Morphology, Life Cycle Biology, and DNA Sequence Analysis of Rust Fungi on Garlic and Chives from California

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Accepted for publication 13 January 2004.

ABSTRACT

Anikster, Y., Szabo, L. J., Eilam, T., Manisterski, J., Koike, S. T., and Bushnell, W. R. 2004. Morphology, life cycle biology, and DNA sequence analysis of rust fungi on garlic and chives from California. Phytopathology 94:569-577.

In the late 1990s, commercial garlic fields in California (CA) were devastated by an outbreak of rust caused by *Puccinia allii*. We compared collections of the pathogen from garlic (*Allium sativum*) and chives (*A. schoenoprasum*) in central CA and Oregon (OR) to collections from garlic and leek (*A. porrum* and *A. ampeloprasum*) in the Middle East. Teliospores from the CA and OR collections were smaller in length, width, and projected cross-sectional area compared with collections from the Middle East. CA and OR collections had a shortened life cycle, in which pycnia and aecia were not formed. Germinating teliospores produced a two-celled promycelium, resulting in two basidiospores, each initially with two nuclei, indicating that this rust was homothallic. In addition, the morphology of the substomatal vesicles was different between the CA-OR (fusiform) and the Middle Eastern (bulbous) collections. DNA sequence analysis of the nuclear ribosomal internal transcribed spacer region showed that the CA and OR rust collections formed a well-supported cluster distinct from the Middle Eastern and European samples. These results suggest that the rust on garlic and chives in CA and OR is a different species than the rust fungus on garlic and leek in the Middle East.

Additional keywords: hemicyclic, infection structure.

Historically, rust has not been a major disease on cultivated garlic and chives in California. However, in 1998, a major outbreak of rust devastated the garlic crop in central California (28). Continuing rust epidemics in 1999 and 2000 led to reduction of garlic production in California by almost 90%. Rust on garlic also was observed in Oregon in 2000, but caused little damage (20). The pathogen causing these epidemics was identified as *Puccinia allii*. In addition, *P. allii* was found on chives near the Pacific coast in Santa Barbara in 1999 in a home garden. The rust also occurred in commercial fields of chives in northern Monterey County in 2001. One of the fields was so heavily damaged that the entire crop was plowed under. In contrast, chives in fields located further east (inland) showed little or no rust.

P. allii causes disease on many *Allium* spp., including chives (*Allium schoenoprasum*), garlic (*A. sativum*), leek (cultivated leek, *A. porrum*; wild leek, *A. ampeloprasum*), and bulb onion (*A. cepa*) (30). *P. allii* is found worldwide and has caused significant losses to garlic, leek, and onion production.

P. allii is a macrocyclic (full cycled), autoecious rust, usually producing pycnia, aecia, uredinia, telia, and basidia (promycelia) on a single host. However, variants of this life cycle have been reported in which pycnia and aecia are lacking (11,16,23,38,39). The garlic rust from California was thought to have a similar life cycle, in that neither pycnia nor aecia were found (28). This shortened life cycle is different from both demicyclic rusts in which uredinia are lacking and microcyclic rusts in which aecia and uredinia are missing (12,22). The shortened life cycle form, lacking pycnia and aecia, has been labeled "hemi-form" (16).

The taxonomic delineation of *P. allii* has been in flux over the last century due to variation in the host range of isolates and in morphological characteristics such as teliospore type (two-celled versus one-celled) and paraphyses. Gäumann (16) reviewed the early literature and divided this species-complex (designated *P. porri* complex) into two species, based on characteristics of telia, teliospores, and life cycle: *P. allii* (DC) Rud. with mostly two-celled teliospores, telia with dark-brown, fused paraphyses, and hemicyclic life cycle; and *P. porri* (Sow) Wint. with more one-celled teliospores (mesospores) than two-celled teliospores, telia mostly lacking dark-brown paraphyses, and macrocyclic life cycle. Others have split *P. porri* into two species (*P. porri* and *Uromyces ambiguus*) based on the percentage of one-celled teliospores (25,39). *U. ambiguus* (DC) Lév. was defined by having essentially only one-celled teliospores, whereas *P. porri* (*P. mixta*) contained a mixture of two- and one-celled teliospores. However, these characteristics often are variable and have been shown to change with host and environmental conditions. In addition, collections of rust on leek and garlic in Europe often lack telia, thus making species classification difficult. Laundon and Waterston (30) grouped these species (as well as *P. blasdalei* and *U. duris*) under *P. allii* as currently accepted (14). We have followed this classification, using *P. allii* for all samples collected from garlic, chives, and leek. However, *P. allii* is a species complex rather than a single species.

Given the difficulty in delineation of different members of this species complex, alternative approaches have been used recently. Jennings et al. (25) used multivariate analysis of urediniospore

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Publication no. P-2004-0402-01R

characteristics as quantitative rather than qualitative traits and was able to divide the complex into two groups ("leek" and "non-leek"), which generally corresponded with the *P. allii-P. porri* classification by Gäumann (16). Niks and Butler (32) examined infection structures and found two distinct morphologies of substomatal vesicles, which supported the division of the *P. allii* species complex into two groups, similar to the results of Jennings et al. (25).

The objective of this study was to clarify the relationship of *P. allii* on *Allium* spp. in California and Oregon with samples of *P. allii* from the Middle East and Europe using biological, morphological, and molecular characteristics.

MATERIALS AND METHODS

Rust collections. The rust collections used in this study, their hosts, and geographical origins are listed in Table 1. Samples of *P. allii* were collected from commercial garlic fields (*A. sativum*) in California (CA) during 1999 and 2000. Eight collections were made from coastal locations (Monterey, San Benito, Santa Barbara, Santa Clara, and Santa Cruz counties) and the inland San Joaquin Valley. One collection was made from a commercial garlic field in Oregon (OR). Three additional collections were made from garlic fields in England, Israel, and Turkey. Two samples were collected from chives (*A. schoenoprasum*) in CA. Seven samples of *P. allii* were collected from leek (*A. ampeloprasum* and *A. porrum*) in Israel and one from Germany.

Teliospores were induced to germinate by the methods of Anikster (3). Dried leaf pieces bearing telia were floated on water in petri dishes at 5 to 6°C for 2 to 4 weeks. Leaves of *A. sativum* (garlic) and *A. ampeloprasum* (wild leek) were inoculated with basidiospores ejected from germinating teliospores by suspending filter paper with pretreated telia over plants as described by Anikster et al. (4). Ten North American and nine Middle Eastern *P. allii* collections were used (Table 1).

