Description of the Demicyclic life Cycle of Puccinia Sherardiana in Sphaeralcea Angustifolia in Mexico.

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Title Page

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

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Abstract. During 2017-2019, leaves and stems with dark brown lesions containing hypophyllous telia surrounded by chlorotic halos were collected from *Sphaeralcea angustifolia* plants located in Axapusco, State of Mexico. Based on the morphological characteristics of pycnia, aecia and telia observed by light microscopy and scanning electron microscopy, the fungus *Puccinia sherardiana* was identified. Uredial stage was not present during the observation period. Identity verification was carried out by phylogenetic analysis with sequences of part of the 28S gene from ribosomal DNA. In addition, pathogenicity tests were done on *S. angustifolia* leaves by inoculating teliospores. The inoculated plants developed symptoms 15 days after inoculation, the signs beginning with the presence of aecia in the epidermis of the host and later telia were formed, completing the Koch 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 revealed that this plant pathogenic fungus has a demicyclic life cycle.

Key words. Morphology, pathogenicity test, phylogenetic analysis, rust.

The *Sphaeralcea* genus (Malvaceae) 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, disturbed areas, abandoned cultivation fields, urban areas and frequently as weeds along railroad tracks (Fryxell 1992; McVaugh 2001).

Pucciniales (Basidiomycota) is 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, with Puccinia the most economically destructive genus (Toome-Heller 2016) and the one that comprises the largest number of species reported, approximately 4,000 (Kirk et al. 2008). Due to all the above, and to the high specificity with their hosts, the interpretation of rust species 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 *Sphaeralcea, Sidalceae, Althaea, Malvastrum,* and *Alcea* (Malvaceae) 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 consisting of 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 lifecycle of *Puccinia sherardiana* on *Sphaeralcea angustifolia*, besides performing a detailed morphological description and verification of 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.

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).

DNA extraction, PCR amplification and sequencing.— Aeciospores were scraped from one aecium for DNA sequencing 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 from seeds were grown 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 they continued their development.

Phylogenetic analysis.— Sequences obtained were clipped in CodonCode Aligner v. 5.1.5 (http://www.codoncode.com/aligner/) and corrected for missing or miscalled nucleotides by referencing chromatograms. The obtained consensus sequences were compared with homologous sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) using the Basic Local Alignment Sequencing Tool for nucleotide sequence queries (BLASTN) (Altschul et al., 1990), for preliminary identification and to obtain reference sequences for phylogenetic analyses. Subsequently, with representative sequences of rust species (Table 1), a multiple alignment was done using the MEGA X program (v10.1.8) with the ClustalW algorithm (Kumar et al., 2018). The matrix obtained was used to perform a phylogenetic analysis based on the Maximum Parsimony (MP) method, with heuristic search and SPR (Subtree Pruning and Regrafting). The support of the internal topology of the phylogenetic tree was done by bootstrap analysis with 1000 iterations (Felsenstein, 1985). The accession number of the sequences in this work are: MT514509.1 and MN967778.2.

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) (Figs. 1a–1b). Small light brown dots were developed in the center, corresponding to the formation of spermogonia; then, the spots turned light yellow and grown irregularly in size, at this stage in the abaxial part the development of yellow aecia and aeciospores was observed (Figs. 1c–1e). Later, telia were formed (Fig. 1f) and these gave rise to teliospores, both cases occur only in the abaxial part, as well as in the stem (Fig. 1g). Telia were delimited by chlorotic halos, in the adaxial part brown lesions delimited by the chlorotic halo were 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 was observed.

