Ecological Genetic Divergence of the Fungal Pathogen *Didymella rabiei* on Sympatric Wild and Domesticated *Cicer* spp. (Chickpea)[∇]

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For millennia, chickpea (*Cicer arietinum*) has been grown in the Levant sympatrically with wild *Cicer* species. Chickpea is traditionally spring-sown, while its wild relatives germinate in the autumn and develop in the winter. It has been hypothesized that the human-directed shift of domesticated chickpea to summer production was an attempt to escape the devastating *Ascochyta* disease caused by *Didymella rabiei*. We estimated genetic divergence between *D. rabiei* isolates sampled from wild *Cicer judaicum* and domesticated *C. arietinum* and the potential role of temperature adaptation in this divergence. Neutral genetic markers showed strong differentiation between pathogen samples from the two hosts. Isolates from domesticated chickpea demonstrated increased adaptation to higher temperatures when grown *in vitro* compared with isolates from the wild host. The distribution of temperature responses among progeny from crosses of isolates from *C. judaicum* with isolates from *C. arietinum* was continuous, suggesting polygenic control of this trait. *In vivo* inoculations of host plants indicated that pathogenic fitness of the native isolates was higher than that of their hybrid progeny. The results indicate that there is a potential for adaptation to higher temperatures; however, the chances for formation of hybrids which are capable of parasitizing both hosts over a broad temperature range are low. We hypothesize that this pathogenic fitness cost is due to breakdown of coadapted gene complexes controlling pathogenic fitness on each host and may be responsible for maintenance of genetic differentiation between the pathogen demes.

Environmental heterogeneity and genetic variability in host populations are major factors distinguishing natural from agricultural habitats. These differences exert powerful selective forces on plants and their pathogens, shaping the biology of pathosystems, epidemiological patterns, and pathogenic fitness (11, 21). Plant pathogens are dependent upon the abiotic environment as well as on their host plants and are subjected to strong selective forces exerted by their hosts. This process is shaped especially (but not exclusively) by genetic variation at loci controlling differential host specificity, which may ultimately be an important driver in speciation (37, 48, 49).

The Neolithic revolution and the adoption of farming have had a large impact on plant communities as well as their related pathogens (11, 34, 57). The long-term interplay between plant pathogens and their hosts and the resulting evolutionary trajectories may have different patterns in natural plant communities as compared to agro-ecosystems (12). One striking observation is that pathogens of natural plant populations, although prevalent, rarely cause the destruction of their hosts

(21). Therefore, investigations of the epidemiological and biological differences between pathogen populations from wild and domesticated origins are of fundamental interest and are highly relevant to understanding disease patterns, parasite evolution, and host resistance in agricultural systems. Such studies are expected to be especially fruitful in the centers of origin of crop species, because these regions are generally considered to be pathogen centers of origin as well (40, 57).

Throughout West Asia, wild cereals and legumes and their domesticated derivatives have been growing sympatrically since the beginning of Near Eastern farming systems (41, 61). Domesticated chickpea, *Cicer arietinum* L., is grown sympatrically with a number of annual and perennial *Cicer* relatives, including the immediate wild progenitor of domesticated chickpea, *C. reticulatum* Ladiz (39, 58). Following the Neolithic agricultural revolution in southeastern Turkey (41), the Near Eastern crop package spread in all directions throughout the east Mediterranean and reached the southern Levant within 1 millennium (2, 3). This “passage” of the cultigens, from their core region in southeast Turkey into the southern Levant, traversed populations of many of their wild progenitors and more distantly related wild relatives (e.g., wild barley, wild emmer wheat, wild bitter vetch, wild lentils, and wild peas), (2, 3). Presumably, these natural populations were infested by pathogens capable of infecting the domesticated forms (2, 20, 24).

Domesticated chickpea differs from the Near Eastern founder crops in its seasonal growth pattern. While most founder crops have retained the autumnal germination/spring

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maturation cycle like their wild relatives, domesticated chickpea is a spring-sown crop, germinating and developing up to 4 months later than its wild relatives (1, 3). This shift of life cycle is puzzling since water availability in the Levant is a major yield-limiting factor and autumn-sown crops enjoy a substantial yield benefit. It has been recently hypothesized that this shift was driven by the extreme vulnerability of chickpea to *Ascochyta* blight during the rainy season and was the only means to secure stable yields in ancient times (3). *Didymella rabiei* (Kovachevski) var. *Arx*. (Anamorph: *Ascochyta rabiei* (Pass) Labr.) is one of the most destructive diseases of domesticated chickpea, affecting all above-ground parts of the plant. Secondary spread of *D. rabiei* conidia occurs through rain splash, and epidemic intensity is governed by rain frequency and quantity. As *Ascochyta* blight epidemics proceed, foci of diseased plants become visible. Unlike other *Ascochyta* diseases of legumes and *Septoria* diseases of cereals, *Ascochyta* blight of chickpea may cause total yield loss under the appropriate environmental conditions (43). Autumn-sown chickpea is severely affected by *Ascochyta* blight because the crop growth period coincides with the rainy season and optimum environmental conditions for pathogen development and spread (3, 56).

