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Evaluation of the rust fungus *Puccinia rapipes* for biological control of *Lycium ferocissimum* (African boxthorn) in Australia: Life cycle, taxonomy and pathogenicity

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ABSTRACT

Fungal plant pathogens are increasingly recognised as being among the most effective and safe agents in classical weed biological control programs worldwide. Suitability of the rust fungus *P. rapipes* as a classical biological control agent for *Lycium ferocissimum* (African boxthorn) in Australia was assessed using a streamlined agent selection framework. Studies with *P. rapipes* were undertaken to elucidate its life cycle, confirm its taxonomic placement and determine its pathogenicity to *L. ferocissimum* and seven closely-related Solanaceae species that occur in Australia. Field surveys in the native range of South Africa, experiments in a containment facility in Australia and DNA sequencing confirmed that *P. rapipes* is macrocyclic and autoecious, producing all five spore stages on *L. ferocissimum*. The stages not previously encountered, spermogonia and aecia, are described. Sequencing also confirmed that *P. rapipes* is sister to *Puccinia afra*, in the 'Old World Lineage' of *Puccinia* species on Lycieae. Two purified isolates of the fungus, representing the Eastern and Western Cape distributions of *P. rapipes* in South Africa, were cultured in the containment facility for use in pathogenicity testing. *L. ferocissimum* and all of the *Lycium* species of Eurasian origin tested – *Lycium barbarum* (goji berry), *Lycium chinense* (goji berry 'chinense') and *Lycium ruthenicum* (black goji berry) – were susceptible to both isolates of *P. rapipes*. The Australian native *L. australe* and three more distantly related species in different genera tested were resistant to both isolates. The isolate from the Western Cape was significantly more pathogenic on *L. ferocissimum* from Australia, than the Eastern Cape isolate. Our results indicate that *P. rapipes* may be sufficiently host specific to pursue as a biological control agent in an Australian context, should regulators be willing to accept damage to the Eurasian goji berries being grown, albeit to a limited extent, in Australia.

1. Introduction

Fungal plant pathogens are increasingly recognised as being among the most effective and safe agents in classical weed biological control programs worldwide (Barton, 2012; Charudattan, 2001; Morin et al., 2006). These programs can be costly and time consuming, with decades and millions of dollars being invested in native range surveys and host-specificity testing for a variety of target weeds over the past century (Gutierrez et al., 1999; McFadyen, 1998). This has led to calls for streamlining of agent selection, encouraging investment in less costly and time consuming initial assessments of candidate agents to quickly exclude and/or prioritise candidate agents (Berner and Bruckart, 2005; Louda et al., 2003; Sheppard, 2003). Initial assessments for plant pathogens should ideally consider pathogenicity and impact, taxonomy and life cycle, and specificity testing of a small number of closely-related non-target plants in the first instance (Morin et al., 2006).

Lycium ferocissimum (African boxthorn), native to South Africa, is a widespread and significant invasive weed in Australia and New Zealand (Weber, 2017). In Australia it is designated as a Weed of National Significance (Australian Weeds Committee, 2013), occurring predominantly in coastal to semi-arid inland habitats of southern Australia (GBIF.org, 19 March 2019; Parsons and

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Cuthbertson, 2001). Infestations of *L. ferocissimum* in Australia are considered a major problem in both agricultural and natural systems (Noble and Rose, 2013). Dense stands are known to exclude most other species, impede stock movement and access to water, harbour vermin (i.e. rabbits and starlings) and host agricultural pests such as Queensland fruit fly (*Bactrocera tryoni*) in Australia and tomato potato psyllid (*Bactericera cockerelli*) in New Zealand (Noble and Rose, 2013; Vereijssen et al., 2018). Conventional physical and chemical control measures are considered inadequate to manage *L. ferocissimum* infestations, particularly on broad scales, in regional settings where infestations may be difficult to access, and in sensitive natural environments (Noble and Rose, 2013).

Biological control offers promise for the management of *L. ferocissimum* as it is a safe, cost effective and sustainable method of control. The relative taxonomic isolation of *L. ferocissimum* from native Australian flora and other naturalised *Lycium* species in Australia (Fukuda et al., 2001; Levin et al., 2007), together with its negative impact at the landscape scale, makes the species a suitable target for biological control (Adair, 2013). Reports of natural enemies of *L. ferocissimum* in South Africa prior to 2016 indicated the species hosted a number of potential biological control agents, including the rust fungus *Puccinia rapipes* (Berndt and Uhlmann, 2006), leaf-feeding tortoise beetles (*Cassida* spp.) (Heron and Borowiec, 1997) and phloem-feeding bugs (*Schuhistes* spp.) (Menard, 2010). *P. rapipes* is considered a promising biological control agent as it has only ever been recorded on *L. ferocissimum*, despite the diversity of native *Lycium* species occurring in South Africa (Venter, 2000). This indicates that this rust fungus could be sufficiently host specific to be used as a classical biological control agent for *L. ferocissimum* in Australia. Some rust fungi are quite specialised, only attacking a single plant species or certain genotypes within a species (Evans et al., 2011), and many have historically performed well as biological control agents (Cullen et al., 1973; Hayes et al., 2013; Morin and Scott, 2012; Morris, 1997).

Studies of *P. rapipes* were initiated to assess its suitability as a classical biological control agent for *L. ferocissimum* in Australia. An initial assessment framework (Morin et al., 2006) was utilised, with a view to streamline the gathering of key information. To this end, we report on the life cycle, taxonomic placement and pathogenicity of *P. rapipes* on the genus *Lycium* and a few other related Solanaceae species that occur in Australia, and discuss these results in the context of biological control of *L. ferocissimum* in Australia.

2. Materials and methods

2.1. Field surveys

A comprehensive survey for diseases on *L. ferocissimum* was performed in October 2017 at 28 sites across the Eastern (13 sites) and Western (15 sites) Cape provinces of South Africa. Two sites were visited multiple times between November 2016 and October 2017 to source material of *P. rapipes* to establish cultures in the Australian containment facility and track life cycle development. The first of these sites was in the Western Cape, at Miller's Point south of Simon's Town (34°13′53.18″ S; 18°28′28.35″ E) and the other was in the Eastern Cape, in the south-eastern suburbs of Grahamstown (33°19′10.56′ S; 26°32′17.16″ E). On each field visit, rust life cycle stages and evidence of any other disease symptoms were recorded, and representative material collected.

2.2. Morphological and molecular characterisation

2.2.1. Morphological characterisation

Material comprising the uredinia and telia observed on *L. ferocissimum* in the field was microscopically examined and compared morphologically with the description of *P. rapipes* given by Berndt and Uhlmann (2006). Spermogonia and aecia, stages not previously observed, were described once their identity was confirmed by molecular characterisation. Voucher material of all stages (spermogonia, aecia, uredinia, telia) observed at the Western Cape site, and telia and uredinia stages from the Eastern Cape site were deposited in the dried herbarium collection (PREM) of the South African National Collection of Fungi, ARC-PHP, Pretoria, South Africa.

2.2.2. Molecular characterisation

Four rust fungus samples from *L. ferocissimum* were subjected to DNA-based molecular characterisation: three morphologically confirmed samples of *P. rapipes*, i.e. urediniospore samples of the purified Eastern and Western Cape isolates used in our studies (see below), and one telia sample cut from sections of dried leaf material sourced from the site where the Western Cape isolate was collected; and one aecia sample on leaf material from a rust fungus species recovered from the same site as the Western Cape isolate, and suspected to be *P. rapipes*. All samples were placed in a 2 % cetyltrimethylammonium bromide (CTAB) solution prior to removal from the containment facility for DNA extraction.

2.2.2.1. DNA extraction, amplification and sequencing. Urediniospores and leaf samples with sori were homogenised in a FastPrep®-24 Classic Tissue and Cell Homogeniser (MP Biomedicals LLC, USA), and genomic DNA was extracted using the DNeasy UltraClean® Microbial kit (QIAGEN, Germany), following the manufacturer's instructions. Isolated DNA was diluted (1:100; 1:1000) and used in PCR amplifications targeting the ITS2 and a portion of the large subunit (LSU) region of the nuclear ribosomal RNA operon using oligonucleotide primers Rust2inv and LR6, and sequenced using primers Rust2inv, LR0R, LR3 and LR6 (Aime, 2006; Moncalvo et al., 1995; Vilgalys and Hester, 1990), and the cytochrome oxidase subunit 3 (CO3) using primers CO3_F1 and CO3_F2 (Vialle et al., 2009). PCRs were carried out using the MyTaq™ HS Mix (Bioline, London), according to the manufacturer's instructions, and following the parameters outlined in Aime (2006) for the ITS2/LSU and Vialle et al. (2009) for the CO3 gene region. All PCR amplicons were sequenced using the ABI BigDye ® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI 3730xl DNA Analyser (Applied Biosystems, USA) at Macrogen Inc. (Seoul, South Korea). DNA sequence chromatograms were checked and edited using GeneStudioTM Professional (GeneStudio Inc., USA). Sequence data were deposited in GenBank (Table A.4).

