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ORIGINAL PAPER

Invaded range of the blackberry pathogen *Phragmidium violaceum* in the Pacific Northwest of the USA and the search for its provenance

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Abstract Field surveys in 2006 confirmed that the exotic rust fungus *Phragmidium violaceum* was widespread on *Rubus armeniacus* and *Rubus laciniatus* in the Pacific Northwest of the USA. The origin and dispersal pattern of this obligate biotrophic pathogen in the USA were investigated by comparing the genetic diversity and structure of 27 isolates each from the USA and Europe, and 20 isolates from Australia where an invasion occurred in 1984. Analysis of 11 microsatellite loci revealed 74 unique genotypes, with the European population having a significantly higher level of allelic diversity and number of private alleles compared to populations from the USA and Australia. Principal coordinate analysis (PCA), analysis of molecular variance and pairwise comparisons of Φ confirmed a strong level of differentiation among continental populations, with little divergence between isolates from the USA and Europe, but a high level of differentiation between these isolates and those from Australia. These results were broadly supported by the Bayesian cluster analysis, which indicated that at $K = 3$ the clustering of the isolates corresponds to their geographic origin. Bayesian clustering, PCA as well as insignificant migration estimates from Europe to the USA suggest that the USA population is not a direct descendant from the European *P. violaceum* population. There was a weak association between genetic and geographic distance among the USA isolates, suggesting invasion was initially localized prior to dispersal or that the population may have been present for some time prior to first detection in 2005.

Keywords Blackberry rust · Genetic diversity · Microsatellites · *Rubus armeniacus* · *Rubus fruticosus* aggregate · *Rubus laciniatus*

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Introduction

Invasions by exotic plant pathogenic fungi can have major ecological, economic and social consequences

in the regions of their introduction (Desprez-Loustau et al. 2007; Rossman 2009). In contrast to invasive plants and animals, the processes underpinning fungal invasions are infrequently investigated in detail except for high profile cases such as potato late blight, Dutch elm disease, chestnut blight and banana black leaf streak disease (Brasier and Buck 2001; Smart and Fry 2001; Dutech et al. 2010; Robert et al. 2012). Population genetic analysis is increasingly being applied to provide insight into factors contributing to invasion success and to understand processes underpinning the genetic diversification of invasive populations (Sakai et al. 2001; Estoup and Guillemaud 2010). The provenance of the invading population, the number of founding genotypes and their movement across the landscape can be inferred from the genetic structure of populations of exotic organisms. While population genetic data can be useful in helping to understand the origin and spread of an invading pathogen, interpretation of these data needs to be approached cautiously due to the limits and potential violations of assumptions of the statistical analyses employed (Fitzpatrick et al. 2012).

Indigenous to Europe and holarctic Asia (Tykhonenko 2007), the leaf-rust fungus *Phragmidium violaceum* (Uredinales) is a macrocyclic and autoecious rust fungus that completes the asexual and sexual components of its life cycle exclusively on taxa belonging to the *Rubus fruticosus* aggregate, hereafter referred to as blackberry (Laundon and Rainbow 1969). Each year, new rust genotypes emerge at the beginning of the growing season as a result of sexual recombination and undergo repeated clonal reproduction during spring and summer, leading to extensive proliferation of wind-dispersed urediniospores (Gomez et al. 2008).

Phragmidium violaceum was first reported in the USA in April 2005 on naturalized populations of the invasive plant *Rubus armeniacus* along the southern coast of Oregon (Osterbauer et al. 2005) and on *Rubus pensilvanicus* in the far north coast of California (Aime and Rossman 2007). The latter species is most likely a misidentification because *R. pensilvanicus* originates from North America and does not belong to *R. fruticosus* agg., which comprises the hosts of this rust fungus (Evans et al. 2007). Later in 2005, *P. violaceum* was found across the Pacific Northwest causing devastating epidemics in nearly all commercial plantings of the blackberry cultivars Thornless Evergreen and Everthornless, both derived from *Rubus laciniatus*, another species within *R. fruticosus* agg. (Johnson and Mahaffee 2010). Although information on the epidemiology and management of this pathogen in cropping systems is growing (Johnson and Mahaffee 2010), little is known about the nature of this incursion into the USA.

Phragmidium violaceum was deliberately introduced to Chile from Germany in 1973 for the biological control of invasive blackberry (Oehrens and González 1974, 1977; Oehrens 1977). The fungus was subsequently recorded in Australia in 1984 following a suspected unauthorized human-mediated introduction to the state of Victoria (Marks et al. 1984). This founding population rapidly became widespread across blackberry infestations in Victoria (Bruzzese and Field 1985) and the fungus is now present in all states and territories where susceptible host plants occur. Commercial blackberry production in Australia, however, has not been affected by *P. violaceum* due to cultivation of resistant varieties or varieties without *R. fructicosus* agg. in their pedigree. Subsequent efforts to enhance its biological control potential led to the authorized introduction of an additional isolate (F15) in the early 1990s (Evans et al. 2000) and eight isolates in 2004 (Morin et al. 2011). Genetic diversity among isolates of *P. violaceum* collected across Australia between 1997 and 1999 revealed low levels of genetic variation compared to isolates from the native range, Europe, suggesting a founder effect (Gomez et al. 2006).

In this paper, we first document the invaded range of *P. violaceum* 1 year after the first record in the USA. We then use a population genetic approach to investigate the origin of the incursion in the USA. We also investigate whether there is a possible association between genetic and geographic distance among isolates to infer gene flow in the USA population. It is noteworthy that *P. violaceum* is an obligate biotroph and it is thus difficult to obtain and maintain large sample sizes for genetic analysis. The study involves comparing the genetic diversity and structure of the rust population in the USA with that of Australia, another introduced range, and Europe, which is part of the native range, using recently developed microsatellite markers (Molecular Ecology Resources Primer Development Consortium et al. 2010). Possible pathways of introduction of *P. violaceum* into the USA are also discussed.

