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# New morphological variants of infective juveniles associated with mutations in four sex-linked genes of *Steinernema feltiae* (Filipjev) (Nematoda: Steinernematidae)

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**Summary** - The research reported here provides a detailed genetic and morphological characterization of the first eight mutations artificially induced in *Steinernema feltiae*. All newly identified alleles proved to be sex-linked and recessive to wild-type counterparts. They define four independent genes designated as *Sfdpy-1*, *Sfdpy-2*, *Sfseg-1*, and *Sfvab-1*. All mutations affected the morphology of the nematode infective juveniles and except for the *pn32* allele they showed complete penetrance. Modifications of the gross morphology observed among identified mutants included visible reduction of infective juvenile length to width ratio (dumpy phenotype), regular serial swellings present along the nematode body line (segmented phenotype), and distinct constrictions of the juvenile body which occurred irregularly on its dorsal side (variable abnormal phenotype). A number of other minor modifications such as helical twist of the nematode body, transverse foldings of its cuticle, various changes of head and tail region, rearrangements of lateral fields and others have also been associated with individual mutations. The wide range of distinctive phenotypes produced in this research provides excellent comparative material for detailed study on the significance of morphological characters to the entomopathogenic activity of *S. feltiae* infective juveniles.

**Résumé** - ***Nouveaux variants de juvéniles infestants associés aux mutations de six gènes liés au sexe chez* Steinernema feltiae *(Filipjev) (Nematoda: Steinernematidae)*** - Une caractérisation détaillée, morphologique et génétique, est relatée concernant les huit premières mutations induites artificiellement chez le nématode *Steinernema feltiae*. Il est démontré que tous les allèles nouvellement identifiés sont liés au sexe et récessifs vis-à-vis de leurs homologues du type sauvage. Ils se rapportent à quatre gènes indépendants désignés comme *Sfdpy-1*, *Sfdpy-2*, *Sfseg-1* et *Sfvab-1*. Toutes les mutations affectent la morphologie des juvéniles infestants et, à l'exception de l'allèle *pn32*, ils montrent une pénétration complète. Les modifications affectant la morphologie générale observées chez les mutants touchent à une nette réduction de taille des juvéniles infestants par rapport à la largeur du corps (phénotype obèse), une série régulière de renflements le long du corps des juvéniles (phénotype segmenté) et des constrictions irrégulières prononcées sur la face dorsale du corps des juvéniles (phénotype anormal variable). D'autres modifications sont associées à des mutations individuelles : courbure hélicoïdale du corps, plis transverses de la cuticule, anomalies des régions céphalique et caudale, structure aberrante du champ latéral, etc. Ce vaste éventail de phénotypes caractérisés ainsi produits fournit un excellent matériel de comparaison pour une étude détaillée de la signification des caractères morphologiques au regard de l'activité entomopathogène des juvéniles infestants de *S. feltiae*.

**Key words:** biological control, entomopathogenic nematode, morphological mutants, mutagenesis, nematode genetics, *Steinernema feltiae*.

The growing economic importance of entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae as biocontrol agents generates a strong demand for strains presenting high reproductive potential *in vitro*, good storage stability, and field efficacy against target insects which is better then or at least comparable to chemical insecticides. Results obtained with natural isolates are, however, sometimes disappointing (Georgis & Gaugler, 1991). Artificial selection, strain hybridization, and genetic engineering have been suggested as possible approaches to improve the parasites' performance (Gaugler, 1987). Successful attempts to use artificial selection in order to improve host-finding, overall pest control efficacy and infectivity at lower temperatures have been undertaken in several species, including *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, and *Heterorhabditis* sp. (Shapiro *et al.*, 1985; Gaugler *et al.*, 1989; Tomalak, 1989, 1994*a*; Gaugler & Campbell, 1991; Selvan *et al.*, 1994; Griffin & Downes, 1994). The genetic background of the characteristics modified in such studies remains, however, mostly unclear.

In both families the infective juvenile is the only developmental stage that can live free in the soil environment, survive prolonged storage, actively search for the insect host, and penetrate into its haemocoel (Ishibashi & Kondo, 1990; Kaya, 1990). It is also the major component of commercial nematode bioinsecticides. Therefore, better understanding of the genetic control of infective juvenile biological functions should facilitate research on nematode improvement.

A number of recent studies have shown that the comprehensive model system developed for genetic analysis of a free-living species, *Caenorhabditis elegans* (Brenner, 1974) could be easily adapted for studies on entomopathogenic nematodes (Fodor *et al.*, 1990;

Zioni *et al.*, 1992; Rahimi *et al.*, 1993; Koltai *et al.*, 1994; Tomalak, 1994*b*). In our recent work on *S. feltiae*, individual components of selected, critical functions of the nematode infective juveniles are extensively studied with the aid of spontaneous and chemically induced mutations affecting genes controlling these functions (Tomalak, 1994*c*). The main objectives are to: *i*) induce, identify and characterize mutations affecting morphology and/or behaviour of infective juveniles, *ii*) identify the genes affected by these mutations, and *iii*) evaluate the effects of the identified mutations on activity of the nematode and on its potential as a biocontrol agent against pest insects. This paper reports results of a systematic study on genetic characterization of a series of mutations affecting morphology of *S. feltiae* infective juveniles.

