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Final Report

Classification of the onion rust complex and development of rapid diagnostic assays

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Content

Summary

Rust diseases, caused by fungi in the genus *Puccinia*, are considered the most serious biosecurity threats to the Australian bulb onion industry. However, there is decades-old confusion about the taxonomy of the onion rusts, mainly because many of the economically-important species were described in the 19th century in Europe, and morphologies were not described in sufficient detail, type specimens were not lodged, and the hosts were sometimes not identified to species level. In the absence of a workable classification scheme, it is impossible to implement any kind of a biosecurity plan in Australia for exotic onion rusts. Rust diseases already occur here on close relatives of the bulb onion but at the beginning of this project, it was unclear as to the precise identity of these fungi and how they could be differentiated from exotic onion rust species, if in fact there were any.

During the course of this project, a large panel of rust specimens from *Allium* species in Australia and overseas were assembled and the fungi classified using DNA sequence data, morphological features and host range. Six fungal species were recognized, but it was only possible to confidently assign names to two, namely *Puccinia porri* and *Puccinia mixta*, for leek and chive-infecting isolates, respectively. Three rust species resembled previous descriptions of *Puccinia allii*, but given the ambiguity in what constituted *P. allii* in the strictest sense, a conservative taxonomic approach was taken and the species informally named *P. allii sensu* Gäumann, *P. allii sensu* Koike and *P. allii sensu lato*. The final species that was described was only distantly related to the previous five, and was represented by two specimens of garlic chives from Thailand and the Philippines. Of the three *P. allii*-like species, two were present in Australia, and the third, *P. allii* sensu Koike, only recorded from California, where it caused major yield losses in garlic crops in 1989-90. It was demonstrated that *P. mixta* had been recorded once in Australia, in 1971, but likely failed to establish given the lack of subsequent records. *P. porri* was common in Europe, but our evidence suggested it was absent from Australia.

To aid in identification of *P. porri*, a diagnostic PCR assay was developed and demonstrated to be specific for its target. An attempt was also made to develop a similar assay for *P. allii sensu* Koike. However, we were unable to locate preserved specimens of this species from California, and in contemporary surveys, only *P. porri* was found.

We were unable to find any specimens of rust disease on bulb onion, either from Australia or overseas, and in a literature search, there were no recent records of disease on this plant species. We conclude that the risk posed to the Australian bulb onion industry by exotic rust species has likely been overstated in the Industry Biosecurity Plan.

Keywords

Onions, *Allium*, rust disease, *Puccinia porri*, *Puccinia allii*, molecular systematics, taxonomy, phylogeny, classification, diagnostics, rust disease.

Introduction

Rust disease, caused by *Puccinia allii* and its relatives, is considered the most important plant pest threat to the Australian onion industry (Anonymous 2012). This assessment is based on several criteria including entry potential, establishment potential, spread potential and economic impact, for which the risk rating for onion rust is either high or extreme. As an example of the economic impact of this disease, rust epidemics in 1999 and 2000 led to a reduction of garlic production in California by almost 90% (Anikster *et al*., 2004). Infection by onion rust is favoured by cool, wet conditions and the emergence of rust as a serious disease in California coincided with a La Niña weather event, which brought record-high rainfall and long periods of cool temperatures (Koike et al. 2001a).

Control of onion rust is based on a multipronged approach, including the use of clean seed, the reduction of inoculum levels (removal of diseased crop residue, infected volunteer plants, older diseased plantings and weedy onion relatives) and when environmental conditions are favourable for disease development, multiple applications of fungicides such as tebuconazole and azoxystrobin (Koike et al. 2001b; Schwartz and Mohan 2008). Some useful sources of resistance have been identified in *Allium* germplasm and in the future this could be incorporated into commercial onion cultivars through plant breeding although the genetics of resistance have not been determined (Fernández-Aparicio et al. 2011).

The taxonomy of the rusts on *Allium* ssp. is in a state of flux and a variable number of species are recognized by different authorities: eight species are listed in the Australian biosecurity target list. What seems likely is that there are at least two species of onion rust in the world, one from Europe and the Middle East and one from western USA, based on genetic and host range differences (Anikster et al. 2004). However, the authors of this study stopped short of proposing new species until a more comprehensive study of the *Allium* rusts had been completed. Part of the difficulty associated with classification of the onion rusts is that their life cycles are shortened (hemicyclic) in North America, Japan and Europe (hemicyclic) and only two spore types, uredinia and telia, are observed (Anikster et al. 2004; Schwartz and Mohan 2008). Special emphasis has been given to teliospore size in recognizing the different onion rust species but overlapping size ranges are observed and teliospores are not always present such as on leek rust samples from Europe (Anikster *et al*., 2004). The uredinial stage, which is the prevalent, cyclic stage in the rust life cycle, is often found, but is not useful in distinguishing between the known rusts on *Allium*. A form of onion rust is found in NSW, Queensland, Tasmania and Victoria (Anonymous 2012) but it is not known how this fungus relates to those from overseas, or whether in fact there is more than one species in Australia.

For the Australian quarantine system to be effective and for control options to be successful, it is essential to properly resolve the taxonomy of the onion rusts. The correct label must be put on a pathogen in order to know what its geographic distribution or host range may be or whether a fungicide or plant resistance gene will be efficacious. DNA barcoding and other molecular markers offer a rapid and accurate means to identify fungi and other organisms in comparison to traditional, morphological methods (Geering 2012). In this project, the diversity of onion rust species in Australia has been analyzed and a classification scheme devised. *Puccinia porri*, a common *Allium* rust in Europe and the Middle East, as been determined to be exotic to Australia and a rapid, molecular diagnostic test devised for this species.

Methodology

1. Specimens

Rust specimens used in this study are listed in Table 1. These specimens were either borrowed from the major Australian plant disease herbaria or freshly collected by team members or collaborators; all new specimens have been deposited in the Brisbane Pathology Herbarium, both as dried plant samples and as DNA extracts from the spores. Specimens from overseas were imported under Australian Government Permit IP15001999 and devitalized by gamma irradiation prior to release into the quarantine approved premise at the Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4073.

2. Morphological examinations

Fungal structures were scraped from leaf material, mounted in lactic acid and gently heated to boiling. Preparations were examined with a Leica DMLB microscope and digital images were taken with a Leica DFC500 camera. Measurements were made from the imaged spores.