Unlike the often massive stands of wild cereals, *C. reticulatum* has a very narrow and fragmented distribution (2, 8, 38). However, other wild annual *Cicer* taxa are more common across the region and can be found in close proximity to the domesticated crop (1, 8). In the southern Levant, domesticated chickpea is grown sympatrically, often just few meters apart from *C. judaicum* (27). *C. judaicum* grows in patchy distributions in stony/rocky habitats in Israel and neighboring territories, mostly in sites with annual precipitation of >480 mm and altitude of <900 m (6). Unlike *C. judaicum*, modern chickpea cropping in Israel spans large tracts of land employing a 5-year rotation in individual fields. Recently, *D. rabiei* isolates sampled from *C. judaicum* and isolates sampled from *C. arietinum* were studied and found to be better adapted to their respective original host than to the other *Cicer* species (26, 27). In addition, *in vitro* hyphal growth rate experiments exposed an adaptation to higher temperatures among isolates originating from *C. arietinum* compared to isolates from *C. judaicum* (26). Given that the natural growing season of *C. judaicum* occurs during the Levantine winter and that chickpea is a traditional spring-sown crop in the region, it is likely that the apparent adaptation to higher temperatures of *D. rabiei* isolates from domesticated chickpea may represent an ecological shift following the introduction of summer cropping practices in the Near East (3). These sympatric wild and domesticated pathosystems of *Cicer*/*Ascochyta* represent a unique opportunity for studying the genetic basis of the pathogen's ecological adaptation and its association with pathogenic fitness. Such a system may also help to determine the role of ecological factors and pathogenic fitness in pathogenic divergence and the evolutionary relationships among pathogen populations in natural and human-directed agro-ecosystems (57).

In this context, our underlying hypotheses were as follows: (i) isolates sampled from *C. arietinum* and *C. judaicum* are conspecific but represent genetically distinct populations; (ii) the temperature growth response of *D. rabiei* isolates from *C. judaicum* and *C. arietinum* has a heritable genetic basis; (iii)

TABLE 1. *Didymella rabiei* isolates sampled from *Cicer judaicum* (wild chickpea) and *C. arietinum* (domesticated chickpea) in Israel during 2004 to 2007

Region and collection site(s) ^a	Longitude ^b	Latitude ^b	Host	No. of isolates
Northern Israel				
Northern valleys	35°17'	32°36'	<i>C. arietinum</i>	7
Central Israel				
Wadi Ara	35°09'	32°33'	<i>C. judaicum</i>	9
Ramot Menashe	35°03'	32°33'	<i>C. judaicum</i>	9
YW forest	35°04'	32°34'	<i>C. judaicum</i>	9
Sharon	34°56'	32°05'	<i>C. arietinum</i>	7
South Judea				
Wadi Sansan	35°00'	31°41'	<i>C. judaicum</i>	8
Southern coast	34°33'	31°35'	<i>C. arietinum</i>	7
Judean hills	34°58'	31°44'	<i>C. arietinum</i>	10

^a The sampling area of isolates from *C. arietinum* is ~100 km². The sampling area of isolates from *C. judaicum* is ~1 km². YW, Yoop Wasterveel.

^b Longitude and latitude positions in the center of the sample site/area.

the temperature growth response plays an important role in the ongoing pathogen divergence process and, therefore, it is expected to have high heritability values; and (iv) the existence of two sympatric *D. rabiei* populations (demes) requires the action of one or more genetic isolation mechanisms. In accord with the above hypotheses, the aims of this study were (i) to assess the genetic differentiation between *D. rabiei* isolates originating from *C. judaicum* versus *C. arietinum*, (ii) to determine the genetic basis of temperature response and estimate its heritability, and (iii) to assess the relationship between temperature adaptation and pathogenic fitness among progeny from crosses between *D. rabiei* isolates from *C. judaicum* and *C. arietinum* on the two original hosts.

MATERIALS AND METHODS

Collection, maintenance, and identification of fungal and plant material. Infected plant organs (leaves and stems) exhibiting symptoms typical of *Ascochyta* blight were sampled from geographically separated sites in Israel from March to May in each year from 2004 to 2007. In total, 35 isolates were sampled from four geographically separated populations of *C. judaicum* across Israel and 31 isolates were sampled from *C. arietinum* in three different production areas in Israel (Table 1).

Samples were surface sterilized, placed on petri dishes containing potato dextrose agar (PDA; Difco, France), and maintained in incubators at 20°C. Single-conidial colonies were prepared from colonies, and all 66 isolates were identified as *D. rabiei* based on culture morphology and conidial shape as described previously (27).

DNA extraction, STMS scoring, and gene sequencing. Liquid cultures of the 66 isolates were initiated from single-conidial isolates in 250-ml flasks containing 100 ml of 2-YEG medium (2 g liter⁻¹ yeast extract and 10 g liter⁻¹ glucose). Flasks were incubated for 7 days on a rotary shaker at 22°C. Mycelium was vacuum-filtered, lyophilized, and stored at -80°C. DNA was extracted from ground mycelium using the DNeasy plant minikit (Qiagen, Ltd.) or the Master Pure yeast DNA purification kit (Epicenter Biotechnologies). DNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies) and adjusted to 20 ng µl⁻¹ for PCR. In order to confirm the morphological classification of the fungi as *D. rabiei*, we amplified and sequenced part of the nuclear ribosomal internal transcribed spacer (ITS) region. Primers ITS1 and ITS4 were used to amplify the ITS1-5.8S-ITS2 region following the protocol described by White et al. (59). Sequences of 10 randomly selected isolates from *C. judaicum* were compared to sequences of 10 randomly selected isolates from *C. arietinum*. All 20 isolates had identical ITS1-5.8S-ITS2 sequences (27). These 20 isolates were further characterized using 10 locus-specific, sequence-tagged microsatellite markers (STMS) developed for *D. rabiei* by Geistlinger et al. (30)

and by specific primers for each mating-type allele developed by Barve et al. (5). These markers are specific to *D. rabiei* and do not cross-amplify from closely related *Didymella* species colonizing related cool season food legumes (5; T. L. Peever, unpublished data).

