Expansion of Genetic Diversity in Randomly Mating Founder Populations of *Alternaria brassicicola* Infecting *Cakile maritima* in Australia[∇]

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Founder populations of fungal plant pathogens are expected to have low levels of genetic diversity coupled with further genetic drift due to, e.g., limited host availability, which should result in additional population bottlenecks. This study used microsatellite markers in the interaction between *Cakile maritima* and the fungal pathogen *Alternaria brassicicola* to explore genetic expectations associated with such situations. The host, *C. maritima*, was introduced into Australia approximately 100 years ago, but it is unknown whether the pathogen was already present in Australia, as it has a wide occurrence, or whether it was introduced to Australia on brassicaceous hosts. Eleven *A. brassicicola* populations were studied, and all showed moderate levels of gene and genotypic diversity. Chi-square tests of the frequencies of mating type alleles, a large number of genotypes, and linkage equilibrium among microsatellite loci all suggest *A. brassicicola* reproduces sexually. Significant genetic differentiation was found among populations, but there was no evidence for isolation by distance effects. Bayesian analyses identified eight clusters where the inferred clusters did not represent geographical populations but instead consisted of individuals admixed from all populations. Further analysis indicated that fungal populations were more likely to have experienced a recent population expansion than a population bottleneck. It is suggested that *A. brassicicola* has been introduced into Australia multiple times, potentially increasing the diversity and size of any *A. brassicicola* populations already present there. Combined with its ability to reproduce sexually, such processes appear to have increased the evolutionary potential of the pathogen through recent population expansions.

Analyses of population genetic diversity provide useful information on the epidemiology and evolutionary history of infectious diseases (6). The effective population sizes, transmission abilities, and reproduction of infectious agents vary, all of which can be used to infer their population histories (25). Such inferences can in turn be used to determine whether the pathogen has been recently introduced (i.e., a founder population), as well as the number of introductions that have occurred. Introductions of plant pathogens into new geographic areas have occurred commonly in the past for a multitude of organisms, reducing their population diversity in the founder populations but sometimes also causing an epidemic in naïve hosts in the areas of introduction. For example, *Phytophthora infestans*, a pathogen of the potato, was introduced from Mexico into the United States, and then only one genotype was introduced into Europe (24), causing the death of more than 1.5 million people due to starvation (12). *Cryphonectria parasitica*, which causes chestnut blight, was introduced from Asia into North America (2), where it underwent a host shift from Asian chestnuts to North American chestnuts, threatening the existence of North American chestnut trees.

For both plant and animal pathogens, introduction into new

areas can be a major driver of emerging diseases, i.e., host shifts (3, 44, 66). In the case of plant pathogens, range expansion can occur after the introduction of an invasive host species (7, 41), resulting in a pathogen with evolutionary potential higher than that represented in the original founder population. Population genetic expansions for plant pathogens could result in the pathogen being able to infect a larger number of plant genotypes or even new host species following a host shift (38).

In agricultural systems, many fungal plant pathogens have genetic structures consistent with having experienced a population bottleneck due to founder events (24, 33, 41, 49); others, such as *Rhynchosporium secalis* on barley (32) and *Mycosphaerella graminicola* on wheat (5, 32), eventually underwent a population expansion, possibly due to an increase in host availability. One consequence of this is the accumulation of additional mutations, thereby creating novel genotypes and increasing the effective population size of the pathogen. However, even in well-studied agricultural-plant-pathogen systems, tests for population expansions have rarely been conducted. In most cases where there is evidence for the genetic expansion of pathogen populations, it was a result of additional migrants coming into the founder population (e.g., *P. infestans* [23]) or the occurrence of sexual reproduction, as in European populations of *C. parasitica* (41). An increase in genotypic diversity has also been demonstrated in a founder population of the Dutch elm disease pathogen *Ophiostoma novo-ulmi* in Portugal, which was able to reproduce sexually following the

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introduction of the other mating type (13). The evolution of founder pathogen populations is likely to be influenced by a range of ecological and life history parameters, including host plant population density, host longevity, and host and pathogen mating systems and dispersal abilities (6, 45).

In theory, bottlenecks reduce gene diversity and the number of alleles in populations. Empirically, it has been shown that founder events frequently reduce allelic diversity and, more rarely, gene diversity (15, 21, 28). Thus, we can detect whether a population has experienced a recent population expansion after a demographic bottleneck by using genetic information to determine whether the population has a deficit in gene diversity relative to the number of alleles (16, 35), or whether it experienced a recent population bottleneck, in which case an excess in gene diversity relative to the number of alleles under a mutation drift model will be present (31).