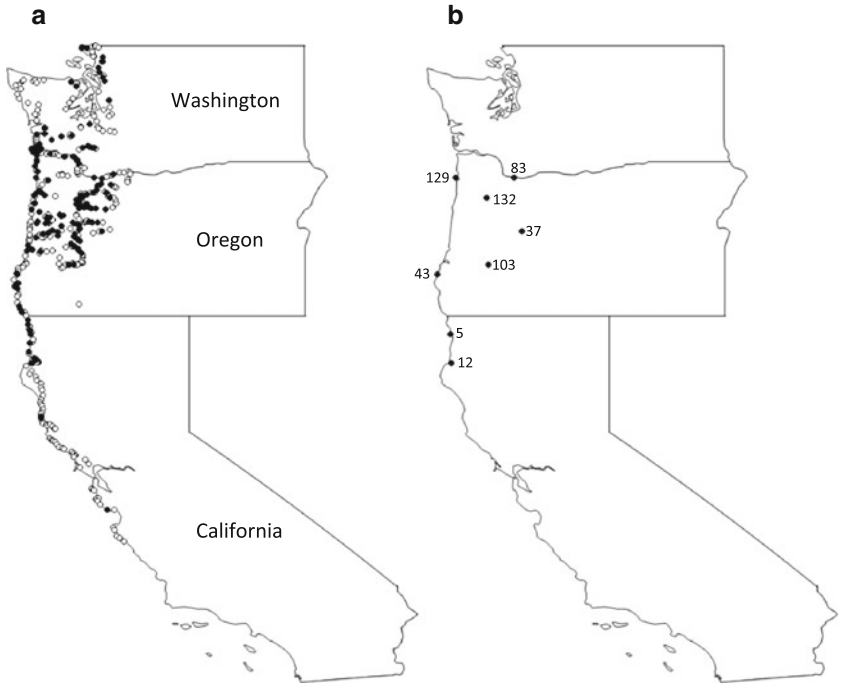

Fig. 1 **a** Locations surveyed in the Pacific Northwest USA in September through October 2006 for presence of *Phragmidium violaceum* on *Rubus* spp., *solid circles* = *P. violaceum* present (n = 158), *open circles* = *P. violaceum* absent (n = 335), **b** Locations where *P. violaceum* isolates (ex. *Rubus armeniacus*) used in this study were collected in 2007 (Table 1)

Materials and methods

Field surveys

From September to October 2006, blackberry infestations noticeable from the road or in frequently visited areas (e.g., parks, rest stops) along major thoroughfares in the Pacific Northwest were examined for any sign of rust infection. Sites sampled were between the Pacific Coast and the Cascade or Sierra Mountains in Washington/Oregon or California, respectively, and from the Canadian border to south of Santa Cruz, CA (Fig. 1a). The host species were identified based on morphological characters of species known to occur in the range, predominately *R. armeniacus*, less frequently *R. laciniatus*, and rarely *R. ulmifolius* (DiTomaso and Healy 2007). *Rubus laciniatus* has deeply cut leaflets in contrast to *R. armeniacus* and *R. ulmifolius* that have elliptic to broadly elliptic leaflets. The inflorescence of *R. ulmifolius* is truncate pyramidal or compact cylindrical; petals are distinctly pink. In contrast, the inflorescence of *R. armeniacus* is very broad and petals are slightly pink. The infestations examined consisted of either *R. laciniatus* or *R. armeniacus*. *Rubus ulmifolius* was not encountered during the surveys. Some infestations identified as *R. armeniacus* were possibly *Rubus anglocandicans* or hybrids, as the two species are closely related and difficult to distinguish without DNA analyses (Evans and Weber 2003; Clark et al. 2013). At each location, GPS coordinates were recorded and the perimeter of the infestation searched for disease symptoms. When rust infection was found, the blackberry species of affected plants were recorded and infected leaves collected and placed in plastic bags, transported in a cooler with ice, and maintained at 4 °C until examined microscopically. The morphology of urediospores and teliospores (overwintering spore stage) was used to confirm presence of *P. violaceum* (Laundon and Rainbow 1969; Washington 1991).

USA isolates

Only isolates of *P. violaceum* originating from *R. armeniacus* were genetically analyzed. In 2007, two sites in California and five in Oregon where *P. violaceum* had been found in 2006 on *R. armeniacus* were resurveyed. These sites were selected on the basis of their geographic location (Table 1, Fig. 1b). Another site in Oregon was surveyed where *P. violaceum* was observed on *R. armeniacus* (Site ID 132, Table 1) for the first time in 2007.

At each site, 4–20 infected leaves, at least 3 m apart, were collected and transported back to the laboratory as described above. For each leaf sample,

Table 1 Isolates of *Phragmidium violaceum* recovered from *Rubus armeniacus* in the Pacific Northwest USA used in this study (see Fig. 1b)

Isolate accession no.[a]	Collection date	Site ID.[b]	State	Latitude	Longitude
CA06-005-001	26/05/2007	5	California	41.510183	−124.079133
CA06-005-003B	26/05/2007	5	California	41.510133	−124.079167
CA06-005-004B	26/05/2007	5	California	41.510067	−124.079233
CA06-005-006A	26/05/2007	5	California	41.510117	−124.0792
CA06-005-007	26/05/2007	5	California	41.510133	−124.079183
CA06-012-013A	26/05/2007	12	California	40.75625	−124.050217
CA06-012-016	26/05/2007	12	California	40.75625	−124.050183
OR06-129-025A	01/06/2007	129	Oregon	45.56435	−123.93685
OR06-129-026A	01/06/2007	129	Oregon	45.564283	−123.936833
OR06-129-026B	01/06/2007	129	Oregon	45.564283	−123.936833
OR06-129-027A	01/06/2007	129	Oregon	45.564283	−123.9369
OR06-129-028A	01/06/2007	129	Oregon	45.564317	−123.9369
OR06-129-029	01/06/2007	129	Oregon	45.56465	−123.936683
OR06-129-030B	01/06/2007	129	Oregon	45.564633	−123.936667
OR06-083-036	01/06/2007	83	Oregon	45.56005	−122.439583
OR06-083-037A	01/06/2007	83	Oregon	45.560033	−122.43955
OR06-083-037B	01/06/2007	83	Oregon	45.560033	−122.43955
OR06-083-040	01/06/2007	83	Oregon	45.56	−122.4394
OR06-083-041	01/06/2007	83	Oregon	45.56005	−122.4394
OR06-037-054	07/06/2007	37	Oregon	44.169933	−122.244033
OR06-043-060A	13/06/2007	43	Oregon	43.0439	−124.414983
OR06-043-064B	13/06/2007	43	Oregon	43.0437	−124.414983
OR06-043-067A	13/06/2007	43	Oregon	43.043583	−124.414817
OR06-103-072A	13/06/2007	103	Oregon	43.2964	−123.106
TH07-080	09/08/2007	132	Oregon	45.039117	−123.137967
TH07-081	09/08/2007	132	Oregon	45.039017	−123.13805
TH07-086	09/08/2007	132	Oregon	45.03925	−123.13835

