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Population Biology

e-Xtra*

Population Structure of *Pyrenophora teres* f. *teres* Barley Pathogens From Different Continents

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ABSTRACT

Net form net blotch disease, caused by *Pyrenophora teres* f. *teres*, results in significant yield losses to barley industries. Up-to-date knowledge of the genetic diversity and structure of pathogen populations is critical for elucidating the disease epidemiology and unraveling pathogen survival and dispersal mechanisms. Thus, this study investigated long-distance dispersal and adaptation by analyzing the genetic structure of 250 *P. teres* f. *teres* isolates collected from Australia, Canada, Hungary, and Republic of South Africa (RSA), and historical isolates from Canada, Denmark, Japan, and Sweden. The population genetic structure detected by discriminant analysis of principal components, with the use of 5,890 Diversity Arrays Technology markers, revealed the presence of four clusters. Two of these contained isolates from all regions, and all isolates from RSA were grouped in these two. Australia and Hungary showed three clusters each. One of the Australian clusters contained only Australian isolates. One of the Hungarian clusters contained only Hungarian isolates and one Danish isolate. STRUCTURE analysis indicated that some isolates from Australia and Hungary shared recent ancestry with RSA, Canada, and historical isolates and were thus admixed. Subdivisions of the neighbor joining network indicated that isolates from distinct countries were closely related, suggesting that multiple introduction events conferred genetic heterogeneity in these countries. Through a neighbor joining analysis and amplification with form-specific DNA markers, we detected two hybrid isolates, CBS 281.31 from Japan and H-919 from Hungary, collected in 1931 and 2018, respectively. These results provide a foundation for exploring improved management of disease incursions and pathogen control through strategic deployment of resistance.

Keywords: Australia, Canada, Diversity Arrays Technology, historical isolates, Hungary, hybrids, net form net blotch, Republic of South Africa

The net blotch diseases, caused by *Pyrenophora teres*, are major fungal foliar diseases of barley, causing devastating production losses throughout the world (Mathre 1997). Yield loss due to *P. teres* in susceptible barley varieties can range from 10 to 70% (Jayasena et al. 2007; Wallwork et al. 2016). Additionally, total plant death may occur in the absence of suitable fungicide treatments (Mathre 1997; Murray and Brennan 2010; Steffenson et al. 1991). Net blotch can appear as two forms, net form net blotch, caused by *P. teres* f. *teres*, and spot form net blotch, caused by *P. teres* f. *maculata*. Phylogenetically these two forms are closely related to each other (Marin-Felix et al. 2019), but in terms of population genetic analyses, the two forms represent two genetically distinct populations (Ellwood and Wallwork 2018; Liu et al. 2011; McLean et al. 2009). Although hybrids between *P. teres* f. *teres* and *P. teres* f. *maculata* have been produced successfully under laboratory conditions (Smedegård-Petersen 1971), hybrids in the field are considered to be absent or rare because of the genetic distance between these two forms (Ellwood et al. 2012; Lehmensiek et al. 2010; Poudel et al. 2017).

Net form net blotch is characterized by streaks or net-like dark brown necrotic lesions along barley leaf veins, comprising longitudinal and transverse striations (Liu et al. 2011; Smedegård-Petersen 1971). Outbreaks of *P. teres* f. *teres* have occurred across a wide range of barley growing regions and climates (Van den Berg 1988). Short-distance dispersal of *P. teres* f. *teres* by air turbulence and water splashing (Deadman and Cooke 1989) can occur through ascospores and conidia produced during sexual and asexual reproduction, respectively (Liu et al. 2011). Because *P. teres* f. *teres* is a seedborne fungus (Liu et al. 2011), long-distance transmission could result from exchange of infected seeds between geographically remote areas (Martin and Clough 1984; Shipton 1966). Furthermore, because sexual recombination is known to play a major role in the life cycle of *P. teres* f. *teres*, integration and adaptation of novel pathotypes into local areas from another geographic region is possible. Introduction of a novel pathotype may greatly shape the local *P. teres* f. *teres* genetic structure.

Knowledge of population diversity and structure is essential for understanding population dynamics and improving disease control methods. The genetic structure of a *P. teres* f. *teres* population depends on a number of factors such as mutations, genetic drift, gene flow, selection, and the relative significance of sexual and asexual stages in the life cycle of the pathogen (Akhavan et al. 2016b). With the advent of molecular genotyping technologies, *P. teres* f. *teres* populations from different geographic locations have been characterized with molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSRs). Genetic characterization studies in Australia (Bogacki et al. 2010; Ellwood et al. 2019; Lehmensiek et al. 2010; Serenius et al. 2007), Europe (Bakonyi and Justesen 2007; Ficsor et al. 2014; Jonsson et al. 2000; Rau et al. 2003; Serenius et al. 2005), North America (Akhavan et al. 2016b; Jonsson et al. 2000; Peever and Milgroom 1994), and the Republic of South Africa (RSA) (Campbell et al. 2002; Lehmensiek et al. 2010) have detected high genetic diversity within *P. teres* f. *teres* populations.

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Studies conducted on Australian *P. teres* populations with AFLP and SSR markers revealed high genetic variation within *P. teres* isolates collected from New South Wales, Queensland, South Australia, Victoria, and Western Australia (Ellwood et al. 2019; Lehmensiek et al. 2010; McLean et al. 2010). To date, two studies have characterized the genetic structure of *P. teres* populations from the RSA with AFLP and RAPD markers (Campbell et al. 2002; Lehmensiek et al. 2010), which revealed high genetic diversity in the *P. teres* f. *teres* populations. Ficsor et al. (2014) used RAPD markers to detect greater genotypic variability and genetic diversities within sampling units than between sampling units (mating type, field type, geographic region, and year) and significant temporal genetic differentiation between seasons in Hungarian *P. teres* f. *teres* populations. Although each of these studies provides valuable information on the biology and epidemiology of *P. teres* f. *teres* in the respective regions, it is not possible to compare the genetic diversity and structure of *P. teres* f. *teres* populations between these geographic areas because different studies used different marker and analysis systems. Hence, application of a single marker system is necessary to enable valid comparisons of the genetic diversity and structure in *P. teres* f. *teres* populations from different parts of the world.

Use of less efficient markers such as AFLPs and RAPDs limits the reproducibility of the results (Mondini et al. 2009). Alternative marker systems, such as Diversity Arrays Technology (DArT), have become available, which produce a large number of reproducible genome-wide markers, some of which are located in gene regions (Diversity Arrays Technology, University of Canberra 2020). DArT is a high-throughput, efficient molecular marker technology that, unlike SSR markers, does not require prior knowledge of the genome sequence (Wenzl et al. 2004). With DArT, polymorphisms are detected at restriction enzyme recognition sites, and the presence or absence of individual DNA fragments in the genome is detected through microarray hybridization (Jaccoud et al. 2001). The advanced DArT technology also identifies single nucleotide polymorphisms (SNPs) within sequences. This technology has been previously implemented for genetic population analysis of fungal species including *P. teres* (Martin et al. 2020; Poudel et al. 2019; Syme et al. 2018).

The genetic diversity of a pathogen can affect its ability to adapt to host resistances and control strategies (McDonald and Linde 2002). Therefore, pathogens that are genetically more diverse may also have a more diverse profile of virulence (Linde and Smith 2019) and a greater ability to respond to environmental changes and control measures, which may affect the resistance to fungicides or pathogenicity on the host (Peltonen et al. 1996). A recent study revealed rapid changes in the genetic structure of *P. teres* f. *teres* populations collected over 3 years from barley fields in Australia, suggesting potential adaptation and indicating the necessity of using multiple sources of host plant resistance for defense against the pathogen (Poudel et al. 2019). The continued evolution of fungal pathogen populations driven by the selection pressure applied by host resistance will probably lead to a decline in the efficiency of the deployed resistance (Suffert et al. 2018).

The worldwide occurrence of *P. teres* f. *teres* in barley and its potential for rapid genetic change through sexual recombination over a short period of time demonstrates the necessity of understanding its population diversity and structure in order to achieve efficient disease management strategies, including the development of resistant barley varieties (Liu et al. 2011; McDonald and McDermott 1993). Therefore, this study was designed to characterize the genetic diversity and structure of *P. teres* f. *teres* populations from Australia, Canada, Hungary, and the RSA and explore the potential for long-distance dispersal and geographic adaptation of the pathogen.

MATERIALS AND METHODS

Sample collection and fungal isolation. The terms *entire collection*, *population*, and *subpopulation* in this study refer to the isolates from all countries included in the study, a collection of isolates from a country, and a collection of isolates from a region or state within a country, respectively. All isolates used in this study were monoconidial isolates and collected randomly. Isolates originated mostly from barley leaves (except two isolates, H-374 and H-376 from Hungary, originated from wheat and one, CG16015 from RSA, originated from rye grass) exhibiting net form net blotch symptoms, collected from Australia, Canada, Hungary, and RSA. Five additional historic isolates were included in this study from Canada (WRS858; Serenius et al. 2007), Denmark (Pt-Pastorale; Justesen et al. 2008), Japan (CBS 282.31 and CBS 281.31; Bakonyi and Justesen 2007), and Sweden (UPSC1838; Bakonyi and Justesen 2007).

