

ORIGINAL ARTICLE

Comparative genomics identifies genetic markers linked to structural variations that differentiate *Puccinia graminis tritici* and *secalis formae speciales*

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Funding information

H2020 European Research Council, Grant/Award Number: 715638 and 773311; Biotechnology and Biological Sciences Research Council, Grant/Award Number: BB/P012574/1 and BB/P016855/1; John Innes Foundation

Abstract

Stem rust is a serious disease of many gramineous plants including small grain staples such as wheat, barley, rye and triticale. Separate *formae speciales* (ff. spp.) of the causal fungus, *Puccinia graminis*, predominantly infect certain host plant genera. However, despite these taxonomic subdivisions, many *P. graminis* ff. spp. are genetically too similar to distinguish using existing genetic markers. For those infecting cereals, this is particularly challenging for *P. graminis* f. sp. *tritici* (Pgt) and *P. graminis* f. sp. *secalis* (Pgs). Herein we harnessed newly available genomic data for 39 Pgt and Pgs isolates and identified four regions of structural variation that were used to design four simple PCR-based assays to distinguish the aforementioned *formae speciales*. These genomic regions display large presence/absence variation between Pgt and Pgs isolates, and yet a high degree of sequence conservation within shared neighbouring regions, facilitating primer design. We also confirmed lack of amplification in host plant genera analysed, which included assessment of the shared alternate host of Pgt and Pgs, *Berberis vulgaris*. Accurate classification of *P. graminis* ff. spp. infections on *B. vulgaris* is exceptionally valuable to rapidly define plants harbouring *P. graminis* inoculum when adjacent to cereal crops. Finally, we demonstrated utility of these four genetic markers to correctly distinguish a genetically diverse array of Pgt and Pgs isolates. This strategy could now be readily applied to other pathogens of interest, which will be of increasing value as genomic resources continue to rapidly expand for many key biotic threats to agricultural productivity.

KEYWORDS

formae speciales, genetic markers, *Puccinia graminis*, stem rust

Anthony Bryan and Andrey Korolev contributed equally to this study.

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1 | INTRODUCTION

Stem (black) rust is a serious disease of gramineous plants including many small grain crops such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereale*) and triticale (*xTritosecale*), as well as other grasses (Leonard & Szabo, 2005). The causal fungus, *Puccinia graminis*, has posed a long-standing threat to cereal production, causing dramatic crop failures throughout history. It was so feared in ancient Rome that for over 1700 years the rust god Robigus was honoured each spring in the hope that grain fields would be spared from infection (Peterson, 1974). Despite the historical significance of the disease, the *Puccinia* genus was only first described in the 18th century (Micheli, 1729) and since, mycologists have grappled with the taxonomic complexity and high diversity of the stem rust pathogen. This led to the division of the *P. graminis* species into a series of *formae speciales* (ff. spp.) to reflect the most commonly associated host plant genera of the dikaryon stage of the fungus (Eriksson, 1898). Today, the most widely recognized *formae speciales* that infect cereal crops include *P. graminis* f. sp. *tritici* (Pgt), *P. graminis* f. sp. *avenae* (Pga) and *P. graminis* f. sp. *secalis* (Pgs), which are predominantly found to infect wheat/triticale/barley, oat and rye/barley, respectively (Boshoff et al., 2019). Within each *forma specialis* there are also genetic variants (termed physiologic forms or races) that differ in virulence to different resistance genes in the host plant species (Roelfs, 1985). Although these subdivisions have been useful to encapsulate the high degree of host specificity displayed by *P. graminis* when infecting its gramineous host, many species in the unrelated *Berberis* genus act as a common alternate host for the *P. graminis* ff. spp. (Barnes et al., 2020).

Like many rust fungi, *P. graminis* is a heteroecious pathogen, requiring two hosts to complete its life cycle. The asexual stage occurs on its gramineous host, where infection is typified by the presence of reddish-brown elongated pustules that are most frequent and abundant on stems and leaf sheaths and contain asexual *P. graminis* urediniospores (McKay, 1957). As the host reaches maturity, urediniospores are often replaced by black teliospores that facilitate overwintering of the pathogen in cooler climates. After a period of rest, teliospores then germinate to create basidiospores that can go on to infect the alternate host of the pathogen, *Berberis* spp. At this point, the pathogen completes its sexual cycle, generating new genetic variants in the form of aeciospores within 'cluster cups'. Aeciospores are then mechanically released from 'cluster cups' and dispersed on the wind to carry the infection back to the gramineous host (Bueno-Sancho et al., 2021). The importance of *Berberis* spp. in the epidemiology of stem rust, and principally for wheat stem rust, historically led to extensive exclusion campaigns and legislation particularly across Europe, the United States and Canada to restrict its prevalence and thereby propensity to act as a nursery bed of inoculum and new genetic variants. These campaigns largely focused on eradication of *Berberis vulgaris*, which is particularly susceptible to Pgt infection (Barnes et al., 2020). However, planting restrictions have

long since lapsed in many countries across Europe, and Western Europe is currently experiencing a resurgence of wheat stem rust (Barnes et al., 2020; Patpour et al., 2022; Saunders et al., 2019). Hence, there is an urgent need to hasten monitoring of the presence of Pgt on *Berberis* spp., which could be achieved by employing molecular markers that can differentiate *P. graminis* ff. spp. However, to date the genetic boundaries between *P. graminis* ff. spp. have remained ambiguous, inhibiting marker design.

Traditionally, gene sequence analysis of *P. graminis* ff. spp. has shown that many are genetically too similar to distinguish (Berlin et al., 2013; Lewis et al., 2018). For those infecting cereals, this is particularly challenging for Pgt and Pgs. These two *formae speciales* are known to be very closely related, as illustrated by successful crossing experiments in controlled conditions (Anikster, 1985; Johnson et al., 1932). Accordingly, when classifying rust species found on *Berberis* in Sweden, sequence analysis of the rDNA internal transcribed spacer (ITS) region and *translation elongation factor 1- α* (*EF1- α*) gene, combined with detailed morphological characterization, failed to differentiate the Pgt and Pgs taxa (Berlin et al., 2013). The distinction between Pgs and Pgt is also confounded by the ability of both *formae speciales* to infect barley to differing degrees (Schumann & Leonard, 2000) and the general inconsistencies found between analysis of classical gene sequence markers and host specificity for *P. graminis* isolates (Abbasi et al., 2005). However, recent analysis using the genome sequence of Pgt enabled the development of an abundance of simple-sequence repeat (SSR) markers aimed at characterizing Pgt population diversity. Within this study, a large proportion of SSR markers differed in amplification between Pgt and Pgs isolates (Karaoglu et al., 2013), suggesting that a genomic-based approach could potentially be fruitful for defining new genetic markers to distinguish these closely related *formae speciales*. Accordingly, herein we used a comparative genomics approach to successfully develop four genetic markers that we showed can distinguish a genetically diverse collection of Pgt and Pgs isolates using a simple PCR-based assay. These simple markers may now enable quick molecular distinction of Pgt and Pgs isolates, which will be particularly valuable when assessing *P. graminis* infections on *Berberis* spp.

