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Use of a trap garden to find additional genetically distinct isolates of the rust fungus *Phragmidium violaceum* to enhance biological control of European blackberry in Australia

Louise Morin · Katherine J. Evans · Mireille Jourdan · Don R. Gomez · John K. Scott

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Abstract Biological control agents can be more effective if their populations are genetically diverse, particularly when the target invasive plant comprises a range of genotypes with different susceptibilities and occurs across various microclimates. We report on the use of an efficient approach to find, in the native range, diverse isolates of a rust fungus for biological control. An outdoor trap garden containing various clones of invasive European blackberry (*Rubus fruticosus* agg.) collected in Australia, each with a different DNA phenotype, was established in France. Within 4 weeks of establishment, the leaf-rust fungus *Phragmidium violaceum* was recovered from all clones in the garden. Molecular analyses of eight recovered and purified isolates of the fungus from the garden revealed that they were genetically distinct from each other and from isolates already present in Australia. These garden isolates also represented a subset of the population existing in Europe, when compared to isolates collected about 30 years ago. Two pathogenicity phenotypes were observed among the garden isolates in bioassays consisting of representative blackberry clones from Australia, and together the isolates were capable of infecting all clones. Results from host-specificity tests on key non-target plant species closely related to European blackberry concurred with previous findings that the leaf-rust fungus does not pose a threat to commercial blackberry cultivars and *Rubus* species native to Australia. The release and establishment of the garden isolates in Australia has potential to increase the genetic diversity and evolutionary potential of the leaf-rust fungus for more effective biological control.

Keywords Agent selection · Genetic diversity · Invasive plant · Microsatellites · *Rubus fruticosus* agg

L. Morin (✉) · D. R. Gomez
CSIRO Ecosystem Sciences,
GPO Box 1700, Canberra, ACT 2601, Australia
e-mail: louise.morin@csiro.au

K. J. Evans
Perennial Horticulture Centre,
Tasmanian Institute of Agricultural Research,
University of Tasmania,
13 St Johns Avenue,
New Town, Tasmanian 7008, Australia

M. Jourdan · J. K. Scott
CSIRO Ecosystem Sciences, European Laboratory,
Campus International de Baillarguet,
34980 Montferrier-sur-Lez Cedex, France

Present Address:
D. R. Gomez
Department of Innovation, Industry, Science and Research,
GPO Box 9839, Canberra, ACT 2601, Australia

Present Address:
J. K. Scott
CSIRO Ecosystem Sciences,
Private Bag 5, P.O., Wembley, WA 6913, Australia

Abbreviations
PCoA principal coordinate analysis

Introduction

Rust fungi are preferentially used over other pathogens in classical biological control of invasive plants principally because of their characteristically high level of specificity (Morin et al. 2006). The disadvantage of such high specificity however, is that in situations where the target invasive plant consists of a complex of genotypes with different susceptibilities, the introduction of multiple rust isolates, which together infect the range of target plant genotypes present, is required to adversely impact on the overall plant population (Ellison et al. 2004). A broad genetic base in the introduced pathogen population may also enable natural selection to take place (Barrett et al. 2008). This can lead to the dominance of the fittest pathogen genotypes or emergence of novel genotypes better adapted to the varying set of hosts and environmental conditions across the landscape.

The Australian biological control program against European blackberry (*Rubus fruticosus* aggregate; hereafter referred to as blackberry) was initiated in the mid-1970s (Bruzzese and Hasan 1986b). In 1984, while isolates of the blackberry leaf-rust fungus *Phragmidium violaceum* were being collected and assessed in Europe (hereafter referred to as European isolates), the fungus was found in Victoria, Australia (Bruzzese and Field 1985). Spread of the disease throughout southern Australia was rapid and the leaf-rust fungus is now present in all states and territories where blackberry occurs. Authorised releases of one of the European isolates (F15) (Bruzzese and Hasan 1986b) were subsequently made in 1991 and 1992. This isolate was selected and approved for release by the authorities because it produced high densities of uredinia on three of the four widespread species tested within *R. fruticosus* agg.

Since its first occurrence in Australia, the leaf-rust fungus has had a significant deleterious impact on some infestations of blackberry (Mahr and Bruzzese 1998). Isolates of the fungus recovered in Australia are hereafter referred to as Australian isolates. Unsuitable climatic conditions and host resistance, however, are believed to have limited the debilitating effect of the fungus in some situations (Evans et al. 2005). We now know that blackberry in Australia comprises at least 15 polyploid agamospecies, each with one or more genotypes, and one diploid, sexual species (Evans et al. 1998, 2007), with *Rubus anglocandicans* the most widespread of these agamospecies (Evans and Weber 2003). Evans et al. (2011) identified at least one Australian isolate capable of infecting the range of blackberry genotypes that have been characterised so far. Nonetheless, blackberry patches are regularly escaping severe disease at some locations where weather is favourable for rust epidemics, indicating that this isolate may not be widespread or fails to colonise its host early enough in the growing season following aerial dispersal to a particular site.

Broadening the genetic base of the leaf-rust fungus to increase its diversity and evolutionary potential has been proposed to enhance the effectiveness of this biological control agent in controlling blackberry populations in Australia (Gomez et al. 2008). This strategy is particularly appropriate since recombination plays an important role in maintaining genotypic variation within populations of this fungus (Gomez et al. 2008). Augmentation of genetic diversity in populations of endemic organisms, particularly those at risk of extinction, has been shown to increase their fitness and persistence (e.g. Ahlroth et al. 2003). Similarly, multiple introductions over time of an invasive species through a variety of sources that bring about large amounts of genetic variation contribute to their colonisation success (Crawford and Whitney 2010).

