

Origins and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*

George O. POINAR, Jr.

Department of Entomology, University of California, Berkeley, CA 94720, USA.

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Summary – On the basis of morphological, physiological, distributional and biological evidence, it is concluded that the genera *Steinernema* and *Heterorhabditis* are widely divergent and similarities of life cycles can be attributed to convergence. Data is presented which indicates that *Heterorhabditis* evolved from a *Pellioditis*-like-ancestor in an arenicolous marine environment whereas *Steinernema* probably evolved from a “proto-*Rhabditonema*” ancestor in a terrestrial environment.

Résumé – Origine et relations phylogéniques des Rhabditides entomophiles *Heterorhabditis* et *Steinernema* – En se fondant sur des arguments morphologiques, physiologiques, biologiques ainsi que sur leur répartition, il est conclu que les genres *Steinernema* et *Heterorhabditis* sont très largement divergents et que les similarités de leurs cycles biologiques doivent être attribuées à des phénomènes de convergence. Les données présentées indiquent qu’*Heterorhabditis* aurait évolué à partir d’un ancêtre de type *Pellioditis*, vivant dans des sables marins, tandis que *Steinernema* tirerait son origine d’un « proto-*Rhabditonema* », vivant en sol ferme.

Key-words : Entomophilic nematodes, phylogenetics, *Heterorhabditis*, *Steinernema*.

Members of the insect parasitic rhabditoid genera *Steinernema* Travassos, 1927 (Steinernematidae) and *Heterorhabditis* Poinar, 1976 (Heterorhabditidae Poinar, 1976) are similar in having evolved a system of carrying and introducing specialized symbiotic bacteria into living invertebrates. These bacteria initiate a septicemia which normally kills the host in 24 hours and the insect-bacterial remains provide the necessary nutrition for the nematodes to develop and reproduce (Poinar & Thomas, 1966; Poinar, 1983). The origins of these nematodes, their phylogenetic relationships, and their relationship to each other, have received little attention, yet could explain basic aspects of their ecology.

The present study provides morphological, physiological, biological and distributional evidence for separate origins of these two nematode genera. The similarities of the life cycles and host relationships of *Steinernema* and *Heterorhabditis* can be attributed to convergent evolution.

Materials and methods

Morphological studies were conducted on adults of the genera *Heterorhabditis* Poinar, 1976 and *Steinernema* Travassos, 1927, with special emphasis on the type species, *H. bacteriophora* Poinar, 1976 and *S. glaseri* (Steiner, 1929). Other species examined included *H. megidis* Poinar, Jackson & Klein, 1987; *H. zealandica* Poinar, 1990; *S. feltiae* (Filipjev, 1934); *S. affinis* (Bovien, 1937); *S. carpocapsae* (Weiser, 1955); *S. intermedia* (Poinar, 1985); and *S. anomali* Kozodoi, 1984. Adult nematodes of the above species were reared in larvae of the wax moth *Galleria mellonella*. After removal from

wax moth larvae (5–7 days after infection (20 °C), the nematodes were heat-killed at 55 °C, fixed in TAF for 96 hours and processed to glycerin. Illustrations were made with a drawing tube mounted on a Nikon optiphot microscope equipped with differential interference contrast.

Morphological characters of free-living microbrotrophic rhabditids were taken from the literature, especially from the works of Andrassy (1983) and Sudhaus (1976).

Results and discussion

The results obtained with *Heterorhabditis* and *Steinernema* are presented separately below. Plesiomorphic and apomorphic characters, indicating stable ancestral and more recently derived features, respectively, are indicated.

