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Phylogeny and Taxonomic Revision of Tylenchidae with Emphasis on the Genus *Cephalenchus* (Tylenchina: Tylenchomorpha)

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Dedication

My PhD is dedicated to my lovely family members back in Brazil, my father Manoel, my mother Nair, and my siblings Lucas and Angela; and also to what is now my closest family, my lovely wife Mirayana and my two beautiful children, Arthur and Gabriel.

ABSTRACT OF THE DISSERTATION

Phylogeny and Taxonomic Revision of Tylenchidae with Emphasis on the Genus *Cephalenchus* (Tylenchina: Tylenchomorpha)

by

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Doctor in Philosophy, Graduate Program in Entomology
University of California, Riverside, June 2016
Dr. James G. Baldwin, Chairperson

Historically, nematode systematics has been heavily driven by morphology, and for many groups, morphology-based classifications are the basis for establishing species relationships. Plant parasitic nematodes of the family Tylenchidae are a great example; they are highly diverse but their phylogenetic relationships remain poorly studied. Molecular phylogenetic analyses of Tylenchidae, including the redescribed *Filenchus annulatus*, differ in results depending on the gene: 28S ribosomal RNA (rRNA) gene strongly supports *Filenchus* as monophyletic, whereas 18S rRNA shows *Filenchus* as polyphyletic. Relationships between *Filenchus* and other Tylenchidae genera are also gene dependent. Molecular phylogenies also suggest that *Cephalenchus* and *Eutylenchus* belong to a separate clade other than Tylenchidae. rRNA phylogenies often assume concerted evolution, so that intraspecific polymorphism for these genes is expected to be eliminated. This phenomenon is further explored in the genus *Cephalenchus*, another

intriguing genus within Tylenchomorpha. Sequence variation of 28S and ITS rRNA genes suggest that not all *Cephalenchus* species undergo concerted evolution. High levels of intraspecific polymorphism are found in *Cephalenchus* sp1 (BRA-01). Secondary structure analyses suggest the functionality of these rRNA copies (*i.e.* not a pseudogene); and potential cross-fertilization in some *Cephalenchus* species, might contribute to both intragenomic and intraspecific polymorphism. These results reinforce the implications of intragenomic and intraspecific genetic diversity on species delimitation, especially in studies based solely on molecular approaches. The phylogenetic position of *Cephalenchus* within Tylenchomorpha is further investigated based on 26 populations (11 species) sampled worldwide. Molecular analyses based on three rRNA genes and different alignment methods always supported *Cephalenchus* as a monophyletic group. A sister relationship between *Cephalenchus* and *Eutylenchus* is also recovered; branch support for this relationship varies depending on the method used. Placement of *Cephalenchus* + *Eutylenchus* within Tylenchidae is not supported by 18S and 28S genes; nevertheless, the position of both taxa within Tylenchomorpha remains ambiguous and highlights the importance of sampling additional genes and taxa. Within *Cephalenchus*, amphid opening morphology shows congruence with molecular-based phylogenetic relationships. All three rRNA genes support the non-monophyly of four morphologically defined *Cephalenchus* species. In light of this evidence, recommendations for the synonymization of *Cephalenchus* species are given.

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General Introduction

Phylum Nematoda: a brief overview

Nematodes (Phylum Nematoda), as all major ecdysozoan lineages, are considered to be ancient (Rota-Stabelli *et al.*, 2013), with a possible late-Ediacaran/early-Cambrian origin [~ 587-543 million years ago (mya)]. Molecular analyses of Ecdysozoa based on multiple datasets also suggest that diversification within Nematoda[1] (*i.e.* split between the major classes, Enoplea and Chromadorea) might have taken place in the early Silurian ~ 442 mya (Rota-Stabelli *et al.*, 2013). This early origin has allowed nematodes to diversify and colonize a wide range of niches and ecosystems, and in this regard it has become an evolutionary successful group (Blaxter, 1998; Bongers and Ferris, 1999; De Ley, 2006). These organisms present an amazing diversity of life styles including free-living and parasitic (*i.e.* of animals and plants) species (Blaxter *et al.*, 1998; Baldwin *et al.*, 2004a; Ottesen *et al.*, 2008).

The first comprehensive molecular phylogeny of Nematoda was established by Blaxter *et al.* (1998) where, based on 18S rDNA of 53 taxa, they identified five major clades comprising the phylum. Moreover, the study suggested extensive morphological convergence and that parasitism (*e.g.* of plants and animals) arose independently multiple times within the phylum. Additional molecular phylogenies of Nematoda, also based on the 18S gene (Holterman *et al.*, 2006; Meldal *et al.*, 2007; Van Megen *et al.*, 2009), have

[1] Taxonomic references herein adhere to the classification of Nematoda by De Ley and Blaxter (2002), unless otherwise stated in the text.

further resolved some conflicting relationships and consequently recognized additional major clades.

Van Megen *et al.* (2009) presented what is so far the largest molecular phylogeny (also based on 18S gene) for the phylum Nematoda, including 1215 nematode sequences. This study also recognized 12 major clades, as proposed by Holterman *et al.* (2006), but with additional subdivisions. For example, the infraorder Tylenchomorpha [clade 12 in Holterman *et al.* (2006)], which represents most of the important plant-parasitic species, was further divided by Van Megen *et al.* (2009) to include 12A (including mostly species of fungal or root hairs feeders) and 12B (specialized economically important endo- and ecto- plant parasites).

Plant parasitic nematodes

The infraorder Tylenchomorpha includes most plant-parasitic nematodes (PPN) as well as some less-studied species presumed to be fungal feeders, root-hair feeders, and parasites of insects. The main morphological feature characterizing Tylenchomorpha is the presence of a stylet in the anterior region (*i.e.* stoma) that is used to pierce surface and deep plant tissues (Fig. 0.1). Typically the stylet (*i.e.* stomatostylet) consists anteriorly of a tapering cone, followed posteriorly by a cylindrical shaft and one dorsal and two subventral stylet knobs (Baldwin *et al.*, 2004b). A lumen through the stylet allows ingestion of host cytoplasm, whereas the knobs provide a point of attachment for protractor muscles. Some classical ideas of relationships of Tylenchomorpha (*i.e.* based

exclusively on morphology) suggest transformations of feeding structures from the open stoma of microbivores to a piercing stomatostylet of parasites (Ragsdale and Baldwin, 2010). On this basis some authors have hypothesized that the ancestor for all tylenchs should exhibit a small or weakly developed stylet [*e.g.* the genus *Psilenchus*; (Ryss, 1993)].

In Tylenchomorpha, molecular phylogenetic studies have been focused mostly on agriculturally important plant parasites such as the cyst (*Globodera* and *Heterodera* spp.), root-knot (*Meloidogyne* spp.), and lesion (*Pratylenchus* spp.) nematodes. However, these phylogenies often ignore the wide diversity of non-plant parasites (*i.e.* no agricultural pests) within Tylenchomorpha such as representatives of the family Tylenchidae (Holterman *et al.*, 2006; Subbotin *et al.*, 2006; Bert *et al.*, 2008; Hunt *et al.*, 2012). Nevertheless, information and taxonomic resolution of the infraorder Tylenchomorpha requires broader representation, including these little known groups (Bert *et al.*, 2010; Bert *et al.*, 2011; Atighi *et al.*, 2013; Qing *et al.*, 2015b).

Such resolution is crucial to fully understanding the phylogeny of Tylenchomorpha as well as the diversity of complex feeding traits of PPN. It is also essential to testing the classical hypothesis of a transition from fungivorous lifestyles to facultative plant-parasitism culminating in obligatory plant parasites. According to Bert *et al.* (2011), new efforts on the families Tylenchidae, Psilenchidae, and Belonolaimidae are crucial to achieve these goals. Finally, this broader resolution is essential to developing a strong phylogenetic framework for the entire group and specifically to understanding the evolution of plant parasitism in nematodes.

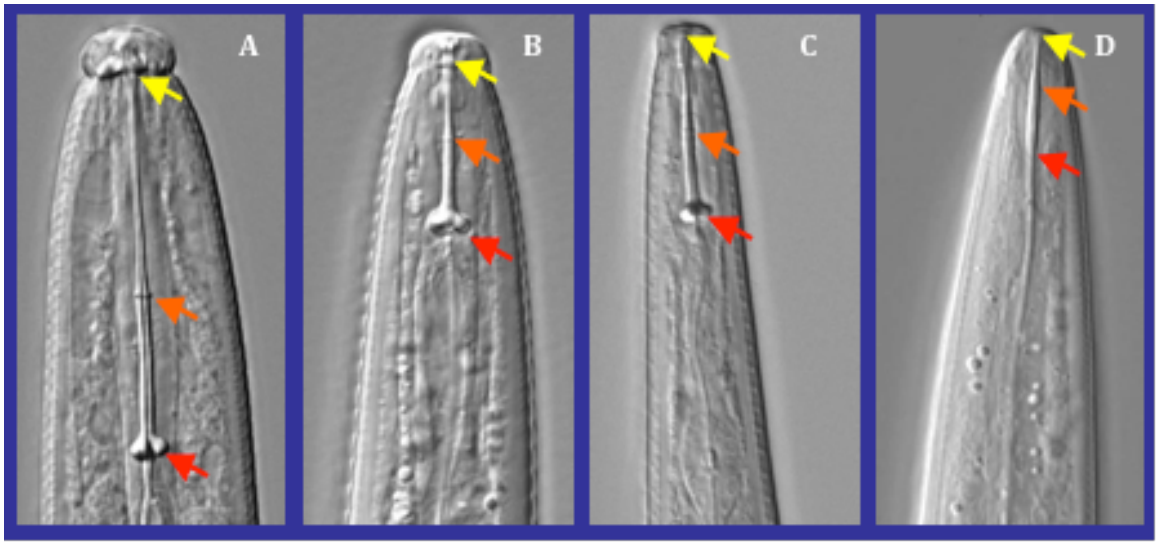

Figure 0.1. Light microscope (LM) photographs showing the anterior region of some Tylenchomorpha genera. Stylet components include knobs (red arrow), shaft (delimited by orange arrow) and conus (delimited by yellow arrow). **A.** *Dolichodorus*. **B**. *Tylenchorhynchus*. **C.** *Cephalenchus*. **D.** *Psilenchus*. Note that in *Psilenchus* stylet knobs are reduced.

In general, molecular phylogenies focusing on Tylenchomorpha have shown congruent results. This is partially due to consistency in the molecular marker used, mostly 18S of the rRNA, as well as in the taxa sampled, relying heavily on species of agricultural importance (Bert *et al.*, 2008; Holterman *et al.*, 2009; Van Megen *et al.*, 2009). Interestingly, the superfamily Aphelenchoidea (fungal feeders and insect associates), which was incorporated into Tylenchomorpha by De Ley and Blaxter (2002) has been supported as paraphyletic: Aphelenchidae (*e.g. Aphelenchus avenae*) is consistently placed as sister of Tylenchomorpha whereas Aphelenchoididae (*e.g. Aphelenchoides*) is grouped with representatives of Panagrolaimomorpha. On the basis of mtDNA, Kim *et al.* (2015) recovered the monophyly of Aphelenchoidea (*A. avenae* + *Bursaphelenchus* spp.), however not as sister of Tylenchomorpha.

Based on the 28S region of rRNA, Subbotin *et al.* (2006) further explored relationships within Tylenchomorpha and introduced some controversy on the position of some Tylenchidae; specifically genera of the Tylenchidae (*i.e. Aglenchus*, *Coslenchus*, *Boleodorus*, and *Basiria*) were grouped together but not as sister to all other tylenchs as in Bert *et al.* (2008) and Holterman *et al.* (2006; 2009). Instead, representatives of the family Anguinidae as well as other insect-associated nematode species were recovered as earlier branching lineages. In addition, the genus *Psilenchus*, typically recognized under Tylenchidae, was grouped with strong support with genera of the subfamily Merliniinae *sensu* Siddiqi (2000).

As a general picture, it seems that most molecular phylogenies show economically important plant-parasite nematodes nested within clades of fungal-feeding Tylenchomorpha. However, these phylogenies have been based on a single gene approach, either the 18S or 28S gene. Thus, some relationships within Tylenchomorpha remain unresolved, especially at the lower taxonomic ranks (*i.e.* family and genus level). In order to rigorously test these hypotheses, a more comprehensive phylogeny is required, especially including underrepresented taxa of Tylenchidae as well as DNA sequences from multiple genes.

Nematode morphology and taxonomy

Nematode systematics and taxonomy has been strongly based on morphological features (De Ley, 2000; De Ley *et al.*, 2005), mostly through the use of light microscopy (LM) and scanning electron microscopy (SEM). Using LM, the internal morphology of these organisms can be observed and measured to obtain qualitative and quantitative data; and SEM techniques, while primarily limited to qualitative aspects of surface structures, can be extremely useful for resolving cuticle patterns, labial features, and minute details including surface expressions of sense organs (*e.g.* amphids, deirids, and phasmids; Fig. 0.2). Nematode species boundaries are thus classically defined on the basis of these qualitative and quantitative characters (Siddiqi, 2000).

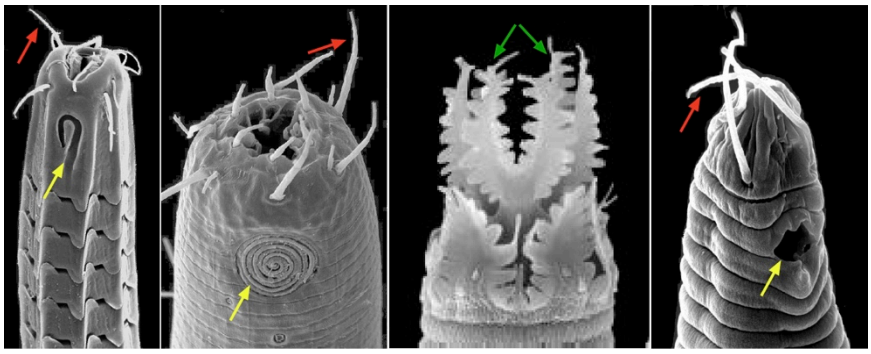

Figure 0.2. Scanning Electron Micrographs (SEM) showing morphological diversity of the anterior region of some nematodes. Left to right: marine nematodes *Ceramonema* sp. and *Paracanthonchus* sp.; terrestrial nematodes *Acrobeles* sp. and *Chronogaster* sp. Sensory organs such as amphid openings (yellow arrow) and setae (red arrow) are shown. *Acrobeles* sp. expresses modifications of the lips, designated probolae (green arrow).

Although morphological information is relevant to species diagnostics, these traditional characters are in many cases homoplasious features, and such convergence often confounds interpretation of evolution (Ragsdale and Baldwin, 2010). Recognition of the extent of such morphological convergence (*e.g.* feeding apparatus) was among the most striking outcomes from the introduction of molecular phylogeny to the phylum. These molecular phylogenies further highlighted convergence of "life style" including, for example, that plant parasitism arose independently at least three times (Blaxter *et al.*, 1998; Bert *et al.*, 2011). Often naïve understanding of morphology, including convergence, has compromised, the interpretation of true relationships among nematode species (phylogeny). Unfortunately, poor morphologically-based species descriptions have also accumulated, making nematode taxonomy and systematics unstable and unreliable. This is particularly true for less-studied tylench nematodes, specifically those Tylenchomorpha that are not considered agricultural pests such as the family Tylenchidae.

In the last two decades, molecular phylogenetic and diagnostic approaches have been embraced by most nematologists (Blaxter *et al.*, 1998; De Ley and Blaxter, 2004, Holterman *et al.*, 2006; Subbotin *et al.*, 2006; Bert *et al.*, 2008; Van Megen *et al.*, 2009). As a result, new nematode species descriptions are increasingly supported by molecular evidence (Palomares-Rius *et al.*, 2009; Ragsdale *et al.*, 2011). Consequently, this molecular information not only serves as a molecular tag for nematode species but also helps to further explore the phylogenetic context. Trends in systematics of nematodes have significantly impacted molecular databases and the number of nematode DNA sequences and represented species continues to grow (De Ley and Blaxter 2004).

On the other hand, morphological studies testing such molecular hypotheses are still limited (Bert *at al.*, 2008; Subbotin *et al.*, 2008; Ragsdale and Baldwin, 2010). However, morphology is the primary interface of an organism with its environment with key implications for development and ecology, and as such, its study remains critical to understanding (Ragsdale and Baldwin, 2010). Therefore, a more robust phylogeny based on morphological and molecular approaches is needed to clarify relationships, and particularly within some of the little-studied groups of key phylogenetic importance, within Tylenchomorpha. Herein, species delimitation relies on independent evidence (*i.e.* morphology and molecules) and nematode species are defined as independent evolutionary (exclusive) lineages. Hypotheses of species are tested through evolutionary (phylogenetic) analysis (Nadler, 2002).

Review and contrast traditional Tylenchidae phylogeny

A beginning point for advancing systematics of Tylenchidae is provided by Siddiqi (1986, 2000) who draws primarily on the literature for a comprehensive morphology-based overview of the group (Table 0.1). More recently, Geraert (2008) compiled all species descriptions for Tylenchidae (about 400 spp.).

The work of Geraert (2008) is strictly taxonomic, in that he does not address relationships among taxa (including different taxonomic levels). However, the author does point out particular groups that need special attention, either owing to their large number of species (*e.g. Basiria* and *Filenchus*) or their ambiguous phylogenetic position

based on DNA sequences (*i.e. Cephalenchus*, *Malenchus*, and *Psilenchus*). Preliminary molecular phylogenetic analysis (18S and 28S genes, with limited representatives) suggests divergence of Tylenchidae near the root of the clades of major agricultural pathogens (Subbotin *et al.*, 2006). This is consistent with classical hypotheses that ascribe within Tylenchidae putative progenitors of the Tylenchomorpha [*e.g.* a *Psilenchus* like ancestor, Ryss (1993) and Siddiqi (2000)].

Priorities of this dissertation encompass a molecular analysis of the family Tylenchidae, including clades of genera that are putatively facultative fungal feeders (*e.g. Coslenchus*, *Filenchus*, etc.) in relation to other Tylenchomorpha. In this sense, the first chapter focuses on the genus *Filenchus*, one of the largest within Tylenchidae. Although, *Filenchus* is commonly found in terrestrial habitats, identification of species is often challenging (Hunt *et al.*, 2012). In this chapter, *F. annulatus* from Iran is redescribed based on morphological and molecular data. Additional *Filenchus* species as well as other Tylenchidae genera are included in the molecular analyses for phylogenetic context.

The resultant phylogenies, based on 18S and 28S rRNA genes, showed different scenarios with respect to the monophyly of *Filenchus*. On the other hand, the family Tylenchidae is not monophyletic regardless of the gene used. Nevertheless, some genera formerly designed as Tylenchidae, and representing different subfamilies (Tylenchinae: *Aglenchus*, *Coslenchus*, *Filenchus*, *Tylenchus*; Boleodorinae: *Basiria*, *Boleodorus*, *Neopsilenchus*) grouped together within each subfamily in the phylogenetic estimations, thus suggesting that these taxa might represent the formerly Tylenchidae (Geraert, 2008; Siddiqi, 2000).

Table 0.1. Morphology-based classification of the family Tylenchidae according to Geraert (2008). These nematode genera are either treated under a different family or subfamily by Siddiqi (2000). Highlighted taxa (**bold**) are commonly encountered in soil samples according to Hunt *et al.* (2012).

Siddiqi (2000)			Geraert (2008)	
Family	**Subfamily**	**Genus**	**Subfamily**	**Genus**
Tylenchidae	Tylenchinae	***Aglenchus***	Atylenchinae	***Aglenchus***
Tylenchidae	Duosulciinae	*Allotylenchus*	Tylenchinae	*Allotylenchus*
-	-	-	Atylenchinae	*Antarctenchus*[1]
Tylodoridae	Tylodorinae	*Arborityncnus*	Tylodorinae	*Arborityncnus*
-	-	-	Boleodorinae	*Atetylenchus*[1]
Atylenchidae	Atylenchinae	*Atylenchus*	Atylenchinae	*Atylenchus*
Tylenchidae	Boleodorinae	***Basiria***	Boleodorinae	***Basiria***
Tylenchidae	Boleodorinae	***Boleodorus***	Boleodorinae	***Boleodorus***
Tylodoridae	Pleurotylenchinae	*Campbellenchus*	Tylodorinae	*Campbellenchus*
Tylodoridae	Pleurotylenchinae	*Cephalenchus*	Tylodorinae	*Cephalenchus*
Tylenchidae	Tylenchinae	*Cervoannulatus*	Tylenchinae	*Cervoannulatus*
Tylenchidae	Ecphyadophoroidinae	*Chilenchus*	Ecphyadophorinae	*Chilenchus*
Tylenchidae	Tylenchinae	***Coslenchus***	Atylenchinae	***Coslenchus***
Tylenchidae	Tylenchinae	*Cucullitylenchus*	Tylenchinae	*Cucullitylenchus*
Tylenchidae	Tylenchinae	*Discotylenchus*	Tylenchinae	*Discotylenchus*
Tylenchidae	Duosulciinae	*Duosulcius*[2]	-	-
Tylenchidae	Ecphyadophorinae	*Ecphyadophora*	Ecphyadophorinae	*Ecphyadophora*
Tylenchidae	Ecphyadophoroidinae	*Ecphyadophoroides*	Ecphyadophorinae	*Ecphyadophoroides*
Tylenchidae	Ecphyadophoroidinae	*Epicharinema*	Ecphyadophorinae	*Epicharinema*
Atylenchidae	Eutylenchinae	*Eutylenchus*	Tylodorinae	*Eutylenchus*
Tylenchidae	Tylenchinae	***Filenchus***	Tylenchinae	***Filenchus***
Tylenchidae	Tylenchinae	*Fraglenchus*	Tylenchinae	*Fraglenchus*
Tylenchidae	Epicharinematinae	*Gracilancea*	Tylenchinae	*Gracilancea*
Tylenchidae	Tylenchinae	*Irantylenchus*	Tylenchinae	*Irantylenchus*
Tylenchidae	Ecphyadophoroidinae	*Lelenchus*	Ecphyadophorinae	*Lelenchus*
Tylenchidae	Duosulciinae	*Malenchus*	Tylenchinae	*Malenchus*
Tylenchidae	Duosulciinae	*Miculenchus*	Tylenchinae	*Miculenchus*
Tylenchidae	Ecphyadophoroidinae	*Mitranema*	Ecphyadophorinae	*Mitranema*
Tylenchidae	Duosulciinae	*Mukazia*[3]	-	-
Tylenchidae	Boleodorinae	*Neopsilenchus*	Boleodorinae	*Neopsilenchus*
Tylenchidae	Thadinae	*Neothada*	Boleodorinae	*Neothada*
Tylenchidae	Duosulciinae	*Ottolenchus*[2]	-	-
Tylodoridae	Pleurotylenchinae	*Pleurotylenchus*	Atylenchinae	*Pleurotylenchus*
Tylenchidae	Tylenchinae	*Polenchus*	Tylenchinae	*Polenchus*
-	-	-	Boleodorinae	*Psilenchus*[1]
Tylenchidae	Duosulciinae	*Ridgellus*	Boleodorinae	*Ridgellus*
Tylenchidae	Tylenchinae	*Sakia*	Tylenchinae	*Sakia*
Tylenchidae	Tanzaniinae	*Tanzanius*	Tylenchinae	*Tanzanius*
Tylenchidae	Ecphyadophoroidinae	*Tenunemellus*	Ecphyadophorinae	*Tenunemellus*
Tylenchidae	Thadinae	*Thada*	Boleodorinae	*Thada*
Tylenchidae	Ecphyadophoroidinae	*Tremonema*	Ecphyadophorinae	*Tremonema*
Tylenchidae	Tylenchinae	***Tylenchus***	Tylenchinae	***Tylenchus***
Tylodoridae	Tylodorinae	*Tylodorus*	Tylodorinae	*Tylodorus*
Tylenchidae	Ecphyadophorinae	*Ultratenlla*	Ecphyadophorinae	*Ultratenlla*
Tylenchidae	Duosulciinae	*Zanenchus*[2]	-	-

[1] These genera are treated under the family Psilenchidae in Siddiqi (2000).
[2] These taxa are treated as synonyms of *Filenchus* in Geraert (2008).
[3] This taxa is considered a synonymy of *Malenchus* in Geraert (2008).

In the second chapter, the implications of delimiting species solely based on molecular data are discussed. Thus, the genus *Cephalenchus*, which has an ambiguous phylogenetic position within Tylenchomorpha, is further explored. *Cephalenchus* species (morphologically identified) are compared at three different levels: intragenomic, intraspecific, and interspecific. Data from two rRNA genes (28S and ITS) suggests that not all *Cephalenchus* species undergo concerted evolution; in fact some species were characterized by high levels of sequence divergence in their rRNA repeats, which turned out to be mostly due to intragenomic (*i.e.* intra-individual) variation. Different approaches, including phylogenetic analyses, rRNA secondary structure analyses, as well as morphological observations of the female reproductive system, are used to explain these findings. The evidence suggests that polymorphism in the rRNA of *Cephalenchus* can be extremely high, and that formation of pseudogenes is unlikely to be responsible for such high levels of sequence variation. Therefore, caution is needed when defining species solely on molecular basis, particularly so when these sequences are not linked to morphological vouchers.

Presented in the third chapter is a thorough analysis of the genus *Cephalenchus* including morphological data from 26 different populations, representing 11 nominal species, sampled worldwide. In addition to the LM and SEM morphological work, 20 *Cephalenchus* populations are included in the molecular phylogenetic analyses. Phylogenetic reconstructions are based on three rRNA genes (*i.e.* 18S, 28S, ITS) as well as on a combined dataset. Different alignment procedures are employed to specifically evaluate the monophyly of *Cephalenchus*, and to test a morphology-based proposed sister

relationship with the genus *Eutylenchus* as well as the phylogenetic position of such a clade (*i.e. Cephalenchus* + *Eutylenchus*) within Tylenchomorpha.

In all molecular analyses (full and reduced alignments), *Cephalenchus* is recovered as a monophyletic group. Furthermore, branch support for a clade of *Cephalenchus* is usually high, particularly so when extremely divergent taxa are excluded from the analyses. A sister relationship between *Cephalenchus* and *Eutylenchus* is also recovered in most of the analyses, however, branch support for this sister relationship varies considerably, which seems to be more sensitive to both taxon sampling and removal of sites from the alignment. Although, a sister relationship between *Cephalenchus* and *Eutylenchus* remains plausible, as suggested by the molecular analyses, their relation with respect to other Tylenchomorpha is still unsolved and needs further investigation. The third chapter also discusses the morphological variation across *Cephalenchus* species, more specifically in the tail length as well as labial region. Integrating morphological and molecular data provides a basis for the synonymization of some *Cephalenchus* species.

Chapter 1

Redescription of *Filenchus annulatus* (Siddiqui & Khan, 1983) Siddiqi, 1986 based on specimens from Iran with contributions to the molecular phylogeny of the family Tylenchidae[2]

ABSTRACT

Filenchus annulatus (Siddiqui & Khan, 1983) Siddiqi, 1986 is redescribed and males are characterized for the first time based on a population found in Northern Khorasan province, Iran. New morphological characterization is based on light and scanning electron microscopy. In addition, molecular analyses based on 18S and 28S genes are included to test monophyly of the genus. Females from the Iranian population have a spermatheca typically filled with sperm. Generally males are similar to females, ranging from 306 to 426 μm long. Spicules are arcuate, cephalated and 11.5-14.0 μm long; the gubernaculum is minute and trough-shaped and caudal alae are adanal. Phylogenetic analyses differed in results depending on the gene used: 28S gene strongly supports *Filenchus* as monophyletic whereas 18S shows *Filenchus* as polyphyletic. In both gene phylogenies, *F. annulatus* is placed as a sister taxon of *F. quartus* from Wyoming, USA. Although, sequence divergence between these two species is only three base pairs and

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one base pair for 28S and 18S genes, respectively, strong morphological differences support their species status. Relationships between *Filenchus* and other Tylenchidae genera are also gene dependent. Such differences in tree topologies and branch support are related with the number of *Filenchus* species used in the analyses (greater for 18S gene) and gene resolution (greater for 28S gene). Molecular phylogenies also suggest that other Tylenchidae genera (*i.e. Psilenchus*, *Cephalenchus*, and *Eutylenchus*) belong to separate clades, as is also suggested by some morphology-based classifications. The inclusion of more taxa and perhaps additional genes is needed to further clarify Filenchus relationships and to further test its monophyly.

INTRODUCTION

The genus *Filenchus* Andrássy, 1954 belongs to the family Tylenchidae Örley, 1880 which includes five subfamilies, Boleodorinae, Duosulciinae, Tanzaniinae, Thadinae, and Tylenchinae (Siddiqi, 2000). Besides Boleodorinae and Tylenchinae, Geraert (2008) recognizes three other subfamilies under Tylenchidae: Atylenchinae, Ecphyadophorinae and Tylodorinae. On the other hand, De Ley and Blaxter (2002), using molecular data to revise Nematoda phylogeny, did not recognize lower taxonomic levels under Tylenchidae (*sensu* De Ley & Blaxter, 2002). This omission underscores the need for further molecular studies to resolve relationships within this family especially including specious genera such as *Filenchus*.

Tylenchidae is mostly comprised of small tylenchid nematodes bearing a relatively short stylet and an elongated-filiform tail. Based on the weak stylet, it has been suggested that Tylenchidae representatives mostly feed on algae, lichens, mosses, and root surfaces and therefore are not considered agricultural pests (Siddiqi, 2000). However, few studies have tested such feeding hypotheses. In the case of *Filenchus*, Okada *et al.* (2002; 2005) showed that some species can reproduce by feeding on fungi present in the soil. On that basis, the authors considered that nematodes in the genus *Filenchus* should be classified as fungal feeders (including root associated fungi) rather than only plant feeders thus bringing new insight to nematode soil ecology (Bert *et al.*, 2010).

According to Geraert (2008), a majority of *Filenchus* species are synonyms previously described under other genera of Tylenchidae including *Tylenchus* Bastian, 1865, *Ottolenchus* Husain and Khan, 1967, and *Lelenchus* Andrássy, 1954. Siddiqi (2000), defining *Filenchus* as having only four incisures in the lateral field, lists 55 valid species for the genus. In a more recent review, Geraert (2008) follows Raski and Geraert (1987) in defining *Filenchus* as having two, three or four incisures in the lateral field and, by including this criterion, lists 95 valid species. This makes *Filenchus* by far the most diverse genus in the family Tylenchidae.

Unfortunately, descriptions of many of these species are based on few specimens, with inadequate morphological descriptions, and with types that are no longer available as well as for localities that are altered or inaccessible. Clearly, descriptions of some species must be reviewed and extended; this may become possible with surveys and discovery of new populations of a given species. The process is further improved by new

tools including molecular approaches that support testing hypotheses of monophyly of existing genera and by new understanding of phylogenetic relationships (Carta *et al.*, 2010), especially for those genera such as *Filenchus* which is not clearly resolved taxonomically under light microscopy alone (Bert *et al.*, 2010). Herein, we present the case of *F. annulatus* that was originally described as *Lelenchus annulatus* by Siddiqui and Khan (1983b) and later transferred to the genus *Filenchus* by Siddiqi (1986). This description was based on morphological characters of only five females recovered from the rhizosphere of potato (*Solanum tuberosum*) from Ooti, Tamil Nadu, India; male specimens were unavailable.

In this study, we redescribe *F. annulatus* based on morphology of 20 females and 15 males of a new population of the species from Iran. Morphology is evaluated based on both light microscopy (LM) and scanning electron microscopy (SEM). We further characterize the Iran population of *F. annulatus* based on 18S (SSU, small subunit) and 28S (LSU, long subunit) rDNA sequences and relative to additional *Filenchus* species from the USA including representative isolates from California, Utah, and Wyoming. For phylogenetic context, additional Tylenchidae genera (*Aglenchus, Basiria, Boleodorus,* and *Neopsilenchus*) are also included in the analyses.

Previous molecular phylogenies including *Filenchus* species were based only on 18S rDNA sequences and have shown a close relationship of *Filenchus* with either *Tylenchus* or *Coslenchus* (Bert *et al.*, 2008; Holterman *et al.*, 2009; Van Megen *et al.*, 2009) as well as being a polyphyletic genus (Bert *et al.*, 2010). The family Tylenchidae was considered paraphyletic in both studies. The present work is based on the 18S and

28S rDNA genes; the later is thought to be more variable (rapidly evolving) than the former. Therefore we believe the 28S gene could be more informative to solve relationships at the genus and species levels and thereby further test hypotheses of the monophyly of the genus *Filenchus*. Moreover, a comparison between the two genes provides a basis for further testing phylogenetic relationships within Tylenchidae.

MATERIALS AND METHODS

Sampling and nematode extraction

Soil was sampled in September, 2010 from the rhizosphere of *Prunus* sp. in the Esfarayen region (37º 02.138' N 57º 29.930' E) of Northern Khorasan Province, in northeastern Iran. *Filenchus* samples from the USA were collected in June, 2009 from Bolsa Chica, California (BC: 33º 42.678' N 118º 03.327' W), September, 2009 from Yellowstone National Park, Wyoming (YLS BY-04: 44º 52.960' N 110º 44.074' W; YLS BY-07: 44º 57.372' N 110º 42.745' W; YLS PY-09: 44º 52.940' N 110º 44.078' W) and Salt Lake City, Utah (SLC: 41º 29.221' N 112º 3.33 W). Nematodes were extracted from soil either by using Baermann funnels or the sugar-flotation method (Jenkins, 1964; Viglierchio and Schmitt, 1983).

Identification of isolates from the USA and Mexico

From each USA *Filenchus* population we selected and measured ten adult females for LM morphological identification to species. The population from Bolsa Chica, California, designated as *Filenchus* sp1, was an exception. In this case, specimens were only available for DNA procedures, although photo vouchers made prior to DNA extraction were sufficient for genus identification. For all other isolates morphometrics were based on the features mentioned above. In addition, if the population included males, at least five of these were measured. *Filenchus* populations from the USA were identified as follows: Wyoming State: YLS BY-07, *F. quartus* (Szczygiel, 1969) Lownsbery & Lownsbery, 1985; YLS BY-04, *F. thornei*; YLS PY-09, *F. sindhicus* Shahina & Maqbool, 1994; Utah: SLC, *F. vulgaris* (Brzeski, 1963) Lownsbery & Lownsbery, 1985 (Table 1.1). Henceforth, *Filenchus* populations are solely referred to by the corresponding species name.

Additional Tylenchidae representatives were identified to genus using published descriptions and especially the key of Geraert (2008), and populations likely to be different species of a single genus, (also based on 28S data) were differentiated by "sp" followed by numbers (Table 1.1). Since, De Ley and Blaxter (2002) do not recognize subfamilies and genera for the family Tylenchidae, we contrast the taxonomic classifications proposed by Siddiqi (2000) and Geraert (2008) in order to discuss phylogenetic relationships of the group.

Table 1.1. Newly sequences for Tylenchidae genera and *F. annulatus* used for phylogenetic analyses. Sampling sites for *Filenchus* specimens are followed by locality code numbers.

Genus/Species*	Specimen ID	Sampled sites	State Country	28S Accession	18S Accession
Aglenchus sp1	9T11F09	Ensenada[1]	BN, Mexico	JQ004997	-
Aglenchus sp1	2T11M08	Riverside[1]	CA, USA	JQ004996	-
Basiria sp1	9T07H09	Cuarnavaca	MR, Mexico	JQ004999	-
Basiria sp2	16T05J09	Yellowstone National Park	WY, USA	JQ005000	-
Basiria sp3	1T11M08	Riverside	CA, USA	JQ004998	-
Boleodorus sp1	10T11M08	Riverside	CA, USA	JQ005001	-
Boleodorus sp2	3T05J09	Yellowstone National Park	WY, USA	JQ005003	-
Boleodorus sp3	15T07H09	Cuarnavaca	MR, Mexico	JQ005002	-
Boleodorus sp4	4T26F09	Coachella Valley	CA, USA	JQ005021	-
Coslenchus sp1	18T05J09	Yellowstone National Park [2]	WY, USA	JQ005007	-
Coslenchus sp2	11T28I09	Yellowstone National Park	WY, USA	JQ005004	-
Coslenchus sp3	14T28I09	Yellowstone National Park	WY, USA	JQ005008	-
Coslenchus sp4	7T11G09	Bolsa Chica[3]	CA, USA	JQ005005	-
Coslenchus sp4	7T03F09	Santa Rosa Plateau Reserve[3]	CA, USA	JQ005011	-
Coslenchus sp4	2T07H09	Cuarnavaca[3]	MR, Mexico	JQ005006	-
Coslenchus sp5	6T12H09	Guasave	SI , Mexico	JQ005010	-
Filenchus annulatus	3T08B11	Esfarayen, (ESF)	KS, Iran	JQ005017	JQ814880
Filenchus quartus	1T28I09	Yellowstone National Park, (YLS BY-07)	WY, USA	JQ005016	-
Filenchus quartus	2T28I09	Yellowstone National Park, (YLS BY-07)	WY, USA	-	JQ814879
Filenchus sindhicus	11T05J09	Yellowstone National Park, (YLS PY-09)	WY, USA	JQ005012	JQ814875
Filenchus sp1	6T11G09	Bolsa Chica, (BC)	CA, USA	JQ005015	JQ814876
Filenchus thornei	21T16I09	Yellowstone National Park, (YLS BY-04)	WY, USA	JQ005014	JQ814878
Filenchus vulgaris	32T16I09	Salt Lake City, (SLC)	UT, USA	JQ005013	JQ814877
Neopsilenchus sp1	12T11G09	Bolsa Chica	CA, USA	JQ005018	-
Neopsilenchus sp2	5T11G09	Santa Rosa Plateau Reserve	CA, USA	JQ005019	-
Neopsilenchus sp3	15T11G09	Cuarnavaca	MR, Mexico	JQ005020	-

* Genera are classified under Tylenchidae by Siddiqi (2000) and Geraert (2008).
[1] Identical D2-D3 sequences within the genus *Aglenchus*.
[2-3] Identical D2-D3 sequences within the genus *Coslenchus*.

LM and SEM procedures for *F. annulatus*

Specimens were initially washed in distilled water to remove any debris attached to the cuticle; they were heat-killed at 65ºC for 2.0 min, and then fixed in 5% formaldehyde solution. For permanent slide mounts, specimens were prepared by dehydration in a graduated series of glycerin/ethanol solutions to pure glycerin (Seinhorst, 1959). Permanent slides were mounted and examined under a Nikon Eclipse E600 microscope for morphometrics. Additional slides were examined using a Zeiss Axioskop microscope equipped with a drawing tube. Initial camera lucida drawings were scanned as a basis for preparing plates using Adobe Illustrator® CS4 (version 14) software. Photomicroscopy was carried out using Openlab® (version 5.0) software and a digital camera (RT-Color Spot®, Diagnostic Instruments, inc.) coupled to the compound microscope. Photographs were saved as Tiff files and later developed as plates using Adobe Photoshop® CS4 (version 11). Identification of *F. annulatus* was based on the original description (Siddiqui and Khan, 1983b) supplemented by a key to Tylenchidae (Geraert, 2008).

Measurements and ratios (Table 1.2) included were partly determined by relevance for comparison with previous descriptions of *Filenchus* species. Abbreviations primarily were as defined by Siddiqi (2000): L (body length); a (L/maximum body width); b (L/pharyngeal length); c (L/tail length); c' (tail length/body width at anus); V (distance from head to vulva/L); T (distance from cloaca to anterior end of testes/L); MB (distant from anterior end of body to center of median bulb as % of pharyngeal length);

excretory pore to anterior end of body; pharynx (from anterior end to pharyngeal-intestinal valve).

For SEM, specimens available for this purpose were limited to a few females. These were repeatedly rinsed in distilled water for 5 min to remove all traces of formalin and then post-fixed for 4 h in 2.0% osmium tetroxide. Post-fixed specimens were dehydrated through a series of aqueous dilutions of 20-100% ethanol. Dehydrated specimens were critical point dried in a Tousimis (Rockville, MD, USA) Autosamdri-810®. Specimens were mounted on double-sticking copper tape attached to aluminum stubs, coated for 1-3 min with a 25 nm layer of gold palladium in a Cressington (Watford, UK) 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).