The most commonly used approach to obtain different isolates of a pathogen for biological control is to visit a range of locations in the native range where the target invasive plant is present and to collect infected material (Morin et al. 2006). The pathogen is then isolated from the material and isolates are screened for pathogenicity on accessions of the target plant from the introduced range. There is no certainty however, that the recovered isolates will be compatible with the invasive genotypes of the plant and cause disease. To circumvent such eventuality, research on the genetic structure of the target plant species in the introduced and native ranges has been recommended to pinpoint the

geographical source of plants that invaded the new range (Prentis et al. 2009) and facilitate collection of suitable pathogenic isolates. The high level of genetic diversity in blackberry in Australia and the possibility of novel plant genotypes having evolved since introduction of the species (Evans et al. 1998) made it impractical to locate their origins in the native range. Therefore, the trap garden approach was employed to find additional isolates of the leaf-rust fungus (hereafter referred to as garden isolates). A preliminary report on this garden can be found in Scott et al. (2002).

The trap garden approach, which is particularly adequate for pathogen species that have wind-borne propagules like rust fungi, involves the establishment in the native range of an outdoor garden planted with genotypes of the target plant species that occur in the introduced range (Morin et al. 2006). Such a garden is placed preferably in the area of highest diversity of the pathogen, although this is not known for the blackberry leaf-rust fungus. Isolates of the pathogen are then recovered from plants as they become naturally infected over one or more growing seasons. In the 1980s, such a garden planted with skeleton weed (*Chondrilla juncea*) clones from Australia and North America was established in Turkey, the putative centre of origin of this invasive plant, to find isolates of the biological control agent *Puccinia chondrillina* (Hasan et al. 1995). No data, however, are available to assess how effective the approach was for this pathosystem.

There has been no report of the blackberry leaf-rust fungus on non-target plant species since its introduction to Australia (K.J. Evans unpublished data). Nevertheless, our plan to collect additional isolates of the fungus for introduction to Australia raised concerns because of the possibility that they could exhibit a slightly different host range to that of the existing rust population. Host-specificity tests with the garden isolates had thus to be performed to assess this risk. A proposal to test the garden isolates only on a restricted number of non-target plant taxa closely related to blackberry was presented to the authorities and accepted. The rational behind this proposal was that the garden isolates were likely to have a similar host-plant range to the pool of 15 European isolates of this rust species that were previously tested (Bruzzese and Hasan 1986b). These 15 isolates had been collected across a wide geographical range in Europe and therefore likely represented a broad genetic base of the species (Bruzzese and Hasan 1986a).

The first objective of this study was to determine the efficiency of the trap garden approach in finding isolates of the blackberry leaf-rust fungus in the native range that are genetically distinct and pathogenic across a range of representative blackberry clones from Australia. The second objective was to determine if the garden isolates would pose a greater threat to non-target plant species than that of European isolates investigated in the 1980s.

Materials and methods

Origin, propagation and establishment of blackberry clones in the garden

During 1999 and 2000, 12 clones (each with a different DNA phenotype) of at least 6 blackberry species occurring in Australia (Table 1) were micropropagated by tissue culture at the University of Adelaide, Australia. The origin of each clone is specified in Evans et al. (2005) and the method used for micropropagation detailed in Evans et al. (2011). For transport to France, single plantlets were micropropagated on 20 ml of firm maintenance medium in 140 ml polycarbonate containers. Cuttings from plants of each clone maintained in the glasshouse were also sent to France. Leafless cuttings with three nodes were dipped in a solution of sodium hypochlorite (0.1% *v*/*v*) for 30 min. Their cut end was then treated with a rooting hormone (3 g kg^{-1} indol-butyric acid) and the entire cuttings buried in a 1:1 perlite and vermiculite mixture moistened with sterile water and contained in plastic bags for transportation.

On arrival, each micropropagated plantlet was removed from the agar and placed into steam-sterilised potting mixture (Table 2). They were then exposed to a cultivation procedure for 'hardening' plants that involved a one-off watering with a fungicide solution of 2 g l^{-1} Fongarid® (250 g kg^{-1} Furalaxyl) and 0.3 g l^{-1} Benlate® (50% Benomyl). Plantlets were initially placed in a controlled-environment room at 25°C with a 16 h photoperiod (fluorescent and filtered natural light) and transferred 2 weeks later in a glasshouse (15–25°C, only natural light). Each cutting without roots was soaked in an insecticide solution of 0.9 ml l^{-1} Torque® S (Fenbutatin oxide 550 g l^{-1})

Table 1 Clones of European blackberry from Australia planted in the trap garden in France and monitored for presence of *Phragmidium violaceum* during the 2000 growing season. Details of single-uredinium isolates made from urediniospores collected on clones are included

Blackberry			No. of isolates purified	Investigated isolates[c]		Isolate voucher specimen[f]
Taxon	Clone[a]	DNA phenotype[b]		Full ID no.[d]	Abbreviated ID no.[e]	
R. sp.	EB18	2	1	–[g]	–	–
R. leucostachys	EB19	6	1	G6–TG–00–4–1	6–4	DAR 76667
R. leucostachys	EB16	7	1	–	–	–
R. leucostachys	JH1669	9	1	–	–	–
R. sp.	SR43	14	2	G14–TG–00–4–1	14–4	DAR 76668
R. sp. Tasmanian form	981901/02	16	1	–	–	–
R. rubritinctus	SR18	18	1	G18–TG–00–4–1	18–4	DAR 76670
R. phaeocarpus	15734	19	1	–	–	–
R. leucostachys	972101	21	2	G21–TG–00–1–1	21–1	DAR 76671
R. vestitus	EB21	28	3	G28–TG–00–2–1	28–2	DAR 76672
R. anglocandicans	9607	32	9	G32–TG–00–1–1	32–1	DAR 76673
				G32–TG–00–2–2	32–2	DAR 76674
				G32–TG–00–3–1	32–3	DAR 76675
R. sp.	971606	39	1	–	–	–

[a] Origin of each clone is detailed in Evans et al. (2005)

[b] Based on DNA phenotypes published in Evans et al. (2007)

[c] Only eight isolates could be cultured for subsequent investigations, because of the overall low viability of stored urediniospores

