Genetic Studies on Selected Traits of *Nectria haematococca*

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ABSTRACT

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The feasibility of using the sexual stage of the fungus *Nectria haematococca* mating population VI (MP VI) for genetic studies was examined by studying segregation patterns among the progeny from crosses between parents differing in mating type, perithecial color, growth habits (specifically, sporulating colonial and mycelial), and female fertility. All of the traits except female fertility appeared to be monogenically controlled, and no linkage was detected. Female sterility required at least two genes for expression in the progeny of one cross. In two crosses, traits that were expected to segregate did not. In one of these crosses, the failure of segregation was restricted to one tetrad of the eight tetrads analyzed, and could be attributed to selfing. In the other cross, however, two traits failed to segregate in any of the progeny while a third trait (mating type) segregated normally. Despite the unexplained results obtained from this cross, we concluded that *N. haematococca* MP VI is amenable to classical genetic study, since the majority of the crosses examined showed simple segregation patterns.

Additional key words: Fusarium solani.

Nectria haematococca Berk. and Br. mating population VI (MP VI) (imperfect stage: *Fusarium solani*) appears to be well suited to genetic studies of physiological attributes implicated in plant pathogenesis. This fungus is a heterothallic ascomycete and its sexual stage is easily induced in culture (7,9). A number of traits of interest for plant pathological studies have been reported for *N. haematococca* MP VI including: phytoalexin tolerance (2,15), cutinase activity (8), production of phytotoxic compounds (3), and formamide hydrolyase activity (5). The wide host range of this pathogen (16,18) suggests that variability in these traits may occur naturally.

We have demonstrated a genetic association between the virulence of this fungus on pea and its tolerance of the pea phytoalexin pisatin (14). In conjunction with that study, it was

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important to examine the inheritance of a variety of other traits to verify that the meiotic processes of *N. haematococca* MP VI are normal and suitable for standard genetic analysis.

MATERIALS AND METHODS

Maintenance of fungal cultures. Cultures of *N. haematococca* MP VI isolates were routinely grown on V-8 agar medium slants (medium M29 [13]) under continuous fluorescent light. All cultures originated from single conidia, hyphal tips, or ascospores. For long-term storage, isolates were maintained on V-8 agar slants at 6 C, stored in dried soil (1), or preserved as lyophilized conidial suspensions in skimmed milk (1).

Crossing of isolates and isolation of ascospores. The procedure of VanEtten (16) was used for making crosses and isolating random ascospores. Conidia of the maternal parent were spread onto V-8 agar slants and grown 10–14 days before spermatization by the paternal parent.

For tetrad analysis, perithecia were collected on Miracloth filters (Chicopee Mills, Inc., 1450 Broadway, New York 10018) and washed to remove conidia and free ascospores from their surfaces. Clusters of asci were expressed into sterile water in glass depression slides. Mature ascospores were present in asci 13–23 days after spermatization. Asci were separated from each other, and those containing six to eight spores were collected by using micropipets and placed on agar medium (M100 minimal or M100 complete [13]). Spores from individual asci were separated by using a fine hand-held tungsten needle (made by electropolishing the end of 0.3-mm-diameter tungsten wire in 2.0% NaOH at 10 V for ~1 hr). Once germinated, the ascospores were transferred to individual culture tubes. The major technical difficulties in tetrad analysis were the resistance of the clusters of asci to dissection and the small size of the ascospores (approximately $6 \times 14\ \mu m$).

Asci of *N. haematococca* contain a maximum of eight unordered ascospores, although most of the asci contain fewer than the maximum. As described below, the results were generally consistent with the expectation that the eight ascospores represent four sets of identical twins resulting from a mitotic division after meiosis, but before spore delimitation. Due to the low frequency of asci containing all eight spores, it was necessary to use some asci with as few as six spores for tetrad analysis. When the different combinations of markers allowed identification of all four meiotic products, the phenotypic ratios are written as if all eight spores were viable. However, the possibility of gene conversions that would have produced odd-numbered ratios (eg, 5:3 or 7:1) cannot be excluded for asci of fewer than eight spores.