The Australian population included 118 isolates collected between 1985 and 2017 from New South Wales (NSW, $n = 20$), Queensland (QLD, $n = 43$), South Australia (SA, $n = 24$), Victoria (VIC, $n = 6$), and Western Australia (WA, $n = 25$), including the previously reported hybrid WAC17021 (McLean et al. 2014) (Supplementary Table S1; Fig. 1). Sample collection and fungal isolation of Australian samples were performed according to the method described by Martin et al. (2020). Six isolates from Canada, collected by Akhavan et al. (2016a) in 2010 and 2011 from Alberta, Manitoba, and Saskatchewan, were also included in the study.

The Hungarian population consisted of 85 isolates derived from naturally infected barley ($n = 83$) and wheat leaves ($n = 2$) collected from 2006 to 2018 (Supplementary Table S1; Fig. 1). Seventy-eight isolates were collected from experimental fields at the Centre for Agricultural Research or National Food Chain Safety Office (NFCSO) in the Martonvásár region ($n = 31$), Fleischmann Rudolf Research Institute, Eszterházy Károly University, Kompolt ($n = 17$), Institute for Agricultural Research and Educational Farm, University of Debrecen, Karcag ($n = 22$), and a commercial field or experimental plots of NFCSO and Cereal Research Non-Profit Ltd. in the Szombathely region ($n = 8$). The remaining seven isolates were collected from five commercial and two NFCSO barley fields (Bőny, $n = 1$; Kölcse, $n = 3$; Márok, $n = 2$; and Székkutas, $n = 1$).

Fungal isolation of Hungarian isolates was performed by inducing conidiogenesis. Leaf segments with necrotic lesions were placed in glass Petri plates and kept on a laboratory bench at ambient temperature or incubated under white light (OSRAM model L36W/640) for 16/8 h light/dark cycles for 1 to 3 days at 18 to 20°C. We made monoconidial isolates by transferring single conidia from the conidiophores to V8 juice agar medium (16 g of agar, 3 g of $CaCO_3$, 177 ml of Campbell's V8 juice, and 900 ml of distilled water) (Miller 1955) with a sterile needle, using a Leica MZ6 stereomicroscope at 300 to 400× magnification in a laminar air flow cabinet. Single-conidial isolates were incubated for 10 to 14 days in the dark at 18 to 20°C and used as inocula for stock and pea broth cultures. Stock cultures were grown on V8 juice agar slants for 7 to 10 days in the dark at 20 to 22°C, then kept under mineral oil at 15°C. Mycelium for DNA extraction was grown on pea broth (Erwin and Ribeiro 1996) in steady cultures for 7 to 10 days at 18 to 20°C in the dark. Liquid cultures were then harvested by filtration, washed with deionized water, freeze-dried, and ground in liquid nitrogen. Pulverized mycelia were kept at −70°C for DNA extraction.

The RSA population contained 72 isolates collected from leaves of barley ($n = 71$) and rye grass ($n = 1$) from eight regions (Bredasdorp, $n = 11$; Caledon, $n = 28$; Greyton, $n = 6$; Klipdale, $n = 8$; Napier, $n = 12$; Protem, $n = 4$; Rietpoel, $n = 2$; and Riviersonderend, $n = 1$) around the Western Cape Province of RSA in October 2016 (Supplementary Table S1; Fig. 1). Fungal isolation was performed by sterilizing the surface of leaf samples in 70% (vol/vol) ethanol for 5 s, 5 g/liter NaOCl for 2 min and washing three times in sterile water. These were placed on water agar (10 g/liter) or moist filter paper (×2) and incubated at room temperature and natural day/night light conditions for 1 to 4 days to allow the growth of conidia. Monoconidial culture production was performed by transferring single conidia to potato dextrose agar (39 g/liter; Biolab Merck, Modderfontein, RSA) and Solustrep (0.3 ml/liter) plates. Plates were incubated for 4 to 5 days, and a single colony was subcultured onto a new potato dextrose

agar plate. After 7 days, agar plugs were collected and stored in 15% glycerol at −80°C, and the remaining mycelium was harvested for DNA extraction.

DNA extraction for DArTseq. DNA from Australian isolates was extracted from single-conidium cultures via the method described by Martin et al. (2020). DNA of Hungarian isolates was extracted from lyophilized mycelium powder via the cetyl trimethyl ammonium bromide method (Richards et al. 1997), and DNA of all other isolates was extracted via a similar cetyl trimethyl ammonium bromide method (Saghai-Maroof et al. 1984).

The integrity of DNA extracted from each isolate was assessed under ultraviolet light (Fusion FX, VILBER, Marne-la-Vallée, France) after electrophoresis at 100 V for 30 min on a 0.8 g/liter agarose gel (Bioline, London, United Kingdom) containing 0.03% GelRed (Biotium Inc., Fremont, CA). DNA quantity was measured with a NanoPhotometer P300 spectrophotometer (IMPLEN, Munich, Germany). For each isolate, 20 µl of DNA solution (>50 ng/µl) was submitted to Diversity Arrays Technology Pty. Ltd. (Canberra, ACT, Australia) for DArTseq.

Data filtering and clone correction. Data obtained from DArTseq consisted of SNPs and SilicoDArTs (equivalent to microarray markers scored for the presence or absence of sequences obtained from genomic representations). Both forms of data were filtered manually with 10% used as the cutoff value for the maximum number of missing data points for markers and isolates. Markers with a minimum allele frequency of <1% were removed from the dataset

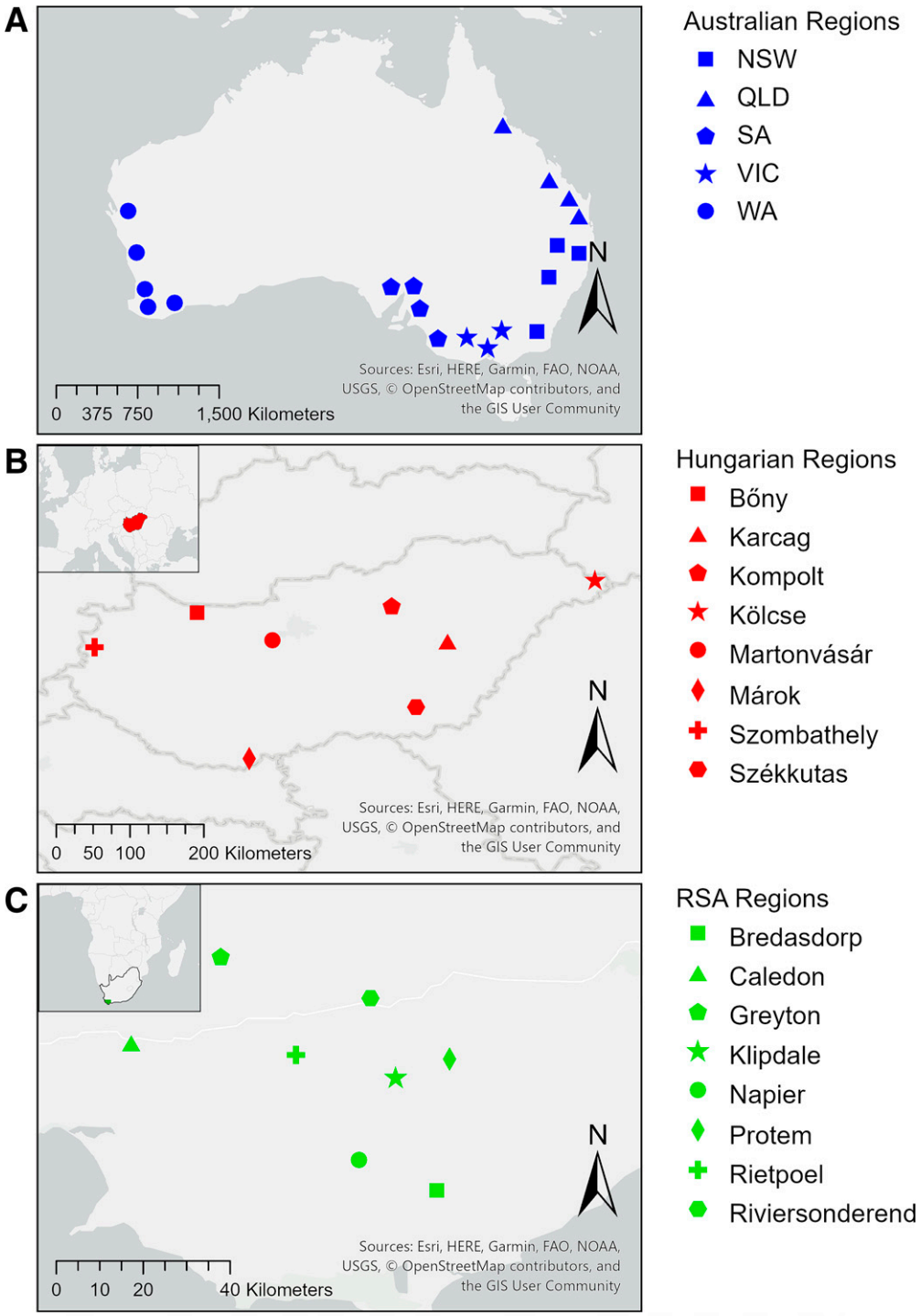

Fig. 1. Sample collection regions of *Pyrenophora teres* f. *teres* isolates in **A,** Australia, **B,** Hungary and **C,** Republic of South Africa (RSA) (ArcGISPro version 2.3, Esri, California, USA).

