

# Mutants of *Nectria haematococca* Created by a Site-Directed Chromosome Breakage Are Greatly Reduced in Virulence Toward Pea

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**Pathogenicity of *Nectria haematococca* toward *Pisum sativum* is determined by a 1.6 million base pair (Mb) supernumerary chromosome. Repeats of the simple telomeric DNA sequence (TTAGGG) and a gene (*PDA1*) from the 1.6-Mb chromosome were used to direct a chromosomal break at the *PDA1* locus. Mutant strains contained a novel 1.5-Mb chromosome that appeared to result from an approximately 100-kb deletion from the 1.6-Mb chromosome. These transformants (but not others that retain an apparently intact and mitotically stable 1.6-Mb chromosome) were essentially nonpathogenic on common pea, a host for the original isolate. Mutants containing the 100-kb deletion grow at a rate similar to the original strain in culture and retain the ability to colonize host tissue. Based on these results, our hypothesis is that genetic determinants of pathogenicity toward pea reside on the deleted portion of the 1.6-Mb chromosome. To our knowledge, this is the first reported use of site-directed chromosome breakage in filamentous fungi. Telomere-mediated chromosome breakage may speed genetic analysis of asexual fungi and be valuable for determining the consequences of duplicated and/or unique dispensable chromosomes in other pathogenic fungi.**

*Additional keywords:* B chromosome, *Fusarium solani*.

Some filamentous fungi contain chromosomes that are variably distributed within a species and nonessential for determining basic morphology, cellular structure, growth or primary metabolic functions (Kistler and Miao 1992; Zolan 1995). In some instances, these dispensable supernumerary chromosomes have been associated with virulence or determining host range for certain plant pathogenic fungi (Masel et al. 1993; Miao et al. 1991; VanEtten et al. 1994). *Nectria haematococca* Berk. & Br., a fungus that causes a stem and root rot disease on the common pea (*Pisum sativum* L.) has pathogenicity genes located on dispensable chromosomes (Miao et al. 1991; VanEtten et al. 1994; Wasmann and VanEtten 1996).

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Simple repeated DNA sequences are found at the ends of many eukaryotic chromosomes. Chromosome breakage may occur when these repeats are placed at interstitial sites on the chromosome by transformation- or transfection-based methods (Brown et al. 1994; Farr et al. 1991; Nimmo et al. 1994; Vollrath et al. 1988). Such chromosome breakage has been used to create hemizygous mutants in diploid mammalian cell lines and in diploid or aneuploid ( $n + 1$ ) yeasts but site-directed breakage never has been used to study the genetics of naturally occurring supernumerary chromosomes. Previously we reported a technique for telomere-mediated chromosome breakage in *N. haematococca* (Kistler and Benny 1992). The transformation-based technique involves a plasmid (pLD) containing a telomere repeat sequence from the fungus *Fusarium oxysporum* and a gene conferring drug resistance (Cullen et al. 1987; Powell and Kistler 1990). When linearized, the plasmid has the telomere sequence at one end and, at the other end, a site for cloning random or specific genomic DNA fragments. Directed integration of the plasmid into the fungal genome causes large deletions in certain chromosomes (Kistler and Benny 1992). Presumably, surviving colonies contain deletions only in dispensable chromosomes or chromosomes capable of sustaining terminal deletions.