Source of isolates. The heterothallic isolates of *N. haematococca* comprise seven distinct intrafertile (but not interfertile [7]) mating populations (MP). The isolates used in this study were from MP VI. The original field, mutant, and ascospore isolates used were first reported in previous studies (16–18). Isolates obtained directly from natural habitats (field isolates) are designated by the letter T followed by an isolate number. Isolates obtained from crosses are referred to as ascospore isolates. Random ascospore isolates are designated by the number of the cross followed by the ascospore number (eg, 105-10). Ascospores isolated for tetrad analysis are distinguished by a series of three numbers: the cross number, the tetrad number, and the ascospore number (eg, 103-3-7).

The markers analyzed genetically were obtained from field isolates and from mutants isolated in the laboratory (Table 1). The wild-type (+) counterparts of the laboratory-derived markers have the phenotypes of appressed growth habit (myc$^+$), red perithecia (red$^+$), and rapidly elongating hyphae with relatively infrequent branching (spc$^+$). All isolates tested in this laboratory are able to function as males in crosses. Mating type, female fertility, and perithecial color of an isolate were determined by crossing it reciprocally to tester isolates 6-36 (mat$^+$, fem$^+$, red) and 6-94 (mat$^-$, fem$^+$, red) (16). Mutant strains having the myc phenotype were selected as mycelial sectors from isolates 6-36 and 6-94. The myc phenotype was most evident on M100 minimal agar medium. The ability to demethylate pisatin was scored as in Tegtmeier and VanEtten (14).

RESULTS

Mating type segregated as a single pair of alleles in the random ascospore progeny of all crosses (Table 2). Tetrad analysis was performed on crosses 103 and 105. The progeny from all eight tetrads from cross 103 and seven of eight tetrads from cross 105 segregated in a 4 mat$^+$:4 mat$^-$ ratio (Table 3). These findings are consistent with a previous report of single-gene segregation for mating type in this fungus (9).

One tetrad from cross 105 failed to segregate for any trait analyzed; all seven spores from this ascus were phenotypically identical to the paternal parent. The nonsegregating tetrad apparently resulted from the selfing of the isolate intended as the paternal parent. Three of the progeny from this tetrad were crossed to an isolate carrying the spc marker discussed below. Random ascospore progeny from these crosses segregated normally (1 spc$^+$: 1 spc), demonstrating that the original progeny had not lost the ability to cross. The frequency of selfing in cross 105 appeared to be low; the ratio of parental to nonparental classes in the random ascospore progeny of this cross was within the expected range ($\chi^2 =$ 0.62).

All seven segregating tetrads from cross 105 segregated for perithecial color in a 4:4 ratio, indicating monogenic control of perithecial color in this cross. A representative tetrad is shown in Table 3. The segregation ratio among random ascospore progeny from cross 105 was also reasonably consistent with monogenic control of this trait; this was not, however, the case for several other crosses (Table 4).

The female fertility trait appears to be controlled by two genes. In cross 103, five tetrads segregated in a 4:4, two segregated in a 6:2, and one segregated in an 8:0 ratio of fem$^+$:fem$^-$. Representatives of each type are given in Table 3. This result suggests that at least two

TABLE 1. Source and description of genetic traits of *Nectria haematococca* used in this study

Trait	Description	Source
mat$^+$	+ Mating type	Field isolates (9,16)
mat$^-$	− Mating type	Field isolates (9,16)
fem$^+$	Female fertile	Field isolates (9,16)
fem$^-$	Female sterile	Field isolates (9,16)
pda$^+$	Having pisatin-demethylating ability	Field isolates (18)
pda$^-$	Lacking pisatin-demethylating ability	Field isolates (18)
myc	Mycelial:aerial hyphae few macroconidia	Spontaneous sectors
red	White perithecia	NTG[a] mutagenesis (16)
spc	Sporulating colonial: restricted growth, heavy conidiation	NTG mutagenesis (designated as sc in 17)

[a]NTG = nitrosoguanidine.