(Vaghefi et al. 2017). Reproducibility (the proportion of technical replicate assay pairs for which the marker score is consistent) and the CallRate (the proportion of samples for which the genotype call is either present or absent rather than missing) of each marker was evaluated, and markers with reproducibility of <1 and CallRate <85% were removed. SNPs and SilicoDArTs were combined for further analyses.

A small number of genotyping errors may occur while DArTseq marker data are generated, which may result in clonal isolates being identified as unique multilocus genotypes (MLGs). To remove potential genotyping errors, we contracted all genotypes by using the furthermost bitwise distance (Kamvar et al. 2015) among five control DNA samples from the same isolate (NB63~~i~~; extracted from an original culture by using five different samples of single-conidium derived mycelia) by the *bitwise.dist* function in *poppr* package version 2.8.3 (Kamvar et al. 2014) in R version 3.0.2 (R Team 2013). The furthermost bitwise distance among five control samples (0.000925) was set as the threshold value to contract genotypes within the entire population. All populations were clone corrected at the subpopulation stratum with the *clonecorrect* function in *poppr* to collapse clonal groups into a single MLG for all subsequent analyses except for the estimation of genetic diversity indices. Multilocus genotypes shared between subpopulations were calculated by the *cross.pop* function in *poppr*.

Dendrogram construction. All isolates were assigned to genetic clusters without a priori assumptions in DARwin version 6.0.021 (Perrier and Jacquemoud-Collet 2006). A dendrogram was produced based on the Jaccard similarity coefficient according to the unweighted neighbor joining clustering method. Bootstrap analysis with 1,000 replicates was used to test the support of the branches on the dendrogram.

Form-specific primer amplification to confirm hybrids. After the dendrogram was assessed, two isolates forming a group with the previously reported *P. teres* f. *teres*–*P. teres* f. *maculata* hybrid isolate WAC17021 were subjected to PCR amplification with six *P. teres* f. *teres* and six *P. teres* f. *maculata* specific primer pairs, according to the method of Poudel et al. (2017) with modifications. A combination of *P. teres* f. *teres* and *P. teres* f. *maculata* specific primer pairs were expected to be amplified in hybrid isolates (Poudel et al. 2017). DNA of three *P. teres* f. *teres* isolates (NB63~~i~~, NB29, and NB50) (Lehmensiek et al. 2010; Martin et al. 2020), three *P. teres* f. *maculata* isolates (HRS06033, SNB113, and HRS07033) (Lehmensiek et al. 2010; McLean et al. 2014), and three laboratory-produced hybrids (37.1, 37.4, and 37.16) (*unpublished data*) were also amplified with the primer pairs as positive controls. Each real-time PCR assay was prepared with 2 µl (about 50 ng/µl) of DNA, 5 µl of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (BIORAD, CA), 0.25 µM of each primer, and 2 µl of molecular water (MilliporeSigma, Fisher Scientific, Waltham, MA) to a final volume of 10 µl. Amplifications were conducted in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with an initial denaturation at 98°C for 3 min followed by 35 cycles of denaturation at 98°C for 15 s and annealing at 60°C for 30 s. We performed a melt curve analysis after PCR completion by ramping the temperature from 65 to 95°C, increasing by 0.5°C with each step. The presence or absence of specific loci in isolates was assessed by comparing the quantitative data generated by the melt curves and the melt temperatures of the positive controls.

Analysis of molecular variance. To identify significant variation between populations and subpopulations, we used the *amova* function in *Ade4* version 1.7.13 (Dray and Dufour 2007) in R. Analysis of molecular variance (AMOVA) was conducted on the combined Australia, Hungary, and RSA populations with the *poppr.amova* function in *poppr* with 1,000 permutations. Isolates were stratified based on the country of origin, region or state, and year of collection. Analysis was conducted to identify the amount of genetic variation within and between countries, year of collection, and region or state within countries. During AMOVA for the separate Australian, Hungarian, and RSA populations, subpopulations consisting of fewer than five isolates were removed. Analysis was performed for genetic variation within and between states or fields and year of collection for Australia and Hungary populations.

Population structure by multivariate cluster analyses. Two multivariate analyses, principal component analysis (PCA) followed by discriminant analysis of principal components (DAPC), were conducted to identify the genetic structure of the entire clone-corrected collection without a priori assumptions. For PCA, the optimum numbers of principal components and principal coordinates were found, and plots were drawn with the *pcadapt* function in *pcadapt* version 4.3.3 (Luu et al. 2017). DAPC was calculated with the *dapc* function in the R package *adegenet* version 2.1.2 (Jombart 2008) and was performed for individual populations in order to detect the population structure and number of clusters within countries. The optimum number of clusters in the population was obtained with the Bayesian information criterion function *find.clusters*, and the optimal numbers of principal component axes to retain in DAPC were estimated via the *xvalDapc* function in *adegenet.*

Population structure by model-based cluster analyses. Population structure without a priori assumption was investigated in STRUCTURE version 2.3.4 (Pritchard et al. 2000), in which the Bayesian unsupervised genetic clustering algorithm was implemented for the entire clone-corrected collection (100 Australian, 78 Hungarian, 59 RSA, six Canadian and one historical isolate each from Canada, Japan, Sweden, and Denmark). The analysis was conducted according to an admixture model with a burn-in period of 10,000 Markov chain Monte Carlo and 100,000 iterations. Ten independent runs were conducted for each potential number of genetic clusters (K), where K ranged from 1 to 10. The analysis was performed independently for Australian, Hungarian, and RSA populations with the previously mentioned criteria to identify the genetic structure within populations. Values extracted from STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012) were used to identify the optimal number of clusters for the entire clone-corrected collection as well as Australian, Hungarian, and RSA populations (Evanno et al. 2005). Each replicate for the optimal delta K (ΔK) value was entered into CLUMPAK version 1.1 (Kopelman et al. 2015) to generate the graphical representation of the optimal K. A cutoff value of 70% was considered the minimum value of an individual to be included in each population.

Population structure based on phylogenetic network. A NeighborNet phylogenetic network was built for the entire collection in SplitsTree version 4.13 (Huson 1998) to identify the subdivisions of the clone-corrected *P. teres* population. The NeighborNet network was produced based on neighbor joining algorithm described by Saitou and Nei (1987) according to the method described by Bryant and Moulton (2004). Bootstrap analysis with 1,000 replicates was used to test the support of branches on the network.

Identification of mating type and sexual recombination. Amplification of mating type primer pairs *pttMAT1-1* and *pttMAT1-2* (Lu et al. 2010) was assessed across all isolates. A χ^2 test of the ratio of *pttMAT1-1* and *pttMAT1-2* was manually calculated for *P. teres* f. *teres* clusters identified by individual DAPCs from Australia, Hungary, and RSA to determine whether there was a significant deviation from the expected 1:1 ratio under panmixia. In order to identify the mating type of the hybrids, all mating type primer pairs (*pttMAT1-1*, *pttMAT1-2*, *ptmMAT1-1*, and *ptmMAT1-2* (Lu et al. 2010) were amplified across hybrids.

Pairwise homoplasy index (PHI) test, which tests the null hypothesis of no recombination available in SplitsTree 4.13, was also implemented for the same clusters detected in individual DAPCs for Australia, Hungary, and RSA to identify the potential sexual recombination within the countries as described by Bruen et al. (2006).

Genetic diversity of populations. The nonclone corrected dataset was used to calculate the number of MLGs, expected MLGs after

rarefaction, Simpson's complement index of multilocus genotypic diversity (1 − λ), and Nei's unbiased gene diversity (genetic variation within the population, defined as the probability that two randomly sampled alleles are different) (Nei 1973; Nei and Chesser 1983) in *poppr*. The normalized Shannon–Wiener index (*H*) was calculated manually according to the method described by Spellerberg and Fedor (2003). Simpson's complement index is given based on the probability of two random isolates drawn from a subpopulation to be of a different genotype (Morris et al. 2014; Simpson 1949), and the Shannon–Wiener index measures the genotypic diversity of the population by richness (number of MLGs in the population) and relative abundance in a defined location (Shannon 2001; Spellerberg and Fedor 2003). Expected MLG, Simpson's complement index of multilocus genotypic diversity (1 − λ), Nei's unbiased gene diversity, and the normalized Shannon–Wiener index were also calculated for the clusters identified from individual DAPCs of Australia, Hungary, and RSA.

