Molecular Assessment of the Introduction and Spread of Potato Cyst Nematode, *Globodera rostochiensis*, in Victoria, Australia

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ABSTRACT

Potato cyst nematodes (PCN) are damaging soilborne quarantine pests of potato in many parts of the world. There are two recognized species, *Globodera pallida* and *G. rostochiensis*, with only the latter species—the golden cyst nematode—present in Australia. PCN was first discovered in Australia in 1986 in Western Australia, where it was subsequently eradicated and area freedom for market access was reinstated. In Victoria, PCN was first detected in 1991 east of Melbourne. Since then, it has been found in a small number of localized regions to the south and east. Strict quarantine controls have been in place since each new detection. It has previously been speculated that there were multiple separate introductions of PCN into Victoria. Our study utilized a historic (years 2001 to 2014) PCN cyst reference collection to examine genetic variability of Victorian PCN populations to investigate potential historical origins and subsequent changes in the populations that might inform patterns of spread. DNA was extracted from single larvae dissected from eggs within cysts

and screened using nine previously described polymorphic microsatellite markers in two multiplex polymerase chain reaction assays. Sequence variation of the internal transcribed spacer region of the DNA was also assessed and compared with previously published data. A hierarchical sampling strategy was used, comparing variability of larvae within cysts, within paddocks, and between local regions. This sampling revealed very little differentiation between Victorian populations, which share the same microsatellite allelic variation, with differences between local regions probably reflecting changes in allele frequencies over time. Our molecular assessment supports a probable single localized introduction into Victoria followed by limited spread to nearby areas. The Australian PCN examined appear genetically distinct from populations previously sampled worldwide; thus, any new exotic incursions, potentially bringing in additional PCN pathotypes, should be easily differentiated from existing established local PCN populations.

Potato cyst nematodes (PCN) are damaging soilborne quarantine pests of potato in many parts of the world, including Australia (EPPO/CABI 1997; Moens et al. 2018). There are two recognized species—*Globodera pallida*, pale cyst nematode, and *G. rostochiensis*, golden cyst nematode—although only *G. rostochiensis* is present in Australia (Faggian et al. 2012). PCN are obligate, sedentary endoparasites that can cause stunting of plants, reduced yields, and sometimes complete crop failure. PCN causes losses of 9% of total potato production in Europe and can cause total losses in other regions of the world, or when no control strategies are employed (Turner and Subbotin 2013). When soil populations of PCN are high, potato yields can be less than the seed planted (Mai 1977; Whitehead and Turner 1998). This severe impact and the small size, cryptic nature, and long-term survival ability of PCN make it a very difficult pest to manage. PCN is recognized throughout the temperate regions of the world as one of the most difficult crop pests to control (Spears 1968).

Hosts of PCN are restricted to plants within the family Solanaceae. The potato (*Solanum tuberosum*) is by far the most important host. Tomato (*S. lycopersicum*) and eggplant (*S. melongena*)

are also attacked, and 90 other *Solanum* spp. and their hybrids can also act as hosts, including *Solanum* weed species (CABI 2016; van Riel and Mulder 1998) and the Australian native kangaroo apple (*S. aviculare*) (Faggian et al. 2012). The significance of weed species as refuges for PCN severely affects the success of any regulatory procedures (Mimee et al. 2014).

PCN is recognized as a significant global threat to food security (Cotton et al. 2014) and, as such, is the subject of stringent quarantine regulations in most countries where it occurs (EPPO/CABI 1997). Countries not known to be infested with PCN have rigid regulations governing the importation of host material or other products that might carry the pest (Brodie 1998; Hockland et al. 2013; Spears 1968). Therefore, the presence of the golden cyst nematode in Australia has had serious implications for market access and international trade of seed potato, which is managed accordingly (DEDJTR 2016). For example, exacting restrictions are placed on the movement of seed potato grown in Victoria (DPIPWE 2015; PBPI-BQ 2016; PBPPIS 2016; PIRSA 2015).

The Australian potato industry produces approximately 1.33 million t of processed, fresh, and seed potato from approximately 28,000 ha. In 2016 to 2017, Australian production was valued at AUS\$717 million; the Victorian potato industry produced 282,000 t, approximately 21% of the total Australian crop, which was valued at AUS\$152 million (HIA 2018). During the same time period, Australia exported 38,000 t of potato worth AUS\$38 million, which was Australia's third most valuable vegetable export commodity (AUS-VEG 2018); however, Victoria contributed only 3% to this export market supply. The estimated losses to the Australian potato industry if no quarantine actions were taken to prevent PCN spread or entry to new areas were estimated by Hodda and Cook (2009) to be AUS\$20 million annually, and as much as AUS\$370 million over 20 years.