[a] USDA-ARS accession numbers

[b] Site locations are shown in Fig. 1b

urediniospores from a single-uredinium were transferred with a sterile fine artist brush to the abaxial surface of a surface-sterilized young detached leaf of commercially grown *R. laciniatus* (cv. Thornless Evergreen) floating on sterile distilled water in a 10 cm Petri dish. A commercial cultivar of *R. laciniatus* was used for culturing the isolates because leaflets developed abundant uredinia following inoculation and rooted cuttings were more easily obtained than wild-growing *R. armeniacus*. Prior to inoculation, batches of *R. laciniatus* leaves were surface-sterilized by submerging them in 20 mM phosphate buffer and 0.05 % Tween20 and shaking (300 rpm) for 5 min. Leaves were then transferred to a solution of 1 % sodium hypochlorite and 0.05 % Tween20, sonicated for 15 min (Branson 5210 sonication bath Danbury, CT) and shaken for 5 min (300 rpm) before rinsing 3–5 times in sterile distilled water. Inoculated leaves were incubated at 18 °C with a 16 h photoperiod under full-spectrum fluorescent lights for 24 h, air dried in a laminar flow hood for approx. 30 min and incubated for another 2 days. Each leaf was then individually surface-sterilized as above, with the exception that only a 5 min sonication was carried out. A 2–3 mm segment at the end of the petiole was aseptically removed before the leaf was transferred to a 10 cm Petri dish containing 1.5 % water agar set to create a slant and the petiole gently embedded into the agar

Table 2 Isolates of *Phragmidium violaceum* from Europe and Australia used in this study

Isolate accession no.[a]	Location[b]	Host (species within the *R. fruticosus* agg. complex)[c]	Collection year	References
Europe				
BG25 (IMI 393552)	Rila, Bulgaria	*R. procerus*	1979	Bruzzese and Hasan (1986)
BG26 (IMI 393553)	Kalojanovo, Bulgaria	*R. procerus*	1979	Bruzzese and Hasan (1986)
GR23	Rentina, Greece	*R. ulmifolius*	1979	Bruzzese and Hasan (1986)
I19 (IMI 393558)	Tovo Santa Agata, Italy	*R. procerus*	1978	Bruzzese and Hasan (1986)
I17	Aramengo, Italy	*R. ulmifolius*	1978	Bruzzese and Hasan (1986)
E4	Zaragoza, Spain	*R. ulmifolius*	1978	Bruzzese and Hasan (1986)
E3 (IMI 393554)	Navaleno, Spain	*R. procerus*	1978	Bruzzese and Hasan (1986)
E6 (IMI 393555)	San Jorge, Spain	*R. ulmifolius*	1978	Bruzzese and Hasan (1986)
YU20 (IMI 393559)	Prozor, Yugoslavia	*R. procerus*	1979	Bruzzese and Hasan (1986)
YU21 (IMI 393560)	Mostar, Yugoslavia	*R. ulmifolius*	1979	Bruzzese and Hasan (1986)
YU229 (IMI 393561)	Skopje, Yugoslavia	*R. procerus*	1979	Bruzzese and Hasan (1986)
F15 (DAR 76669)	Chalon-sur-Saône, France	*R. procerus*	1978	Bruzzese and Hasan (1986)
F7	Barrerre, France	*R. fruticosus* subg. *Discolores*	1978	Bruzzese and Hasan (1986)
F12 (IMI 393556)	La Mejanelle, France	*R. fruticosus* subg. *Vestiti*	1980	Bruzzese and Hasan (1986)
F14 (IMI 393557)	St. Victor, France	*R. ulmifolius*	1980	Bruzzese and Hasan (1986)
G6-TG-00-4-1 (DAR 76667)	Trap garden, Montpellier, France	*R. leucostachys*	2000	Morin et al. (2011)
G14-TG-00-4-1 (DAR 76668)	Trap garden, Montpellier, France	*R.* sp. clone SR43	2000	Morin et al. (2011)
G18-TG-00-4-1 (DAR 76670)	Trap garden, Montpellier, France	*R. rubritinctus*	2000	Morin et al. (2011)
G21-TG-00-1-1 (DAR 76671)	Trap garden, Montpellier, France	*R. leucostachys*	2000	Morin et al. (2011)
G28-TG-00-2-1 (DAR 76672)	Trap garden, Montpellier, France	*R. vestitus*	2000	Morin et al. (2011)
G32-TG-00-1-1 (DAR 76673)	Trap garden, Montpellier, France	*R. anglocandicans*	2000	Morin et al. (2011)
G32-TG-00-2-2 (DAR 76674)	Trap garden, Montpellier, France	*R. anglocandicans*	2000	Morin et al. (2011)
G32-TG-00-3-1 (DAR 76675)	Trap garden, Montpellier, France	*R. anglocandicans*	2000	Morin et al. (2011)
G2-TG-00-2-1	Trap garden, Montpellier, France	*R. leucostachys*	2000	Scott et al. (2002)
G7-TG-00-2-1	Trap garden, Montpellier, France	*R. leucostachys*	2000	Scott et al. (2002)
G39-TG-00-2-1	Trap garden, Montpellier, France	*R.* sp. clone 971606	2000	Scott et al. (2002)
C23	Comporta, Portugal	*R. ulmifolius*	2000	Scott et al. (2002)
Australia				
V1 (DAR 77369)	Cobden to Port Campbell Road, VIC	*R. leucostachys*	1997	Evans et al. (2000)
EB19-14	Cobden to Port Campbell Road, VIC	*R. leucostachys*	1997	KJ Evans unpubl.
SA1 (DAR 75508, 75509, 75561)	Cleland Conservation Park, SA	*R. anglocandicans*	1998	Evans et al. (2000)

