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PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL GENOMES OF FILARIAL NEMATODES IN THE SUBFAMILY WALTONELLINAE

by

Sarina Marie Bauer
Bachelor of Science, University of North Dakota, 2011

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Master of Science

Grand Forks, North Dakota
August
2015

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This thesis, submitted by Sarina Marie Bauer in partial fulfillment of the requirements for the Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done, and is hereby approved.

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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the Graduate School at the University of North Dakota and is hereby approved.

Wayne Swisher
Dean of the Graduate School

July 7, 2015

PERMISSION

Title	Phylogenetic Analysis of Mitochondrial Genomes of Filarial Nematodes in the Subfamily Waltonellinae
Department	Biology
Degree	Master of Science

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Name: Sarina Bauer

Date: June 23, 2015

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To my family,
Without whom this would not have been possible.

ABSTRACT

The family Onchocercidae is a group of parasitic vector born nematodes that are split up into eight subfamilies. Although the subfamilies containing species that parasitize humans and livestock (e.g., Onchocercinae) have been well-studied, there is a considerable lack of information for the lesser studied subfamilies in Onchocercidae (e.g. Waltonellinae). This uncertainty leaves some doubt as to the phylogenetic and evolutionary relationships among the different taxonomic groupings of filarioid nematodes.

I worked with two species of the subfamily Waltonellinae, *Foleyellides* n. sp. and *F. flexicauda*. The primers used in this study for polymerase chain reactions (PCR) and DNA sequencing reactions were based on previously published sources and newly designed as needed. DNA products having the predicted molecular size were used for sequencing reactions. Sequencing was performed using appropriate primers in order to cover the entire mitochondrial genome. Obtained sequences were assembled into a complete circular mitochondrial genome with exception of a small AT-rich region. Phylogenetic analyses using Baysian inference and other algorithms was performed to determine phylogenetic affinities of newly sequenced nematodes with the previously sequenced filarioids. Both genome-level and gene-level phylogenies were produced and compared.

Foleyellides n. sp. and *F. flexicauda* were often found to be the basal branch of the phylogenies. This, along with the fact that neither species is *Wolbachia*-dependent, suggests Waltonellinae was one of the first filarioid nematode groups to exist within the nematode family.

CHAPTER I

INTRODUCTION

Mitochondria are cellular organelles found in most eukaryotes. They are thought to have originated from endosymbiotic bacteria and are involved in respiratory metabolism. Mitochondria have their own genome which is distinct from the nuclear genome. The mitochondrial genome is a closed, circular molecule that is typically inherited maternally in animals. Because of this, mitochondrial sequences are not exposed to sexual re-assortment brought on by diversity generating mechanisms [1]. Also, the mitochondrial DNA (mtDNA) of many animals mutates faster than nuclear DNA. These traits may be advantageous when differentiating between closely related taxa [2,3]. One group of animals in particular that has been difficult to phylogenetically analyze for this reason is the filarioid nematode family Onchocercidae.

Filarioids are parasitic roundworms found in clade III (Spiruria) of the Phylum Nematoda [4]. They have an indirect lifecycle, requiring two different hosts. One host is a vertebrate, including all vertebrate classes except fishes. The other host is a bloodsucking arthropod, such as mosquitoes, blackflies, and ticks, and is responsible for transmitting the parasite from one vertebrate host to another. Within the vertebrate, adult worms are found in the lymphatic system, subcutaneous tissues, or the peritoneal cavity.

After mating, the adult females release thousands of larvae, known as microfilariae, which inhabit the blood or skin, depending on species. Microfilariae are the infective stage for the arthropod vectors [5]. This life cycle is summarized in Figure 1.

