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Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet

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Cercospora beticola is the main causal agent of cercospora leaf spot on sugar beet and has a large negative impact on the yield and quality of sugar beet production worldwide. Previous studies have shown that both mating type idiomorphs of *C. beticola* are present in natural populations, suggesting that *C. beticola* is heterothallic and may be reproducing sexually. *Cercospora beticola* isolates are diverse in the morphology of their conidia, onset of disease symptoms and fungicide resistance. To find the source of this diversity and to determine if sexual reproduction occurs in this fungus, *C. beticola* populations were collected from Western Europe, Iran and New Zealand. The mating types of these isolates were determined and AFLP analyses were used to study the genetic diversity in these populations. The mating type ratios did not deviate significantly from a 1:1 ratio in most of the populations and AFLP analyses showed high levels of genetic variation within and between the populations, with 86·4% of the isolates having unique genotypes. All populations were in significant linkage disequilibrium but levels of disequilibrium were low, and loci from only one primer pair were in significant gametic equilibrium in populations from the Netherlands and Italy. From these results there is the possibility that *C. beticola* reproduces sexually. High levels of gene flow among the samples from Europe demonstrated a single panmictic European population. This study confirms *C. beticola* to be a genetically highly diverse species, supporting the assumption that some populations are reproducing sexually.

Keywords: AFLP, *Beta vulgaris*, gene flow, genetic diversity, mating type idiomorphs, population structure

Introduction

More than 3000 species have been named in the genus *Cercospora* (Pollack, 1987), which is currently regarded as one of the largest genera of hyphomycetes. Following the recent revision by Crous & Braun (2003), this number was significantly reduced to 659 species, with a further 281 species that are treated as morphologically indistinguishable from *C. apii sensu lato*. *Cercospora beticola* belongs to the *C. apii* complex (Crous & Braun, 2003) and is the main causal agent of cercospora leaf spot of sugar beet (Saccardo, 1876; Groenewald *et al*., 2005, 2006a). Some confusion existed in the past about whether *C. beticola* and *C. apii*, the main leaf spot causing agent of *Apium* species, are synonymous. Groenewald *et al*. (2005) conducted a detailed study of the cultural characteristics, cardinal temperature requirements for growth and molecular analyses to demonstrate that these two *Cercospora* species are indeed distinct.

Cercospora beticola is considered to be one of the most destructive foliar pathogens of sugar beet, causing yield losses of up to 40% (Shane & Teng, 1992; Holtschulte, 2000). For most *Cercospora* species, including *C. beticola*, no sexual stage is known from nature and *in vitro* pairing studies have not been successful in producing a teleomorph for *C. beticola* (unpublished data). The genus *Cercospora* is a well-established anamorph of *Mycosphaerella* (Crous & Braun, 2003), and phylogenetic analyses on a variety of *Cercospora* species have placed them as a well-defined clade within *Mycosphaerella* (Crous *et al*., 2001, 2006a, 2006b; Goodwin *et al*., 2001). Therefore, if a sexual stage does exist for *C. beticola*, it would be a species of *Mycosphaerella*.

A wide array of phenotypic diversity has been described for *C. beticola* that includes variation in spore morphology and production, cultural characteristics, pathogenicity and fungicide resistance (Rossi, 1995; Moretti *et al*., 2004). In fungi, gene diversity is not necessarily affected by the mating structure (McDonald, 1997), but sexually

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reproducing fungi usually have high levels of genotypic diversity and alleles among loci should be randomly associated (Milgroom, 1996). Even though phenotypic markers indicate high levels of variation, little is known about the genetic structure of *C. beticola* populations.

Recently a few studies attempted to determine the population genetic structure of *C. beticola* and a substantial amount of genetic variation was found within *Cercospora* strains isolated from sugar beet fields in Italy (Moretti *et al*., 2006), and genetic variation was also observed in *C. beticola* isolates from lesions of the same plant (Moretti *et al*., 2004). This is in contrast to the data available for other *Cercospora* species which have low levels of genetic diversity, e.g. *C. sorghii* (Okori *et al*., 2004). This species also shows low genetic differentiation between populations from Uganda, suggesting a close genetic relatedness among populations (Okori *et al*., 2004). Similarly, genetic variation among isolates of *C. zeae-maydis* from Africa (Okori *et al*., 2003) and the United States (Wang *et al*., 1998; Crous *et al*., 2006a) was also found to be low, with little genetic differentiation either within or between populations.