In this study, we investigated the introduced *Cakile maritima*-*Alternaria brassicicola* host-pathogen interaction in Australia. *A. brassicicola* is a heterothallic haploid fungus that causes black spots on the leaves, stems, and fruits of a wide range of brassicaceous hosts. The pathogen has a wide distribution and is commonly found on both agricultural brassicaceous crops and wild species (53, 61, 65). The pathogen naturally occurs on *C. maritima*, a succulent foredune annual native to the Mediterranean and Western Europe that was introduced into Australia more than 100 years ago (50). *C. maritima* is a self-incompatible, obligate outcrossing species with low levels of self-fertilization (62) and produces seeds that can survive for long periods immersed in seawater. Therefore, seed dispersal between populations is possible, both in terms of seed survival and ocean current patterns (22), and it is estimated that in Australia, *C. maritima* has spread along the coast at rates of 50 to 100 km per year (51). The conidia (asexual spores) of *A. brassicicola* are airborne and are dispersed through wind and rain splash, as well as vertically via infected seeds transported by ocean currents. In such interactions, where there is significant potential for seed-borne pathogen transmission, it is expected that the pathogen population should track the dispersal of the host and, to some extent, display a population structure that reflects the host's geography.

Whether *A. brassicicola* is able to reproduce sexually is still unknown, although it is presumed to be an asexual fungus, as it has no known sexual cycle. However, studies using amplified fragment length polymorphisms (AFLPs) have shown high levels of gene and genotypic diversity in Australian populations of *A. brassicicola* infecting *C. maritima* (9, 10). Although the populations were in significant gametic disequilibrium, the authors deemed the levels of gametic disequilibrium to be low and predicted that a sexual stage should be present based on the high genotypic diversity observed (9, 10). Asexual organisms are generally characterized by an overrepresentation of particular genotypes, and therefore, genotype diversity is generally low, whereas recombination of alleles creates numerous new genotypes in sexually reproducing populations (37, 40, 63). It is worth noting, in the context of the preceding discussion regarding pathogen dispersal, that sexual reproduction in *A. brassicicola* would result in the formation of sexual spores (ascospores), which are smaller than asexual spores, potentially leading to longer-distance wind dispersal.

The aims of this study were to further investigate the pop-

ulation genetic structure of *A. brassicicola* and to determine whether populations are randomly mating. The mating type frequencies of *A. brassicicola* in either natural or agricultural host populations have never been determined. Random mating was investigated by (i) analyzing levels of genetic diversity and linkage disequilibrium in *Alternaria* populations characterized with microsatellite markers and (ii) quantifying the extent of frequency-dependent selection of pathogen mating type alleles in these populations. Sexual reproduction can occur in heterothallic fungi only when isolates of opposite mating types are in close physical proximity to each other; therefore, the finding that both mating types occur in a population at equal frequencies (i.e., through frequency-dependent selection of the mating types) would imply that random mating is occurring. Furthermore, we investigated the evolution and genetic structures of fungal populations. Specifically, we investigated whether (i) local populations belong to one panmictic population representing one population; (ii) populations have undergone a recent (in the last 100 years) population bottleneck, typical of founder populations; or (iii) whether populations have expanded genetically, as well as geographically, resulting in pathogen populations with an evolutionary potential higher than that in the original population (38).

MATERIALS AND METHODS

Populations. Populations of *A. brassicicola* were collected from *C. maritima* growing in three regions along the southern New South Wales (NSW) coast of Australia. Three populations were collected previously from Central Tilba (CT) and four each from Durras (DU) and Moruya-Bodalla (MB), spanning 34°40'S, 150°17'E to 36°19'S, 150°7'E (61). All of the populations occur on beaches and are separated by rocky outcrops and headlands. The sites chosen for this study represent a subset of populations that form part of ongoing work on the meta-population dynamics of the *Cakile-Alternaria* interaction (60, 61). The collection, isolation, and maintenance of isolates were as described previously (60). Infected plants were sampled randomly within populations. A total of 401 isolates were analyzed in this study (Table 1).

Mating type determination. DNA was extracted either using the protocol (39) for a previous AFLP study (9) or, for isolates that were not used in the AFLP study, with a DNeasy Plant Mini Kit (Qiagen) according to the recommendations of the manufacturer. Initial attempts to determine the mating types of the *A. brassicicola* isolates were made using primers designed by Berbee and coworkers (8). Primers BPH05 and BPH04 were used to amplify the *MAT1-1* gene and BPHMG1 and BPHMG2 were used to amplify the *MAT1-2* gene in a multiplex PCR. Because amplification was poor for the majority of the isolates, new primers were designed from the GenBank accessions of the *MAT1-1* (AY042093) and *MAT1-2* (AY042092) loci. These primers were MAT1-1F (5'-CTCAATGCCTTTGTCTGGATT-3'), MAT1-1R (5'-CCGAGTGTCGAAGGAATGTT-3'), MAT1-2F (5'-TCTTCAGAGATGCGATGCAC-3'), and MAT1-2R (5'-CTCTTCTTCGCGGACTGTG-3'). The new primers were designed to amplify a 683-bp fragment in MAT1-1 and a 276-bp fragment in MAT1-2 isolates to provide unambiguous size distinctions on gels. A multiplex PCR mixture contained 5 to 20 ng DNA in 20- μ l reaction mixtures, 2.5 mM MgCl₂, 2.5 mM each deoxynucleotide triphosphate (dNTP), 0.5 U *Taq* polymerase (Fisher Biotec), 2 μ l of 10 $\times$ reaction buffer (Fisher Biotec), and 2 μ M (each) of the four primers. PCRs were performed on an Eppendorf Mastercycler with the following conditions: 95°C for 2 min and 30 cycles of 95°C for 30 s and 55°C for 1 min, followed by a final extension step at 72°C for 45 s. The PCR products were visualized on 1% agarose gels (0.5 $\times$ Tris-borate-EDTA [TBE]) and stained with ethidium bromide. Mating type analysis was conducted only for isolates deemed to belong to different haplotypes within populations (e.g., clone-corrected populations), as determined by microsatellite analyses (see below).

