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Markers linked to vegetative incompatibility (*vic*) genes and a region of high heterogeneity and reduced recombination near the mating type locus (*MAT*) in *Cryphonectria parasitica*

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Abstract

To find markers linked to vegetative incompatibility (*vic*) genes in the chestnut blight fungus, *Cryphonectria parasitica*, we constructed a preliminary linkage map. In general, this map is characterized by low levels of polymorphism, as evident from the more than 24 linkage groups observed, compared to seven expected from electrophoretic karyotyping. Nonetheless, we found markers closely linked to two *vic* genes (*vic1* and *vic2*) making them candidates for positional cloning. Two markers were found to be linked to *vic2*: one cosegregated with *vic2*, i.e., it is 0.0 cM from *vic2*, the other was at a distance of 4.5 cM; a single marker was found 4.0 cM from *vic1*. The closest markers linked to three other *vic* genes (*vic4*, *vic6*, and *vic7*) were >15 cM away; additional markers are needed before efficient positional cloning of these three *vic* genes can be realized. In contrast to the low levels of polymorphism observed across most of the *C. parasitica* genome, the linkage group containing the *MAT* locus appears to harbor an extremely high level of RAPD heterogeneity and reduced recombination. Markers within this highly heterogeneous region are in linkage disequilibrium in some natural populations; however, recombination is clearly evident between this region and the *MAT* locus.
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1. Introduction

Vegetative and heterokaryon incompatibility function as a self/non-self recognition system during asexual interactions between individuals in filamentous fungi (Glass and Kaneko, 2003; Leslie, 1993; Saupe, 2000). Recognition is controlled by multiple vegetative incompatibility (*vic*) or heterokaryon incompatibility (*het*) genes, where individuals heteroallelic at one or more genes are incompatible. Heterokaryon incompatibility genes, to date, have been cloned only from *Neurospora crassa* and *Podospora anserina*. In *N. crassa*, three *het* genes, *het-6*, *un-24*, and *het-c* were cloned by positional cloning (Saupe et al., 1996; Smith et al., 2000). In *P. anserina*, four have been cloned, *het-s*, *het-c*, *het-d* and *het-e*, without the benefit of positional cloning, by functional complementation (Coustou et al., 1997; Espagne et al., 2002; Saupe et al., 1994, 1995). Cloning *het* (or *vic*) genes in other species has not been accomplished, in part, because of the lack of genetic analyses and linked markers for positional cloning.

Cryphonectria parasitica, the ascomycete fungus that causes chestnut blight, is a species for which vegetative incompatibility has been studied extensively, although *vic* genes have not yet been cloned. *C. parasitica* caused devastating epidemics to American and European chestnut trees (*Castanea dentata* and *Castanea sativa*, respectively) after being introduced from east Asia into North America and Europe (Anagnostakis, 1987; Griffin, 1986). Interest in vegetative incompatibility in *C. parasitica* derives mainly from

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its potential impact on biological control of chestnut blight by fungal viruses, a phenomenon known as hypovirulence (Anagnostakis, 1982; Heiniger and Rigling, 1994; Milgroom and Cortesi, 2004; Van Alfen et al., 1975). In *C. parasitica*, vegetative incompatibility is controlled by at least six unlinked *vic* genes (Cortesi and Milgroom, 1998). The success of hypovirulence depends largely on transmission of viruses between individuals (Milgroom, 1999). However, horizontal transmission is restricted between *vic* genotypes (vegetative compatibility, or vc, types) (Anagnostakis, 1983; Cortesi et al., 2001). Although some transmission does occur between *vic* genotypes in the field (Carbone et al., 2004), hypovirulence appears to be more effective in populations that are less diverse for *vic* genotypes (Anagnostakis et al., 1986), presumably because of fewer barriers to horizontal transmission (Milgroom, 1999; Milgroom and Cortesi, 2004).

To understand more about the mechanisms of vegetative incompatibility and virus transmission, and to understand the evolutionary forces acting on *vic* genes, it would be advantageous to clone and sequence *vic* genes. An additional advantage of cloning *vic* genes would be to expedite *vic* genotyping for assessing vc type diversity. One approach to cloning is to find markers linked to *vic* genes by constructing a preliminary linkage map of *C. parasitica*. Over the past few decades, genetic linkage maps have been developed for a number of filamentous fungi including several ascomycetes (Jurgenson et al., 2002a,b; Kaye et al., 2003; Metzenberg et al., 1985; Pedersen et al., 2002; Tzeng et al., 1992; Xu and Leslie, 1996; Zhong et al., 2002). Markers from these maps have been very useful in positional cloning of genes involved in particular phenotypes (Attard et al., 2002; Zhong and Steffenson, 2002).

Currently, relatively little is known about the genome and genome organization in *C. parasitica*. Previous linkage studies in *C. parasitica* have been limited to only a few loci (Anagnostakis, 1982; Cortesi and Milgroom, 1998; Milgroom et al., 1992, 1996). In this study, we used random amplified polymorphic DNAs (RAPDs) (Williams et al., 1990), which have been extensively used for mapping within defined pedigrees. RAPD markers have been identified throughout most portions of various genomes, repetitive as well as gene-rich regions (Welsh and McClelland, 1990; Williams et al., 1990). This is particularly significant in mapping genomes of organisms where very little genome sequence polymorphism data is available.

Sequence data for approximately 2200 individual open reading frames from a mixed cDNA library of *C. parasitica* were recently made available (Dawe et al., 2003). Twelve of these sequences were found to have high similarities to *het* genes or *mod* genes, modifiers of vegetative incompatibility, from *N. crassa* and *P. anserina*. However, polymorphisms within these gene sequences in *C. parasitica*, and their linkage relationship with specific known *vic* genes have yet to be determined.