2.2.2.2. Phylogenetic analyses. For phylogenetic analyses only the ITS2 and CO3 regions were included. DNA sequences (ITS2 and CO3 gene regions) of *Puccinia* species known to infect plant species from the Lycieae (Otálora and Berndt, 2018) were downloaded from NCBI and aligned with those of the rust fungus samples collected from *L. ferocissimum* in MAFFT (Katoh and Standley, 2013). Alignments were manually adjusted in Mesquite (Maddison and Maddison, 2018) and ambiguously aligned regions were excluded. Analyses of the ITS2 and CO3 regions were first run separately using Maximum Likelihood to test for congruence between loci. No conflicts were detected, and the two regions were combined. Phylogenetic relationships were inferred using a Bayesian approach with Mr Bayes v 3.2.2 (Ronquist et al., 2012). MrModeltest v 2.4 (Nylander, 2004) was used to determine the best-fit nucleotide substitution model for ITS2 and the three codon positions of CO3. The GTR + G model was selected for ITS2 while the SYM + I model was selected for the first codon position of CO3, F81 for the second codon position and HKY + G for the third codon position of CO3. The Bayesian analysis was run on the CIPRES Science Gateway (Miller et al., 2010), using two runs of 4 chains, 5,000,000 generations and sampling every 500 generations. Tracer

v1.7.1 (Rambaut et al., 2018) was used to verify stationarity and a burn-in of 5000 trees was applied to each run. Posterior probabilities were estimated using the remaining trees in PAUP v 4.0a (Swofford, 2003). The most likely tree was obtained in Mr Bayes using 'sumt', then visualised in PAUP. Additional support values were obtained using a Maximum Likelihood approach with RAxML v8 on CIPRES (Stamatakis, 2014). For this bootstrap analysis, GTRGAMMA was used as the substitution model and 1000 bootstrap iterations were implemented. Bayesian and RAxML support values, as well as the New and Old World lineage designations of Otálora and Berndt (2018), were added to the Bayesian consensus tree by editing the output in Adobe Illustrator (Adobe Inc., USA). All trees were rooted with *Puccinia hemerocallidis*, *Puccinia pampeana* and *Puccinia dichondrae*. The combined CO3 and ITS2 dataset was deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S24445).

2.3. Plant production and identity

Plants were produced from seed (*Hyoscyamus albus*, *Hyoscyamus aureus*, *Lycium barbarum*, *L. ferocissimum*, *Lycium ruthenicum* and *Solanum aviculare*), clonally from root suckers (*Lycium australe*) or cuttings (*L. australe* and *Lycium chinense*), or purchased from nurseries as mature plants (*L. chinense*). Species were selected based on their close phylogenetic relationship to *L. ferocissimum*, according to the centrifugal phylogenetic approach of Wapshere (1974) and based on recent Solanaceae phylogenetics (Särkinen et al., 2013; Stevens, 2019). The centrifugal phylogenetic method prioritises test species according to their degree of phylogenetic separation from the target weed, rather than by classical taxonomic circumscription (see Briese, 2003, for more information). Only *L. australe* and *S. aviculare* are considered native to Australia. All *Lycium* species were identified morphologically in the first instance and confirmed by DNA sequencing (to preliminary haplotype level for *L. ferocissimum*), using three chloroplast and one nuclear marker. Seeds were soaked in 250 ppm (*L. barbarum and L. ruthenicum*) or 500 ppm (*H. albus*, *H. aureus*, *L. ferocissimum*, *S. aviculare*) gibberellic acid (Sigma–Aldrich, St Louis, Missouri, USA) for 24-h to promote germination, and sown and germinated in a seedling mix (Plugger 111 Seedraising Mix, Australian Growing Solutions, Tyabb, Vic., Australia). Root suckers of *L. australe* were separated from the main stem of mature plants and transplanted into potting mix (5:1:1:3 straw-based compost, peat moss, river sand, perlite, with at least a teaspoon of slow-release fertiliser at the soil surface [Osmocote, Bella Vista, NSW, Australia; NPK 19.4:1.6:5]). Stem cuttings of *L. australe* and *L. chinense* were treated with a hormone rooting gel (Yates Clonex Rooting Hormonal Gel Purple, Yates, Clayton, Vic., Australia; 3 g L^{-1} Indole-3-Butyric Acid), planted in a 1:1 perlite and vermiculite mixture, and maintained wet with intermittent overhead misting to encourage root development. All seedlings with true leaves and rooted cuttings were transplanted into potting mix in plastic pots of at least 5 cm diam. × 5 cm high and transferred to larger pots as necessary to support developing plants. Plants were grown in greenhouses maintained at 16–26 °C, under natural light (and, if required, additional lighting with fluorescent lights to maintain at least a 12-h photoperiod) and/or in 20 °C controlled environment constant temperature (CT) rooms with a 14-h photoperiod provided by fluorescent lights or LED plant growth lights, for a minimum of four weeks before use in experiments. All plants were grown in CT rooms for at least two weeks prior to experimental use to reduce random effects of growth conditions. All plants were at least six weeks old, with at least eight fully developed leaves, when used in experiments. Plants were fertilised fortnightly with Aquasol (Yates, Clayton, Vic., Australia; NPK 23:3.95:14), trimmed regularly to encourage active and healthy plant growth, and treated as necessary with pesticides (never fungicides; most commonly Confidor, Yates, Clayton, Vic., Australia [15 mg L^{-1} Imidacloprid] as a soil drench, or Vertimec, Syngenta, Macquarie Park, NSW, Australia [18 g L^{-1} Abamectin] as a spray) to reduce pest pressure. All plants treated with pesticides were withheld from experiments for a minimum of two weeks following application, to reduce the likelihood of the pesticide interacting with fungal infection.

2.4. Isolate purification and inoculum production

Two isolates of *P. rapipes* were purified from material collected at the regularly visited sites in the Eastern and Western Capes and imported into the containment facility in Australia. *L. ferocissimum* plants were inoculated with urediniospores from a single uredinium by transferring them onto leaves with a moist fine camel hair brush. Inoculated plants were placed in separate moist boxes (plastic boxes with a film of water covering the base, placed within large sealed clear plastic bags), misted with deionised water and placed in the dark (i.e. covered with a large black plastic bag and put under a bench) for 24 h, at which time they were placed in clear plastic isolation boxes (80–120 L clear plastic boxes either cracked open at the lid or with 15 cm diam. windows cut out and covered with paper towel to allow for air flow). Plants were kept in a CT room (same conditions as above) within the containment facility. Development of uredinia on each plant was assessed regularly and additional inoculations of other plants, using fresh urediniospores produced on uredinia resulting from the initial inoculations, were performed to bulk-up the purified isolates. Once established, the two cultures were maintained by further inoculations as required. Inoculations were performed either by brush transfer of urediniospores or by spraying urediniospore suspensions in either deionised water or a solution of 0.1 % Tween 80 (Sigma–Aldrich, Castle Hill, NSW, Australia) in deionised water. Urediniospores were collected weekly from three weeks after the plants were inoculated until uredinia ceased producing high levels of urediniospores or developed into telia. These urediniospores were either used immediately, dried overnight over silica gel beads and used the following day in experiments, or stored at either 4 °C for use within 1 week or - 20 °C for longer term storage.

2.5. Life cycle investigations in the laboratory

2.5.1. Inducement of teliospore germination

Teliospores extracted from telia or still within telia on leaf material collected from the two regularly visited sites (ex. Eastern Cape, July and October 2017, and ex. Western Cape, November 2016, January, September and October 2017), and from the culture in the containment facility (ex. Eastern Cape isolate, November 2017), were used to attempt breaking their potential dormancy and induce germination. The material was subjected to different treatments and exposure to a range of temperatures in the dark or under a 14-h photoperiod, until teliospore germination was induced or for a maximum of six months (~24 weeks). One or a combination of two of the following treatments was performed: i) teliospores transferred to water agar plates following rinsing of telia in 70 % ethanol or sterile deionised water, ii) immersion of telia in 15 or 30 % hydrogen peroxide for 30 or 60 min, iii) telia placed under dripping tap water for 3 d, iv) telia continuously maintained under dry or wet conditions and v) telia exposed to alternating cycles of wetting and drying (Table A.1). Teliospores for each treatment or treatment combination were assessed generally at 2, 4, 6, 8, 12, 16, 20 and 24 week intervals, unless stated otherwise (Table A.1). Germination was assessed by transferring up to two ~1 cm^2 agar blocks cut from water agar plates with teliospores or by spreading teliospores extracted from a minimum of three telia

(from two to three leaf sections, washed in 70 % ethanol and rinsed in sterile deionised water) onto blocks of water agar, and placing these agar blocks on glass slides in moist chambers (petri dishes or small plastic boxes with moist paper towel) in the dark at 20 °C for 24–72 h. At this time a drop of blue-lacto-glycerol stain (0.02 g aniline blue, 10 ml lactic acid, 10 ml glycerol, 10 ml deionised water) was generally placed on top of the agar block to stop the germination process, though some samples were examined without the aid of the stain, and percentage germination (i.e. when the length of the germ tube was at least half as long as the width of the teliospore) was assessed using a light microscope. Germination was assessed for 30 to 100 teliospores (with less than 100 assessed only when this constituted all of the teliospores plated). Basidiospore production was noted for germinated teliospores, but not quantified.

