Hindawi
Journal of Zoological Systematics and Evolutionary Research
Volume 2022, Article ID 6216266, 11 pages
https://doi.org/10.1155/2022/6216266

Research Article

A New Interactive Web-Based Polytomous Key for Species Identification of Pin Nematodes of the Genus *Paratylenchus* Micoletzky, 1922 (Nematoda: Paratylenchinae) with the Use of Ribosomal and Mitochondrial Genes

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Received 19 April 2022; Accepted 17 June 2022; Published 4 July 2022

Academic Editor: Miroslawa Dabert

Pin nematodes of the genus *Paratylenchus* comprise 140 species. This group of nematodes is characterized by a quite homogeneous morphology and cosmopolitan distribution and is prevalent in cultivated and natural soil ecosystems. The present study describes the first interactive and illustrated web-assisted polytomous identification key for the genus *Paratylenchus*. The updated *Paratylenchus* species polytomous key was based on a wide list of 24 diagnostic characters generated for the 140 species comprising this genus. Here we developed a web-assisted method to achieve an easy and accurate *Paratylenchus* species characterization that will greatly improve the identification of these plant-parasitic nematodes for many diagnostic laboratories and researchers. However, this identification needs to be completed with the use of molecular markers available for the species due to the existence of species complexes studied in former researches. This idea is pointed in the polytomous key in the specific species complexes up-to-know. In some cases, the presence in the soil as survival stage of few individuals in the fourth-stage juvenile (J4) required the use of molecular markers for species identification. We suggest the use of at least a fragment of mitochondrial COI gene for species identification or the combination of nuclear D2-D3 regions of the 28S rRNA and the COI to complement each marker. However, for some species complexes, the use of the D2-D3 regions alone has not enough resolution to separate the putative species inside the species complex. Web-based polytomous key was constructed using the free software *Xper3*, for computers and mobile devices (smartphones, tablets, and pocket PCS).

1. Introduction

Pin nematodes of the genus *Paratylenchus* Micoletzky, 1922 [1] are obligate plant ectoparasitic nematodes of small body length (≤600 μm) with wide variable stylet length range (10-120 μm), extensively distributed worldwide in different natural environments and crops [2–5]. Tarjan [6] reviewed the genus *Paratylenchus* for the first time and proposed the first dichotomic key for 16 nominal species identification and based on 7 main diagnostic characters. During the last decades, there were numerous taxonomical discussions debating about the validity of the genera *Gracilacus*, *Paratylenchoides*, and *Gracilpaurus*, based on stylet length, heavy sclerotization in the lip region, or the presence of tubercles or punctations on the cuticle of mature females [7–9]. However, recent integrative taxonomic studies including ribosomal and mitochondrial genes questioned the monophyly of Tylenchulidae and do not support the validity of these

genera that were considered synonyms of *Paratylenchus* [3–5, 10]. In any case, the *Paratylenchus* species description increased exponentially to 140 nominal species in the last 61 years (Table S1, 58 of them molecularly characterized), including the confirmation of examples of cryptic diversity [3–5, 10]. Although several diagnostic keys have been published during this period [2, 8, 11–14], given the large number of *Paratylenchus* species and their high morphological and morphometric similarity, the species identification of these nematodes is often a very difficult task. Ghaderi et al. [2, 14] pragmatically divided the genus into eleven groups based on the three most stable characters, such as stylet length, number of lines in the lateral field, and the presence/absence of vulval flaps in females. Within each group, species can be identified using dichotomic keys based on presence/absence of males, shape of the spermatheca, tail terminus, etc., following a hierarchical system. In addition, Ghaderi et al. [14] proposed a diagnostic compendium based on seven items: pragmatical group, lip region from lateral view, males and their stylets, spermatheca shape, fourth-juvenile stylet, cuticle annulation, and tail terminus shape. Although these keys can help in the morphometrical identification of *Paratylenchus* spp., it still lacks a useful, wider, holistic, and accurate key using a wide list of morphological and morphometrical traits, included in a comprehensive web-based polytomous key which may help in the identification of known and new species within this genus, as well as the potential separation of species complexes differing in a few traits. Over the last two decades, the advance of new web-based techniques represents an excellent opportunity to generate useful species key tools for helping in nematode identification [15]. These tools, such as *Xper3*, allow the integration of large amounts of quantitative data, as well as accurate and precise descriptions using a wider set of qualitative and quantitative characters that can be applied simultaneously with no restrictions to the specimen to be identified [15, 16]. They are available in every browser at no charge [15, 16]. In *Xper3*, species identification is achieved in several steps by gradual elimination of species names that do not meet the selection criteria at each step of the process.