The primary objective of this study was to identify markers that might aid in the eventual positional cloning of *vic* genes in *C. parasitica*. The specific objectives were: (i) to employ RAPD PCR to identify segregating polymorphisms in a laboratory cross of *C. parasitica*; (ii) to identify single nucleotide polymorphisms (SNPs) in previously identified *het/mod* gene homologs; (iii) to examine the segregation of these markers; and (iv) to construct a preliminary genetic linkage map with the goal of placing *vic* genes within this recombination-based framework.

2. Materials and methods

2.1. Crosses and DNA extraction

To maximize heterogeneity, a wide cross was performed between Japanese isolate JA17 (*MAT-2*) and Italian isolate P17-8 (*MAT-1*) of *C. parasitica* (referred to as cross MJ1; Cortesi and Milgroom, 1998). A Japanese parent was used because *C. parasitica* is thought to be native to Japan and Japanese populations are more polymorphic than populations in either the United States or Europe (Milgroom et al., 1996). JA17 was one of three isolates found in a sample from Japan that was not compatible with any of the known *vic* genotype testers (Liu and Milgroom, unpublished data). A total of 194 ascospore progenies were generated and genotyped for five segregating *vic* genes by Cortesi and Milgroom (1998). All progeny were also assayed for mating type by PCR, using methods described previously (Marra and Milgroom, 1999; McGuire et al., 2004). Total genomic DNA was prepared as described in Milgroom et al. (1992) and sent to the Southern Institute of Forest Genetics in Saucier, Mississippi for RAPD PCR analysis.

2.2. RAPD and SCAR marker amplification and detection

RAPD amplification was based on a modification of the protocol reported by Williams et al. (1990). RAPD reaction mixtures consisted of the following in 24 μL total volume: 1.25 ng of template DNA, 1 μL of 10-mer primer DNA (5 μM stock), 3.6 μL of dNTPs (1 mM stock), 2.4 μL 10× *Taq* DNA polymerase reaction buffer (500 mM KCl, 100 mM Tris–HCl, 1.0% Triton X-100, 15 mM $MgCl_2$), and 0.8 U of *Taq* DNA polymerase. Reactions were loaded in flexible microtitre plates and overlaid with 25 μL mineral oil. Microtitre plates were placed in preheated (85 °C), programmable temperature cyclers (MJ Research PTC-100) and covered with mylar film. DNA samples were amplified using the following thermal profile: 5 s at 95 °C; 1 min 55 s at 92 °C; followed by 45 cycles of 5 s at 95 °C, 55 s at 92 °C, 1 min at 35 °C, and 9 min at 72 °C. Completed reactions were electrophoresed in 2% agarose gels and TAE buffer (40 mM Tris base, 20 mM sodium acetate, 2.0 mM EDTA, and glacial acetic acid to pH 7.2) for 3.5 h at 3 V/cm (150 V). About 3.0 μL of loading buffer (10× TAE, 50% glycerol, and 0.25%

bromophenol blue) was added to each reaction prior to electrophoresis. After electrophoresis, the gels were stained with ethidium bromide (0.4 μg/mL) for 45 min, washed in dH_2O for 1.0 h, and photographed using UV light. Codominant SCAR markers were amplified as described by Davis et al. (2005) and separated in 3% Trevigels as described by the manufacturer (Trevigen, Inc. Gaithersburg, MD). Polymorphisms were visualized as described above for RAPDs.

2.3. EST marker development, amplification, and detection

Primers were designed for 11 of the 12 expressed sequence tags (ESTs) in *C. parasitica* found by Dawe et al. (2003) to have significant matches to five different vegetative incompatibility-related genes (*het* and *mod*) previously identified in *N. crassa* and *P. anserina*. Visual inspection of the sequence submitted for two clones (13B11 and 12D10; GenBank Accession Nos. CB687222 and CB688502, respectively) revealed that they differed by only 3 of 604 overlapping bases. Two of the three differences were the direct result of ambiguous base calls ("N"), one of which created a questionable single base insertion. Therefore, we felt that they likely represented transcripts from the same ortholog (vs. different paralogs), and only the transcript with the lower *E*-value was further evaluated. Primers were designed using Primer 3 (Rozen and Skaletsky, 1998) to cover as much of the published sequence as possible. DNA from the parents of the mapping cross (JA17 and P17-8) were PCR amplified using the following "touchdown" profile: 2 min at 95 °C; followed by 10 cycles of 1 min at 92 °C, 20 s at *X*, and 20 s at 72 °C, where $X = 73$ °C in the first cycle and decreases by 2 °C every cycle thereafter; followed by 25 cycles of 20 s at 92 °C, 20 s at 55 °C, 20 s at 72 °C; followed by a 5 min extension at 72 °C and an indefinite hold at 4 °C. About 1 μL of PCR product was sequenced in both the forward and reverse directions on an ABI PRISM® 3100 Genetic Analyzer using the BigDye® Terminator v 3.1 Cycle Sequencing Kit as described by the manufacturer (Applied Biosystems, Inc. Foster City, CA). Single nucleotide polymorphisms (SNPs) were identified by comparing the sequence data obtained for each of the parental isolates using the software Sequencher version 5.1.1 (Gene Codes Corporation, Ann Arbor, MI). All SNPs observed were transitions (A>G or T>C). Therefore, to increase primer specificity, a SNP-specific primer (of similar T_m to the original flanking primers) was developed for each polymorphic EST that contained either the G or C base at the 3′ end. Three-primer PCR was then performed using the above-mentioned thermal profile.

