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Scanning electron microscope study of morphological modifications of lateral fields in infective juveniles of mutant *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae)

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Summary - A detailed SEM study on morphological modifications of lateral fields in infective juveniles of dumpy *Sfdpy-1 (pn7, pn11, pn29,* and *pn31)*, segmented *Sfseg-1(pn12)*, and double dumpy segmented *Sfdpy-1(pn7)Sfseg-1(pn12)* mutants of *Steinernema feltiae* is presented. Lateral fields of all mutants examined differed significantly from those of wild-type individuals. A wide spectrum of changes in morphology, arrangement, and number of individual ridges were observed. Combined action of mutations of both *Sfdpy-1* and *Sfseg-1* genes examined in double mutants further widened the range of observed variation. This produced much greater irregularity in morphology and arrangement of individual ridges than any of the parental mutations separately. The range of variation in mutant nematode lateral fields appeared to be gene specific but not allele specific: morphological modifications in infective juveniles carrying mutations of the *Sfdpy-1* gene were significantly different from the modifications in juveniles with mutations of the *Sfseg-1* gene, whereas individuals with different mutant alleles of the *Sfdpy-1* gene had rather similar modifications. Since distinctive lateral fields are dauer-specific in *S. feltiae*, this suggests that both genes may play a significant role in the process of cuticle formation in dauers of this nematode.

Résumé - ***Étude en microscopie électronique à balayage des modifications des champs latéraux chez les juvéniles infestants de mutants de* Steinernema feltiae *(Filipjev) (Rhabditida: Steinernematidae)*** - Les résultats relatés concernent une étude détaillée en microscopie électronique à balayage des modifications morphologiques observées dans les champs latéraux des juvéniles infestants de divers mutants de *Steinernema feltiae* obèses *Sfdpy-1 (pn7, pn11, pn29* et *pn31)*, segmentés *Sfseg-1(pn12)*, et un double mutant, obèse + segmenté *Sfdpy-1(pn7)Sfseg-1(pn12)*. Les champs latéraux de tous les mutants examinés diffèrent significativement de ceux des individus de type sauvage. Les modifications observées concernent un large spectre de changements morphologiques, ainsi que la disparition ou le nombre des lignes individuelles. Une action mutagène combinée des deux gènes *Sfdpy-1* et *Sfseg-1* observée chez les mutants doubles élargit de plus la variabilité observée. Cette action produit en effet des irrégularités plus prononcées dans la morphologie et la disposition des lignes que celles produites par l'action des mutations parentales agissant seules. L'étendue de la variation provoquée chez le nématode mutant apparaît spécifique du gène, mais non de l'allèle. Bien que les modificationss morphologiques diffèrent significativement entre les juvéniles infestants comportant des mutations liées aux gènes *Sfdy-1* et *Sfseg-1*, les changements observés chez des spécimens comportant différents allèles mutants du gène *Sfdy-1* sont assez semblables. Etant donné que des champs latéraux distincts sont, chez *S. feltiae*, spécifiques des "dauer larvae", les résultats obtenus suggèrent que l'un et l'autre gènes mis en cause pourraient jouer un rôle significatif dans le processus de formation de la cuticule chez les "dauer larvae" de ces nématodes.

Key words: entomopathogenic nematode, infective juvenile, lateral fields, mutant nematode, *Steinernema feltiae*.

Among nematode cuticular structures, the lateral fields or alae are one of the most distinctive characters of species belonging to Chromadoria and Secernentea (Maggenti, 1981). They are present over the lateral hypodermal cords and extend along the whole length of the body. The cuticle of the lateral fields forms one or several characteristic parallel ridges separated by incisures. Although the function of these structures for nematode activity remains unclear, the number of ridges and their arrangement are considered as important diagnostic characters in various nematode genera, particularly those of Tylenchida (Siddiqi, 1986; Sturhan & Brzeski, 1991). Recently, lateral fields characters have been used also for taxonomic differentiation in Steinernematidae (Rhabditida) as their morphology remains fairly stable within species groups (Mrácek & Bednarek, 1991; Mráček, 1994). Generally, 6-8 ridges are present in this family and they can be identified exclusively in infective juveniles (IJs) (Mráček & Bednarek, 1991).

