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Phylogeny and biogeography of the genus *Cephalenchus* (Tylenchomorpha, Nematoda)

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The phylogenetic position of *Cephalenchus* is enigmatic with respect to other tylench nematodes. In this study, *Cephalenchus* populations representing 11 nominal species were sampled worldwide for molecular and morphological characterization. Species identification was based on light microscopy (LM) and scanning electron microscopy (SEM). Molecular analyses were based on the genes (i.e. 18S, 28S, 5.8S) and internal transcribed spacers (ITS-1 and ITS-2) of the ribosomal RNA (rRNA). Phylogenetic analyses (i.e. full and reduced alignments) of either concatenated or single genes always supported the monophyly of *Cephalenchus*. A sister relationship between *Cephalenchus* and *Eutylenchus excretorius* was recovered by most analyses, although branch support varies depending on the dataset used. The position of *Cephalenchus* + *E. excretorius* within Tylenchomorpha nevertheless remains ambiguous, thus highlighting the importance of sampling additional genes as well as taxa. Placement of *Cephalenchus* + *E. excretorius* as sister of Tylenchinae or Boleodorinae could not be rejected on the basis of 18S and 28S rRNA genes. Within *Cephalenchus*, amphidial opening morphology shows congruence with molecular-based phylogenetic relationships, whereas the number of lines in the lateral field is likely to be a convergent trait. Morphometric analyses clearly distinguished short tail from medium–long tail species, and SEM observations seem to suggest a relation between tail length and amphidial opening. In addition, molecular phylogenies support the non-monophyly of *Cephalenchus cephalodiscus*, *Cephalenchus cylindricus*, *Cephalenchus daisuce* and *Cephalenchus leptus*. The known extent of *Cephalenchus* diversity is increased with the inclusion of two new species, and the biogeography of the genus is discussed.

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Introduction

Plant parasitic nematodes, especially 'tylenchs' (infraorder Tylenchomorpha De Ley & Blaxter, 2002), are responsible for major crop losses worldwide. Hence, there has been broad interest in understanding phylogenetic relationships of these agricultural pests and particularly so with the advance of molecular methods (Subbotin *et al.* 2006; Bert *et al.* 2008; Holterman *et al.* 2009). However, many tylench species not directly implicated in plant damage are underrepresented in extant molecular phylogenies, and therefore, their phylogenetic associations remain poorly understood (Bert *et al.* 2011; Hunt *et al.* 2012).

The family Tylenchidae Örley, 1880, with over 400 species, is one of the most diverse groups and yet, with respect to phylogeny, it remains understudied (Siddiqi 2000; Geraert 2008; Hunt *et al.* 2012). Extant phylogenetic analyses suggest a hypothesis of non-monophyly of Tylenchidae and some of its genera [e.g. *Filenchus* Andrássy, 1954 (Bert *et al.* 2010; Atighi *et al.* 2013), *Malenchus* Andrássy, 1968 (Qing *et al.* 2015b)]. They also provide evidence that genera such as *Cephalenchus* (Goodey 1962) Golden, 1971 are potentially misclassified within Tylenchidae (Palomares-Rius *et al.* 2009; Van Megen *et al.* 2009). Uncertainty regarding the phylogenetic position of *Cephalenchus* underscores the need to reevaluate classical morphology-based systems in Tylenchomorpha.

Cephalenchus was originally proposed as a new subgenus and species [*Tylenchus (Cephalenchus) megacephalus* Goodey, 1962] of *Tylenchus* Bastian, 1865 by Goodey (1962). Golden (1971) raised it to generic status and named *Cephalenchus hexalineatus* (Geraert, 1962) Geraert & Goodey, 1964 as the type species. The genus is relatively small with about 20 valid species, and its placement under subfamily and family ranks remains controversial (Siddiqi 2000; Geraert 2008). Goodey (1962) placed *Cephalenchus* in the subfamily Tylenchinae Örley, 1880 within Tylenchidae, a scheme recognized by other authors (Geraert & Goodey 1964; Geraert 1968; Andrássy 1984; Raski & Geraert 1986). Conversely, Dhanachand & Jairajpuri (1980) transferred it to Tylodorinae Paramonov, 1967, although still within Tylenchidae. Siddiqi (1986) first supported this action but subsequently transferred *Cephalenchus* to Pleurotylenchinae Andrássy, 1976 within a revised Tylodoridae (Paramonov, 1967) Siddiqi, 1976 along with *Campbellenchus* Wouts, 1978 and *Pleurotylenchus* Szczygieł, 1969 (Siddiqi 2000).

Molecular phylogenies based on the 18S ribosomal RNA (rRNA) gene have shown *Cephalenchus* to be a unique lineage sister to most of the economically important tylenchs. Nevertheless, the placement of *Cephalenchus* in these phylogenies varies with the inference method used, and it is often poorly supported (Bert *et al.* 2008; Holterman *et al.* 2009; Van Megen *et al.* 2009). Additional studies using the 28S rRNA gene (Palomares-Rius *et al.* 2009; Atighi *et al.* 2013; Qing *et al.* 2015b) strongly support a sister relationship between *C. hexalineatus* and *Eutylenchus excretorius* Ebsary & Eveleigh, 1981, although not within Tylenchidae. Recently, Panahandeh *et al.* (2015a,b) and Yaghoubi *et al.* (2015), also based on the 28S rRNA gene, showed *Cephalenchus* (i.e. including *C. hexalinetus* and *C. leptus* Siddiqi, 1963) + *E. excretorius* to be closely related to *Malenchus* and *Lelenchus* Andrássy, 1954. In these studies, however, taxon sampling was biased towards the family Tylenchidae, thus sidestepping a much needed more rigorous testing of the position of *Cephalenchus* relative to other tylenchs.

Herein, the phylogenetic position of *Cephalenchus* is investigated using several populations sampled worldwide. Based on morphological and molecular data, this study aims to (i) investigate the monophyly of *Cephalenchus*, (ii) the monophyly of a clade formed by *Cephalenchus* + *E. excretorius*, (iii) their phylogenetic position in relation to other tylenchs, (iv) to evaluate the morphological patterns in the labial region of *Cephalenchus* and its congruence with species relationships as defined by molecular characters and (v) to discuss the biogeography of *Cephalenchus*.

Material and methods

Sampling and nematode extraction

Soil samples, each about 300 g, were collected with a small shovel and stored in labelled plastic bags for further laboratory work (Table S1). Nematode specimens were extracted from soil using either a Baermann funnel or plastic tray method (Whitehead & Hemming 1965; Viglierchio & Schmitt 1983). Samples collected outside the USA were fixed in DESS (Yoder *et al.* 2006) and 5% formalin solutions for molecular and morphological procedures, respectively. Specimens were sorted under a dissecting microscope (Olympus SZX16, Olympus Corporation, Tokyo, Japan) for further morphological and molecular characterization; when needed, *Cephalenchus* identity was determined using a compound microscope (Nikon Eclipse E600, Nikon Corporation, Kawasaki, Japan). Samples collected in the USA were processed at the University of California, Riverside (UCR), so that fresh specimens were used for DNA extraction. A morphological voucher of each specimen was digitally recorded as photographs or through-focus videos prior to DNA extraction (De Ley & Bert 2002).

Search of curated samples from the UCR Nematode Collection (UCRNC)

In addition to newly collected samples, formalin-fixed material from UCRNC was also consulted. Five additional *Cephalenchus* populations, not previously identified to species, were included for morphological and morphometric characterization (Table S1).

