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Two previously unreported species of steinernematids from woodlands in Belgium

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Summary. Two steinernematid species, *Steinernema kraussei* and *Steinernema* sp. E1, which were not reported during previous surveys in Belgium, were recovered from woodland soils by direct extraction of infective stages from soil samples. Identification of the juveniles was based on their morphology and confirmed by RFLP analysis of the PCR amplified ITS-rDNA region. Baiting by *Galleria mellonella* larvae of soil samples in which these two species were present, did not result in the normal infection development and was not followed by juvenile migration. It is concluded that the presence of some populations of these two species in soil may not be revealed by *Galleria* baiting.
Key words: Steinernematidae, Belgium, *Galleria* baiting, direct extraction.

Belgian soils were intensively sampled for the presence of entomopathogenic nematodes (epns) using the *Galleria*-baiting technique (Miduturi *et al.* 1996a, 1996b, 1997). As a result of these surveys, the presence of three steinernematid species, *S. feltiae*, *S. affine* and the undescribed *Steinernema* sp. B3, and also *Heterorhabditis megidis* was reported. In recent years methods for direct extraction of epn infective juveniles (IJs) from soil and their identification to species level have been developed (Sturhan, 1992, 1995, 1997). During the summer of 1998 further investigations were made of steinernematid diversity in Belgian soils using a direct extraction method to obtain IJs from soil samples.

MATERIAL AND METHODS

Isolation of epns. A total of 36 soil samples were collected, mainly from woodlands in different provinces of Belgium. Each sample was homogenised and divided into 200 g subsamples. Nematodes were extracted from these subsamples by the sieving-decanting technique. Soil aliquots were agitated in a 1.5l measuring cylinder. After 30 sec sedimentation, the supernatant was poured onto a 70 μm nylon sieve. This procedure was repeated 3 times. The combined residues of the three decantations were spread over a 70 μm sieve (15x25 cm). The sieve was placed in a tray filled with water sufficient to cover the soil to a depth of at least 1 mm. Nematodes were allowed to pass through the sieve during 4-12 hours and thereafter were examined under the dissecting microscope to detect epn specimens. All nematodes which corresponded in size, structure and behaviour to that of epn IJs were subsequently examined under a compound microscope (magnification x100). To examine living IJs, individuals were placed on a thin (< 1 mm) layer of 1.5% agarose and a cover slip added. The agarose pad significantly slowed the movement of the juveniles without causing damage to the specimens. This method enabled the characteristic features of steinernematid juveniles e.g. closed mouth and anal opening, absence of pulsations in the pharynx, prominent bacterial vesicle, and number and appearance of lines in the lateral field to be readily distinguished.

Culturing isolated epns. Juveniles examined under the compound microscope were removed and transferred for storage in Syracuse watch glasses. When the soil sample contained only small numbers of IJs, the nematodes were divided into groups of 4-6 specimens. Each group was subsequently injected by a microsyringe into a last instar larvae of *Galleria mellonella*. When larger numbers of IJs were available, the infestation of *G. mellonella* was made on wet filter paper (20-30 IJs per insect) in chambers of immunological multiwell plates. Several 200 g aliquots of each sample were baited in plastic containers by adding 5 *G. mellonella* last instar larvae. The infestation process was monitored daily. Insect ca-

davers with features of successful infestation were transferred onto filter paper in Petri dishes.

Molecular identification. RFLP analysis of the ITS region was used to confirm the morphological identification of juveniles extracted from the soil and of the progeny obtained from culturing in the laboratory. DNA was extracted from 10-40 infective juveniles and used for PCR amplification of the rDNA-ITS region. The methods for DNA extraction and PCR amplification were similar to those of Subbotin *et al.* (1997). Primers were those prepared by Vrain *et al.* (1992). Amplified products were stored at -20 °C until used. Three enzymes, *Alu*I, *Dde*I and *Cfo*I, which are particularly useful for steinernematids, were used for the RFLP analysis and the RFLP profiles obtained were compared with those published by Reid *et al.* (1997).