3. DNA extractions, PCR amplifications and DNA sequencing

Fungal tissue was selectively removed from fresh leaf material with a filtered pipette tip attached to a vacuum pump. DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA). The ITS region was amplified with ITS1F/ITS4B (Gardes and Bruns 1993) and the LSU region was amplified with Rust 2INV/LR6 (Vilgalys and Hester 1990; Aime 2006). PCRs were done with high fidelity Phusion DNA polymerase (New England Biolabs Inc.) using the following thermocycler conditions: initial denaturation step of 1 min at 98 °C; 35 cycles of 10 s at 98 °C, 30 s at 62 °C and 45 s at 72 °C; a final extension step for 5 min at 72 °C. Amplified products were purified and sequenced by Macrogen Korea, Seoul. Sequences were deposited in GenBank with the accession codes provided in Table 1.

4. Phylogenetic analyses

LSU sequences of rusts on Allium and species of *Puccinia* and *Uromyces* representative of the Pucciniaceae were aligned using the MAFFT algorithm (Katoh and Standley 2013) in SATé (Liu et al. 2012). The alignment was examined with two phylogenetic criteria, Bayesian inference and maximum likelihood. MrBayes was used to conduct a Markov Chain Monte Carlo (MCMC) search with Bayesian inference (Ronquist and Huelsenbeck 2003). Four runs, each consisting of four chains, were implemented for 10 million generations. The cold chain was heated at a temperature of 0.25. Substitution model parameters were sampled every 1000 generations and trees were saved every 1000 generations. Convergence of the Bayesian analyses was confirmed using the cumulative and compare functions in AWTY (Nylander et al. 2008) (available at: ceb.csit.fsu.edu/awty/) and 30,000 trees were summarized. Maximum likelihood (ML) was implemented as a search criterion in RAxML (Stamatakis 2014). GTRGAMMA was specified as the model of evolution in both criteria. The RAxML analyses were run with a rapid Bootstrap analysis (command -f a) using a random starting tree and 1000 maximum likelihood bootstrap replicates.

Further phylogenetic analyses were made with an alignment of the ITS and LSU regions. Isolates without these data were excluded from the analyses, except for BRIP 62011, BRIP 62277 and DAR 38182. Partitions were set for the ITS and LSU regions, and run under Bayesian inference and maximum likelihood. Indels of the ITS and LSU regions were scored as present or absent characters that corresponded to 20 homologous sites in the alignment. The indels were deleted

and run as standard characters in Bayesian inference and multistate characters in RAxML analyses as a third partition. The same parameters as above were used for MrBayes and RAxML. The ML analysis included a Markov model for the multistate characters (command -K MK).

Table 1. Host, isolate number, country and GenBank numbers of taxa included in the phylogenetic analyses. GenBank numbers of fresh specimens obtained for this study in **bold** font

Species	Isolate no.	Country	Host	Host Family	ITS/LSU GenBank accession
Ceratocoma jacksoniae	BRIP 57762	Australia	*Daviesia* sp.	Fabaceae	NA/KT199394[1]
P. allii s. Gäumann (1959)	BRIP 61619	Australia	*A. cepa* var. *aggregatum*	Amaryllidaceae	**KU296866/ KU296866**
P. allii s. Gäumann (1959)	BRIP 61620	Australia	*A. cepa* var. *aggregatum*	Amaryllidaceae	**KU296867/ KU296867**
P. allii s. Gäumann (1959)	DAR 25710	Australia	*A. cepa* var. *aggregatum*	Amaryllidaceae	**KU296868**/NA
P. allii s. Gäumann (1959)	DAR 67439	Australia	*A. fistulosum*	Amaryllidaceae	**KU296869/ KU296869**
P. allii s. Gäumann (1959)	BRIP 61618	Australia	*A. sativum*	Amaryllidaceae	**KU296870/ KU296870**
P. allii s. Gäumann (1959)	BRIP 62277	Australia	*A. sativum*	Amaryllidaceae	**KU296871/ KU296871**
P. allii s. Koike et al. (2001)	HSZ0162	USA	*A. sativum*	Amaryllidaceae	AF511077/AF511077[2]
P. allii s. Koike et al. (2001)	HSZ0343	USA	*A. sativum*	Amaryllidaceae	AF511076/AF511076[2]
P. allii s. Koike et al. (2001)	HSZ0344	USA	*A. sativum*	Amaryllidaceae	AF511078/AF511078[2]
P. allii s. Koike et al. (2001)	HSZ0508	USA	*A. sativum*	Amaryllidaceae	AF511075/AF511075[2]
P. allii s. Koike et al. (2001)	HSZ0341	USA	*A. schoenoprasum*	Amaryllidaceae	AF511080/AF511080[2]
P. allii s. lat.	BRIP 62279	Australia	*A. fistulosum*	Amaryllidaceae	**KU296872/ KU296872**
P. allii s. lat.	BRIP 62010	China	*A. fistulosum*	Amaryllidaceae	**KU296873/ KU296873**
P. allii s. lat.	BRIP 62011	China	*A. fistulosum*	Amaryllidaceae	**KU296874**/NA
P. allii s. lat.	BRIP 62269	China	*A. fistulosum*	Amaryllidaceae	**KU296875/ KU296875**
P. allii s. lat.	BRIP 62271	China	*A. fistulosum*	Amaryllidaceae	**KU296876/ KU296876**
P. allii s. lat.	BRIP 62272	China	*A. fistulosum*	Amaryllidaceae	**KU296877/ KU296877**
P. allii s. lat.	BRIP 62273	China	*A. fistulosum*	Amaryllidaceae	**KU296878/ KU296878**
P. allii s. lat.	BRIP 59595	Australia	*A. sativum*	Amaryllidaceae	**KM249849/KM249849**
P. allii s. lat.	BRIP 59655	Australia	*A. sativum*	Amaryllidaceae	**KM249850/KM249850**
P. allii s. lat.	BRIP 59724	Australia	*A. sativum*	Amaryllidaceae	**KM249851/KM249851**
P. allii s. lat.	BRIP 61815	Australia	*A. sativum*	Amaryllidaceae	**KU296879/ KU296879**
P. allii s. lat.	BRIP 61962	Australia	*A. sativum*	Amaryllidaceae	**KU296880/ KU296880**
P. allii s. lat.	BRIP 61966	Australia	*A. sativum*	Amaryllidaceae	**KU296881/ KU296881**
P. allii s. lat.	VPRI 41965	Australia	*A. sativum*	Amaryllidaceae	**KU296882/ KU296882**
P. allii s. lat.	BRIP 62270	China	*A. sativum*	Amaryllidaceae	**KU296883/ KU296883**
P. boroniae	BRIP 57783	Australia	*Chorilaena quercifolia*	Rutaceae	NA/**KU296884**
P. cyperi	BRIP 60997	Australia	*Cyperus iria*	Cyperaceae	NA/**KU296885**
P. graminis	BRIP 60137	Australia	*Glyceria maxima*	Poaceae	NA/**KM249852**
P. haemodorii	BRIP 57767	Australia	*Anigozanthus* sp.	Haemodoraceae	KF690675/KF690693[3]
P. hemerocallidis	BRIP 53476	Australia	*Hemerocallis* sp.	Hemerocallidaceae	**KM249855/KM249855**
P. heterospora	BRIP 60937	Australia	*Abutilon* sp.	Malvaceae	NA/**KU296886**