Our study of spontaneous and artificially induced mutants in *Steinernema feltiae* revealed a series of new morphological variants among the IJs (Tomalak, 1994 *a*, *b*, 1997). The recorded gross morphological modifications often were associated with changes of nematode motility and infectivity. Surprisingly, signi-

ficant differences in activity have also been observed between mutants presenting apparently similar body shapes (Tomalak, unpubl.). Subsequently, more detailed light microscope examination showed that in addition to changes in nematode gross morphology individual cuticular structures were affected by the mutations.

The main objective of the present scanning electron microscope (SEM) study was to examine in detail the range of morphological modifications of lateral fields of *S. feltiae* caused by separate mutations of dumpy (*Sfdpy-1)* and segmented (*Sfseg-1)* genes, and by the joint action of mutations of both genes in *Sfdpy-1Sfseg-1* double mutants. We believe that this information can help us understand the effect of lateral field morphology on IJ movement, efficacy, and infectivity to insect hosts.

Materials and methods

The study was conducted on IJs of dumpy *Sfdpy-1 (pn7, pn11 pn29,* and *pn31)*, segmented *Sfseg-1 (pn12))*, and dumpy segmented *Sfdpy-1(pn7)Sfseg-1 (pn12)* mutant and double mutant strains of *S. feltiae*. The induction, isolation, and genetic characterization of those mutants were described earlier (Tomalak, 1994, 1997). All individuals examined originated from strains homozygous for one of the mutant alleles. The *Sfdpy-1(pn7)Sfseg-1(pn12)* double mutant population was obtained by individual, *in vitro* crossbreeding of nematodes carrying *Sfdpy-1(pn7)* mutation with nematodes carrying *Sfseg-1(pn12)* mutation. The phenotypically recombinant IJs were isolated from F2 generation offspring. As both of the genes under consideration are sex-linked and recessive to wild type (Tomalak, 1994, 1997), all isolated recombinant infective juveniles developed as males. Such males were then individually backcrossed to wild-type females and the heterozygous F1 females obtained were backcrossed again to recombinant males. Phenotypically recombinant infectives isolated from F2 generation offspring of the last crosses developed into both males and females. To obtain homozygous double mutant populations, the recombinant IJs were subsequently allowed to mate and reproduce *in vitro* for one or two generations.

Prior to the SEM study, all nematodes were mass produced *in vivo* in *Galleria mellonella* larvae, according to the method described by Dutky *et al.* (1964). Due to the extremely low motility and infectivity of the *pn7pn12* double mutants, it was necessary to inject IJs of this strain directly into the insect hemocoel. Before injection, the nematodes were surface sterilized in 0.1% sodium hypochlorite for 15 min and washed in five changes of sterilized distilled water. Approximately ten IJs were injected per insect larva. Freshly harvested and washed IJs of the new generation offspring were stored for 2 weeks at 20°C. Additional washing in five changes of distilled sterilized water was performed shortly before fixation. The live IJs were fixed in 0.5% glutaraldehyde solution in 0.2% cacodylate buffer and postfixed in 0.1% osmic acid. Subsequently, the nematodes were subjected to a serial alcohol dehydration, critical point drying and gold coating. Microscopic examination of mounted specimens was performed on JEOL JSM 6300 scanning electron microscope at the Institute of Parasitology, Czech Academy of Sciences in Ceske Budejovice. Morphological observations were made from several series of mounts and at least 30 specimens from each mutant strain to exclude the risk of cuticular artifacts caused by the preparation process.

Results

The number and arrangement of ridges in the lateral fields of *S. feltiae* wild-type (ScP, UK76) IJs did not differ from those observed by Kozodoi and Spiridonov (1988) and Mráček and Bednarek (1991). Eight parallel ridges, with less prominent submarginal pair, could be distinguished in the nematode midbody region (Fig. 1A). In the tail region proximal to anus, only four ridges were present and they gradually merged towards the tail end (Fig. 1E).