Table 2 continued

Isolate accession no.[a]	Location[b]	Host (species within the *R. fruticosus* agg. complex)[c]	Collection year	References
WA10 (DAR 77371)	Nanarup, WA	*R. anglocandicans*	1998	Evans et al. (2000)
WA11 (DAR 77372)	Yakamia drain, WA	*R. anglocandicans*	1998	Evans et al. (2000)
WA12 (DAR 77373)	West of Hall, WA	*R. anglocandicans*	1998	Evans et al. (2000)
WA9 (DAR 77374)	Albany, WA	*R. anglocandicans*	1998	Evans et al. (2000)
WA18	Chester Pass Rd, Albany, WA	*R. anglocandicans*	1998	KJ Evans unpubl.
WP69 (DAR 77376)	Princetown, VIC	*R. anglocandicans*	1998	Evans et al. (2000)
WP43 (DAR 77377)	Strzelecki Ranges, VIC	*R. cissburiensis*	1998	Evans et al. (2000)
WP59 (DAR 77378)	Creswick, VIC	*R. laciniatus*	1998	Evans et al. (2000)
WP52 (DAR 77379)	Foster, VIC	*R. leucostachys*	1998	Evans et al. (2000)
WP70 (DAR 77380)	Gellibrand, VIC	*R. vestitus*	1998	Evans et al. (2000)
WP42 (DAR 77381)	Jeeralong, VIC	*R.* sp.	1998	Evans et al. (2000)
WP63	Cobden, VIC	*R. leucostachys*	1998	KJ Evans unpubl.
R3WP5	Mornington Flinders Rd, VIC	*R.* sp.	1998	KJ Evans unpubl.
WP90 (DAR 77382)	Hiawatha, VIC	*R. anglocandicans*	1999	Evans et al. (2000)
9901 (DAR 77383)	Belair National Park, SA	*R. anglocandicans*	1999	Evans et al. (2000)
9902 (DAR 77384)	Mount Lofty, SA	*R. anglocandicans*	1999	Evans et al. (2000)
R3WP3 (DAR 77385)	Red Hill, VIC	*R. leucostachys*	1999	Evans et al. (2000)

[a] Numbers with a 'DAR' and 'IMI' prefix represent specimens lodged at the Plant Pathology Herbarium (HERB-DAR) of the New South Wales Department of Primary Industries (Orange) and CABI Bioscience Genetic Resource Collection (Egham, UK), respectively

[b] *VIC* Victoria, *WA* Western Australia, *SA* South Australia

[c] The taxon described as *R.* affin. *armeniacus* by Evans et al. (2000) has been renamed here *R. anglocandicans*, according to Evans and Weber (2003)

All plants in the CSIRO Montpellier trap garden originated from naturalized infestations of *R. fruticosus* agg. in Australia

slant. Leaves were incubated as above until sporulating uredinia developed. Urediniospores from each of the single-uredinium isolates were collected using a cyclonic spore collector and used to inoculate more detached leaves following the protocol above to bulk-up quantities for storage at −80 °C (Evans et al. 2000). A total of 27 isolates (ex *R. armeniacus*), 1–7 isolates per site, were used for genetic analyses (Table 1).

European and Australian isolates

In total, 27 European isolates of *P. violaceum* were genetically analyzed (Table 2). These included 15 isolates collected between 1978 and 1980 across continental Europe (Bruzzese and Hasan 1986), 11 isolates collected in 2000 from a *Rubus* trap garden at the CSIRO European Laboratory near Montpellier, France (Scott et al. 2002; Morin et al. 2011) and 1 isolate collected in Portugal in 2000 (Scott et al. 2002). The 20 Australian isolates used in this study were collected between 1997 and 1999 (Evans et al. 2000; KJ Evans unpublished) (Table 2).

Each isolate, with the exception of C23 from Portugal (Scott et al. 2002), was made from a single uredinium and propagated as described by Bruzzese and Hasan (1986) (for European isolates collected in 1978–1980), Morin et al. (2011) (for all isolates recovered from the trap garden) or Evans et al. (2000) (for Australian isolates). Urediniospores of 1978–1980 isolates from Europe were lyophilized and stored at both 4 and −20 °C (Bruzzese and Hasan 1986; M Jourdan pers. comm.), while all other isolates were stored at −80 °C (Evans et al. 2000).

Microsatellite analyses

Total nucleic acid from stored urediniospores of each USA, European and Australian isolate was extracted using the method described in Morin et al. (2011). Each isolate was then characterized at 11 microsatellite loci as described in Molecular Ecology Resources Primer Development Consortium et al. (2010).

Data analyses

Allelic diversity among groups of isolates from the USA, Europe and Australia were compared using the following indices: the number of different alleles observed, the effective number of alleles (Kimura and Crow 1964), the number of private alleles (unique alleles observed for each population) and Shannon's Information index (Brown and Weir 1983). The indices were calculated using GENALEX v6 (Peakall and Smouse 2006) and averaged over 11 loci. Tests for linkage disequilibrium were also performed using GENEPOP (Rousset 2008).

GENALEX v6 was also used to assess genetic relatedness among all genotypes using a principal coordinate analysis (PCA), an ordination analysis that can be useful for recovering non-hierarchical patterns of genetic variation (Lessa 1990). The PCA was based on the pairwise genotypic distance measure described by Smouse and Peakall (1999).

An analysis of molecular variance (AMOVA) was used to assess the distribution of genetic variation within and among populations of the fungus. AMOVA estimates variance components for diploid (and dikaryotic in this case) genotypes and partitions genotypic variation within and among populations based on the proportion of allelic differences between all pairs of isolates. The parameters estimated by AMOVA, referred to as Φ statistics (Excoffier et al. 1992) are analogous to Wright's F-statistics (Wright 1969), which describes the level of differentiation among populations. The degree of differentiation between each pair of populations was also measured by calculating pairwise Φ statistics. Under the null hypothesis of no genetic difference between isolate populations, the test statistic Φ ranges between 1, indicating maximum differentiation, through to 0, indicating no differentiation (Excoffier et al. 1992). Calculations for Φ were performed for each population comparison using GENALEX v6. The significance against the null hypothesis of zero differentiation ($\Phi = 0$) was tested via comparisons to randomized distributions created from 999 permutations.