HETERORHABDITIS

This genus is placed in the monotypic family Heterorhabditidae of the order Rhabditida. The family clearly falls in the suborder Rhabditina, which with its 47 genera (the number is variable depending on whether the groups are considered genera or subgenera) represents a wide range of free-living and animal-associated nematodes. *Heterorhabditis* has retained many plesiomorphic characters such as small amphids, a simple non-cuticularized cheilostom, a didelphic female gonad with the vulva at mid-body and a peloderan bursa. These characters are shared by members of the subfamily Peloderinae in the family Rhabditidae. Additional plesiomorphic characters such as an open bursa, separate spicules and

nine pairs of bursal papillae show that of the free-living members of the subfamily Peloderinae, *Heterorhabditis* most closely resembles the genera *Pellioiditis* (Douglherty, 1953) and *Dolichorhabditis* Andrassy, 1983. *Heterorhabditis* adults differ morphologically from these free-living genera mainly in apomorphic characters related to the manner of food uptake. In *Heterorhabditis*, the meso- and metastom have shifted forward and inward and the mesostom is shorter than wide (see Fig. 1). Such an adaptation is probably related to the uptake of food in a semi-liquid habitat as is found in an insect cadaver.

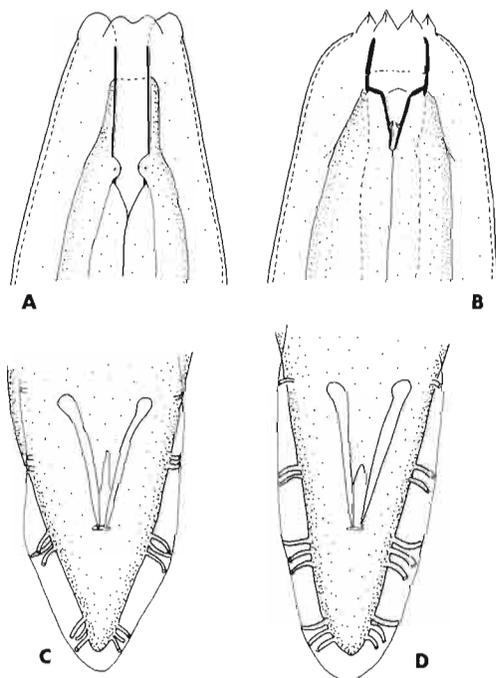


Fig. 1. *Heterorhabditis* and probable ancestral form (*Pellioidites*). A: Stomal region of a female *Pellioidites marina* (redrawn from Osche, 1954); B: Stomal region of a female *Heterorhabditis bacteriophora*; C: Ventral view of male tail of *Pellioidites marina*. (redrawn from Osche, 1954); D: Ventral view of male tail of *Heterorhabditis bacteriophora*.

The genera *Dolichorhabditis* and *Pellioidites* are very closely related, differing only in the amount of swelling in the corpus of the pharynx and the size of the armature (warts or denticles) on the glottoid apparatus. Both genera have representatives which are associates of invertebrates (Table 1). *Dolichorhabditis dolichura* (Schneider, 1866) occurs in ant nests and the dauer stages are found in the head glands of adult ants (Wahab, 1962). *Pellioidites pelliio* (Schneider, 1866) invades earthworms as dauer juveniles, then feeds in the cadavers after death.

Phasmarhabditis papillosa (Schneider, 1866), *P. neopapillosa* (Mengert, 1952) and *P. hermaphrodita* (Schneider, 1866) in the same subfamily (Peloderinae) are associated with terrestrial snails, the invasive stage again being the dauer juvenile (Mengert, 1953; Andrassy, 1983). On the basis of morphological comparisons, *Heterorhabditis* most closely resembles marine nematodes of the genus *Pellioidites*. Only a few marine rhabditids occur in the intertidal and coastal regions, and most of them are closely-related members of the subfamily Peloderinae. These include *Phasmarhabditis nidrosiensis* (Allgén, 1933), *Pellioidites bengalensis* (Timm, 1956), *P. marina* (Bastian, 1865) and *P. mediterranea* (Sudhaus, 1974). One of these, *P. marina* is cosmopolitan and has been collected on the coasts of Europe, Africa, North and South America, Australia and New Zealand (Andrassy, 1983).