Microsatellites. Eleven primer pairs previously designed for *A. brassicicola* (4), were used to characterize the Australian *A. brassicicola* populations. The forward primer of each microsatellite marker was labeled with an M13 (-21) tail (5'-TGTAAACGACGGCCAGT-3'). The reverse primers were retained in their original forms, and a third universal M13 (-21) reporter primer that was fluorescently labeled with either VIC, FAM (6-carboxyfluorescein), or NED (In-

TABLE 1. Sample size (N), number of unique haplotypes, genotypic diversity, clonal fraction, mean number of alleles, and gene diversity, as determined with 11 microsatellite loci on 11 populations of *A. brassicicola* representing three regions in New South Wales, Australia^a

Region	Population	N	No. of haplotypes	$\hat{G}/N\%$	Clonal fraction	Clone corrected for identical genotypes on the same leaf				N_a	H
						N	No. of haplotypes	$\hat{G}/N\%$	Clonal fraction		
Central Tilba	CT17B	27	18	49	0.33	24	18	63	0.25	2.1	0.33
	CT18	18	14	56	0.22	16	14	68	0.12	2.4	0.35
	CT0S	51	24	25	0.53	41	24	29	0.41	2.5	0.27
Durras	DUD	25	20	61	0.20	25	20	61	0.20	2.2	0.36
	DUG	59	25	19	0.58	41	22	24	0.39	2.4	0.30
	DUI	37	11	17	0.70	34	11	19	0.68	2.0	0.27
	DUL	35	26	49	0.26	33	26	60	0.21	2.5	0.35
Moruya-Bodalla	MBH	35	17	27	0.51	34	17	28	0.50	1.8	0.22
	MBJ	25	15	42	0.40	23	15	49	0.35	2.1	0.28
	MBON	57	24	20	0.58	50	24	25	0.52	2.3	0.25
	MBS	32	17	21	0.47	25	17	35	0.32	2.2	0.30
Total		401	211	15	0.59	346	208	20	0.53	4.1	0.38

^a $\hat{G}/N\%$, genotypic diversity (59); N_a , mean number of alleles across 11 loci; H , Nei's gene diversity (42).

vitrogen) was added (54). The following PCR conditions were used for each locus: each 20- μ l PCR mixture contained 5 to 20 ng of DNA template, 1 $\times$ PCR buffer, 0.25 mM dNTPs, 2.0 mM MgCl₂, 0.5 units of *Taq* polymerase, 0.1 μ M –21M13-labeled forward primer, 0.25 μ M reverse primer, and 0.2 μ M fluorescently labeled (VIC, FAM, or NED) –21M13 universal primer. The PCR conditions were an initial 3-min denaturation at 94°C, followed by 25 to 35 cycles of 94°C for 30 s, 30 s at annealing temperature (T_a ; see below), and 72°C for 30 s to amplify a locus-specific fragment. The primers used (4) were Abmic-5 and Abmic-8 in a multiplex reaction with a T_a of 60°C, whereas Abmic-1, -3, -7, -9, -10, and -12 were amplified at a T_a of 55°C and Abmic-2, -6, and -11 at a T_a of 60°C. In order to amplify fragments with the universal M13 (–21) reporter primer, another eight cycles were carried out at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. All loci were amplified in single reactions, but only Abmic-5 and Abmic-8 were multiplexed. Only one allele was amplified per locus, as expected for a haploid organism. For fragment analyses, 1 to 5 μ l of PCR product was added to 0.35 μ l of GeneScan –500 LIZ size standard (Applied Biosystems) and 9 μ l of Hi-Di Formamide. The mixture was denatured for 3 min at 95°C and then cooled on ice. Samples were loaded onto an ABI 310 Prism Genetic Analyzer. Fragments were analyzed using Genescan software (Applied Biosystems). Individual alleles at each locus were assigned using fragment lengths.