RESULTS

Steinernematid juveniles were present in 19 of the 36 soil samples. Morphological examination revealed characteristic patterns of lateral fields, bacterial vesicle, and tail structure corresponding to *S. feltiae*, *S. affine*, *S. kraussei* and an unknown steinernematid species belonging to the *S. affine-intermedium* group. The IJs of *S. kraussei* and *S. feltiae* were characterised by three bands (four lines) and four bands (five lines) in the central part of the lateral field, respectively. In both species the submarginal lines were poorly visible. *Steinernema affine* and the unknown steinernematid were characterised by two central bands in the lateral field. *S. affine* juveniles possessed the refractile drop-like inclusion in the tail tip, but this inclusion was absent in the unknown species.

Results from the molecular study supported the morphological identification of the specimens. The RFLP patterns of populations identified as *S. feltiae*, *S. kraussei* and *S. affine* corresponded to those characteristic for the species (Fig. 1). The only deviation from this concordance was the presence of an additional band in the profile of the *S. feltiae* laboratory culture isolated in Merelbeke. The DNA patterns of the unknown steinernematid species suggested that these nematodes were conspecific with the isolate E1 from the British Isles (Reid *et al.*, 1997).

Juveniles of *S. feltiae*, *S. affine*, *S. kraussei* and *Steinernema* sp. E1 were present in 9, 3, 7 and 3 of the samples collected. The occurrence together of two steinernematid species was recorded in each of 3 samples. *Steinernema affine* IJs were found only in soil collected from grassland adjacent to or within woodland. *Steinernema feltiae* was present outside and inside woodlands and *S. kraussei*, not previously reported from Belgium, was common in woodlands, especially under coniferous trees. *Steinernema* sp. E1 was recovered from soil collected from the canopy of deciduous trees.

Sufficient number of *Steinernema feltiae* and *S. kraussei* IJs were obtained for the infection via filter paper. Juveniles of *S. kraussei* (4 samples) and of *Steinernema* sp. E1 (2 samples) were used for injections of *Galleria* caterpillars. Soil baiting experiments were successful only for the isolation of *S. feltiae*. Attempts to isolate other epn species by baiting from known positive soil samples were unsuccessful. In several cases as the first indication of infestation by *S. kraussei* or *Steinernema* sp. E1 of *Galleria* caterpillars, these were evenly coloured and odourless, but these initially successful infestations did not result in juvenile migration, even though some cadavers were kept for up to 3-4 weeks.

Infestations via filter paper or injections were also mainly unsuccessful. Five to ten days after exposure to epns, *Galleria* cadavers showing signs of successful infection were dissected and found to contain giant females and males of steinernematids. However, uteri of these females were free of eggs and the female gonad lumen was filled with lipid globules, some cells of different sizes, and other non-characteristic bodies. All *Galleria* cadavers infected with *Steinernema* sp. E1 were characteristically yellow or yellowish. Only a single infestation of *Galleria* with *S. kraussei* juveniles on filter paper and a single injection of *Galleria* caterpillar with 5 juveniles of *Steinernema* sp. E1 resulted in the production of numerous IJs. In both cases the caterpillars were pierced accidentally during the early stage of the infestation process; the cadavers were visibly colonised by foreign (non-*Xenorhabdus*) micro-organisms.

DISCUSSION

Despite previous very intensive sampling for the presence of steinernematids in Belgian soils with the *Galleria* baiting technique, two relatively common species, *S. kraussei* and *Steinernema* sp. E1, were not detected (Miduturi *et al.*, 1996a, 1996b, 1997). Our attempts to bait, with *Galleria* caterpillars, soils samples in which specimens of these two species were discovered by a direct extraction technique, failed. Such baiting did not yield laboratory cultures of these species. IJs of both these species, however, were able to infect *Galleria* caterpillars and even to develop to become giant females and males. Further development was stopped because of unknown factors. Both of these species have previosly been successfully isolated and established as cultures (Reid *et al.*, 1997). It is concluded that only under some circumstances can these steinernematids be revealed by a baiting technique.