DNA extraction, amplification, and sequencing

DNA was extracted from single individuals using proteinase K protocol and Worm Lyses Buffer (WLB). Each nematode was placed in a drop containing 5 µl of WLB and 2 µl of proteinase K (10 mg/ml), cut in pieces, and transferred to a 0.2 mL PCR tube with an additional 15 µl of WLB. Samples were incubated for 1 h at 65 º C followed by 10 min at 95 º C. The D2-D3 domains of the 28S rDNA gene were amplified by the polymerase chain reaction (PCR) with the primers D2Ab (forward) and D3B (reverse) (De Ley *et al.*, 2005) in combination with Pure Taq-Ready to Go kit (GE Health Care®). PCR reactions of 25 µl were made with 5 µl of DNA template, 0.2 µl of primers (20 µM),

and 19.6 µl of PCR purified water. In addition, the 18S rDNA gene was amplified for all *Filenchus* species using three overlapping sets of primers (G18S4 and 4R; 22F and 13R; 4F and 18P; Blaxter *et al.*, 1998; Bert *et al.*, 2008). Prior to 18S amplification, DNA extract from the same individuals was subjected to a GenomiPhi protocol (GenomiPhi V2 DNA Amplification Kit, GE Health Care®) to increase the amount of DNA in the samples. For *F. quartus*, an additional specimen was used (specimen 2T28I09 instead 1T28I09, Table 1.1) owing to problems in the sequencing process. These two specimens had identical 28S sequences; therefore we also would expect identical sequences for the 18S gene since this is a more conserved gene. Then, PCR reactions were performed as previously described. Amplification success was evaluated electrophoretically on 1% agarose gel. PCR products were purified for sequencing using the QIAquick PCR purification kit (Qiagen®) following the manufacturer's protocol. Finally, the 28S and 18S genes were sequenced in both directions with PCR primers using ABI-PRISM® Dye- DeoxyTerminator Big DyeTM v3.1 (Applied Biosystems Inc, CA) with an automatic sequencer Gene Analyzer ®ABI 3100 (Applied Biosystems Inc, CA) at the University of California, Riverside, Genomics Center.

Phylogenetic analyses

In order to evaluate the phylogenetic position of *F. annulatus* within the family Tylenchidae, 18S and 28S sequences from additional taxa were downloaded from GenBank. To these, we added *Filenchus* sequences from newly collected and sequenced

species from three localities in the USA as well as new sequences from additional Tylenchidae genera from Mexico and the USA (Appendix A). In total, sequences of 104 and 74 taxa for the 28S and 18S genes, respectively, were separately aligned on ClustalX 2.0 using the default parameters (Thompson *et al.*, 1997). Outgroup taxa for phylogenetic analyses (28S gene) were chosen according to the results of previous studies (Subbotin *et al.*, 2006, Palomares-Rius *et al.*, 2009). For the 18S gene, representatives of the family Aphelenchidae (*i.e. Aphelenchus avenae* and *Paraphelenchus acontioides*) were also included as outgroups (Megen *et al.*, 2009; Bert *et al.*, 2010). Additional phylogenetic analyses for the 28S gene (not shown) including Aphelenchidae as the outgroup did not result in significant differences in tree topology.

Phylogenetic relationships among sequences (18S and 28S datasets) were estimated with maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI). MP analysis was performed in PAUP* 4.0b10 using heuristic searches and TBR branch swapping to seek the most parsimonious trees (max. tree number = 1000). Gaps in the alignment were treated as missing data. Nonparametric bootstrap analysis (BS), 1000 pseudoreplicates, was used to assess branch support (Swofford, 1998). For ML analysis, we used a fast maximum likelihood method, RAxML-HPC Black Box (Randomized Axelerated Maximum Likelihood) through the server CIPRES (http://www.phylo.org/) under the GTR model. Gamma parameters were estimated from Log Likelihood units and bootstrap support (BS) values (stopped after 400 and 500 replicates for 28S and 18S genes, respectively) were automatically calculated for the best-scoring ML tree (Stamatakis, 2006). BI analysis was performed on MrBayes 3.1.2

(Huelsenbeck and Ronquist, 2001) under the GTR+I+G model with the settings: random starting tree, two independent runs with four chains (1.0×10^6 and 4.0×10^6 generations for 28S and 18S genes, respectively). Markov chains were sampled at intervals of 100 generations. The log-likelihood values of the sample points stabilized after approximately 1000 generations. A 50% majority rule consensus tree was generated and posterior probabilities (PP) were calculated for each clade. The best fit model of DNA evolution (28S and 18S dataset) for BI analysis was obtained using the program Modeltest 3.7 based on the Akaike Information Criterion in conjunction with PAUP* 4.0b10 (Swofford, 1998; Posada and Buckley, 2004).

RESULTS

Filenchus annulatus (Siddiqui & Khan, 1983) Siddiqi, 1986

Redescription of female

Body straight or slightly ventrally arcuate after fixation. Cuticle *ca* 1.0 µm thick, annuli 0.5-1.0 µm wide and widest near tail tip. Lateral field about one third of the maximum body width, and outer lines partly areolated (Table 1.2, Figs. 1.1-1.3). Head quadrangular, continuous with body contour in submedial positions, but deeply inset dorsally, ventrally and laterally, annulated (4-5 annuli as shown by SEM). Labial disc with stoma opening surrounded by openings of six labial papillae and demarcated from surrounding lip region by a deep invagination.

Table 1.2. Morphometrics for *Filenchus annulatus* isolated from Iran. All measurements are in µm and in the format: mean ± standard deviation (range)*.

Character	Iranian population		After Siddiqui and Khan (1983)
	Female (20)	Male (15)	Female (5)
L	402 ± 24.5 (370-450)	377 ± 37.9 (306-426)	380-410
a	33 ± 2.4 (28.7-37.4)	33.2 ± 2.5 (29.7-38.7)	30.7-36.6
b	4.9 ± 0.3 (4.5-5.6)	4.7 ± 0.5 (3.7-5.6)	5.4-5.7
c	5.3 ± 0.6 (4.3-6.7)	5.0 ± 0.2 (4.7-5.3)	3.08-4.2
c'	10.3 ± 1.6 (6.8-13.1)	9.4 ± 1.2 (7.5-11.3)	10
V or T	63 ± 2.1 (60.8-67.8)	32.9 ± 5.5 (25.8-44.1)	61.9-63.7
V'**	78 ± 1.3 (75.6-80.1)	-	84
Head height	2.0 ± 0.1 (1.5-2.0)	1.9 ± 0.2 (1.5-2.0)	-
Head width	4.4 ± 0.3 (4.0-5.0)	4.5 ± 0.3 (4.0-5.0)	-
Stylet length***	8.2 ± 0.7 (7.0-10)	7.7 ± 0.7 (7.0-9.0)	7-8
DGO position	1.2 ± 0.2 (1.0-1.5)	1.2 ± 0.2 (1.0-1.5)	
MB	47.1 ± 2.4 (42.5-51.1)	47.4 ± 2.8 (38.6-50.7)	41
Excretory pore	58.3 ± 2.5 (54-63)	56.3 ± 3.7 (51-65)	58
Pharynx	82 ± 4.6 (75-94)	79.9 ± 5.6 (71-91)	72
Head-vulva	253 ± 13.6 (233-285)	-	-
Maximum body width	12.2 ± 0.7 (11-14)	11.4 ± 1.0 (10-13)	-
Gonad length	95 ± 12.1 (72-120)	-	-
Postuterine sac (PUS)	6.6 ± 1.7 (4.0-11)	-	-
PUS/vulval body width	0.5 ± 0.1 (0.3-0.9)	-	-
Anal body width	7.6 ± 0.7 (6.5-9.0)	8.1 ± 0.8 (7.0-10)	-
Vulva – anus (V-a)	71.3 ± 6.9 (62-85)	-	-
Tail length	77.5 ± 10.9 (58-93)	75.9 ± 9.1 (60-91)	100
Tail/V-a	1.1 ± 0.2 (0.9-1.5)	-	1.9
Spicule length	-	12.8 ± 0.8 (11.5-14)	-
Gubernaculum length	-	4.6 ± 0.7 (3.5-6.0)	-

*Abbreviations according to Siddiqi (2000).
** Character measured on 21 specimens.
*** Value taken from Geraert (2008) for *F. annulatus* Siddiqui and Khan (1983).

Fused lips are enlarged submedially, lateral lips are reduced and enclose deep set, oval amphid apertures positioned near base of lip region. Cephalic framework delicate, but with distinct vestibule and vestibule extension. Stylet small with well-developed knobs posteriorly directed. Stylet *ca* 1.9 of body diameter at level of cephalic framework, conus shorter than shaft, *ca* 36.2 % of the entire stylet. Dorsal gland orifice opening about 1.2 µm ± 0.2 (1.0-1.5) µm from the base of stylet.

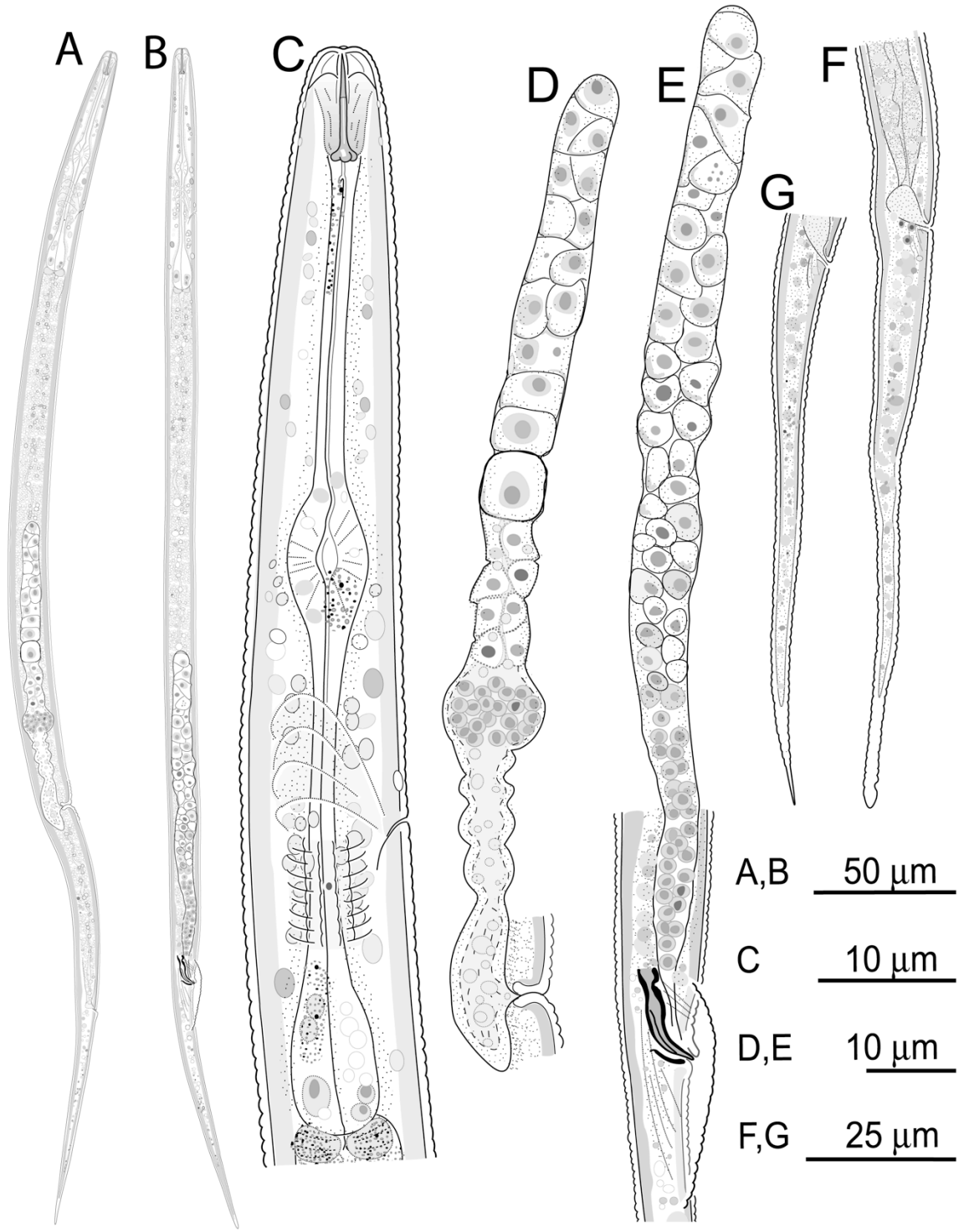

Figure 1.1. *Filenchus annulatus*, Iranian isolate. **A.** Entire female. **B.** Entire male. **C**. Anterior end, female. **D.** Reproductive tract including ovary. **E.** Reproductive tract including testis and portion of tail showing copulatory structures. **F.** Female tail with rounded tip. **G.** Female tail with pointed tip.

Neck region, from anterior to the base of pharynx, comprising *ca* 18-22% of total body length. Procorpus narrow cylindrical and posteriorly joining a fusiform, slightly muscular median bulb with small faintly cuticularized valve. Posterior glandular region bulbular, pyriform, not overlapping the intestine; pharyngeal-intestinal valve rounded, posterior to contour of basal bulb, and embedded in anterior end of intestine. Nerve ring encircling a portion of anterior half of isthmus. Deirids very small, at level of posterior region of isthmus, slightly posterior to level of excretory pore. Excretory pore slightly posterior to nerve ring position *ca* 51-65 µm from lip region, duct weakly cuticularized. Hemizonid distinct, *ca* 1.0-3.0 annuli anterior to excretory pore. Two cephalids faintly visible; respectively 2-4 annuli and 7-11 annuli from the base of the cephalic framework. Ovary single, straight, bluntly rounded at distal end, spermatheca nearly spherical and typically filled with rounded sperm, 11-15 µm long and 8.0-12 µm wide. Postuterine sac usually shorter than the corresponding body width. Vagina perpendicular to the body axis with a thin cuticular lining *ca* 40-51% of corresponding body diameter. Tail filiform with a sharp or minute rounded terminus, anus as a minute pore. Phasmids not observed with LM or SEM.

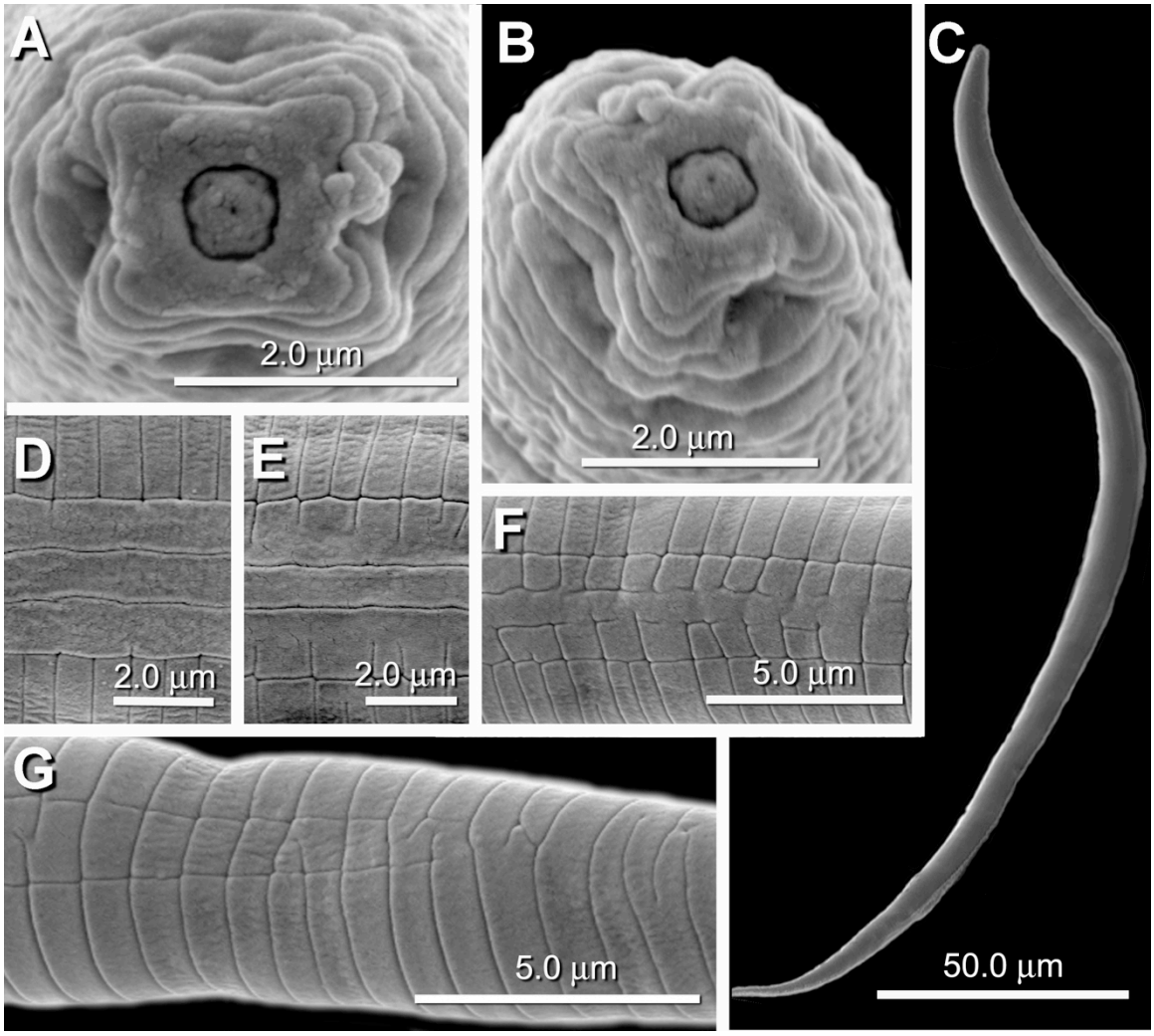

Figure 1.2. Scanning electron micrographs of females of *Filenchus annulatus*, Iranian isolate. **A.** En face view of lip region; dorsal is toward the top of the plate. **B**. Left subdorsal view of A. **C.** Left subventral view of entire female. **D.** Lateral field near midbody, note near lack of areolation. **E.** Lateral field slightly anterior to vulva, note areolation. **F.** Lateral field near anus. **G.** Posterior terminus of lateral field on tail.

Description of male

Generally similar to female. Body straight after fixation. Cuticle 0.8–1.0 µm thick, annuli 0.5-1.0 µm wide, and widest on tail. Lateral field partly areolated, about one third of corresponding body width. Head continuous, faintly annulated, labial disc as in

females, cephalic framework delicate, but with distinct vestibule and vestibule extension. Amphid apertures near base of lip region are deep set and oval. Stylet length *ca* 1.8 of body diameter at level of cephalic framework, conus shorter than shaft, about 37 % of the entire stylet. Stylet with well developed posteriorly directed knobs. Dorsal gland orifice opening 1.2 µm ± 0.2 (1.0-1.5) from the base of the stylet. Neck region comprising *ca* 18-27% of total body length. Procorpus narrow broadening posteriorly to join fusiform median bulb with delicate valvular apparatus. Posterior glandular region of pharynx bulbular, pyriform, not overlapping intestine; pharyngeal-intestinal valve rounded and embedded in anterior end of intestine. Nerve ring encircling a portion of anterior half of isthmus. Deirids very small at level of posterior region of isthmus and slightly posterior to level of excretory pore. Two cephalids as in females. Excretory pore 51-65 µm from lip region. Hemizonid distinct, *ca* 1.0-3.0 annuli anterior to excretory pore. Testes a single continuous tube, bluntly rounded at distal end, spermatogonia and spermatocytes primarily in two rows and proximally with vas deferens full of small rounded sperm. Cloaca opening protruding slightly. Spicules small, slender, slightly curved. Gubernaculum small, trough-shaped, caudal alae adanal with crenated margin and faintly annulated, *ca* 23-27 µm long. Tail filiform as in female. Phasmids not observed with LM or SEM.

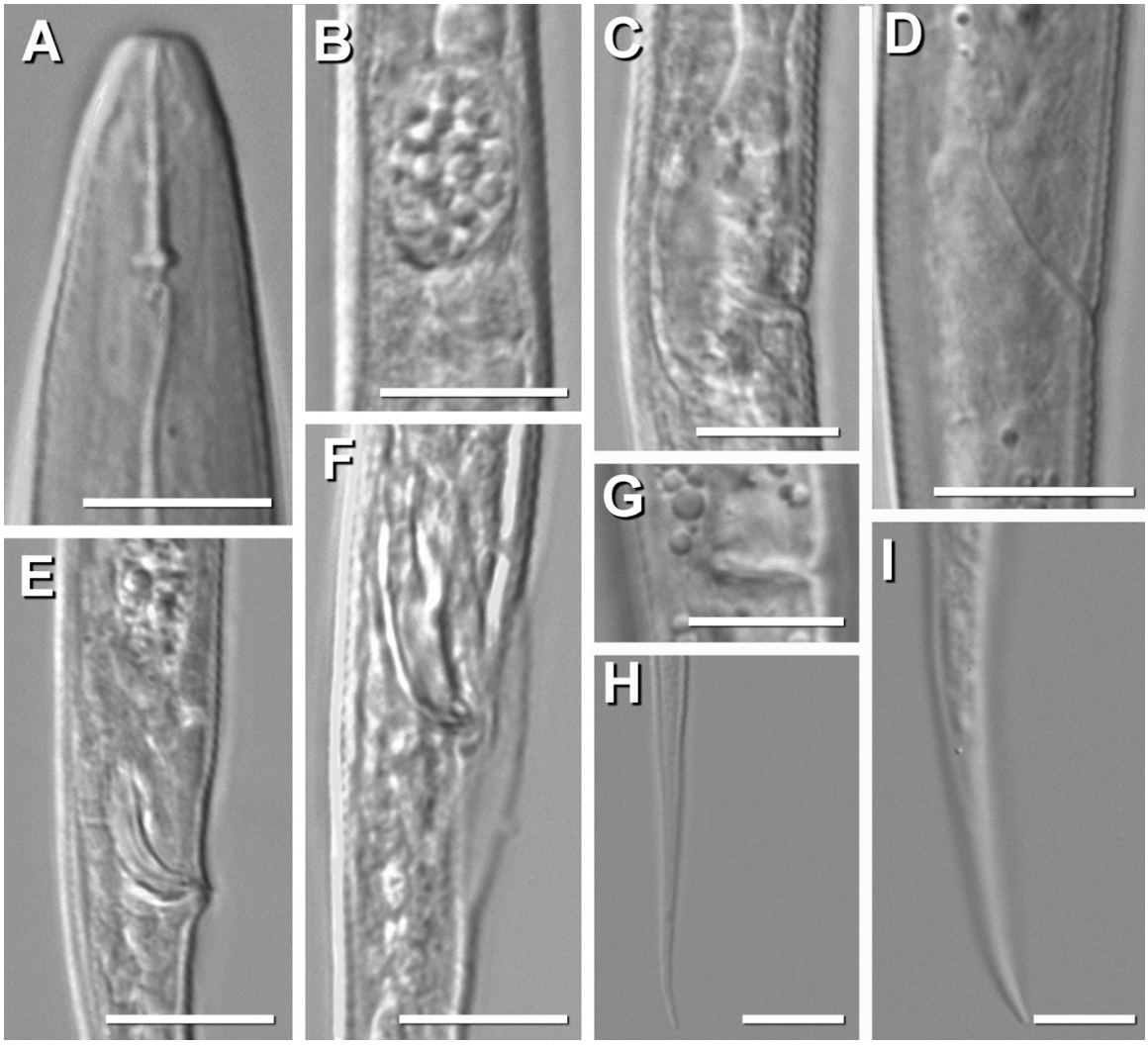

Figure 1.3. Light photomicrographs of right lateral views of *Filenchus annulatus*, Iranian isolate. Scale bars represent 10 µm. Females A-D, G-I; males E, F. **A.** Head including stylet and dorsal gland orifice. **B.** Spermatheca. **C.** Uterus and postuterine sac. **D.** Rectum and anus. **G.** Vulva and vagina. **H.** Tail end with sharply pointed tip. **I.** Tail end with slightly rounded tip.

Remarks on the morphology of *F. annulatus* from Iran

The general morphology of *F. annulatus* from Iran closely resembles the original description of *F. annulatus* by Siddiqui and Khan (1983b). However, the tail differs

slightly being shorter in the Iranian population (58-93 µm *vs.* 100 µm), c value is higher (4.3-6.7 *vs.* 3.08-4.2), and V' value is lower in the Iranian population (80 *vs.* 84). Such variation on V' (*ca* 4 %) seems to be common for *Filenchus* species when large numbers of specimens are measured (Okada *et al.*, 2002; Bert *et al.*, 2010). Siddiqui and Khan (1983b) did not include V' value in their description of *F. annulatus*. In his Tylenchidae revision Geraert (2008), on the other hand, mentioned the V' value probably calculating this only from the holotype original description. In the absence of a range for V', comparison, in this regard, with the Iranian population is made more difficult. In addition, stylet length (7.0-10 µm vs. 7.0-8.0 µm) and total body length (370-450 µm *vs.* 380-410 µm) have a greater range in the Iranian population. These small differences can be attributed to the larger number of specimens measured in this study compared with the original description. Based on morphology, Brzeski (1997) considered *F. annulatus* as a junior synonym of *F. misellus*. Furthermore, he synonymized the latter species with *F. ditissimus*. However, tail shapes substantially differ between *F. annulatus* and *F. misellus* (filiform *vs.* conical, respectively). Moreover, SEM of the labial pattern of *F. annulatus* is clearly distinct from those of *F. misellus* and *F. ditissimus* (Geraert, 2008). Okada *et al.* (2002) described the feeding of *F. misellus* and improved the morphological description of this species.

The presence of males as well as a developed spermatheca filled with sperm suggests amphimictic reproduction in the Iranian population. Besides the type locality, *F. annulatus* has been reported in Punjab, India (Sultan *et al.*, 1991). This is the first record of the species for Iran and the first description of males for *F. annulatus*. In addition

previous studies have not included SEM of the lip region of *F. annulatus*, which herein show that the amphid openings are somewhat oval and positioned slightly posteriorly from the labial disc. Although *Filenchus neonanus* has been shown to have more anteriorly positioned ovoid openings, many other species of *Filenchus* are shown by SEM to have amphid openings that are elongated on the longitudinal axis (Raski and Geraert, 1987; Bert *et al.*, 2010).

Phylogenetic position

For phylogenetic analyses, BI, ML, and MP were used to estimate the position of *F. annulatus* within the family Tylenchidae, Örley, 1880 (*sensu* De Ley & Blaxter, 2002). For this purpose, the analyses were limited to Tylenchina representatives especially including taxa deemed from earlier analysis to be closely related with *F. annulatus*. The total alignment lengths used in the phylogenetic analyses were of 780 and 1830 base pair (bp) for 28S and 18S genes, respectively (gaps included). The number of parsimony-informative characters used for MP analysis was 497 and 730 for 28S and 18S genes, respectively. Results of phylogenetic analysis using the 28S gene were congruent in tree topologies among the three different methods (BI, ML, and MP), although some small differences in branch support were noted (mostly with MP). On the other hand, three topologies based on the 18S gene had a greater level of inconsistency, mostly characterized by low branch support values (BI and ML) and presence of politomies (MP). Herein, we mostly focus on the relationships among *F. annulatus* relative to other

Filenchus species as well as to other Tylenchidae representatives. We proceed with the analyses in a gene-based comparison.

28S Phylogeny

All *Filenchus* sequences, including *F. annulatus* from Iran, were grouped together by all phylogenetic methods with maximum support values (BI=1.00, ML=100, and MP=100), forming a monophyletic clade (Fig. 1.4). Specifically, the *Filenchus* clade was divided into two subclades: (1) *F. vulgaris* and *Filenchus* sp1 were sister taxa; and (2) *F. sindhicus* and *F. thornei* were sister taxa of *F. annulatus* and *F. quartus*. Such relationships were consistent in all phylogenetic analyses and highly supported by (BI=1.00 ML=100, and MP=100).

Sequences of the D2-D3 domains of the 28S gene showed different levels of divergence among the *Filenchus* species and their sister relationships: *F. vulgaris* and *Filenchus* sp1 diverged in 21 bp; *F. sindhicus* and *F. thornei* diverged in 7 bp; *F. annulatus* and *F. quartus* diverged by only 3 bp. Although, *F. annulatus* and *F. quartus* were very similar in their D2-D3 sequences, striking morphological differences suggest that they are indeed different species. For example, body length, stylet length, tail length, and oesophagus length differed substantially between these two species, with higher values occurring for *F. quartus*. In addition, multivariate analysis (MDS) based on morphological data clearly separates these two species (ANOSIM, $p<0.05$, Fig. 1.5).

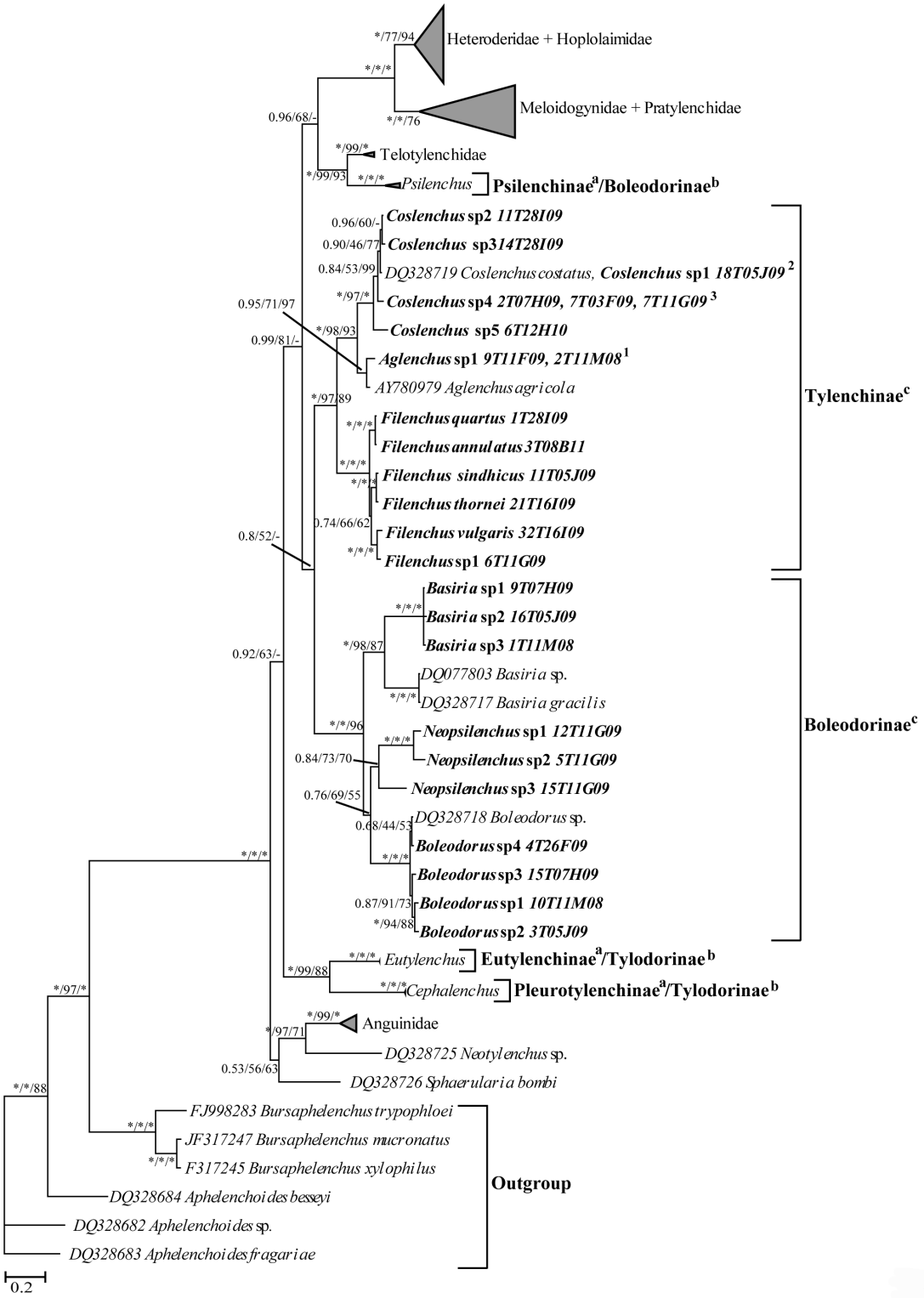
*/77/94
Heteroderidae + Hoplolaimidae
//*
Meloidogynidae + Pratylenchidae
0.96/68/-
//76
/99/
Telotylenchidae
*/99/93
//*
Psilenchus
Psilenchinae[a]/Boleodorinae[b]
0.96/60/-
Coslenchus sp2 11T28I09
0.90/46/77
Coslenchus sp314T28I09
0.84/53/99
DQ328719 Coslenchus costatus, Coslenchus sp1 18T05J09 [2]
/97/
Coslenchus sp4 2T07H09, 7T03F09, 7T11G09 [3]
0.95/71/97
*/98/93
Coslenchus sp5 6T12H10
0.99/81/-
Aglenchus sp1 9T11F09, 2T11M08 [1]
AY780979 Aglenchus agricola
*/97/89
Filenchus quartus 1T28I09
//*
Filenchus annulatus 3T08B11
//*
Filenchus sindhicus 11T05J09
//*
Filenchus thornei 21T16I09
0.74/66/62
Filenchus vulgaris 32T16I09
0.8/52/-
//*
Filenchus sp1 6T11G09
Tylenchinae[c]
Basiria sp1 9T07H09
//*
Basiria sp2 16T05J09
*/98/87
Basiria sp3 1T11M08
0.92/63/-
DQ077803 Basiria sp.
//*
DQ328717 Basiria gracilis
//96
Neopsilenchus sp1 12T11G09
//*
0.84/73/70
Neopsilenchus sp2 5T11G09
Neopsilenchus sp3 15T11G09
0.76/69/55
0.68/44/53
DQ328718 Boleodorus sp.
Boleodorus sp4 4T26F09
//*
//*
Boleodorus sp3 15T07H09
0.87/91/73
Boleodorus sp1 10T11M08
*/94/88
Boleodorus sp2 3T05J09
Boleodorinae[c]
//*
Eutylenchus
Eutylenchinae[a]/Tylodorinae[b]
*/99/88
//*
Cephalenchus
Pleurotylenchinae[a]/Tylodorinae[b]
/97/
/99/
Anguinidae
*/97/71
DQ328725 Neotylenchus sp.
0.53/56/63
DQ328726 Sphaerularia bombi
//88
FJ998283 Bursaphelenchus trypophloei
//*
JF317247 Bursaphelenchus mucronatus
//*
F317245 Bursaphelenchus xylophilus
DQ328684 Aphelenchoides besseyi
DQ328682 Aphelenchoides sp.
DQ328683 Aphelenchoides fragariae
Outgroup
0.2

Figure 1.4. Phylogenetic analysis focused on the family Tylenchidae. Bayesian 50% majority rule consensus tree inferred from 104 sequences of the D2-D3 domains of the 28S rDNA gene under the GTR+I+G model. Branch support values are given in the following order: BI, ML, and MP. An asterisk (*) in any position denotes maximum branch support for that method; (–) indicates no branch support in MP. Subfamilies are as proposed by (a) Siddiqi (2000) and (b) Geraert (2008) or by (c) both authors. New sequences specific to this study are indicated in bold. [1-3] denotes identical sequences according to Table 1.1. Specimen localities are given in Table 1.1. For collapsed families see Appendix A for what is included.

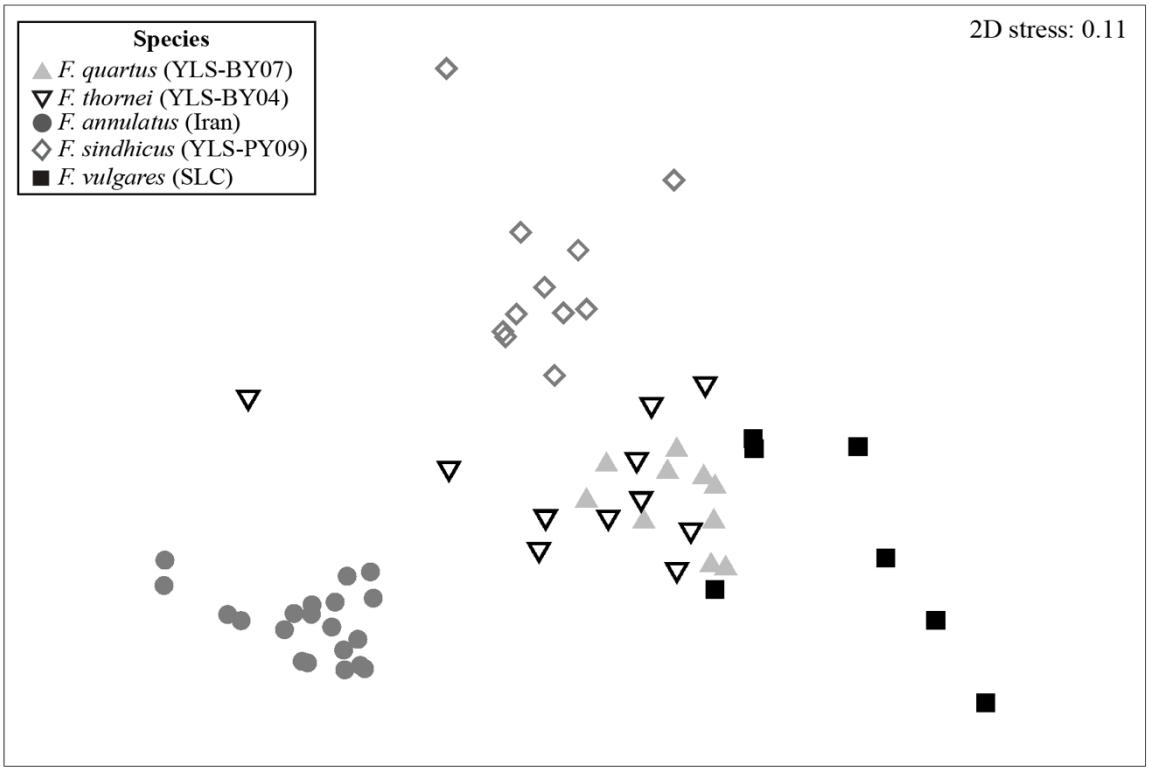

Figure 1.5. Multi-dimensional scaling (MDS) plot obtained from the morphometric data of five different *Filenchus* species (Distance (Euclidean) matrix is based on normalized).

The clade *Filenchus* was grouped with high branch support values (BI=1.00 and ML=97) as a sister taxon of *Aglenchus* and *Coslenchus* clades providing support for the monophyly of the subfamily Tylenchinae (excluding *Psilenchus*) that is recognized by Siddiqi (2000) and Geraert (2008). MP analysis did not contradict such relationships, although branch support for the Tylenchinae clade was slightly lower (MP=89) compared to BI and ML results. Similarly, *Boleodorus*, *Neopsilenchus*, and *Basiria*, comprise a well-supported clade (BI=1.00, ML=100, and MP=96) providing support for monophyly of the subfamily Boleodorinae. Tylenchinae and Boleodorinae were grouped as sister

taxa with relatively weak support (BI=0.85 and ML=52) under Tylenchidae but this relationship had no support with MP.

The genera *Cephalenchus* and *Eutylenchus* are traditionally classified in different subfamilies and families based on their morphology. Geraert (2008) placed both genera in the subfamily Tylodorinae under Tylenchidae, whereas Siddiqi (2000) placed each genus in a separate family; that is, he placed *Cephalenchus* and *Eutylenchus* in Pleurotylenchinae (Tylodoridae) and Eutylenchinae (Atylenchidae), respectively. A sister relationship between these genera is strongly (BI=1.00 and ML=99) or moderately (MP=88) supported by the different phylogenetic methods. BI and ML analyses place these genera in a more basal position outside of Tylenchidae with relatively high support (BI=0.99 and ML=81). On the other hand, MP analysis did not support this basal position.

The genus *Psilenchus* is placed under Psilenchinae (Psilenchidae) by Siddiqi (2000) and under Boleodorinae (Tylenchidae) by Geraert (2008). However, it is herein grouped with representatives of Telotylenchidae (BI=1.00, ML=99, and MP=100) thereby suggesting that the classification of the family Tylenchidae proposed by Geraert (2008) is artificial (Fig. 1.4), a consideration recognized by Geraert (2008).

18S Phylogeny

For the 18S gene, we incorporated additional *Filenchus* species from the GenBank database to further evaluate the monophyly of the group. Eighteen unique *Filenchus* sequences were incorporated in the phylogenetic analyses for this gene

(Appendix A, Fig. 1.6A-C). Overall, the genus *Filenchus* was characterized as polyphyletic: *F. misellus* grouped with representatives of Criconematina; *F. hamatus* grouped with representatives of Anguinidae; and *F. ditissimus* with the subfamily Boleodorinae + *E. excretorius*. In general, these relationships were weakly supported by BI and ML methods or not supported at all in the MP analysis, except in the position of *F. misellus* (BI=0.96 and ML=70, Fig. 1.6A).

In addition, 2 main *Filenchus* clades, being well and moderately supported by BI and ML methods, respectively, were identified as grouping with other Tylenchidae genera: a *Malenchus* + *Filenchus* spp. clade A (BI=1.0 and ML=84) and a *Tylenchus* spp. + *Filenchus* spp. clade B (BI=0.91, ML=71, and MP=75). MP did not support the *Malenchus* + *Filenchus* spp. clade A relationships. Specifically, under MP analysis clade A was reduced to *M. andrassyi* as sister of *F. fungivorus* + *Filenchus* sp. (GB=FJ949565, Fig. 1.6B). The four other *Filenchus*, including two *F. discrepans*, one *F. helenae*, and one *Filenchus* sp. (GB=AY912036), were excluded from this clade. Both *F. discrepans* sequences were grouped together in the MP analysis, however joining a deeper polytomy in the phylogenetic tree. The same was the case for *F. helenae*, and *Filenchus* sp. (GB=AY912036).