Portions of two genes coding for the proteins translation elongation factor 1 alpha (*EF1α*) and glyceraldehyde-3-phosphate dehydrogenase (*G3PD*) were sequenced for eight isolates from *C. judaicum* and eight isolates from *C. arietinum*, randomly sampled from our collection. These regions were used previously to estimate the phylogeny among *Ascochyta* spp. and proved to be informative at or below the species level (48). Primers EF1-728F and EF1-986R (14) were used to amplify a portion of the translation elongation factor 1 alpha gene (*EF1α*). The conditions consisted of 96°C for 1 min, followed by 35 cycles of 96°C for 15 s, 53°C for 15 s, and 72°C for 15 s. A portion of the *G3PD* gene was amplified with 0.4 mM each of the primers *gpd-1* and *gpd-2* (7). The cycling conditions consisted of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by 5 min at 72°C, as described by Peever et al. (49). PCR products were detected on 1% agarose gel and purified with ExoSap according to the manufacturer's protocol (Amersham). PCR products were sequenced by Macrogen, Korea, using an ABI PRISM 3100 DNA sequencer. Raw sequences were edited using Vector NTI (Invitrogen). Alignment of sequences was performed using DNAMAN (Lynnon BioSoft).

Isolates were screened for allelic variation at six sequence-tagged microsatellite (STMS) loci (30), which have been demonstrated to be genetically unlinked (50, 52; T. L. Peever, unpublished data). PCR amplifications were performed as described by Peever et al. (50) with minor modifications. Amplifications were performed in a DNA Engine Tetrad 2 thermocycler (MJ Research) in 25-μl volumes containing 20 ng DNA, 0.2 mM deoxynucleoside triphosphates (dNTPs) (Sigma), 0.2 μM unlabeled reverse primer (Sigma), 0.2 μM 5'-fluorescent-labeled forward primer (Agentek, Israel), 1 U of Super-Therm *Taq* polymerase, and 2.5 μl buffer X1 (JMR Holding, United Kingdom). The cycling conditions were as follows: initial denaturation at 96°C for 2 min followed by 35 cycles of 96°C for 15 s, annealing at 53 to 59°C for 15 s and then 72°C for 45 s, and a final extension phase of 72°C for 20 min. Microsatellite alleles were scored by the Center for Genomic Technologies at the Hebrew University of Jerusalem using an ABI PRISM 3100 capillary sequencer (Applied Biosystems). Chromatograms were analyzed using Peak Scanner v1.0 (Applied Biosystems). Isolates AR738 and WSFS003-1 were previously genotyped using these markers (T. L. Peever, unpublished data) and served as reference isolates for allele sizes.

Microsatellite markers were used to estimate genetic differentiation between isolates infecting wild versus domesticated hosts. Genalex v6.1 software (46) was used to estimate the observed number of alleles and gene diversity across the entire sample (from both hosts). Since isolates from *C. judaicum* are relatively rare, the final number of wild isolates from each site was low (≤ 10). Therefore, we could not consider individual sampling site as a subpopulation. Individual pairwise genetic distances (47) were estimated, and a principal coordinates analysis (PCoA) was performed. A Bayesian model-based clustering method using multilocus genotype data implemented in the STRUCTURE software package v2.2 (51) was used to assign individuals to clusters. An admixed model using independent allele frequencies was adopted, and the analysis was performed using 5×10^4 burn-in generations and a run length of 5×10^5 Markov chain Monte Carlo (MCMC) generations. Log likelihood values and posterior probabilities were estimated assuming one to eight clusters ($K = 1, 2, \dots, 8$). Five independent runs were performed for each analysis in order to verify the convergence of parameter estimates. The most probable number of clusters was estimated using the method described by Evanno et al. (25), based on the rate of change in the log probability of data between successive K values (ΔK).

Temperature adaptation of isolates from wild and domesticated origins. *In vitro* growth of the isolates that survived until 2007 (31 isolates from *C. judaicum* and 29 isolates from *C. arietinum*) was estimated at 15 and 25°C. These two temperatures were chosen because isolates from *C. judaicum* can be found only in the late winter to early spring (late February to the beginning of April) and 15°C is the average temperature in central Israel at that period. *Ascochyta* blight in traditional domesticated fields is found in mid-April to late May, and 25°C represents an average daily temperature to which the isolates from *C. arietinum* are usually exposed. Agar pieces (8 mm in diameter) were cut with a cork borer from the edge of 14-day-old colonies, placed in the center of petri dishes containing PDA, sealed with parafilm, and placed in growth chambers at 15 and 25°C under a 12-h-dark–12-h-light cycle. The diameter of each colony was measured after 9 days. Since the two diameters of the colony were often uneven, we measured the radius in two perpendicular directions (a and b) and calculated the area as an ellipsoid (πab in cm²). The initial area of the agar piece (2.01 cm²) was subtracted from the ellipsoid area. Phenotypes of each isolate (Δ) were estimated by subtracting colony area at 15°C from the colony area at 25°C for each isolate.

TABLE 2. Segregation of mating type and an STMS marker among ascospore progeny of two crosses between isolates sampled from *C. arietinum* and *C. judaicum*

Cross	Locus ^a	Ratio ^b	χ^2 ^c	P^d
N04 × M305	<i>MAT</i>	9:6	0.60	0.43
	<i>ArR01D</i>	10:5	1.70	0.19
WSFS 003-1 × Y1105	<i>MAT</i>	7:8	0.06	0.79
	<i>ArR01D</i>	6:9	0.60	0.43

^a There were 15 progeny analyzed for each locus. *MAT*, mating type locus scored using a mating-type-specific PCR (5); *ArR01D*, STMS locus scored on an ABI sequencer (30).