Here we report chromosome breakage directed to a particular locus on a dispensable chromosome in *N. haematococca*. Earlier genetic studies had suggested that the gene for pisatin demethylase, located on this chromosome, was the sole factor responsible for controlling pathogenicity to pea in *N. haematococca* (VanEtten et al. 1989). Pea plants synthesize the antimicrobial isoflavonoid compound (phytoalexin) called pisatin, in response to microbial challenge. Some strains of the fungus, however, upon exposure to pisatin, produce a cytochrome P-450 class enzyme called pisatin demethylase, that is capable of oxidatively demethylating and, thus, detoxifying the pea phytoalexin. Virulence of the fungus was thought to be mediated by detoxification of the host's specific phytoalexin (Kistler and VanEtten 1984a; Mackintosh et al. 1989; Tegtmeyer and VanEtten 1982). However, VanEtten and co-workers have succeeded in specifically eliminating pisatin demethylase activity in the fungus by disruption of the major pisatin demethylase gene (*PDA1*) in some isolates (VanEtten et al. 1994; Wasmann and VanEtten 1996). A strain called Tr18.5 is a gene-disrupted mutant of wild-type strain 77-13-7. Although Tr18.5 is reduced in its ability to cause lesions on

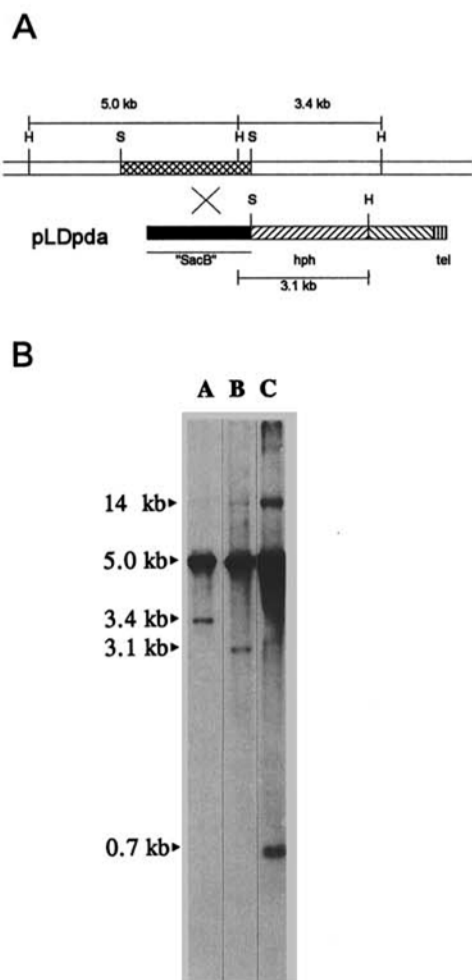
pea epicotyls compared to 77-13-7, it retains a large degree of its ability to cause necrosis on host tissue compared to authentic nonpathogenic isolates. This fact has caused VanEtten and co-workers to hypothesize the presence of other pea pathogenicity genes, closely linked to *PDA1* on the 1.6-Mb chromosome (VanEtten et al. 1994).

## RESULTS

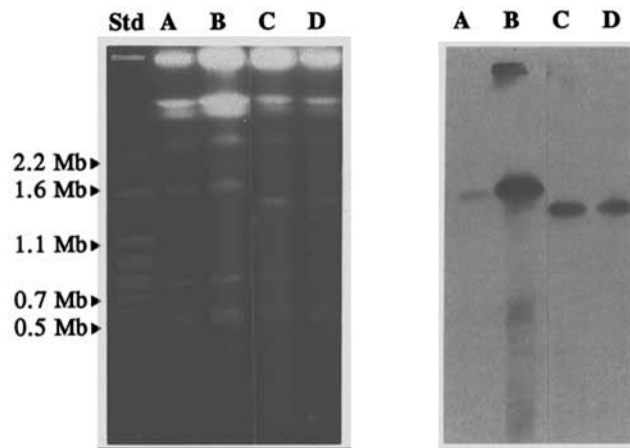
In an effort to identify other pathogenicity determinants linked to *PDA1*, an internal restriction fragment from a pisatin

