BRILL

Nematology 20 (2018) 423-439

Nematology
brill.com/nemy

On the morphometric identity of populations of *Helicotylenchus pseudorobustus* (Steiner, 1914) Golden, 1956 (Tylenchida: Hoplolaimidae)

Renaud FORTUNER [1,*], Pierre-Yves LOUIS [2] and Dominique GENIET [3]

[1] *Laboratoire Ecologie et Biologie des Interactions, Equipe Ecologie Evolution Symbiose, Université de Poitiers, CNRS, UMR 7267, Bât. B 36 – 6 rue Michel Brunet BP 633, 86022 Poitiers Cedex, France*
[2] *Laboratoire de Mathématiques et Applications, Université de Poitiers, CNRS, UMR 7348, 11 Boulevard Marie et Pierre Curie, Téléport 2 – BP 30179, 86962 Futuroscope de Poitiers-Chasseneuil Cedex, France*
[3] *Laboratoire d'Informatique et d'Automatique pour les Systèmes, Université de Poitiers, EA 6315, Bât. B2, 2 rue Charles-Claude Chenou, TSA 51106, 86073 Poitiers Cedex, France*

Received: 31 October 2017; revised: 15 December 2017
Accepted for publication: 15 December 2017; available online: 7 February 2018

Summary – *Helicotylenchus microlobus* is considered to be a junior synonym of *H. pseudorobustus* by several authors while others consider it as valid. To clarify the status of both species, 39 samples collected from various countries were subjected to statistical analyses that showed they could be grouped into six groups. Topotypes of *H. pseudorobustus* and *H. microlobus* belong to two different groups. However, samples in the other groups were morphologically intermediate between these two groups. Characters used in the past to uphold the validity of *H. microlobus* were variable and overlapping from group to group. The 28 samples studied are identified as *H. pseudorobustus*. *Helicotylenchus microlobus*, *H. bradys* and *H. phalerus* are confirmed as junior synonyms of *H. pseudorobustus*. There was no complete congruence between the morphological groups and molecular groups proposed by other authors. For these, two MOTU (Molecular Operational Taxonomic Unit) are accepted within *H. pseudorobustus*.

Keywords – *Helicotylenchus bradys*, *Helicotylenchus microlobus*, *Helicotylenchus phalerus*, hierarchical clustering, Microlobus-group, mixed data, MOTU, multivariate statistics, Pseudorobustus-group, synonymy, taxonomy.

A study conducted 30 years ago (Fortuner *et al*., 1984; referred to as 'the 1984 study' below) on various samples of *H. pseudorobustus* (Steiner, 1914) Golden, 1956 and other species (*H. microlobus* Perry *in* Perry, Darling & Thorne, 1959, *H. bradys* Thorne & Malek, 1968, *H. phalerus* Anderson, 1974, *H. egyptiensis* Tarjan, 1964, *H. africanus* (Micoletzky, 1916) Andrássy, 1958, and *H. dihystera* (Cobb, 1893) Sher, 1961) found some morphological differences among the samples of *H. pseudorobustus* that made it possible to distinguish two main groups within that species:

i) the Pseudorobustus-group, including topotypes from Switzerland, other European samples from France and Germany, two samples from Africa, and one sample from New Zealand; and
ii) the Microlobus-group, with several American samples from New York State, Maryland, Indiana and Iowa, plus paratypes of *H. microlobus* from Wisconsin, *H. phalerus* from Canada, and *H. bradys* from South Dakota and Iowa.

However, other samples were found to be morphologically intermediate between these two main groups. Some samples from France and Florida were close to the Microlobus-group, but differed by a small difference in the position of phasmids (six annuli anterior to anus instead of four). Conversely, samples from Iran and Florida were closer to the Pseudorobustus-group but with phasmids four annuli anterior to the anus instead of six, and samples from California and Venezuela had the vulva slightly more posterior. Finally, samples from Portugal and Israel were considered as "questionable pseudorobustus".

In addition, intra-sample variability was high among all samples. The latter observation was consistent with

* Corresponding author, e-mail: fortuner@wanadoo.fr

DOI 10.1163/15685411-00003148

the high morphological variability observed within the progeny of a single *H. dihystera* female when raised on different hosts (Fortuner & Quénéhervé, 1980).

Because of the high intra-sample variability and the presence of samples morphologically intermediate between the two main groups, Fortuner *et al*. (1984) identified all the studied samples as belonging to the species, namely *H. pseudorobustus*, and accepted the synonymy of *H. microlobus* with *H. pseudorobustus*, as already proposed by Sher (1966) and Sauer & Winoto (1975) and later by Firoza & Maqbool (1994), the only dissenting voices being those of Siddiqi (1972, 2000) and Krall' (1978). *Helicotylenchus bradys* and *H. phalerus* were also considered by Fortuner *et al*. (1984) to be junior synonyms of *H. pseudorobustus*.

Subbotin *et al*. (2011) analysed 89 sequences of the D2-D3 expansion segments of 28S rRNA gene sequences from 54 *Helicotylenchus* isolates, including eight isolates morphologically identified as *H. pseudorobustus* (from Kansas, Nebraska, Illinois, California, Florida, Germany, Italy and China). They proposed a species group complex for *H. pseudorobustus* with four possible candidates, *viz*., *H. pseudorobustus* types A, B, C and D. Type A was found in Germany and New Zealand. Type B was more widely distributed, in Italy, China and the USA. Types C and D were found only in North American prairies, in Kansas and Nebraska, respectively. These authors considered that it is most likely that ". . . the type B found in Europe and having a wider distribution represents the true *H. pseudorobustus*", although the specimens studied by these authors did not include topotypes from Switzerland.

A second morphological and molecular study was conducted by Subbotin and other colleagues (Subbotin *et al*., 2015; referred to as "the 2015 study" below) on a number of *Helicotylenchus* samples, including topotypes of *H. pseudorobustus* and samples from many other species. These authors considered that some of their American and European samples were representative of *H. microlobus* rather than *H. pseudorobustus*, and that their "data clearly indicate that these two species are valid and morphologically and molecularly different." They also reported that DNA sequences of a sample from Kansas identified as *H. digonicus* matched those of samples identified by Subbotin *et al*. (2011) as *H. pseudorobustus* type C and type D. Consequently, they considered all these samples ". . . as representative of *H. digonicus*." Finally, they considered that the new genetic analyses showed that type A represents *H. pseudorobustus sensu stricto*, and not type B as proposed by Subbotin *et al*. (2011).

In view of the different conclusions reached in the three articles mentioned above, the present study was initiated to take a new look at morphological data from a taxonomist's perspective and decide: first, whether or not *H. pseudorobustus* and *H. microlobus* are separate species; and second, whether the molecular types described by Subbotin *et al*. (2011, 2015) correspond to morphologically distinct species.

Materials and methods

Populations

The samples used in the present study are listed in Table 1.

The origin and processing of the nematode samples used by Fortuner *et al*. (1984) and Subbotin *et al*. (2011, 2015) were described in the corresponding articles.

The specimen morphometric data used in the 1984 study were still available and were used as such in the present analyses. In addition, specimens from some of the isolates in Subbotin *et al*. (2011, 2015) were available for the present study and were also included. Slides prepared as described in these articles were observed by the present senior author at the Laboratoire Ecologie et Biologie des Interactions, Université de Poitiers, CNRS, UMR 7267, France, using an OpTec B6 microscope at 450× and 1000× magnifications. Measurements were made directly on the images taken by a video recording camera and displayed on screen.

Several samples from the 1984 study were excluded because of low sample size ($n < 10$). Sample E2 was excluded because it differs from *H. pseudorobustus* in the shape of the lip region (truncate instead of hemispherical) – the original identification of it belonging to that species was clearly erroneous. Eleven *H. dihystera* samples were used as the outgroup.

Statistics

Both quantitative and qualitative characters were included in the statistical analyses using a statistical approach different from that used in Fortuner *et al*. (1984). In the 1984 study, inclusion of both kinds of characters was achieved by transforming the quantitative data into categorical data. The present study used the opposite ap-

proach as qualitative characters at individual level were treated as quantitative characters at sample level by considering only two states for each character and using the percentage of one state among specimens in each sample. In order to reduce variability in the new study, quantitative characters were averaged over each of the 39 samples representing a total of 770 individuals. The 1984 study dealt with 24 samples for 427 individuals. In both studies, the median size of samples was 17 individuals. There were no missing data in the selected set of variables.

For each dataset, characters values were standardised. Means over the whole sample set, at individual level, were used for centering. The standard deviation at individual level of each quantitative variable was used for normalisation. For qualitative variables, the square root of the proportion multiplied by 1 minus the proportion was used. This is the usual standard deviation of a Bernoulli distributed random variable. Analyses were made on standardised values aggregated by samples. Sample size (n) varied from five to 32 specimens (Table 1). The n values were used as weight when working on aggregated data for the sake of consistency. Hierarchical clustering was implemented. Euclidean distance was used between samples. The agglomeration method was the Ward linkage. In Figures 1 and 2, the label 'inertia (Ward)' refers to that choice. It means that the height function is related to the within-cluster variance in the constituted clusters of (normalised) samples at that level. For more details about that method see, for instance, Chapter 4 in Everitt *et al*. (2011). The R command hclust was used on the distance matrix to build the agglomerative clustering sequence.

