Neoaplectana glaseri and *N. anomali* : sibling species or parallelism?

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SUMMARY

A comparison was made between *Neoaplectana glaseri* which occurs in North and South America and *N. anomali* which has been reported only from Central Europe. Both nematodes parasitize members of the Scarabaeidae and are morphologically very similar. However they do not interbreed and differences occur in their symbiotic bacteria, electrophoresis enzyme patterns and spicule shape. The question as to whether these two species are an example of parallelism or divergence is addressed.

RÉSUMÉ

Neoaplectana glaseri *et* N. anomali : *espèces jumelles ou parallélisme?*

Neoplectana glaseri, présent en Amérique du Nord et du Sud, et *N. anomali,* connu seulement d'Europe centrale, sont comparés. L'un et l'autre nématodes parasitent des Scarabéides et sont morphologiquement très semblables. Cependant ils ne se croisent pas et des différences existent qui concernent la bactérie symbionte, les diagrammes enzymatiques électrophorétiques et la forme du spicule. La question est posée de savoir si ces deux espèces représentent un cas de parallélisme ou de divergence.

Neoaplectana glaseri was originally described by Steiner (1929) as a parasite of the Japanese beetle *(Popillia japonica)* in New Jersey. It has been considered a New World species, having been subsequently recovered from North Carolina and Florida (Poinar, 1986) in North America and Brazil in South America (Pizano *et al.,* 1985). *N. anomali* was described as a parasite of the chafer, *Anomala dubia,* in the Riazan and Voronez provinces of the Soviet Union (Kozodoi, 1984).

The similarity of these two species, which do not interbreed under laboratory conditions, in respect to morphology, rate of development and host preference, is striking. These similarities as well as morphological and biochemical differences between the two species are discussed in the present paper and the question whether these species are an example of divergence leading to sibling species or parallelism is addressed.

Materials and methods

The Florida and North Carolina strains of *N. glaseri* and the Riazan and Voronez strains of *N. anomali* were used in the present study. Crossing experiments were performed using the hanging blood drop method (Poinar, 1966) by the first author and the *Galleria* injection method (Akhurst & Bedding, 1979) by the second author. Fifty crosses were attempted, using males and females of each species. Measurements of the infective juveniles were made on nematodes recovered from larvae of *Galleria mellonella.* These juveniles had been heat-killed (60°), fixed in TAF and processed to glycerin. Ratios employed were A (total length divided by width), B (total length divided by distance from head to base of pharynx), C (total length divided by length of tail), D (distance from head to excretory pore divided by distance from head to base of pharynx), and E (distance from head to excretory pore divided by length of tail).

Fifty one nematodes used for disc electrophoresis in polyacrilamide gels were harvested from *Galleria mellonella* and concentrated by centrifugation in plastic tubes. After being alternately frozen and thawed five times at - 30°, the nematodes were crushed in a tissue grinder and then centrifuged at 20 000 *g* for 30 mm in a 40 % sucrose solution with a 0.01 % brom-phenol blue marker dye. The technique of vertical electrophoresis in parallel layers of polyacrilamide gel was used (Truwelle & Nephedow, 1974). The gels were fixed in a 15 % solution of tri-chloracetic acid and stained with 0.01 % Coomasy G-250 in 7 % acetic acid. Methods used to observe visualization of phosphatase, esterase and malatedehydrogenase were employed.

A comparison of the development of the two species was performed by infecting *Galleria mellonella* larvae with a similar dose of infective juveniles (n = 20) and maintaining them at 22°. At 24 h intervals, a *Galleria* larva infected with each species was dissected and

nematode development noted. Comparisons of the cultural characteristics of the respective symbiotic bacteria (*Xenorhabdus* spp.) were made on nutrient and tergitol-7 agar at 72 h after inoculation (at 22°). Measurements were made on cells grown in nutrient broth shaker cultures for 18 h at 22°.

Results

Hybridization experiments

None of the fifty crosses showed evidence that *glaseri* and *anomali* were interfertile, however all of the controls (using nematodes of the same species) resulted in fertile offspring. In the initial hanging drop breeding experiments, it was noted that each nematode species poorly tolerated the conditions present in the blood drops initiated by the opposite species. Those conditions were established by the respective symbiotic (*Xenorhabdus* spp.) bacteria of each nematode. To correct this situation, further crossing attempts were conducted by placing respective adult nematodes in a fresh drop of *Galleria* blood. This provided a balance of the respective bacteria which the nematodes could tolerate for two weeks when the experiments were terminated.