Additionally to the use of morphological data for species identification, molecular-based approaches integrated with morphology and morphometric data have revealed an exponential increase in the number of cryptic species in the phylum Nematoda over recent decades [17–19]. In plant-parasitic nematodes, this phenomenon of cryptic speciation can be explained on the basis of genetic mutations and/or ecological adaptations to geographical location or host range [20, 21]. More specifically, pin nematodes are characterized by a conserved morphology that has led to the use of DNA barcoding with different fragments of nuclear and mitochondrial DNA to clarify different species groups [3–5, 10]. Several studies demonstrated the existence of cryptic diversity within the genus *Paratylenchus* and highlighted the need for correct and accurate species identification in food security and pest management strategies [3–5, 10]. The knowledge of intraspecific and interspecific sequence variability of the 28S rRNA and the mitochondrial cytochrome c oxidase subunit I (COI) genes within *Paratylenchus* is important to detect species misidentifications/incorrect identifications deposited in GenBank (https://www.ncbi.nlm.nih.gov/) or cryptic speciation [3–5, 10]. However, morphological data of nematode individuals prior to DNA extraction significantly improve species delimitation accuracy in comparison to simply molecular taxonomy [19]. An emblematic example of high cryptic species diversity within *Paratylenchus* comprises *P. straeleni*-complex species, distinguishing 4-9 putative species [3–5, 10], and one new species recently described from southern Spain, *viz*., *P. parastraeleni* [4], which 33 years before was identified as *P. straeleni* only based on morphometry and morphology [22]. Similarly, Van den Berg et al. [10] and Singh et al. [3] studied numerous *Paratylenchus* populations revealing several species complexes with few morphological differences in the main diagnostic characters, but clearly differing in molecular markers. Some examples of these species' complexes include *P. aquaticus* complex (*P. humilis*, *P. pandus*, *P. triincisus*, and *P. variatus*), *P. hamatus* complex (*Paratylenchus* sp. 2 and *Paratylenchus* sp. CaD), and *Paratylenchus nanus* complex (*P. projectus*, *P. neoprojectus*, and *P. neoamblycephalus*). Another excellent example includes *P. microdorus*-complex species, extensively reported in Spain in cultivated and natural environments [23–26]. Recently, analyses based on integrative taxonomy identified different species with the basic morphology of *P. microdorus*, but molecularly well separated, including *P. recisus*, *P. variabilis*, *P. veruculatus*, and *P. zurgenerus* [4, 5, 27]. Probably, these potential misidentifications can also be referred to the numerous records of *P. microdorus* in other countries, such as Bulgaria, Germany, Hungary, Poland, and Romania [2], which need further investigation. Species belonging to the genus *Paratylenchus* display a particular resting stage which accumulates in soil under adverse environmental conditions [2, 4]. This state is nonfeeding, molting to adults after stimulation by host-plant roots, and may provide some useful data for species identification [2]. Usually the resting stage is fourth-stage juvenile (J4), but third-stage (J3) appears in other species, recognized by granular body contents and presence/absence of stylet [2]. This survival strategy makes an identification based on only morphological and morphometrical characters difficult. Thus, the establishment of clear and unequivocal molecular markers for species identification is even more necessary. The identification of *Paratylenchus* spp. became more complicated with the usual presence of several species in the same soil sample. Using an integrative taxonomy, we detected even four species within a soil sample and the coexistence of almost identical species sharing the same niche and the same host in our recent studies in cultivated *Prunus* spp. and natural environments in Spain [4, 5]. For these reasons, it is important to include molecular markers and more than one individual in the integrative taxonomy of this genus.

Therefore, the objectives of this research were as follows: (1) to facilitate *Paratylenchus* species identification, developing an open access web-assisted, interactive, and illustrated polytomous key based on a wide list of diagnostic and

morphometric traits (freely accessible at https://nemabioli .ias.csic.es/paratylenchus/index.html), and (2) to evaluate and review the usefulness of D2-D3 regions of the 28S rRNA gene and the COI gene fragment for *Paratylenchus* species identification for some groups of species complexes within this genus.

2. Materials and Methods

2.1. Database of Paratylenchus Species and Diagnostic Characters. For constructing the *Paratylenchus* database, we considered only the type population for each *Paratylenchus* nominal species according to the latest monograph of the genus published by Ghaderi et al. [2], as well as other *Paratylenchus* species populations based only on integrative taxonomical identification [3–5, 10, 28]. This is because of the great cryptic diversity detected in the recent studies of pin nematodes [3–5, 10, 28], which may alter the real morphometric delimitations for each species. For that, we analysed all main diagnostic *Paratylenchus* characters, comprising 11 numerical and 13 categorical characters (Table 1 and Figure 1). Selection for quantitative character division in subgroups within each diagnostic numerical character was based on K-means clustering method [29]. All data analyses were performed with the R software, version 4.1.0 [30]. The library NbClust v. 2.0.4 [31] was used to perform the clustering using Euclidean distances. We tested from 2 to 6 clusters. The best number of clusters was selected testing all indexes to optimize the K-means cluster number for one variable each time based on the NbClust library. The best number of groups was obtained as the number with the major quantity of accepted indexes. Once the best number of clusters was obtained, we calculated it using the library factoextra v. 1.0.7. using Euclidean distances. Range of the clusters for each character was checked for incongruences and limits included in the figures (Figure 2). All figures were created using the ggplot2 package version 3.3.3 [32]. All database characters could be obtained upon reasonable request to the authors.