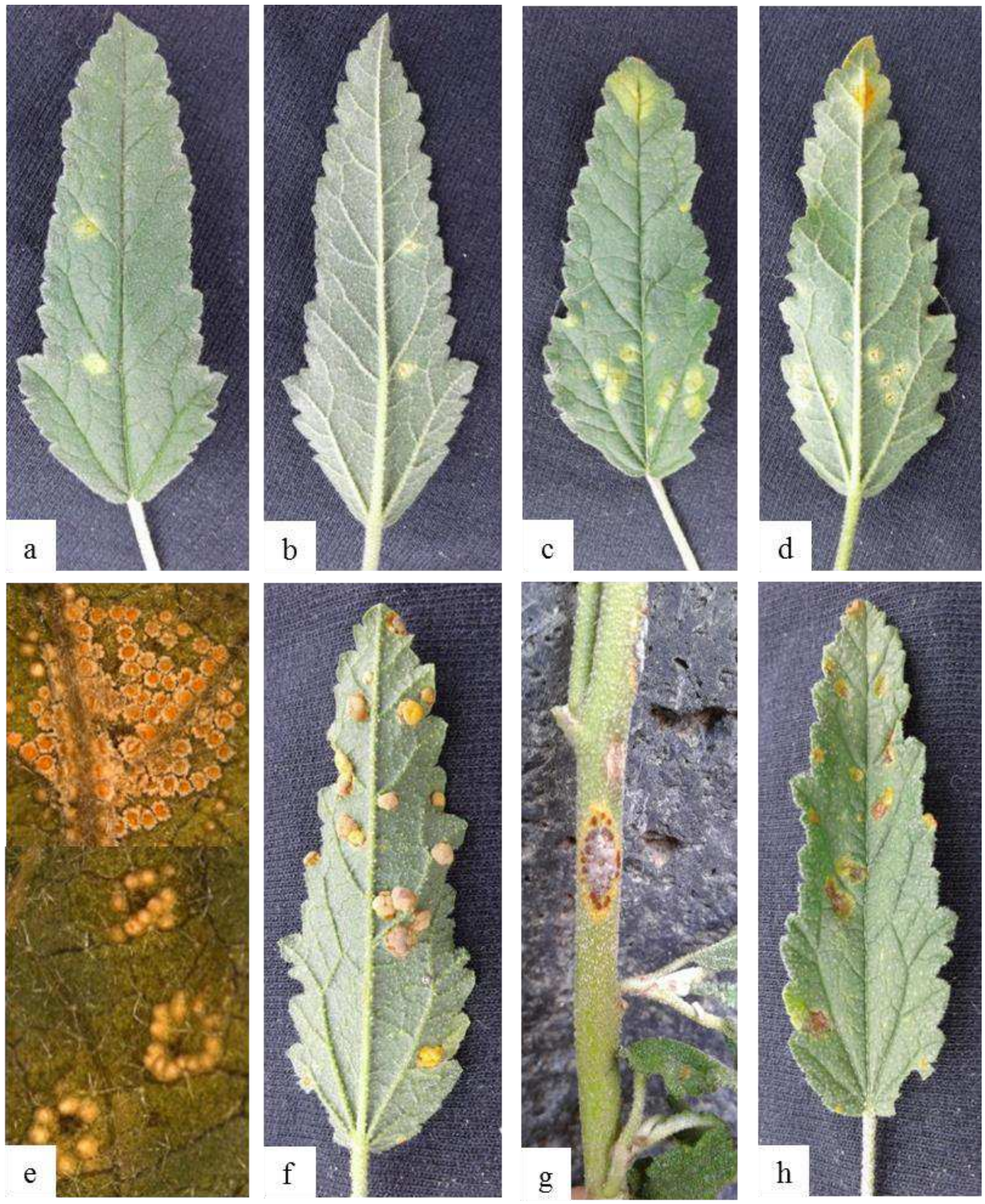

Fig. 1 Symptoms and signs induced by *Puccinia sherardiana* in tissues of *Sphaeralcea angustifolia*. a, b. Light green circular spots. c, d, e. Development of aecia and aeciospores of yellow color. f, g. Development of telia in leaf and stem. h. Telia delimited by a chlorotic halo

Morphological description.— Pycnia (spermogonia), in small groups, epiphyllous, subepidermal, group V-type 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 × 2.5 µm (Fig. 2a*). Aecia, hypophyllous, cupulate, closely grouped, light brown, and 219 to 230 µm in diameter (Figs. 2b–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 × 14.38 µm (Figs. 2d–2e). Telia on the stem and hypophyllous, pulvinate, irregularly formed, 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 15 × 42 µm, smooth, dark brown or brownish, the wall 0.5–1.5 µm thick at sides, 1.5–3 µm apically, in clusters (Figs. 2f –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. The description of spermogonia, spermatia, aecia and aeciospores was made for the first time.