Morphological measurements

Quantitative measurements of the adults of *N. glaseri* and *N. anomali* were too variable to be used for differentiating between the two species. Quantitative measurements of the infective stages demonstrated less variation and indicated that the distance from the head to the excretory pore could possibly be used as a diagnostic character (see Tab. 1). As a result of this difference, ratio D (distance from head to excretory pore divided by the distance from the head to the base of the pharynx) also showed very little overlap. Ratio E (distance from the head to excretory pore divided by the length of the tail) was too variable to be used in this case. The males possessed one character which was constant. This was the tip of the spicule, which in *N. anomali* is slightly swollen giving an offset appearance (Fig. 1 A). The tip of the spicule of *N. glaseri* is usually slightly notched (sometimes the notch is deep enough to term the condition " hooked ") (Fig. 1 B). This character was the only consistent morphological difference that could be used to separate the adults.

Electrophoresis

Disc electrophoresis revealed distinct differences in proteins (Fig. 2 A), malatedehydrogenase (Fig. 2 B),

Table 1

Measurements of the infective stages of *Neoaplectana glaseri* and *N. anomali*

Measurement	N. glaseri	N. anomali	
	Poinar, 1986 (*n* = 35)	*Kozodoi, 1984* (*n* = 10)	*Poinar, present study* (*n* = 15)
Total length	1130 (864-1448)	1097 (724-1408)	970 (928-1088)
Greatest width	42 (31-50)	63 (41-77)	29 (28-35)
Distance : head to excretory pore	102 (87-110)	—	83 (76-86)
Distance : head to nerve ring	120 (112-126)	—	109 (100-120)
Distance : head to pharynx base	162 (158-168)	125 (123-131)	151 (138-160)
Tail length	80 (72-86)	80 (77-84)	70 (64-77)
Anal diameter	23 (19-24)	—	20 (16-22)
Ratio A	29 (26-35)	21.7 (17.2-25.6)	30.6 (26.4-34.4)
Ratio B	7.3 (6.3-7.8)	8.8 (5.9-10.8)	6.4 (5.9-7.0)
Ratio C	14.7 (13.6-15.7)	13.7 (9.4-16.9)	13.9 (13.2-15.0)
Ratio D	0.65 (0.58-0.71)	—	0.55 (0.52-0.59)
Ratio E	1.31 (1.22-1.38)	—	1.19 (1.06-1.30)

