

Microcyclic rusts of hollyhock (*Alcea rosea*)

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Abstract: Rust fungi infecting hollyhock and other plants in *Malveae* are frequently intercepted at ports of entry to the USA, particularly *Puccinia malvacearum* and *P. heterogenea*. These two species can be difficult to distinguish and can be further confused with other, less common species of microcyclic rust fungi infecting hollyhock: *P. heterospora*, *P. lobata*, *P. platyspora*, and *P. sherardiana*. Molecular phylogenetic analysis revealed that *P. malvacearum* and *P. heterogenea* are closely related, along with *P. sherardiana* and *P. platyspora*. A key to the six microcyclic *Puccinia* species infecting hollyhock is presented.

Key words:

Pucciniales
Puccinia
pathogen interceptions
identification
taxonomy

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INTRODUCTION

Plants and plant products imported into the USA are inspected at ports of entry for pests including insects, mollusks, nematodes, bacteria, and fungi (McCullough *et al.* 2006). Plant pests that are found during inspections may be barred from entering the USA depending on their risk to agriculture and natural ecosystems and whether the pests are already established within the country. Some frequently imported plants include species of *Malvoideae*, the mallow subfamily that includes economically important plants such as cotton (*Gossypium hirsutum*), kenaf (*Hibiscus cannabinus*), okra (*Abelmoschus esculentus*), and many ornamental plants including hollyhock (*Alcea rosea*) (Baum *et al.* 2004). *Alcea rosea*, a plant grown worldwide, is often intercepted at ports of entry infected with rust fungi.

Many rust fungi can infect *A. rosea*. Six species of *Puccinia* have been reported to produce telia on *A. rosea*: *P. heterogenea*, *P. heterospora*, *P. lobata*, *P. malvacearum*, *P. platyspora*, and *P. sherardiana* (Arthur 1922, Lindquist 1982, Farr & Rossman 2015). All of these species are microcyclic and do not produce uredinia or aecia, have no known alternate hosts, and produce both one-celled and two-celled teliospores with different spore morphologies predominating in different species (Arthur 1922, Lindquist 1982). Another two species of *Puccinia*, *P. interveniens* and *P. schedonnardi*, are reported to produce aecia on *A. rosea*, and *A. rosea* is also reported to be infected by two other rust fungi, *Endophyllum tuberculatum* and *Kuehneola malvicola* (Arthur 1934, Farr

& Rossman 2015). Of these fungi, *P. malvacearum* is the most widely reported (Farr & Rossman 2015) and the most frequently intercepted at US borders. *Puccinia malvacearum* has been reported worldwide on a large number of hosts in *Malvoideae*, primarily in tribe *Malveae*, including the genera *Abutilon*, *Alcea*, *Anoda*, *Hibiscus*, *Lavatera*, *Malva*, *Malvastrum*, *Pavonia*, *Sida*, and *Sidalcea* (Farr & Rossman 2015). *Puccinia heterogenea*, although less commonly reported than *P. malvacearum*, has also been intercepted at US ports of entry multiple times. *Puccinia heterogenea* has only been reported from Ecuador and Peru on hosts in *Alcea*, *Malva*, and *Fuertesimalva* (Farr & Rossman 2015) but is morphologically similar to *P. malvacearum* and frequently misidentified.

In this study, *P. malvacearum*, *P. heterogenea*, and the other microcyclic rusts on *A. rosea*, *P. heterospora*, *P. lobata*, *P. platyspora*, and *P. sherardiana*, were characterized morphologically and the LSU rDNA region was sequenced. These data are presented as a guide to identification of the rusts of *A. rosea*. In addition, the phylogenetic placement of these six *Puccinia* species was determined.

MATERIALS AND METHODS

Specimens for this study were obtained from interceptions at US borders and from reference material, from the years 1890 to 2015. Morphological features of all specimens were characterized, and measurements were made using Zeiss AxioVision SE64 4.9.1 software (Carl Zeiss Microscopy, Jena). A minimum of 20 spores was examined from each specimen, with multiple specimens examined per species. DNA was extracted from small (approximately 0.5–1 cm²) leaf fragments containing telia using the Qiagen DNeasy Plant Mini kit (Gaithersburg, MD). PCRs were done using the KAPA2G Robust PCR kit (Wilmington,