***Analysis of Cephalenchus* global distribution**

Because *Cephalenchus* has been almost entirely ignored in molecular phylogenies, little is known about its biogeography. As a first attempt to better understand the biogeography of this group, the geographic distribution of *Cephalenchus*, based on geographical coordinates representing the studied populations and species, was plotted on a world map (Fig. S1). For those populations retrieved from the UCRNC, approximate geographical coordinates were estimated based on verbal descriptions from collecting logs. Additionally, a literature search on the genus indicated 89 sites where *Cephalenchus* has been documented. The geographic distribution of *Cephalenchus* is summarized in Fig. S1.

Morphological characterization

LM procedures. For permanent slides, fixed specimens were initially washed in distilled water to remove debris attached to the cuticle and then dehydrated and infiltrated in a graduated series of glycerine/ethanol solutions to pure glycerine (Seinhorst 1959). Specimens were examined using a Zeiss Axioskop microscope equipped with a drawing tube and morphological parameters measured using a micrometre following Geraert (2008). Identification of *Cephalenchus* species was based on original descriptions and supplemented by available keys (Andrássy 1984; Raski & Geraert 1986; Geraert 2008).

SEM procedures. Specimens were repeatedly (3 times) rinsed in distilled water for 5 min to remove traces of formalin and then postfixed overnight in an aqueous solution of 2.0% osmium tetroxide. Postfixed specimens were dehydrated through a series of aqueous dilutions of 10–100% ethanol. Dehydrated specimens were critical point dried in a Tousimis Autosamdri-810®, mounted on double-sticking copper tape attached to aluminium stubs, coated for 1.5 min with a 25 nm layer of gold palladium in a Cressington 108 Auto® sputter coater and then observed with an XL 30-FEG Phillips 35® scanning electron microscope operating at 10 kV (Mundo-Ocampo *et al.* 2003).

Morphological analyses. Morphometrics were based on measurements of female specimens. Statistical analyses were carried out to evaluate the significance of morphological distinction between different *Cephalenchus* species and populations. Because tail length has been commonly used for identification of *Cephalenchus*, variation of this morphological feature (mean ± SD) was characterized, so that species could potentially be grouped accordingly to tail length (i.e. short, medium and long). Morphological data were first normalized and then used to compute pair-wise Euclidean distances among individuals. Missing data were replaced by the mean value of that particular *Cephalenchus* population. Non-metric multidimensional scaling (nMDS) was used to assess morphological differentiation among species and populations. Significant differences ($P < 0.05$) among groups were assessed with analysis of similarity (ANOSIM, Clarke & Gorley 2006). All morphological analyses were performed using PRIMER v6.0 (Clarke & Gorley 2006).

Characterization of the labial region. Based on SEM micrographs, the labial region of each species was evaluated; regardless of variation, three typical patterns were recognized. Morphological structures of the labial region were 3D modelled using Autodesk® Maya® following the procedure of Qing *et al.* (2015a). The terminology used in this study to describe the anterior region of *Cephalenchus* is largely consistent with previous authors (Raski & Geraert 1986; Geraert 2008).

Molecular analyses

DNA extraction, amplification and sequencing. DNA was extracted from single individuals using proteinase K protocol and Worm Lysis Buffer (WLB) following Pereira *et al.* (2010). Samples were incubated for 1 h at 65 °C followed by 10 min at 95 °C and then submitted to polymerase chain reaction (PCR). The D2-D3 domains of the 28S rRNA gene were amplified with primers D2Ab and D3B (De Ley *et al.* 1999). The 18S rRNA gene was amplified using primers G18S4 and 18P (Blaxter *et al.* 1998). Additional internal primers, 4R, 22F, 13R and 4F, were also used for sequencing the 18S rRNA (Bert *et al.* 2008). The 5.8S rRNA gene and its flanking regions ITS-1 and ITS-2 were amplified using primers N93 and N94 (Nadler *et al.* 2000). All PCRs were 25 μL made of 5 μL of DNA template, 0.2 μL of each primer (20 μM) and 19.6 μL of PCR purified water in combination with Pure Taq-Ready to Go kit (GE Health Care®, Buckinghamshire, UK). Low DNA samples were also subjected to a GenomiPhi protocol (GenomiPhi V2 DNA Amplification Kit; GE Health Care®) to increase DNA concentration prior to PCR. Amplification success was evaluated electrophoretically on 1% agarose gel stained with ethidium bromide (0.5 μgmL^{-1}). Positive PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, USA) and then cloned with the pGEM®-T Easy Vector cloning Kit (Promega, Madison, WI, USA). Cloning used JM109 high competent cells following the manufacturer's instructions before sequencing. Sequencing was performed in both directions with PCR primers using ABI-PRISM® Dye-DeoxyTerminator BIG DYE™ v3.1 (Applied Biosystems, Foster City, CA, USA) with an automatic sequencer Gene Analyzer® ABI 3100 (Applied Biosystems) at the Institute for Integrative Genome Biology, UCR.

Phylogenetic analyses. To evaluate the phylogenetic position of *Cephalenchus* and its validity as a natural group (i.e. its monophyly), 18S, 28S and ITS sequences from additional Tylenchomorpha and outgroup taxa were downloaded from GenBank (Table S2–S3). Outgroup taxa for broader phylogenetic analyses (28S and 18S data sets) were chosen based on previous studies (Subbotin *et al.* 2006; Bert *et al.* 2008; Palomares-Rius *et al.* 2009; Van Megen *et al.* 2009). These analyses (i.e. large datasets) included 159 taxa and covered the major lineages within Tylenchomorpha (Table S3). On the other hand, ITS sequences were only used to explore relationships within *Cephalenchus* owing to the high variability of ITS as well as its paucity for Tylenchomorpha in GenBank. For those analyses solely focused on *Cephalenchus*, only a few Tylenchomorpha taxa (i.e. 18S, 28S and ITS reduced data sets) were used as phylogenetic context (Table S2, Figs 3A–C).

Sequences representing the different data sets were separately aligned on MAFFT v7.0 (http://mafft.cbrc.jp/alignment/server) using three iterative refinement methods: E-INS-i, G-INS-i and Q-INS-i (Katoh & Standley 2013). These alignments were also submitted to Gblocks 0.91b so that poorly aligned and divergent regions could be identified (based on all three less stringent criteria) and deleted from the original datasets (Castresana 2000). Both full and reduced alignments (after Gblocks treatment) of each refinement method were used for further phylogenetic analyses. In this sense, the influence of alignment procedures on the overall phylogeny, and particularly so on the position of *Cephalenchus* + *E. excretorius* in relation to other tylenchs, could be evaluated. Previous studies have shown that nematode phylogenies based on rRNA genes can be influenced by alignment parameters (Smythe *et al.* 2006; Subbotin *et al.* 2006).

Phylogenetic analyses were inferred by maximum likelihood (ML) and Bayesian inference (BI) on the CIPRES Science Gateway (http://www.phylo.org/). The best-fitting substitution model for the different datasets was estimated using jModelTest 2.1.2 (Darriba *et al.* 2012) based on the Akaike Information Criterion. ML analyses were performed using RAXML-HPC 8.2.4 under the GTRCAT model. Gamma parameters were estimated from log-likelihood units, and bootstrap support (1000 pseudoreplicates) was automatically calculated for the best-scoring ML tree (Stamatakis 2006, 2014). BI analyses were performed on MRBAYES 3.2.6 (Huelsenbeck & Ronquist 2001) under the GTR + I + G model with the settings: random starting tree, two independent runs with four chains (2.0×10^8 generations for broader analyses, 2.0×10^6 generations for analyses solely focused on *Cephalenchus*). Markov chains were sampled at intervals of 1000 generations. After assessing chain convergence using the standard deviation of split frequencies (<0.01) and Potential Scale Reduction Factors (PSRF, close to 1.0), burn-in phase was set at 25% of the results. A 50% majority rule consensus tree was generated, and posterior probabilities (PP) were calculated for each clade. Concatenated analyses (18S + 28S, a total of 2597 sites) were performed also using the settings of broader phylogenetic analyses. Alternative topology hypotheses (i.e. *Cephalenchus* + *E. excretorius* as sister to Tylenchinae or to Boleodorinae) were tested using the 18S and 28S data sets. Constrained Bayesian analyses were run in MRBAYES 3.2.6 using the same parameters as the original analyses. Site-specific likelihoods were calculated for the unconstrained and constrained Bayesian trees using PAML v4.8 with the same models of the original analyses, but with the model parameters optimized by BASEML (Yang 2007). These likelihoods were compared based on Shimodaira-Hasegawa (SH) and approximately unbiased (AU) tests (Shimodaira & Hasegawa 1999; Shimodaira 2002) using CONSEL v0.20 (Shimodaira & Hasegawa 2001).