Data analysis. Isolates with the same alleles at all loci and the same mating type were considered to be clones of the same multilocus haplotype. To determine the population genotypic diversity, the maximum possible genotypic diversity ($\hat{G}/N$) (59) and clone fraction were calculated for each population and region. The clonal fraction was calculated as the occurrence and frequency of clones within a population, $(N - G)/N$, where N is the sample size and G is the number of haplotypes present. In some cases, multiple pathogen isolates were derived from the same individual plant. To avoid sampling bias, where the likelihood of finding the same clone at a small scale increases due to splash dispersal of conidia, an analysis was conducted using only one isolate per genotype per plant. For all other analyses, populations were clone corrected to avoid overrepresentation of alleles present in clones. For genotypic analyses, local populations were also pooled to represent regional populations for Central Tilba, Durras, and Moruya-Bodalla.

The selective neutrality of loci was examined with the Ewens-Watterson test (57, 67) in Popgene v3.2 (68). Nei's gene diversity (42) and the number of alleles present in populations were determined in Popgene v3.2 (68). To measure population relatedness, a modified version of Wright's *F*_{st} for haploids called theta (θ) was used in pairwise estimates of θ in Multilocus v1.3 (1). Theta was calculated between all pairs of clone-corrected populations, as well as between the three regions (Central Tilba, Durras, and Moruya-Bodalla). To examine whether observations deviated significantly from the hypothesis of no linkage disequilibrium among loci, the observed value was compared to the results of 1,000 randomized data sets. Relationships between genetic relatedness (θ) and among-population distances (i.e., isolation by distance) were estimated with Mantel tests, in which the significance of correlation was estimated with 1,000-

random-permutation tests using GenALex (46). The distributions of genetic variance within and among populations were determined with an analysis of molecular variance (AMOVA) in GenALex (46). The data were partitioned into regional populations representing Central Tilba, Durras, and Moruya-Bodalla.

Random mating in populations was investigated with a chi-square (χ^2) test on mating type ratios with the null hypothesis that each *A. brassicicola* population did not significantly differ from a 1:1 mating type ratio. Microsatellite data were used to test whether populations were in gametic disequilibrium using the index of association (I_A) (14) test in Multilocus v1.3 (1) running 1,000 randomizations. An alternative measure of the index of association ($\bar{r}_d$) that was less sensitive to the number of loci (1) was also performed in Multilocus v1.3.

To examine the levels of admixture of genotypes among populations, the populations were analyzed with Structure v2.2 (19, 20, 48). In this Bayesian approach, multilocus genotypic data are used to define a set of populations with distinct allele frequencies and to assign individuals probabilistically to them. The degree of population substructure was investigated using microsatellite data, as well as AFLP data from a previous study (10). Only populations from Durras were included in the earlier study; however, the inclusion of additional AFLP analyses conducted on the populations from Central Tilba and Moruya-Bodalla provided AFLP data for a total of 380 isolates. In the program Structure v2.2, an admixture ancestry model-based clustering method (allowing mixed ancestry among individuals from different populations [K]) with correlated allele frequencies (i.e., allowing allele frequencies among populations to be similar) was used. Four independent runs of 1 to 20 subpopulations ($K = 1$ to 20) were performed using 100,000 Markov chain steps after a burn-in period of 50,000 steps. We compared the likelihood estimate of each of the K values assayed to determine the number of K populations present in *A. brassicicola*. The number of genetically discrete populations was estimated based on the maximum log probability of data $\ln P(D)$ for different values of K and by using the statistic ΔK (18), which considers the rate of change in $\ln P(D)$ values among successive K runs to account for patterns of dispersal that are not homogeneous among populations.

To determine whether there was an excess (a recent population bottleneck) or deficit (a recent population expansion) in H (gene diversity) relative to the number of alleles present in *A. brassicicola* populations, we used Bottleneck v1.2 (16, 47). Rare alleles are lost faster than gene diversity (H) and can be further reduced in founder populations or bottlenecked populations that have experienced recent reductions in their effective population sizes. Therefore, H becomes larger than the gene diversity expected under mutation drift equilibrium (H_{EQ}) after going through a bottleneck, because H_{EQ} is based on the observed number of alleles. When the population size is restored, the average number of alleles is predicted to increase faster than the gene diversity until it reaches mutation drift equilibrium (43). The sign and Wilcoxon significance tests were used to determine whether loci displayed a significant excess ($H > H_{EQ}$) or deficit in gene diversity under a mutation drift equilibrium for loci evolving under the stepwise mutation (SMM) and two-phase mutation models (TPM) (70% SMM and 30% IAM: infinite-allele model) (16). A qualitative descriptor of the allele frequency

distribution ("mode shift" indicator) that distinguishes bottlenecked populations from stable populations (34) was also investigated.

RESULTS

Gene and genotypic diversity. A total of 401 isolates were analyzed with 11 microsatellite markers, all but one of which (Abmic-12) were polymorphic. All polymorphic loci were selectively neutral according to the Ewens-Watterson test for neutrality (data not shown). The highest number of alleles per locus within populations was seven in population CT0S (locus, Abmic-2). The number of alleles observed among populations as an average across loci ranged from 1.8 to 2.5 (Table 1). Gene diversity (H) values were similar across all populations and ranged from $H = 0.22$ to 0.36 (Table 1).