In some countries, land in which PCN is detected is not eligible for seed potato production for many years. For example, infested

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land in Northern Ireland is not eligible even to be sampled until at least 15 years from the last potato crop and, if it is still infested thereafter, it is only eligible to be resampled every 5 years until declared free from PCN (Lehman 2004; Whitehead and Turner 1998). In Scotland, land is scheduled if official sampling finds one or more viable cysts of PCN. Thereafter, the land becomes eligible for descheduling after a period of between 1 and 6 years, depending upon the species and number of cysts found (Pickup and Hockland 2002). The Canadian Food Inspection Agency and the United States Department of Agriculture Animal and Plant Health Inspection Service have jointly prepared guidelines on the surveillance and phytosanitary actions for PCN (USDA 2014). These guidelines provide detailed instructions for the surveillance and phytosanitary measures that are required to deregulate infested, adjacent, and exposed fields. In summary, infested fields are eligible for the lifting of some restrictions when intensive surveys, viability assays, and bioassays are completed with no live detections. Seed potato production in a field which was determined to be infested with PCN is not allowed if they are going to be planted outside of the PCN regulated area. If results are negative, an additional four full field surveys must be conducted before most restrictions can be lifted. Kepich (2011) reported that, in the United States, state and federal

regulatory agencies had a 5-year plan with the goal of deregulating up to 90% of quarantined area by the end of 2014.

The golden cyst nematode (*G. rostochiensis*) was first discovered in Australia in September 1986 in Munster, a suburb near Perth, Western Australia (Fig. 1A). Western Australia had a total of six PCN-infested properties within a 5-km radius (Stanton 1986). An eradication program was implemented soon after detection, resulting in no further detections of PCN since 1989. After 24 years of continued surveillance, eradication of the nematode from Western Australia was declared and area freedom from PCN for market access was successfully reinstated in 2010 (Collins et al. 2010).

In Victoria, *G. rostochiensis* was first detected in a potato crop showing early senescence in a small market garden at Wandin in February 1991 (Guy et al. 1992). As a result of subsequent intensive surveys, PCN was found at several additional locations east and south of Melbourne, including Gembrook (in 1991), Emerald (1992), Keysborough (1992), Boneo (1992), Koo Wee Rup/Cora Lynn (2003), and, most recently, near Thorpdale (2008) (Fig. 1B). The second economically significant species of PCN, pale cyst nematode (*G. pallida*) has never been recorded in Australia (Faggian et al. 2012). Similarly, other species of *Globodera* that can feed on potato (e.g., *G. tabacum solanacearum*, *G. ellingtonae*, and *G. leptonepia*) have not been recorded in Australia.

In both Australian states, the first detections were the result of growers observing poor growth and plant collapse in their fields. Such visible symptoms (Fig. 2A and B) suggest that the infestations may have been present for some time before detection (Mai 1977; Spears 1968).

In Victoria, quarantine control areas were established around the Gembrook, Wandin, Koo Wee Rup, Keysborough, Thorpdale, and Boneo PCN infestations (Fig. 1B). Area freedom for the remainder of Victoria is based on 57,000 PCN soil tests taken between 1991 and 2018 from surveys covering 29,000 ha of potato production throughout Victoria. In 2013, all Australian states except Western Australia accepted a risk-based PCN management program, which provides industry and government a harmonized management system to minimize the risk of spreading the pest while allowing market access for potato (AUSVEG 2012). An important implication of this recently implemented risk-based model is that interstate quarantine controls which were based on the treatment and certification of all host produce sourced from within 20 km of a known PCN detection were replaced by property-based controls. Prior to 2013, phytosanitary certificates for export of Victorian potato to overseas markets were only issued for potato grown outside a 20-km radius zone of an infested property (AUSVEG 2012). The changes aimed to ease trade restrictions for properties within the 20-km zone that were not historically infested or linked in some way (e.g., by ownership, shared machinery, or shared watercourses) to infested properties. The changes also created a national standard for the distribution of PCN host material and management practices for reducing the biosecurity risks associated with spreading the pest (AUSVEG 2012). Both infested and associated linked lands are still subject to several prohibitions, restrictions, and requirements that affect the use of that land and market access for produce grown there. PCN host material originating from land classified as uninfested no longer requires certification for interstate access, except for Western Australia, whereas all potato tubers marketed as seed or for planting are tested for PCN prior to interstate movement (DEPI 2013).

The Victorian Certified Seed Potato Authority (ViCSPA) Seed Potato Certification Scheme provides for production of high-quality certified seed potato reliably free of PCN, allowing for exportation clearance of seed potato for trade. All ViCSPA-certified seed crops must be soil sampled for PCN and tested negative. Certified seed potato production under the ViCSPA scheme covers an area of 1,900 ha with approximately 85 growers (ViCSPA 2015). The traceability and high health record of Australia's seed potato certification system led to the acquisition in 2018 of new market

Fig. 1. A, Map of Australia indicating the locations of potato cyst nematode (PCN) incursions in Western Australia (Perth, now eradicated) and Victoria (Melbourne, currently established); lines on map indicate Australian state boundaries. B, Victorian quarantine zones (gray) and 20-km controlled areas (circles) which historically involved restricted movement of potato material and extra hygiene restrictions around PCN outbreaks. Location abbreviations: Bo = Boneo, Em = Emerald, Ge = Gembrook, Ke = Keysborough, Ko = Koo Wee Rup/Cora Lynn, To = Thorpdale, and Wa = Wandin.

access to Indonesia for approximately 85,000 t of Australian seed potato worth AUS\$110 million (Laskie 2018).