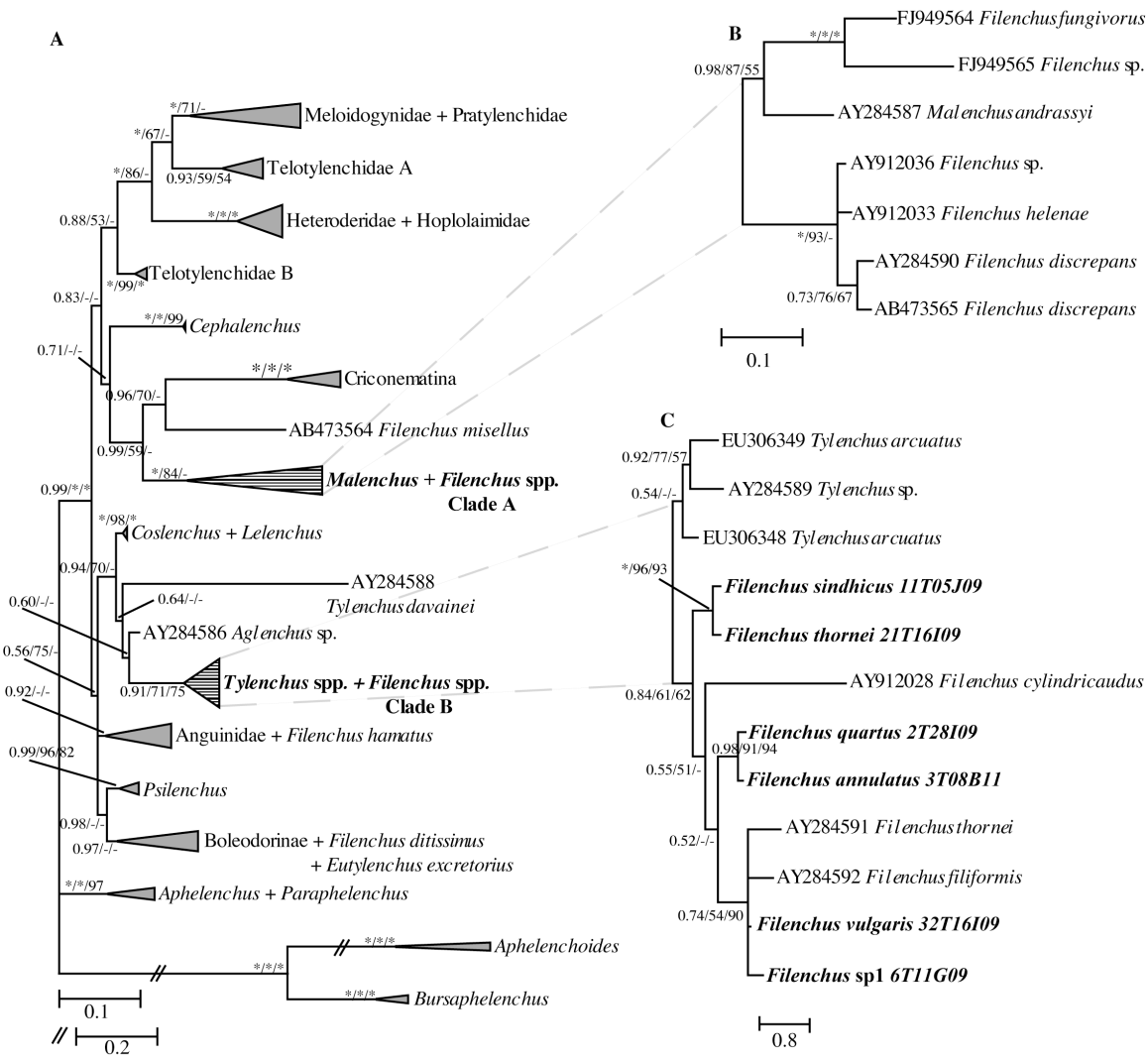

Figure 1.6. Phylogenetic analysis focused on the family Tylenchidae. **A.** Bayesian 50% majority rule consensus tree inferred from 74 sequences of the 18S rDNA gene under the GTR+I+G model. Branch support values are given in the following order: BI, ML, and MP. An asterisk (*) or a dash (–) in any position denotes maximum branch support and no branch support for that method, respectively. **B-C.** Phylogenetic relationships in detail for *Malenchus* + *Filenchus* spp. Clade A and *Tylenchus* spp. + *Filenchus* spp. Clade B. Taxonomic classification follows that given in Fig. 1.4. New sequences specific to this study are indicated in bold and specimen localities can be consulted in Table 1.2. For collapsed families see Appendix A for what is included.

All *Filenchus* species from USA as well as *F. annulatus* from Iran were grouped in the *Tylenchus* spp. + *Filenchus* spp. clade B (Fig. 1.6C). Relationships among these sequences were fairly consistent with the 28S results: *F. sindhicus* was grouped as a sister

taxon of *F. thornei* and *F. annulatus* as a sister taxon of *F. quartus*. These relationships were strongly supported by all three methods of analysis (Fig. 1.6C). *Filenchus* sp1 and *F. vulgaris*, which were sister taxon in the 28S phylogenies, in the 18S phylogeny were grouped (weakly supported by BI and ML methods) with *F. cylindricaudus*, *F. thornei*, and *F. filiformis* (Fig. 1.6C). With few exceptions MP supported the same relationships for *Filenchus* clade B (MP=62). For example, *F. cylindricaudus* joined the main tree polytomy as was previously noted to be the case for some other *Filenchu*s spp. from clade A; furthermore, some internal nodes were weakly (MP<50) supported.

Although the relationships among 18S *Filenchus* sequences from the USA as well as *F. annulatus* from Iran (clade B) were consistent with 28S results, sequence divergence values were much lower for 18S: *F. vulgaris* and *Filenchus* sp1 diverged by 4 bp; *F. sindhicus* and *F. thornei* diverged in 2 bp; *F. annulatus* and *F. quartus* diverged by only 1 bp. Regarding other Tylenchidae genera, 18S phylogenies were not able to recover the same relationships as shown by the 28S phylogeny: the subfamily Boleodorinae including *Basiria*, *Boleodorus*, and *Neopsilenchus* grouped with weak supported with *E. excretorius* + *F. ditissimus* (Fig. 1.6A). In the MP analysis, such genera were grouped within a main tree polytomy. Furthermore, the genus *Tylenchus* was also paraphyletic; the relationship of *Aglenchus* and *Tylenchus* spp. + *Filenchus* spp. clade B was weak supported. In this sense, both subfamily, Boleodorinae and Tylenchinae, were paraphyletic and polyphyletic, respectively. Other genera traditionally classified under the family Tylenchidae (*i.e. Psilenchus* and *Cephalenchus*) were weakly supported by BI and ML analyses or they were not resolved in the MP tree.

DISCUSSION

The phylogenies based on the 18S and 28S genes substantially differed regarding the relationships among the genus *Filenchus* and other Tylenchidae genera. Overall, the 28S phylogeny showed high branch support values for the monophyly of *Filenchus* as well as for the relationships among *Filenchus* and other Tylenchidae genera (*e.g.* *Aglenchus* and *Coslenchus*). However, it is important to recognize that such results are tentative pending the inclusion of additional *Filenchus* representatives in the analyses; such tests are especially crucial to further evaluate monophyly of *Filenchus*. Monophyly of other genera under the subfamilies Tylenchinae and Boleodorinae is also recovered as well as the sister relationship of these two subfamilies under Tylenchidae (weakly support). Furthermore, previous BI analysis using the 28S rDNA gene strongly supported (BI=0.99) these subfamilies as sister taxa (Subbotin *et al.*, 2006). The differences in clade support between our results and Subbotin *et al.* (2006) might be related to the number of taxa included within the Tylenchidae clade, being much higher in the present study.

Based on the 18S phylogeny, the genus *Filenchus* was polyphyletic. *Filenchus* species were grouped, generally with lower support when compared to 28S, with different tylench groups. Previous studies carried out by Van Megen *et al.* (2009) and Bert *et al.* (2010), both based on 18S rDNA gene sequences, also showed *Filenchus* to be paraphyletic and polyphyletic, respectively. However, it is important to mention that some of the *Filenchus* sequences used in the present study for the 18S analyses were represented by a shorter length (635-890 bp) compared to the total alignment length

(1830 bp); these shorter sequences could thus affect the final tree topology. Surprisingly, our two main *Filenchus* clades (Fig. 1.6B-C) on the 18S phylogeny resemble the findings of Bert *et al.* (2010) and therefore bring attention to the possible artificiality of the genus *Filenchus*.

Holterman *et al.* (2006), Bert *et al.* (2008), Van Megen *et al.* (2009), and Bert *et al.* (2010) considered that 18S rRNA-based phylogenies did not support a Tylenchinae plus Boleodorinae sister relationship. Differences in resolution, and the relative information content of phylogenies based on 18S versus 28S genes, especially at the subfamily and genus levels, have been also documented in other studies (Palomares-Rius *et al.*, 2009; Bik *et al.*, 2010; Carta *et al.*, 2010). These have demonstrated the importance of considering resolution from faster evolving genes to further test putative monophyly as well as classical versus molecular phylogenies. The low resolution of 18S compared to 28S at these taxonomical levels is also evident in the present study.

The phylogenetic position of *Cephalenchus*, *Eutylenchus*, and *Psilenchus* has been inconsistent and controversial among previous molecular studies (Subbotin *et al.*, 2006; Bert *et al.*, 2008; Van Megen *et al.*, 2009). Palomares-Rius *et al.* (2009) found a sister relationship between the genera *Cephalenchus* and *Eutylenchus* positioned outside the family Tylenchidae, a relationship that might prove justified as also argued by Siddiqi (2000).

The controversy regarding the classification of some Tylenchidae genera underscores the need for broader taxon sampling within this family, especially including the most specious genera such as *Basiria*, *Filenchus*, and *Tylenchus*. In addition, other

genera with conflicting phylogenetic positions in previous molecular studies (*e.g.* *Ecphyadophora*, *Malenchus*, and *Ottolenchus*) should be further investigated (Van Megen *et al.*, 2009).

Bert *et al.* (2010) suggested that *Filenchus* is most likely polyphyletic and perhaps this also might be the case for certain other genera of Tylenchidae. In the present study, the genus *Filenchus* was also polyphyletic in the 18S phylogeny but strongly supported as monophyletic by the 28S phylogeny. These contrasting results are mainly due to the number of sequences included in the analyses (lower in the 28S) as well as relative differences in the potential of these genes to revolve relationships at these lower taxonomic levels (greater in the 28S). Clearly, hypotheses of monophyly must be further tested based on additional representatives in order to clarify some traditional morphologically-based classifications. We propose that multiple genes will be particularly informative to increase phylogenetic resolution and thus clarify the relationships within and among extant clades.

Chapter 2

Contrasting evolutionary patterns of 28S and ITS rRNA genes reveal high intragenomic variation in *Cephalenchus* (Nematoda): Implications for species delimitation[3]

ABSTRACT

Concerted evolution is often assumed to be the evolutionary force driving multi-family genes, including those from ribosomal DNA (rDNA) repeat, to complete homogenization within a species, although cases of non-concerted evolution have been also documented. In this study, sequence variation of 28S and ITS ribosomal RNA (rRNA) genes in the genus *Cephalenchus* is assessed at three different levels, intragenomic, intraspecific, and interspecific. The findings suggest that not all *Cephalenchus* species undergo concerted evolution. High levels of intraspecific polymorphism, mostly due to intragenomic variation, are found in *Cephalenchus* sp1 (BRA-01). Secondary structure analyses of both rRNA genes and across different species show a similar substitution pattern, including mostly compensatory (CBC) and semi-compensatory (SBC) base changes, thus suggesting the functionality of these rRNA copies despite the variation found in some species. This view is also supported by low sequence variation in the 5.8S gene in relation to the flanking ITS-1 and ITS-2 as well as by the existence of conserved motifs

[3] Published as:

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in the former gene. It is suggested that potential cross-fertilization in some *Cephalenchus* species, based on inspection of female reproductive system, might contribute to both intragenomic and intraspecific polymorphism of their rRNA genes. These results reinforce the potential implications of intragenomic and intraspecific genetic diversity on species delimitation, especially in biodiversity studies based solely on metagenetic approaches. Knowledge of sequence variation will be crucial for accurate species diversity estimation using molecular methods.

INTRODUCTION

Biodiversity surveys have exploited metagenetics (*sensu* Creer *et al.* 2010) for identification and discovery of species in terrestrial and marine environments. These studies have demonstrated substantial previously hidden diversity for many groups including bacteria (Tringe *et al.*, 2005), nematodes (Fonseca *et al.*, 2010), zooplankton (Lindeque *et al.*, 2013), protists (Bachy *et al.*, 2013), and arthropods (Gibson *et al.*, 2014). Metagenetic studies of microorganisms have targeted primarily ribosomal RNA (rRNA) genes owing to both theoretical and practical reasons. Despite impressive claims of new insight into diversity, the method is still subject to potentially misleading PCR aspects such as chimera formation as well as amplification and sequencing errors (Bachy *et al.*, 2013). Moreover, ribosomal DNA (rDNA), being a multi-family gene, can exacerbate problems of interpretation due to intragenomic and intraspecific polymorphism; these characteristics have important implications for species estimation and delimitation as well

as for phylogenetic reconstruction (Buckler *et al.*, 1997; Wörheide *et al.*, 2004; André *et al.*, 2014; Weber and Pawlowski, 2014).

The rDNA is believed to evolve in a concerted manner, such that its different repeats are not independent from one another but instead are homogenized by different mechanisms (*e.g.* gene conversion, unequal crossing over) collectively termed concerted evolution (Dover, 1982). As a result, rDNA polymorphism within a species is expected to be very low or absent; yet, comparison among different species often reveals substantial variation (Hillis and Dixon, 1991). Nevertheless, rDNA variation within a given species has also been widely reported and high levels of intragenomic and intraspecific polymorphism have been attributed to the formation of pseudogenes (Márquez *et al.*, 2003), species with hybrid origins (Hugall *et al.*, 1999), multiple functional rDNA variants in the genome, and non-concerted evolution (Crease and Lynch, 1991; Carranza *et al.*, 1996; Keller *et al.*, 2006).

Despite these issues, rDNA remains widely used as a molecular marker for phylogenetic interpretation at diverse taxonomic levels and throughout the tree of life (Hillis and Dixon, 1991; Bik *et al.*, 2013). Whereas phyla to family level studies are mostly based on the 18S and 28S genes, species phylogenies as well as population level studies have focused on the ITS region (Blaxter *et al.*, 1998; Hugall *et al.*, 1999; Beszteri *et al.*, 2005; Mallatt and Giribet, 2006; Xu *et al.*, 2015a). For nematodes, molecular phylogenies have been mostly based on rRNA genes. In particular, plant parasitic nematodes of infraorder Tylenchomorpha commonly referred to as “tylenchs”, have received great attention owing to their worldwide impact on agriculture (Subbotin *et al.*,

2006; Bert *et al.*, 2008). However, tylenchs not directly associated with plant damage have been underrepresented and therefore their phylogenetic relationships remain poorly understood thus hampering our understanding of the overall phylogeny as well as evolution of parasitism in this group.

The present study focuses on the rDNA repeat variation of nematode species belonging to the genus *Cephalenchus*, which are not considered plant pests (Siddiqi, 2000), and where most of the species have been described or reported associated with non-agricultural vegetation. The monophyly of *Cephalenchus* in relation to other tylenchs has been proposed based on rDNA sequences (Pereira, unpublished data). Herein, using sequences from the 28S and ITS genes, rDNA repeat variation for *Cephalenchus* is evaluated at three different levels: (i) intragenomic, variation within an individual (among clones of the same nematode specimen); (ii) intraspecific, variation within a species (clones from different specimens within the same species); and (iii) interspecific, variation among different species. Additionally, (iv) the levels of sequence differentiation between the two rRNA genes across the different species are compared. Potential sources of rDNA polymorphism such as the formation of pseudogenes, weak concerted evolution as well as the mode of reproduction (cross-fertilization *vs.* parthenogenesis) are addressed in relation to our findings. Furthermore, the implications of rDNA polymorphism are discussed in the context of metagenetic studies where species delimitation and identification rely mostly on distance-based methods.

MATERIAL AND METHODS

Sampling and nematode extraction

Soil samples, about 300 g, were collected using a small shovel and stored in labeled plastic bags with respective GPS coordinates. In total, 12 *Cephalenchus* populations (including GenBank sequences), representing eight morphological species, were recovered from different geographic regions and analyzed (Table 2.1). Nematodes were extracted from soil using either a Baermann funnel or plastic tray method (Viglierchio and Schmitt, 1983; Hunt and De Ley, 1996). Samples, except those from the USA, were split with subsamples fixed in DESS solution (Yoder *et al.*, 2006) and 5% formalin solution for molecular and morphological procedures, respectively. Samples collected in the USA were processed at the University of California, Riverside (UCR) for DNA extraction before fixation. *Cephalenchus* specimens were sorted under a dissecting microscope (Olympus SZX16).

Morphological identification

For permanent slide mounts, nematodes were dehydrated and infiltrated in a graduated series of glycerin/ethanol solutions to pure glycerin (Seinhorst, 1959). Specimens were subsequently examined under light microscopy (LM) with a Zeiss Axioskop microscope for morphometrics following Geraert (2008).

Table 2.1. Sampling information for the *Cephalenchus* species used in this study.

Species	28S rRNA		ITS rRNA		Gender structure[c]	Sampling locality/code	GPS coordinates	Sampling date
	N	S	N	S				
***Cephalenchus* sp1**	8	58	6	36	F and M	Jaguaruna, SC, Brazil, (BRA-01)	*S 28° 36′ 02.43″* *W 48° 56′ 44.64″*	07/01/2009 25/05/2010
***Cephalenchus* sp.**[a]	2	10	-	-	F, M rare	Benjamin Constante, AM, Brazil (BRA-02)	*S 04° 20′ 59.8″* *W 69° 36′ 29.4″*	10/02/2010
C. cylindricus Sultan & Jairajpuri, 1982	6	19	6	21	F, M rare	Ensenada, BC, Mexico (MEX)	*N 31° 54′ 03″* *W 116° 36′ 32″*	30/05/2009
C. cephalodiscus Sultan & Jairajpuri, 1982	7	34	4	17	F, M rare	Yellowstone, WY, United States (USA-01)	*N 44° 52′ 57.19″* *W 110° 44′ 04.89″*	01/07/2009
C. daisuce Mizukobo & Minagawa, 1985	5	34	5	28	F only	Woods Hole, MA, United States (USA-02)	*N 41° 31′ 42″* *W 70° 40′ 30.80″*	30/07/2013
C. daisuce Mizukobo & Minagawa, 1985	4	31	5	27	F, M rare	Riverside, CA, United States (USA-03)	*N 33° 59′ 7″* *W 117° 18′ 18″*	22/02/2014
C. daisuce Mizukobo & Minagawa, 1985	5	29	4	14	F, M rare	Cabin Creek, OR, United States (USA-04)	*N 43° 28′ 10.72″* *W 123° 18′ 52.63″*	17/12/2013
C. daisuce Mizukobo & Minagawa, 1985	5	28	5	25	F only	Vancouver, BC, Canada (CAN)	*N 49° 19′ 41.24″* *W 122° 56′ 57.40″*	29/08/2013
***Cephalenchus* sp2**	4	30	2	8	F, M rare	Nam Cat Tien, Vietnam (VIE-01)	*E 107°20′ 25′′* *N 11°27′ 48′′*	26/05/2010
C. nemoralis Mizukobo & Minagawa, 1985	4	13	4	12	F and M	Cuc Phuong, Vietnam (VIE-02)	*W 105°35′ 36′′* *N 20°20′ 28′′*	05/06/2010
C. hexalineatus[b] (Geraert, 1962) Geraert & Goodey, 1964	3	3	2	8	F only			
Total	**53**	**289**	**43**	**196**				

[a] Species not identified due to small number of available individuals.

[b] 28S rRNA sequences for *C. hexalineatus*, which represent two populations from the USA (Florida: EU915491, EU915492; Oregon: EU915493), were retrieved from GenBank (Palomares-Rius *et al.*, 2009). Sequences of the ITS rRNA for *C. hexalineatus* also represent DNA extracts from the same individuals and localities and were courteously provided by Dr. Sergei Subbotin (see Appendix B for more details).

[c] Gender structure was determined based on individuals (F: female, M: male) used for morphological (permanent slides) identification, scanning electron microscopy, and PCR. The meaning of "rare" is used to denote that only one male individual (out of 20-25 specimens) was observed during sorting.

Identification of *Cephalenchus* species was based on the original descriptions and supplemented by available keys (Andrássy, 1984; Geraert and Raski, 1987; Geraert, 2008). Additionally, 5-10 nematodes of each species were processed for scanning electron microscopy (SEM) following the procedures described in Mundo-Ocampo *et al.* (2003). Nematodes were observed on a XL 30-FEG Phillips 35® scanning electron microscope operating at 10 kV.

DNA extraction, amplification, and sequencing

To evaluate sequence variation (*i.e.* intragenomic, intraspecific, and interspecific levels) of the rRNA genes in the genus *Cephalenchus*, sequencing approaches were designed to obtain multiple clones per individual. Based on preliminary observations, such approaches were concentrated on species likely to show high levels of rDNA polymorphism. Also, owing to differences in sample quality (fixed *vs.* fresh material), *Cephalenchus* species were necessarily represented by different numbers of sequences (Table 2.1). DNA was extracted from single individuals using proteinase K protocol and Worm Lysis Buffer (WLB) as described in Pereira *et al.* (2010). The D2-D3 domains of the 28S gene were amplified with primers D2Ab and D3B (De Ley *et al.*, 2005) and the ITS region (including ITS-1, 5.8S, and ITS-2) with primers N93 and N94 (Nadler *et al.*, 2000). All PCR reactions were 25 µl made as follows: 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®). Samples with low DNA template were also

subjected to a GenomiPhi protocol (GenomiPhi V2 DNA Amplification Kit, GE Health Care®) to increase the amount of DNA prior to PCR. Amplification success and amplicon size were verified in 1.0% agarose gel stained with ethidium bromide (0.5 mg ml^{-1}). Positive PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen) and then cloned with the pGEM®-T Easy Vector cloning Kit (Promega) using JM109 high competent cells following the manufacturer's instructions before sequencing. PCR products were sequenced in both directions with PCR primers using ABI-PRISM® Dye- DeoxyTerminator Big Dye™ v3.1 (Applied Biosystems) on an automatic sequencer Gene Analyzer® ABI 3100 (Applied Biosystems) at the Institute for Integrative Genome Biology, UCR. Additionally, direct sequencing was performed for some *Cephalenchus* species (see Appendix B).

Analysis of sequences

Sequence edition, polymorphism detection, and haplotype estimation

Newly obtained sequences were manually checked, edited, and assembled using CodonCode Aligner v. 4.2.7 (CodonCode Corporation, LI-COR, Inc.). After removing primers and carefully checking for ambiguous sites, contigs were produced for each clone. Posteriorly, multiple clones from the same nematode specimen were aligned using the MUSCLE algorithm (Edgar, 2004) as built in CodonCode Aligner for a second round of sequence edition and for evaluating intragenomic polymorphism. These nematode specimen alignments were then submitted to the Bellerophon web server for chimera

checking using Huber-Hugenholtz correction and window sizes of 200 bp and 300 bp (Huber *et al.*, 2004). Sequences revealed to be potential chimera were removed from the dataset for subsequent molecular analysis. Sequence divergence were estimated using p-distance and raw distance (bp differences) measures on MEGA 6 (Tamura *et al.*, 2013) and present as boxplot graphics produced with R software (R-Core Team, 2014). Molecular variation was also characterized by the number of haplotypes, haplotype diversity (h), and nucleotide diversity (π) as calculated with DnaSP (Rozas *et al.*, 2003). Additional alignment statistics within species and populations were also calculated with DnaSP (Rozas *et al.*, 2003).

A haplotype network including all *Cephalenchus* species was reconstructed with PopART (Leigh and Bryant, 2015) using the TCS method and having gaps and missing data excluded (Clement *et al.*, 2000). Haplotype estimation curves were also constructed using EstimateS v. 9.1.0 (Colwell, 2013) to determine haplotype coverage. Randomization was based on both with and without sample replacement. A total of 100 randomizations were performed. Haplotype curves were extrapolated by a population factor of two. The estimators used were first-order and second-order jackknife (Jack1 and Jack2) and first-order and second-order unbiased Chao richness estimators (Chao1 and Chao2).

Phylogenetic analyses

The phylogenetic position of the genus *Cephalenchus* in relation to other tylenchs will be revised elsewhere (Pereira, unpublished data). Herein, we focus only on the branching pattern within and between *Cephalenchus* species, and in relation to its closest available outgroup, *Eutylenchus excretorius*. Both genera have been classified either in the same family or subfamily by different authors (Siddiqi, 2000; Geraert, 2008), and their close association is further supported by molecular data (Palomares-Rius *et al.*, 2009).

Sequences (outgroup included), 291 and 197 for the 28S and ITS genes, respectively, were separately aligned on MAFFT version 7.0 (http://mafft.cbrc.jp/alignment/server/) using the iterative refinement method G-INS-i (Katoh and Standley, 2013). Default parameters were used, except that the scoring matrix for nucleotide sequences was set for 1PAM/ k=2 as suggested for closely related sequences. Outgroup sequences were either simultaneously aligned with the ingroup or subsequently added into the alignment using the option mafft-add in MAFFT version 7.0 with parameters set as described above, however no effects on topology were observed. For the ITS region, additional phylogenetic analyses were performed, extending outgroup taxa (*i.e.* anguinids) and including only two most divergent sequences representing each *Cephalenchus* species (see RESULTS).

Phylogenetic relationships among sequences were estimated with maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI). Maximum

parsimony analyses were performed in PAUP* 4.0a146 using heuristic searches and TBR branch swapping to seek the most parsimonious trees (max. tree number =100). Gaps in the alignment were treated as missing data. Nonparametric bootstrap analysis (BS), 1000 pseudoreplicates, was used to assess branch support (Swofford, 2002). For ML analyses, we used a fast ML method, RAxML-HPC v.8 (Randomized Axelerated Maximum Likelihood), through the server CIPRES (http://www.phylo.org/) under the GTR + G model. Gamma parameters were estimated from Log Likelihood units and bootstrap support was automatically calculated for the best-scoring ML tree (Stamatakis, 2014).

Bayesian analyses were performed on MrBayes 3.2 (Ronquist *et al.*, 2012) under the GTR + G model with the following settings: random starting tree, two independent runs with four simultaneous chains (three heated and one cold chain) for 10 x 10^6 generations. Markov chains were sampled at intervals of 1000 generations. Convergence was assessed using standard deviation of split frequencies (less than 0.01) and PSRF (Potential Scale Reduction Factors, 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. The best fitting substitution model for both datasets was estimated using jModelTest 2.1.2 (Darriba *et al.*, 2012) based on the Akaike Information Criterion. Concatenated analyses (28S + ITS, a total of 1507 sites) were also performed for a select group of sequences in addition to outgroup *E. excretorius* with the above-described methods.

Secondary structure analyses

Cephalenchus species-specific alignments, including only unique haplotypes (gaps/missing data included) were used for secondary structure analyses. The D2-D3 domains of the 28S rRNA were identified based on previously published studies (Subbotin *et al.*, 2007; Douda *et al.*, 2013). In the case of the ITS region, nematode sequences were retrieved from GenBank to establish gene identity. Additionally, ITS secondary structures were consulted to confirm gene boundaries (Subbotin *et al.*, 2011; Subbotin *et al.*, 2015). Alignments were trimmed accordingly and redundant sequences were removed from datasets.

Secondary structures were predicted with LocARNA (Will *et al.*, 2012) using global alignment and LocARNA-P as the alignment type and mode, respectively, in the Freiburg RNA tools server (Smith *et al.*, 2010). For *Cephalenchus* sp1 (BRA-01), the number of 28S sequences was reduced to 30 (maximum accepted by LocARNA tool) based on their identity (sequences with similarity > 99.3 were removed). A consensus secondary structure with probabilistic base pairing was predicted for each *Cephalenchus* species. Variability of sites for both rRNA genes were calculated following Subbotin *et al.* (2007) and mapped on the predicted secondary structures provided by LocARNA. Putative secondary structures (not necessarily representing the in-situ structure) were finally refined using PseudoViewer3 (Byun and Han, 2009) and Adobe Illustrator® v. 16.0.4.

RESULTS

Species diagnosis and determination

Identification of *Cephalenchus* species from widely divergent localities (Table 2.1) was based on morphological observations from LM and SEM. Based on this species concept (*i.e.* morphology), *Cephalenchus* from sites BRA-01 and VIE-01, respectively designated "sp1" and "sp2", were determined to be new species and will be properly described elsewhere (Pereira, unpublished data). Yet, *Cephalenchus* from site BRA-02 was designated "sp." because insufficient material was available for species determination. Although *C. daisuce* was recovered from multiple localities, including the USA and Canada, results are separately described for each population. Thus, sequence varation in the rDNA of 12 *Cephalenchus* populations, representing eight morphologically defined species, is addressed below. Furthermore, morphological species hypotheses are also confronted in light of molecular evidence.

Molecular representation of *Cephalenchus* species

For the 28S gene, the number of sequences per species ranged from 10 (from two nematode specimens) for *Cephalenchus* sp. (BRA-02) to 58 (from eight specimens) for *Cephalenchus* sp1 (BRA-01). Except for *C. cylindricus* (MEX) and *C. nemoralis* (VIE-02), respectively with 19 (from six specimens) and 13 (from four specimens) sequences,

other *Cephalenchus* species were each represented by about 30 sequences (Table 2.1). The number of sequences for the ITS region ranged from eight for *Cephalenchus* sp2 (VIE-01) and *C. hexalineatus* (both with two individuals) to 36 (from six specimens) for *Cephalenchus* sp1 (BRA-01). Sequences, including those obtained from direct sequencing, produced in the present study and have been deposited on GenBank (28S: KU722973-KU723258, ITS: KU723259- KU723454, see also Appendix B).

Intraspecific variation as a result of intragenomic polymorphism

Results demonstrated a surprisingly high level of intraspecific variation for certain species and this proved to be primarily not a result of variation among individuals within a given species, but rather due to of intragenomic polymorphism within individual nematodes (Fig. 2.1 and Appendix B). For *Cephalenchus* sp1 (BRA-01), intragenomic polymorphism ranged from 0 to 10% (0 to 63 bp difference) and from 0 to 13% (0 to 67 bp difference) for the 28S and ITS genes, respectively (Fig. 2.1). In *Cephalenchus* sp1 (BRA-01), most individuals (except 5T12G10) have values of intragenomic polymorphism that extend to at least 7% for the 28S gene. The presence of indels in the alignments at the intragenomic and intraspecific levels in *Cephalenchus* sp1 showed a similar pattern, up to five indel events. Intraspecific sequence divergence, for *Cephalenchus* sp1 (BRA-01), was as high as 10% or 65 bp difference. In the case of the ITS region, intragenomic polymorphism in *Cephalenchus* sp1 (BRA-01) was always greater than 4% and as high as 13%. Indel events and indel event length for

Cephalenchus sp1 (BRA-01) were generally greater in the ITS than in the 28S gene, with up to 15 indel events. Intraspecific sequence divergence for *Cephalenchus* sp1 (BRA-01) in the ITS region was as high as 13% or 71 bp difference (Appendix B).

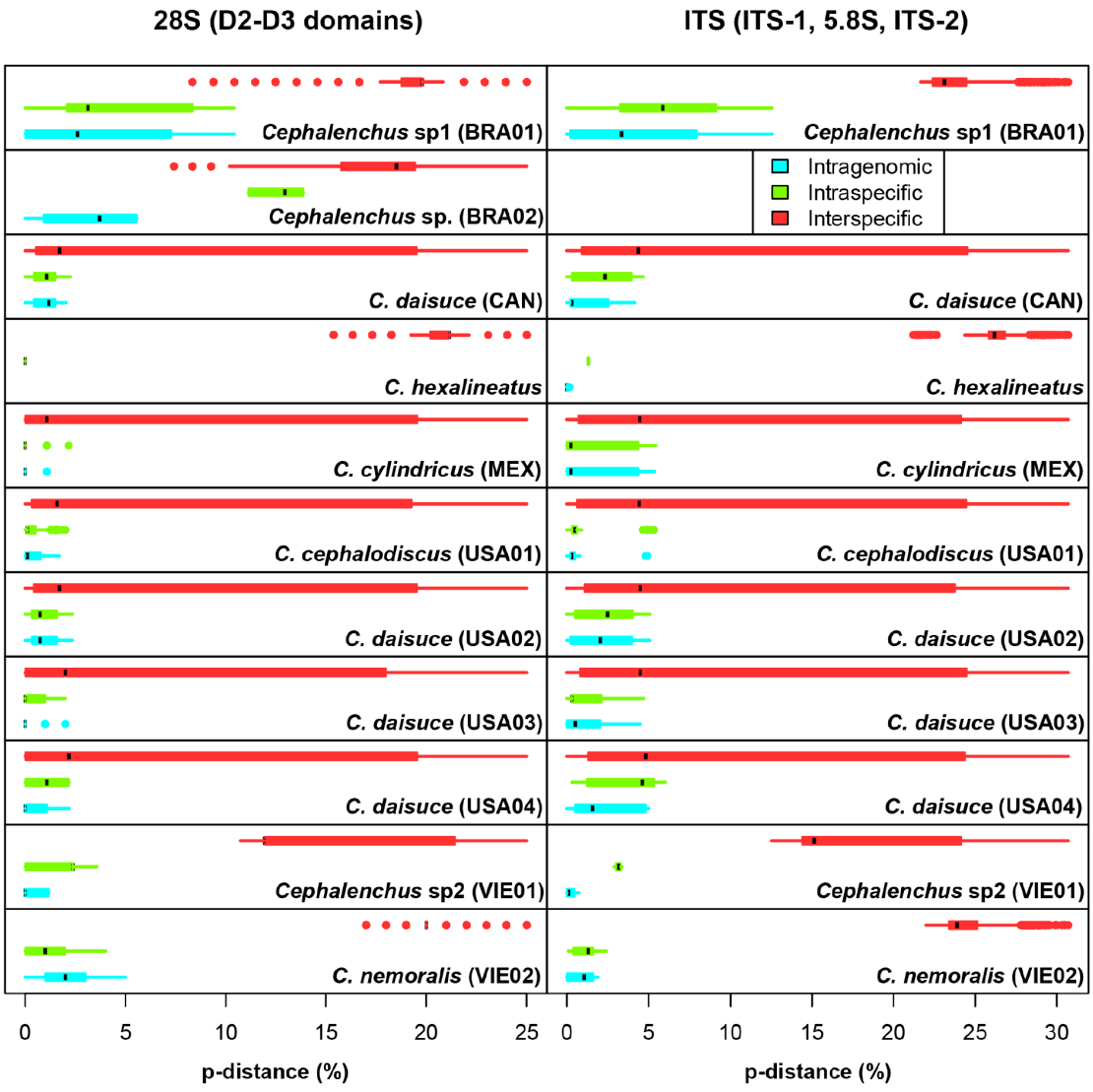


Figure 2.1. Boxplot distribution of 28S (left panel) and ITS (right panel) rRNA genetic divergence (p-distances, expressed as percent of nucleotide change) among the *Cephalenchus* species used in this study. Central box represents the upper and lower quartiles; whiskers represent the extreme of the data with points exceeding Q3+1.5IQ or below Q1-1.5IQ (Q1: 1st quartile, Q3: 3rd quartile and IQ: Q3-Q1) considered as outliers; the central mark represents the position of the median. Intragenomic distance for *C. hexalineatus* is not given (28S gene) due to the limited number of sequences.

Cephalenchus sp. (BRA-02) also showed high levels of intraspecific variation for the 28S gene (Fig. 2.1), however not as a result of intragenomic polymorphism as seen for *Cephalenchus* sp1 (BRA-01); that is, intra-individual sequence divergence was much lower (0 to 6%) than intraspecific (as high as 15%). In contrast to *Cephalenchus* sp1 (BRA-01), *Cephalenchus* sp. (BRA-02) was shown to be monophyletic at the individual level, but not at the species level (Fig. 2.2). Also, the intraspecific alignment of *Cephalenchus* sp. (BRA-02) substantially differed from the intragenomic. Notable values of intragenomic polymorphism in the 28S gene were also found for *C. nemoralis* (VIE-02), ranging from 0 to 5% (0 to 34 bp difference). By contrast, *C. nemoralis* (VIE-02) together with *C. hexalineatus* displayed the lowest values of sequence variation for the ITS region (Fig. 2.1).

Regarding the 28S gene, all other *Cephalenchus* species including *C. cephalodiscus* (USA-01), *C. cylindricus* (MEX), *C. daisuce* (USA-02 to USA-04 and CAN), *C. hexalineatus*, and *Cephalenchus* sp2 (VIE-01), showed very low intragenomic and intraspecific sequence variation. Conversely, for the ITS region these same species, except *Cephalenchus* sp2 (VIE-01) and *C. hexalineatus*, displayed relatively higher levels of intragenomic and intraspecific polymorphism (as high as 5%; up to 33 bp difference) when compared with the 28S gene. Interestingly, the species and populations representing the North America region showed a similar pattern with respect to the presence of indels in the ITS alignments (see Appendix B). More importantly, these indel events (sites 63-74, 83-99 of the ITS-1) were present across the different species and

populations. Consequently, sequences representing *C. cephalodiscus* (USA-01), *C. cylindricus* (MEX), and *C. daisuce* (USA-02 to USA-04 and CAN) did not partition into clades in the rRNA phylogenies discussed below, but instead clustered collectively into a single clade referred hereafter as the North American clade.

Interspecific variation among linages: species delimitation through phylogenetic analysis

In general, both rRNA phylogenies were congruent with respect to the number of inferred clades and the relationships among *Cephalenchus* species (Figs. 2.2 and 2.3). For the 28S tree, five main clades were recovered. Clades I and II are represented by *C. nemoralis* (VIE-02) and *C. hexalineatus*, respectively. Monophyly of both species (BI = 1.0, MP = 99, ML = 100) as well as their sister relationship (BI = 1.0, MP = 90, ML = 100) is strongly supported by all phylogenetic analyses. Clade III harbors two species from Brazil, *Cephalenchus* sp1 (BRA-01) and *Cephalenchus* sp. (BRA-02). Within clade III, the monophyly of *Cephalenchus* sp1 (BRA-01, clade IIIa) is strongly supported by all analyses (BI= 1.0, MP=91, ML=80). On the other hand, *Cephalenchus* sp. (BRA-02) was paraphyletic in relation to *Cephalenchus* sp1 (BRA-01). Clones from one nematode specimen (clade IIIb) of *Cephalenchus* sp. (BRA-02) were more closely related to *Cephalenchus* sp1 (BRA-01) and strongly supported by BI and ML, but not by MP (BI = 1.0, ML = 84; Fig. 2.2).