^b Ratio of *MAT1-1* to *MAT1-2* progeny for the *MAT* locus and ratio of alleles for STMS locus *ArR01D*.

^c χ^2 value for test of 1:1 ratio.

^d Probability of a greater χ^2 value under the null hypothesis of 1:1 segregation (1 df).

A Δ value of >0 indicated increased hyphal growth at 25°C relative to that at 15°C; $\Delta < 0$ indicated increased hyphal growth at 15°C relative to that at 25°C, and $\Delta = 0$ indicates that the isolate grew equally at both temperatures. The experiment was laid out in a completely randomized design. Each isolate was replicated four times, and the experiment was conducted twice. Statistical analyses of the data were performed with the JMP 5.0 software for windows (SAS Institute, Cary, NC). To facilitate analysis of variance and multiple means comparisons, data were normalized by the square root of the colony area ($\sqrt{\text{colony area}}$). The following general linear model was used: $Y_{kj} = \mu + C_k + F_j(C_k)$, where Y_{kj} represents the expected colony area (in cm²), μ represents the true mean, C_k is the “isolate origin” effect (*C. judaicum* or *C. arietinum*), and $F_j(C_k)$ stands for the individual “isolate's” effect nested within each “isolate origin.” Multiple comparisons of means were performed using the Tukey-Kramer honestly significant differences (HSD) test at $\alpha = 0.05$. Data from the two experiments were pooled and analyzed jointly since the variation between independent experiments was not significant ($P = 0.09$).

Genetic analysis of temperature adaptation and its association with pathogenic fitness. Two *MAT1-1* isolates with high Δ values from *C. arietinum* (N04 and WSFS 003-1), and two *MAT1-2* isolates with low Δ values from *C. judaicum* (M305 and Y1105) were chosen randomly among isolates with high and low Δ values. The isolates were crossed using procedures described previously (36, 60), and 105 ascospore progeny were collected from each cross. Cross 1 involved isolates N04 from *C. arietinum* and M305 from *C. judaicum* in Israel, and cross 2 involved isolates WSFS 003-1 from *C. arietinum* in California and Y1105 from *C. judaicum* in Israel. The Californian isolate was used to determine if the genetic basis of the temperature adaptation seen in Israeli isolates was similar to that of isolates sampled outside Israel. Segregation of the two *MAT* alleles and the two alleles of STMS marker *ArR01D* was used to demonstrate the hybrid status of the progeny. PCR amplification of the *MAT* locus and the STMS markers was conducted according to Peever et al. (50). Segregation ratios among 15 progeny from each cross were tested under the null hypothesis of 1:1 segregation using a χ^2 test. We failed to reject the null hypothesis of 1:1 segregation (Table 2), and segregation at both loci confirmed the hybrid status of the progeny. Hyphal growth responses of the parental and progeny isolates were examined *in vitro* at 25 and 15°C. Experiments were conducted as described above, and the Δ values were estimated for each progeny isolate.

Based on the Δ data from the previous experiments, three progeny isolates adapted to low temperatures ($\Delta < 0.40$) and four progeny isolates adapted to high temperatures ($\Delta > 1.86$) were sampled randomly from cross 1. Differences between Δ values of the high-temperature-adapted (H-Temp) progeny and the low-temperature-adapted (L-Temp) progeny and between the parental isolates were significant using the Tukey Kramer HSD test ($P < 0.001$). Isolates were grown on chickpea seed meal agar (CSMA) (60) for 2 weeks as described by Frenkel et al. (27) and pathogenic fitness was determined on both hosts as follows.

Two chickpea cultivars (*C. arietinum*) and two *C. judaicum* accessions were used in this study. Cultivar “Spanish White” is highly susceptible and cv. “Yarden” is moderately resistant to *D. rabiei*. Seeds were planted in 0.5-liter pots, and the plants were maintained in a greenhouse at 15 to 23°C under natural light for 21 days. *C. judaicum* accessions were chosen from the working collection of Ben-David et al. (6), and their response to *D. rabiei* was previously determined by Frenkel et al. (26). Accession Cj 25 is highly resistant and Cj 19 is moderately

FIG. 1. Genetic associations among 31 *D. rabiei* isolates sampled from four populations of *C. arietinum* (North, Sharon, South, and Judean hills) and 35 *D. rabiei* isolates sampled from four populations of *C. judaicum* (Wadi Ara, Sansan, Yoop Wasterveel [YW] forest, and R. Menashe) as depicted by principal coordinates (Coord.) analysis (PCoA) of pairwise individual genetic distances. Arrows indicate individual “mismatched” isolates (i.e., isolates with multilocus genotype that does not match the predominant genotype of isolates sampled from that host).

resistant to *D. rabiei* isolates from both hosts. *C. judaicum* seeds were scarified to enhance germination and planted in 0.5-liter pots and grown for 28 days under the same conditions as the domesticated chickpea cultivars.

