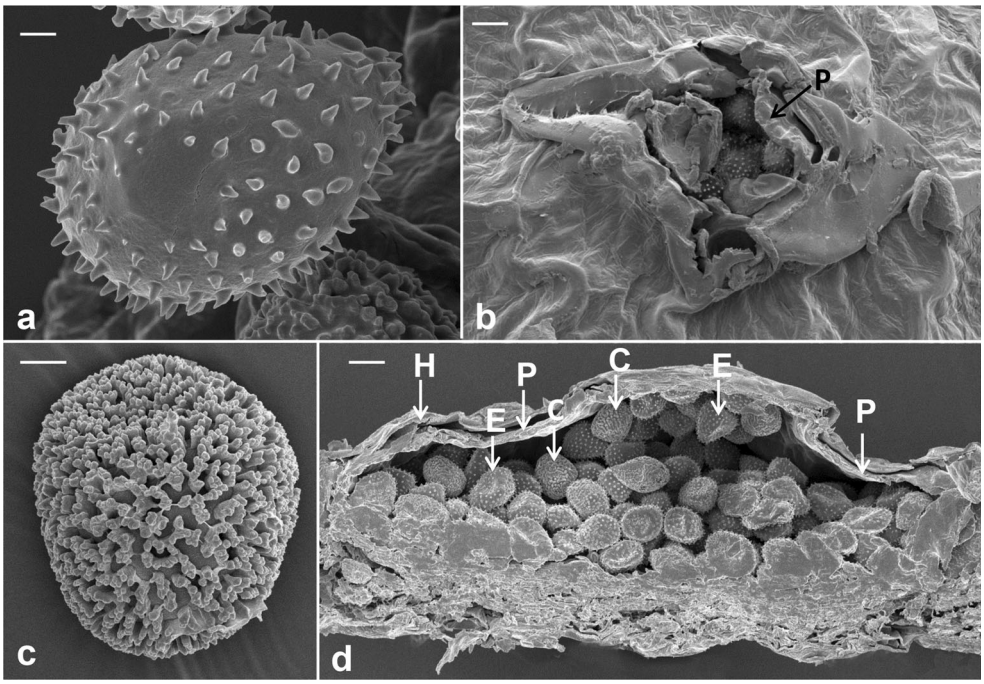

Fig. 5 Morphology of *Pucciniastrum coronisporum* observed by SEM. **a** Echinulate urediniospore. **b** Small type of uredinium with inconspicuous peridium (P), opening by an apical pore. **c** Coronate urediniospore. **d** Large type of uredinium with peridium (P) produced under the epidermis of plant (H). Coronate (C) and echinulate spores (E) are produced in the uredinium. Bars **a** 3 μm; **b**, **d** 15 μm; **c** 4 μm