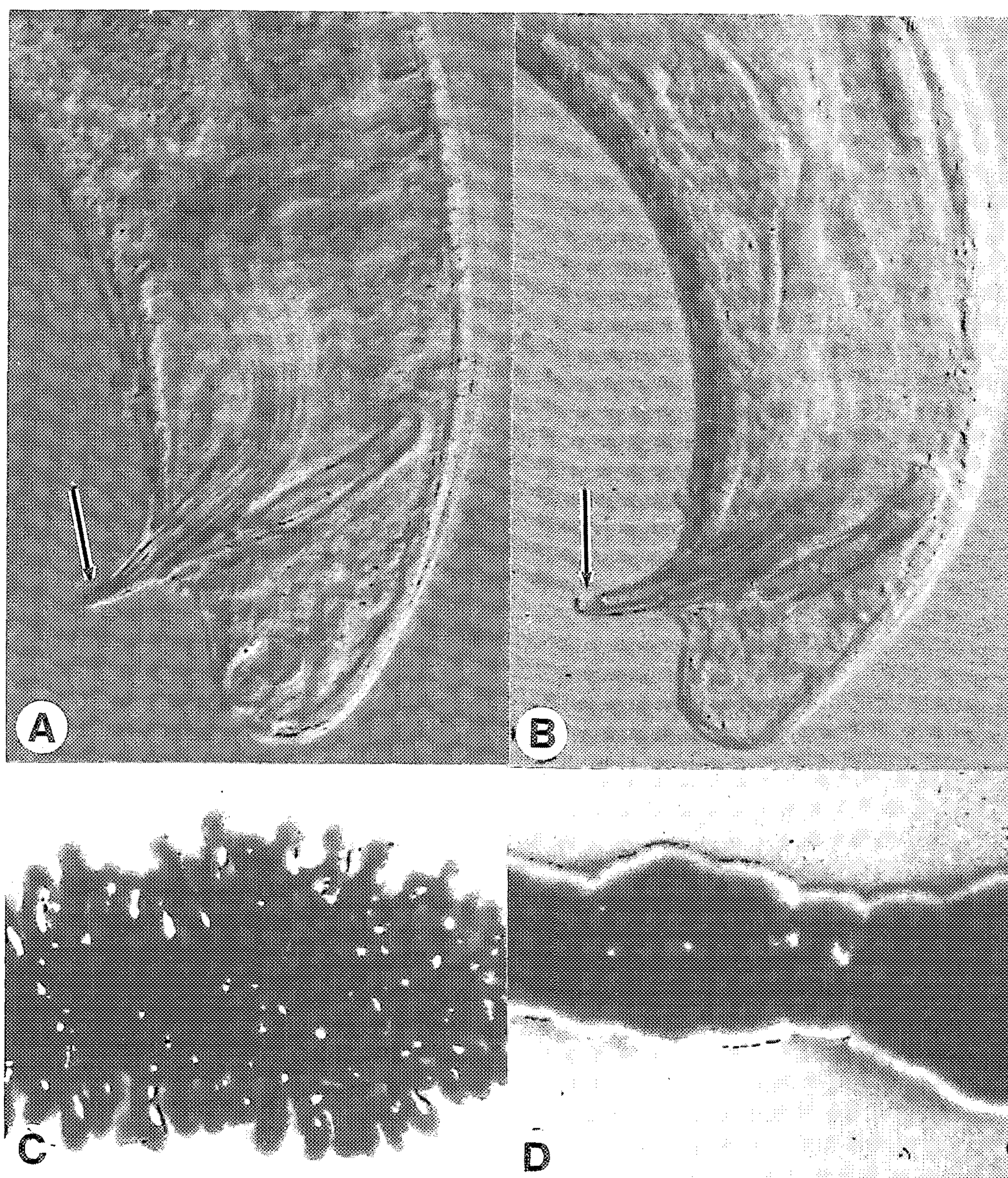

Fig. 1. Male tail of *N. anomali* (arrow shows swollen spicule tip); B : Male tail of *N. glaseri* (arrow shows notched spicule tip); C : Crenulate border of *Xenorhabdus* from *N. anomali* on tergitol-7 agar; D : Slightly wavy border of *Xenorhabdus nematophilus poinarii* from *N. glaseri* on tergitol-7 agar.