Bayesian cluster analysis was undertaken using STRUCTURE v2.3.2 (Pritchard et al. 2000) to examine population substructure and the levels of admixture of genotypes among populations. STRUCTURE v2.3.2 uses multilocus genotypic data to define a set of populations with distinct allele frequencies and to assign individuals probabilistically to these populations. Simulations were replicated using 10 runs for each value of K between 1 and 15, with the following software settings: a burn-in length of 50,000, a Markov Chain Monte Carlo length of 50,000 steps, no population information, correlated allele frequency and an admixture model. For each independent run,

Table 3 Comparison of allelic patterns, averaged over the 11 microsatellite loci analyzed, for the populations of *Phragmidium violaceum* from three continents

Population	Sample size	No of multilocus genotypes	Na[a]	Ne[b]	Private alleles[c]	I[d]
USA	27	27	7.91	4.69	3.27	1.62
Europe	27	27	13.00	7.22	7.55	2.15
Australia	20	20	3.73	2.15	0.91	0.81

[a] Number of different alleles

[b] Effective number of alleles. This is an estimate of the number of equally frequent alleles in an ideal population with homozygosity equivalent to the actual population

[c] Number of different alleles unique to each population

[d] Shannon's Information index

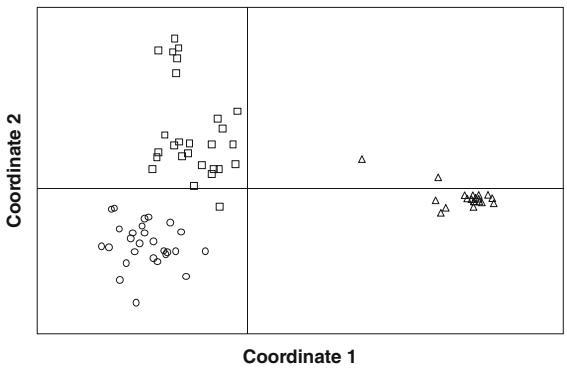

Fig. 2 Genetic relatedness among *Phragmidium violaceum* isolates from the USA (*circle*), Europe (*square*) and Australia (*triangle*) based on a principal coordinate analysis of the genetic distance measure described by Smouse and Peakall (1999)

the posterior probability, Ln$P(D)$, was estimated for a set value of K. This was also used to calculate the second order rate of change in Ln$P(D)$, denoted ΔK. The value of K when ΔK no longer improves represents a good indicator of the "real" number of populations (Evanno et al. 2005). Once values of K had been chosen, the genetic contribution of each inferred cluster to the populations, as well as to each individual, was investigated.

To investigate migration patterns among *P. violaceum* populations and identify a possible source population, we used the isolation-with-migration model implemented in the program IMa (Hey and Nielsen 2007). Markov Chain Monte Carlo simulations started with a burn-in period of 10^6 steps so that the state of the chain was independent of the starting point. After the burn-in period, simulations were continued for at least 30 million steps and genealogies were recorded every 100 steps over the course of the run, with a geometric heating-scheme. Analyses were considered to have converged upon the stationary distribution if three independent runs generated similar 95 % highest posterior densities (HPD), with each estimated parameter exceeding an effective sample size of 50 as recommended in Hey and Nielsen (2004). To account for differences in effective population sizes, migration parameters *m1* and *m2* were converted into population migration rates (Nm = the number of gene copies that migrate in each generation) using the equation $Nm = (\theta\ m)/4$. The effective population size (Ne) for each population was also estimated assuming one generation per year. To calculate Ne, ($\theta = 4Ne\mu$) we chose a microsatellite mutation rate (μ) of 5×10^{-4}, which was calculated for *Venturia inaequalis* (Gladieux et al. 2010) and is an average mutation rate for SSRs as suggested by Estoup et al. (2002) and is similar to a rate calculated for the smut fungus, *Ustilago maydis* (Munkacsi et al. 2008).

The dispersal pattern of *P. violaceum* in the USA was compared to that observed in Australia 13–15 years after the first record of the rust in this country, using Mantel tests to investigate the hypothesis of isolation by distance. GENALEX v6 was used to construct a genetic distance matrix between all pairs of isolates within a country, which was compared to a corresponding geographic distance matrix. Under the null hypothesis that genotypic distance is independent of geographic distance, the estimated correlation coefficient, r, is small. The significance of r was determined by comparisons to randomized distributions of r created from 999 permutations, where genetic distance data was randomly interchanged with geographic distance.

Table 4 AMOVA among 74 isolates of *Phragmidium violaceum* collected from the USA, European and Australian populations

Source	df	Sums of squares	Estimated variance	% variance	Φ	*P*
Among populations	2	120.55	2.23	28	0.28	0.001
Within populations	71	411.74	5.80	72		
Total	73	532.29	8.03	100		

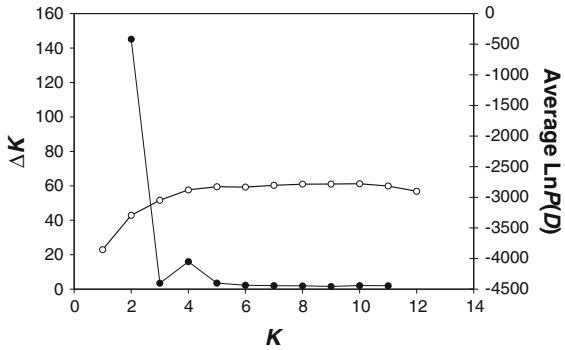

Fig. 3 Comparison of Ln*P*(*D*) (*open circle*) and the second order rate of change in Ln*P*(*D*), denoted Δ*K* (*solid circle*) (Evanno et al. 2005) for 74 isolates of *Phragmidium violaceum* from the USA, Europe and Australia

Results

Field surveys

Phragmidium violaceum was found on naturally-occurring blackberries at 32 % of the 493 locations surveyed in Washington (92), Oregon (306) and California (95) in 2006 (Fig. 1a). It was recorded at the northern limit of the surveyed region near the Canadian border in Washington, and only at four locations between Eureka and Santa Cruz, CA, the southern limit of the survey. The rust was found at 37, 34, and 15 % of locations surveyed in Oregon, Washington and California, respectively. Across the three states, it was predominantly found on