The most likely way that PCN is transported to new localities is by the cysts in soil adhering to potato tubers, because loose cysts can be embedded in tuber eyes or other irregularities on the tuber (Anon 2007). In addition, cysts can be carried in soil adhering to farm machinery, implements, boots, potato bins, plant roots, and, particularly, bulbs (Been and Schomaker 2013; Pendrigh 1993; Turner and Evans 1998). Cysts can also be spread by wind and water (CABI 2016; Persley et al. 2010; Riggs and Schuster 1998; Turner and Evans 1998). On a local level, PCN is usually spread by farming activities such as sharing farm equipment contaminated with infested soil. Initial sampling after the first Victorian detection of PCN led Marshall (1998) to comment that “Family connections and the sharing of seed and potato boxes linked all properties. Two of the infested properties at Wandin East were adjoining, and two were farmed in association”.

Understanding dispersal modes and genetic links between populations is essential to prevent further spread and to develop effective control strategies. Picard et al. (2004) found that, in its native habitat, *G. pallida* showed extensive gene flow between fields, and they suggested that this was a result of passive dispersal of cysts by natural means (wind, running water, or wild animals) or by anthropogenic means (tillage or movement of infected seed tubers). On the other hand, they noted that it is difficult to study the population genetics of an introduced organism such as PCN because the current genetic structure could have already been influenced by several factors such as (i) number of individuals that were introduced; (ii) number of generations since introduction; (iii) origin and, thus, genotypes of the individuals introduced; and (iv) stochastic effect of genetic drift.

At least five *G. rostochiensis* pathotypes are known to exist (Ro1 to Ro5), which have different abilities to infect certain tuberous *Solanum* hosts (which are used as differentials to distinguish pathotypes) (EPPO/CABI 1997; Kort et al. 1977). The only pathotype recorded in Australia is Ro1 (Hinch et al. 1998; Nambiar et al. 2008), to which most commonly grown resistant cultivars have resistance (Faggian et al. 2012).

In a molecular examination of the internal transcribed spacer (ITS) region of DNA in *G. rostochiensis*, Bulman and Marshall (1997) found no variation within Australasian collections, which they suggested originated in Europe. Conversely, sequence analysis by Nambiar et al. (2008) revealed the presence of genetically diverse populations in infested fields in Victoria, from which they suggested the possibility of up to seven separate introductions. Determining whether there has been more than one introduction has practical importance for the management of PCN. It is important to be able to recognize new outbreaks, because the genetic basis of PCN virulence (i.e., pathotypes) may differ (Grenier et al. 2001). Nambiar et al. (2008) examined genetic variation of the ITS region by sequencing whole cysts containing potentially up to several hundred eggs, rather than single larvae. They chose 87 cysts from seven locations representing two localized regions in Victoria (Gembrook and Koo Wee Rup) separated from each other by approximately 20 km. All isolates were grouped into six genotypic groups. Victorian isolates were found to be different from the European and American genotypes recorded in GenBank at that time. They noted that *G. rostochiensis* from different properties of the same grower were placed into separate genotypic groups.

Fertilization is essential for reproduction in *Globodera* spp. (Turner and Evans 1998) and, because cyst nematodes practice polyandry, the genetic diversity within a single cyst that can contain hundreds of individuals should be high (Mimee et al. 2015). In the *G. pallida* invasion of Western Europe, as much genetic variability occurred in the PCN on a single plant as in a field or even a whole region (Plantard et al. 2008). Hence, introduction via a single infested potato plant could result in relatively high genetic variability.

To study the genetic structure and evolution of *G. rostochiensis* and *G. pallida* in Canada, Boucher et al. (2013) used whole-genome sequencing of individual larvae to identify 12 polymorphic microsatellite markers informative enough for the purpose. Extracting DNA from individual juveniles dissected from cysts, they could genotype populations originating from 15 populations from around the world. This enabled them to characterize genetic

Fig. 2. A, Potato cyst nematode (PCN) infestation symptoms in a Victorian market crop; plants on left are stunted due to a heavy PCN infestation. B, PCN-infested roots under high magnification. Cysts, the bodies of female nematodes containing nematode eggs, are initially yellow and darken with age. C, Cyst dissection showing eggs and released juveniles. Images: Agriculture Victoria.

links between these populations, and to reveal the history of introductions and dispersal within Canada. Subsequently, Alenda et al. (2014) used the nine most informative of these microsatellite marker sets to explore the dispersal and genetic structure of populations of another nematode species, *G. tabacum*, in France. This study showed that human activity in the form of soil transport during harvest was the major factor contributing to the passive dispersal of these organisms.