P. iridis	BRIP 56852	Australia	*Iris* sp.	Iridaceae	NA/**KM249853**
P. kuehnii	BRIP 59200	Australia	*Saccharum officinarum*	Poaceae	NA/**KU296887**
P. lagenophorae	BRIP 57563	Australia	*Emilia sonchifolia*	Asteraceae	NA/KF690696[3]
P. malvacearum	BRIP 57522	Australia	*Malva parviflora*	Malvaceae	NA/**KU296888**
P. melanocephala	BRIP 59199	Australia	*Saccharum officinarum*	Poaceae	NA/**KU296889**
P. menthae	BRIP 59667	Australia	*Mentha spicata*	Lamiaceae	NA/**KU296890**
P. mixta	DAR 38182	Australia	*A. schoenoprasum*	Amaryllidaceae	**KU296891**/NA
P. mixta	BRIP 61581	England	*A. schoenoprasum*	Amaryllidaceae	**KU296892/KU296892**
P. mixta	BRIP 61576	Germany	*A. schoenoprasum*	Amaryllidaceae	**KU296893/KU296893**
P. mixta	BRIP 61577	Germany	*A. schoenoprasum*	Amaryllidaceae	**KU296894/KU296894**
P. mixta	BRIP 61589	Germany	*A. schoenoprasum*	Amaryllidaceae	**KU296895/KU296895**
P. mixta	BRIP 61556	Netherlands	*A. schoenoprasum*	Amaryllidaceae	**KU296896/KU296896**
P. mixta	HSZ0509	USA	*A. schoenoprasum*	Amaryllidaceae	AF511087/AF511087[2]
P. myrsiphylli	BRIP 57782	Australia	*Asparagus asparagoides*	Asparagaceae	NA/**KM249854**
P. porri	BRIP 61586	England	*A. ampeloprasum*	Amaryllidaceae	**KU296897/KU296897**
P. porri	BRIP 61575	Germany	*A. ampeloprasum*	Amaryllidaceae	**KU296898/KU296898**
P. porri	BRIP 61588	England	*A. fistulosum*	Amaryllidaceae	**KU296899/KU296899**
P. porri	BRIP 61590	Germany	*A. fistulosum*	Amaryllidaceae	**KU296900/KU296900**
P. porri	BRIP 61570	Albania	*A. porrum*	Amaryllidaceae	**KU296901/KU296901**
P. porri	BRIP 61579	England	*A. porrum*	Amaryllidaceae	**KU296902/KU296902**
P. porri	BRIP 61580	England	*A. porrum*	Amaryllidaceae	**KU296903/KU296903**
P. porri	BRIP 61583	England	*A. porrum*	Amaryllidaceae	**KU296904/KU296904**
P. porri	MS165/92	Germany	*A. porrum*	Amaryllidaceae	AY187090/AY187090[2]
P. porri	BRIP 61573	Germany	*A. porrum*	Amaryllidaceae	**KU296905/KU296905**
P. porri	BRIP 61591	Germany	*A. porrum*	Amaryllidaceae	**KU296906/KU296906**
P. porri	BRIP 61592	Germany	*A. porrum*	Amaryllidaceae	**KU296907/KU296907**
P. porri	BRIP 61567	Greece	*A. porrum*	Amaryllidaceae	**KU296908/KU296908**

P. porri	BRIP 61571	Montenegro	*A. porrum*	Amaryllidaceae	**KU296909/KU296909**
P. porri	HSZ0004	England	*A. sativum*	Amaryllidaceae	AF511079/AF511079[2]
P. porri	YA8799	Turkey	*A. sativum*	Amaryllidaceae	AF511073/AF511073[2]
P. porri	YA8827	Israel	*A. ampeloprasum*	Amaryllidaceae	AF511074/AF511074[2]
P. psidii	BRIP 58517	Australia	*Myrtus communis*	Myrtaceae	KF318430/KF318447[4]
P. stylidii	BRIP 60107	Australia	*Stylidium armeria*	Stylidiaceae	NA/KJ622215[3]
P. ursiniae	BRIP 57993	Australia	*Ursinia anthemoides*	Asteraceae	NA/KF690705[3]
Puccinia sp.	BRIP 62274	Thailand	*A. tuberosum*	Amaryllidaceae	**KU296910/KU296910**
Puccinia sp.	BRIP 61458	Philippines	*A. tuberosum*	Amaryllidaceae	**KM249856/KM249856**
Puccinia sp.	BRIP 56915	Australia	*Smilax australis*	Smilacaceae	**KM249857/KM249857**
Puccinia sp.	BRIP 59643	Australia	*Smilax australis*	Smilacaceae	**KM249858/KM249858**
Uredo dianellae	BRIP 57433	Philippines	*Dianella javanica*	Hemerocallidaceae	NA/**KM249859**
Uredo geitonoplesii	BRIP 57603	Australia	*Geitonoplesium cymosum*	Hemerocallidaceae	NA/**KM249861**
Uromyces appendiculatus	BRIP 60020	Australia	*Phaesolus vulgaris*	Fabaceae	NA/KM249870[5]
Uro. appendiculatus var. *crassitunicatus*	BRIP 60929	Australia	*Macroptilium atropurpureum*	Fabaceae	NA/**KU296911**
Uro. lomandracearum	BRIP 59022	Australia	*Lomandra* sp.	Asparagaceae	NA/**KM249862**
Uro. transversalis	BRIP 59244	Australia	*Gladiolus* sp.	Iridaceae	NA/**KM249864**
Uro. vicia-fabae	BRIP 59246	Australia	*Vicia faba*	Fabaceae	NA/**KM249865**