Mating type genes are often under frequency-dependent selection in randomly mating populations (Milgroom, 1996; May *et al*., 1999). Mating type genes (*MAT1-1-1* and *MAT1-2*) of *C. beticola* were isolated and characterized to show that the fungus has a bipolar mating system (Groenewald *et al*., 2006b). However, the putative intron splicing sites, gene predictions and functionality of these genes in *C. beticola* have not yet been confirmed and additional studies are necessary to show whether these genes are functional. Ascomycetes that are heterothallic have a single locus, two allele mating system which requires two nuclei of opposite mating types to fuse in order for sexual reproduction to occur (Kronstad & Staben, 1997). *Cercospora* mating type-specific primers were developed for use in a multiplex PCR to determine the frequencies of these idiomorphs in field populations (Groenewald *et al*., 2006b). They found that mating types occurred in similar frequencies in *C. beticola* field populations, a phenomenon that is commonly accepted as indicative of random mating, such as in *Mycosphaerella graminicola* (Waalwijk *et al*., 2002; Zhan *et al*., 2002). Groenewald *et al*. (2006b) therefore suggested that some *Cercospora* species cannot be strictly asexual and that another method of reproduction has to occur to account for the frequency-dependent selection of the mating type genes observed within field populations.

Although previous studies showed that high levels of genotypic variation could be found in populations of *C. beticola* (Moretti *et al*., 2004, 2006), these studies were all based on small sample sizes ($N \leq 13$ per population). Knowledge of the distribution of the mating types, together with the amount of genotypic variation observed within a specific fungal population, can provide a strong indication whether or not sexual reproduction is likely to occur. The main objectives of this study were therefore to (i) determine the genetic structure of *C. beticola* populations with AFLPs, including genotypic diversity and gametic disequilibrium, and (ii) to determine whether there is frequency-dependent selection on mating types. This knowledge will provide indirect evidence for the possible presence of a sexual cycle occurring in this fungus. In order to achieve these objectives populations from Western Europe, Iran and New Zealand were analysed.

Materials and methods

Fungal isolation and DNA extraction

Beta vulgaris leaves were sampled during the 2003 growing season from single sugar beet fields in four European countries (Netherlands, Germany, France and Italy) as well as in New Zealand (Table 1). The samples from Iran were collected during the 2004 growing season. The sampling was done in an X figure across each field. For each population, leaves with symptoms were collected from 10 plants in each leg of the cross. Single-spore isolations were made and cultures were established on 2% malt extract agar (MEA). The isolates were examined morphologically to confirm their identity as *C. apii sensu lato* as described by Crous & Braun (2003). All isolates were also screened with *C. beticola*-specific primers to confirm that they were truly *C. beticola* before being included in the analyses (Groenewald *et al*., 2005). Isolates were cultured on MEA plates for 8 days at 24°C, and 200–400 mg mycelium were used in the DNA extraction using the FastDNA kit (BIO 101, Carlsbad) according to the manufacturer's instructions.

Screening of markers

Degenerate mating type idiomorph primers designed by Groenewald *et al*. (2006b) were used to screen all isolates from the six *C. beticola* populations as described previously. AFLP analyses were performed according to Vos *et al*. (1995), with minor modifications as described by Groenewald *et al*. (2005). Genomic DNA (30 ng) from 250 isolates was digested with the restriction enzymes *Eco*RI and *Mse*I and ligated to the corresponding adaptors. Four selective primer combinations were used, namely *Eco*RI-A-[FAM]/*Mse*I-CT, *Eco*RI-AT-[JOE]/*Mse*I-C, *Eco*RI-AG-[NED]/*Mse*I-C and *Eco*RI-G-[JOE]/*Mse*I-CG (Applied Biosystems), for the final amplification step. To test the reproducibility of the AFLP profiles, separate DNA extractions, PCR amplifications and AFLP analyses were performed in duplicate on 10 isolates (using the four

Table 1 *Cercospora beticola* populations included in this study

Country of origin	Sample size	Location	Collector
France (Fr)	46	Longvic	S. Garressus
Germany (Ger)	39	Niedersachsen	S. Mittler
Italy (It)	32	Ravenna	V. Rossi
Netherlands (Neth)	48	Bergen op Zoom	Unknown
New Zealand (NZ)	35	Unknown	C.F. Hill
Iran (Ir)	50	Pakajik	A.A. Ravanlou

primer combinations). An error rate of 1% (1 to 2 bands difference per isolate among 206 loci) was observed. Only polymorphic loci (78) were included in the analyses.