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MA), following the manufacturer's suggested protocol. A portion of the LSU rDNA gene was amplified using two overlapping primer sets, LR0R (5'-GTACCCGCTGAACTTAAGC-3'; <http://sites.biology.duke.edu/fungi/mycolab/primers.htm>) and PucLSUr (5'-GCTTACTGCCTTCCTCAATCT-3'; designed by JED in this study), and LR5 (5'-ATCCTGAGGGAACTTC-3'; Vilgalys & Hester 1990) and PucLSUf (5'-TGGGTGTTGGAGAAGGGTTT-3'; designed by JED in this study). Host identifications of reference material were made by the original collector based on morphology and confirmed at the generic level using rDNA ITS2 sequences. Some intercepted plants could not be identified based on morphology due to the small size of the specimens, and identification was then based solely on plant ITS2 sequences. Host ITS2 sequences were amplified with the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al.* 1990) and ITS2S2F (5'-ATGCGATACTTGGTGTGAAT-3'; Chen *et al.* 2010). Amplifications for all primer sets were performed using the conditions 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 30 s, followed by 10 min at 72 °C, with annealing temperatures of 53 °C for PucLSUf/LR5, 55 °C for ITS4/ITS2S2F, and 58 °C for LR0R/PucLSUr. Sequencing was performed using the BigDye Terminator v. 3.1 cycle sequencing kit (Life Technologies, Grand Island, NY), and reactions were run on an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, Grand Island, NY). Sequences were edited with Sequencher v. 5.0 (Gene Codes Corp., Ann Arbor, MI) and aligned with MAFFT v. 7 (Katoh & Standley

2013). Maximum likelihood trees were inferred using RAxML BlackBox (Stamatakis *et al.* 2008).

RESULTS

Both *Puccinia malvacearum* and *P. heterogena* were commonly intercepted at ports of entry into the USA between 1950 and 2015. *Puccinia malvacearum* was frequently intercepted on *Alcea rosea*, but also on *Malva arborea*, *M. multiflora*, *M. neglecta*, *M. nicaeensis*, and *M. sylvestris*. *Puccinia heterogena* was intercepted less often than *P. malvacearum*, although still intercepted multiple times, and was primarily found on *M. arborea*, as well as on *A. rosea*. Other rust fungi intercepted on *A. rosea* were *P. heterospora*, *P. lobata*, and *P. sherardiana*.

LSU sequences were generated for 29 specimens of rust fungi on *Malveae* (GenBank accession nos. KT827287–KT827315). Phylogenetic analysis revealed that *P. malvacearum*, *P. heterogena*, *P. sherardiana*, and *P. platyspora* are closely related to each other in one clade (Fig. 1). No other species of rust fungi were identified in that clade, based on LSU sequences available in GenBank. Little intra-specific variation was found among specimens of *P. malvacearum* and *P. heterogena*, while a high degree of intra-specific variation was found within *P. heterospora* as compared to the other rust fungi infecting *A. rosea*.

TAXONOMY

Key to the microcyclic rusts of hollyhock

- | | | |
|-------|--|-----------------------------|
| 1 | Teliospores predominately one-celled | 2 |
| | Teliospores predominately two-celled | 4 |
| 2 (1) | One-celled teliospores mostly < 25 µm in length, globose | Puccinia heterospora |
| | One-celled teliospores mostly > 25 µm in length, ovoid to oblong | 3 |
| 3 (2) | Teliospores with apical walls to 13 µm thick | Puccinia platyspora |
| | Teliospores with apical walls to 5 µm thick | Puccinia heterogena |
| 4 (1) | Telia light reddish brown, scattered | Puccinia malvacearum |
| | Telia dark brown, in clusters | 5 |
| 5 (4) | Two-celled teliospores with no or little central constriction | Puccinia lobata |
| | Two-celled teliospores with a central constriction | Puccinia sherardiana |

Puccinia heterospora Berk. & M. A. Curtis, *Bot. J. Linn. Soc.* **10**: 356 (1869).
(Figs 2–3)

Description: Telia dark brown, mainly hypophyllous, in tight clusters. Teliospores light brown to reddish-brown, predominately one-celled. One-celled teliospores globose, highly variable in size, 17–30 × 15–29 µm, on average 21 × 19 µm. Two-celled teliospores globose to oblong, frequently have septa perpendicular to the pedicel attachment point; length of

the two-celled teliospores parallel to pedicel attachment point (17–35 µm, on average 22 µm) often shorter than the length of the spores perpendicular to the pedicel attachment point (16–34 µm, on average 20 µm). Side walls of teliospores 1.5–3 µm thick, apical walls 3–7 µm thick. Pedicels hyaline, to 100 µm long.

