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Phenotypic and Genetic Characterization of Two New Mutants of *Heterorhabditis bacteriophora*

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Abstract: Two new "dumpy" mutants (*Hbdpy-2* and *Hbdpy-3*) of *Heterorhabditis bacteriophora* were induced and characterized. Mutants (hermaphrodites and males) that hatched from eggs were shorter and wider than the wild-type strain. This phenotype was not discernible in young animals until 24 hours after hatching from eggs or in mutants that developed from infective juveniles. Scanning electron microscopy revealed that the tails of the two mutants are much more slender than in the wild-type. In addition, the vulva of *Hbdpy-3* nematodes appeared to be sunken; that of *Hbdpy-2* animals was protruding, like in the wild-type. Upon self fertilization, individual *Hbdpy-3* hermaphrodites produced fewer progeny than the wild-type. Crosses between virgin *Hbdpy-2* and *Hbdpy-3* hermaphrodites and wild-type males indicated that the two mutations are recessive. Complementation tests indicated that *Hbdpy-1, Hbdpy-2,* and *Hbdpy-3* affect different genes. The ratio (1.03:1) of wild-type to dumpy phenotype among the F_2 progeny of self-fertilizing heterozygotes suggested linkage among the three genes. The genetic map distance was estimated only between *Hbdpy-1* and *Hbdpy-2* genes, approximately 29 map units.

Key words: dumpy mutant, genetics, *Heterorhabditis bacteriophora,* linkage map, mutagenesis, nematode.

The lack of mammalian pathogenicity and the availability of relatively simple and inexpensive methods for commercially culturing *Heterorhabditis bacteriophora* have made this nematode species an attractive candidate for biological insect control (8). However, environmental extremes such as high temperatures, low humidity, and solar radiation reduce nematode efficacy considerably (7,12). One approach for overcoming susceptibility to harsh environmental conditions is through genetic improvement (4–6), either by i) selection of an existing variant displaying the desired trait from a genetically heterogenous wild-type population or ii) induction of random mutations in genes controlling the desired trait, in individuals from a genetically homogenous wild-type population.

Heterorhabditis bacteriophora strain HP88 is especially suited for genetic analysis and improvement. It has a short generation time and large number of offspring. This species is easily cultured in the laboratory and reproduces either by self- or cross-fertilization (19), two processes crucial for genetic analysis and improvement (4,9). Self-fertilization enables establishment of inbred lines, whereas cross-fertilization allows exchange of genetic material between individuals.

Isolation of mutants with unique phenotypic characteristics that would serve as genetic markers is essential for future mapping and genetic analysis of mutants displaying improved beneficial traits. Furthermore, establishment of a linkage map of the marker mutations would facilitate mapping of beneficial genes.

Zioni et al. (20) and Rahimi et al. (17) recently isolated morphological mutants of *H. bacteriophora.* The present study describes the induction and isolation of two additional morphological mutants of *H. bacteriophora,* a further step towards establishing a genetic linkage map in *H. bacteriophora.*

MATERIALS AND METHODS

Nematode strains and culture: The HP88 strain of *H. bacteriophora* was obtained from Biosys (Palo Alto, CA). The homogeneous strain "6Dy" was derived from the

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wild-type population by 15 repeated cycles of self-fertilization, each initiated from a single hermaphrodite of the previous generation. The 6Dy strain was thus estimated to be >95% homozygous for the wild-type alleles (20). In vivo culture of the nematodes was carried out on last-instar larvae of the greater wax moth, *Galleria mellonella* (3). In vitro culture of the nematodes was in 3- or 5-cm-d petri dishes containing dog food agar (DFA) (9) or nematode growth agar (NGA) (1). The DFA or NGA plates were preinoculated with *Photorhabdus luminescens* (=*Xenorhabdus luminescens*), the bacterial associate of *H. bacteriophora* (14,15). The bacteria were isolated and propagated according to Poinar and Thomas (15).

Mutagenesis, screening, and establishment of mutant lines: Ethyl methanesulfonate (EMS) mutagenesis and stereomicroscopic screening of the F_2 generation of the mutagen-treated population for morphological and behavioral abnormalities were performed by described procedures (20). The putative mutant individuals thus identified were transferred singly to a new NGA plate to verify transmission of the mutant phenotype to subsequent generations and to initiate a homozygous line.