Results

Cephalenchus geographic distribution

Cephalenchus has been reported from all continents, except Antarctica, and it has been found as far north as 62.9°N in Finland (reported as *Cephalenchus* sp.) and as far south as 55.4°S in Chile (type locality of *Cephalenchus chilensis* Raski & Geraert 1986). However, the worldwide distribution of *Cephalenchus* has been mostly in the northern hemisphere. Except for a few sites sampled in the Australasian region (Australia, New Zealand and some islands in the Pacific Ocean), Congo and Chile (Geraert 2008), all other sites where *Cephalenchus* occurred were reported from north of the equator (Fig. S1). Herein, two additional sites in the southern hemisphere, south (BRA-01) and north of Brazil (BRA-02), are added to the known geographic distribution of *Cephalenchus*. Among *Cephalenchus* species, *C. hexalineatus* (26 entries) and *C. leptus* (16 entries) are the most widely distributed. Also, from numerous sites (19 entries), *Cephalenchus* is only reported as *Cephalenchus* sp. In summary, most *Cephalenchus* species have been originally described from India (eight species), Japan (three species), Australia and Pakistan (both with two species). Besides type localities, *Cephalenchus* species have been mostly reported from two other geographic regions likely to harbour considerable *Cephalenchus* diversity, North America (eight species) and Europe (five species, Fig. S1).

Species identity and morphological variation across Cephalenchus

Based on published morphological descriptions, 11 species of *Cephalenchus* were identified in this study including *C. cephalodiscus* Sultan & Jairajpuri, 1981 (CHN-02 and 03,

USA-01), *C. cylindricus* Sultan & Jairajpuri, 1981 (MEX), *C. daisuce* Mizukubo & Minagawa, 1985 (CAN-01, USA-02 to 04, and BEL-02), *C. hexalineatus* (BEL-01, CAN-02, CRI, USA-08), *Cephalenchus illustris* Andrássy 1984 (USA-06), *C. leptus* (CHN-01, USA-05 and 09), *Cephalenchus longicaudatus* Maqbool & Ghazala, 1986 (USA-07), *Cephalenchus nemoralis* Mizukubo & Minagawa, 1985 (VIE-02) and *Cephalenchus planus* Siddiqui & Khan 1983 (THA). Owing to the low number of adult specimens, one population from Brazil (BRA-02) was only identified as *Cephalenchus* sp. Additionally, two species, designated herein as *Cephalenchus* 'sp1' from Brazil (BRA-01) and *Cephalenchus* 'sp2' from Vietnam (VIE-01), were found to be new to science and will be formally described elsewhere.

The nMDS, based on morphometric data of 23 *Cephalenchus* populations (11 nominal species), showed that, in general, individuals representing specific populations clustered together with substantial overlap (Fig. 1A). An exception is *Cephalenchus* sp1 (BRA-01), which was much more dispersed throughout morphological space. In some cases, clusters were very cohesive, indicative of little morphological variation (e.g. *C. longicaudatus*). Morphometric differences between species were significant in all comparisons except in a few cases (Table 1). Also, populations identified as the same species showed slightly more overlap (e.g. *C. hexalineatus* from CAN-01, USA-07 and BEL-01; some populations of *C. daisuce*) compared to those belonging to different species. Such overlap was also the case for species considered to be morphologically similar (e.g. *C. cylindricus* and *C. cephalodiscus*).

A separation between species (e.g. *C. hexalineatus*, *C. illustris*, *C. nemoralis*, *C. planus* and *C. longicaudatus*) with a short tail (mean ≤ 155 μm) relative to species with a medium (mean > 155 to ≤ 202 μm) to long (mean > 202 μm) tail is also shown by the nMDS analysis (Fig. 1B). Yet, the transition between species with a medium to long tail is less evident. For example, species with medium tail length such as *C. daisuce* and *Cephalenchus* sp2 showed a certain degree of overlap with species possessing longer tails. Nevertheless, significant differences between tail groups were

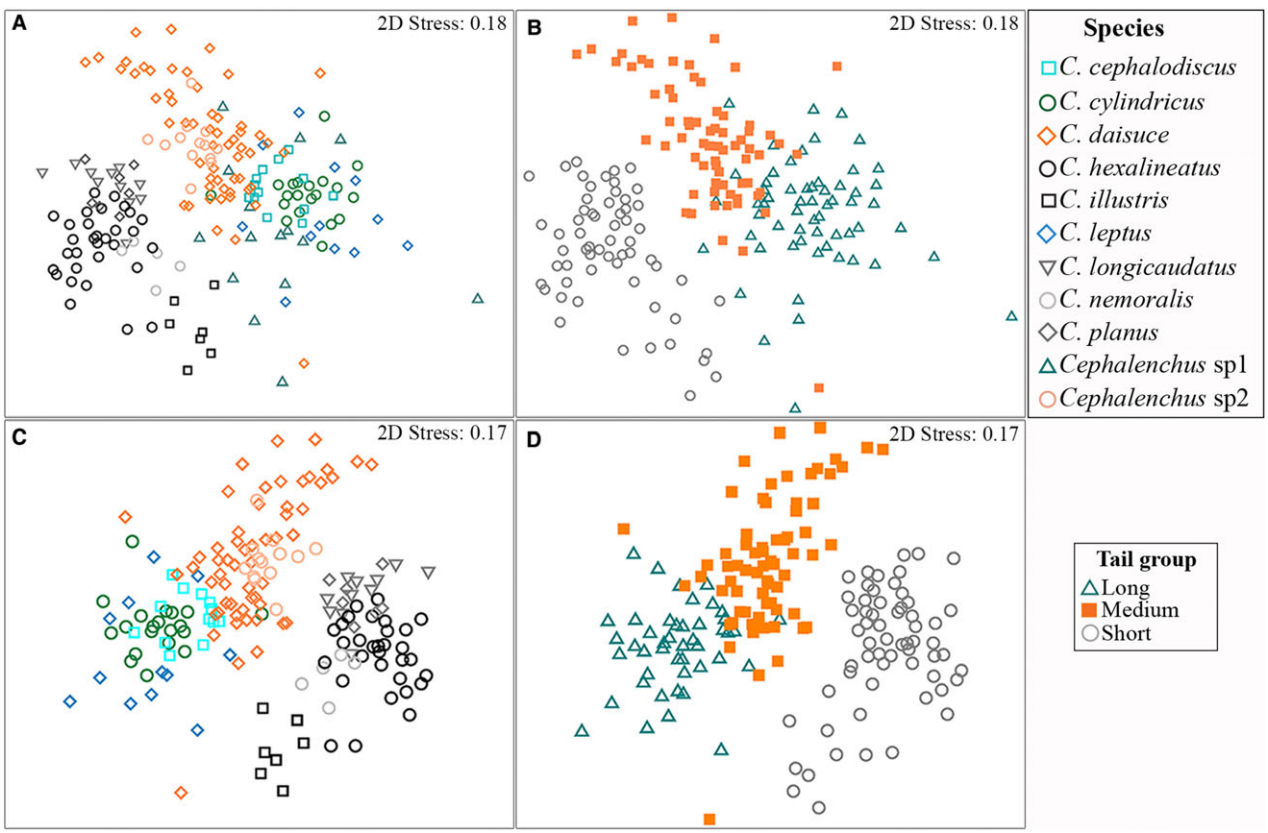

Fig. 1 Nonmetric multidimensional scaling (nMDS) plot obtained from the morphometric data of *Cephalenchus* species. —A. Spatial distribution of all *Cephalenchus* species in the morphological space. —B. *Cephalenchus* species are clustered according to tail group. —C. Spatial distribution of all *Cephalenchus* species, but excluding *Cephalenchus* sp1 (BRA-01) from the data set. —D. *Cephalenchus* tail grouping, but also excluding *Cephalenchus* sp1 (BRA-01) from the data set.