There is both distributional and physiological evidence that the heterorhabditids evolved in a coastal habitat from free-living, microbotrophic marine rhabditids. First, natural populations of heterorhabditids frequently occur in sandy beach areas. In sampling the Hawaiian islands, Hara *et al.* (1991) found that nearly all *Heterorhabditis* positive samples (95.5% or 21 out of 22) were collected at sea level within 100 m of ocean beaches. In Ireland and Britain, heterorhabditids are entirely restricted to sandy coastal soils (Griffin & Dounes, 1991) and all of the three *Heterorhabditis* positive soil samples from the Azores island of Terceira came from coastal areas (Lawrence Lacey, *in litt.*). Several factors could account for this (pH, availability of oxygen, absence of enemies) including that the infective stages of *Heterorhabditis* may actually survive longer in a saline environment as a result of their origin from halophilous ancestors. On the Isle of Sylt (off the Western Coast of Schleswig-Holstein, Germany) Blome (*in Heip et al.*, 1985) noted that populations of *P. marina* were recovered in the sandy beach slope between 10 and 20 m from the high tide mark, corresponding to sandy areas where researchers have recovered heterorhabditids. In studying *H. bacteriophora*, Milstead and Poinar (1978) noted that the infective stages would survive longer when stored in Ringers solution than in water. Further studies corroborated these findings and showed that infectives of *H. bacteriophora* survive in seawater for a longer period than in freshwater (Poinar & Hess-Poinar, unpubl.).

Xenorhabdus luminescens Thomas & Poinar, 1979, the bacterial symbiont of *Heterorhabditis* nematodes probably originated from a marine shore habitat. *Pellioidites marina* is a selective bacterial feeder which has rather specific nutritional requirements. In their studies on the nutritional requirements of *P. marina*, Tietjen *et al.* (1970) discovered that populations of *P. marina* from North Sea Harbor in Southampton, New York were naturally associated with three bacterial species, *Pseudomonas* sp., *Micrococcus* sp. and *Flavobacterium mari-*

num. Continuous monoxenic cultures were obtained with *Pseudomonas* sp. but were unsuccessful with *Micrococcus* sp. With *F. marinum*, monoxenic cultures were only successful for three transfers and the authors suspected that this bacterium lacked some nutrient required by the nematode. It is interesting to note that *Flavobacterium* species are peritrichously flagellate, gram-negative, facultatively anaerobic rods similar to *Xenorhabdus* (Buchanan & Gibbons, 1974). Actually *Flavobacterium* Bergey *et al.*, 1923 is considered a group of uncertain affiliation by Buchanan and Gibbons (1974), who place it near *Lucibacterium* Hendrie, Hodgkiss & Shewan. The latter genus is characterized by gram negative, non spore forming rods, motile by peritrichous flagella, the presence of pleomorphic forms in adverse culture conditions and the ability to produce light. All of these characters are also possessed by *X. luminescens*. Most strains of the latter species also normally produce a yellow to red pigment, a character shared with species of *Flavobacterium*. The species *L. harveyi* (Johnson & Shunk, 1936), which was originally described in the genus *Achromobacter* (as was *Xenorhabdus nematophilus* Poinar & Thomas, 1965), occurs in sea-water and on the surfaces of dead marine animals.

Reports of living and dead marine invertebrates containing luminescent bacteria have been summarized by Harvey (1952). These include luminous beach fleas (*Gammarus* Fab.) from the Mediterranean near Genoa as well as sand fleas of the genera *Talitrus* and *Orchestia* Leach near Wood's Hole, Massachusetts. Individuals of the latter genera were noted to die a few days after becoming infected with a luminous bacterium. The bacterium was isolated and experimentally introduced into crab and shrimp. These inoculated individuals also became luminous and died.