2 | MATERIALS AND METHODS

2.1 | Alignment of reads to the Pgt reference genome

Publicly available genomic data for a total of 35 Pgt, four Pgs and two *P. graminis* f. sp. *avenae* (Pga) isolates was collated (Table S1). For each dataset, raw sequencing reads were trimmed to remove the first 13 bases using *fastx_trimmer* from the FASTX-Toolkit v. 0.0.14. Reads were then aligned to the Pgt reference genome (isolate 21-0, haplotype B; Li et al., 2019) using BWA v. 0.7.5 (Li & Durbin, 2009) and summarized in pileup format using *samtools* v. 1.10 (Li et al., 2009). Single-nucleotide polymorphisms (SNPs) were identified using a

custom Python script (Bryan & Saunders, 2023), with a minimum depth of coverage of 10x. Sites that matched the reference genome were recorded if they had a minimum of 2x depth of coverage.

2.2 | Phylogenetic analysis

To create the phylogeny, first consensus gene sequences were generated that independently incorporated sequence variation for each of the 39 Pgt and Pgs isolates and 2 Pga isolates to be used as outliers to root the phylogenetic tree. The third codon position for each of the 18,843 gene models annotated in the Pgt reference genome was extracted using custom Python scripts (Bryan & Saunders, 2023) and filtered to extract only sites with bases recorded in at least 80% of *P. graminis* isolates. A total of 20 starting phylogenetic trees were generated (10 random and 10 parsimony-based) using RAxML-NG v. 1.1.0 (Kozlov et al., 2019) using the GTR-G substitution model, and a maximum-likelihood search conducted. The results of 100 bootstraps were mapped to the best-scoring tree and the phylogeny visualized using a custom Python script (Bryan & Saunders, 2023) that used Biopython 1.78 (Cock et al., 2009), Pandas 1.1.5 (McKinney, 2012) and Matplotlib 3.2.2 (Hunter, 2007).

2.3 | Identifying genomic regions of structural variation

For each of the 39 Pgt and Pgs isolates, read depth data were extracted and combined from the pileup files following alignment to the Pgt reference genome (see above). The minimum and maximum depth of coverage at each nucleotide position across all Pgt or Pgs isolates was determined for each chromosome using Pandas 1.1.5 (McKinney, 2012). The minimum depth of coverage for all Pgt or Pgs isolates were divided independently into 20bp segments and if the 10th percentile for a given 20bp segment was at least 3, then the segment was considered to have high coverage. By using the 10th percentile of the minimum depth of coverage values, this allowed for small deletions to occur (up to 2bp) within each segment. To generate a PCR marker that was of sufficient length to be distinguished using agarose gel electrophoresis, groups of eight or more contiguous segments (minimum 160bp) of high Pgs coverage were identified using Pandas 1.1.5 (McKinney, 2012) and NumPy (Harris et al., 2020). A similar process was used to identify regions of contiguous high coverage for all Pgt isolates, with a higher minimum length threshold of 16 segments (320bp).

To identify regions of low Pgs coverage, the maximum depth of coverage for all Pgs isolates was divided into 20bp segments as described above. A segment was considered to have low coverage when the maximum depth of coverage was 0 for all Pgs isolates. Regions with at least eight contiguous segments were selected. Nearby regions of high or low Pgs coverage (within 400bp) that overlapped a region of high Pgt coverage of at least 160bp were defined as suitable for primer design. Each marker region was also

assessed for overlap with any gene annotations using Pandas 1.1.5 (McKinney, 2012). Further details are provided in Data S1.

2.4 | Primer design

For each candidate marker region, a trio of primers was designed (Table 1) using Primer-BLAST that would create a maximum of two amplicons: (a) one when either Pgs or Pgt DNA was present, and (b) one when only Pgt DNA was present. Default parameters were used for Primer-BLAST, except the use of a maximum primer size of 30, GC clamp of 1 and specifying the number of primers to return as 30 (Ye et al., 2012). Primer pairs that shared a common primer between the two runs were then further assessed for conservation across a genetically diverse array of Pgt isolates and to prevent nonspecific amplification in the plant host. We also designed primers for amplification of the Pgt *EF1- α* gene (accession XM_003333024.2) using Primer-BLAST with the aforementioned settings and searched for an amplicon in the middle of the mRNA with a product size of between 150 and 400bp. The Pgs *EF1- α* gene sequence was extracted from alignment of the Pgs isolate DE-06 to the Pgt reference genome and sequence alignments of the Pgt and Pgs *EF1- α* genes were generated using MUSCLE v. 5.1 (Edgar, 2004) and visualized in Jalview (Procter et al., 2021).

2.5 | Assessment of primer specificity

To assess conservation of the primer trios across a diverse set of Pgt isolates, we gathered publicly available genome assemblies for four additional Pgt isolates: UK-01 (Lewis et al., 2018), 99KS76A-1 (Salcedo et al., 2017), CRL-75-36-700-3 (Duplessis et al., 2011) and a Pgt isolate belonging to the Ug99 race group (Li et al., 2019). Amplification of the primer trios for each of the four markers (M1, M2, M3 and M4) was assessed in each Pgt genome assembly using Primer-BLAST with the aforementioned parameters (Ye et al., 2012). We then extracted the predicted PCR products from each of the five Pgt genome assemblies using ipcrs from exonerate v. 2.4.0 (Slater & Birney, 2005), allowing up to four mismatches between the template and any primer sequence, with up to one mismatch allowed in the last five bases of the 3' region. Multiple sequence alignments were generated using MUSCLE v. 5.1 (Edgar, 2004) and visualized with the python pyMSAviz package v. 0.4.0 (Shimoyama, 2022).