Data analyses

The presence and absence of bands obtained from AFLP analyses were scored as 1 and 0, respectively, and these results were combined for the statistical analyses. Isolates were considered members of the same clone or clonal lineage if they had 99% similar bands. Clones identified with AFLPs which had different mating type idiomorphs were considered different haplotypes. To quantify genotypic variation within populations, the genotype richness was measured with a Shannon-Wiener index (Grünwald & Hoheisel, 2006).

To evaluate the associations among loci in each sample, the index of association (I_A) and an unbiased estimate of multilocus linkage disequilibrium ($\bar{r}_d$) were used. I_A and $\bar{r}_d$ values were calculated by using Multilocus 1·3 software, and 1000 artificially recombined data sets were used to determine the statistical values of the test (Agapow & Burt, 2001). Significant departures from an expected 1:1 ratio in mating type frequencies were tested with a chi-squared test.

TFPGA (Miller, 1997) and POPGENE v1·32 (Yeh *et al*., 1997) were used to analyse the 0/1 matrix. The population genetic analyses program TFPGA was used to calculate the gene diversity (Nei, 1978), percentage of polymorphic loci, *F*-statistics, genetic distances and the exact tests. The percentage polymorphic loci were based on 99% criteria. The population differentiation was calculated using the method of Weir & Cockerham (1984), jackknife over loci was done with 10 000 iterations using a confidence interval (C.I.) of 95%. Genetic distances between the populations were calculated using Wright's (1978) modification of Rogers' (1972) distance. For this study, a value of < 0·1 indicates small genetic distances, 0·10–0·15 indicates moderate genetic distances, 0·15–0·2 indicates high genetic distances and > 0·2 indicates very large genetic distances. A graphical representation of the genetic distance data (Nei, 1978) was done using the UPGMA algorithm. Bootstrap support values were calculated over all the loci using 1000 repetitions. The exact test was used to determine if significant differences in allele frequencies exist between populations (Sokal & Rohlf, 1995). The Markov Chain Monte Carlo approach that was used to calculate the exact test values gives an approximation of the exact probability of the observed differences in allele frequencies (Raymond & Rousset, 1995).

POPGENE was used to calculate the gene flow (*Nm*) between any two populations, between the four Western European populations, between the five Eurasian populations and between all six populations. The grouping of populations into major geographic areas of Asia (Iran), Europe (Netherlands, France, Italy and Germany) and New Zealand allowed the analysis of variation (analysis of molecular variance, or AMOVA) at three levels: within individual populations, between populations within geographic regions, and between geographic regions. All calculations, including random-permutation procedures to assess statistical significance, were performed using the GenALEx 6 package (Peakall & Smouse, 2005).

Results

AFLP markers

Moderate levels of polymorphism were obtained from the four AFLP primer combinations used in this study (Table 2). In total, 208 bands could be scored unambiguously. The number of polymorphic bands obtained from all six populations varied from 15 to 22 (Table 2) and the band sizes ranged from 50 to 500 base pairs. The AFLP primer sets *Eco*RI-AG/*Mse*I-C amplified the largest number of polymorphic bands (22) whereas AFLP primer pair *Eco*RI-G/*Mse*I-CG amplified the lowest number of polymorphic bands (15) (Table 2). The percentage polymorphic loci ranged from 20·9% in the New Zealand population to 30·6% in the German population (Table 2).

Population genetic analyses

Genotypic diversity (*H*) ranged from 3·25 (New Zealand) to 3·82 (France, Table 3). Among 250 isolates, 217 (86·4%) unique genotypes were obtained. Unique genotypes refer to isolates with dissimilar AFLP profiles, but also to isolates with identical AFLP profiles but different mating types.