Specimens examined: **Argentina**: Tucuman Province: San Miguel de Tucuman, on *Anoda cristata*, 23 Jun. 1995, J. R. Hernandez (BPI 863576). – **Intercepted specimens (origin unknown)**: Intercepted

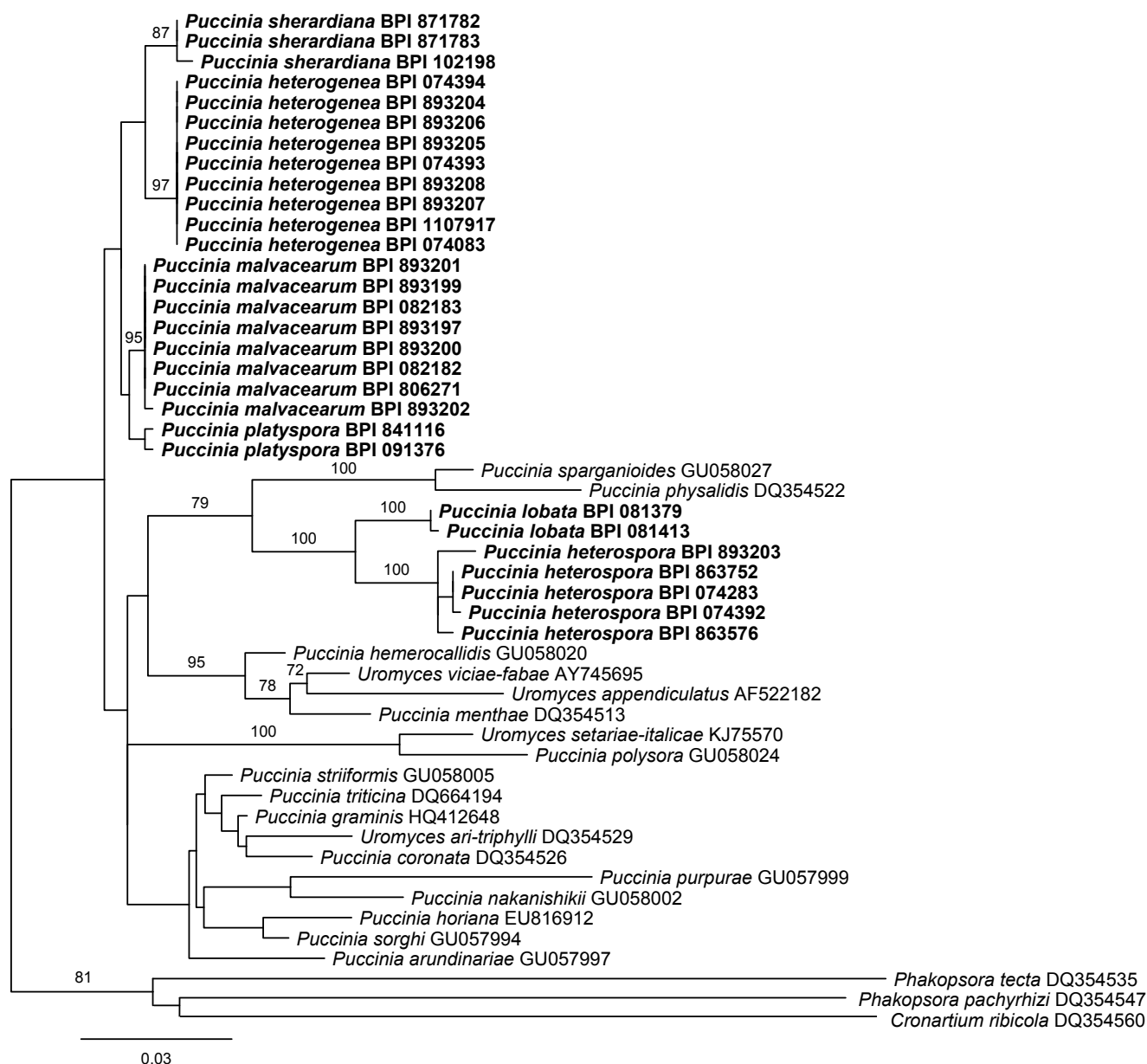


Fig. 1. Most likely phylogenetic tree of the microcyclic rusts infecting *Malveae* (in bold). Likelihood values $\geq 70\%$ are shown above branches. The tree is rooted with *Phakopsora tecta*, *Phakopsora pachyrhizi*, and *Cronartium ribicola*.