Phenotypic analysis: Infective juveniles (IJ) were surface sterilized with 1% methylbenzethonium chloride (Sigma Chemical Co., St. Louis, MO) (13) and were transferred to DFA plates. Eggs were subsequently obtained from the bodies of gravid hermaphrodites (16). The length of nematodes developed from IJ or eggs was determined at 24-hour intervals for a period of 96 hours, with a calibrated stereomicroscope (Wild, Heerbrugg, Switzerland). Fine structural and cuticular surface details were examined with a scanning electron microscope (SEM) (18).

The infectivity of IJ from wild-type or mutant strains toward the last-instar larvae of *Galleria mellonella* was determined as follows. A suspension of 0, 25, 50, 100, 200, or 400 IJ in 0.5 ml deionized water was applied to a 5-cm-d filter paper (Whatman No. 1), which was then placed in a 5-cm-d petri dish. Three to five minutes later, 4 last-instar larvae of *G. mellonella* were placed in each of the dishes and maintained at 25 C in the dark. Insect mortality was recorded 72 hours after inoculation. Four dishes were utilized for each nematode strain and number of IJ. The experiment was performed three times.

For determination of reproductive potential of the different strains, young virgin hermaphrodites originating from IJ or eggs were transferred individually to separate 3-cm-d NGA plates containing symbiotic bacteria. Five days later, the number of progeny was recorded. Forty-four, 43, and 15 individual hermaphrodites were thus examined for the wild-type, *Hbdpy-2*, and *Hbdpy-3* strains, respectively.

Genetic crosses: Separate cultures of mutant and wild-type (6Dy) strains were initiated from IJ on DFA plates. Virgin hermaphrodites and males were rinsed from the DFA plates with autoclaved saline (8 mg/ml NaCl in distilled water). Reciprocal crosses between wild-type and mutant nematodes were conducted by first transferring five to six males and one virgin hermaphrodite to a 3-cm-d NGA plate. Four to five days later, the adult F_1 progeny were phenotypically examined under a stereomicroscope and used for production of subsequent generations. Because single hermaphrodites were used in the crosses, we could determine accurately the wild-type to dumpy phenotypic ratio among their progeny. Crosses between dumpy males from one mutant strain and dumpy hermaphrodites from another mutant strain were conducted similarly, except five to six virgin hermaphrodites were used in each cross.

Chi-square tests were performed to test frequency ratios. For other measurements, one-way analysis of variance was done followed by Duncan's multiple-range test.

Results and Discussion

Phenotypic characterization of the mutants: The mutagenesis yielded two independent "dumpy" mutants (Fig. 1B,C) named *Hbdpy-2* and *Hbdpy-3* in accordance with

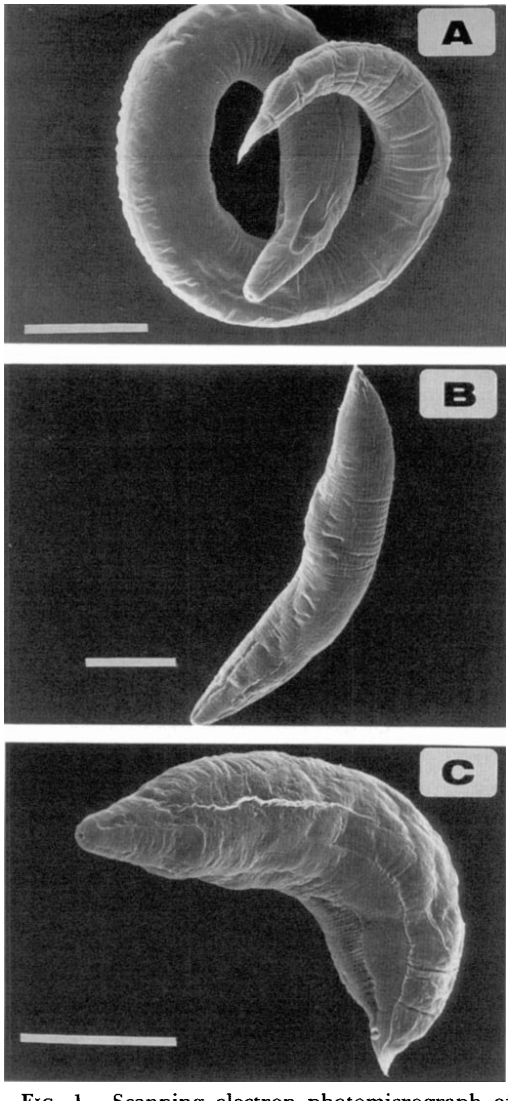

FIG. 1. Scanning electron photomicrograph of hermaphrodites of *Heterorhabditis bacteriophora* HP88. A) Wild-type. B) Dumpy mutant *Hbdpy-2*. C) Dumpy mutant *Hbdpy-3*. Scale bar = 100 μm.