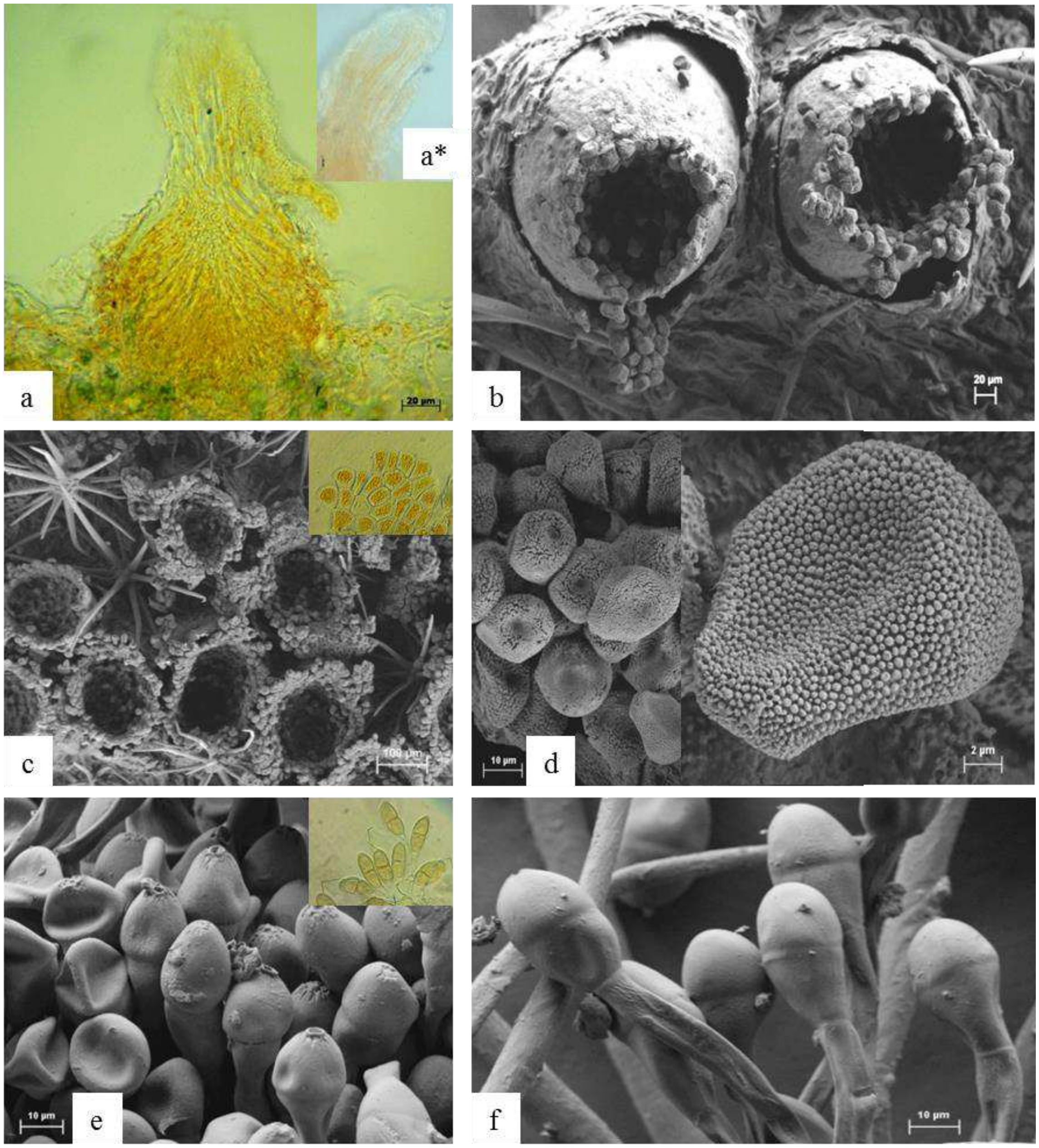

Fig. 2 Reproduction structures of *Puccinia sherardiana* observed by light microscopy (a) and scanning electron microscopy (b-f). a. Longitudinal section of a spermogonium. b-c. Aecia. d. Aeciospores. e-f. Teliospores

Pathogenicity tests.— Rust symptoms on leaf 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 BLAST analysis of the consensus sequences obtained in this work allowed to confirm their identity as *Puccinia sherardiana*. The phylogenetic tree was built with the consensus sequences; 16 Pucciniales sequences deposited in GenBank, were selected for their high level of identity with the large subunit RNA ribosomal region in the genus *Puccinia* spp., also *Taphrina deformans* (accession: MH867217) was used as an external analysis group (outgroup), and selected for not being phylogenetically related to the fungus under study (Table 1).

The final alignment of the sequences was 695 sites, of which 129 were conserved, 551 variable but not informative, 434 informative for parsimony. The length of the phylogenetic tree with maximum parsimony was 1261 steps, with a consistency index 0.718477 (0.676685), retention index 0.739736 (0.739736), and the composite index 0.531484 (0.500568) for all sites and parsimony informative sites (in parenthesis). This analysis involved 18 nucleotide sequences. There were a total of 695 positions in the final data set (Kumar et al. 2018). The phylogenetic tree has six well-defined clades. The percentage of replicated trees in which the associated taxa were grouped in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The maximum parsimony tree was obtained by means of the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar 2000) with search level 1 in which the initial trees were obtained by means of the random addition sequences (10 repetitions) [Fig. 3]. These clades made it possible to identify at the species level that the fungus corresponds to *Puccinia sherardiana*, thus confirming the results obtained by morphology.