Spore morphology. For microscopic examination, teliospores of *P. allii* were scraped from dry leaves of *A. ampeloprasum*, *A. porrum*, *A. sativum*, and *A. schoenoprasum*. The teliospores were mounted in 50% glycerol on glass slides and examined with a Zeiss Axioskop microscope. Video images, obtained with a CCD B/W video camera (LIS-700 Applitec, Israel), were digitized and analyzed using image analysis software (NIH Image, 1.62). The length, width, and projected cross-sectional area were determined as described by Anikster et al. (4,5). Dimensions of at least 50 teliospores were measured, excluding pedicels, for each collection.

Basidiospores were collected by placing glass slides under telia-bearing leaf pieces that had been pretreated to induce teliospore germination. The basidiospores were mounted in lactophenol cotton blue and slightly heated for 5 min. Urediniospores were mounted in 50% glycerol. Spores were measured as described previously.

Germ pores of urediniospores were counted using a modification of the squash technique of Jennings et al. (24) as described (5). Germ pores were viewed with a Zeiss microscope using differential interference contrast optics. Germ pores of at least 50 spores of each collection were counted.

Substomatal vesicles. Leaves of *Hordeum vulgare* (L-94), a nonhost for *P. allii*, were inoculated with a thick suspension of *P. allii* urediniospores and then incubated in a dew chamber for 24 h. Niks and Butler (32) showed that development of substomatal vesicles and other infection structures by *P. allii* on *H. vulgare* was normal up to the formation of the haustoria. Substomatal vesicles were examined in cleared, stained leaf segments using a modification of the procedure of Niks and Butler (32) as described by Anikster et al. (4). Four collections of *P. allii* from CA (HSZ0339, HSZ0343, HSZ0162, and HSZ0509) and three collections from Israel (YA8884, YA8890, and YA8893) were tested.

Nuclear staining of promycelia and basidiospores. Teliospores were germinated from 10 North American (CA and OR) and 9 Middle Eastern collections (Table 1). The North American collections were from chives and garlic. The Middle Eastern collections were from garlic, leek, and wild leek. Teliospores that

TABLE 1. Collections of *Puccinia allii* used in this study

Hosts, collections[a]	Location, date	GenBank accession no.
Allium ampeloprasum (wild leek)		
YA8827*	Yavne, Israel, 1996	AF511074
YA8866*	Israel, 2000	…
YA8884*	Mavkiim, Israel, 2001	…
YA8885*	Yavne, Israel, 2001	…
YA8888*	Yavne, Israel, 2002	…
YA8893*	Kamun, Galilee, Israel, 2002	…
A. porrum (cultivated leek)		
MS165/92	Germany, 1992	AY187090
YA8890*	Israel, 2001	…
A. sativum (cultivated garlic)		
YA8799*	Tarsus, Turkey, 1996	AF511073
YA8837*	Kefar Hemeham, Israel, 1997	…
HSZ0004	Oxon, England, 2001	AF511079
HSZ0075*	King City, Monterey Co., CA, 1999	…
HSZ0077*	Santa Clara Co., CA, 1999	…
HSZ0078*	San Joaquin Valley, CA, 1999	…
HSZ0080*/PUR N2540[b]	Santa Barbara Co., CA, 1999	…
HSZ0162*/PUR N2537[b]	San Benito Co., CA, 1999	AF511077
HSZ0163*	San Benito Co., CA, 1999	…
HSZ0343*	Salinas, Monterey Co., CA, 2000	AF511076
HSZ0344	Santa Cruz Co., CA, 2000	AF511078
HSZ0508*	Marion Co., OR, 1999	AF511075
A. schoenoprasum (cultivated chives)		
HSZ0341*/PUR N2539[b]	Santa Barbara, CA, 1999	AF511080
HSZ0509*/PUR N2536[b]	Monterey Co., CA, 2001	AF511087

[a] Prefix designations and sources for collections: HSZ, L. J. Szabo; MS, Markus Scholler, Staatliches Museum für Karlsruhe, Karlsruhe, Germany; PUR, The Arthur Rust Herbarium, Purdue University; and YA, Y. Anikster. Asterisks indicate collection was used in study of promycelium and basidiospore development and in inoculation experiments.

[b] Collection of L. J. Szabo was placed in the Arthur Rust Herbarium, Purdue University.

had been pretreated to induce germination were scraped from host leaf tissue and transferred to the surface of water agar in petri dishes for observation. Germinating teliospores were stained with a solution of 0.18 M Tris-HCl (pH 7.2), propidium iodide (PI) at 4 µg/ml, RNase at 50 µg/ml, and Triton X-100 at 4 µg/ml for 30 min at room temperature. Basidiospores were collected on glass slides in a petri dish by placing pieces of host leaves with telia on wet filter paper attached to the lid of the petri dish. Basidiospores on the glass slides were stained with PI as described previously. Stained nuclei in promycelia and basidiospores were examined with a Zeiss epi-fluorescence microscope with filter set (510-560, FT580, LP590).

PCR amplification and DNA sequencing. DNA was extracted from either dried urediniospores (1 to 20 mg) collected from infected host material or from dried host leaf tissue (20 to 30 mg) containing uredinial or telial pustules. Dried wheat leaf tissue (20 mg) was added to samples containing <5 mg of urediniospores as a carrier. Urediniospores and infected leaf tissue were pulverized by shaking samples in tubes with 1-mm glass beads (Lysing matrix C; Bio 101, Carlsbad, CA) and 25 mg of diatomaceous earth (Sigma-Aldrich, St. Louis) in a Savant FastPrep shaker (FP120, Holbrook, NY) for 10 s at a speed setting of 5 (40). This step was repeated once. Samples were chilled on ice prior to and between pulverization steps. DNA was extracted from the pulverized samples using a modification of the cetyltrimethylammonium bromide (CTAB) method described by Liu and Kolmer (31). Extraction buffer (600 µl of: 0.165 M Tris-HCl, pH 8.0; 66 mM EDTA, pH 8.0; 1.54 M NaCl; 1.1% CTAB; and proteinase K at 50 µg/ml) was added and mixed by vortexing. To each tube, 66 µl of 20% sodium dodecyl sulfate was added. The tube was inverted to mix the contents and incubated at 65°C for 1.5 h. During this time, the tubes were inverted every 15 to 20 min. After incubation, the samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol), followed by an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol). DNA was precipitated with 0.6 volume of isopropanol. DNA pellets were washed with 70% ethanol and resuspended in 100 µl of 0.1 TE (10 mM Tris-HCl, pH 8; 0.1 mM EDTA, pH 8). RNA was removed by digestion with RNase A (40 to 100 µg/ml) at 37°C for 1 h. DNA was reprecipitated using ethanol and resuspended in 100 µl of 0.1 TE.