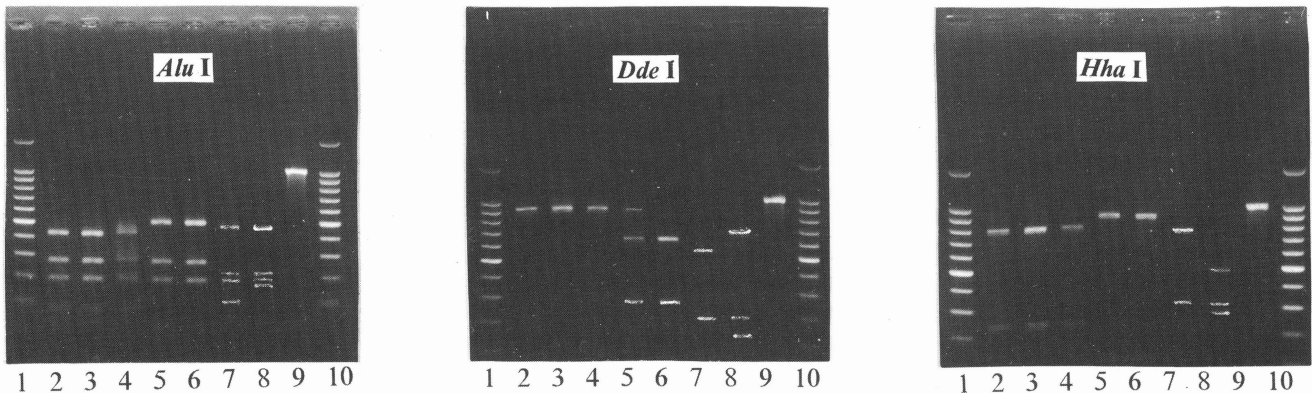

Fig. 1. RFLPs obtained by digestion of the ITS region of steinernematids from Belgium. Lanes 1 and 10 molecular weight markers (DNA ladder 100 bp); 2- *S. kraussei*, PCR product of 20 IJs extracted from soil, spruce wood near the Ourthe river, Luxembourg province; 3 - *S. kraussei*, PCR product of 20 IJs extracted from soil, Leignon, spruce wood, Namur province; 4 - *S. kraussei*, PCR product of 20 IJs, laboratory culture, origin Leignon, Namur province; 5 - *S. feltiae*, PCR product of 20 IJs, laboratory culture, origin Merelbeke, East Flanders; 6 - *S. feltiae*, PCR product of 20 IJs extracted from soil, Merelbeke, woodland, East Flanders; 7 - *S. affine*, PCR product of 40 IJs extracted from soil, Merelbeke, grassland, East Flanders; 8 - *Steinernema* sp. E1, PCR product of 40 IJs, laboratory culture, origin-deciduous wood, Rochefort, Namur; 9 - Unrestricted PCR product of *S. feltiae*.

Our study has revealed that these two species are not rare in Belgium and results from previous surveys to isolate these epns suggests that at least some populations of these species cannot develop in *Galleria*. It is concluded, that the *Galleria* baiting technique is not a reliable method for determining steinernematid diversity in soils.

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Спиридонов С.Э., Мунс М. Два ранее не отмеченных вида штейнернематид из лесов Бельгии.
Резюме. Два новых вида штейнернематид *Steinernema kraussei* и *Steinernema* sp. E1 были обнаружены при обследовании методами прямой экстракции почвенных проб из лесных ценозов Бельгии. Определение выделенных из почвы личинок основывалось на морфологических признаках и было затем подтверждено анализом различий в длинах рестрикционных фрагментов ПЦР-амплифицированного ITS участка рибосомальной ДНК. Попытки выделить эти два вида штейнернематид из проб с помощью живых приманок, гусениц большой вощинной моли *Galleria mellonella*, оказались безуспешными, т.к. не приводили к нормальному развитию штейнерматид и миграции личинок. Делается вывод о том, что в некоторых случаях присутствие в почве этих двух видов не может быть выявлено с использованием гусениц моли как приманок.