To evaluate the potential for nonspecific amplification of host plant DNA, we used Primer-BLAST with the aforementioned settings and the Chinese Spring wheat genome (IWGSC RefSeq v1.0; International Wheat Genome Sequencing Consortium, 2018) and rye line Lo7 genome (Rabanus-Wallace et al., 2021). To assess off-target amplification of the primer trios for the alternate host *B. vulgaris*, we extracted DNA from *B. vulgaris* leaves using the DNeasy Plant Mini Kit following the manufacturer's guidelines (Qiagen). We then conducted PCRs to amplify each of the four marker regions (M1, M2, M3 and M4), two universal DNA plant barcodes (*psbA-trnH* and *rpoC1*; Chen et al., 2010) and the *P. graminis EF1- α* gene. The resulting PCR products were

TABLE 1 Primers used in this study.

Locus/gene	Species	Sequence (5'-3')	GC (%)	Tm (°C)	Tm (°C) used in PCRs
M1 (F)	Pgt	TTTGAAGCCCCAGACTGCTATG	50.00	60.62	55
M1 (F)	Pgs, Pgt	GTGTAACACTCTCCAATTATTGAG	40.00	57.65	–
M1 (R)	Pgs, Pgt	CCCATAATTTCACTGCTCTCCTTG	45.83	59.66	–
M2 (F)	Pgs, Pgt	AATGCCTTCAGTCCCTATCTGTACTC	46.15	61.87	58
M2 (R)	Pgs, Pgt	ACTACAGAGGAGCAGTGATCAAG	47.83	59.55	–
M2 (R)	Pgt	CCACCATTGAAGCAAGACCC	52.38	59.73	–
M3 (F)	Pgt	TTCCTTCTCACTGAGTCACAC	47.62	57.26	55
M3 (F)	Pgs, Pgt	TGTACATGGAGTGGAGATTGCG	50.00	60.42	–
M3 (R)	Pgs, Pgt	GCTCACCAGCTTTGGTATGTTG	50.00	60.10	–
M4 (F)	Pgs, Pgt	ATGCCAACTGTAGTGGTAGCACAG	50.00	62.90	58
M4 (R)	Pgs, Pgt	TGATAAACTAGCATGAGTGCATACTGAC	37.93	61.73	–
M4 (R)	Pgt	AACCCTGGTCTAGTCTTTATCAACC	44.00	60.05	–
eEF1a1 (F)	Pgs, Pgt	GACCCTCCTCGATGCTATTG	55.00	57.55	55
eEF1a1 (R)	Pgs, Pgt	CAGACTTGACTTCAGTCGTCAC	50.00	58.95	–
psbA-trnH (F) ^a	Plant	GTTATGCATGAACGTAATGCTC	40.91	56.23	55
psbA-trnH (R) ^a	Plant	CGCGCATGGTGGATTACAATCC	56.52	64.97	–
rpoC1 (F) ^a	Plant	GGCAAAGAGGGAAGATTTCG	50.00	56.79	55
rpoC1 (R) ^a	Plant	CCATAAGCATATCTTGAGTTGG	40.91	54.68	–

Note: Primers are indicated as forward (F) or reverse (R). M1–M4, marker regions 1–4. Pgs, *Puccinia graminis* f. sp. *secalis*, Pgt, *P. graminis* f. sp. *tritici*.

^aPrimers are from Chen et al. (2010).

subjected to agarose gel electrophoresis, where DNA fragments were run on approximately 1% (wt/vol) agarose gels and visualized by staining with ethidium bromide (0.5 mg/mL) or GelRed (1 unit/gel; Merck) and viewed under UV light on a transilluminator.

2.6 | PCR amplification of the four marker regions

DNA were extracted from dried urediniospores for all Pgt and Pgs isolates (Table S2) using methods described previously (Tsushima et al., 2022). We also extracted DNA from leaves of the hexaploid wheat (*T. aestivum*) variety Santiago or Avocet line, an organic rye variety (Microscience) and *P. graminis*-infected *Berberis* leaves using the DNeasy Plant Mini Kit following the manufacturer's guidelines (Qiagen). For each marker (M1, M2, M3 and M4) a PCR was performed with the primer trios (Table 1) and DNA from Pgt and/or Pgs isolates, *P. graminis*-infected *Berberis*, wheat and rye. The size and number of resulting PCR products were assessed following agarose gel electrophoresis.

3 | RESULTS

3.1 | Genetic divergence in Pgt and Pgs isolates

To assess the level of genetic divergence between Pgt and Pgs isolates, we sought to gather publicly available genome sequence data for comparative genomic analysis. We obtained raw read data for a

total of four Pgs isolates, sourced from a single country (Germany), and 35 Pgt isolates, sourced from 11 countries (Table S1). Following processing, raw reads for each of the 39 *P. graminis* isolates were independently aligned to the Pgt reference genome (isolate 21-0, haplotype B; Li et al., 2019), where an average of 72.0% (standard deviation 13.5%) of reads from Pgt isolates and 80.1% (standard deviation 7.9%) reads derived from Pgs isolates aligned (Table S1). To assess the genetic similarity between *P. graminis* isolates we then conducted phylogenetic analysis (6,103,822 sites) of the 39 Pgt and Pgs isolates, with two Pga isolates included as outliers for rooting the tree. In the phylogeny, Pgs isolates formed their own distinct clade separate from all Pgt isolates, as shown previously (Tsushima et al., 2022; Figure 1a). This analysis confirmed a clear genetic distinction between isolates from the Pgs and Pgt ff. spp., supporting the potential for identifying genetic markers to differentiate Pgs and Pgt isolates.

3.2 | Using genomic variation between Pgs and Pgt isolates to guide marker design

To identify genomic loci that could be used to develop genetic markers to differentiate Pgs and Pgt isolates, we focused on searching for genomic structural variation. We surmised that by focusing on regions of structural variation between Pgt and Pgs isolates, this would enable the design of sets of differential PCR primers for Pgt and Pgs isolates. Specifically, we set out to identify genomic regions displaying large presence/absence variation, indicated by regions of abrupt low/no coverage for all Pgs isolates when aligned to the Pgt reference genome.

