# Towards the First Linkage Map of the *Didymella rabiei* Genome

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A genetic map was developed for the ascomycete *Didymella rabiei* (Kovachevski) v. Arx (anamorph: *Ascochyta rabiei* Pass. Labr.), the causal agent of Ascochyta blight in chickpea (*Cicer arietinum* L.). The map was generated with 77 $F_1$ progeny derived from crossing an isolate from the U.S.A. and an isolate from Syria. A total of 232 DAF (DNA Amplification Fingerprinting) primers and 37 STMS (Sequence-Tagged Microsatellite Site) primer pairs were tested for polymorphism between the parental isolates; 50 markers were mapped, 36 DAFs and 14 STMSs. These markers cover 261.4cM in ten linkage groups. Nineteen markers remained unlinked. Significant deviation from the expected 1:1 segregation ratios was observed for only two markers (Prob. of $\chi^2 < 0.05$). The implications of our results on ploidy level of the asexual spores are discussed.
KEY WORDS: Ascochyta blight; chickpea; DAF; linkage map; molecular markers; ploidy level; STMS.

## INTRODUCTION

The necrotrophic fungus *Didymella rabiei* (Kovachevski) v. Arx (anamorph: *Ascochyta rabiei* Pass. Labr.) is the causal agent of Ascochyta blight, one of the most important diseases of chickpea (*Cicer arietinum* L.). Scarce information is available on the genetic factors involved in the host–pathogen interaction. Recently it was demonstrated that the Ascochyta blight resistance in chickpea is quantitatively inherited and controlled by a number of loci (10,12). Although no studies have been conducted regarding the genetics of pathogenicity in *D. rabiei*, evidence from the inoculation of sets of cultivars with a range of isolates suggests a quantitative interaction (1,8).

Little is known about the genetics of this pathogen. Ascospores and conidia are commonly multinucleated (3,15). The ploidy level of *A. rabiei* isolates is still subject to controversy (3,15) and the number and size of chromosomes and genome size are not known.

Several types of molecular marker have been developed and shown to be reliable tools for the characterization of *D. rabiei* isolates. RAPD analysis (Random Amplified Polymorphic DNA), a PCR-based approach also called DAF analysis (DNA Amplification

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Fingerprinting) (4) used to amplify anonymous regions of the genome, was applied to detect genomic diversity among major pathotypes (11,14). Oligonucleotide sequences complementary to microsatellites were efficiently used as probes in RFLP analysis and as primers in locus-specific microsatellite analysis, the so-called STMS analysis (Sequenced-Tagged Microsatellite Site) (2), to genotype *D. rabiei* isolates (5,6,7,14). However, there is no information available on the linkage relationships of these markers.

Knowledge of the genomic organization of *D. rabiei* might provide an insight into the high phenotypic variability observed among isolates, specifically, the variability in pathogenicity. Moreover, any genetic data concerning the pathogenicity of the fungus would be of great help in understanding the *D. rabiei – C. arietinum* interaction and, consequently, the mechanisms of resistance to the pathogen in chickpea. To that end, we present a low-density genetic map of the *D. rabiei* genome with specific co-dominant STMS markers and non-specific dominant DAF markers, utilizing monoascosporic $F_1$ progeny derived from a cross between isolates with different aggressiveness levels to chickpea.

## MATERIALS AND METHODS

The $F_1$ population derived from a cross between the North American isolate ATCC76501 and isolate AA13 (AR628) from Syria (ICARDA, Aleppo), which were tested to be highly polymorphic at the genome level and to differ in their aggressiveness (5). All the 77 isolates of the population originated from single ascospores. The compatible cross and mono-ascosporic cultures were prepared as described by Wilson and Kaiser (15). Isolates were germinated and grown on chickpea seed meal agar (CSMA) (5). DNA was extracted from fresh fungal mycelia and spores obtained by scraping the CSMA surface with a glass slide. The material was immediately frozen in liquid nitrogen and ground to a fine powder. DNA isolation was performed using a modified CTAB protocol (5).