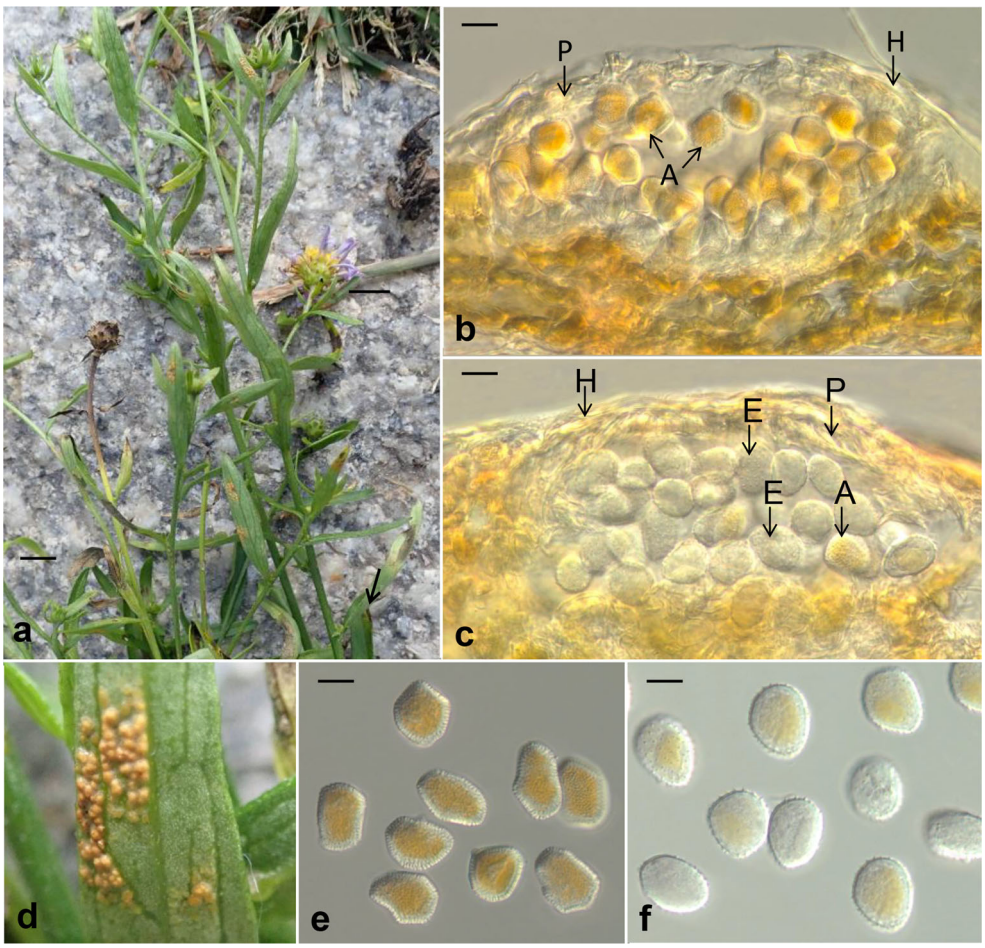

Fig. 6 *Pucciniastrum verruculosum*. **a** *Kalimeris integrifolia* producing sori. **b** Vertical section of large type of uredinium with peridium (P) consisting of firmly attached cells, produced under the epidermis of plant (H) containing verrucose to annulate urediniospores (A). **c** Vertical section of small type of uredinium with peridium (P) consisting of firmly attached cells, produced under the epidermis of plant (H) with verrucose to annulate (V) and echinulate urediniospores (E). **d** Large type uredinia produced on a leaf of *K. integrifolia*. **e** Verrucose to annulate urediniospores mostly produced in large type uredinium. **f** Echinulate spores mostly produced in small type uredinium. Bars: **b**, **c** 15 μm; **e**, **f** 10 μm