2.4. Marker identification and naming

To identify segregating polymorphisms, RAPD 10-mer primers (primer sets A through Z, Operon Technologies, Inc. Alameda, CA; and various primers 001-799, University of British Columbia), the SCAR primers, and the EST-SNP primers were screened against a panel of DNAs consisting of eight randomly selected ascospore progenies from cross MJ1. Assuming 1:1 segregation, eight progenies gave us greater than a 99% probability ($=1-(0.5)^7$) of detecting segregation. RAPD markers were chosen based on the intensity of amplification (only brightly stained bands were scored), the absence of co-migrating DNA fragments, and their reproducibility with the same eight ascospore progenies. Segregating RAPD markers were denoted by the manufacturer primer code (corresponding to the 10-mer primer responsible for their amplification), followed by a four-digit number indicating the fragment size in base pairs. A prefix 'j' for parent JA17 or 'p' for parent P17-8 is used to refer to the parental origin of the band-present allele. Codominant RAPD markers, i.e., those markers amplified by the same 10-mer primer, but found to be in complete repulsion phase linkage, were reduced to single markers and named according to the larger of the two alleles observed. A prefix 'b' for 'both' is used to refer to the parental origin of these markers. Since the SCAR markers were developed from RAPDs (Davis et al., 2005), they are named similarly to the RAPDs but uniquely identified in this paper by a prefix 's'. The EST-SNP markers were named according to their EST clone ID (Dawe et al., 2003). All markers were further characterized on an additional 184 ascospore progenies ($n = 192$ total).

2.5. Segregation and linkage analysis

Each marker was tested for goodness-of-fit to the expected Mendelian segregation ratio (1:1) using chi-square (χ^2) analysis. Markers with significant segregation distortion ($P < 0.05$) were initially excluded from preliminary mapping analyses, but then added later to the dataset once a framework was established. The software package MAPMAKER/EXP version 3.0 (Lincoln et al., 1992) was used to produce a genetic linkage map. Linkage groups were established using a minimum log of the odds (LOD) threshold of 5.0 and a maximum recombination distance of 45 cM. If a putative linkage group consisted of fewer than nine loci, an exhaustive search for the "best" marker order was performed using the COMPARE command. For linkage groups consisting of nine or more markers, the BIG LODS command was used to identify marker bins in an attempt to make exhaustive searches more manageable. Marker orders were further verified using the RIPPLE command and a maximum window size of eight loci. Once a "best" order was determined, potentially spurious genotypes, i.e., suspect double-recombinants or highly recombinant progeny, were identified using the GENOTYPES command with error detection "on". All spurious genotypes were re-verified from archived gel images and the data (if changed) subject to re-analysis. All marker orders suggested by MAPMAKER/EXP were further investigated using the software package JoinMap® version 3.0 (Van Ooijen and Voorrips, 2001).

2.6. Genome length estimates

We calculated observed genome length as $G=\sum G_I$, where G_I is the total genetic distance of linkage group or pair I. We also estimated expected genome length using the method-of-moments estimator (Hulbert et al., 1988). This estimate is based on our partial linkage data, as $G=m(m-1)X/K$, where m is the number of markers or loci typed, X is the map distance corresponding to the LOD threshold Z for declaring linkage, and K is the number of marker pairs having LOD values at or above Z. As this method assumes a random distribution of markers, some adjustments were made for non-randomly associated loci. Genome size estimates using this method were performed over a range of LOD thresholds. We estimated the standard error associated with these estimates by resampling 90% of the markers without replacement and calculating genome size for 1000 iterations. Observed and expected genome lengths were used to estimate the percentage of the genome likely to be covered by the markers, as well as to estimate the relationship between physical and genetic distance. The probability for the expected number of loci per linkage group was evaluated under the cumulative Poisson distribution using a one-tailed test at $\alpha \leqslant 0.05$. Deviations from this distribution were used as an indication of significant clustering or dispersion of markers.

3. Results

3.1. Preliminary linkage map

In an initial screen of 1128 unique 10-mer RAPD primers against eight ascospore progenies from cross MJ1, 119 primers amplified a total of 140 RAPDs that met our grading criteria for polymorphic markers (brightly stained, lack of co-migrating DNAs, and reproducible within the screening set). Among these 119 primers, 100 amplified a single RAPD, 17 amplified two RAPDs, and two amplified three RAPDs. Out of 11 co-dominant SCAR markers reported by Davis et al. (2005), eight were segregating in this cross and hence informative for mapping. Of the eleven EST products sequenced, only four were found to contain SNPs between the parents of cross MJ1 (Table 1). Data for these 152 markers were scored on a total of 192 ascospore progenies.

Chi-square analysis indicated that all but 10 of the markers segregated according to the expected 1:1 Mendelian inheritance ratio. Only 10 markers were significantly distorted from 1:1 at $P<0.05$, which is not much greater than the number expected by chance (5% of $152=7.6$). In total, 138 of the markers, all five *vic* genes and the *MAT* locus mapped at a LOD score of 5.0–17 linkage groups, consisting of three or more loci, and seven pairs (Fig. 1). The 138 markers included 128 RAPDs, seven SCARs, and three ESTs; 12 RAPDs, one SCAR, and one EST remained unlinked. The number of markers and genes mapped per linkage group ranged from 3 to 38, with a median of 6. The genetic distance spanned per linkage group ranged from 14.1 to 143.0 cM, with a median of 38.6 cM. Altogether the 17 groups and seven pairs spanned a total genetic distance of 1207.3 cM (Table 2 and Fig. 1). Nine of ten loci with segregation distorted at $P<0.05$ were found to map to four linkage groups and one pair (V,VI, IX, XVII, and p6). In every case, the distorted markers were located distally (markers followed by asterisks in Fig. 1).

