Description of the demicyclic life cycle of *Puccinia sherardiana* in *Sphaeralcea angustifolia* in Mexico.

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Abstract

During 2017 and 2018, plants with dark brown lesions containing hypophilic telia surrounded by chlorotic halos were collected on leaves and stems of *Sphaeralcea angustifolia* located in Axapusco, Estado de México. Based on the morphological characteristics of pycnia, aecia and telia observed in light microscopy and scanning electron microscopy, the fungus *Puccinia sherardiana* was identified. No uredinal status was present during the observation period. Verification of identity was performed by phylogenetic analysis with a ribosomal DNA 28S gene sequence. Additionally, pathogenicity tests were performed on leaves of *S. angustifolia* by inoculation of teliospores. The inoculated plants developed symptoms 15 days after inoculation, beginning with the presence of errupent aecia in the epidermis of the host and later telia were formed, completing Koch's Postulates. *Puccinia sherardiana* was previously described as a rust with a microcyclic life cycle on species of the genera *Alcea, Malvastrum, Sidalcea* and *Sphaeralcea*, belonging to the Malvaceae family, however, this study showed that this phytopathogenic fungus has a demicyclic life cycle.

Introduction

The *Sphaeralcea* genus belong to the Malvaceae family is represented by around 40 species that are found mainly in the western part of North America, and more than 50% are distributed in Mexico. Some species are used in traditional medicine, for example, the Navajo Indians use the leaves of *S. coccinea* to treat skin diseases (Wyman and Stuart 1941). In Mexico, the most representative species is *Sphaeralcea angustifolia* Cav. G. Don. which is a plant commonly known as Vara de San José, black herb or tlixihuitl in the Náhuatl language (Rzedowski and Rzedowski 2005). This plant is widely distributed in Mexico (Villaseñor and Espinosa 1998; McVaugh 2001), and it is used because of its anti-inflammatory effect in diseases such as tonsillitis, bronchitis, conjunctivitis, rheumatism, contusions and hemorrhoids (Díaz 1976; Andrade-Ceto 2009). It is also used to treat scars, gastrointestinal disorders such as diarrhea and dysentery (García-Rodríguez et al. 2012; Calzada et al. 2017) and osteoarthritis (Romero-Cerecero et al. 2013). *Sphaeralcea angustifolia* grows in areas of pine-oak forest, low deciduous forest and grasslands, but mainly in arid areas. It is also found on roadsides, riot areas, abandoned cultivation fields, urban areas and frequently as weeds along railroad tracks (Fryxell 1992; McVaugh 2001).

Pucciniales (Basidiomycota) are an order of obligate parasitic fungi of vascular plants, with complex life cycles that cause diseases known as rusts (Romero-Cova 1988; Toome-Heller 2016; Aime et al. 2018). Currently, it is estimated that there are from 7,500 to 8,500 described species of Pucciniales, being *Puccinia* genus the most economically destructive (Toome-Heller 2016) and the one that comprises the largest number of species reported with approximately 4,000 (Kirk et al. 2008). Due to all the above, and to the high specificity with its hosts, the interpretation of its evolution is complicated (Aime et al. 2018). Most Pucciniales require two specific but unrelated hosts to complete their life cycle. This type of cycle can be differentiated into two phases (aecial and telial) where each of them occurs in its associated host. The aecial stage represents the part of the life cycle in which haploid monokaryon (i.e. spermatia) are united by fertilization (plasmogamy) to form the dikaryon. Then, dikaryotic aeciospores are formed and

dispersed to the host where the telial phase will develop. In this host, asexual propagation occurs through the production of urediniospores. Ultimately, the dikaryon will cease asexual sporulation and will form teliospores, generally in response to environmental conditions. It is during this stage that karyogamy takes place, followed by meiosis. Lastly, haploid basidiospores are produced from the germination of teliospore, which carry the new monokaryon back to the aecial host (Aime et al. 2018).

Different species of the *Sphaeralcea, Sidalceae, Althaea, Malvastrum, Alcea* (Malvaceae) genus are affected by *Puccinia sherardiana* Körn (Horst 2013; Demers et al. 2015); which is a rust reported as microcyclic (Arthur 1962; Briere and Franc 1998; Dugan and Nazaire 2011). This rust is characterized by producing symptoms and signs both on the stem and on the leaves that consist on the presence of dark brown telia, surrounded by chlorotic halos. These symptoms have been reported in *Sphaeralcea grossulariaefolia, S. munroana*, and *Sidalcea malviflora* in the USA (Briere and Franc 1998; Sampangi et al. 2010).