2.2. Xper3 Software Polytomous Key. The web-based polytomous key was constructed using the free software *Xper3* version 1.4.0 (https://www.xper3.fr), it is compatible for computers and mobile devices (smartphones, tablets, and pocket PCS), and it is freely accessible at https://nemabioli .ias.csic.es/paratylenchus/index.html. This software allows for the selection of any character (morphological or morphometrical) within a set of descriptors for the identification of *Paratylenchus*. Descriptors were ranked with the built-in ranking system of *Xper3* (rank 1: little important and 5: highly important) according to their distinct nature and relative simplicity to study. Measurements and indexes for all species were read in as quantitative data with fixed ranges. Completeness of the database was checked using the inbuilt tools for analysis of the data. The tool "Checkbase" is an automatic search for various errors in the database, for instance, items with identical descriptions, undescribed or inapplicable descriptors, and descriptors described as "unknown." A direct link to these errors provides a handy means for quick and easy revision. *Xper3* lists all *Paratylenchus* species including all descriptors which can be edited and weighted. A view of the complete description matrix with the possibility to search for undescribed items and the revision of these is of great help to make sure that each species is completely described. We ranked descriptors because some characters showed greater stability inside the same species and also less prone to create artefacts during nematode fixation prior measurements, such as female stylet length, number or lines in lateral fields, and the presence or absence of advulval flaps. Moreover, these characters were recommended by other authors to create groups [14]. This is also a great way to simplify and speed up the identification process, allowing quick and reliable results, for instance, rank 5 for female stylet length as a descriptor highly important, rank 4 for the number of lines in lateral field and advulval flap, and rank 3 for the rest of morphometric and morphological characters (Table 1). In addition, the morphological and morphometric characters of the male (*viz*., male tail shape, male stylet, and spicule length) were conditioned to the presence of males (Table 1). Finally, in *Xper3*, it is possible to select various species or groups for comparison of all characters. Although users can decide the starting character to initiating the *Paratylenchus* identification, the authors suggest to follow the order and weighted proposed. In any case, the section "History" provides a helpful means to look up the identification process and correct potential mismatches without resetting the complete selection. In the case that the aided key leads to several species, the resulting identification will be based on the individual comparison of the target species with original descriptions and the help of molecular markers.

Graphical picture/s available in an illustrated window will appear for categorical characters (*viz*., advulval flap, excretory pore level, lip region shape, submedian lobes, female and male tail shape, female tail terminus, and cuticle annulation) when the user clicks on these web characters; then, the user enters the corresponding appropriate state for his/her sample data.

The present interactive and illustrated web-based polytomous key has been successfully tested on popular browsers in Windows, Linux, and MacOS.

2.3. Sequence Variability Analyses of D2-D3 Regions and the COI Fragment of Paratylenchus Species. A total of 340 and 292 sequences assigned to the genus *Paratylenchus* were retrieved from GenBank and used to calculate the intraspecific and interspecific sequence variability of D2-D3 regions and the COI gene fragment, respectively. For sequence selection from NCBI, we have taken into consideration all the annotated misidentifications indicated by recent papers based on integrative taxonomical approaches [3–5, 10]. These molecular markers are commonly used in the molecular identification of species in this genus. Internal transcribed spacer (ITS) region and partial 18S rRNA are also used, but the first one has less sequences deposited in GenBank in comparison to D2-D3 regions, and partial 18S rRNA has a lower resolution for species identification than the other most frequently used markers [3].

Table 1: Morphometric and morphological characters used to distinguish *Paratylenchus* spp. in the web-assisted polytomous key. Some characters are ranked in the program (shown in the table), and the rest are ranked to 3.

	(I) (*Female stylet lenght*/*L*) × 100	
	I1: ≤12.20%	
(A) Female stylet length (rank 5)	I2: 12.21-21.8%	
A1: <23.5 μm	I3: >21.8%	
A2: 23.5-40.0 μm	*(J) Male*	*(Q) Female tail terminus shape*
A3: 40.1-69.5 μm	J1: unknown	Q1: acute or pointed
A4: >69.5 μm	J2: present	Q2: subacute to finely rounded
(B) Excretory pore level	*(K) Male tail shape*	Q3: bluntly rounded
B1: at median bulb level or anterior	K1: conoid	Q4: digitate, lobed, or indented
B2: at isthmus level	K2: rounded, cylindrical	Q5: serrated
B3: at basal bulb level or posterior	K3: filiform terminus	*(R) V ratio (%)*
(C) Number of lines in the lateral field (rank 4)	K4: unknown	R1: ≤76.0%
C1: 2 lines	*(L) Male stylet*	R2: 76.1-81.0%
C2: 3 lines	L1: unknown	R3: >81.0%
C3: 4 lines	L2: without stylet	*(S) Female tail length*
C4: indistinct lines	L3: with stylet	S1: ≤26.4 μm
(D) Advulval flap (rank 4)	*(M) c' ratio*	S2: >26.4 μm
D1: present	M1: ≤2.25	*(T) J4 stylet*
D2: absent	M2: 2.26-2.85	T1: absent
(E) Lip region shape in lateral view	M3: 2.86-3.35	T2: present
E1: conoid	M4: 3.36-3.95	T3: unknown
E2: rounded	M5: >3.95	*(U) c ratio*
E3: disc-shape	*(N) b ratio*	U1: ≤13.25
E4: truncate, concave, anteriorly flattened	N1: ≤2.85	U2: 13.26-17.25
E5: cap-like structure	N2: 2.86-3.65	U3: >17.25
(F) Submedian lobes in lateral view	N3: 3.66-4.25	*(V) Female tail shape*
F1: without submedian lobes	N4: >4.25	V1: conoid
F2: with small submedian lobes	*(O) Body length (L)*	V2: cylindrical
F3: with prominent submedian lobes	O1: ≤253 μm	V3: subcylindrical, elongate
(G) Vulva-anus distance	O2: 253-303 μm	*(W) Cuticle annulation*
G1: ≤32.75 μm	O3: 304-356 μm	W1: normal annulation, *ca.* 1 μm
G2: 32.76-47.50 μm	O4: 357-426 μm	W2: cuticle with punctations
G3: >47.50 μm	O5: >426 μm	W3: cuticle annuli crenate
(H) Spermatheca shape	*(P) Spicules' length*	*(X) a ratio*
H1: rounded to spherical	P1: ≤14.25 μm	X1: ≤21.75
H2: elongated, oval	P2: 14.26-16.75 μm	X2: >21.7
H3: not developed, inconspicuous	P3: 16.76-19.75 μm	
	P4: 19.76-23.0 μm	
	P5: >23.0 μm	