Nuclear ribosomal internal transcribed spacer (ITS) region and the 5′ end of the large subunit was polymerase chain reaction

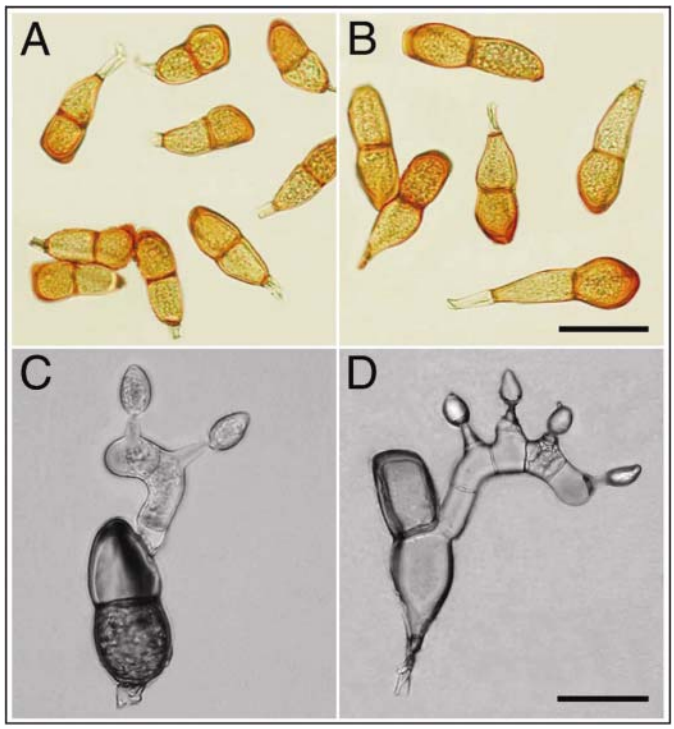

Fig. 1. Teliospores, promycelia, and basidiospores produced by North American and Middle Eastern collections of *Puccinia allii*. **A,** Teliospores of collection HSZ0343 from garlic in California; **B,** teliospores of collection YA8866 from wild leek in Israel. Collections from North America (as in **A)** have smaller teliospores than collections from the Middle East (as in **B**). **C,** Two-celled promycelium with two basidiospores produced by collection HSZ0343 from garlic in California; and **D,** four-celled promycelium with four basidiospores produced by collection YA8866 from wild leek in Israel. Bars = 50 µm (**A and B**) and 25 µm (**C and D**).

TABLE 2. Morphological characteristics of *Puccinia allii* teliospores and telia[a]

		Teliospore dimensions			Telia	
Collection	Host	Length (µm)	Width (µm)	Area (µm^2)	Mesospores (%)[b]	Paraphyses (pigmented)[c]
North America						
HSZ0077	Garlic	53 ± 7	18 ± 2	744 ± 101	1–3	Many
HSZ0078	Garlic	51 ± 4	18 ± 2	700 ± 90	1–3	Many
HSZ0162	Garlic	49 ± 4	19 ± 2	743 ± 65	1–3	Many
HSZ0163	Garlic	45 ± 5	21 ± 2	703 ± 69	1–3	Many
HSZ0508	Garlic	47 ± 3	20 ± 2	749 ± 91	1–3	Many
HSZ0343	Garlic	53 ± 5	17 ± 2	732 ± 95	1–3	Many
HSZ0080	Garlic	53 ± 5	16 ± 1	725 ± 92	1–3	Many
HSZ0341	Chives	47 ± 5	20 ± 2	745 ± 91	32–38	None[d]
HSZ0509	Chives	45 ± 5	20 ± 2	700 ± 72	5–30	None[d]
Mean	…	49.2[e]	18.7[f]	727[f]	…	…
Middle East						
YA8799	Garlic	59 ± 7	23 ± 2	1,067 ± 133	2	nd
YA8827	Wild leek	64 ± 8	23 ± 2	1,105 ± 147	1–3	Many
YA8837	Garlic	57 ± 5	24 ± 2	1,070 ± 121	1–3	Many
YA8866	Wild leek	61 ± 7	29 ± 3	1,048 ± 127	1–3	Many
YA8884	Wild leek	65 ± 8	21 ± 2	1,042 ± 130	1–3	Many
YA8885	Wild leek	53 ± 6	24 ± 2	1,014 ± 150	1–3	Many
YA8890	Leek	50 ± 4	23 ± 2	923 ± 112	1–3	Few
YA8893	Wild leek	nd	nd	nd	1–3	Many
Mean	…	58.4[e]	23.9[f]	1,038[f]	…	…

[a] nd = Not determined.
[b] Single-celled spores as percentage of spores in telium.
[c] Presence of brown or black pigmented paraphyses.
[d] A few nonpigmented paraphyses were present.
[e] Means for North American and Middle Eastern collections are significantly different ($P < 0.01$) using a one-tailed t test.
[f] Means for North American and Middle Eastern collections are significantly different ($P < 0.001$) using a one-tailed t test.

(PCR) amplified in a 50-µl reaction mixture as described by Zambino and Szabo (41) with the following modifications: concentration of primers was reduced to 25 pmol and the amount of Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA) was reduced to 1.25 units per reaction mix. Primers pairs used for amplification were ITS1F and ITS4B (15) for the ITS region and ITS1F and R-635 (21) or ITS1F and RUST1 (29) for the ITS and 5′ end of the large subunit. DNA extract (5 µl) diluted from 1:10 to 1:200 in 0.1 TE was used in each PCR reaction. DNA was amplified using a thermocycler (PT100; MJ Research, Reno, NV) with the following parameters: 5 min at 94°C; 45 s at 94°C; 1 min 15 s at 44°C; 2 min at 72°C; cycle to step 2, 30 times; 10 min at 70°C; hold at 4°C. PCR amplification products were purified using a StratPrep PCR purification kit (Stratagene, La Jolla, CA) and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). DNA sequencing reactions were performed using a Thermo Sequenase Prime Cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) and analyzed on an automated DNA sequencer (LI-COR, Lincoln, NE). At least three clones were sequenced for each sample and the DNA sequence data was assembled and edited with Sequencer (Genecodes, Ann Arbor, MI). In some cases, up to nine clones were analyzed to verify the DNA sequence.

Ten collections of *P. allii* from North America, Europe, and the Middle East were selected for DNA sequence analysis (Table 1). These samples represented collections from cultivated chives, garlic, leek, and wild leek. For two of the collections (YA8827 and YA8799), only the complete ITS region was amplified. Blast analysis of the ITS region of *P. allii* (HSZ0343) against GenBank DNA sequences identified that the ITS region of barley leaf rust *P. hordei* (L08697) and its correlated species *U. scillarum* (L08733) were the closest matches to *P. allii*. Both of these ITS sequences were not complete; therefore, the ITS region was cloned and sequenced for two collections each of *P. hordei* (CDL64-2B and CDL22/81) and *U. scillarum* (YA3464 and YA3465) for inclusion in the analysis. Nucleotide sequence data have been submitted as GenBank accession nos. AF511073 to AF511080, AF511085 to AF511087, AY187087, AY187089, AY187090, and AY302495 (Table 1).