The aims of this work were to determine the cycle of *Puccinia sherardiana* in *Sphaeralcea angustifolia*, besides performing a detailed morphological description and verify its pathogenicity.

Materials And Methods

Sample collection.— During 2017 and 2018, *S. angustifolia* plants with typical rust infection were collected in the Santa María region, Axapusco, State of Mexico, Mexico. Additionally, samples of flowering plants were collected and conditioned for subsequent identification based on taxonomic keys and comparison with specimens present in the herbarium.

Morphological characterization.— For the morphometric description, spermatia, aeciospores and teliospores, as well as sections of the spermogonia, aecia and telia were mounted separately in a drop of lactophenol cotton blue on slides for observation by light microscopy. The morphology and size of 100 spores were determined at a 40X magnification. The surface structures of the aeciospores and teliospores were observed directly by field emission scanning electron microscopy (Carls Zeiss). The characteristics of telia and teliospores were compared with taxonomic keys and descriptions previously made by Hotson (1934) and Arthur (1962). The description of spermogonia, spermatia, aecia and aeciospores was made for the first time.

DNA extraction, PCR amplification and sequencing.— Aeciospores were scraped from one aecium in order to confirm the identity of the fungus. Genomic DNA was extracted using the commercial DNeasy Plant kit (Qiagen, USA) following manufacturer's specifications. DNA quality was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized under UV light using an M-26X transilluminator (UVP Ltd, USA). DNA concentrations were quantified using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA).

Part of the 28S gene of the ribosomal DNA (including the D1 and D2 domains) was amplified by PCR using the LR0R and LR6 primers (Vilgalys and Hester 1990). Each reaction mixture (50 µL) containing 1X

PCR Buffer, 0.02 U μL^{-1} DNA polymerase (Promega, Madison, Wisconsin), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 mM of each primer and 2 ng of template DNA. PCR was carried out in a Bio-Rad C1000 thermocycler (Bio-Rad Labs, USA) under the following conditions: initial denaturation step at 95°C for 3 min, 35 cycles at 95°C for 90 sec, 50°C for 60 sec, 72°C for 90 sec, followed by a final extension at 72°C for 7 min. Amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, USA), and they sequenced at Macrogen (Seoul, Korea).

Pathogenicity tests.—S. angustifolia plants were reproduced under isolated conditions in a greenhouse located at the Universidad Autónoma Chapingo. The plants were placed in polyethylene bags with a substrate based on mineral perlite, peat and bush soil in a proportion of 20, 30 and 50%, respectively; the mixture was autoclaved three times for 30 min each. When the plants reached an age of six weeks, the leaves were inoculated with a suspension of teliospores at a concentration of $1´10^4$ teliospores mL^{-1}, these were obtained from plants with signs of the pathogen. Immediately after inoculation, the plants were placed in a greenhouse were continued their development.

Phylogenetic analysis.— Searches were performed using the BLASTn algorithm to obtain similar sequences from GenBank (www.ncbi.nlm.nih.gov/genbank). The accession numbers of these sequences are shown in the corresponding phylogenetic tree (Figure 3). The matrices were compiled using MEGA 5.0.5. The alignments were made online using MAFFT 7 (Katoh 2013) using the E-INS-i and FFT-NS-i strategies and the default parameters. The sequence generated in this study will be deposited at the GenBank. The matrices were analyzed using Bayesian inference criteria. For the analyzes, four MCMC chains ran simultaneously from a randomly selected starting tree for 1,000,000 generations. The trees obtained were stored in memory every 100 generations. The trees sampled during the 'burn in' (15%) were discarded, and the posterior probabilities were calculated using the rule of majority of a consensus tree generated by using the remaining trees (excluding the 'burn in').

Results

Description of symptoms and signs.— Symptoms begin with a light green discoloration in the form of circular spots that are observed on both sides of the leaf (adaxial and abaxial) (FIG. 1A and FIG. 1B). Small light brown dots develop in the center, corresponding to the formation of spermogonia; then, the spots turn light yellow and grow irregularly in size, at this stage in the abaxial part the development of yellow aecia and aeciospores is observed (FIG. 1C, FIG. 1D and FIG. 1E). Later, telia are formed (FIG. 1F) and these give rise to teliospores, both cases occur only in the abaxial part, as well as in the stem (FIG. 1G). Telia are delimited by chlorotic halos, in the adaxial part brown lesions delimited by the chlorotic halo are distinguished (FIG. 1H), it is common to observe the two phases on the same leaf; however, at the end of the cycle only the telial phase is observed.