Across all populations, 164 distinct haplotypes were identified ($\hat{G}/N\% = 15$; clonal fraction = 0.59) (Table 1). Genotypic diversities varied among populations, with the highest genotypic diversity observed in DUD ($\hat{G}/N\% = 61$; clonal fraction = 0.20) and the lowest in DUI ($\hat{G}/N\% = 17$; clonal fraction = 0.70). When populations were clone corrected to represent only one member of a clone per plant to avoid overrepresentation of clones (e.g., due to splash dispersal of asexual spores), genotypic diversities were always higher due to the smaller sample sizes and ranged from the lowest in DUI ($\hat{G}/N\% = 19$; clonal fraction = 0.68) to the highest in CT18 ($\hat{G}/N\% = 68$; clonal fraction = 0.12) (Table 1).

A number of haplotypes were shared among different populations within regions, and even across all three geographic regions examined. Thirty-nine of the haplotypes occurred at least twice in two or more different populations. The most common haplotype was found in six populations and was isolated 29 times, which accounted for 7% of the total sample. This haplotype was found in two adjacent regions, Durras and Moruya-Bodalla, which are approximately 34 km apart. A total of 83 haplotypes were found only once in the entire metapopulation.

Mating types and linkage disequilibrium. Haplotypes representing individual populations only were assayed for their mating type alleles with the new primers designed from the GenBank accessions of the mating type locus. Of the 211 isolates analyzed, 210 produced a single amplicon of the expected size corresponding to a MAT1-1 (683-bp) or MAT1-2 (276-bp) haplotype. One isolate produced a double band. This isolate most likely was contaminated during PCR preparation and was excluded from the analyses.

Both mating types were found in each of the 11 populations of *A. brassicicola*. In 7 out of the 11 populations, both mating types were found on the same plant. Four of the 11 populations (CT18, DUD, DUI, and MBS) differed significantly from a 1:1 mating type ratio based on a χ^2 test (Table 2). On the regional scale, only Central Tilba deviated significantly from a 1:1 mating type ratio ($P = 0.01$). On average, across all populations analyzed, mating type frequencies did not differ significantly from a 1:1 ratio (Table 2). Using the I_A and $\bar{r}_d$ tests, only two populations were not in linkage equilibrium, DUI and DUL (Table 2).

Population structure. Estimates of θ (among-population differentiation) showed low to moderate levels of differentiation in pairwise comparisons between the majority of populations

TABLE 2. Mating type frequencies and multilocus association tests for 11 *A. brassicicola* populations representing three regions of the southern New South Wales coast, Australia^a

Population or region	No. of isolates			Multilocus association ^a		
	MAT1-1	MAT1-2	Total	χ^2	I_A	$\bar{r}_d$
Population						
CT0S	16	8	24	2.667	0.088	0.012
C17B	11	7	18	0.889	0.004	0.001
CT18	11	3	14	4.571a	-0.037	-0.005
DUD	4	16	20	7.200b	0.166	0.021
DUG	12	13	25	0.040	0.078	0.013
DUI	9	2	11	4.455a	0.559a	0.094a
DUL	17	9	26	2.462	0.191a	0.024a
MBH	9	8	17	0.059	-0.131	-0.023
MBON	10	14	24	0.667	-0.006	-0.001
MBJ	7	7	14	0.000	0.214	0.036
MBS	2	15	17	9.941b	-0.028	-0.028
Region						
Central	38	18	56	7.143a		
Tilba						
Durras	42	40	82	0.049		
Moruya-Bodalla	28	44	72	3.556		
Total	108	102	210	0.171		

^a χ^2 was based on a 1:1 ratio mating type with 1 degree of freedom. Mating type frequencies followed by "a" ($P < 0.05$; significant multilocus association at $P < 0.001$) and "b" ($P < 0.01$) deviated significantly from a 1:1 ratio; I_A , index of association as calculated in Multilocus v3.1 (1); $\bar{r}_d$, multilocus association as calculated in Multilocus v3.1 (1). The number of randomizations was 1,000 in all multilocus association tests.

(Table 3). In general, θ values were higher among populations from different regions than within the same region. When populations were combined into regional populations, the θ values were all significant but were higher between the two regions that are geographically the furthest separated, i.e., Central Tilba and Durras (approximately 83 km apart) ($\theta = 0.10$; $P = 0.001$) than between Central Tilba and Moruya-Bodalla ($\theta = 0.06$; $P = 0.001$) or Durras and Moruya-Bodalla ($\theta = 0.06$; $P = 0.001$). Despite this, there was no significant relationship between genetic distance and geographic distance ($R_{xy} = 0.128$; $P = 0.157$) or Φ_{pt} (population genetic differentiation) and geographic distance ($R_{xy} = 0.05$; $P = 0.30$) in a Mantel test, indicating that the populations have not reached a genetic-drift-gene flow equilibrium, typical of recent founder populations.