Table 1 Accession table for sequences used in the phylogenetic analyses of this study. Sequences were either acquired from NCBI or generated as part of this study. Host species and location are also provided where available.

Species	Host	GenBank accession number	Country	Reference
Coleosporium sp.	*Tussilago farfara*	KY783667	Alemania	Beenken et al. 2017
Neophysopella meliosmae-myrianthae	NA	MK290822	Brasil	Santos et al. 2018 (Unpublished)
Gymnosporangium yamadae	*Malus* sp.	MN605738.1	China	Zhao et al. 2020
Puccinia graminis	*Anthoxanthum* sp.	MN686236	Ecuador	Barnes et al. 2019 (Unpublished)
Melampsora larici-populina	*Populus* sp.	KY617835	Eslovenia	Piskur 2017 (Unpublished)
Melampsora larici-epitea	*Salix viminalis*	KY617852.1	Eslovenia	Piskur 2017 (Unpublished)
Taphrina deformans	NA	MH867217.1	Holanda	Vu et al. 2019
Aecidium raphiolepidis	NA	MT419967.1	Japón	Kasuya et al. 2020
Puccinia sherardiana	*Sphaeralcea angustifolia*	MT514509.1	México	This study
Puccinia sherardiana	*Sphaeralcea angustifolia*	MN967778.2	México	This study
Puccinia graminis	*Hordeum* sp.	HQ412648	Omán	Deadman et al. 2011
Hemileia vastatrix	*Coffea arabica*	MN386222	Perú	Gamarra-Gamarra et al. 2019 (Unpublished)
Thekopsora minima	*Vaccinium corymbosum*	MN736468	Perú	Huarhua et al. 2020
Gymnosporangium asiaticum	*Juniperus chinensis*	KX355285	Taiwan	Shen et al. 2016
Puccinia sp.	*Marrubium globosum* subsp. *globosum*	KU872004.1	Turquía	Kabaktepe et al. 2016
Puccinia arundinariae	*Arundinaria* sp.	DQ415277	USA	Aime et al. 2006
Puccinia sherardiana	*Alcea rosea*	KT827313.1	USA	Demers et al. 2015
Coleosporium vernoniae	*Elephantopus*	MG907230	USA	Aime et al. 2018