For DAF analyses (4), each 15 $\mu l$ PCR reaction contained 1.5 $\mu l$ 10xPCR buffer, 2.5 mM $MgCl_2$, 15 mM dNTPs, 0.2 U 'Silverstar' DNA polymerase, 30 pmol of a random decamer primer, and 5 ng of template DNA. After initial denaturation (95°C, 2 min), PCR was run for 40 cycles (95°C for 15 sec, 35°C for 1 min, 72°C for 1 min), with a final elongation of 7 min at 72°C. The reaction products were separated in 1.8% agarose gels and stained with ethidium bromide. For STMS analyses (2), each 15 $\mu l$ PCR reaction contained 1.5 $\mu l$ 10$\times$PCR buffer, 1.5 mM $MgCl_2$, 20 mM dNTPs, 0.5 U DNA polymerase, 5 pmol of each microsatellite-flanking primer and 10 ng of template DNA. The PCR program was run according to Geistlinger *et al.* (6). All amplification products were electrophoresed in 2.4% agarose gels and stained with ethidium bromide. The sequences of the 37 clones containing microsatellites and the respective primer pairs were published in the NCBI website (http://www.ncbi.nlm.nih.gov) accessions AJ246946 to AJ246982. Marker order and map distances were calculated with Mapmaker V2.0 (9,13) at a minimum LOD (log of odds) of 3.0 and a maximum recombination fraction of 0.25. The Kosambi mapping function was employed to compute recombination distances in centiMorgans (cM).

## RESULTS AND DISCUSSION

Of the 232 DAF primers tested for polymorphism between the parental isolates, only 22 (9.5%) resulted in reproducible polymorphic bands, producing 36 informative DNA

markers. Most DAF primers produced patterns with four to 15 amplicons per genotype (Fig. 1A). However, for most primers only one of these bands showed a polymorphism between the parental isolates (Fig. 1A). In cases where more than one locus per primer was detected, Roman numerals (I–IV) indicate the different loci. A total of 37 STMS primer pairs were tested; 13 (35%) showed clear and reproducible polymorphic amplicons between the parental isolates (Fig. 1B), resulting in 14 useful markers. The polymorphism level observed in general, and specifically the polymorphism shown by the non-specific DAF markers, was much lower than expected considering that the isolates originated from different word regions (5).

TABLE 1. Segregation of 50 DAF and STMS markers among $F_1$ progeny of a cross between *Didymella rabiei* isolates ATCC76501 from the U.S.A. and AA13 (AR628) from Syria

| Marker[z] | A:B[y] | Prob. $\chi^{2}$[x] | Marker[z] | A:B[y] | Prob. $\chi^{2}$[x] |
|---|---|---|---|---|---|
| OpA9 I | 35:42 | 0.573 | R370/1 I | 43:34 | 0.468 |
| OpA9 II | 36:41 | 0.687 | R370/1 II | 41:36 | 0.687 |
| OpB17 I | 35:42 | 0.573 | OpX10 I | 39:35 | 0.742 |
| OpB18 | 38:39 | 0.936 | OpX10 II | 33:42 | 0.462 |
| OpB20 I | 37:40 | 0.809 | OpX10 IV | 51:25 | 0.035 |
| OpB20 II | 37:37 | 1.000 | OpX11 | 41:36 | 0.687 |
| OpB20 III | 38:37 | 0.935 | OpX15 | 42:35 | 0.573 |
| OpB20 IV | 37:36 | 0.934 | OpX9 | 39:38 | 0.936 |
| OpE15 I | 39:37 | 0.871 | OpY3 I | 37:39 | 0.871 |
| OpE15 II | 42:34 | 0.516 | OpY3 II | 36:40 | 0.746 |
| OpI14 | 36:37 | 0.934 | OpY3 III | 34:42 | 0.516 |
| OpI16 | 39:36 | 0.806 | *Ar*A03T | 46:31 | 0.227 |
| OpI18 I | 39:37 | 0.871 | *Ar*A04T | 37:40 | 0.809 |
| OpI18 II | 43:33 | 0.417 | *Ar*A06T | 31:45 | 0.256 |
| OpK8 | 39:37 | 0.871 | *Ar*A10T | 43:34 | 0.468 |
| OpL15 I | 29:44 | 0.214 | *Ar*H05T | 41:35 | 0.626 |
| OpL15 II | 37:36 | 0.934 | *Ar*H06T | 30:47 | 0.171 |
| OpL15 III | 39:31 | 0.499 | *Ar*H08D | 42:35 | 0.573 |
| OpL6 | 40:35 | 0.683 | *Ar*R01D | 35:42 | 0.573 |
| OpL8 II | 27:46 | 0.116 | *Ar*R04D | 45:31 | 0.256 |
| OpM16 | 40:36 | 0.746 | *Ar*R07T | 44:33 | 0.375 |
| OpN2 | 17:59 | 0.001 | *Ar*R10T | 35:42 | 0.573 |
| OpX6 | 37:39 | 0.871 | *Ar*R12D | 36:41 | 0.687 |
| R170/3 | 31:44 | 0.288 | *Ar*S01T I | 43:33 | 0.417 |
| R260/7 | 50:27 | 0.064 | *Ar*S01T II | 47:29 | 0.144 |