[d] Each isolate was identified using the same coding system. Each component of the isolate ID number corresponds to the following: DNA phenotype number of the blackberry plant from which urediniospores were collected; Location (TG=Trap Garden); Year of collection (00=2000); Block number location of the plant; Uredinium isolation number

[e] Full isolate ID numbers have been abbreviated for use in the paper and only include the DNA phenotype number of the blackberry plant from which urediniospores were collected and block number location of the plant in the trap garden

[f] Single-uredinium isolates permanently preserved as uredinia on infected leaves in the Plant Pathology Herbarium (HERB-DAR) of Industry & Investment, New South Wales

[g] Not applicable

and the basal end dipped into rooting hormone (8 g kg^{-1} indol-butyric acid), before planting in steam-sterilised potting mixture (Table 2) and watered with a fungicide solution of 1 g l^{-1} Fongarid®. Pots were placed under high relative humidity conditions in the controlled-environment room (as above) until roots formed and then transferred to the glasshouse.

Authorisation to grow blackberry clones from Australia outdoor in a garden at the CSIRO European Laboratory was obtained from the relevant French authorities. The garden consisted of four blocks (approx. 10×4 m) set 7 m apart and oriented north-south to maximise exposure to prevailing north-west and north-east winds. Two trenches (approx. 80 cm wide and 50 cm deep) were dug along the long axis of each block and lined with polypropylene to contain roots and facilitate the removal of plants at the end of the experiment. Each trench was partly filled with soil and divided into six equal plots. During the 2000 growing season, groups of four plants for each of the 12 blackberry clones were allocated randomly to a plot in each of the block. Plants grown in the glasshouse for 2 to 9 months were transplanted into large pots (Table 2), which were buried almost up to soil surface level in the centre of each plot. Plants were watered by drip irrigation when required. Plant spread by tip rooting was prevented by the use of weed matting and by coiling canes around

Table 2 Details of materials and methods specific to the different locations where the study occurred

	Montpellier protocols	Canberra protocols
Plant propagation		
Glasshouse-maintained plants	Micropagated plantlets	
	Growing medium: 60% sphagnum peat moss, 20% composted pine bark, 10% river washed sand, 10% perlite Pot size: 9×9×10 cm	Growing medium: 5:1:1:3 compost base, peat moss, river sand, perlite, with 1.4 kg slow-release fertiliser m^{-3} [Aboska®: *N*=15.16%, *P*=6.93%, *K*=5.19%] Pot size: 19.5 cm diam.
	Cuttings	Fertiliser: Aquasol™ (*N*=23, *P*=4, *K*=18)
	Growing medium: 50% sphagnum peat moss, 50% river washed sand Pot size: 18×18×19 cm	
	Fertiliser: Algospeed® (*N*=18, *P*=18, *K*=18)	
Outdoor plants	Growing medium: 50% ground garden waste, 30% composted pinebark and 20% sphagnum peat moss Pot size: 37 L	
	Fertiliser: Multicote 8® (slow-release granules; *N*=18, *P*=6, *K*=12)	
Production of inoculum		
Source of compound leaves used	Glasshouse-maintained plants of the clone on which the isolate to be multiplied was recovered from	*Rubus anglocandicans* plants (ex Black Mountain, Canberra) grown from field-collected crowns planted in a glasshouse (16–26°C; natural light)
Surface-sterilisation of inoculated leaves	Immersed in 70% ethanol for 1 s followed by one rinse in sterile distilled water and 1.5 min in 7% bleach solution (0.64% *w*/*v* available chlorine)	Immersed in 0.15% (*w*/*v*) solution of mercuric chloride for 1.5 min
Agar medium used	Knop's agar (0.5 g $Ca(NO_3)_2$, 0.125 g KNO_3, 0.125 g $MgSO_4 \cdot 7H_2O$, 0.125 g K_2HPO_4, 0.005 g $FeCl_2$, 13 g agar, 1 L water)	1% water agar
Pathogenicity bioassays		
Isolates tested	6–4, 21–1, 28–2, 32–2	14–4, 18–4, 32–1, 32–3
Test to assess viability of urediniospores	A microscope slide covered with a film of 2% water agar was sprayed with each urediniospore suspension and placed on a moist filter paper inside a 9 cm plastic Petri plate	A block (≈1 cm^2) of 2% water agar placed on a glass slide was sprayed with each urediniospore suspension. The slide was then placed on a moist filter paper inside a 9 cm plastic Petri plate

pots. Flowers were removed as they formed, and all vegetative material destroyed and potting mixture autoclaved at the end of the experiment to prevent dispersal of plant material.

Collection and purification of isolates from the garden

Plants were monitored for presence of leaf-rust symptoms at weekly intervals from May to November 2000. Once plants of a clone in a block were severely infected (compound leaves with >50 sporulating uredinia), all urediniospores from an excised leaf were collected with a custom-built suction device and dusted with a camel hair paint brush onto the abaxial surface of detached, young, fully-expanded leaflets from a plant of the same clone maintained in the glasshouse. Inoculated leaflets were incubated and processed as described below. Once uredinia were produced on the detached leaflets, a single-uredinium isolate was made by removing urediniospores from one large uredinium with a fine camel hair paint brush, with the aid of magnification from a stereomicroscope,

and dusting them onto detached leaflets. For clones that were not severely infected by the rust, single-uredinium isolates were made directly from field-infected leaves. The isolates were multiplied on detached leaflets at least once and urediniospores stored in the refrigerator (4°C) and in freezers at −20 and −80°C.