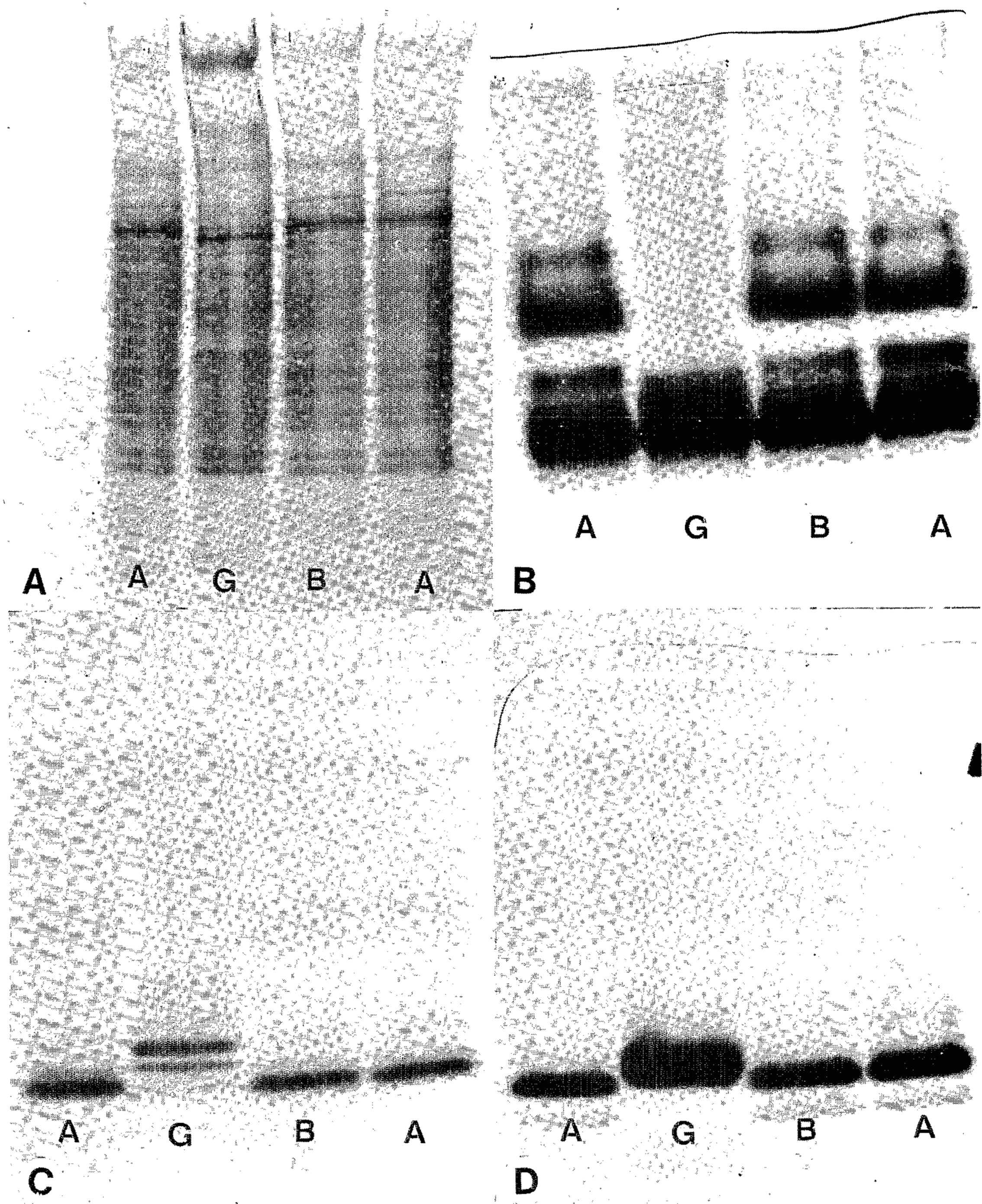

Fig. 2. A : Electrophoresis of total proteins of *N. anomali* from Riazan (A) and Voronez (B) provinces and *N. glaseri* (G); B : Electrophoresis of malatedehydrogenase of *N. anomali* from Riazan (A) and Voronez (B) provinces and *N. glaseri* (G); C : Electrophoresis of esterases of *N. anomali* from Riazan (A) and Voronez (B) provinces and *N. glaseri* (G); D : Electrophoresis of acid phosphatase of *N. anomali* from Riazan (A) and Voronez (B) provinces and *N. glaseri* (G).

esterases (Fig. 2 C) and acid phosphatase (Fig. 2 D). It should be noted that the patterns for *N. anomali* were identical in separate populations that originated from the Riazan and Voronez provinces.

BACTERIAL CHARACTERISTICS

The symbiotic bacterium of *N. glaseri* has been characterized and named *Xenorhabdus nematophilus poinarii* by Akhurst (1983). In subsequent studies Akhurst (1985) showed that in contrast to other *Xenorhabdus* species associated with *Neoaplectana, X. nematophilus poinarii* produced, morphologically, only one type of colony which was roughly equivalent to type II of the dimorphic subspecies of *X. nematophilus.* On nutrient agar, the colonies are brown or reddish-brown pigmented. The symbiotic bacterium from *N. anomali* is probably a new subspecies of *Xenorhabdus nematophilus.* Although on nutrient agar plates the colonies of both bacteria are similar in color and form, distinct changes occur when the symbiotic bacteria are grown on tergitol-7 agar. Colonies of *X. nematophilus poinarii* tend to be red pigmented, whereas those from *N. anomali* tend to vary from dark red to purple. The margins and surface of the latter isolate are usually wavy and rugose (Fig. 1 C) whereas those of the former subspecies are usually smooth or slightly wavy (Fig. 1 D). The average and range in cell length of the two isolates of *Xenorhabdus* made on 18 h shake cultures in nutrient broth (N = 50) were 7.0 (3.7-24.4) µm for the isolate from *N. anomali* and 8.3 (3.7-14.6) µm for *X. n. poinarii.* The differences were not significant.

DEVELOPMENT

Both *N. anomali* and *N. glaseri* develop more rapidly in *Galleria mellonella* larvae than do other species of *Neoaplectana* (Poinar, 1979). A comparison of their development under similar conditions shows some striking resemblances (Tab. 2). The development of *N. anomali* is slightly faster than that of *glaseri* with second stage juveniles emerging from the insect 132 hours after initial contact. Both nematode species share the characteristic of emergence from the insect cadaver as predauer or mature second stage juveniles instead of fully formed third stage infective juveniles. The final molt to infective juveniles usually occurs within 48-72 hours after leaving the host. With the relatively high doses used in this study, second generation adults occurred in small numbers and a second generation was never completed.