at Brownsville, Texas, entering from Mexico, on *Alcea rosea*, 2 Feb. 1950, Gayden (BPI 074286); Intercepted at Brownsville, Texas, entering from Mexico, on *Alcea rosea*, 12 Mar. 1950, C. B. Brown (BPI 074283); Intercepted at Brownsville, Texas, entering from Mexico, on *Abutilon trisulcatum*, 25 Dec. 1951, G. A. Pfaffman (BPI 074275); Intercepted at El Paso, Texas, entering from Mexico, on *Sida* sp., 25 Aug. 2003, M. A. Mendoza (BPI 893203). – **Mexico:** Veracruz: El Higo, on *Abutilon trisulcatum*, 13 Dec. 2003, J. R. Hernandez (BPI 863752).

Puccinia platyspora (Speg.) H. S. Jacks. & Holw., *Mycologia* **23**: 480 (1931). (Figs 2–3)

Description: *Telia* reddish brown, hypophyllous, scattered. *Teliospores* yellowish brown, often with apical pores or split walls at the apices, predominately one-celled; some two-

celled teliospores with very thin septa or partial septa. *One-celled teliospores* $34\text{--}53 \times 15\text{--}22 \mu\text{m}$, on average $42 \times 17 \mu\text{m}$. *Two-celled teliospores* $34\text{--}57 \times 13\text{--}21 \mu\text{m}$, on average $46 \times 16 \mu\text{m}$. *Side walls of teliospores* $1.5\text{--}2 \mu\text{m}$ thick, apical walls $3\text{--}13 \mu\text{m}$ thick. *Pedicels* hyaline, to $70 \mu\text{m}$ long.

Specimens examined: **Argentina:** La Falda, on *Sphaeralcea* sp., 21 Aug. 1922, E. W. D. & M. M. Holway (BPI 091376); **Jujuy Province:** Tilcara, on *Alcea rosea*, 3 Feb. 1996, J. R. Hernandez (BPI 841116).

Puccinia heterogenea Lagerh., *J. Mycol.* **7**: 44 (1891). (Figs 2–3)

Description: *Telia* dark reddish brown, mainly hypophyllous, scattered. *Teliospores* yellowish brown, often with apical pores or split walls at the apices; one- and two-celled