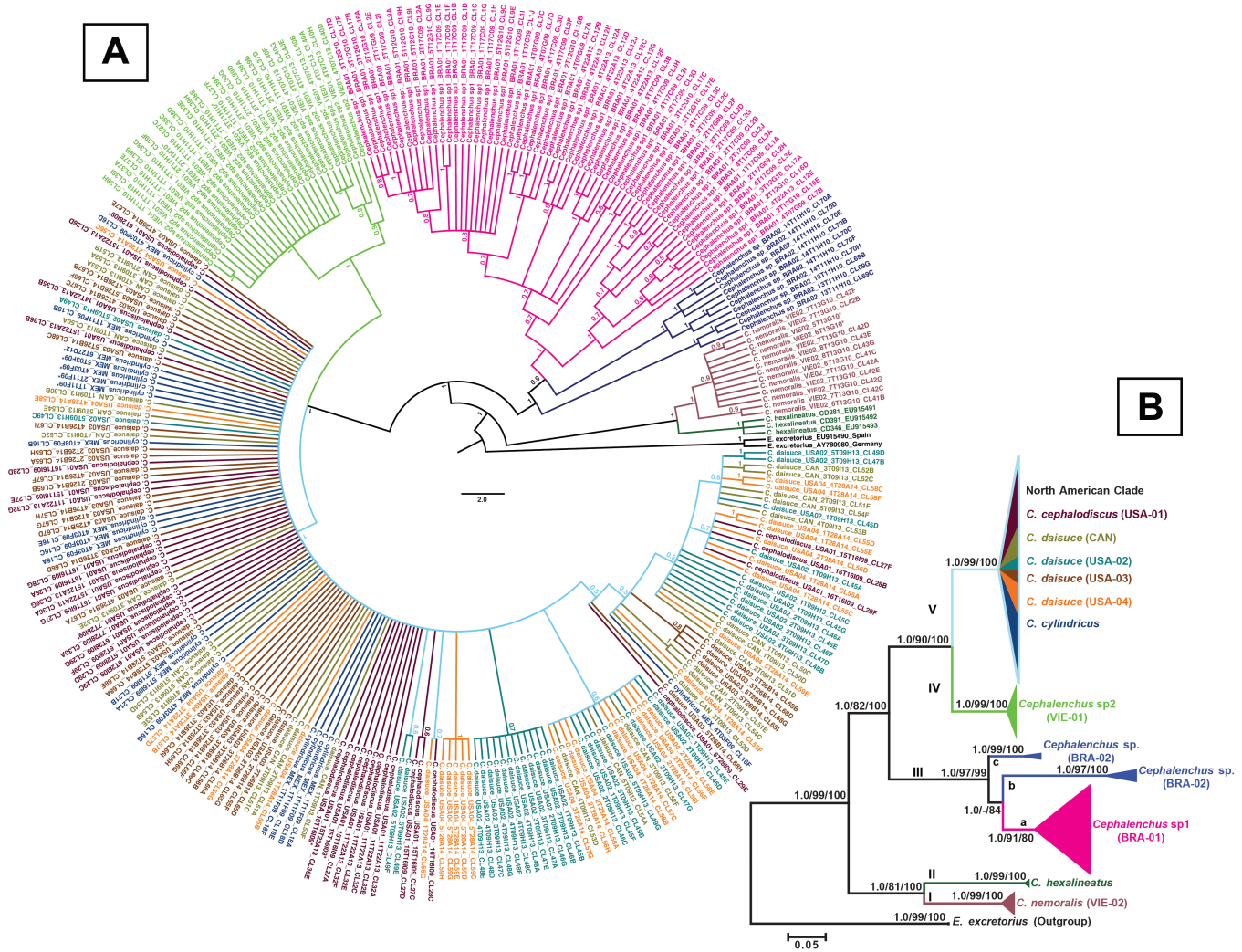

A
B
North American Clade
C. cephalodiscus (USA-01)
C. daisuce (CAN)
C. daisuce (USA-02)
C. daisuce (USA-03)
C. daisuce (USA-04)
C. cylindricus
Cephalenchus sp2 (VIE-01)
Cephalenchus sp. (BRA-02)
Cephalenchus sp. (BRA-02)
Cephalenchus sp1 (BRA-01)
C. hexalineatus
C. nemoralis (VIE-02)
E. excretorius (Outgroup)
1.0/99/100
1.0/90/100
1.0/82/100
1.0/99/100
1.0/97/99
1.0/-/84
1.0/91/80
1.0/81/100
1.0/99/100
1.0/99/100
1.0/99/100
1.0/99/100
1.0/97/100
1.0/99/100
V
IV
III
II
I
c
b
a
0.05

Figure 2.2. Molecular phylogeny of the *Cephalenchus* species and populations (color-coded) used in this study based on the 28S rRNA gene. **A.** The 50% majority rule consensus tree (Cladogram) from Bayesian analysis based on 291 sequences of the 28S rRNA gene showing the branching pattern among *Cephalenchus* species. **B.** Five clades (I-V) are identified among *Cephalenchus* sequences. Branch support (BI/MP/ML) is shown on branches (only for the collapsed branches). Both trees are rooted to *E. excretorius*.

Clade IV is comprised of sequences from *Cephalenchus* sp2 (VIE-01), which is also strongly supported as a monophyletic group (BI = 1.0, MP = 99, ML=100). Clade V is represented by six populations that encompass three morphologically defined species [*C. cephalodiscus* (USA-01), *C. cylindricus* (MEX), and *C. daisuce* (USA-02 to USA-04 and CAN)], collected in North America. Phylogenetic analyses based on either rRNA gene did not support monophyly of the different populations nor species (Figs. 2.2 and 2.3). For the 28S gene, mean genetic divergence between clades ranged from 10% (clades IV and V) to 23% (clades II and III; clades II and V).

For the ITS-based phylogeny, the same five clades were recovered (Fig. 2.3), but with a few changes of relationships among *Cephalenchus* species: (i) *Cephalenchus* sp1 (BRA-01, clade III) is placed as sister to all other *Cephalenchus* species after the tree is rooted to the outgroup *E. excretorius* (only well supported by BI analysis); (ii) *C. nemoralis* (VIE-02, clade I) and C. *hexalineatus* (clade II) are more closely related to *Cephalenchus* sp2 (VIE-01, clade IV) and North American species (clade V); (iii) the relationship of clades I and II relative to clades IV and V is also weakly supported by MP and ML analyses (MP = 62 and ML = 67). The potential close association of *Cephalenchus* sp1 (BRA-01, clade III) and *E. excretorius* was further tested by extending outgroup taxa in the ITS dataset. These analyses recovered: (i) *Cephalenchus* as monophyletic (BI = 1.0, MP = 100, ML = 96) with respect to *E.* excretorius; (ii) *Cephalenchus* sp1 (BRA-01, clade III) as sister to all *Cephalenchus* species; (iii) the relationship of *C. nemoralis* (VIE-02, clade I) and *C. hexalineatus* (clade II) to clades IV and V is moderately or poorly supported (BI = 0.8, MP = 81, ML = 56, data not shown).

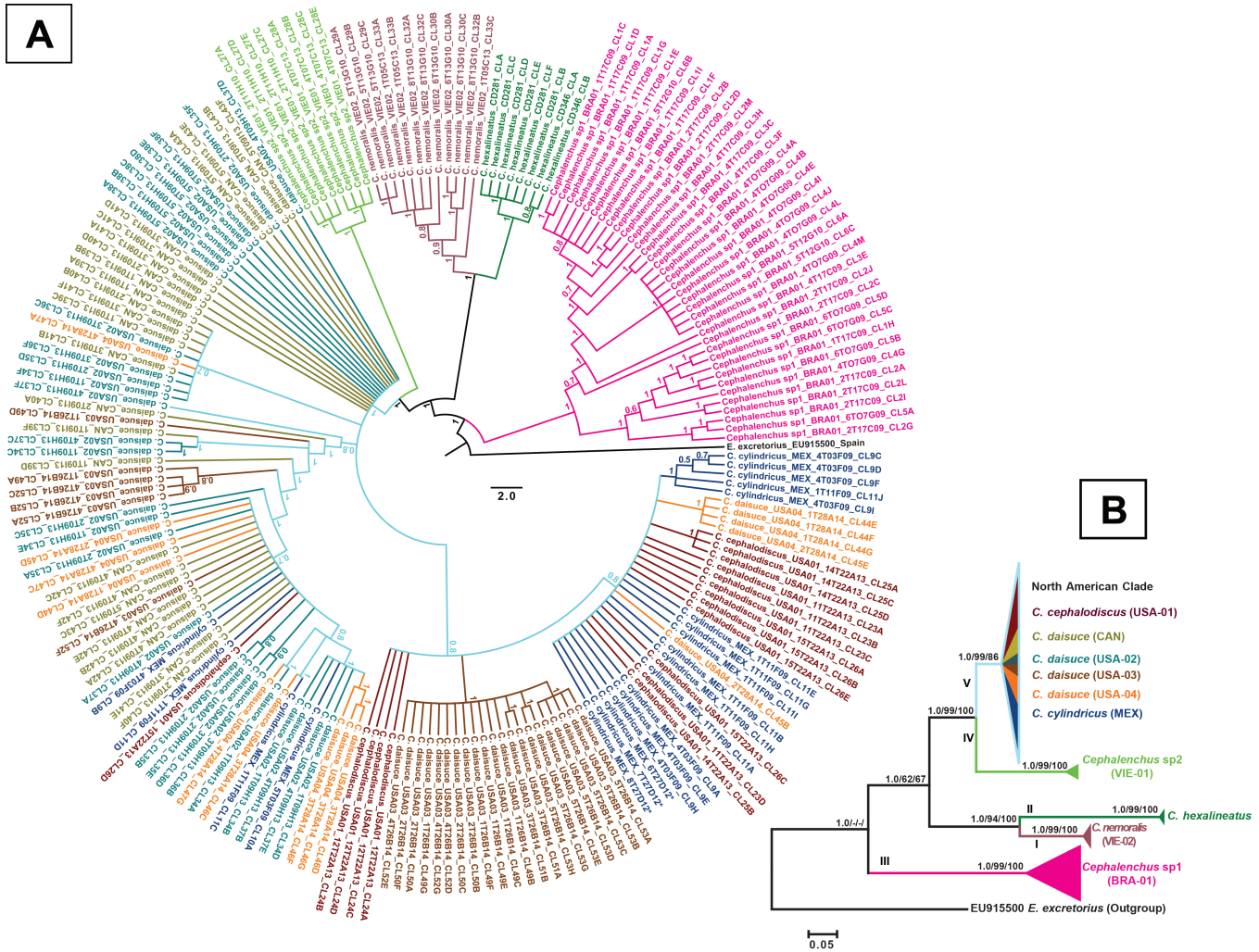

A
B
2.0
0.05
North American Clade
C. cephalodiscus (USA-01)
C. daisuce (CAN)
C. daisuce (USA-02)
C. daisuce (USA-03)
C. daisuce (USA-04)
C. cylindricus (MEX)
Cephalenchus sp2 (VIE-01)
C. hexalineatus
C. nemoralis (VIE-02)
Cephalenchus sp1 (BRA-01)
EU915500 E. excretorius (Outgroup)
I
II
III
IV
V
1.0/99/86
1.0/99/100
1.0/62/67
1.0/-/-/
1.0/94/100

Figure 2.3. Molecular phylogeny of the *Cephalenchus* species and populations (color-coded) used in this study based on the ITS rRNA region. **A.** The 50% majority rule consensus tree (Cladogram) from Bayesian analysis based on based on 197 sequences of the ITS rRNA region. **B.** Five clades (I-V) are identified among *Cephalenchus* sequences. Notations are as for Fig. 2.2. *Cephalenchus* sp. (BRA-02) is not included.

Conversely, concatenated analyses based on both genes are generally congruent with the 28S phylogeny, that is, clades I and II are sister to all other *Cephalenchus* species (BI = 1.0, ML = 100, but unresolved in MP, Fig. 2.4). Mean genetic divergence between clades, based on the ITS region, was higher than that based on the 28S, ranging from 16% (clades IV and V) to 32% (clades II and III).

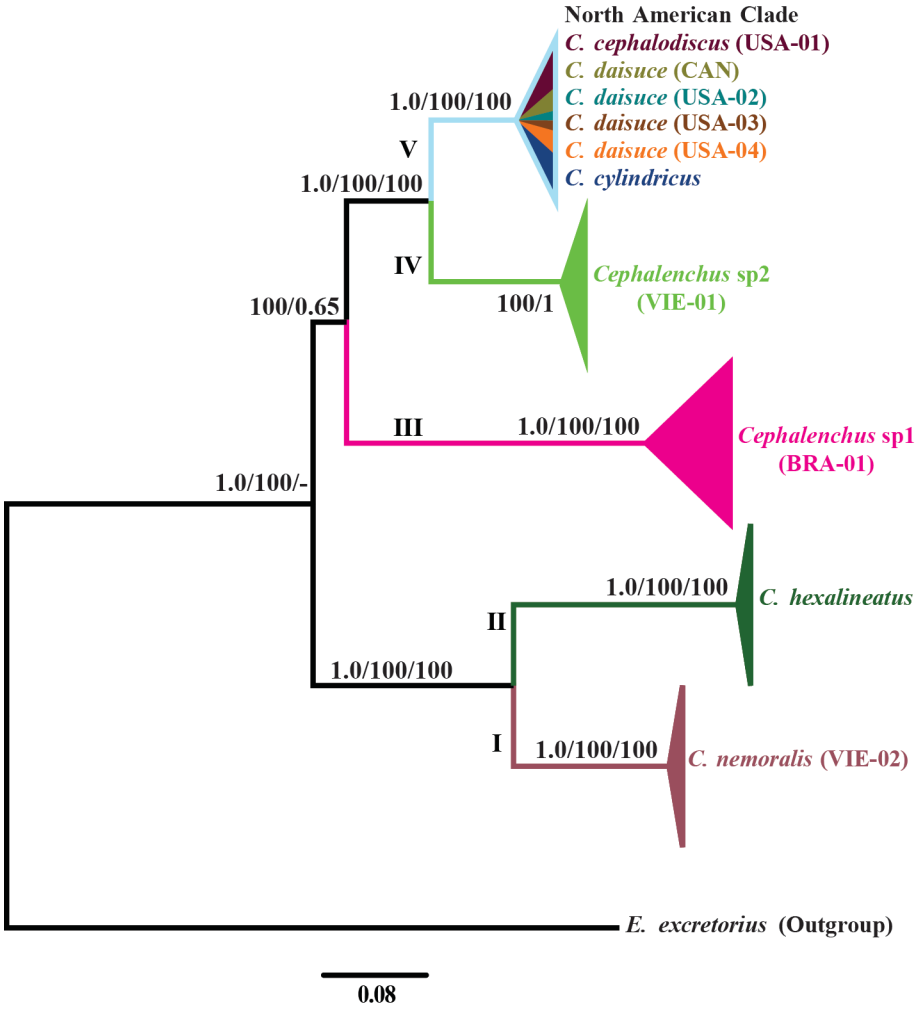


Figure 2.4. Molecular phylogeny of the *Cephalenchus* species and populations (color-coded) used in this study based on the concatenated dataset (28S + ITS genes). Five clades (I-V) are identified among *Cephalenchus* sequences. Notations are as for Fig. 2.2 *Cephalenchus* sp. (BRA-02) is not included.

Patterns of haplotype diversity among Cephalenchus species

Haplotype (h) and nucleotide (π) diversity were estimated within *Cephalenchus* species and a summary of the genetic diversity, including other alignment parameters, is given in Tables 2.2 and 2.3 for 28S and ITS genes, respectively. For the 28S gene, the highest values of haplotype diversity were found in *C. hexalineatus*, *Cephalenchus* sp1 (BRA-01), and *Cephalenchus* sp. (BRA-02). For *C. hexalineatus*, which is only represented by three sequences and characterized by low intraspecific sequence variation, this high haplotype diversity could drastically decrease with greater representation. A network haplotype based on the 28S gene shows that most of the sequences from *Cephalenchus* sp1 (BRA-01) and *Cephalenchus* sp. (BRA-02) are unique and private haplotypes (Fig. 2.5 and Table 2.2). Additionally, these two *Cephalenchus* species were characterized by high mean values of pairwise distance between haplotypes. Shared haplotypes, on the other hand, were only observed among species and populations comprising clade V. In particular, one haplotype (central haplotype in Fig. 2.5) was very frequent and more or less equally shared by all three species. Most of the haplotypes in the North American clade differed from the central and highly frequent haplotype by a distance of one to three base pairs. The two Vietnamese species, *Cephalenchus* sp2 (VIE-01) and *C. nemoralis* (VIE-02), were also characterized by private haplotypes and high haplotype diversity (Table 2.2).

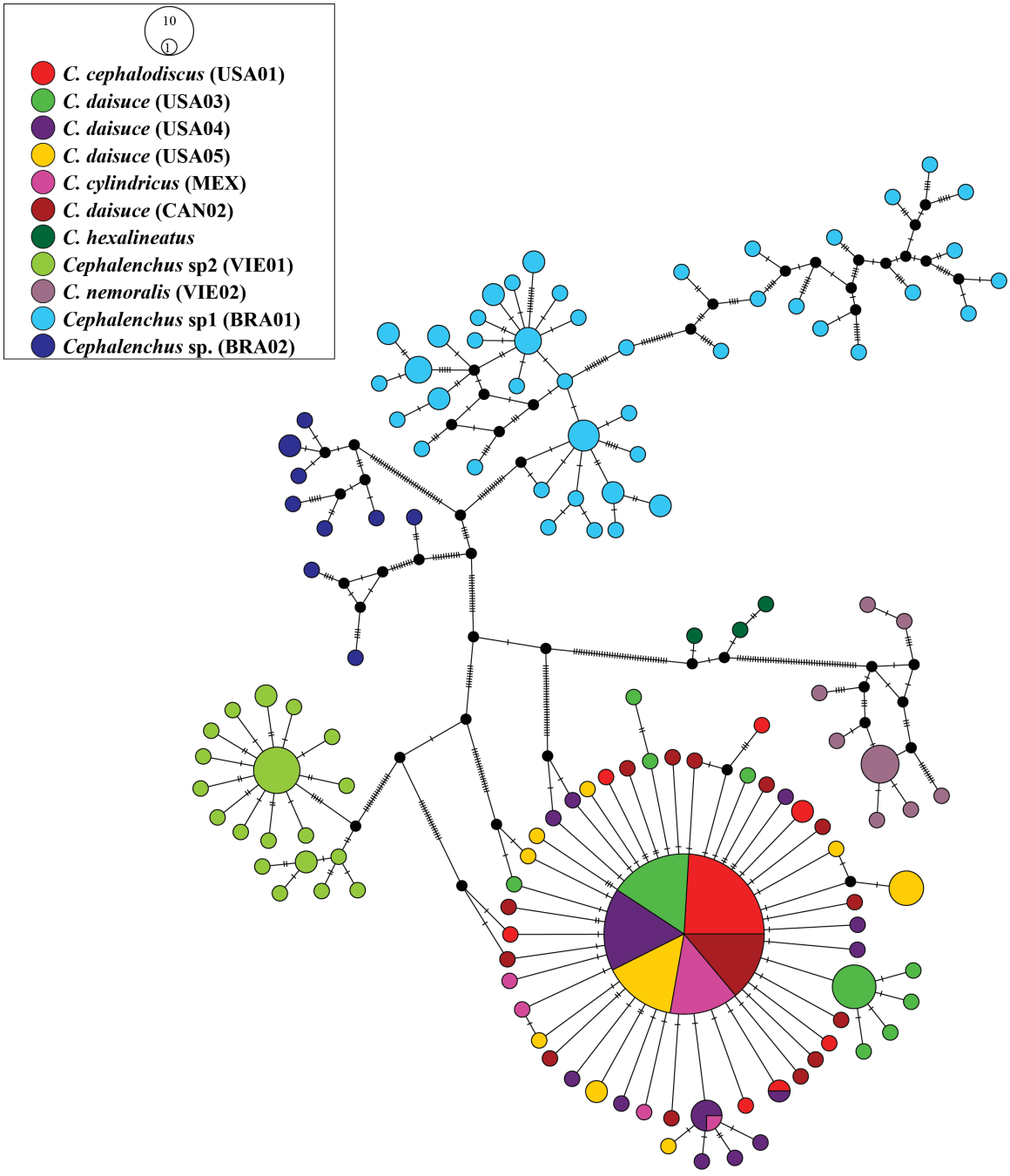


Figure 2.5. Minimum spanning haplotype network (gaps and missing data excluded) based on the 28S rRNA gene. *Cephalenchus* species and populations are color-coded. Pie size is proportional to the haplotype frequency. Vertical bars at the edges represent differences between haplotypes. Black nodes connecting haplotypes are considered intermedian haplotypes in the network.

Table 2.2. 28S genetic diversity, mutation types, alignment characteristics, and base composition for each *Cephalenchus* species used in the present study. Values are calculated either including (left) or excluding (right) gaps/missing data from the alignments. Single values indicate no differences between alignments with or without gaps/missing data (No. sequences = number of sequences, No. hap. = number of haplotypes, Hap. diversity = haplotype diversity, Hap. mean diff. = haplotype mean difference, No. Ts = number of transitions, No. Tv = number of transversions, No. indels = number of indels, C = conserved sites, V = variable sites, S = singleton sites, Pi = parsimony informative sites).

	***Cephalenchus* sp1 (BRA01)**	***Cephalenchus* sp. (BRA02)**	***C. cylindricus* (MEX)**	***C. cephalodiscus* (USA01)**	***C. daisuce* (USA02)**	***C. daisuce* (USA03)**	***C. daisuce* (USA04)**	***C. daisuce* (CAN)**	***Cephalenchus* sp2 (VIE01)**	***C. nemoralis* (VIE02)**	***C. hexadlineatus* (GenBank)**	**All *Cephalenchus***
No. sequences	58	10	19	34	34	31	29	28	29	13	3	289
Genetic diversity												
No. hap.	51/50	9	7	18	22/21	19/16	16/15	20	22	10	3	193/162
Hap. diversity (*h*)	0.996/0.995	0.978	0.544	0.758	0.939/0.927	0.832/0.742	0.923/0.911	0.923	0.96	0.949	1	0.963/0.941
Hap. mean diff.	30.9	82.8	1.7	3.1	6.5	3.3/3.7	7.1	6.8	8.1	11.1	5.3	98.3/98.2
Nuc. diversity (π)	0.045/0.046	0.115/0.072	0.002	0.004	0.009	0.005/0.006	0.01	0.009	0.012/0.009	0.015	0.008	0.131/0.112
Mutations												
No. Ts	127/126	90	12	24	30	23/22	27	31	30	31	6	350/342
No. Tv	40	33	3	8	7	7	11	7	10	20	2	186/184
Ts/Tv ratio	3.2/3.1	2.7	4	3	4.3	3.3/3.1	2.5	4.4	3	1.5	3	1.9/1.9
No. indels	8	61	0	0	0	0	2	5	6	2	0	104
Alignment parameters												
Length	687/622	720/659	730/699	730/700	730/690	730/525	730/682	730/558	676/640	735/720	653	748/469
C	530/473	579/546	715/684	699/669	694/655	699/497	694/649	689/527	634/601	684/670	645	331/194
V	157/149	141/113	15	31	36/35	31/28	36/33	41/31	42/39	50	8	416/275
S	51/46	18/18	14	21	23/22	22/19	19/17	26/20	29/27	32	8	103/67
Pi	106/103	123/95		10	13	9	17/16	15/11	13/12	18	0	313/208
Base composition												
A (%)	23.07	22.3	22.18	21.88	22.09	21.92	22.22	22.14	24.45	22.34	21.29	22.56
C (%)	19.39	21.81	19.61	19.56	19.56	19.43	19.53	19.53	19.44	20.82	21.9	19.66
G (%)	32.00	32.53	31.90	32.04	32.03	32.09	31.92	31.98	30.58	31.79	32.41	31.85
T (%)	25.54	23.36	26.31	26.52	26.32	26.56	26.33	26.35	25.53	25.05	24.4	25.93
G + C (%)	51.36	54.34	51.51	51.60	51.59	51.52	51.45	51.51	50.02	52.61	54.31	51.51

For the ITS region, *Cephalenchus* sp1 (BRA-01) was also characterized by high haplotype diversity (*h*= 0.95 or 0.96), with a haplotype number ranging from 25 to 26 (Table 2.3), and mostly represented by unique/private haplotypes in the ITS haplotype network, thus in agreement with the 28S dataset. Although, *Cephalenchus* species from the North American clade showed greater intraspecific variation for the ITS region, their haplotype network pattern was relatively similar to that of the 28S gene, that is, thus including shared and some highly frequent haplotypes (Fig. 2.6).

Despite the high haplotype diversity found in most of the *Cephalenchus* species, haplotype estimation curves based on both rRNA genes show that sampling effort seems to be limited. Also, estimation curves differed substantially when sampling was performed with or without replacement. In particular, haplotype coverage based on sampling without replacement was very low, whereas sampling with replacement produced better coverage (Table 2.4, Fig. 2.7).

Variation in the 28S and ITS predicted secondary structure among *Cephalenchus* species

Predicted secondary structures of both rRNA genes were compared among all *Cephalenchus* species to further explore sequence variation. The length of the D2 and D3 domains (28S gene), excluding the junction sequences between these two segments, ranged from 303 bp to 360 bp and from 146 bp to 165 bp, respectively (Table 2.5).

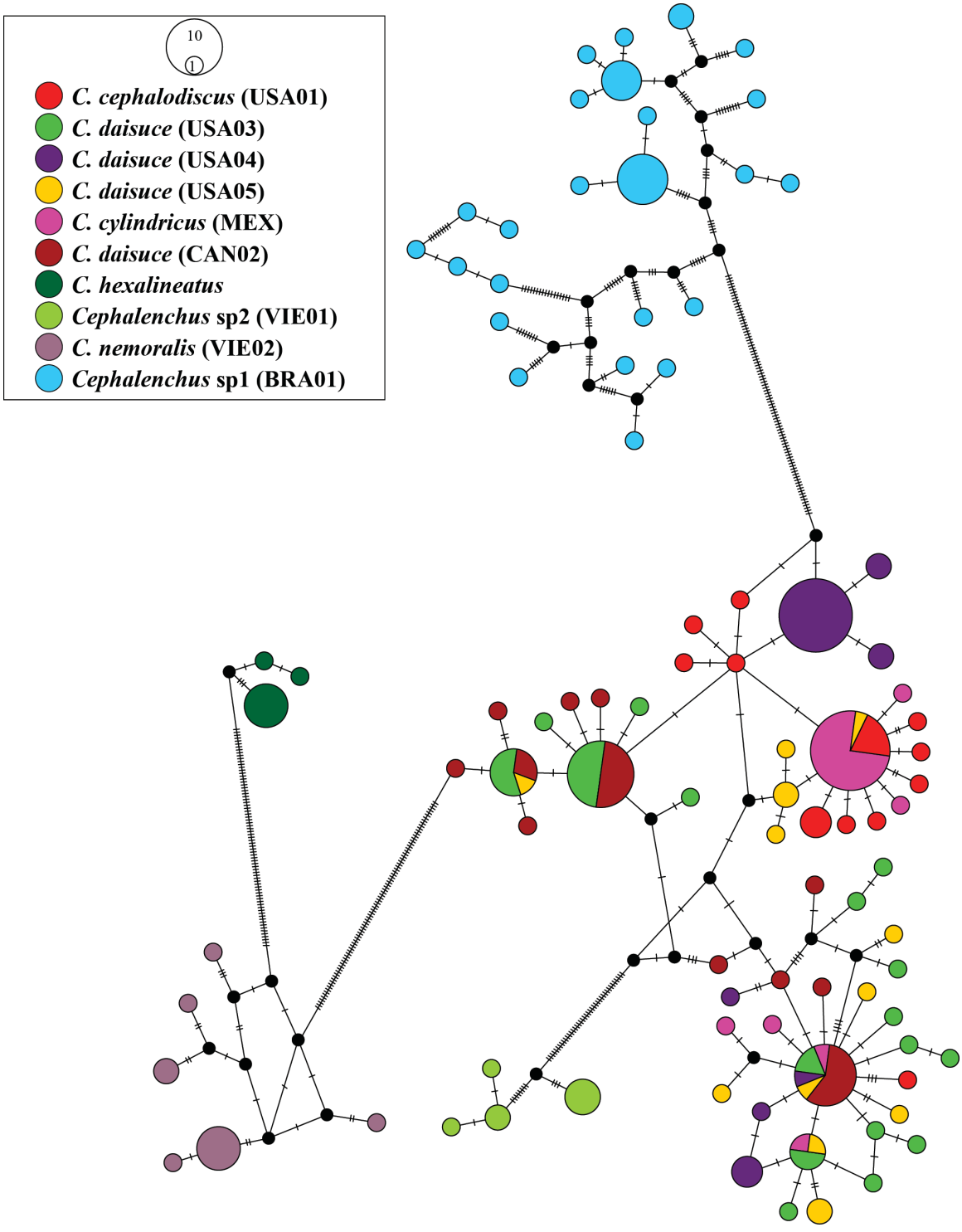


Figure 2.6. Minimum spanning haplotype network (gaps and missing data excluded) based on the ITS region. Notations are as for Fig. 2.6. *Cephalenchus* sp. (BRA-02) is not included in the ITS haplotype network.

Table 2.3. ITS genetic diversity, mutation types, alignment characteristics, and base composition for each *Cephalenchus* species used in the present study. Values are calculated either including (left) or excluding (right) gaps/missing data from the alignments. Single values indicate no differences between alignments with or without gaps/missing data (No. sequences = number of sequences, No. hap. = number of haplotypes, Hap. diversity = haplotype diversity, Hap. mean diff. = haplotype mean difference, No. Ts = number of transitions, No. Tv = number of transversions, No. indels = number of indels, C = conserved sites, V = variable sites, S = singleton sites, Pi = parsimony informative sites).

	***Cephalenchus* sp1 (BRA01)**	***C. cylindricus* (MEX)**	***C. cephalodiscus* (USA01)**	***C. daisuce* (USA02)**	***C. daisuce* (USA03)**	***C. daisuce* (USA04)**	***C. daisuce* (CAN)**	***Cephalenchus* sp2 (VIE01)**	***C. nemoralis* (VIE02)**	***C. hexalineatus***	**All *Cephalenchus***
No. sequences	36	21	17	28	27	14	25	8	12	8	196
Genetic diversity											
No. hap.	26/25	14/8	12	19	10/9	12	14/13	5	8	3	115/93
Hap. diversity (*h*)	0.959/0.948	0.933/0.710	0.934	0.942	0.83/0.8	0.978	0.88/0.86	0.786	0.924	0.464	0.985/0.969
Hap. mean diff.	45.2/47.7	18.29	8.63	28.8	22.0	34.1	24.4	13.5	10.5	3.7	126.9/127.3
Nuc. diversity (π)	0.08/0.069	0.031	0.014	0.05	0.035/0.01	0.05	0.04/0.02	0.02	0.02/0.01	0.007	0.189/0.132
Mutations											
No. Ts	86	23	21	29	21	35	27	30/19	23	9	277/274
No. Tv	50	10	19	14	12/11	16	11	10/7	5	0	175/174
Ts/Tv Ratio	1.7	2.3	1.1	2.1	1.8/1.9	2.2	2.5	3/2.7	4.6	-	1.6
Indels	41	31	29	31	54	37	30	1	7	0	210
Alignment parameters											
Length	580/469	639/564	638/573	638/557	638/579	638/601	638/592	637/636	679/664	616/562	717/400
C	455/353	605/531	598/533	596/516	605/555	590/556	601/557	611/610	652/637	607/553	317/163
V	125/116	34/33	40	42/41	33/24	48/45	37/35	26	27	9	399/237
S	27/22	8/8	38	12/11	15/6	18/16	16/14	6	16	1	55/36
Pi	98/94	26/25	2	30	18	30/29	21	20	11	8	344/201
Base composition											
A (%)	27.13	24.98	25.12	25.21	25.23	25.21	25.16	25.04	25.09	28.51	25.44
C (%)	21.75	22.49	22.4	22.22	22.46	22.29	22.22	22.01	22.77	22.33	22.31
G (%)	23.67	23.84	23.85	23.48	23.59	23.56	23.6	23.59	23.83	22.98	23.61
T (%)	27.45	28.69	28.63	29.09	28.72	28.94	29.02	29.36	28.31	26.18	28.64
G + C (%)	45.42	46.33	46.25	45.70	46.05	45.85	45.82	45.6	46.6	45.31	45.92

Table 2.4. Haplotype curve estimations based on the number of observed haplotypes in each rDNA gene for all *Cephalenchus* species used in the present study. Estimations were performed with and without (*in italics*) sample replacement. Values are based on 100 independent runs.

Species	Gene	Observed haplotypes[a]	Chao1 richness estimator mean[b]	Jack1 richness estimator mean	Jack2 richness estimator mean	Proportion of observed to estimated (%)
***Cephalenchus* sp1 (BRA01)**	28S	51	52.48 +/- 10.31 *167.21 +/- 53.38*	54.22 +/- 3.54 *94.24 +/- 3.23*	62.9 +/- 9.38 *130.08*	81.08 – 97.18 *30.5 – 54.12*
	ITS	26	26.39 +/- 7.37 *104.43 +/- 56.98*	26.67 +/- 2.53 *47.39 +/- 2.88*	29.87 +/- 5.66 *65.41*	87.0 – 98.5 *24.9 – 54.9*
***Cephalenchus* sp. (BRA02)**	28S	9	7.18 +/- 2.42 *17.33 +/- 9.8*	8.1 +/- 1.31 *14.3 +/- 1.37*	9.26 +/- 3.08 *19.19*	97.2 – 100 *46.9 – 62.9*
***C. cylindricus* (MEX)**	28S	7	7.63 +/- 3.97 *21.21 +/- 12.78*	7.34 +/- 1.36 *12.68 +/- 1.97*	8.56 +/- 3.07 *18.05*	97.2 – 100 *33.0 – 55.2*
	ITS	14	17.0 +/- 8.71 *71.62 +/- 67.71*	14.51 +/- 1.83 *24.48 +/- 2.23*	16.59 +/- 7.19 *33.57*	82.3 – 96.5 *19.5 – 57.2*
***C. cephalodiscus* (USA01)**	28S	18	20.3 +/- 8.6 *150.0 +/- 66.04*	18.5 +/- 2.23 *34.5 +/- 2.87*	21.33 +/- 4.51 *50.5*	84.4 – 97.3 *12.0 – 35.6*
	ITS	12	15.44 +/- 8.67 *54.35 +/- 28.3*	12.69 +/- 1.72 *21.41 +/- 1.97*	15.12 +/- 3.59 *30.24*	77.7 – 94.6 *22.1 – 56.0*
***C. daisuce* (USA02)**	28S	22	25.37 +/- 10.71 *187.97 +/- 78.29*	22.2 +/- 2.31 *40.44 +/- 2.85*	25.23 +/- 6.1 *58.32*	86.7 – 99.1 *11.7 – 54.4*
	ITS	19	26.71 +/- 12.93 *142.43 +/- 138.44*	20.27 +/- 2.22 *34.43 +/- 2.57*	24.02 +/- 5.1 *48.39*	86.7 – 99.1 *13.3 – 55.2*
***C. daisuce* (USA03)**	28S	19	23.46 +/- 11.87 *167.06 +/- 71.89*	19.21 +/- 2.23 *36.42 +/- 2.7*	22.67 +/- 5.4 *53.26*	80.9 – 98.9 *11.4 – 54.4*
	ITS	10	11.08 +/- 4.59 *16.02 +/- 7.26*	10.36 +/- 1.35 *14.81 +/- 1.98*	10.9 +/- 4.16 *17.66*	90.2 – 96.5 *56.6 – 67.5*
***C. daisuce* (USA04)**	28S	16	18.3 +/- 7.91 *85.52 +/- 80.7*	16.15 +/- 1.92 *27.59 +/- 2.61*	18.42 +/- 4.73 *37.86*	80.9 – 98.9 *18.7 – 58.0*
	ITS	12	18.28 +/- 11.13 *35.21 +/- 22.54*	13.53 +/- 1.66 *21.29 +/- 1.63*	16.43 +/- 5.05 *28.27*	65.1 – 88.7 *34.1 – 56.4*
***C. daisuce* (CAN)**	28S	20	23.49 +/- 9.58 *176.21 +/- 173.15*	20.41 +/- 2.24 *37.36 +/- 2.49*	24.05 +/- 4.87 *53.18*	83.2 – 98.0 *11.3 – 53.5*
	ITS	14	16.29 +/- 8.17 *77.36 +/- 38.26*	13.82 +/- 1.83 *25.52 +/- 2.45*	16.13 +/- 4.1 *36.56*	85.9 – 100 *18.1 – 54.9*
***Cephalenchus* sp2 (VIE01)**	28S	22	28.67 +/- 13.98 *109.14 +/- 74.04*	22.92 +/- 2.34 *40.34 +/- 2.52*	27.06 +/- 5.73 *56.24*	60.8 – 87.3 *20.2 – 49.6*
	ITS	5	4.93 +/- 2.3 *10.25 +/- 6.16*	5.11 +/- 0.96 *8.5 +/- 1.32*	5.84 +/- 2.44 *11.5*	85.6 – 100 *43.5 – 58.8*
***C. nemoralis* (VIE02)**	28S	10	10.14 +/- 3.54 *22.92 +/- 12.27*	10.36 +/- 1.46 *17.38 +/- 1.69*	11.96 +/- 4.19 *23.38*	83.6 – 98.6 *42.8 – 58.0*
	ITS	8	9.33 +/- 4.7 *13.73 +/- 6.96*	8.65 +/- 1.32 *12.58 +/- 1.64*	9.72 +/- 3.34 *15.23*	82.3 – 92.5 *52.5 – 63.6*
***C. hexalineatus*[c]**	ITS	3	2.49 +/- 0.71 *3.88 +/- 1.88*	2.97 +/- 0.58 *4.75 +/- 1.15*	3.31 +/- 1.73 *6.25*	90.6 – 100 *48 – 77.3*

[a] Number of observed haplotypes includes gaps/missing data in the alignments.
[b] Chao1 and Chao2 estimators produced identical values and therefore are shown in the same column.
[c] Data for *C. hexalineatus* is presented only for the ITS region due to the low number of sequences for the 28S gene.

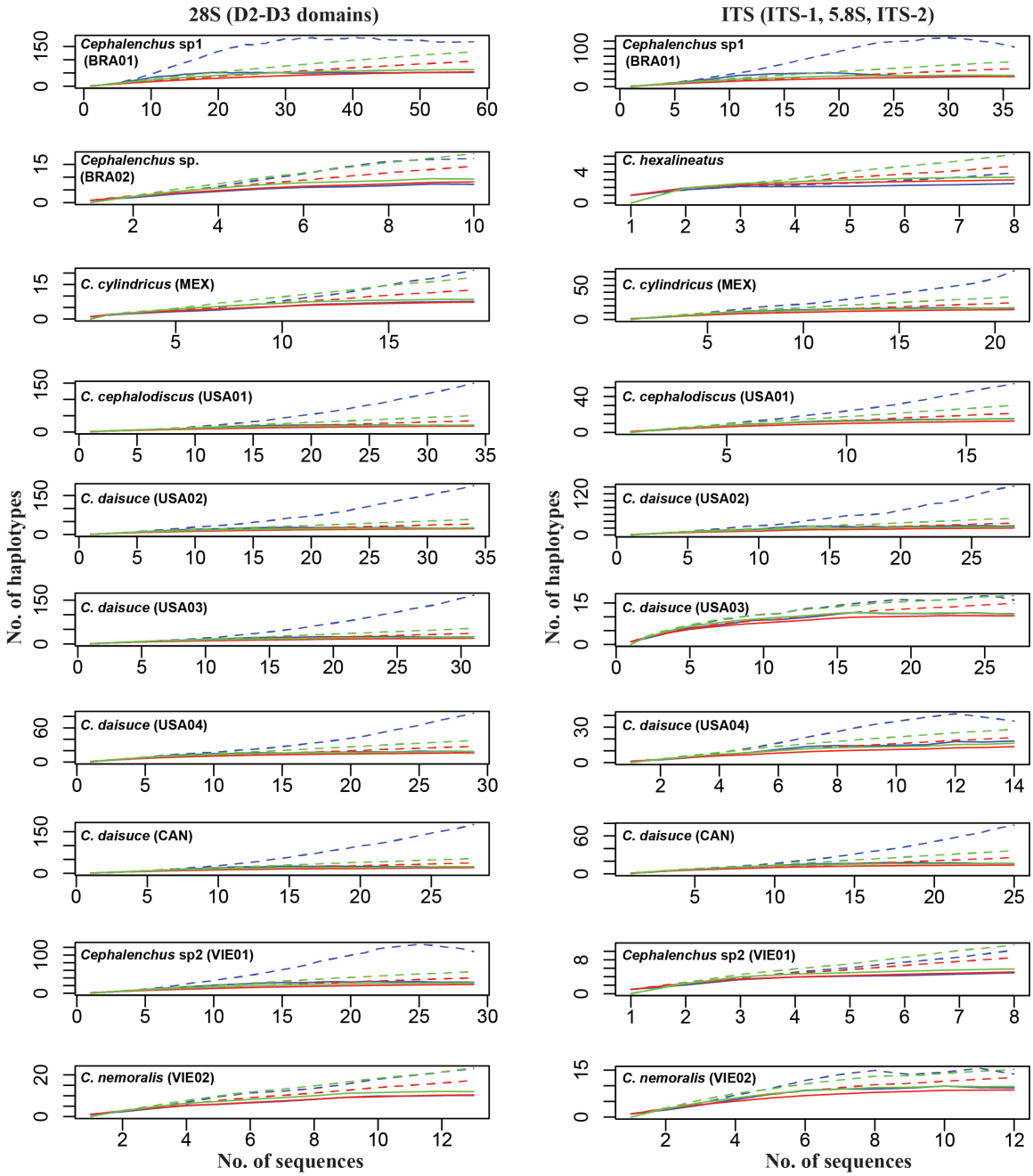


Figure 2.7. Haplotype estimation curves for all *Cephalenchus* species and populations used in this study based on the 28S (left) and ITS (right) rRNA genes. Estimators used were first-order and second-order jackknife (Jack1 and Jack2) and first-order and second-order unbiased Chao richness estimators (Chao1 and Chao2 but only Chao1 is presented since they have identical behaviours). Randomizations (100 runs) are based on both with (solid lines) and without (dashed lines) sample replacement. Blue, red, and green lines represent estimators Chao1, Jack1, and Jack 2, respectively.