The Victorian government plant diagnostic laboratory, Crop Health Services, has been responsible for all PCN testing undertaken in Victoria. As a result, a reference collection of cysts from each positive detection since 1991 has been maintained, with full collection and location details for each. Early cyst collections were preserved in formaldehyde and were unable to be utilized but more recent collections (post-2000) have been stored dry at room temperature. In this investigation, our objectives were to use this historic cyst reference collection to genetically characterize Victorian populations of *G. rostochiensis* using polymorphic microsatellite markers as well as sequences from the ITS region to investigate the introduction, history, and spread of this pest in Victoria.

MATERIALS AND METHODS

Field sampling and nematode extraction. During monitoring and detection surveys for PCN, soil was collected using a 10-by-10-m sampling pattern, where 100-by-10-cm³ cores to a depth of 10 cm were taken per hectare. Two hundred cores per 2 ha were combined and mixed, from which a subsample of 0.5 kg was used for PCN cyst extraction (Hinch 1991). Cysts were extracted from soil using Fenwick cans (Fenwick 1940). Cysts containing unhatched eggs and larvae were placed each in a drop of purified water on a cleaned microscope slide and were broken using two sets of fine forceps to release the contents. Using a Leica M205C microscope (magnification ×30), single larvae were extracted from eggs within the cysts using a fine probe and placed in 20 µl of purified water in 1.1-ml eight-strip minitubes (Neptune). When cysts were abundant, one larva was selected from each of 16 cysts from each paddock. Where fewer than 16 cysts were available per paddock, a total of eight larvae were selected from each cyst. From

one of the Gembrook paddocks where plentiful cysts were available, eight larvae were selected from each of 24 cysts.

Nematode populations used in this study. In this study, we utilized the PCN cyst reference collection (VPRI) held by Crop Health Services, Agriculture Victoria, to examine the genetic variability of Victorian PCN populations. A hierarchical sampling strategy was used to compare variability of juveniles within cysts, within and across paddocks, and between regions (Supplementary Table S1). In total, 944 juveniles were collected from four regions: Gembrook (*n* = 592), including a large sample from one crop variety trial site within Gembrook (*n* = 384); Koo Wee Rup (*n* = 208); Boneo (*n* = 24); and Thorpdale (*n* = 120).

DNA extraction and detection by real-time quantitative polymerase chain reaction. The total genomic DNA was extracted from single juveniles following the method described by Boucher et al. (2013). The real-time quantitative polymerase chain reaction (qPCR) primer (Sigma) and probe set (Applied Biosystems) designed by Reid et al. (2010) was used to check the quality and quantity of the PCN DNA samples (Table 1). Real-time qPCR was performed in a Rotor-Gene Q (Qiagen) in a total volume of 12.5 µl. Each reaction mixture included 6.25 µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.3 µM each forward and reverse primer, 0.1 µM Taqman probe, and 2 µl of template DNA, made up to 12.5 µl with RNA-free water. A nontemplate control with 2 µl of water instead of DNA and a positive control (DNA extracted from 100 PCN juveniles) was included in each run. The PCR thermal cycling conditions consisted of a two-step denaturation: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of amplification in a two-step procedure: 95°C for 15 s and 60°C for 30 s. The threshold cycle (Ct) value of the positive control was used as a comparison to determine the presence or absence of PCN DNA and eventually estimate its quality and quantity in unknown samples.

PCR amplification and genotyping. DNA samples which were detected as positive (qPCR) with good DNA yields were then processed for microsatellite PCR amplification and genotyping. PCR was conducted using nine microsatellite markers developed by Boucher et al. (2013): seven developed from *G. rostochiensis* (Gr50, Gr67, Gr75, Gr85, Gr90, Gr91, and Gr96) and two from *G. pallida* (Gp116 and Gp118) (Table 2). We originally tested four additional

TABLE 1. List of primers and probe used for potato cyst nematode DNA detection (quantitative polymerase chain reaction) and for sequencing the ribosomal gene spacer region^a

Oligo name ^b	Forward primer sequences (5'–3')	Reverse primer sequences (5'–3')	Probe (5'–3')	Reference
<i>Globodera rostochiensis</i>	CGTTTGTGTTGACGGACAYA	GGCGCTGTCCRTACATTGTTG	CCGCTGTGTATKGGC	Reid et al. 2010
ITS5	GGAAGTAAAAGTCGTAACAAG	...	...	White et al. 1990
ITS26	...	ATATGCTTAAGTTCACGGGGT	...	Howlett et al. 1992

^a All primers were ordered online from Sigma-Aldrich and the TaqMan probe from Applied Biosystems.

^b ITS = internal transcribed spacer.