demethylase gene was used to target integration at this locus on the dispensable 1.6-Mb chromosome (Fig. 1). The sequence from the pisatin demethylase gene (called SacB) was placed in tandem with a gene for resistance to hygromycin B and a repeated sequence (TTAGGG)<sub>n</sub> found at chromosome ends from all tested filamentous fungi (Coleman et al. 1993; Connelly and Arst 1991; Farman and Leong 1995; Guzmán and Sánchez 1994; Javerzat et al. 1993; Woods and Goldman 1992). The plasmid was then linearized and used to transform *N. haematococca* strain Tr18.5. As a gene disruption mutant, Tr18.5 contains plasmid sequences at the *PDA1* locus (Wasmann and VanEtten 1996) and thus provides a large target for integration of pLD based on both plasmid and *PDA1* sequence similarity. Several transformants were selected by drug resistance and then screened for alteration at the *PDA1* locus and deletion.

Two independent transformants of Tr18.5 were found both to be altered at the *PDA1* locus and associated with a deletion on the 1.6-Mb chromosome. These transformants were called N-15 and 0601-2. Southern hybridization of DNA from the recipient and transformant strains with the SacB probe illustrates the change (Fig. 1). Based on the restriction map of the transformation plasmid, pLDpda, and the *PDA1* locus defined by this probe, simple integration occurred in the case of N-15, while a DNA rearrangement appears to have occurred in 0601-2. The SacB probe hybridized to 5.0- and 3.4-kb *Hind*III fragments in recipient strain Tr18.5, but a 5.0- and 3.1-kb band in N-15. The hybridization pattern of the SacB probe is consistent with replacement of the 3.4-kb *Hind*III chromosomal fragment with the 3.1-kb *Hind*III fragment derived from pLDpda. 0601-2 also lacked the 3.4-kb *Hind*III fragment but contained a 0.7-kb band that is inconsistent with a simple integration event. All strains also contained a 14-kb band that hybridized to SacB apparently at a site located slightly upstream from deletions noted in N-15 and 0601-2 (see below). SacB-hybridizing restriction fragments, distinct from the



**Fig. 1.** Targeted insertion at the *PDA1* locus. **A**, Diagram of pLDpda, a plasmid constructed for directing integration at the *PDA1* locus and the presumptive recombination event leading to the observed hybridization patterns. tel, the telomere repeat sequence; H, *Hind*III site; S, *Sac*I site. Diagonally marked region labeled "hph" represents the hygromycin B phosphotransferase gene; the adjacent sequence marked with diagonals is derived from pUC119 (Vieira and Messing, 1987). The cross-hatched portion of the diagram indicates sequences that hybridize to "SacB" (see Materials and Methods) in the genome of Tr18.5. The SacB homologous sequences are not identical and the copy in Tr18.5 contains an internal *Hind*III site, whereas the sequence on the plasmid does not. Simple recombination could produce a novel 3.1-kb *Hind*III band if the site is retained or a single 8.1-kb *Hind*III band if this site is lost. **B**, Southern hybridization of DNAs from recipient and transformant strains. DNAs from Tr18.5 (A), N-15 (B) or 0601-2 (C), were digested with *Hind*III, fractionated on a 0.7% agarose gel, and blotted by standard methods (Sambrook et al. 1989). The Southern blot was probed with the labeled SacB fragment.



**Fig. 2.** Electrophoretic karyotypes of recipient and transformant strains illustrating the loss of 100 kb from the 1.6-Mb chromosome. Left, photograph of an ethidium bromide-stained gel illuminated by UV light. The gel was run at conditions described in the Materials and Methods. Lanes contain chromosome-sized DNAs from *Saccharomyces cerevisiae* strain YNN295 as a size standard (Std) and *Nectria haematococca* strain 77-13-7 (A), Tr18.5 (B), and transformants N-15 (C), and 0601-2 (D). Right, autoradiogram of blot probed with a plasmid containing the SacB fragment.

*PDA1* locus, have been observed previously for a sibling ascospore isolate 77-2-3 (Maloney and VanEtten 1994).

