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Phylogenetic analysis of the genus *Steinernema* by morphological characters and randomly amplified polymorphic DNA fragments [1]

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Summary – Morphological characters and randomly amplified polymorphic DNA (RAPD) fragments were used for phylogenetic analysis in entomopathogenic nematodes of the genus *Steinernema*. The phylogenetic relationships of *Steinernema* species based on different suites of data were not congruent. We found that RAPD fragments were an effective means of differentiating species, but the divergence among species at this level was so great that these fragments may provide little information about the phylogenetic relationships among the species. However, the RAPD fragments may be used to produce phylogeny for different isolates of the same species because more common RAPD fragments were found among isolates of the same species. Phylogeny inferred from morphological characters was similar to that inferred from the repeat unit of ribosomal DNA. Ratio D (distance from anterior end to excretory pore divided by distance from anterior end to base of pharynx) of males and infective juveniles had an individual consistency index of 1, which may be more important for the estimation of phylogeny.

Résumé – ***Analyse phylogénique du genre* Steinernema *fondée sur les caractères morphologiques et la longueur de fragments d'ADN amplifiés au hasard*** – Des caractères morphologiques et la longueur de fragments d'ADN amplifiés au hasard (RAPD) ont été utilisés pour réaliser une analyse phylogénique des nématodes entomopathogènes du genre *Steinernema*. Les résultats concernant les relations phylogéniques entre espèces de *Steinernema* fondées sur différentes bases de données n'ont pas été concluants. Les fragments de RAPD ont correctement séparé les espèces, mais la divergence entre espèces est si importante que cette analyse n'a apporté que peu d'information sur les relations phylogéniques entre espèces. Néanmoins, les fragments de RAPD peuvent être utilisés pour étudier la phylogénie des populations d'une même espèce car nombre de fragments de RAPD communs ont été trouvés parmi les populations d'une même espèce. La phylogénie déduite des caractères morphologiques est similaire à celle déduite de l'analyse de polymorphisme de fragments d'ADN ribosomal. Le rapport D (distance de l'avant au pore excréteur divisée par la distance de l'avant à la base du pharynx) chez les mâles et les juvéniles infestants montre un index de consistance individuelle de 1, ce qui pourrait se révéler important pour l'évaluation de la phylogénie.

Key-words : Entomopathogenic nematodes, morphological characters, phylogenetic analysis, RAPD fragments, *Steinernema*.

Entomopathogenic nematodes belonging to the genus *Steinernema* are obligate and lethal parasites of insects. Their potential as biological control agents for a variety of pests is great, because different species and strains exhibit different efficacies for particular pests and conditions. Unfortunately, when new isolates are discovered, their identification is not always staightforward. Until 1990, many species in the genus *Steinernema* were diagnosed as species in the genus *Neoaplectana*. Several taxonomic changes at the generic and species levels have resulted in confusion in the literature. Present classification of the genus *Steinernema* recognizes species largely by overall similarity; taxonomic rank is determined by the degree of phenetic differentiation, which is designed to provide identification guides, not to reflect evolutionary history. The phylogenetic relationships of these nematodes have received little attention (Poinar, 1993).

From an evolutionary point of view, *Steinernema* nematodes probably arose from a terrestrial proto-*Rhabditophanes* line (Poinar, 1993). The phylogenetic relationships of the genus *Steinernema* have been investigated from the relative positions of restriction enzyme recognition sites within the repeat unit of ribosomal DNA (Reid, 1994). The purpose of this study was to investigate the phylogeny of the genus *Steinernema* based on morphological characters and randomly amplified polymorphic DNA (RAPD) fragments. We also analyzed congruence of phylogenetic relationships of these nematodes based on different suites of characters.

Materials and methods

For the phylogenetic analysis based on morphological data, eleven characters that have diagnostic value (see Poinar, 1986; Doucet & Doucet, 1990; Nguyen & Smart, 1992) were selected and coded from fourteen *Steinernema* species.