the terminology used for *Caenorhabditis elegans* (11) and the first mutant of *H. bacteriophora, Hbdpy-1* (20). The "Hb" stands for *Heterorhabditis bacteriophora,* "dpy" for the dumpy phenotype, and "2" and "3" for the corresponding loci (distinct loci, as subsequently determined). The dumpy phenotype of the mutants was apparent in the adults obtained from eggs on DFA plates. However, in adults developed from IJ, the dumpy phenotype was much less extreme (not significantly different from the wild-type phenotype, Fig. 1, Table 1, $P < 0.05$, Duncan's multiple-range test).

The dumpy phenotype was fully penetrant in the adult stage of both hermaphrodite and male *Hbdpy-2* and *Hbdpy-3* hatching from eggs (data not shown). However, this phenotype in young animals was not discernible until 24 hours after nematode hatching from eggs. This delay in expression of the dumpy phenotype could indicate that the normal function of the *Hbdpy-2* and *Hbdpy-3* genes is required in a rather late stage of development.

Examination by SEM revealed differences in the tails of mutants vs. that of the wild-type, the former being sharper (Fig. 2). In addition, the vulva of *Hbdpy-3* animals appeared to be sunken, whereas in wild-type and *Hbdpy-2* animals, it was typically protruding (Fig. 3).

Infective juveniles of *Hbdpy-2* caused as much mortality as the wild-type in *G. mellonella* exposed to IJ (Table 2, $P < 0.05$, Duncan's multiple-range test). Because *Hbdpy-3* produced too few IJ, its ability to induce mortality could not be determined.

Upon self-fertilization on NGA plates, individual hermaphrodites of *Hbdpy-3* tended to produce fewer progeny than the wild-type and *Hbdpy-2* (15 ± 2, 78 ± 31, and 51 ± 32 respectively, $P < 0.05$, Duncan's multiple-range test).

Genetic characterization of the mutants: Crosses were conducted between virgin *Hbdpy-2* or *Hbdpy-3* hermaphrodites and wild-type males to determine if the mutations are dominant or recessive, autosomal or sex-linked. Because both wild-type and dumpy phenotypes were observed among F_1 hermaphrodites and males (Table 3), the two mutations are recessive.

The basis of sex determination of this nematode species has not been critically tested. Therefore, our results do not allow determination of whether the mutations are autosomal or sex-linked. In the related species *Caenorhabditis elegans* and in many other nematodes, sex determination is chromosomal: females (or hermaphrodites) are XX and males are XO (10). If an XX-XO mechanism were operational in *H.*

TABLE 1. Width and length (mean ± SD) of hermaphrodite adults of wild-type nematodes and dumpy mutants (*Hbdpy-2* and *Hbdpy-3*) of *Heterorhabditis bacteriophora* strain HP88, 96 hours after transfer of eggs or infective juveniles (IJ) to dog food agar plates.

	Adults developed from eggs		Adults developed from IJ	
Strain	Width (mm)	Length (mm)	Width (mm)	Length (mm)
Wild-type	0.13 ± 0.01	2.30 ± 0.09	0.21 ± 0.06	2.45 ± 0.06
Hbdpy-2	0.19 ± 0.01	1.24 ± 0.04	0.23 ± 0.03	2.39 ± 0.04
Hbdpy-3	0.20 ± 0.01	1.02 ± 0.06	†	†

Ten adults were measured for each sample.
† Because *Hbdpy-3* produced too few IJ, this value could not be determined.

bacteriophora, then our results would be consistent with the hypothesis that the two mutations are autosomal. However, results from studies by Dix et al. (2) and the following observations suggest that the basis of sex determination in *H. bacteriophora* and *C. elegans* might be different. In a spontaneous wild-type population of *H. bacteriophora,* the frequency of males is 9.6% (19). In this population it is impossible to determine whether the males originated from self- or cross-fertilization. In our crosses between virgin *Hbdpy-2* hermaphrodites and wild-type males, 8.78 ± 6.12% males originated from self-fertilization and 0.55 ± 0.43% males originated from cross-fertilization (Table 3). These frequencies are different from those obtained for *C. elegans,* where <0.5% males were obtained in self-fertilization and 50% males were obtained in cross-fertilization (10). Self- and cross-fertilization of *Hbdpy-*