DNA sequences were aligned using the program CLUSTAL W (35) and the multiple sequence editor in MacVector (version 7.0; Genetics Computer Group, Madison, WI). Phylogenetic analysis of the data set (812 characters that included the complete ITS1, 5.8S, and ITS2 region) was performed using a heuristic parsimony program (PAUP version 4.04b) with random stepwise addition option with 10 replicas (33). Deletions were scored as a "fifth base" and counted as a single character. Nucleotide substitutions and deletions were treated as unordered. Support for the nodes of the shortest trees was determined by analysis of 1,000 bootstrap replicas. A *P. triticina* ITS sequence (GenBank accession no. AY187087) was used as an outgroup. DNA sequence alignment and trees have been submitted to TreeBASE: study accession no. S1067, matrix accession no. M1819.

RESULTS

Morphology of telia and teliospores. We examined the morphology of teliospores and telia in collections from North America (CA and OR) and the Middle East (Table 2). Teliospores of all collections had attached pedicels although, in many cases, the pedicles were broken off when spores were harvested (Fig. 1A and B). Furthermore, teliospores of most collections, whether from North America or the Middle East, were largely two-celled; only 1 to 3% of teliospores of most collections were one-celled (mesospores) (Table 2). Exceptions were the two collections of chives from CA that had higher percentages of mesospores: 5 to 30% for collection HSZ0509 and 32 to 38% for HSZ0341 (Table 2).

Telia of most collections from North America and the Middle East had many black or brown paraphyses surrounding clusters of teliospores (Table 2). However, Middle Eastern collection YA8890 differed in having only a few such pigmented paraphyses and the two CA chives collections (HSZ0509 and HSZ0341) had only a few nonpigmented paraphyses (Table 2).

Teliospore dimensions of the North American collections were significantly smaller than those from the Middle East (Table 2). In mean length, width, and projected cross-sectional area, North American teliospores were 49.2 µm, 18.7 µm, and 727 µm^2, respectively, compared with 58.4 µm, 23.9 µm, and 1,038 µm^2 for Middle Eastern teliospores (Table 2). Differences between the two groups were significant ($P < 0.01$ or 0.001, Table 2) and the differences were especially clear cut for width and projected cross-sectional area, in that values for individual collections in the North American group did not overlap with any of those in the Middle Eastern group.

Morphology of urediniospores. Urediniospore dimensions did not differ significantly between collections from North America and the Middle East (Table 3). Mean values for length, width, and projected cross-sectional area were 25.8 µm, 22.6 µm, and 460 µm^2, respectively, for the North American collections compared with 25.0 µm, 21.7 µm, and 431 µm^2 for the Middle Eastern collections. Furthermore, the two groups were similar in number of germ pores, typically nine per spore (Table 3).

Morphology and nuclear number of promycelia and basidiospores. Teliospores were germinated so that resulting promycelia could be examined microscopically for cell number and number of nuclei. Promycelia from North American collections had only two cells (Fig. 1C). Each of these cells, in turn, contained two nuclei, giving a total of four nuclei per promycelium (Fig. 2A). Each cell of the promycelium then produced one

TABLE 3. Morphological characteristics of *Puccinia allii* urediniospores[a]

Collection no.	Hosts	Length (µm)	Width (µm)	Area (µm^2)	No. of germ pores[b]
North America					
HSZ0075	Garlic	27 ± 1	24 ± 1	507 ± 35	8 (7–10)
HSZ0163	Garlic	26 ± 2	23 ± 2	474 ± 59	9 (8–12)
HSZ0508	Garlic	25 ± 6	22 ± 2	443 ± 43	9 (7–12)
HSZ0080	Garlic	nd	nd	nd	9 (7–11)
HSZ0341	Chives	24 ± 5	22 ± 2	412 ± 50	9 (7–12)
HSZ0509	Chives	27 ± 3	22 ± 2	464 ± 65	9 (7–13)
Mean	…	25.8	22.6	460	9
Middle East					
YA8866	Wild leek	25 ± 2	22 ± 2	449 ± 75	nd
YA8884	Wild leek	24 ± 2	21 ± 2	393 ± 54	9 (8–12)
YA8890	Cultivated leek	26 ± 2	22 ± 1	452 ± 41	nd
Mean	…	25.0	21.7	431	9

[a] nd = Not determined.
[b] Most frequent number of germ pores per spore followed by the range.

basidiospore, which contained two nuclei that had migrated from the basal promycelial cell (Fig. 2B). Basidiospores examined after they were ejected from the promycelium contained four nuclei (Figs. 2C and 3A), apparently the result of a mitotic nuclear division. In contrast to those of North American collections, the promycelia produced by Middle Eastern collections each had four cells (Fig. 1D), each containing a single nucleus, resulting in four uninucleate basidiospores (Fig. 2E). After basidiospores were ejected, each contained two nuclei (Figs. 2F and 3B).

Basidiospores from North American collections were significantly longer and had greater projected cross-sectional area than those from the Middle East (Table 4). Length and projected cross-sectional area values were 13.2 µm and 89.4 µm^2, respectively, for the North American collections compared with 10.5 µm and 64.5 µm^2 for those from the Middle East. The differences were significant at $P < 0.05$ (Table 4). No significant difference was found in the width between the samples.

Inoculation studies. Leaves of *A. sativum* (garlic) and *A. ampeloprasum* (wild leek) were inoculated with basidiospores. On *A. sativum*, all North American collections produced uredinia 9 to 13 days after inoculation (Fig. 4A), followed by telia 7 to 21 days later (Fig. 4B). No pycnia or aecia were seen. On *A. ampeloprasum,* none of the North American collections produced infection (i.e., no uredinia, telia, pycnia, or aecia were seen). In contrast, inoculation with basidiospores from all Middle Eastern *P. allii* collections led to formation of pycnia in 7 to 12 days (Fig. 4C) on both *A. sativum* and *A. ampeloprasum*. If nectar from several of these pycnia were intermixed on the leaves, aecia appeared 3 to 4 days later and were well developed by 10 days (Fig. 4D). If aeciospores harvested from these aecia were used to inoculate additional plants, uredinia were produced in 10 to 15 days and telia 10 to 30 days later (data not shown).