N/A not applicable

[1]McTaggart et al. (2016); [2]Anikster et al. (2004); [3]McTaggart et al. (2014a); [4]Pegg et al. (2014); [5]McTaggart et al. (2014b)

5. Development of diagnostic PCR assays for *Puccinia porri*

To generate new DNA sequence data for uncharacterized regions of the onion rust genomes, gene sequences of related rust fungi (*Puccinia graminis f.sp. tritici*, *P. striiformis f.sp. tritici*, *P. triticina*, *P. sorghi*, *Melampsora larici-populina*, *Uromyces viciae-fabae* and *Cronartium ribicola*) were extracted from the published genomes of these fungi, the amino sequences aligned using the MUSCLE algorithm in MEGA6, and then back translated to give the DNA sequence alignments. Conserved regions in the alignment were then identified either by eye or using the program GPRIME, and chosen for PCR primer design (Table 2). PCR amplifications were done using Bioline MyTaq Hot Start DNA polymerase as per the manufacterer's instructions and the amplicons directly sequenced at Macrogen using the amplification primers.

Table 2. Universal PCR primers used to amplify uncharacterized regions of the onion rust genomes

Target gene	Primer name	Primer sequence[1]
AGC/PKC protein kinase	AGC-PKC-F1	TGGTGYTGYCATTGTGGRATG
	AGC-PKC-R1	AAGTT4CCYTTACCYAAGACAGC
Bestrophin	Bestrophin-F1	TAYGG4ATMGAYTATGAAGA
	Bestrophin-R1	GTRCAYTGYTTGAGRTGRAT
Ca2+:Cation Antiporter	Ca2antiporter-F1	TTTGG4AACGCAGTYGAAGC
	Ca2antiporter-R1	AC4CCRATAGC4AGYTCCAT
Fatty acid synthase	FAS-F1	TGYTGRCCTTCGACGTTGAA
	FAS-R1	ACSATYACKCAYGG4ATGTGGTC
Phospholipase C	PLC-F1	GAYTGYTGGGATGGAGAT
	PLC-R1	TCYGAATCTGAACTSGTAGATGA
Utp14 protein	UTP14-F1	ATGGCCAGAGAACTGATGTT
	UTP14-R1	CARTCTACCCGGRTTTCC

[1]4=deoxyinosine.

From the Ca2+:Cation Antiporter gene sequences, the primers Ca2-F2 (TTTATCTCTTAACTAACCACTT) and and Ca2-R3 (GCTGTATGTGTAACGCAGAYA) were used in a PCR assay to identify *P. porri*. PCR amplifications were done using Bioline MyTaq Hot Start DNA polymerase as per the manufacterer's instructions and thermocycling conditions comprised an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 30 s at 95 °C, 30 seconds at 51 °C, and 1 minute at 72 °C, with a final extension at 72 °C for 5 minutes. As a control to ensure there was amplifiable DNA, the primers Ca2-F (GCCTTTATCTCTTAACTAACC) and Ca2-R (CAGCTGTATGTGTAACGC) were used.

Outputs

1. Classification scheme for the onion rusts from Australia and overseas.

DNA sequences from the ITS and LSU loci, which are standard DNA barcodes for rust fungi in the family Pucciniaceae, were obtained from 54 rust specimens originating from various parts of the world, including Australia (South Australia, Tasmania, Victoria, NSW and Queensland), Europe, the Middle East, USA, China, Thailand and the Philippines (Table 1). Host species from Australia and overseas included shallot (*A. cepa* var. *aggregatum*), garlic (*A. sativum*), Welsh onion (*A. fistulosum*) and *A. schoenoprasum* (chives). Additionally, rust specimens from leek (*P. porri*) and garlic chives (*A. tuberosum*) were collected overseas. A much broader assemblage of rust specimens from Australia, dating back to 1971, were available for morphological examination but PCR amplifiable DNA was generally only obtainable from specimens that were five years old or younger. We found no records of rust on bulb onion (*A. cepa* var. *cepa*) following an interrogation of the Australian Plant Disease Database and from an associated literature survey, nor did we find any

evidence of infection of crops from field surveys during this project.

The onion rust species examined in this study were polyphyletic in the Pucciniaceae based on the LSU region (Fig. 1). Most of the rusts on *Allium*, including all of the Australian specimens, formed a well-supported clade within Pucciniaceae group 2 sensu Dixon et al. (2010). These rusts occurred in five clades, which were recovered in identical topologies by maximum likelihood and Bayesian inference based on the ITS and LSU regions of rDNA (Fig. 3). These five clades corresponded to (i) rusts on *A. porrum*, *A. ampeloprasum* and *A. fistulosum* from Europe, considered *Puccinia porri*; (ii) rusts on *A. fistulosum* and *A. sativum* in Australia and China, considered *P. allii sensu lato*; (iii) rusts on several species of *Allium* used in the study of Metcalf (2002), considered *P. allii sensu* Gäumann (1959); (iv) specimens from the USA on *A. sativum* and *A. schoenoprasum* included in the study by Anikster et al. (2004), considered *Puccinia allii* sensu Koike et al. (2001), and (v) rusts on *A. schoenoprasum* from Europe and one specimen from Australia, considered *Puccinia mixta*. With the exception of *Puccinia porri*, these rusts were differentiated by indels in the ITS2 region and were identical in the LSU region.

Light microscopic studies were also undertaken to determine if there were morphological features of the spores that could be used for identification (Table 2). The percentage of 2-celled teliospores was found to be a useful diagnostic character for the two rust species that currently occur in Australia. However, when considering all clades that were recovered, including species from overseas, the most reliable characters for identification were the degree of thickening of teliospore apex, as well as the host species.