Production of inoculum

A modified version of the method of Evans et al. (2000) was used. Young, fully-expanded compound leaves of blackberry (Table 2) were placed, abaxial surface facing upward, onto paper towel moistened with sterile distilled water contained in plastic Petri plates (10 or 14 cm diam.). Leaves were inoculated by spraying a suspension containing approximately 0.25 mg fresh urediniospores (or 0.5 mg if heat shocked urediniospores from the freezer were used (Evans et al. 2000)) per ml of sterile distilled water using a hand-held sprayer until the surface was covered with fine droplets. Plates were covered and placed in a controlled-environment room at 20°C with a 16 h photoperiod (fluorescent lights). Between 3 and 5 days after inoculation, leaves were surface sterilised (Table 2) and then rinsed three times in sterile distilled water and placed, abaxial side up, in plastic Petri plates (9 cm diam.) containing agar medium (Table 2). Plates were covered and placed back in the controlled-environment room for about 20 days. Urediniospores from each isolate were then harvested and used in bioassays.

Nucleic acid extraction and genetic analysis of garden isolates

The genotypes of the garden isolates were compared with those of 15 European isolates collected between 1978 and 1980 (all isolates listed in Gomez et al. (2006) and isolates GR23, I17, E4, and F7 of Bruzzese and Hasan (1986b)) and 16 Australian isolates collected between 1997 and 1999 (isolates listed in Gomez et al. (2006), with exception of isolates V2 and WP21).

For each isolate, DNA was extracted from 50–100 mg of urediniospores using the ChargeSwitch gDNA Plant Kit (Invitrogen) with a modification to manufacturer's protocol. Briefly, 1 ml of supplied Lysis Buffer and 2 µl of supplied RNase A were added to a 2 ml Safe-Lock Eppendorf Tube containing urediniospores and 20 sterilised 2 mm diameter borosilicate glass beads. Tubes were placed on a flat-bed vortex and samples homogenised at maximum velocity for 2 min. The manufacturer's protocol was then used in the remaining steps to attain the final elution of DNA.

Each isolate was characterised across 11 micro-satellite loci as described in Molecular Ecology Resources Primer Development Consortium (2010). Genetic relatedness among all genotypes was determined using a principal coordinate analysis (PCoA) in GENALEX V6 (Peakall and Smouse 2006). The PCoA was based on the pairwise genetic distance measure described in Smouse and Peakall (1999). Comparison of gene diversity was made using raw allele counts averaged across the 11 loci for each group of isolates. The number of unique alleles observed for each group of isolates was also determined.

Pathogenicity bioassays

Representative blackberry clones from each of the three groups identified by Evans et al. (2011), based on the relative density of uredinia produced on leaflets of whole plants and/or leaflet disks by four isolates of the leaf-rust fungus (isolate F15 and three Australian isolates) (Table 3), were used to characterise pathogenicity phenotype of garden isolates. Each clone was imported to the CSIRO European laboratory, processed and grown as described above. Each clone was also propagated in Canberra using crowns obtained from the University of Adelaide (Evans et al. 2005) and under the same conditions as for *R. anglocandicans* plants used for inoculum production (Table 2). The pathogenicity of garden isolates on each clone was first tested in bioassays using detached leaflets, with half of the isolates tested in Montpellier and the other half in a quarantine facility in Canberra to share the workload among the research team and streamline testing (Table 2).

For each clone, groups of three terminal leaflets were excised from three successive compound leaves (starting with the youngest, fully-expanded leaf) of one cane from different potted plants. Four to six groups (replicates) of three leaflets were then placed, abaxial surface facing upward, onto paper towel moistened with sterile distilled water contained in 14 cm diam. plastic Petri plates. Small notches at

Table 3 Pathogenicity of *Phragmidium violaceum* isolates on various representative blackberry clones. Pathogenicity of isolates from the trap garden in France was classified as high (H) when>60% of inoculated (detached) leaflets or at least one of the inoculated leaves on whole shoots in each of the replicates were given a disease score of 3 or 4 (Table 4). Pathogenicity was classified as low (L) for all other isolate-clone combinations, or non-pathogenic (N) if no uredinia whatsoever were produced on any of the leaflets inoculated in either bioassays (disease score of 0 or 1; Table 4)

Blackberry				*Phragmidium violaceum*											
Group[a]	Taxon	Clone[b]	DNA phenotype[c]	Relative density of uredinia produced by four reference isolates in Evans et al. (2011)[a]				Pathogenicity of garden isolates[d]							
				V1	V2	SA1	F15	6–4	14–4	18–4	21–1	28–2	32–1	32–2	32–3
1	*R. anglocandicans*	9607	32	M/H	M/H	M/H	M/H	H	H	H	H	**H**	H	H	H
1	*R. leucostachys*	972101	21	M/H	M/H	M/H	M/H	H	H	H	H	**H**	H	H	H
1	*R. vestitus*	EB21	28	M/H	M/H	M/H	M/H	H	H	H	H	**H**	H	H	H
2	*R. laciniatus*	KE1[e]	37	M/H	M/H	M/H	L/nil	**L**	**N**	**H**	**L**	**N**[f]	**L**	**N**	**L**
2	*R. laciniatus*	EB22	37	M/H	M/H	M/H	L/nil	**N**	**N**	**H**	**N**	**N**	**L**	**N**	**L**
2	*R. polyanthemus*	961107	36	M/H	M/H	M/H	L/nil	**H**	**H**	H	**H**	**H**	H	H	H
3	*R. erythrops*	EB20	25	M/H	L/nil	L/nil	M/H	H	H	H	H	**H**	H	H	H
3	*R. leucostachys*	EB9	7	M/H	L/nil	L/nil	M/H	H	**H**	**H**	H	H	H	H	H
3	*R. leucostachys*	EB19	6	M/H	L/nil	L/nil	M/H	H	**H**	**H**	H	H	H	H	H
3	*R.* sp.	SR43	14	M/H	L/nil	L/nil	M/H	H	H	**H**	H	H	H	H	H

[a] Grouping of blackberry clones identified by Evans et al. (2011) based on variation in the relative density of uredinia produced by four isolates of *P. violaceum* (isolates V1, V2 and SA1 from Australia, and F15 from Europe) on leaflet disks and/or leaflets still attached to plants. M/H=moderate to high number of uredinia; L/nil=low number or zero uredinia

[b] Origin of each clone is detailed in Evans et al. (2005)