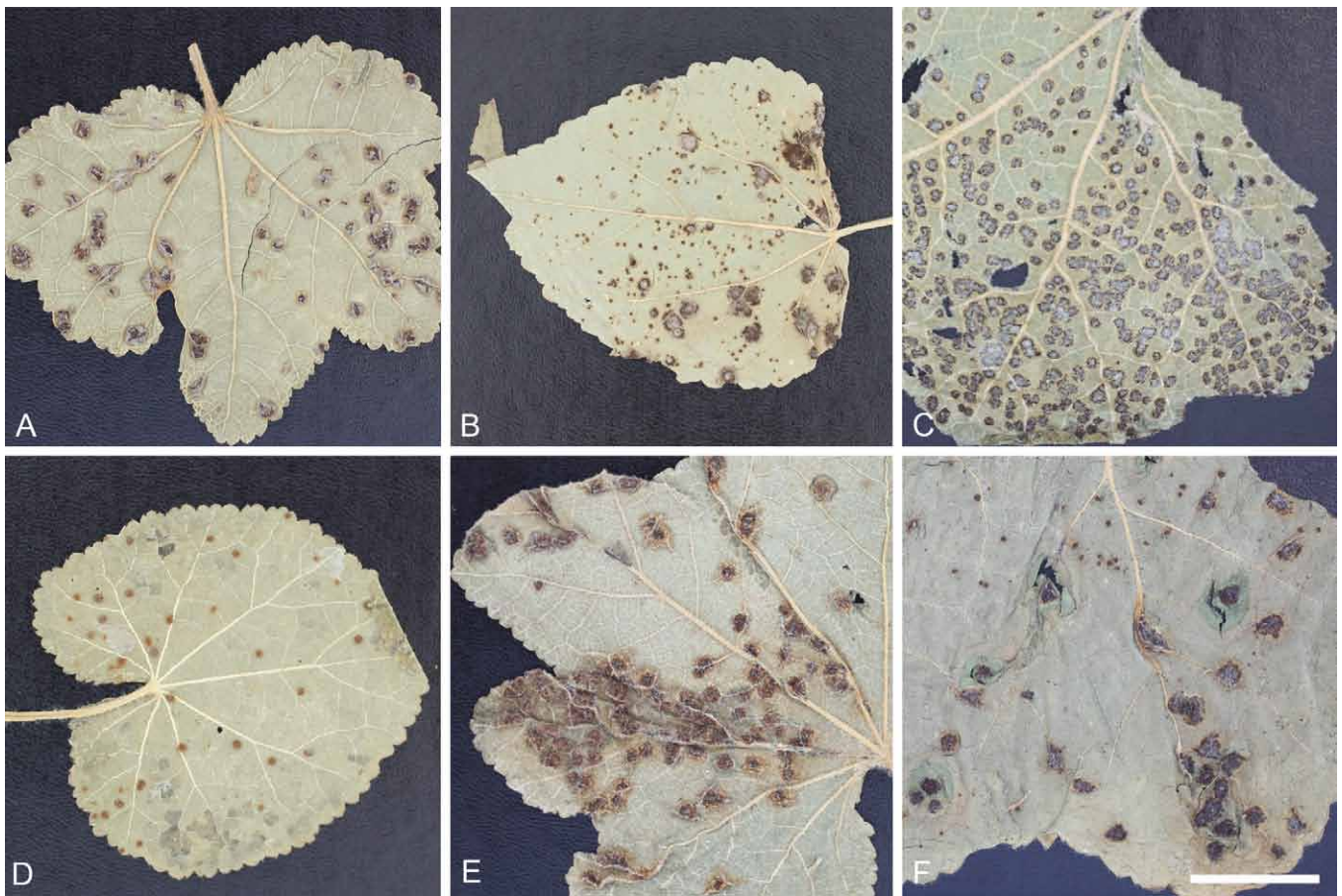


Fig. 2. Macroscopic features. **A.** *Puccinia heterogenea* (BPI 074083 – holotype). **B.** *Puccinia heterospora* (BPI 074275). **C.** *Puccinia lobata* (BPI 081381). **D.** *Puccinia malvacearum* (BPI 074281). **E.** *Puccinia platyspora* (BPI 841116). **F.** *Puccinia sherardiana* (BPI 102200). Bar = 2 cm.

spores mixed, one-celled spores predominating in most samples. *One-celled teliospores* 24–57 × 15–34 μm, on average 32 × 21 μm. *Two-celled teliospores* 28–57 × 15–34 μm, on average 42 × 24 μm. *Side walls of teliospores* 2–3 μm thick, apical walls 2–5 μm thick. *Pedicels* hyaline, to 140 μm long.

Specimens examined: **Ecuador:** Quito, on *Alcea rosea*, Nov. 1890, G. Lagerheim (BPI 074083 – holotype). – **Intercepted specimens (origin unknown):** Intercepted at Miami, Florida, entering from Ecuador, on *Malva arborea*, 9 Mar. 1967, P. R. Whitley (BPI 074394); Intercepted at Miami, Florida, entering from Ecuador, on *Malva arborea*, 5 Aug. 1967, J. Bache-Wiig (BPI 074393); Intercepted at Los Angeles, California, entering from Ecuador, on *Malva arborea*, 9 Aug. 1986, R. Heliczler (BPI 1107917); Intercepted at Atlanta, Georgia, entering from Ecuador, on *Malva arborea*, 14 Aug. 2010, M. Burgos (BPI 893206); Intercepted at Miami, Florida, entering from Ecuador, on *Malva arborea*, 12 Mar. 2013, J. E. Lopez (BPI 893204); Intercepted at Miami, Florida, entering from Ecuador, on *Malva arborea*, 13 Mar. 2013, M. Polanco (BPI 893205); Intercepted at Miami, Florida, entering from Ecuador, on *Malva arborea*, 5 Jul. 2014, L. Pizarro (BPI 893207); Intercepted at Miami, Florida, entering from Jamaica, on *Malva arborea*, 13 Aug. 2014, D. B. Leimer (BPI 893208).