Discussion

Geographic isolation has been cited as the most probable mode of speciation among nematodes (Mayr,

Table 2

Comparison of development of *Neoaplectana anomali* and *N. glaseri* in mature larvae of *Galleria mellonella*

Hours after placing insects and infective stages together	*Development**	
	N. glaseri	N. anomali
32	1 *Galleria* dead; 4th stage juv. present	19 *Galleria* dead 4th stage juv. present
48	13 *Galleria* dead; adult and preadult nematodes	All 20 *Galleria* dead; adult and preadult nematodes
72	First and second stage juveniles in dead insects	First and second stage juveniles in dead insects
96	Mostly 2nd and 3rd stage juveniles	Mostly 2nd and 3rd stage juveniles
132	No predauer emerging yet	Predauer emerging from insect
156	Predauer emerging from insect	Most predauer already emerged from insect

*20 larvae of *Galleria* were challenged with 6-month-old infective stages of both nematodes species (dose = approximately 200 nemas *Galleria*).

1969). In the concept of speciation, do *N. anomali* and *N. glaseri* represent sibling species or a case of parallelism? Sibling species would suggest a recent divergence from a common ancestor whereas parallelism would indicate a far distant divergence followed by the appearance of morphological differences which eventually become less distinct as the result of similar selection pressure.

Both species appear to be geographically isolated. *N. glaseri* is presumably a neotropical species (never has been recorded in Europe) which has entered eastern North America at some time in the past when North and South America were connected (separation occurred in early Cenozoic) (Cox & Moore, 1985). Although North America and Europe were connected at one time, their separation came much earlier (possibly sometime in the mid-Jurassic) (Cox & Moore, 1985). *N. anomali* is known only from Central Europe.

Crossing experiments confirm the distinctness of *N. glaseri* and *N. anomali* as separate biological species. This is supported by distinct gel electrophoresis patterns and differences in the symbiotic bacteria. Both species resemble each other morphologically enough to be considered sibling species. This term is used to describe similar and closely related sympatric or allopatric species which do not interbreed. Sibling species are usually allopatric populations which recently diverged from a single common source. Extrinsic or physical geographical barriers are usually cited as the most common factors maintaining the populations apart until the genetic changes in each population have reached a level which prevents mating, fertilization or the like (Futuyma, 1979).

However, we believe that the similarity observed in the two species is a result of parallelism or the acquisition of similar characters in related evolutionary lines that diverged as long ago as the mid Jurassic (Mayr, 1969). If the genus began its expansion in the middle Mesozoic, then *N. anomali* and *N. glaseri* could have had a common stock in the late Permian-Triassic, then become separated in the mid-Jurassic, resulting now in distinct species which exhibit parallelism. The natural selection favoring parallelism could result from host selection. Although neoaplectanid species are capable of attacking a wide variety of insects, each nematode species is probably adapted to a particular microhabitat in the soil. This particular " niche " encompasses both a particular environment and the hosts associated with that environment. Both *N. anomali* and *N. glaseri* prefer to attack larvae of Scarabaeidae in nature. Natural selection favoring development in larvae of this insect group may favor large infective stages, rapid development and symbiotic bacteria which lack two clearly distinct (primary and secondary) stages. Since larvae of Scarabaeidae normally contain soil (with foreign bacteria) in their rectum which would be released in the insect's hemocoel upon death, a rapid development period would be advantageous to the nematode. Maturation from a mature second stage to an infective juvenile can occur after the nematodes leave the host, but development from the infective stage to adults and the following generation second stage depends on relatively pure hemolymph inoculated with the symbiotic bacterium. Also, the fact that the primary form of *X. n. poinarii* is relatively unstable (Akhurst, 1985) and can lose its ability to produce antimicrobial compounds is another reason for the rapid development of these nematodes. After the first 72 h the nematodes may not be able to depend on the antimicrobial effect of *Xenorhabdus* to maintain a relatively pure environment and the natural gut flora could establish itself and hinder nematode development. It has been assumed here, on the basis of comparative observations, that the symbiont of *N. anomali* is very similar, from the standpoint of conversion and antimicrobial production, to that of *X. n. poinarii.* This still remains to be shown experimentally.

Mayr (1969) states that similarities arising from parallelism are usually due to similar demands of the environment. Since the physical environment of South America and the southern United States is quite different from that of central Europe, we consider that adaptation to a particulat host group, in this case, representatives of the family Scarabaeidae, is the major factor responsible for the morphological and behavioral similarity of the two nematodes.

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