Within species, length variation of both domains is observed in *C. daisuce* (CAN), *Cephalenchus* sp1 (BRA-01), *Cephalenchus* sp. (BRA-02), and *C. nemoralis* (VIE-02). A similar folding pattern for the predicted consensus secondary structures of D2 and D3 domains is observed across all *Cephalenchus* species (Figs. 2.8 and 2.9), including folding of the D2 domain into helices C1-C1/e4 and the D3 into helices D2-D6, but excluding helix D4, following the scheme of Wuyts *et al.* (2001).

Table 2.5. Length variation (bp) of the amplified rRNA genes across the different *Cephalenchus* species used in this study.

Species	28S rRNA		ITS rRNA		
	D2-domain	**D3-domain**	**ITS-1**	**ITS-2**	**5.8S**
Cephalenchus sp1 (BRA-01)	308-311	161-164	209-221	175-195	157-158
Cephalenchus sp. (BRA-02)[a]	307-353	146-150	-	-	-
C. cylindricus (MEX)	357	162	249-279	202-203	157
C. cephalodiscus (USA-01)	357	162	249-278	203	157
C. daisuce (USA-02)	357	162	249-278	202-203	157
C. daisuce (USA-03)	357	162	249-278	202-203	156-157
C. daisuce (USA-04)	357	161-162	246-278	202-203	156-157
C. daisuce (CAN)	354-357	161-162	249-278	203	157
Cephalenchus sp2 (VIE-01)	303	156-162	279-280	200	157
C. nemoralis (VIE-02)	358-359	164-165	305-309	210-213	157
C. hexalineatus	360	163	283	176	157

[a] For *Cephalenchus* sp. (BRA-02), D2-domain varies from 307-309 bp and from 352-353 bp for haplotypes with reduced and long C1/e1 helix, respectively. Variation in the D3-domain for the same haplotypes is 150 bp and 146-147 bp, respectively.
(-) Data not available

Base changes were mostly observed on the stem instead of the loop regions, especially for the D2 domain (for complete secondary structures of D2 domain, see Appendix C), however, they were either compensatory (CBCs) or semi-compensatory (SBCs) base changes. This substitution pattern was observed in all species regardless of their levels of intraspecific polymorphism.

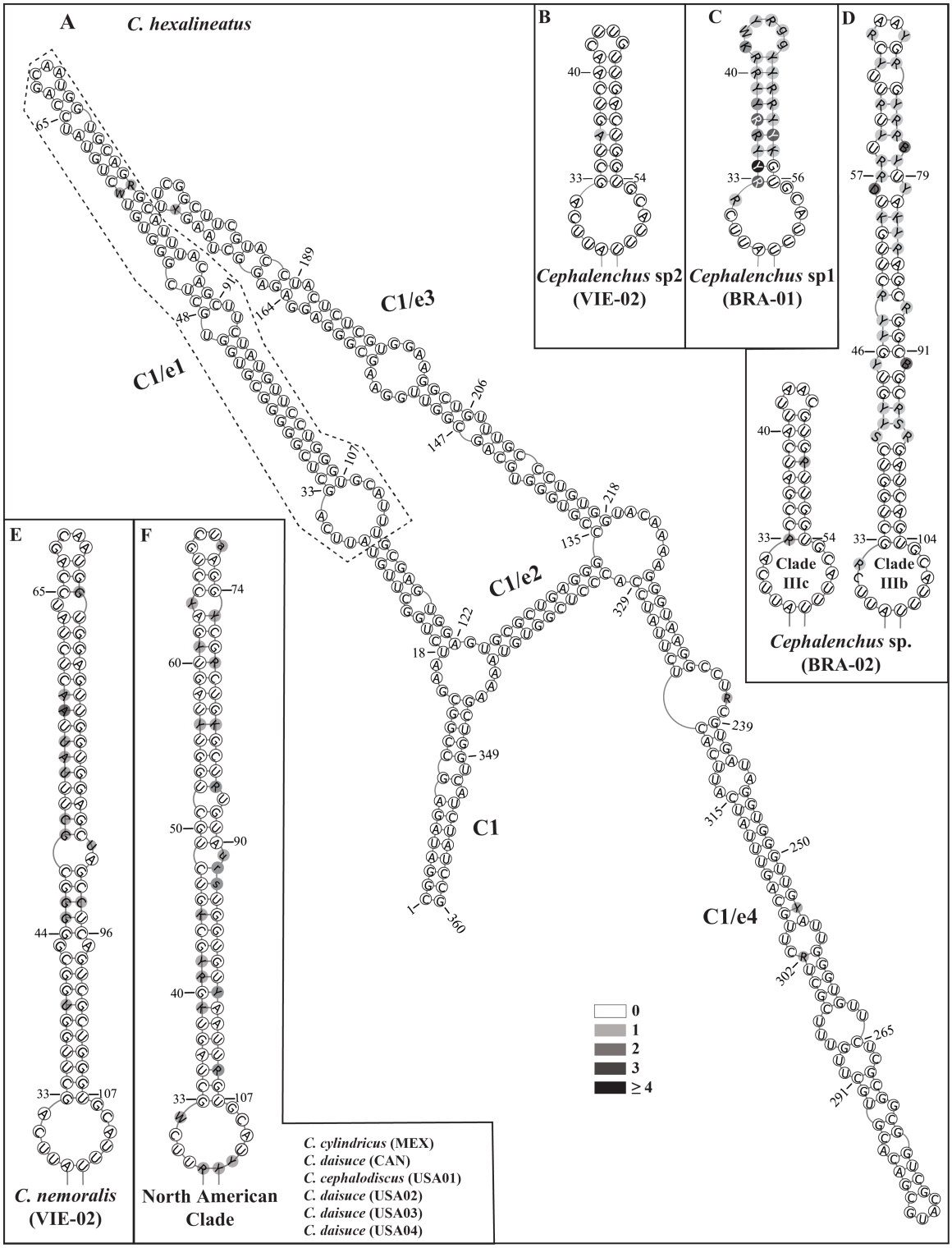

A
C. hexalineatus
B
Cephalenchus sp2
(VIE-02)
C
Cephalenchus sp1
(BRA-01)
D
Clade IIIc
Clade IIIb
Cephalenchus sp.
(BRA-02)
C1/e1
C1/e3
C1/e2
C1
C1/e4
E
C. nemoralis
(VIE-02)
F
North American
Clade
C. cylindricus (MEX)
C. daisuce (CAN)
C. cephalodiscus (USA01)
C. daisuce (USA02)
C. daisuce (USA03)
C. daisuce (USA04)
0
1
2
3
≥ 4

Figure 2.8. Variability map of D2 expansion fragment of the 28S rRNA gene superimposed on the putative consensus secondary structure provided by LocARNA. **A.** Complete predicted secondary structure for *C. hexalineatus* showing all five helices (C1 to C1/e4). **B-F.** Variation on the C1/e1 helix (reduced *vs.* long types) across *Cephalenchus* species. Variability sites were calculated using MP analysis (gap = fifth character). Variability sites are divided into five categories: 0, constant; 1, one change; 2, two changes; 3, three changes; 4, four or more changes. Lower case letters indicate deletion/insertion events. The standard ambiguity code for nucleotides is used.

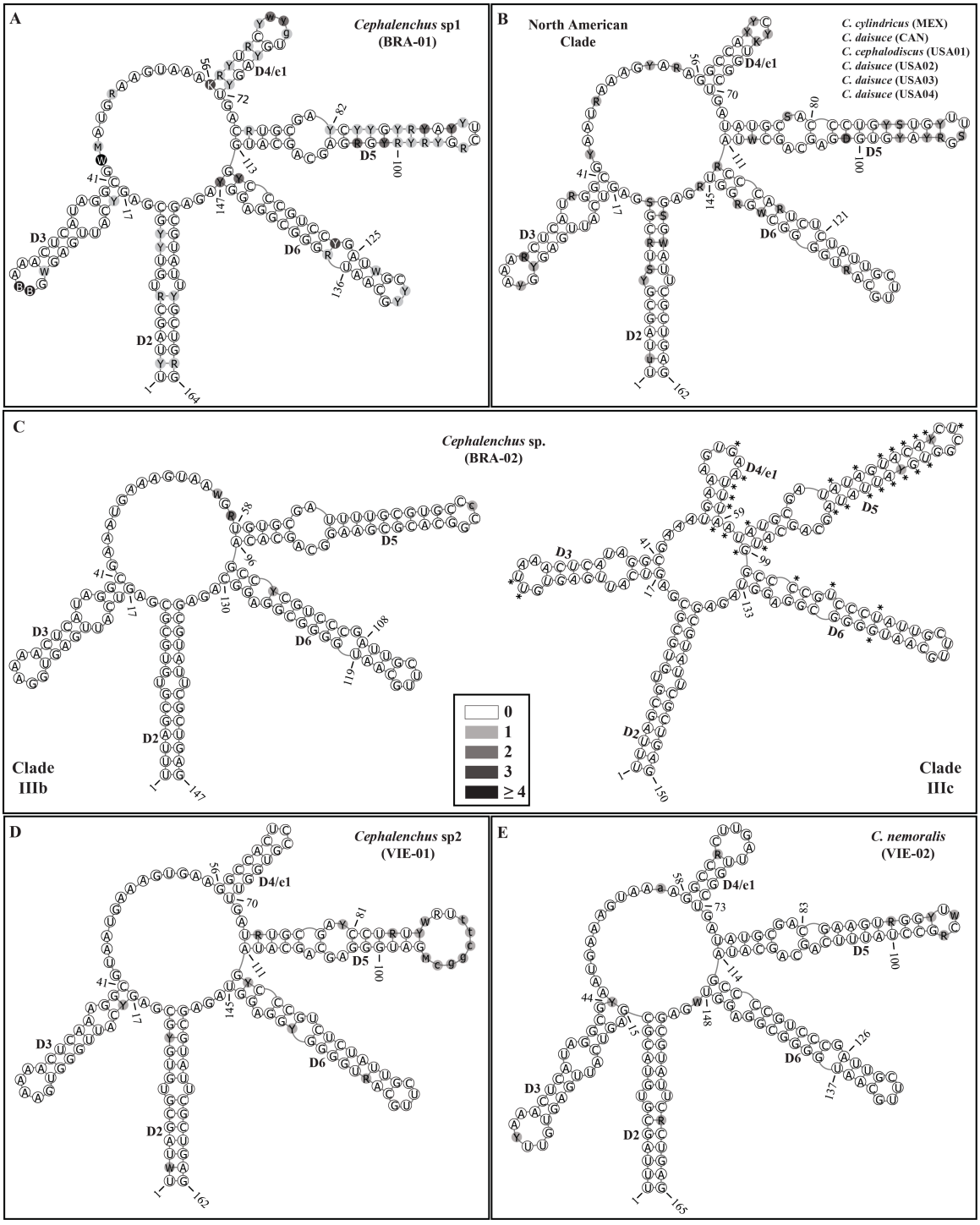


Figure 2.9. Variability map of D3 expansion fragment of the 28S rRNA gene superimposed on the putative consensus secondary structure provided by LocARNA for each *Cephalenchus* species. **A-E.** Predicted secondary structure for *Cephalenchus* species showing all five helices (D2 to D6, excluding D4). Black asterisks (**2.9C**) show the differences between the two predicted structures for *Cephalenchus* sp. (BRA-02). Codes and notations are as for Fig. 2.8.

Due to the length variation in the D2 domain, two secondary structure types, including a reduced and a long C1/e1 helix, were found among *Cephalenchus* species (Fig. 2.8). The reduced C1/e1 helix is found in *Cephalenchus* sp2 (VIE-01) and *Cephalenchus* sp1 (BRA-01), thus including species with high and low intragenomic/intraspecific sequence variation, respectively (Fig. 2.8B and 2.8C). Variation in the C1/e1 helix length is also observed within a single species. This was the case of *Cephalenchus* sp. (BRA-02) where the long C1/e1 helix represented clade IIIb and the reduced clade IIIc (Figs. 2.2 and 2.8D). Despite the length variation in the D3 domain, the predicted secondary structures were very similar across all *Cephalenchus* species (Fig. 2.9). A slight variation of the D3 domain was only detected for *Cephalenchus* sp. (BRA-02); sequences representing clade IIIb had helix D4/e1 fused into the central loop (Fig. 2.9C).

In the 5.8S gene, length variation within species was minimal and only found in *Cephalenchus* sp1 (BRA-01) and *C. daisuce* (USA-03 and USA-04). Likewise, 5.8S alignment variation between species was only one bp difference. The regions flanking 5.8S gene, ITS-1 and ITS-2, were more variable among *Cephalenchus* species, especially the ITS-1 where length variation is found in all species and populations, except *C. hexalineatus* (Table 2.5). In particular, the species and populations representing the North American clade displayed great variation (up 29 bp) in the length of the ITS-1 gene, a result of a large deletion event in some of the haplotypes from that clade. Yet, for the ITS-2 gene, considerable length variation was only found in *Cephalenchus* sp1 (BRA-01). Moreover, the predicted secondary structures for 5.8S gene and ITS-2 region were very

consistent across the different *Cephalenchus* species, except when *Cephalenchus* sp1 (BRA-01) is compared with the other species (data not shown). Although sequence variation in the ITS regions is much more pronounced than in the 5.8S and 28S coding regions, a close inspection shows the pattern of variation to be consistent among *Cephalenchus* species. Except for *C. cylindricus* (MEX) and *C. daisuce* (USA-04), ITS sequence divergences showed the following pattern: ITS-2 > ITS-1 > 5.8S. Additionally, three conserved 5.8S motifs identified by Harpke and Peterson (2008) for Nematoda are also found in all *Cephalenchus* species (Table 2.6).

Table 2.6. Comparison of 5.8S rRNA motifs across *Cephalenchus* species. Differences from Harpke and Peterson (2008) are highlighted (bold). Nucleotide ambiguity code is used to denote intraspecific polymorphism. Genetic divergence is separately given for 5.8S, ITS-1, and ITS-2.

Species	Motifs defined for Nematoda by Harpke and Peterson (2008) (based on two nematode species)			5.8S Motif variation within species[b]	p-distance[d] (%)		
	Motif-1 (16 bp) Position: site 35 CGATGAAGAACGCAGC	Motif-2 (13 bp, 1 gap)[a] Position: site 70 GAATTGCAG-ACAC	Motif-3 (10 bp) Position: site 98 TTCGAACGCA		5.8S	ITS-1	ITS-2
***Cephalenchus* sp1 BRA01**	CGAT**A**AAGAACGC**R**GC	GAA**C**TGCAG-A**T**AC	TT**Y**GAA**T**GCA	1-G; 0; 1-T	1.7 (0.0-3.8)	6.0 (0.0-15.0)	10.9 (0.0-27.8)
***C. cylindricus* MEX**	CGATGAAGAACGCAGC	GAA**C**TGCAG-A**T**A**T**	TTCGA**RT**GCA	0; 0; 1-G	0.1 (0.0-1.3)	2.1 (0.0-6.6)	1.8 (0.0-7.1)
***C. cephalodiscus* USA01**	CGA**Y**GA**M**GAACGCAGC	G**W**A**C**TGCAG-A**T**A**T**	TTCGAA**A**GCA	1-C, 1-C; 1-T; 0	0.6 (0.0-1.3)	0.9 (0.0-6.1)	1.0 (0.0-6.4)
***C. daisuce* USA02**	CGATGAAGAACGCAGC	GAA**C**TGCAG-A**T**A**T**	TTCGAA**T**GCA	0; 0; 0	0.3 (0.0-1.3)	2.8 (0.0-6.7)	3.1 (0.0-6.4)
***C. daisuce* USA03**	CGATGAAGAACGCAGC	**R**AA**C**TGCA**g**-A**T**A**T**[c]	TTCGAA**T**GCA	0; 1-A, 4-gap; 0	0.2 (0.0-1.3)	0.9 (0.0-5.6)	1.9 (0.0-5.4)
***C. daisuce* USA04**	CGATGAAGAACGCA**K**C	GAA**C**TGC**R**G-A**TRT**	TTCGAA**T**GCA	2-T; 1-G, 1-G; 0	0.8 (0.0-1.9)	3.3 (0.0-7.2)	0.4 (0.0-7.9)
***C. daisuce* CAN**	CGATGAAGAACGCAGC	GAA**C**TGCAG-A**T**A**T**	TTCGA**RT**G**M**A	0; 0; 1-G, 1-A	0.3 (0.0-1.9)	2.3 (0.0-5.6)	2.5 (0.0-6.9)
***Cephalenchus* sp2 VIE01**	CGATGAAGAACGCAGC	GAA**C**TGCAG-A**T**A**T**	TTCGAA**T**GCA	0; 0; 0	1.1 (0.0-1.9)	2.0 (0.0-3.6)	2.8 (0.0-5.0)
***C. nemoralis* VIE02**	CGATGAAGAACGCAGC	GAA**C**TGCAG-A**T**A**T**	TTCGAA**T**GCA	0; 0; 0	0.5 (0.0-1.9)	1.0 (0.0-2.0)	1.6 (0.0-5.2)
C. hexalineatus	CGATGAAG**R**ACGCAGC	GAA**C**TGCAG-A**T**A**T**	TTCGAA**T**GCA	1-G; 0; 0	0.2 (0.0-0.6)	0.6 (0.0-1.6)	1.1 (0.0-2.6)

[a] Gaps were included on *Cephalenchus* sequences for consistency purposes only.
[b] Number of sequences (and base type) differing from the consensus motif (order: motif-1; motif-2; motif-3).
[c] Lower case indicates a deletion/insertion event.
[d] Genetic divergence (p-distance %) is presented as mean (min-max) values.

DISCUSSION

Relaxed concerted evolution in some *Cephalenchus* species

Our findings suggest that not all *Cephalenchus* species used in this study undergo strict concerted evolution with respect to 28S and ITS genes. In fact, the levels of intragenomic and intraspecific polymorphism were surprisingly high for two *Cephalenchus* species found in Brazil when compared to those reported for other nematodes [*e.g.* 5.5% in the 18S gene of *Rotylenchulus reniformis* (Nyaku *et al.*, 2013); 2.7% in the 28S gene of *Halicephalobus gingivalis* (Yoshiga *et al.*, 2014)] and metazoans [2.2% in the ITS-1 region of *Myzus persicae* (Fenton *et al.*, 1998); 8% in the 18S gene of *Dugesia* (*Schmidtea*) *mediterranea* (Carranza *et al.*, 1996); 7.7% in the ITS-1 region of *Podisma pedestris* (Keller *et al.*, 2006)]. Accordingly, these studies have suggested the simultaneously co-existence of two divergent variants in the genome of the aforementioned species.

In the case of *Cephalenchus* sp1 (BRA-01), intragenomic polymorphism was the main source of sequence variation; that is, intragenomic and intraspecific divergences displayed exactly the same pattern for both genes (see Fig. 2.1). Variation in the 28S and ITS rRNA of *Cephalenchus* sp1 (BRA-01), however, did not result in dominance of one or two variants. In fact, sequences recovered for this species were mostly unique haplotypes, and sometimes highly divergent. Contrary to the high rRNA diversity, partial sequences of the cytochrome *c* oxidase subunit I (COI) of the mitochondrial DNA

(mtDNA) for *Cephalenchus* sp1 (BRA-01) showed very little intraspecific variation (< 1% on average based on 16 sequences, seven specimens) suggesting that for this marker intraspecific polymorphism has been sorted out (Pereira, unpublished data). High intragenomic polymorphism has been associated with the existence of pseudogenes in the genome, which are less likely to be homogenized by concerted evolution, and particularly so where genomes are large (Keller *et al.*, 2006; Xu *et al.*, 2015b). Pseudogenes are usually characterized by non CBCs that can disrupt the secondary structure as shown by Márquez *et al.* (2003) in the coral *Acropora*. Our secondary structure analyses, however, suggest that sequence polymorphism observed in *Cephalenchus* may not be related to the formation of pseudogenes.

Intraspecific variation in the 28S dataset was also high in *Cephalenchus* sp. (BRA-02), which was represented by two specimens. In contrast to *Cephalenchus* sp1 (BRA-01), this high variation was not a result of intragenomic polymorphism, but instead of variation among individuals. The large gap between intragenomic and intraspecific divergences in *Cephalenchus* sp. (BRA-02) is somewhat intriguing as these levels of sequence variation are typically in the same range or at least they display a degree of overlap (Harris and Crandall, 2000; André *et al.*, 2014; Weber and Pawlowski, 2014; Yasuda *et al.*, 2015). Alternatively, the existence of two cryptic *Cephalenchus* species, each represented by one specimen, may explain the pattern of variation observed in this geographic locality.

The distribution of rRNA arrays in different regions of the genome can also affect the ability of concerted evolution to remove polymorphisms within species and

particularly so when found in different chromosomes (Fenton *et al.*, 1998; Keller *et al.*, 2006). In C*aenorhabditis elegans*, rRNA array estimation ranges from 100-150 copies located exclusively in chromosome I. However, rRNA repeat numbers seems to vary substantially, from 56 to 323, as estimated by Bik *et al.* (2013). It is possible that the high intragenomic and intraspecific variation found in *Cephalenchus* sp1 (BRA-01) may relate to both rRNA copy number and rRNA chromosomal loci. This hypothesis can be further explored using fluorescent in situ hybridization (FISH) and genomic in situ hybridization (GISH) techniques to comparatively map the rRNA arrays of *Cephalenchus* species displaying different levels of polymorphism.

Predicted secondary structures do not support the existence of pseudogenes

For nematodes, several studies have analyzed the secondary structure of the D2 and D3 domains of the 28S gene (Subbotin *et al.*, 2005; Subbotin *et al.*, 2007; Bae *et al.*, 2010; Subbotin *et al.*, 2011; Douda *et al.*, 2013). The present findings regarding the general secondary structure of *Cephalenchus* for both domains are consistent with those from previous studies. Yet, a few features found among *Cephalenchus* species are notable for further discussion. Among the different predicted secondary structures for the D2-domain, *Cephalenchus* sp1 (BRA-01) had the highest number of base pair changes and these were mostly transitions (C⇔T and G⇔A) occurring in the stem regions, specifically on the C1/e1 and C1/e4 helices (Appendix C). Despite a large number of mutations, these changes were mostly CBCs or SBCs thus maintaining the base pairing

and consequently the stability of the secondary structure. This pattern has also been observed among species of Hoplolaimidae where the integrity of the D2-D3 secondary structures is maintained via CBCs or SBCs changes (Subbotin *et al.*, 2007; Bae *et al.*, 2010).

Another interesting feature is the shortening of the C1/e1 helix in some *Cephalenchus* species, a phenomenon observed in *Cephalenchus* sp1 (BRA-01) and *Cephalenchus* sp2 (VIE-01), thus including species characterized both by high and low levels of rDNA polymorphism, respectively (Fig. 2.8 and Appendix C). Although, length variation in the D2-D3 domains of the 28S gene is often documented among congeneric species of plant parasitic nematodes, these variations are usually low (Subbotin *et al.*, 2007; Bae *et al.*, 2010; Douda *et al.*, 2013; Subbotin *et al.*, 2015).

For criconematids (suborder Criconematina), (Subbotin *et al.*, 2005) found the C1/e1 helix to be the most variable region in the D2-domain; the authors also documented a large deletion event in the C1/e1 helix of *Trophonema arenarium* that closely resembles the reduced C1/e1 helix found in *Cephalenchus* sp1 (BRA-01) and *Cephalenchus* sp2 (VIE-01). Likewise, Nagahama *et al.* (2006) reported two types of C1/e1 helix (long: 52 bp; short: 21-27 bp) in the 28S gene of different yeast species, and Gillespie *et al.* (2004) characterized regions of expansion and contraction (REC), supposedly responsible for length variation in the C1/e1 helix of leaf beetles.

Thus, variation found in the C1/e1 helix of *Cephalenchus* can be interpreted as a true phenomenon and not as an artifact owing to the formation of pseudogenes. Variation in the C1/e1 helix also occurred within a species, as was the case of *Cephalenchus* sp.

(BRA-02). Length variation, resulting in different secondary structures, has been reported for species of the nematode genus *Ditylenchus*, and particularly so for the ITS region (Marek *et al.*, 2010; Subbotin *et al.*, 2011). That this length variation is found in the more conserved 28S gene supports the hypothesis of two cryptic species representing this geographic locality. Nevertheless, these findings suggest that remarkable variation of the D2-domain can occur among congeneric species.

Except for *Cephalenchus* sp1 (BRA-01), sequence variation of the 5.8S rRNA gene was minimal within *Cephalenchus* species, suggesting that this region is heavily constrained. Additionally, the pattern of sequence divergence, with ITS-1 and ITS-2 always being greater than that of 5.8S, also supports the idea that variation found in some *Cephalenchus* species is not due to the formation of pseudogenes. Moreover, the three 5.8S conserved motifs identified for nematodes by Harpke and Peterson (2008), which might suggest the functionality of the ITS transcribed spacers, are also found in all *Cephalenchus* species, even on those ITS-1 sequences with a large gap representing the North America clade. Differences between the motifs defined by Harpke and Peterson (2008) and those found in *Cephalenchus* are also detected, but may be due to the low number of nematode species analyzed by these authors (Table 2.6).

Implications of sequence variation for the *Cephalenchus* phylogeny and species delimitation

In this study, phylogenetic analyses showed the existence of five well-supported clades (found in both rRNA phylogenies) within *Cephalenchus* (Figs. 2.2 and 2.3). Moreover, for the *Cephalenchus* species included in this study, 28S and ITS phylogenies were fairly congruent with respect to presence or absence of monophyly, regardless of the levels of intraspecific variation. However, a few differences between these two phylogenies are noted. In the ITS phylogeny, *Cephalenchus* sp1 (BRA-01) seems to be more closely associated to the outgroup species, represented by *E. excretorius*, although this relationship is only supported by BI. Further analyses with the ITS region, however, recovered *Cephalenchus* as monophyletic with respect to *E. excretorius.*

This suggests that the ITS gene may be too variable (*i.e.* likely to display homoplasy) to resolve relationships within *Cephalenchus* considering extant limited representation of its species diversity. In fact, substitution saturation plots based on the number of transversions and transitions versus genetic distance showed that for this region, transversions outnumber transitions when the genetic divergence is high thus indicating saturation (data not shown). Additionally, concatenated analyses support (i) *Cephalenchus* sp1 (BRA-01, clade III) as sister of clades IV and V (BI = 0.7, MP = 100, ML = 79) and (ii) *C. hexalineatus* (clade I) and *C. nemoralis* (VIE-02, clade II) as sister to all other *Cephalenchus* species (BI = 1.0, ML = 100, but unresolved in MP) in agreement with the 28S phylogeny. Based on the less variable 18S gene, clades I and II

are also recovered as sister to all other *Cephalenchus* species, and this is further supported by unique morphology [*e.g.* character states of the amphid (*i.e.* sensory organ) slit opening (Pereira, unpublished data)].

The lack of reciprocal monophyly for the species representing the North American clade can also be attributed to the low resolving power of rRNA genes, which can be less sensitive to detect recent speciation events when compared to genes of the mtDNA genome (Blouin *et al.*, 1998; Blouin, 2002; Nieberding *et al.*, 2008). Limited data from partial COI sequences of the mtDNA for *Cephalenchus* sp1 (BRA-01) and *Cephalenchus* sp2 (VIE-01) show that the levels of interspecific divergence can be as high as those found in the ITS region [*Cephalenchus* sp1 (BRA-01) *vs. Cephalenchus* sp2 (VIE-01), ITS: 23.7%; COI: 22.4%], however with the absence of long gaps that make more difficult the alignment procedures (Pereira, unpublished data). Alternatively, extant morphological variation among species and populations in the North American clade can also result from phenotypic plasticity subject to environmental conditions. Molecular analyses (*i.e.* phylogeny and sequence divergence plots) support a single species with a broad distribution range (Figs. 2.1-2.3), although additional analyses based mtDNA would be needed to propose a strong case of synonymy for these *Cephalenchus* species.

Notwithstanding the high levels of intraspecific variation detected for *Cephalenchus* sp1 (BRA-01), the branch pattern exhibited by this species in both phylogenetic reconstructions is monophyletic (Figs. 2.2 and 2.3), suggesting a potential case of "shallow paralogy" as defined by Bailey *et al.* (2003). In this sense, a gene duplication event lacking concerted evolution had occurred subsequent to the most recent

speciation event [after the split between *Cephalenchus* sp1 (BRA-01) and *Cephalenchus* sp. (BRA-02)], thus not affecting the phylogeny reconstruction. Yet, *Cephalenchus* sp. (BRA-02) showed monophyly at the individual level, but not at the species level being paraphyletic with respect to *Cephalenchus* sp1 (BRA-01). Nematode specimens representing site BRA-02 (Amazon) were extracted from a composite soil sample, which was comprised of 12 subsamples as part of an ecological survey (Cares and Huang, 2008). The distance between these subsamples was up to 12 m and this scale for soil nematodes might increase chances of mixing species, which can be exacerbated if cryptic species are present in the area. Thus, the possibility of two *Cephalenchus* species, contrary to the morphological assumption, representing this locality cannot be completely ruled out.

How cross-fertilization may impact genetic diversity

Mode of reproduction has also been associated with rRNA sequence variation. Some studies support that cross-fertilization may increase intraspecific variation due to recombination [*e.g. Daphnia pulex* (Crease and Lynch, 1991); *M. persicae* (Vorburger *et al.*, 2003); *Lobaria pulmonaria* (Zoller *et al.*, 1999)]. On the other hand, Márquez *et al.* (2003) reported extreme rDNA diversity in the asexually reproducing coral *Acropora millepora* attributed to the inability of concerted evolution to homogenize divergent rDNA in asexual species. This has also been suggested by Pringle *et al.* (2000) to explain the high ITS variation in the fungus *Acaulospora colossica*. For root knot nematodes (genus *Meloidogyne*), Hugall *et al.* (1999) showed that parthenogenetic species were

characterized by two groups (12-26% divergence) of ITS sequences whereas sexual reproducing species had only one. Accordingly, Hugall et al. (1999) suggested that the reticulate pattern observed in the ITS region of parthenogenetic *Meloidogyne* species was due to hybrid origins.

Although, sex ratio was not precisely determined in the present study, males were fairly common in *Cephalenchus* sp1 (BRA-01) and *C. nemoralis* (VIE-02), and rare or not observed in the other species (Appendix B). For the 28S gene, sequence variation within *Cephalenchus* sp1 (BRA-01) and *C. nemoralis* (VIE-02) were considered high and still notable (Fig. 2.1), thus suggesting that potential cross-fertilization might foster maintenance of intraspecific polymorphism. At least for *Cephalenchus* sp1 (BRA-01), this view is also supported by the ITS region. Two hypotheses might explain why sequence variation in the ITS region for *C. nemoralis* (VIE-02) was not congruent with that of the 28S: (i) this species was represented by a lower number of individuals and clones relative to *Cephalenchus* sp1 (BRA-01) and (ii) specimen 7T13G10, which displayed the highest 28S polymorphism was not included in the ITS dataset due to failure of PCR. These results also highlight that biased datasets might obscure the true levels of rDNA polymorphism within a species.

Reproduction in *C. emarginatus* is thought to be via parthenogenesis as documented by Gowen (1970). Yet, *C. hexalineatus* is believed to be a species complex with both sexually and parthenogenetic reproducing species (Sutherland, 1967; Geraert, 2008). Among the 20 *Cephalenchus* species recognized by Geraert (2008), only three (*C. cylindricus*, *C. daisuce*, and *C. imphalus*) were described based solely on female

specimens. In this study, the same *Cephalenchus* species were also characterized by low intraspecific variation in both rRNA genes thus supporting the view that males might play an important role in the genetic diversity of some *Cephalenchus* species. Inspection of the female reproductive system showed reduced or atrophied spermatheca with no sperm in species where males were rare or not observed, but well defined and filled with sperm in *Cephalenchus* sp1 (BRA-01) and *C. nemoralis* (VIE-02) in agreement with observations made by Geraert (1968) on the genus *Cephalenchus*. A more comprehensive study, including additional *Cephalenchus* species in which males occur, is needed to further explore the relationships between cross-fertilization and high genetic diversity.

Implications for metagenetics and metabarcode studies

The findings presented herein underscore difficulties in establishing clear cut-offs when using molecular data for species delimitations. Overlap between intraspecific and interspecific (clade) genetic distances was clearly identified in *Cephalenchus* sp1 (BRA-01) for the 28S gene thus highlighting the limitations of distance methods when solely used for species delimitation (André *et al.*, 2014; Weber and Pawlowski, 2014; Subbotin *et al.*, 2015). Pattern of mutations in highly polymorphic species such as *Cephalenchus* sp1 (BRA-01) differ when intragenomic and intraspecific genetic divergences are compared, in particular mutations became informative when a larger number of sequences is evaluated. It also suggests that singletons observed at the intragenomic level are unlikely to be due to PCR or sequencing errors. For diatoms, Alverson and Kolnick

(2005) highlighted the importance of understanding intraspecific variation to properly define species boundaries. Likewise, Carranza *et al.* (1996) stressed the need for a large sample when dealing with rDNA sequences. Certainly, improving sample size (*i.e.* more specimens and clones per species) will increase the likelihood of recovering the true genetic diversity within a species as well as promote reliable interpretation of sequence artifacts.

As metagenetic techniques become commonplace for biodiversity surveys, caution on interpreting species diversity is crucial. Comparative studies in tintinnid ciliates have demonstrated discrepancies up to several orders of magnitude in species estimation, largely composed of artifacts produced by the OTU defining methods (Bachy *et al.*, 2013). Similarly, Lücking *et al.* (2014) found that, at the 95% similarity threshold, 454 pyrosequencing overestimated the diversity in *Cora inversa* 35-fold in comparison to the Sanger sequencing. When biodiversity is exclusively assessed using metagenetics, artifacts are likely to be overlooked due to lack of morphological vouchers and reference sequences. Thus, a set of Sanger reference sequences, preferable linked to a morphological voucher should also be considered in metagenetic biodiversity studies to avoid misinterpretations due to lack of calibration procedures and existence of intragenomic and intraspecific variation (Lücking *et al.*, 2014; Cowart *et al.*, 2015).

Chapter 3

Phylogeny and biogeography of the genus *Cephalenchus* (Tylenchomorpha, Nematoda): inferring species relationships from morphological and molecular data

ABSTRACT

The phylogenetic position of *Cephalenchus* in relation to other tylenchs is revisited. *Cephalenchus* populations, representing 11 nominal species, were sampled worldwide for molecular and morphological characterization. Morphological identification and exploration of extant and new characters was based on LM and SEM observations of multiple individuals and by following published diagnostic keys. Molecular analyses were based on three ribosomal (rRNA) genes (*i.e.* 18S, 28S, ITS) and using different alignment procedures (*i.e.* full *vs*. reduced alignments). Phylogenetic analyses (either combined or on a gene basis) always supported *Cephalenchus* as a monophyletic group. A sister relationship between *Cephalenchus* and *Eutylenchus* is recovered by most analyses; however branch support for this relationship varies depending on the dataset used. The position of *Cephalenchus* + *Eutylenchus* within Tylenchomorpha nevertheless remains ambiguous, thus highlighting the importance of using additional genes as well as increasing taxon sampling. Placement of *Cephalenchus* within Tylenchidae is not supported on the basis of two rRNA genes (*i.e.* 18S and 28S). Within *Cephalenchus*, amphid opening morphology (lateral *vs*. dorso-ventral) shows congruence with molecular-based phylogenetic relationships, whereas the number of lines in the lateral

field (six or four lines) can be interpreted only as an autapomorphy. Furthermore, all three rRNA genes support the non-monophyly of four morphologically defined *Cephalenchus* species. Morphometric analyses clearly distinguished short tail species from medium-long tail species and SEM observations suggest that *Cephalenchus* species with a shorter tail also might be characterized by a laterally oriented amphid opening. The range of *Cephalenchus* diversity is increased with the inclusion of two new species and the biogeography of the genus is further discussed.

INTRODUCTION

Plant parasitic nematodes (PPN), especially "tylenchs" (infraorder Tylenchomorpha De Ley and Blaxter, 2002), are responsible for worldwide crop losses. 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). On the other hand, many tylench species, not directly implicated in plant damage (*i.e.* presumably feeding on fungi, mosses, root hairs, or superficial root cells), are underrepresented in extant molecular phylogenies and therefore their phylogenetic associations remain poorly understood (Bert *et al.*, 2011).

Within Tylenchomorpha, 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 some taxa such as *Cephalenchus* (Goodey, 1962) Golden, 1971, are putatively misclassified within Tylenchidae (Palomares-Rius *et al.*, 2009; Van Megen *et al.*, 2009). Uncertainty regarding the phylogenetic position of *Cephalenchus*, as suggested by molecular data, underscores the need to re-evaluate classical morphology-based systems.

Cephalenchus was originally proposed by Goodey (1962) who described *Tylenchus (Cephalenchus) megacephalus* Goodey, 1962 as a new subgenus as well as species of *Tylenchus* Bastian, 1865. Subsequently, Golden (1971) raised *Cephalenchus* to genus level and named *C. hexalineatus* (Geraert, 1962) Geraert & Goodey, 1964 as the type species. The genus *Cephalenchus* is a relatively small group with about 20 valid species and its phylogenetic position under subfamily and family ranks remains controversial (Siddiqi, 2000; Geraert, 2008). Originally, Goodey (1962) placed *Cephalenchus* in the subfamily Tylenchinae Örley, 1880 within Tylenchidae, a scheme recognized by other authors (Geraert and Goodey, 1963; Geraert, 1968; Andrássy, 1984; Raski and Geraert, 1986). Conversely, Dhanachand and Jairajpuri (1980) transferred *Cephalenchus* 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 genera such as *Campbellenchus* Wouts, 1978 and *Pleurotylenchus* Szcygiel, 1969 (Siddiqi, 2000).

Molecular evidence based on the 18S ribosomal (rRNA) gene has shown *Cephalenchus* (*C. hexalineatus* only) to be a unique lineage and sister to most of the economically important PPN, however this position depends on and 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 evidence from the 28S rRNA gene (Palomares-Rius *et al.*, 2009; Atighi *et al.*, 2013; Qing *et al.*, 2015b) strongly supports a sister relationship between *C. hexalineatus* and *Eutylenchus excretorius* Ebsary & Eveleigh, 1981, although these are not closely related to other genera of Tylenchidae. Recently, Yaghoubi *et al.* (2015), also based on the 28S gene, showed *Cephalenchus* (*C. hexalineatus* + *C. leptus* Siddiqi, 1969) + *E. excretorius* closely related to *Malenchus* Andrássy, 1968 and *Lelenchus* Andrássy, 1954; however, these authors only included representative taxa of the Tylenchidae, which has been shown in broader molecular phylogenies to be polyphyletic, thus sidestepping a much needed more rigorous testing of the position of *Cephalenchus* relative to other PPN.

Herein, the phylogenetic position of *Cephalenchus* in relation to other PPN is revisited using several populations sampled worldwide. Species identification is based on morphological characters traditionally used for species diagnostics. Morphological observations are based on light microscopy (LM) and scanning electron microscopy (SEM). Molecular phylogenetic analyses are based on three (18S, 28S and ITS) rRNA genes. This study aims to (i) investigate the monophyly of *Cephalenchus*, (ii) the monophyly of a clade formed by *Cephalenchus* + *Eutylenchus* Cobb, 1913, and (iii) their phylogenetic position in relation to other Tylenchomorpha. Additionally, (iv)

morphological variation in the labial region of *Cephalenchus* is evaluated to explore speciation and congruence with species relationships as defined by molecular characters, and (v) the biogeography of *Cephalenchus*, based on the populations considered in this study together with those available in the literature, is further discussed.

MATERIAL AND METHODS

Sampling and nematode extraction

Soil samples, each about 300 g, were collected with a small shovel and stored in labeled plastic bags. Additionally, GPS coordinates, soil features, and associated plants/vegetation were documented in the field (Table 3.1). Nematode specimens were extracted from soil using either a Baermann funnel or plastic tray (Viglierchio and Schmitt, 1983; Hunt and De Ley, 1996). For those localities sampled outside the US, samples were split with subsamples fixed in DESS solution (Yoder *et al.*, 2006) and 5% formalin solution for molecular and morphological procedures, respectively. Specimens were sorted under a dissecting microscope (Olympus SZX16) for further morphological and molecular characterization; when needed, *Cephalenchus* identity was determined using a compound microscope (Nikon Eclipse E600). Samples collected in the US, were processed at the University of California, Riverside (UCR), so that fresh specimens were used for DNA extraction and PCR procedures. In addition, prior to DNA extraction a

morphological voucher of that specimen was digitally recorded as photos or through-focus videos (De Ley, 2000).

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

In addition to freshly collected samples, preserved (formalin fixed) wet collections from UCRNC were also consulted revealing five additional *Cephalenchus* populations, collected worldwide (Table 3.1). These *Cephalenchus* populations had not yet been identified to species and therefore were included for morphological characterization.

Analysis of *Cephalenchus* global distribution

In order to characterize the geographic distribution of *Cephalenchus*, GPS coordinates representing the studied populations were plotted on a world map (Fig. 3.1). For those populations retrieved from the UCRNC, an approximate GPS coordinate was estimated using Google Earth based on verbal descriptions (*i.e.* province/state and country names) from collecting logs. Additionally, a search on the genus was performed on the Web of Science website to include 89 sites where *Cephalenchus* has been documented from ecological and taxonomic studies. The geography distribution of *Cephalenchus*, based on 112 entries, is summarized in Figure 3.1.