In a hierarchical analysis of genetic distribution (AMOVA), genetic differentiation was low, although it was significant among regions ($df = 2$; $\Phi_{pt} = 0.017$; $P = 0.010$). Significant genetic differentiation ($P = 0.010$) was also observed among populations within regions ($df = 8$; $\Phi_{pt} = 0.137$) and within populations ($df = 200$; $\Phi_{pt} = 0.151$). Most of the genetic diversity observed (85%) was distributed among individuals within a population. Thirteen percent of the genetic variation was distributed among populations and only 2% among regions.

A cluster-based method was used to infer the minimum number of clusters (K) required to explain the total sum of genetic variation observed (48) in the microsatellite and AFLP data sets. The results from Structure indicated that $K = 8$ or $K = 9$ population groups exist among *A. brassicicola* isolates

TABLE 3. Pairwise comparisons of population differentiation among clone-corrected populations of *A. brassicicola*

Population	Differentiation									
	CT17B	CT18	CT0S	DUD	DUG	DUI	DUL	MBH	MBJ	MBON
CT18	0.19 ^a									
CT0S	0.20 ^a	0.15 ^a								
DUD	0.04	0.19 ^a	0.19 ^a							
DUG	0.12 ^a	0.15 ^a	0.15 ^a	0.10 ^a						
DUI	0.03	0.29 ^a	0.22 ^a	0.05 ^a	0.14 ^a					
DUL	0.16 ^a	0.29 ^a	0.30 ^a	0.13 ^a	0.15 ^a	0.08				
MBH	0.17 ^a	0.30 ^a	0.18 ^a	0.17 ^a	0.09 ^a	0.10 ^a	0.14 ^a			
MBJ	0.07 ^a	0.28 ^a	0.21 ^a	0.07	0.11 ^a	0.04	0.07 ^a	0.02 ^a		
MBON	0.18 ^a	0.17 ^a	0.14 ^a	0.26 ^a	0.18 ^a	0.26 ^a	0.34 ^a	0.27 ^a	0.28 ^a	
MBS	0.17 ^a	0.19 ^a	0.20 ^a	0.13 ^a	0.04	0.18 ^a	0.15 ^a	0.06	0.09 ^a	0.23 ^a

^a Theta value was significant at $P < 0.001$.

along the NSW coast, as estimated from the microsatellite or AFLP data, respectively (Fig. 1). In both data sets, ΔK gave the strongest indicator of the number of populations estimated (Fig. 1). In the AFLP data set, the populations mostly corresponded to the broad geographic regions from which isolates were collected. Clusters 1, 2, 5, 6, and 7 consisted exclusively of isolates collected from Durras, although the clusters did not strictly represent individual subpopulations (i.e., DUD, DUI, DUG, and DUL), as these were admixed among clusters. Clus-

ters 3 and 4 consisted of a mixture of Moruya and Durras isolates, whereas clusters 8 and 9 consisted of a mixture of Central Tilba and Moruya isolates. Patterns were less clear in the microsatellite data set, with all clusters having representatives of most of the subpopulations analyzed (Fig. 2). Grouping of individuals by geographical location (populations) clearly showed admixture among populations, as most populations consisted of individuals from all eight inferred clusters (Fig. 3).

Evidence for population expansion. All microsatellite loci followed a stepwise mutation method (SMM) of evolution (Table 4). When all local populations were combined into a single population representing the southern coast of NSW, the sign tests in Bottleneck showed a significant H deficit in 10 of the 11 loci under both an SMM ($P = 0.0015$) and a TPM ($P = 0.0018$) model of evolution, indicating recent population expansion. Only one locus (locus 9) under both models of evolution showed a significant gene diversity excess, indicating a recent population bottleneck (Table 4). With the Wilcoxon test, the probability that all loci had an H deficit was $P = 0.0017$ for TPM and $P = 0.0005$ for SMM. In regional populations, the Wilcoxon test also showed a significant H deficit in 10 of the 11 loci under an SMM in Durras ($P = 0.0017$) and Moruya-Bodalla ($P = 0.0005$) and in 8 of the 11 loci in Central Tilba ($P = 0.0105$), indicating recent population expansion in regional populations. Sign values gave similar probability values for regional populations. Allele frequencies followed a normal L-shaped distribution, indicating that the populations are at mutation drift equilibrium and did not experience a recent population bottleneck in all comparisons.

DISCUSSION

In an earlier study, Bock and colleagues (9, 10) examined the genetic diversity of *A. brassicicola* populations along the south-eastern coast of Australia, using AFLPs as molecular markers. The current study substantially expands on that earlier analysis by additionally including pathogen populations from two other regions, Central Tilba and Moruya-Bodalla, thus allowing evaluation of genetic patterns across multiple spatial scales. These data were complemented with additional analyses using microsatellites, which are expected to be more sensitive to recent phylogeographic and demographic events (e.g., bottlenecks or population expansions) due to their higher rate of evolution. The high variation and codominant nature of microsatellites

FIG. 1. Comparison of $\ln(K)$ and ΔK values for the microsatellite data set (A) and the AFLP data set (B) calculated from the Structure v2.2 output, where the hypothesized number of populations ranged from 1 to 20.