The analyses were validated depending on a correct separation of the *H. dihystera* outgroup. Validation failed when using classification methods such as k-means or dimension reduction (through PCA) on the raw and standardised values.

For a better visual discrimination of the samples studied, dendrograms were preferred over ordination plots because dendrograms integrate the whole variability instead of only the first two principal factors as often presented in similar studies. Here, no dimension reduction was used for classification. Moreover, the hierarchical structure of dendrograms represents the distances between the different clusters. Also, a dendrogram was used to present the results of molecular analysis in Figure 9 of Subbotin *et al*. (2015), and we thought it best to use the same type of presentation.

The coefficient of determination R^2 was computed for validating the clustering we proposed. R^2 is the ratio of the sum of square between-clusters (explained sum of squares) by the total sum of squares. R^2 varies between 0 and 1, and the higher it is the better the classification is at explaining the variability of the character.

Several clustering validity indices (Calinski-Harabasz, Dunn, Silhouette) were computed using the R packages NbClust, clValid and clusterSim (see Table 1 in Liu *et al*., 2010 and references therein). The package randomForest (based on the random forest method using decision trees to explain the observed clustering) was used to compute the contribution of each variable for explaining the chosen classification. The CH (Calinski-Harabasz) index was computed with the clusterSim package; Silhouette with the cluster package (see section 8.7.2 in Everitt *et al*., 2011); and Dunn with the NbClust package. The pvclust R package was used to check the robustness of the clusters observed (see section 9.5.1 in Everitt *et al*., 2011).

The univariate error bars represented in the figures for different samples were computed using the usual and well-known normal confidence interval with 95% significance. This is driven by the need for simplicity and the homogeneous treatment of all variables. To deal with normality defects, Bootstrap confidence intervals were also computed and confirmed the results.

The normal confidence interval values were computed from individual data aggregated by selected group of samples (Tables 2-6). In these tables, qualitative characters are represented by the percentage in the various samples and groups. Confidence intervals are given in Tables 2-6 instead of the traditional way to state measurements as mean ± S.D. (min-max) because they give a better idea of the true value of the mean. Fortuner (1984) explained that "The interval $+i$ or $-i$ around the mean X observed in the sample has 95% probability of including the true value of the mean in the population." The sample mean and standard deviation are statistically correct parameters, but they give the reader a false impression of precision. The confidence interval of the mean emphasises the fact that the exact population mean is not known.

Results

Multivariate statistical analyses

A preliminary multivariate analysis was made on the 24 samples used in the analysis depicted in Figure 4

Table 1. List of *Helicotylenchus* samples and species used in the present study.

Code/Molecular type	Identification	n	Host	Locality	Source
Populations from the Fortuner *et al.* (1984) study					
A1	*H. pseudorobustus*	20	Moss	Altmatt, Switzerland,	Topotypes from Sher (1966)
A2	*H. pseudorobustus*	24	Pine	Hünxe, Germany	B. Weischer
A3	*H. pseudorobustus*	11	Chestnut	Torino, Italy	G. Mancini
A4	*H. pseudorobustus*	28	Apple	Bergerac, France	ORSTOM coll.
A5	*H. pseudorobustus*	17	Tomato	Carpentras, France	ORSTOM coll.
B2	*H. pseudorobustus*	15	Kikuyu Grass	Israel	ORSTOM coll.
C1	*H. dihystera*	14	Citrus	Ibadan, Nigeria	F.E. Caveness
C2	*H. dihystera*	18	Plantain banana	Ibadan, Nigeria	CDFA coll.
El	*H. pseudorobustus*	27	Blue Grass	West Point, New York	A.M. Golden
E3	*H. pseudorobustus*	25	Corn	Near La Fayette, Indiana	R. McSorley
E4	*H. pseudorobustus*	17	Corn	Boone County, Iowa	D.C. Norton
F1	*H. pseudorobustus*	16	Homestead, Florida	R. McSorley	
F2	*H. pseudorobustus*	30	Itchgrass	Sulphur Springs, St Lucia	D.J. Hunt
G1	*H. pseudorobustus*	30	Philodendron	San Francisco, California	CDFA coll.
I1	*H. pseudorobustus*	24	Pasture	Kaitoke, New Zealand	G.W. Yeates
Brad	*H. pseudorobustus*	14	Soybean	South Dakota and Iowa, paratypes of *H. bradys*	A.M. Golden
Micro	*H. pseudorobustus*	5	*Poa pratensis*	Madison, Wisconsin, paratypes of *H. microlobus*	A.M. Golden
Phal	*H. pseudorobustus*	9	Grass	Canada, paratypes of *H. phalerus*	R.V. Anderson
Egypt	*H. egyptiensis*	4	Sugarcane	Egypt	A.C. Tarjan
HdA	*H. dihystera*	19	Cocoa	Madagascar	ORSTOM coll.
HdB	*H. dihystera*	20	Banana	Canary Island	ORSTOM coll.
HdC	*H. dihystera*	17	Forest	Senegal	ORSTOM coll.
HdD	*H. dihystera*	16	Millet	Senegal	ORSTOM coll.
HdE	*H. dihystera*	17	Upland rice	Senegal	ORSTOM coll.
HdF	*H. dihystera*	19	Peanut	Gambia	ORSTOM coll.
HdG	*H. dihystera*	17	Corn	Gambia	ORSTOM coll.
HdH	*H. dihystera*	18	Tobacco	Senegal	ORSTOM coll.
HdI	*H. dihystera*	16	Peanut	Senegal	ORSTOM coll.
HdJ	*H. dihystera*	16	Papaya	Mauritania	ORSTOM coll.
HdK	*H. dihystera*	15	Potato	California	ORSTOM coll.

of the 1984 article and using the same seven characters (distance head to vulva, stylet length, tail length, number of tail annuli, length of terminal process, type of fusion of inner incisures on tail, and areolations in lateral field). A first attempt made using only the inter-sample variance was not satisfactory. A second attempt including both inter-sample variance and intra-sample variance was more conclusive. That analysis yielded a dendrogram (Fig. 1) similar to the 1984 dendrogram. It shows a group with the *H. dihystera* samples (including samples C1 and C2), clearly separated from two subgroups,

- one with the type population of *H. pseudorobustus* (Pseudo-a, named A1 in the 1984 study), A2, A3, A4 and, slightly separate, F2, G1, Egypt;
- the other with the type population of *H. microlobus*, A5, E3, E4 and, slightly separate Brad, Phal, F1, B2 and E1.

The only difference between the present analysis and the 1984 study is that sample F1, which belonged to the group with the topotypes of *H. pseudorobustus* in the 1984 study, is now in the group with the paratypes of *H. microlobus*.

Table 1. (Continued.)

Code/Molecular type	Identification	n	Host	Locality	Source
H. pseudorobustus populations with molecular types as identified by Subbotin *et al.* (2015)					
Pseudo-b-A	*H. pseudorobustus*	6	Moss/swamp	Altmatt (type locality), Switzerland	S. Kiewnick
Pseudo-c-A	*H. pseudorobustus*	28	Altmatt (type locality), Switzerland	S. Kiewnick	
CD704-A	*H. pseudorobustus*	28	*Lolium perenne*, *Trifolium repens*	Kaitoke, New Zealand	G.W. Yeates
CD785-A	*H. pseudorobustus*	15	Grasses	Mendocino County, California	S.A. Subbotin
CD881-A	*H. pseudorobustus*	20	Grasses	Tomales (Marin county), California	S.A. Subbotin
J238-B	*H. microlobus*	22	*Olea europaea*	Andújar, Spain	P. Castillo
Locubin-B	*H. microlobus*	29	*Populus nigra*	Castillo de Locubín, Spain	P. Castillo
J94-B	*H. microlobus*	19	*Ceratonia siliqua*	Jerez de la Frontera, Spain	P. Castillo
Palag-B	*H. microlobus*	26	*Vitis vinifera*	Palagiano, Italy	N. Vovlas
Bari-B	*H. microlobus*	32	*Olea europaea* subsp. *europaea*	Bari, Italy	N. Vovlas
CD743-B	*H. microlobus*	4	Grasses	Riverside, Fairmount Park, California	S.A. Subbotin
CD694-pax	*H. paxilli*	27	*Paspalum vaginatum*	Hague, Florida	R. Inserra
CD761-IV2	*Helicotylenchus* spIV-2	28	*Calathea* sp.	Gainesville, Winter Garden, Florida	R. Inserra

Other analyses were made including some samples from the 1984 study and other nematode samples (Table 1), including specimens from the same localities as used by Subbotin *et al*. (2015) who determined their molecular 'types'. The molecular types determined by these authors (A, B, paxilli and IV-2) were added to the code names of the samples. Samples C1 and C2 from the 1984 study were not included since both the 1984 analyses and the present ones show that they are closer to *H. dihystera* than to *H. pseudorobustus*.