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These characters are the following :

1. Mean body length of infective juveniles : Longer > 800 (0); shorter < 800 (1).
2. Ratio A (total length of body divided by width of body) of infective juveniles : Robust, a < 25 (0); slender, a > 25 (1).
3. Ratio D (distance from anterior end to excretory pore divided by distance from anterior end to base of pharynx) of infective juveniles : Smaller, d < 0.3 (0); medium, d = 0.3-0.4 (1); greater, d > 0.4 (2).
4. Ratio E (distance from anterior end to excretory pore divided by length of tail) of infective juveniles : Smaller, e < 0.5 (0); medium, e = 0.5-1.0 (1); greater, e > 1.0 (2).
5. Mucro of first generation males : Present (0); absent (1).
6. Ratio D of first generation males; Smaller, D < 0.5 (0); greater, D > 0.5 (1).
7. Ratio E of first generation males : Smaller, E < 2.0 (0); medium, E = 2.0-4.0 (1); greater, E > 4.0 (2).
8. Ratio SW (length of spicule divided by width of body at cloaca) of first generation males : Smaller, SW < 1.0 (0); medium, SW = 1.0-2.0 (1); greater, SW > 2.0 (2).
9. Ratio GS (length of gubernaculum divided by length of spicule) of first generation males : Smaller, GS < 0.7 (0); greater, GS > 0.7 (1).
10. Curvature of spicules of first generation males : Moderate, < 70° (0); strong, > 70° (1).
11. Mucro of second generation males : Present (0); absent (1).

The resulting data matrix (Table 1) was used to reconstruct the phylogeny of the genus *Steinernema.*

For the phylogenetic analysis based on molecular data, genomic DNA was isolated from the following nematodes : *S. anomali; S. carpocapsae* (Agriotos, British, Mexican); *S. feltiae* SN; *S. glaseri* KG, NJ; *S. oregonensis* OS21 (Liu & Berry, 1996); *Steinernema* spp. OH1S (Newport, Oregon), OS22 (Hermiston, Oregon), and OS23 (Madras, Oregon) by the methods described by Curran *et al.* (1985). Four decamer oligonucleotides, OPA-02, -03, -09, -10 (Kit A, Operon Technologies, Alameda, CA), were used to amplify random DNA fragments. Amplification was performed according to the methods described by Liu and Berry (1995 *a*). Blurred or less intense RAPD fragments were eliminated in our analysis. The RAPD fragments that were reproducible and with sharpness were scored for four primers as present (1) or absent (0) to form a discrete data matrix. Genetic similarity between each pair of the species was calculated using the method of King *et al.* (1993), which ranged from 0 (no common RAPD frag-

Table 1. *Data matrix of coded character states for* Steinernema *species.*

Nematode	Character											Reference
	1	2	3	4	5	6	7	8	9	10	11	
S. affinis	0	0	2	1	0	1	0	1	0	1	?	Poinar, 1988
S. anomali	2	1	2	2	1	1	1	2	0	0	?	Poinar & Kozodoi, 1988
S. carpocapsae	0	0	0	1	0	0	1	1	0	0	?	Poinar, 1967
S. cubana	2	1	2	2	1	1	1	1	0	0	1	Mracek *et al.*, 1994
S. feltiae	1	1	2	1	0	1	1	1	1	0	1	Wouts, 1980
S. neocurtillis	1	1	0	0	0	0	0	1	1	0	1	Nguyen & Smart, 1992
S. glaseri	2	1	2	2	1	1	2	1	0	0	?	Poinar, 1978
S. intermedia	0	0	2	1	1	1	1	1	1	0	0	Poinar, 1985
S. kushidai	0	0	2	1	1	1	1	1	1	0	0	Mamiya, 1988
S. oregonensis	1	1	2	1	1	1	1	1	1	0	0	Liu & Berry, 1996
S. rara	0	0	1	1	0	0	0	0	1	1	0	Doucet, 1986
S. riobravis	0	0	2	2	1	1	1	1	1	1	1	Cabanillas *et al.*, 1994
S. ritteri	0	0	2	1	1	0	1	1	0	0	0	Doucet & Doucet, 1990
S. scapterisci	0	0	1	1	0	0	1	2	1	0	0	Nguyen & Smart, 1990

? : unknown character states.