FIG. 2. Cluster analyses of *A. brassicicola* populations from the NSW coast of Australia (results from Structure v2.2). Each individual is represented by a bar, divided into K colors, where K is the number of clusters assumed. Individuals are sorted according to Q , the inferred clusters. (A) Microsatellite data; $K = 8$. (B) AFLP data; $K = 9$.

make them particularly sensitive for detecting population changes (58). For example, microsatellite mutation rates were estimated to be several orders of magnitude greater than those of regular nonrepetitive DNA for the ascomycetous fungus *Neurospora* (17).

The results from the analyses of microsatellite data indicated moderate levels of gene and genotypic diversity among all populations. Of the 401 isolates included in this analysis, 211 haplotypes (52%) could be identified. Given the high variability of microsatellite markers, it was somewhat surprising to find that AFLPs were able to distinguish more haplotypes (91%) in a previous study (202 from 222) (10). However, only 12 microsatellite loci were used in this study, one of which was monomorphic. Compared to three primer combinations used with AFLPs, resulting in 47 to 69 loci per primer combination, in this case, AFLPs clearly provided a locus-rich marker with higher resolution.

Population structure and mating. Significant pairwise population differentiation among most populations indicates significant spatial structure. Local beach populations of *Cakile maritima* are separated by natural barriers (rocky outcrops), which most likely provide enough isolation for population structure to develop in the pathogen, despite effective dispersal of seeds in ocean currents. At the same time, the absence of isolation by distance (there was no significant association between population differentiation and geographic distance) ($R_{xy} = 0.05$; $P = 0.30$) indicates that the populations have not

reached a gene flow/genetic-drift equilibrium (56). This might be because these populations represent recent founder populations (29), where a sufficient number of generations may not have passed since colonization by *C. maritima* 100 years ago. Alternatively, dispersal in ocean currents is not restricted to adjacent populations and therefore does not represent a stepping stone dispersal gradient among populations.

Further evidence for population structuring in the NSW population, complementing analyses by the population-based method (8 estimates), is found in the number of clusters produced by Structure. Although Structure identified fewer clusters ($K = 8$ with microsatellite data; $K = 9$ with AFLP data) than the 11 populations analyzed, significant structure does exist. The structure is made up of clusters that are admixed from individuals representing different populations within a region, or even individuals from different regions, indicating a common source population(s) for the southern NSW populations or gene flow/migration among populations. A common founder source is also suggested by the AMOVA analyses, where most (85%) genetic diversity was found within populations and only 2% among regions.

Linkage equilibrium, equal mating type allele frequencies, and moderate levels of genotypic diversity in most populations indicate that *A. brassicicola* is reproducing sexually and that mating is not hampered by an absence of a particular mating type allele. This result is in contrast to an earlier AFLP analysis of a more limited sample, where the results suggested that the

FIG. 3. Cluster analyses of *A. brassicicola* populations from the NSW coast of Australia. Shown are the results of microsatellite data from Structure v2.2. Each individual is represented as a bar, divided into K colors where K is the number of clusters assumed, i.e., $K = 8$. Individuals are sorted according to geographic region and original population (1 to 12). pop1, CT05; pop2, CT17B; pop3, CT18; pop4, DUD; pop5, DUG; pop6, DUI; pop7, DUL; pop8, MBC; pop9, MBH; pop10, MBI; pop11, MBON; pop12, MBS.

TABLE 4. Comparison of observed gene diversity (H) with expected gene diversity at mutation drift equilibrium (H_{EQ}) calculated from the observed number of alleles under the SMM and TPM (16)^a

Group	Mutation model	D/E	
		SMM	TPM
Central Tilba	SMM ($P = 0.0000$)	8/3 ^b	7/4
Durras	SMM ($P = 0.0000$)	10/1 ^b	10/1 ^b
Moruya-Bodalla	SMM ($P = 0.0000$)	10/1 ^b	9/2 ^b
Total	SMM ($P = 0.0000$)	10/1 ^b	10/1 ^b

^a D/E is the number of loci showing a deficit/excess of gene diversity. Significance estimates of gene diversity excess ($H > H_{EQ}$) or deficiency, on average, across loci were obtained using the one-tailed Wilcoxon test and the sign test (16, 47).

^b $P \leq 0.01$.

populations were all in linkage disequilibrium (10), although the authors deemed the populations to be randomly mating due to the high genotypic diversities observed. Only two populations from Durras (DUI and DUL) showed significant linkage disequilibrium among microsatellite loci. In the case of DUI, the small sample size would have reduced the power of the analyses. Both mating types were detected in all populations, and most populations also had equal proportions of mating type alleles, indicating frequency-dependent selection (36) and providing further support for the occurrence of sexual reproduction in *A. brassicicola*.