Morphological description.— Pycnia (spermogonia), in small groups, epiphyllous, subepidermal, group V-tipe 4 (according with the classification of Cummins and Hiratsuka, 2003), light brown, located on the opposite side of the aecia, globose 120-150 µm in diameter (FIG. 2A), with abundant and outward

growing periphyses. Pycniospore (spermatia), hyaline to yellowish, not septate, ellipsoidal to subglobose, 3.5 x 2.5 µm (FIG. 2A*). Aecia, hypophyllous, cupulate, closely grouped, light brown, and 219 to 230 µm in diameter (FIG. 2B and FIG. 2C). Aeciospore, oblong to ellipsoid, the wall colorless (1.0-)1.5 µm thick, but the inner light brown or yellowish, finely echinulate, 16.46 x 14.38 µm (FIG. 2D and FIG. 2E). Telia on the stem and hypophyllous, pulvinate, irregularly form, verrucose, regularly confluent, reddish-brown to dark-brown. Teliospore, mostly ellipsoid or oblong-ellipsoid, with a central constriction, two-celled, with apical pore, average size 15x42 µm, smooth, dark brown or brownish, the wall 0.5-1.5 µm thick at sides, 1.5-3 µm apically, in clusters (FIG. 2F and FIG. 2G). Pedicels usually yellowish, persistents, 105 µm long, smooth side walls 1.5 µm thick (FIG. 2G). Based on the morphological characteristics of teliospores, rust was identified as Puccinia sherardiana Körn in according to Arthur (1962), Demers et al. (2015), Dugan and Nazaire (2011). The uredial stage was not observed.

Pathogenicity tests.— Leaf symptoms were detected 15 days after inoculation in the greenhouse, during this time aecia in formation were observed, which later gave rise to the formation of aeciospores after breaking the epidermis of the leaf. Five days later, formation of telia and teliospores was observed to complete Koch's Postulates. Their characteristics corresponded to those previously observed for *P. sherardiana*.

Phylogeny.- The sequence generated in this study was deposited at the GenBank database with accession number MN967778.1, isolate CNRF 3. Phylogenetic analysis generated with the partial sequence of the 28S gene, and using the Bayesian inference criterion showed a value of 0.99 of posterior probability with sequences from *P. sherardiana* isolates BPI871783 and BPI102198 (FIG. 3).

Discussion

Puccinia sherardiana has been reported in the USA (Arizona, California, Colorado, Nevada, Oregon, Washington, Idaho, Nebraska, New Mexico, Texas, Utah, Wyoming, Montana, and North Dakota) infecting different species of the genus *Alcea, Malvastrum, Sidalcea* and *Sphaeralcea,* within the Malvaceae family (Arthur 1962; Briere and Franc 1998; Horst 2008; Dugan and Nazaire 2011; Demers et al. 2015). In the case of Mexico, Arthur (1962) mentioned that *P. sherardiana* is found in the country; however, data on the collection site or the host were not provided; meanwhile Demers et al. (2015), identified this rust in two specimens (of unknown origin) of *Sphaeralcea* sp., in interceptions made in El Paso, Texas, in merchandise that entered through the Mexican border. However, there is no scientific evidence of the presence of *P. sherardiana* in Mexico to date, so to our knowledge this work represents the first confirmed report of this plant pathogen fungus in Mexico.

Regarding morphometry, the characteristics of the *P. sherardiana* teliospores identified on *S. angustifolia* were similar to those reported by Arthur (1962), Briere and Franc (1998), Dugan and Nazaire (2011) and Demers et al. (2015). However, there were some differences, for example, in this study, teliospores were narrower (15.8 µm), compared to the size reported (27–30 µm) by the previously mentioned authors in different hosts, 30.85 µm in *Sphaeralcea grossulariaefolia* and *S. munroana*, 21–25 µm in *Sidalcea*

malviflora and 18–30 µm in *Alcea rosea*. Regarding the telia development, Briere and Franc (1998) reported that they are present on both sides of the leaf in *S. grossulariaefolia* and *S. munroana*. On the other hand, Dugan and Nazaire (2011) observed telia mainly in the adaxial part of *Sidalcea malviflora* leaves, while in this study they only appeared in the abaxial part.