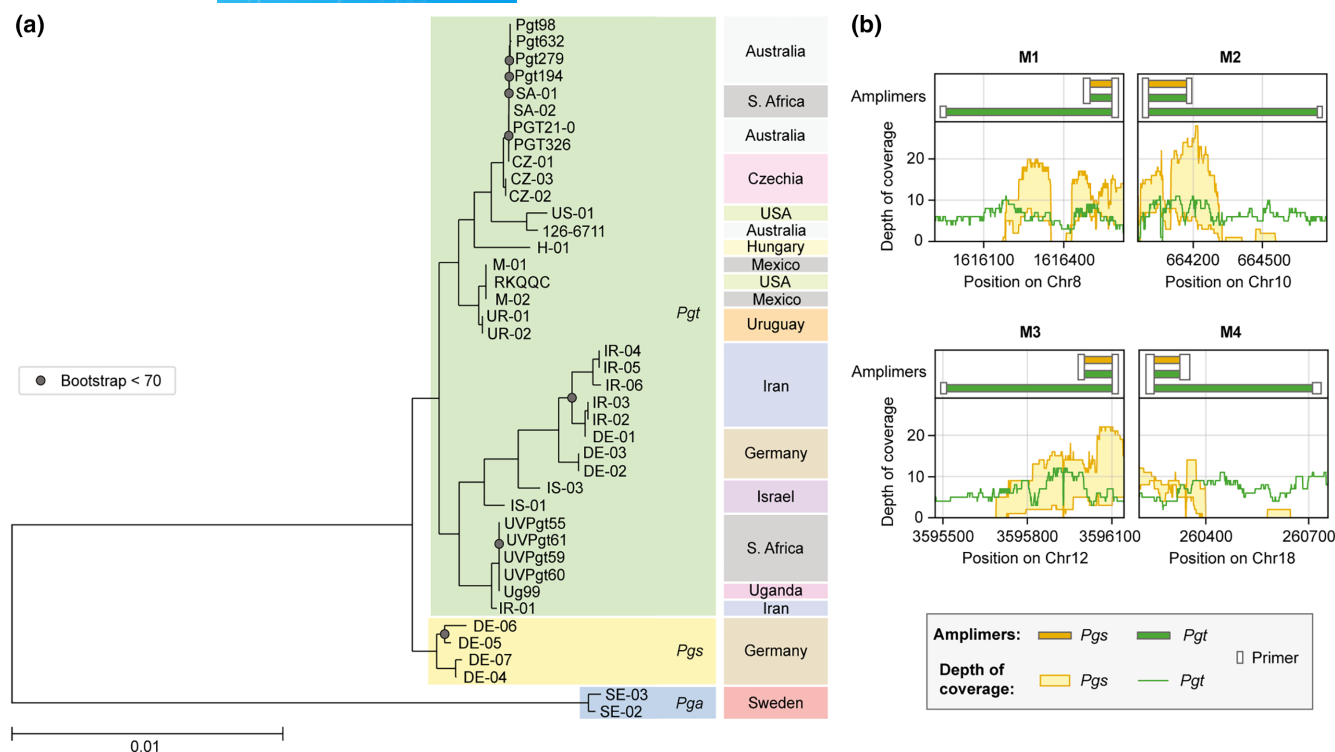


FIGURE 1 *Puccinia graminis* f. sp. *tritici* (Pgt) and *P. graminis* f. sp. *secalis* (Pgs) isolates are genetically distinct, facilitating the design of four genetic markers to distinguish isolates from the two *formae speciales*. (a) Phylogenetic analysis of 35 Pgt, 4 Pgs and 2 *P. graminis* f. sp. *avenae* (Pga) isolates illustrated that all Pgs isolates formed their own distinct clade separate to all Pgt isolates. The third codon position of 18,843 gene models were selected and sites with coverage across at least 80% of all *P. graminis* isolates (6,103,822 sites) used for phylogenetic analysis using a maximum-likelihood approach. Two Pga isolates were included as outliers in the phylogeny. Source countries for each Pgt, Pgs and Pga isolate are indicated. (b) Four genomic loci were identified for marker design (M1, M2, M3 and M4) that displayed structural variation between Pgt and Pgs isolates. The minimum depth of coverage for Pgt isolates and depth of coverage range for Pgs isolates are illustrated for each of the marker regions identified on Pgt chromosomes (Chr) 8, 10, 12 and 18. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/ppa.13890)]

This would support the design of PCR primers to generate (a) an amplicon for both Pgt and Pgs isolates from within a preceding or succeeding conserved high coverage region and (b) a longer amplicon that spanned the conserved region of high coverage and the Pgs-specific low coverage region that would only be PCR amplified from Pgt DNA.

First, to identify regions of high coverage for Pgs and Pgt isolates, we assessed the minimum coverage at each nucleotide position for all Pgs and Pgt isolates when aligned to the Pgt reference genome. To also account for the potential for small deletions (up to 2bp) to occur within regions of high coverage, we divided the genome into segments of 20bp and assessed the level of coverage for all Pgs or Pgt isolates across each segment. Where the 10th percentile depth of coverage across the 20bp segment for all Pgt or Pgs isolates was at least 3, we considered the segment to have high coverage. We then selected Pgs high coverage regions with eight or more contiguous segments (minimum 160bp) as these would be sufficient in length to be distinguished using agarose gel electrophoresis. For Pgt, we used a higher minimum length threshold of 16 segments (320bp) for classifying high coverage regions, as the subsequent PCR fragment would need to overlap the Pgs high coverage region and be distinguishable in length. Pgs-specific low coverage regions were defined as eight or more contiguous 20bp segments with no coverage for all Pgs isolates.

We selected regions of high or low Pgs coverage within 400bp as potentially suitable for marker design, because the Pgt-specific amplicon needed to stretch from a region of high Pgs coverage to a region of low Pgs coverage. To facilitate primer design for the Pgt-specific amplicon, we also only considered regions of high Pgt coverage that overlapped by at least 160bp of a low coverage Pgs region (Figure 1b). Following this strategy, we identified 12 potential marker loci that were distributed across seven chromosomes (Table S3). We were able to design an appropriate trio of primers for four of the identified marker loci (designated M1, M2, M3 and M4; Table 1), with each being present on a separate chromosome. Comparing the boundaries of each marker to annotated regions, we found that only M3 overlapped with an annotated gene sequence. The marker is entirely within the gene, which is predicted to encode a protein with kinase activity (Protein ID 4218) (Li et al., 2019).