TABLE 2. Characteristics of nine microsatellite markers used in this study for Australian population genetic diversity analysis of *Globodera rostochiensis* (Boucher et al. 2013)

Locus name ^a	Species developed on	Fluor ^b	Primer left sequence	Primer right sequence	Repeat motif	Size (bp)
Gr96	<i>G. rostochiensis</i>	FAM	CGGATAAAAAGCTTAAACCCCT	TGCAAAACGTTTCAGATATTTT	(AC)8	103
Gr90	<i>G. rostochiensis</i>	FAM	CGTAGTACGACGCGTTCAAG	GATCCGGCACTGGAGTACAT	(AC)12	115
Gr91	<i>G. rostochiensis</i>	HEX	GGTTGAAATGACGCAAGTGA	GATCTAATCCTTCCGGGCTC	(GT)5	112
Gp118	<i>G. pallida</i>	HEX	ACCGATGAAGAACATCGTCC	TCGTTCCGCTCTCGTAATCC	(TCCG)4	133
Gr85	<i>G. rostochiensis</i>	FAM	CCAAAAATTGATTGGCATCC	AATATCGCGTTGTTCCCAAG	(CA)7	123
Gr75	<i>G. rostochiensis</i>	HEX	CCAAAAATGGCACATCCACT	GCTGCTGTCGTACGCAAGAT	(CA)10	147
Gp116	<i>G. pallida</i>	FAM	ATTCAATTCGCAATGTTTCCC	TGGAAATGTGAGAAAGGGCT	(CGTC)4	141
Gr67	<i>G. rostochiensis</i>	FAM	ACCTGAACGTCGTCATTTC	TTTTCTTACCCGAATGGCAC	(GT)5	180
Gr50	<i>G. rostochiensis</i>	HEX	TTTGTCGGGCCTAAAAAGTG	CAAGTTCCTCTCCTATGCCG	(ACCA)5	322
Gr56	<i>G. rostochiensis</i>	HEX	TACAGATGTGCTGAATGGC	CTTCCTAAAGCAACAACGGC	(GT)6	314
Gr60	<i>G. rostochiensis</i>	HEX	ACAAATAAATGGATTGTAGATGAA	GAGAACAGATTGGCACTCG	(AC)5	225
Gr68	<i>G. rostochiensis</i>	FAM	TGCAAAAGACAATCCATGAAA	GAACGTGTCTGTCTGAACGC	(TG)5	177
Gr82	<i>G. rostochiensis</i>	FAM	CGTCTGCATTTTGTCTGTGT	GTTCCGGCCAAATCCGTC	(GA)6	131

^a Abbreviations: Gr = isolated from *G. rostochiensis* and Gp = isolated from *G. pallida*.

^b Fluorophore. Note that HEX is equivalent to VIC.

G. rostochiensis microsatellite markers employed by Alenda et al. (2014) (Gr56, Gr60, Gr68, and Gr82) but these were found to possess insufficient variation to discriminate Victorian *G. rostochiensis* populations (data not shown). All primers were synthesized (Sigma) and the 5' ends of the forward primers were labeled with two fluorescent dyes (6-FAM and HEX). PCR reagents, volume, and cycling conditions used were according to Boucher et al. (2013). All PCR amplifications were conducted on a Veriti 96-well Thermal Cycler (Applied Biosystems). Each primer pair was first tested by running PCR in simplex. The PCR products were diluted 1:10 in ultrapure water, with 2 µl of the dilution mixed with 8 µl of GeneScan-500 LIZ size standard (Applied Biosystems) plus Hi-Di formamide. Chromatograms showing PCR product sizes were produced using an ABI PRISM 3730xl automated sequencer (Applied Biosystems). Allele sizes of the fragments were scored by manual identification of the peaks for the presence or absence of the corresponding chromatogram using the software Geneious v9.1.3. Following this, all DNA samples were genotyped in two multiplex combinations: multiplex 1 had four markers (Gr67, Gr75, Gr90, and Gp118) and multiplex 2 had five markers (Gr50, Gr85, Gr91, Gr96, and Gp116).

To ensure that base pair sizes were determined accurately, allele sizes were confirmed through sequencing PCR products of homozygous alleles commercially through MacroGen. The forward primers for each of the nine primer sets were synthesized (Sigma) without the fluorescent dyes. Duplicate DNA samples were processed for primers Gr50, Gr67, Gr75, Gr85, Gr90, and Gp118 and quadruplicates for primers Gr91, Gr96, and Gp116. Standard PCR was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) in a total volume of 25 µl. Each PCR contained 1× Standard Taq reaction buffer, 200 µM dNTP, 5 mM MgCl₂, 2 U of Taq DNA Polymerase (Invitrogen), 0.18 µM each forward and reverse primers, and 5 µl of template DNA, and was made up to 25 µl with RNA-free water. A nontemplate control with 5 µl of water instead of DNA was included for each primer set. The PCR thermal cycling conditions were as follows: initial denaturation step at 95°C for 10 min; followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 1 min 30 s, and extension at 72°C for 30 s; and a final extension at 60°C for 30 min. To confirm the presence and the size of the band, 5 µl of the PCR product of all the samples was separated on a 2% agarose gel stained with 1× SYBR Safe DNA (Invitrogen). The gel was visualized under UV light using Gel Doc EZ System (Bio-Rad). The sizes of the amplicons were estimated using a 100-bp ladder (Invitrogen). Each PCR product (20 µl) was sent for sequencing to MacroGen. Forward and reverse sequences for each sample were analyzed using the software Geneious v9.1.3 and allele size was determined.