NA = Not Available

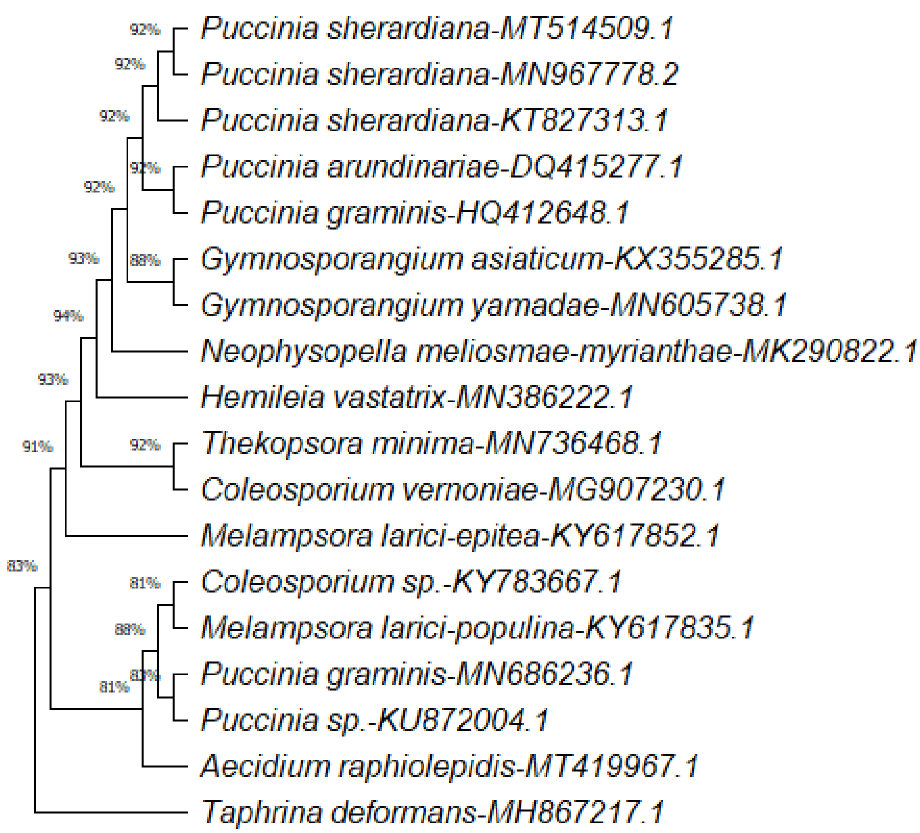

Fig. 3 Phylogenetic tree based on large subunit ribosomal RNA sequences for *Puccinia sherardiana* obtained from *Sphaeralcea angustifolia* plant, at Santa Maria region, Axapusco, State of Mexico, Mexico, and sequences obtained from GenBank. The branch lengths were calculated using the average path method and are in units of the number of changes in the entire sequence. They are shown next to the branches

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 genera *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; also, many specimens are stored in various herbaria on the hosts *Sphaeralcea endlichii*, *Malvastrum coromandelianum*, and *Sphaeralcea angustifolia* collected in different localities of Chihuahua, Coahuila, Durango, Zacatecas, San Luis Potosí, Guanajuato, Veracruz, Hidalgo, Mexico

City, and Mexico State; 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.

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. Which indicates that variations may occur depending on the host.

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.

Declarations

Funding: No funding was received for conducting this study.

Conflicts of interest/Competing interests: The authors declare that they have no conflict of interest.

Availability of data and material (data transparency): All data generated or analysed during this study are included in this article.

Code availability (software application or custom code): Not applicable.