2.5.2. Inoculation with basidiospores

Once treatments that induced germination in teliospores were identified, inoculation of *L. ferocissimum* plants with basidiospores was undertaken. Blocks of agar with treated teliospores were placed in the base of 5 cm diam. Petri dishes, which were suspended without lids (so that the teliospores were face down) above young, fleshy leaves either abaxial or adaxial side up, using metal clips attached to fine bamboo sticks inserted in the potting mix. Narrow strips of masking tape were used to ensure leaves remained lined up with the dish containing teliospores for the duration of the inoculation. Plants with inoculation set-ups were then misted with deionised water and placed in moist boxes in a CT room (same conditions as above). After 48 h, plants were removed from the boxes, inoculation set-ups were dismantled, and germination of teliospores was confirmed by assessing as described previously. Inoculated leaves were inspected daily for symptom development. Developing spermogonia were cross-fertilised by hand using a sterile fine camel hair brush moistened with deionised water to encourage development of aecia.

2.5.3. Inoculation with aeciospores

Following aecia development, aeciospore release was encouraged by placing leaves with aecia still attached to plants over fleshy, young leaves of other non-infected *L. ferocissimum* plants misted with deionised water, and placing these plants in a moist box in a CT room (same conditions as above) for 24 h. When aecia split open, deposited aeciospores were then dispersed further to other leaves using a sterile fine camel hair brush moistened with deionised water and plants were again placed in a moist box in the same CT room for 24 h. Germination of aeciospores was assessed by brushing a portion of aeciospores onto a block of water agar, which was placed in a moist chamber in the dark for 24 h and assessed as reported above for teliospore germination. Leaves were then inspected regularly for development of uredinia and telia.

2.6. Isolate pathogenicity and species susceptibility

2.6.1. Experimental design

The pathogenicity of *P. rapipes* was tested on *L. ferocissimum*, the closely-related *L. australe* (Australian boxthorn), *L. barbarum* (goji berry), *L. chinense* (goji berry 'chinense'), *L. ruthenicum* (black goji berry), and more distantly related *H. albus* (white henbane), *H. aureus* (golden henbane) and *S. aviculare* (kangaroo apple). Seven experiments (exp.) on whole plants were conducted in total: Five 'isolate pathogenicity' experiments to ascertain the pathogenicity of the two rust isolates on the plant species (exp. 1–5), and two 'species susceptibility' experiments to compare the relative susceptibility of these plant species to the Western Cape isolate (exp. 6 and 7; Table A.2). *L. ferocissimum* was included in each experiment, using the healthiest available plants of either haplotype. Five to ten leaves per isolate on five to ten plant replicates per species were marked for inoculation in each experiment, depending on plant availability and leaf quality (i.e. expanded leaves with no abrasions) at the time of inoculation (Table A.2). In the isolate pathogenicity experiments, leaves for inoculation with each isolate were chosen on opposite sides of a single branch of each plant to reduce branch effect on measures of pathogenicity and prevent contamination drip between isolates. For each species in each experiment, a 0.1 % Tween 80-deionised water solution was applied on the same number of leaves on a separate single branch (with a separate plastic bag cover to prevent contamination from inoculated branches) or plant (placed in a separate moist box) to act as a control. Randomised complete block designs at the plant replicate level were used in all experiments.

2.6.2. Inoculum preparation and application

Inoculum for each experiment was prepared by suspending urediniospores in a 0.1 % Tween 80-deionised water solution at the beginning of each experimental block and applying within 45 min. Density of the suspensions was determined using a haemocytometer and adjusted to 2×10^4 urediniospores ml^{-1}. For the isolate pathogenicity experiments (exp. 1–5) the suspension was applied to marked leaves using fine camel hair brushes. A single dip of the brush was used for each side of the leaf, and spread over the surface of the whole leaf in at least three brush strokes, with separate brushes of similar size used for each isolate. For the species susceptibility experiments (exp. 6, 7), the suspension was sprayed onto the foliage using a hand-held manual spraying device, spraying down from the top of the plant to drip for exp. 6 and to drip for both sides of marked leaves in exp. 7. All plants were then sprayed with additional deionised water and placed in moist boxes in a CT room (same conditions as above) for approximately 24 h, before transferring them to the bench of the same CT room.

The viability of urediniospores used in each experiment was assessed by applying an aliquot of the suspension with a camel hair brush onto the surface of a water agar block placed on a microscope slide. The slide was placed in a moist chamber under the same CT room conditions as for inoculated plants. Germination was assessed after 24 h, as described above for teliospore germination. At least one germination assessment was made per isolate per experiment, with multiple assessments made at the beginning and end of each experimental block for the majority of experiments.

2.6.3. Determination of infection process

Additional inoculations of single leaves of all plant species with both the Eastern and Western Cape isolates were performed in parallel with the isolate pathogenicity experiments to provide material for microscopic examination of rust development at 1, 5, 12 and 21 d after inoculation (dai). At each time point for each plant species, at least one inoculated leaf per species was excised and cut into small pieces (approx. 0.5–1 cm^2). Inoculated leaves that were abscised by plants or displayed a hypersensitive response were also prepared for microscopic examination. Leaf pieces were cleared and stained in a solution containing aniline blue, ethanol, chloroform, lactic acid, phenol and chloral hydrate for 2–5 d (Bruzzese and Hasan, 1983). They were then rinsed in water, placed in a saturated solution of chloral hydrate for 1 d and transferred back to water for storage. Prior to microscopic examination, the pieces were placed in blue-lacto-glycerol stain on a microscope glass slide for 2–5 min. Excess stain was then gently removed with blotting paper and pieces were mounted in water and examined under a light microscope. At least 50 urediniospores per species were examined.

2.6.4. Disease assessments

Three weeks after inoculation, images of the abaxial and adaxial side of each marked leaf were taken using a flatbed scanner at 300 dpi. Images were processed using the image analysis software ASSESS v2 (APS Press, St Paul, MN, USA). Disease incidence was recorded as presence or absence of uredinia on either side of the leaf. Where chlorotic and/or necrotic spots occurred without the development of uredinia, leaves were classified as having a hypersensitive response, with a disease incidence of zero. Disease severity was recorded as the number of uredinia per cm^2 of leaf area, calculated using data from image analyses and summarised from the abaxial and adaxial measures to give a single per leaf measure (i.e. sum of uredinia on both sides of the leaves divided by leaf area in cm^2). Each species was then assigned a response rating, based on macroscopic and microscopic development of the rust fungus (Table 1), using a classification system adapted from previous studies on other rust fungi (e.g. Evans and Tomley, 1994).

2.6.5. Statistical analyses

Statistical analyses were conducted for each experiment in the R statistical environment, version 3.5.3 (R Core Team, 2019), using fixed and mixed effects models. Disease incidence was analysed using a binomial generalised linear model with a logit link using the base R function glm with a chi squared test of significance, with experimental block, species, isolate (only for isolate pathogenicity experiments), plant replicate, leaf position and their interactions as fixed effects. A simplified glm testing only the effect of species on disease incidence and a post-hoc Tukey's test was then conducted using the glht function of the package multcomp (Hothorn et al., 2008), as a post-hoc analysis of differences between species. Disease severity was analysed using a log + 100 transformation and analysis of variance (ANOVA) using the base R function aov, with species, treatment (only for isolate pathogenicity experiments), leaf position and their interactions as fixed effects and plant nested within block as random effects. Residuals and fitted values from the model were extracted using the package dae ver. 3.0–23 (Brien, 2018) to use in testing model assumptions. Least significant differences were used to compare differences amongst mean disease severity between species and isolate treatments using the package agricolae ver. 1.2–8 (de Mendiburu, 2017). Visual inspection of residual plots were used to confirm there were no substantial deviations from homoscedasticity or normality for any of the statistical analyses.