Variant annotation and associated genes. Markers with the largest contribution to the genetic variation detected in DAPC of the entire clone-corrected collection were detected with the function *loadingplot* in *adegenet* (Jombart et al. 2010). The largest contributing markers for the genetic clusters in PCA for the entire collection were also determined at the 0.0001 significance level with the *outliers.pcadapt* function in the *pcadapt* package and compared with the markers detected from DAPC. Sequences (68 bp reads produced by DArTseq) harboring markers significantly ($P < 0.0001$) responsible for the genetic variation were aligned by National Center for Biotechnology Information BLAST and NBLSTX (*EnsemblFungi*) to the reference genomes of *P. teres* f. *teres* isolates W1-1 (GenBank accession number OCTH00000000 and BioProject PRJEB18107) and 0-1 (GenBank accession number AEEY01000000 and BioProject PRJNA66337) and partial genomic regions of 13A (GenBank accession numbers JQ837863 and JQ582646). This method enabled identification of possible genes linked to markers with the largest contribution to the genetic clustering during DAPC and PCA. The putative proteins for the respective genes were predicted by the Universal Protein knowledgebase (UniProt).

RESULTS

Genetic data and marker filtering. Across 286 isolates, a total of 6,440 SNPs and 14,829 SilicoDArTs were reported, with 891 SNPs and 4,999 SilicoDArTs retained for the analysis after filtering (Supplementary Material S2). After contraction (collapsing genotypes by genetic distance in order to remove genotypes identified as unique due to genotyping errors) of the entire collection, 286 genotypes were contracted to 250 genotypes. No clonal genotypes were identified after clone correction of 250 MLGs, and no MLGs were shared across any regions or states within a country. Of these, 101 MLGs were from the Australian population collected from 1985 to 2017 (including a previously reported hybrid WAC17021), seven were Canadian isolates collected in 2010 and 2011 including one historical isolate collected in 1973, 59 were RSA isolates collected in 2016, and 79 were Hungarian isolates (16 collected from 2006 to 2009 and 63 in 2017 and 2018). Four historical isolates representing four different MLGs, two from Japan (collected in 1931) and one each from Denmark (1976) and Sweden (1986), were also included (Supplementary Table S1).

Dendrogram construction. The distance-based dendrogram obtained from DARwin showed the presence of a distinct group of three isolates (Supplementary Fig. S1). This group showed distinct genetic separation from the rest of the *P. teres* f. *teres* isolates and contained the previously reported hybrid WAC10721 from Australia along with H-919 from Hungary and CBS 281.31 from Japan, thus suggesting that these two isolates may also be hybrids.

Form-specific primer amplification to confirm hybrids. PCR amplification of six *P. teres* f. *teres* and six *P. teres* f. *maculata* specific primer pairs (Poudel et al. 2017) confirmed the hybrid identity of isolates H-919 and CBS 281.31. PCR results of the isolate H-919 with 12 primer pairs showed amplification for *PttQ1*, *PttQ3*, *PttQ5*, *PtmQ7*, *PtmQ8*, and *PtmQ12*, and CBS 281.31 showed amplification for *PttQ1*, *PttQ2*, *PttQ5*, *PtmQ7*, *PtmQ8*, and *PtmQ9*. The *P. teres* f. *teres* positive control isolates NB63~~i~~, NB29, and NB50 and the *P. teres* f. *maculata* positive controls HRS06033, SNB113, and HRS07033 showed amplification for the six *P. teres* f. *teres* specific primers pairs and the six *P. teres* f. *maculata* specific primer pairs, respectively. Isolate WAC10721 and the laboratory-produced hybrid isolates used as controls amplified a mixture of both *P. teres* f. *teres* and *P. teres* f. *maculata* specific primer pairs. The two hybrid isolates H-919 and CBS 281.31, along with the previously reported hybrid WAC10721, were removed from subsequent analyses characterizing the genetic structure and genetic diversity of *P. teres* f. *teres*.

Analysis of molecular variance. AMOVA showed significant genetic variation between countries, accounting for 19.13% ($P = 0.001$) of the total genetic variation, and variation between isolates within populations was 82.59% ($P = 0.001$) (Table 1). Within populations, variation between regions or states accounted for 17.40% ($P = 0.001$) of the total genetic variation. Considering the country and the year of collection, no significant genetic variation ($P = 0.259$) was observed between populations (0.52%). Of the total genetic variation in Australia, 7.01% ($P = 0.001$) was observed between states in Australia, whereas genetic variation between regions in Hungary (2.08%) and RSA (1.78%) was not significant ($P = 0.072$). The percentage variations for the year of collection of *P. teres* f. *teres* isolates of the total genetic variation in Australia (0.12%) and Hungary (0.99%) were not significant ($P = 0.415$ and 0.192, respectively).

Population structure based on multivariate cluster analyses. In the PCA plot, principal component 1 (PC1) separated a group of Australian isolates ($n = 45$) and another cluster of Hungarian isolates ($n = 55$) along with the historical Danish isolate Pt-Pastorale from the rest of the collection. Separation of the 45 Australian isolates from the rest of the collection was further supported by PC2 (Fig. 2).

DAPC without a priori population assignment indicated the presence of four clusters for the entire clone-corrected collection (Fig. 3). All isolates in cluster 1 ($n = 46$) were from Australia, and cluster 3 consisted of 55 Hungarian isolates and isolate Pt-Pastorale from Denmark. Cluster 4 consisted of isolates from Australia ($n = 44$), Canada ($n = 6$), Hungary ($n = 5$), and RSA ($n = 40$). Cluster 2 contained isolates from Australia ($n = 10$), Hungary ($n = 18$), RSA ($n = 19$), and one each from Canada (MB05), Japan (CSB 282.31), and Sweden (UPSC1838).

Individual DAPC results obtained for each population from Australia, Hungary, and RSA showed three clusters each (Supplementary Fig. S2A, S2B, and S2C). These clusters contained isolates from different regions or states within the respective countries, except for cluster 3 in the Australian population, which contained isolates only from QLD, SA, and WA (Supplementary Fig. S2A). Cluster 1 and cluster 3 obtained from the individual DAPC plot of Australian isolates consisted of isolates present in cluster 1 from the entire clone-corrected DAPC plot. Cluster 2 contained isolates present in cluster 2 and cluster 4 from the entire clone-corrected DAPC plot. Cluster 1 and cluster 3 isolates from the individual Hungarian DAPC plot contained isolates present in cluster 3 from the entire clone-corrected DAPC plot, and cluster 2 contained isolates present in cluster 2, cluster 3, and cluster 4 from the entire clone-corrected DAPC plot. Cluster 1 and cluster 2 from the individual RSA DAPC plot contained isolates present in cluster 4 and cluster 2 from the entire clone-corrected DAPC plot, respectively, and cluster 3 contained isolates present in clusters 4 and 2 from the entire clone-corrected DAPC plot.

Population structure based on model-based cluster analyses. STRUCTURE analysis of 247 isolates determined that three clusters best described the data (Supplementary Fig. S3A). In the three-cluster STRUCTURE model, genotypes from Australia tended to have intermediate membership in multiple clusters, whereas genotypes from

RSA and Hungary tended to have high membership proportions in a single cluster. With a 70% cutoff on membership proportions to assign a genotype into a cluster, a first cluster (cluster I) consisted of 46 isolates from Australia, a second cluster (cluster II) consisted of 55 isolates from Hungary and one isolate (Pt-Pastorale) from Denmark, and a third cluster (cluster III) consisted of 145 isolates from Australia (n = 54), Canada (n = 6), Hungary (n = 23), RSA (n = 59), and historical isolates (n = 3) (Supplementary Table S1; Fig. 4). Many genotypes from Australia (cluster III) had shared ancestry with genotypes from RSA and are thus admixed in the three-cluster model. The six Canadian isolates along with the historical Canadian isolate were also found to be admixed (cluster III). At K = 3, historical isolates from Japan and Sweden had high membership in the cluster present in RSA, Hungary, and Australia, and the historical isolate from Denmark had high membership in the cluster specific to Hungary.

Genetic structure was also analyzed independently for each population to identify further subdivision within countries. The mode of ΔK was observed at K = 2 for the Australian, Hungarian, and RSA populations (Supplementary Fig. S3B, S3C, and S3D). The individual STRUCTURE analysis for Australian isolates showed that 50 and 43% of the isolates clustered into either cluster I or cluster II, with

TABLE 1. Analysis of molecular variance of *Pyrenophora teres* f. *teres* isolates from Australia, Hungary, and the Republic of South Africa (RSA)

Source of variance	Degrees of freedom	Variation (%)	Sum of squares	Mean square
Australia, Hungary, and RSA				
Between countries	2	19.13[a]	6,788.27	3,394.13
Year between countries	4	0.52[b]	846.12	211.53
Between regions or states within countries	13	17.40[a]	10,088.81	776.06
Between isolates within populations	211	82.59[a]	38,143.65	180.78
Australia				
Between states	4	7.01[a]	1,794.25	448.56
Within states	95	92.99[a]	17,670.53	186.01
Year within Australia	3	0.12[b]	601.16	200.39
Hungary				
Between fields	3	2.08[b]	775.19	258.40
Within fields	67	97.92[a]	12,822.33	191.38
Year within Hungary	1	0.99[b]	244.96	244.96
RSA				
Between fields	4	1.78[b]	731.09	182.77
Within fields	49	97.90[a]	9,576.68	195.44

[a] Significant at $P \leq 0.001$.
[b] Not significant.