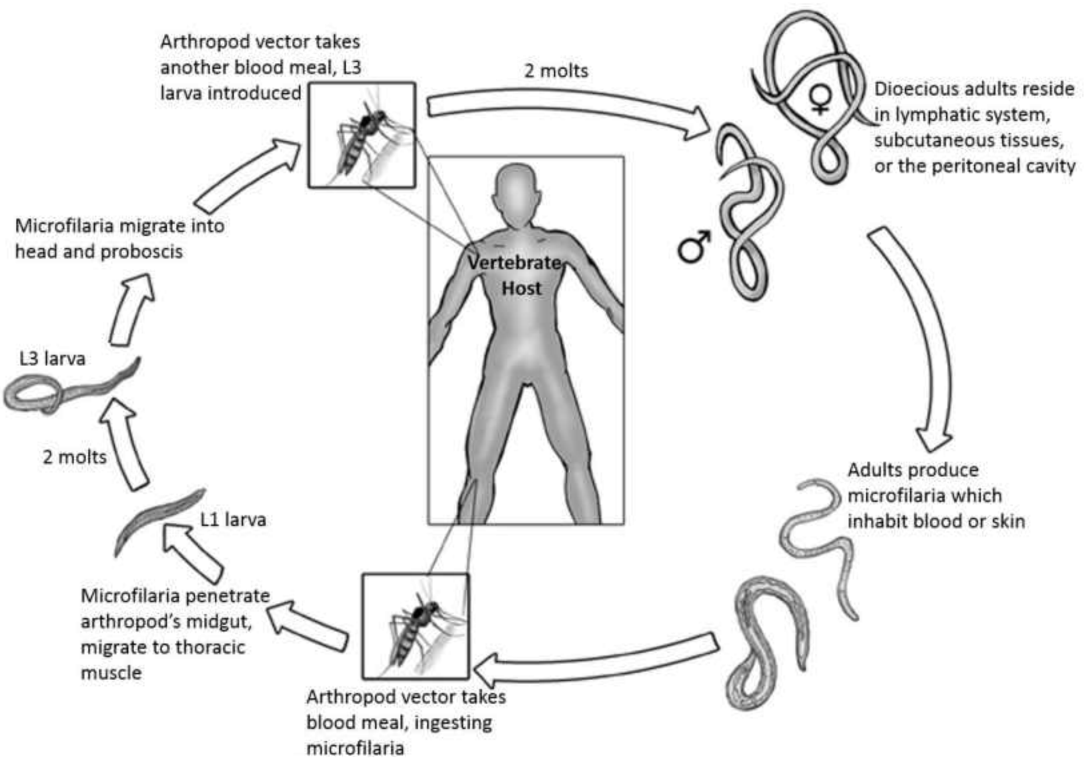

Figure 1 Filarioid life cycle depicting the transmission from definitive host (vertebrate) to vector (arthropod). Modified from rowdy.msudenver.edu.

The Onchocercidae family is organized into 8 subfamilies [5]. These subfamilies are listed in Table 1, along with their definitive host. The first three subfamilies listed infect humans and domestic animals causing human suffering and economic losses throughout the world. Because of this, these subfamilies have the most medical and veterinary importance [6], and therefore most research thus far has been focused on them.

Table 1. Filarioid subfamilies with *Wolbachia* prevalence and their definitive hosts.

Subfamilies (approx. no# of genera)	Percentage with *Wolbachia* (no# species examined)	Host animals
Onchocercinae (17)	61% (39)	Mammals, including humans
Dirofilariinae (10)	50% (4)	Mostly mammals
Setariinae (1)	0% (7)	Large hoofed mammals
Waltonellinae (3)	0% (2)	Amphibians
Splendidofilariinae (6)	25% (4)	Mostly songbirds
Oswaldofilarinae (2)	0% (1)	Reptiles
Iscosiellinae (1)	Unknown	Amphibians
Lemdaninae (4)	Unknown	Birds, reptiles, mammals

Several different species of filarioids from the subfamily Onchocercinae are among leading causes of parasite-induced disability in humans [7]. *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori* are the cause of lymphatic filariasis (elephantiasis) [8]. These nematodes dwell in the lymph nodes of the host, slowing blocking the pathway [9]. Over time, certain areas of the body, especially the feet, legs, and scrotum, become severely swollen and disfigured, to the point where mobility becomes difficult [10]. *Onchocerca volvulus* is the cause of onchocerciasis (river blindness) [8]. The microfilaria can migrate to the cornea, causing inflammation and, ultimately, blindness [11].