Table 2. Host and morphological characters for differentiation of the five taxa recovered in the present study

Species	Host	Location	Percentage of 2-celled teliospores	Teliospore apex
Puccinia allii s. lat.	*Allium fistulosum*, *A. sativum*	Australia (QLD, SA, TAS,VIC), China	90–95 %	Usually thickened, up to 7 µm
Puccinia allii sensu Koike *et al.* (2001)[1]	*A. sativum*, *A. schoenoprasum*	USA	97–99 % (on garlic) 62–95 % (on chives)	Not thickened
P. allii sensu Gäumann (1959)	*Allium* spp.	Australia (QLD, TAS)	50–60 %	Slightly thickened up to 4.5 µm
P. mixta	*A. schoenoprasum*	Europe	80–90 %	Not thickened
P. porri	*A. ampeloprasum*, *A. fistulosum*, *A. porrum*	Europe	90–95 %	Usually thickened, up to 9 µm

Fig. 1. Phylogram obtained from a Bayesian analysis of the LSU region of rDNA in MrBayes. Posterior probabilities (≥0.95) summarized from 30,000 trees shown above nodes. Bootstrap values (≥70 %) from 1000 replicates in a maximum likelihood search with RAxML shown below nodes.

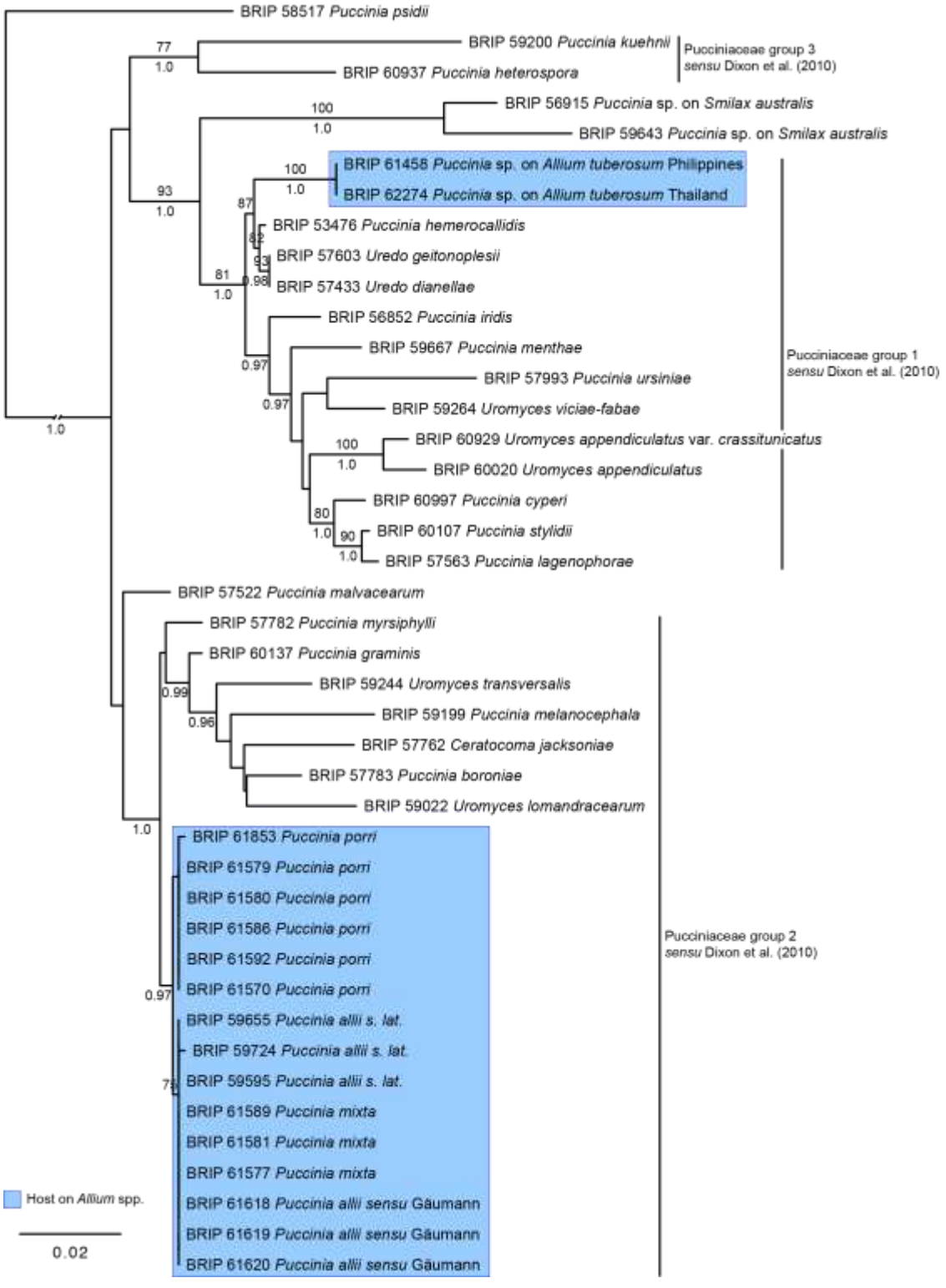
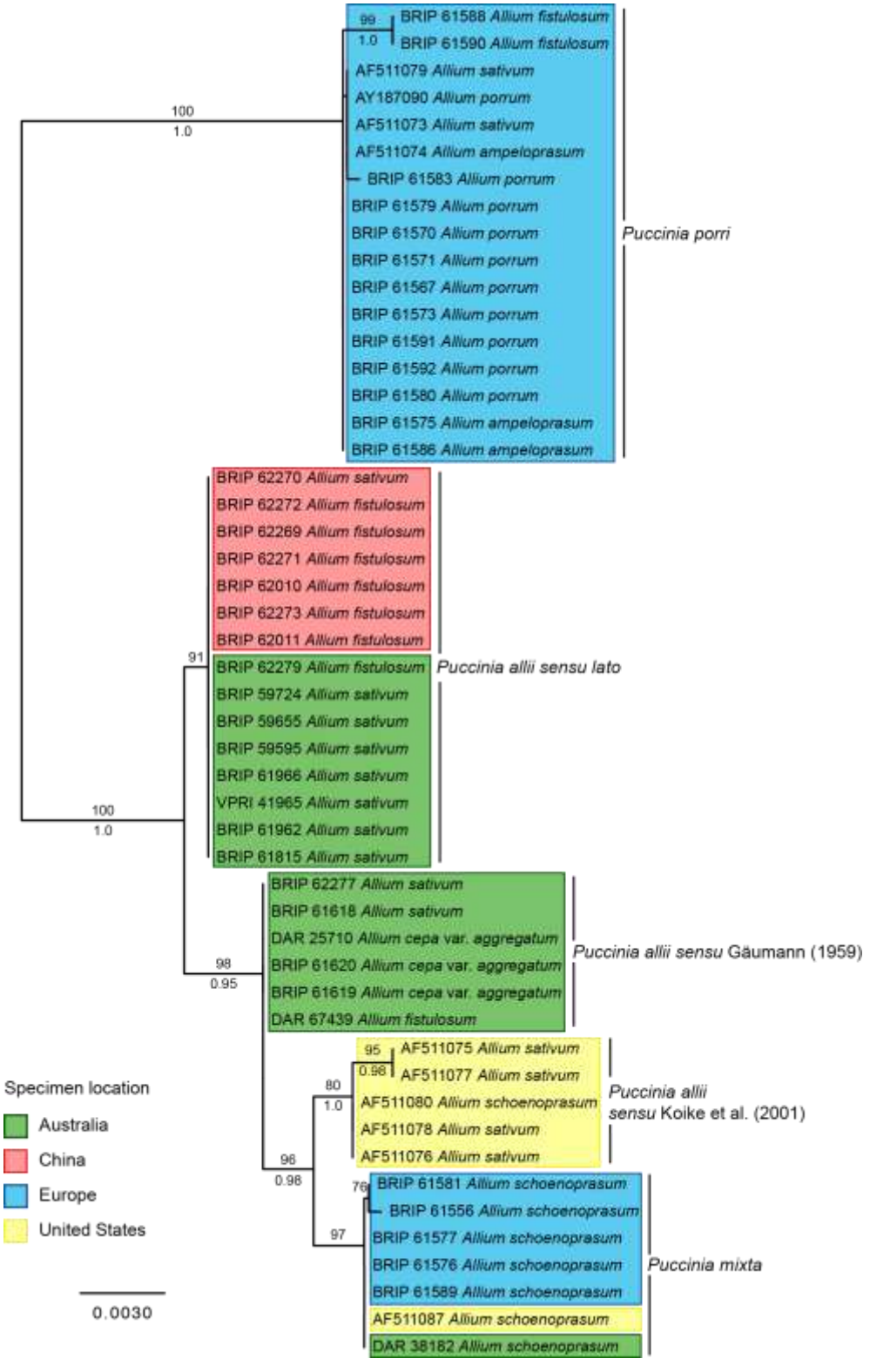