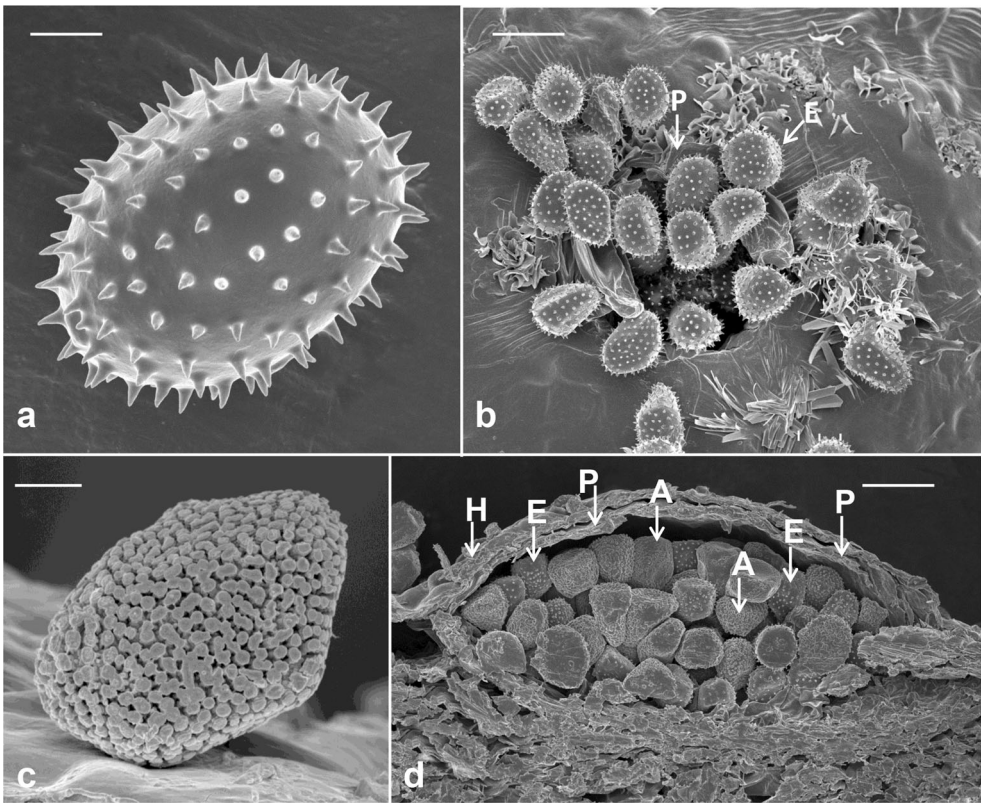

Fig. 7 Morphology of *Pucciniastrum verruculosum* observed by SEM. **a** Echinulate urediniospore. **b** Small type of uredinium with peridium (P), opening from a stoma by apical pore and producing echinulate urediniospores (E). Ostiolar cells are inconspicuous. **c** Verrucose to annulate urdiniospore. **d** Large type of uredinium with peridium (P), produced under the epidermis of plant (H) with verrucose-annulate (A) and echinulate urediniospores (E). Bars **a**, **c** 5 μm; **b**, **d** 25 μm

spores (17.5–22.5 × 14.0–18.5 μm) are smaller than that of verrucose spores of *P. nipponicum* reported on *Galium*.

Typification On *Galium boreale* L., China, Jilin Province, Jilin City, Jiaohe County, 21 Sep 2016, *M. Kakishima and J. X. Ji* (HMJAU 8562, holotype). GenBank: ITS = MG787102; 28S = MG787127.

Etymology Named after the surface structure of the spores.

Descriptions Uredinia and urediniospores dimorphic. The first type of uredinia amphigenous, subepidermal, scattered or sometimes in small groups, small, round, about 0.3 mm in diameter, yellow in color; peridia applanate-hemispherical, firm, erumpent with apical pores and no ostiolar cell; peridial cells small, irregularly polygonal, firmly attached to each other, wall thin, hyaline. The second type of uredinia amphigenous, subepidermal, scattered, large, irregular, about 0.5–2.5 mm in diameter, pale yellow in color; peridia applanate-hemispherical, not opened by pores, firm; peridial cells small, irregularly polygonal, firmly attached to each other, wall thin, hyaline. The first type of urediniospores sessile, subglobose, ovate or ellipsoid, 12.0–20.5 × 9.0–14.0 μm (av. 16.5 × 12.5 μm), wall thin, 0.2–0.8 μm (av. 0.4 μm) thick, hyaline, echinulate. The second type of urediniospores sessile, subglobose, ovate, or ellipsoid, 17.5–22.5 × 14.0–18.5 μm (av. 20.0 × 16.5 μm), wall thick, 0.5–1.5 μm (av. 1.0 μm) thick, hyaline, coronate. The first type of uredinia mostly containing the first type of urediniospores, but sometimes intermingled with the second type of urediniospores. The second type of uredinia mostly containing the second type of urediniospores, but sometimes intermingled with the first type of urediniospores.

Other specimens examined China, Jilin Province: On *Galium davuricum*, Jilin City, Jiaohe County, Hongyegu, 21 Sep 2016, M. Kakishima (MK) and J. X. Ji (JI) (HMJAU 8561); Baishan City, Southern area of Changbai Mountain, 7 July 2017, MK and JI (HMJAU 8564); On *Galium boreale*, Tonghua City, Jian County, Wunvfeng, 3 Oct 2015, JI (HMJAU 8563); Jilin City, Jiaohe County, Qingling Water Falls, 29 June 2017, MK and JI (HMJAU 8562); On *Galium trifidum*, Jilin City, Jiaohe County, Water Qingling Falls, 12 Sep 2017, MK and JI (HMJAU 8565); On *Galium bungei*, Jilin City, Jiaohe County, Bangchuigu, 12 Sep 2017, MK and JI (HMJAU 8566); 1 Jul 2018, MK and JI (HMJAU 8592, HMJAU 8593).