Morphology of substomatal vesicle. A substomatal vesicle is produced after a penetration peg has entered the host plant via a stomatal opening (Fig. 5A). The substomatal vesicles produced by *P. allii* collections from CA were parallel in orientation with respect to the leaf surface and were fusiform in shape (Fig. 5B). Each substomatal vesicle produced two infection hyphae, one from each end (data not shown). The substomatal vesicle usually contained a septum that divided it into two cells. In contrast, substomatal vesicles produced by *P. allii* collections from Israel were oriented at right angles to the leaf surface, forming a bulbous, one celled structure (Fig. 5C). Each substomatal vesicle produced a single infection hyphae at the end distal to the penetration peg (data not shown).

DNA analysis. Parsimony analysis resulted in three optimal trees, each with a tree length of 111 steps, one of which is shown in Figure 6. A cluster containing all *P. allii* sequences from CA and OR collections was well supported by bootstrap analysis (90%). No separation was seen between the chives and garlic DNA sequences and the ITS sequence from one chives collection (HSZ0341) was identical to the ITS sequences from two garlic samples (HSZ0343 and HSZ0344). Minor variation in the DNA sequences (1 to 4 changes) was observed among several of the collections from CA and OR. The differences among the three optimal trees resulted from differences in the specific arrangements of six CA and OR collections within this group. The DNA sequences of *P. allii* from the European and Middle Eastern collections formed a separate cluster, which was well supported by

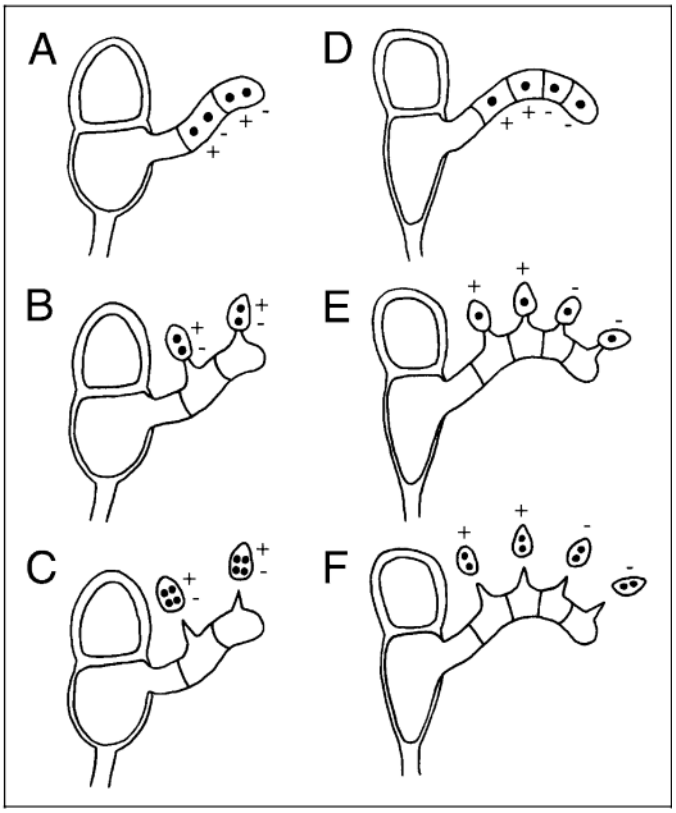

Fig. 2. Development of promycelium and basidiospores by *Puccinia allii* collections from North America and the Middle East with postulated mating types (+ and –) of nuclei. **A to C,** North American collections from cultivated garlic and chives. **A,** Germinating teliospore produces a two-celled promycelium; each cell containing two nuclei; **B,** each promycelial cell produces one basidiospore, each with two nuclei; **C,** ejected basidiospores each have four nuclei. **D to F,** Middle Eastern collections from wild leek and cultivated garlic; **D,** germinating teliospore produces a four-celled promycelium; each cell contains one nucleus; **E,** each cell of the promycelium produces one basidiospore, each with one nucleus; and **F,** ejected basidiospores each have two nuclei. Nuclei of ejected basidiospores as viewed in Figure 3.

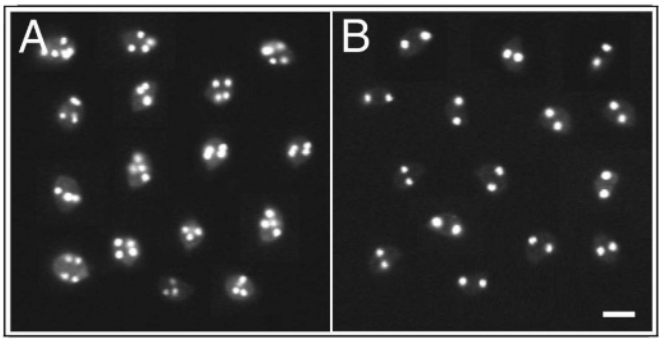

Fig. 3. Fluorescing nuclei in mature basidiospores of *Puccinia allii.* The basidiospores were stained with propidium iodide after they were ejected onto glass slides from promycelia. Viewed by epifluorescence microscopy. **A,** Four nuclei per spore in a California collection from garlic (HSZ0163); **B,** Two nuclei per spore in an Israeli collection from wild leek (YA8885). Bars = 10 µm.

TABLE 4. Dimensions of *Puccinia allii* basidiospores

Collection no.	Hosts	Length (µm)	Width (µm)	Area (µm^2)
North America				
HSZ0163	Garlic	12 ± 1	8 ± 1	76 ± 9
HSZ0508	Garlic	13 ± 1	8 ± 1	88 ± 12
HSZ0343	Garlic	13 ± 1	10 ± 1	103 ± 13
HSZ0341	Chives	14 ± 1	9 ± 1	98 ± 9
HSZ0509	Chives	14 ± 2	7 ± 1	82 ± 9
Mean	…	13.2[a]	8.4	89.4[a]
Middle East				
YA8884	Wild leek	11 ± 1	7 ± 1	62 ± 7
YA8893	Wild leek	10 ± 1	8 ± 1	67 ± 1
Mean	…	10.5[a]	7.5	64.5[a]

[a] Means for North American and Middle Eastern collections are significantly different ($P < 0.05$) using a one-tailed *t* test.

bootstrap analysis (100%). Collections from cultivated garlic and leek had identical sequences, whereas a collection from wild leek (YA8827) had a single change. Together, the two *P. allii* clusters formed a group that was closely related and well supported (92% bootstrap value). Blast analysis of ITS sequences of *P. allii* identified the barley leaf rust (*P. hordei*) and its correlated species (*U. scillarum*) as the closest matches (data not shown). This was supported by the parsimony analysis showing that *P. allii* is more closely related to *P. hordei* and *U. scillarum* than to *P. triticina* or other rust fungi infecting grasses or Liliaceous hosts (*P. asparagi*, *P. coronata*, *P. graminis*, *P. striiformis*, or *U. gagae*) (data not shown).