The filarioids from the subfamily Dirofilariinae are responsible for deadly veterinary diseases such as dirofilariasis (dog heartworm) [12]. Adult *Dirofilaria immitis* nematodes reside in the heart. Infections can become very dangerous with many adult worms, severely impacting the heart and health of the animal host [13].

The nematodes mentioned thus far have a unique relationship with an endosymbiotic intracellular bacterium known as *Wolbachia.* When the worms die within the vertebrate host, they release the bacteria. This causes the host to launch an immune response, which in turn causes increased and prolonged inflammation. This inflammation is the root cause of the most debilitating symptoms of these filarial diseases[14].

Wolbachia, an obligate mutualistic endosymbiont, is found to be mutually dependent with many filarioids [15]. This dependency is so important that the bacteria are required for such things as growth, molting, fertility, and even survival of the worm [16]. *Wolbachia* are found in both sexes and in all developmental stages. In females, they are found in the reproductive organs where they are transmitted transovarially, from mother to offspring [7]. Some filarioid species have been found to be naturally free of *Wolbachia,* but there are differences within subfamilies and even genera (e.g., *Onchocerca*), of filarioids. The evolutionary dynamics of *Wolbachia* in filarioids is complex and not completely understood [17]. Table 1 depicts an up-to-date summary of the known taxonomy of filarioid species that are either *Wolbachia*-dependent or -independent. Table 1 also illustrates the amount of information that still needs to be collected. There is a considerable lack of information for the lesser studied subfamilies in Onchocercidae. This lack of knowledge leaves some doubt in the phylogenetic relationships amongst the different taxonomic groupings of filarioids.

All filarioid mitochondrial genomes encode two ribosomal RNA genes, 22 transfer RNA genes, and 12 protein-coding genes [18]. This is different from other metazoan mitochondrial genomes which encode 13 protein genes [19]. All nematodes are missing the atp8 gene except for *Trichinella spiralis* [20]. This information about the mitochondrial genome allows for easier evolutionary comparison of different species and lineages because it gives a template to read from and compare new sequences to. To date, there have been nine published mitochondrial genomes of filarioid parasites that have been used in phylogenetic analyses. One example is shown in Figure 2 [17].

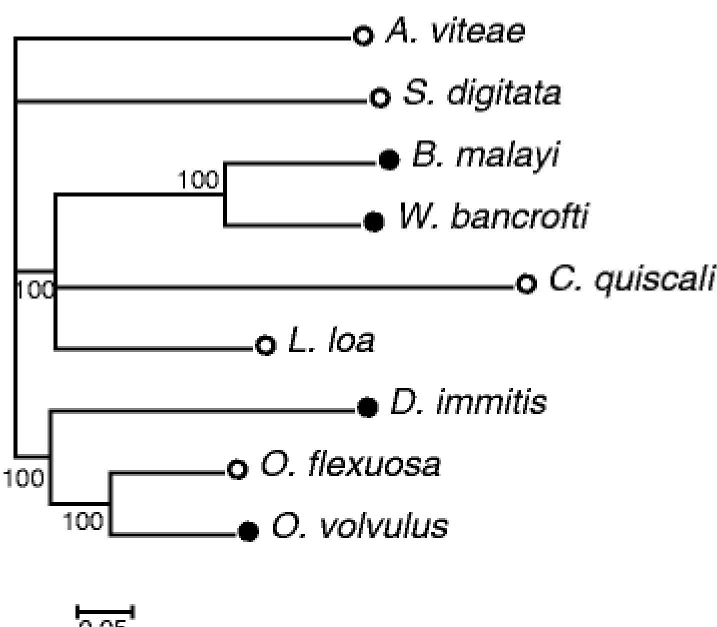

Figure 2 Phylogeny based on the 12 protein coding genes of the mitochondrial (mtDNA) genome sequences of nine species of filarioids. Percentages of Bayesian posterior probabilities are displayed at nodes. Black circles indicate *Wolbachia*-dependence while white circles indicate *Wolbachia*-independence [17].