The electrophoretic karyotypes of both N-15 and 0601-2 indicated that a 100-kb deletion occurred in the 1.6-Mb dispensable chromosome (Figs. 2 and 3). Both N-15 and 0601-2 have 1.5-Mb chromosomes that hybridized strongly to the *SacB*-containing plasmid. From these results we hypothesize that *PDA1* is 100 kb from the telomere of the 1.6-Mb chromosome. The *SacB*-hybridizing DNA is sensitive to *Bal31* exonuclease digestion (see Materials and Methods), suggesting a telomere-proximal location for the *PDA1* locus in N-15 and 0601-2.

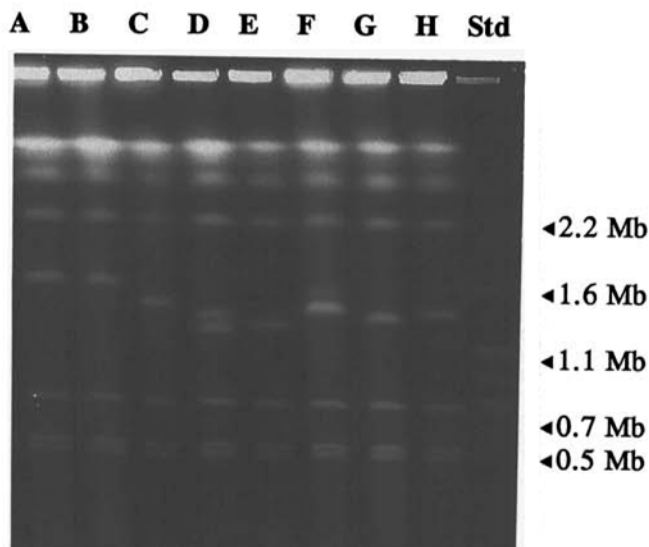
Transformants N-15 and 0601-2 also were altered in their pathogenicity toward pea. Whereas the recipient strain Tr18.5 was capable of causing  $8.7 \pm 2.7$  mm lesions on wounded pea epicotyls, the mean lesion sizes for N-15 and 0601-2 were  $1.6 \pm 0.3$  and  $1.9 \pm 0.3$  mm, respectively (Fig. 4). Mean lesion sizes for the recipient and mutants differed significantly ( $P = 0.05$ ). All of the other 14 tested transformants arising due to ectopic integration of the plasmid and/or retaining a 1.6-Mb chromosome based on electrophoretic karyotype, produced lesions on pea that were not significantly different from the recipient strain (Fig. 4, data not shown).

Isolates N-15 and 0601-2 had radial growth rates similar to Tr18.5 on a complete agar medium. Mean growth rates were 0.241, 0.229, and 0.237 mm/h for Tr18.5, N-15 and 0601-2 respectively. The mean growth rates for Tr18.5 and 0601-2 were not significantly different; the growth rate for N-15 was significantly less than Tr18.5 ( $P = 0.02$ ) but not significantly different than 0601-2. Both N-15 and 0601-2 were observed microscopically, growing within nonsymptomatic pea plants. Transformants were recovered on hygromycin-containing (50  $\mu\text{g/ml}$ ) medium by plating slices of the pea epicotyl in 3 mm intervals away from the site of inoculation. Both transfor-

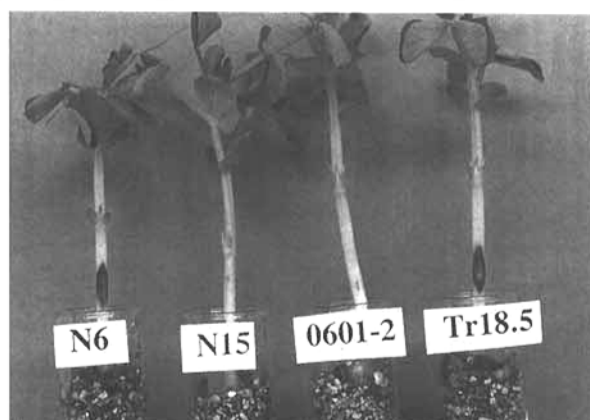
ants were easily recovered from plants 1 week after inoculation, and greater than 1 cm from the site of inoculation. Therefore, although the mutants caused little or no disease, they still were capable of colonizing plant tissue in a non-destructive manner. The electrophoretic karyotype of 77-13-7 and Tr18.5 and hygromycin-resistant single-spore derived cultures of N-15 or 0601-2 recovered from inoculated peas were identical to the karyotype found in the original isolates (data not shown).