For interspecific sequence variability, one consensus sequence was obtained from *Paratylenchus* each species, resulting an alignment of 69 and 57 consensus sequences of 575 and 320 bp from D2-D3 regions and the COI fragment, respectively. Sequence alignment was manually edited using BioEdit [33] in order to improve the default multialignment. Then, pairwise identity expressed in percentage among taxa was computed using Sequence Demarcation Tool version 1.2 (SDT v1.2) with MAFFT alignment options and adjustment for missing data [34]. On the other hand, for the intraspecific sequence variability, one dataset from each species with more than one available sequence (Tables S2-S3) was created and aligned using MAFFT v. 7.450 using the FFT-NS-2 algorithm [35]. Then, intraspecific sequence variability was calculated for both molecular markers in the number of nucleotide differences using BioEdit [33].

3. Results

3.1. Interactive Web-Based Polytomous Key of Paratylenchus spp. The interactive and illustrated web-based *Paratylenchus* polytomous key (version 1, April 2022, 140 species) is hosted and freely available on the server of the Spanish National Research Council (CSIC), Madrid, Spain at https://nemabioli.ias.csic.es/paratylenchus/index.html, and it is freely accessible to any user.

Quantitative character division in subgroups within each diagnostic numerical character was based on K-means clustering method (Figure 2). All data analyses were performed testing from 2 to 6 clusters, and the best number of groups was obtained as the number with the major number of indexes, checking for incongruences and limits included in the figures (Figure 2). The best subgroups for these characters comprised 5 subgroups for female body length (*L*), c'

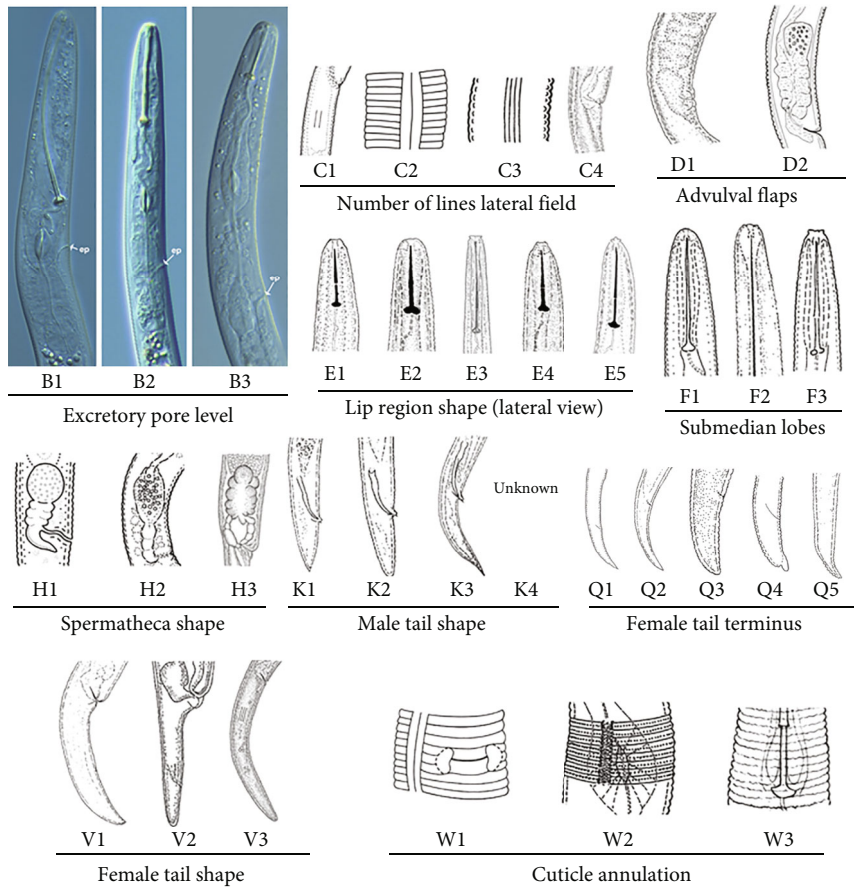

Figure 1: Morphological categorical characters and subgroups used in the web-assisted *Paratylenchus* species polytomous key.

ratio, and spicule length; 4 subgroups for female stylet length and b ratio; 3 subgroups for percentage of female stylet length/*L*, vulva-anus distance, *V* ratio, and c ratio; and 2 subgroups for female tail length and a ratio (Figure 2).