Plants were sprayed to runoff with conidial suspensions of each isolate (3×10^5 spores ml^{-1}) with an air pressure hand-sprayer. Plants were covered with polyethylene bags for 24 h and placed into growth chambers with 10-h photoperiods. Temperatures in one chamber cycled between 12 and 20°C to simulate Israeli late winter temperatures, and those in the other cycled between 21 and 29°C to simulate Israeli late spring temperatures. After 24 h, the polyethylene bags were removed and the plants remained in the same growth chambers for the duration of the experiments. Plants were visually inspected for *Ascochyta* blight symptoms at 5, 6, 7, 8, 9, 11, and 14 days postinoculation. The first appearance of disease symptoms was recorded, and disease severity (proportion of affected plant area) was assessed visually. Each treatment (*D. rabiei* isolate $\times$ chickpea cultivar/*C. judaicum* accession $\times$ growth temperature) was replicated three times. The experiment was laid out in a randomized block and was repeated once. Two different pathogenic fitness measures representing different stages of the infection process and disease development were used: (i) incubation period was determined as the time (in days) between inoculation and appearance of the first disease symptoms, and (ii) disease intensity in the entire epidemics was determined in terms of the relative area under the disease progress curve (RAUDPC; in %). To enable analyses of variance (ANOVA), the RAUDPC values were approximately normalized as follows: $\text{tRAUDPC} = \text{inverse sine} [\text{square root (RAUDPC)}]$, where $\text{tRAUDPC} = \text{transformed RAUDPC}$. Differences between the parental isolates and their progeny at each of the tested temperatures were determined using one-way analysis of variance (ANOVA). Multiple comparisons of means were performed with the Tukey-Kramer honestly significant differences (HSD) test at $\alpha = 0.05$.

Data from each experiment were analyzed independently. Conclusions obtained from the two experiments were similar; therefore, results of only one of the experiments are presented.

Nucleotide sequence accession numbers. Sequences were deposited into GenBank under the following accession numbers: for *G3PD*, FJ514779 (*D. rabiei* from *C. arietinum*) and FJ514778 (*D. rabiei* from *C. judaicum*); and for *EF1 α* , FJ514777 (*D. rabiei* from *C. arietinum*) and FJ525953 (*D. rabiei* from *C. judaicum*).

RESULTS

Genetic differentiation between *D. rabiei* isolates from *C. judaicum* and *C. arietinum*. Genetic associations among the isolates, based on 70 alleles from six STMS loci across sites and both host, were investigated using principal coordinates analysis (PCoA). Two principal coordinates, PCo1 and PCo2, jointly accounted for 65% of the observed genetic variation (Fig. 1). The PCoA scatter plot shows clear differentiation between isolates from *C. judaicum* and *C. arietinum*. However,

one isolate sampled from *C. judaicum* clustered with the majority of isolates from *C. arietinum* and two isolates sampled from *C. arietinum* clustered with the majority of isolates from *C. judaicum*. The PCoA did not show any differentiation among isolates or sampling site from the same host (Fig. 1). STRUCTURE (v2.2) analysis corroborated these data by indicating two clusters ($K = 2$) with the highest posterior probability. More than 95% of isolates from *C. arietinum* were assigned to cluster 1, and more than 93% of the isolates from *C. judaicum* were assigned to cluster 2 (Fig. 2). This analysis follows the PCoA analysis by identifying the same three misidentified isolates (i.e., isolates sampled from one host with the dominant diagnostic genotypes of isolates from the other host).

Alignment of partial *G3PD* sequences (547 bp) showed 100% similarity among all 16 isolates from both wild and domesticated hosts. In contrast, alignment of *EF1 α* (288 bp) detected 98% similarity among the same 16 isolates. Four polymorphic nucleotides (positions 27, 177, 220, and 222) distinguished isolates from *C. judaicum* and *C. arietinum*. All four polymorphisms were fixed within samples of isolates from each host.

Genetic basis and heritability of temperature adaptation. Isolates sampled from *C. judaicum* and *C. arietinum* had significant differences in temperature adaptation *in vitro*. All isolates from *C. arietinum* had a higher growth rate at 25°C compared to 15°C, whereas only 62% of the isolates from *C. judaicum* grew faster at 25°C (Fig. 3). Isolates sampled from *C. arietinum* had a significantly higher Δ values ($P = 0.01$; mean = 1.38; standard deviation [SD] = 0.76 cm^2 ; range, 0.35 to 3.35 cm^2) than isolates from *C. judaicum* (mean = 0.15; SD = 0.85 cm^2 ; range, -1.85 to 1.99 cm^2). In addition to the differences in temperature adaptation, morphological differences were detected between isolates from each host when grown on PDA. Isolates from *C. arietinum* produced large amounts of red-pink conidial ooze, while those from *C. judaicum* produced little conidial ooze and sporulated weakly (no quantitative data recorded).

Data from the *in vitro* hyphal growth experiments were used to estimate the genetic component of the growth rate trait and its interaction with temperature ($G \times E$ interaction). The R^2 of

FIG. 2. Estimated membership coefficient for each *Didymella rabiei* isolate inferred from the multilocus STMS haplotype. Each isolate is represented by a single vertical bar divided into maximum of two colors (black and gray). Isolates are classified by their original host (*C. arietinum* and *C. judaicum*). The majority of isolates sampled from *C. arietinum* had the “gray” genotype (with two exceptions), and the majority of isolates sampled from *C. judaicum* had the “black” genotype (with one exception).

the model used to analyze the data was 0.83, and all factors except “host” were highly significant ($P < 0.001$). The “isolate (origin)” factor, which accounts for the genetic variance component of the growth trait, explained 34% of the total variation. The haploid nature of *D. rabiei* indicates that this value is the narrow sense heritability of the growth phenotype. The “isolate (origin)” $\times$ “temperature” factor, which represents the $G \times E$ variance component, contributed 40% of the total variation. This considerable variance component is reflected in the scattering of colony area values of the two isolate groups at the two temperatures, with all isolates from *C. arietinum* responding in a similar manner (below the 1:1 line) to the temperature change and a wider differential temperature growth response among isolates from *C. judaicum* (Fig. 3). Given

the above interaction, we analyzed the narrow sense heritability of the growth phenotype for each combination of isolate origin (*C. judaicum* versus *C. arietinum*) and growth temperature (15 versus 25°C) in a one-way model, which included “isolate” as a random effect and the residual. R^2 of the four ANOVA models ranged between 0.72 and 0.78, and in all cases the “isolate” was highly significant ($P < 0.0001$). As in the above complex model, the variance component of “isolate” may be used as an estimate of the narrow sense heritability of the growth phenotype of each isolate group at each temperature. The heritability values were 0.74, 0.68, 0.71, and 0.68 for the isolates from *C. judaicum* at 25°C and 15°C and for the isolates from *C. arietinum* at 25°C and 15°C, respectively.