Fig. 2. Phylogram obtained from a maximum likelihood search of the ITS and LSU regions of rDNA, and indels from these regions scored as standard characters in RAxML. Bootstrap values (≥70 %) from 1000 replicates in a maximum likelihood search shown above nodes. Posterior probabilites (≥0.95) summarized from 30,000 trees shown below nodes.



2. Online, diagnostic diagnostic guides for the onion rusts in Australia, to be found at http://collections.daff.qld.gov.au/web/key/rustfungi/Media/Html/pucciniaallii-sl.html and http://collections.daff.qld.gov.au/web/key/rustfungi/Media/Html/pucciniaallii-sg.html.

3. Diagnostic PCR assays to distinguish the exotic *Puccinia porri* from the endemic *P. allii*.

Within the rDNA, the internally transcribed spacer regions 1 and 2 (ITS1 and 2) are the most variable regions for the rust species examined. However, these variable regions are not very useful for PCR primer design, as they are characterized by long strings of cytosine, adenosine or thymine. Alternative, rapidly evolving regions of genes were therefore sought for PCR primer design. The six, single copy genes that were examined were (i) AGC/PKC protein kinase; (ii) Bestrophin; (iii) Ca2+:Cation Antiporter; (iv) Fatty acid synthase; (v) Phospholipase C; (vi) Utp14 protein. To obtain these gene sequences, 'universal' PCR primers were designed by searching for conserved regions in an alignment of sequences extracted from whole genome shotgun libraries of related rust species. The PCR products that were obtained with the onion rusts were then sequenced and compared for polymorphisms. Minor variation (single nucleotide polymorphisms and indels) between *P. porri* and *P. allii* was observed for each gene sequence. However, the two genetic lineages of *P. allii* in Australia, *P. allii sensu Gäumann* and *P. allii sensu lato* were invariant for all genes examined, except for the Ca2+:cation antiporter (CaCA) gene. This gene was therefore targeted for PCR diagnostic assay design and two pairs designed, one for specific detection of *P. porri* (Ca2-F2 and R3) and the second (Ca2-F and R) for detection of all onion rust species (Fig. 4).

The region of the CaCA gene that was PCR-amplified from the *Allium* rust specimens spanned the coding regions for the hypervariable N-terminus of the protein, the first, conserved, α-repeat, the transmembrane spanning domain, and a portion of the second α-repeat (Emery et al. 2012). Comparisons of the mRNA and DNA sequences of this gene in *Puccinia graminis* f. sp. *tritici* suggested the presence of two, short introns, one in the first α-repeat and the second, in the transmembrane spanning domain.

Dried herbarium specimens of the rust fungus that caused such devastation to the Californian garlic industry in 1999/2000, here referred to as *P. allii sensu Koike*, were not available. Hence, Steven Koike (University of California Davis) was approached to collect fresh specimens from California in the hope that it was the same genetic lineage (Table 3). However, to our initial surprise, these specimens tested positive using the *P. porri* diagnostic assay. To investigate whether this was a false positive, due to the non-specificity of the primers, the ITS and LSU regions of the rRNA of the Californian specimens was sequenced, and the specimens confirmed to be *P. porri*, rather than *P. allii sensu Koike*. This result correlates with field observations, as since 2007, a severe rust disease has been afflicting leek crops in California in addition to garlic, suggesting the introduction of *P. porri* (S. Koike, pers. comm.).