DISCUSSION

In the late 1990s, an outbreak of rust disease, caused by *P. allii*, devastated the garlic crop in CA. This epidemic was unusual, in that rust had not been a disease problem of cultivated garlic (28,38). Data presented here demonstrate that the *P. allii* samples collected from garlic and chives in CA and OR represent a different species than the samples collected from the Middle East. This separation is based on teliospore and basidiospore size, substomatal vesicle morphology, life cycle biology, and nuclear ribosomal ITS DNA sequences.

Differences in teliospore size provided a reliable means to differentiate between the North American and Middle Eastern collections of *P. allii*. Compared with collections from the Middle East, teliospores from CA and OR collections were significantly smaller in length, width, and projected cross-sectional area (Table 2). In the subgroups that Gäumann (16) had described within the European *P. allii* complex, teliospores of *P. porri* (=*P. mixta*) were somewhat smaller in length and width (32 to 38 by 17 to 24 μm) than those of *P. allii* (45 to 60 by 15 to 26 μm). This is similar to the differences in teliospore dimensions that we observed between North American (45 to 53 by 16 to 21 μm) and Middle Eastern (50 to 65 by 21 to 29 μm) *P. allii* collections (Table 2).

Another characteristic that separated the North American and Middle Eastern collections was substomatal vesicle (SSV) morphology. SSVs of *P. allii* from CA and OR collections were two-celled, fusiform, and oriented parallel to the leaf surface (Fig. 5B). In contrast, SSVs from Middle Eastern collections were one-celled, bulbous, and oriented perpendicular to the leaf surface (Fig. 5C). Davis and Butler (13), who were the first to examine infection structures of *P. allii,* found bulbous SSVs in collections from leek similar to those we observed in Middle Eastern collections. In a subsequent study, Niks and Butler (32) likewise reported single-celled, bulbous SSVs in collections from *A. porrum* (Table 5). In addition, they found the two-celled fusiform type (similar to the type we found in CA and OR collections) in European collections from *A. schoenoprasum* (chives), *A. ampeloprasum* (wild leek), *A. fistulosum*, *A. roylei*, *A. scorodoprasum*, and *A. vineale.*

The division of *P. allii* into two different groups based on SSVs supported the findings of Jennings et al. (25), in which principal component analysis of six quantitative characteristics of urediniospores was used to divide *P. allii* into two groups, leek and nonleek. The characteristic that was most informative in their study was urediniospore spine density. Leek types had spine densities of 16 to 18 spines per 100 μm^{-2}, whereas nonleek types had 22 to 26 per 100 μm^{-2}. The leek type samples were collected from cultivated leek (*A. porrum*) and the nonleek type samples were collected from *A. schoenoprasum*, *A. babingtonii*, and *A. scorodoprasum.* Jennings et al. (25) suggested that the leek type should be *P. allii* and the nonleek type be *P. mixta* or *U. ambiguus* (Table 5).

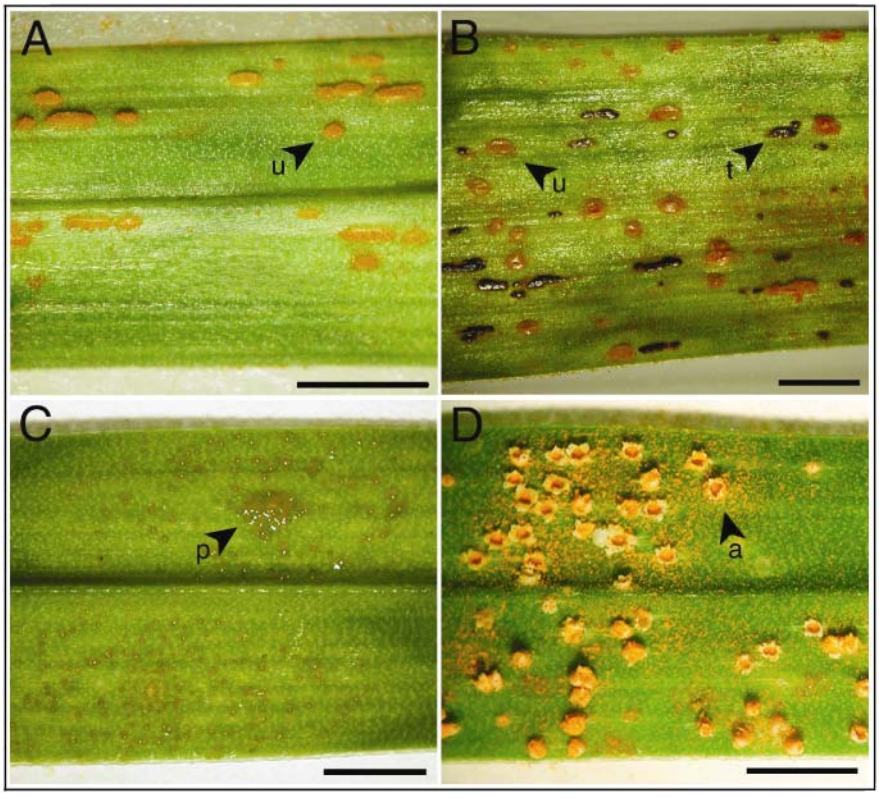

Fig. 4. Stages of life cycle produced by North American and Middle Eastern collections of *Puccinia allii* on host tissues. **A to B,** North American collection HSZ0343 on *Allium sativum* (garlic); **A,** uredinia (u), 12 days after inoculation with basidiospores; **B,** telia (t) and uredinia, 22 days after inoculation with basidiospores (neither pycnia nor aecia were produced). **C to D,** Middle Eastern collection YA8888 on *A. ampeloprasum* (wild leek); **C,** pycnia (p), 12 days after inoculation with basidiospores; **D,** aecia (a), produced 10 days after nectar from several pycnia clusters (as in **C**) was intermixed. Aeciospores from these aecia used to inoculate plants produced uredinia by 12 days and telia by 22 days after inoculation (data not shown). Bars = 2 mm (**A, B, and D**) and 1.5 mm (**C**).

DNA sequence analysis of the nuclear ITS region also separated *P. allii* into two distinct clusters, one containing collections from North America (CA and OR) and the second collections from the Middle East and Europe (Fig. 6). Each of these clusters was well supported by bootstrap analysis and correlated with differences in teliospore dimensions, nuclear condition of the promycelium, and substomatal vesicle morphology as discussed here, indicating that these two populations of rust on *Allium* spp. are distinct species. This is consistent with the separation of *P. allii* into leek and nonleek types.