Even though our power for detecting linkage was quite high with 192 progenies, a large number of linkage groups, each with relatively few markers, and pairs were inferred. This observation could be explained by unusually high rates of recombination across much of the *C. parasitica* genome and/or an insufficient number of polymorphisms to establish significant linkage. The latter seems more probable because the observed number of cross-overs per ascospore progeny is close to the expected. Although

Table 1
C. parasitica single nucleotide polymorphisms (SNPs) in sequences with high similarities to *het* and *mod* genes from *P. anserina* (Dawe et al., 2003)

CEST clone/ marker[a]	Name of match	Primer[b] sequence 5′–3′	Expected size (bp)[c]	Observed size (bp)	# of introns (intron size in bp)	SNP in coding or non-coding DNA (intron)
CEST-12A07	HET-C	F: TCTCCAGACGACCACAGACA F-SNP[d]: CCCCTCGCGACGACTC R: GTAGAAATCAGCACGCTTGG	F+R: 560 F-SNP+R: 224	F+R: 825 F-SNP+R: 224	3 (81, 91, 93)	Non-coding (93)
CEST-55F11	HET-C	F: CAACGCTACGAAAATCGACA F-SNP: AACCCCCTCGCGACGACTC R: CTTGAGGATGCCGACAATTT	F+R:398 F-SNP+R: 321	F+R: 491 F-SNP+R:321	1 (93)	Non-coding (93)
CEST-12E11	HET-C	F: TACCTCCGTCCCGTACTTTG F-SNP: ATGAGTCGACGGACTAATTGG R: TGGTGAAGGAGACGGATAGC	F+R: 383 F-SNP+R: 336	F+R: 648 F-SNP+R: 336	3 (81, 91, 93)	Non-coding (91)
CEST-25F07	MOD-E	F: AAGCCCAAGATTGAGGAGGT F-SNP: CAACCCCTCCGACATCACC R: CAAACTGCTCCCTTGTCCTC	F+R:368 F-SNP+R: 408	F+R: 533 F-SNP+R: 408	1 (165)	Coding

[a] Name of EST from Dawe et al. (2003).
[b] F, forward flanking primer, F-SNP, forward SNP-specific, and R, reverse flanking primer.
[c] Expected size of PCR product based on GenBank Accession Nos. CB688497, CB689732, CB687186, and CB688049.
[d] The underlined base identifies the single nucleotide polymorphism.