## Materials and methods

### Nematode strains

The presently reported mutagenesis studies were conducted on the ScP, UK-76, and 93.05.19/10 strains of *S. feltiae* (Filipjev) (=*S. bibionis* Bovien). The ScP strain originated from a Polish isolate (PL0), selected subsequently for 34 generations to improve its biocontrol efficacy against *Lycoriella solani* larvae in mushroom cultures (Tomalak, 1994*a*). The UK-76 strain was kindly provided by Dr. D. Gouge from the nematode collection of Reading University. The 93.05.19/10 strain was originally derived from morphologically wild-type revertants, which arise spontaneously in a dumpy infective juvenile strain homozygotic for the *Sfdpy-1(pn7ij)X* mutant allele (Tomalak, 1994*b*). Prior mutagenesis the nematode strains were inbred through individual sib-mating *in vitro* for at least ten generations to increase their homozygosity.

### Induction and isolation of mutants

Mutagenesis was induced chemically with 0.05 M ethyl methanesulphonate (EMS), 0.8 mM methyl nitrosourea (MNUA) or 0.07 mM sodium azide (SAz), or with the aid of a helium-neon laser light with a wavelength of 6328 Å and power density of 1 $mW/cm^2$. The chemical mutagenesis was induced as described by Brenner (1974) with some modifications. Before the mutagen treatment the nematode infective juveniles (IJs) were allowed to develop *in vitro* for 48 h. For this purpose about 10 000 IJs were surface sterilized in 1% sodium hypochlorite for 15 min and washed in three changes of sterilized, distilled water. The concentrated suspension of IJs was then transferred with a sterile pipette into five 9 cm Difco nutrient agar (NA) plates with 3-day-old culture of *Xenorhabdus bovieni*. The plates were incubated for 2 days at 24°C, until most nematodes developed to L4 or adult stage. Then, all worms were washed off the plates and the suspension was cleaned in three changes of M9 buffer. Three ml of this nematode suspension were immediately transferred to 3 ml of a double strength solution of a selected mutagen and incubated for 4 h at 20°C. After subsequent washing five times in M9 buffer, the final suspension of nematodes was pipetted into ten 9 cm NA/*X. bovieni* plates and incubated at 24°C. In the laser-induced mutagenesis, NA/*X. bovieni* plates with developed L4 juveniles and adults were uncovered and directly exposed to the laser light for 30 min. Subsequently, they were incubated at 24°C. After 15-16 days the F2 IJs produced in each plate were collected separately. They were cleaned and stored in tap water at 2°C for 2 weeks. Each F2 nematode population was divided into two parts. One part was screened for mutant phenotypes immediately, while the second part was further cultured *in vivo*, in *Galleria mellonella* (Dutky *et al.*, 1964) and subjected to the mutant screening as F2(F4) generation IJs, harvested 12-14 days after infection of the insect. The preliminary, *en masse* screening for morphological mutants involved microscopic examination of IJs in water. All individuals phenotypically differing from wild-type nematodes were collected, backcrossed to wild-type partners and the resultant F2 offspring examined in subsequent tests. If proved to segregate mutants, the nematodes were allowed to reproduce further to obtain populations homozygotic for the mutant allele. For autosomal recessive alleles, phenotypically mutant individuals backcrossed to a wild-type partner should automatically segregate mutant nematodes of both sexes in the F2 generation. For sex-linked genes, to obtain females homozygotic for the mutant allele requires an additional cross. In our study isolated mutant males were individually backcrossed with wild-type females. Heterozygotic F1 females produced from these crosses were then mated with mutant males. Morphologically mutant IJs obtained from this cross in the F1 generation could develop into adults of either sex. All homozygotic mutant populations used in this study were initiated by a mating of two mutant individuals.

### Nematode crosses *in vitro* for gene linkage and complementation analysis

Since this study concerned mutations affecting morphology of IJs, only phenotypes of this developmental stage were considered in the genetic analysis of the new mutant alleles. Infective juveniles of the F1 generation were obtained by pairwise crossbreeding *in vitro* using a modification of the method described by Tomalak (1994*b*). Plates were prepared by pouring 25 ml of sterilized 2% agar into 9 cm Petri dishes. After 1 h, two drops of warm sterilized NA were carefully pipetted on the surface of the solidified agar in the center of each dish. One hour later those islets

were smeared with a loopful of *X. bovieni* taken from 3-day-old colony. IJs chosen for crosses were predeveloped individually *in vitro*, for 2-3 days, as described earlier (Tomalak, 1994*b*). The adult nematodes were then transferred to freshly prepared 2% agar/NA/*X. bovieni* plates and set in pairs according to the breeding scheme. They were allowed to mate and reproduce for 7 days at 24°C. In breeding dishes prepared in this manner, the layer of 2% agar served as a source of moisture, while the small islet of NA placed in the center of each dish restricted the growth of *X. bovieni* to this region only. The limited amount of food caused all F1 generation nematodes to develop into IJs. The only exceptions were populations with a brood size of only a few juvenile (usually less than ten individuals). In those cases juveniles developed directly to adults and infectives could be collected in F2 generation only.

Linkage of the new alleles was examined *in vitro* by evaluation of IJ phenotypes segregating in the F1 generation. As all identified mutant alleles were recessive to wild type, for these tests homozygotic mutant females were individually crossed to wild-type males. The presence of both wild-type and mutant phenotypes in approximately equal proportions in the F1 offspring was considered to be conclusive evidence for sex-linkage of the mutant gene locus. Such phenotypic ratio would be in agreement with the sex ratio observed in the wild-type strain (ScP) cultured *in vitro*, where the proportion of female to male individuals was as 1.10±0.03:1.00 (mean ± S.E.) and did not significantly differ from the 1:1 ratio (Chi square test: $\chi^2 = 2.54$; $P \leq 0.05$; df = 1) (Tomalak, unpublish.). Unfortunately, due to the variable recovery rate of mutant infective juveniles *in vitro*, the sex ratio of these nematodes could not be precisely evaluated yet. The presence of only wild-type individuals in the F1 offspring would indicate an autosomal linkage.