Several analyses were made with different sets of characters but failed to separate the outgroup (*H. dihystera* samples) from the other samples. This was achieved when 17 characters were used (ratios a, c, c′, m, V, and MB, stylet length, distance head to dorsal gland opening (DGO), distance head to excretory pore, number of tail annuli, number of annuli between anus and phasmids, anal body diam., length of tail terminal process, habitus in number of degrees, percentage of specimens with hemispherical lips, percentage of specimens with U- and m-shaped inner incisure fusion, and percentage of specimens with flat/rounded stylet knobs). Analyses made using either the inter-sample variance only or both inter-sample and intra-sample variances yielded identical results, demonstrating the robustness of the approach. The character set was constituted considering the correlations and preferring some ratios over the original characters. Some characters (distance head to end of pharyngeal glands, ratio MB, and distance stylet base to DGO) were discarded because of high variability. Presence of areolation in lateral field was not included because that character could not be properly observed in the present specimens. Ratios were preferred over their constituent characters with the exception of anal body diam. that was included in addition to ratios c and c′ because it was used by Subbotin *et al*. (2015) to differentiate *H. microlobus* from *H. pseudorobustus*. Two qualitative characters (habitus and tail shape) were replaced by quantitative values. Habitus was included as the number of turns, in degrees, described by each specimen body. Tail shape was replaced by the length of the terminal process as tails typical of *H. pseudorobustus* have a process length positive (>0), whereas in tails without a terminal process, more representative of *H. dihystera*, the process length is equal to 0.

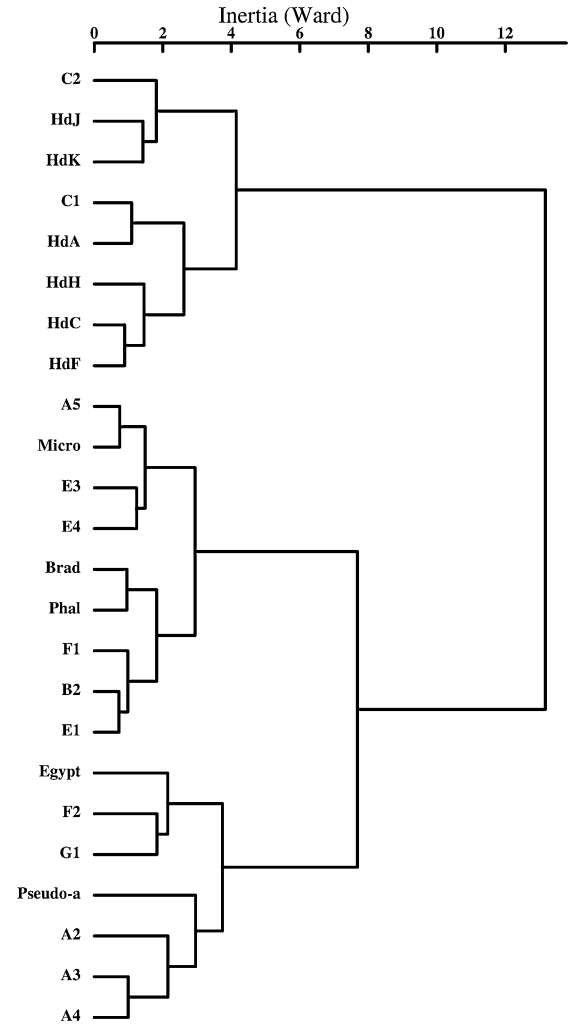

Fig. 1. Multivariate analysis of 24 populations from Fortuner *et al*. (1984) using seven characters.

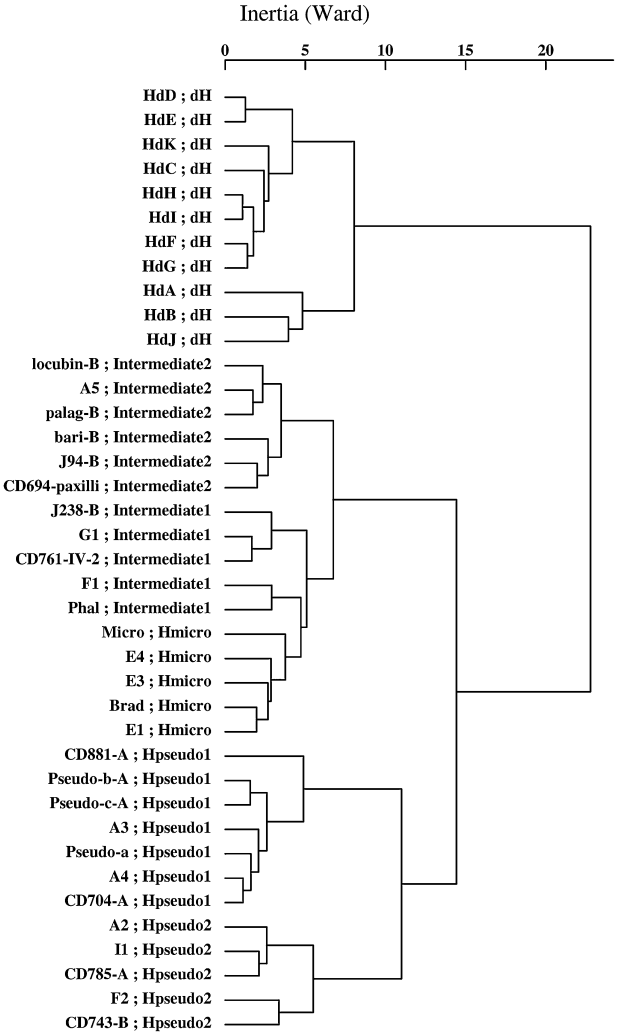

Fig. 2. Hierarchical classification of 39 populations from Fortuner *et al*. (1984) and Subbotin *et al*. (2015) (see Table 1) based on a multivariate analysis using 17 characters.

Only two states were noted for each of the three other qualitative characters (shape of anterior end, type of fusion of inner incisures on tail, and shape of stylet knobs), and each character was included in the analyses as the percentage of one of the states in the sample.

The resulting dendrogram is shown in Figure 2. The 11 *H. dihystera* ('Hd') samples are clearly separated from the other samples (at a level corresponding to Inertia = 23). Among these other samples, a second separation was found at Inertia = 14, corresponding to the two major groups observed in the 1984 study, the Pseudorobustus-group and the Microlobus-group. At lower inertia values it is possible to identify several subgroups (the names of groups and subgroups below have no nomenclatural value and are used in this article only for convenience):

Pseudo-1: the three samples from the type locality (Pseudo-a, Pseudo-b-A and Pseudo-c-A), A3, A4, CD704-A and CD881-A;

Pseudo-2: A2, F2, I1, CD785-A and CD743-B;

Intermediate-1: Phal, F1, G1, J238-B and CD761-IV2;

Intermediate-2: CD694-paxilli, A5, J94-B, bari-B, locubin-B and palag-B;

Micro: Micro, Brad, E1, E3 and E4.

Some of the most commonly used indices of the internal validity of the clustering were computed to

compare several possible clustering levels: two groups (*H. dihystera vs* the rest of the sample), three groups (*H. dihystera* plus two groups with the rest of the samples: Micro, Intermediate-1 and Intermediate-2 into one group, H. pseudo 1 and H. pseudo 2 into another group), or six groups (as presented in Fig. 2):

Cluster	CH index	Dunn coeff.	Silhouette profile average
2 groups	11.81	0.33	0.23
3 groups	10.61	0.33	0.21
6 groups	8.12	0.41	0.19

These are weak values. For reference purposes the highest Dunn coefficient obtained was equal to 0.55 for a clustering of 14 groups using the medoid method (pam), which is hardly higher than the value for six groups. In any case, it would be impossible to differentiate 14 separate species among the samples studied using either morphological or molecular data. Moreover, the R function clValid indicates that the hierarchical clustering method has a higher index of consistency than other methods such as medoid and k-means, and that the majority of indices indicate that the best choice for clustering is with only two groups.

The influence of the selected clustering on each character's variability is represented through R^2 coefficients in Figure 3. The most significant characters were process length (related to tail shape) and distance head to median bulb, with R^2 respectively equal to 62.5 and 34.8%. The package randomForest shows that the variables that contribute the most to the ranking were process length, stylet length, and distance head to bulb, with values equal to 32.95, 16.5 and 12.7, respectively. The values for the other characters were lower than 8.5.

The same situation as observed in 1984 was seen in the present analysis with the outgroup (*H. dihystera*) clearly separate from the rest of the samples. Among these other samples, two 'core' groups for *H. pseudorobustus* and *H. microlobus* are separate when considered alone but are linked by intermediate groups that close the gap between the type samples of the two species. The statistical validation indices support that interpretation as the best clustering is obtained with only two groups, the *H. dihystera* samples *vs* the rest of the samples, including type populations of *H. pseudorobustus* and *H. microlobus*. Sample F1 belongs to one of the intermediate groups, which might explain its change of position noted above in the preliminary study.