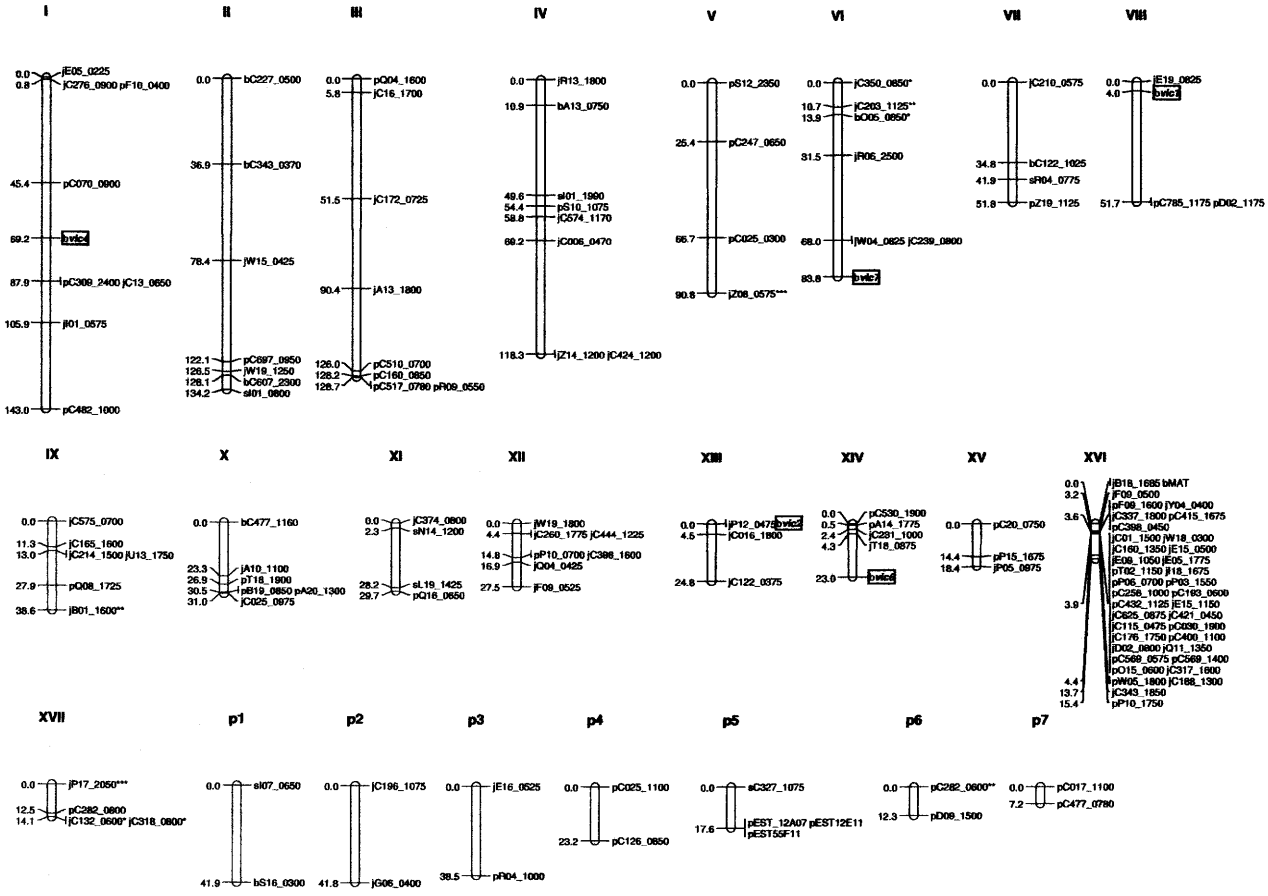

Fig. 1. Genetic linkage for cross MJ1 (JA17 × P17-8) of *C. parasitica* based on segregation of markers and genes in 192 ascospore progenies. The map consists of 128 RAPDs, seven SCARs, three ESTs, five *vic* genes, and the mating-type (*MAT*) locus linked at a minimum LOD score of 5.0. Dominant markers inherited from parent JA17 are identified by a prefix 'j' and those from parent P17-8 are identified with a prefix 'p'. Codominant RAPD loci with alleles inherited from each parent are identified with a prefix 'b'. Codominant SCAR markers are identified with a prefix 's'. Markers with distorted segregation ratios are identified by asterisks.

considerable variation has been observed within as well as among chromosomes, on average at least one chiasma (or site of crossing-over) per bivalent arm appears to be quite consistent across very diverse genera (Anderson et al., 2003; Dumas and Britton-Davidian, 2002; Jurgenson et al., 2002b; Sherman and Stack, 1995; Yin et al., 2004). Among the linked markers on our map, we estimated an average 8.69 (SD ± 3.25) recombination events per ascospore progeny (Fig. 2). If we include the 14 unlinked loci, the average number of recombination events per ascospore progeny is 15.69, which is not much greater than the 12–14 expected based on the observation of six to seven chromosomes in electrophoretic karyotyping (Milgroom et al., 1992).

3.1.1. Markers linked to vic genes

We observed RAPD markers significantly linked to all five of the *vic* genes (*vic1*, *vic2*, *vic4*, *vic6*, and *vic7*) segregating in cross MJ1. However, the distances between markers and *vic* loci varied considerably (Fig. 1). Marker P12_0475 cosegregated with *vic2*, i.e., it is 0.0 cM from *vic2* on LG XIII; marker C016_1800 mapped at a distance of 4.5 cM from *vic2*. Marker E19_0825 is the second closest to a *vic* gene, estimated to be 4.0 cM from *vic1* on LG VIII. RAPD markers linked to the remaining three *vic* genes (*vic4*, *vic6*, and *vic7*) were observed to be at distances >15 cM; the closest marker to *vic7* is 15.8 cM; the closest to *vic6* is 18.7 cM, and the closest to *vic4* is 18.7 cM.

3.1.2. Markers linked to the mating-type (MAT) locus

The linkage group containing the *MAT* locus (LG XVI) appears to harbor a region of high heterogeneity and reduced recombination. A significant excess of markers—37 of 152 (24.3%) markers—mapped to this linkage group (Table 2 and Fig. 1). Of these 37 markers, 26 were found to map to a single locus, i.e., they were inherited together as a single non-recombining group. Interestingly, this locus was found to be only 3.9 cM from the *MAT* locus. Although roughly equal numbers of polymorphisms were observed to be present in and inherited from each parent within this non-recombining region (15 and 11 in JA17 and P17-8, respectively), we could not rule out the possibility that the observed linkage pattern might be the result of an anomaly specific to the cross chosen for mapping.

To investigate this anomalous linkage group further, we mapped or screened markers in two additional crosses previously studied for inheritance of RFLP markers

Table 2
Marker distribution across the *C. parasitica* genome

Linkage group	Map length (cM)	Expected # markers	Observed # markers[a]	Parental origin of markers		
				JA17	P17-8	Both[b]
I[c]	143.0	17.06	9*	4	4	1
II	134.2	16.01	7**	2	1	4
III	128.7	15.36	8*	3	5	0
IV	118.3	14.11	8	5	1	2
V	90.8	10.83	4*	1	3	0
VI	83.8	10.00	7	5	0	2
VIII	51.8	6.18	4	1	1	2
VII	51.7	6.17	4	1	2	1
IX	38.6	4.61	6	5	1	0
X	31.0	3.70	6	2	3	1
XI	29.7	3.54	4	1	1	2
XII	27.5	3.28	7*	6	1	0
XIII	24.8	2.96	4	3	0	1
XIV	23.0	2.74	5	2	2	1
XV	18.4	2.19	3	1	2	0
XVI	15.4	1.83	38***	21	16	1
XVII	14.1	1.68	4*	3	1	0
P1	41.9	5.00	2	0	0	2
P2	41.8	4.99	2	2	0	0
P3	38.5	4.59	2	1	1	0
P4	23.2	2.76	2	0	2	0
P5	17.6	2.10	4	1	3	0
P6	12.3	1.47	2	0	2	0
P7	7.2	0.86	2	0	2	0
Total	1207.3	144	144	69	51	20
Avg. Spacing	8.38					

[a] The probabilities for the expected vs. observed number of markers were evaluated under the cumulative Poisson distribution. Deviations from this distribution were used as an indication of significant clustering or dispersion of markers.
[b] Codominant RAPDs, codominant RAPD-derived SCARs, *vic* genes and the *MAT* gene were considered as being inherited from both parents.
[c] *vic* genes and the *MAT* locus were included in both expected and observed marker counts.
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.
*** $P \ll 0.0001$.

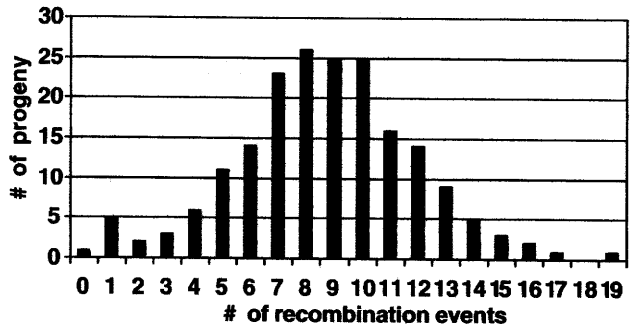

Fig. 2. Distribution of the number of inferred recombination events for 192 ascospore progeny of cross MJ1 of *C. parasitica*. Recombinations were inferred using the GENOTYPES command in MAPMAKER/EXP (Lincoln et al., 1992).