3. Results

3.1. Field surveys

Disease symptoms caused by *P. rapipes* were observed on *L. ferocissimum* at 4 of the 13 sites (5 individual plants) in the Eastern Cape and 10 of the 15 sites (13 individual plants) in the Western Cape when surveyed in October 2017 (Fig. 1 and Table A.3). These sites included multiple haplotypes of *L. ferocissimum* (Fig. 1 and Table A.3). The rust fungus was not observed on any other *Lycium* species. The most severe rust symptoms were observed on *L. ferocissimum* at coastal sites in the Western Cape (Fig. 2A). Uredinia, telia, spermogonia and aecia were observed on *L. ferocissimum* at both sites where the Eastern and Western Cape purified isolates of *P. rapipes* were collected from (Table A.3). Disease symptoms of any other primary pathogens were not observed at any of the sites surveyed.

3.2. Morphological and molecular characterisation

3.2.1. Morphological characterisation

Uredinia and telia observed on *L. ferocissimum* in the field matched the description of *P. rapipes* given by Berndt and Uhlmann (2006) (Fig. 2A, B, D–F). Descriptions of spermogonia (Fig. 2H) and aecia (Fig. 2I–L) are provided in the taxonomy section below, as these were not previously described.

3.2.2. Molecular characterisation

The ITS2 dataset consisted of 22 sequences of 396 characters, while the CO3 dataset contained 16 sequences of 591 characters. The total number of characters in the combined dataset was 987. The 50 % majority rule consensus tree of the Bayesian analysis grouped the aecia collected on *L. ferocissimum* in the field with the urediniospore and telia samples of *P. rapipes* (confirmed by morphological characters) into a well-supported clade sister to *Puccinia afra*, in the 'Old World Lineage' of *Puccinia* species on Lycieae (Otálora and Berndt, 2018) (Fig. 3).

3.3. Life cycle investigations in the laboratory

The macrocyclic and autoecious life cycle of *P. rapipes* was confirmed experimentally in the laboratory, following observations in the field (Table A.3, Table A.5 and Figure A.1).

Table 1
Categories used to classify the response of test plant species to *Puccinia rapipes*.

Category	Macro-symptoms	Developmental stage of the fungus and micro-symptoms
Immune	None	No sign of penetration
Highly resistant	None	Abnormal penetration (necrotic/collapsed penetration hyphae and vesicle, none or very short or necrotic/collapsed primary infection hyphae); plant defence reaction sometimes visible at the cellular level.
Resistant	Discoloration, chlorosis sometimes present	Successful penetration and development of some infection hyphae. Haustorium mother cells sometimes developed but generally no haustoria present.
Moderately resistant	Chlorotic or necrotic spots present	Restricted network of infection hyphae developed. Haustoria present, but generally non-functional/collapsed. Plant host cell plasmolysis often present.
Moderately susceptible	Chlorotic or necrotic spots present. Underdeveloped, non-eruptive uredinia present. No sporulation.	Extensive network of infection hyphae; haustoria abundant but often non-functional/collapsed. Development of uredinia initiated but aborted.
Susceptible	Normal uredinia present but restricted in numbers (<5 uredinia/cm^2). Sporulation.	Extensive network of infection hyphae; functional haustoria abundant.
Highly susceptible	Large number of normal uredinia present (≥5 uredinia/cm^2). Abundant sporulation.	Extensive network of infection hyphae; functional haustoria abundant.

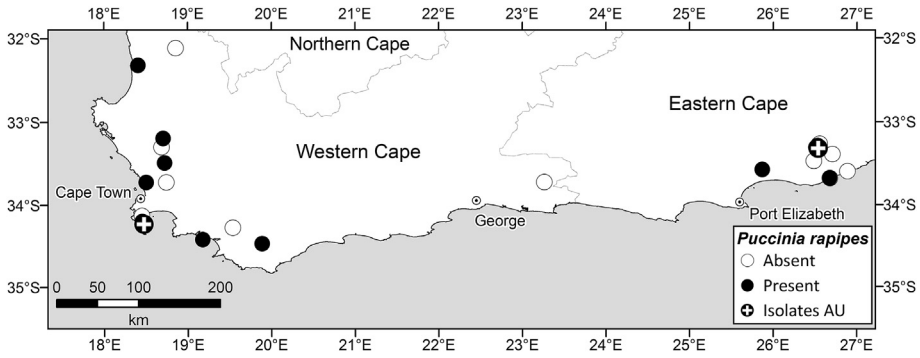

Fig. 1. *Puccinia rapipes* survey sites in South Africa, October 2017. Filled symbols indicate sites where rust symptoms were observed on *Lycium ferocissimum* (black circles with and without white crosses; 4 sites in the Eastern Cape, and 10 sites in the Western Cape, 2 of which overlap on the map at this scale). Black circles with a white cross indicate the locations where the two purified isolates of *P. rapipes* used in our studies originate from, while the open circles indicate *L. ferocissimum* sites where *P. rapipes* was not observed.

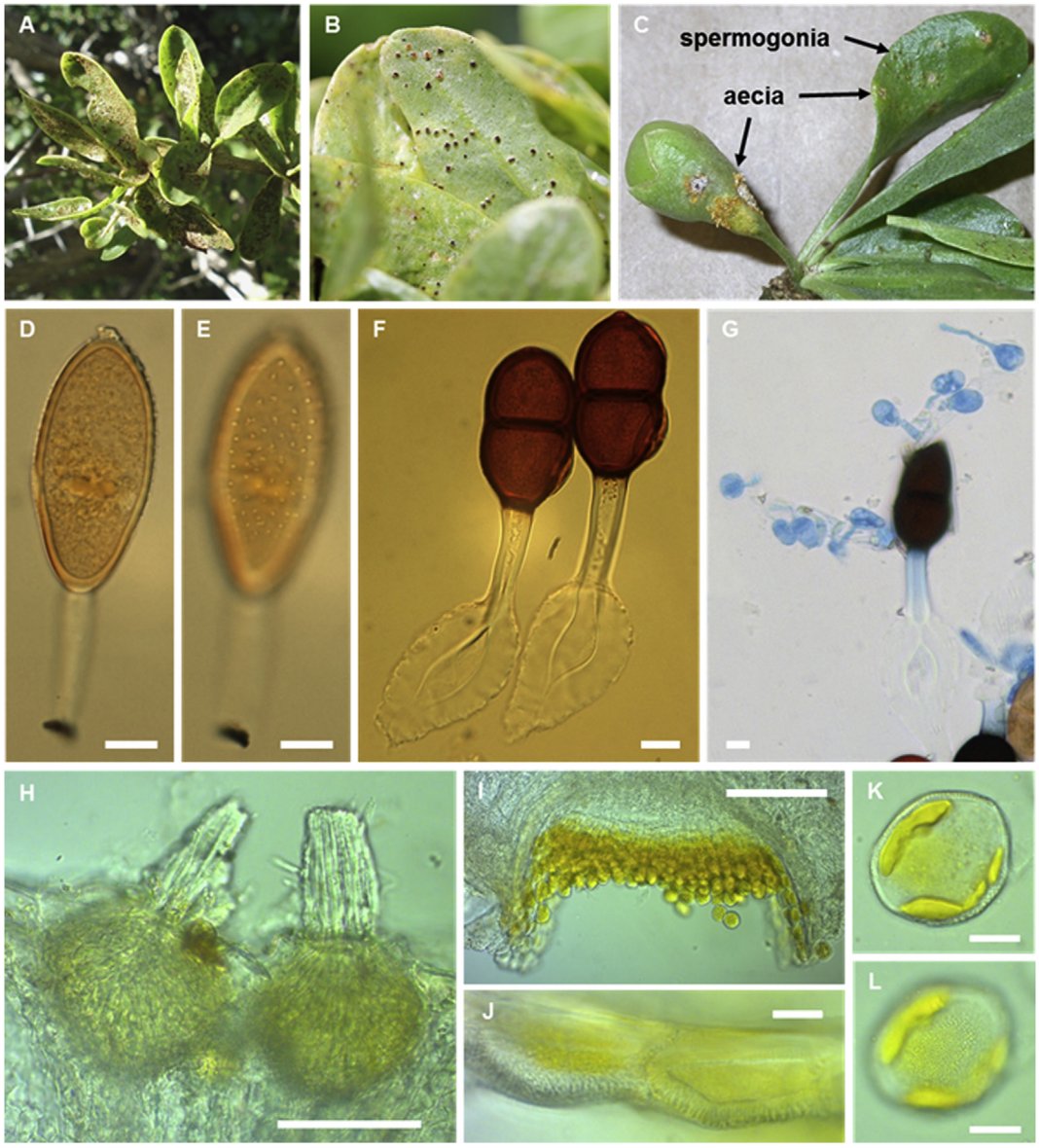

Fig. 2. *Puccinia rapipes* (A) uredinia and (B) uredinia (orange) and telia (dark brown) on leaves of *Lycium ferocissimum* in the Western Cape of South Africa, (C) spermogonia (tan) and aecia (yellow) on leaves and sepals of *L. ferocissimum* in the Eastern Cape of South Africa, (D–E) urediniospore showing wall ornamentation, (F) teliospores and (G) germinated teliospore with basidia and germinated basidiospores, (H) spermogonia, (I) cross section through an aecium, showing catenulate aeciospores and bounding peridium, (J) peridial cell wall ornamentation and (K–L) aeciospores showing wall ornamentation. Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