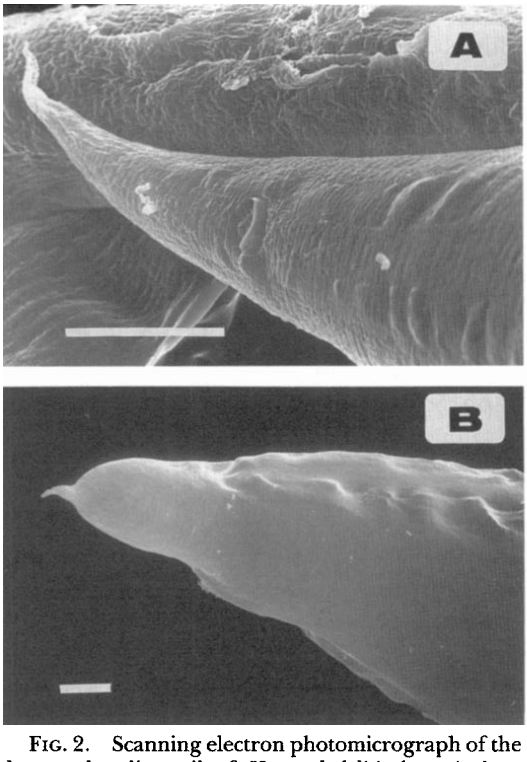

FIG. 2. Scanning electron photomicrograph of the hermaphrodite tail of *Heterorhabditis bacteriophora* HP88. A) Wild-type. B) Dumpy mutant *Hbdpy-2.* Scale bar = 10 μm.

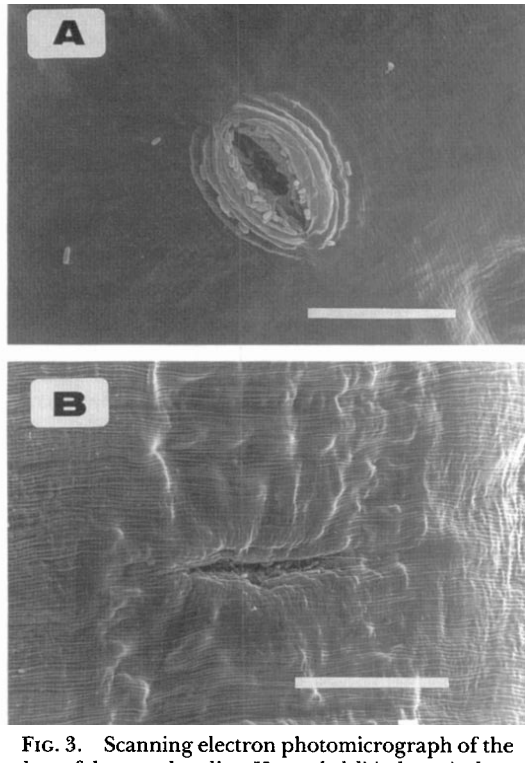

FIG. 3. Scanning electron photomicrograph of the vulva of hermaphrodite *Heterorhabditis bacteriophora* HP88. A) Wild-type. B) Dumpy mutant *Hbdpy-3.* Scale bar = 10 μm.

TABLE 2. Percentage mortality of last-instar larvae of the wax moth *Galleria mellonella* following 72-hour exposure to different numbers of infective juveniles (IJ) of wild-type and *Hbdpy-2* mutants of *Heterorhabditis bacteriophora* HP88 in petri dishes containing moist filter paper.

Nematodes (IJ/dish)	Insect mortality (%)	
	Wild-type	*Hbdpy-2*
0	0	0
25	55.2 ± 10.4	71.1 ± 6.3
50	81.4 ± 6.7	75.5 ± 6.8
100	100.0 ± 0.0	100.0 ± 0.0
200	100.0 ± 0.0	100.0 ± 0.0
400	100.0 ± 0.0	100.0 ± 0.0

Data are means of 12 replicates (with 4 replicates in each of three experiments). Each replicate comprised four insect larvae.

3 produced very few males, possibly a result of the small number of offspring (Table 3).

We also conducted the reciprocal crosses between *Hbdpy-2* or *Hbdpy-3* males and wild-type virgin hermaphrodites. In both cases only the wild-type phenotype was observed among F_1 hermaphrodites and males. These results indicate that the two mutations are recessive. Had they been dominant, some of the progeny of these crosses would have had a dumpy phenotype. An alternative interpretation of these results is that mutant males cannot fertilize wild-type hermaphrodites; all of the observed progeny originated solely from self-fertilization of the wild-type hermaphrodites.