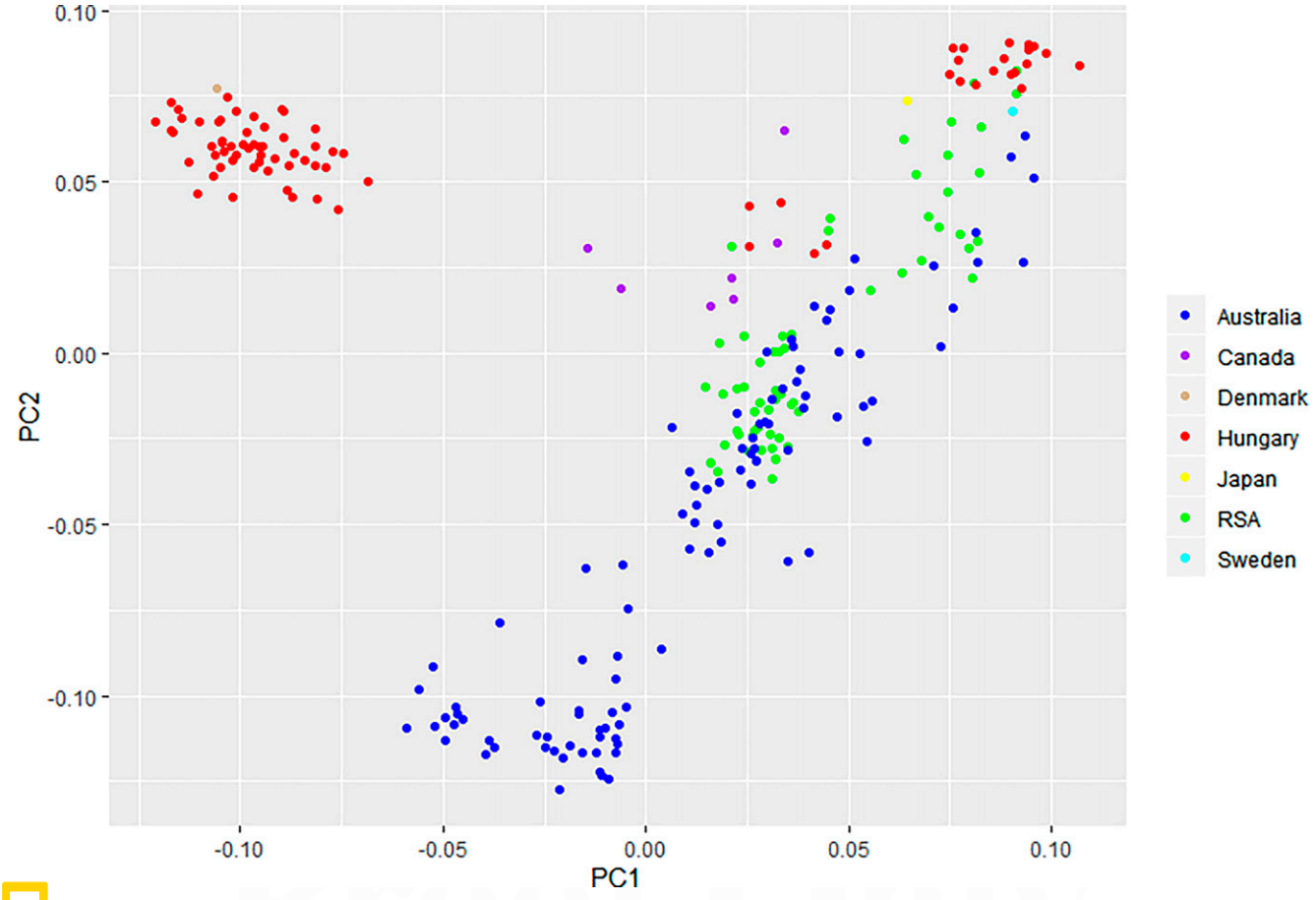

Fig. Principal components analysis of *Pyrenophora teres* f. *teres* isolates collected from Australia, Canada, Denmark, Hungary, Japan, Republic of South Africa (RSA) and Sweden. Principal component axis 1 (PC1) and principal component axis 2 (PC2) explained 13.6% and 9.3% variation, respectively, for the genetic clusters.

membership proportions of >70% for the respective clusters, and 7% of isolates were considered admixed due to membership proportions of <70% for both clusters (Supplementary Fig. S4A). The Hungarian isolates showed two clusters, cluster I and cluster II, containing 71 and 29% of the isolates, respectively, with no admixed individuals. The two clusters, cluster I and cluster II, from the STRUCTURE analysis of RSA isolates contained 68 and 17% of the isolates with membership proportions of >70% for the respective clusters and 9% admixed isolates that were not assigned to either of the clusters. The clusters obtained for the Australian, Hungarian, and RSA populations were compared with the year and field or state of collection, and no association was found.

Cluster I and cluster II obtained from the individual Australian STRUCTURE analysis consisted of isolates present in cluster III and I from the entire clone-corrected STRUCTURE analysis, respectively. Cluster I and cluster II from the Hungarian STRUCTURE analysis contained isolates present in cluster II and cluster III from the entire clone-corrected STRUCTURE analysis, respectively. Cluster I, cluster II, and admixed isolates from the individual RSA STRUCTURE analysis contained isolates present in cluster III from the entire clone-corrected STRUCTURE analysis.

The DAPC and STRUCTURE analyses of the entire clone-corrected collection resulted in identification of four and three clusters, respectively. Cluster 1 and cluster 3 from the DAPC corresponded to cluster I and cluster II from the STRUCTURE analysis, respectively. Isolates present in cluster 2 and cluster 4 from the DAPC corresponded to the isolates in cluster III from the STRUCTURE analysis. Therefore, DAPC characterized the population subdivision in the dataset with higher resolution than STRUCTURE analyses (Jombart et al. 2010); thus, clusters detected by DAPC were further used to calculate the sexual recombination and genetic diversity.

Population structure based on phylogenetic network. The NeighborNet phylogenetic network inferred from Splitstree showed extensive reticulation connecting all isolates (Fig. 5), consistent with a history of recombination. The structure of the network indicated that genotypes from different countries could be closely related (Fig. 5). Historical Danish isolate Pt-Pastorale, Japanese isolate CBS282.31, and Swedish isolate UPSC1838 grouped with Hungarian genotypes.

Identification of mating type and sexual recombination. Amplification of *P. teres* f. *teres* isolates with mating type primers indicated that 47 Australian isolates had the *MAT1-1* idiomorph (mating type 1), and the remaining 53 carried the *MAT1-2* idiomorph (mating type 2) (Supplementary Table S3). For Hungary, 37 isolates were found to be *MAT1-1*, and 41 isolates were *MAT1-2*. Of 59 RSA isolates, 39 were *MAT1-1* and 21 were *MAT1-2*. Mating type ratios calculated for populations from Australia, Hungary, and RSA based on clusters identified with country-specific DAPCs (Supplementary Fig. S2) showed that except cluster 2 from RSA (P = 0.021), the χ^2 values for the clusters from Australia, Hungary, and RSA did not significantly differ from the expected ratio of 1:1 under panmixia. PHI rejected the null hypothesis of clonality in cluster 2 (P = 0.014) in Australia and cluster 1 (P = 4.8E-4) and cluster 3 (P = 0.007) in