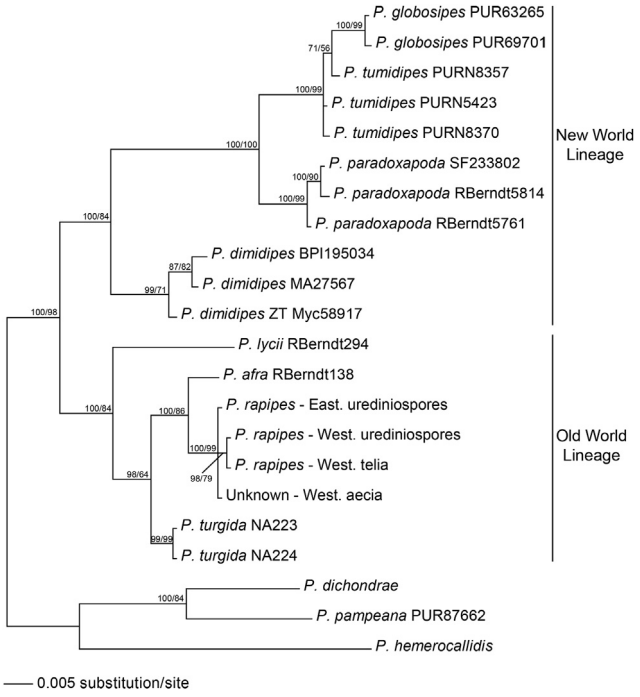

Fig. 3. Phylogenetic relationships based on Bayesian Inference of combined ITS2 and CO3 sequences of urediniospore and telia samples of *Puccinia rapipes* (confirmed by morphological characters) and an aecia sample collected on *Lycium ferocissimum* in the field in South Africa, to other *Puccinia* species occurring on Lycieae. *P. rapipes* - East. urediniospores = urediniospores of the purified isolate from the Eastern Cape; *P. rapipes* - West. urediniospores = urediniospores of the purified isolate from the Western Cape; *P. rapipes* - West. telia = telia from the site where the Western Cape isolate was collected; Unknown - West. aecia = aecia from the site where the Western Cape isolate of *P. rapipes* was collected (Table A.4). Support values are shown above each branch, ordered as Bayesian posterior probabilities followed by RAxML bootstrap values (PP/BS). New and Old World lineages indicated as per Otálora and Berndt (2018).

3.3.1. Inducement of teliospore germination

Teliospores most often germinated when derived from dried field-collected material stored in the refrigerator at 4 °C for 1 week to 14 m or freshly collected from the *P. rapipes* culture in the containment facility (Fig. 2G), washed with 70 % ethanol or sterile deionised water, plated on water agar, and subjected to a period of cold temperature (refrigeration at 4 °C in the dark) for at least three weeks before being transferred to 20 °C for germination to occur (Table 2). Teliospore germination under these conditions were observed from 1 day to 24 weeks post plating onto water agar, peaking within the 3–8 week range, with highest maximum germination rates of 86–96 %. Basidiospores were usually observed where germination was more than 10 %. Some teliospores extracted from telia on excised leaves did germinate after being stored under a continuous dry condition at 20 °C in the dark for less than 2 m and subjected to a secondary treatment at 4 °C in the dark for 3 (Eastern Cape material collected January 2017, 1–3 %) or 13 m (Western Cape material collected July 2017, 6 %). None of the other treatments (Table A.1) were effective in inducing teliospore germination.

3.3.2. Life cycle stages

Spermogonia developed within 14 d of inoculation with basidiospores from teliospores germinated in April 2018, on the side of the leaf exposed to the basidiospores, but more commonly on the abaxial side of the leaf. Aecia developed 5–11 d after spermogonia were cross-fertilised when they were glossy and raised, and matured over the course of three weeks. Aeciospore release was found to occur when aecia on plants were placed in a moist box in a CT room (same conditions as above) for approximately 24 h. Twenty-one percent of these aeciospores germinated on water agar. Following plant inoculations with aeciospores, uredinia developed on the adaxial surface of leaves within 17–23 d. Despite maintaining the uredinia in this culture for another six months telia never developed. However, telia were observed to develop from uredinia in laboratory cultures of both the Eastern and Western Cape isolates at other times, including at the same time as this aeciospore-derived culture persisted as uredinia.

3.4. Isolate pathogenicity & species susceptibility

The inoculum used in all experiments was viable (Table A.6). Germination varied from 34 to 100 % for the Eastern Cape isolate and 37–100 % for the Western Cape isolate across the experiments. Statistical analyses were only conducted for the susceptible *Lycium* species in each experiment.

3.4.1. Isolate pathogenicity

Both the Eastern and Western Cape isolates of *P. rapipes* were pathogenic on both haplotypes of *L. ferocissimum* tested, *L. barbarum*, *L. chinense* and *L. ruthenicum*, and non-pathogenic on *L. australe*, *H. albus*, *H. aureus* and *S. aviculare*. The Western Cape isolate was significantly more pathogenic on *L. ferocissimum* than the Eastern Cape isolate in four of the five experiments, based on disease severity (Table 3). Where significant, disease incidence was 1.3–2.3 times higher and disease severity was 1.6–12.4 times higher for leaves of *L. ferocissimum* inoculated with the Western Cape isolate. Measures of disease incidence and severity varied greatly across all experiments for all hosts, even when inoculated with the same isolate (Table 3). Measures for L. *ferocissimum* leaves inoculated with the Western Cape isolate were less variable than the measures for other species and isolate combinations (Table 3).

The origin of the isolate was found to significantly influence disease incidence in only two of the experiments (exp. 1: χ^2 (1, N = 294) = 25.37, P < 0.001; exp. 2: χ^2 (1, N = 330) = 14.56, P < 0.001), although the species*isolate interaction in exp. 3 was found to be significant (χ^2 (2, N = 254) = 8.45, P = 0.015). Isolate origin was also found to significantly affect disease severity in exp. 1 and 2 (exp. 1: F (1,256) = 14.31, P < 0.001; exp. 2 F (1,292) = 85.33, P < 0.001). Similarly the species*isolate interaction in exp. 3 for disease severity was found to be significant (F (2,216) = 3.46, P = 0.033). This interaction was also marginally significant for disease severity in exp. 4 (F (1,55) = 3.52, P = 0.066) and exp. 5 (F (1,56) = 3.79, P = 0.057). Leaf position was also found to significantly affect disease severity in exp. 1 (F (4,256) = 2.75, P = 0.029) and exp. 2 (F (4,292) = 10.71, P < 0.001), with younger leaves at the crown of the plants likely to express greater disease severity than older leaves toward the base of the crown.

Hypersensitive plant responses were observed for both isolates of *P. rapipes* on 17–39 % of *L. australe* leaves in exp. 1 and 2 (across both isolates), 4 % of *L. barbarum* leaves inoculated with the Western Cape isolate in exp. 1, and 2–39 % of *L. ferocissimum* leaves (across both isolates and all experiments) (Table A.7).

3.4.2. Species susceptibility

P. rapipes uredinia developed on *L. barbarum*, *L. chinense* and *L. ferocissimum* across both experiments (Fig. 4). Uredinia did not develop on *L. ruthenicum* in exp. 6, but did in exp. 7. As observed in the isolate pathogenicity experiments, no uredinia developed on *L. australe*, *H. albus*, *H. aureus* and *S. aviculare* in either species susceptibility experiment.

Disease incidence and severity measures differed greatly across both experiments (Fig. 4). Notably, much smaller percentages of urediniospores germinated in exp. 6 (37–65 %) compared to those

Table 2
Maximum germination of teliospores extracted from telia on dried field-collected material stored in the refrigerator at 4 °C for 1 week to 14 m or freshly collected from a laboratory culture, following washing with 70 % ethanol or sterile deionised water, plating on water agar, exposure to a period of cold temperature (4 °C in the dark) and transfer to 20 °C in the dark for 24–72 h.