Congruence between morphological and genetic data

The samples with 'A' molecular type are generally found in the two Pseudo-groups and those with 'B' molecular type in the two intermediate groups but not in the Micro-group. Some overlaps do occur: Pseudo-2 group includes one sample with a molecular type A (CD758) and one with a molecular type B (CD743). Sample CD694, identified because of its molecular type as *H. paxilli* Yuen, 1964 in the 2015 study, is found in the Intermediate-2 group together with four samples with molecular type B. CD761, identified because of its

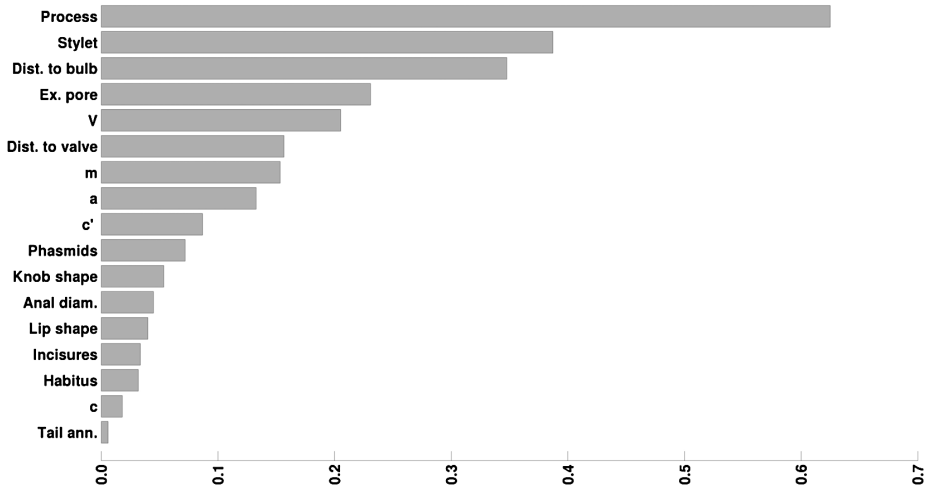

Fig. 3. Proportion of variance explained by the clustering presented in Figure 2 for each character (R^2).

molecular type as species IV-2 in the 2015 study, is found in the Intermediate-1 group, together with one sample with a molecular type B.

Detailed morphology of some samples

Tables 2-6 give the confidence intervals of the means of quantitative and qualitative characters for each of the populations studied and the group means in the five groups defined by the statistical analysis.

Sample CD694

In the 2015 study, sample CD694 was identified as *H. paxilli* based on its molecular type. *Helicotylenchus paxilli* was said by Yuen (1964) to differ from *H. microlobus* by the greater number of head annuli, shape of the head and stylet knobs, and distance of the DGO from the stylet base. Sher (1966) distinguished *H. paxilli* from *H. pseudorobustus* by position of the DGO and the usually more posterior position of the phasmids in relation to anus level:

	H. paxilli in Yuen	*H. paxilli* in Sher	CD694
Head annuli	6	4-5	4-5
Head	Bulbous	Hemispherical	Hemispherical
Stylet knobs	Slightly concave	Slightly indented	Rarely indented (15%)
Dist. stylet-DGO	7-8 μm	8.5 μm	8.5-10 μm
Phasmids	-3 to $+3$	-3 to $+3$	4-5 anterior
Stylet length	29-32 (30.3) μm	28-31 μm	26-27 μm

As described in Table 5, CD694 is clearly different from *H. paxilli* because of the smaller stylet (26-27 *vs* 29-32 μm), more anterior position of phasmids (4-5 annuli anterior to anus level *vs* three annuli posterior to three annuli anterior to anus level), and longer distance between the stylet base and the dorsal gland opening (8.5-10 *vs* 7-8 μm). CD694 does not show any marked difference with the diagnostic characters of *H. pseudorobustus* and that sample is here considered to belong to that species.

Sample CD761

Sample CD761 was considered by Subbotin *et al.* (2015) as belonging to another (undetermined) species because it has a different molecular type. Its morphological

Table 2. Morphological description of seven samples in group Pseudo-1, with the range of the population mean for each character (range = confidence interval centered on sample mean).

Character	Pseudo-a	Pseudo-b-A	Pseudo-c-A	A3	A4	CD704-A	CD881-A	Group Pseudo-1
n	20	6	28	11	28	28	20	141
L	736-791	693-786	712-756	683-743	713-765	699-731	742-782	729-747
V	60.8-62.4	61.4-64.1	62.2-63.0	60.8-62.9	60.4-62.2	61.5-62.6	60.5-61.6	61.6-62.1
m	45.9-47.5	44.6-47.6	46.5-47.6	47.8-49.6	47.3-48.5	47.8-48.8	46.7-48.0	47.3-47.8
a	26.1-29.7	26.7-28.2	27.1-28.2	29.5-32.1	27.3-29.0	27.0-28.3	26.9-28.8	27.7-28.4
c	46.2-50.3	40.9-56.9	42.7-45.8	42.4-47.0	41.9-45.1	39.7-43.0	39.7-43.4	43.1-44.7
c′	0.97-1.12	0.84-1.16	1.07-1.16	1.03-1.13	1.03-1.11	1.05-1.16	1.07-1.19	1.07-1.11
Stylet length	26.8-27.4	25.5-27.1	25.8-26.6	25.9-26.7	26.6-27.4	26.7-27.2	26.4-27.5	26.6-26.9
Dist. to bulb	77-80	71-80	75-78	76-81	78-81	77-81	69-73	76-78
Dist. to int. valve	113-119	108-121	110-115	108-117	113-117	110-116	92-100	110-113
Dist. to end of glands	138-146	137-148	141-149	136-146	142-147	140-147	125-133	140-143
MB	0.54-0.56	0.49-0.57	0.52-0.54	0.54-0.57	0.54-0.56	0.54-0.56	0.54-0.56	0.54-0.55
DGO	8.3-9.3	7.2-9.3	8.7-9.5	8.7-9.8	8.4-9.2	8.2-9.2	7.9-8.6	8.6-9.0
Dist. to excretory pore	112-116	114-121	112-117	106-113	108-112	110-115	95-104	110-112
Tail annuli	8.6-9.6	6.9-9.4	8.5-9.5	7.7-9.6	9.3-10.4	8.2-9.3	8.4-9.7	8.8-9.3
Anus-phasmid annuli	4.9-7.7	6.8-9.6	7.3-8.3	5.8-8.5	6.3-7.5	6.8-8.0	5.7-7.2	6.8-7.4
Anal body diam.	14.7-16.4	14.8-15.9	14.6-15.3	14.3-15.2	15.5-16.6	15.4-16.3	16.3-17.0	15.5-15.9
Length tail term. process	1.8-2.6	1.1-3.2	2.7-3.4	1.7-2.3	1.9-2.4	2.1-2.9	2.1-3.1	2.3-2.6
Habitus (in degrees)	651-725	561-747	604-662	629-692	673-707	658-722	522-597	642-670
Hemisph. lips (%)	100	100	100	100	100	100	100	100
U- and m-incisure fusion (%)	85	67	64	91	96	79	100	84
Flat/round stylet knobs (%)	80	100	86	91	93	75	90	86

Table 3. Morphological description of five samples in group Pseudo-2, with the range of the population mean for each character (range = confidence interval centered on sample mean).

Character	A2	F2	I1	CD785-A	CD743-B	Group Pseudo-2
n	24	29	24	15	4	96
L	745-806	682-717	726-771	782-824	718-873	737-764
V	59.9-62.3	62.8-63.7	60.5-62.3	60.6-61.6	59.2-63.2	61.4-62.2
m	47.7-48.7	46.3-47.0	47.3-48.6	46.3-47.4	45.1-47.1	47.1-47.6
a	29.7-31.0	30.8-32.0	28.3-29.9	29.3-32.0	27.4-32.5	30.0-30.8
c	39.8-42.9	34.3-36.1	38.2-41.8	36.9-40.4	28.3-44.0	37.7-39.4
c′	1.14-1.22	1.43-1.52	1.11-1.20	1.20-1.30	1.37-1.78	1.25-1.33
Stylet length	26.9-27.6	26.6-27.1	27.1-28.0	27.0-27.5	25.2-29.0	27.0-27.4
Dist. to bulb	80-82	83-85	85-89	88-92	84-89	84-86
Dist. to int. valve	115-119	124-127	125-129	125-131	119-125	123-125
Dist. to end of glands	143-149	155-160	154-159	155-163	153-158	153-156
MB	0.5-0.6	0.5-0.5	0.5-0.6	0.6-0.6	0.5-0.6	27.0-27.4
DGO	9.0-9.7	11.9-13.1	9.8-11.0	8.6-10.2	6.2-8.6	10.1-10.9
Dist. to excretory pore	113-117	113-117	117-122	117-123	118-124	116-118
Tail annuli	11.3-13.0	9.8-11.1	10.3-11.4	11.1-12.9	7.7-13.8	10.9-11.6
Anus-phasmid annuli	5.0-6.8	6.3-7.7	7.2-9.4	5.3-6.9	0.0-7.0	6.3-7.2
Anal body diam.	15.6-16.7	13.2-13.9	16.0-16.9	16.1-17.1	12.9-15.2	15.1-15.8
Length tail term. process	1.9-2.3	3.3-4.0	2.0-2.7	2.8-4.0	4.2-4.2	2.7-3.1
Habitus (in degrees)	658-718	501-546	615-676	643-703	517-781	604-643
Hemisph. lips (%)	100	100	100	100	100	100
U- and m-incisure fusion (%)	96	100	92	40	0	83
Flat/round stylet knobs (%)	83	59	71	73	25	69

Table 4. Morphological description of five samples in group Intermediate-1, with the range of the population mean for each character (range = confidence interval centered on sample mean).