3.3 | Sequence conservation of four marker regions across genetically diverse Pgt isolates

To assess the robustness of the primer trios in amplification of Pgt and Pgs DNA, we generated sequence alignments of the four marker regions for all 39 Pgt and Pgs isolates. This analysis illustrated a high

level of sequence conservation across Pgt and Pgs isolates within the primer trio annealing sites, except for the reverse primer for M4 that had a 7bp insertion following position 19 in all Pgs isolates. When excluding the Pgs alignments for this one primer, 95.2% of remaining comparisons had a sequence conservation level with a maximum of three mismatches to the primer sequence (Figure 2a). To further evaluate the robustness of the primer trios in amplification of Pgt DNA, we also checked their conservation across a series of genetically diverse Pgt isolates where genome assemblies are publicly available. This included (a) the Pgt isolate used as a reference genome that originated from Australia (isolate 21-0; Li et al., 2019), (b) a UK Pgt isolate collected in 2013 (UK-01, pathotype TKTTF; Lewis et al., 2018), (c) a US Pgt isolate collected in Kansas in 1999 (99KS76A-1, pathotype RKQQC; Salcedo et al., 2017), (d) a US Pgt isolate collected in Pennsylvania in 1975 (CRL-75-36-700-3, pathotype SCCL; Duplessis et al., 2011) and (e) a Pgt isolate belonging to the Ug99 race group that was collected in Uganda (Li et al., 2019). Sequence alignments of the genomic regions relating to the four primer trios illustrated that the primer regions were highly conserved (Figure 2b) and found as single products in 65% of the comparisons, with only M4 lacking in the assembly for Pgt isolate CRL-75-36-700-3.

3.4 | Assessing the potential for nonspecific amplification in Pgt and Pgs plant hosts

To evaluate the chance of nonspecific amplification of host plant DNA when using the four markers, we also assessed primer specificity against the Chinese Spring wheat genome (IWGSC RefSeq v1.0; International Wheat Genome Sequencing Consortium, 2018) and rye line Lo7 genome (Rabanus-Wallace et al., 2021). BLAST searches failed to predict any amplicons being formed by the chosen primers in either plant assembly. To also analyse the potential for nonspecific amplification in the alternate *Berberis* host, we tested the amplification of the four primer trios on DNA extracted from *B. vulgaris*, as no genome assembly is currently available for any *Berberis* spp. We found no evidence of amplification of the four primer trios from *B. vulgaris* DNA (Figure S1), while two universal plant DNA barcodes (*psbA-trnH* and *rpoC1*; Table 1) successfully amplified at the expected sizes (c. 100–1000bp or 462–556bp, respectively; Chen et al., 2010), confirming the presence of plant DNA. Overall, this analysis confirmed the specificity of the four selected primer trios, which appeared unlikely to amplify nonspecific regions from host plant DNA from wheat, rye or *B. vulgaris*.

3.5 | Differentiation of Pgt and Pgs isolates using four genetic markers

To determine the utility of the four PCR-based markers to differentiate Pgs and Pgt isolates, we initially selected three Pgt isolates (11-13a, 24-13a and 25-13c) and three Pgs isolates (11-4, 46-2 and

106-5) for testing (Table S2) and DNA was extracted from purified urediniospores for each *P. graminis* isolate. In addition, we also included DNA extracted from wheat and rye leaves in our analyses to assess any nonspecific amplification from the respective plant hosts. The eight DNA samples were then used to perform PCRs for the four target marker regions. Amplicon band patterns and sizes for each of the four markers were as expected (Figure 3a). All four markers produced a single amplicon between 128 and 214bp for all Pgs isolates and two amplicons at 128–214bp and 511–780bp for all Pgt isolates. However, it was noted that a third amplicon was evident in Pgt samples for M2. There was also evidence of nonspecific amplification when using wheat DNA, producing a small fragment similar in size to the 128bp amplicon for M4 (Figure 3b). As this could potentially confuse results, we decided to design a further marker that could be used to specifically confirm presence of *P. graminis* DNA in a sample. We targeted the highly conserved *EF1-α* gene from Pgt and confirmed that the sequence is conserved across the two *P. graminis* ff. spp. (Figure S2; Table 1). We then tested the amplification of a 197bp region of the *EF1-α* gene for each of the eight DNA samples and found, as expected, a single amplicon of approximately 200bp was present for all *P. graminis* samples, with no amplification detected in the wheat and rye samples (Figure 3b). This confirmed the suitability of *EF1-α* amplification as a positive control for *P. graminis* presence in DNA samples. These results indicated that the four markers could be used to differentiate Pgs and Pgt isolates using the isolates tested.

3.6 | Ability to distinguish genetically diverse Pgt and Pgs isolates

To evaluate the ability of the four genetic markers to differentiate genetically diverse isolates of Pgt and Pgs, we expanded our analysis to include an array of diverse isolates. We focused on Pgt and Pgs isolates that had been collected on their respective hosts, with DNA extracted from purified urediniospores for each isolate. We first widened our analysis of German Pgt and Pgs isolates to evaluate an additional set of 11 Pgt and 12 Pgs isolates (Pgt isolates WSR 1/17, 2/17, 1/19, 2/19, 1/20-1, 1/20-2, 1/20-3, 1/21, 2/21, 3/21 and 4/21; Pgs isolates RSR1–12). For all Pgt and 11 of the 12 Pgs isolates, amplicon band patterns were consistent with those expected for the known *formae speciales*, with only one Pgs isolate giving inconsistent band patterns across markers, which could be reflective of impurity of this *P. graminis* isolate (Figure 4a). Next, we conducted analysis of three Pgt and three Pgs isolates sourced from South Africa that had been assigned to the aforementioned *formae speciales* through pathology analysis (Boshoff et al., 2019; Pgt isolates UVPgt56, UVPgt63 and UVPgt64; Pgs isolates UVPgs1, UVPgs2 and UVPgs3), alongside DNA from wheat as a control. In all cases, we found that the amplicon band patterns from the South African *P. graminis* isolates were consistent with the defined *formae speciales* (Figure 4b).