[z] Marker identification: DAF markers are identified either by Op (obtained from Operon, Germany) or by R (obtained from Roth, Germany) and STMS markers are identified by *Ar* (full description of loci abbreviations in reference #7).

[y] Ratio of ATCC76501 (A) to AA13 (B) alleles.

[x] Probability of $\chi^2$ values. The null hypothesis of the $\chi^2$ test is that the A:B segregation equals the 1:1 ratio. A Prob. of $\chi^2 < 0.05$ indicates a significant deviation from the expected 1:1 ratio.

All markers used for mapping (except OpN2 and OpX10 IV) segregated in a 1:1 Mendelian ratio among the $F_1$ individuals (Prob. of $\chi^2 > 0.05$, Table 1). Heterokaryosis (1) or polyploid heterozygous genomes (3) had been suggested as possible reasons for the high phenotypic variability between and within *D. rabiei* isolates. However, our results speak against these assumptions. No heterozygosity was detected with codominant STMS markers (Fig. 1B) and almost all progeny alleles segregated 1:1, as expected for any haploid $F_1$ population and as observed in other ascomycetous fungi, *e.g. Magnaporthe*

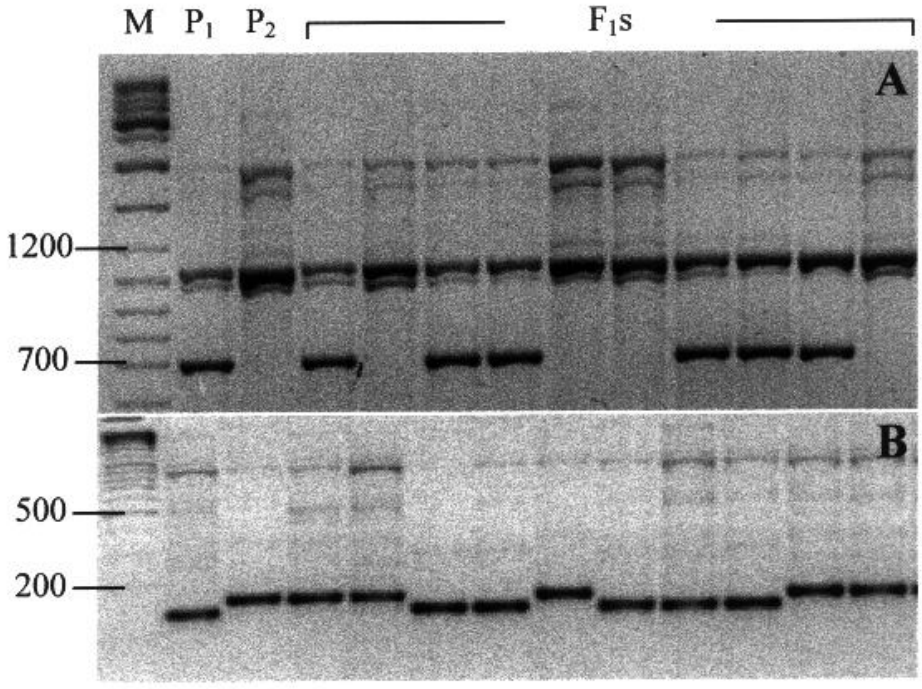


Fig. 1. PCR amplification profiles of *Didymella rabiei* genomic DNA from the parental isolates ATCC76501 ($P_1$) and AA13 ($P_2$), and from ten $F_1$ progeny ($F_1$s). A: Amplification products using the DAF primer OpI16, as observed in 1.8% agarose gel; the polymorphic amplicon (700 bp) is present in the $P_1$ genome and absent in the $P_2$ genome. B: Amplification products using the STMS primer pair A06T, as observed in 2.4% agarose gel; $P_1$ shows a smaller amplicon (around 160 bp) than that shown in $P_2$. The Gene Ruler$^{TM}$ DNA Ladder Mix (MBI Fermentas) was used as the molecular weight marker (M) in both gels.