The introduction of sample data in *Xper*3 implies that the software will exclude every taxon which does not fit the set of states and give a list of the remaining possible species names. After sample data introduction, the interactive key enables the simultaneous comparison of all characters of the unknown *Paratylenchus* population with the data of *Paratylenchus*-database species and provides and displays the identified species if all characters are coincident with those of species in the database. Alternatively, the web application will provide a list of species that are the most similar to the user's nematode sample. In addition, the identified species or the relevant species group provides a link to NCBI accessions in case they have any available molecular markers for species confirmation. Authors recommend verifying species identification and potential grouping with other species using molecular markers if the species selected have available sequence data or species-specific PCR primers. This key will be updated if new species are described or any taxonomical change is provided for any of the 140 nominal valid species. Some of these species' complexes will be studied in more detail in the next point, but in this interactive polytomous key species, we included the original descriptions as well as those reports based on molecular markers, but we did not include species not described formally. However, the topotype and the measured populations are included in the key (see the example of *P. straeleni* with topotype population, Belgium and USA, respectively).

3.2. Sequence Variability of D2-D3 Regions and the COI Fragment Genes within Paratylenchus spp. and Species Complexes. Intraspecific and interspecific sequence variability within the genus *Paratylenchus* was analysed based on nuclear and mitochondrial DNA (D2-D3 regions of 28S rRNA and the COI fragment). The intraspecific sequence identity of D2-D3 regions detected in the 67 studied *Paratylenchus* species ranged from 100 to 97% (from 0 to 28 different nucleotides per 575 nucleotide positions), while for the 63 *Paratylenchus* species included in the COI fragment analyses, these values ranged from 100 to 95% (from 0 to 31 different nucleotides per 320 nucleotide positions). Interestingly, the amount of intraspecific variability was found to be highly variable, and the majority of species with more than one sequence available in GenBank showed low or none intraspecific variability (Tables S2-S3). For example, for both markers, D2-D3 regions and the COI fragment, no variability was detected for some species,

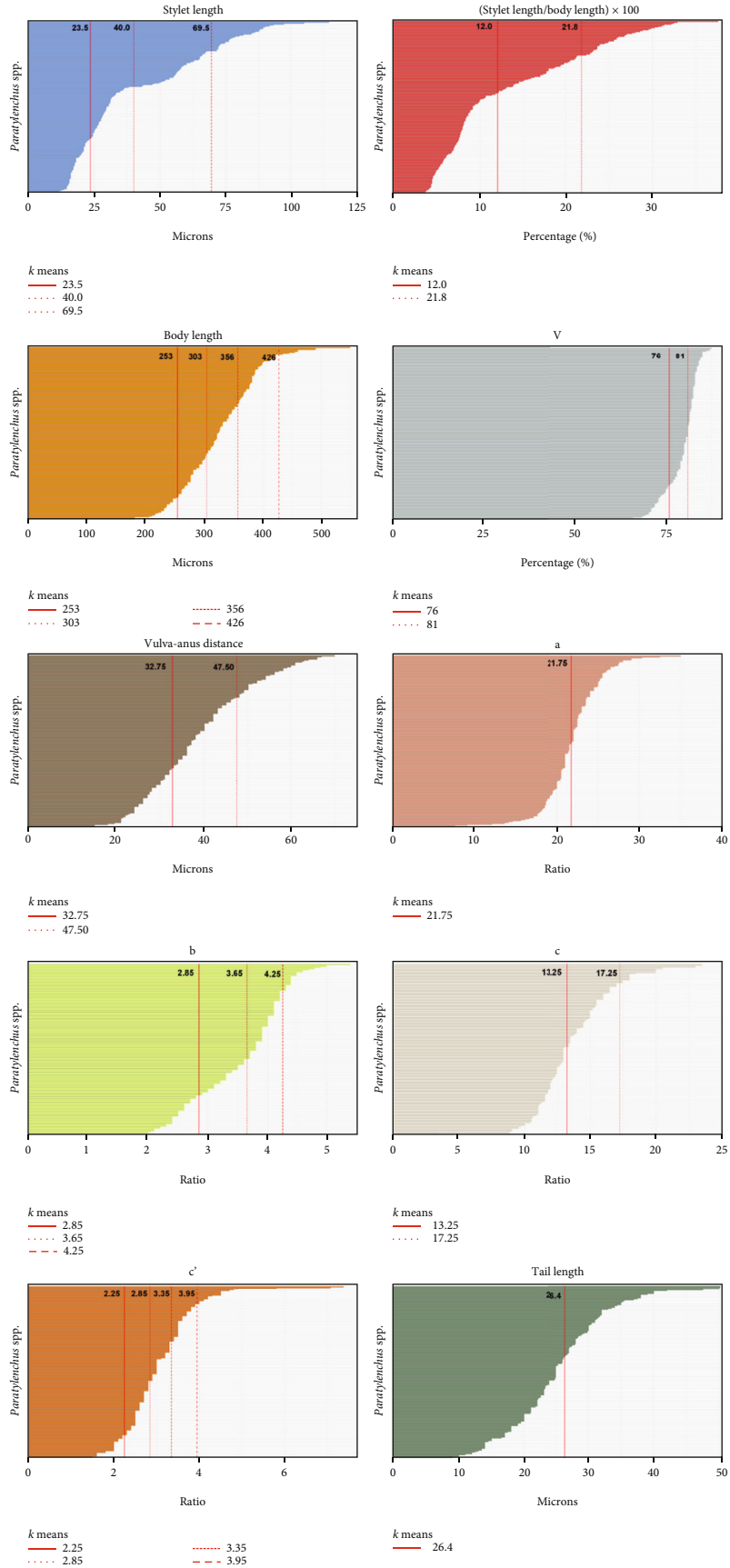

Figure 2: K-means of morphometric numerical characters of *Paratylenchus* species for subgroup separation.