Next, 10 phenotypically wild-type F_1 hermaphrodites originating from crosses between *Hbdpy-2* hermaphrodites and wild-type males, and five phenotypically wild-type F_1 hermaphrodites originating from crosses between *Hbdpy-3* hermaphrodites and wild-type males were transferred individually to separate DFA plates for reproduction by self fertilization. Because each of these F_1 hermaphrodites produced both wild-type and dumpy progeny, these F_1 hermaphrodites were heterozygous. Therefore, the phenotypic data of their progeny (F_2) were pooled, for each mutant separately, and consisted of 23.7%–25.7% dumpy and 74.3%–76.3% wild-type animals among both males and hermaphrodites (Table 4, frequencies were consistent with a ratio of 1:3, $P > 0.05$, χ^2 test). These frequencies further support the conclusion that the mutations are recessive. If the basis of sex determination in *H. bacteriophora* were as in *C. elegans,* then such a 1:3 ratio would be expected among the F_2 hermaphrodites, whether the recessive mutations are autosomal or sex-linked. Among the F_2 male progeny this ratio is informative: it suggests that *Hbdpy-2* and *Hbdpy-3* are autosomal (had they been sex-linked the dumpy:wild-type ratio among the F_2 males would be 1:1).

Complementation tests between Hbdpy-1 *and* Hbdpy-2: Because *Hbdpy-1* (20), *Hbdpy-2,* and *Hbdpy-3* have a similar phenotype, they could represent different mutations in the same gene. Therefore, crosses were conducted among the three mutants to investigate this possibility.

Crosses (n = 99) between virgin *Hbdpy-1* hermaphrodites and *Hbdpy-2* males gave F_1 offspring of both wild-type and dumpy phenotypes (in both hermaphrodites and males). Therefore these two recessive mutations complement each other, i.e., they affect different genes. Had the two mutations affected the same gene, all progeny would have had a dumpy phenotype.

Fifteen phenotypically wild-type F_1 hermaphrodites originating from these crosses were transferred to separate DFA plates for reproduction by self-fertilization. Again, because each produced wild-type and dumpy progeny, the F_1 hermaphrodites were heterozygous, and the F_2 phenotypic data were pooled. The phenotypic ratio of wild-type to dumpy among F_2 indicates whether *Hbdpy-1* and *Hbdpy-2* are linked. Independent assortment (i.e., no linkage) should yield an expected 1.286:1 wild-type:dumpy ratio among the F_2, whereas a ratio of 1:1 is suggestive of linkage (1). The actual ratio obtained in the F_2 was 1.03:1 (Table 5) which is significantly different from 1.286:1 ($P < 0.05$, χ^2 test), suggesting linkage.

The genetic map distance between the two linked genes can be estimated from

TABLE 3. Phenotypes (mean number of offspring ± SD) of F_1 progeny originating from crosses between single mutant hemaphrodites (*Hbdpy-2* or *Hbdpy-3*) and five to six wild-type (WT) males of *Heterorhabditis bacteriophora* strain HP88.

Cross (♀ × ♂)	n†	WT phenotype		% WT males‡	Dumpy phenotype		% dumpy males§
		Hermaphrodite	Male		Hermaphrodite	Male	
Hbdpy-2 × WT	18	106 ± 60	9 ± 7	8.8 ± 6.1	53 ± 48	0.3 ± 1	0.5 ± 0.4
Hbdpy-3 × WT	6	6 ± 8	0 ± 0	0	3 ± 3	‖	‖

† Number of fertile crosses.
‡ Mean ± SD of [(number of WT males)/[(number of WT hermaphrodites) + (number of WT males)]].
§ Mean ± SD of [(number of dumpy males)/[(number of dumpy hermaphrodites) + (number of dumpy males)]].
‖ Dumpy males were seen occasionally, only when several *Hbdpy-3* hermaphrodites were crossed with males (data not presented).

the proportion of the recombinants in the F_2 generation. Given that the genotype of the F_1 hermaphrodites was *Hbdpy-1* +/+ *Hbdpy-2*, progeny homozygous for the recombinant chromosomes would be either the double mutants (*Hbdpy-1 Hbdpy-2*/*Hbdpy-1 Hbdpy-2*) or the homozygous wild-type animals. Unfortunately, the double mutants could not be distinguished phenotypically from the other mutant offspring in this F_2 generation. Therefore, we resorted to estimating the number of homozygous wild-type animals by transferring 45 of the 163 phenotypically wild-type F_2 hermaphrodites (Table 5) to separate DFA plates for self-fertilization. Only homozygous wild-types yield exclusively wild-type progeny in such a test. Only 2 of the 45 hermaphrodites belonged to this category; because the rest gave rise to phenotypically wild-type and dumpy offspring, these 43 others were heterozygous. Although extrapolating from a ratio of 2:45 cannot yield a precise calculation, an approximate distance of 29 map units is indicated (1).