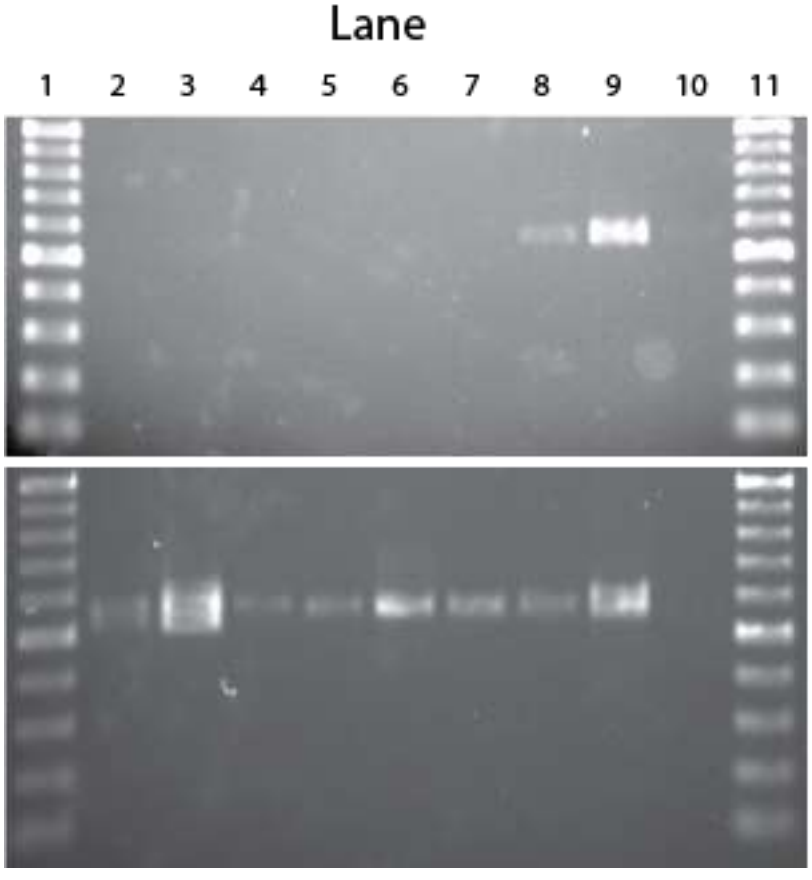
Table 3. Garlic rust specimens from California

Accession	Collection Date	Locality
BRIP 64665	26 Apr 2016	Huron, California, USA
BRIP 64666	26 Apr 2016	Shandon, California, USA
BRIP 64667	26 Apr 2016	Coalinga, California, USA
BRIP 64668	26 Apr 2016	Patterson, California, USA
BRIP 64669	26 Apr 2016	Huron, California, USA
BRIP 64670	19 May 2016	Hollister, California, USA
BRIP 64671	19 May 2016	Hollister, California, USA
BRIP 64672	24 May 2016	San Ardo, California, USA

Fig. 3. Alignments of primer sequences in the Ca2+:Cation antiporter gene. Nucleotides highlighted in red indicate sequence mismatches relative to *Puccinia porri*.

	Forward primer	Reverse primer
P. porri	TTTATCTCTTAACTAACCACTT	GCTGTATGTGTAACGCAGATA
P. allii sensu Gäumann	TTTATCTCTTAACTAACCCCT**C**	GCTGTATGTGTAACGCAGAYA
P. allii sensu lato	TTTATCTCTTAACTAACCCCT**C**	GCTGTATGTGTAACGCAGAT**G**
P. mixta	TTTATCTCTTAACTAACCCCT**C**	GCTGTATGTGTAACGCAGAT**G**

Fig. 4. Detection of *Puccinia porri* by PCR. The upper panel shows results using Ca2-F2 and R3 primers and the lower panel, Ca2-F and R primers. Lanes are: (1) 100 bp marker; (2) *Puccinia allii sensu lato* (BRIP 59724); (3) *P. allii s. lato* (BRIP 59655); (4) *P. allii s.* Gäumann (BRIP 61618); (5) *P. allii s.* Gäumann (BRIP 61620); (6) *P. mixta* (BRIP 61577); (7) *P. mixta* (BRIP 61581); (8) *P. porri* (BRIP 61570); (9) *P. porri* (BRIP 61575); (10) No template; (11) 100 bp marker.



4. Two extension articles about the project were published in the Onions Australia Magazine:

Geering, A., McTaggart, A., Doungsa-ard, C., Shivas, R. (2015) Deciphering the onion rust complex. *Onions Australia Magazine* **2016**, 26-27.

Geering, A., Weese, T., McTaggart, A., Doungsa-ard, C., Shivas, R. (2016). Identifying species of onion rust. *Onions Australia Magazine* **2016**, 24.

Outcomes

The outcomes of this project are as follows:

1. For the first time, an accurate overview of the diversity of onion rusts in Australia has been obtained. It is impossible to implement a biosecurity system without knowledge of which pathogens are already in the country, as this is a premise for judging which are exotic. According to the International Plant Protection Convention, claims of pathogen-freedom must be evidence-based, and without this evidence, quarantine conditions cannot be placed on the import of plants or plant commodities because of unacceptable risks of introducing the pathogen in question.
2. A classification scheme for the onion rusts is provided, which is essential for disease management in Australia. The outbreak of an onion rust in Adelaide in 2013 led to an incursion response by Biosecurity SA. At the time, it was uncertain whether the outbreak was a range extension of the existing rust species that was present in Victoria, NSW and Queensland, or the introduction of a new species altogether. Knowing which of these scenarios existed may have greatly changed the incursion response.
   It is also difficult to collate and extract information from independent scientific studies in Australia or overseas if the pathogen under study is not accurately identified. For example, a 'leek-infecting strain of *Puccinia allii*' was used by Jennings et al. (1990) to screen *Allium* germplasm for utilizable rust resistance. From this project, it can be concluded that the subject of the study was *P. porri*, a species of rust that is exotic to Australia.
3. A method for rapid identification of *P. porri* is presented, which can be used by a molecular diagnostician without specialist training in mycological taxonomy. The discipline of plant pathology in Australia is suffering an erosion in specialist skills: new science graduates may have a general training in molecular biology, but normally do not have more than a few hours theoretical experience in any particular plant pathology discipline. There are less than a handful of scientists in Australia who could expertly identify rust species using morphological features, but many who could follow a PCR method such as that developed in this project.
4. Information has been provide that will allow much more accurate pest risk analyses to be done, which can be incorporated in the next version of the Onion Industry Biosecurity Plan.