As all identified alleles turned out to be sex-linked complementation testing between *a* and *b* alleles was conducted *in vitro* by individually crossing a female homozygotic for the *a* allele with a male hemizygotic for the *b* allele. For all tested alleles reciprocal crosses, which were replicated at least five times, were performed. The presence of only the mutant phenotype in the F1 offspring was conclusive evidence for a lack of complementation and indicated that both tested alleles were of the same gene. The appearance of approximately equal proportions of both the wild-type and mutant IJs in the F1 offspring indicated that complementation had occurred between the tested alleles and that the alleles represented two separate genes.

### Morphological analysis

The mutant morphology was examined in infective juveniles obtained from *in vivo* culturing in *G. mellonella*. Freshly harvested nematodes were stored in a tap water for 1 week at 20°C and then subjected to 1% sodium dodecyl sulfate (SDS) treatment for 1 h to kill all noninfective developmental stages (Cassada & Russel, 1975). After subsequent washing in three changes of distilled water and storage for an additional week the infective juveniles were heat killed, fixed in TAF and processed using the glycerin-ethanol method (Seinhorst, 1959). The specimens were examined under a compound Olympus BX50 microscope at a magnification ranging between 40 to 1000 ×. The measurements of mutant IJs are presented according to the modified de Man's formula (Poinar, 1986) and compared with those recorded for ScP strain. The means were based on measurements of n=50 individuals for each strain (Tomalak, 1994*b*).

### Statistics

The phenotypic segregation data obtained in linkage and complementation experiments were subjected to the Chi square test. The significance of differences was tested at $P \leq 0.05$.

## Results

### Isolation of morphological mutants

The microscopic screening conducted on the offspring of nematodes treated with all mutagens revealed a series of morphologically and behaviorally new phenotypes among F2 and F4 IJs. Most of the individuals tentatively classified as mutants did not, however, segregate the mutant phenotype in the backcross F2 generation offspring or the mutant phenotype showed a very low penetrance. Therefore, only eight independent mutations affecting morphology of infective juveniles could be analyzed genetically in detail. All of the examined individuals proved to be IJs as they survived SDS treatment and presented other characteristics, including a distinct bacterial sack, collapsed intestine, lack of pharyngeal pumping and ability to survive prolonged storage in distilled water, which are found exclusively in this developmental stage. The mutants reported here have been placed into three general phenotypic categories designated as dumpy (Dpy), segmented (Seg) and variable abnormal (Vab). The dumpy mutants were easily separated from other morphological categories by the shortened length and increased width of their body. Such changed proportions of measurements made the nematode IJs look stout or dumpy. The morphology of segmented mutants was distinguished by almost regular serial swellings present along the nematode body line. In general viewing such altered nematodes appeared segmented. The variable abnormal phenotype category corresponds with that described earlier for *C. elegans* (Brenner, 1974). Such mutations produced a wide spectrum of morphologically altered phenotypes ranging from almost wild type to severely abnormal individuals.

Body measurements of all identified mutants differed in many aspects from those recorded for wild-type individuals. The data obtained for IJs from the mutant and wild-type parental strains reared *in vivo*, in *G. mellonella* larvae are presented in Table 1.

Besides the body measurements a number of other distinctive morphological characters has been recorded in the examined populations. The following section provides a short characterization of the new mutant strains.

The gross morphology of *S. feltiae* wild-type infective juvenile (ScP strain) is showed in Figs 1A, 1F and 2D.

*pn11* - This mutation was induced in ScP strain by SAz treatment. The mutant IJs present a clear dumpy phenotype (Fig. 1B) and their gross morphology resembles that of the *pn7* mutant described earlier (Tomalak, 1994*b*). The juvenile head region is protruded and forms a short snout (Fig. 1G). The tail region presents numerous transverse foldings posterior to the anus (Fig. 2E). When relaxed in water 60.6-68.2% of individuals show a helical twist of their body around the long axis. Although it was not reported earlier, a similar twist was observed in 12.2-14.7% of *pn7* mutants. Moving on a flat surface such nematode rotates the whole body around its main axis. This phenotype resembles the dumpy roller

***Table 1.*** *Comparison of body measurements of wild type (ScP) and mutants pn7, pn11, pn12, pn29, pn31, pn32, pn33, pn34, and pn35 infective juveniles of* Steinernema feltiae *(all measurements in μm).*