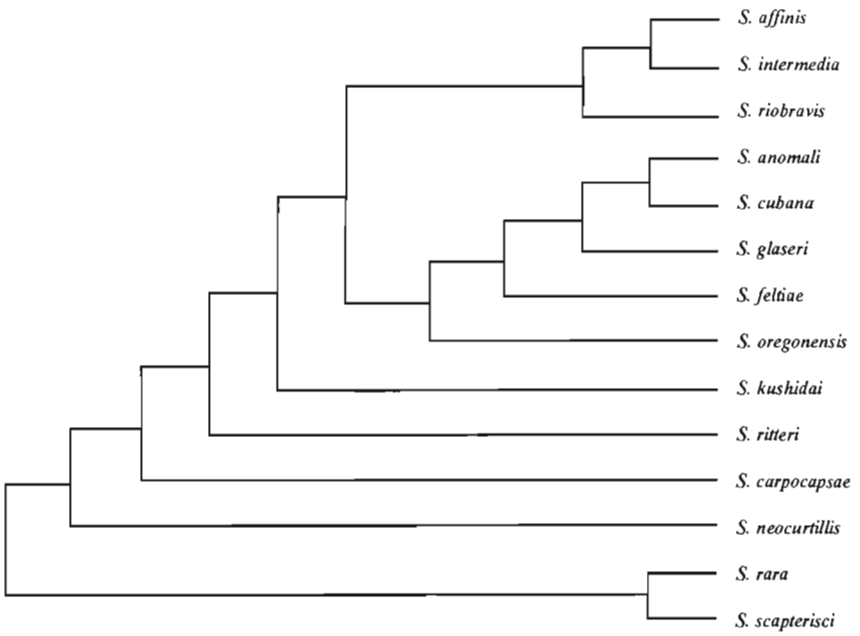

Fig. 1. *Dendrogram of 50 % majority-rule consensus tree showing the relationships among* Steinernema *species based on morphological character data.*

ments) to 1 (all RAPD fragments were common to both species).

Phylogenetic analyses were performed with the computer program PAUP, version 3.or. (Swofford, 1992). The branch-and-bound method was used to search the most parsimonious trees. Consistency index (Kluge & Farris, 1969) wase calculated without uninformative characters. Fifty percent majority-rule consensus trees were constructed. No assumptions were made on the more ancestral taxon, and the trees obtained are unrooted.

Results

Based on morphological data, PAUP yielded 22 of the most parsimonious trees with a length of 31 and a consistency index of 0.52. Characters 3 and 6 had an individual consistency index of 1, which were more useful for the estimation of phylogeny. Characters 1, 2, 4, 7, 8, 10, and 11 had a moderate individual consistency index ranging from 0.5 to 0.7. Characters 5 and 9 had a lower consistency index, and their importance in determining diversification of the genus may be smaller. When all characters were weighted by their consistency indexes, we found six trees with a length of 158 and a consistency index of 0.62. A dendrogram of a 50 % majority-rule consensus tree showed three species groups : *i) S. affinis, S. intermedia,* and *S. riobravis; ii) S. anomali, S. cubana, S. glaseri, S. feltiae,* and *S. oregonensis; iii) S. rara* and *S. scapterisci.* The remaining species *(S. carpocapsae, S. kushidai, S. neocurtillis and S. ritteri)* formed distinct groups (Fig. 1).

The four primers used in this study produced species-specific RAPD fragments. Forty-four RAPD fragments from eleven isolates were detected and scored for reconstructing phylogenetic trees. Each species gave different RAPD fragments, and only a few fragments were common to two or more species. There were more fragments in common among isolates of the same species than between species. Genetic similarity among different isolates of the same species ranged from 0.67 to 0.80; these among different species ranged from 0.00 to 0.29 (Table 2). PAUP analyses yielded two of the most parsimonious trees, with a length of 58 and a consistency index of 0.76. A dendrogram of a 50 % majority-rule consensus tree showed five species groups : *i)* three isolates of *S. carpocapsae; ii) S. anomali* and *Steinernema* sp. OS23; *iii)* the two isolates of *S. glaseri; iv) S. feltiae, S. oregonensis,* and *Steinernema* sp. OH1S; and *v) Steinernema* sp. OS22 (Fig. 2).

Only four species were analyzed in both morphological and RAPD fragment data, we found that topologies of two dendrograms (Figs 1, 2) were different. The phylogenetic relationship derived from the RAPD fragments was different from that derived from morphological characters in those species. For example, *S. anomali* and *S. glaseri* belonged to the same species group on the basis of morphological characters and formed distinct species groups on the basis of RAPD fragments. No

Table 2. *Genetic similarity between* Steinernema *species.*

	SCM	SCA	SCB	SAN	SGK	SGN	SFS	OS22	OS23	OH1S	OS21
SCM[a]	1.00										
SCA	0.74	1.00									
SCB	0.67	0.80	1.00								
SAN	0.00	0.00	0.00	1.00							
SGK	0.00	0.13	0.12	0.00	1.00						
SGN	0.11	0.22	0.10	0.00	0.67	1.00					
SFS	0.25	0.13	0.24	0.14	0.17	0.26	1.00				
OS22	0.00	0.00	0.00	0.00	0.15	0.13	0.00	1.00			
OS23	0.00	0.00	0.00	0.13	0.15	0.13	0.15	0.00	1.00		
OH1S	0.21	0.33	0.30	0.12	0.26	0.44	0.66	0.00	0.13	1.00	
OS21	0.25	0.27	0.12	0.29	0.17	0.27	0.50	0.00	0.15	0.40	1.00