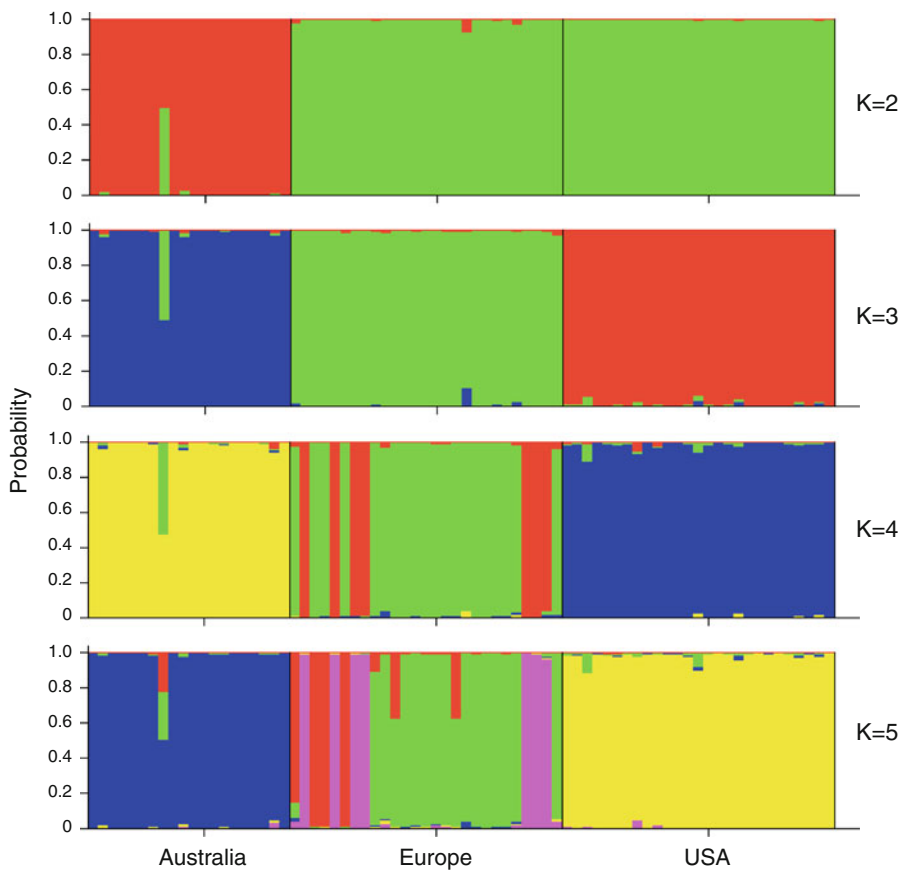

Fig. 4 Bayesian assignment probabilities of 74 *Phragmidium violaceum* multilocus SSR genotypes using STRUCTURE v2.3.2 across the three groups of isolates (USA, Europe and Australia). Several outputs are presented, each corresponding to the different values of *K* (from 2 to 5) that most efficiently summarize the data. The *vertical line* represents an individual and *colors* indicate the probability that the genotype belong to each of *K* clusters (particular lineage)

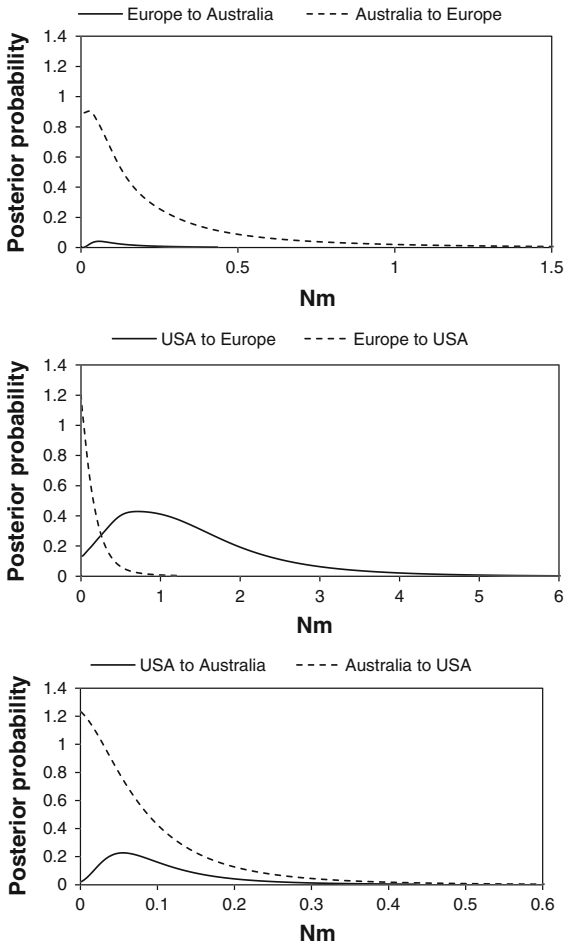

Fig. 5 Posterior probability distributions of *Phragmidium violaceum* population migration rates (Nm = per gene copy per year) as determined under the isolation-with-migration model in IMa

R. armeniacus (66 % of total records) and to a lesser extent on *R. laciniatus* (34 %).

Comparison of genetic diversity between populations

Each of the 74 isolates of *P. violaceum* investigated in this study represented a unique SSR multilocus genotype. Allelic diversity differed between populations. The Shannon's Information index (*I*), the number of different alleles (Na), the effective number of alleles (Ne) and the number of private alleles, averaged across 11 microsatellite loci, were significantly higher (*t* tests, $P < 0.01$ for all comparisons; Table 3) for the European population and lower (*t* test, $P < 0.01$; Table 3) for the Australian population. Linkage disequilibrium was not observed for any of the loci.