Notes Specimens on 4 species of *Galium* collected from Jilin Province were phylogenetically included in *Pucciniastrum*, but they were distinct from other species, although no telial stage was found in the specimens. The phylogenetic results were also supported by morphological observations. This new species is characterized by dimorphic sori and spores. The first

Table 1 Comparative morphology of *Pucciniastrum* species reported on Rubiaceae and Compositae

Species	Host plant genus	Sorus (uredinium)		Spore (urediniospore)				Reference[a]
		Type	Peridium	Ostiolar cell	Shape	Size (μm)	Wall thickness (μm)	Surface	
P. verruculosum	*Aster*, *Kalimeris*	Small	Firm	Not present or inconspicuous	Subglobose, ovate, or ellipsoid	15.5–26.0 × 13.5–19.0	0.5–1.5	Echinulate	A
		Large	Firm	Not present	Subglobose, ellipsoid, or polygonal	16.5–25.5 × 13.0–20.0	1.0–2.5	Verrucose to annulate	A
P. coronisporum	*Galium*	Small	Firm	Not present	Subglobose, ovate, or ellipsoid	12.0–20.5 × 9.0–14.0	0.2–0.8	Echinulate	A
		Large	Firm	Not present	Subglobose, ovate, or ellipsoid	17.5–22.5 × 14.0–18.5	0.5–1.5	Coronate	A
P. asterum	*Aster*, *Heteropappus*, *Kalimeris*		Firm	Not present	Globose, ellopsoid, or obovate	17.5–25 × 15–20	1.5	Echinulate	B
P. guttatum	*Asperula*, *Galium*, *Sherardia*		Firm or delicate	Round shape	Subglobose, ovate, or ellipsoid	12–24 × 10–18	1–1.5	Echinulate	B
P. nipponicum	*Galium*		Firm	Round shape	Globose, subglobose, or obovate	16–24 × 15–22	2–2.5	Verrucose	B
P. rubiae	*Rubia*		Firm	Thick cell	Subglobose, ovate, or ellipsoid	18–27 × 12–18	1–1.5	Echinulate	B

[a] A: Present paper; B: Hiratsuka (1936, 1958) and Hiratsuka et al. (1992)

type of sori and spores is morphologically similar to uredinia and urediniospores of *P. guttatum* and *P. rubiae* reported on Rubiaceae, but it is different from them in peridial structures and size of spores. *Pucciniastrum coronispora* has no ostiolar cell in peridia, whereas the other two species have conspicuous ostiolar cells, and spores of *P. coronisporum* (12.0–20.5 × 9.0–14.0 μm) are smaller than urediniospores of *P. guttatum* (12–24 × 10–18 μm after Hiratsuka et al. 1992) and *P. rubiae* (18–27 × 12–18 μm after Hiratsuka et al. 1992) (Table 1) (Hiratsuka 1936, 1958; Gäumann 1959; Arthur 1962; Wilson and Henderson 1966; Hiratsuka et al. 1992; Termorshuizen and Swertz 2011; Azbukina 2015). We also examined specimens of *P. guttatum* and *T. rubiae* borrowed from TSH and confirmed these morphological differences. Sori and spores of the second type are similar to uredinia and urediniospores of *P. nipponicum* reported on *Galium*, but *P. coronisporum* is different from *P. nipponicum* in peridial structures, and size and surface of spores (Table 1). The sori of *P. coronisporum* are not opened by pores and have no ostiolar cell, but uredinia of *P. nipponicum* have round-shaped ostiolar cells (Hiratsuka 1958; Hiratsuka et al. 1992). The spores of *P. coronisporum* (17.5–22.5 × 14.0–18.5 μm) are smaller than urediniospores of *P. nipponicum* (16–24 × 15–22 μm), and the spore surface is coronate whereas urediniospores of *P. nipponicum* are verrucose (Hiratsuka et al. 1992; Azbukina 2015). These morphological differences between *P. coronisporum* and *P. nipponicum* were also supported by phylogenetic analyses (Figs. 1, 2).

Several species of *Puccinia* and *Uromyces* have been reported on *Galium* (Tai 1979; Hiratsuka et al. 1992; Termorshuizen and Swertz 2011; Okane et al. 2014; Azbukina 2015). However, they are morphologically different from this species in sorus structures (Cummins and Hiratsuka 2003).

Pucciniastrum verruculosum Jing X. Ji & Kakish., **sp. nov.** Figs. 6, 7.

MycoBank no. MB 854852

This species is characterized by the dimorphic sori with peridia, and spores with echinulate and verrucose to nail-headed surfaces. This species differs from *P. asterum* (Tranzschel) Jørst. reported on Compositae by having spores with verrucose to nail-headed surfaces.