Table 2: Species complexes included in the polytomous key with molecular markers needed for putative species separation. Many of these putative species are not formally described as a new species. Species separation data were obtained from Singh et al. [3] and Clavero-Camacho et al. [4, 5].

Species	D2-D3 regions				COI			
	n. species[a]	% similarity	Seq. GenBank[b]	Suitability[c]	n. species[a]	% similarity	Seq. GenBank[b]	Suitability[c]
P. aquaticus	2	73.2-98.5	4 (4)	Yes	2	81.1	2 (2)	Yes
P. enigmaticus	2	99.8-100	17 (2)	No	3	97.1-100	7 (4)	Yes
P. goodeyi	1	98.3-100	32 (9)	No	7	94.3-100	19 (15)	Yes
P. hamatus/*P.* sp. 2/*P.* sp. CaD/*P. tenuicaudatus*	4	94.4-100	49 (15)	Yes	4	93.7-100	17 (4)	Yes
P. sheri	1*	99.6-100	6 (3)	No	2	94.3-100	9 (3)	Yes
P. straeleni/*P. parastraeleni*	6	93.0-100	14 (9)	No	10	91.1-100	29 (14)	Yes
P. veruculatus	2	97.4-100	17 (7)	No	3	93.7-100	13 (6)	Yes

[a]Based on species separation methods [3] or similarity cut-off (approx. 98% for D2-D3 regions and 97.5% for the COI fragment). [b]Number of different haplotypes in brackets. [c]Suitability to separate species inside the species-complex taking the number of species with COI marker for the D2D3 regions. * This marker cannot separate *P. sheri* from *P. israelensis* and *P. neoamblycephalus*.

such as *P. amundseni*, *P. elachistus*, *P. holdemani*, *P. tateae*, *P. variabilis*, or *P. verus*, while other species, such as *P. enigmaticus*, *P. goodeyi*, *P. straeleni*, and *P. veruculatus*, presented highly diverse haplotypes. These species have been previously studied, and some of them were identified as a complex of cryptic species by Singh et al. [3] and Clavero-Camacho et al. [5]. Therefore, these species are difficult to be separated morphologically. The study of these complexes is shown in Table 2.

Multiple alignments of D2-D3 regions and the COI fragment consensus sequences between *Paratylenchus* spp. showed identities ranging from 68% to 99% and 70% to 98%, respectively (Figure 3). The nucleotide differences among *Paratylenchus* species ranged from 2 to 188 nucleotides for D2-D3 region and from 7 to 88 nucleotides for COI from an alignment of 575 and 320 bp length, respectively. The pairwise similarity observed between *Paratylenchus* species was, in almost all cases, below 95% (Figure 3), except for some species complexes, such as *P. pandatus*, *P. macrodorus*, and *P. wuae,* which showed similarity values of 98% for the D2-D3 regions; however, for the COI fragment, this value was of 94% (Figure 3). These three species comprise a species complex with similar morphology in several diagnostic characters including stylet length, excretory pore level, lateral field lines, and female tail shape, but clearly differing in others, such as advulval flap (absent in *P. pandatus* vs. present), lip region shape (conoid in *P. wuae* vs. rounded), submedian lobes (prominent in *P. wuae* vs. small), spermatheca (rounded in *P. wuae* vs. elongated-oval), and shorter vulva-anus distance in *P. wuae* vs. large in *P. macrodorus* and *P. pandatus* [2, 36].

Almost identical pairwise identity was found for *P. sheri*, *P. israelensis*, and *P. neoamblycephalus.* The D2-D3 region sequences of these species were 98% similar among them, but the similarities among the COI fragment sequences were 93-94%. However, these three species, although sharing some similar morphology in several diagnostic characters including stylet length, lateral field lines, presence of vulval flaps, female tail shape, and tail terminus, clearly differed in others, such as spermatheca (rounded in *P. sheri* vs. elongated-oval), submedian lobes (prominent in *P. israelensis* vs. small and absent in *P. sheri* and *P. neoamblycephalus*, respectively), and shorter vulva-anus distance in *P. sheri* vs. large in *P. israelensis* and *P. neoamblycephalus* [2, 5].

The species complex comprising *P. colinus*, *P. aciculus*, *P. aculentus*, *P. audriellus*, and *P. paralatescens* showed a 99% similarity in D2-D3 regions, but unfortunately, only the COI fragment sequences of *P. aciculus* and *P. aculentus* were available in GenBank, with 90% similarity between them. This case has previously been studied by Singh et al. [3] and Munawar *et al.* [37], who distinguished *P. colinus*, *P. aciculus*, *P. aculentus*, and *P. paralatescens* according to some morphological characters, including stylet length (larger in *P. paralatescens*), excretory pore position (at the isthmus level in *P. audriellus* and *P. colinus*), lateral field lines (four in *P. audriellus* vs. three), advulval flaps (present in *P. audriellus* vs. absent), vulva-anus distance (larger in *P. aciculus* and *P. paralatescens* vs. shorter), and spermatheca (elongate-oval in *P. aciculus* and *P. paralatescens* vs. rounded) [3, 5, 38].