Table 1 Results from the ANOSIM. Significant differences are highlighted in bold. In the case of species and populations comparisons, only the non-significant differences are listed

Comparisons	R statistic	Significance level %
Between tail groups		
Long vs. medium tail	0.413	**0.001**
Long vs. short tail	0.82	**0.001**
Medium vs. short tail	0.635	**0.001**
Between populations		
Cephalenchus sp1 (BRA-01) vs. *Cephalenchus daisuce* (BEL-02)	0.039	0.401
C. daisuce (USA-02) vs. *C. daisuce* (BEL-02)	0.183	0.123
Cephalenchus longicaudatus (USA-07) vs. *Cephalenchus planus* (THA)	0.105	0.133
Between species		
Cephalenchus cephalodiscus vs. *C. daisuce*	0.086	0.133
C. daisuce vs. *Cephalenchus* sp2	−0.083	0.844
C. longicaudatus vs. *C. planus*	0.105	0.128

detected by the ANOSIM for all comparisons (Table 1). Four species (*Cephalenchus* sp1, *C. cephalodiscus*, *C. cylindricus* and *C. leptus*) were characterized as having a long tail. Except for *Cephalenchus* sp1, which was more dispersed (Fig. 1A), the other three species were distributed in the same region of the morphological space. Species cohesiveness and tail group clustering become more evident when *Cephalenchus* sp1 is removed from the nMDS analysis (Fig. 1C–D).

Patterns in the labial region of Cephalenchus

With the exception of *C. illustris*, all other *Cephalenchus* species were observed under SEM. Although some of the populations were particularly fragile and prone to distortion, comparable species-specific lip patterns could be accurately determined based on a combination of specimens and micrographs. From these observations, two clear patterns emerged: (group 1) *Cephalenchus* species (i.e. *C. hexalineatus*, *C. longicaudatus*, *C. nemoralis* and *C. planus*) with a small and laterally oriented amphidial opening (Fig. 2A–B); and (group 2) *Cephalenchus* species (all other studied species) with a large dorso-ventrally oriented amphidial opening (Fig. 2C–D).

In addition to the amphidial aperture (dorso-ventral vs. lateral), the oral opening was also distinct between these two groups. *Cephalenchus* species with a dorso-ventral amphidial opening have a narrow dorso-ventral slit on the oral disc (Fig. 2A–B). These *Cephalenchus* species are also characterized by a medium or long tail. *Cephalenchus* species with a lateral amphidial opening displayed a small rounded-oval oral opening on the oral disc (Fig. 2C–D) and short tail. All studied *Cephalenchus* species displayed button like cephalic papilla in the labial disc and had no annulations on the cephalic region (smooth head).

For context, the labial region of *Cephalenchus brevicaudatus* Raski & Geraert 1986 was also determined based on SEM micrographs available in the literature (Raski & Geraert 1986). This species has 2–3 annuli on the cephalic region, with the most anterior one outlining the labial plate, thus contrasting with the abovementioned species (Fig. 2E–F). Despite the annuli, the amphidial and oral openings of *C. brevicaudatus* as well as the tail length (short, 54–93 μm) agreed with that of species in group 1.

Molecular characterization of Cephalenchus species

Molecular data are presented for 20 *Cephalenchus* populations, including 12 previously studied by Pereira & Baldwin (2016). In that study, Pereira & Baldwin (2016) reported high levels of intraspecific rRNA sequence variation for some *Cephalenchus* populations. With the exception of *C. leptus* (CHN-01) and *C. hexalineatus* (CRI), DNA sequences produced in this study were obtained from multiple specimens and clones, thus providing insight into intraspecific variation which was determined following Pereira & Baldwin (2016). Sequences produced in this study have been deposited on GenBank (28S: KX462018-KX462071 and KX685166, ITS: KX462072-KX462115, 18S: KX685160-KX685165, see also Table S2).

Intraspecific variation, although very low, was observed in all newly sampled *Cephalenchus* species. For the 28S rRNA gene, sequence divergence was in the range of 0.0–1.9% and 0.0–1.5% in *C. cephalodiscus* (CHN-02 and 03, respectively), 0.1–1.8% in *C. daisuce* (BEL-02), 0.4–0.8% for *C. hexalineatus* (BEL-01) and 0.0–0.5% in *C. leptus* (USA-05). Yet, for the ITS region, this variation was slightly higher in all populations, except *C. hexalineatus* (BEL-01). For example, it was in the range of 0.0–2.7% in *C. cephalodiscus* (CHN-03), 0.0–4.2% in *C. daisuce* (BEL-02) and 0.0–4% in *C. leptus* (USA-05). Two newly sampled populations of *C. hexalineatus* (BEL-01 and CRI) grouped with *C. hexalineatus* from the USA (FL and OR) in a strongly supported clade (Fig. 3A–B). The three other species including *C. cephalodiscus* (CHN-02 and 03), *C. daisuce* (BEL-02) and *C. leptus* (CHN-01 and USA-05) all grouped together into a large clade with other sequences representing the same species (see below).

Overall, the tree topologies (18S, 28S and ITS data sets) were congruent with respect to the number of clades as well as the monophyly of the different *Cephalenchus* species. Therefore, only the trees based on the full G-INS-I alignments are given (but see Fig. 3C). Five well-supported clades are recovered in the 28S and ITS phylogenies: clades I and II, represented by sequences of *C. nemoralis* (VIE-02) and *C. hexalineatus* (BEL-01, CRI and USA),