To widen the genetic diversity of Pgt isolates even further, we selected four additional Pgt isolates that are representative of four

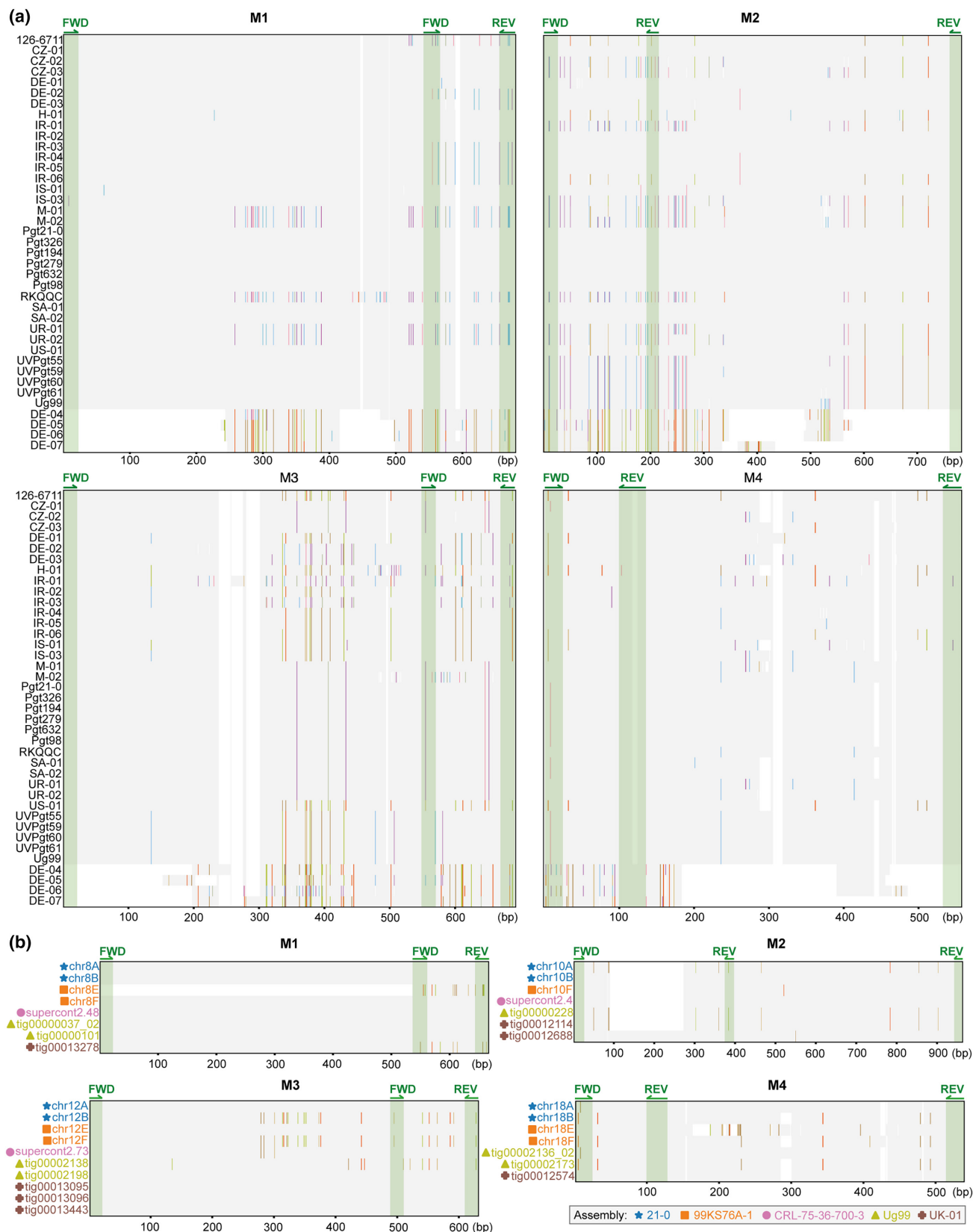


FIGURE 2 Primer trios for the four marker regions were conserved across a diverse range of *Puccinia graminis* f. sp. *tritici* (Pgt) isolates. A high degree of sequence conservation was observed within the primer trio annealing sites (green shaded areas) for all 39 Pgt and *P. graminis* f. sp. *secalis* (Pgs) isolates (a) and a genetically diverse set of five Pgt isolates where genome assemblies are publicly available (b). Multiple sequence alignments were generated using MUSCLE v. 5.1 (Edgar, 2004) and visualized with the python pyMSAviz package v. 0.4.0 (Shimoyama, 2022). bp, base pairs. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

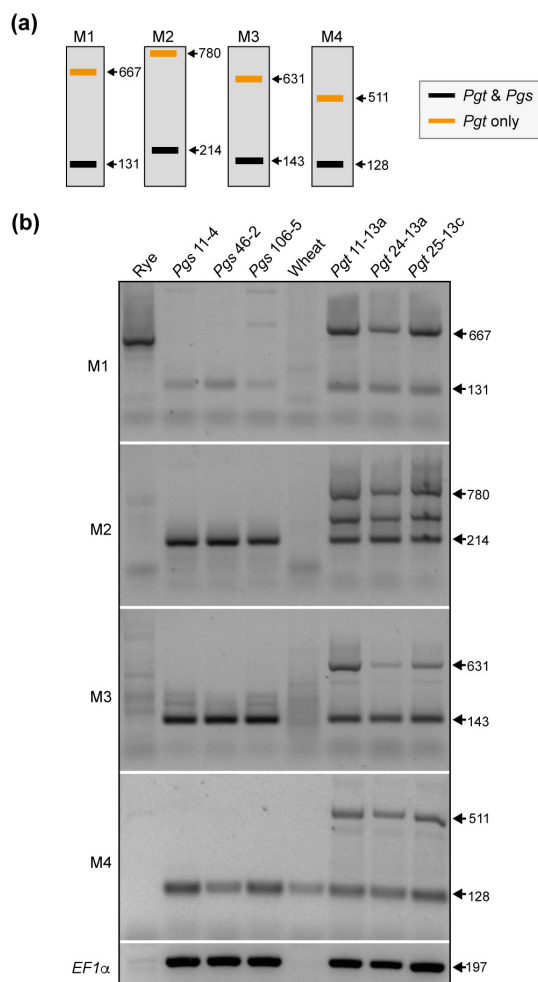


FIGURE 3 The four selected genetic markers can be used to correctly distinguish *Puccinia graminis* f. sp. tritici (Pgt) and *P. graminis* f. sp. secalis (Pgs) isolates. (a) Genomic regions displaying large presence/absence variation between Pgt and Pgs isolates were selected, and PCR primers designed to generate (i) an amplicon for both Pgt and Pgs isolates from within a preceding or succeeding conserved high coverage region and (ii) a longer amplicon that spanned the conserved region of high coverage and the Pgs-specific low coverage region that would only be PCR amplified from Pgt DNA. Predicted amplicon sizes are indicated for the four markers (M1, M2, M3 and M4). (b) The amplicon band patterns and sizes generated following PCR of DNA from three Pgt isolates (11-13a, 24-13a and 25-13c) and three Pgs isolates (11-4, 46-2 and 106-5) were as expected, with no amplification of wheat and rye DNA for M1, 2 and 3 and only a faint band when using wheat DNA for M4. The presence of *P. graminis* DNA was further confirmed by amplification of the *EF1- α* gene exclusively in all Pgt and Pgs lanes. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