PCR amplification for ITS sequencing. The ribosomal gene spacer region was amplified using primers ITS5 and ITS26 (Table 1), as described by Bulman and Marshall (1997) and Nambiar et al. (2008). In total, 28 DNA samples were processed for sequencing from the four regions: Gembrook ($n = 13$), Koo Wee Rup ($n = 7$), Boneo ($n = 2$), and Thorpdale ($n = 6$).

Standard PCR was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) in a total volume of 25 µl. Each PCR contained 1× Standard Taq reaction buffer, 200 µM dNTP, 5 mM MgCl₂, 2 U of Taq DNA Polymerase (Invitrogen), 0.18 µM each forward and reverse primers, and 5 µl of template DNA, and was made up to 25 µl with RNA-free water. A nontemplate control with 5 µl of water instead of DNA was included in the run. PCR cycling conditions were as follows: initial denaturation step at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. To confirm the presence and the size of the band, 5 µl of the PCR product of all 28 samples was separated on a 2% agarose gel stained with 1× SYBR Safe DNA (Invitrogen). The gel was visualized under UV light using Gel Doc EZ System (Bio-Rad). The sizes of the amplicons were estimated using a 100-bp ladder (Invitrogen).

Each PCR product (20 µl) was sent for sequencing to MacroGen. Forward and reverse sequences for each sample were aligned and edited using the Geneious software v9.1.3.

Analyses of molecular variation. Microsatellite variation was analyzed for both population differentiation, estimated through pairwise F_{st} and levels of population structure, and levels of gene flow between populations, calculated from an analysis of molecular variance (AMOVA), conducted in GenAlEx 6.502 (Peakall and Smouse 2006, 2012). Patterns of population structure and admixture were also estimated using a Bayesian model-based clustering method implemented in STRUCTURE, version 2.3.4 (Falush et al. 2003; Pritchard et al. 2000), employing 25,000 replicates, with a burn-in of 500 and up to 10 populations ($K = 1$ to 10). Relationships between Australian PCN and worldwide populations (Boucher et al. 2013) were also examined through generating Nei's genetic distances between populations in GenAlEx, analyzed as a neighbor-joining tree in MEGA, version 7.0.26 (Kumar et al. 2016). Departures from Hardy-Weinberg equilibrium within each Australian population were examined by performing a χ^2 test in Excel 2016 to examine variation of microsatellite genotype frequencies at a site examined over time (2008 to 2010).

All publicly available ITS DNA sequences, with greater than 70% query coverage of the ITS sequences generated in the current study, were obtained from GenBank. Combined sequences were aligned using ClustalW with genetic relationships analyzed through generating a neighbor-joining tree, in MEGA.

Further genetic analyses were conducted in R (R Core Team 2017). Distance-based redundancy analysis was used to test for differences (i) among all populations and (ii) between all Australian and all non-Australian populations using the Adonis function in the R vegan package (Anderson 2001; Oksanen et al. 2016; Zapala and Schork 2006). Nonmetric multidimensional scaling ordination was used to visualize variation, similarities, and differences among samples and populations using the metaMDS function in the R vegan package (Minchin 1987).

RESULTS

Genotyping. Of the 944 juveniles extracted, approximately one-third (32%) produced DNA that could be amplified for examination of microsatellites. Of these, 88% (265 individuals) produced complete genotypes for all microsatellite markers examined (Supplementary Table S2). DNA was successfully extracted from cysts from 14 paddocks at Gembrook, 12 at Koo Wee Rup, 6 at Thorpdale, and 1 at Boneo.

Absolute microsatellite allele sizes (in base pairs) were determined through DNA sequencing homozygotes (Table 3). However, it was found necessary to adjust the size of alleles to match previously published allele sizes for comparison with worldwide genotypic data (Boucher et al. 2013) (see below).

Population structure. Analyses of population structure between Australian and worldwide PCN populations, using the nine microsatellites screened in the current study (Fig. 3; Supplementary Fig. S1), detected the same clusters of populations worldwide as had been previously reported through analyses of a larger number of microsatellites (Boucher et al. 2013). However, these analyses did not reveal any apparent population structure between Australian populations. A close association of Australian populations to any worldwide population included in the analyses was also not indicated (Figs. 3, 4, and 5). Analyses allowing for a larger number of potential populations (e.g., $K = 10$) (Fig. 3) still did not provide strong evidence of population differentiation within Australia.

No microsatellite alleles were found to be unique among Australian putative populations. Additional evidence for a lack of population structure within Australian populations was also shown by the percentage of AMOVA. AMOVA revealed that, when overall PCN populations (worldwide and Australian) were

analyzed together, a large amount of variation was observed among populations (51%) and individuals (41%), with very little within individuals (8%). On the other hand, when only the four Australian regions were analyzed together, the variation among populations was found to be very low (10%), with most variation being found within populations (37%) and individuals (53%). Distance-based redundancy analysis provided strong support for differences among populations ($P < 0.001$) and between Australian and non-Australian populations ($P < 0.001$), and this was supported by the nonmetric multidimensional scaling ordination (Fig. 5).