Gene diversity (*H*) is lowest in the New Zealand population (0·19) and highest in the German and Italian populations (0·27) (Table 3). The theta value shows high population differentiation (0·17) across the six populations, and moderate population differentiation across the four European populations (0·07) and five Eurasian populations (0·07) (Table 4). The pairwise comparisons of population differentiation between the New Zealand population and other populations was high (theta = 0·33–0·41), even though the New Zealand population had only two private alleles. The theta values from pairwise comparisons between the remaining populations varied between 0·02 (Dutch/Italian) and 0·13 (French/German).

Table 2 The number of polymorphic bands analysed with four AFLP primer combinations on 250 *Cercospora beticola* isolates

Primer pair	No. of bands	No. of polymorphic bands[a]						
		NZ	Fr	Ger	Ir	It	Neth	All
*Eco*RI-A/*Mse*I-CT	54	14	14	16	14	16	16	21
*Eco*RI-AG/*Mse*I-C	52	11	16	17	17	16	15	22
*Eco*RI-G/*Mse*I-CG	52	8	11	13	12	12	12	15
*Eco*RI-AT/*Mse*I-C	48	10	11	17	9	14	16	21
Total	206	43	52	63	52	58	59	79
% Polymorphic loci		20·9	25·2	30·6	25·2	28·6	28·6	38·3

[a]NZ = New Zealand, Fr = France, Ger = Germany, Ir = Iran, It = Italy, Neth = Netherlands, All = total of all six populations.

Table 3 The number of isolates, (number of haplotypes), genotypic and gene diversity, tests of multilocus association and mating type frequencies of *Cercospora beticola* clone corrected populations

Population	*n*	*H*'[a]	*H*[b]	MAT frequency		χ^{2c}	I_A^d	$\bar{r}_d^d$
				Mat1-1-1	Mat1-2			
NZ	35 (27)	3·25	0·19	0·52	0·48	0·037	1·530*	0·037*
Fr	46 (46)	3·82	0·23	0·54	0·46	0·347	0·729*	0·015*
Ger	39 (32)	3·39	0·27	0·59	0·41	1·125	1·934*	0·032*
Ir	50 (43)	3·67	0·24	0·49	0·51	0·023	1·025*	0·021*
It	32 (32)	3·47	0·27	0·31	0·69	4·500*	0·377*	0·006*
Neth	48 (37)	3·29	0·25	0·57	0·43	0·675	0·214*	0·004*
Total	250 (217)	–	–	0·51	0·49	6·668	1·135*	0·016*

[a]Shannon – Wiener index for genotype richness.
[b]Gene diversity (Nei, 1978).
[c]χ^2 value based on 1:1 ratio and 1 degree of freedom for clone corrected populations and 5 degrees of freedom for the contingency χ^2 analyses of the total data set. *Indicates mating type frequencies which are significantly different at $P < 0{\cdot}05$.
[d]*indicates significant I_A and $\bar{r}_d$ values at $P < 0{\cdot}01$.

Table 4 Gene flow (*Nm*) (below diagonal) and theta (population differentiation, above diagonal) for pair-wise comparisons among the six *Cercospora beticola* populations, among all combined populations, and among populations from Europe or Eurasia

	NZ	Fr	Ger	Ir	It	Neth
NZ	–	0·41*	0·33*	0·40*	0·36*	0·36*
Fr	1·3	–	0·13*	0·08*	0·06	0·06
Ger	1·8	5·7	–	0·10	0·05	0·06
Ir	1·4	8·5	6·6	–	0·06	0·09
It	1·6	11·1	10·9	11·3	–	0·02
Neth	1·6	11·4	10·4	8·0	19·1	–
Nm (all populations)	2·2					
Nm (European)	6·8					
Nm (Eurasian)	5·8					
theta (all populations)	0·17*					
theta (European)	0·07*					
theta (Eurasian)	0·07*					

*$P \leq 0{\cdot}01$, *P*-values obtained with 1000 randomizations in Multilocus v1·3.