Table 3.1. List of *Cephalenchus* species studied in the present study.

Species/Authority Name	Locality/Country	GPS Coordinates	Sampling Date	Study*	Habitat/host	Source
***Cephalenchus* sp1**	Jaguaruna, SC, Brazil (BRA-01)	*S 28° 36′ 02.43″ W 48° 56′ 44.64″*	07/01/2009 25/05/2010	Both	Moist soil, high organic matter, grass vegetation.	Pereira, T. J.
***Cephalenchus* sp.**	Benjamin Constante, AM, Brazil (BRA-02)	*S 04° 20′ 59.8″ W 69° 36′ 29.4″*	10/02/2010	Mol.	Clay soil, banana trees.	Cares, J.
C. cylindricus Sultan & Jairajpuri, 1982	Ensenada, BC, Mexico (MEX)	*N 31° 54′ 03″ W 116° 36′ 32″*	30/05/2009	Both	Moist soil, high organic matter, edge of a creek.	Pereira, T. J.
C. cephalodiscus Sultan & Jairajpuri, 1982	Yellowstone, WY, USA (USA-01)	*N 44° 52′ 57.19″ W 110° 44′ 04.89″*	01/07/2009	Both	Moist soil, high organic matter, edge of a creek.	Pereira, T. J.
C. daisuce Mizukubo & Minagawa, 1985	Woods Hole, MA, USA (USA-02)	*N 41° 31′ 42″ W 70° 40′ 30.80″*	30/07/2013	Both	Moist soil, associated to roots of trees.	Pereira, T. J.
C. daisuce Mizukubo & Minagawa, 1985	Riverside, CA, USA (USA-03)	*N 33° 59′ 7″ W 117° 18′ 18″*	22/02/2014	Both	Moist soil, high organic matter, edge of a creek.	Pereira, T. J.
C. daisuce Mizukubo & Minagawa, 1985	Cabin Creek, OR, USA (USA-04)	*N 43° 28′ 10.72″ W 123° 18′ 52.63″*	17/12/2013	Both	Small forest of mixed oak spp. Marshy area, 50 m from a creek.	Burr, J.
C. leptus Siddiqi, 1963	Sequoia National Park, CA, USA (USA-05)	*N 36° 33′ 37.08″ W 118° 44′ 33.36″*	08/06/2015	Both.	Moist soil, grass vegetation. Next to Tarp Log area.	Baldwin, J.
C. illustris Andrássy, 1984	Shell Mound, FL, USA (USA-06)	*N 29° 12′ 24.43″ W 83° 03′ 55.72″*	01/12/2008	Morph.	Silt/sand, *Juncus* sp. brackish marsh area	Holovachov, O.
C. longicaudatus Maqbool & Ghazala, 1986	Upland, CA, USA (USA-07)	-	22/10/1956	Morph.	Associated to azalea flower	UCRNC
C. hexalineatus (Geraert, 1962) Geraert & Goodey, 1964	Watsonville, CA, USA (USA-08)	-	22/02/1973	Morph.	Associated to apple roots	UCRNC
C. leptus Siddiqi, 1963	San Jacinto Mountains, CA, USA (USA-09)	-	05/06/1963	Morph.	Associated to *Veratrum* sp., *Pinus ponderosa*	UCRNC
C. daisuce Mizukubo & Minagawa, 1985	Deep Cove, Vancouver, BC, Canada (CAN-01)	*N 49° 19′ 41.24″ W 122° 56′ 57.40″*	29/08/2013	Both	Moist soil, grass area.	Lum, J.
C. hexalineatus (Geraert, 1962) Geraert & Goodey, 1964	Victoria, BC, Canada (CAN-02)	-	27/07/1967	Morph.	Associated to *Piceaabies*.	UCRNC
C. planus Siddiqui & Khan, 1983	Chantaburi, Thailand (THA)	-	06/06/1968	Morph.	Clay soil, associated to coffee and banana trees.	UCRNC
***Cephalenchus* sp2**	Nam Cat Tien, Vietnam (VIE-01)	*E 107° 20′ 25′′ N 11° 27′ 48′′*	26/05/2010	Both.	Moist soil, associated to Forest bamboo (*Bambusaprocera*).	Ragsdale, E.; Nguyen, C.
C. nemoralis Mizukubo & Minagawa, 1985	Cuc Phuong, Vietnam (VIE-02)	*E 105° 35′ 36′′ N 20° 20′ 28′′*	05/06/2010	Both.	Moist soil associated to forest banana tree.	Ragsdale, E.; Nguyen, C.
C. hexalineatus (Geraert, 1962) Geraert & Goodey, 1964	Poeke, Belgium (BEL-01)	*N 51° 02′ 34.5″ E 03° 27′ 18.0″*	05/11/2014	Both.	Forest soil near *Fagus* sp.	Qing, X.
C. daisuce Mizukubo & Minagawa, 1985	Ghent, Belgium (BEL-02)	*N 51° 02′ 08.99″ E 03° 43′ 19.16″*	10/10/2014	Both.	Forest in botanic garden of UGent, near bamboo soil.	Qing, X.
C. leptus Siddiqi, 1963	Jinping, China (CHN-01)	*N 22° 58′ 48.9″ E 103° 23′ 33.5″*	15/07/2013	Both.	Subtropical rain forest, associated to bushes soil.	Qing, X.
C. cephalodiscus Sultan & Jairajpuri, 1982	Pingbian, China (CHN-02)	*N 23°02'53.1″ E 103°25'27.0″*	25/05/2014	Both.	Subtropical rain forest, associated to bushes soil.	Qing, X.

Table 3.1. (Continued).

Species/Authority Name	Locality/Country	GPS Coordinates	Sampling Date	Study*	Habitat/host	Source
C. cephalodiscus Sultan & Jairajpuri, 1982	Pingbian, China (CHN-03)	*N 23°00'49.0" E 103°25'20.5"*	25/05/2014	Both.	Subtropical rain forest, associated to bushes soil.	Qing, X.
C. hexalineatus (Geraert, 1962) Geraert & Goodey, 1964	San José, Costa Rica (CRI)	*N 9°53'41.1" W 84°04'27.2"*	30/06/2014	Both	Soil around *Acnistus arborescens* tree	Qing, X.
C. hexalineatus (Geraert, 1962) Geraert & Goodey, 1964	OR, USA FL, USA	- -		Mol. Mol.	GenBank	Palomares-Rius *et al.* 2009
C. leptus Siddiqi, 1963	Northwestern, Iran	-		Mol.	GenBank	Panahandeh *et al.* 2015

Mol.: molecular; Morph.: morphological, Both: molecular and morphological.
(-) Data not available

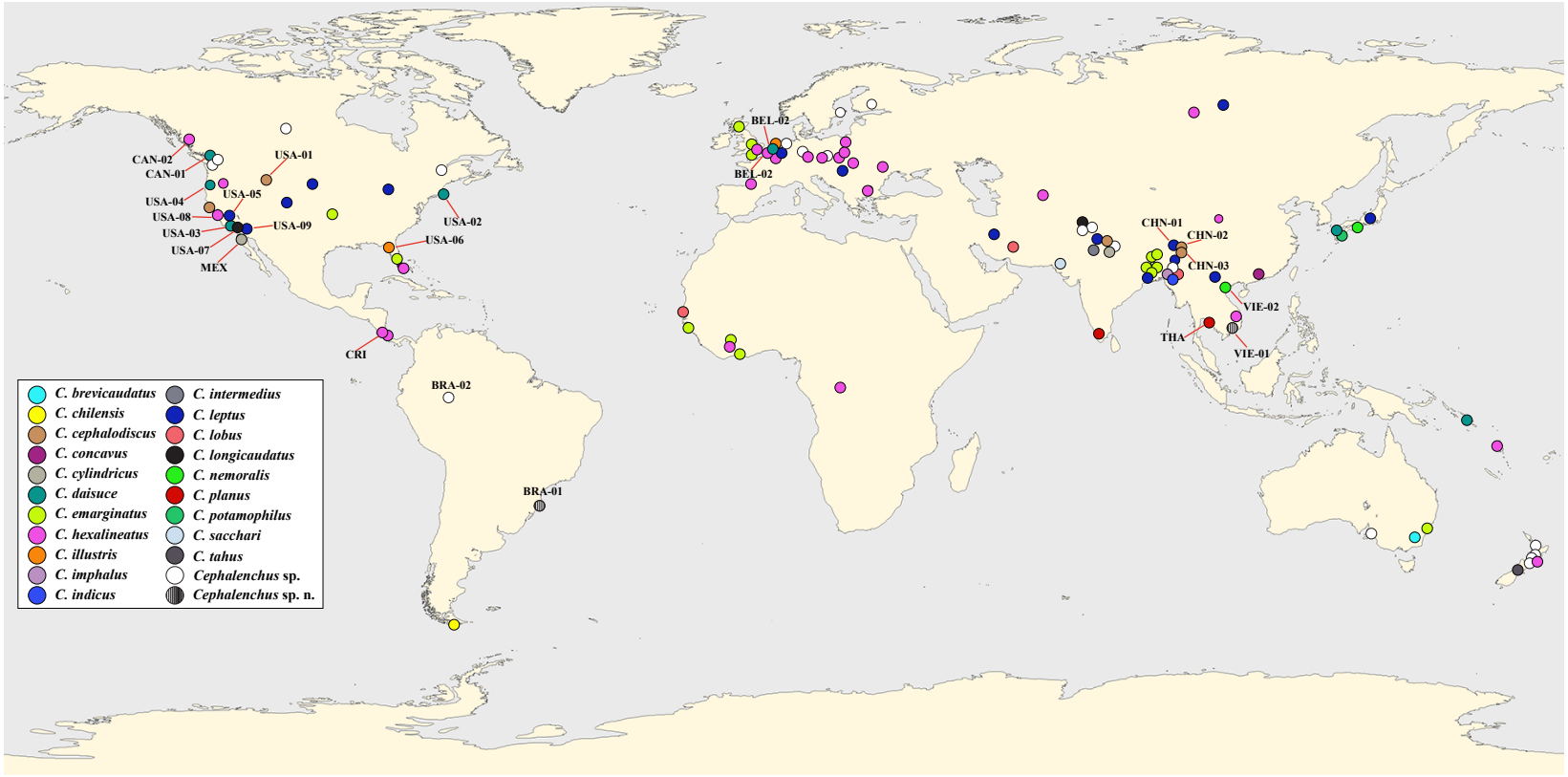


Figure 3.1. Worldwide distribution of the genus *Cephalenchus* based on the published literature and new collections (this study). *Cephalenchus* species are color-coded. New *Cephalenchus* sampled sites and samples retrieved from the UCRNC are indicated by their locality code.

Morphological characterization

LM procedures

For permanent slide mounts, 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 glycerin/ethanol solutions to pure glycerin (Seinhorst, 1959). Permanent slides were examined using a Zeiss Axioskop microscope equipped with a drawing tube. Morphological parameters were manually measured using a micrometer and following Geraert (2008). Identification of *Cephalenchus* species was based on original descriptions and supplemented by available keys (Andrássy, 1984; Geraert and Raski, 1987; Geraert, 2008).

SEM procedures

Specimens of each population, including 5-10 individuals of males and females, were processed for SEM. Specimens were repeatedly rinsed in distilled water for 5 min to remove all traces of formalin and then post-fixed overnight in an aqueous solution of 2.0% osmium tetroxide. Post-fixed specimens were dehydrated through a series of aqueous dilutions of 10-100% ethanol. Dehydrated specimens were critical point dried in a Tousimis Autosamdri-810®. Specimens were mounted on double-sticking copper tape attached to aluminum 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

Morphometric analyses were based on measurements of female specimens. Statistical analyses were carried out to evaluate the significance of morphological distinction (dissimilarity) between different *Cephalenchus* species as well as to explore patterns of phenotypic variation. Morphological data was first normalized and then used to compute pair-wise Euclidean distances among individuals. Non-metric multidimensional scaling (MDS) was used to assess morphological differentiation among species. Significant differences ($p < 0.05$) among groups were assessed with analysis of similarity (ANOSIM, Clarke and Gorley 2006). Analysis of similar percentages (SIMPER) was used to identify which morphological characters contributed most to the differentiation among groups. All morphological analyses were performed using Primer version 6.0 (Clarke and Gorley, 2006). Missing data for morphological characters were replaced by the mean value of that particular *Cephalenchus* population.

Labial pattern reconstruction

Few studies have examined the diversity of lip morphology of *Cephalenchus* using either SEM or LM. The terminology applied in the present study to describe the anterior region of *Cephalenchus* is largely consistent with previous authors (Raski and Geraert, 1986; Geraert, 2008), but with some modifications for more consistent clarity of apparent homologies among *Cephalenchus* species and potential outgroup taxa. Based on SEM micrographs, the labial pattern (frontal view) of each species was evaluated; regardless of variation, three typical patterns were recognized. As a complement to SEM

observations, 3D structures representing these three patterns were modeled using Autodesk® Maya® following the procedure of Qing *et al.* (2015a). This reconstruction further allows exploring morphological characters that might inform phylogeny among species as well as to distinguish character polarity (*e.g.* basal and derived character states) within the genus.

Molecular analysis

DNA extraction, amplification, and sequencing

DNA was extracted from single individuals using proteinase K protocol and Worm Lysis Buffer (WLB). Each nematode was placed in a drop containing 5 µl of WLB, cut in pieces, and transferred to a 0.2 ml PCR tube with an additional 15 µl of WLB and 2µl of proteinase K (10 mg/ml). 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 rDNA were amplified with primers D2Ab and D3B (De Ley *et al.*, 2005). The 18S rDNA gene was amplified either using 3 overlapping sets of primers (G18S4 and 4R; 22F and 13R; 4F and 18P; see (Blaxter *et al.*, 1998; Bert *et al.*, 2008) or using only primers G18S4 and 18P. The ITS region (ITS-1, 5.8S, and ITS-2) of the rDNA was also amplified using the primers N93 and N94 (Nadler *et al.*, 2000). All PCR reactions were 25 µl made of as it follows: 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®). Samples with low DNA template were also subjected to a

GenomiPhi protocol (GenomiPhi V2 DNA Amplification Kit, GE Health Care®) to increase the amount of DNA in the samples prior to PCR. Amplification success was evaluated electrophoretically on 1% agarose gel. PCR products were purified for sequencing using the QIAquick PCR purification kit (Qiagen®) following the manufacturer's protocol. Finally, the 18S, 28S, and ITS genes were sequenced in both directions with PCR primers using ABI-PRISM® Dye-DeoxyTerminator Big Dye™ v3.1 (Applied Biosystems) with an automatic sequencer Gene Analyzer® ABI 3100 (Applied Biosystems) in the UCR Genomics Center.

Phylogenetic analyses

In order to evaluate the phylogenetic position of *Cephalenchus* and its validity as a natural group (*i.e.* monophyly), 18S, 28S, and ITS sequences from additional Tylenchomorpha and outgroup taxa were downloaded from GenBank (see Appendix D). The selection of outgroup taxa was informed by results of previous molecular phylogenies (Subbotin *et al.*, 2006; Bert *et al.*, 2008; Palomares-Rius *et al.*, 2009; Van Megen *et al.*, 2009). Broader phylogenetic analyses were based on sequences of 159 (for 18S and 28S genes) taxa covering the major lineages within Tylenchomorpha. On the other hand, ITS sequences were only used to explore relationships within *Cephalenchus* owing to the high variability found in the ITS region. Sequences from all three rRNA genes were separately aligned using Mafft using and different alignment strategies [http://mafft.cbrc.jp/alignment/server, (Katoh and Standley, 2013). 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) were used for further molecular phylogenetic analyses.

Phylogenetic relationships among sequences were estimated with maximum likelihood (ML) and Bayesian inference (BI) on the CIPRES Science Gateway (http://www.phylo.org/); 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 replicates) was automatically calculated for the best-scoring ML tree (Stamatakis, 2006,2014). BI analysis was performed using MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) under the GTR + I + G model with the settings: random starting tree, 2 independent runs with 4 chains (2.0×10^8 generations for both genes). Markov chains were sampled at intervals of 1000 generations. Convergence was assessed using standard deviation of split frequencies (less than 0.01) and PSRF (Potential Scale Reduction Factors, 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. The best fitting substitution model for the different datasets (18S, 28S and ITS genes) was estimated using jModelTest 2.1.2 (Darriba *et al.*, 2012) based on the Akaike Information Criterion. Concatenated analyses (18S + 28S + 5.8S, a total of 2758 sites) were also performed for a select group of sequences representing the major PPN groups.

RESULTS

Cephalenchus geographic distribution

Cephalenchus has been reported from all continents, except Antarctic, and has been found as far north as 62.9° N in Finland (reported only as *Cephalenchus* sp.) and as far south as 55.4° S in Chile (type locality of *C. chilensis* Raski and Geraert, 1986). Based on the published literature retrieved from Web of Science, however, the worldwide distribution of *Cephalenchus* seems to be mostly concentrated 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, all other sites where *Cephalenchus* occurred were reported north of the equator (Fig. 3.1). Herein, two additional sites in the Southern Hemisphere, south (BRA-01) and north of Brazil (BRA-02), are added to the geographic distribution of *Cephalenchus*. Among *Cephalenchus* species, *C. hexalineatus* (26 entries) and *C. leptus* Siddiqi, 1963 (16 entries) are the most widely distributed. Also, from numerous sites (19 entries), *Cephalenchus* is only reported as *Cephalenchus* sp. Although, most *Cephalenchus* species were described from India (eight species), two other geographic regions, USA (eight species) and Europe (five species), also harbor considerable *Cephalenchus* diversity (Fig. 3.1).

Species identity and morphological variation across *Cephalenchus*

Based on published morphological descriptions, 11 species of *Cephalenchus* were identified including *C. cephalodiscus* Sultan & Jairajpuri, 1981 (USA-01, CHN-02 and CHN-03), *C. cyclindricus* 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), *C. illustris* Andrássy, 1984 (USA-06), *C. leptus* (CHN-01, USA-05 and USA-09), *C. longicaudatus* Maqbool & Ghazala, 1986 (USA-07), *C. nemoralis* Mizukubo & Minagawa, 1985 (VIE-02), and *C. 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 properly described elsewhere (Pereira, unpublished data).

Multidimensional scaling analysis (MDS, Fig. 3.2A) based on morphometric data of 23 *Cephalenchus* populations (11 nominal species), showed that, in general, nematode specimens representing specific populations clustered together with substantial overlap. An exception is *Cephalenchus* sp1 (BRA-01) which was much more widely dispersed throughout the 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. 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*).

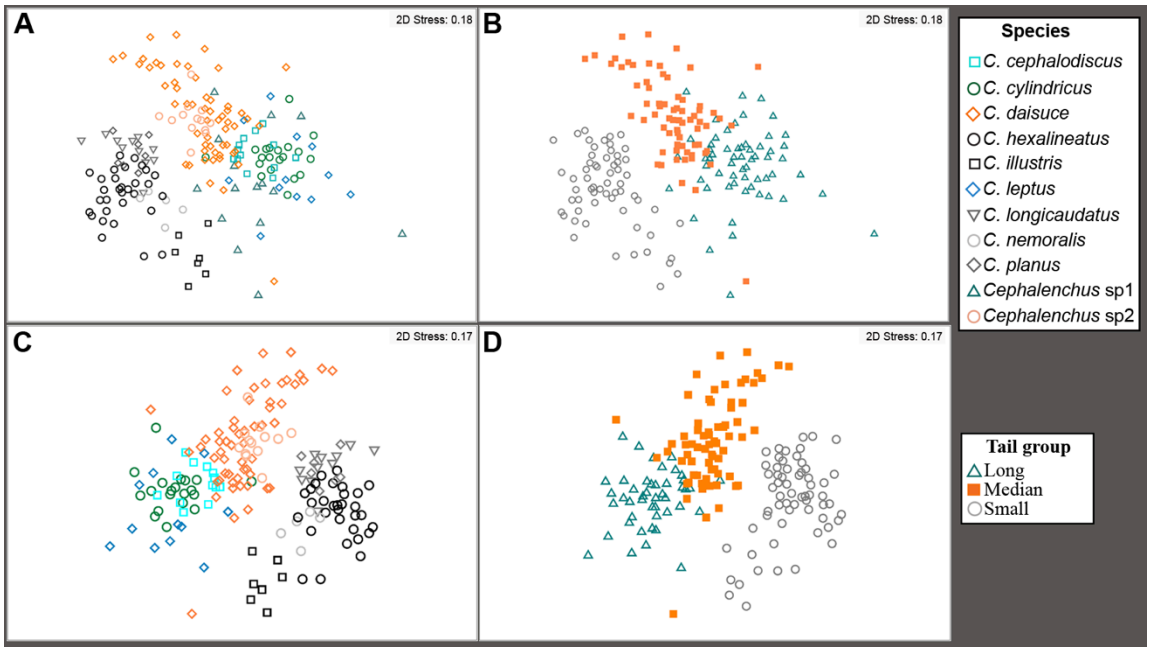


Figure 3.2. Multi-dimensional scaling (MDS) plot obtained from the morphometric data of *Cephalenchus* species. **A.** Spatial distribution of all *Cephalenchus* species in the morphological space. **B.** *Cephalenchus* species are clustered according to tail group. **C.** Spatial distribution of all *Cephalenchus* species, but excluding *Cephalenchus* sp1 (BRA-01) from the dataset. **D.** *Cephalenchus* tail grouping, but also excluding *Cephalenchus* sp1 (BRA-01) from the dataset.

The MDS analysis also showed a clear 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 median (mean ≤ 202 µm) to long (mean ≥ 206 µm) tail (Fig. 3.2B). On the other hand, the transition between species with a median to long tail is less clear; species with median tail length such as *C. daisuce* (multiple

populations) and *Cephalenchus* sp2 showed a certain degree of overlap with species possessing longer tails. Nevertheless, significant differences between tail groups were detected by the ANOSIM for all comparisons (Table 3.2). Only 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. 3.2A), the other three species were distributed in the same region of the morphological space. These patterns of species cohesiveness and tail group aggregation become more evident when *Cephalenchus* sp1 is removed from the MDS analysis (Fig. 3.2C-D).

Table 3.2. 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.* median tail	0.413	**0.001**
Long *vs.* small tail	0.82	**0.001**
Median *vs.* small tail	0.635	**0.001**
Between populations		
Cephalenchus sp1 (BRA-01) *vs. C. daisuce* (BEL-02)	0.039	0.401
C. daisuce (USA-02) *vs. C. daisuce* (BEL-02)	0.183	0.123
C. longicaudatus (USA-07) *vs. C. planus* (THA)	0.105	0.133
Between species		
C. 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

Characterization of labial pattern in *Cephalenchus*

With the exception of *C. illustris*, all other *Cephalenchus* species were observed under SEM. Although specimens from some of the populations were particularly fragile and prone to distortion, comparable species-specific lip patterns could be accurately reconstructed and illustrated based on a combination of specimens and micrographs. From these observations, two clear patterns emerged: (1) *Cephalenchus* species (*i.e. C. hexalineatus*, *C. longicaudatus*, *C. nemoralis*, and *C. planus*) with a small and laterally oriented amphid opening; and (2) *Cephalenchus* species (all other species) with a large dorso-ventrally oriented amphid opening (Fig. 3.3A-D).

As well as the amphid orifice (dorsal-ventral *vs*. lateral), the oral opening was also distinct between these two groups. *Cephalenchus* species with a dorso-ventrally oriented amphid have a very narrow dorsoventral slit on the oral disc (Fig. 3.3A-B). These *Cephalenchus* species are also characterized by a short tail. *Cephalenchus* species with a laterally oriented amphid displayed a small rounded-oval oral opening on the oral disc (Fig. 3.3C-D) and the tail length is median or long. All *Cephalenchus* species displayed a button like cephalic papilla in each of the four subventral wings of the labial disc (butterfly face view pattern) and had no annulations on the cephalic region (smooth head).

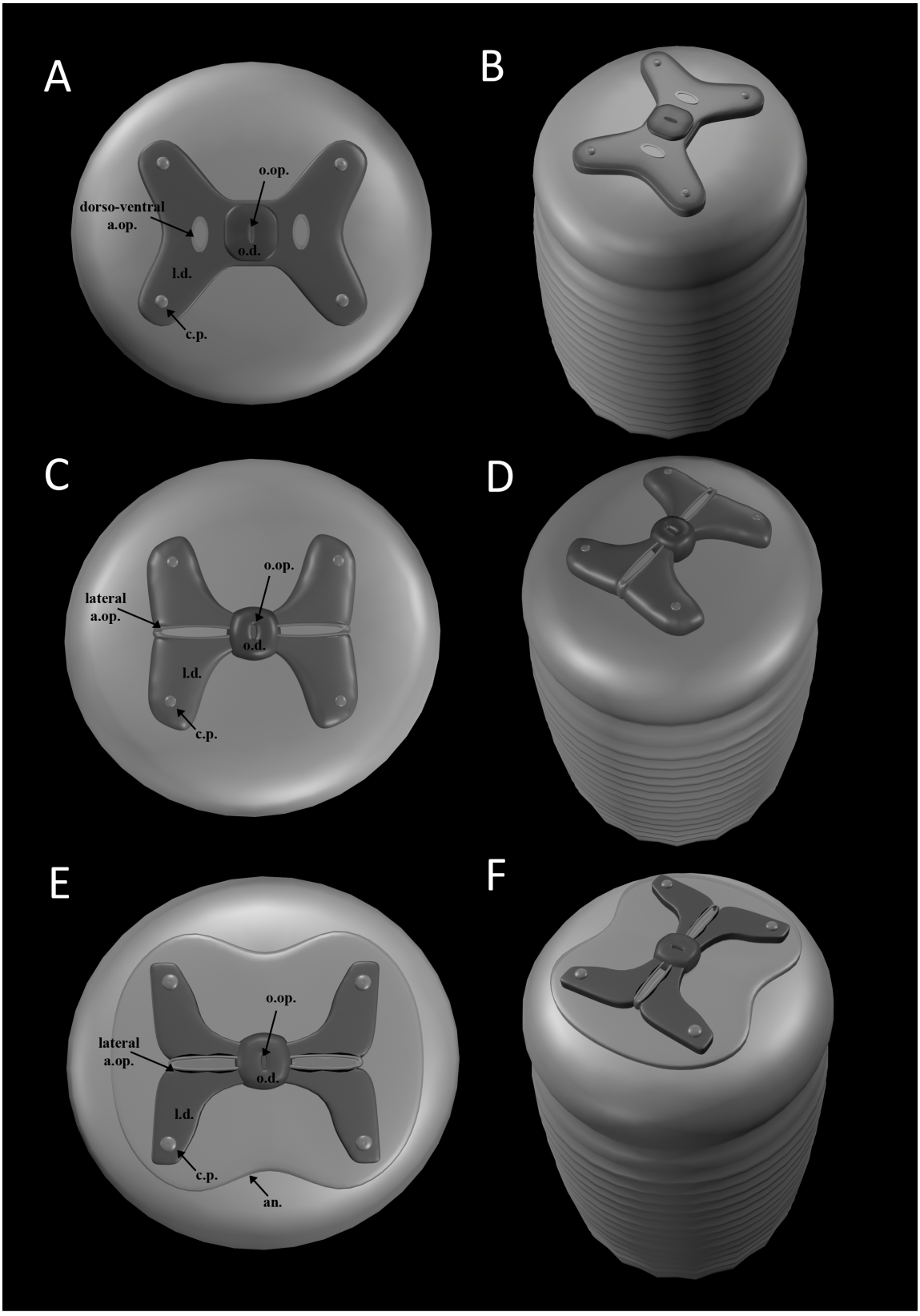

A
o.op.
dorso-ventral
a.op.
o.d.
l.d.
c.p.
B
C
o.op.
lateral
a.op.
o.d.
l.d.
c.p.
D
E
o.op.
lateral
a.op.
o.d.
l.d.
c.p.
an.
F

Figure. 3.3. Labial patterns among *Cephalenchus* species. **A-B.** 3-D reconstruction based on SEM micrographs of *Cephalenchus* sp1 (BRA-01) showing the dorsal-ventrally oriented amphid opening. **C-D.** Reconstruction based on SEM micrographs of *C. hexalineatus* (USA-08) showing the laterally oriented amphid opening. **E-F.** Reconstruction of *C. brevicaudatus* based on SEM micrographs included in Raski and Geraert (1986). Labels are included on the face view images (*i.e.* 3.3A, 3.3C, 3.3F) to better explaing the labial (Abbreviations: o.op = oral opening, a.op = amphid opening, o.d = oral disc, l.d. = labial disc, c.p. = cephalic papilla, an. = first annulation).

For context, *C. brevicaudatus* Raski and Geraert, 1986 was also redrawn based on SEM micrographs available in the literature (Raski and Geraert, 1986) and included in this comparison. By contrast, *C. brevicaudatus* has annulations on the cephalic region, but its amphid and oral openings agreed with that of species in the first group (Fig. 3.3E-F). This species is also characterized by a short tail (54-93 μm).

Molecular characterization of *Cephalenchus* species

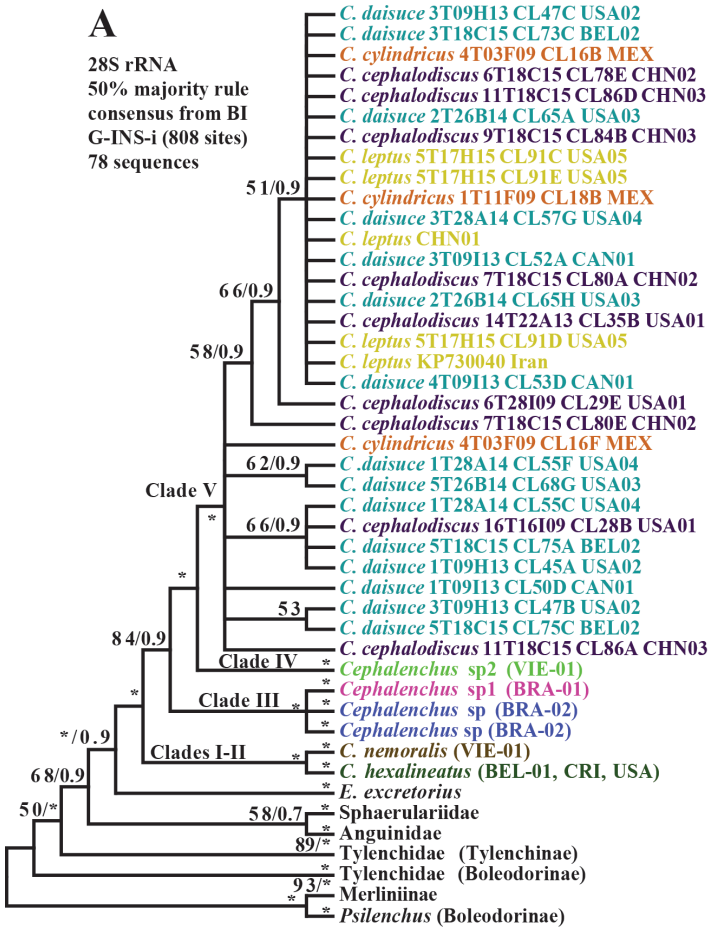
Molecular data are presented for 20 *Cephalenchus* populations (Table 3.1). From these, 12 populations were previously studied by Pereira and Baldwin (2016); accordingly, some *Cephalenchus* populations have been characterized by high levels of intragenomic and intraspecific rRNA sequence variation. With the exception of *C. leptus* (CHN-01) and *C. hexalineatus* (CRI), DNA sequences produced in the present study were obtained from multiple specimens and clones, thus providing insight into intraspecific variation. GenBank accession numbers for the newly sampled *Cephalenchus* species as well as previously published Tylenchomorpha sequences used in the phylogenetic analysis are given in Appendix D.

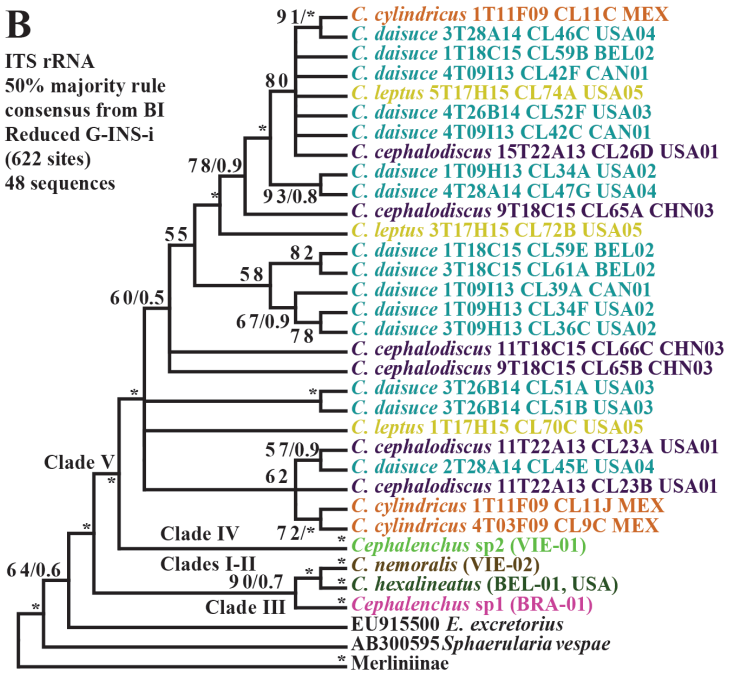
Intraspecific variation, although very low, was observed in all newly sampled *Cephalenchus* species. For the 28S gene, sequence divergence ranged from 0-1.9% and 0-1.5% in *C. cephalodiscus* (CHN-02 and CHN-03, respectively), 0.1-1.8% in *C. daisuce* (BEL-02), 0.4-0.8% for *C. hexalineatus* (BEL-01), and 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 ranged from 0-2.7% in *C. cephalodiscus* (CHN-03), 0-4.2% in *C. daisuce* (BEL-02), and 0-4% in *C. leptus* (USA-05). The two new populations of *C. hexalineatus* (BEL-01 and CRI) grouped with *C. hexalineatus* from the USA in a strongly supported clade (Fig. 3.4A-B). The three other species including *C. cephalodiscus* (CHN-02 and CHN-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 were congruent among the three rRNA genes with respect to the number of clades as well as the monophyly of the different *Cephalenchus* species (Fig. 3.4A-C). Five well-supported clades are recovered in the 28S and ITS phylogenies: clades I and II, represented by *C. nemoralis* (VIE-02) and *C. hexalineatus* (BEL-01, CRI, USA), 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. 3.4A). In the 18S and 28S phylogenies, *Cephalenchus* sp1 (BRA-01) is sister to clades IV + V with relatively high support. Yet in the ITS phylogeny, this species is recovered as sister to clades II and III.

Clade IV is represented by *Cephalenchus* sp2 (VIE-01) and its sister relationship with clade V is strongly supported by all phylogenetic analyses. Clade V comprises four morphologically defined species including multiple populations of *C. cephalodiscus*, *C. daisuce, C. leptus,* and *C. cylindricus* (MEX). The non-monophyly of these four species is supported in both 28S and ITS rRNA phylogenies (Fig. 3.4A-B).


A
28S rRNA
50% majority rule
consensus from BI
G-INS-i (808 sites)
78 sequences
C. daisuce 3T09H13 CL47C USA02
C. daisuce 3T18C15 CL73C BEL02
C. cylindricus 4T03F09 CL16B MEX
C. cephalodiscus 6T18C15 CL78E CHN02
C. cephalodiscus 11T18C15 CL86D CHN03
C. daisuce 2T26B14 CL65A USA03
C. cephalodiscus 9T18C15 CL84B CHN03
C. leptus 5T17H15 CL91C USA05
C. leptus 5T17H15 CL91E USA05
C. cylindricus 1T11F09 CL18B MEX
C. daisuce 3T28A14 CL57G USA04
C. leptus CHN01
C. daisuce 3T09I13 CL52A CAN01
C. cephalodiscus 7T18C15 CL80A CHN02
C. daisuce 2T26B14 CL65H USA03
C. cephalodiscus 14T22A13 CL35B USA01
C. leptus 5T17H15 CL91D USA05
C. leptus KP730040 Iran
C. daisuce 4T09I13 CL53D CAN01
C. cephalodiscus 6T28I09 CL29E USA01
C. cephalodiscus 7T18C15 CL80E CHN02
C. cylindricus 4T03F09 CL16F MEX
C .daisuce 1T28A14 CL55F USA04
C. daisuce 5T26B14 CL68G USA03
C. daisuce 1T28A14 CL55C USA04
C. cephalodiscus 16T16I09 CL28B USA01
C. daisuce 5T18C15 CL75A BEL02
C. daisuce 1T09H13 CL45A USA02
C. daisuce 1T09I13 CL50D CAN01
C. daisuce 3T09H13 CL47B USA02
C. daisuce 5T18C15 CL75C BEL02
C. cephalodiscus 11T18C15 CL86A CHN03
Cephalenchus sp2 (VIE-01)
Cephalenchus sp1 (BRA-01)
Cephalenchus sp (BRA-02)
Cephalenchus sp (BRA-02)
C. nemoralis (VIE-01)
C. hexalineatus (BEL-01, CRI, USA)
E. excretorius
Sphaerulariidae
Anguinidae
Tylenchidae (Tylenchinae)
Tylenchidae (Boleodorinae)
Merliniinae
Psilenchus (Boleodorinae)
Clade V
Clade IV
Clade III
Clades I-II


B
ITS rRNA
50% majority rule
consensus from BI
Reduced G-INS-i
(622 sites)
48 sequences
C. cylindricus 1T11F09 CL11C MEX
C. daisuce 3T28A14 CL46C USA04
C. daisuce 1T18C15 CL59B BEL02
C. daisuce 4T09I13 CL42F CAN01
C. leptus 5T17H15 CL74A USA05
C. daisuce 4T26B14 CL52F USA03
C. daisuce 4T09I13 CL42C CAN01
C. cephalodiscus 15T22A13 CL26D USA01
C. daisuce 1T09H13 CL34A USA02
C. daisuce 4T28A14 CL47G USA04
C. cephalodiscus 9T18C15 CL65A CHN03
C. leptus 3T17H15 CL72B USA05
C. daisuce 1T18C15 CL59E BEL02
C. daisuce 3T18C15 CL61A BEL02
C. daisuce 1T09I13 CL39A CAN01
C. daisuce 1T09H13 CL34F USA02
C. daisuce 3T09H13 CL36C USA02
C. cephalodiscus 11T18C15 CL66C CHN03
C. cephalodiscus 9T18C15 CL65B CHN03
C. daisuce 3T26B14 CL51A USA03
C. daisuce 3T26B14 CL51B USA03
C. leptus 1T17H15 CL70C USA05
C. cephalodiscus 11T22A13 CL23A USA01
C. daisuce 2T28A14 CL45E USA04
C. cephalodiscus 11T22A13 CL23B USA01
C. cylindricus 1T11F09 CL11J MEX
C. cylindricus 4T03F09 CL9C MEX
Cephalenchus sp2 (VIE-01)
C. nemoralis (VIE-02)
C. hexalineatus (BEL-01, USA)
Cephalenchus sp1 (BRA-01)
EU915500 E. excretorius
AB300595 Sphaerularia vespae
Merliniinae
Clade V
Clade IV
Clade III
Clades I-II

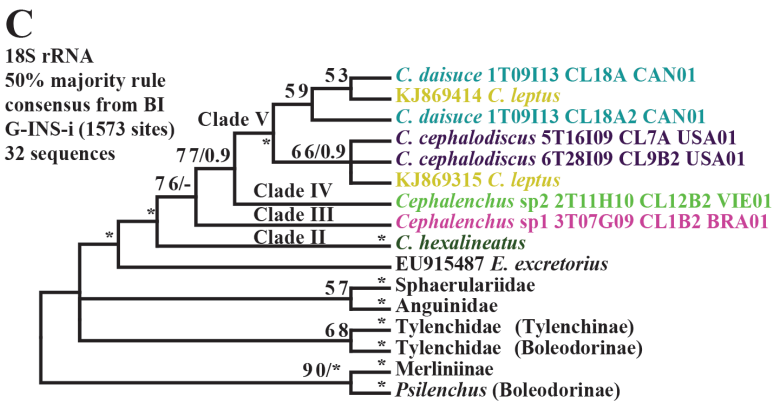

C
18S rRNA
50% majority rule
consensus from BI
G-INS-i (1573 sites)
32 sequences
C. daisuce 1T09I13 CL18A CAN01
KJ869414 C. leptus
C. daisuce 1T09I13 CL18A2 CAN01
C. cephalodiscus 5T16I09 CL7A USA01
C. cephalodiscus 6T28I09 CL9B2 USA01
KJ869315 C. leptus
Cephalenchus sp2 2T11H10 CL12B2 VIE01
Cephalenchus sp1 3T07G09 CL1B2 BRA01
C. hexalineatus
EU915487 E. excretorius
Sphaerulariidae
Anguinidae
Tylenchidae (Tylenchinae)
Tylenchidae (Boleodorinae)
Merliniinae
Psilenchus (Boleodorinae)
Clade V
Clade IV
Clade III
Clade II

Figure 3.4. Molecular phylogeny of the *Cephalenchus* species (color-coded) used in this study. The 50% majority rule consensus tree (Cladogram) from the Bayesian analysis is presented. **A.** Molecular analysis based on the 28S gene. **B.** Molecular analysis based on the ITS region. **C.** Molecular analysis based on the 18S gene. Five clades (I–V) are identified among *Cephalenchus* sequences. Branch support (ML/BI) is shown on branches. An asterisk, *, indicates that support for ML and BI are ≥ 95% and 0.95, respectively. Trees are rooted on the branch leading to *Psilenchus* + Merliniinae. Tylenchidae subfamilies are according to Geraert (2008).