The main objectives for this study are:

- sequence the mitochondrial genomes of filarioid species in the lesser studied Waltonellinae subfamily
- compare the structure and gene organization of these mitochondrial genomes with those of other more well-studied filarioid species
- construct revised phylogenies of filarioids based on completed mitochondrial genomes and specific filarioid genes (e.g. 12s rRNA and Cox1 genes) currently available from a greater selection of filarioid species (GenBank)
- screen the newly sequenced Waltonellinae for the bacterial endosymbiont *Wolbachia.*

The hypotheses of the study are:

- Waltonellinae will be a basal branch
- Waltonellinae will not possess the endosymbiont *Wolbachia.*

Support for these hypothesis arise from the fact that the subfamily Waltonellinae are parasites of amphibians. Amphibians are the most phylogenetically basal group of vertebrates that are parasitized by filarioid nematodes.

CHAPTER II

MATERIALS AND METHODS

Parasite materials and DNA isolation

Adult female and male filarioid nematodes were collected from the peritoneal cavities of two leopard frogs (*Rana* [=*Lithobates*] sp.), obtained at two different times from teaching laboratories at the University of North Dakota. Both nematodes were identified as belonging to the genus *Foleyellides* using the taxonomic keys of Skrjabin and Shikhobalova 1948. One of the nematodes was determined to be *Foleyellides flexicauda* using the key of Schacher and Crans 1973, while the other was determined to be a new species. The new species, designated as *Foleyellides* n. sp., was collected in 2006 from the Animal Biology laboratory. The location of the supply company in which the host species were ordered is unknown. *Foleyellides flexicauda* was collected in 2011 from the Parasitology laboratory. The frogs were ordered from Niles Biological, Inc. in Sacramento, CA. DNA was isolated from the adult filarial nematodes using the DNeasy blood and tissue kit (Qiagen, Valencia, CA).

Morphology of collected specimens was examined under DIC-equipped Olympus BX51 compound microscope using Rincon digital imaging and measurement software.

Primer design

Primers used to amplify and sequence mtDNA are based on previously published sources [17] and were newly designed as needed (Table 2). The design method used for the primers was primer walking. Initial sequences were acquired using the previously published primers. Primers were designed on either side of the gaps that were left after initial sequencing was done. It was often difficult to find ample place to design acceptable primers because filarial nematode mitochondrial genomes are high in AT content, making adequate binding difficult. Primers were designed to be 15-20 bp in length and have a relatively even mixture of A's and T's versus G's and C's. Using this method, the gaps were slowly filled in from both sides until they met and overlapped in the middle.

Table 2. Primers used to amplify and sequence the mitochondrial genomes of *Foleyellides* n. sp. (A) and *Foleyellides flexicauda* (B).