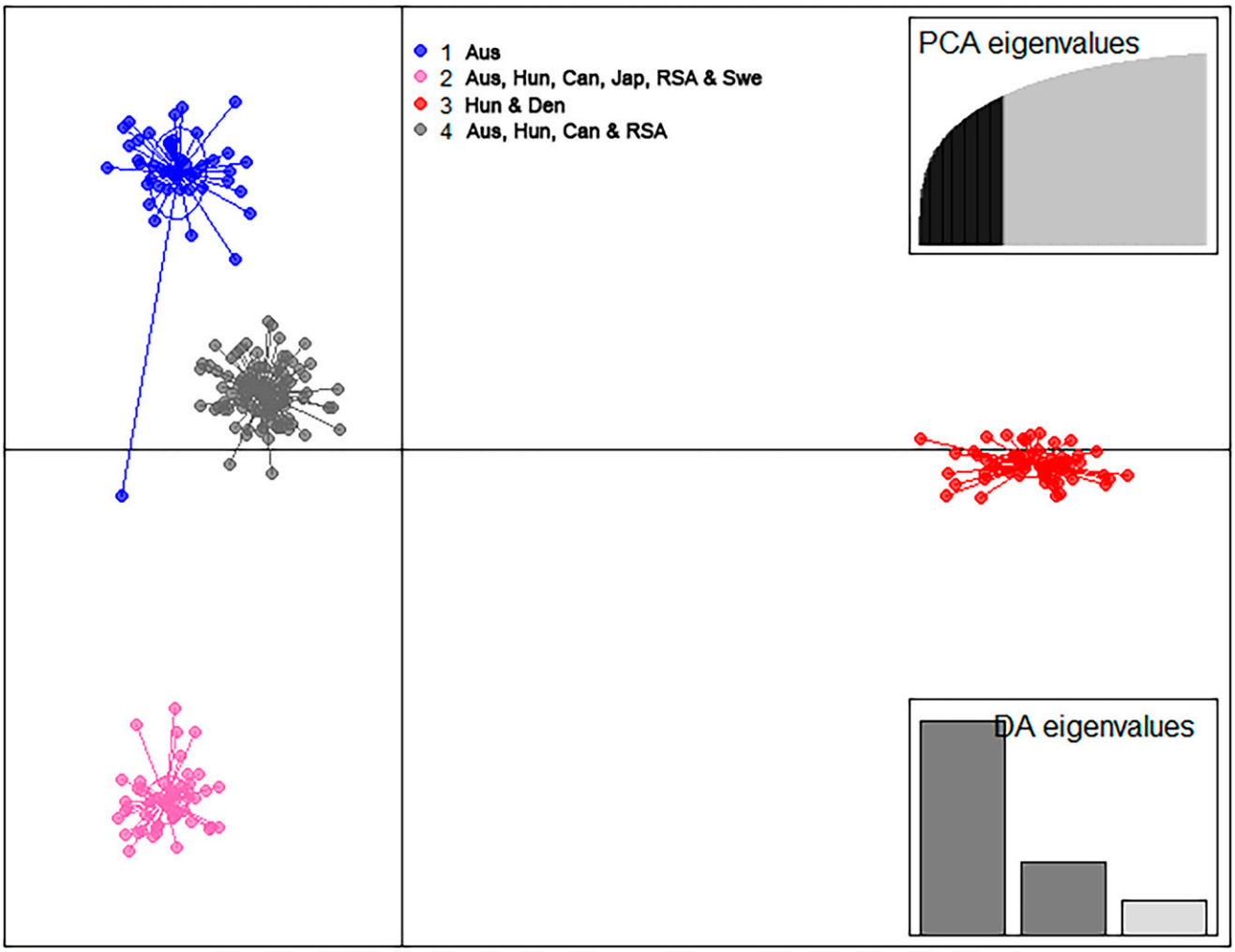

Fig. 3. Discriminant analysis of principal components of the entire collection of *Pyrenophora teres* f. *teres* from Australia (Aus), Republic of South Africa (RSA), Hungary (Hun), Canada (Can), Japan (Jap), Sweden (Swe) and Denmark (Den). The distribution of the eigenvalues of principal component analysis (PCA) and discriminant analysis (DA) indicate that the first two principal components explain 25% of the genetic structure of the clusters.

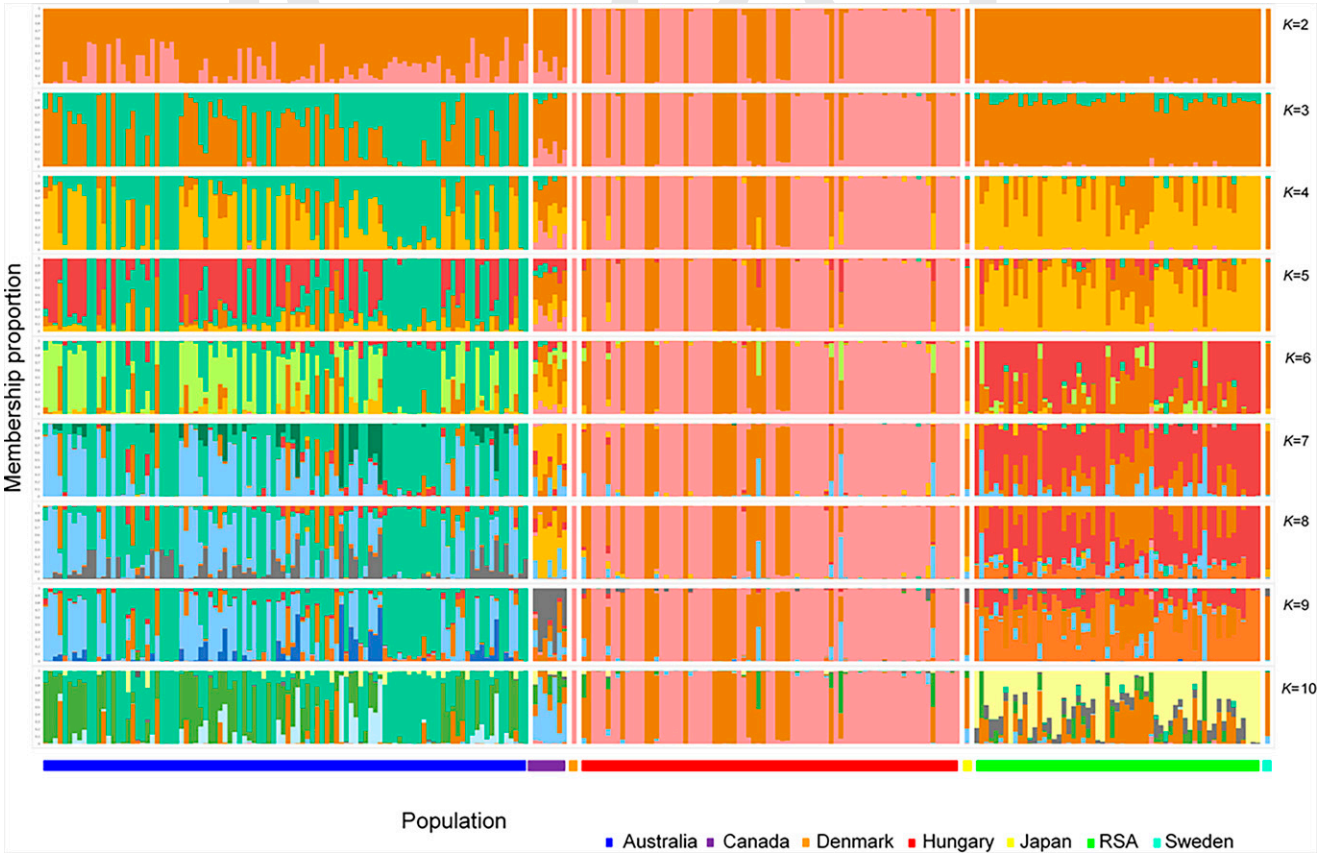

Fig. 4. Estimates of genetic structuring in the entire clone-corrected *Pyrenophora teres* f. *teres* collection grouped into clusters (K = 2-10) using the model-based clustering method in STRUCTURE. Population color bars represent isolates from Australia (n = 100), Canada (n = 7; including the historical Canadian isolate), Denmark (n = 1), Hungary (n = 78), Japan (n = 1), Republic of South Africa (n = 59) and Sweden (n = 1) respectively. Bars represent individual isolates and the color and height of each bar depicts the estimated membership fraction of each individual into the corresponding cluster.

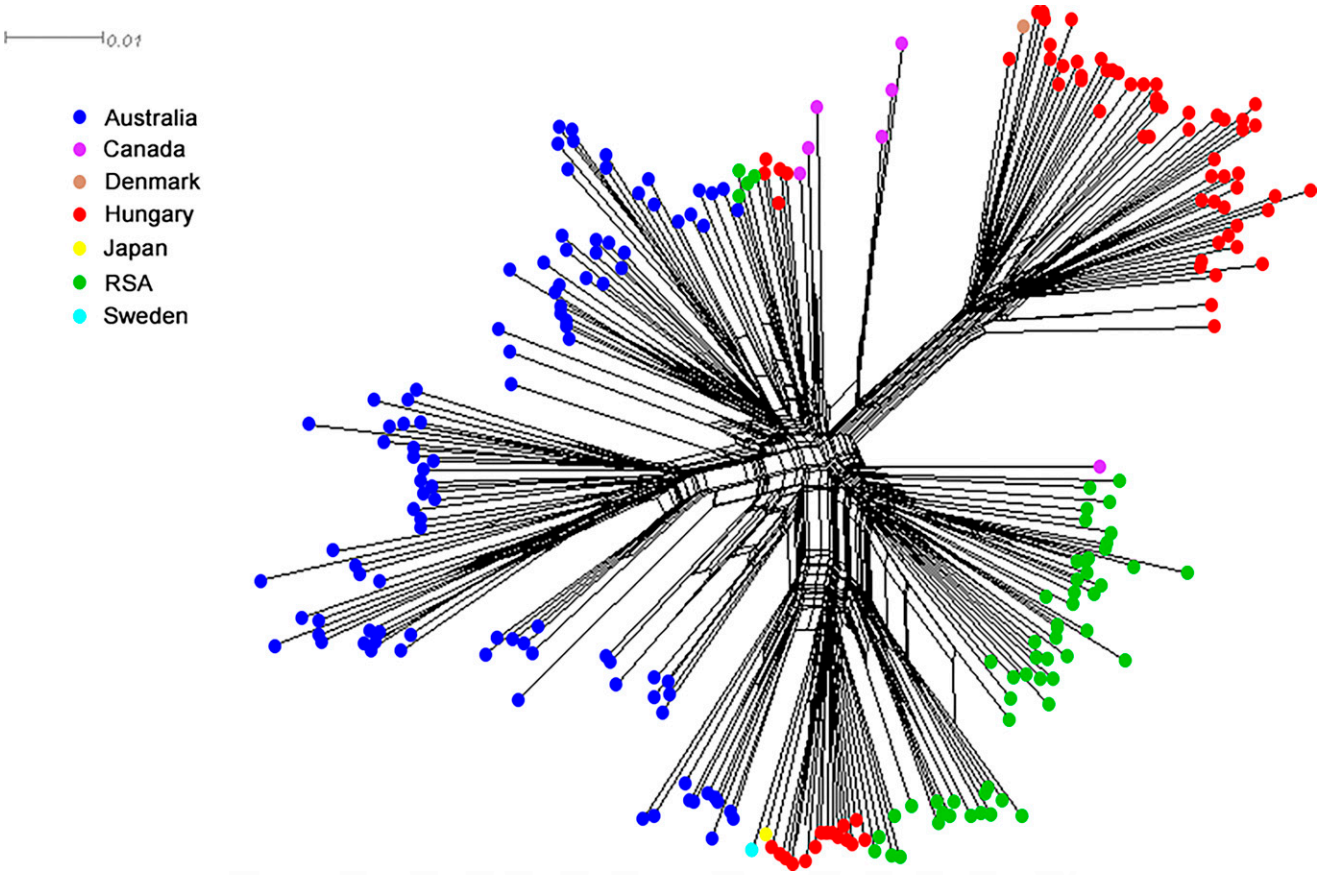

Fig. 5. NeighborNet phylogenetic network based on DArTseq data for *Pyrenophora teres* f. *teres* isolates from Australia (n = 100), Canada (n = 7; including the historical Canadian isolate), Denmark (n = 1), Hungary (n = 78), Japan (n = 1), Republic of South Africa (n = 59), and Sweden (n = 1).