*grisea* (Hebert) Barr (13). Our results agree with the assumption that the numerous nuclei in ascospores are haploid and derived from several mitotic divisions (15).

Ten linkage groups could be established, while 19 markers remained unlinked at a minimum LOD of 3.0 and a maximum recombination fraction of 0.25 (Fig. 2). By relaxing the maximum recombination fraction up to 0.5 (the highest limit value for linkage tests), the linkage results remained the same. The linkage map comprised 25 DAF markers and six STMS markers and covered a total of 261.4 cM. The fact that 38% of the markers were not linked to any group implies that the markers are not randomly distributed throughout the genome and that large regions remained uncovered. Addition of more markers to the present genetic map is needed to allow efficient tagging of important traits in this pathogen.

Despite the economic importance of *D. rabiei*, little is known about its genetics. Backcrossing the $F_1$ progeny will enable mapping of the mating type locus. Using the markers linked to this locus to characterize the fungus populations should be faster and more efficient than the traditional compatibility tests. Aggressiveness tests will enable mapping of the loci involved in pathogenicity, providing an extended base to understand the *D. rabiei – C. arietinum* interaction. Furthermore, this will render more efficient resistance breeding in chickpea.

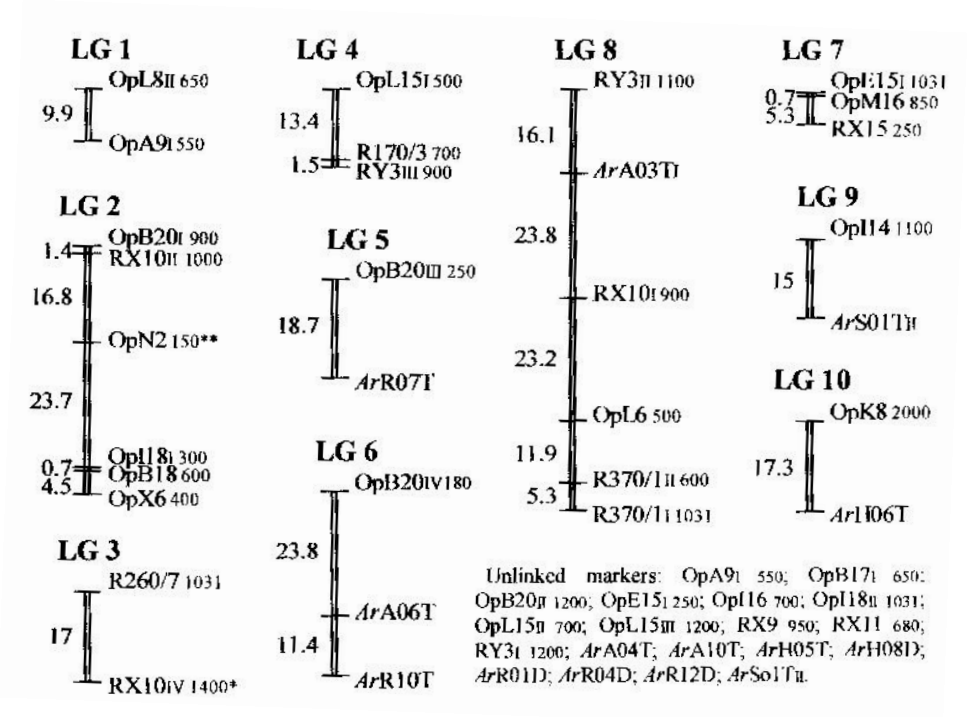


Fig. 2. Linkage map of the *Didymella rabiei* genome obtained from an $F_1$ mapping population. Ten linkage groups (LGs) and unlinked markers are presented. DAF marker identification: Op (obtained from Operon, Germany) or R (obtained from Roth, Germany); STMS markers are identified by *Ar.* Approximated DAF-fragment sizes are given by the marker name in base pairs.

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