(Milgroom et al., 1996). In a cross ($n = 53$) between the Japanese isolate JA19 (*MAT-1*) and the US isolate EP155 (*MAT-2*), 120 of 137 segregating RAPDs mapped to 15 groups, consisting of three or more loci, and 10 pairs at a LOD threshold of 3.0 (data not shown). Thirty-eight of the 137 RAPDs (27.7%) mapped to the linkage group containing the *MAT* locus. Of these 38 markers, 29 were found to map to a single locus 3.0 cM from *MAT*. As in cross MJ1 reported above, roughly equal proportions of the RAPDs were present in and inherited from each parent (18 and 11 in JA19 and EP155, respectively). In a third cross between US isolates RH10-4 (*MAT-1*) × EP155 ($n = 6$), 49 of 149 RAPD markers (32.9%) cosegregated with the *MAT* locus. As above, approximately half the markers were present in and inherited from each parent (29 and 20 from RH10-4 and EP155, respectively). Taken together, these data suggest that within at least three crosses involving five different isolates of *C. parasitica* there is evidence for a region of high heterogeneity and reduced recombination that maps near the *MAT* locus.

Of the 37 markers found to map to the *MAT*-containing linkage group in cross MJ1, 18 were segregating in one or both of the other two crosses (**15** in JA19 × EP155 and **16** in RH10-4 × EP155, respectively; Table 3). Only marker B18_1685 was found to be completely linked with the *MAT* locus. In all three crosses, the band-present allele for marker B18_1685 was found to be in coupling with *MAT-2*. The sequence for primer B18 matches a region within the *MAT-2* idiomorph and another (reverse complement) in the flanking region (McGuire et al., 2001). The B18 priming sites were found to be 1665 base pairs apart, and therefore appear to amplify a DNA fragment containing a

Table 3
Linkage phase associations between *MAT* idiomorphs and RAPD markers common across three different laboratory crosses of *C. parasitica* in a region of high heterogeneity in linkage group XVI

Locus	Cross					
	JA17 × P17-8		JA19 × EP155		RH10-4 × EP155	
MAT	*MAT-1*	*MAT-2*	*MAT-1*	*MAT-2*	*MAT-1*	*MAT-2*
B18_1685	0	1	0	1	0	1
C115_0475	1	0	1	0	1	0
C160_1350	1	0	1	0	1	0
C337_1800	1	0	1	0	1	0
C421_0450	1	0	—		1	0
C625_0875	1	0	1	0	1	0
D02_0800	1	0	1	0	1	0
E09_1050	1	0	—		1	0
E15_1150	1	0	1	0	1	0
F09_0500	1	0	1	0	—	
C030_1900	0	1	1	0	—	
C258_1000	0	1	0	1	0	1
C400_1100	0	1	0	1	0	1
C432_1125	0	1	0	1	0	1
F09_1600	0	1	0	1	0	1
P03_1550	0	1	—		0	1
T02_1150	0	1	0	1	0	1
W05_1800	0	1	0	1	0	1

For RAPD loci: '1', band present; '0', band absent; —, not scored because of lack of segregation.

portion of the *MAT-2* idiomorph. The estimated distances of 3.0 and 3.9 cM between the non-recombining region and *MAT* suggested that we might observe recombination between markers in the highly heterogeneous region and the *MAT* locus in natural populations. To further examine this question, marker data were collected on four *MAT-1* and four *MAT-2* isolates from each of six natural populations previously genotyped for *MAT* (McGuire et al., 2004). These isolates were genotyped for 17 markers from the *MAT*-containing linkage group (LG XVI) that mapped to a 1-cM interval, as well as five unlinked markers from other linkage groups as controls. Among 32 isolates from four populations in the US, 14 *MAT-1* and 13 *MAT-2* isolates had the same alleles at 16 of the 17 loci (Table 4); alleles at the 17th locus were variable (data not shown). The five markers from other linkage groups were in linkage equilibrium among these 27 isolates (data not shown), even though they all shared the same alleles at 16 loci from LG XVI. Five other US isolates were found with three other haplotypes for these 16 loci: isolate WV48 differed from the majority haplotype at one locus; isolates BF31, DU2, and V21 showed the alternate alleles at most of the 16 loci compared to the majority haplotype; and isolate BF93 appeared to have a recombinant haplotype for these 16 loci (Table 4). Among 16 isolates from two populations in Japan, we found 12 haplotypes based on the same 16 loci. There was no apparent association of haplotypes to mating types (Table 4). These results clearly demonstrate that recombination occurs between the non-recombining region and the *MAT* locus, rejecting the hypothesis that this region represents a sex chromosome.

3.2. Genome length estimates

Observed and expected genome lengths were used to estimate the percentage of the genome likely to be covered by the markers. Using our partial linkage data, estimates for expected genome size for *C. parasitica* were found to vary from an average of 2234–3356 cM at LODs 5 through 10 (Fig. 3). Using our estimate of observed genome coverage and assuming that *C. parasitica* has seven chromosomes (Milgroom et al., 1992; W. Powell, personal communication), so that 14 of the 48 ends of our 17 groups and seven pairs cover true telomeric regions (25 cM/unaccounted end), and that each unlinked locus accounts for 50 cM (25 cM on either side), the total map coverage is estimated to be approximately 2757.3 cM, or 82% if one assumes a 3350 cM genome.