Character	F1	G1	J238-B	CD761-IV-2	Phal	Group Intermediate-1
n	16	30	22	28	9	105
L	669-735	648-673	690-731	612-640	654-714	660-680
V	59.6-62.5	61.7-62.8	60.4-61.8	62.3-63.5	59.1-61.1	61.4-62.2
m	45.6-46.8	49.2-50.0	47.0-48.5	48.8-49.6	48.4-50.6	48.2-48.9
a	24.5-26.4	27.6-28.4	26.8-28.4	25.8-26.9	25.9-28.6	26.7-27.4
c	37.1-43.3	33.7-35.7	33.5-36.7	33.6-36.9	34.9-39.2	35.1-36.8
c′	1.06-1.25	1.27-1.36	1.37-1.48	1.24-1.35	1.19-1.32	1.27-1.33
Stylet length	25.9-27.1	26.6-27.0	26.3-26.7	25.0-25.8	26.4-27.7	26.2-26.6
Dist. to bulb	82-87	77-79	76-82	76-79	77-82	78-80
Dist. to int. valve	120-130	116-120	107-113	111-117	114-123	115-118
Dist. to end of glands	160-170	147-152	140-147	142-148	146-158	148-152
MB	0.50-0.52	0.52-0.53	0.53-0.57	0.52-0.54	0.50-0.54	0.52-0.54
DGO	10.4-11.5	11.2-12.1	10.9-11.8	8.6-9.8	9.9-11.0	10.4-11.0
Dist. to excretory pore	113-120	99-102	109-113	99-103	109-115	105-108
Tail annuli	8.8-11.2	8.2-9.3	9.7-11.2	8.2-9.4	10.4-12.9	9.2-9.9
Anus-phasmid annuli	2.4-5.2	4.5-6.3	2.1-3.5	4.7-5.8	2.9-4.9	4.0-4.9
Anal body diam.	14.8-16.2	14.2-15.0	13.9-15.0	13.4-14.5	14.3-15.3	14.3-14.8
Length tail term. process	1.9-3.2	2.7-3.4	3.4-4.2	3.3-4.0	0.9-2.0	2.9-3.4
Habitus (in degrees)	417-512	554-596	461-539	515-569	413-528	508-541
Hemisph. lips (%)	100	100	100	100	100	100
U- and m-incisure fusion (%)	19	40	45	18	0	29
Flat/round stylet knobs (%)	31	30	45	46	78	42

Table 5. Morphological description of six samples in group Intermediate-2, with the range of the population mean for each character (range = confidence interval centered on sample mean).

Character	A5	locubin-B	J94-B	palag-B	bari-B	CD694-paxilli	Group Intermediate-2
n	17	29	19	26	32	27	150
L	700-740	672-707	677-719	680-718	727-765	682-732	702-720
V	60.4-61.3	60.4-61.5	61.2-62.8	60.5-61.5	59.9-61.0	62.4-63.3	61.1-61.6
m	46.8-48.3	46.1-48.0	46.9-48.0	46.1-46.9	47.6-48.5	44.9-46.0	46.7-47.3
a	27.1-28.4	25.7-27.3	27.5-29.9	27.1-28.9	27.8-28.9	28.0-30.1	27.7-28.4
c	42.0-50.0	40.2-44.8	38.7-43.0	44.0-47.7	39.9-42.2	39.0-43.6	41.8-43.7
c′	1.02-1.21	1.10-1.21	1.19-1.37	1.14-1.24	1.23-1.30	1.21-1.34	1.19-1.24
Stylet length	26.8-27.7	26.2-26.8	25.5-26.4	26.4-27.0	26.7-27.3	26.0-27.0	26.5-26.8
Dist. to bulb	76-79	75-78	78-81	78-80	81-83	80-83	79-80
Dist. to int. valve	110-115	109-113	112-117	113-117	119-123	115-121	115-117
Dist. to end of glands	143-148	142-148	138-147	145-150	149-153	145-153	146-148
MB	0.5-0.5	0.5-0.5	0.5-0.6	0.5-0.5	0.5-0.6	0.5-0.6	0.5-0.5
DGO	9.6-10.9	10.2-11.2	8.3-9.8	10.1-10.9	10.2-11.4	8.5-9.9	9.9-10.4
Dist. to excretory pore	111.3-114.6	106.8-110.9	108.2-113.0	111.8-115.5	114.1-118.4	113.2-119.0	112.3-114.2
Tail annuli	6.7-8.1	7.7-9.0	8.4-9.9	7.2-8.0	9.4-10.2	6.8-8.2	8.1-8.6
Anus-phasmid annuli	5.1-7.0	5.3-6.4	2.7-4.4	5.3-6.0	1.6-2.8	4.1-4.9	4.2-4.9
Anal body diam.	13.7-14.7	13.8-14.6	13.1-14.0	12.6-13.3	14.1-14.8	13.3-14.2	13.7-14.1
Length tail term. process	1.8-2.7	3.3-4.1	3.1-3.9	3.2-3.8	2.9-3.3	3.6-4.2	3.2-3.5
Habitus (in degrees)	571-608	498-557	623-718	624-701	610-673	643-684	610-640
Hemisph. lips (%)	100	97	100	100	100	100	99
U- and m-incisure fusion (%)	0	41	11	4	0	4	11
Flat/round stylet knobs (%)	65	34	37	54	0	85	43

Table 6. Morphological description of five samples in group Micro, with the range of the population mean for each character (range = confidence interval centered on sample mean).

Character	Brad	E1	E3	E4	Micro	Group Micro
n	14	27	25	17	5	88
L	706-754	670-705	704-757	629-676	647-751	688-713
V	58.4-59.7	59.9-61.0	59.4-61.3	60.1-62.0	58.8-60.4	59.9-60.7
m	47.3-49.3	47.4-48.5	46.4-47.5	47.4-48.6	45.3-49.1	47.4-48.0
a	25.4-27.9	24.8-26.2	23.7-25.6	24.3-26.7	26.7-30.5	25.1-26.1
c	34.8-40.7	37.3-40.0	39.6-43.5	34.8-38.0	36.7-48.4	38.1-40.1
c′	1.24-1.42	1.17-1.27	1.18-1.36	1.19-1.27	1.15-1.45	1.23-1.29
Stylet length	25.9-26.9	26.0-26.5	27.3-28.0	26.7-27.3	26.0-27.8	26.7-27.0
Dist. to bulb	76-80	71-74	77-80	74-77	64-78	75-76
Dist. to int. valve	111-117	106-109	109-114	108-112	92-111	109-111
Dist. to end of glands	138-146	136-140	138-144	136-141	125-146	138-141
MB	0.5-0.6	0.5-0.5	0.6-0.6	0.5-0.6	0.5-0.6	0.5-0.5
DGO	9.4-10.9	10.5-11.4	10.2-11.1	11.7-12.5	6.0-10.8	10.5-11.1
Dist. to excretory pore	105-112	110-114	104-108	107-113	95-101	107-110
Tail annuli	9.7-11.8	9.2-10.4	8.6-10.1	8.5-10.0	6.6-9.8	9.2-10.0
Anus-phasmid annuli	3.3-5.4	3.0-4.1	2.8-4.9	1.5-2.8	1.8-6.6	3.1-3.9
Anal body diam.	14.2-15.6	14.2-14.9	13.5-14.8	14.1-15.0	12.1-13.3	14.1-14.7
Length tail term. process	1.4-2.1	1.6-2.2	1.7-2.5	1.7-2.2	0.8-3.6	1.8-2.1
Habitus (in degrees)	516-647	627-721	446-511	597-678	507-583	563-616
Hemisph. lips (%)	100	100	100	82	100	97
U- and m-incisure fusion (%)	0	0	0	6	0	1
Flat/round stylet knobs (%)	0	11	12	71	20	22

characteristics (Table 4) do not show marked differences with other *H. pseudorobustus* samples in Pseudo-1 group, except for a slightly higher V-value (62-63.5 *vs* 61.5-62 in Pseudo-1 group) and a slightly shorter stylet (25-26 *vs* 26.5-27 μm in Pseudo-1 group). That sample is herein identified as *H. pseudorobustus*.

Sample CD881

Sample CD881 changes group (from Pseudo-1 to Micro-group) depending on whether the anal body diam. is included or not in the multivariate analyses. That character is part of ratios c and c′, also included in the analyses, which gives an extra weight to body anal diam. because of co-linearity. The values in Table 2 show that CD881 is close to *H. pseudorobustus* in position of the phasmids, type of incisure fusion, and shape of stylet knobs. Its anal body diam. at anus level (16.3-17 μm) is larger than that of the Pseudo-1 group (15-16.2 μm) and much larger than in the Micro-group (12.2-13.2 μm). The variability of anal body diam. is discussed below. The V-value of sample CD881 is intermediate between the V-values in the various groups.

Samples described in Subbotin et al. *(2015)*

Eight of the samples measured for the present analyses were also described in Subbotin *et al*. (2015). For most samples and most variables there are either no or very small differences between the means published in the 2015 article and the present data describing the same samples, despite the fact that these data were measured by different scientists working with different microscopes. Table 2 in Subbotin *et al*. (2015) does not include the whole set of 17 characters used for the present analysis. A clustering analysis was made with 11 characters available from that table and the present samples. The analysis broadly confirmed the results obtained with the complete set of 17 characters, but we consider that, as it is based on a smaller set of variables, it does not add anything to the topic being discussed and it is not given here.