[c] Based on DNA phenotypes published in Evans et al. (2007)

[d] Detached-leaflet bioassays were performed in Montpellier (for isolates 6–4, 21–1, 28–2 and 32–2) or Canberra (for remaining isolates), while all whole-shoot bioassays were performed in Canberra only. Bold letters correspond to final ratings obtained in whole-shoot bioassays, except those for isolates on clones KE1 and EB22, which are based on results from both the detached-leaflet and whole-shoot bioassays unless indicated otherwise. All other ratings (non-bold) were obtained in detached-leaflet bioassays

[e] Non-laciniate leaflets

[f] Bioassay only performed on whole-shoot for this isolate-clone combination

specific locations on the side of the leaflets were made to keep track of the relative age of each leaflet and the replicate number. Leaflets were inoculated, incubated and surface sterilised following the same protocols used to produce inoculum (Table 2), with each group of three leaflets placed in a separate 9 cm diam. plate containing 1% water agar. For each bioassay, an additional four groups of three leaflets from *R. anglocandicans* (clone 9607) were used as a negative control and sprayed with water only.

The viability of urediniospores of each isolate was assessed by spraying the spore suspension on water agar (Table 2) and incubating in the controlled-environment room where inoculated leaflets were located. After 24 h, a drop of blue-lacto-glycerol stain was applied to the agar to stop the germination process. Percentage germination was assessed using a light microscope. At 3 to 4 weeks after inoculation, detached leaflets were examined for presence of macroscopic symptoms and rated according to scores presented in Table 4. The effect of an isolate on a clone was classified in the high pathogenicity category when more than 60% of inoculated leaflets (across all replicates) were given a disease score of 3 or 4.

A second set of bioassays using compound leaves still attached to shoots of potted plants was carried out in Canberra for clone-isolate combinations that were

Table 4 Scoring system used to categorise macroscopic symptoms observed on blackberry leaflets or leaves 3 to 4 weeks after inoculation with *Phragmidium violaceum* isolates

Disease score	Symptoms
0	No macroscopic symptoms.
1	Discolouration and chlorosis present but no uredinia. Pin size chlorotic or necrotic spots.
2	Purple blotches on upper surface of leaflet(s). Underdeveloped, non-eruptive uredinia. An occasional sporulating uredinium usually restricted in size ($\leq$ 2 uredinia/leaflet) [a].
3	Normal sporulating uredinia present but number restricted ($>$ 2 uredinia/leaflet but$<$5 uredinia/cm^2) [a]. Sporulation. Purple/red halos present or not on upper leaf surface.
4	Large number of normal uredinia present ($\geq$ 5 uredinia/cm^2) [a]. Abundant sporulation. Purple/red halos present or not on upper leaf surface.

[a] A visual qualitative assessment of the number of uredinia per cm^2 was made using as reference photographs of infected leaflets for which number of uredinia per cm^2 had been determined

not classified in the high pathogenicity category in the detached-leaflet bioassays. For each selected clone-isolate combination, three to four canes (replicates), each from a different potted plant, were inoculated by spraying the urediniospore suspension (approx. 0.5 mg fresh urediniospores per ml sterile distilled water) onto the abaxial surface of the three youngest fully-expanded compound leaves per cane. The inoculated portion of each cane was then sprayed with tap water and covered with a plastic bag, which was then sealed with tape around the stem. Inoculated plants were placed in a controlled-environment room (same conditions as those used for inoculum production) and plastic bags were removed 18 h after inoculation. The viability of urediniospores of each isolate was assessed as described under Canberra protocols in Table 2. Macroscopic symptoms were assessed as described above. The effect of an isolate on a clone was classified in the high pathogenicity category when at least one of the inoculated leaves in each of the replicates was given a disease score of 3 or 4 (Table 4).

Host-specificity tests

The non-target plant species/cultivars used to test the specificity of the garden isolates were: (a) commercial blackberry cultivars with *R. fruticosus* agg. in their breeding pedigree, (b) *Rubus* taxa indigenous to Australia that were shown to be resistant or moderately susceptible to the leaf-rust fungus in previous tests (*R. moorei*, *R. gunnianus*) (Bruzzese and Hasan 1986a), and (c) recently described *Rubus* taxa indigenous to Australia that had not been tested previously (*R. nebulosus*, *R. probus*, *R. queenslandicus*).

Non-target plant species/cultivars were grown in Canberra from cuttings, field-collected root pieces, or crowns planted in potting mixture contained in plastic pots of various sizes and maintained in a glasshouse (as described for *R. anglocandicans* in the Canberra protocols in Table 2). Cuttings were treated with a hormone rooting gel (4 g l^{-1} indole-butyric acid), planted in a 1:1 perlite and vermiculite mixture, placed in the glasshouse and maintained wet with intermittent overhead misting to encourage root development prior to planting into potting mixture. *Rubus gunnianus* plants from an alpine region in Tasmania were transplanted in peat moss and placed under intermittent overhead misting to simulate its preferred habitat. In each test, three to four plants (replicates) (unless indicated otherwise) of each of the positive control (*R. anglocandicans* ex Black Mountain, Canberra) and test species were inoculated with a single garden isolate or a group of three or four isolates arbitrarily selected. Spores were produced as detailed in the Canberra protocols (Table 2) and an equal amount of urediniospores from each isolate was used in suspensions containing a group of isolates. Each test was conducted on at least two separate occasions.

Each plant (except *R. gunnianus*) was inoculated and processed as in whole-shoot bioassays, with the exception that both leaf surfaces were sprayed with a suspension of approx. 0.25 mg per ml distilled water, plastic bags over canes/branches were left for 24 h

and a 12 h photoperiod was used. Given the prostrate growth habit of *R. gunnianus*, all leaves of this species were inoculated, misted with tap water, and placed for 24 h in a humid chamber (40×30×29 cm covered plastic box containing water to a depth of 3 cm and an inverted metal wire tray onto which pots were placed) within the controlled-environment room. All inoculated plants were kept in the controlled-environment room for 3 weeks and then examined for macroscopic symptoms as described above. The viability of urediniospores of each individual isolate, applied singly or in a mixture with spores from other isolates during inoculations, was assessed for each test following the Canberra protocols (Table 2).