The subdivision of *P. allii* into leek and nonleek types generally is supported by host inoculation studies in our investigation. The North American isolates collected from garlic and chives did not infect wild leek, whereas the Middle Eastern collections did. A more extensive host range study showed that *P. allii* collections from CA would infect garlic, onion (*A. cepa*) and chives, but not *A. porrum*, *A. ampeloprasum*, or *A. cepa* var. *ascalonicum* (shallot) (28). Similar results were observed by Jennings et al. (26), in which samples collected from leek did not infect chives and samples collected from chives and *A. babingtonii* did not infect leek (*A. porrum*). However, there were a few exceptions (e.g., one isolate from chives infected one cultivar of leek) (26). Furthermore, some hosts (*A. sativum*, *A. babingtonii*, *A. vineale*, *A. cepa*, and *A. fistulosum*) were susceptible to both leek and nonleek types of *P. allii* (26). This range of susceptibility of some *Allium* hosts has been a source of confusion in defining species in the *P. allii* complex.

Collections of *P. allii* from North America have been reported to lack pycnia and aecia (11,28,38). We confirmed these observations in greenhouse tests with respect to collections from CA and OR. Plants exposed to germinating teliospores from these collections developed uredinia but no pycnia or aecia, whereas inoculations with collections from the Middle East produced pycnia and aecia prior to uredinia (Fig. 4). Examination of germinated teliospores from CA showed that these collections produced a two-celled promycelium, instead of the usual four-celled promycelium as seen with Middle Eastern collections (Figs. 1 and 2). As a result, each of the cells of the promycelium contained two nuclei (Fig. 2B) and produced tetra-nucleate basidiospores (Fig. 3A). These basidiospores, in turn, were greater in length and projected cross-sectional area than were basidiospores of collections from the Middle East (Table 4).

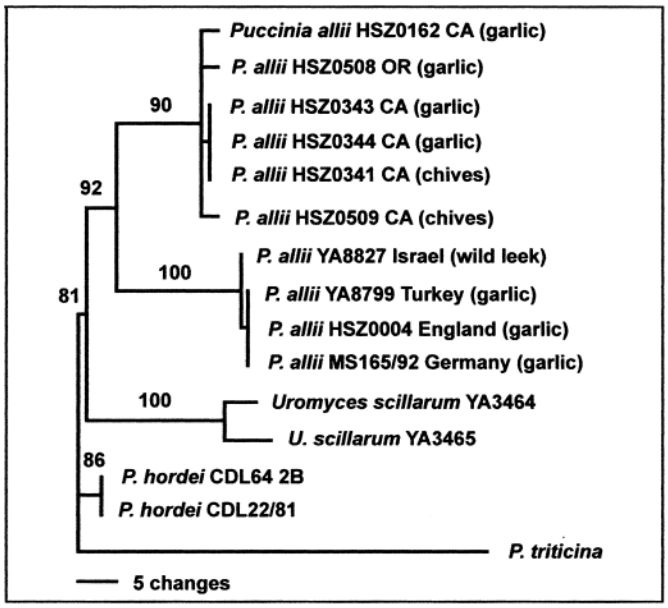

Fig. 6. Parsimony tree from analysis of nuclear ribosomal internal transcribed spacer (ITS) sequence data for *Puccinia allii* and closely related rust species. Aligned DNA sequences of 812 characters included the complete ITS region (ITS1, 5.8S, and ITS2). An ITS DNA sequence from *P. triticina* was used as an outgroup. Parsimony analysis resulted in three optimal trees, each with a tree length of 111 steps, CI = 0.9550, HI = 0.0450, and RI = 0.9038, one of which is shown. Numbers above branches indicate percentage of congruent clusters in 1,000 bootstrap trials. Nucleotide sequence data have been submitted as GenBank accession numbers AF511073 to AF511080, AF511085 to AF511087, AY187087, AY187089, AY187090, and AY302495.

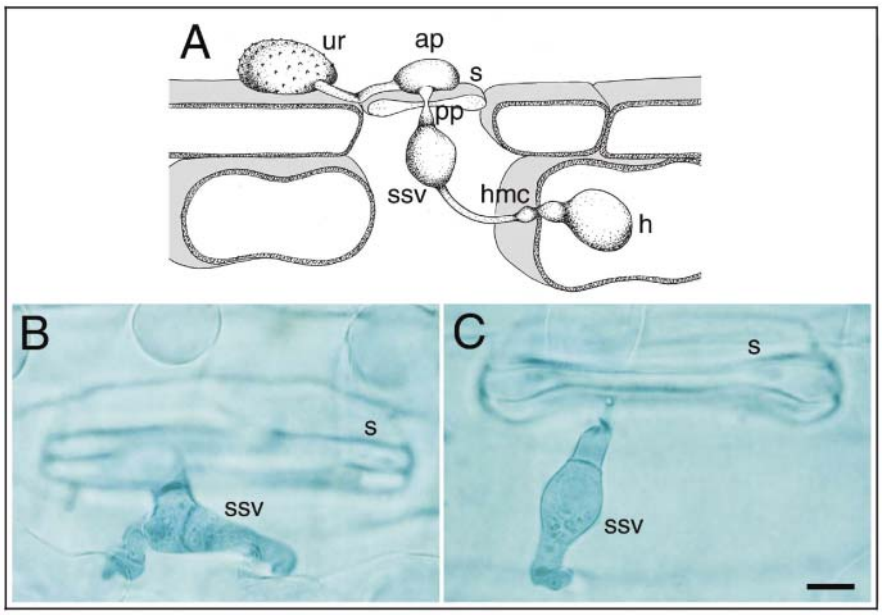

Fig. 5. Substomatal vesicles (SSVs). **A,** Position of the SSV in the sequence of infection structures produced by germinating urediniospores. The urediniospore (ur) produces an appressorium (ap), a penetration peg (pp) which traverses the opening of a stomate (s), the SSV, followed by a haustorial mother cell (hmc), and a haustorium (h). **B,** SSV produced by North American collection HSZ0163 of *Puccinia allii* from garlic; the SSV is fusiform, two-celled, and is oriented parallel to the long axis of the stomate (s); a hypha is produced at each of the two ends of the SSV (only traces of these hyphae show). **C,** SSV produced by Middle Eastern collection YA8885 of *P. allii* from wild leek; the SSV is globular, one-celled, and extends into the substomatal cavity perpendicular to the long axis of the stomate(s); a single infection hypha is produced at the end of the SSV, distal from the stomate. Bars = 10 µm.

Allium rusts that lack pycnia and aecia have been observed before in Europe (16,39), Japan (23), and the United States (11,28, 38). Tranzschel (36) was the first to experimentally demonstrate this shortened life-cycle form by inoculating *A. schoenoprasum* with *P. allii* telia from the same host, which resulted in the formation of uredinia and telia, but not pycnia and aecia. Although some were skeptical of these experiments, suggesting that the telia were contaminated with urediniospores (11), the results reported here conform to the work of Tranzschel (36).