Although *R. marina* is an amphimictic species, hermaphroditic species do occur in the same subfamily e.g.,

P. hermaphrodita, *Caenorhabditis elegans* (Maupas, 1900), and *C. briggsae* (Dougherty & Nigon, 1949). Also hermaphroditism is a phenomenon related to, among other things, the availability of food. In developmental studies with various heterorhabditids, growth on low-protein culture media (sub-optimum nutritional conditions) results in a continuous cycle of hermaphrodites, without the intervention of the amphimictic cycle. The sexual cycle occurs under optimum nutritional conditions as is found in the insect hemolymph (Poinar, unpubl.).

One of the major evolutionary developments which would allow a free-living bacterial feeder like *R. marina* to evolve into an insect parasitic heterorhabditid was the retention of a bacterium by the infective stage nematode. This co-evolutionary development required adaptations by the nematode and bacterium to prevent the latter from being destroyed by the nematode's digestive secretions and to maintain its position during the gut evacuation phase which normally occurs just before a second stage rhabditid enters a dauer or infective stage (Poinar, unpubl. data). This was accommodated by the development of a bacterial-receptive "pouch" region in the anterior portion of the nematode's intestine as well as a thick capsular deposit on the bacterial cell wall (Poinar, unpubl.).

In an arenicolous marine habitat, *Heterorhabditis* could have parasitized littoral and beach dwelling crustaceans such as isopods, amphipods and possibly small crabs. Reports of dead amphipods containing luminescent bacteria were mentioned earlier. Also, it has been experimentally shown that *Heterorhabditis* can infect and develop in terrestrial crustaceans (Poinar & Paff, 1985). A shift from crustaceans to sabulicolous insects would not have been difficult. Probable insect hosts would include the arenicolous weevils, *Otiiorhynchus atroapterus* De G. (Curculionidae : Coleoptera) and *O. ligneus* Ol

Table 1. Characteristics of nematode genera in the Peloderine most closely related to *Heterorhabditis*.

Genus	Cheilostom	Bursa	Number (pairs) bursal papillae	Spicules	Phasmids	Habitats	Invertebrate associates
<i>Caenorhabditis</i>	not cuticularized	closed	9	separate	minute	terrestrial	snails
<i>Dolichorhabditis</i>	not cuticularized	open	9	separate	minute	terrestrial	insects
<i>Phasmarhabditis</i>	cuticularized	open	9	separate	prominent	terrestrial, marine	snails
<i>Pellioditis</i>	not cuticularized	open	9	separate	minute	terrestrial, marine	earthworms
<i>Pelodera</i>	not cuticularized	open	10	fused	minute	terrestrial, fresh water	snails
<i>Heterorhabditis</i>	not cuticularized	open	9	separate	inconspicuous	terrestrial, sandy beaches	insects

which are widely distributed along the seacoasts of Europe and the British Isles. Larvae of the former species feed on the roots of European beachgrass, *Ammophila arenaria* (L.) Link (Gramineae) while the latter species attacks roots of the seashore wormwood, *Artemisia maritima* L. (Compositae). Another potential host would be larvae of the scarabaeid, *Aegialia arenaria* Fab. (Scarabaeidae: Coleoptera), occurring along the Atlantic seaboard in Europe and the British Isles and feeding on the roots of European beachgrass (Lengerken, 1929; Walsh, 1925). It is interesting to note that representatives of the Curculionidae and Scarabaeidae are the most susceptible soil insects of many *Heterorhabditis* species (Poinar & Georgis, 1990).

Other potential insect hosts occur along most seacoasts, including even tropical islands (Usinger & La Rivers, 1953). The discovery of *Heterorhabditis* in desert soils (Glazer *et al.*, 1991) may reflect a tolerance to halomorphic soils which resemble those occurring on sandy beaches.

In conclusion, the heterorhabditids, which form a cohesive group, probably arose from a marine rhabditid such as *Pellioiditis marina* or a precursor that fed on free-living bacteria. The preference of *Heterorhabditis* infectives to saline solutions, the location of luminous bacteria in beach areas and the presence of heterorhabditids in beach sand, all support these conclusions. The present wide distribution of heterorhabditids away from beach areas could indicate their adaptation to low saline soils although there may be a preference to those soils that formed the border of seas that extended well inland during past geological periods. It would be interesting to see if those populations of heterorhabditids living in a completely terrestrial habitat still are halophilic and whether the salt content of the soil has any influence on their distribution.