FIG. 3. Scatter plots and frequency distributions of temperature phenotypes of 29 *D. rabiei* isolates sampled from *Cicer arietinum* (A and B) and 31 *D. rabiei* isolates sampled from *C. judaicum* (C and D). Scatter plots show the relationship between colony area of each isolate at 25 and 15°C (A and C). The phenotype of each isolate was estimated as Δ by subtracting for each isolate its colony area at 25°C by its colony area at 15°C (B and D). Average Δ values (cm^2) $\pm$ SD for each distribution are depicted in histograms (B and D).

FIG. 4. Frequency distributions of Δ , the differences in colony area of *D. rabiei* progeny from crosses between isolates from *C. judaicum* and *C. arietinum* at 25°C and 15°C. (A) Frequency distribution of 105 progeny from cross 1 between isolate M305 from *C. judaicum* and isolate N04 from *C. arietinum*; (B) Frequency distribution of 105 progeny from cross 2 between isolate Y1105 from *C. judaicum* and isolate WSFS 003-1 from *C. arietinum*. Average Δ values (in cm²) $\pm$ SD for each distribution are depicted in the histograms. Arrows indicate the parental phenotypes.

Association between temperature adaptation and pathogenic fitness. Average Δ values for progeny from cross 1 were 1.18 ± 0.72 cm² (range, 0.13 to 2.78 cm²). The parental isolates from *C. judaicum* (M305) and from *C. arietinum* (N04) had Δ values of 0.19 ± 0.17 cm² and 1.07 ± 0.28 cm², respectively, and these values were significantly different ($P = 0.001$) (Fig. 4A). Frequency distributions of Δ values of the progeny were continuous and differed from normality according to the Shapiro-Wilk test ($W < 0.001$), with positive skewness toward higher temperature. Fourteen progeny (with $\Delta > 1.86$) had significantly higher Δ values than the parental isolate obtained from *C. arietinum* (N04). Δ values of all progeny with low Δ did not differ significantly ($P = 0.05$) from that of the parental isolate obtained from *C. judaicum* (M305). The frequency distribution of Δ values among the progeny derived from cross 2 had a similar pattern. Δ values ranged between 0 and 4.06 cm², with an average of 1.24 ± 0.86 cm² (Fig. 4B). The distribution of the Δ values differed from normality ($W < 0.001$) with positive skewness. The parental isolate from *C. arietinum* (WSFS 003-1) had an average Δ value of 1.26 ± 0.28 cm², which was significantly lower ($P < 0.05$) than that of the 15 progeny with $\Delta > 2.01$ cm². The parental isolate from *C. judaicum* (Y1105) had an average Δ value of 0.42 ± 0.25 cm²,

which differed significantly from that of isolate WSFS 003-1 ($P = 0.003$) but not from any of the progeny with lower Δ values.

Pathogenic fitness of parental isolates M305 and N04 and seven of their progeny was determined on two chickpea cultivars and two *C. judaicum* accessions. Typical disease symptoms appeared on leaves and stems of all cultivars and accessions. Symptoms caused by the parental isolates and the tested progeny were visually similar; however, differences were detected in RAUDPC and the incubation period. On the domesticated chickpea hosts (cv. Spanish White and cv. Yarden), the largest RAUDPC values were observed for the parental isolate collected from *C. arietinum* (N04) when maintained at late spring temperatures (Fig. 5). RAUDPC values of N04 at late spring temperatures were significantly higher ($P = 0.01$) than those recorded for the other tested progeny and isolates (including N04 itself at winter temperatures). At late spring temperatures, the RAUDPC values of all seven progeny fell between those of the parents. Similar comparisons conducted for the incubation period showed that the parental isolate N04 (obtained from domesticated chickpea) had the shortest incubation period (5 days on both cv. Spanish White and cv. Yarden) when plants were maintained at late spring temperatures. However, due to large variation, significant differences were only detected between this parental isolate and two L-Temp progeny (Fig. 5).

The parental isolate from *C. judaicum* (M305) induced the highest RAUDPC values on *C. judaicum* accessions maintained at winter temperatures; these RAUDPC values differed significantly from most of the cross progeny. The parental isolate from *C. arietinum* (N04) induced very low RAUDPC values on plants maintained at winter temperatures; these values did not differ from those of the least-aggressive progeny tested (Fig. 6).

Data were used to test the hypothesis of genetic association (linkage) between temperature adaptation and pathogenic fitness on each host. If temperature adaptation and pathogenic fitness are genetically linked, then the H-Temp progeny would have shorter incubation periods and higher RAUDPC values on *C. arietinum* plants maintained at late spring temperatures compared with those on plants maintained at winter temperatures. Likewise, the L-Temp hybrid progeny would have a shorter incubation period and higher RAUDPC values on *C. judaicum* plants maintained at winter temperatures compared with those recorded for plants maintained at late spring temperatures. Interestingly, the one-way ANOVA comparison for incubation period and RAUDPC did not detect any significant differences between the different temperatures, regardless of the progeny phenotype (L-Temp/H-Temp) and the host (*C. arietinum*/*C. judaicum*). Probability (P) values ranged between 0.11 and 0.46 for incubation period and between 0.08 and 0.77 for RAUDPC values.