Two-celled promycelia and tetra-nucleate basidiospores have been observed in several rust fungi, including *P. coronata* f. sp. *bromi* (5), *P. horiana* (27), *P. mesnieriana* (10), *U. christensenii* (7), *U. oliveirae* (2), and *U. viennot-bourginii* (6). The basidiospores apparently are self-fertile (homothallic) as demonstrated experimentally for *U. christensenii* (7), *P. mesnieriana* (10), and *U. viennot-bourginii* (6). We postulate, therefore, that our North American collections of *P. allii* are likewise homothallic, with mating types as shown in Figure 2. At present, the exact mechanism is unclear by which homothallic rusts produce two cells in the promycelium, each containing both mating types, leading to a dikaryotic state. In the near future, DNA probes to the mating type loci for rust fungi should be available and may help resolve this issue.

Homothallism in rust fungi occurs in three different types of life cycles in which tetra-nucleate basidiospores are produced. These include demicyclic rusts (as mentioned earlier), which lack pycnia, as in the heteroaecious of *P. coronata* f. sp. *bromi* (5); rusts which form only telia and promycelia are produced, as in microcyclic *P. mesnieriana* (10); and the hemicyclic rusts, which lack pycnia and aecia (16), as in North American *P. allii* from garlic as characterized in this investigation. However, in many reviews of rust life cycles, the hemicyclic form is overlooked (12,22).

Considerable variation in telia morphology was observed with respect to the leek and nonleek groups in our investigation (Table 5). Both groups contained a range among collections in prevalence of dark-brown paraphyses and mesospores. For example, the North American collections from garlic contained many dark-brown paraphyses and only a few mesospores, characteristics that have been associated with leek types (16,25). Furthermore, despite these morphological differences among North American *P. allii* collections from chives and garlic, the ITS DNA sequences are almost identical. The instability of these characteristics has been noted (25,37) and, given the results presented here, these characteristics should not be considered reliable for taxonomic differentiation of species of Allium rusts.

Teliospore size has been used consistently as a taxonomic characteristic of Allium rusts (11,16), emphasizing the importance of a reliable method for spore size determination. However, this has been problematic in the past due the overlapping ranges in spore size (16,37) and lack of telia in samples of leek rust in Europe (30). We found that the use of digital image analysis increased the speed and accuracy of length and width measurements, reduced subjective bias, and, most importantly, allowed the projected cross-sectional area of spores to be determined directly instead of by approximations calculated from length and width. In addition, we used statistical analysis to establish that differences between North American and Middle Eastern collections were significant, much as had been done earlier by Goto (17–19) for comparison of onion rusts collected in different regions of Japan. Projected cross-sectional area was especially useful for teliospores, often resulting in larger relative differences between collections than either length or width (4,5).

Urediniospore morphology was not useful in distinguishing between the North American and Middle Eastern collections of *P. allii*. We found no significant differences in urediniospore dimensions or germ pore number (Table 3). Jennings et al. (25) likewise found no significant differences in dimensions among urediniospore collections from several *Allium* hosts. In preliminary studies of urediniospore spine density (data not shown), we found no differences between CA-OR and Middle Eastern collections (16 to 18 per 100 μm^{-2}). These results are contrary to those of Jennings et al. (25), in which urediniospore spine density separated *P. allii* into leek and nonleek types. Additional data needs to be obtained to confirm these observations and to determine the relative usefulness of urediniospore density as a taxonomic characteristic.

Phylogenetic analysis demonstrated that *P. allii* is closely related to the macrocyclic heteroecious leaf rust fungus, *P. hordei* (Fig. 6), which has an alternate host in the family *Alliaceae*, and also is related to a correlated microcyclic rust, *U. scillarum* which occurs on a Liliaceous host (1,8,9). These results are in line with previous work (34) showing a close phylogenetic relationship between the North American barley leaf rust *U. hordeinus* and a *P. allii* collection from *A. cernuum*. Preliminary DNA analysis of North American rust samples collected from *A. canadense* and *A. cernuum* indicated that these rusts represent species distinctly different from the two groups of *P. allii* analyzed in this study and are more closely related to *U. hordeinus* than to *P. hordei* (data not shown).

The data presented here clearly indicate that the rust fungi collected from garlic and chives in North America represents a

TABLE 5. Telia and substomatal vesicle morphology of the *Puccinia allii* complex[a]

Source	Species	Host	Telia		Substomatal vesicles
			Paraphyses	Mesospores (%)	
Nonleek type					
Present study	*P. allii*	*Allium sativum*	Many dark	1–3	Fusiform
(North American)		*A. schoenoprasum*	Few pale	32–38	Fusiform
Jennings et al./Niks & Butler[b]	*P. mixta*	*A. vineale*	Many dark	27	Fusiform
(European)		*A. schoenoprasum*	Few pale	94	Fusiform
	Uromyces ambiguus	*A. scorodoprasum*	None	100	Fusiform
Gäumann (16)	*P. porri*	*A. ampeloprasum*[c]	Few	>50	nd
(European)		*A. schoenprasum*[c]	…	…	…
Leek type					
Present study	*P. allii*	*A. sativum*	Many dark	1–3	Bulbous
(Middle Eastern)		*A. ampeloprasum*	Many dark	1–3	Bulbous
		A. porrum	Few dark	1–3	Bulbous
Jennings et al./Niks & Butler[b]	*P. allii*	*A. porrum*	nd	nd	Bulbous
(European)		…	…	…	
Gäumann (18)	*P. allii*	*A. pulchellum*[c]	Many dark	<50	nd
(European)		*A. sphaerocephalum*[c]	…	…	…

[a] nd = Not determined.
[b] Combined data from common isolates used by Jennings et al (25) and Niks and Butler (32).
[c] Representative hosts that were used to distinguish between different members of species complex.

different species of Allium rust than the samples from the Middle East. In addition, based on SSV morphology and limited host range, these two groups are consistent with the leek and nonleek types as defined by Jennings et al. (25). Currently, we are expanding our analysis to include additional samples of Allium rust from other hosts and geographical locations as well as from type collections. Therefore, we feel that it is important to await the completion of an expanded study of Allium rusts before classifying the two groups taxonomically and potentially adding more confusion to the *P. allii* complex.

ACKNOWLEDGMENTS

We thank S. Dobler, T. McGovern, J. J. Nunez, J. Rava, and H. J. Scheck for their assistance; M. Scholler and G. Wells for providing samples of *Puccinia allii*; J. Morrison for graphical assistance; K.-P. Nguyen for technical assistance; and K. Leonard and two anonymous reviewers for comments on the manuscript.

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