Puccinia malvacearum Bertero ex Mont., *Hist. fis. y polit. Chile* 8: 43 (1852). (Figs 2–3)

Description: *Telia* light reddish brown, mainly hypophyllous, scattered. *Teliospores* yellowish brown, often with apical pores or split walls at the apices; most specimens predominately with two-celled spores, but some with predominately one-celled spores. *One-celled teliospores* 30–54 × 14–24 μm, on average 42 × 18 μm. *Two-celled teliospores* 29–82 × 12–32 μm, on average 51 × 21 μm. *Side walls of teliospores* 1–4 μm thick, apical walls 3–7 μm thick. *Pedicels* hyaline, to 130 μm long.

Specimens examined: **Intercepted specimens (origin unknown):** Intercepted at Chicago, Illinois, entering from Israel, on *Malva nicaeensis*, 11 Mar. 2004, N. A. Jarrett (BPI 893202); Intercepted at Hoboken, New Jersey, entering from The Netherlands, on *Alcea rosea*, 25 Jan. 2005, P. Milicia (BPI 893199); Intercepted at San Luis, Arizona, entering from Mexico, on *Malva neglecta*, 29 May 2010, C. G. Vazquez (BPI 893201); Intercepted at Boston, Massachusetts, entering from Portugal, on *Malva multiflora*, 21 May 2013, B. Nichols (BPI 893200); Intercepted at San Ysidro, California, entering from Mexico, on *Alcea* sp., 23 Feb. 2015, A. Ashley (BPI 893197). – **USA:** *California:* Stanford, on *M. arborea* var. *variegata*, 7 Apr. 1926, J. W. Gillespie (BPI 082182); *California:* Felton, on *M. arborea* var. *variegata*, 26 Apr. 1948, N. R. Hunt (BPI 082183); *Oregon:* Linn Co., Corvallis, on *M. sylvestris*, 12 Sep. 1997, R. R. Halse (BPI 806271).

Puccinia lobata Berk. & M. A. Curtis, *Grevillea* 3: 54 (1874). (Figs 2–3)