| | | | | | | | modified | w-t-like | modified | dpy-like | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ScP* | *pn7** | *pn11* | *pn12* | *pn29* | *pn31* | *pn32* | *pn32* | *pn33* | *pn33* | *pn34* | *pn35* |
| Body length | 825 (660-920) | 620 (510-710) | 650 (470-830) | 729 (640-800) | 604 (380-760) | 590 (400-600) | 693 (630-820) | 807 (600-1010) | 511 (400-600) | 573 (480-690) | 647 (540-780) | 661 (490-770) |
| Maximum body diameter | 28 (24-30) | 36 (34-38) | 34 (26-40) | 34 (30-40) | 30 (23-36) | 32 (25-40) | 27 (25-30) | 27 (21-33) | 32 (26-39) | 32 (27-38) | 33 (28-38) | 30 (24-35) |
| Anterior end to excretory pore | 58 (53-63) | 47 (43-53) | 49 (43-55) | 54 (48-60) | 45 (33-53) | 44 (40-50) | 54 (48-59) | 56 (48-63) | 46 (38-53) | 47 (40-53) | 48 (40-55) | 48 (40-58) |
| Anterior end to nerve ring | 91 (85-98) | 80 (75-83) | 88 (80-98) | 85 (75-93) | 84 (68-100) | 80 (70-88) | 88 (78-98) | 96 (85-105) | 79 (73-90) | 82 (68-88) | 84 (75-95) | 86 (75- 95) |
| Anterior end to pharynx base | 139 (128-150) | 135 (130-143) | 142 (128-155) | 128 (110-145) | 136 (110-155) | 131 (115-145) | 129 (118-138) | 135 (113-148) | 121 (105-143) | 130 (105-145) | 135 (118-145) | 132 (113-145) |
| Tail length | 78 (60-88) | 64 (58-70) | 59 (43-75) | 74 (58-83) | 55 (35-68) | 53 (40-63) | 64 (55-73) | 72 (50-90) | 50 (38-65) | 52 (40-58) | 61 (48-75) | 68 (50-80) |
| a | 30 (24-31) | 17 (15-19) | 19 (15-23) | 22 (16-25) | 20 (16-25) | 19 (14-22) | 26 (23-28) | 30 (27-32) | 16 (13-19) | 18 (15-21) | 20 (15-25) | 22 (18-26) |
| b | 5.9 (4.9-6.6) | 4.6 (3.9-5.4) | 4.6 (3.7-5.5) | 5.7 (4.4-6.3) | 4.5 (3.5-5.1) | 4.5 (3.5-5.5) | 5.4 (4.9-6.1) | 6 (4.8-7.1) | 4.2 (3.6-4.9) | 4.4 (3.9-5.2) | 4.8 (3.9-5.6) | 5 (4.1-5.7) |
| c | 10.6 (9.5-11.6) | 9.7 (8.4-10.9) | 11.1 (9.4-14.4) | 9.9 (8.8-12.0) | 10.9 (9.0-14.7) | 11.2 (9.5-14.7) | 10.8 (9.4-12.4) | 11.2 (10.3-14.8) | 10.2 (8.8-12.5) | 11.1 (9.6-13.6) | 10.7 (8.8-13.1) | 9.7 (8.2-11.0) |
| d | 0.41 (0.39-0.43) | 0.35 (0.32-0.40) | 0.34 (0.32-0.39) | 0.42 (0.37-0.46) | 0.33 (0.30-0.38) | 0.34 (0.29-0.40) | 0.42 (0.38-0.46) | 0.42 (0.38-0.46) | 0.38 (0.31-0.43) | 0.36 (0.33-0.43) | 0.36 (0.31-0.40) | 0.37 (0.32-0.42) |
| e | 0.74 (0.68-0.90) | 0.74 (0.61-0.83) | 0.83 (0.70-1.00) | 0.73 (0.61-0.96) | 0.81 (0.67-0.90) | 0.84 (0.72-1.12) | 0.84 (0.77-0.96) | 0.79 (0.66-1.00) | 0.92 (0.75-1.20) | 0.91 (0.82-1.19) | 0.80 (0.68-1.00) | 0.71 (0.56-0.86) |

* Data after Tomalak (1994*b*).

mutants described earlier in *C. elegans* (Brenner, 1974). The penetrance of the roller character is not complete, however, and crosses conducted within phenotypic groups of dumpy roller, or normal dumpy individuals always produce both dumpy and dumpy roller phenotypes in the offspring. A particularly interesting characteristic of this mutation is an unusually high rate of spontaneous reversions to wild-type morphology of IJs which can be regularly observed in all populations homozygotic for the *pn11* allele. Such revertants bred within the phenotypic group behave as those described earlier for the *pn7* allele (Tomalak, 1994*b*). The highest reversion rate recorded in F2 generation obtained from phenotypically mutant IJs injected into *G. mellonella* was 0.52%.

*pn12* - This mutation was isolated from the 93.05.19/10 strain treated with EMS. IJs homozygotic for the *pn12* allele differ from other mutants in their characteristically changed body dimensions and almost regular 12(±1) serial swellings along the body line (Figs 1C; 2A, F). This phenotype was quite stable in all examined individuals. The described morphological changes are apparently caused by modifications of the nematode hypodermis and cuticle. Detailed microscopic observations of IJ's cuticular structures also revealed differences between wild-type and mutant individuals in the organization of the lateral fields. In wild-type *S. feltiae* IJs lateral fields have eight almost straight parallel ridges extending throughout the body length. In the *pn12* mutant IJs lateral fields are wavy with six to eight ridges in regions between body swellings and with only four distinct ridges in the swollen regions. This mutation showed complete penetrance in all examined populations.

*pn29* - This mutation was induced in the ScP strain by MNUA treatment. The dumpy phenotype caused by the *pn29* allele resembles that observed in nematodes carrying *pn7* or *pn11* alleles. The most obvious differences can be recorded in the predominance (85.7-91.3%) of dumpy roller phenotype in populations of *pn29* IJs and the very low rate of spontaneous reversions to the wild-type morphology. So far no revertant individual has been isolated from over $8 \times 10^6$ IJs examined in our study.

*pn31* - The parental strain, mutagen treatment, gross morphology and reversion rate of this mutant are similar to those described for the *pn29* mutant. However, the *pn29* and *pn31* alleles have been induced in different populations and they represent separate mutational events.