[a]SCM, *S. carpocapsae* Mexican; SCA, *S. carpocapsae* Agriotos; SCB, *S. carpocapsae* British; SAN, *S. anomali;* SGK, *S. glaseri* KG; SGN, *S. glaseri* NJ; SFS, *S. feltiae* SN; OS22, *Steinernema* sp. (isolated from Oregon); OS23, *Steinernema* sp. (isolated from Oregon); OH1S, *Steinernema* sp. (isolated from Oregon); OS21, *S. oregonensis.*

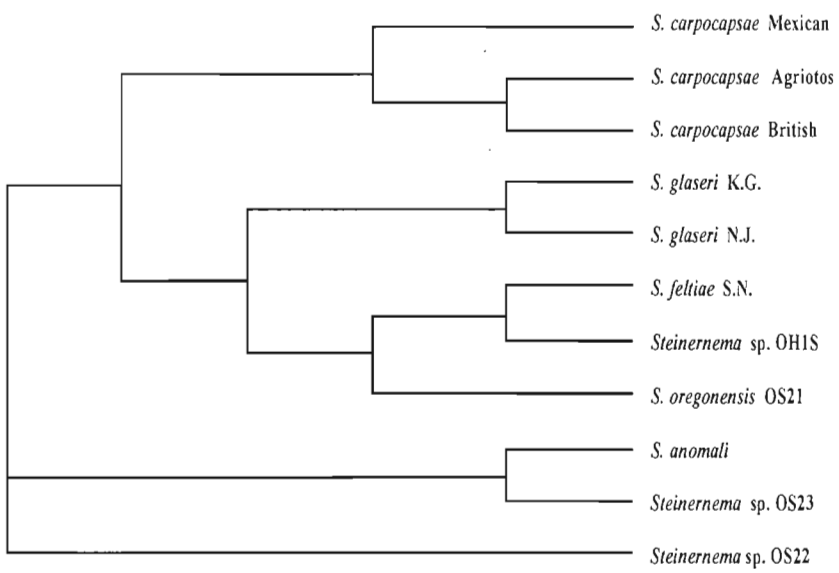

Fig. 2. *Dendrogram of 50 % majority-rule consensus tree showing the relationships among* Steinernema *species and isolates based on RAPD fragment data.*

obvious correlation was detected between the geographic region of isolate origin and their phylogenetic relationships based on morphological characters and RAPD fragments. Four isolates from Oregon soils belonged to three different species groups (Fig. 2).

Discussion

Estimating phylogenetic relationships among *Steinernema* species on the basis of morphological characters is difficult, because most of the diagnostic characters are quantitative and continuous, their character states are difficult to delimit, and there is a large degree of intraspecific morphological variation. Many of the characters normally used to describe species have a wide range. Continuously varying characters have rarely been used because of difficulties in reducing the range of variation to discrete states. Pimentel and Riggins (1987) and Cranston and Humphries (1988) doubted whether such characters can provide valid phylogenetic information.

We agree with Chappill (1989) that phylogeneticists should be interested in all heritable features of the organisms they are studying. Any observed trends that have a genetic basis should potentially be available for analysis (Chappill, 1989). Even though quantitative characters of organisms are more difficult to deal with than qualitative characters in phylogenetic analysis, they should not be ignored. Several methods of discrete coding of continuous characters and ratios for phylogenetic analysis have been proposed (*e.g.,* Archie, 1985; Thiele, 1993). In general, these methods cannot be used in the genus *Steinernema* because the morphometrics of some species have been described without standard deviations, and no variability is known. A method of arbitrarily choosing cut-off points between ranges (Quicke, 1993) was used in our analysis. This method usually works well based on previous knowledge of taxonomy (Quicke, 1993). We used the consistency index (CI) as our measure of homoplasy and fit of character data on a tree. The CI was defined as the minimum number of character state changes required by a given data set divided by the number of character state changes required for the same data given the tree in question and a CI of 1.0 indicates no homoplasy in the character (s) in question for a given tree and higher CIs indicate greater usefulness for the estimation of phylogeny (De Queiroz & Wimberger, 1993). The CI was affected by a variety of parameters and retention index is a superior measure of character data fit to a tree (Crother & Presch, 1992). However, CI is easily calculated, its meaning is generally understood by systematists, and its inherent biases can be accounted for in comparative analyses (De Queiroz & Wimberger, 1993). We found that ratio D of first-generation males and infective juveniles may be important for reconstruction of phylogeny because of their higher consistency index. For character weighting scheme, some more reliable characters could be given higher weight than the rest based on previous knowledge of taxonomy (Swofford, 1992). In our analysis, all characters were weighted by their CIs because the characters with higher CIs may be more reliable and have greater usefulness in phylogenetic analysis. Similar weighting scheme also was used by Guirado and Arcos (1994). The phylogenetic relationships between *S. glaseri* and *S. anomali;* and between *S. affinis* and *S. intermedia* were the same as that based on the repeat unit of ribosomal DNA (Reid, 1994). The CI of phylogeny based on morphological characters was not high (0.62). More characters and their non independence should be examined because our morphological data set was small and most of them were ratios. Ratio can be misleading, in that very different values can provide equivalent ratios. However, morphological characters available for phylogenetic analysis in *Steinernema* species are very limited.