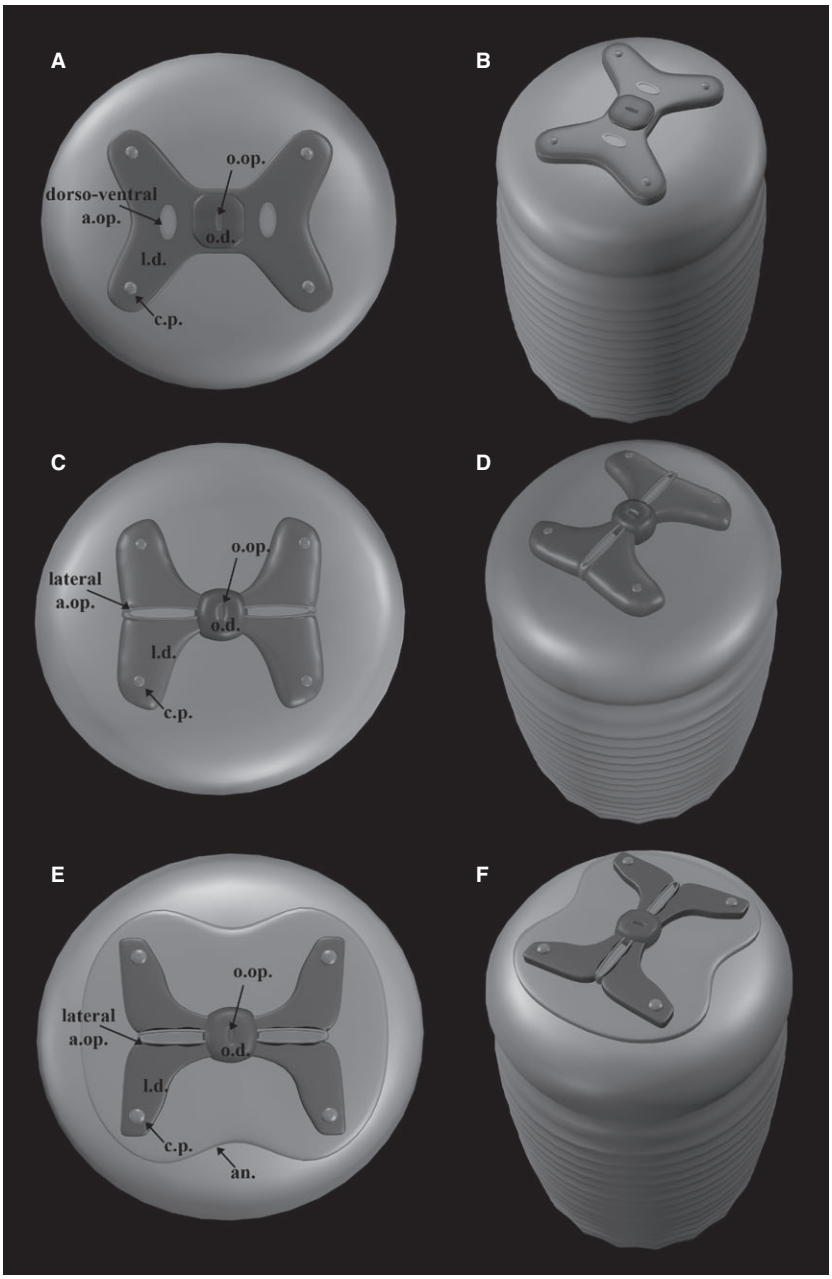

Fig. 2 Characterization of the labial region among *Cephalenchus* species. A–B. 3-D model based on scanning electron microscopy (SEM) micrographs of *Cephalenchus* sp1 (BRA-01) showing the dorsal–ventral amphidial opening. C–D. 3-D model based on SEM micrographs of *Cephalenchus hexalineatus* (USA-08) showing the lateral amphidial opening. E–F. 3-D model based on SEM of *Cephalenchus brevicaudatus* based on SEM micrographs included in Raski & Geraert (1986). Labels are included on the face view images (i.e. 3A, 3C, 3F) to better explaining the labial patterns (Abbreviations: o.op, oral opening; a.op, amphidial opening; o.d, oral disc; l.d., labial disc; c.p., cephalic papilla; an., first annulation).

respectively, are strongly supported as sister taxa. In the 28S phylogeny, clade III is represented by both populations from Brazil (BRA-01 and BRA-02); however, only *Cephalenchus* sp1 (BRA-01) is supported as a monophyletic group (Fig. 3A). In the 28S phylogeny, *Cephalenchus* sp1 (BRA-01) is sister to clades IV + V with high support (Fig. 3A). Yet, in the ITS and 18S phylogenies, this species is recovered as sister to clades II and III and sister to clade IV, respectively (Fig. 3B–C).

Clade IV is represented by sequences of *Cephalenchus* sp2 (VIE-01), and its sister relationship with clade V is strongly supported in the 28S and ITS phylogenetic analyses. Clade V comprises four morphologically defined species including multiple populations of *C. cephalodiscus*, *C. daisuce*, *C. leptus* and a single population of *C. cylindricus* from Mexico. The non-monophyly of these four species is supported in both 28S and ITS rRNA phylogenies (Fig. 3A–B). Although fewer *Cephalenchus* sequences are included in the 18S

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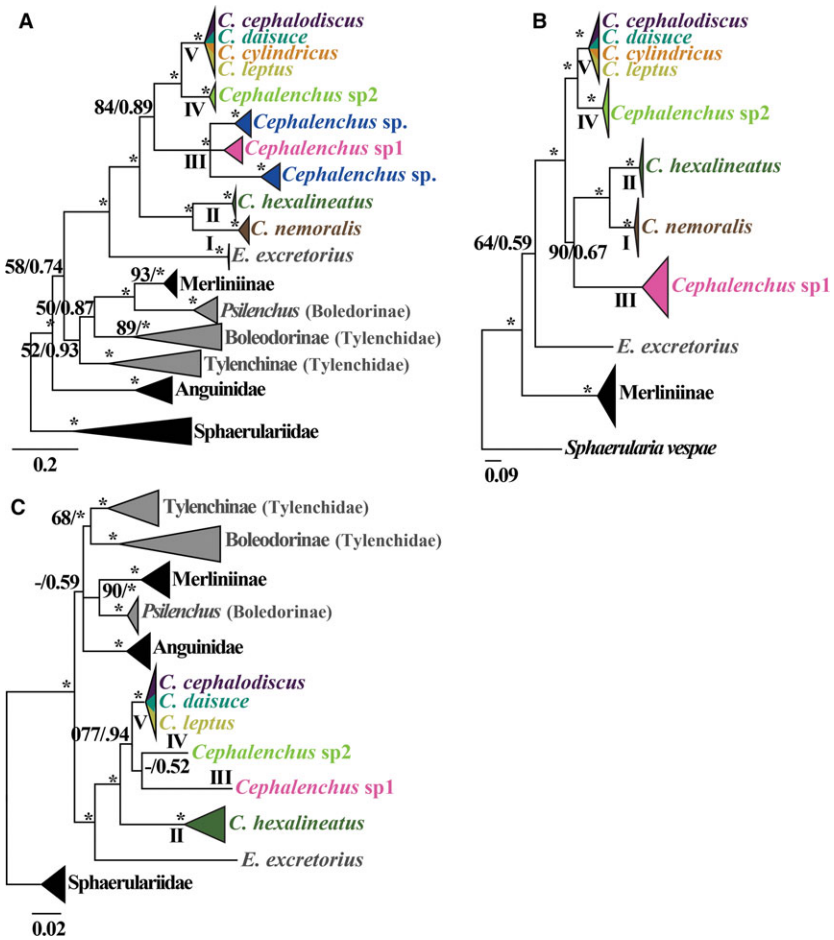

Fig. 3 Molecular phylogeny of the *Cephalenchus* species (reduced datasets) used in this study. The 50% majority rule consensus tree from the Bayesian analysis is presented. A–C Molecular analysis based on the 28S rRNA gene/full G-INS-i alignment method, ITS regions/reduced G-INS-i alignment method and 18S rRNA gene/full G-INS-i alignment method, respectively. Five clades (I–V) are identified among *Cephalenchus* sequences. Branch support (only above 50%) is shown on branches as ML/BI. An asterisk indicates support for ML and BI ≥ 95%. Trees were arbitrarily rooted to Sphaerulariidae taxa. Subfamilies of Tylenchidae (in grey) are according to Geraert (2008). Taxonomy scheme of additional outgroups (in black) is in accordance with Siddiqi (2000). *Cephalenchus* species are colour-coded.

analyses, non-monophyly of the same species is also supported (Fig. 3C).