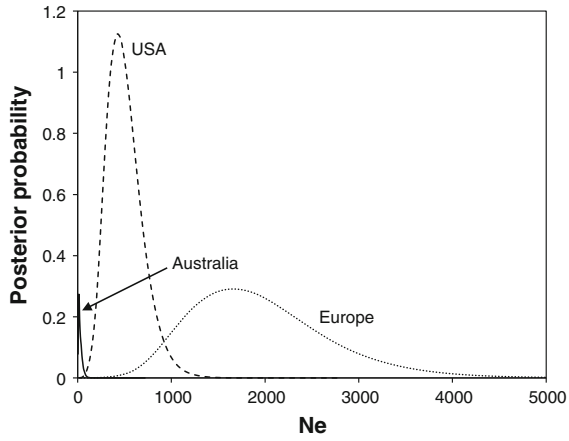

Fig. 6 Estimated effective population sizes (Ne) of *Phragmidium violaceum* populations from the USA, Europe and Australia

Comparison of populations to infer putative origin of USA population

PCA ordination revealed three clusters of isolates that were congruent with geographic origin (Fig. 2). Isolates from Australia formed a distinct cluster, whereas populations from the USA and Europe showed a higher genetic relatedness, although clusters did not overlap. The relative contributions of the two first coordinate axes to the total genetic variability were 43.5 and 17.6 %, respectively, whereas it was 11.6 % for the third axis, which gave no interpretable information (data not shown).

AMOVA revealed a strong level of differentiation among populations (overall $\Phi = 0.28$, $P < 0.001$). Twenty-eight percent of the variation distinguished the three populations, while approximately 72 % of the variation was attributed to structure within populations (Table 4). Pairwise calculations of Φ revealed strong genetic differentiation between isolates from Australia and those from Europe ($\Phi = 0.35$; $P < 0.01$) and the USA ($\Phi = 0.40$; $P < 0.01$) and moderate differentiation between isolates from the USA and Europe ($\Phi = 0.12$; $P < 0.01$).

The results of the Bayesian cluster analysis are summarized in Figs. 3 and 4. Even though probability values at $K > 5$ were similar to that at $K = 5$, standard deviations increased significantly, suggesting that $K > 5$ was not the most likely number of *K* populations. On the other hand, the ΔK statistics supported $K = 2$ or $K = 4$, depending on how the signal is interpreted.

Although there is debate on how to estimate the number of clusters (Pritchard et al. 2000; Evanno et al. 2005; Waples and Gaggiotti 2006; François and Durand 2010), we focus on $K = 2$–4 detected by the ΔK statistics as an uppermost level of population structure. At $K = 2$ ($\mathrm{Ln}P(D) = -3{,}293$), estimated proportional assignment of each predefined group of isolates to the two identified clusters (K_1 and K_2) showed a strong correlation to geographic origin (Fig. 4). The Australian isolates were assigned almost exclusively to a single cluster (K_1, overall proportional assignment = 96.7 %), while the isolates from Europe and the USA were assigned to the second cluster, K_2, with overall proportional assignment to this cluster exceeding 99 %. At $K = 3$ ($\mathrm{Ln}P(D) = -3{,}047$), proportional assignment of individuals to clusters corresponded to geographic origin of isolates. At $K = 4$ and 5 ($\mathrm{Ln}P(D) = -2{,}878$), clusters still corresponded to geographic origin of isolates, except that the population from Europe is now divided into two or three clusters (admixed).

IMa was applied to estimate the migration (per gene copy per generation) among populations to identify a possible source population. All migration estimates were low and close to zero, except for migration from the USA to Europe, which was still below 1 per gene copy per generation (Fig. 5). Migration estimates were; from Europe to the USA (Nm = 0.001; 95 % HPD = 0.000–0.738: Opposite direction Nm = 0.790; 95 % HPD = 0.091–3.850), from Australia to the USA (Nm = 0.001; 95 % HPD = 0.000–0.002: Opposite direction Nm = 0.001; 95 % HPD = 0.000–0.298) and from Europe to Australia (Nm = 0.058; 95 % HPD = 0.025–0.388: Opposite direction Nm = 0.032; 95 % HPD = 0.011–1.185). Population sizes as determined with IMa were large for Europe (Ne = 1663; 95 HPD = 776–3,659), followed by the USA (Ne = 423; 95 HPD = 202–938) and very small for Australia (Ne = 12; 95 HPD = 4–79) (Fig. 6).

Isolation by distance analysis

There was a significant, but weak association between genetic and geographic distance among isolates from the USA (Mantel test: $r = 0.127$, $P = 0.025$). In contrast, isolation by distance was not observed for isolates belonging to the Australian population (Mantel test: $r = 0.177$, $P = 0.102$).

Discussion

Biological invasions are an all too common phenomenon for plant pathogenic fungi, often with devastating effects. The invasion can occur through natural dispersal, especially for species with spores that can be wind dispersed over long distances, such as the rusts (e.g., Brown and Hovmøller 2002). However, most fungal plant pathogen invasions are generally a result of human-mediated dispersal events. *Phragmidium violaceum* was recorded for the first time in the USA in 2005 and soon after found to be widespread in the Pacific Northwest. This study aimed to shed light on this invasion by comparing the USA population of the pathogen with a population from Europe (a putative centre of diversity) and Australia (a known founder population). The biotrophic nature of the pathogen limited considerably the sample size used in the study because DNA had to be extracted from bulked-up urediniospores of single-uredinium isolates. While it was possible in preliminary work to extract DNA directly from single uredinium of *P. violaceum* on field-collected leaves using a variety of commercial plant DNA extraction kits from Invitrogen, Qiagen and MoBio, microsatellite loci failed to amplify from these samples, possibly due to inhibition caused by plant-related compounds (data not shown).

The significantly lower levels of allelic diversity, i.e., low number of alleles, low effective number of alleles, low number of private alleles, low Shannon's Information index, low effective population sizes observed among isolates from the USA and Australia compared to isolates from Europe are typical of a founder effect observed with invading populations (Dlugosch and Parker 2008; Fitzpatrick et al. 2012). The putative genetic bottleneck in the USA population may imply that the invasion is the result of a single or a very few colonization events. Unfortunately, an empirical test to confirm the suspected bottleneck in the USA and Australian populations could not be performed (e.g., with Bottleneck; Cornuet and Luikart 1996; Piry et al. 1999), because of the mixed mating system of the fungus. Mixed mating systems may influence the mutation-drift equilibrium and may thus bias the estimates of bottlenecks (Cornuet and Luikart 1996).