Morphological study of selected characters

For a better understanding of the taxonomic status of some of the present populations, a more detailed study was made on the four characters used by Subbotin *et al*. (2015) to differentiate *H. microlobus* from *H. pseudorobustus*. In Figures 4-6, the variables in each sample are represented by a bar giving the 95% confidence interval of the population mean. The various samples are grouped in the five morphological groups defined above (Figs 4A; 5A; 6A) or in the two molecular groups defined in the 2015 study (Figs 4B; 5B; 6B).

Anal body diameter

Figure 4A shows that the anal body diam. values are often larger in some populations from the Pseudo-1 and Pseudo-2 groups than in populations from the Micro-group (in particular the paratypes of *H. microlobus*). However, overlaps do occur in these two groups and even more so when samples in Intermediate-1 and -2 groups are considered. Subbotin *et al*. (2015) already noted that the anal body diam. of a sample identified as *H. microlobus* (CD740) did not differ from that of *H. pseudorobustus*.

In Figure 4B the anal body diam. mean values in populations with molecular type A are generally larger than in populations with molecular type B, but, in topotypes of *H. pseudorobustus*, that character overlaps several populations with molecular type B.

Position of phasmids in relation to anus level

The position of phasmids relative to anus level varies continuously from group to group with no definite gap between the various groups (Fig. 5A). In populations belonging to Pseudo-1 group, the phasmids are located a little more anteriorly than those in the other populations but overlaps occur here again.

In molecular groups, populations with molecular type A have phasmids more anterior than three populations with molecular type B but there is no difference in phasmid position with the other B populations (Fig. 5B).

Position of dorsal gland opening

There is considerable overlap among all morphological groups (Fig. 6A). Pseudo-1 group has distance stylet base-DGO a little smaller than in one population of the Micro-group, but the other groups completely fill the gap.

In molecular groups (Fig. 6B), distance stylet base-DGO in populations with molecular type A range from 8.2 to 9.4 and in those with molecular type B from 7.4 to 11.3.

Fusion of inner lateral field lines on tail

The last character used in the 2015 study to differentiate *H. microlobus* from *H. pseudorobustus* was the type of fusion of the inner incisures of the lateral field. Inner incisures fuse on the tail in a Y-shaped pattern in the former species *vs* a U-, μ- or M-shaped pattern in the latter. This qualitative character cannot be subjected to the same statistical procedures as used for the three quantitative characters.

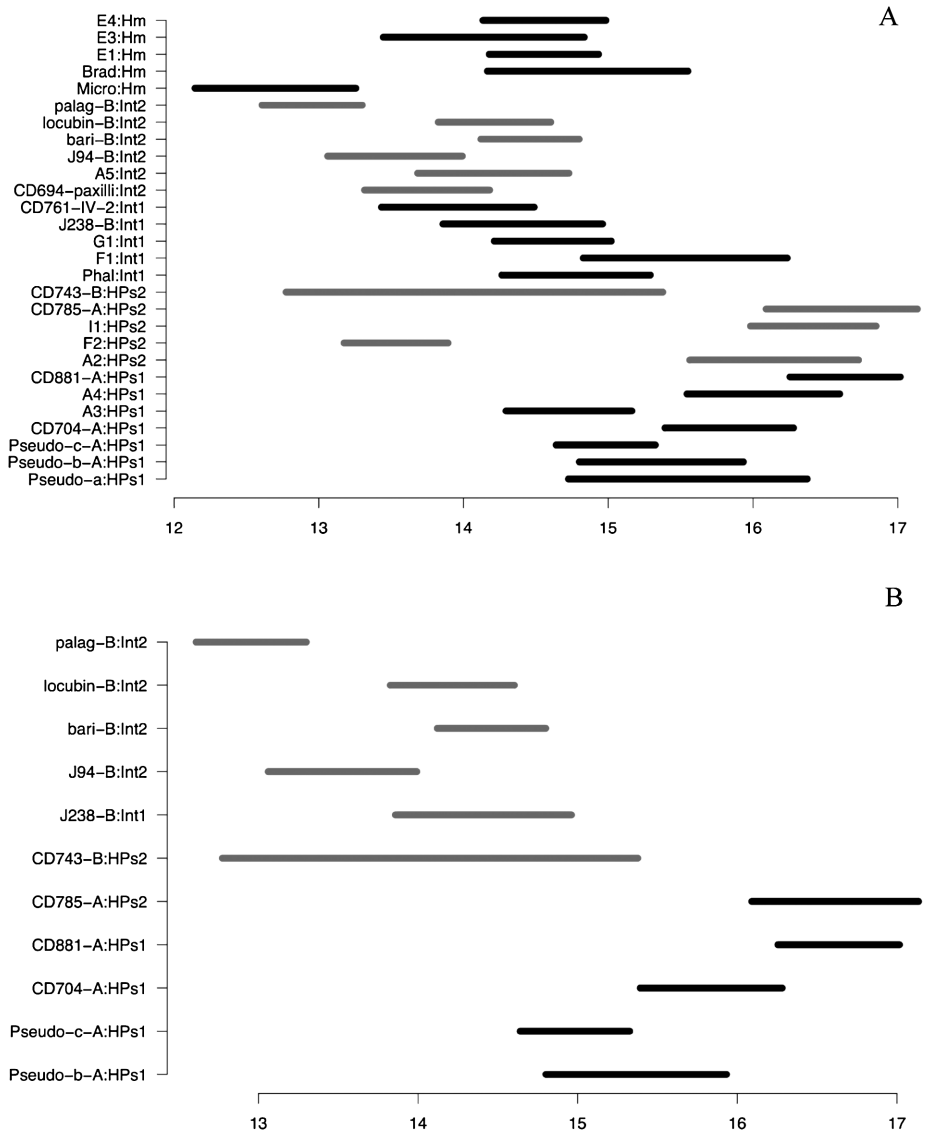

Fig. 4. Confidence interval of the mean of anal body diam. A: Twenty-eight populations in five morphological groups; B: Eleven populations in two molecular groups. y-axis: population codes and groups; x-axis: anal body diam. in μm.

The article by Subbotin *et al*. (2015) includes five figures with photographs of tails, but the inner lines are seen only in their Figures 7J and 8G and these two photos are too fuzzy to make it possible to determine the type of fusion. In *H. pseudorobustus*, these authors state that "the U- and M-shaped patterns were observed in equal proportions". In *H. microlobus*, they state that the inner lines were "... usually fusing distally for *ca* two annuli" (which corresponds to what is called here a Y-shaped pattern).

The actual fusion of inner lines on tail is difficult to observe under an optical microscope and that character is rarely used for species identification as it is very variable. For example, in the dichotomous key of Siddiqi (1972), it is used in only three out of 71 lines and then only at key ends, for differentiating between two very similar species. A striking example of that variability was observed during the 1984 study when one specimen from the type locality of *H. pseudorobustus* was found with a Y-shaped fusion