Evaluation and discussion

The aims of this project have been successfully achieved, as a classification scheme for the onion rusts in Australia has been devised, as well as diagnostic methods developed to detect the exotic *Puccinia porri*. Prior to this project, there had been much confusion as to what species of Allium rust occurred in Australia, and conversely, what were exotic. Furthermore, there had not been a thorough assessment of the risks posed to the Australian bulb onion industry by onion rusts. The problems of diagnosis were exemplified by the Industry Biosecurity Plan for the Onion Industry, in which it was reported that *P. porri* was the only species of onion rust to occur in Australia, albeit only in the eastern states. Furthermore, *P. mixta* and *P. allii* were reduced to synonyms of *P. porri*. Ironically, the results of this project suggest that *P. porri* is one of the few common species of onion rust in the world that is actually absent from Australia.

Two different genetic lineages of *P. allii* were found in Australia, as well as *P. mixta*, although the latter was represented by a single record from 1971, and does not appear to have established in this country. Whether the two genetic lineages of *P. allii* should be considered strains of the same species or two distinct species, is beyond the scope of this project and may require host range studies and investigations of gene flow between populations using microsatellite or single nucleotide polymorphism markers.

Rust fungi are economically-important pathogens of *Allium* species in some regions, including on *A. sativum* in the USA (Koike et al. 2001a) and Ethiopia (Worku and Dejene 2012), *A. tuberosum* in Taiwan (Ko and Sun 1993), *A. fistulosum* in Japan (Furuya et al. 2009) and *A. porri* in Western Europe. However, there are no verifiable records of major rust epidemics in *A. cepa* var. *cepa* that have been reported in the scientific literature, although the dogma is that this plant species is a natural host (Schwartz and Mohan 2008). Experimental inoculations of *A. cepa* var. *cepa* with *P. porri* in the United Kingdom suggested that this species has a high degree of disease resistance, with pustules rarely developing to sporulation stage (Jennings et al. 1990). Similarly, in Japan, *A. cepa* var. *cepa* showed a high degree of disease resistance at different ages to a *P. allii* isolate derived from *A. fistulosum* (Wako et al. 2015). Results of glasshouse studies in Tasmania (Metcalf 2002), which showed that *A. cepa* var. *cepa* is susceptible to infection by a local isolate of *P. allii*, have not translated to field observations as no naturally infected plants have ever been reported in this state (D. Metcalf, pers. comm.). It is possible that rust disease in bulb onion crops in Tasmania has been suppressed by routine applications of fungicide for other pathogens, as suggested by Metcalf (2002). However, it is more likely that the natural resistance of the plants is preventing disease. In conclusion, rust pathogens are unlikely to represent a major threat to Australian bulb onion industry according to current knowledge. A caveat to this statement is that rust pathogens can mutate to allow a major host range shift, but this is just as likely to occur with endemic as with exotic species. Moreover, environmental changes or the expansion of cropping to new regions can lead to the emergence of new diseases, as when eucalypts were first grown in South America and succumbed to a rust disease for the first time (Glen et al. 2007).

An interesting finding in this project was the discovery of a second genetic lineage of *P. allii* in Australia, which was detected for the first time in Adelaide in 2013 and has now become the dominant genotype on the east coast of Australia. This result does suggest that an incursion pathway still exists for onion rust species into Australia. The only other country that this new *P. allii* genotype has been found in is China and it is tempting to speculate that this country was the most recent origin of the organism. Australia imports large amounts of garlic bulbs from Australia, and even though they should be devitalized as part of quarantine import conditions, the treatments are not always completely effective (Wylie et al. 2014). However, until more extensive sampling is done around the world, it remains unclear which route the pathogen entered.

In summary, this project perfectly illustrates the problem of cryptic speciation of plant pathogens, and the biosecurity implications that arise when only morphological methods have been used for fungal identification. Scientific literature emanating from the past century of research on onion rusts is very difficult to interpret as species names have been incorrectly applied and names used interchangeably. Even as recently as a few years ago, new host records of Allium rusts have been published, for which the fungal identifications are incorrect (e.g. Sansford et al. (2015)). Precision in naming of pathogens is absolutely fundamental to plant biosecurity, and it is hoped that this project has provided a rational classification system that can be enlarged and refined in the future.

Recommendations

1. To provide final resolution of the taxonomy of the onion rusts, more extensive surveys in Europe will be needed, as this is where many of the species were originally described. Replacements (epitypes) of the holotypes will have to be collected, and according to the taxonomic rules, these should derive from the same plant species and location as the holotype in order that they can be considered to form part of the same population.
2. The Industry Biosecurity Plan for the Onion Industry needs to be revised. In this document, *P. porri* is listed as occurring in Australia but with a limited distribution. Conversely, *P. allii* is not considered to be present in

Australia. These statements are erroneous. Furthermore, the threat rating of exotic rusts to the Australian bulb industry is exaggerated, as worldwide, rust fungi are not important pathogens of *A. cepa* var. *cepa*; virtually all records of rust on *A. cepa* are on shallot (*A. cepa* var. *aggregatum*). Although rust is presently not a threat to the bulb onion industry, vigilance needs to be maintained in the event of the emergence of new strains.

3. The risk of introduction of rust into Western Australia on bulb onions is extremely small and therefore quarantine conditions on the domestic trade of the commodity pertaining to this group of pathogens, are judged to be not necessary.

Scientific refereed publications

McTaggart, A.R., Shivas, R.G., Doungsa-ard, C., Weese, T.L., Beasley, D.R., Hall, B.H., Metcalf, D.A., Geering, A.D.W., 2016. Identification of rust fungi (Pucciniales) on species of *Allium* in Australia. *Australasian Plant Pathology* **45**, 581–92.

Intellectual property/commercialisation

No commercial IP generated.

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