Without selection in culture, most single-spore derived isolates of N-15 and 0601-2 did not retain the hygromycin-resistant phenotype but did retain the 1.5-Mb chromosome. When grown for 10 days in culture without hygromycin selection, a total of 86% (99/115) or 97% (101/104) of single spore isolates of N-15 or 0601-2, respectively, no longer grew in the presence of 50  $\mu\text{g}$  of hygromycin per ml. However, all 131 hygromycin-sensitive isolates examined by Southern hybridization (78 derived from N-15 and 53 derived from 0601-2) retained sequence similarity to the gene for hygromycin B phosphotransferase (not shown). Curiously, further single-spore of hygromycin-sensitive isolates led to isolates again resistant to 50  $\mu\text{g/ml}$  hygromycin. The similar phenomenon known as "phenotype switching" has been observed in yeasts when genes are juxtaposed to telomeres. In the case of yeast, the telomere position effect results in variable gene expression due to transcriptional silencing (Sandell et al. 1994; Gottschling et al. 1990; Nimmo et al. 1994). A similar situation may exist for the *hph* gene in the transformants analyzed here.

In some instances, hygromycin-sensitive isolates had further chromosome rearrangements. A few single-spore isolates of both N-15 and 0601-2 showed additional change in the 1.5-Mb chromosome, most commonly a further reduction in the size of the chromosome or the appearance of an additional, novel-sized chromosome (Fig. 3). When it was used as a probe for Southern hybridization, the gene for hygromycin B phosphotransferase hybridized to the altered chromosomes in these strains as well as to the 1.5-Mb chromosome, as it did in



**Fig. 3.** Electrophoretic karyotypes of recipient and transformant strains illustrating instability of the 1.5-Mb chromosome in culture. Conditions for the gel are described in Materials and Methods. Lanes contain chromosome-sized DNAs from strain Tr18.5 (A), 77-13-7 (B), transformant 0601-2 (C), two independent hygromycin-sensitive single-spore isolates of 0601-2 (D,E), two independent hygromycin-sensitive single-spore isolates of N-15 (F,G) and N-15 (H). *Saccharomyces cerevisiae* strain YNN295 was used as a size standard (Std).



**Fig. 4.** The disease phenotypes of recipient and transformant strains. Photo illustrates the results of a stem lesion bioassay conducted as described previously (Kistler and VanEtten 1984a). Plants are inoculated with strain Tr18.5, 0601-2, N-15, and N-6. N-6 is a transformant isolated from the same experiment as N-15 but with pLDpd integrated at an ectopic site in the genome. N-6 showed no signs of deletion from the 1.6 Mb chromosome (data not shown).

all other tested hygromycin-sensitive or -resistant strains derived from N-15 or 0601-2 (not shown). However since cultures obtained from inoculated plants retained both hygromycin resistance and the 1.5-Mb chromosome, loss of pathogenicity in N-15 and 0601-2 did not appear to be due to further destabilization of the entire, or a portion of, the dispensable chromosome.