Lateral fields of all examined mutants significantly differed from those of wild-type individuals. In nematodes with mutations of the *Sfdpy-1* gene (*pn7*, *pn11*, *pn29* and *pn31*), the lateral fields presented a wide range of morphological modifications (Fig. 1B, C, D, F). In general, all ridges lost their regular parallel arrangement. They were undulated and frilled throughout their length. The width of individual ridges and incisures differed significantly within and between nematode specimens. In some mutant IJs, no longitudinal ridges could be distinguished in the lateral fields. Instead, a series of irregular oblique folds extended throughout the nematode length (Fig. 1D). In spite of numerous variations in the arrangement of ridges, the region of the lateral field was always clearly separated from the rest of the nematode body surface in all individuals studied. In general, none of the morphological modifications observed was specifically associated with a particular mutant allele. With minor exceptions, all mutations of the *Sfdpy-1* gene produced relatively similar changes in the organization of the lateral fields. However, the proportions of the various morphological forms differed widely between the populations examined.

All populations homozygous for mutations of the *Sfdpy-1* gene produced both dumpy and dumpy roller infective juveniles (Tomalak, 1997). Additional examination of individuals presenting the dumpy roller phenotype revealed that their lateral fields were helically twisted around the body axis (Fig. 2A, B). Other

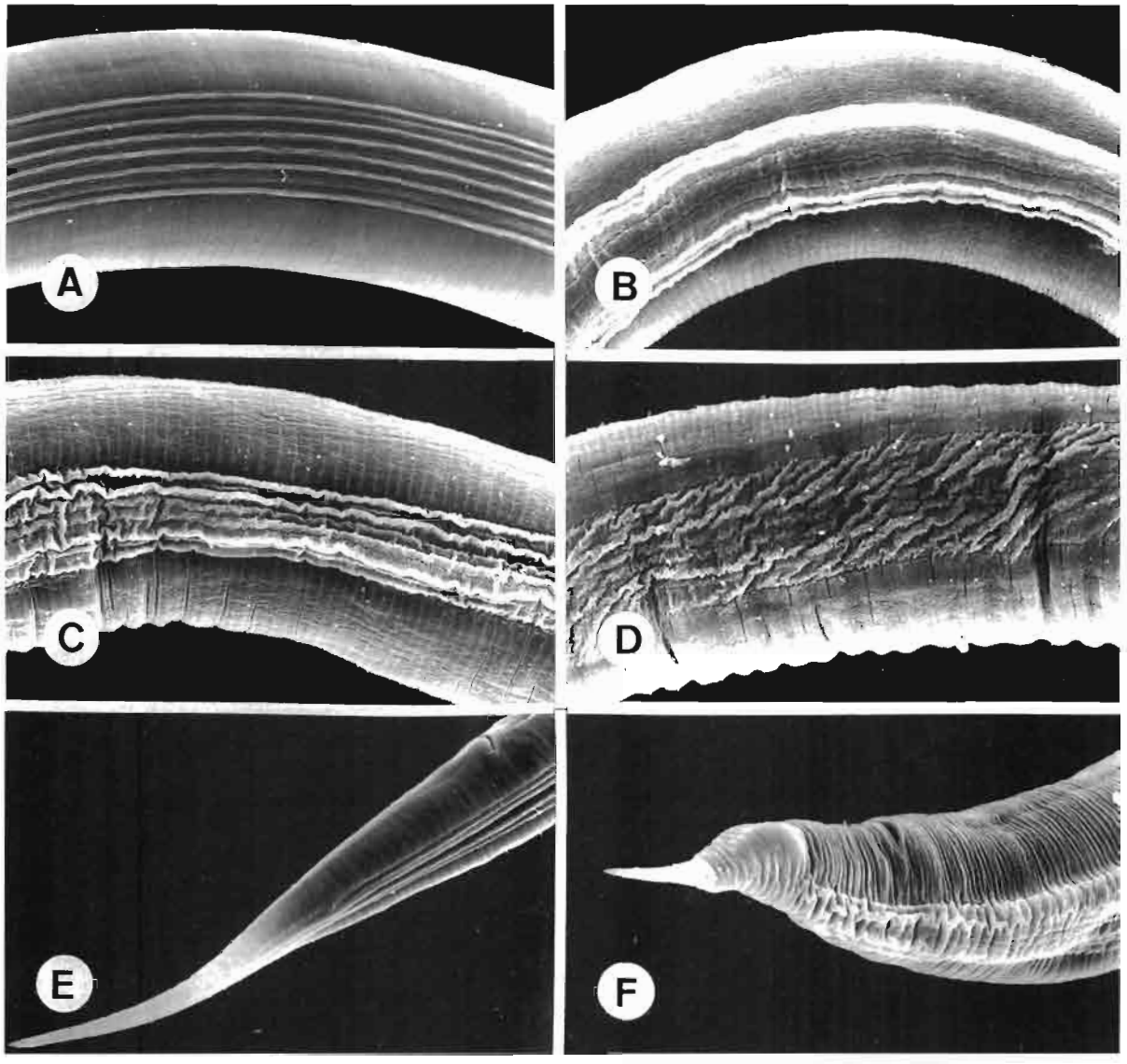

Fig. 1. *Morphology of lateral fields in wild-type (A, E) and* Sfdpy-1 *mutant (B, C, D & F) infective juveniles of* Steinernema feltiae *(SEM). A-D: Mid-body region; E-F: Tail region.*

modifications of ridges and incisures were as in normal dumpy IJs described earlier.

Infective juveniles homozygous for *Sfseg-1(pn12)* mutation showed still another type of modification of lateral fields (Fig. 2C, D). The number and arrangement of lateral ridges were strongly correlated with swellings and constrictions alternately present along the nematode body. Up to eight irregular ridges could be observed in the constricted regions. They partially merged in the swollen regions and formed only four separate ridges (Fig. 2C). The swelling located just posterior to the pharynx base often was totally devoid of lateral fields ornamentation (Fig. 2D). The ridges adjacent to this region appeared to diffuse.

The phenotype of *Sfdpy-1(pn7)Sfseg-1(pn12)* double mutant infective juveniles (Fig. 2E, F) was clearly dumpy as in *dpy-1(pn7)* mutants, and with numerous distinct swellings as in the *seg-1(pn12)* mutant parent. However, the swellings were much more protruding and irregularly distributed than in the *pn12* parental mutant strain. Joint action of the mutations of the two genes also led to further modifications in the