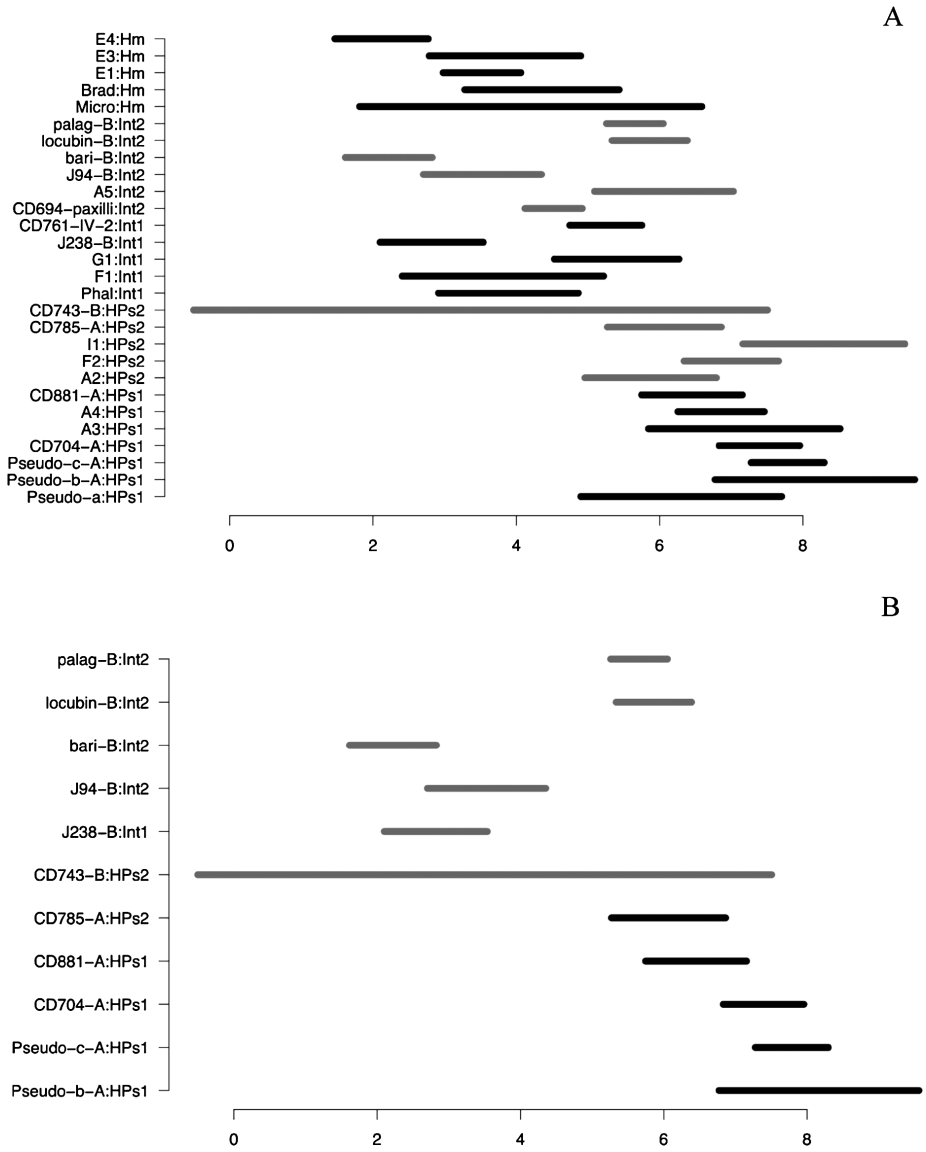

Fig. 5. Confidence interval of the mean of position of phasmids. A: Twenty-eight populations in five morphological groups; B: Eleven populations in two molecular groups. *y*-axis: population codes and groups; *x*-axis: number of annuli between anus and phasmid levels.

on the left side of the body and an m-shaped fusion on the right side (Fig. 7).

A possible explanation to this variability might be found in Figure 7 of Marais (1998), with SEM views of tails of *H. paracanalis*. The inner lines fuse in a U-shaped pattern in Figure 7D and a Y-shaped pattern in Figure 7E. However, it can be seen in Figure 7C that the two outer bands of the lateral field are raised above the inner band and they come together at some point, squeezing the inner band from sight. Depending on the haphazard position of these bands, the overall picture as seen under the optical microscope takes one or the other of the shapes often described.

Because of its variability within a sample, and even in a single specimen, and because of the overlapping observed during the present study within both morphological groups and molecular groups (Tables 2-6), the fusion of inner lines on the tail cannot be trusted as the single character differentiating two species.

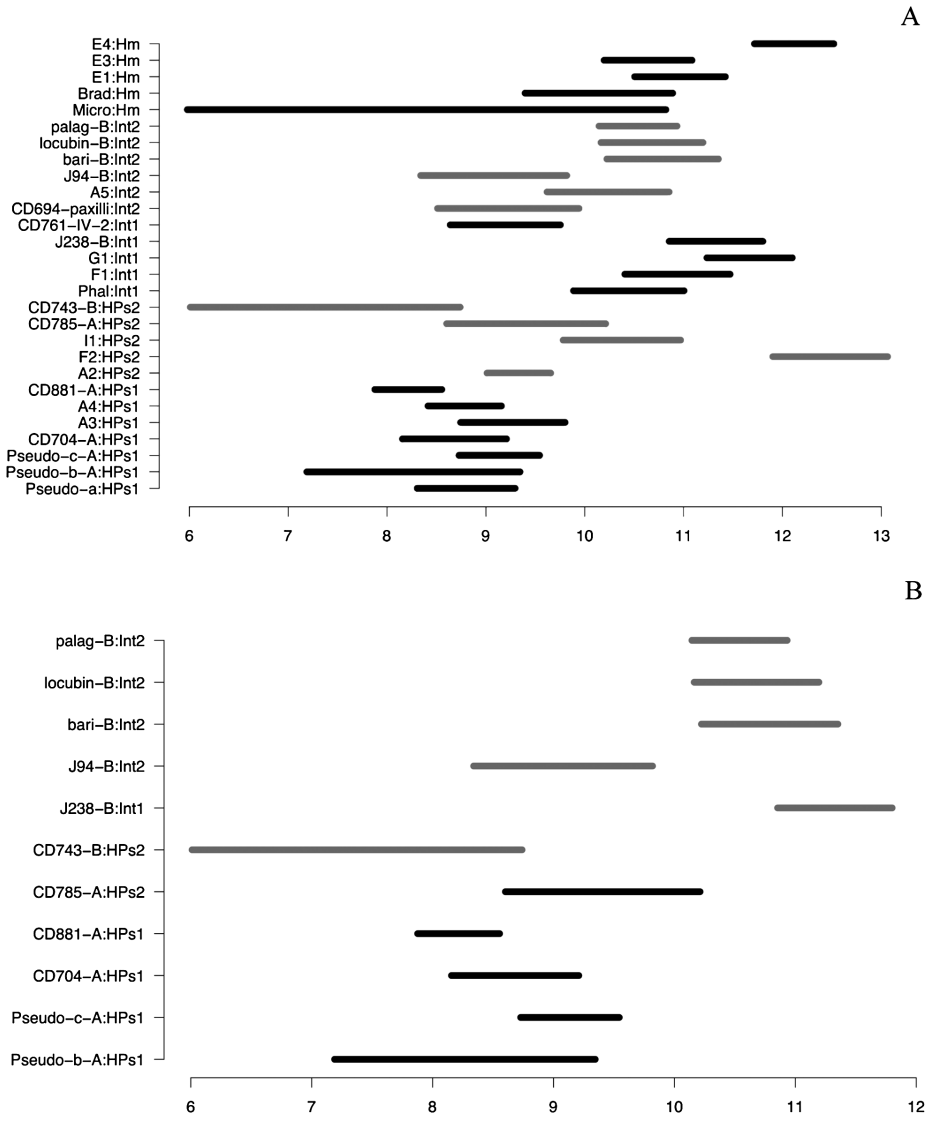

Fig. 6. Confidence interval of the mean of position of dorsal gland opening. A: Twenty-eight populations in five morphological groups; B: Eleven populations in two molecular groups. *y*-axis: population codes and groups; *x*-axis: distance between stylet base and dorsal gland opening in μm.

Conclusions

The first analysis of the present study generally confirms the conclusions of the 1984 study, in spite of the fact that it used a very different statistical approach. In 1984, quantitative characters were transformed into qualitative characters, whereas the opposite approach was followed here. This validates the new approach used for the present article, resulting in a dendrogram based on a multivariate analysis of 17 morphological characters.

IDENTITY OF THE POPULATIONS STUDIED

Variability of all morphological characters blur any small differences that exist among the various morphological groups. This is true for the four variables used in the 2015 study to differentiate *H. microlobus* from *H. pseudorobustus*.

According to Fortuner (1985, 1986), *H. pseudorobustus* is characterised as follows: body spiral 650-775 μm long, lip region hemispherical, with 4-5 annuli, stylet

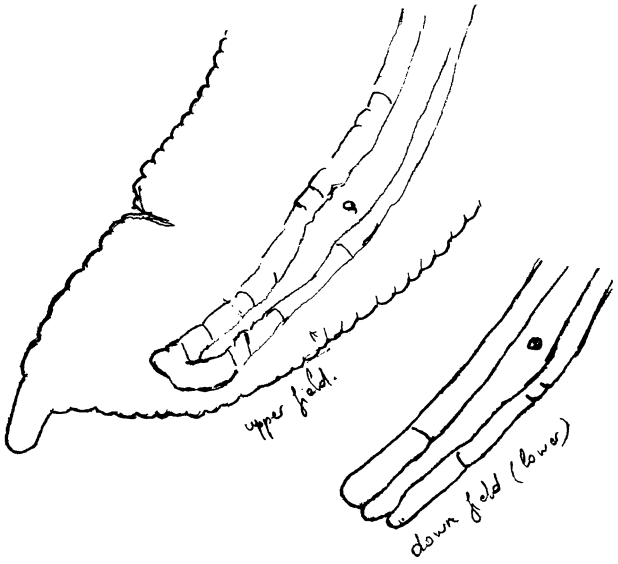

Fig. 7. Fusion of inner incisures in the left and right sides of a topotype specimen of *H. pseudorobustus*. Reproduction of a 1984 pencil drawing in the archives of the senior author.

25.5-28 μm long, stylet knobs often flattened, rarely rounded or indented anteriorly, tail dorsally convex-conoid, terminus with a definite rounded projection 1-4 μm long, phasmids 2-8 annuli anterior to anus level, junction of the inner incisures on tail very variable, often u-, μ-, or m-shaped in topotypes and European samples, y- or v-shaped in North American samples. Males absent. The samples in the Micro-group (Table 6) fit with this diagnosis.

Consequently, the conclusions of Subbotin *et al*. (2015) are rejected and *H. microlobus* is once again considered to be a junior synonym of *H. pseudorobustus*.

All samples in Table 1, except the 11 *H. dihystera* samples HdA-HdK, are identified as *H. pseudorobustus*, including samples CD761 and CD694, identified by Subbotin *et al*. (2015) as *Helicotylenchus* spIV-2 and *H. paxilli*, respectively.