Results

Isolates recovered from the garden

The first uredinium on blackberry clones planted in the garden was observed in mid-June 2000; almost 4 weeks after the first plants were transferred to the garden. By the end of the growing season all clones had developed some uredinia. The most severely infected clones were 9607, EB21 and 972101, which had been the first to be transferred to the garden in May. At least one rust isolate per clone was obtained from infected leaves collected in the garden by initiating cultures from urediniospores of a single uredinium (Table 1). However, due to the overall low viability of urediniospores stored at 4, –20 and −80°C, only eight of the isolates were subsequently cultured.

Genetic discrimination among garden isolates

Each of the European (15), Australian (16) and garden (8) isolates characterised represented a unique multilocus genotype with between 2 to 16 alleles observed per locus. Gene diversity, expressed as the number of alleles averaged across the 11 microsatellite loci, was greatest for European isolates followed by garden isolates. Australian isolates had the lowest levels of gene diversity of the three groups of isolates (Table 5). The garden isolates possessed eight alleles not present among Australian and European isolates, and shared 38 and 9 alleles exclusively with isolates from Europe and Australia, respectively (data not shown).

Table 5 Number of total and unique alleles observed across 11 microsatellite loci in each group of *Phragmidium violaceum* isolates based on origin

Isolate origin	Number of isolates	No. of different alleles observed in each population	No. of alleles unique to each population
Australia	16	40 (3.64) [a]	13 (1.18)[a]
Europe	15	124 (11.27)	70 (6.36)
Trap garden	8	52 (4.73)	8 (0.73)

[a] Numbers in brackets refer to allele counts averaged across 11 loci

In the PCoA, the relative contributions of the two first coordinate axes to the total genetic variability were 45.5 and 16.9%, respectively (Fig. 1), whereas it was 11.7% for the third axis, which gave no interpretable information (data not shown). One group on the first coordinate axis was composed of genotypes of European isolates, including the garden isolates, and the other group consisted of genotypes of Australian isolates, with isolate WA9 in an intermediate position between the two groups. The second coordinate axis was less discriminating, although it revealed that half of the garden isolates (28–2, 14–4, 32–2, 32–3) formed a tight cluster with isolate YU22 collected at Skopje in the Republic of Macedonia in the late 1970s. This suggested that there was little genetic divergence between these isolates (Fig. 1). The other four garden isolates (6–4, 18–4, 21–1, 32–1) clustered with the other European isolates.

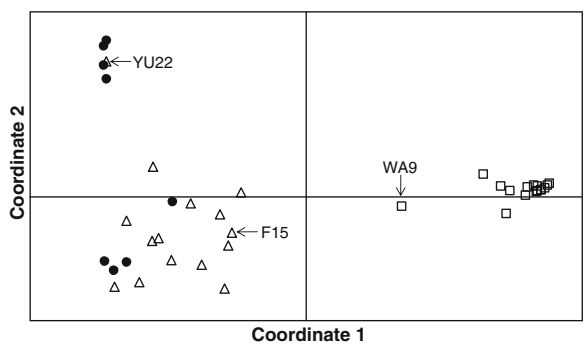

Fig. 1 Genetic relatedness among *Phragmidium violaceum* isolates recovered from the trap garden in France (*full circle*), from a range of locations in Europe between 1978 and 1980 (*open triangle*) and in Australia between 1997 and 1999 (*open square*) based on a principal coordinate analysis of the genetic distance measure described in Smouse and Peakall (1999). The position of specific isolates mentioned in the text is indicated

Table 6 Macroscopic symptoms on non-target *Rubus* species and cultivars closely related to blackberry following inoculation with *Phragmidium violaceum* isolates from the trap garden in France. Plants were inoculated with urediniospores of a single isolate or an equal mixture of urediniospores from each of the isolates in a group

Plant species	Percentage of inoculated leaves[a]											
	Isolates											
	28–2				14–4, 32–2, 32–3				6–4, 18–4, 21–1, 32–1			
	Disease score				Disease score				Disease score			
	0	1 & 2	3	4	0	1 & 2	3	4	0	1 & 2	3	4
Control species												
R. anglocandicans	12	25	63	0	0	24	43	33	0	0	87.5	12.5
Native species												
R. gunnianus	83	17	0	0	83	17	0	0	96	4	0	0
R. moorei	100	0	0	0	50	50	0	0	37.5	37.5	25	0
R. nebulosus	95	5	0	0	88	12	0	0	50	50	0	0
R. probus	100	0	0	0	100	0	0	0	100	0	0	0
R. queenslandicus	100	0	0	0	100	0	0	0	100	0	0	0
Commercial blackberry cultivars												
American Thornfree	100	0	0	0	71	29	0	0	25	67	8	0
Chester	61	39	0	0	61	39	0	0	50	50	0	0
Dirksen Thornless	83	17	0	0	100	0	0	0	75	25	0	0
Loch Ness	76	24	0	0	100	0	0	0	46	54	0	0
Silvan	100	0	0	0	76	24	0	0	50	50	0	0
Smoothstem[b]	100	0	0	0	84	16	0	0	59	41	0	0

[a] Pooled results from two trials presented for each isolate or group of isolates. Three or four replicates (plants) of each species were used unless indicated otherwise. On each plant, three compound leaves of different ages (i.e. the three fully opened leaves from the tip of a shoot) were inoculated and assessed for macroscopic symptoms. See Table 4 for description of symptoms for each disease score

[b] Cultivar was included in only one of the trials (three replicates/plants) with isolate 28–2. Only two replicates (plants) of this cultivar were used in each of the trials conducted with the two groups of pooled isolates

Pathogenicity bioassays

Germination tests on agar confirmed that urediniospores of the eight garden isolates used in the detached-leaflet and whole-shoot bioassays were viable, with germination percentages ranging from 20 to 92% (data not shown). In both types of bioassay, inoculated leaflets of the youngest leaves of a susceptible blackberry clone generally developed more uredinia than leaflets of older leaves (data not shown).