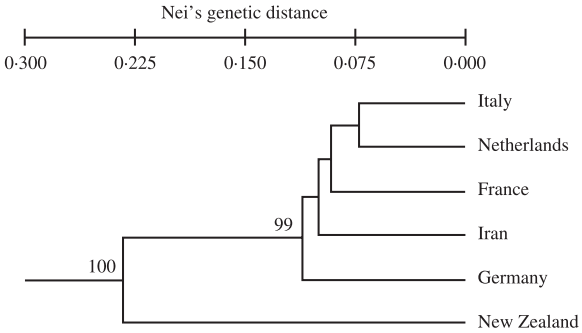

Figure 1 Graphical representation of the genetic distance data (Nei, 1978) generated by UPGMA clustering in the software TFPGA. The scale bar shows the genetic distance, and bootstrap support values in percentage from 1000 replicates are shown at the nodes (only bootstrap support values of 70% and higher are shown).

The high gene flow (*Nm*) values of 6·8 and 5·8 across the four European and five Eurasian populations, respectively, indicate high genetic exchange between these populations, but *Nm* was low when the New Zealand population was included in the calculation (*Nm* = 2·2) (Table 4). Low *Nm* values (1·3–1·8) were observed between the New Zealand population and every other population analysed. The highest *Nm* values were obtained in pairwise comparisons between Italy and the Netherlands (*Nm* = 19·1), followed by Netherlands/France (*Nm* = 11·4) (Table 4).

AMOVA analyses revealed that the percentage of genetic variation among individuals within populations was 75%. Only 4% of the variation was due to differences among populations within a region (European populations) and 21% to differences among geographic regions.

In 14 cases, isolates with the same multilocus AFLP haplotype had different mating type idiomorphs. Mating type ratios did not deviate significantly from a 1:1 ratio suggesting frequency-dependent selection, except in the population from Italy where MAT1-2 isolates were more predominant (Table 3). Multilocus measures of association (I_A = index of association and $\bar{r}_d$ = multilocus linkage disequilibrium) were significant ($P < 0{\cdot}01$) for all populations (Table 3). All four loci showed significant ($P < 0{\cdot}01$) I_A and $\bar{r}_d$ values for the New Zealand, German and Iranian populations. Loci from only two primer combinations were in gametic disequilibrium for the population from France, and only one primer combination A/CT was in significant ($P < 0{\cdot}01$) gametic disequilibrium for the *C. beticola* populations from Italy and the Netherlands (data not shown).

Cluster analysis

Figure 1 represents the genetic distance data obtained between populations using the TFPGA program with UPGMA clustering, and bootstrap support values from 1000 replicates are shown. Genetic distances between the New Zealand population and all other populations were high (0·22–0·25) (Fig. 1). The genetic distance values between the remaining populations were lower and varied

between 0·07 and 0·13. The Exact test showed significant differences between the New Zealand populations and the rest ($P < 0{\cdot}001$) as well as for the pair-wise comparison between populations of France/Germany ($P = 0{\cdot}02$).

Discussion

This study is the first to report on the genetic structure and mating type distribution of *C. beticola* populations from different geographic localities. The results obtained from population differentiation, gene flow and genetic distance analyses suggest that the populations from Europe and Iran are genetically similar, whereas the New Zealand population is significantly different. High levels of genetic variation were found among the *C. beticola* isolates tested. This variation, illustrated by the high number of distinct haplotypes obtained with the AFLP analyses, compares well with earlier studies that also reported high levels of genetic variation among isolates obtained from the same lesion on a sugar beet plant in Italy (Moretti *et al*., 2004), and between isolates from Italy (Moretti *et al*., 2006). Most of the isolates that were obtained from one plant during the present study also had a distinct multilocus AFLP haplotype (data not shown). The sampling allowed partitioning of genetic variation and showed that most variation could be found within populations (75%), whereas only 4% of the variation was due to differences among populations within a region (European populations) and 21% to differences among geographic regions.

To date no teleomorph has been found for *C. beticola* (Groenewald *et al*., 2006b) and the reproductive structure of this pathogen has been considered clonal. However, this study found high levels of genotypic diversity in all six populations analysed. It is known that populations that regularly undergo sexual reproduction should have many genotypes that result in higher levels of genotypic diversity compared to those that reproduce only asexually (Milgroom, 1996). This type of genetic structure is seen in most populations of *M. graminicola* (Linde *et al*., 2002; Zhan *et al*., 2003; Zhan & McDonald, 2004). Thus, the genotypic diversity observed for *C. beticola* is exceptionally high for a presumed asexually reproducing organism.