Typification On *Aster tataricus* L. f., China, Jilin Province, Changchun City, Jingyue Forest Park, 16 Sep 2017, *M. Kakishima* and *J. X. Ji* (HMJAU 8568, holotype). GenBank: ITS = MG787109; 28S = MG787134.

Etymology Named after the surface structure of the spores.

Descriptions Uredinia and urediniospores dimorphic. The first type of uredinia hypophyllous, subepidermal, scattered or

sometimes in small groups, small, round, about 0.2 mm in diameter, white to pale yellow in color; peridia applanate-hemispherical, firm, usually erumpent from stomata with inconspicuous ostiolar cells or without ostiolar cells; peridial cells small, irregularly polygonal, firmly attached to each other, wall thin, hyaline. The second type of uredinia hypophyllous, subepidermal, scattered, large, irregular, about 1–2 mm in diameter, orange yellow in color; peridia applanate-hemispherical, not opened by pores, firm; peridial cells small, irregularly polygonal, firmly attached to each other, wall thin, hyaline. The first type of urediniospores sessile, subglobose, ovate, or ellipsoid, 15.5–26.0 × 13.5–19.0 μm (av. 20.0 × 16.0 μm), wall thin, 0.5–1.5 μm (av. 1.0 μm) thick, hyaline, echinulate. The second type of urediniospores sessile, subglobose, ellipsoid, or polygonal, 16.5–25.5 × 13.0–20.0 μm (av. 21.0 × 16.0 μm), wall 1.0–2.5 μm (av. 1.5 μm) thick, hyaline, verrucose to nail-headed. The first type of uredinia mostly containing the first type of spores, but sometimes intermingled with the second type of urediniospores. The second type of uredinia mostly containing the second type of urediniospores, but sometimes intermingled with the first type of urediniospores.

Other specimens examined China, Heilongjiang Province, Wuchang City, Fenghuangshan Forest Park, on *Kalimeris lautureana*, 9 Sep. 2017, MK and JI (HMJAU 8567). Jilin Province, Changchun City, Jingyue Forest Park, on *Aster tataricus*, 10 Aug. 2017, MK and JI (HMJAU 8568); on *Aster tataricus*, 5 Jul. 2018, MK and JI (HMJAU 8586, HMJAU 8587, HMJAU 8588); Jilin City, Jiaohe County, Lafa Mountain, on *Kalimeris integrifolia*, 11 Sep. 2017, MK and JI (HMJAU 8569);1 Jul. 2018, MK and JI (HMJAU 8589); on *Kalimeris lautureana,* 1 Jul. 2018, MK and JI (HMJAU 8590); Bangchuigu, 1 Jul 2018, MK and JI (HMJAU 8591).

Notes Specimens on 2 species of *Kalimeris* from Jilin or Heilongjiang Province and 1 species of *Aster* from Jilin Province were phylogenetically included in *Pucciniastrum*, but they were distinct from other species, although no telial stage was found in the specimens. The phylogenetic results were also supported by morphological observations. This new species is characterized by dimorphic sori, and spores with echinulate and verrucose to nail-headed surface structures. The first type of sori and spores is morphologically similar to uredinia and urediniospores of *P. asterum* reported on species of *Aster*, *Heteropappus*, and *Kalimeris* (Compositae) in the size and surface of urediniospores, but *P. asterum* has not been reported to have verrucose to annulate urediniospores, which were observed as the second type of this species (Table 1) (Hiratsuka 1936, 1958; Hiratsuka et al. 1992; Azbukina 2015). The type specimen of *P. asterum* collected on *Aster incisus* Fish. by Tanzschel in 1929 is not available. Therefore, we compared their morphology based on the descriptions of Hiratsuka (1936) and Hiratsuka et al. (1992). The second type of sori and spores is similar to uredinia and urediniospores of *P. nipponicum* reported on species of *Galium* in the size and surface of spores; however, *P. nipponicum* has not been reported to have echinulate spores as first type of this species, and also their peridial structures are different to each other; ostiolar cells of *P. verruculosum* are inconspicuous whereas *P. nipponicum* has round-shaped ones (Table 1) (Hiratsuka 1958; Hiratsuka et al. 1992; Azbukina 2015). These sorus structure and spores are also morphologically similar to uredinia and urediniospores of *Coleosporium asterum* (Dietel) P. Syd. & Syd. reported on species of *Aster* and *Kalimeris*, but its uredinia have no peridium and its urediniopsores (20–32 × 14–24 μm) are bigger than spores of *P. verruculosum* although *C. asterum* has verrucose urediniospores (Kaneko 1981). The phylogenetic tree based on 28S sequence data also clearly showed that specimens of this new species on *Aster* and *Kalimeris* are genetically distinct from species of *Coleosporium* including *C. asterum* (Fig. 3).