Complementation tests between Hbdpy-1 *and* Hbdpy-3: A similar approach was applied to examine complementation of *Hbdpy-1* and *Hbdpy-3*. Ten crosses of *Hbdpy-1* males and virgin *Hbdpy-3* hermaphrodites each gave phenotypically wild-type and dumpy animals among hermaphrodites and males of the F_1 generation; thus, the two mutations affect distinct genes. Each of the four F_1 hermaphrodites transferred to separate plates for self-fertilization produced wild-type and dumpy F_2, indicating that all of these hermaphrodites were heterozygous. The ratio of phenotypically wild-type to dumpy animals was 0.84:1 (Table 5), suggesting that the *Hbdpy-1* and *Hbdpy-3* genes are linked ($P < 0.05$, χ^2 test). Map distance between these two genes could not be estimated because too few phenotypically wild-type F_2 hermaphrodites were available for further study.

Complementation tests between Hbdpy-2 *and* Hbdpy-3: Eighteen crosses of *Hbdpy-2* males with virgin *Hbdpy-3* hermaphrodites each gave phenotypically wild-type and

TABLE 4. Phenotypes of F_2 generation of *Heterorhabditis bacteriophora* strain HP88 originating from self-fertilization of phenotypically wild-type (WT) hermaphrodites of the F_1 generation that had originated from crosses between dumpy (*Hbdpy*) hermaphrodites and wild-type males.

Genotype of parent (F_1)	n†	WT phenotype		Dumpy phenotype		Male ratio WT/dumpy
		Hermaphrodite	Male	Hermaphrodite	Male	
Hbdpy-2/+	10	1390	60	476	27	2.22:1
Hbdpy-3/+	5	315	16	97	6	2.67:1

Data represent total numbers in pooled offspring.
† Number of fertile F_1 hermaphrodites studied.

TABLE 5. Phenotypes of F_2 generation of *Heterorhabditis bacteriophora* strain HP88, originating from self-fertilization of phenotypically wild-type (WT) F_1 hermaphrodites originating from crosses between mutant (*Hbdpy*) strains.

Genotype of parent (F_1)	*n*†	WT phenotype	Dumpy phenotype	WT/ dumpy ratio
Hbdpy-1/Hbdpy-2	15	163	158	1.03:1
Hbdpy-3/Hbdpy-1	4	56	66	0.84:1
Hbdpy-3/Hbdpy-2	10	256	278	0.92:1

Data represent total numbers in pooled offspring.
† Number of fertile F_1 hermaphrodites studied.

dumpy F_1 hermaphrodites and males. Thus, the two mutations affect distinct genes. When 10 F_1 hermaphrodites were transferred to separate DFA plates for self-fertilization, each produced wild-type and dumpy progeny, indicating that the F_1 was heterozygous for the two mutations. In the pooled data for the F_2 generation, a phenotypic wild-type to dumpy ratio of 0.92:1 (Table 5) indicated that the *Hbdpy-1* and *Hbdpy-3* genes are linked ($P < 0.05$, χ^2 test). Map distance between these two genes could not be estimated because none of the phenotypically wild-type F_2 hermaphrodites reproduced.

The reciprocal crosses for each of these complementation tests were less successful. However, they would not be expected to contribute different information.

Unfortunately, the *Hbdpy-3* mutant strain became progressively less viable and was lost. Hence additional crosses with it could not be conducted.

The three *Hbdpy* mutations demonstrate the feasibility of conducting genetic studies in *H. bacteriophora* strain HP88. These mutations also served us as useful markers for determining the mode of reproduction of this nematode. The methodology we have developed for mutagenesis and genetic analysis should pave the way for generating additional marker mutations on each chromosome and for generating a genetic map for *H. bacteriophora*. These genetic tools should facilitate the efficient genetic enhancement of the beneficial traits of this species.

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