*pn32* - This mutation was produced in the ScP population exposed to helium-neon laser light for 30 min. In contrast to the other alleles described in this paper the *pn32* mutation shows a very low penetrance. The characteristic phenotype can be regularly observed in 6.5-7.6% individuals of the mutant populations. The morphology of the *pn32* mutant IJs is distinguished by three to six "swellings" present on the dorsal side of the nematode body (Figs 1D; 2B). Occasionally, the number of distinct "swellings" can be reduced to one. As no difference can be observed between the maximum body width of morphologically mutant and wild-type individuals homozygotic for the *pn32* allele (Table 1) the morphological structures referred to here as "swellings' should rather be considered as bumps created in the region located between abrupt constrictions of the nematode body present on its dorsal side (Fig. 2B). The presence of "swellings"

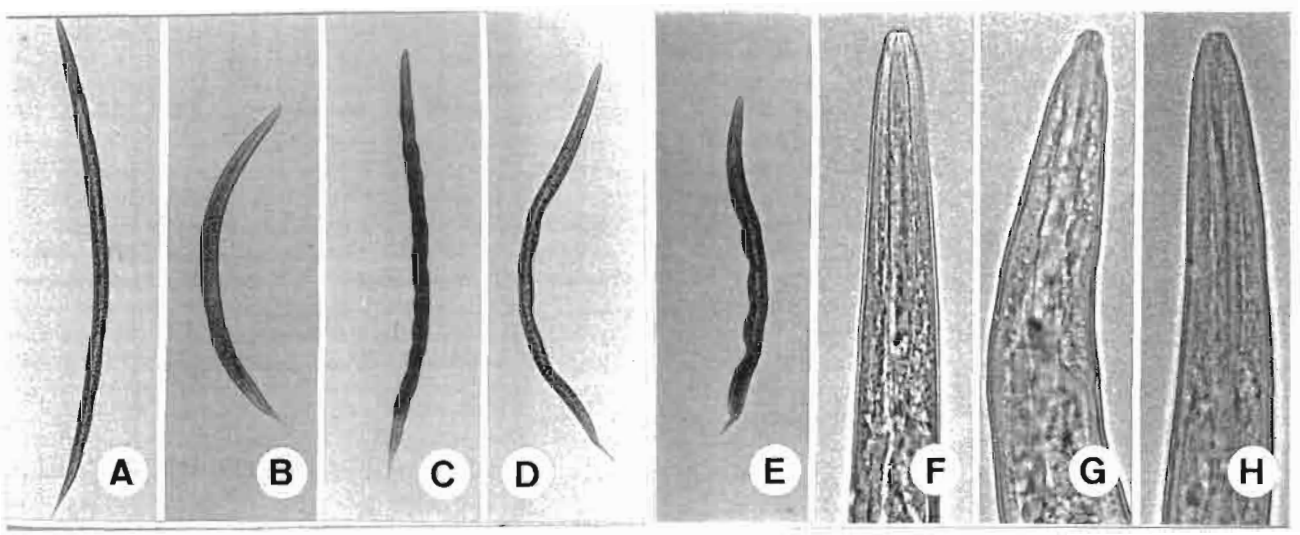


***Fig. 1.*** *Body shape (A-E) and oesophageal region (F-H) of wild-type (A, F) and mutant (B-E, G, H) infective juveniles of* Steinernema feltiae. *(A, F: ScP; B, G: dpy-1[pn11]; C: seg-1[pn12]; D: vab-1[pn32]; E: dpy-1[pn33]; H: dpy-2[pn35]).*

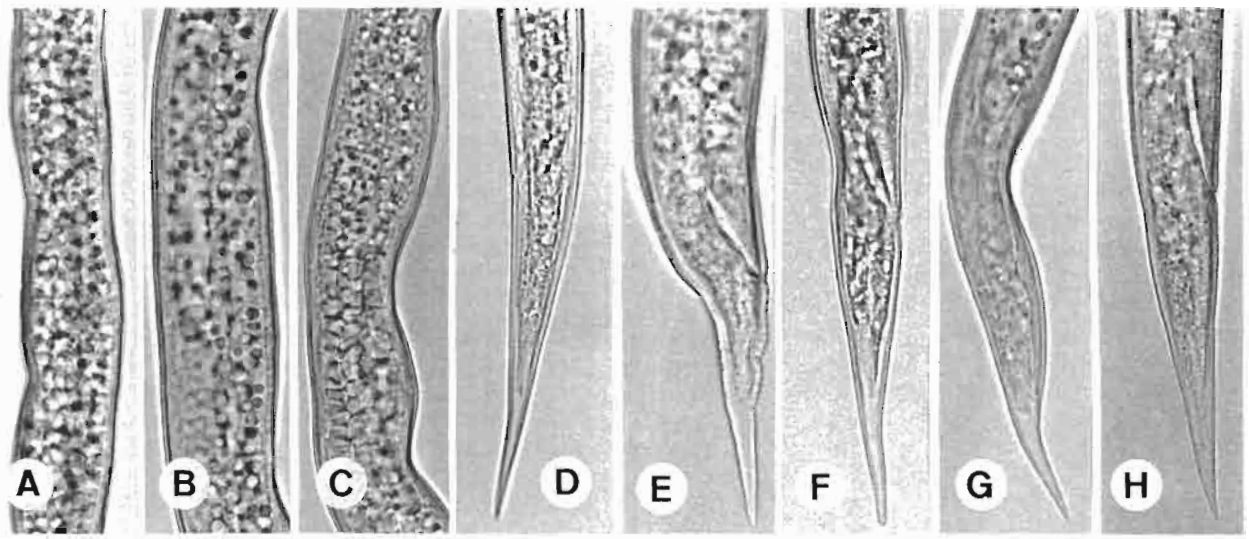


***Fig. 2.*** *Mid body (A-C) and tail (D-H) shapes of wild-type (D) and mutant (A-C, E-H) infective juveniles of* Steinernema feltiae. *(A, F: seg-1[pn12]; B, G: vab-1[pn32]; C: dpy-1[pn33]; D: ScP; E: dpy-1[pn11]; H: dpy-2[pn35]).*

changes also the morphology of the IJ tail region (Fig. 2G). In a relaxed state the *pn32* mutants bend dorsally. Such a posture is unlike that of wild-type individuals and most other mutants which always bend ventrally.