TABLE 2. Segregation of mating type among random ascospore progeny from crosses of *Nectria haematococca* MP VI

Cross	Parents: Female	Parents: Male	Progeny: mat$^+$	Progeny: mat$^-$	χ^{2a}	Ascospore germination (%)
101	6-94 (mat$^-$)	T63 (mat$^+$)	48	49	0.00	ND[c]
103	T161 (mat$^-$)	T110 (mat$^+$)	27	16	2.32	ND
104	6-94 (mat$^-$)	6-36A (mat$^+$)[b]	78	59	2.36	85
108	105-3 (mat$^-$)	6-36 (mat$^+$)	13	10	0.17	73
113	105-43 (mat$^-$)	6-36 (mat$^+$)	32	23	1.16	78
142	105-18 (mat$^-$)	101-66 (mat$^+$)	61	50	0.90	69
105	6-36 (mat$^+$)	T213 (mat$^-$)	24	30	0.46	ND
107	105-1 (mat$^+$)	6-94 (mat$^-$)	11	9	0.05	74
126	T33 (mat$^+$)	T221 (mat$^-$)	61	57	0.76	30
127	T33 (mat$^+$)	T95 (mat$^-$)	12	19	1.16	7

[a]χ^2 For 1:1 ratio is 3.84 at $P = 0.05$ according to Yates' correction (10).
[b]Isolate 6-36A is a myc mutant of isolate 6-36.
[c]ND = not determined.

loci for female fertility are segregating in cross 103, and that a fem⁻ allele at both loci is required for the female sterile phenotype. However, the random ascospore analysis of this cross (Table 5) showed a lower proportion of fem⁺ progeny than would be expected (at least 3 fem⁺:1 fem⁻) if this was true. The fem⁺ progeny may be less viable than the fem⁻ progeny or the *fem* genes may be linked. The segregation of this trait among random ascospore progeny was consistent with single-gene control in crosses 101, 126, and 142 (Table 5). Tetrad analysis was not performed on these crosses, so it is not known whether the parents in these crosses really differed at only one female fertility locus, or whether more than one locus was involved as in cross 103. A few fem⁻ progeny were recovered in the random ascospore analysis of crosses between fem⁺ isolates (Table 5). Some fem⁻ progeny might be expected in particular crosses since tetrad analysis of cross 103 indicates that this organism does have more than one locus controlling the fem⁺ phenotype. However, it is also possible that the progeny classified as fem⁻ actually carried the fem⁺ alleles: the degree of female fertility varied widely among fem⁺ progeny, as well as fem⁺ field isolates, and a few isolates classified as fem⁺ produced only one fertile perithecium in the testcrosses. This quantitative variation in female fertility might obscure the expression of the fem⁺ alleles in some progeny.

Ascospore progeny from the cross 6-36 (spc⁺) × M61 (spc) segregated into 47 spc⁺:40 spc ($\chi^2 = 0.41$ for a 1:1 ratio), indicating monogenic control of sporulating colonial growth. Similar results for the inheritance of this trait in another cross were reported previously (17).

Another single-gene morphological trait, myc, affected not only growth habit, but also female fertility. All four spontaneous mycelial (myc) mutants derived from fem⁺ isolates were unable to function as females in crosses. The random ascospore progeny from a cross between one of these mutants and a fem⁺ myc⁺ isolate segregated into 72 mycelial, female sterile:65 wild-type ($\chi^2 = 0.26$ for a 1:1 ratio). No myc, female fertile progeny or myc⁺, female sterile progeny were recovered, implying that female sterility is a pleiotropic effect of the myc allele. No other linkage was detected among the single-gene traits analyzed in this study.

An unusual phenomenon was observed in the cross between isolates 105-18 (mat⁻, fem⁺, pda⁻) and T57 (mat⁺, fem⁻, pda⁺): one marker segregated normally, but the other two failed to segregate at all. The 74 random ascospore progeny that were analyzed segregated into 40 mat⁺:34 mat⁻, but all were fem⁺ and pda⁻. Germination of ascospores from this cross was 60%. No linkage between *fem* and *pda* genes was detected in any other crosses.