Teliospore source	Collection date[a]	Treatment initiation date	Duration of exposure at 4 °C (wks)	Maximum germination (%)
Eastern Cape	July 2017	August 2017	11	71
			17	22
			19	30
			20	8
		November 2017	1	0
			4	39
			6	37
			16	11
			20	3
			24	7
	October 2017	March 2018	<1 (1 d)	2
			1	7
			2–3	0
			8	20
Western Cape	November 2016	August 2017	6–16	0
		January 2018	6–24	0
	January 2017	February 2017–March 2018[b]	2–16	0
	September 2017	March 2018	2–3	0
			8	7
	October 2017	March 2018	2	0
			3	86
			8	72
Fresh (Eastern Cape laboratory culture)	November 2017	December 2017	4	0
	March 2018	March 2018	1	0
			3	0
			4	88
			8	96

[a] Dry field-collected material was stored in the refrigerator at 4 °C until use.
[b] Monthly initiation dates, excluding July, September, October and December 2017.

in exp. 7 (86–93 %). Species was found to significantly influence disease incidence (exp. 6: χ^2 (2, N = 89) = 17.62, P < 0.001; exp. 7 χ^2 (3, N = 198) = 35.64, P < 0.001), with moderate influences of block*species (χ^2 (8, N = 89) = 30.28, P < 0.001) and species*leaf position (χ^2 (10, N = 89) = 28.27, P = 0.002) in exp. 6 and block (χ^2 (4, N = 198) = 18.51, P < 0.001) and block*species (χ^2 (12, N = 198) = 51.77, P < 0.001) in exp. 7. Species was also found to significantly affect disease severity (exp. 6: F (2,68) = 5.27, P = 0.007); exp. 7: F (3,155) = 16.38, P < 0.001). Leaf position and the leaf position*species interaction had no significant effect on disease incidence or severity for either experiment.

Disease incidence was lower for all species in exp. 6 than in exp. 7. No disease symptoms developed on *L. ruthenicum* in exp. 6, and the species had significantly lower disease incidence compared to all other species in exp. 7 (Fig. 4A). Disease incidence for *L. chinense* was significantly lower than all other infected species in exp. 6, but significantly higher than all other infected species in exp. 7 (Fig. 4A).

Disease severity was much lower and less variable in exp. 6 compared to exp. 7 (Fig. 4B). While disease severity was statistically not significant in exp. 6 between *L. barbarum* and *L. ferocissimum*, both species had significantly higher disease severity than that recorded for *L. chinense*. *L. barbarum* had much greater and statistically significant disease severity than all other species in exp. 7. While not immediately apparent for these species with lower disease severity, due to analysis on the log scale, *L. chinense* had significantly higher disease severity than *L. ruthenicum*, but not *L. ferocissimum*; and there was no significant difference in disease severity between *L. ferocissimum* and *L. ruthenicum* in this experiment.

Hypersensitive plant responses were observed on 16 % of *L. australe* leaves in exp. 7, but not at all in exp. 6. Hypersensitive responses were also observed for *L. barbarum* (7 %), *L. chinense* (17 %) and *L. ruthenicum* (17 %) in exp. 6, and *L. barbarum* (6 %), *L. ferocissimum* (14 %) and *L. ruthenicum* (29 %) in exp. 7.

Table 3
Pathogenicity of Eastern (East.) and Western (West.) Cape isolates of *Puccinia rapipes* to *Lycium ferocissimum* and closely-related susceptible species present in Australia, as a measure of disease incidence (proportion of leaves with uredinia) and severity (mean number of uredinia per cm^2 leaf area ± standard error). Significantly greater measures of disease incidence and severity are identified in bold print. Details of each experiment in Table A.2.

Species (- haplotype)	Disease incidence			Disease severity (uredinia per cm^2)		
	East.	West.	sig.[a]	East.	West.	sig.[a]
Lycium ferocissimum - 5						
Exp. 1	0.44	**0.78**	***	1.54 ± 0.62	**2.50 ± 0.55**	***
Exp. 3	0.19	0.42		1.13 ± 0.64	**5.25 ± 2.00**	**
Exp. 4	0.63	0.75		4.63 ± 1.62	5.33 ± 1.52	
Exp. 5	0.96	0.91		1.47 ± 0.33	**8.62 ± 2.10**	*
L. ferocissimum - hybrid 5*2						
Exp. 1	0.29	**0.68**	***	0.13 ± 0.07	**1.31 ± 0.23**	***
Exp. 2	0.69	**0.93**	*	1.16 ± 0.24	**14.39 ± 1.74**	***
Lycium barbarum						
Exp. 1	0.77	0.67		**3.92 ± 0.77**	3.06 ± 0.77	***
Exp. 2	1.00	1.00		10.68 ± 1.21	13.19 ± 1.17	
Lycium chinense						
Exp. 2	0.96	1.00		5.14 ± 0.63	3.38 ± 0.40	
Exp. 3	0.84	0.82		7.78 ± 0.89	6.31 ± 0.73	
Lycium ruthenicum						
Exp. 3	0.86	0.76		20.55 ± 3.86	**23.41 ± 4.17**	*
Exp. 4	0.75	0.65		6.73 ± 2.32	2.51 ± 0.63	

[a] Asterisks denote statistical significance, P ≤ 0.05 (*), P ≤ 0.01 (**) and P ≤ 0.001 (***).

3.4.3. Determination of infection process

Urediniospores of *P. rapipes* germinated readily on the leaf surface of *L. barbarum*, *L. chinense*, *L. ruthenicum*, and both haplotypes of *L. ferocissimum* (Table 4). Appressoria developed from the germ

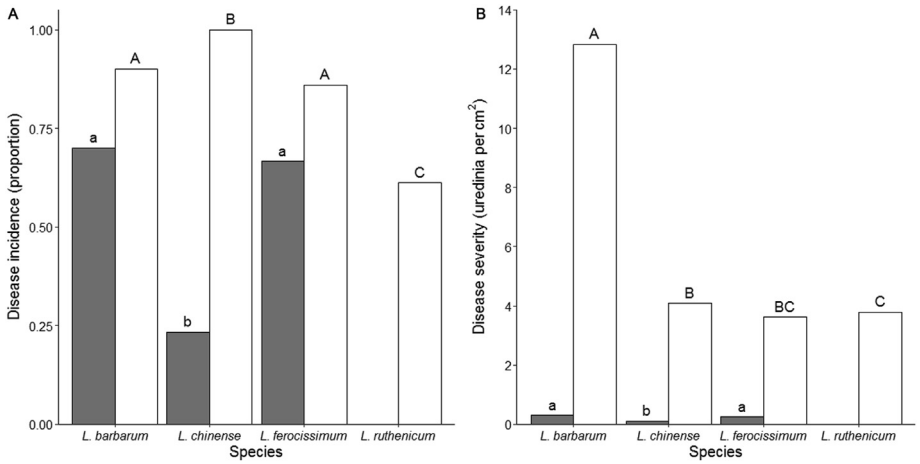

Fig. 4. Susceptibility of *Lycium* species to the Western Cape isolate of *Puccinia rapipes*, as a measure of (A) disease incidence (proportion of leaves with uredinia) and (B) disease severity (mean number of uredinia per cm^2 leaf area). Species with different letters above the bars are significantly different ($P \leq 0.05$) (lower case for exp. 6, dark grey columns, and upper case for exp. 7, white columns). Results for *L. australe* are not shown as the species was not found to be susceptible.

tubes over stomata, producing a penetration peg which penetrated through the stomatal aperture. *P. rapipes* then proceeded to produce sub-stomatal vesicles and an extensive network of intercellular infection hyphae on these susceptible hosts, with numerous and normally developed haustoria, and the development of normal uredinia (Fig. 5A and B; Table 4).

While urediniospore germination and penetration via stomata did occur on *L. australe*, these infections were never observed to progress to uredinia development and, while haustorial mother cells developed very rarely (<1 % of observations), haustoria were never observed. Aborted intercellular hyphae were commonly observed in *L. australe* (Fig. 5C). Closer inspection of necrotic spots on *L. australe* at 21 dai revealed the presence of a restricted network of infection hyphae in these areas, with plant cell death surrounding the infection site (Fig. 5D). While urediniospores were found to occasionally germinate on the leaf surface of *H. albus*, *H. aureus* and *S. aviculare*, no germ tubes were ever observed forming appressoria over stomata (Table 4).

3.4.4. Plant responses

L. barbarum was categorised as highly susceptible to *P. rapipes*, given the large number of uredinia per cm^2 of leaf area formed following spray inoculation of this species in exp. 7 (Fig. 4B; Table 4). Based on results from this experiment, both haplotypes of *L. ferocissimum*, *L. chinense* and *L. ruthenicum* were categorised as susceptible species. Microscopy confirmed *L. australe* as resistant, and *H. albus*, *H. aureus* and *S. aviculare* as immune, to infection by *P. rapipes*.