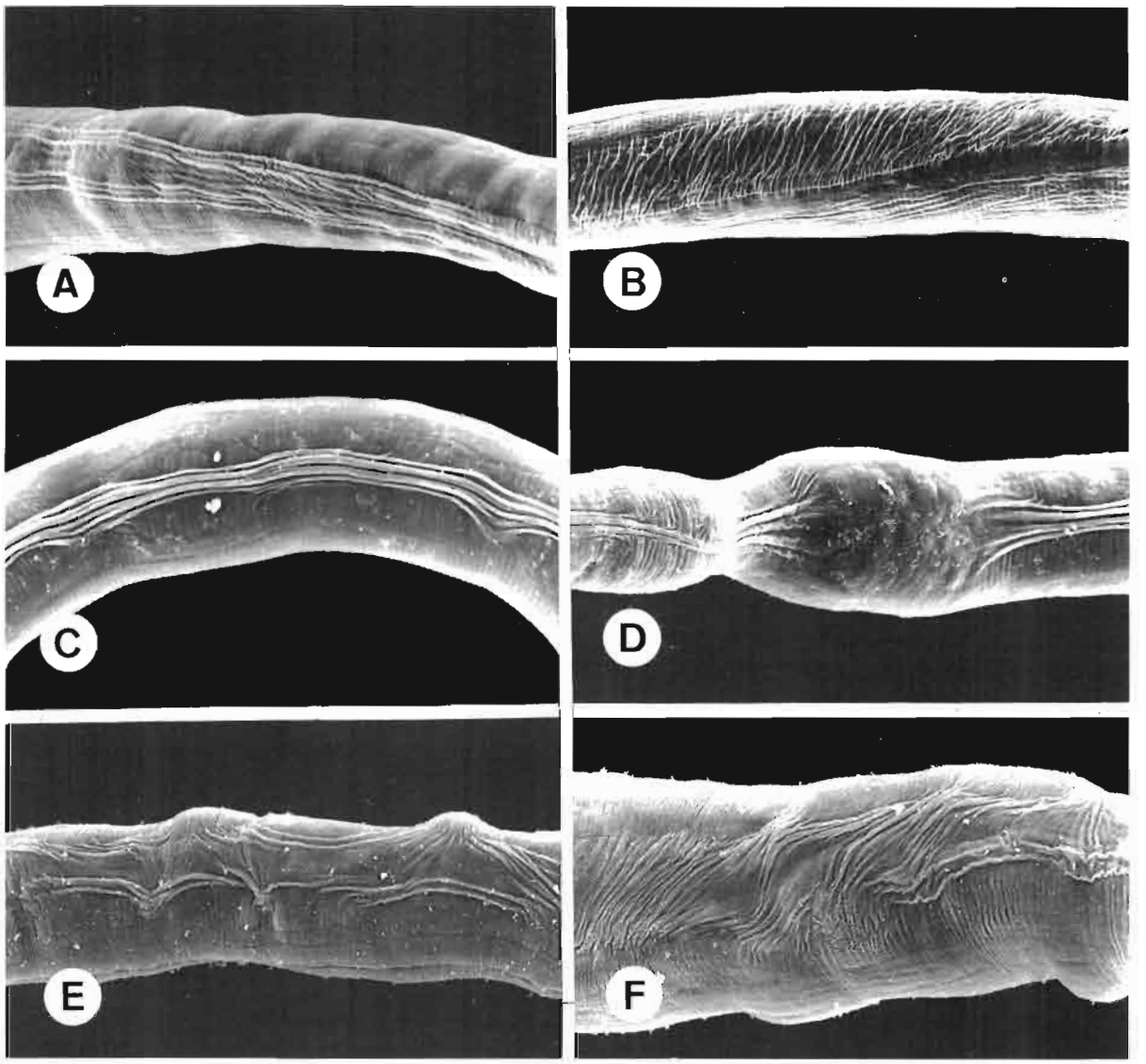

Fig. 2. *Morphology of mid-body region lateral fields in* Sfdpy-1 *(A-B),* Sfseg-1 *(C-D), and* Sfdpy-1Sfseg-1 *double mutant (E-F) infective juveniles of* Steinernema feltiae *(SEM). A, B: Dumpy roller phenotype; C, D:* pn12*; E, F:* pn7pn12.

arrangement and number of the lateral ridges. Lateral fields of the double mutants completely lost their regularity. Contrary to both parental strains, the lateral fields of double mutant infectives were not clearly separated from the rest of the body surface. Individual ridges were strongly deformed, oriented in various directions and scattered over most of the nematode cuticle. They frequently disrupted the transverse annuli normally present in those regions (Fig. 2E, F). They also formed visible aggregations around the swellings occurring over the entire body surface of *pn7pn12* double mutants (Fig. 2E).

Discussion

The nematode dauer or infective juvenile is adapted in several ways to life in harsh environmental conditions. The morphology and ultrastructure of its cuticle are unique (Cassada & Russell, 1975). Our earlier investigations conducted on *S. feltiae* revealed that

mutations of individual genes could lead to gross morphological modifications of the IJs (Tomalak, 1994 *a*, *b*, 1977). The present SEM study provides further evidence that subtle cuticular structures also can be strongly affected by such mutations. Commonly observed modifications of the nematode lateral fields represented a wide spectrum of changes in morphology, arrangement, and number of individual alae. Such high sensitivity of lateral fields to the activity of individual genes is not unusual among nematodes. Cox *et al.* (1980) reported a series of *Caenorhabditis elegans* roller mutants with lateral alae presenting numerous minor interruptions, larger gaps, or irregular loops. In those individuals also, the lateral fields were helically twisted around the nematode body axis. This type of modification is directly related to the helical twist of roller cuticle (Higgins & Hirsh, 1977; Cox *et al.*, 1980) and it was also regularly observed among the dumpy roller mutants examined in our study.