STEINERNEMA

Members of the genus *Steinernema* do not share many morphological characters with any known genus of free living microbotrophic nematodes (Table 2). Plesiomorphic characters of *Steinernema* include the didelphic condition of the female, separate spicules and a gubernaculum. Apomorphic characters include the absence of a bursa and the stoma structure (Fig. 2) which in many respects parallels that of *Heterorhabditis* in showing modifications related to ingesting a semi-liquid diet in the body cavity of dead insects.

The male tail of *Steinernema* most closely resembles that of nematodes of the genus *Rhabditophanes* Fuchs, 1930 (Alloionematidae) and members of the family Panagrolaimidae in lacking a bursa, possessing paired spicules, a gubernaculum and distinct genital papillae. Female representatives of the Panagrolaimidae possess a prodelphic gonad however, which sets them aside from *Steinernema* spp. which are all amphidelphic (as are members of the Alloionematidae). Spicular and gubernacular characters of *Steinernema* spp. are also similar to those of *Rhabditophanes* except that the number of genital papillae differs (21-23 in *Steinernema* and 4-12 in *Rhabditophanes*) as do the number of lips (six lips in *Steinernema* and four in *Rhabditophanes*). However, additional observations are needed to confirm the four-lipped condition of *Rhabditophanes* since Cobb (1924) noted six head papillae suggesting that there actually might be six fused lips.

Alloionema Schneider, 1859, which belongs to the same family (Alloionematidae) as *Rhabditophanes*, possesses six lips and together with the latter genus shares a cuticularized cheilostom, inconspicuous phasmids and incomplete fusion of the rhabdions with *Steinernema*. *Alloionema* shows apomorphy in the structure of the

Table 2. Characters of nematode genera most closely related to *Steinernema*.

Genus	Lips	Cheilostom	Stoma	Number (pairs) of genital papillae	Phasmids	Habitats	Invertebrate associates
<i>Alloionema</i> (Alloionematidae)	6 fused	cuticularized	separate rhabdions	5	inconspicuous in adult	terrestrial	slugs
<i>Rhabditophanes</i> (Alloionematidae)	4 (?) fused	cuticularized	partially separate rhabdions	2-6	inconspicuous	terrestrial	insects
<i>Rhabditonema</i> (Rhabditonematidae)	6 partially fused	not cuticularized	fused rhabdions	6	inconspicuous	terrestrial	insects
<i>Steinernema</i> (Steinernematidae)	6 fused	cuticularized	separate rhabdions	11 (10) (plus a single median)	inconspicuous	terrestrial	insects