Congruence of morphological and molecular groupings

Of the 13 samples included in the present study whose molecular types were determined by Subbotin *et al*. (2015), those with a molecular type A were found mostly in the Pseudo-1 group, and those with a molecular type B in the Intermediate-2 group. However, there is no complete congruence between morphological groups and molecular types: CD743 (type B) is found in the Pseudo-2 group together with CD785 (type A), CD761 (type IV-2) is found in the Intermediate-1 group together with J238 (type B), and CD694 (type paxilli) is found in the Intermediate-2 group together with locubin, J94, palag, and bari samples, all of type B.

Traditional taxonomy *vs* barcoding

The present conclusions differ from those of Subbotin *et al*. (2015). This is a direct consequence of the different approaches taken by traditional and molecular taxonomists.

In the early days of the search for molecular identification, authors were looking for a molecular barcode that would be present in all of the specimens of a given species and absent in specimens belonging to all of the other species. For example, Hebert *et al*. (2003) used 55 'test' taxa to verify that the *COI* sequences they proposed were able to discriminate correctly between known phyla. Clearly, if these sequences had failed the test, they would have been rejected as potential barcodes.

However, over the years, this approach seems to have evolved. Now, if several barcodes are found in specimens morphologically identified as a single species, instead of rejecting the sequences as unreliable, some authors tend to consider that there are as many species (the so-called 'cryptic species') as there are barcodes.

Barcoders and taxonomists stand their ground on their respective positions and it would appear that irreconcilable differences exist between the two approaches. However, the concept of MOTU (Floyd *et al*., 2002) might be a way to reach a consensual solution. Traditional taxonomists could continue to identify species based on their morphological characters and molecular biologists could propose several MOTU within the species recognised by traditional taxonomists.

In the present case, it is proposed to recognise that the morphological species *H. pseudorobustus* includes at least two MOTU called *H. pseudorobustus* A and *H. pseudorobustus* B, corresponding to molecular types A and B described by Subbotin *et al*. (2011, 2015). Other molecular types described in these two articles might represent other MOTU but no samples with such molecular types could be obtained for the present study and no definite proposal can be made here.

Acknowledgements

The authors thank Dr D. Bouchon for allowing the use of the laboratory Ecologie et Biologie des Interactions, Université de Poitiers, CNRS, UMR 7267 France, and all of the scientists who provided the fixed nematode specimens used in the 1984 study and the present study, as listed in Table 1 (column 'Source').

References

Anderson, R.V. (1974). Canadian species of the genus *Helicotylenchus* Steiner, 1945 (Nematoda: Hoplolaimidae), their identifying characteristics and descriptions of three new species. *Canadian Journal of Zoology* 52, 1365-1381. DOI: 10.1139/z74-174

Andrássy, I. (1958). *Hoplolaimus tylenchiformis* Daday, 1905 (syn. *H. coronatus* Cobb, 1923) und die Gattungen der Unterfamilie Hoplolaiminae Filipjev, 1936. *Nematologica* 3, 44-56. DOI: 10.1163/187529258X00337

Cobb, N.A. (1893). Nematode worms found attacking sugar cane. [In: *Plant diseases and their remedies*.] *Agricultural Gazette of New South Wales* 4, 808-833.

Everitt, B.S., Landau, S., Leese, M. & Stahl, D. (2011). *Cluster analysis*, 5th edition. Chichester, UK, Wiley.

Firoza, K. & Maqbool, M.A. (1994). A diagnostic compendium of the genus *Helicotylenchus* Steiner, 1945 (Nematoda: Hoplolaimidae). *Pakistan Journal of Nematology* 12, 11-50.

Floyd, R., Eyualem, A., Papert, A. & Blaxter, M.L. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology* 11, 839-850. DOI: 10.1046/j.1365-294X.2002.01485.x

Fortuner, R. (1984). Statistics in taxonomic descriptions. *Nematologica* 30, 187-192. DOI: 10.1163/187529284X00086

Fortuner, R. (1985). *Helicotylenchus pseudorobustus*. *CIH Descriptions Vol. 8, No. 109, plant-parasitic nematodes*. Farnham Royal, UK, Commonwealth Agricultural Bureaux.

Fortuner, R. (1986). *Variabilité et identification des espèces chez les nématodes du genre* Helicotylenchus. Thèse de Doctorat, Université Claude Bernard, Lyon, France.

Fortuner, R. & Quénéhervé, P. (1980). Morphometrical variability in *Helicotylenchus* Steiner, 1945. 2. influence of the host on *H. dihystera* (Cobb, 1893) Sher, 1961. *Revue de Nématologie* 3, 291-296.

Fortuner, R., Maggenti, A.R. & Whittaker, L.M. (1984). Morphometrical variability in *Helicotylenchus* Steiner, 1945. 4: study of field populations of *H. pseudorobustus* and related species. *Revue de Nématologie* 7, 121-135.

Golden, A.M. (1956). Taxonomy of the spiral nematodes (*Rotylenchus* and *Helicotylenchus*), and the developmental stages and host-parasite relationships of *R. buxophilus* n. sp., attacking boxwood. *Maryland Agricultural Experimental Station Bulletin* A-85.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B* 270, 313-321. DOI: 10.1098/rspb.2002.2218

Krall', E.L. (1978). [*Parasitic root nematodes. Family Hoplolaimidae*.] Leningrad, USSR, Nauka. Translated in 1990: *Root parasitic nematodes. Family Hoplolaimidae*. Leiden, The Netherlands, Brill.

Liu, Y., Li, Z., Xiong, H., Gao, J. & Wu, J. (2010). Understanding of internal clustering validation measures. In: *2010 IEEE international conference on data mining*. DOI: 10.1109/ICDM.2010.35

Marais, M. (1998). Some species of *Helicotylenchus* Steiner, 1945 from South Africa (Nematoda: Hoplolaimidae). *Fundamental and Applied Nematology* 21, 327-352.

Micoletzky, H. (1916). Ergebnisse einer botanischen Forschungsreise nach Deutsch-ostafrika und Südafrika (Kapland, Natal und Rhodesien). Süsswasser-nematoden aus Südafrika. *Denkschrift der Kaiserlichen Akademie Wissenschaft* 92, 149-171.

Perry, V.G., Darling, H.M. & Thorne, G. (1959). Anatomy, taxonomy, and control of certain spiral nematodes attacking blue grass in Wisconsin. *Bulletin of the Wisconsin Agriculture Experiment Station* 207, 1-24.

Sauer, M.R. & Winoto, R. (1975). The genus *Helicotylenchus* Steiner, 1945 in West Malaysia. *Nematologica* 21, 341-350. DOI: 10.1163/187529275X00077

Sher, S.A. (1961). Revision of the Hoplolaiminae (Nematoda). I. Classification of nominal genera and nominal species. *Nematologica* 6, 155-169. DOI: 10.1163/187529261X00414

Sher, S.A. (1966). Revision of the Hoplolaiminae (Nematoda). VI. *Helicotylenchus* Steiner, 1945. *Nematologica* 12, 1-56. DOI: 10.1163/187529266X00013

Siddiqi, M.R. (1972). On the genus *Helicotylenchus* Steiner, 1945 (Nematoda: Tylenchida), with descriptions of nine new species. *Nematologica* 18, 74-91. DOI: 10.1163/187529272X00278

Siddiqi, M.R. (2000). *Tylenchida parasites of plants and insects*, 2nd edition. Wallingford, UK, CAB International, pp. 86-121.

Steiner, G. (1914). Freilebende Nematodes aus der Schweiz. 1. Teil einer vorlaufigen Mitteilung. *Archiv für Hydrobiologie und Planktonkunde* 9, 259-276.

Subbotin, S.A., Inserra, R.N., Marais, M., Mullin, P., Powers, T., Roberts, P.A., Van den Berg, E., Yeates, G.W. & Baldwin, J.G. (2011). Diversity and phylogenetic relationships within the spiral nematodes of *Helicotylenchus* Steiner, 1945 (Tylenchida: Hoplolaimidae) as inferred from analysis of the D2-D3 expansion segments of 28S rRNA gene sequences. *Nematology* 13, 333-345. DOI: 10.1163/138855410X520936

Subbotin, S.A., Vovlas, N., Yeates, G.W., Hallmann, J., Kiewnick, S., Chizhov, V.N., Manzanilla-López, R.H., In-

serra, R.N. & Castillo, P. (2015). Morphological and molecular characterisation of *Helicotylenchus pseudorobustus* (Steiner, 1914) Golden, 1956 and related species (Tylenchida: Hoplolaimidae) with a phylogeny of the genus. *Nematology* 17, 25-52. DOI: 10.1163/15685411-00002850

Tarjan, A.C. (1964). Two new mucronate-tailed spiral nematodes (*Helicotylenchus*: Hoplolaiminae). *Nematologica* 10, 185-191. DOI: 10.1163/187529264X00015

Thorne, G. & Malek, R.B. (1968). Nematodes of the Northern Great Plains. Part I. Tylenchida (Nemata: Secernentea). *South Dakota Agricultural Experiment Station Technical Bulletin* 31, 1-111.

Yuen, P.H. (1964). Four new species of *Helicotylenchus* Steiner (Hoplolaiminae: Tylenchida) and a redescription of *H. canadensis* Waseem, 1961. *Nematologica* 10, 373-387. DOI: 10.1163/187529264X00385