Combined results from the two bioassays revealed two pathogenicity phenotypes for the garden isolates based on macroscopic symptoms on representative blackberry clones from each of the three groups identified by Evans et al. (2011) (Table 3). Isolate 18–4 was highly pathogenic on all blackberry clones from the three groups, while other garden isolates were classified in either the low pathogenicity or non-pathogenic category on the two clones of *R. laciniatus* (KE1 and EB22). In the initial detached-leaflet bioassay, isolates 6–4, 21–1, 32–1 and 32–3 developed a small number of normal uredinia on a few of the leaflets of one or both of the *R. laciniatus* clones (disease score 3). However, none of the leaves inoculated with these isolates in the subsequent whole-shoot bioassay developed any uredinia, with the exception of some of the leaves inoculated with isolate 32–3. For clone KE1, 78% of total number of leaves inoculated with this isolate

developed the occasional sporulating uredinium (≤ 2 uredinia/leaflet; disease score 2) and 22% developed slightly more than two uredinia per leaflet and were given a disease score of 3. Similarly, 17% of leaves of clone EB22 were given a disease score of 3, 75% a disease score of 2 and 17% a disease score of 1 because they did not develop any uredinia. Nonetheless, isolate 32–3 was classified in the low pathogenicity category on these two clones, on the basis of our predefined criterion classifying an isolate in the high pathogenicity category on a clone when at least one of the inoculated leaves in each of the replicates was given a disease score of 3 or 4.

Host-specificity tests

Germination on agar of urediniospores used in host-specificity tests ranged from 26 to 90% (data not shown). The isolates were found to be highly pathogenic (disease score 3 or 4) on *R. anglocandicans*, the invasive blackberry species used as a positive control in each of the tests (Table 6). By 3 weeks after inoculation, the rust had produced large uredinia with abundant urediniospores on this host.

The majority of leaves of the non-target plant species inoculated developed either no macroscopic symptoms (disease score 0) or only a few chlorotic or necrotic flecks (disease score 1) or underdeveloped, non-eruptive uredinia (disease score 2) (Table 6). Isolate 28–2 and isolates 14–4, 32–2 and 32–3 applied as an equal mixture of urediniospores were non-pathogenic (disease score 0 or 1) on all non-target species inoculated (Table 6). In contrast, 25% of total leaves of the native species *R. moorei* inoculated with a mixture of isolates 6–4, 18–4, 21–1 and 32–1 developed several normal uredinia and were given a disease score of 3. The cultivar American Thornfree was the only cultivated blackberry with some leaves given a disease score of 3 following inoculation with this group of isolates. Nonetheless, in both of these non-target plant species, the uredinia that developed were considerably smaller in diameter compared to those produced on the control *R. anglocandicans*. Necrosis of tissue surrounding a few uredinia was observed on leaves of cultivar American Thornfree and may have played a role in restricting development of the rust.

Discussion

The overarching goal of our recent research activities on the biological control of blackberry in Australia has been to find and release additional isolates of the leaf-rust fungus, genetically distinct from the existing population, in an attempt to enhance the effectiveness of the rust in controlling its highly variable host across a wide geographical range. Comparison between multilocus microsatellite genotypes of the garden isolates and other isolates collected from a wide geographic range in Europe and Australia demonstrated that the trap garden approach was an efficient means of obtaining additional isolates of the leaf-rust fungus in the native range. It showed that the garden isolates are genetically distinct to isolates recovered from the rust population in Australia in 1997–1999. It also indicated that the garden isolates represent a subset of the potential genetic diversity of the rust in Europe, assuming that the rust population in Europe in 2000 was just as diverse as it was in 1978–1980 when the isolates used in this study were collected. AFLP-SAMPL characterisation of a subset of these isolates performed in a previous study also indicated that the garden isolates are distinct from each other and nested within the isolates collected in previous years from Europe (Gomez et al. 2006). This finding was expected for two reasons. Firstly, the rust genotypes available for selection by the garden in 2000 were restricted to spores present in air currents over southern France in a single growing season. Secondly, a selection pressure was imposed on the rust fungus, whereby only rust genotypes capable of developing a compatible interaction with blackberry clones from Australia planted in the garden could be recovered.

It is noteworthy that isolate WA9 from Western Australia did not cluster with the other isolates recovered from Australia. The PCoA ordination suggests WA9 bears ancestry to both European and Australian isolates, supporting a previous finding by Evans at al. (2000) that isolate WA9 shared DNA restriction fragments with isolate F15 released in Australia. This finding is also further evidence to support the hypothesis that WA9 emerged following recombination between isolate F15 and an individual from the rust population that established in the mid 1980s as a result of an unauthorised introduction.

Two different pathogenicity phenotypes, based on macroscopic symptoms that developed on inoculated blackberry clones, were observed for the garden isolates and did not correlate with groupings generated from microsatellite data. Only isolate 18–4 was found to be pathogenic across all clones inoculated—a phenotype similar to what Evans et al. (2011) observed for isolate V1, which originated from the western part of the state of Victoria in Australia. In contrast, the pathogenicity phenotype of all other seven garden isolates resembled observations made with isolate F15 by Evans et al. (2011), with the exception that the garden isolates developed numerous sporulating uredinia on *Rubus polyanthemus* (clone 961107) in the detached-leaflet or whole-plant bioassays and were thus classified in the high pathogenicity category for this host. Evans et al. (2011) observed that only a few uredinia developed on this clone after inoculation with isolate F15. The lack of a relationship between pathogenicity and molecular data in our study was not surprising and has been observed for other rust pathogens (Villaréal et al. 2002; Keiper et al. 2003).