DISCUSSION

For several millennia across the Mediterranean basin, chickpea has been sown in the warm and dry late spring (3). Unlike domesticated chickpea, populations of its sympatric wild relatives kept their autumnal germination (1, 3). Therefore, it is reasonable to hypothesize that this shift in the

FIG. 5. Pathogenic fitness of two *D. rabiei* parental isolates and seven of their progeny on two *C. arietinum* cultivars: cv. Spanish White (highly susceptible) and cv. Yarden (moderately resistant). Parental isolate N04 was sampled from *C. arietinum*, and parental isolate M305 was sampled from *C. judaicum*. H-Temp, four progeny adapted to high temperatures (25°C); L-Temp, three progeny adapted to low temperatures (15°C). After inoculation, plants were maintained at temperatures typical of Israeli late spring (filled bars) or winter (empty bars). RAUDPC, relative area of the disease progress curve, a measure of epidemic intensity. Vertical bars represent the least significant differences (at $\alpha = 0.05$) as determined by the HSD test.

hosts' life cycle imposed directional selection on *D. rabiei*, forcing isolates parasitizing domesticated chickpea to survive and disperse under hotter and drier conditions compared with isolates parasitizing the populations of *C.*

judaicum or any other wild *Cicer* species in the east Mediterranean.

Supporting the above hypothesis, *in vitro* hyphal growth of isolates sampled from *C. arietinum* showed better adaptation

FIG. 6. Pathogenic fitness of two *D. rabiei* parental isolates and seven of their progeny on two *C. judaicum* accessions: Cj 19 (moderately resistant) and Cj 25 (highly resistant). Parental isolate N04 was sampled from *C. arietinum*, and parental isolate M305 was sampled from *C. judaicum*. H-Temp, four progeny adapted to high temperatures (25°C); L-Temp, three progeny adapted to low temperatures (15°C). After inoculation, plants were maintained at temperatures typical of Israeli late spring (filled bars) or winter (empty bars). RAUDPC, relative area of the disease progress curve, a measure of epidemic intensity. Vertical bars represent the least significant differences (at $\alpha = 0.05$) as determined by the HSD test.

to higher temperatures (i.e., 25°C which reflects the late spring growth temperatures) compared with isolates sampled from *C. judaicum*. Our *in vivo* experiment shows the relationship between data obtained from the *in vitro* experiments and pathogenic fitness of these isolates on wild and domesticated hosts (Fig. 5 and Fig. 6) (O. Frenkel, unpublished data). Although only a small subset of *D. rabiei* isolates was used in this study, isolates from *C. judaicum* were better adapted to winter temperatures and isolates from *C. arietinum* were better adapted to late spring temperatures on potted plants. These data support our hypothesis regarding the evolutionary effect of the summer cropping shift of chickpea on its major pathogen, *D. rabiei*.

Significant genetic differentiation was detected between *D. rabiei* isolates sampled from *C. arietinum* and *C. judaicum*. Even isolates from *C. arietinum* and *C. judaicum* growing as close as 15 m apart were genetically distinct, demonstrating that differentiation occurred on a small scale. The PCoA and the Bayesian clustering analysis supported the above claim by grouping *D. rabiei* samples from *C. arietinum* and *C. judaicum* into two distinct clusters. Therefore, based on the observed different genetic clusters, the *EF1 α* polymorphism, and their responses to different hosts and temperatures, we suggest that *D. rabiei* isolates from *C. judaicum* and *C. arietinum* belong to two separate demes.

Despite the temporal gap between the life cycle of domesticated chickpea and its Israeli wild relative, sporadic rain events still occur in the spring (March to April), which may provide the environmental conditions required for natural hybridization between the two pathogen demes (26). The emergence and long-term survival of distinct sympatric *D. rabiei* demes are therefore dependent upon a certain degree of reproductive isolation (32, 37). Wild chickpea populations are very small relative to the large tracts of land devoted to chickpea cropping in the Near East since the Neolithic revolution. Therefore, our observation that *D. rabiei* populations colonizing *C. judaicum* were not swamped by massive gene flow from *D. rabiei* populations colonizing sympatric domesticated chickpea is strong indirect evidence for the existence of such reproductive isolation.

The cross between isolates from the two *D. rabiei* demes allowed us to explore the genetic basis of this putative reproductive isolation mechanism operating in this system. Previous host specificity studies involved inter- and intraspecific hybridizations of fungal pathogens (15, 36). However, despite the potential importance of ecological factors driving genetic divergence, they are poorly understood in fungi (18, 22, 32, 33), and most of the existing data have been obtained from highly artificial experiments (18). In our work, the differences between the two parental isolates included both pathogenic fitness (RAUDPC and incubation period) and temperature adaptation. This enabled us to address the question of a genetic association between pathogenic fitness and temperature adaptation in this system. This question is highly relevant to understanding the *Cicer-D. rabiei* coevolution since we attribute the different seasonal growth temperature profiles of *C. judaicum* and *C. arietinum* as playing a selective role in the emergence of the two demes.

As predicted by the morphological criteria and molecular data, we were able to obtain viable progeny from crosses between isolates from *C. judaicum* and *C. arietinum* and these

were capable of infecting both hosts. The 1:1 segregation of DNA markers shows that the progeny used in our experiments were of hybrid origin.