Finally, similarity values from 97 to 99% were found for the D2-D3 regions among *P. neoprojectus*, *P. projectus*, *P. coronatus*, *Paratylenchus* sp. C SAS 2019, and *Paratylenchus* sp. 4 (Figure 3). However, *Paratylenchus* sp. 4 and *P. projectus* can be separated on the basis of COI sequences (91% similar to each other). Sequence data on 28S rRNA and ITS from *P. neoprojectus* and *P. projectus* suggest that both species can be synonymized, since minor morphological differences can be detected between them [3, 37]; however, additional data on the COI for *P. neoprojectus* are needed for confirming this new status. Likewise, D2-D3 from *P. coronatus* is highly similar to *P. neoprojectus* (98.3% similarity), and the detailed comparison of morphometrics from both species suggests that both can be synonymized [37, 39]. Unfortunately, both species have no available COI sequences for confirmation.

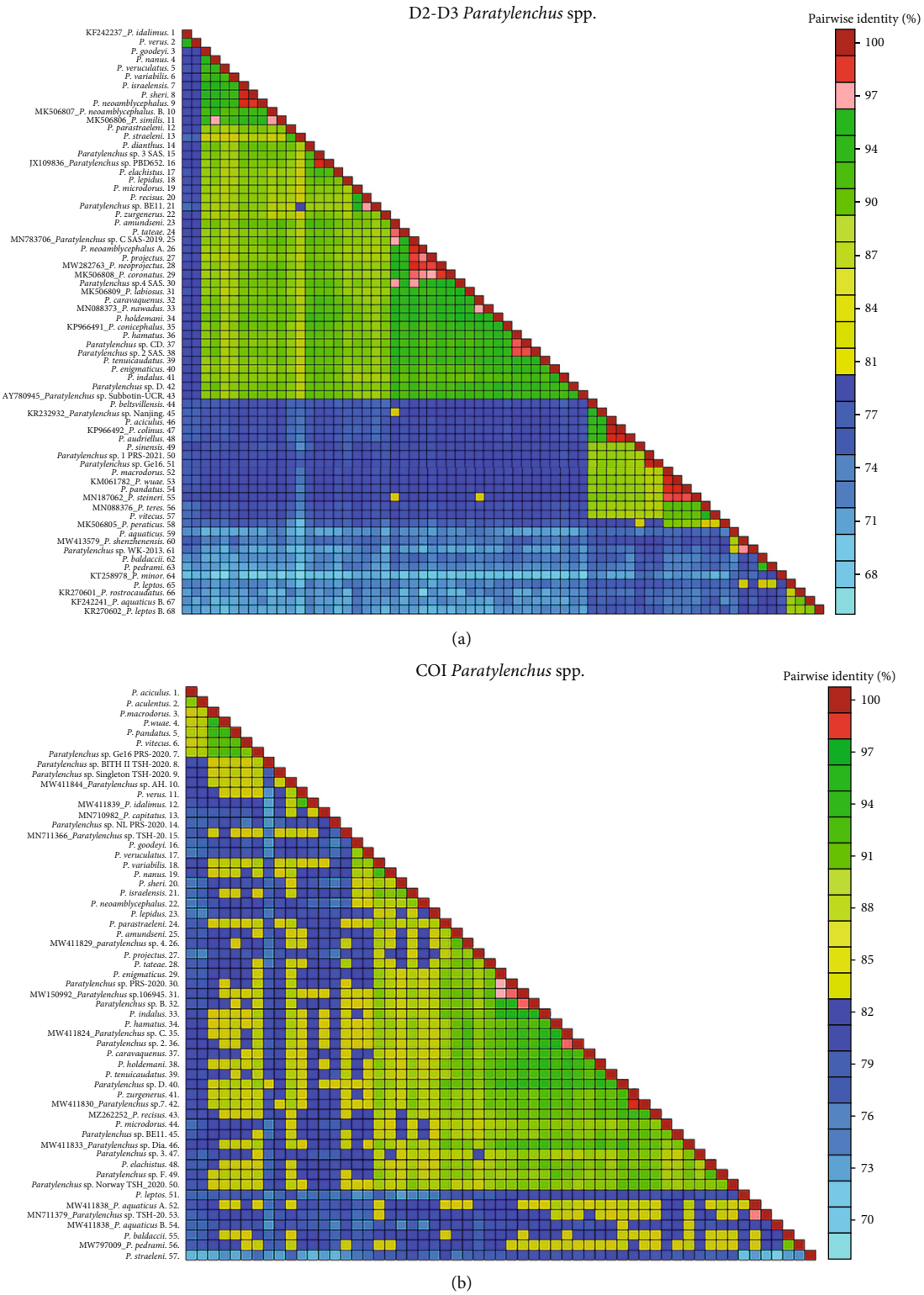

Figure 3: Colour-coded pairwise nucleotide sequence identity matrix of *Paratylenchus* spp. of (a) D2-D3 regions and the (b) COI fragment sequences from GenBank using SDT v1.2. Each coloured cell represents a percentage identity score between two sequences (one indicated horizontally to the left and the other vertically at the bottom).