Cephalenchus species of clades I and II are characterized by a lateral amphidial opening (Fig. 2C–D) and also by a short tail. Conversely, *Cephalenchus* species in clades III-V have either a medium (e.g. *C. daisuce* and *Cephalenchus* sp2) or a long tail (e.g. *C. cephalodiscus*, *C. cylindricus*, *C. leptus* and *Cephalenchus* sp1) and are characterized by a dorso-ventral amphidial opening (Fig. 2A–B). Although, phylogenetic analyses (Fig. 3A–C) are not fully congruent (i.e. placement of *Cephalenchus* sp1), combined analyses (18S + 28S data sets) recover clade II as sister to all *Cephalenchus* species (ML = 98%, BI = 1.0, Fig. 4).

Phylogenetic position of Cephalenchus within Tylenchomorpha

Regardless of the alignment strategy used, all phylogenetic analyses strongly recovered *Cephalenchus* as a monophyletic group (Table S4). In addition, a sister relationship between *Cephalenchus* and *E. excretorius* is often recovered, although branch support for this relationship is usually low (bootstrap <60%) for ML-based phylogenies and sometimes inconsistent for BI phylogenies (i.e. not supported by both full and reduced alignments). Removal of poorly aligned sites/divergent regions from the data sets seems to limit resolution on broader phylogenetic analyses (Table S4). When taxon sampling is limited to *Cephalenchus* species, *E. excretorius* and a few Tylenchomorpha taxa, branch support values for the clade *Cephalenchus* + *E. excretorius* are increased (ML ≥ 95%, BI ≥ 0.95, Figs 3A, C, but see ITS phylogeny).

In most of the phylogenetic analyses, the position of clade *Cephalenchus* + *Eutylenchus* in relation to other tylenchs is unresolved or poorly supported. Additionally, a presumably close association of *Cephalenchus* + *Eutylenchus*, as previously suggested by morphology, with other Tylenchidae genera is not observed. The monophyly of Tylenchidae is not recovered in the 18S and 28S phylogenies. However, some Tylenchidae genera grouped together in the molecular analyses, which resulted in the monophyly of subfamilies Boleodorinae (but excluding *Psilenchus* de Man, 1921) and Tylenchinae (Figs 3A, B, 4).

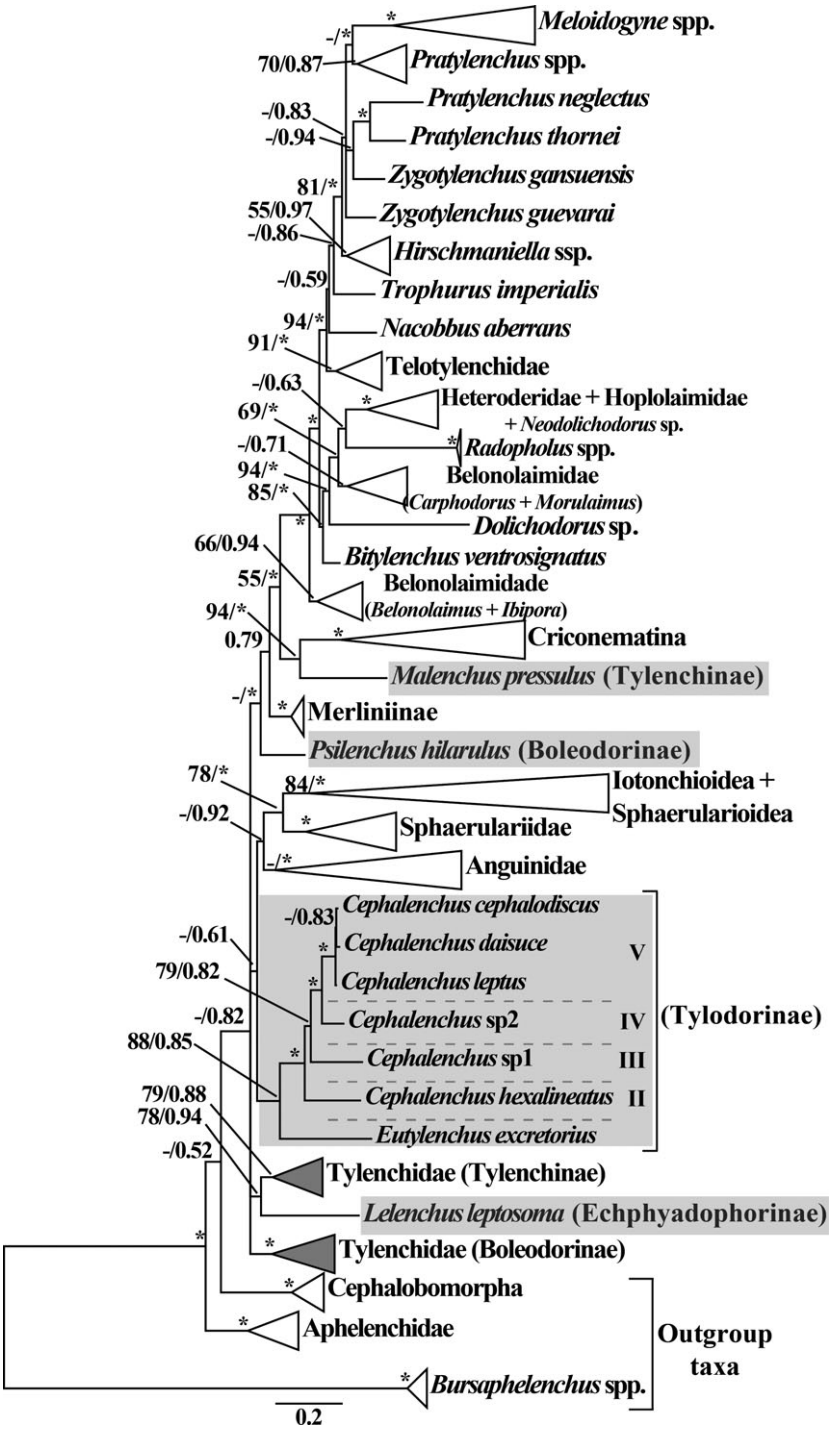

Fig. 4 A combined analysis based on two rRNA genes (18S + 28S, 2597 sites). The 50% majority rule consensus tree from Bayesian analysis is presented. Tylenchidae taxa, including *Cephalenchus* + *Eutylenchus*, as defined in Geraert (2008), are highlighted in the grey box. Branch support (only above 50%) is shown on branches as ML/BI. An asterisk indicates support for ML and BI ≥ 95%. (-) indicates no support for that node. Taxonomy scheme of families, except Tylenchidae, and higher taxa is in accordance with Siddiqi (2000); subfamilies of Tylenchidae follow Geraert (2008). The tree was rooted using *Bursaphelenchus* species.

In a few cases (28S rRNA gene), BI analyses including broader taxon sampling recovered *Cephalenchus* + *Eutylenchus* as sister of Anguinidae (full Q-INS-i, BI = 0.81) or of Sphaerulariidae (full and reduced G-INS-i align, BI = 0.96/0.76). There were also cases where *Cephalenchus* + *Eutylenchus* are recovered as a unique lineage in the Tylenchomorpha tree, although branch support is always poor (BI < 0.6, Table S4). Alternative topology hypotheses (i.e. *Cephalenchus* + *E. excretorius* + Boleodorinae; *Cephalenchus* + *E. excretorius* + Tylenchinae) for the

placement of the clade *Cephalenchus* + *E. excretorius* in Tylenchomorpha were also tested. Both the AU and SH tests were not able to reject the alternative hypotheses at the significant level of 95%. These results were consistent across the 18S and 28S data sets (Table S4).

Concatenated analyses based on the 18S and 28S rRNA genes recovered *Cephalenchus* as a monophyletic group with strong branch support (ML = 98, BI = 1.0). Additionally, a sister relationship between *Cephalenchus* and *E. excretorius* is also recovered by the concatenated analyses (ML = 88, BI = 0.85). Although support for *Cephalenchus* + *Eutylenchus* is improved, their placement within Tylenchomorpha is still unresolved or poorly supported (Fig. 4).