Origin of Australian PCN populations. The microsatellite analyses did not indicate a strong relationship between the Australian PCN populations and any of the worldwide populations that have been previously screened (Figs. 3, 4, 5, and 6; Supplementary Fig. S2), with the allelic variation observed in the current study consisting predominantly of the most common alleles known worldwide (Table 3). Analyses of ITS DNA sequence variation confirmed Australian PCN to be *G. rostochiensis*

(Fig. 7), with Australian PCN samples exhibiting relatively low genetic variation for the ITS gene and appearing to be most closely related to samples that were predominantly collected from Europe (Fig. 7). GenBank accession numbers for ITS DNA sequences obtained in the current study are MH820358 to MH820362.

Variation in an Australian PCN population. The most comprehensively sampled Australian region, both temporally and spatially, was Gembrook. At the Gembrook potato variety trial site, sampled between 2008 and 2010, microsatellite data were successfully obtained from 62 juveniles from 16 cysts. An AMOVA of these samples reveals that only 6% of the genetic variation was localized within paddocks, while the great majority existed within individuals (78%) and among individual cysts (16%). Differences between crop varieties were observed in microsatellite genotype frequencies, including several departures from Hardy-Weinberg equilibrium within paddocks (Fig. 8). Additionally, microsatellite allele frequency variation was observed within PCN cysts collected from Gembrook (Fig. 9).

TABLE 3. Comparison between worldwide published alleles (Boucher et al. 2013), Australian genotypes (scored in Geneious), and direct DNA sequencing of alleles

Locus name	Original sequenced allele (base pairs, repeat motif)	Published allele size (bp)	Geneious allele size (bp)	DNA sequence allele size (bp)	Observed repeat motif ^b	GenBank accession ^b	Most common allele worldwide (bp)	Genotypes (bp) in Australia ^a			
								1	2	3	4
Gr96	103, (AC)8	93	92	93	(AC)3	MH820363	Yes: 93	9393	9399	9999	...
Gr90	115, (AC)12	106 and 121	104 and 119	Not determined	N/A	N/A	Yes: 106, Rare: 121	106106	106121	121121	106117
Gr91	112, (GT)5	114	111	113	(GT)6	MH820364	Yes: 114	114114	114116	116116	...
Gp118	133, (TCCG)4	123	124	126	(TCCG)4	MH820365	No: 127 is most common	123123	...	...	...
Gr85	123, (CA)7	125	125	127	(CA)3C(CA)7	MH820366	Yes: 125	125125	...	...	...
Gr75	147, (CA)10	137	137	140	(CA)7	MH820367	Yes: 137	137137	...	...	...
Gp116	141, (CGTC)4	131/139	131/139	133/141	(CGTC)2, (CATT)5/7	MH820368, MH820369	Yes: 131	131131	131139	139139	...
Gr67	180, (GT)5	169	168	168	(GT)3	MH820380	Yes: 169	166166	166169	169169	...
Gr50	322, (ACCA)5	317	317	Not determined	N/A	N/A	Yes: 317	317317	...	...	...

^a Bold text indicates the alleles that were successfully sequenced.
^b N/A = not available.

Fig. 3. Population structure analyses (STRUCTURE) for all potato cyst nematode individuals from worldwide and Australian populations ($K = 7$ and $K = 10$), using data from nine microsatellite loci. Cluster names B2, B4, and C1 to C3 are from Boucher et al. (2013).

DISCUSSION

Our study clearly demonstrates that PCN in Victoria, Australia, possesses limited genetic variation compared with known potential source populations (Boucher et al. 2013). In contrast to the hypothesis proposed by Nambiar et al. (2008), the populations in

Victoria do not appear to indicate multiple independent origins. All populations screened appeared to share genetic variation, suggesting a single introduction and subsequent limited spread. Although we could not match the Australian PCN to any particular worldwide population, the microsatellite and ITS genetic variation observed in Australian PCN is present within European PCN populations; thus,

Fig. 4. World map showing distribution of genetic groups determined by STRUCTURE analyses using nine microsatellite loci. **A**, Microsatellite data from Boucher et al. (2013) reanalyzed using only nine loci. Cluster names, B2, B4, and C1 to C3 are from Boucher et al. (2013). **B**, Microsatellite data obtained from Australian populations (current study).

Fig. 5. Nonmetric multidimensional scaling ordinations illustrating genetic variation, similarities, and differences among samples and populations. Each point represents a single sample. All samples are included in the ordination on the left whereas, on the right, the outlier populations B2 and B4 have been left out to better illustrate the remaining populations and the Australian populations shown in a different color.

frequencies, which differentiates Australian populations from all other known worldwide populations.