4. Discussion

The primary objective of this study was to find markers closely linked to *vic* genes for the eventual purpose of positional cloning. Alleles were segregating at five *vic* loci in our mapping population, but we found markers $\leqslant$4.5 cM for only two genes (*vic1* and *vic2*). Although the genetic map reported herein represents the most detailed linkage analysis of the *C. parasitica* genome to date, and for the first time illustrates the map positions of the *MAT* locus and five of the six currently known *vic* genes, it must still be considered preliminary. Given the large number of linkage groups observed compared to that found by electrophoretic karyotyping analyses (Milgroom et al., 1992), this map is characterized by low levels of RAPD polymorphism across most of the genome. Based on electrophoretic karyotyping, *C. parasitica* has only six or seven chromosomes, therefore our 24 linkage groups do not represent whole chromosomes. In this regard, our results are similar to those reported for another ascomycete fungus, *Blumeria graminis*, where over 350 markers were mapped to 34 linkage groups spanning over 2100 cM (Pedersen et al., 2002).

In contrast to the low levels of RAPD polymorphism observed across most of the *C. parasitica* genome, one linkage group (XVI)—containing the *MAT* locus—appears to harbor extremely high levels of RAPD heterogeneity and reduced recombination (Fig. 1). Markers in this linkage group were inherited in roughly equal proportions from either parent, suggesting that this highly polymorphic region is common to both parents in this cross. In addition, we observed this same pattern of high heterogeneity and reduced recombination in two additional crosses involving three other parental isolates. Finding the same pattern in three crosses in the linkage group containing the *MAT* locus suggested that it is not just an anomaly, but instead may have some biological significance. For example, we thought this linkage group might have represented something similar to a sex chromosome where recombination is highly suppressed (Hood, 2002; Merino et al., 1996).

Table 4
Mating type (*MAT*) and RAPD haplotypes for loci in a region of suppressed recombination in linkage group XVI in *C. parasitica* isolates from the US and Japan

Population[b]	Isolate	*MAT*	*MAT*-linked markers																Unlinked RAPDs[a]				
			C115_ 0475	C160_ 1350	C337_ 1800	D02_ 0800	E09_ 1050	E15_ 1150	Q11_ 1350	C030_ 1900	C193_ 0600	C258_ 1000	C400_ 1100	C432_ 1125	F09_ 1600	P06_ 0700	T02_ 1150	W05_ 1800	C01_ 1300	C160_ 0850	C231_ 1000	D02_ 1175	S10_ 1075
US	27 isolates[c]	1 and 2	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	*	*	*	*	*
	WV48	2	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1
	BF31	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1
	DU2	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	.[d]	1	0	0	0
	V21	2	0	0	0	0	0	0	.	0	1	1	1	1	1	1	1	1	0	1	0	0	0
	BF93	2	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0
Japan	OK3	2	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0
	OK5	1	1	1	1	1	1	1	1	0	1	0	0	0	.	0	1	0	1	0	0	0	0
	CD7	2	1	1	1	1	1	1	1	0	1	0	0	0	0	1	0	0	1	0	1	0	0
	OK7	2	1	.	1	0	1	1	0	1	1	0	0	1	0	1	0	0	1	.	1	0	0
	CD4	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	0	0
	CD5	2	1	1	0	1	1	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	0
	OK11	1	1	0	1	1	1	1	1	1	1	0	0	1	0	1	0	0	1	0	0	0	0
	CD1	1	1	0	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	0	1	0
	CD8	2	1	0	1	1	1	1	.	1	1	0	0	1	0	1	0	0	1	0	1	1	0
	CD2	1	1	0	1	1	1	1	1	.	1	0	0	1	0	1	0	0	1	1	1	1	0
	OK1	1	1	0	1	1	1	1	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0
	CD6	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	CD3	2	1	0	1	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0
	OK10	1	1	0	1	1	1	0	1	1	1	0	0	1	0	1	0	0	1	0	1	0	0
	OK2	2	0	0	1	1	0	0	1	1	1	0	0	1	0	1	0	0	1	0	1	0	0
	OK8	2	0	0	1	1	0	0	1	1	1	0	0	1	0	1	0	0	1	0	0	0	0
Parental Isolates[e]																							
USA	EP155	2	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	1	0	1	0
	RH10-4	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1
Italy	P17-8	2	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1
Japan	JA17	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0
	JA19	1	1	1	1	1	.	1	1	1	1	0	0	0	0	1	0	0	1	0	0	0	0

[a] RAPD markers (found to be linked in other groups or unlinked).

[b] Isolates sampled from US and Japan were genotyped by McGuire et al. (2004) for mating type (*MAT*). US populations included Danby, NY (BF), Depot Hill, NY (DU), Parsons, WV (WV), and Mountain Lake, VA (V). Japanese populations included Chudai, Kyoto (CD) and Okobe, Kumamoto (OK).

[c] 27 US isolates from four populations had the identical RAPD haplotype for linkage group XVI markers, but varied for mating type and at unlinked RAPD loci. Fourteen isolates were *MAT-1*, and 13 isolates were *MAT-2*. Alleles at unlinked loci for these 27 isolates were in linkage equilibrium (data not shown).

[d] Missing data.

[e] Haplotypes for parental isolates used in crosses in this study.