Hungary, whereas other clusters from Australia (cluster 1 and cluster 3), Hungary (cluster 2), and RSA (cluster 1, cluster 2, and cluster 3) did not show evidence of recombination (Supplementary Table S3).

Genetic diversity. The number of expected MLGs calculated for Australia, Hungary, and RSA was 10. The highest genetic diversity indices among three countries for the nonclone corrected dataset were observed for the population from Hungary, with a normalized Shannon–Wiener index and Nei's unbiased gene diversity index of 0.992 and 0.184, respectively (Table 2). The lowest normalized Shannon–Wiener index, 0.973, and Nei's unbiased gene diversity index, 0.143, were calculated for the population from RSA. The highest value for Simpson's complement index of multilocus genotypic diversity was 0.991, exhibited by the Australian population, and the lowest value, 0.986, was reported for the population from RSA. However, the overall genetic diversity within the populations was high.

The highest genetic diversity indices for the clusters detected by DAPC were observed for Hungarian isolates with a normalized Shannon–Wiener index and Nei's unbiased gene diversity index of 0.935 and 0.279, respectively (Supplementary Table S4). The lowest total normalized Shannon–Wiener index, 0.859, and Nei's unbiased gene diversity index, 0.224, were observed for the clusters from RSA. The highest Simpson's complement index of multilocus genotypic diversity was 0.990, exhibited by the Australian population, and the lowest value, 0.983, was reported for the population from RSA.

Variant annotation and associated genes. Of 5,890 markers used for the DAPC and PCA of the entire clone-corrected collection, 66 were found to be significantly associated with the genetic differences of clusters and subdivisions ($P < 0.0001$) detected by DAPC and PCA, respectively. Of 66 markers, 34 were aligned with reference genomes with the E-values (expected value) ranging from $8.4E^{-33}$ to 1.7. Of these 34 markers, four markers aligned with known genes, another four were not situated near genes, five aligned with genes of uncharacterized proteins, and 21 aligned with genes for hypothetical proteins in the reference *P. teres* f. *teres* genomes (Supplementary Table S2). The four markers aligned with genes were associated with ND89-9 nonribosomal peptide synthetase 2 (GenBank accession number JQ582646), glyceraldehyde-3-phosphate dehydrogenase-like protein (*GPD1*) gene (GenBank accession number JQ837863), endo-1,4-beta-xylanase A mRNA (GenBank accession number JX900135) and cytochrome P450 lanosterol 14 alpha-demethylase (*CYP51A*) gene (GenBank accession number KX778121). The identified hypothetical genes represented seven different hypothetical proteins: ANK_REP_REGION domain-containing protein, DDE-1 domain-containing protein, SET domain-containing protein, DUF1996 domain-containing protein, peptidase A1 domain-containing protein, AAA domain-containing protein, and MFS domain-containing protein in *P. teres* f. *teres*.

DISCUSSION

The present study investigates the most geographically diverse collection of *P. teres* f. *teres* isolates analyzed in a single study to date. It provides a comprehensive investigation of the genetic structure of *P. teres* f. *teres* populations from different geographic areas through the implementation of the genome-wide marker system DArTseq and inclusion of a higher number of isolates compared with previous studies. In this study, 247 *P. teres* f. *teres* MLGs, predominantly from

TABLE 2. Indices of genetic diversity for *Pyrenophora teres* f. *teres* populations from Australia, Hungary, and Republic of South Africa (RSA)

Population	n[a]	MLG[b]	eMLG[c]	H[d]	$1 - \lambda$[e]	H_{exp}[f]	CF[g]
Australia							
New South Wales	20	17	10	0.974	0.941	0.164	0.150
Queensland	43	37	10	0.981	0.973	0.172	0.140
South Australia	24	23	10	0.995	0.957	0.184	0.042
Victoria	6	6	6	1.000	0.833	0.167	0
Western Australia	24	17	10	0.633	0.941	0.177	0.292
Australia total	117	100	10	0.986	0.991	0.183	0.145
Hungary							
Bony	1	1	1	NaN[h]	NaN[h]	NaN[h]	NaN[h]
Karcag	22	19	10	0.610	0.947	0.162	0.136
Kölcse	3	3	3	NaN[h]	NaN[h]	NaN[h]	NaN[h]
Kompolt	16	14	10	0.511	0.929	0.188	0.125
Márok	2	2	2	NaN[h]	NaN[h]	NaN[h]	NaN[h]
Martonvásár	31	30	10	0.996	0.967	0.186	0.032
Székkutas	1	1	1	NaN[h]	NaN[h]	NaN[h]	NaN[h]
Szombathely	8	8	8	1.000	0.875	0.190	0
Hungary total	84	78	10	0.992	0.988	0.184	0.071
RSA							
Bredasdorp	11	6	6	0.960	0.833	0.120	0.455
Caledon	28	26	10	0.992	0.962	0.149	0.071
Greyton	6	5	5	0.970	0.800	0.145	0.167
Klipdale	8	8	8	1.000	0.875	0.157	0
Napier	12	9	9	0.973	0.889	0.150	0.250
Protem	4	3	3	NaN[h]	NaN[h]	NaN[h]	NaN[h]
Rietpoel	2	2	2	NaN[h]	NaN[h]	NaN[h]	NaN[h]
Riviersonderend	1	1	1	NaN[h]	NaN[h]	NaN[h]	NaN[h]
RSA total	72	59	10	0.973	0.986	0.143	0.181
Total	273	237	10	0.987	0.996	0.202	0.132

[a] Number of isolates.
[b] Number of multilocus genotypes (MLGs).
[c] The number of expected MLGs based on rarefaction at the smallest sample size of ≥10.
[d] *H*, Normalized Shannon–Wiener index of MLG genotypic diversity, the genotypic diversity of the population by richness and relative abundance in a defined location.
[e] Simpson's complement index of multilocus genotypic diversity, the probability of two random isolates drawn from a subpopulation to be of a different genotype.
[f] H_{exp}, Nei's unbiased gene diversity, the probability that two randomly chosen alleles are different.
[g] CF, clonal fraction, calculated as 1 − MLG/*n*, where MLG equals the number of MLGs and *n* equals the number of isolates of the population or subpopulation.
[h] NaN, not calculated due to <5 isolates.

Australia, Hungary, and RSA, were assessed to describe the genetic structure of *P. teres* f. *teres* isolates in distinct geographic areas.

The genetic structure of the entire clone-corrected collection detected by the DAPC revealed the presence of four clusters. Two clusters contained some isolates from Australia and Hungary, all isolates from Canada and RSA, and all historical isolates except Pt-Pastorale from Denmark. The other two clusters were specific to Australian isolates and Hungarian isolates along with the historical Danish isolate. STRUCTURE analysis also revealed the presence of two distinct clusters for Australia ($n = 46$) and Hungary ($n = 55$), reflecting their genetic isolation from each other based on geographic origin. Furthermore, NeighborNet phylogenetic network showed a distinct Hungarian cluster. In the NeighborNet phylogenetic network, the *P. teres* f. *teres* isolates from Australia, Canada, Hungary, and RSA formed more than one subdivision per country. The isolates from these subdivisions did not relate to their year of collection or the region or state of origin. Therefore, the underlying factor for the genetic isolation of *P. teres* f. *teres* populations from the same geographic area might include other variables such as varietal differences (Fowler et al. 2017), fungicide regimes, geographic isolation, or environmental factors.