*pn33* - This mutation was isolated from UK76 strain subjected to EMS treatment. All the *pn33* mutant IJs clearly show the dumpy (63.9-65.0%) or dumpy roller (35.0-36.1%) phenotype. In gross morphology most of the individuals resemble nematodes homozygotic for *pn7*, *pn11*, *pn29*, *pn31* or *pn34* alleles. Distinctive, however, is the presence of a new class of IJ dumpy morphology characterized by three to five "swellings' located on the dorsal side of the nematode body (Fig. 1E). As in *pn32* mutants the "swellings" are produced between the IJ body constrictions present on its dorsal side (Fig. 2C). This phenotype was recorded in 14.6-17.9% of individuals. Such individuals mated within the phenotypic group always produce normal dumpy, dumpy with dorsal swellings, and dumpy roller IJs in the offspring.

*pn34* - This mutation was induced in ScP strain by EMS treatment. All *pn34* mutant IJs display a dumpy (65.9-68.7%) or dumpy roller (31.3-34.1%) phenotype and morphologically resemble nematodes carrying *pn7*, *pn11*, *pn29* or *pn31* alleles. Penetrance of this mutation is complete.

*pn35* - This mutant was originally isolated from ScP population treated with EMS. Its IJs present dumpy (47.7-61.6%) or dumpy roller (38.4-52.3%) phenotype. Despite a close resemblance of IJ gross morphology to other dumpy mutants the *pn35* differs from the rest in details of head and tail regions (Figs 1H; 2H). The head of individuals carrying the *pn35* allele is similar to wild type while in the remaining dumpy mutants the lip region is protruded (Fig. 1G). Although the tail of *pn35* IJs is less slender than in wild-type individuals, the cuticle of this region is quite smooth. This characteristic distinguishes the *pn35* IJs from all other dumpy mutants which present numerous transverse foldings posterior to the anus (Fig. 2E). The penetrance of this mutation is complete.

The characteristic phenotype of all dumpy mutants can be clearly observed in infective juveniles only. The gross morphology of other developmental stages is similar to that of wild-type *S. feltiae*, with only minor modifications of adult nematodes. This leads to the unusual situation where the J2 cuticle remaining on infective juveniles is as much as 30-50% bigger than the infective juvenile itself. Although in *pn12* mutants the characteristic segmented morphology can be observed in infective juveniles only, the adult individuals of both sexes display an extremely dumpy phenotype which is unusual compared to normal *S. feltiae* adults. In *pn32* mutants the cuticular "swellings" can be observed in all developmental stages.

### Tests for dominance/recessiveness of the identified alleles

All the newly identified mutations were recessive to the respective wild-type alleles. For all tested alleles wild-type homozygotic females crossed to mutant males always produced only wild type IJs in the F1 offspring.

GENE LINKAGE

All of the originally isolated mutant IJs cultured *in vitro* turned out to be males. Also all of the phenotypically mutant juveniles segregating in F2 generation offspring of wild-type females crossed to mutant males developed into males only. Furthermore, individual crosses of females homozygotic for the mutant allele with wild-type males always segregated both mutant and wild-type IJs in F1 generation offspring (Table 2), thus indicating that all the examined mutations were located on sex chromosomes. The phenotypic segregation ratios recorded in backcrosses with the *pn12* and *pn32* mutants were, however, significantly different from the expected 1:1 ratio (Chi square test: $\chi^2$=10.26 and $\chi^2$=95.11, respectively; $P \leq 0.05$; df=1).

GENETIC COMPLEMENTATION

Results of crosses conducted to test genetic complementation between the identified mutant alleles revealed that the examined mutations could be assigned to four gene loci. All crosses performed between individuals bearing *pn7*, *pn11*, *pn29*, *pn31*, *pn33*, and *pn34* alleles produced uniform populations of dumpy IJs in the F1 generation offspring. Those results indicated the lack of complementation between tested alleles and proved that all respective mutations affected the same *Sfdpy-1* gene, identified earlier by the *pn7* allele (Tomalak, 1994*b*). Therefore, the new mutant alleles and their gene loci have been designated as *Sfdpy-1(pn11ij)X*, *Sfdpy-1(pn29ij)X*, *Sfdpy-1(pn31ij)X*, *Sfdpy-1(pn33ij)X*, and *Sfdpy-1(pn34ij)X*, respectively, based on the genetic nomenclature used for *Caenorhabditis elegans* (Horvitz *et al.*, 1979). To avoid possible confusion with names already used in other nematodes a specific prefix "*Sf*" standing for *S. feltiae* has been added to the gene designation (Tomalak, 1994*b*). The "*pn*" is a specific symbol used for mutations identified at the Institute of Plant Protection, Poznan, Poland.

Phenotypic segregation data of F1 IJs obtained in crosses performed to test complementation of the remaining mutant alleles (*i.e.*, *pn12*, *pn32* and *pn35*) are presented in Table 3. All of the examined crosses segregated both mutant and wild-type IJs in the F1 offspring, thus indicating complementation of the tested alleles. Segregation ratios of four of the crosses which involved *pn12* or *pn32* mutants revealed, however, significant differences from the expected 1:1 ratio (Chi square test: $P \leq 0.05$; df=1).

By complementing the *pn7*, *pn31*, *pn32*, *pn33*, *pn34* and *pn35* mutations the *pn12* allele proved to represent a separate gene. Due to the characteristic and quite unusual morphology we named this phenotype segmented (Seg), and the respective allele and gene *Sfseg-1(pn12ij)X*.