Discussion

Cephalenchus species distribution and diversity

Although *Cephalenchus* has been reported worldwide (Andrássy 1984; Geraert 2008), information retrieved from published studies in conjunction with samples collected in the present study suggests a distribution of *Cephalenchus* throughout the Northern Hemisphere. Furthermore, its deep latitudinal range (about 60°N and 55°S) further suggests that its absence in many regions of the Southern Hemisphere is mostly related to inadequate sampling, especially in habitats likely to harbour *Cephalenchus* species. Sampling of tylenchs is mostly carried out on specific field crops and to a lesser degree in natural areas where *Cephalenchus* might be more commonly found. Although a few laboratory studies reported *Cephalenchus* as feeding on root cells (Sutherland 1967; Gowen 1970), severe plant damage has not yet been attributed to *Cephalenchus* spp. and the genus is not regarded as a significant plant pathogen (Siddiqi 2000).

All *Cephalenchus* species collected in the present work were found in natural (i.e. not agrarian) sites, usually in moist, organically rich soils (Table S1). Additionally, sampling site descriptions from previous collections further underscores that *Cephalenchus* has a preference for moist habits. In fact, several *Cephalenchus* species were specifically collected at the edge of streams where moisture conditions are even higher. A few studies have reported *Cephalenchus* occurring on the rhizosphere of banana (Choudhury & Phukan 1990; Abedin *et al.* 2012) and that was also the case for three *Cephalenchus* species found in this study (i.e. *C. nemoralis*, *C. planus* and *Cephalenchus* sp.). *Cephalenchus* also has been reported from grassland and deciduous forest (Dhanachand & Jairajpuri 1980; Andrássy 1984; Ladislav 2003).

Although *C. hexalineatus* and *C. leptus* are currently regarded as the most widely distributed species, some authors have suggested each of these to be a complex representing multiple species and thus potentially increasing the number of known species for the genus (Raski & Geraert 1986; Geraert 2008). Increased sampling of natural areas, particularly in the Southern Hemisphere, is likely to result in a more complete picture of *Cephalenchus* diversity and species distribution. Furthermore, the inclusion of molecular data from additional *Cephalenchus* species representing different geographic regions will allow the use of more comprehensive biogeographic analyses, thus improving our understanding of distribution patterns and speciation.

Defining species based on continuous and qualitative morphological characters

About 25 nominal species of *Cephalenchus* have been described, but species synonymizations by different authors, while controversial, have reduced the number of species to 20 (Siddiqi 2000; Geraert 2008). Based on morphometric analyses, significant differences are found in most of the comparisons between *Cephalenchus* species and even between populations representing the same morphological species (e.g. within *C. daisuce* and *C. hexalineatus*). Population and species level comparisons also differed. Specifically, non-significant differences between *Cephalenchus* sp1 (BRA-01) vs. *C. daisuce* (BEL-02) can be attributed to the morphometric variability found in the former species as well as to the low number of specimens representing the latter species (5 specimens). On the other hand, non-significant differences between *C. daisuce* vs. *C. cephalodiscus* and *C. daisuce* vs. *Cephalenchus* sp2 were largely affected by the variation found within *C. daisuce* (only populations BEL-02 and USA-02 were not significantly different).

Within *Cephalenchus*, tail length is probably the main morphological character to separate species (Geraert 1968). Morphometric analyses performed in this study also showed that *Cephalenchus* species clearly fall into three main tail groups (i.e. short, medium and long). Although tail length defining these species groups is somewhat arbitrary (i.e. broad range in some species), *Cephalenchus* identification keys commonly rely on this morphological feature as typically diagnostic (Raski & Geraert 1986; Geraert 2008). Nevertheless, overlap between median and long tail groups is also observed, thus suggesting the difficulty in separating species from these two groups based alone on designated cut-offs. In this sense, molecular data have become essential to appropriately establishing and testing species hypotheses.

Most *Cephalenchus* species are characterized by having six lines in the lateral field (LF), which led Dhanachand & Jairajpuri (1980) to propose the genus *Imphalenchus* to accommodate similar species bearing only four lines in the LF. However, this action was not supported by Raski & Geraert (1986) who synonymized *Imphalenchus* with

Cephalenchus. Since then, four species characterized by four lines in the LF have been described in *Cephalenchus*, but relationships among these species as inferred by molecular data have not yet been explored. In this study, *Cephalenchus* sp2 (VIE-01) was the only species characterized by having four lines in the LF, and although it showed some overlap with *C. daisuce* (both fall in the medium tail group) in the morphometric analyses, molecular phylogenetic analyses always recovered it as separate lineage within *Cephalenchus*, which supports the synonymy of *Imphalenchus* with *Cephalenchus* (Raski & Geraert 1986).

With respect to tail length, *Cephalenchus* species bearing four lines in the LF show great variability, ranging from short (115–155 μm) as in *Cephalenchus imphalus* Dhanachand, Renubala & Annandi, 1993, to medium length (134–190 μm) as in *Cephalenchus concavus* Xie & Feng, 1994 and (184–202 μm) as in *Cephalenchus indicus* (Dhanachand & Jairajpuri 1980) Raski & Geraert 1986, to long (214–280 μm), as in *Cephalenchus intermedius* Kanwar, Bajaj & Dabur, 1993, thus, representing all three tail groups. Therefore, number of lines in the LF of *Cephalenchus*, although useful to distinguish species, is not linked to a specific tail group. Moreover, based on the molecular analyses, number of lines in the LF (six evolving to four lines) can be interpreted only as homoplastic, and its utility to explain phylogeny needs further investigation of additional species bearing four lines in the LF.

Information on the labial pattern of *Cephalenchus* is limited to a few studies. In fact, only Raski & Geraert (1986) have observed its morphology using SEM techniques. The authors characterized the labial region of three species, *C. chilensis*, *C. brevicaudatus* and *C. leptus* as well as provided a general overview of the labial morphology for the genus. Other studies (Siddiqi 1963; Dhanachand & Jairajpuri 1980; Siddiqui & Khan 1983) have based their conclusions upon cross sections of the anterior region of *Cephalenchus* under LM observation. However, LM resolution is often limited and might not fully represent the labial pattern of *Cephalenchus*, thus obscuring interpretation of morphological features as well as characters that define species relationships.

Among potential characters of the labial region, orientation of the amphidial opening (i.e. dorso-ventral or lateral) was the primary difference observed among *Cephalenchus* species, dividing the genus into two groups of species. Variation in the oral opening (i.e. slit vs. oval shape) is also congruent with groups defined on the basis of the amphidial opening. More importantly, molecular phylogenetic analyses recovered sister relationships between clades displaying the same labial morphology (e.g. clades I-II and clades III-V) suggesting that such features might explain species/clade relationships in *Cephalenchus*. Notably, similar methods have proven to be useful to explain phenotypic evolution and species relationships in other tylench groups (Subbotin *et al.* 2008). Although *C. brevicaudatus* also has a laterally oriented amphidial opening, molecular data are not yet available for it to test its phylogenetic position within *Cephalenchus*, either as sister to clades I and II (as they all share lateral amphidial opening) or as sister to all *Cephalenchus* species (*C. brevicaudatus* has one annulation in the labial region).