Many species of *Puccinia* and *Uromyces* have been reported on Compositae in the world (Gäumann 1959; Arthur 1962; Wilson and Henderson 1966; Cummins 1978; Tai 1979; Hiratsuka et al. 1992); however, they are morphologically different from this new species in sorus structures (Cummins and Hiratsuka 2003).

Discussion

Species of *Pucciniastrum* with dimorphic sori and spores have not been reported before (Hiratsuka 1936, 1958; Gäumann 1959; Arthur 1962; Wilson and Henderson 1966; Hiratsuka et al. 1992; Azbukina 2015). The two types differed morphologically from each other in sorus structures and surface structure of spores, but two types of spores produced in one type of sori are also observed in another type of sori. Therefore, they are produced by same species and not contaminated with different species. The species identity of these different types of spores was also supported by molecular analyses because all direct sequencing data from same host specimens including different types of spores were identical. It will be better way to analyze sequence of each spore type separately, but, two types of spores are intermingled in same sorus and also two types of sori are produced closely on the same leaf. Therefore, we confirmed their identity by direct sequencing from samples on same host plant.

The sori and spores, which are small and opened by apical pores, were suspected as uredinial stages because these structures were morphologically similar to those of *Pucciniastrum*. In our field observations, these small sizes of sori with mostly echinulate spores appears firstly in spring and then gradually changes to large size of sori with mostly coronate or verrucose

to nail-headed spores in summer and autumn. However, no spermogonium, aecium, and teium were found in any season until plants died. Therefore, it is suspected that these echinulate spores play a role in dispersal of the species. The larger sori are usually not opened as they are covered by strong peridia consisting of firmly attached cells. They produce relatively thick-walled spores with coronate or verrucose to nail-headed surface structures. Their functions in the life cycle are not clear although it is suspected that this type of spore may survive winter as a resting spore for infections in the next spring, as no telial stage is found in specimens collected in autumn. The spores produced in uredinia and survived during unfavorable climate conditions are known in some genera of rust fungi as amphispores, which have darkly pigmented thick walls and smooth surfaces (Hiratsuka and Sato 1982). However, they are morphologically more similar to urediniospores than amphispores because walls of the spores with coronate or verrucose to nail-headed surface structures are hyaline (Figs. 4e, 6e). The presence of dimorphic urediniospores has been reported in *Melampsora dimorphospora* Kaneko & Hirats. f. (Hiratsuka and Kaneko 1982; Hiratsuka et al. 1992). The one type of its urediniospores is echinulate and produced on pedicels, and another type, similar to aeciospores of *Melampsora*, is verrucose and produced in chains. Both types are morphologically different, but formed in same sorus.

In the phylogenetic analyses, *P. guttatum* and *P. rubiae* are genetically not separated clearly (Figs. 1, 2). They are also morphologically similar to each other and their distribution overlaps in Asia and Russia, although their host plants are clearly separated; host plants of *P. guttatum* and *P. rubiae* are species of *Galium* and *Rubia*, respectively (Hiratsuka 1936, 1958; Hiratsuka et al. 1992). *Thekopsora ostryae* Y.M. Liang & T. Yang, *T. lanpingensis* Y.M. Liang & T. Yang, and *T. triangula* Y.M. Liang & T. Yang (Yang et al. 2014, 2015) used for comparative phylogeny were genetically close to species of *Pucciniastrum*, and the monophyly of *Thekopsora* was not recognized, as suggested by Padamsee and McKenzie (2014) and Aime et al. (2017) (Figs. 1, 2).

Acknowledgments We wish to thank Dr. E.H.C. McKenzie, Landcare Research, Auckland, New Zealand, and Dr. M. C. Aime, Department of Botany and Plant Pathology, Purdue University, IN, USA, for critical reading of the manuscript and suggestions.

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