## CONCLUSIONS

Our results are consistent with the hypothesis that an additional gene or genes linked to the pisatin demethylase locus are involved in determining virulence to pea. Deletion of 100 kb from the 1.6-Mb chromosome in Tr18.5, a strain already lacking pisatin demethylase activity, resulted in the near complete loss of the ability to cause lesions on pea epicotyls. Although one of the two mutants grew at rate in culture that was slightly reduced compared with the original isolate, it is unlikely that this reduction greatly influenced virulence. Growth rates of nonmutagenized strains *in vitro* are not correlated with virulence and the growth of N-15 was still 95% that of Tr18.5; well within the range of other cultures virulent toward pea (Kistler and VanEtten 1984b, and unpublished). Additionally, both N-15 and 0601-2 still colonized nonsymptomatic plant tissue so loss of lesion-causing ability seemed not to result from the inability to grow within the plant. Both N-15 and 0601-2 also retained the ability to cause lesions on ripe tomato fruit (C. C. Wasmann and H. D. VanEtten, personal communication) similar to that observed for Tr18.5 (Wasmann and VanEtten 1996). Reduction in virulence of N-15 and 0601-2 thus appears to be specific for pea.

Our results are complementary to those of VanEtten and coworkers who have found that disruption of the pisatin demethylase gene in *N. haematococca* does not lead to complete loss of virulence (Wasmann and VanEtten 1996), nor does introduction of a gene for pisatin demethylase completely restore virulence to strains lacking the *PDA1* locus and the 1.6-Mb chromosome (Ciuffetti and VanEtten 1996). Also, loss of the *PDA1*-containing 1.6-Mb chromosome as a consequence of transformation or by benomyl treatment leads to reduced virulence similar to what we have observed in N-15 and 0601-2 (C. Jorgenson and H. D. VanEtten, unpublished). Presumably novel virulence determinants are located on the 100-kb portion deleted from the 1.6-Mb chromosome.

Although essentially nonpathogenic, N-15 and 0601-2 still were capable of colonizing host tissue and growing well beyond the site of inoculation. Similar results have been noted for a nonpathogenic mutant of *Colletotrichum magna* (Freeman and Rodriguez 1993) where a single mutation converted a pathogenic isolate into a harmless endophytic fungus. It remains to be determined if the *N. haematococca* mutants, like those of *C. magna*, also protect plants from subsequent challenge by pathogenic fungi (Prusky et al. 1994).

Genes associated with host specialization in a few instances have been localized to unique supernumerary chromosomes. Without these chromosomes *N. haematococca* isolates may be able to survive as soil saprophytes. However in nature the chromosomes may confer fitness when appropriate plant hosts are present by serving as reservoirs for pathogenicity genes and host range determinants. Reports suggest that pathogenicity genes of *Colletotrichum gloeosporioides* also may be on

unique supernumerary chromosomes (Masel et al. 1993). Telomere-mediated chromosome breakage may supplement existing methodologies for genetic analysis of asexual fungi for which meiotic recombination analysis is impossible. This approach may be valuable for examining the biological consequences of duplicated and/or unique dispensable chromosomes in other pathogenic fungi of plants and animals.

## MATERIALS AND METHODS

### *N. haematococca* transformation and bioassays.

The strain of *N. haematococca* used for this investigation was Tr18.5 (Wasmann and VanEtten 1996). Tr18.5 was derived from strain 77-13-7 that contains a single gene for pisatin demethylase (*PDA1*) and is virulent toward pea (Kistler 1983; Kistler and VanEtten 1984b). Tr18.5 has a replacement of an internal fragment of *PDA1* with a hygromycin B resistance (*hph*) cassette from transforming plasmid pKO1 (Wasmann and VanEtten 1996). This strain was chosen because it lacks pisatin demethylase yet still retains virulence toward pea. Mutations resulting in lowered virulence thus could not be attributed to changes in expression of pisatin demethylase. Despite having a functional *hph* gene at the *PDA1* locus, the strain remains sensitive to 50 µg of hygromycin B per ml under conditions previously used by us for transformation of *N. haematococca* (Kistler and Benny 1992). Thus transformation of Tr18.5 by pLD (Kistler and Benny 1992) with hygromycin selection was possible and easily achieved.