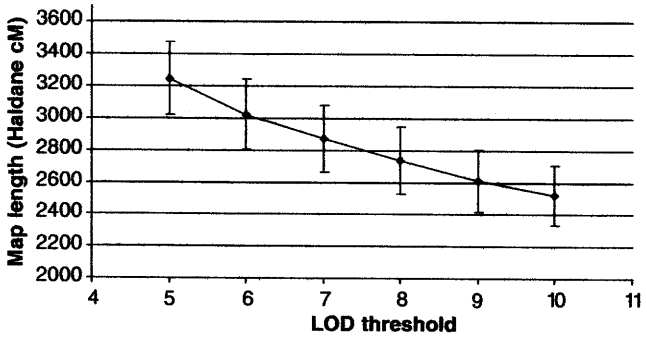

Fig. 3. Estimates of the total map length for *C. parasitica* based on different LOD thresholds for determining pairwise linkage. Bars represent one standard deviation based on 1,000 iterations.

However, data from natural populations (Table 4) showed clearly that recombination occurs between this heterogeneous region and the *MAT* locus, and that this region is in no way associated with a sex chromosome.

Although recombination between this region and the *MAT* locus is evident, recombination within this region is significantly suppressed and a high level of linkage disequilibrium among markers is maintained in populations in the US. In contrast, no such pattern was observed in two populations in Japan. We speculate that suppressed recombination could be caused by an ancient inversion, the two phases of which have not recombined but instead have diverged by mutation over a long time. Only one form of inversion is present in the small sample from two Japanese populations, but by chance, both phases are present in the US, possibly because of separate founder events in *C. parasitica*. Additional evidence will be needed to confirm if this region represents an inversion, and to determine the physical length of chromosome involved.

Some filamentous ascomycetes are reported to have relatively small genomes of around 35 Mb or smaller (Kupfer et al., 1997) and map sizes less than 1500 map units (Farman and Leong, 1995; Jurgenson et al., 2002a; Radford and Parish, 1997; Sweigard et al., 1993; Tzeng et al., 1992; Zhong et al., 2002). However, map sizes approaching 2,000 map units or more have also been reported in ascomycetes (Jurgenson et al., 2002b; Pedersen et al., 2002; Xu and Leslie, 1996). Pulsed-field electrophoresis data for a number of *C. parasitica* isolates suggest the presence of six or seven chromosomes with an estimated average genome size of approximately 40 Mb (B.I. Hillman, personal communication; Milgroom et al., 1992; W.A. Powell, personal communication). Although the physical size of the *C. parasitica* genome appears to be similar to other ascomycetes, its estimated map size, in contrast, is quite large (possibly > 3000 cM).

The discrepancy between the large number of linkage groups in our map and the estimated number of chromosomes in *C. parasitica* cannot be resolved without further study. In theory, more markers would be needed to fill gaps between linkage groups found in this study. However, we screened a large number of RAPD primers and found a fair percentage of the polymorphisms identified to cluster into a single highly heterogeneous region, making the screening of additional RAPD primers unpromising. Moreover, given the relatively large number of progenies we analyzed ($n = 192$), mapping more progenies is not likely to solve the problem. A potential option might be to exploit the parasexual cycle for mapping (Fincham et al., 1979). For example, we could use the parents from MJ1 to produce heterokaryons with vegetatively compatible isolates and follow the inheritance of the parental markers in single conidial isolates. Parasexuality has been demonstrated in *C. parasitica* in the laboratory (Puhalla and Anagnostakis, 1971; Rizwana and Powell, 1995; Vannacci et al., 1997), and may also occur in nature (McGuire et al., 2005). Because recombination occurs infrequently in parasexuality, this method might facilitate the grouping of mapped linkage groups, locus pairs, and some of the currently unlinked markers that reside on the same chromosomes. An alternative might be to separate chromosomes by pulsed-field electrophoresis and probe blots with labeled RAPD marker bands. However, this method could prove problematic as RAPDs can contain repeated sequences, which precludes screening by hybridization (Bohm and Zyprian, 1998; Dioh et al., 1997; Paran and Michelmore, 1993).

The original goal of this study was to construct a genetic linkage map for *C. parasitica* to find markers linked to *vic* genes to facilitate their positional cloning. However, only a single marker (P12_0475), was found to cosegregate completely with a *vic* gene (*vic2*). Of the four putative vegetative incompatibility-related cDNAs that were found to contain SNPs between the parents of cross MJ1, only the three *P. anserina het-c* analogs (12A07, 55F11, 12E11; Dawe et al., 2003) could be placed on the map. All three of these ESTs represent the same gene based on sequence identity within both the coding and non-coding regions (data not shown) and complete cosegregation of the SNPs in the ascospore progeny. However, none were observed to cosegregate with *vic* genes.

At this time, only two *vic* genes (*vic1* and *vic2*) may be reasonable candidates for positional cloning because we found markers ≤ 4.5 cM from each of them. Given a physical genome size of 40 Mb and a minimum recombination-based genome size of 2757 cM (total map coverage estimated in this study), an upper bound estimate for the number of kilobase pairs (kb) per cM might be only 14.5. Assuming that the relationship between genetic and physical maps are constant within as well as between chromosomes, we estimated that marker P12_0475 is within 14.5 kb of *vic2*, and could possibly be found on the same cosmid clone (average length in library is ca. 34 kb). The other two linked markers are estimated to be within approximately 65 kb or less of *vic1* and *vic2*, making it feasible to find them by chromosome walking. The band-present allele for each of these markers has been cloned and sequenced. No evidence for repetitive motifs was

found in any of the cloned markers by sequence analysis, and Southern blotting revealed that all three markers are found only in single copy (unpublished data). SCAR primers have been designed and will be used to amplify DNAs contained in a cosmid library (Dioh et al., 1997) for *C. parasitica* isolate EP44 (kindly provided by A. Churchill, Cornell University). Although markers closely linked to *vic1* and *vic2* were observed, it is clear that additional markers are needed for *C. parasitica* before efficient positional cloning of the remaining segregating *vic* genes (*vic4*, *vic6*, *vic7*) can be attempted.

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