***Table 2.*** *Linkage of identified mutations affecting morphology of* Steinernema feltiae *infective juveniles (Phenotypic segregation of F1 infective juveniles obtained from backcrosses between homozygotic mutant female and wild-type male).*

| Tested allele | Mean ±S.E phenotypic segregation | Phenotypic ratio mutant : wild type |
|---|---|---|
| *pn7* | 108±6.14 Dpy : 85±3.09 W-t | 1.26 : 1.00 |
| *pn11* | 119±3.24 Dpy : 98±2.96 W-t | 1.22 : 1.00 |
| *pn12* | 11±1.58 Seg : 32±2.63 W-t | 0.35 : 1.00* |
| *pn29* | 36±1.82 Dpy : 24±1.48 W-t | 1.55 : 1.00 |
| *pn31* | 80±2.5 Dpy : 60±2.14 W-t | 1.34 : 1.00 |
| *pn32* | 35±1.84 Vab : 177±6.58 W-t | 0.20 : 1.00* |
| *pn33* | 121±4.86 Dpy : 111±3.95 W-t | 1.08 : 1.00 |
| *pn34* | 43±1.83 Dpy : 27±1.28 W-t | 1.64 : 1.00 |
| *pn35* | 118±3.48 Dpy : 101±3.46 W-t | 1.17 : 1.00 |

* Segregation significantly different from expected 1:1 ratio, Chi square test at $P \leq 0.05$.

Although the phenotypic segregation ratios recorded for *pn32* crosses were strongly affected by the low penetrance of this mutation, the obtained data indicated that this allele represented still another gene. Due to the variable mutant phenotype ranging from almost wild-type morphology to IJs with one to six distinct dorsal "swellings" observed in the mutant population we designated this phenotype as variable abnormal (Vab) and the corresponding allele and gene as *Sfvab-1(pn32ij)X*.

Finally, the phenotypically dumpy *pn35* allele complemented the *pn31* and *pn34* dumpy, as well as the *pn12* and *pn32* mutations. These results indicate that the *pn35* allele represents another dumpy gene. The-refore, the complete designation of this allele and its gene locus is *Sfdpy-2(pn35ij)X*.

## Discussion

Extensive research conducted on a model, free-living species, *C. elegans* has provided a thorough insight into many aspects of nematode genetics. Nevertheless, the broad ecological and behavioral diversity present among nematode families and orders requires further detailed studies which would better explain numerous specific characteristics of particular nematode groups. In steinernematids and heterorhabditids the genetic control of infective (dauer) juvenile entomopathogenic activity is probably the most urgent area for such investigations. This complex mechanism must, however, be dissected into relatively simple components before such research could be possible.

In our study on *S. feltiae* we have approached this task by inducing point mutations responsible for visible modifications of morphology and/or behavior in

***Table 3.*** *Genetic complementation of identified mutations affecting morphology of* Steinernema feltiae *infective juveniles (Phenotypic segregation of F1 infective juveniles obtained* in vitro *from crosses between individuals carrying mutant alleles).*

| Combination of alleles (female × male) | Mean ±S.E. phenotypic segregation | Phenotypic ratio mutant : wild type | Identified genes |
|---|---|---|---|
| *pn7* x *pn12* | 27±1.34 Dpy : 24±1.06 W-t | 1.11 : 1.00 | *dpy-1*; *seg-1* |
| *pn12* x *pn34* | 10±0.92 Seg : 37±1.42 W-t | 0.28 : 1.00* | *seg-1*; *dpy-1* |
| *pn31* x *pn12* | 41±1.86 Dpy : 28±1.16 W-t | 1.46 : 1.00 | *dpy-1*; *seg-1* |
| *pn31* x *pn35* | 28±1.56 Dpy : 44±2.07 W-t | 0.62 : 1.00 | *dpy-1*; *dpy-2* |
| *pn32* x *pn12* | 11±1.10 Vab : 47±1.86 W-t | 0.23 : 1.00* | *vab-1*; *seg-1* |
| *pn32 x pn31* | 10±0.86 Vab : 53±2.31 W-t | 0.19 : 1.00* | *vab-1; dpy-1* |
| *pn33* x *pn12* | 18±1.21 Dpy : 27±1.24 W-t | 0.64 : 1.00 | *dpy-1*; *seg-1* |
| *pn33* x *pn32* | 50±2.34 Dpy : 42±1.75 W-t | 1.19 : 1.00 | *dpy-1*; *vab-1* |
| pn34 x pn35 | 38±1.62 Dpy : 48±1.84 W-t | 0.79 : 1.00 | *dpy-1; dpy-2* |
| *pn35* x *pn12* | 18±1.14 Dpy : 35±1.18 W-t | 0.52 : 1.00* | *dpy-2*; *seg-1* |
| *pn35* x *pn32* | 28±1.08 Dpy : 37±1.36 W-t | 0.77 : 1.00 | *dpy-2*; *vab-1* |

* Segregation significantly different from expected 1:1 ratio, Chi square test at $P \leq 0.05$

the nematode IJs. The value of mutant analysis in studying functional relationships between the genetic background and phenotypic characters have been already well documented in *C. elegans* (*e.g.*, Brenner, 1974; Waterson *et al.*, 1977; Culotti & Russell, 1978; Cox *et al.*, 1980). We believe that identification of visible mutations, genetic analysis of responsible genes and detailed examination of phenotypic expression of these genes can help us to understand their significance to the behavior of *S. feltiae* IJ and, particularly, to its entomopathogenic activity. Such information could be invaluable to further work on genetic improvement of beneficial traits in the whole group of entomopathogenic nematodes.

The research reported here provides a detailed genetic and morphological characterization of eight new mutations artificially induced in *S. feltiae*. All these mutations have been located on the sex chromosomes and they define four independent genes designated as *Sfdpy-1*, *Sfdpy-2*, *Sfseg-1*, and *Sfvab-1*. Although the identification of mutant alleles was based on morphological modifications in IJs, the respective mutations also affected other aspects of the nematode phenotype, including IJ behaviour and occasionally morphology and behaviour of the remaining developmental stages. The latter aspects of this research will be reported elsewhere.