Authors' contributions: All authors contributed to the study conception and design. Also, all authors read and approved the final manuscript. Specific contributions by author are: **Magnolia Moreno-Velázquez:** conceptualization, methodology, investigation, writing-original draft. **Jesús Ricardo Sánchez-Pale:** methodology, investigation, interpretation of data. **Ricardo Tapia Nuño:** morphological identification, field work. **Moisés Camacho-Tapia:** formal analysis (molecular analysis: DNA extraction, sequencing, and alignment). **José Manuel Cambrón-Crisantos:** formal analysis (phylogenetic analysis, molecular methodology). **Santos Gerardo Leyva-Mir:** methodology, investigation. **Juan Manuel Tovar-Pedraza:** investigation, morphological identification. **Andres Quezada-Salinas:** investigation, project administration, supervision, writing-review and editing.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Ethics approval: Not applicable.

Consent to participate (include appropriate statements): Not applicable.

Consent for publication (include appropriate statements): Not applicable.

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Figures

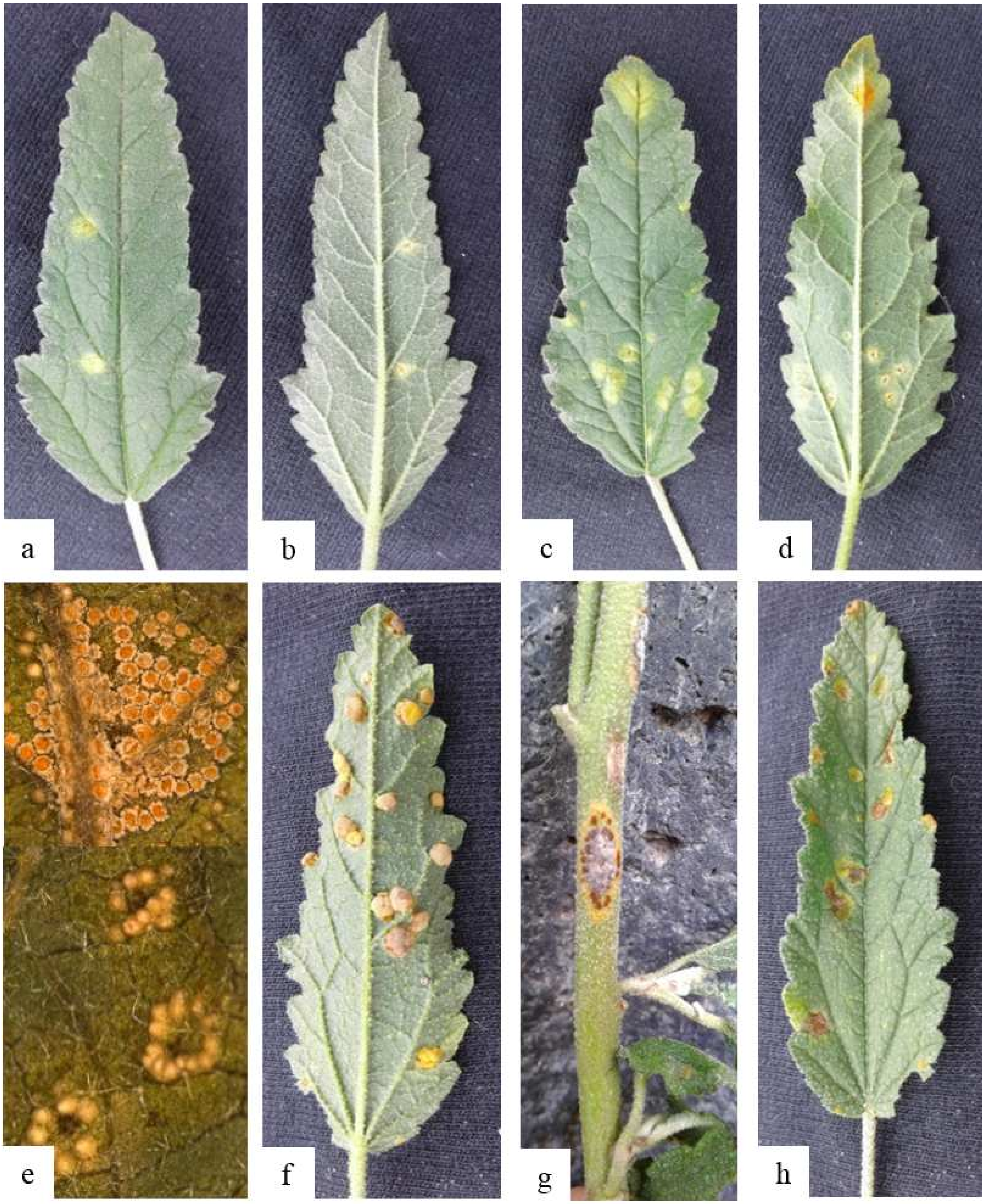

Figure 1

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