The plasmid used for chromosome breakage was constructed using a 1.2-kb *SacI* fragment ("SacB") from *N. haematococca* strain T9 containing an internal portion of the pisatin demethylase gene (Maloney and VanEtten 1994), largely retained in Tr18.5. This fragment was ligated in both orientations into the *SacI* site of pLD and one such plasmid, called pLDpda, was used to transform protoplasts of Tr18.5. Digestion at a unique *KpnI* site linearizes the plasmid such that the pisatin demethylase sequence was at one end and the telomere repeat sequence (marked tel in Fig. 1) was in the correct orientation (5'[TTAGGG]<sub>18</sub>3' on the top strand as illustrated here) near the other end. Transformation procedures have been described previously (Kistler and Benny 1988).

The electrophoretic karyotypes of 20 single-spored transformant cultures were examined. Based on Southern analysis, most if not all strains had integration/interaction with the 1.6-Mb chromosome. The most common alteration (6/20 transformants) was the presence of a supernumerary 1.5-Mb chromosome. These results are consistent with a 100-kb deletion mutation of the 1.6-Mb chromosome along with retention of the original-sized chromosome. In one case (the initially recovered transformant strain N15) both a 1.6- and 1.5-Mb chromosome were found but the 1.6-Mb band was present at much less than stoichiometric amounts compared to other chromosome bands. A further single spore isolation of strain N15 contained the 1.5-Mb chromosome but no evidence of the original 1.6-Mb chromosome.

Pathogenicity tests were conducted by wound-inoculating pea epicotyls using the *Pisum sativum* cultivar Little Marvel (Kistler and VanEtten 1984a). Lesion length values given are mean and standard deviation of three independent tests; five or more plants were observed for each test. Comparison of means was by two-sample Student *t*-tests. Tests for hygromy-

cin resistance or sensitivity (Kistler and Benny 1992) and comparison of fungal growth rates (Kistler and VanEtten 1984a) also were described previously. Growth rates were determined on V8 juice agar (167 ml of V8 juice, 3 g of CaCO<sub>3</sub>, 15 g of agar per liter, pH 6) by taking the slope of the linear regression equation obtained by plotting growth (in mm) over time (in h). R<sup>2</sup> values for all equations were 99.9% or greater. Growth rate tests were conducted three times, with four replicates per strain. Comparison of mean growth rates was by two-sample Student *t*-tests.

### Pulsed field gel electrophoresis and Southern hybridization.

Chromosome preparations and Southern hybridizations were as described previously (Kistler et al. 1995). Chromosome-sized DNAs from *N. haematococca* strains were resolved using a commercially available apparatus (BioRad CHEF DR-II, Hercules, CA). The 0.8% agarose gels were run in 0.25× TBE buffer (Sambrook et al. 1989) for 90 h at 2.4 V/cm with a linear ramped 40- to 800-s pulse interval. Probes were made by priming the large subunit of DNA polymerase I with random hexanucleotides in the presence of deoxynucleotides plus fluorescein-conjugated dUTP. Chemiluminescent detection was performed by manufacturers's recommendations (RENAISSANCE, DuPont NEN). Hybridization and washes were carried out at 68°C.

### Bal31 digestion of DNA.

Ten micrograms of DNA from N15 or 0601-2 was incubated with 2 U of Bal31 in a total reaction volume of 80 µl. Twenty microliters aliquots were removed at 0, 2, 8, and 20 min time points and the reaction stopped by addition of 3 µl, 200 mM EGTA and heating 10 min at 70°C. Samples were digested with *Hind*III, fractionated on a 0.7% agarose gel, and blotted. The Southern blot was probed with the labeled SacB fragment. Strong hybridization was noted to DNA at the zero time points, much less at 2 min and no hybridization was seen to DNA after 8- or 20-min Bal31 incubations. In contrast, ethidium bromide-stained gels indicated little or no DNA degradation.

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