The continuous distributions of the *in vitro* temperature adaptation point to polygenic control of this trait. The transgressive segregation of this character is most probably the combined result of the polygenic control of the trait and complementary gene action. The hybridization allows the recombination of rare alleles which were masked in the two parental backgrounds to be expressed among some progeny lines. Indeed, this phenomenon is quite common in wide crosses in plants and also among certain animal hybrid populations (54).

It also appears that concerning the temperature adaptation, the studied isolates have not reached their selection limits. This is in accordance with the relatively high heritability values of the temperature response, which suggests a potential for emergence of more extreme phenotypes. However, the two demes apparently have evolved with distinct adaptive pathogenic fitness gene complexes conferring partial host specificity. The progeny do not show higher pathogenic fitness than their parents on either host, probably because recombination following hybridization broke up adaptive gene complexes (e.g., see references 18 and 23) controlling fitness on each host. A similar phenomenon was reported by Hernandez-Bello et al. (36), who tested hybrids between two closely related *Ascochyta* species parasitizing faba bean and pea. Among 120 progeny from that cross, only three were pathogenic on pea and none were pathogenic on faba bean, suggesting that host specificity is under polygenic control and crossing these two host-adapted forms broke up gene complexes controlling host specificity. In our study, no loss of pathogenicity was observed but a decrease in pathogenic fitness (virulence) relative to that of the parents was detected among the seven tested progeny lines.

Notwithstanding our small sample of hybrid progeny tested (the H-Temp and L-Temp lines), the loci controlling *in vitro* temperature adaptation do not appear to be strongly associated with pathogenic fitness loci. Indirect support of this interpretation can be obtained from the fact that while temperature adaptation showed transgressive segregation, the pathogenic fitness of selected low- and high temperature-adapted isolates always fell between that of the two parents, suggesting independent genetic control. Temperature adaptation distributions of the progeny from both crosses clearly demonstrated that transgressive segregation was only observed toward Δ values higher than the Δ values of the parental isolates from *C. arietinum* but not toward Δ values lower than the Δ values of the parental isolates from *C. judaicum*. This suggests a potential for adaptation of *D. rabiei* isolates to yet higher temperatures. This is especially relevant when facing the prospect of future global warming and its implications for the ecology of wild and agro-ecosystems and their interaction with pathogens (18, 35). For example, it was previously reported that high temperatures may increase susceptibility of plants to their pathogens (16, 29, 31). Hybridization between fungal species (or demes) occupying different ecological niches or with unique host specificities may dramatically reduce progeny fitness (19, 28) but may also have the potential to generate "superpathogens" with novel host specificities (10, 45) or with the combined host ranges of the parents (37, 44, 55). There-

fore, it seems prudent to ask whether there is a risk of formation of a *D. rabiei* hybrid with high pathogenic fitness on both *C. judaicum* and *C. arietinum* and adapted to a wider temperature range. Emergence of such hypothetical hybrids in sympatric pathosystems of wild and domesticated plant species in general, and in legumes in particular, has great ecological and economic relevance. According to our data, the likelihood of such an event is very low. Parental isolates had higher pathogenic fitness than their progeny on their main host and under their favorable conditions: that is, isolates from *C. judaicum* had larger RAUDPC values and shorter incubation periods on their original host when the plants were maintained at winter temperatures and isolates from *C. arietinum* had larger RAUDPC values and shorter incubation periods on their original host when the plants were maintained at late spring temperatures. Under such conditions, hybrid progeny can survive and cause disease but will mostly have lower fitness under competition with the native parental isolates. However, the principal coordinate analysis and the Bayesian algorithm demonstrate that individuals from one deme can be found on the opposite host and vice versa. This implies that the incomplete host specificity in this sympatric pathosystem is true to life and is not an artifact of our laboratory conditions. Such migration of presumably less-fit individuals may occur for example in the host population where members of the more-adapted deme occur in low frequency and/or while parasitizing highly susceptible host lines/accessions. In the same manner, we cannot totally rule out the potential of *D. rabiei* hybrids to survive under conditions which are unfavorable for both parents, as suggested for pathogenic fungi and oomycetes (4, 10, 55). In this context, it is possible that H-Temp progeny may successfully compete with their parents under high and unfavorable conditions such as warm spring conditions.

Further questions emerge from the current study. Since we obtained evidence for niche separation and for postzygotic barriers in the form of virulence reduction, are we observing two lineages on their way to perhaps become different species? In order to answer this question, future phylogenetic analyses employing fast-evolving regions of the genome might be needed to estimate the divergence level and its time scale, as has recently been done for *Ustilago maydis* (42) and *Mycosphaerella graminicola* (57). In addition, cytogenetic studies of chromosomal rearrangements, which are a frequent event during speciation (53), may provide us with further indications. Despite the sympatric distribution of the two hosts and pathogen demes across Israel, we cannot determine if the assumed ongoing speciation process is sympatric or allopatric. In other words, did the divergence take place in the southern Levant or further north? According to Coyne and Orr (17) and Bolnick and Fitzpatrick (9), sympatric speciation requires that the involved taxa are sisters and have largely or completely overlapping geographic ranges. Domesticated chickpea is found side-by-side with more than four wild *Cicer* spp. across a wide geographical area where summer cropping is still being practiced (between the southern Levant and southeastern Turkey). Since three of these wild *Cicer* spp. (*C. judaicum*, *C. pinnatifidum*, and *C. bijugum*) are genetically close (38), it will be important to determine the phylogenetic relationships among *D. rabiei* isolates from those of wild *Cicer* spp. to ensure that the fungi colonizing *C. arietinum* and *C. judaicum* are sister

taxa. The recent isolation of *D. rabiei* from *C. pinnatifidum* in southeastern Turkey (13) is an important first step in this direction.

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