Leaf symptoms are feasible to reproduce by inoculating *S. angustifolia* plants with *P. sherardiana* teliospores under greenhouse conditions, over a period of 15 days with an average temperature of 22°C during the day and 18°C during the night. For their part, Briere and Franc (1998) completed Koch's postulates in a period of 13 days when using 8-week-old plants and temperatures of 20 and 15°C during the day and night, respectively.

It is important to mention that in this work, the morphometric characteristics of the pycnia and pycniospores are described for the first time. The aecial phase is reported with its respective description of the aecia and aeciospores, thus, *P. sherardiana* is classified as a demicylic rust and not as a microcyclic rust as previously reported by Arthur (1962), Briere and Franc (1998) and Dugan and Nazaire (2011). These authors also mentioned that the rust lacks the aecial and uredinial phase. However, it is still unknown whether or not it presents the uredinial stage.

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Figures

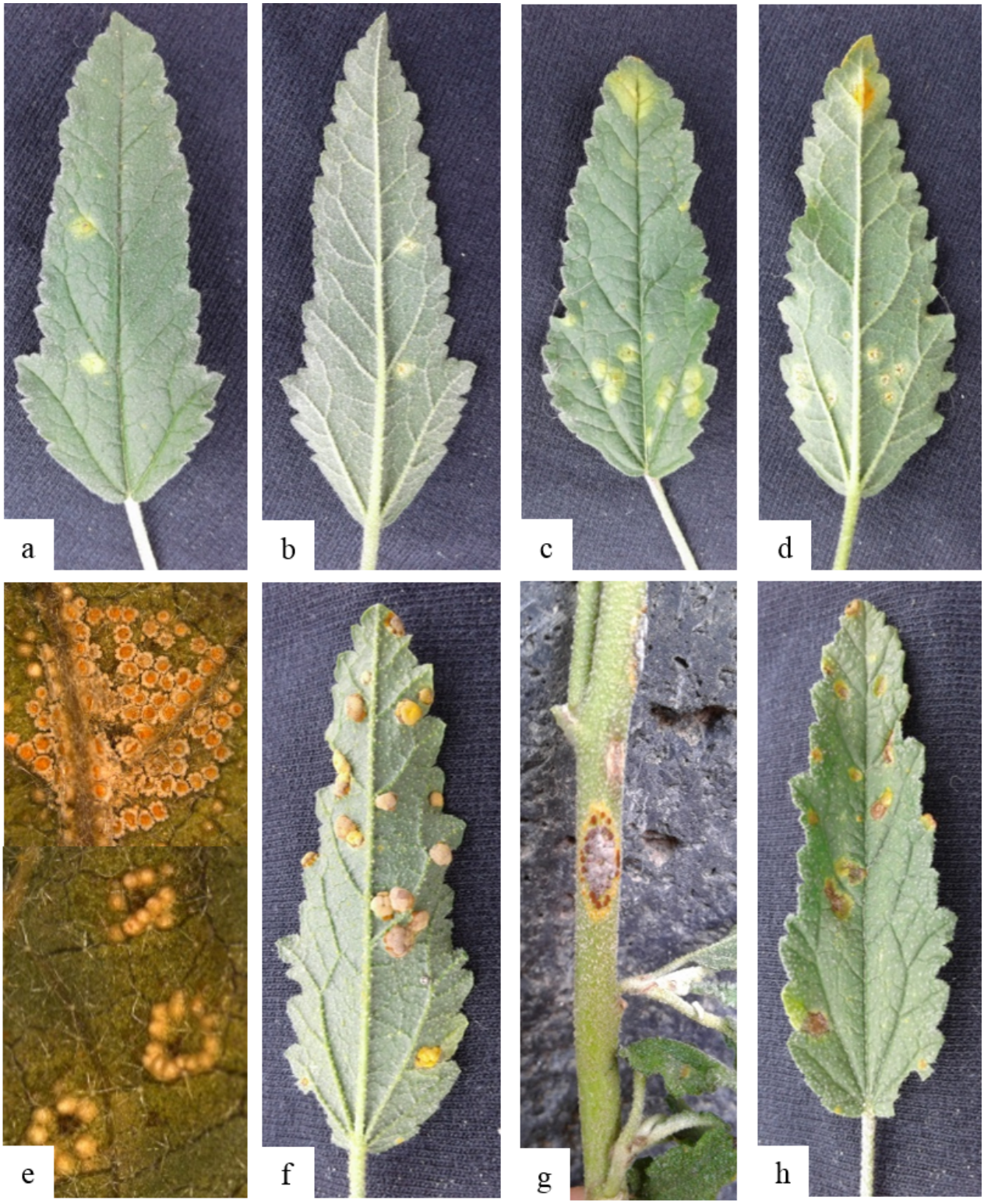

Figure 1

Symptoms and signs induced by *Puccinia sherardiana* in tissues of *Sphaeralcea angustifolia*. A, B. Light green circular spots. C, D, E. Development of aecia and aeciospores of yellow color. F, G. Development of telia in leaf and stem. H. Telia delimited by a chlorotic halo.

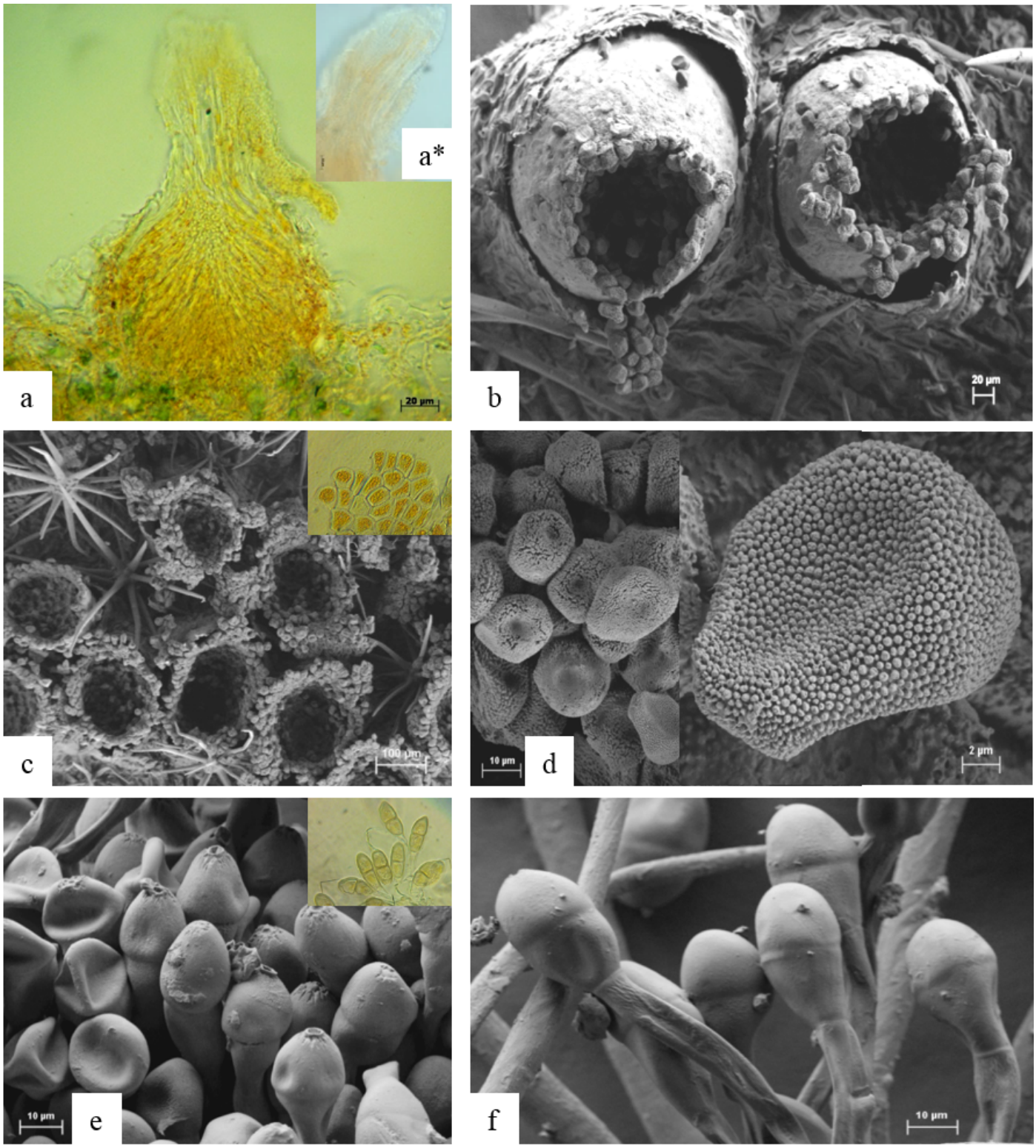

Figure 2

Reproduction structures of *Puccinia sherardiana* observed by light microscopy (A) and scanning electron microscopy (B-F). A. Longitudinal section of a spermogonium. B, C. Aecia. D. Aeciospores. E, F. Teliospores.