The range of variation produced in the mutant nematode lateral fields was gene specific but not allele specific. Morphological modifications in infective juveniles with mutations of the *Sfdpy-1* gene were significantly different from the modifications in juveniles with mutations of the *Sfseg-1* gene, whereas individuals with different mutant alleles of the *Sfdpy-1* gene had rather similar modifications. Although both genes are involved in the dauer morphogenesis, they apparently control different steps of this process. The joint action of mutations of both *Sfdpy-1* and *Sfseg-1* genes seen in double mutants produced clearly new recombinant phenotype and further widened the range of observed variation. The morphology and arrangement of individual ridges were much more deformed than those individually caused by any of the parental mutations.

As cuticular structures, the lateral alae are produced by the underlying hypodermis. Therefore, the modifications observed suggest that both *Sfdpy-1* and *Sfseg-1* genes can affect the hypodermis itself or the process of cuticle formation. So far, no detailed study on the morphogenesis of IJ has been made in steinernematid nematodes. However, Singh and Sulston (1978) showed that, in *C. elegans*, hypodermal seam cells were specifically responsible for the formation of lateral alae and for the nematode radial shrinkage that normally occurs during its dauer molt. Mutants with locally missing or damaged seam cells did not develop lateral alae in adjacent regions of the cuticle. The corresponding sections of the nematode body were wider than sections with lateral alae. The modifications of lateral fields observed in *S. feltiae* IJs appear to be of the same general type as in *C. elegans*. Local gaps recorded in lateral alae of *Sfseg-1* mutants could result from the absence or altered activity of seam cells normally present under these regions. Cuticular modifications in this mutant support the concept of a direct correlation between radial shrinkage and formation of lateral alae in the IJs (Singh & Sulston, 1978). Along the nematode body, the number of distinct ridges alternated from four to eight in swollen and constricted regions, respectively. After the dauer molt, IJs of dumpy mutants remain much wider than wild type individuals (Tomalak, 1994*a*, 1997). Therefore, the incomplete radial shrinkage in dumpy nematodes also could account for the observed morphological distortion and rearrangement of lateral fields. Those deformations were apparently exaggerated by longitudinal compression of the IJ body caused by mutations of *Sfdpy-1* gene (Tomalak, 1994*a*).

The significance of morphologically distinctive lateral fields to nematode activity remains unclear. As these animals lie and move on their sides, the lateral alae are in contact with the substrate and probably assist locomotion by increasing traction and preventing slipping (Bird & Bird, 1991). The absence of alae does not, however, inhibit movement activity. In the family Steinernematidae, the dauer or infective juvenile is the only developmental stage that can live free in the soil, disperse in this environment, and actively reach the hemocoel of new insect host. Therefore, the presence of lateral alae exclusively in this stage suggests that they have a particular role in the IJ movement and dispersion. Previous studies (Ishibashi *et al.*, 1987; Ishibashi & Kondo, 1990) revealed that movement of IJs of *S. carpocapsae* was twice as effective as movement of third-stage juveniles during the normal developmental cycle. Our preliminary observations on movement efficacy and infectivity of *S. feltiae* IJs revealed significant differences between mutant strains presenting different morphology (Tomalak, unpubl.). How large a part of these differences can be explained by modifications of lateral fields remains to be elucidated. Earlier research on *C. elegans* showed that mutants defective for the function to be investigated are particularly useful for such studies (Herman, 1988). We believe that the increasing range of mutant phenotypes available in *S. feltiae* (Tomalak, 1994*a*, *b*, 1997) will make it possible to compare and explain the effects of individual morphological and anatomical characters on movement and perhaps infectivity of IJs. Such information would be invaluable for future works on the genetic improvement of entomopathogenic nematodes.

This article is the first report on the construction of a double mutant of an entomopathogenic nematode and on the resulting recombinant phenotype. Due to very limited movement and low infectivity to insect hosts, the double mutant strain presents significantly reduced biocontrol potential. However, the protocol developed for *in vitro* crosses of *S. feltiae* (Tomalak,

1994 *a*, 1997) has proved its validity for this kind of breeding work. We hope that, with the growing number of morphological and behavioural mutants identified in steinernematid nematodes, this method can be effectively used in the construction of new and perhaps more effective strains for biological control of insect pests.

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