RAPD fragments have been used to produce phylogenies for a variety of different nematode groups (Caswell-Chen *et al.,* 1992; Kaukas *et al.,* 1994). Within our data set, we found that the RAPD fragments were an effective means of identifying species and can be used to differentiate isolates of the same nematode species (Liu & Berry, 1995 *b*), but the divergence among species at this level is so great that these characters provide little information about the phylogenetic relationships among the species. However, the RAPD fragments may be used to produce phylogeny for different isolates of the same species because more common RAPD fragments were found between isolates of the same species. The RAPD fragments also may be useful to confirm the parallel relationship between *S. anomali* and *S. glaseri,* which was proposed by Poinar and Kozodoi (1988), because these species belonged to distinct species groups in RAPD fragment-based phylogenetic tree. The major criticism of using RAPD fragments as characters for phylogenetic reconstruction relates to fragment homology among the species and the utility of RAPD fragments in deriving phylogenies depends on the primer used (Cognato *et al.,* 1995). However, several other problems may also be encountered in using RAPD fragments for phylogenetic analysis (Smith *et al.,* 1994), and care must be taken to ensure that the use of RAPD fragments as characters in phylogenetic studies produces valid results.

As an alternative to RAPD-PCR, specific portions of the genome can be isolated and the sequences of these regions can be compared. The repeat unit of ribosomal DNA is an ideal choice for identification purposes because it contains potentially highly variable regions. The repeat unit also may be useful for phylogenetic studies because it contains highly conserved regions (Nadler, 1990). The phylogenetic relationships of *Steinernema* species derived from the relative positions of restriction enzyme recognition sites DNA within their ribosomal repeat unit (Reid, 1994) showed more similarity to that derived from our morphological character data. For ex-

ample, both trees linked *S. anomali* and *S. glaseri,* which are morphologically very similar. The same is true for *S. affinis* and *S. intermedia.* However, the two trees were not congruent between *S. riobravis* and other species. The relationship between *S. anomali* and *S. glaseri* was not congruent with that estimated from RAPD fragments. No single phylogeny is likely to explain all the events from various origins connected with hypothetical relationships among *Steinernema* species.

The search for the best estimate of phylogenetic relationships among taxa is the ultimate goal in the reconstruction of the pattern of evolutionary history. The appropriate methods to achieve that goal have been and will remain the subject of much debate (Crother & Presch, 1992), but almost all practicing phylogeneticists would agree on the appropriate data for obtaining the best estimate of phylogeny : use all the available data. This means multiple data sets including information on behavioral, morphological, biochemical, and molecular characteristics, and the myriad data sets within these broad categories. Because a group of organisms has only one evolutionary history, phylogenetic studies based on molecular characters should, in theory, be congruent with and additive to studies based on morphological characters. The congruence among independent studies based on different suites of characters is strong evidence for a particular phylogenetic estimate. Incongruence between studies may have many causes, including homoplasy, low resolving power of the technique, or use of tree building algorithms with different evolutionary assumptions (Nadler, 1990). We are unable to make a robust conclusion about phylogenetic relationships among *Steinernema* species, but molecular sequencing techniques show promise for determining these relationships. Morphological characters that have traditionally been used to classify the *Steinernema* species may need to be reevaluated. Through rigorous reexamination of type species with ultrastructural, ecological, and molecular studies, a more meaningful classification can be created.

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