Name	Primer (5'-3')	Forward (F) or Reverse (R)	PCR, Sequence (Seq), or Both	Species	Reference
1RN1	CAACCCTAAACAACC	R	Both	A,B	This study
1F	TTATTTTTAATTTTCGATTA	F	Both	A	McNulty et al.[17]
1FN	CTTCTAAACTAATATCGAG	F	Both	A,B	This study
1F2	GTTGTGGGGCTTTGG	F	Seq	B	This study
1F4	CTTGTTGTGGGGCTTTGG	F	Both	A	This study
1R3	CCAAAACACTTTCACAATTC	R	Both	A	This study
1R	ACACTCATAAAAGACAAAA	R	Both	A,B	This study
2F	TTTTTAATGTTCCTTTGAGTATTACTTTT	F	Both	A,B	McNulty et al.[17]
2R	TAGCAATATGATAAAACTCACCAATAAA	R	Seq	A,B	McNulty et al.[17]
3F	TTGTAAGTCTTTGGCTGCTTA	F	Seq	A,B	McNulty et al.[17]
3R	TCATAAAAAGAAGTATTAAAATTACGAT	R	Both	A,B	McNulty et al.[17]
4F	TGTTGGGTGCTATTAATTTT	F	Both	A,B	McNulty et al.[17]
4R	CTACAAAAATAAGTTAACAAAGAAA	R	Both	A,B	McNulty et al.[17]
4F3	GTGAGGTTAAGATGCTTG	F	Both	A	This study
5F	GTCGTGTTGGTTATGGTTATCA	F	Both	A,B	McNulty et al.[17]
4R1	GCCCACCTAAAACTTAATAG	R	Both	A	This study
5RN	CCGTAATCAGATATAG	R	Seq	A	This study
5R	AATAAGGAACAGAAGTTATCAAACTAGTAAT	R	Both	A,B	McNulty et al.[17]
6R2	CTGAATCCGCCCCACACTC	R	Both	A	This study
5F4	CCTGCCAGGTTTTCTTTG	F	Both	A	This study
6FN1	CTATTTTACGTTCTG	F	Both	A	This study
6FN	GGTTTGATTAATGGAAG	F	Both	A,B	This study
6RN	TCCAGTATAACATCC	R	Both	A,B	This study
6R	GAACCTCAAACATATAATAAACAA	R	Both	A,B	McNulty et al.[17]
7R3	CTTAAAAACCAACCGCGC	R	Seq	A	This study
6FN5	GGGGCTCATGTTTTTATCGGGG	F	Seq	A	This study
8R3	GCCACAACAAATAGAG	R	Seq	A	This study

Table 2. cont.

Name	Primer (5'-3')	Forward (F) or Reverse (R)	PCR, Sequence (Seq), or Both	Species	Reference
7R4	GTAACTAAAAATGCCACAAC	R	Seq	A	This study
12SR1	GGTACTAATCCAGTTC	R	Seq	A	This study
7F4	GTGTTCCTAGAATAATCGGC	F	Seq	A	This study
8F	AGATAGTATTACTTATTTTTAGTTT	F	Both	B	McNulty et al.[17]
8F4	GATTAATGAGGCATG	F	Seq	B	This study
8FN	ATGAGGCATGTGTTAC	F	Both	A	This study
8FN1	TTTGGTTTACTTTGCC	F	Both	A,B	This study
8RN1	TCTAATCCGCAATGTC	R	Both	A,B	This study
8RN	GACTTTATAAAAGAATC	R	Both	A	This study
8R	AAATATAACTCTGCAAAAA	R	Both	B	McNulty et al.[17]
8F5	GGCGCGTACTTTTAC	F	Both	B	This study
8F18	GCAAAGTTATATTTTTTAAC	F	Both	B	This study
8FN4	CAGTATCAGGGTCGG	F	Seq	A	This study
8FN2	ACTGATACAGTATCAGG	F	Both	A,B	This study
8R14	CTATGACAAAAACTTATACTGATATGC	R	Both	B	This study
8F6	CTCTAAGTTGTTTGTC	F	Both	B	This study
8F7	GTCTTCTGCCCTATG	F	Both	A,B	This study
8F9	CAGGAATGAATGAAG	F	Both	B	This study
8R12	CAAATCACTTACGCTAC	R	Both	A	This study
8F22	GGGATGTATGTTTTTTC	F	Both	B	This study
8FN3	GGTATTGCATATCAG	F	Both	A	This study
8F7	GTCTTCTGCCCTATG	F	Both	A,B	This study
8F11	GTTCGTCGTGAGACAGAGCGG	F	Both	A	This study
8F22	GGGATGTATGTTTTTTC	F	Both	B	This study
9F4	GGGGGCTAGTACGAAAGGAAAG	F	Both	A	This study
8R6	CTATCTCAGCAAGTTAAAG	R	Both	A	This study
8F12	GTGGTATTTAGGTAATTTGCC	F	Both	A	This study
9R6	CTTTATTACCCCTTTTATCG	R	Both	A,B	This study
9R3	CCCCTTTTATCGTAA	R	Both	A,B	This study

Table 2. cont.