A number of different analyses used in this study identified the admixed nature of multiple isolates mainly from Australia. STRUCTURE-based cluster analysis revealed that there were population subdivisions in Hungary and Australia and that one of the clusters present in each of these countries shared recent ancestry with the cluster containing the Canadian, RSA, and most of the historical isolates. Cluster analyses also revealed more admixture in Australia than in Hungary. DAPC and a highly reticulated NeighborNet phylogenetic network also gave evidence that these isolates are of mixed origin. In the NeighborNet phylogenetic network, some of the isolates from the same countries were closely related to isolates from other countries. Even though some isolates from subdivisions of Australia and RSA showed mixed origin or multiple origins, others showed ancestry in a single group, suggesting that these isolates could have evolved from a common ancestor or an introduction of isolates from a common population and then adapted to the respective environments through sexual reproduction. The admixed origin of isolates could have resulted from gene flow between countries. Gene flow is one of the main evolutionary forces affecting in the genetic structure of a pathogen (Rogers and Rogers 1999). Because *P. teres* f. *teres* is a seedborne pathogen (Liu et al. 2011), gene flow or introduction of isolates from one geographic area to another is possible through seed exchange and then adaptation to local environments. This may have occurred in the case of Australian *P. teres* f. *teres* isolates, which have been suggested by Fowler et al. (2017) to have evolved and adapted to regional barley cultivars in Australia.

Individual STRUCTURE analyses of Australian, Hungarian, and RSA isolates indicated that some of the isolates from Australia and RSA were admixed, whereas isolates from Hungary showed no admixture. The potential admixture found in Australian and RSA isolates could have resulted from the dispersion of the pathogen through sexual reproduction and lack of varietal specialization within the country. The absence of admixed ~~in~~ Hungarian isolates might have been caused by physical and reproductive barriers in the dispersion of the pathogen, host specialization, or recent introduction of isolates.

The genetic structure of *P. teres* f. *teres* populations detected in model-based cluster analyses did not correspond to the region or state or the year of collection of the isolates; therefore, factors contributing to the genetic structure of *P. teres* f. *teres* populations were investigated by identifying the markers underlying the genetic structure detected in DAPC and PCA. One of these markers was located in the gene responsible for the nonribosomal peptide synthetase protein. Nonribosomal peptide synthetases are responsible for the production of nonribosomal peptides, which are bioactive secondary metabolites known to be involved in cellular development, pathogenicity, and stress responses in plant fungal pathogens (Keller et al. 2005; Sayari et al. 2019). The potential role of this locus in differential aggressiveness of *P. teres* f. *teres* isolates warrants further investigation. Other markers that were significantly associated with genetic structuring of the *P. teres* f. *teres* populations included a glyceraldehyde-3-phosphate dehydrogenase-like protein (*GPD1*) gene, an endo-1,4-beta-xylanase A mRNA gene, and a cytochrome P450 lanosterol 14 alpha-demethylase (*CYP51A*) gene. The *GPD1* gene has been frequently used as a genetic marker in phylogenetic studies to differentiate fungal pathogens including *P. teres* (Andrie et al. 2008; Lu et al. 2013; Zhang and Berbee 2001). *GPD1* plays a major role in fungal metabolic pathways such as energy synthesis and biomass synthesis (Larsson et al. 1998). It has been suggested that mutations in the ~~glyceraldeyde-3-phosphate dehydrogenase~~ gene contribute to the nutrient uptake of phytopathogenic *Colletotrichum* spp. during their biotrophic phase in the infection process on many perennial plants including olive, citrus, and tomato (Materatski et al. 2019; Wei et al. 2004). The enzyme endo-1,4-beta-xylanase plays a vital role in the breakdown of xylan, a major component of plant cell walls (Nguyen et al. 2011), and the degradation of the plant cell wall has been correlated with virulence and pathogenicity of phytopathogenic *Fusarium* spp. and *Valsa* spp. on tomato and apple (Gómez-Gómez et al. 2001; Wang et al. 2014). Cytochrome P450 lanosterol 14 alpha-demethylase is important for the biosynthesis of ergosterol, a primary fungal cell membrane sterol that is responsible for maintaining membrane fluidity and stability (Koch et al. 2013; Luo and Schnabel 2008; Parks and Casey 1995; Rodriguez et al. 1985). Mutations of this gene have been associated with the demethylase inhibitor or group 3 fungicide resistance in *P. teres* (Ellwood et al. 2019; Mair et al. 2019). Considering the importance of these genes for fungal virulence or pathogenicity, it is plausible that mutations at these loci are caused by external effects such as environmental factors and fungicide regimes. These factors may have driven local or host adaptation of *P. teres* f. *teres* isolates in different regions, resulting in the distinct genetic substructuring detected in this study.

Sexual recombination plays a major role in the evolution and adaptation of a pathogen, which may influence the genetic structure (Lee et al. 2010). *P. teres* f. *teres* is a well-known sexually reproducing fungus (Liu et al. 2011). A mating type ratio of 1:1 is expected in the absence of segregation distortion and clonal selection among mating types, and the mating type ratio is equalized through sexual recombination in *P. teres* (Milgroom 1996; Rau et al. 2005). In the current study, except for cluster 2 from RSA, other clusters collected from Australia, Hungary, and RSA did not deviate from the expected 1:1 ratio. Studies of Finish, Australian, and Canadian *P. teres* f. *teres* populations reported that the mating type ratio did not deviate from the expected 1:1 ratio (Akhavan et al. 2016b; Linde and Smith 2019; Rau et al. 2005; Serenius et al. 2005), whereas studies of *P. teres* f. *teres* populations from Czech Republic and Slovakia, and Krasnodar, Russia deviated from a 1:1 ratio (Leišová-Svobodová et al. 2014; Serenius et al. 2007). Deviation of mating type ratio in cluster 2 from RSA and absence of sexual recombination evidence for clusters 1 and 3 from Australia, cluster 2 from Hungary, and all clusters from RSA based on PHI test results might have occurred through unsystematic sampling or introduction of primary inoculum such as contaminated seeds or conidia to the fields. In the current study, *P. teres* f. *teres* isolates from Australia and Hungary have been collected from different years. Therefore, further studies with a higher number of isolates and intensive sampling methods are necessary to confirm the evidence for sexual reproduction of Australian and Hungarian *P. teres* f. *teres* populations.

Previous studies have suggested that hybridization between the two types of *P. teres* is rare or absent under field conditions because of the apparent genetic isolation of both forms (Ellwood et al. 2012; Lehmensiek et al. 2010). Before this study, only four naturally occurring putative hybrids had been detected from barley fields: one putative hybrid from the southwestern Cape of RSA (Campbell et al. 2002), two from Tovacov, Czech Republic (PTM-15 and PTM-16) (Leišová et al. 2005), and one from a barley field in Western Australia (WAC10721) (McLean et al. 2014). In the current study, additional isolates from Hungary (H-919) and Japan (CBS 281.31) were identified as putative hybrids based on distinct genetic subdivision

compared with the *P. teres* f. *teres* population and genetic similarity to the previously identified hybrid WAC10721 in the NeighborNet phylogenetic network. Amplification with *P. teres* f. *teres* and *P. teres* f. *maculata* specific DNA markers confirmed that these two isolates were hybrids. The isolate CBS 281.31 was originally identified as *Pyrenophora japonica* by Ito (Crous et al. 1995). Crous et al. (1995) found a high degree of homology in restriction digestion (*Hae*III and *Msp*I) DNA banding patterns and similar symptom expression on differential cultivars when comparing CBS 281.31 with *P. teres* f. *maculata* isolates. In addition, similar morphological characterizations between these isolates led Crous et al. (1995) to conclude that *P. japonica* was a synonym of *P. teres*. A recent study by Marin-Felix et al. (2019) also referred to isolate CBS 281.31 as *P. japonica* and found that the isolate grouped together with *P. teres* based on phylogenetic similarities. Marin-Felix et al. (2019) agreed with the conclusion of Crous et al. (1995) that *P. japonica* was a synonym of *P. teres* based on CBS 281.31 as the sole representative of *P. japonica*. A previous distance-based cluster analysis study, using seven RAPD markers and complemented with the two *P. teres* from specific PCR markers developed by Williams et al. (2001), identified CBS 281.31 as a *P. teres* f. *teres* isolate (Bakonyi and Justesen 2007). The types and small number of markers used might be the reason for not detecting this isolate as a hybrid in previous studies. The Japanese isolate, CBS 281.31, collected in 1931 was found to be a hybrid nearly a century after it was collected. In the 89 years since it was collected, this hybrid could have crossed with many other Japanese *P. teres* isolates, potentially influencing the genetic structure of the population. Sexual recombination or hybridization between and within the forms of *P. teres* can potentially lead to the generation of novel pathotypes. This may increase the genetic diversity of the population and make disease management more challenging through changes in traits such as fungicide resistance of the pathogen (Syme et al. 2018). Therefore, further population genetics studies and pathotyping of *P. teres* f. *teres* populations are warranted.

In conclusion, the genetic structure and the genetic relationships of *P. teres* f. *teres* isolates collected from different continents reported in this study indicated that some isolates from Australia, Canada, Hungary, and RSA shared ancestry with other countries, and some of the isolates from Australia and Hungary showed no admixture. Admixed origins between populations provide crucial evidence for the spread of the pathogen. Identification of naturally occurring hybrids supports the fact that the hybridization between two forms of *P. teres* is possible, which may lead to novel and more complex pathotypes and may cause unpredicted yield losses to the barley industry. Therefore, up-to-date knowledge about genetic structure and the genetic diversity of geographically diverse *P. teres* populations is important to predict and implement efficient disease management strategies and to develop resistant barley cultivars.

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Q: 5_39 + 21 = 60, not 59; please revise as needed.