distinct lineages of the pathogen that were previously genetically defined using SNP analysis (Olivera Firpo et al., 2017). This included representatives of Clade I (isolate KE178a_18), known to contain Pgt isolates of the Ug99 race group (Olivera et al., 2015); Clade III_B (IT16a_16), which represents the race TTRTF causing the epidemic outbreak in Sicily in 2016; Clade IV_B (isolate IT200b_18), which

includes the TKTTF Pgt race which is currently common in Western Europe; and Clade IV_F (isolate IT29a_18), that represents the TKKTF race found initially in Georgia (Szabo et al., 2022) and is now widespread in Europe (Patpour et al., 2022; Tsushima et al., 2022). In all cases, amplicon patterns for each of the Pgt isolates were consistent with the previously assigned *formae speciales* (Figure 4c). Finally, we tested the four markers directly on *P. graminis*-infected *Berberis* leaf tissue and showed that we were able to identify band patterns consistent with Pgt or Pgs from the six samples analysed (Figure S3). To ensure assignment of *P. graminis* isolates to the correct *formae speciales*, we also used infected *Berberis* leaf tissue to derive single pustule isolates that were assigned to Pgt and Pgs ff. spp. through pathology tests. For the 11 samples where subsequent pathology analysis was conducted, the *P. graminis* f. sp. indicated from evaluating the amplicon size and number from the four PCR markers was consistent with the *formae speciales* indicated through pathology analysis (Figure 4d).

4 | DISCUSSION

Over the past decade, an increase in wheat stem rust infections has been recorded across Western Europe. This has coincided with an increase in the abundance of the alternate host (*Berberis* spp.) that can act as a reservoir of highly diverse early season inoculum with the potential to seed future epidemics in the region. With an array of *P. graminis* ff. spp. able to infect the same *Berberis* species, deciphering the precise identity of aecial structures formed on *Berberis* and correctly assigning them to *P. graminis* ff. spp. is vital to assess the risk they could pose to neighbouring cereal crops and inform disease management strategies. However, distinguishing the closely related Pgt and Pgs ff. spp. in particular has proved challenging, with a lack of differential genetic markers (Berlin et al., 2013; Lewis et al., 2018). To address this gap, we set out to harness newly available genome sequence data for Pgt and Pgs isolates and identified four regions of genomic structural variation that could be used to design simple PCR-based assays to distinguish the aforementioned *formae speciales*. These genomic regions had abrupt losses in coverage across all Pgs isolates and consistently high coverage across all Pgt isolates.

We focused on the design of DNA marker protocols mediated by PCR application to distinguish Pgt and Pgs isolates due to their ease of use and simplicity, which has led to their widespread use in diagnostic tests for various fungi (Henry, 1997). For instance, simple PCR assays have been developed to rapidly detect the presence of the wheat pathogen *Fusarium poae* (Parry & Nicholson, 1996), determine the distribution and frequency of mating-type idiomorphs in *Sporothrix* species that can infect humans and animals (de Carvalho et al., 2021) and detect the presence of *Paecilomyces lilacinus*, which can act as a biological control agent of plant-parasitic nematodes (Atkins et al., 2005), among many other applications. For the wheat rust pathogens, simple molecular markers such as SSRs have been widely adopted by the community due to their ability to distinguish between