PCN is a declared notifiable pest in Victoria and, until 2012, strict movement restrictions were in place for any host material, soil, and equipment within PCN control areas (i.e., within a 20-km radius from a PCN-positive parcel of land). Since 2013, a Risk-Based Regulatory Reform Model has been utilized instead, with regulated movement restrictions based on pathways in the production and supply chain that have been identified as being at high risk for the spread and establishment of PCN (Agriculture Victoria 2017). Since the first PCN detection in 1991, extensive surveillance based on soil sampling has taken place across all potato-growing regions of Victoria. All PCN soil testing has been undertaken by Agriculture Victoria's diagnostic laboratory, Crop Health Services, and, with more than 57,000 soil samples tested, only 66 parcels of land have tested positive for PCN. All of the positive detections are within a small region south and east of Melbourne. Therefore, the strict quarantine control measures involving increased hygiene and enforcing restricted movement appear to have been effective in stopping the spread of PCN, with the last detections from new properties from each control area being in 2007 (Koo Wee Rup and Boneo), 2011 (Thorpdale), and 2014 (Gembrook). In addition, Australia's strict requirements around potato imports since the 1980s (DAWR 2013) appear to have been effective in preventing any further introductions of the pest, with Australian PCN possessing stable ITS and microsatellite genetic variation both temporally and spatially.

Many previously examined PCN populations worldwide appear to contain microsatellite allelic variation within the population that is not especially common in other populations (e.g., C3 populations share a very high frequency of 137, 117, and 139 alleles at Gr75, Gr90, and Gp116 loci, respectively) (Boucher et al. 2013). However, Australian PCN populations appear to possess alleles that are relatively common worldwide but are present at unusual

Fig. 7. Neighbor-joining tree of genetic relationships among worldwide *Globodera rostochiensis* potato cyst nematode populations assessed from internal transcribed spacer DNA sequence data (730 bp). Australian populations are indicated in bold, with Australian specimens examined in the current study labeled as “new” and those from Nambiar et al. (2008) indicated by an asterisk (*).

current study. However, the microsatellite allele size required adjustment in our study to provide the best chance of matching the Australian data with known allele sizes from other worldwide populations (Boucher et al. 2013). It is common for small size shifts to be observed between alleles scored between different laboratories (Ellis et al. 2011; Moran et al. 2006), which are believed to

result from incremental differences in determining the size of alleles between equipment and screening platforms. Size shifts of microsatellite alleles have even been previously shown to occur with changes between different fluorophores (Sutton et al. 2011). As in previous studies of the related *G. pallida* (Plantard et al. 2008), it appears that the amount of genetic variability observed

Fig. 8. Genotype frequencies of three variable microsatellites (Gr 90, Gr 91, and Gr 116) in three plots of different potato varieties, collected from Gembrook between 2008 and 2010. Previous crop = original susceptible crop being grown on property ($n = 30$), Plot 1 = susceptible Trent variety ($n = 8$), and Plot 2 = resistant Crop13 variety ($n = 23$). Gray indicates heterozygotes and asterisks indicate significant departures from Hardy-Weinberg equilibrium.

Fig. 9. Allele frequencies of three variable microsatellites (Gr 90, Gr 91, and Gr 116) within individual potato cyst nematode cysts ($\times 12$), collected at Gembrook between 2008 and 2010. Three to six juveniles per cyst, $n = 56$ total.

within individual PCN cysts can be similar to that observed among entire populations. In the current study, a great deal of variation was observed in the frequency of alleles present within and between cysts, with changes in allele frequencies differing from Hardy-Weinberg equilibrium, possibly relating to the crop variety being utilized by PCN populations.

Our study was based on PCN samples collected through biosecurity sampling and retained (dry) in a long-term reference collection (VPRI, Agriculture Victoria), making our study different from most previous studies which have specifically collected PCN samples through short-term targeted surveys (Boucher et al. 2013; Bulman and Marshall 1997; Grenier et al. 2001; Mimee et al. 2015; Nambiar et al. 2008; Picard et al. 2004; Plantard et al. 2008). The reference collection samples collected over an extended period provide a historical element to our study. The number of samples genotyped was much less than the number of cysts extracted and examined, with the success rate of DNA genotyping greatly reduced with sample age. This is probably due to the need for juvenile PCN to be well preserved within cysts. Larvae within cysts preserved dry in the laboratory may perish sooner than those within soilborne cysts in the field, where larvae can remain viable for more than 20 years (Perry et al. 2013; Turner 1996). Indeed, some older samples from areas of interest in Australia, including samples from Western Australia, could not be used because they were found to have been not suitably preserved for DNA analyses (i.e., in formalin). Other regions such as New Zealand and the Netherlands represent current gaps in our knowledge of PCN population genetic relationships, which will hopefully be addressed through future studies.

The molecular assessments conducted in our study have revealed very little differentiation between Victorian populations, which share ITS DNA sequence and microsatellite allelic variation, with differences between local regions probably reflecting changes in allele frequencies over time. Our study suggests a probable single localized introduction into Victoria followed by limited spread to nearby areas, with Australian PCN appearing genetically distinct from populations previously sampled worldwide. Additionally, all of the Australian ITS sequences were of *G. rostochiensis*, confirming that *G. pallida* has not been recorded in Australia (Faggian et al. 2012). Any new exotic incursions, which potentially might bring in additional PCN pathotypes, should now be easily differentiated from existing established local PCN populations.

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