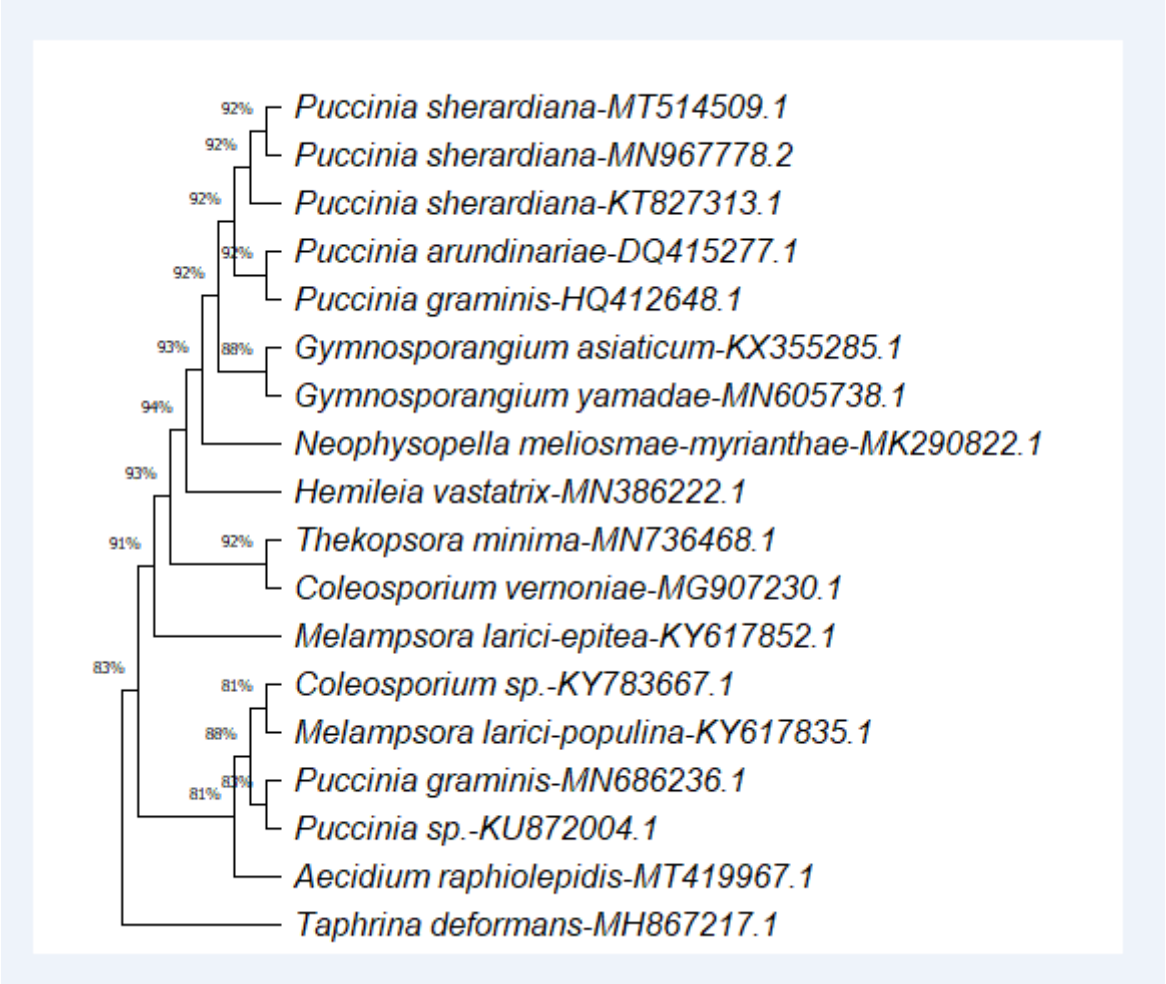

Figure 3

Bayesian tree generated from alignment of partial sequences of the 28S gene of *Puccinia*species. Bayesian posterior probability values above 0.50 are shown in the nodes. *The Pucciniosira pallidula*isolate BPI863541 was used as the external group.

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