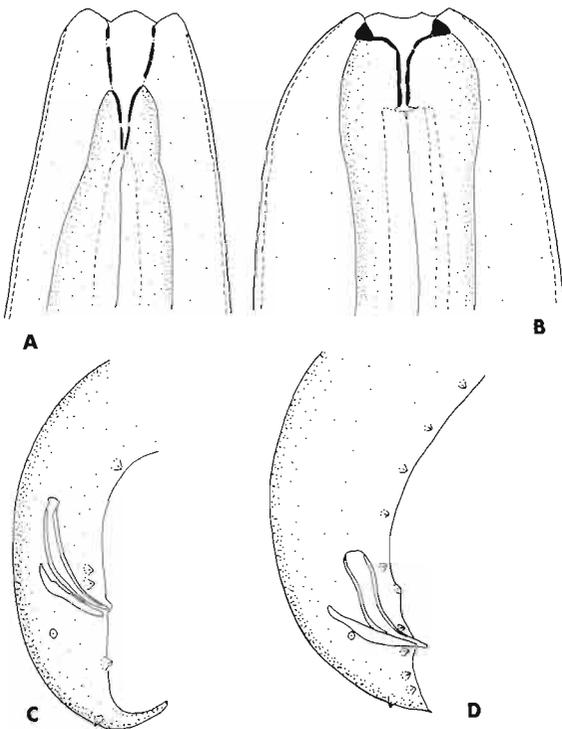


Fig. 2. *Steinernema* and possible ancestral form (*Rhabditophanes*). A: Stomal region of *Rhabditophanes schneideri* (redrawn from Fuchs, 1930); B: Stomal region of a female *Steinernema carpocapsae*; C: Lateral view of male tail of *Rhabditophanes aphodii* (redrawn from Sachs, 1950); D: Lateral view of male tail of *Steinernema feltiae*.

metastom which is anisomorphic and anisoglottid, with the dorsal metarhabdion swollen and covered with denticles. This is probably related to its biology as a facultative parasite of slugs (Mengert, 1953).

There are three described species of *Rhabditophanes*: *R. aphodii* (Sachs, 1950) which is associated with *Aphodius* beetles in dung, *R. cobbi* (Hnatewytch, 1929) which occurs in saprobic habitats and is probably associated with insects in at least a phoretic relationship and *R. schneideri* (Bütschli, 1873) which is associated with beetles (Staphylinidae and Curculionidae) as well as occurring in other saprobic habitats (Andrássy, 1983). The male tail of *R. aphodii* shows similarities to that of *Steinernema* spp. in lacking a bursa, possessing large genital papillae and well developed curved spicules with a prominent gubernaculum (Fig. 2 C). The stoma of *R. aphodii* shows some similarity to that of *Steinernema* spp. in having a cuticularized cheilostom with incomplete fusion of the remaining rhabdions (Fig. 2 A).

Bovien (1937) recovered *R. schneideri* from decaying plants but noted that the dauer juveniles were phoretically associated with adult and larval staphylinid beetles. The dauer were coiled up and attached to the insect

by a hyaline peduncle which was in contact with the tip of the nematode's head. These dauer juveniles would leave the insect under favorable conditions, multiply in the environment and produce another generation of dauer juveniles which would seek out and attach themselves to the surface of newly emerged staphylinid beetles.

Steinernema shows no evidence of a present or past association with a marine environment and probably evolved from a terrestrial proto-*Rhabditophanes* line which also gave rise to *Alloionema*. The steinernematid species show more morphological variability than the heterorhabditids and this may be associated with their type of reproduction (essentially amphimictic in *Steinernema* and hermaphroditic in *Heterorhabditis*) as well as the age of their lineage. Poinar (1983) speculated that both families probably evolved at roughly the same time in the mid-Paleozoic, some 375 million years ago. Molecular genetic studies could better elucidate the time of ancestral separation between these entomogenous rhabditids and their free-living counterparts.

The symbiotic bacteria of both *Steinernema* and *Heterorhabditis* are considered as separate species in the genus *Xenorhabdus* and differ enough to be separate genera. The similarities of these bacteria are superficial and probably a result of convergent evolution arising from their mutualistic association with the nematodes. It is now clear that they arose from fairly distant lineages. Recent molecular studies on *Xenorhabdus* using oligonucleotide catalogs of the 16S rRNAs of *X. nematophilus* and *X. luminescens* (Ehlers *et al.*, 1988) showed that the relatedness of *X. luminescens* to *X. nematophilus* is the same as that of *X. luminescens* to *Escherichia coli*. On the basis of DNA/DNA homology data, *X. nematophilus* and *X. luminescens* appear to be as different from one another as they are from other Enterobacteriaceae (Grimont *et al.*, 1983; Akhurst, *in litt.*).

The available morphological, distributional, physiological and biological evidence also shows that the genera *Steinernema* and *Heterorhabditis* are widely divergent and had completely separate origins (polyphyletic), the former evolving in a terrestrial environment and the latter in a coastal habitat. Similar patterns of infectivity, life cycle and association with a bacterial symbiont are considered to be the result of convergence.

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