Although, fewer *Cephalenchus* sequences are included in the 18S analyses, non-monophyly of the same species, except for *C. cylindricus* (MEX), is also supported (Fig. 3.4C).

Cephalenchus species of clades I and II are characterized by a lateral amphid opening (Fig. 3.3C-D) such as is found in *C. hexalineatus* and *C. nemoralis,* and also by a short tail. Conversely, *Cephalenchus* species in clades III-V have either a median tail (*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 dorsal-ventral amphid opening (Fig. 3.3A-B). Although, phylogenetic analysis are not fully congruent with respect to *Cephalenchus* clade relationships with ITS differing from the 18S and 28S trees (*i.e.* placement of *Cephalenchus* sp1), a combined analysis of all three genes recovers clades I and II as sister to all *Cephalenchus* species; this agrees with single gene phylogenies (*i.e.* 18S and 28S, Fig. 3.5).

Phylogenetic position of *Cephalenchus* within Tylenchomorpha

Regardless of the alignment method used, all phylogenetic analyses strongly recovered *Cephalenchus* as a monophyletic group (Table 3.3). A sister relationship between *Cephalenchus* and *E. excretorius* is also recovered, however, branch support for this relationship is usually low (ML < 60%, BI < 0.6) and varies considerably on broader phylogenetic analyses, particularly so when poorly aligned sites/divergent regions are removed from the datasets (Table 3.3).

Table 3.3 Parameters for the alignments used for broader (159 sequences) phylogenetic analyses of Tylenchomorpha and the support for the monophyly of *Cephalenchus* as well as the clade *Cephalenchus* + *Eutylenchus* in the ML and BI analyses. Values are presented in the format full/reduced alignment (L = alignment length, C = conserved sites, V = variable sites, Pi = parsimony informative sites, S = singleton sites).

Alignment Strategy	L[a]	C	V	Pi	S	Monophyly of *Cephalenchus*	*Cephalenchus* + *Eutylenchus*
18S rRNA							
E-INS-i	2115/1573 (74%)	700/663	1121/910	807/652	296/258	ML= 93/94, BI= 1.0/0.95	ML= 57/40, BI= 0.6/NA
G-INS-i	2126/1550 (73%)	694/657	1128/893	804/630	306/263	ML= 94/92, BI= 1.0/0.99	ML= 54/36, BI= 0.65/NA
Q-INS-i	2107/1560 (74%)	702/652	1129/908	803/641	308/267	ML= 95/89, BI= 1.0/0.98	ML= 50/39, BI= 0.53/NA
28S rRNA							
E-INS-i	1111/547 (49%)	248/146	735/401	573/337	136/67	ML= 88/92, BI= 1.0/1.0	ML= 57/52, BI=0.54/0.99
G-INS-i	1047/579 (55%)	222/146	719/433	579/364	125/69	ML= 93/92, BI= 1.0/1.0	ML= 56/54, BI=0.86/0.80
Q-INS-i	1166/463 (40%)	266/146	745/317	547/254	163/63	ML= 98/51, BI= 1.0/0.74	ML= 37/NA, BI=0.56/NA

[a] Percentage of reduced alignment in relation to full alignment is given.
NA: not recovered as sister taxa by the analysis.

When taxon sampling is limited to *Cephalenchus*, *Eutylenchus*, and a few potential outgroup taxa, branch support values for the clade *Cephalenchus* + *Eutylenchus* improved (ML ≥ 95%, BI ≥ 0.95, Figs. 3.4A and 3.4C, but see ITS phylogeny).

In most of the phylogenetic analyses, the position of clade *Cephalenchus* + *Eutylenchus* in relation to other PPN is unresolved or poorly supported. Additionally, a presumably close association of *Cephalenchus* + *Eutylenchus*, as suggested by morphology, with other Tylenchidae genera is not observed. The monophyly of Tylenchidae is not recovered in the 18S and 28S molecular 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 (Fig. 3.4A and 3.4C). In a few cases (28S gene), BI analyses including broader taxon sampling recovered the clade *Cephalenchus* + *Eutylenchus* as sister of Anguinidae (Full Q-INS-i, BI=0.81) or of Sphaerulariidae (Full G-INS-i align, BI=0.86).

Concatenated analyses based on the rRNA genes (18S + 28S + 5.8S) recovered *Cephalenchus* as a monophyletic group with strong branch support (ML=98, BI = 1.0). In contrast to the single gene analyses, monophyly of *Cephalenchus* is always recovered, regardless of taxon sampling. 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. 3.5).

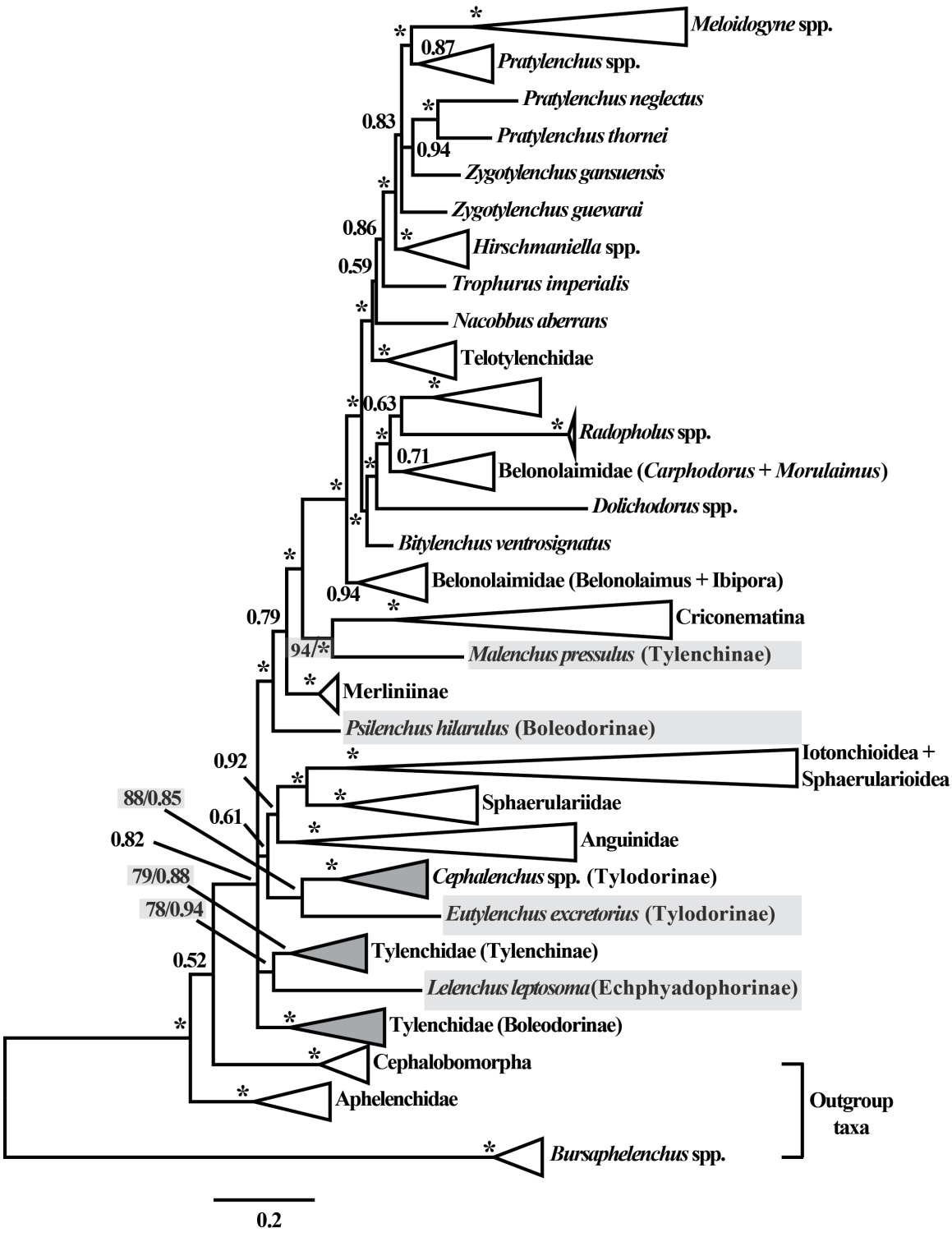

Meloidogyne spp.
0.87
Pratylenchus spp.
Pratylenchus neglectus
0.83
Pratylenchus thornei
0.94
Zygotylenchus gansuensis
Zygotylenchus guevarai
0.86
Hirschmaniella spp.
0.59
Trophurus imperialis
Nacobbus aberrans
Telotylenchidae
0.63
Radopholus spp.
0.71
Belonolaimidae (Carphodorus + Morulaimus)
Dolichodorus spp.
Bitylenchus ventrosignatus
Belonolaimidae (Belonolaimus + Ibipora)
0.94
0.79
Criconematina
94/*
Malenchus pressulus (Tylenchinae)
Merliniinae
Psilenchus hilarulus (Boleodorinae)
0.92
Iotonchioidea + Sphaerularioidea
88/0.85
Sphaerulariidae
0.61
0.82
Anguinidae
Cephalenchus spp. (Tylodorinae)
79/0.88
Eutylenchus excretorius (Tylodorinae)
78/0.94
Tylenchidae (Tylenchinae)
0.52
Lelenchus leptosoma (Echphyadophorinae)
Tylenchidae (Boleodorinae)
Cephalobomorpha
Aphelenchidae
Outgroup taxa
Bursaphelenchus spp.
0.2

Figure 3.5. A combined analysis based on three rRNA genes (18S + 28S + 5.8S, 2758 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 gray. Branch support is shown on branches (ML/BI). An asterisk, *, indicates that support for ML and BI are ≥ 95% and 0.95, respectively. ML support values are only given for Tylenchidae taxa (in the ML phylogeny *Psilenchus* is recovered as sister to Merliniinae, ML=72). Taxonomy scheme of families, except Tylenchidae, and higher taxa is in accordance with Siddiqi (2000). The tree is rooted to *Bursaphelenchus* species.

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 that *Cephalenchus* is mostly concentrated in the Northern Hemisphere. However, its wide 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 harbor *Cephalenchus* species. Sampling of PPN 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* 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 areas (*i.e.* not on a crop), usually in moist, organically rich soils (Table 3.1). Additionally sampling site descriptions from previous collections further underscores that *Cephalenchus* has a preference for moist habitas; 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 and 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 and Jairajpuri, 1980; Andrássy, 1984; Ladislav, 2003).

Increased sampling of natural areas, particularly so in the Southern Hemisphere, is likely to result in a more complete picture of *Cephalenchus* diversity and species distribution. Furthermore, 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 of species for the genus (Raski and Geraert, 1986; Geraert, 2008).

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. 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 are 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.* designated short, median, and long). Although ranges defining these categories are somewhat arbitrary, *Cephalenchus* identification keys commonly rely on these ranges, regardless, as typically diagnostic (Raski and 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 cutoffs. In this sense, molecular data has become essential to appropriately establishing and testing species hypotheses.

Most *Cephalenchus* species are characterized by having 6 lines in the lateral field (LF), which led Dhanachand and Jairajpuri (1980) to propose the genus *Imphalenchus* to accommodate similar species bearing only 4 lines in the LF. However, this action was not supported by Raski and Geraert (1986) who synonymized *Imphalenchus* with *Cephalenchus*. Since then, four species characterized by 4 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 4 lines in the LF, and although it showed some overlap with *C. daisuce* (both fall in the median tail group) in the morphometric analyses, molecular phylogenetic analyses always recovered it as separate lineage within

Cephalenchus, which thus supports the synonymy of *Imphalenchus* with *Cephalenchus* (Raski and Geraert, 1986).

With respect to tail length, *Cephalenchus* species bearing 4 lines in the LF show great variability, ranging from short (115-155 µm) as in *C. imphalus* Dhanachand, Renubala & Annandi, 1993, to median (134-190 µm) as in *C. concavus* Xie & Feng, 1994 and (184-202 µm) as in *C. indicus* (Dhanachand and Jairajpuri, 1980) Raski & Geraert, 1986, to long (214-280 µm), as in *C. 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 an autapomorphy, and its utility to explain phylogeny needs further investigation of additional species bearing 4 lines in the LF.

Information on the labial pattern of *Cephalenchus* is limited to a few studies. In fact, only Raski and 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 and Jairajpuri, 1980; Siddiqui and Khan, 1983a) have based their conclusions upon cross sections of the anterior region of *Cephalenchus* under LM observation. However, the LM resolution is often limited and might not fully represent the labial pattern of *Cephalenchus* thus obscuring interpretation of morphological features.

With respect to the labial region, orientation of the amphid opening as either dorsal-ventral or lateral, was the primary difference observed among *Cephalenchus* species, thus dividing the genus into two groups of species. Variation in the oral disc (*i.e.* slit *vs.* oval shape) is also congruent with groups defined on the basis of the amphid 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 track phylogeny 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 amphid opening, molecular data is not yet available to test its phylogenetic position within *Cephalenchus*, either as sister to clades I and II or sister to all *Cephalenchus* species.

Cephalenchus is monophyletic but its position within Tylenchomorpha remains unresolved.

Extant molecular phylogenies of Tylenchomorpha have included only a few *Cephalenchus* species and thus hypotheses of monophyly for the genus remained untested. 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; Yaghoubi *et al.*, 2015).

In this study, molecular phylogenetic analyses included multiple *Cephalenchus* populations, representing 11 nominal species, sampled worldwide. All three rRNA genes as well as a combined phylogenetic analysis strongly (ML ≥ 88, BI ≥ 0.95) supported *Cephalenchus* as a monophyletic group. By contrast, analyses based on one dataset (28S gene, reduced Q-INS-I, Table 3.3) showed fairly low support for the monophyly of *Cephalenchus*; this can be explained by the drastic reduction in the number of sites (only 40% from the full alignment). Interestingly, the branch support for the monophyly of *Cephalenchus* is improved (always with ML ≥ 95 and BI ≥ 0.95) when a reduced number of taxa, that is, only *Cephalenchus* and potential outgroups, are analyzed (Fig. 3.4). 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 between the different molecular analyses. In this sense, non-monophyly of four defined morphological species is supported. A similar result was obtained by Pereira and Baldwin (2016), in which *C. cephalodiscus*, *C. cylindricus* and *C. daisuce* all grouped together in the North American clade as defined by these authors. Herein, *C. leptus* from three different geographic regions including China, Iran, and US, 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 (i) morphological variation in *Cephalenchus* might happen at a much faster pace than molecular variation, perhaps mostly due to environment conditions (*i.e.* infraspecific character variation), and (ii)

Cephalenchus species defined solely by morphology might be more prone to inconsistences in a such homogenous group, which is in agreement with the overlap found between tail groups. Additionally, molecular analyses also support the synonymization of *C. cephalodiscus* and *C. cylindricus* as proposed by Raski and Geraert (1986).

It is argued that mitochondrial genes (*e.g.* COI) might be more suitable for resolving recent speciation events when compared to rRNA genes (Blouin, 2002; Nieberding *et al.*, 2008). In fact, Pereira and Baldwin (2016) suggested that the lack of reciprocal monophyly for the species representing the North American clade could be explained by the low resolving power of rRNA genes. A closer look at *C. hexalineatus* (clade II), however, shows some structure at the population level, supported by both 28S and ITS genes. For example, in the ITS region, *C. hexalineatus* from Oregon, US, is characterized by six fixed autapomorphies, and a similar pattern is not observed in any species of clade V (data not shown). Existence of fixed autapomorphies from independent loci can potentially guide species delimitation in closely related species as suggested by Nadler (2002).

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 a sister 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 gene, and with a

single exception (Palomares-Rius *et al.*, 2009), have not treated both *Cephalenchus* and *Eutylenchus* together (Bert *et al.*, 2008; Van Megen *et al.*, 2009; Bert *et al.*, 2010). 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 phylogenies, but not by the *hsp* 90 gene. In this study, branch support for a clade of *Cephalenchus* + *Eutylenchus* is also improved when taxon sampling is reduced (Fig. 3.4A-C). Additionally, a combined analyses of all three rRNA genes recovered *Cephalenchus* + *Eutylenchus* with relatively high support (Fig. 3.5).

Although a clade of *Cephalenchus* + *Eutylenchus* seems to be convincing, as recovered by most of the analyses, neither single gene nor a combined analysis was able to unequivocally determine the position of these taxa within Tylenchomorpha. However, in two analyses based on the 28S gene, the clade *Cephalenchus* + *Eutylenchus* was recovered with at least relatively high support as sister taxa of Anguinidae or Sphaerulariidae. Similar results, although with lower branch support, were also found by Palomares-Rius *et al.* (2009) in a 28S phylogeny. Moreover, Subbotin *et al.* (2006) reported *E. excretorius* (*Cephalenchus* not included) in a clade containing both, Anguinidae and Sphaerulariidae representatives.

This study also showed that *Cephalenchus* + *Eutylenchus* are not closely related to other Tylenchidae genera as suggested by morphology (Geraert and Raski, 1987; Geraert, 2008), 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, particularly including genera believed to be closely related. As Tylenchidae representation in molecular phylogenies improves, the validity of its genera as natural groups as well as its relationships among other tylenchs can be adequately tested. Ultimately, a revision of the entire group formerly designated as Tylenchidae will be needed to accommodate new insights gained by molecular phylogenies.

GENERAL CONCLUSIONS

To date, broad molecular phylogenetic analyses of phylum Nematoda have been almost exclusively based on rRNA genes, particularly 18S and 28S. These genes also have been primarily used for phylogenic resolution of tylenchs, although studies focusing on lower taxonomic ranks (*i.e.* family or genus) and population level studies have also used the ITS region. Furthermore, molecular studies of tylenchs are usually based on a single gene region, or in those few cases using multiple genes, a combined analysis is often omitted. Certainly, the number of DNA sequences representing nematodes in molecular phylogenetic studies has considerably grown; from 53 as in the first molecular phylogeny of the phylum to over 1200 sequences (Blaxter *et al.*, 1998; Van Megen *et al.*, 2009). This number continues to expand, with some current 18S molecular phylogenies (Quist *et al.*, 2015) including about 2700 DNA sequences representing the major nematodes clades.

Plant parasitic nematodes are frequently well represented in these broad molecular phylogenetic studies; however, taxon sampling is heavily biased towards species of agricultural importance. In this sense, groups such as the family Tylenchidae are still ignored. As late as February of 2016, 121 (18S gene) and 69 (28S) DNA sequences representing the family Tylenchidae had been deposited in GenBank (excluding doubtful accessions). Although these numbers are promising, they only represent 18 (18S gene) or 13 (28S gene) genera of Tylenchidae, that is, 30-40% of extant genera of Tylenchidae genus diversity. The scenario, however, is worse when considering extant species

diversity; that is, DNA sequences representing only 8-14% of species of Tylenchidae have been reported.

Based on morphological observations (LM and SEM), nematode specimens of the present study were identified to genus or species level. In the molecular analyses presented in the first chapter, the family Tylenchidae was represented by the following genera: *Aglenchus, Basiria, Boleodorus, Cephalenchus, Coslenchus, Eutylenchus, Filenchus, Lelenchus, Malenchus, Neopsilenchus, Psilenchus*, and *Tylenchus*. In general, the phylogenetic analyses (BI and ML) based on D2-D3 domains of the 28S rRNA showed similar results. In both analyses, the monophyly of the family Tylenchidae *sensu* Siddiqi (2000) and *sensu* Geraert (2008) is rejected.

Although, complete monophyly of Tylenchidae is not resolved, some clades within this family are well defined and highly supported. For example, the genera *Aglenchus*, *Coslenchus*, and *Filenchus* are recovered as monophyletic with high branch support in the 28S phylogeny. Likewise, the genera *Basiria, Boleodorus*, and *Neopsilenchus* are also strongly supported as monophyletic. Both Siddiqi (2000) and Geraert (2008) classified the former three genera under the subfamily Tylenchinae and the latter under Boleodorinae. Furthermore, a sister relationship between these subfamilies is also recovered in the 28S phylogeny, thus partially supporting some of the morphologically-based systems (Siddiqi, 2000; Geraert, 2008). With respect to the monophyly of Boleodorinae and Tylenchinae, similar results were also reported by Subbotin *et al.* (2006), Qing *et al.* (2015b), and Yaghoubi *et al.* (2015).

Some of these findings, however, were challenged by the results of the 18S phylogeny. For example, the genus *Filenchus*, which is monophyletic in the 28S phylogeny, is polyphyletic in the 18S tree. Although some relations between *Filenchus* and other tylenchs might be considered spurious (*i.e.* short 18S sequences representing these species), two clades containing *Filenchus* sequences emerged from the analyses: one clade grouped with other Tylenchinae genera (*i.e. Aglenchus*, *Coslenchus*, *Lelenchus*, and *Tylenchus*); the second clade grouped with the genus *Malenchus* [also Tylenchidae under the schemes of Siddiqi (2000) and Geraert (2008)] and was more closely related to the suborder Criconematina *sensu* Siddiqi (2000).

In Van Megen *et al.* (2009), *M. andrassyi* and *F. discrepans* (presented as *O. discrepans*) showed some affinities with Criconematina. In addition, this clade included sequences representing the genus *Ecphyadophora* [considered to be in the Tylenchidae by Geraert (2008)]. The phylogenies presented in Qing *et al.* (2015b) and Yaghoubi *et al.* (2015), although limited in taxon sampling, suggest that *Malenchus* might represent a lineage outside of the formerly Tylenchidae. Moreover, the phylogenetic affinities between genera *Malenchus* and *Filenchus* (18S gene), as shown in the first chapter, are corroborated by analyses of the 28S gene. Morphology of the lateral field (*i.e.* off-set vs. continuous) seems to be an informative character to properly separate *Filenchus* from *Malenchus*. Nevertheless, a thorough revision of the genera *Filenchus* and *Malenchus*, the largest groups in Tylenchidae, will be needed to add insights from molecular based phylogenies (Qing, personal communication).

In the first chapter, it was also shown that genera *Psilenchus*, *Cephalenchus* and *Eutylenchus*, although monophyletic, were not related to other Tylenchidae. For example, *Psilenchus* grouped with high support (BI= 1.00 and ML= 94) as sister taxa of some Telotylenchidae. Surprisingly, genera in the family Telotylenchidae (subfamily Merliniinae *sensu* Siddiqi, 2000) includes only stunt nematode genera that bear deirids (except *Scutylenchus* which lacks deirids) and phasmids. These are sense organs laterally located on the anterior (level of basal bulb) and posterior (tail) region of the body, respectively. Such morphological features are also found in *Psilenchus* as well as in *Antarctenchus* and *Atetylenchus* and therefore the presence of deirids and phasmids ("*complete lateral complex*") are suggested by Ryss (1993) to be a synapormophy that groups these genera.

In fact, Siddiqi (2000) recognized *Psilenchus* and *Atetylenchus* in a separate family (Psilenchidae) and did not consider them to be closely related to other Tylenchidae genera (*i.e. Aglenchus*, *Coslenchus*), but instead placed them within the superfamily Dolichodoroidea (*sensu* Siddiqi, 2000) that includes all the stunt nematodes (*e.g. Nagelus*, *Merlinius*, etc.). Additional phylogenetic analyses based on the 28S, but not included in the first chapter, showed *Psilenchus* and a specimen tentatively identified as "*Atetylenchus"* to group with Merliniinae. Similarly, Yaghoubi *et al.* (2015) recovered a sister relationship between *A. minor* and *Psilenchus* sp., both taxa being sister to Merliniinae on the basis of the 18S gene.

It has been shown in the first chapter that family Tylenchidae, as morphologically defined by different authors is polyphyletic (Siddiqi, 2000; Geraert, 2008). Additionally, evidence from the molecular analyses based on 18S and 28S rRNA genes show that formerly designed Tylenchidae genera are widely spread in the Tylenchomorpha tree; in fact, only a few genera (*e.g. Aglenchus* and *Coslenchus*) seems to represent what can be considered the "authentic" Tylenchidae since they also group with the type genus *Tylenchus*.

In the second chapter, sequence variation of two rRNA genes (28S and ITS) was evaluated across different populations and species of the plant parasitic nematode *Cephalenchus*. Levels of intragenomic and intraspecific variation differed among species and rRNA genes, thus suggesting that not all *Cephalenchus* species undergo strict concerted evolution. In fact, levels of polymorphism in the rRNA genes of some *Cephalenchus* were extremely high when compared to other nematode species as well as metazoans. It was shown that intragenomic polymorphism (*i.e.* within individual) is the main source of sequence variation in highly polymorphic species. Variation on the predicted secondary structures of 28S and ITS rRNA genes are also detected, in particular in the D2-domain of the 28S gene. In this sense, two types of C1/e1 helix (*i.e.* reduced and long) were identified; reduction of C1/e1 helix is found in species with high and low levels of intragenomic/intraspecific polymorphism.

The pattern of base pair change, mostly CBCs and SBCs, was fairly consistent within *Cephalenchus* suggesting that high levels of intragenomic and intraspecific variation in some species are unlikely due to the formation of pseudogenes. This idea is

also supported by (i) the very conserved 5.8S rRNA gene, (ii) the existence of conserved 5.8S motifs in all *Cephalenchus* species, and (iii) the pattern of sequence divergence in the entire ITS region. Moreover, it has been shown that cross-fertilization, as presumed by abundant males, might contribute to the intraspecific diversity found in the same *Cephalenchus* species. Finally, the findings presented in the second chapter show that intragenomic and intraspecific variation of rRNA genes can be high in *Cephalenchus*, sometimes approaching levels of interspecific variation. Knowledge of species genetic diversity, in particular on the targeted genes used on molecular biodiversity studies, becomes essential, and particularly so when using approaches lacking morphological vouchers such as some metagenetic methods.

A broad objective of this dissertation work was to improve the phylogeny of Tylenchomorpha *sensu* De Ley & Blaxter, 2002. In this regard, molecular and morphological evidence were gathered from genera of Tylenchidae. Previous molecular evidence had suggested that Tylenchidae, as presently structured based on morphology, may lack monophyly (Subbotin *et al.* 2006, Bert *et al.* 2008, Van Megen *et al.* 2009). Targeting specific groups with Tylenchidae, perhaps more tractable genera (*i.e.* relatively small groups) might provide a starting point for a more refined revision of Tylenchidae.

A genus presenting such qualities within Tylenchidae is *Cephalenchus* (about 20 species only). Moreover, previous molecular evidence has shown *Cephalenchus* not to be closely related to other Tylenchidae genera (Holterman *et al.*, 2006; Bert *et al.*, 2008) but instead to be more closely associated to insect-associated tylenchs (Palomares-Rius *et al.*, (2009). In the third chapter, extensive broad sampling (26 populations, 11 species) of

Cephalenchus allowed confirmation of the monophyly of the genus. In fact, all molecular analyses (either single gene or combined dataset) strongly supported *Cephalenchus* as monophyletic. Moreover, a sister relationship of *Cephalenchus* + *Eutylenchus*, as suggested by morphology, is also resolved by molecular phylogenies. Nevertheless, support for a clade of *Cephalenchus* + *Eutylenchus* depends on the taxa and method used in the phylogenetic reconstructions. In this sense, the position of both taxa within Tylenchomorpha remains ambiguous and highlights the importance of using additional genes as well as increasing taxon sampling.

Within the genus *Cephalenchus*, morphometric data clusters species into different groups based on categories of tail length (*i.e.* short *vs.* median-long), a morphological feature commonly used in species identification. Moreover, these groups are further supported by congruence with details of labial morphology, especially the amphid opening morphology (lateral *vs*. dorso-ventral orientation). Although, labial morphology, in particular the shape and position of the amphid opening, as well as tail length might explain species relationships, the contribution of the lateral field (LF) to the overall *Cephalenchus* phylogeny needs to be further evaluated, and particularly so by the inclusion of additional species bearing 4 lines in the LF. In inclusion of such species in future molecular phylogenies of *Cephalenchus* might confirm the assertion of LF being an autapomorphy within *Cephalenchus*.

The findings here presented shall be further advanced by including additional taxa and genes to further resolve ambiguous relationships of genera formerly designated as Tylenchidae. For example, within Tylenchidae *sensu* Geraert (2008) candidate genera

include *Malenchus* and *Psilenchus*. In addition, the family Ecphyadophorinae *sensu* Geraert (2008) seems to be an interesting target also due to its ambiguous position in previous molecular phylogenies (Subbotin *et al.* 2006; Megen *et al.* 2009). In regards to the phylogenetic position of *Cephalenchus* + *Eutylenchus* within Tylenchomorpha, the inclusion of crucial taxa such as members of Tylodorinae *sensu* Geraert (2008), might shed light on their relationships with respect to other tylenchs. In particular, the genus *Campbellenchus* looks very promising, as it presents a morphology similar to that of *Cephalenchus*, especially in regards to the labial morphology.

Also, in the inclusion of the genus *Atylenchus*, that shares with *Eutylenchus* the presence of four cephalic setae in the anterior region (instead of homologs expressed as cephalic papillae as in *Cephalenchus* and *Campbellenchus*), should be perused in future in molecular phylogenetic studies. Certainly, increasing sampling of Tylenchidae taxa will provided additional evidence for the morphologically-based classifications proposed by Siddiqi (2000) and Geraert (2008). Furthermore, the inclusion of these taxa in future molecular phylogenies will positively impact our overall understanding of Tylenchomorpha.

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Appendix A

List of species downloaded from GenBank used in the 18S and 28S phylogenetic analyses of chapter 1. Nematode classification is according to Siddiqi (2000).

Family	Genus/Species	28S GenBank Accession	Reference	18S GenBank Accession	Reference
Aphelenchidae (Outgroup)	*Aphelenchus avenae*	-	-	EU306347	Bert *et al.* 2008
	Aphelenchus sp.	-	-	AY284641	Holterman *et al.* 2006
	Paraphelenchus sp.	-	-	AY284642	Holterman *et al.* 2006
Aphelenchoididae (Outgroup)	*Aphelenchoides bicaudatus*	-	-	AY284643	Holterman *et al.* 2006
	Aphelenchoides besseyi	DQ328684	Subbotin *et al.* 2006	-	-
	Aphelenchoides blastophtorus	-	-	AY284644	Holterman *et al.* 2006
	Aphelenchoides fragaria	DQ328683	Subbotin *et al.* 2006	-	-
	Aphelenchoides sp.	DQ328682	Subbotin *et al.* 2006	-	-
Parasitaphelenchidae (Outgroup)	*Bursaphelenchus mucronatus*	JF317247	Zheng *et al.* 2003	AY284648	Holterman *et al.* 2006
	Bursaphelenchus trypophloei	FJ998283	Tomalak and Filipiak 2011	-	-
	Bursaphelenchus xylophilus	F317245	Zheng *et al.* 2003	-	-
	Bursaphelenchus sp.	-	-	AY284649	Holterman *et al.* 2006
Anguinidae	*Anguina tritici*	DQ328723	Subbotin *et al.* 2006	AY593913	Holterman *et al.* 2006
	Ditylenchus destructor	-	-	AY593912	Holterman *et al.* 2006
	Ditylenchus dipsaci	HQ219218	Vovlas *et al.* 2011	AY593906	Holterman *et al.* 2006
	Ditylenchus gigas	HQ219215	Vovlas *et al.* 2011	-	-
	Heteroanguina graminophila	DQ328720	Subbotin *et al.* 2006	-	-
	Mesoanguina millefolii	DQ328722	Subbotin *et al.* 2006	-	-
	Nothotylenchus acris	-	-	AY593914	Holterman *et al.* 2006
	Pseudhalenchus minutus	-	-	AY593916	Holterman *et al.* 2006
	Subanguina chilensis	DQ328724	Subbotin *et al.* 2006	-	-
	Subanguina radicicola	DQ328721	Subbotin *et al.* 2006	-	-
Atylenchidae[1]	*Eutylenchus excretorius*	AY780980 EU915490	Subbotin *et al.* 2005 Palomares-Rius *et al.* 2007	EU915487	Palomares-Rius *et al.* 2009

Appendix A (Continued).

Family	Genus/Species	28S GenBank Accession	Reference	18S GenBank Accession	Reference
Criconematidae	*Hemicriconemoides pseudobracyurus* [C]	-	-	AY284622	Holterman *et al.* 2006
	Mesocriconema xenoplax [C]	-	-	AY284625	Holterman *et al.* 2006
Ecphyadophoridae[1]	*Lelenchus leptosoma*	-	-	AY284584	Holterman *et al.* 2006
Hemicycliophoridae	*Hemicycliophora thienemanni* [C]	-	-	EU306341	Bert *et al.* 2008
Heteroderidae	*Globodera pallida*	GQ294489	Madani *et al.* 2009	AY284618	Holterman *et al.* 2006
	Globodera rostochiensis	GQ294484	Madani *et al.* 2009	AY284619	Holterman *et al.* 2006
	Globodera tabacum	GQ294492	Madani *et al.* 2009	-	-
	Heterodera aucklandica	DQ328688	Subbotin *et al.* 2006	-	-
	Heterodera cajani	DQ328692	Subbotin *et al.* 2006	-	-
	Heterodera cynodontis	DQ328698	Subbotin *et al.* 2006	-	-
	Heterodera glycines	DQ328692	Subbotin *et al.* 2006	-	-
	Heterodera goettingiana	DQ328697	Subbotin *et al.* 2006	-	-
	Heterodera koreana	-	-	EU306357	Bert *et al.* 2008
	Heterodera latipons	DQ328687	Subbotin *et al.* 2006	-	-
	Heterodera litoralis	DQ328691	Subbotin *et al.* 2006	-	-
	Heterodera oryzicola	DQ328694	Subbotin *et al.* 2006	-	-
	Heterodera salixophila	DQ328690	Subbotin *et al.* 2006	-	-
	Heterodera schachtii	-	-	EU306355	Bert *et al.* 2008
	Heterodera sorghi	DQ328689	Subbotin *et al.* 2006	-	-
	Heterodera urticae	DQ328696	Subbotin *et al.* 2006	-	-
	Heterodera zeae	DQ328695	Subbotin *et al.* 2006	-	-
Hoplolaimidae	*Helicotylenchus digonicus*	HM014240	Subbotin *et al.* 2011	-	-
	Helicotylenchus dihystera	HM014242	Subbotin *et al.* 2011	-	-
	Helicotylenchus labiodiscinus	HM014293	Subbotin *et al.* 2011	-	-
	Helicotylenchus leiocephalus	HM014268	Subbotin *et al.* 2011	-	-
	Helicotylenchus multicintus	HM014290	Subbotin *et al.* 2011	-	-
	Helicotylenchus platyurus	HM014265	Subbotin *et al.* 2011	-	-
	Helicotylenchus pseudorobustus	HM014263	Subbotin *et al.* 2011	-	-
	Helicotylenchus varicaudatus	-	-	EU306354	Bert *et al.* 2008

Appendix A (Continued).

Family	Genus/Species	28S GenBank Accession	Reference	18S GenBank Accession	Reference
	Helicotylenchus vulgaris	HM014238	Subbotin *et al.* 2011	AY284607	Holterman *et al.* 2006
	Rotylenchus agnetis	EU280795	Vovlas *et al.* 2008	-	-
	Rotylenchus cazorlaensis	EU280792	Vovlas *et al.* 2008	-	-
	Rotylenchus eximius	EU280794	Vovlas *et al.* 2008	-	-
	Rotylenchus jaeni	EU280791	Vovlas *et al.* 2008	-	-
	Rotylenchus incultus	EU280796	Vovlas *et al.* 2008	-	-
	Rotylenchus laurentinus	EU280798	Vovlas *et al.* 2008	-	-
	Rotylenchus magnus	EU280789	Vovlas *et al.* 2008	-	-
	Rotylenchus robustus	EU280788	Vovlas *et al.* 2008	-	-
	Rotylenchus uniformis	-	-	EU306356	Bert *et al.* 2008
	Rotylenchus unisexus	EU280799	Vovlas *et al.* 2008	-	-
	Rotylenchus sp.	-	-	AY284608	Holterman *et al.* 2006
Meloidogynidae	*Meloidogyne arenaria*	AF435803	De Ley *et al.* 2005	-	-
	Meloidogyne dunensis	EF612712	Palomares-Rius *et al.* 2007	-	-
	Meloidogyne exigua	AF435795	Tenente *et al.* 2004	-	-
	Meloidogyne graminicola	HQ420905	Liu *et al.* (unpubl.)	-	-
	Meloidogyne hapla	DQ328685	Subbotin *et al.* 2006	-	-
	Meloidogyne hapla	DQ145641	Nadler *et al.* 2006	AY593892	Holterman *et al.* 2006
	Meloidogyne hispanica	GQ375158	Castillo *et al.* 2009	-	-
	Meloidogyne incognita	AF435794	De Ley *et al.* 2005	AY284621	Holterman *et al.* 2006
	Meloidogyne konaensis	AF435797	De Ley *et al.* 2005	-	-
	Meloidogyne paranaensis	AF435800	De Ley *et al.* 2005	-	-
	Meloidogyne silvestris	EU570214	Castillo *et al.* 2009	-	-
	Meloidogyne thailandica	EU364890	Skantar *et al.* (unpubl.)	-	-
	Meloidogyne trifoliophila	AF435801	De Ley *et al.* 2005	-	-
Neotylenchidae	*Neotylenchus* sp.	DQ328725	Subbotin *et al.* 2006	-	-
Paratylenchidae	*Paratylenchus microdorus* [C]	-	-	AY284632	Holterman *et al.* 2006
Pratylenchidae	*Hirschmanniella kwazuna*	EU620466	Van Den Berg *et al.* 2009	-	-
	Hirschmanniella loofi	EU620468	Van Den Berg *et al.* 2009	EU306353	Bert *et al.* 2008
	Hirschmanniella sp1	-	-	AY284614	Holterman *et al.* 2006

Appendix A (Continued).

Family	Genus/Species	28S GenBank Accession	Reference	18S GenBank Accession	Reference
	Pratylenchus agilis	EU130841	Subbotin *et al.* 2008	-	-
	Pratylenchus coffeae	EU130843	Subbotin *et al.* 2008	-	-
	Pratylenchus crenatus	EU130852	Subbotin *et al.* 2008	AY284610	Holterman *et al.* 2006
	Pratylenchus neglectus	EU130854	Subbotin *et al.* 2008	-	-
	Pratylenchus thornei	EU130866	Subbotin *et al.* 2008	AY284612	Holterman *et al.* 2006
	Pratylenchus vulnus	EU130882	Subbotin *et al.* 2008	-	-
	Pratylenchus zeae	EU130889	Subbotin *et al.* 2008	-	-
Psilenchidae[1]	*Psilenchus* sp.	DQ328716	Subbotin *et al.* 2006	EU130840	Subbotin *et al.* 2008
	Psilenchus hilarulus	EU915489	Palomares-Rius *et al.* 2009	EU915488	Palomares-Rius *et al.* 2009
				AY284593	Holterman *et al.* 2006
Sphaerulariidae	*Sphaerularia bombi*	DQ328726	Subbotin *et al.* 2006	-	-
Telotylenchidae	*Amplimerlinius icarus*[B]	DQ328714	Subbotin *et al.* 2006	EU306351	Bert *et al.* 2008
	Bitylenchus dubius[A]	-	-	AY284601	Holterman *et al.* 2006
	Macrotrophurus arbusticola[A]	-	-	AY284596	Holterman *et al.* 2006
	Nagelus leptus	DQ328715	Subbotin *et al.* 2006	-	-
	Merlinius brevidens[B]	-	-	AY284597	Holterman *et al.* 2006
	Nagelus obscurus[B]	-	-	EU306350	Bert *et al.* 2008
	Sauertylenchus maximus[A]	-	-	AY284604	Holterman *et al.* 2006
	Telotylenchus ventralis[A]	-	-	AY593905	Holterman *et al.* 2006
	Tylenchorhynchus dubius[A]	-	-	EU306352	Bert *et al.* 2008
Tylenchidae	*Aglenchus agricola*	AY780979	Subbotin *et al.* 2005	-	-
	Aglenchus sp.	-	-	AY284586	Holterman *et al.* 2006
	Basiria gracilis	DQ328717	Subbotin *et al.* 2006	EU130839	Subbotin *et al.* 2008
	Basiria sp.	DQ077803	De Ley *et al.* 2005	-	-
	Boleodorus thylactus	-	-	AY593915	Holterman *et al.* 2006
	Boleodorus sp.	DQ328718	Subbotin *et al.* 2006	-	-
	Coslenchus costatus	DQ328719	Subbotin *et al.* 2006	AY284581	Holterman *et al.* 2006
	Coslenchus franklinae	-	-	AY284583	Holterman *et al.* 2006
	Filenchus cylindricaudus	-	-	AY912028	Powers *et al.* (unpubl.)
	Filenchus discrepans	-	-		

Appendix A (Continued).