4. Discussion

Morphological *Paratylenchus* species identification is particularly complex because of the large number of species within the genus (140), the great phenotypic plasticity (including remarkable cryptic diversity), and the limited availability of molecular markers for many species. Then, the use of available software, such as *Xper*3, allows to analyse large amounts

of descriptive data facilitating the identification process. Morphological identification of *Paratylenchus* species can be reduced in time and steps with respect to dichotomous keys. As an example, the identification of *Paratylenchus ciccaronei* and *P. neoamblycephalus* in morpho-group 3 (stylet < 40 μm, 4 lines in lateral fields and advulval flaps present) by Ghaderi et al. [2] requires 49 steps, whereas with our interactive web-based key, only 8 and 6 steps are needed, respectively. This means an optimization of *Paratylenchus* spp. identification, as well as a potential comparison with closer related species. The free access of any user to the polytomous key assures that everybody accessing to the link will be working with the latest version, since any change or updating of the database will not affect the permanent URL. Because the polytomous key and database are deposited at a public institutional research organization (CSIC) linked to a nematological research group with young researchers, we assure the long-term storage of these data. Although some morphometric characters and indexes showed a narrow range of measurements and ratios (*viz*., $(\text{stylet length/body length}) \times 100$, V, vulva-anus distance, a, c, and tail length), we maintain the separation in subgroups estimated by K-means method (Figure 2), since it can help in the separation of some species groups or cryptic species (i.e., *P. microdorus* complex). The new web-based tool can also help to new species identification. Still, because of the great phenotypic plasticity of these nematodes, the *Xper*3 results must be corroborated with the original description or redescriptions of the species, as well as multilocus molecular markers, to find the correct species. Van den Berg et al. [10] proposed the synonymy of *P. pandus*, *P. triincisus*, and *P. variatus* with *P. aquaticus*; however, the species comparison under the *Xper*3 polytomous key separates these species by the corresponding matrix codes (Table S4). We have confirmed that application of the new *Xper*3 polytomous key is decisive and efficient in the morphological-morphometrical separation of *Paratylenchus* species comprising species closely related morphologically to *P. aquaticus* [10]. Consequently, since no molecular characterization of these proposed synonymized species is provided, we maintain a conservative proposal considering all of them as valid species until molecular data can confirm if these few differences can be attributable to intraspecific variation or to the real cryptic species.

Several recently published studies have contributed to unravelling the species delineation within the genus *Paratylenchus*, resolving many misidentifications, describing new species, and assigning molecular data to known species. This is important because the amount of sequences deposited in GenBank has greatly increased in recent years [3–5, 37]. The use of both D2-D3 regions and the COI fragment sequences appears most promising for species identification of *Paratylenchus* genus, being the COI more effective to separate putative species [3–5, 37]. The sequences of D2-D3 regions showed the resolution necessary to separate the majority of *Paratylenchus* species. However, some species-complex groups remain unresolved with D2-D3 regions (showing 99% similarity), *viz*., *P. wuae-P. pandatus-P. macrodorus*, *P. colinus-P. aciculus-P. aculentus-P. audrielllus-P. paralatescens*, and *P. sheri-P. israelensis-P. neoamblycephalus*, requiring the COI fragment sequences for its correct identification and even the analyses of the morphological data. On the contrary, a high intraspecific variability within this marker was found in some other species, such as *P. straeleni*, *P. enigmaticus*, or *P. veruculatus*. These may suggest intraspecific genetic diversity or even cryptic species that need to be clarified; however, further studies are still needed to determine whether or not this high genetic diversity within the genus *Paratylenchus* is linked to the intrinsic characteristics of this taxonomic group.

5. Conclusions

The new web-based application to identify *Paratylenchus* species allows users to easily use the developed polytomous key for this genus, as well as an efficient tool for characterizing unknown and still undescribed species. In addition, the compatibility of this web application with any operation system and other mobile devices (smartphones, tablets, and pocket PCS) gives a great versatility, facilitating its use for any user irrespective of access to complicated electronic devices. This interactive and illustrated polytomous key will be very useful for nematologists and specialist-diagnosticians to identify *Paratylenchus* species in agricultural, forest, and natural environments. Even so, this key should be combined with nuclear and mitochondrial molecular markers in a multilocus analysis. We propose D2-D3 regions of 28S rRNA gene and the COI gene fragment as molecular markers due to their sequence variability and availability in public databases. In spite of that, some complex-species groups need further studies for confirming the specific status.

Data Availability

Sequence data used for the analyses of molecular diversity within ribosomal and mitochondrial DNA within the genus Paratylenchus can be found at https://www.ncbi.nlm.nih.gov/. In addition, data on polytomous key can be found at https://nemabioli.ias.csic.es/paratylenchus/index.html.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This research was supported by grant (RTI2018-095925-A-100) from Ministerio de Ciencia Innovación y Universidades from Spain and European Union inside the European Regional Development Fund (ERDF). This research is part of the PhD project of the third author. The third author is a recipient of a contract from the Ministry of Science and Innovation for Predoctoral Researchers in Spain (PRE2019-090206). We would like to thank J. Martin Barbarroja (IAS-CSIC) for their excellent technical assistance.

Supplementary Materials

The supporting material includes Table S1: updated checklist of nominal species of the genus *Paratylenchus* Micoletzky, 1922 and Table S2: sequence difference count matrix ranges for D2-D3 regions among *Paratylenchus* species found in NCBI. Values in the diagonal showed the intraspecific variability of the species. Table S3: sequence difference count matrix ranges for the COI fragment among *Paratylenchus* species found in NCBI. Values in the diagonal showed the intraspecific variability of the species. Table S4: matrix codes for *Paratylenchus aquaticus* close related species, including *P. aquaticus*, *P. humilis*, *P. pandus*, *P. triincisus*, and *P. variatus*). *(Supplementary Materials)*

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