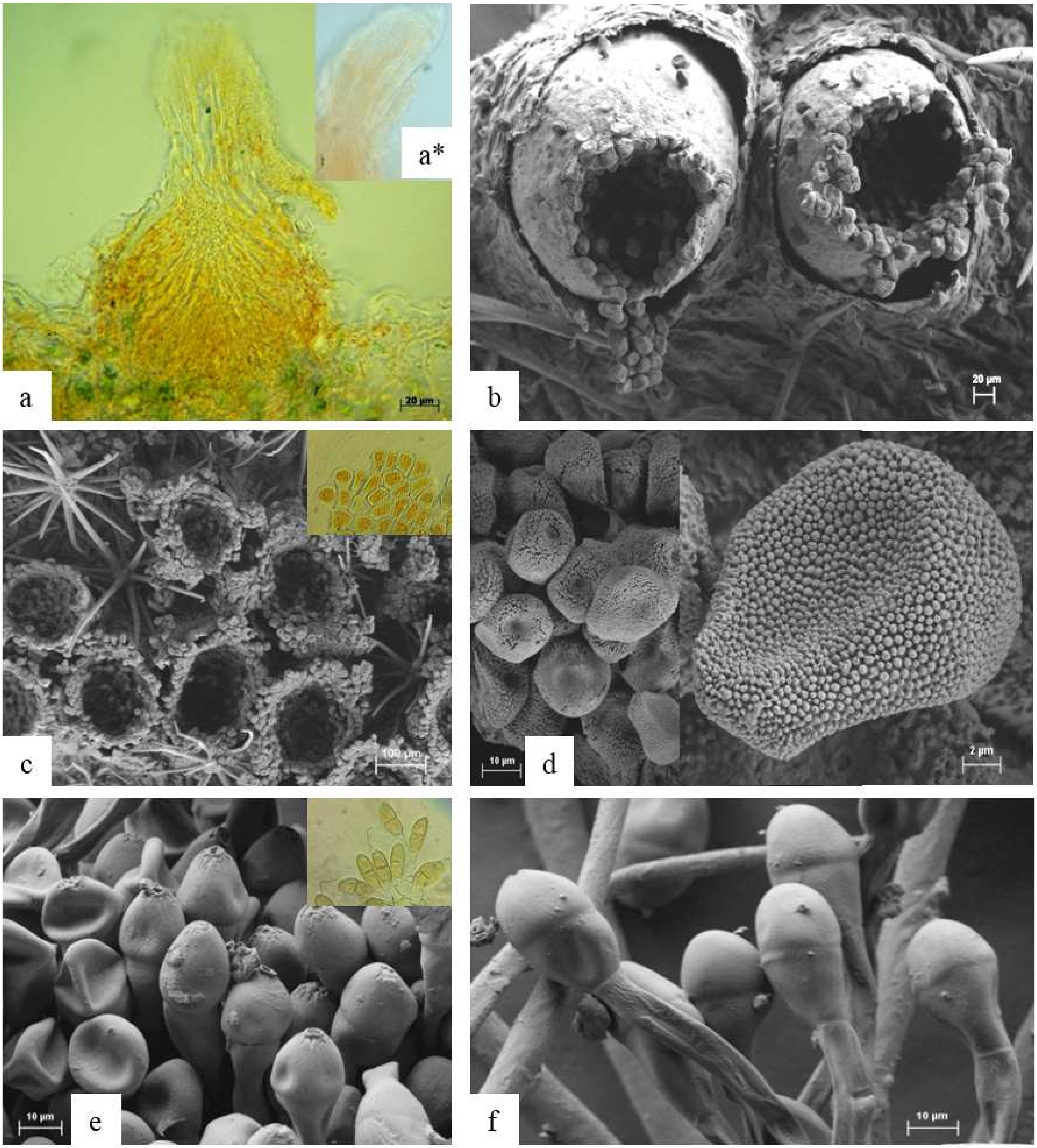

Figure 2

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

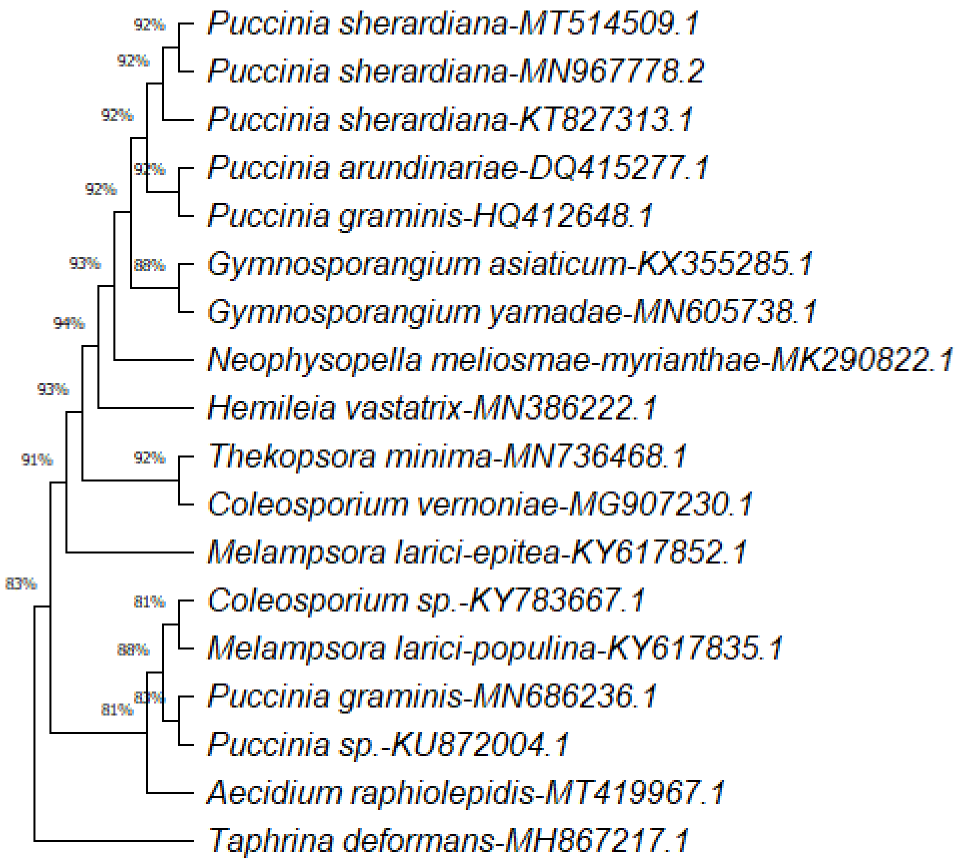

Figure 3

Phylogenetic tree based on large subunit ribosomal RNA sequences for Puccinia sherardiana obtained from Sphaeralcea angustifolia plant, at Santa Maria region, Axapusco, State of Mexico, Mexico, and sequences obtained from GenBank. The branch lengths were calculated using the average path method and are in units of the number of changes in the entire sequence. They are shown next to the branches

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