Milgroom (1996) and Zhan *et al*. (2002) found that a combination of high levels of genetic diversity and the equal distribution of mating types in a given population indicates that sexual recombination occurs. This study therefore screened for the presence and frequency of the mating type idiomorphs in the populations. The equal distribution of mating types in most populations (except Italy) suggests frequency-dependent selection and thus random mating. Both mating types could also be found on the same plant (data not shown), providing opportunity for genetic exchange. Thus, the high levels of genotypic diversity together with equal mating type ratios indicate that this fungus reproduces sexually. If *C. beticola* was strictly asexual, one would expect that, over time, there would be a skewed distribution of the mating types, or that only one mating type would be present, as was found for other *Cercospora* species such as *C. apii* and *C. apiicola* (Groenewald *et al*., 2006b). *Cercospora beticola* has been observed to form spermatogonia on leaf tissues collected during this study, which is also indicative of a possible sexual cycle, although any sexual stage that may exist is, so far, not readily observed in nature nor induced under laboratory conditions.

Tests for multilocus associations (I_A and $\bar{r}_d$) showed that all six populations were in gametic disequilibrium. This suggests that asexual production is predominant and that random mating occurs only rarely, if at all. However, although significant, the values of I_A and $\bar{r}_d$ were low for populations from Italy, France and the Netherlands. Furthermore, $\bar{r}_d$ was similar or even lower in *C. beticola* (0·004–0·037) than that estimated for *Pyrenophora teres* f.sp. *teres* (0·037–0·039), which is known to undergo regular sexual recombination (Rau *et al*., 2003). Furthermore, only one primer combination was in significant gametic disequilibrium in the population from Italy and the Netherlands. This contradicts results on frequency-dependent selection and levels of genotypic diversity which suggest populations undergo regular sexual recombination. There are two possible explanations for gametic disequilibrium in these populations. First, frequent population expansions during epidemics can result in populations dominated by closely related individuals (Maynard-Smith *et al*., 2000). During epidemics, even though populations are recombining, genotypes may arise that are strongly favoured by selection. These genotypes will increase in frequency, generating disequilibrium until recombination has had time to randomize the genetic background (Maynard-Smith *et al*., 2000), presumably at the end of the growing season when sexual reproduction is known to occur as a survival mechanism for many plant pathogens. Unless mating type idiomorphs are linked to pathogenicity factors or fungicide resistance, their frequency should by chance follow a 1:1 ratio during the epidemic. However, the AFLP loci used in this study were selectively neutral.

A second explanation for the observed gametic disequilibrium lies with the type of marker used. AFLPs often represent hypervariable regions that include dispersed repetitive elements (reviewed in Wong *et al*., 2001), resulting in a co-dominant marker. Thus, conventional population genetic approaches to analyse AFLP data will underestimate the variability at each locus and overestimate the number of loci analysed, since each allele will be taken as an independent locus (Wong *et al*., 2001). In a comparison between RFLP and hypervariable AFLP markers, Yan *et al*. (1999) showed that heterozygosity was underestimated in the yellow fever mosquito by AFLP markers, resulting in Hardy-Weinberg disequilibrium. The present results suggest that at least one AFLP primer pair (A/CT) amplified hypervariable regions since it was the only primer combination that showed significant gametic disequilibrium in all *C. beticola* populations analysed. Furthermore, in populations from Italy and the Netherlands, this was the only primer combination that resulted in loci (20 out of 78) in gametic disequilibrium. It is therefore suggested that at least the *C. beticola* populations from Italy and the Netherlands are in gametic equilibrium.