Family	Genus/Species	28S GenBank Accession	Reference	18S GenBank Accession	Reference
				AY284590	Holterman *et al.* 2006
				AB473565	Okada *et al.* 2005
	Filenchus ditissimus	-	-	AY912030	Powers *et al.* (unpubl.)
	Filenchus filiformis	-	-	AY284592	Holterman *et al.* 2006
	Filenchus fungivorus	-	-	FJ949564	Bert *et al.* 2010
	Filenchus hamatus	-	-	AY912031	Powers *et al.* (unpubl.)
	Filenchus helenae	-	-	AY912033	Powers *et al.* (unpubl.)
	Filenchus misellus	-	-	AB473564	Okada *et al.* 2005
	Filenchus thornei	-	-	AY284591	Holterman *et al.* 2006
	Filenchus sp.	-	-	AY912036	Powers *et al.* (unpubl.)
				FJ949565	Bert *et al.* 2010
	Malenchus sindhicus	-	-	AY284587	Holterman *et al.* 2006
	Neopsilenchus magnidens	-	-	AY284585	Holterman *et al.* 2006
	Tylenchus davainei	-	-	AY284588	Holterman *et al.* 2006
	Tylenchus arcuatus	-	-	EU306348	Bert *et al.* 2008
				EU306349	Bert *et al.* 2008
	Tylenchus sp.	-	-	AY284589	Holterman *et al.* 2006
		EU915491	Palomares-Rius *et al.* 2009	AY284594	Holterman *et al.* 2006
Tylodoridae[1]	*Cephalenchus exalineatus*	EU915492		EU915486	Palomares-Rius *et al.* 2009
		EU915493			

[1] Geraert (2008) recognized these genera under the family Tylenchidae.
[A, B] Telotylenchidae clades in Figure 1.6 (18S phylogeny).
[C] Taxa included under the suborder Criconematina in Figure 1.6 (18S phylogeny).

Appendix B

Intragenomic and intraspecific variation of the 28S (D2-D3 domains) and ITS (including ITS-1, 5.8S, and ITS-2) genes in the *Cephalenchus* species used in this study. Genetic divergence (range) between sequences is presented as p-distance (%) and number of base pair (bp) difference. Parameters (N: number of sequences per nematode specimen; Size: alignment length in bp; C: number of conserved sites; V: number of variable sites; Pi: number of parsimony-informative sites; S: number of singleton sites; IT: total number of indels in the alignment; IE/length: number of indel events and indel event average length) are given for each nematode specimen as well as each species (range and mean, values in bold). The highest values of intragenomic polymorphism are highlighted in gray (only one value in the case of ties).

28S (D2-D3 domains) rRNA gene														
Species	**Nematode ID**	**Sex**	**N**	**Accession No. GenBank[d]**	**Size**	**C**	**V**	**Pi**	**S**	**Pi/S**	**IT**	**IE/ length**	**p-dist. (%)**	**bp diff.**
***Cephalenchus* sp1 (BRA-01)**	1T17C09	♀	10	KU722973-982	684	624	60	1	59	0.02	4	2 (2)	0-8	0-54
	2T12G10	♂	4	KU722983-986	686	611	73	30	43	0.7	6	3 (2)	0-10	15-56
	2T17C09	♀	10	KU722987-996	684	599	85	59	26	2.27	5	3 (1.7)	0-9	2-60
	3T12G10	♀	6	KU722997-002	687	612	73	26	47	0.55	6	3 (2)	0-9	2-63
	4T07G09	♀	4	KU723003-006	684	635	49	2	47	0.04	4	2 (2)	0-7	3-43
	4T17C09	♀	9	KU723007-015	684	598	86	83	33	2.52	4	2 (2)	0-9	0-61
	4T22A13	♀	9	KU723016-024	684	618	66	12	54	0.22	4	2 (2)	0-9	0-58
	5T12G10	♂	6	KU723025-030	684	674	10	4	6	0.67	0	0	0-1	0-6
All sequences			**58**		**687**	**530**	**157**	**106**	**51**	**2.08**	**8**	**5 (1.6)**	**0-10 (4.3)**	**0-65 (29)**
***Cephalenchus* sp. (BRA-02)**	13T11H10	♀	3	KU723031-033	670	641	29	0	29	0	1	2	2-3	15-23
	14T11H10	♀	7	KU723034-040	711	658	53	41	12	3.42	2	1	0-6	0-42
All sequences			**10**		**720**	**579**	**141**	**123**	**18**	**6.83**	**61**	**7 (8.7)**	**0-15 (8)**	**0-97 (55)**
***C. daisuce* (CAN)**	1T09I13	♀	5	KU723041-045	730	714	16	9	7	1.29	0	0	0-2	0-14
	2T09I13	♀	6	KU723046-049, 059, 061	730	716	14	6	8	0.75	3	1 (3.0)	0-2	0-11
	3T09I13	♀	6	KU723050-055	730	711	19	9	10	0.9	1	1 (1.0)	0-2	1-14
	4T09I13	♀	5	KU723056-058, 060, 062	730	720	10	0	10	0	1	1 (1.0)	0-1	0-7
	5T09I13	♀	6	KU723063-068	730	713	17	4	13	0.31	3	1 (3.0)	0-2	0-12
All sequences			**28**		**730**	**689**	**41**	**25**	**26**	**0.96**	**4**	**2 (2.0)**	**0-2 (0.9)**	**0-15 (6)**
***C. cylindricus* (MEX)**	1T11F09[a]	♂	6	KU723073, 076, 080, 082-084	730	728	2	0	2	0	0	0	0	0-2
	2T11F09[a]	♀	1	KU723072	730	-	-	-	-	-	-	-	-	-
	4T03F09[a]	♂	8	KU723069-071, 077-079, 081, 085	730	717	13	1	12	0.08	0	0	0-1	0-10
	5T03F09[a]	♀	1	KU723075	730	-	-	-	-	-	-	-	-	-

	5T16I09	♀	2	KU723086-087	730	730	0	0	0	0	0	0	0	0
	6T27D12[a]	♀	1	KU723074	730	-	-	-	-	-	-	-	-	-
All sequences			**19**		**730**	**715**	**15**	**1**	**14**	**0.07**	**0**	**0**	**0-2 (0.2)**	**0-11 (1)**
	11T22A13	♀	6	KU723088-093	730	729	1	0	1	-	0	0	0	0-1
	14T22A13	♀	1	KU723094	730	-	-	-	-	-	-	-	-	-
C. cephalodiscus	15T16I09[a]	♀	7	KU723095-101	730	718	12	1	11	0.09	0	0	0-1	0-10
(USA-01)	15T22A13	♀	5	KU723102-106	730	726	4	0	4	0	0	0	0	0-3
	16T16I09[a]	♀	7	KU723107-113	730	716	14	8	6	1.33	0	0	0-2	1-12
	6T28I09[a]	♀	6	KU723114-119	730	721	9	0	9	0	0	0	0-1	0-9
	7T28I09[a]	♀	2	KU723120-121	730	730	0	0	0	0	0	0	0	0
All sequences			**34**		**730**	**699**	**31**	**10**	**21**	**0.48**	**0**	**0**	**0-2 (0.4)**	**0-14 (3)**
	1T09H13	♀	7	KU723122, 125, 130-131, 136, 139, 146	730	711	19	10	9	1.11	0	0	0-2	0-13
	2T09H13	♀	7	KU723126-127, 132, 137, 140, 148-149	730	716	14	11	3	3.67	0	0	0-2	0-12
C. daisuce														
(USA-02)	3T09H13	♀	6	KU723123, 128, 141, 144, 150-151	730	712	18	10	8	1.25	0	0	0-2	0-16
	4T09H13	♀	7	KU723129, 145, 147, 152-155	730	716	14	0	14	0	0	0	0-2	0-1
	5T09H13	♀	7	KU723124, 133-135, 138, 142-143	730	714	16	3	13	0.23	0	0	0-2	0-12
All sequences			**34**		**730**	**694**	**36**	**13**	**23**	**0.57**	**0**	**0**	**0-2 (0.9)**	**0-16 (6.5)**
	2T26B14	♀	6	KU723156-161	730	725	5	0	5	0	0	0	0-1	0-4
C. daisuce	3T26B14	♀	8	KU723162-169	730	729	1	0	1	0	0	0	0	0-1
(USA-03)	4T26B14	♀	9	KU723170-178	730	721	9	0	9	0	0	0	0	0-3
	5T26B14	♀	8	KU723179-186	730	714	16	9	7	1.3	0	0	0-2	0-12
All sequences			**31**		**730**	**699**	**31**	**9**	**22**	**0.41**	**0**	**0**	**0-2 (0.5)**	**0-14 (3.4)**
	1T28A14	♀	7	KU723187-193	730	709	21	9	12	0.75	0	0	0-2	0-13
	2T28A14	♀	8	KU723194-201	730	717	13	1	12	0.08	0	0	0-2	0-12
C. daisuce	3T28A14	♀	4	KU723202-205	730	726	4	1	3	0.33	1	1 (1.0)	0-1	0-4
(USA-04)	4T28A14	♀	4	KU723206-209	730	717	13	4	9	0.44	0	0	0-2	0-13
	5T28A14	♀	6	KU723210-215	730	718	12	0	12	0	1	1 (1.0)	0-2	0-12
All sequences			**29**		**730**	**694**	**36**	**17**	**19**	**0.89**	**2**	**2 (1.0)**	**0-2 (0.9)**	**0-17 (7)**
	1T11H10	♀	13	KU723216-228	670	655	15	3	12	0.25	0	0	0-1	0-7
***Cephalenchus* sp2**	2T11H10[a]	♀	8	KU723229-236	670	660	10	0	10	0	0	0	0-1	0-5
(VIE-01)	3T11H10	♀	1	KU723237	670	-	-		-	-	-	-	-	-
	4T07C13	♀	8	KU723238-245	676	665	9	1	8	0.12	0	0	0-1	0-6
All sequences			**30**		**676**	**634**	**42**	**14**	**28**	**0.5**	**6**	**1 (6.0)**	**0-3 (0.9)**	**0-17 (6)**
C. nemoralis	5T13G10[a]	♀	1	KU723252	734	-	-	-	-	-	-	-	-	-
(VIE-02)	6T13G10[a]	♂	3	KU723247, 253-254	734	714	16	2	14	0.14	1	1 (1.0)	0-2	0-16

	7T13G10	♀	7	KU723246, 248-251, 255-256	735	688	47	8	39	0.2	2	2 (1.0)	0-5	1-34
	8T13G10	♂	2	KU723257-258	734	734	0	0	0	0	0	0	0	0
All sequences			**13**		**735**	**685**	**50**	**18**	**32**	**0.56**	**2**	**2 (1.0)**	**0-5 (1.4)**	**0-34 (11)**
	CD281	♀	1	EU915491	653	-	-	-	-	-	-	-	-	-
***C. hexalineatus*[b]**	CD391	♀	1	EU915492	653	-	-	-	-	-	-	-	-	-
	CD346	♀	1	EU915493	653	-	-	-	-	-	-	-	-	-
All sequences			**3**		**653**	**645**	**8**	**0**	**8**	**0**	**0**	**0**	**0-1 (0.8)**	**3-7 (5)**

ITS (ITS-1, 5.8S, ITS-2) rRNA gene

Species	Nematode ID	Sex	N	Accession No. GenBank[d]	Size	C	V	Pi	S	Pi/S	IT	IE/ length	p-dist. (%)	bp diff.
	6T07G09	♀	4	KU723259-262	570	522	48	11	37	0.3	27	8 (3.4)	1-8	7-41
	5T12G10	♂	3	KU723263-265	570	547	23	0	23	0	1	1 (1.0)	0-4	2-23
***Cephalenchus* sp1**	4T17C09	♀	4	KU723266-269	564	530	34	5	29	0.17	15	6 (2.5)	0-5	2-27
(BRA-01)	4T07G09	♀	8	KU723270-277	558	511	47	0	47	0	15	10 (1.5)	0-9	0-47
	2T17C09	♀	9	KU723278-286	578	484	94	59	35	1.69	43[c]	17 (2.8)[c]	0-12	0-67
	1T17C09	♀	8	KU723287-294	573	508	65	2	63	0.03	30	9 (3.3)	0-13	0-62
All sequences			**36**		**580**	**455**	**125**	**98**	**27**	**3.63**	**41**	**15 (2.7)**	**0-13 (6.1)**	**0-71 (33)**
	1T09I13	♀	5	KU723303-304, 306, 308, 310	638	617	21	8	13	0.62	1	1 (1.0)	0-2	0-14
	2T09I13	♀	4	KU723297 KU723305, 311, 313	638	616	22	5	17	0.29	29	2 (14.5)	0-4	2-22
C. daisuce	3T09I13	♀	6	KU723298 KU723312, 314-316, 319	638	616	22	1	21	0.05	29	2 (14.5)	0-4	0-22
(CAN)	4T09I13	♀	5	KU723295-296, 299 KU723300-001	609	608	1	0	1	0	0	0	0	0-1
	5T09I13	♀	5	KU723302, 307, 309 KU723317-318	638	615	23	0	23	0	29	2 (14.5)	0-4	0-22
All sequences			**25**		**638**	**601**	**37**	**16**	**21**	**0.76**	**30**	**3 (10.0)**	**0-4 (1.8)**	**0-24 (11)**
***C. hexalineatus*[b]**	CD281	♀	6	KU723320-325	616	610	6	6	0	-	0	0	0	0
	CD346	♀	2	KU723326-327	616	614	2	1	1	1	0	0	0	0-1
All sequences			**8**		**616**	**607**	**9**	**8**	**1**	**8**	**0**	**0**	**0-2 (0.6)**	**0-9 (4)**
	1T11F09	♂	9	KU723331, 335-340, 345-346	638	606	32	23	9	2.56	29	2 (14.5)	0-5	0-30
	4T03F09	♀	8	KU723332-334, 341-344, 347	639	614	25	2	23	0.09	31	4 (7.8)	0-4	0-25
C. cylindricus	5T03F09	♀	1	KU723348	608	608	0	0	0	-	-	-	-	-
(MEX)	6T27D12[a]	♀	1	KU723329	594	594	0	0	0	-	-	-	-	-
	7T27D12[a]	♀	1	KU723328	594	594	0	0	0	-	-	-	-	-
	8T27D12[a]	♀	1	KU723330	594	594	0	0	0	-	-	-	-	-
All sequences			**21**		**639**	**605**	**34**	**26**	**8**	**3.25**	**31**	**4 (7.7)**	**0-5 (1.5)**	**0-30 (9)**
C. cephalodiscus	11T22A13	♂	4	KU723350-352, 354, 358	638	632	6	0	6	0	0	0	0-1	1-5
(USA-01)	12T22A13	♀	4	KU723363-365	638	634	4	0	4	0	0	0	0	1-3

	14T22A13	♀	4	KU723353, 355-357	638	635	3	0	3	0	0	0	0	0-3
	15T22A13	♀	5	KU723349, 359-362	638	611	27	0	27	0	29	2 (14.5)	0-4	0-27
All sequences			**17**		**638**	**598**	**40**	**2**	**38**	**0.05**	**29**	**2 (14.5)**	**0-5 (0.8)**	**0-29 (5)**
***C. daisuce* (USA-02)**	1T09H13	♀	6	KU723366-371	638	605	33	26	7	3.71	31	4 (7.75)	0-5	1-28
	2T09H13	♀	6	KU723372-377	638	612	26	21	5	4.2	29	2 (14.5)	0-4	2-23
	3T09H13	♀	4	KU723378-381	638	615	23	22	1	22	29	2 (14.5)	0-4	0-23
	4T09H13	♀	6	KU723382-387	638	609	29	20	9	2.22	30	3 (10)	0-4	1-24
	5T09H13	♀	6	KU723388-393	638	638	0	0	0	-	0	0	0	0
All sequences			**28**		**638**	**596**	**42**	**30**	**12**	**2.5**	**31**	**4 (7.75)**	**0-5 (2.2)**	**0-28 (13)**
***C. daisuce* (USA-03)**	1T26B14	♀	7	KU723394-400	638	622	16	10	6	1.67	54	3 (18)	0-2	0-13
	2T26B14	♀	4	KU723401-404	638	638	0	0	0	-	0	0	0	0
	3T26B14	♀	2	KU723405-406	638	638	0	0	0	-	0	0	0	0
	4T26B14	♀	7	KU723407-413	638	610	28	12	16	0.75	54[c]	4 (20.75)[c]	0-4	0-27
	5T26B14	♀	7	KU723414-420	638	635	3	2	1	2	0	0	0	0-3
All sequences			**27**		**638**	**605**	**33**	**15**	**18**	**0.83**	**54[c]**	**5 (16.6)[c]**	**0-4 (1.1)**	**0-25 (6)**
***C. daisuce* (USA-04)**	1T28A14	♀	4	KU723424-426, 433	638	611	27	0	27	0	29	2 (14.5)	0-4	0-27
	2T28A14	♀	3	KU723422-23, 434	638	610	28	1	27	0.04	31	4 (7.75)	1-4	5-27
	3T28A14	♀	4	KU723428-431	609	598	11	2	9	0.22	1	1 (1.0)	0-2	0-10
	4T28A14	♀	3	KU723421, 427, 432	638	609	29	2	27	0.07	34	6 (5.7)	0-2	0-13
All sequences			**14**		**638**	**590**	**48**	**30**	**18**	**1.67**	**37**	**9 (4.1)**	**0-5 (2.9)**	**0-33 (17)**
***Cephalenchus* sp2 (VIE-01)**	4T07C13	♀	4	KU723435-438	637	637	0	0	0	-	0	0	0	0
	2T11H10	♀	4	KU723439-442	636	630	6	0	6	0	0	0	0-1	1-5
All sequences			**8**		**637**	**611**	**26**	**20**	**6**	**3.33**	**1**	**1 (1.0)**	**0-4 (2)**	**0-23 (13)**
***C. nemoralis* (VIE-02)**	1T05C13	♂	3	KU723443, 450-451	674	663	11	0	11	0	2	1 (2.0)	0-2	0-11
	5T13G10	♀	3	KU723452-454	672	672	0	0	0	-	0	0	0	0
	6T13G10	♂	3	KU723444-445	679	668	11	0	11	0	5	2 (2.5)	0-2	0-11
	8T13G10	♂	3	KU723446-449	734	734	0	0	0	-	0	0	0	0
All sequences			**12**		**679**	**652**	**27**	**11**	**16**	**0.69**	**7**	**4 (1.75)**	**0-2 (1.1)**	**0-16 (7)**

[a] Alignment also included one DNA sequence obtained by direct sequencing (i.e. not a clone).

[b] Sequences of 28S gene for *C. hexalineatus* were retrieved from GenBank. They represent two localities in the USA: C281 and C391, Florida; C346, Oregon. Yet, for the ITS region, sequences (for nematode specimens CD281 and CD346) were produced in this study.

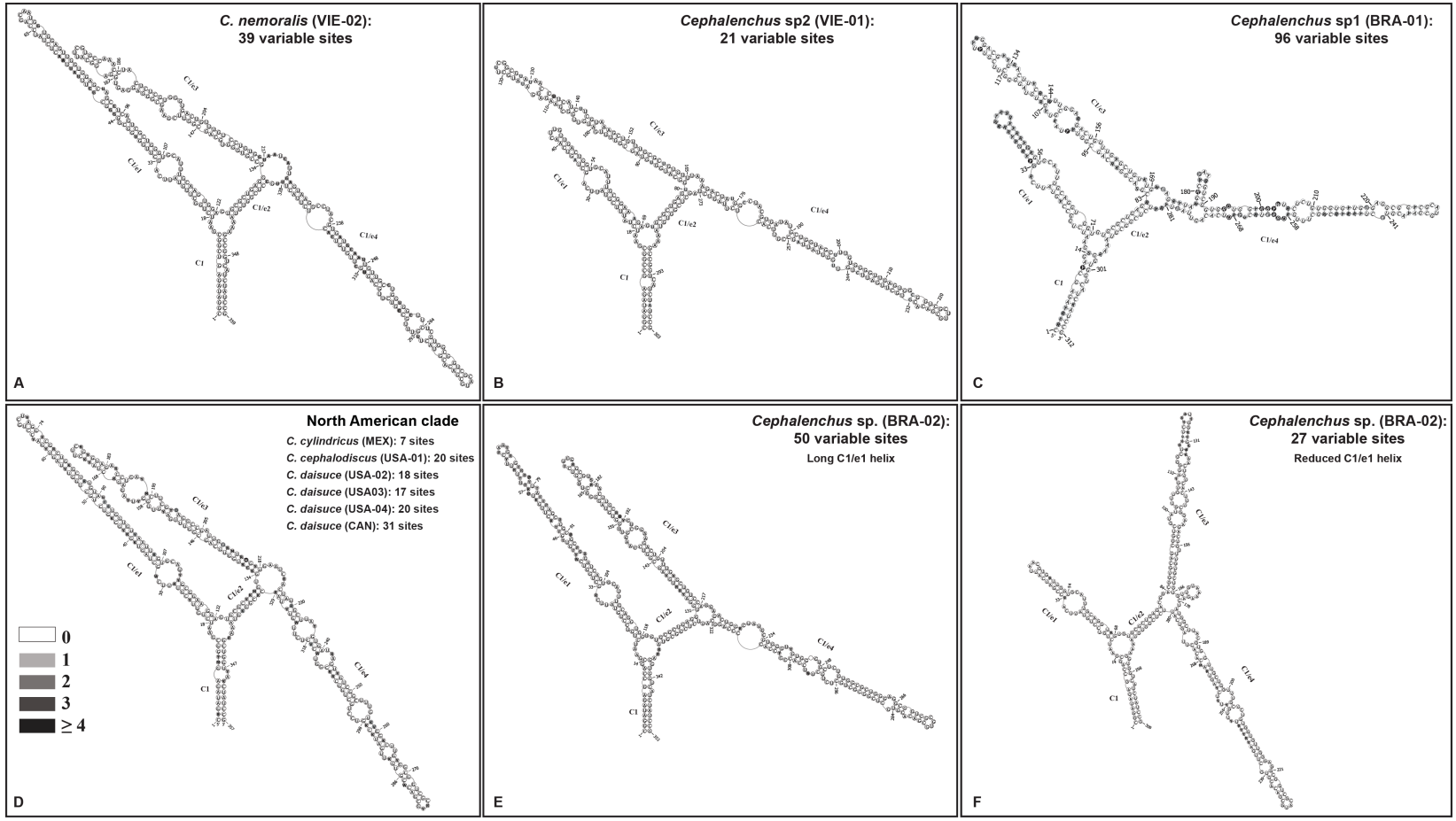
[c] IT and IE/length do not match due to overlapping indel regions that are counted as additional indel events.

[d] GenBank accession numbers are separately given for each nematode specimen. Accession numbers representing different clones from the same individual are shown as ranges (ascending order), so that only the last three digits are provided. Also, note that accession numbers within a species are not always sequential.

(-) Could not be calculated.

Appendix C

Complete secondary structure of D2-domain (28S rRNA) for *Cephalenchus* species presented on chapter 2.



Appendix D

List of species downloaded from GenBank used in the 18S and 28S phylogenetic analyses of chapter 3. Nematode classification is according to Siddiqi (2000). Locality codes are given for the different *Cephalenchus* populations. Sequences produced in the present study are highlighted in bold.

	GenBank Accession Numbers[b]				GenBank Accession Numbers[b]		
Species	**18S rRNA**	**28S rRNA**	**ITS rRNA**	**Species**	**18S rRNA**	**28S rRNA**	**ITS rRNA**
Abursanema iranicum	KF885743	KF885742	-	*Filenchus vulgaris*	JQ814877	JQ005013	-
Acrobeles complexus	AY284671	HM055394	DQ146425	*Globodera pallida*	AY284620	KJ409635	HQ260428
Aglenchus agricola	FJ969113	KP835679	-	*Globodera rostochiensis*	AY284619	KJ409632	DQ847120
Amplimerlinius icarus	EU306351	DQ328714	-	*Globodera tabacum*	FJ040401	AF393845	HQ260404
Amplimerlinius macrurus	FJ969114	KJ585424	-	*Helicotylenchus digonicus*	KM603517	HM014272	GQ906351
Anguina tritici	AY593913	DQ328723	KM114445	*Helicotylenchus dihystera*	AJ966486	HM014262	DQ309585
Aphelenchus avenae	AY284640	JQ348400	AB368919	*Helicotylenchus pseudorobustus*	AY284606	HM014280	KM506874
Basiria gracilis	EU130839	DQ328717	-	*Helicotylenchus vulgaris*	AY284607	HM014239	-
Belonolaimus longicaudatus	AY633449	AB602607	KF963098	*Hemicaloosia vagisclera*	JQ246426	JQ246424	JQ246429
Bitylenchus brevilineatus	KJ461603	KJ461533	-	*Hemicriconemoides alexis*[a]	-	AY780959	-
Bitylenchus dubius	AY284601	DQ328707	-	*Hemicriconemoides cocophillus*[a]	-	-	KM516183
Bitylenchus hispaniensis	KJ461609	KJ461545	KJ461576	*Hemicriconemoides pseudobrachyurus*[a]	AY284623	-	-
Bitylenchus iphilus	KJ461610	KJ461549	KJ461579	*Hemicycliophora conida*	AJ966471	FN433875	KF430580
Bitylenchus maximus	KJ461612	KJ461551	KJ461581	*Hemicycliophora thienemanni*	AY284629	AY780976	KF430568
Bitylenchus ventrosignatus	KJ461617	KJ461567	KJ461596	*Heterodera avenae*	FJ040403	GU083593	AF274395
Boleodorus sp.[a]	-	JQ005021	-	*Heterodera elachista*	KC618471	KC618465	AF498391
Boleodorus thylactus[a]	AY593915	-	-	*Heterodera koreana*	EU306357	EU284032	EU284042
Bradynema listronoti	DQ915805	DQ915804		*Heterodera schachtii*	EU306355	JQ040527	AY166437
Bursaphelenchus mucronatus	AB067759	DQ364688	U93554	*Heterodera trifolii*	FJ040402	GU475089	AF498388
Bursaphelenchus xylophilus	AB067760	EU295504	U92464	*Hirschmanniella loofi*	EU306353	EU620469	EU620472
Cactodera cacti[a]	-	DQ328702	-	*Hirschmanniella mucronata*[a]	-	-	DQ309589
Cactodera milleri[a]	-	-	AF161007	*Hirschmanniella oryzae*	KF366907	JX291142	DQ309588
Cactodera sp.[a]	KJ934187	-	-	*Hirschmanniella santarosae*[a]	EF029855	EF029859	-
Caloosia longicaudata	GU989625	GU989627	GU989621	*Hoplolaimus columbus*	KJ934149	EU554676	DQ309584
Carphodorus sp.	JQ771538	JQ771550	-	*Hoplolaimus galeatus*	KJ934148	EU626788	KP303599
Cephalenchus cephalodiscus (CHN02)	-	**This study**	-	*Howardula aoronymphium*	AY589304	AY589395	AF519224
Cephalenchus cephalodiscus (CHN02)	-	**This study**	-	*Howardula phyllotretae*	JX291137	DQ328728	-

Appendix D (Continued).

	GenBank Accession Numbers[b]				GenBank Accession Numbers[b]		
Species	**18S rRNA**	**28S rRNA**	**ITS rRNA**	**Species**	**18S rRNA**	**28S rRNA**	**ITS rRNA**
Cephalenchus cephalodiscus (CHN02)	-	**This study**	-	*Ibipora lolii*	JQ771535	JQ771542	JQ771558
Cephalenchus cephalodiscus (CHN03)	-	**This study**	**This study**	*Lelenchus leptosoma*	AY284584	KP730042	-
Cephalenchus cephalodiscus (CHN03)	-	**This study**	**This study**	*Litylenchus coprosma*	GU727546	GU727547	GU727548
Cephalenchus cephalodiscus (CHN03)	-	**This study**	**This study**	*Macrotrophurus arbusticola*	AY284595	DQ328708	-
Cephalenchus cephalodiscus (USA01)	**This study**	KU723094	KU723349	*Malenchus pressulus*	KM229333	KM229341	-
Cephalenchus cephalodiscus (USA01)	**This study**	KU723108	KU723350	*Meloidoderita kirjanovae* [a]	-	DQ768428	DQ768427
Cephalenchus cephalodiscus (USA01)	-	KU723116	KU723351	*Meloidoderita salina* [a]	KF751618	-	-
Cephalenchus cylindricus (MEX)	-	KU723069	KU723345	*Meloidogyne arenaria*	AY268118	KC287192	U96301
Cephalenchus cylindricus (MEX)	-	KU723077	KU723331	*Meloidogyne artiellia*	KC875392	AY150369	AF248478
Cephalenchus cylindricus (MEX)	-	KU723076	KU723333	*Meloidogyne chitwoodi*	AY593889	KC241981	U96302
Cephalenchus daisuce (BEL02)	-	**This study**	**This study**	*Meloidogyne ethiopica*	JQ768373	KF482373	EU204644
Cephalenchus daisuce (BEL02)	-	**This study**	**This study**	*Meloidogyne exigua*	AY942627	AF435804	-
Cephalenchus daisuce (BEL02)	-	**This study**	**This study**	*Meloidogyne fallax*	AY593895	KC241975	AY281853
Cephalenchus daisuce (CAN01)	**This study**	KU723044	KU723295	*Meloidogyne graminicola*	KF201168	JN005874	HM581973
Cephalenchus daisuce (CAN01)	**This study**	KU723050	KU723296	*Meloidogyne hapla*	AY593898	KF430798	AF516722
Cephalenchus daisuce (CAN01)	-	KU723058	KU723306	*Meloidogyne hispanica*	HE667741	EU443606	JX885741
Cephalenchus daisuce (USA02)	-	KU723123	KU723366	*Meloidogyne ichinohei*	KC875385	EF029862	-
Cephalenchus daisuce (USA02)	-	KU723144	KU723371	*Meloidogyne incognita*	AY284621	KF482374	U96304
Cephalenchus daisuce (USA02)	-	KU723125	KU723379	*Meloidogyne javanica*	JX100422	KC953092	U96305

Appendix D (Continued).

	GenBank Accession Numbers[b]				GenBank Accession Numbers[b]		
Species	**18S rRNA**	**28S rRNA**	**ITS rRNA**	**Species**	**18S rRNA**	**28S rRNA**	**ITS rRNA**
Cephalenchus daisuce (USA03)	-	KU723156	KU723412	*Meloidogyne konaensis*	HE667744	AF435797	-
Cephalenchus daisuce (USA03)	-	KU723185	KU723405	*Meloidogyne mali*	JX978225	KF895396	JX978228
Cephalenchus daisuce (USA03)	-	KU723161	KU723406	*Meloidogyne marylandi*	JN241856	JN157852	JN241880
Cephalenchus daisuce (USA04)	-	KU723189	KU723428	*Meloidogyne minor*	JN389787	KC241978	AY281855
Cephalenchus daisuce (USA04)	-	KU723192	KU723427	*Meloidogyne naasi*	JN241841	KC241979	EU910042
Cephalenchus daisuce (USA04)	-	KU723205	KU723422	*Meloidogyne paranaensis*	AY942622	AF435800	-
Cephalenchus hexalineatus	AY284594	-	-	*Merlinius brevidens*	AY284597	KJ585416	-
Cephalenchus hexalineatus	KJ869347	-	-	*Mesocriconema xenoplax*	AY284626	AY780966	HM116073
Cephalenchus hexalineatus	KJ869346	-	-	*Morulaimus* sp.	JQ771540	JQ771552	JQ771555
Cephalenchus hexalineatus	KJ869316	-	-	*Nacobbus aberrans*	AJ966494	KF178912	AY254369
Cephalenchus hexalineatus (BEL01)	-	**This study**	**This study**	*Nagelus hexagrammus*[a]	-	-	**This study**
Cephalenchus hexalineatus (BEL01)	-	**This study**	**This study**	*Nagelus leptus*[a]	-	DQ328715	-
Cephalenchus hexalineatus (BEL01)	-	**This study**	**This study**	*Nagelus obscurus*[a]	AY593904	-	-
Cephalenchus hexalineatus (CRI)	-	**This study**	-	*Neodolichodorus* sp.	JQ771537	JQ771549	-
Cephalenchus hexalineatus (FL, USA)	EU915486	EU915491	KU723325	*Neodolichorhynchus lamelliferus*[a]	AY284598	-	-
Cephalenchus hexalineatus (FL, USA)	-	-	KU723321	*Neodolichorhynchus phaseoli*[a]	-	KJ585429	-
Cephalenchus hexalineatus (OR, USA)	-	EU915492	KU723326	*Neopsilenchus magnidens*[a]	AY284585	-	-
Cephalenchus hexalineatus (OR, USA)	-	EU915493	KU723327	*Neopsilenchus* sp.[a]	-	JQ005018	-
Cephalenchus leptus	KJ869414	-	-	*Nothotylenchus acris*[a]	AY593914	-	-
Cephalenchus leptus	KJ869315	-	-	*Nothotylenchus persicus*[a]	-	KT149799	-
Cephalenchus leptus (CHN01)	-	**This study**	-	*Ogma civellae*[a]	-	AY780955	-
Cephalenchus leptus (IRAN)	-	KP730040	-	*Ogma octangulare*[a]	-	-	JQ708141
Cephalenchus leptus (USA05)	-	**This study**	**This study**	*Ogma* sp.[a]	KJ934175	-	-
Cephalenchus leptus (USA05)	-	**This study**	**This study**	*Paraphelenchus acontioides*	HQ218323	HQ218322	-

Appendix D (Continued).

	GenBank Accession Numbers[b]				GenBank Accession Numbers[b]		
Species	**18S rRNA**	**28S rRNA**	**ITS rRNA**	**Species**	**18S rRNA**	**28S rRNA**	**ITS rRNA**
Cephalenchus leptus (USA05)	-	**This study**	**This study**	*Parasitylenchus* sp.	KJ636418	KM245038	-
Cephalenchus nemoralis (VIE02)	-	KU723247	KU723443	*Paratylenchus colinus*	KP966494	KP966492	-
Cephalenchus nemoralis (VIE02)	-	KU723246	KU723452	*Paratylenchus conicephalus*	KP966493	KP966491	-
Cephalenchus nemoralis (VIE02)	-	KU723254	KU723447	*Paratylenchus nanus*	KJ636435	AY780946	KF242264
Cephalenchus sp. (BRA02)	-	KU723031	-	*Paratylenchus straeleni*	AY284630	KM875547	KF242274
Cephalenchus sp. (BRA02)	-	KU723032	-	*Pratylenchoides magnicauda*	AF202157	KF026289	-
Cephalenchus sp. (BRA02)	-	KU723033	-	*Pratylenchoides ritteri*	AJ966497	JX261964	-
Cephalenchus sp. (BRA02)	-	KU723037	-	*Pratylenchus crenatus*	AY284610	EU130853	FJ712912
Cephalenchus sp. (BRA02)	-	KU723035	-	*Pratylenchus japonicus*	KF385443	KF385445	KF452048
Cephalenchus sp. (BRA02)	-	KU723036	-	*Pratylenchus neglectus*	JQ303332	JX261951	FJ712952
Cephalenchus sp1 (BRA01)	**This study**	KU722999	KU723294	*Pratylenchus pratensis*	KC875387	AM231934	-
Cephalenchus sp1 (BRA01)	-	KU723009	KU723279	*Pratylenchus thornei*	AY284613	JX261963	FJ713002
Cephalenchus sp1 (BRA01)	-	KU723004	KU723278	*Pratylenchus vulnus*	KC875389	HM469437	FJ713007
Cephalenchus sp2 (VIE01)	**This study**	KU723231	KU723435	*Psilenchus hilarulus*	AY284593	EU915489	-
Cephalenchus sp2 (VIE01)	-	KU723239	KU723441	*Psyllotylenchus* sp.	KF373734	KF373739	-
Cephalenchus sp2 (VIE01)	-	KU723241	KU723439	*Punctodera punctata*[a]	-	-	AF274416
Cephalobus persegnis	AY284663	DQ903077	-	*Punctodera stonei*[a]	KC852180	KC852182	-
Cervidellus alutus[a]	AF202152	HM055400	-	*Radopholus duriophilus*[a]	-	-	HQ823571
Cervidellus sp.[a]	-	-	DQ146424	*Radopholus similis*	AJ966502	JN091964	GQ281456
Coslenchus costatus	AY284581	DQ328719	-	*Radopholus* sp.[a]	FJ040398	DQ328712	-
Coslenchus franklinae	AY284583	KM817175	-	*Rotylenchulus reniformis*	JX406383	DQ328713	AY335190
Criconema mutabile[a]	-	AY780954	-	*Rotylenchus goodeyi*	AY284609	DQ328756	-
Criconema sp.[a]	AJ966480	-	-	*Rotylenchus jaeni*	JX015428	EU280791	EU373662
Criconema sphagni[a]	-	-	JQ708135	*Rotylenchus paravitis*	JX015429	JX015422	JX015434
Criconemoides brevistylus[a]	-	-	KC937032	*Rotylenchus robustus*	AJ966503	JX015426	JX015439
Criconemoides informis[a]	KF900157	AY780970	-	*Rotylenchus uniformis*	AY593882	DQ328740	-
Cryphodera brinkmani	JQ965679	KF430215	AF274418	*Rotylenchus vitis*	JN032583	JN032581	JN032582
Cryphodera sinensis	JX566453	JX566454	JX566457	*Rubzovinema* sp.	KF373732	KF373736	KF155281
Deladenus proximus[a]	-	-	KF908909	*Scutellonema bradys*	AJ966504	JX472035	AY271722
Deladenus siricidicola	AY633447	AY633444	EF122861	*Skarbilovinema lyoni*	JX291138	DQ328733	-
Deladenus sp.[a]	AJ966481	JX104326	-	*Sphaeronema alni*	GU253916	JQ771954	GU253920
Discotylenchus iranicus	KM502981	KM502982	-	*Sphaerularia bombi*	AB250212	DQ328726	-
Ditylenchus destructor	KJ636422	FJ707365	DQ471334	*Sphaerularia vespae*	AB300595	AB300596	AB300595
Ditylenchus dipsaci	AY593909	FJ707364	AY574289	*Spilotylenchus* sp.	KF373735	KF373740	-
Ditylenchus drepanocercus	JQ429768	JQ429773	JQ429774	*Subanguina radicicola*	AF202164	DQ328721	AF396365

Appendix D (Continued).

	GenBank Accession Numbers[b]				GenBank Accession Numbers[b]		
Species	**18S rRNA**	**28S rRNA**	**ITS rRNA**	**Species**	**18S rRNA**	**28S rRNA**	**ITS rRNA**
Ditylenchus gallaeformans	JQ429767	JQ429771	JQ429777	*Telotylenchus* sp.[a]	-	JX472064	-
Ditylenchus gigas	HQ219211	KC310734	HQ219231	*Telotylenchus ventralis*[a]	AY593905	-	-
Ditylenchus halictus	AY589297	AY589364	EF627047	*Trophurus imperialis*	FJ969144	KJ461529	
Dolichodorus mediterraneus[a]	-	DQ838803	-	*Tylenchorhynchus claytoni*	EU368587	EU368589	**This study**
Dolichodorus sp.[a]	EF025336	-	**This study**	*Tylenchorhynchus leviterminalis*	EU368585	EU368591	EF030984
Eutylenchus excretorius	EU915487	EU915490	EU915500	*Tylenchulus semipenetrans*	AJ966511	JN112252	FJ588909
Fergusobia camaldulensae	AY589294	AY589378	-	*Tylenchus naranensis*	KJ869373	KP730043	-
Fergusobia rileyi	AY589292	AY589335	-	*Veleshkinema iranicum*	KP300015	KM40154	-
Ficotylus congestae	EU018049	EU018047	-	*Vittatidera zeaphila*	JF741962	JF741960	JF741961
Filenchus annulatus	JQ814880	JQ005017	-	*Xenocriconemella macrodora*	JF972482	AY780960	JQ708139
Filenchus quartus	JQ814879	JQ005016	-	*Zeatylenchus pittosporum*	JQ586255	JQ586256	JQ586257
Filenchus sindhicus	JQ814875	JQ005012	-	*Zygotylenchus gansuensis*	KJ129766	KJ129769	KJ129772
Filenchus sp.	JQ814876	JQ005015	-	*Zygotylenchus guevarai*	AF442189	JX261956	FJ717817
Filenchus thornei	JQ814878	JQ005014	-				

[a] Sequences were combined to represent the genus in the concatenated analyses.
[b] Sequences have been submitted to GenBank and accession numbers are still pending.
(-) Sequences not available.