TABLE 3. Analysis of representative unordered tetrads isolated from crosses 103 and 105 of *Nectria haematococca* MP VI. Ascospore isolates with identical phenotypes are grouped

Cross 103[a]	Ascus no.	Ascospore no.	Phenotype[a] of progeny: mat	fem	pda
T161 (mat⁻ fem⁺ pda⁺)					
T110 (mat⁺ fem⁻ pda⁻)	13	4	+	fem⁺	pda⁺
		5	+	fem⁺	pda⁺
		1	−	fem⁺	pda⁻
		3	+	fem⁻	pda⁺
		2	−	fem⁻	pda⁻
		6	−	fem⁻	pda⁻
	9	1	+	fem⁺	pda⁺
		2	+	fem⁺	pda⁺
		4	+	fem⁺	pda⁺
		3	−	fem⁺	pda⁺
		5	−	fem⁺	pda⁺
		6	−	fem⁺	pda⁺
		7	−	fem⁺	pda⁺
	3	1	+	fem⁺	pda⁺
		2	+	fem⁺	pda⁺
		4	+	fem⁺	pda⁺
		7	+	fem⁺	pda⁺
		3	−	fem⁺	pda⁺
		5	−	fem⁻	pda⁺
		6	−	fem⁻	pda⁺

Cross 105[a]	Ascus no.	Ascospore no.	Phenotype[a] of progeny: mat	red	pda
6-36 (mat⁺ red pda⁺) ×					
T213 (mat⁻ red⁺ pda⁻)	13	1	−	red	pda⁺
		2	−	red	pda⁺
		3	+	red⁺	pda⁺
		7	+	red⁺	pda⁺
		4	+	red	pda⁺
		5	+	red	pda⁺
		6	−	red⁺	pda⁻
		8	−	red⁺	pda⁻

[a]See Table 1 for description of abbreviated progeny phenotype traits. Genetic control of the trait pda is discussed in Tegtmeier and VanEtten (14).

DISCUSSION

Mendelian segregations in most crosses indicate that meiosis in *N. haematococca* proceeds normally. Tetrad analysis of two crosses confirmed this conclusion. Low fertility of some crosses and low viability of ascospores do pose some problems for genetic analysis, however. Also selfing can occur, as was seen in one tetrad,

TABLE 4. Segregation of perithecial color among random ascospore progeny from crosses of *Nectria haematococca* MP VI

Cross	Parents: Female	Male	Progeny: red⁺	red	χ^2[a]	Ascospore germination (%)
101	6-94 (red)	T63 (red⁺)	30	67	13.36	ND[b]
105	6-36 (red)	T213 (red⁺)	34	20	3.13	ND
107	105-1 (red⁺)	6-36A (red)	16	5	4.76	74
108	105-3 (red⁺)	6-36 (red)	15	8	1.56	73
113	105-43 (red⁺)	6-36 (red)	24	31	0.65	78
142	105-18 (red⁺)	101-66 (red)	66	26	16.53	69
103	T161 (red⁺)	T110 (red⁺)	93	0		ND
104	6-94 (red)	6-36A (red)	0	137		85

[a]χ^2 For a 1:1 ratio is 3.84 at $P = 0.05$ according to Yates' correction (10).
[b]ND = not determined.

TABLE 5. Segregation of female fertility among random ascospore progeny from various crosses of *Nectria haematococca* MP VI

Cross	Parents: Female	Male	Progeny: fem⁺	fem⁻	Ascospore germination (%)
105	6-36(fem⁺)	T213(fem⁺)	53	1	ND[a]
107	105-1(fem⁺)	6-94(fem⁺)	21	0	74
108	105-3(fem⁺)	6-36(fem⁺)	23	0	73
113	105-43(fem⁺)	6-36(fem⁺)	55	0	78
127	T33(fem⁺)	T95(fem⁺)	25	6	7
101	6-94(fem⁺)	T63(fem⁻)	55	42	ND
103	T161(fem⁺)	T110(fem⁻)	16	27	ND
126	T33(fem⁺)	T221(fem⁻)	65	53	30
142	105-18(fem⁺)	101-66(fem⁻)	67	43	69

[a]ND = not determined.

but that appears to be infrequent since the ratio of parental to nonparental types in progeny of this cross meets theoretical expectations.