Name	Primer (5'-3')	Forward (F) or Reverse (R)	PCR, Sequence (Seq), or Both	Species	Reference
9R5	CCCCAAATTATACTTTCAAACACCATAAGGC	R	Both	A	This study
9R11	CAAACGAGACACCCCCACC	R	Both	B	This study
9R2	CAAAACTACACAAATC	R	Both	B	This study
9R1	CAAACCACTCATACTC	R	Both	A	This study
9RN	AGCGCACAAACACCAG	R	Both	B	This study
9F3	CGGTCTTTACTAATCG	F	Both	B	This study
9RN1	AGCGCACAAACACCAG	R	Both	A	This study
9FN	CCTGTTAGTTGTTTGGTTC	F	Both	A,B	This study
10RN	CTAACAAAAATAAGAGAG	R	Both	A,B	This study
10FN1	CTGGAATTTTTATTG	F	Both	A,B	This study

PCR amplification and sequencing

All polymerase chain reactions (PCRs) were carried out in a final volume of 25µl using 12.5µl *Taq* 2X Master Mix (New England BioLabs Inc., Ipswich, MA), 8.5µl ultrapure water, 2µl DNA template, 1µl of 10µmol/L forward primer, and 1µl of 10µmol/L reverse primer. The PCRs were run in an Eppendorf Mastercycler® using cycling conditions designed specifically for each primer pair and the anticipated amplicon lengths. The initial denaturation was 4-min at 94ºC followed by 40 cycles of 94ºC for 30s, 40ºC-55ºC (depending on primer's melting temperature) for 45s, and 68ºC for 1-3min (depending on desired amplicon length). The amplified DNA resulting from these PCRs were visualized on a 1.5% agarose gel stained with ethidium bromide in a 0.5x Tris-borate-EDTA buffer. The DNA from PCR-positive samples was purified by either; 1) combining 2µl of the PCR product with 5µl of ExoSap and heated to 80ºC for 15-min then 37ºC for 15-min, or 2) by using DNA Clean & Concentrator™-5 (Zymo

Research, Irvine, CA), depending on the strength of the band. For bands with very low DNA concentration, the Zymo kit was used to concentrate the product. For gels showing more than one band, with the correct band length being highly visible and fairly separated from other bands on the gel, a gel cleanup was done on the correct band. The Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA) was used after running the PCR product out on a 2.5% agarose gel stained with ethidium bromide in a 0.5x Tris-borate-EDTA buffer and excising the DNA fragment from the agarose gel using a scalpel blade and UV light box. The remaining purified DNA was then ready for sequencing.

Sequencing reactions were performed using 2µl of the purified PCR product in a 10µl solution containing 1µl of BigDye Terminator ® v3.1 (Life Technologies, Grand Island NY), 2µl 5x buffer, 2µl of 10µmol/L primer, and 3µl ultrapure water in an Eppendorf Mastercycler®. The initial denaturation was 2-min at 94ºC followed by 25 cycles of 96ºC for 15s, 50ºC for 5s, and 60ºC for 4min. Sequencing was conducted using an ABI PRISM® 3100 Genetic Analyzer.

Assembly and annotation of mitochondrial genomes

Sequences were assembled and spliced together using the program Sequencher (v. 4.2.2). Sequences were verified by comparison, and aligned against, the published complete mitochondrial genome sequences of *Acanthocheilonema vitea* (accession number HQ186249), *Chandlerella quiscali* (accession number HM773029), *Loa loa* (accession number HQ186250), and *Onchocerca flexuosa* (accession number HQ214004) using BioEdit Sequence Alignment Editor (v. 7.2.0).

Phylogenetic analysis

I used the program jModelTest [21] to select the appropriate model for the mitochondrial cytochrome oxidase subunit I (Cox1) dataset, mitochondrial 12s rRNA dataset, and the combined dataset with the 12 protein-coding genes in the mitochondrial genome. This program generated Akaike Information Criterion (AIC) scores for 40 different models and the GTR+I+G model had the best AIC score among the different models for both Cox1 and 12s datasets. This components of this model are:

> **GTR** (General Time Reversible) refers to the fact that any base can switch to any other base, or switch back. This model also accounts for the empirically measured frequencies of those bases in the gene. In other words, the model actually counts the number of each base, instead of estimating the frequency of each base.
>
> **I+G** (Inverse Gamma) refers to the probability of a mutation to occur based on the inverted Poisson distribution (i.e. tossing a coin to determine the probability). This is the most common model to decide this probability.