For populations with unequal proportions of mating types (CT18, DUD, DUI, and MBS), other than small sample sizes (as in DUI), this may be due to less frequent sexual reproduction or the possibility that selection may have favored one mating type over the other by chance. Note that either MAT1-1 or MAT1-2 isolates could be in excess, indicating the lack of a selection bias favoring a particular mating type. In either case, it is possible that the populations are reproducing sexually but have not yet reached equilibrium among alleles at loci. For example, if the frequency of recombination is low (e.g., recombination rate [r] = 0.05), it would take more than 50 generations for linkage disequilibrium to disappear (26).

Population expansion. The levels of genetic diversity observed in *A. brassicicola* are much higher than would be generally expected for an organism that has been recently founded, as genetic diversity in such situations is usually purged through bottlenecks and genetic drift. Founder pathogen populations are also subject to genetic drift as a result of, e.g., low host availability or unfavorable climatic conditions that limit their evolutionary potential. However, many fungal pathogens that are founders in new geographic areas exhibit relatively high levels of genetic diversity compared to their centers of origin, e.g., *R. secalis* (32) and *M. graminicola* (33). A similar phenomenon was observed for several aquatic invasive species (52), as well as for invasive plants (64) and animals (11). In these organisms, the genetic diversity of invasive species has been shown to be equal to or higher than that of native populations; in the case of invasive animals, approximately 80% of the genetic diversity present in native populations is maintained in introduced populations (11). This phenomenon is referred to as the “genetic paradox,” i.e., how do newly founded populations overcome low genetic diversity and ex-

pected low evolutionary potential to become established outside their natural ranges?

Three scenarios might explain the high levels of gene and genotypic diversity observed in *A. brassicicola*: (i) there were multiple founder populations, resulting in the admixture of populations; (ii) there was population genetic expansion, where rare alleles accumulated in populations due to mutations, etc.; or (iii) *A. brassicicola* has a worldwide distribution and was present in Australia prior to the introduction of *C. maritima*. All three scenarios are plausible, as evidenced by the high levels of population admixture identified in Structure. Also, many haplotypes were shared among populations within, as well as among, regions, indicating multiple introductions of the same haplotype. This is possible for fungal pathogens with a pronounced asexual reproductive phase. Furthermore, our analysis showed that all individual populations were admixed and consisted of haplotypes from multiple populations within a region or even from other regions (especially SSRs), indicating multiple founder events. Evidence for recent population expansion was detected using Bottleneck (47), where all regional populations showed a significant deficit in gene diversity under the SSM, indicating that the number of alleles increase faster than the gene diversity (16, 34). Because bottleneck detects only recent bottlenecks or expansions occurring within the last ~100 years, i.e., 0.2 to 4.0 N_e (effective population size) generations ago (16, 34), the population expansions observed here are likely to be associated with migration events from neighboring populations. Whether *A. brassicicola* has a natural occurrence in Australia is unknown but is suspected, given its ubiquitous presence; however, it is worth noting that in the coastal zone where *Cakile* occurs, there is a paucity of other potential hosts. Moreover, it is also probable that the pathogen has been reintroduced to Australia with the importation of brassicaceous crops and other exotic hosts, such as *C. maritima*. Naturally, *A. brassicicola* disperses either as airborne spores or with seeds in ocean currents (as can be the case for *C. maritima*), and therefore, population expansion due to admixture by dispersal is likely.

Predicting the ecological and evolutionary dynamics of novel plant-pathogen interactions (or existing interactions occurring in new environments as a result of introductions) is of considerable practical interest (44). For example, genetic population expansion of introduced pathogens could pose a significant threat to native plant communities or agricultural crops as a result of an increase in the pathogen's evolutionary potential. A host shift or host range expansion has to be considered possible for introduced pathogens that are expanding genetically. *Alternaria* produces a suite of toxins (30) that, once introduced into Australia, has the ability to introgress into other *Alternaria* populations through gene flow and sexual recombination, thereby increasing the evolutionary potential and pathogenicity of the pathogen. While the majority of native brassicaceous species in Australia are found in the more arid inland zones (27), there are also a range of introduced weedy species, as well as economically significant agricultural and vegetable crops, that could potentially act as alternative hosts for *A. brassicicola* (e.g., *Brassica napus* [canola] and *Brassica oleracea* [cabbages]) in Australia (55).

In contrast to previous studies of *A. brassicicola* using AFLPs, microsatellites were able to uncover recent population

demographic events that were not detected with the previous markers. Populations that are likely to have experienced significant genetic expansion or admixture as a result of multiple introductions in the last ~100 years were identified. This conclusion is supported by high observed levels of gene and genotypic diversity, as well as the Structure analyses. Microsatellite markers also indicated that most populations were in linkage equilibrium and therefore randomly mating. This study highlights the ability of an invasive fungal plant pathogen to invade, become established, and then genetically expand to form a pathogen population with a high evolutionary potential.

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