Cephalenchus is monophyletic but its position within Tylenchomorpha remains unresolved

Extant molecular phylogenies of Tylenchomorpha have included only a few *Cephalenchus* species, and thus, hypothesis of monophyly for the genus was previously not rigorously tested. Moreover, these studies have either relied on a single gene or they did not appropriately sample the group to infer the position of *Cephalenchus* (Bert *et al.* 2008; Holterman *et al.* 2009; Palomares-Rius *et al.* 2009; Panahandeh *et al.* 2015a,b; Yaghoubi *et al.* 2015). Because *Cephalenchus* may be the sister taxon to a group including some most economically important plant parasites, its study has important implications for comparative analysis of parasitism.

In this study, molecular phylogenetic analyses (single genes and concatenated dataset) strongly supported (ML ≥ 88, BI ≥ 95) *Cephalenchus* as a monophyletic group. By contrast, analyses based on one data set (28S rRNA gene, reduced Q-INS-I, Table S4) showed fairly low support for the monophyly of *Cephalenchus*; this might be explained by the drastic reduction in the number of sites (only 40% from the full alignment). Branch support for the monophyly of *Cephalenchus* is improved (ML ≥ 95 and BI ≥ 0.95) when a reduced number of taxa (i.e. *Cephalenchus* and few Tylenchomorpha) are analysed (Fig. 3A–C). This outcome shows that addition of highly divergent taxa, potentially with long branches, can increase difficulty in making phylogenetic estimations rather than to improve them (Hillis 1998).

Relationships among *Cephalenchus* species (clades I–V) were fairly congruent based on the different molecular analyses. In this sense, non-monophyly of four defined morphological species is supported. In a similar result, *C. cephalodiscus*, *C. cylindricus* and *C. daisuce* all grouped together in the North American clade (Pereira & Baldwin 2016). Herein, *C. leptus* from three different geographic regions including China, Iran and USA also fall into the same clade. Moreover, two additional populations of *C. cephalodiscus* and one of *C. daisuce* were recovered in the same clade. This pattern suggests that morphological variation in *Cephalenchus* might happen at a much faster pace than molecular variation, perhaps mostly due to

environmental conditions. Therefore, species delimitation in such a homogenous group as *Cephalenchus* might be more prone to inconsistencies when solely based on morphology (e.g. the overlap between medium and long tail groups). Additionally, molecular analyses also support the synonymization of *C. cephalodiscus*, *C. cylindricus*, *C. daisuce* and *C. leptus*. Synonymization of *C. cephalodiscus* and *C. cylindricus* has been previously proposed by Raski & Geraert (1986) likewise, Mizukubo (1989) proposed *C. daisuce* as a subspecies of *C. leptus*.

Additional molecular evidence from a faster evolving gene [e.g. cytochrome oxidase *c* subunit I (COI)] of the mitochondrial genome might be needed to make a strong case of synonymy for these species. Mitochondrial genes might be more suitable for resolving recent speciation events when compared to rRNA genes (Blouin 2002; Nieberding *et al.* 2008). Thus, the low resolving power of rRNA genes may explain the lack of reciprocal monophyly for the species representing the North American clade (Pereira & Baldwin 2016). A closer inspection of *C. hexalineatus* sequences, however, shows some structure (i.e. monophyly) at the population level. For example, in the ITS region, *C. hexalineatus* from Oregon (USA) is characterized by six fixed autapomorphies (data not shown), and a similar pattern is not observed in any species of clade V. The inferred existence of fixed autapomorphies can potentially guide species delimitation in closely related species as suggested by Nadler (2002). Thus, the lack of monophyly as well as autapomorphies for *Cephalenchus* species in clade V reinforces their synonymization.

Besides the monophyly of *Cephalenchus*, a sister relationship between *Cephalenchus* and *E. excretorius*, both treated by Geraert (2008) as members of Tylodorinae and Tylenchidae, is recovered by the molecular phylogenies. However, branch support for this relationship is usually low on broader phylogenies based on single genes, particularly so when using reduced alignments. Broader molecular phylogenies of Tylenchomorpha have been mostly based on the 18S rRNA gene, and with a single exception (Palomares-Rius *et al.* 2009), have not treated both *Cephalenchus* and *Eutylenchus* together (Bert *et al.* 2008, 2010; Van Megen *et al.* 2009). In the molecular analyses performed by Palomares-Rius *et al.* (2009), *C. hexalineatus* and *E. excretorius* are recovered as sister taxa with high support (BI = 0.9) on 18S and 28S rRNA phylogenies, but not by the *hsp* 90 gene. In the present study, branch support for a clade of *Cephalenchus* + *Eutylenchus* is also improved when taxon sampling is reduced (Fig. 3A–C). Additionally, combined analyses of two rRNA genes recovered *Cephalenchus* + *Eutylenchus* with relatively high support (Fig. 4).

Although a clade of *Cephalenchus* + *Eutylenchus* seems to be convincing, as recovered by most of the analyses, neither a single-gene nor combined analysis was able to unequivocally determine the position of these taxa within Tylenchomorpha. However, two analyses based on the 28S rRNA gene recovered *Cephalenchus* + *Eutylenchus* with moderate support as sister taxa of Anguinidae or Sphaerulariidae. Similar results with the 28S rRNA, although with lower branch support, were also found by Palomares-Rius *et al.* (2009). Moreover, Subbotin *et al.* (2006) reported *E. excretorius* (*Cephalenchus* not included in the analysis) in a clade containing representatives of both Anguinidae and Sphaerulariidae. Conversely, analyses solely based on the 18S rRNA gene poorly recovered *Cephalenchus* + *Eutylenchus* as a unique lineage sister to a group including most economically important plant parasites in agreement with previous 18S rRNA phylogenies (Bert *et al.* 2008; Holterman *et al.* 2009).

This study also showed that *Cephalenchus* + *Eutylenchus* are possibly not related to other Tylenchidae genera as suggested by morphology (Geraert & Raski 1987; Geraert 2008). Although alternative hypotheses for the placement of *Cephalenchus* + *Eutylenchus* could not be rejected, likelihood tree values suggest that such relationships are less likely and therefore should be accommodated in a separate family. The results partially support Siddiqi (2000) in transferring both genera to Pleurotylenchinae, Tylodoridae. On the other hand, the placement of these genera within Tylenchoidea Örley, 1880, as also suggested by Siddiqi (2000), needs further investigation (Subbotin *et al.* 2006).

The position of *Cephalenchus* + *Eutylenchus* in the tylench tree, although still unresolved, will certainly benefit from inclusion of additional genes and increased taxon sampling. For example, *Campbellenchus* share similarities with *Cephalenchus* with respect to the labial morphology and internal morphology, and *Atylenchus* Cobb, 1913 has four cephalic setae in the anterior region as *Eutylenchus*. The inclusion of genera having similarities with *Cephalenchus* and *Eutylenchus* is likely to improve some of the findings presented in this study. As Tylenchidae representation in molecular phylogenies increases, the validity of its genera as natural groups as well as its relationships among other tylenchs can be adequately tested; as a result our understanding of the Tylenchomorpha phylogeny will be improved. Ultimately, a revision of the entire Tylenchidae will be needed to accommodate new insights gained by molecular phylogenies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Worldwide distribution of the genus *Cephalenchus* based on the published literature and new collected

sites (this study). *Cephalenchus* species are colour-coded. New *Cephalenchus* sampled sites and samples retrieved from the UCRNC are indicated by their locality code.

Table S1. List of *Cephalenchus* species studied in the present study.

Table S2. Sequences used in the molecular phylogenetic analyses focused on the genus *Cephalenchus* (i.e. reduced datasets).

Table S3. List of taxa used in the broad phylogenetic analyses.

Table S4. Parameters for the alignments used for broader (159 sequences) phylogenetic analyses of Tylenchomorpha and the support for the monophyly of *Cephalenchus*, of the clade *Cephalenchus* + *E. excretorius*, and for their position within Tylenchomorpha in the ML and BI analyses.