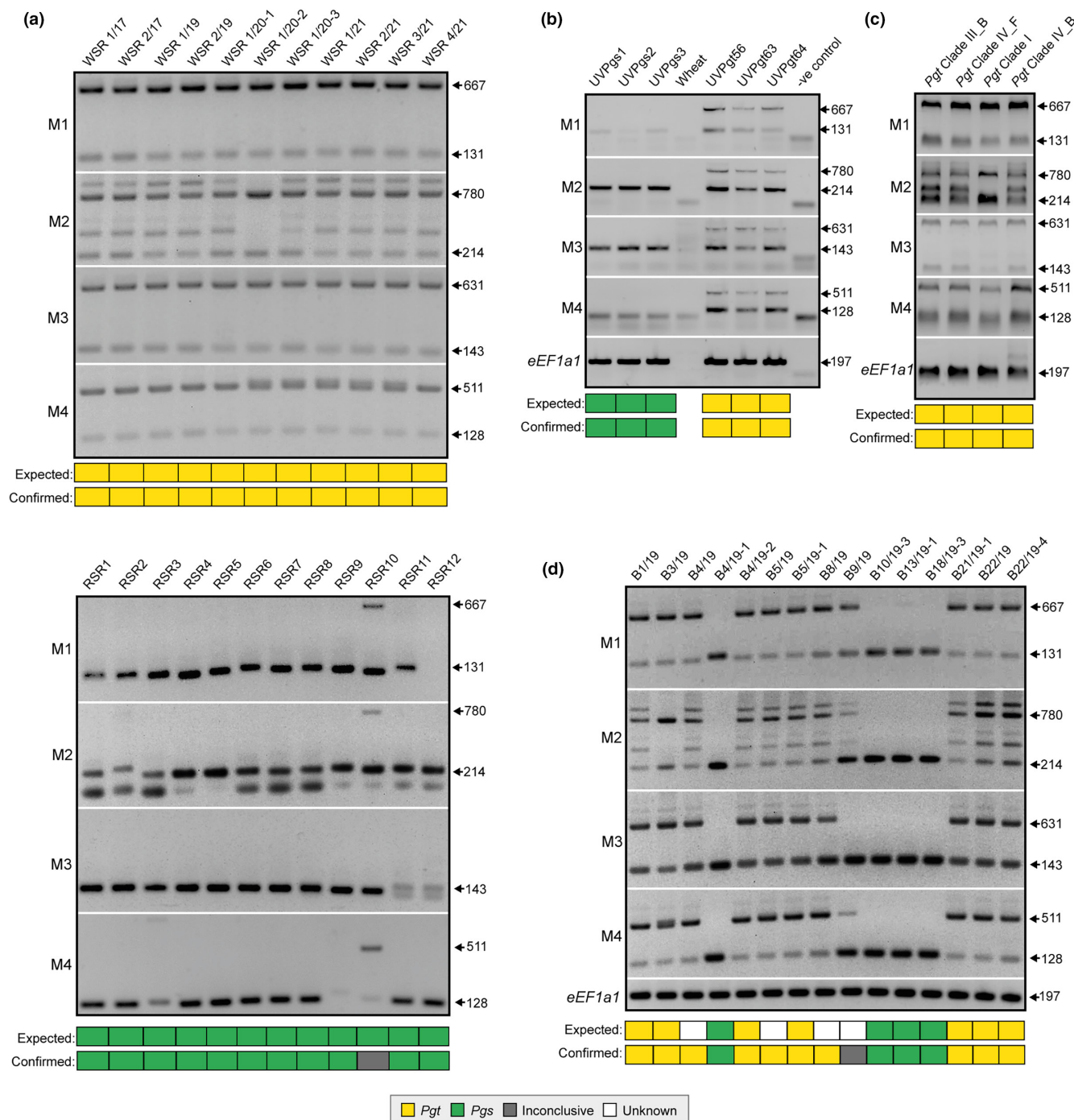


FIGURE 4 A diverse array of *Puccinia graminis* f. sp. *tritici* (Pgt) and *P. graminis* f. sp. *secalis* (Pgs) isolates could be correctly distinguished using the four selected genetic markers. A total of 33 Pgt and Pgs isolates (a–c) and 15 *P. graminis* samples from *Berberis* (d) were assigned to the same *formae speciales* ('confirmed') as determined by pathology analysis ('expected'), with only two samples giving inconclusive results. Expected band sizes in base pair are indicated by arrows. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/ppa.13890)]

physiological race groups, permitting the assessment of population structure and geographic distribution of physiologic forms. For instance, SSR markers were used to attribute the likely origin of newly emergent races of the related wheat yellow (stripe) rust fungus *Puccinia striiformis* f. sp. *tritici* in Europe to the near-Himalayan region (Hovmøller et al., 2016). More recently for Pgt, 17 SNPs were also demonstrated to be sufficient for

distinguishing 12 genetic groups that have recently emerged in Africa, Central Asia and Europe, including the Ug99 race group (Szabo et al., 2022). With PCR-based assays widely used across research groups worldwide for studying the wheat rust, this demonstrates that a simple PCR diagnostic assay for distinguishing Pgs and Pgt isolates would be highly fitting and have the potential for widespread adoption.

To ascertain the robustness of these newly defined genetic markers, we also demonstrated their utility to correctly distinguish Pgt and Pgs isolates when tested on an array of genetically diverse *P. graminis* isolates from Germany and South Africa. However, the *P. graminis* isolates used in this study are probably all highly adapted to parasitizing their respective hosts. For instance, the chosen South African Pgt isolates were previously shown to induce incompatible responses when inoculated on rye (Boshoff et al., 2019). It will be of great interest to see how the markers perform once challenged with more diverse Pgt and Pgs isolates worldwide and more broadly from those belonging to other *P. graminis* ff. spp. It is also possible that sexual recombination or somatic hybridization between Pgt and Pgs isolates could occur in nature, which could generate *P. graminis* isolates that would produce inconsistent results using the designed genetic markers. For instance, it would be of particular interest to see how the markers perform on scabrum rust, which is regarded to have arisen in Australia from somatic hybridization between Pgt and Pgs isolates (Park, 2008). In addition, where DNA is extracted from samples containing mixed Pgt and Pgs infections, these mixed infections would not be distinguishable using the current markers. Yet, once genome assemblies are available for Pgs isolates, the strategy presented could be readily applied to identify additional markers to differentiate mixed Pgt/Pgs infections. Furthermore, the high degree of host specificity displayed generally by Pgt and Pgs isolates, and thereby the importance in distinguishing them, could be eroded by further introgressions of rye chromatin into wheat, as a valuable mechanism of enhancing disease resistance (Molnár-Láng et al., 2015). Accordingly, recent *P. graminis* isolates identified on wheat and rye in Spain were shown to infect wheat, with a subset displaying virulence to Sr31, a stem rust resistance gene derived from translocation of the short arm of chromosome 1 from rye into wheat (Mago et al., 2005). However, the described genetic markers will be incredibly valuable to highlight potential isolates with unusual results that require further investigation and to track any changes in host plasticity for Pgt and Pgs isolates.

In conclusion, across the wheat rust community, the four genetic markers developed in this study will be of particular value for the rapid identification of *Berberis* hedgerows harbouring Pgt infections that need to be carefully monitored. This will be especially valuable in Western Europe where wheat stem rust is re-emerging as a disease of major concern (Saunders et al., 2019). The strategy described herein to define genetic markers for closely related taxa could also be easily applied to other pathogens of interest and will be of increasing value as genomic resources continue to rapidly expand for many key biotic threats to agricultural productivity.

ACKNOWLEDGEMENTS

We thank Clare Lewis (JIC) for assistance with DNA extractions from *B. vulgaris*, Anne Witzke (JKI) and Dr Anne-Kristin Schmitt (JKI) for preparation of Pgt, Pgs and *Berberis* samples from Germany, Janne Holm Hansen (Aarhus University) for assistance with DNA extractions and PCR assays and Mehran Patpour (Aarhus University) for

purification and multiplication of reference Pgt isolates. This project was primarily funded by the European Union's Horizon 2020 research and innovation programme under grant agreement no. 773311 (RustWatch). Additional funding was provided to D.G.O.S. by the European Research Council (no. 715638), BBSRC Institute Strategic Programmes BB/P012574/1 and BB/P016855/1, and the John Innes Foundation.

DATA AVAILABILITY STATEMENT

All data used in this study are publicly available and individual accession numbers for all sequence data used are provided in Table S1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Bryan, A., Korolev, A., Bergmann, S., Boshoff, W.H.P., Flath, K., Justesen, A.F. et al. (2024) Comparative genomics identifies genetic markers linked to structural variations that differentiate *Puccinia graminis tritici* and *secalis formae speciales*. *Plant Pathology*, 73, 1542–1552. Available from: <https://doi.org/10.1111/ppa.13890>