The analysis was run in MrBayes v3.1.2 (www.mrbayes.sourceforge.net). Over the course for 1 million generations, trees were saved at intervals of 100 generations to reduce the size of the final population of trees. The final analysis included a "burn-in" parameter. The reason for including a "burn-in" is because initially the selection process generates a rough estimation starter tree, then keeps the most likely trees. The program builds upon these trees to generate even more refined trees. Therefore, the first 25% of

the analysis are generally discarded (i.e. the “burn-in”) and only the remaining 75% is retained.

The resulting phylogenetic tree was visualized in FigTree v1.4.0. Numbers at specific nodes indicate the percentages of Bayesian posterior probabilities when ≥ 70%. The trees are rooted with *Thelazia callipaeda* as the outgroup. All sequences were obtained from GenBank, except those from *Foleyellides* n. sp. and *F. flexicauda*, which were obtained in this study (Table 3).

Twelve separate phylogenies each based on different mitochondrial protein-coding genes were selected, created and viewed using using Maximum Likelihood (ML) in MEGA 5.05. The mitochondrial protein-coding genes included NADH dehydrogenase 2 (ND2), NADH dehydrogenase 4 (ND4), cytochrome c oxidase I (Cox1), NADH dehydrogenase 6 (ND6), cytochrome b (CytB), cytochrome c oxidase III (Cox3), NADH dehydrogenase subunit (NDL4), NADH dehydrogenase 1 (ND1), ATP synthase 6 (ATP6), cytochrome c oxidase II (Cox2), NADH dehydrogenase 3 (ND3), and NADH dehydrogenase 5 (ND5). Numbers at specific nodes indicate the degree of maximum likelihood bootstrap support (estimated from 500 replicates) when ≥ 50%.

The Cox1, 12s, and combined protein-coding phylogenies were also performed as maximum likelihood searches via GARLI [22] and maximum parsimony searches via PAUP [23].

Wolbachia Detection

Both nematode species were screened for the groEL gene that is specific for *Wolbachia* endosymbionts, using a modification of the PCR methods described by

McNulty et al.[17] The primers provided by Dr. Tkach's lab yielded better amplification than the primers described by McNulty et al.[17] For *Wolbachia* positive control, I extracted the DNA from microfilaria of *Brugia pahangi,* a species known to harbor the endosymbiont. *Brugia pahangi* was obtained from the NIH-NIAID Filariasis Research Reagent Resource Center (www.filariasiscenter.org). Distilled water was used as negative control.

Table 3. List of nematode species, family/subfamily, and accession number used in the phylogenetic analyses of the Cox1 gene (Cox1), the 12s rRNA gene (12s), and all 12 protein-coding genes combined (Total).