All of the described mutants showed various modifications of their cuticle. The noncellular cuticle is produced by the underlying hypodermis. Therefore, the observed changes suggest that the responsible genes and their products could directly affect the hypodermis itself or the process of cuticle formation. Earlier studies on *C. elegans* revealed a large series of genes with potential effects on the nematode cuticle (Brenner, 1974; Higgins & Hirsh, 1977; Cox *et al.*, 1980). Most of these genes were, however, phenotypically expressed in adult worms only. Cox *et al.* (1980) suggested potential involvement of different genes in the formation of the cuticle of each stage. This statement is in agreement with ultrastructural and biochemical differences observed between the nematode cuticle of adult and various juvenile stages (Cassada & Russel, 1975; Singh & Sulston, 1978). Our findings strongly support such developmental stage specificity of genes involved in the formation of the nematode cuticle. In all but the *pn32* mutation, the characteristic mutant phenotypes identified in the present study were expressed in infective juveniles only. Other juvenile stages did not differ from the wild type or produced phenotypes apparently unrelated to mutant characters of IJs (*e.g.*, *pn12*). These results may also provide the first evidence in *S. feltiae* for the presence of a special genetically controlled developmental pathway operating in the formation of dauer (infective) juvenile. Such a pathway has been already described in detail in *C. elegans* (Riddle, 1988).

Genetic analysis of new alleles requires visible mutations which could be used as markers for the necessary crosses. Distinctive phenotypes and complete penetrance of all but one (*pn32*) alleles make the newly described mutations perfect for this purpose. However, for further genetic studies it would be most desirable to have a complete set of marker mutations representing all chromosomes. Surprisingly, despite of a double screening of the mutagenized population in F2 and F4 generations up to the present only sex-linked alleles have been identified in our research. Whether we observe this phenomenon because *i*) genes responsible for the hypodermis and cuticle

formation in infective juveniles are located on sex chromosomes only, *ii*) *S. feltiae* sex chromosomes are more sensitive to mutagen damage, or *iii*) still other factors influence the mutant isolation remains to be elucidated in the future. The distribution of various genes responsible for dumpy and roller phenotypes of *C. elegans* dauer juveniles over autosomes and sex chromosomes (Cox *et al.*, 1980) suggests that the first alternative could be difficult to defend. If the second option is true we may continue isolation of sex-linked mutations with relative ease, while the autosomal mutants will be scarce. Although highly speculative this hypothesis seems to be supported by our present work on *S. feltiae* uncoordinated and roller mutant classes, as most of our newly identified genes are also sex-linked (Tomalak, unpubl.). We need, however, further isolations of still new mutants to verify this hypothesis.

The phenotypic segregation ratio of wild-type and mutant IJs recorded in most crosses involving the *pn12* or *pn32* mutants significantly differed from the expected 1:1 ratio. In crosses with the *pn32* mutant this was apparently caused by the reported earlier low penetrance of the respective gene. In the case of *pn12* mutant this phenomenon seems, however, to be related with the nematode development *in vitro*. Our study (Tomalak, unpubl.) revealed that cuticle of the *pn12* adults and non-infective juveniles has a reduced resistance to osmotic changes in the environment. Developmental stages other than IJs are frequently observed to burst and die when transferred to new bacterial cultures. It seems probable that local differences in osmotic properties of the culture plate can eliminate a portion of mutant juveniles. This would well explain the reduced proportion of Seg phenotypes in the F1 offspring.

In contrast to *C. elegans* which can reproduce through both automixis and amphimixis, all known *Steinernema* spp. are obligatorily amphimictic. The isolation of mutants and establishment of homozygotic mutant populations in *Steinernema* spp. requires therefore additional crosses. This minor disadvantage of using an amphimictic species is compensated for, however, by the relative ease of genetic analysis which does not require complicated crosses with the inclusion of additional markers as is necessary in hermaphroditic species (Brenner, 1974). The methods of *in vitro* crossing developed for *S. feltiae* and described in this and the previous paper (Tomalak, 1994*b*) have proved to be reliable and very effective in our investigations. Although these procedures were specifically designed to produce IJs in F1 or F2 generations, they could be equally effective in evaluation of phenotypes of any other developmental stages in this species. We also believe that by changing the bacterial component these methods could be easily adapted for other *Steinernema* species.

As the cuticle plays an important role in the organization and functioning of the nematode hydrostatic skeleton, any significant changes in this structure can affect both the animal's morphology and its activity (Higgins & Hirsh, 1977; Cox *et al.*, 1980; Tomalak, 1994*b*). In our pilot experiments the morphological modifications reported here were always associated with modifications of various aspects of the IJs behavior, including movement pattern and efficacy, dispersal, host penetration rate and others, which frequently led to significant changes in the nematode infectivity to insect hosts (Tomalak, unpubl.). Further experimental work is needed, however, to show if any of these modifications could lead to the improvement of *S. feltiae* biocontrol potential at a practical level.

The wide range of distinctive IJ phenotypes induced and isolated in this research provides an excellent tool for detailed study on the significance of morphological characters to nematode activity. Together with the *pn7* allele identified earlier (Tomalak, 1994*b*) the alleles described here constitute a set of nine independent, heritable mutations which alter the phenotype of *S. feltiae* IJs. Over 30 other morphological and behavioral mutations are at present being analyzed in our laboratory. These numbers show a great genetic and phenotypic flexibility of *S. feltiae* and holds promise that with the aid of other screening methods still new characters, including those potentially advantageous to biocontrol efficacy, can be easily induced in this species and perhaps in the whole group of entomopathogenic nematodes.

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