Leaf-age related resistance, which has been demonstrated in the blackberry-rust pathosystem (Evans and Bruzzese 2003; Evans et al. 2011), was also observed in both types of bioassay performed in our study. While the detached-leaflet bioassay was an efficient technique to explore the pathogenicity of the garden isolates, in several instances results were highly variable and additional whole shoots were bioassayed. For some of the clones inoculated with particular isolates, none of the inoculated leaves in whole-shoot bioassays developed any uredinia, although uredinia had developed on a few leaflets in the detached-leaflet bioassays. However, in all instances where no uredinia developed on any of the detached leaflets inoculated, none of the leaves in subsequent whole-shoot bioassays developed uredinia. Pei et al. (2004) reported similar observations when comparing reactions of willow clones to the rust *Melampsora larici-epitea* in a leaf-disk assay and in the field. They found that several clones, which showed substantial infections in leaf-disk assays were free of rust in the field, while others that did not support uredinia development in the assay were also free of rust in the field. In their trials, other clones had become infected in the field confirming that environmental conditions were adequate for rust infection. Difference in expression of resistance between leaf disks maintained under optimal experimental conditions and leaves on whole plants in the field was put forward by these authors to explain discrepancies between results. Nonetheless, despite these inconsistencies there was overall a good correlation between leaf-disk and field results. Detached-leaf bioassays have also been shown to produce results comparable to assays performed using whole plants in a growth chamber or greenhouse during screening of soybean genotypes with the rust *Phakopsora pachyrhizi* (Twizeyimana et al. 2007) and oat cultivars with *Puccinia coronata* (Jackson et al. 2008).

Results from host-specificity tests performed with the garden isolates concur with previous findings that the leaf-rust fungus does not pose a threat to commercial blackberry cultivars and *Rubus* species native to Australia (Bruzzese and Hasan 1986b, c). None of the leaves of the blackberry cultivars Loch Ness and Chester, which are now popular among growers in Australia, and the native *Rubus* species from Australia that had not been tested previously (*R. nebulosus*, *R. probus*, *R. queenslandicus*), developed any uredinia following inoculation with the isolates. Of the species and cultivars that had previously been tested using a pool of 15 European isolates in the mid-1980s, only a small proportion of leaves of *R. moorei* and the cultivar American Thornfree developed several normal, albeit small, sporulating uredinia following inoculation with a mixture of four of the isolates. In these previous tests, small, generally non-eruptive uredinia or telia were recorded on *R. moorei* and the cultivar Silvan as well as many sporulating uredinia of varying sizes on *R. gunnianus* and cultivars Dirksen Thornless, Smoothstem and Thornfree (Bruzzese and Hasan 1986b). It is noteworthy that the 1980s host-specificity tests were conducted on detached leaves of the various test plants contained in Petri plates, while we used leaves attached to whole shoots in our study.

We believe that the level of risk associated with the introduction of these additional isolates into Australia is no higher than what was assessed in the mid-1980s. The likelihood of non-target effects in the field is further reduced when considering that, since its first appearance in 1984, the leaf-rust

fungus has never been found on *R. gunnianus* nor has it become an impediment to the cultivation of blackberry cultivars that were found to be slightly (Silvan) and moderately (Dirksen Thornless, Smoothstem and Thornfree) susceptible in previous testing (Bruzzese and Hasan 1986b). While an increase in genetic diversity could favour the emergence of novel rust genotypes better adapted to the marginal hosts identified in our tests, it is not straightforward to predict if expansion or shift to completely new hosts will occur. Host shifts are known to have occurred in the evolutionary history of *Phragmidium* spp. (Ritz et al. 2005) and other fungal species (Giraud et al. 2010). Parker and Gilbert (2004) emphasised that likelihood of host shifts occurring depends on the particular pathogen, host and environmental conditions and that predictions for non-agricultural situations are difficult to make.

The main advantage of the trap garden approach examined in this study is that it enabled collection of rust isolates pathogenic on blackberry clones from Australia under field conditions without the need for extensive screening trials. Conducting such trials is inevitable when *ad hoc* collections of infected material are made in order to determine if the pathogen isolates recovered from collected material can infect plant genotypes from the introduced range. The establishment of trap gardens in various regions in Europe or the use of a mobile trap garden, whereby potted blackberry clones from Australia placed on a vehicle would be moved to different locations within the same growing season (Morin et al. 2006), could have led to the collection of an even more diverse set of isolates. These options however, would have been considerably more costly. While there was no guarantee that isolates recovered at a single location would be genetically and phenotypically different, results presented here support the use of a single-site trap garden, considering the isolates recovered represent unique genotypes possessing alleles not observed among Australian isolates. The approach was cost-effective when compared with travelling to multiple locations across Europe and West Asia to collect a range of isolates with the possibility of some being discarded subsequently if non-pathogenic on plant genotypes from Australia. More genetically distinct isolates may have been recovered from the single-site trap garden if it had comprised a larger set of genetically diverse blackberry clones and collections had been performed beyond a single growing season, as was originally envisaged in this project (Scott et al. 2002).

The garden isolates were approved for introduction to Australia in 2004 and were subsequently released extensively across the range of blackberry (Morin et al. 2008). Together the garden isolates have the potential to affect all (European) blackberry clones present in Australia, assuming they establish and persist long enough to contribute genetic material to the existing rust population. It is highly likely that the isolates will not remain clonal, but rather recombine with each other and with individuals in the existing population (Gomez et al. 2008). This process will generate novel rust genotypes that hopefully will be better adapted to the varying conditions occurring at blackberry-infested sites across Australia. It is anticipated that natural selection will take place with this strategy, possibly leading to over-representation of one or a few fit rust genotypes at different infested sites that can compete with the existing rust population. The rust genotypes that will eventually become dominant at each site may vary, depending on the underlying genetic structure of the blackberry population or other environmental conditions. Research is now focusing on using developed microsatellite markers (Molecular Ecology Resources Primer Development Consortium 2010) to determine if alleles of the garden isolates have been incorporated in the rust population in Australia.

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