Table 4
Response of *Lycium ferocissimum* and closely-related species to *Puccinia rapipes*, based on assessments of microscopic and macroscopic symptom development (Table 1). Microscopic development includes urediniospore germination (germ.), penetration through stomata (pen.) and colonisation by intercellular hyphae (col.), through to full reproductive uredinia development (repr.). Species are listed by approximate phylogenetic relatedness to the target plant, *Lycium ferocissimum* (Levin et al., 2007; Särkinen et al., 2013).

Species (– haplotype)	Germ.	Pen.	Col.	Repr.	Plant response
Lycium ferocissimum – 5	+	+	+	+	Susceptible
L. ferocissimum – hybrid 5*2	+	+	+	+	Susceptible
Lycium barbarum	+	+	+	+	Highly susceptible
Lycium chinense	+	+	+	+	Susceptible
Lycium ruthenicum	+	+	+	+	Susceptible
Lycium australe	+	+	+	–	Resistant
Hyoscyamus albus	+	–	–	–	Immune
Hyoscyamus aureus	+	–	–	–	Immune
Solanum aviculare	+	–	–	–	Immune

4. Taxonomy

P. rapipes Berndt & E. Uhlmann; Mycol. Progress 5:173 (2006).

Spermogonia few in small groups, amphigenous though predominantly hypogenous, subepidermal, flask shaped (type 4), 100–140 μm diameter, ostiolar trichomes apparently adherent forming a short column, spermatia 5–7 × 4 (–5) μm (Fig. 2H). Aecia 1 to few, or developing in rings around spermogonia, *circa* 1 mm, *Aecidium*-type, 350–450 μm wide, not extending far beyond the epidermis, appearing bright yellow due to colour of aeciospores *en mass* (Fig. 2C). Peridium white, edges shortly lacerate; peridial cells irregularly oblong or trapezoid, 25–41 × 15–34 μm (mean 34.3 × 23 μm), wall 4–5 (–6) μm thick; inner wall coarsely verrucose, 4–5 μm thick, verrucae irregularly shaped; outer wall finely verrucose, 6–8 μm thick, verrucae irregularly shaped (Fig. 2I and J). Aeciospores globose to subglobose, 27–34 × 22–28 μm (mean 30.5 × 25.4 μm), length to breadth ratio of 1–1.5 (mean 1.2), cell contents hyaline with large bright yellow oil droplets, wall 1–1.5 μm, finely verruculose, verrucae round and regular in shape and size, no pore plugs present (Fig. 2K and L).

Distribution: South Africa.

Specimens examined: **South Africa**: *Western Cape Province*: Miller's Point, South of Simon's Town, on leaves of *L. ferocissimum* Miers (Solanaceae), DATE 21 Nov. 2008, 11 Jul. 2017 and 11 Jul. 2018, A.R.Wood (PREM 60083, 62240 & 62260). *Eastern Cape Province:* Grahamstown, Hillsview Rd, on leaves of *L. ferocissimum*, DATE 6 Jun. 2017 and 26 Nov. 2018, E. Mauda & L. Chari (PREM 62261 & 62316); Gamtoos, on leaves of *L. ferocissimum*, 12 Nov. 2018, E. Mauda & L. Chari (PREM 62315).

5. Discussion

The research reported here demonstrates how initial assessments can be conducted to streamline the selection of pathogens as candidate weed biological control agents. Such assessments provide the necessary information to decide whether or not extensive and costly host-specificity testing should be undertaken (Morin et al., 2006). Streamlining agent selection has the potential to make the biological control pipeline cheaper and less time

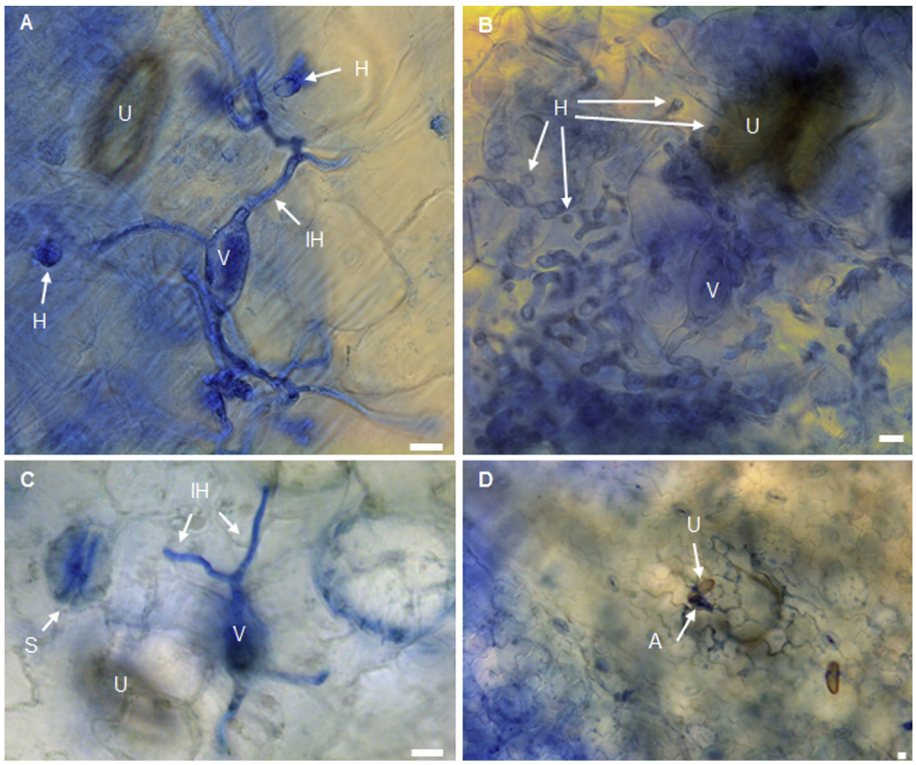

Fig. 5. Microscopic development of *Puccinia rapipes* on (A) *Lycium ferocissimum* at 5 dai (days after inoculation) showing development of haustoria, (B) *Lycium barbarum* at 12 dai showing extensive development of intercellular hyphae and many haustoria, (C) *Lycium australe* at 5 dai, showing aborted intercellular hyphae and (D) *L. australe* at 21 dai, showing plant cell death surrounding site of attempted infection, typical of microscopic symptoms of hypersensitive response. Scale bars = 10 μm. A = appressorium, H = haustorium, IH = intercellular hyphae, S = stomata, U = urediniospore, V = sub-stomatal vesicle.

consuming by discarding, early in the process, candidates that do not meet basic requirements. Further, focussing investment on agents which are likely to be successful once released is also likely to improve the cost-benefit ratio of biological control (Sheppard, 2003).

Sequencing of aecia observed on *L. ferocissimum* in the field has allowed us to categorically confirm that they belonged to *P. rapipes*. The descriptions of aecia and spermogonia of *P. rapipes* and genetic sequence data generated in our study, now complement the original description of *P. rapipes* given by Berndt and Uhlmann (2006). Further, sequencing of *P. rapipes* samples has also allowed us to resolve this species' position within the recent taxonomic revision of *Puccinia* on Lycieae by Otálora and Berndt (2018). *P. rapipes* was not included in their phylogeny as the type collection was only available for microscopic examination and not for destructive sampling for DNA extraction. Species recognition of *Puccinia* on Lycieae relies not only on micromorphology but also host identity and geographic location (Otálora and Berndt, 2018). Considering that several species of *Puccinia* have been recorded infecting a number of *Lycium* species in South Africa (Berndt and Uhlmann, 2006), and that the taxonomy and phylogeny of *Lycium* in South Africa is highly complex, with evidence of hybridisation and many undescribed species within numerous species complexes (Levin et al., 2007; unpublished data), we considered it prudent to confirm with sequencing the identity of the rust fungus life cycle stages we observed on *L. ferocissimum* in the field.

Confirmation of the macrocyclic, autoecious life cycle of *P. rapipes* provided initial confidence that the fungus would likely be host specific within a single plant family, as alternate hosts are not necessary for completion of its life cycle. Rust fungi that do not affect cultivated plants are often poorly studied, with knowledge of their life cycle and host range often limited to taxonomic descriptions, if they are described at all. Indeed, *P. rapipes* was only first described in 2006 (Berndt and Uhlmann, 2006), based on uredinia and telia described from a single specimen collected in 2004 in South Africa. To our knowledge, no macrocyclic, autoecious rust fungus has ever been reported as having a host range encompassing more than a single plant family. The autoecious nature of *P. rapipes*' life cycle thereby simplifies application of the centrifugal phylogenetic method for testing of candidate weed biological control agents (Briese, 2003; Wapshere, 1974), allowing the host test list to be centred on the family of the target weed taxa.