The high level of genotypic variation in *C. beticola* can also be explained by other factors. First, it is possible that *C. beticola* reproduced sexually prior to modern agricultural practices (e.g. burying of plant material during soil cultivation) which prevents sexual reproduction at the end of the growing season. Secondly, Weiland & Koch (2004) showed that the genome of *C. beticola* can undergo chromosome changes after repetitive subculturing. These changes were observed after chromosome separation by gel electrophoreses. Although the authors studied only two isolates and did not mention the number of times the sub-culturing was repeated before these rearrangements were observed, the possibility that such rearrangements can influence results obtained using marker systems, such as AFLPs, has to be taken into account. In order to limit these chromosomal rearrangements in isolates, sub-culturing during this study was kept to a minimum and the DNA was extracted from the cultures directly after the original isolation. It is therefore concluded that the genetic variation observed in the populations screened during this study occurred during the life cycle of the fungus in its natural field environment.

Genetic diversity within a species can also be caused by asexual events that include hyphal anastomosis (Molnar *et al*., 1990), selfing (Anderson & Kohn, 1995), normal mutations (Koenig *et al*., 1997; Bentley *et al*., 1998; O'Donnell *et al*., 1999) and events occurring during parasexual cycles (Kuhn *et al*., 1995; Taylor *et al*., 1999). There is no evidence for parasexual recombination as an important generator of genetic diversity *in vivo* for any fungal system. The high levels of genetic diversity observed in *C. beticola* cannot be explained by mutation only, thus it is proposed that, apart from asexual recombination, a sexual cycle must be present for this pathogen.

No geographic boundaries could be enforced on the European populations based on the country of isolation because of the low population subdivision and low genetic distances between them, and because of shared haplotypes. Also, the Iranian population was not differentiated from the European populations. Sharing of haplotypes among geographic populations could be explained by man-mediated dispersal, as import and export of host material between countries in the European Union readily occurs because of the open borders. The high gene flow and low genetic distance and differentiation values observed between European populations and Iran indicate that genotype transfer also readily takes place between these countries. Based on genetic distance analysis, the Iranian genotypes are intermingled with European isolates, but this was not found for the New Zealand isolates. Therefore, it can be concluded that the European populations and the population from Iran are panmictic.

Pennycook (1989) recorded *C. beticola* on sugar beet in New Zealand, and during the last few years it has been isolated from different localities in New Zealand (New Zealand Fungi Database, 2002). The population from New Zealand is readily distinguished from other populations because of its low gene diversity, high genetic distances and population subdivision. This genetic differentiation could either be due to a founder event, or the New Zealand populations might represent a different species of *Cercospora*. Groenewald *et al*. (2006a) included New Zealand isolates in a multi-gene phylogeny and could not distinguish them from the other *C. beticola* isolates. Also, the *C. beticola*-specific primers (Groenewald *et al*., 2005) amplified a product of the correct size for the New Zealand isolates. Only two private AFLP alleles and two null alleles were found to be specific to the New Zealand populations during this study. From these data it is concluded that the *Cercospora* isolates obtained from sugar beet in New Zealand are indeed *C. beticola*. Small population sizes and genetic drift during founder events could have resulted in genetic subdivision, as has been found for other *Mycosphaerella* populations (Boileau *et al*., 1992; Hayden *et al*., 2003). However, the specific origin of *C. beticola* in New Zealand is unknown. The first strain that was designated a type of *C. beticola* was described from *Beta cicla* in Italy in 1875 (Saccardo, 1876) and it is most likely that earlier sugar beet trade introduced *C. beticola* to New Zealand from Europe.

Several studies have reported high levels of variation during the onset and progression of cercospora leaf spot on sugar beet (Wolf & Verreet, 2002, 2005), and that *C. beticola* has become resistant or has developed an increased tolerance to fungicides (Karaoglanidis *et al*., 2000; Weiland & Koch, 2004). Variation in fungicide resistance and variability in disease symptoms on resistant sugar beet plants make effective disease management difficult. It is likely that the high levels of genetic variation that exists within *C. beticola* plays a role in the variation in pathogenicity that has been reported.

Previous studies showed that some genetic variation exists within *C. beticola*, but it was not known whether this variation was due to sexual recombination. The results here indicate that the genetic variation observed in the isolates studied was most likely caused by recombination events. It is suggested that *C. beticola* has both an asexual and sexual reproduction system and that it is unlikely that only asexual reproduction occurs in *C. beticola*. The high levels of genotypic variation and the equal distribution of the mating types within populations suggest that sexual recombination events most likely play an important role in the reproductive cycle of this species.

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