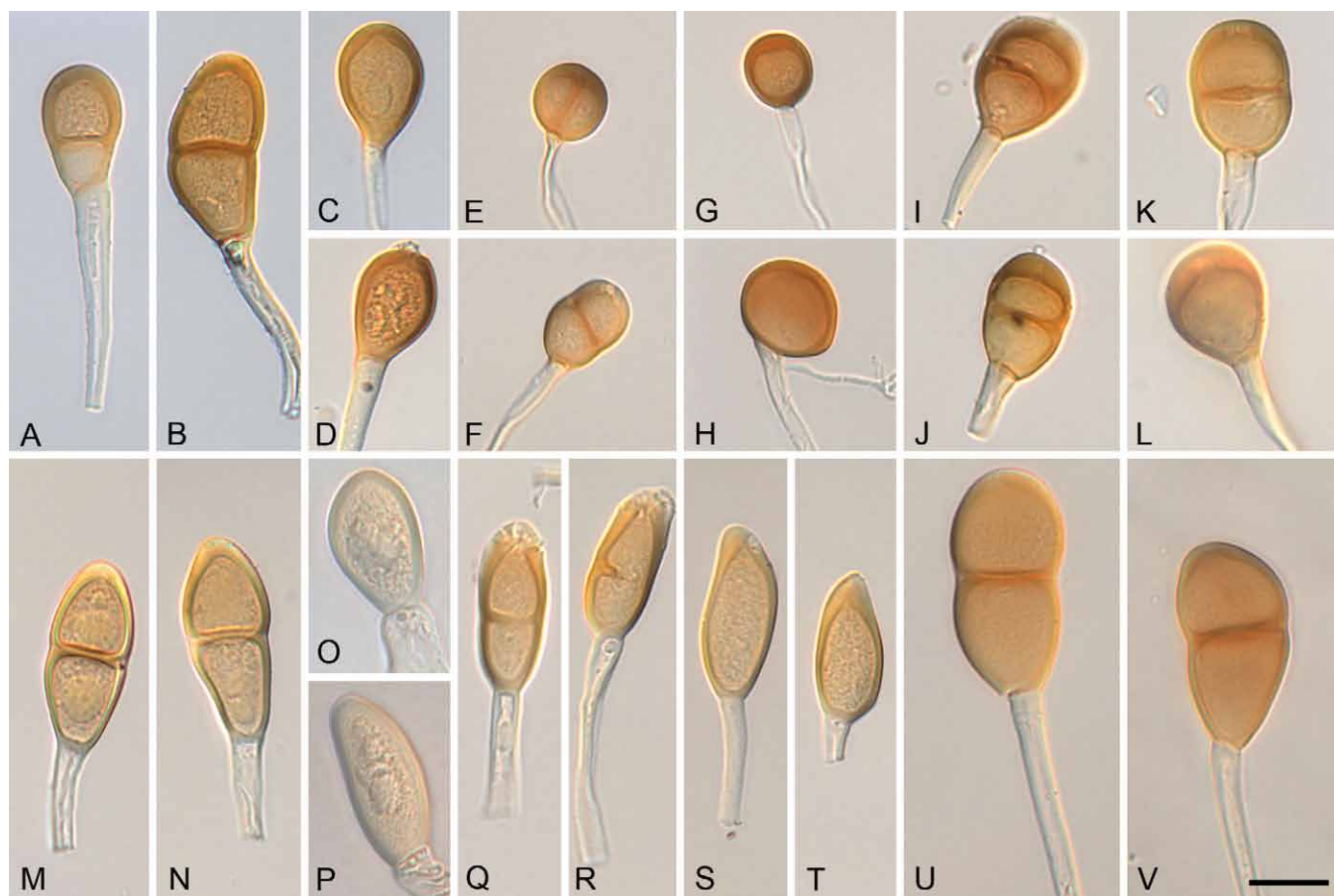


Fig. 3. Teliospores. **A–D.** *Puccinia heterogenea*. **A, B.** Two-celled teliospores (BPI 074393). **C, D.** One-celled teliospores (BPI 074393, BPI 893208). **E–H.** *Puccinia heterospora*. **E.** Two-celled teliospore with septum perpendicular to pedicel attachment point (BPI 074283). **F.** Two-celled teliospore (BPI 074283). **G.** One-celled teliospore (BPI 074283). **H.** Large one-celled teliospore (BPI 863576). **I–L.** *Puccinia lobata*. **I–K.** Two-celled teliospores (BPI 081369, BPI 081369, BPI 018413). **L.** One-celled teliospore (BPI 081369). **M–P.** *Puccinia malvearum*. **M, N.** Two-celled teliospores (BPI 893197). **O, P.** One-celled teliospores (BPI 893199). **Q–T.** *Puccinia platyspora*. **Q.** Two-celled teliospore (BPI 841116). **R.** Two-celled teliospore with partial septum (BPI 841116). **S, T.** One-celled teliospores (BPI 091376, BPI 841116). **U, V.** *Puccinia sherardiana*. **U, V.** Two-celled teliospores (BPI 102198). Bar = 20 μ m.

Description: *Telia* dark brown, hypophyllous, in tight clusters. *Teliospores* yellowish brown, often with apical pores or split walls at the apices, predominately two-celled. *One-celled teliospores* 26–31 \times 21–25 μ m, on average 28 \times 24 μ m. *Two-celled teliospores* 25–40 \times 16–27 μ m, on average 32 \times 22 μ m. *Side walls of teliospores* 1.5–3 μ m thick, apical walls 5–10 μ m thick in one-celled spores and 4–9 μ m thick in two-celled spores. *Pedicels* hyaline, to 120 μ m long.

Specimens examined: Intercepted specimens (origin unknown): Intercepted at Laredo, Texas, entering from Mexico, on *Malvella lepidota*, 26 May 1966, G. E. Reuthinger (BPI 081413). – **USA:** Texas: Presidio, on *Alcea rosea*, 23 Nov. 1955, J. H. Russell (BPI 081369). Texas: Corpus Christi, on *A. rosea*, 28 May 1966, C. M. Locke (BPI 081379).

***Puccinia sherardiana* Körn., *Hedwigia* 16: 19 (1877).** (Figs 2–3)

Description: *Telia* dark brown, hypophyllous, in tight clusters. *Teliospores* light brown, often with apical pores or split walls at the apices, predominately two-celled. *One-celled*

teliospores 34–36 \times 18–30 μ m, on average 35 \times 24 μ m. *Two-celled teliospores* 34–60 \times 17–34 μ m, on average 47 \times 25 μ m. *Side walls of teliospores* 1.5–3 μ m thick, apical walls 3–7 μ m thick. *Pedicels* hyaline, to 200 μ m long.