Improved understanding of the life cycle of *P. rapipes* gathered in our study can now inform future field surveys and laboratory work with the rust fungus. Our understanding of the putative seasonal timeline for the life cycle stages of *P. rapipes* (Table A.5 and Figure A.1) indicate that collections of urediniospores (the most easy to manage propagules) for use in studies of the fungus would be most abundant if made during the winter and spring, from June to October in the Eastern Cape and August to as late as January in the Western Cape of South Africa, dependant on weather conditions prevalent within any year. Germination of dormant teliospores in the laboratory and subsequent completion of the life cycle via laboratory inoculations with basidiospores has historically been a challenging task (Anderson et al., 2011). Our success in achieving this, combined with field observations, has assisted us in not only confirming the macrocyclic, autoecious nature of *P. rapipes*, but has enhanced our understanding of the probable environmental triggers for breaking teliospore dormancy and how rapidly the rust fungus may progress through its various life cycle stages. Our observations indicate that teliospores are likely developed in response to temporal and/or abiotic and biotic conditions such as ageing

plant tissue and/or drier climatic conditions; given that they were commonly occurring in the field at the onset of summer and subsequent spermogonia and aecia were observed at the onset of the first winter rains in the Western Cape. We speculate that cool, wet conditions and time and/or natural weathering, as simulated in the successful treatments that broke teliospore dormancy in the laboratory, most likely breaks dormancy of teliospores and/or encourages their germination in the field. Given that urediniospores were formed within five weeks of teliospore germination in the laboratory, collections of mature urediniospores would likely be most fruitful if collected at least six to eight weeks following the onset of the first winter rains.

Disease symptoms caused by *P. rapipes* on *L. ferocissimum* in the field in South Africa ranged from very low incidence and severity, with only a single uredinium or telium observed on single leaves at some survey sites, through to the high incidence and severity seen at the Miller's Point site in the Western Cape, where greater than 50 % disease incidence and severity (i.e. more than 50 % of leaves per plant were observed to have uredinia and telia, covering at least 50 % of the leaf surface of those leaves) was observed for many infected plants (data not shown). In our laboratory experiments, the Western Cape isolate of *P. rapipes* was clearly more pathogenic on the target weed *L. ferocissimum* than the Eastern Cape isolate, and would therefore be the best candidate isolate for use in further host-specificity testing. Aggressive isolates of fungi are commonly selected during early screening phases of classical biological control work, to ensure that potential damage in the field is adequate to reduce weed populations. Highly aggressive isolates of rust fungi can persist for years within a weed population following release. For example, an isolate of the rust fungus *Puccinia chondrillina* that is highly aggressive on the narrow leaf form of the target weed skeleton weed (*Chondrilla juncea*), which was first released in Australia in 1971 (Cullen et al., 1973), continues to actively infect and provide adequate control of populations of this form of the weed to the present day (Cullen, 2012). The Western Cape isolate of *P. rapipes* may also be better adapted to the haplotypes of *L. ferocissimum* present in Australia, as evidence indicates that Australian populations of the weed are most likely derived from Western Cape populations of *L. ferocissimum* in South Africa (unpublished data). The Western Cape has also been identified as the area with the greatest climatic match index to those areas invaded in Australia (unpublished data). Selecting agents from bioclimatically homologous regions in the native range of the weed, and preferably from populations most closely genetically related to the invasive populations, has long been recognised as an effective strategy to prioritise agent selection (Dhileepan et al., 2006; Rafter et al., 2008; Robertson et al., 2008). Conducting all future host-specificity testing with the Western Cape isolate of *P. rapipes* is therefore recommended, as this isolate is more likely to survive and thrive in Australia, should it be approved for release.

While all *Lycium* of African and Eurasian provenance tested in our experiments were found to be susceptible to *P. rapipes*, the Australian native *L. australe*, and all other closely-related tested species of both exotic (*H. albus* and *H. aureus*) and native (*S. aviculare*) origin were found to be resistant or immune to infection. Phylogenetically, all *Lycium* species included in our studies belong to the Old World clade of *Lycium* (*sensu* Levin et al., 2007). Within this clade, the Eurasian species L. *barbarum*, *L. chinense* and *L. ruthenicum* comprise a clearly monophyletic lineage, which currently nests within a well-supported clade of predominantly African species to which *L. australe* and *L. ferocissimum* belong (Fukuda et al., 2001; Levin et al., 2007). Based on this phylogeny, it was surprising to find that *Lycium* species of Eurasian origin were susceptible to *P. rapipes*, while *L australe*, which is phylogenetically closer to *L. ferocissimum*, was resistant. As only *Lycium* species present in Australia could be used in our studies, it is possible that other species in the genus may also be susceptible to *P. rapipes*. Planting of *Lycium* species found to be susceptible to *P. rapipes* in our study under *L. ferocissimum* naturally infected with the rust fungus in South Africa would be a useful exercise to establish the ability of the fungus to infect and persist on these hosts, outside of ideal laboratory conditions. Host-specificity testing under artificial conditions is often considered to overestimate the realised host range of many agents (Barton, 2012; Hinz et al., 2019).

Within 18 m we were able to generate adequate data to show that *P. rapipes* may be sufficiently host specific to pursue as a potential biological control agent for *L. ferocissimum* in an Australian context, as the rust fungus does not infect the sole native *Lycium* species found in Australia. However, there are other considerations to be taken into account surrounding evident non-target impacts. In releasing *P. rapipes*, regulators would need to accept that the fungus would most likely infect the susceptible Eurasian goji berries, *L. barbarum*, *L. chinense* and *L. ruthenicum*, currently being grown, albeit to a limited extent, in Australia. Further comprehensive host-specificity testing of *P. rapipes* has therefore been suspended at present, while the regulatory implications and social acceptance surrounding the potential release of such an agent are explored.

Release applications for weed biological control agents in Australia are assessed within an entirely risk-based assessment framework, focussed on ensuring an Appropriate Level of Protection (ALOP) is conserved when deliberately introducing a new organism into the country (Biosecurity Act, 2015; Australian Department of Agriculture, 2018; Australian Government Department of Agriculture and Water Resources, 2018). This is in contrast to New Zealand, who conducts a cost-benefit analysis prior to an agent release, allowing regulators to weigh the benefits of releasing the agent against the risks of any non-target impacts (Hazardous Substances and New Organisms Act (Methodology) Order 1998; Hazardous Substances and New Organisms Act 1996; Environmental Protection Authority, 2013). The ALOP for Australia is defined by the Biosecurity Act (2015) as "a high level of sanitary and phytosanitary protection aimed at reducing biosecurity risks to a very low level, but not zero." The expected infection of goji berries by *P. rapipes* in the field could well be considered as sufficient risk to prevent this fungus being approved for release in Australia. This will largely depend on what the threshold for this very low level risk is for the Australian Government. While *L. barbarum*, *L. chinense* and *L. ruthenicum* are all sold as either seed or seedlings by the nursery and garden industry in Australia, there appears to be little to no commercial production of these species at present or planned in the near future (Wainwright, 2015); and two of these species, *L. barbarum* and *L. chinense*, are also recognised as weeds in Australia and globally (Randall, 2017). Non-target damage on these species associated with the introduction of a rust fungus such as *P. rapipes* could be mitigated through the use of fungicides already registered to target other *Puccinia* species in Australia. Efficacy studies of such fungicides could form a component of any future scientific work, to underpin a more comprehensive risk assessment for the release of *P. rapipes* in the Australian environment.

Our initial results indicate that *P. rapipes* may be sufficiently host specific to pursue as a potential biological control agent for *L. ferocissimum* in an Australian context, should regulators be willing to accept damage to the Eurasian goji berries being cultivated in Australia. Prior to conducting any further host-specificity testing, it is essential to assess the acceptability of potentially introducing *P. rapipes* as a biological control agent in Australia. To do so it is crucial to engage with regulators and goji berry stakeholders (such as nursery producers and niche market growers), to

assess acceptance of the damage the fungus could inflict on Eurasian goji berries, and any management implications the release of such a pathogen may have in home garden and production systems. *L. ferocissimum* is also known to be invasive in New Zealand (Weber, 2017), aside naturalised populations of *L. barbarum* and *L. chinense* (GBIF.org, 19 March 2019). Our results may therefore also be of interest when considering biological control efforts against *L. ferocissimum* in a New Zealand context, particularly given their cost-benefit approach to target vs. non-target damage when evaluating release applications for classical biological control agents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.08.007.

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