The search for the origin of the USA incursion was investigated with a PCA, which identified three separate clusters of isolates congruent to their

geographic location. Isolates from the USA and Europe formed separate clusters that were in close proximity, which suggested little divergence between these two groups. Australian isolates formed a distinct cluster separated from the other two groups by a large distance across coordinate 1. The AMOVA and pairwise comparisons of Φ supported these results. The AMOVA revealed a high overall level of differentiation among populations, while the pairwise calculation of Φ indicated a moderate level of differentiation between isolates from Europe and USA, and strong differentiation between these isolates and those from Australia.

The results from the PCA were also broadly supported by the Bayesian cluster analysis, which showed that at $K = 2$ the isolates are assigned to two distinct populations: one group comprising the USA and European isolates and the other the Australian isolates. At $K = 3$ though, clusters correspond to their geographic origin. Furthermore, at $K = 4$ and 5, some isolates from the European population are assigned to a fourth or fifth cluster, suggesting a high diversity among isolates from Europe. Thus, although a European origin, or source population, for the USA population seems plausible at $K = 2$, Bayesian clustering at $K = 3$ and 4 suggest that while the USA population is genetically related to the European population as determined by the PCA, the USA population is not a direct descendant from the European population. This is further supported by insignificant estimates of migration from Europe to the USA as revealed by the IMa analysis.

Given that the *P. violaceum* population in the USA is not a direct descendent from Europe, it is likely that the USA population was established from a population that was not sampled in this study, such as the population established in Chile following introduction of the pathogen from Bonn, Germany in the 1970s (Oehrens 1977). A Chilean origin of the USA population could explain the low genetic differentiation between the USA and European populations, whereas independent evolution of the population in Chile prior to its introduction to the USA could explain the separate Bayesian clustering and low migration estimates obtained from Europe. An alternative hypothesis for the origin of the USA population could be that it was introduced from another unsampled population of geographic and/or genetic proximity to Europe. Molecular characterization of Chilean isolates as well as additional isolates from the native range, which extends from Europe to China (Tykhonenko 2007), will be required to clarify the provenance of the incursion in the USA. This information would be beneficial to identify the pathway of introduction and assess biosecurity risks relating to other aerial pathogens. Such a large scale study, however, would be logistically challenging with *P. violaceum* because isolates would have to be cultured on living tissue of their particular host, in the country of origin or in a quarantine facility in another country, to obtain sufficient urediniospores for DNA extraction.

While the pathway of introduction of *P. violaceum* into the USA cannot be elucidated from our study, the pathogen was probably introduced via incoming passengers (Sheridan 1989) or infected plant material. *Phragmidium violaceum* was intercepted twice at USA customs borders over an 11-year period (Farr and Rossman 2012). The first interception was plant material of a *Rubus* sp. infected with *P. violaceum* in 1979 at JFK International Airport, New York, in a consignment arriving from Germany. The second interception was in Texas in 1990 when the pathogen was found on diseased *Rubus* leaves imported from Chile. On the other hand, long-distance transport of *P. violaceum* urediniospores on wind currents from Chile or holarctic Asia to the Pacific Northwest is an unlikely possibility considering the recognized global long-distance aerial dispersal routes for rust diseases (Nagarajan and Singh 1990). Movement of rust fungi between continents along recognized dispersal routes has been associated with extreme weather events (Brown and Hovmøller 2002). For example, sugarcane rust (*Puccinia melanocephala*) was most likely dispersed by cyclonic winds from Cameron, Africa, to the Dominican Republic in the Caribbean in the late 1970s (Purdy et al. 1985). More recently, aerobiology modelling showed that airflows associated with Hurrican Ivan in southern USA in September 2004 have most likely transported urediniospores of soybean rust (*Phakopsora pachyrhizi*) from Columbia, South America, to Louisiana, USA (Isard et al. 2005).

The association between genetic and geographic distance among the USA isolates was significantly different from zero (null hypothesis of no isolation by distance) and suggested that the invasion of *P. violaceum* into the Pacific Northwest was initially localized prior to dispersal. However, the association was weak, and care should be taken in the interpretation of this result. The weak level of isolation by

distance and the relatively large effective population size as determined by the IMa analyses in the USA population most likely reflects considerable gene flow, indicating that the pathogen may have rapidly dispersed soon after its introduction in the Pacific Northwest or has been present in the region for much longer than it is believed. More than one incursion into the USA, which would distort the isolation by distance analysis, also cannot be ruled out. Concurrent initial reports of *P. violaceum* in Oregon and California in April 2005 (Osterbauer et al. 2005; Aime and Rossman 2007) suggested that the fungus was possibly present in the USA for some time before it was officially recorded. Indeed, there is anecdotal evidence from ranchers in south-western Oregon that *R. armeniacus* in the wild was suffering from foliage dieback in May and June of 2004 (Osterbauer et al. 2005). The field surveys carried out in 2006 as part of this study confirmed that *P. violaceum* was widespread in the states of Oregon and Washington, and present, but restricted in distribution, in California. The extent of this geographic distribution is more than 1,300 km from north to south. A long period between arrival and detection of *P. violaceum* in the USA is a possibility considering that the same species only spread radially about 70 km in 9 months following its introduction to Chile (Oehrens and González 1974, 1977). However, rapid spread over a large distance soon after its incursion in the USA cannot be ruled out when considering the documented natural dispersion of other rust fungi that also produce wind-borne spores such as *Melampsora medusae* and *Puccinia chondrillina*, which spread 350 km within 4 months and 320 km in 1 year, respectively, after their introduction to Australia (Cullen et al. 1973; Walker et al. 1974).

The establishment of a rust fungus in a new environment depends not only on the introduction of infective propagules, but also on the availability of susceptible hosts growing in environments conducive to infection and disease development. After the initial discovery of *P. violaceum* in the Pacific Northwest, it was found to be widespread on invasive blackberry across the region and in areas of commercial blackberry production where susceptible cultivars are grown. The disease was most severe during the 2005 growing season, which was characterized by an early budbreak and weather particularly favorable for infection (Johnson and Mahaffee 2010). Since then, severe outbreaks of *P. violaceaum* have not been observed, likely due to climatic conditions that have been less conducive to epidemic development. Improved timing of fungicide applications in commercial plantations and the apparent loss of highly susceptible *R. armeniacus* biotypes in the wild, possibly as a result of pathogen-mediated selection, may also have led to a considerable reduction of the inoculum potential in the region (Johnson and Mahaffee 2010).

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