Segregation ratios for mating type, perithecial color, and the mycelial growth and sporulating colonial morphologies were consistent with single-gene control of each trait. In one cross, the fem$^-$ phenotype required the presence of fem$^-$ alleles at two different loci. The relationship of the mycelial locus to the two female fertility loci was not determined. Since both of these traits result in female sterility, direct crosses between fem$^-$ and myc strains cannot be performed.

Male sterility in field isolates of *N. haematococca* MP VI has been reported by Matuo and Snyder (6) and Reichle et al (9), but no male-sterile isolates were isolated by VanEtten (16) or during the present investigations. Several isolates (Matuo's isolates I-2, III-13, SUF 224-2, and SUF 255) classified as male-sterile by Matuo, functioned normally when crossed reciprocally to the tester isolates used in this study (K. J. Tegtmeier and M. F. Dietert, *unpublished*). The high fertility of these tester isolates may account for the contradictory results. Naturally occurring male-sterile isolates are well documented in the sexually incompatible, but morphologically similar, *N. haematococca* MP I (11).

In one cross, progeny failed to segregate for traits other than mating type. Selfing alone would not account for this observation unless a concomitant change in mating type occurred. Such a change appears unlikely since single-spored isolates were never observed to produce mature perithecia in pure culture, even when grown under conditions optimal for crossing. Furthermore, pure culture isolates have never been shown to change mating type. El-Ani (4) reported secondary fertilization of perithecia of *N. haematococca* MP I. Apparently ascospores from perithecia of the initial cross fertilized later-developing perithecia. Secondary crosses were detected by the lack of expected segregation of traits, production of new perithecia later than expected, and presence of perithecia not of the maternal color. However, it is unlikely that this phenomenon occurred in *N. haematococca* MP VI crosses since, in crosses between parents differing in perithecial color, perithecia of the paternal color were extremely rare even quite late (up to 6 mo after crossing). While there is no satisfactory explanation for the failure of the progeny from this cross to segregate for all markers, such crosses can be eliminated from genetic analyses by selecting progeny from perithecia in which segregation occurs for at least one trait in addition to mating type and the other character(s) under consideration. In the four crosses reported in the accompanying paper (14) in which pda$^+$ was associated with virulence on pea, the traits mat and either female fertility or red segregated independently of each other and also independently of pda$^+$.

The typical segregation patterns in most crosses indicate that *N. haematococca* MP VI is amenable to sexual genetic studies. However, the segregation ratios for some traits deviated significantly from the expected results in some crosses (see Table 5). One possible cause for aberrant ratios may be structural (chromosomal) differences between isolates. The isolates used in this study were collected from many diverse habitats and geographic locations. Aberrant segregation ratios for a trait known to be controlled by a single gene have also been observed in progeny of crosses between isolates of *Neurospora* from widely different backgrounds (12).

Accurate measurement of segregation ratios is of most importance for determining the number of loci controlling a given trait or the distance between linked loci. For many studies in plant pathology, the number of genes controlling a physiological trait is a less important question than whether that trait is genetically correlated with pathogenicity. The latter question can be addressed using even crosses that give irregular segregation ratios for some markers, if it can be demonstrated that genes known to be unlinked do segregate independently in those crosses. As shown in this study, independent segregation is usually observed in *N. haematococca* MP VI, even in crosses between isolates obtained directly from the field. For more detailed studies of traits of particular interest, further crosses among the siblings and backcrosses to the parents should produce more reliable segregation ratios, as well as providing independent tests of the genotypes suggested by random ascospore and tetrad analysis of the original crosses.

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