Specimens examined: Intercepted specimens (origin unknown): Intercepted at El Paso, Texas, entering from Mexico, on *Sphaeralcea* sp., 30 Nov. 2004, R. A. Garner-Lozoya (BPI 871782); Intercepted at El Paso, Texas, entering from Mexico, on *Sphaeralcea* sp., 8 Dec. 2004, J. Gasca (BPI 871783). – **USA:** Colorado: Las Animas Co., Trinidad, on *Alcea rosea*, 4 Sep. 1965, J. A. Parmalee & D. I. Hammond, (BPI 102198).

DISCUSSION

Multiple rust fungi can infect plants in *Malveae*, and plants from this tribe imported into the USA are often intercepted with visible rust disease, generally caused by *Puccinia malvearum* and *P. heterogenea*. *Puccinia malvearum* has been reported to have a much wider geographical distribution than *P. heterogenea* (Farr & Rossman 2015). The exact origin

of many of the specimens in this study is, however, uncertain because they were intercepted from luggage and cargo and may have been extensively moved during travel and trade. The reported origins of the intercepted *P. malvacearum* specimens include Israel, Mexico, The Netherlands, and Portugal, with additional specimens with verified origins from the USA, agreeing with the reported worldwide distribution of *P. malvacearum*. In contrast, all intercepted *P. heterogenea* specimens were reported as originating from Ecuador, except for one reported as from Jamaica, which was possibly not its true origin. *Puccinia heterogenea* has only been previously reported from Ecuador and Peru (Farr & Rossman 2015), and the samples collected in this study suggest that it has not spread further.

Puccinia malvacearum has been intercepted on many species of plants and is reported to have a very large host range in *Malvoideae* (Farr & Rossman 2015). This study focused on intercepted rust fungi of *Malveae*, especially *Alcea rosea*, so a comprehensive survey of other possible hosts of *P. malvacearum* was not completed. In this limited sampling, *P. malvacearum* was detected on *A. rosea* and several *Malva* species. *Puccinia heterogenea* is reported on a much smaller host range than *P. malvacearum*, only *A. rosea* and various *Malva* species (Farr & Rossman 2015). In this study, *P. heterogenea* was mainly detected on plants identified based on ITS sequences as *Malva arborea*, tree mallow. Tree mallow is considered medicinal (Germplasm Resources Information Network; <http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?455281>), so it is more likely to be imported and intercepted than other, less ethnobotanically useful, hosts of *P. heterogenea*.

The other four microcyclic *Puccinia* species reported on *A. rosea* are *P. heterospora*, *P. lobata*, *P. platyspora*, and *P. sherardiana*. Sequencing a representative sample of each confirmed that they are different from both *P. malvacearum* and *P. heterogenea*. These rusts are morphologically similar and may be confused with each other, as well as with other *Puccinia* species on malvaceous plants such as *P. anodae*, *P. exilis*, *P. modiolae*, *P. sidalceae*, and *P. sphaeralceae*. *Puccinia heterospora*, *P. lobata*, *P. platyspora*, and *P. sherardiana* appear to have wide host ranges like *P. malvacearum*, as all four were found on hosts not closely related to *A. rosea* (Escobar García et al. 2009): *Sphaeralcea* for *P. platyspora* and *P. sherardiana*; *Malvella* for *P. lobata*; and *Abutilon*, *Anoda*, and *Sida* for *P. heterospora* – although the high degree of intraspecific variation in *P. heterospora* raises the possibility that it is composed of cryptic species with smaller, more specific host ranges. As with *P. heterogenea*, *P. heterospora*, *P. lobata*, *P. platyspora*, and *P. sherardiana* have a smaller reported geographic distribution than *P. malvacearum*. *P. heterospora* has been reported worldwide except for Europe, *P. lobata* and *P. sherardiana* have been reported in North and South America, and *P. platyspora* has been reported only in South America (Farr & Rossman 2015). Although *P. platyspora* and *P. heterogenea* have not been reported in the US, and *P. lobata* and *P. sherardiana*

have only been reported in the western US, their similarity to *P. malvacearum* suggests that they would not cause more damage than *P. malvacearum* if spread to new areas and that they could be similarly managed. The study of intercepted fungi can provide useful information about their biology, systematics, and long-distance movement in trade.

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