Family/Subfamily	Species	Cox1	12s	Total
Onchocercidae/Onchocercinae	*Acanthocheilonema reconditum*	AJ544876	AJ544853	
	Acanthocheilonema viteae	AJ272117	AJ544852	HQ186249
	Brugia malayi	AJ271610	AJ544843	NC_004298
	Brugia pahangi	AJ271611		
	Cercopithifilaria crassa	AM749260	AM779791	
	Cercopithifilaria japonica	AM749262	AM779794	
	Cercopithifilaria multicauda	AM749254	AM779799	
	Dipetalonema gracile	AJ544877	AM779824	
	Litomosoides hamletti	AJ544868	AJ544847	
	Litomosoides westi	AJ544871	AJ544851	
	Litomosoides yutajensis	AJ544869	AJ544846	
	Loxodontofilaria caprini	AM749237	AM779821	
	Mansonella perforate	AM749265	AM779803	
	Mansonella ozzardi		JF412318	
	Onchocerca dewittei japonica	AM749266	AM779815	
	Onchocerca flexuosa	NC_016172	JQ733523	HQ214004
	Onchocerca gibsoni	AJ271616	AY462913	
	Onchocerca lupi	JX080029	JN863696	
	Onchocerca ochengi	AJ271618	KC167333	
	Onchocerca suzukii		AM779811	
	Onchocerca volvulus	AM749285	KC167339	NC_001861
	Wuchereria bancrofti	AJ271612	AJ544844	HQ184469
Onchocercidae/Dirofilariinae	*Dirofilaria immitis*	EU169124	EU182327	NC_005305
	Dirofilaria repens	AM749231		
	Foleyella candezei	FR823336	FR827906	
	Foleyella furcate	AJ544879	AJ544841	
	Loa Loa	AJ544875	AJ544845	HQ186250
Onchocercidae/Setariinae	*Setaria digitata*	EF174427	EF179382	NC_014282
	Setaria equina	AJ544873	AJ544835	
	Setaria labiotopapillosa	AJ544872	AJ544833	
	Setaria tundra	AJ544874	AM779848	
Onchocercidae/Splendidofilariinae	*Aproctella* sp	FR823335	FR827905	
	Chandlerella quiscali	NC_014486	NC_014486	HM773029
	Madathamugadia hiepei	JQ888272	JQ888290	
	Rumenfilaria andersoni	JQ888279	JQ888296	
Onchocercidae/Waltonellinae	*Ochoterenella* sp. 1	AJ544878		
	Ochoterenella sp. 2	FR823337		
	Ochoterenlla sp.		AJ544836	
	Ochoterenella royi		AM779830	
	Foleyellides n. sp.	TBD	TBD	TBD
	Foleyellides flexuosa	TBD	TBD	
Onchocercidae/Oswaldofilariinae	*Piratuba scaffi*	AM749281	AM779831	
Onchocercidae/-	*Filaria martis*	AJ544880	AJ544855	
Thelaziidae/-	*Thelazia callipaeda*	NC_018363	NC_018363	NC_018363

CHAPTER III

RESULTS

Genome Features and Organization

The mitochondrial genome of *Foleyellides* n. sp. and *F. flexicauda* was sequenced, annotated, and will be deposited to Genbank prior to publication. The genome of *Foleyellides* n. sp. is slightly smaller in size (13,312 bp) compared to the other species including *O. volvulus* reported at 13,474 bp (Table 4), though it is unclear if the genome of *Foleyellides* n. sp. was completely sequenced. The AT content, at 72.0% is also slightly lower than *O. volvulus,* which is at 73.3% (Table 4). Compared to preliminary data for *F. flexicauda*, the new species of *Foleyellides* has similar ratios of AT/GC content (Table 4).

All 10 filarial mtDNAs, including *Foleyellides* n. sp., encode the same 12 protein-coding genes, two rRNAs, 22 tRNAs, and an AT-rich non-coding region (Fig. 3). Due to sequencing gaps for *Foleyellides* n. sp., two tRNA genes were not able to be placed in the genome (Met and Ala). They are mapped where they are expected to be present based on the positions in related species (*C. quiscali* and *A. viteae*; Fig. 3); however, it is possible that these positions could shift once the complete genome is sequenced. There is also a small, 62 bp insertion found in *Foleyellides* n.sp not known to be present in other species.

Table 4. Comparison of the mitochondrial genomes of the ten filarioid nematode species with *Foleyellides* n. sp.

Species	Subfamily	Size of mt genome (bp)	A%	T%	G%	C%	A+T%	Reference
F. flexicauda	Waltonellinae	N/A	20.16%	51.38%	20.01%	8.00%	71.53%	This study
Foleyellides n. sp.	Waltonellinae	13,312	20.86%	51.13%	20.12%	7.89%	71.99%	This study
O. volvulus	Onchocercinae	13,474	19.30%	54.00%	19.80%	6.90%	73.30%	Keddie et al.[24]
L. loa	Dirofilariinae	13,590	20.80%	54.80%	17.70%	6.70%	75.60%	McNulty et al.[17]
B. malayi	Onchocercinae	13,657	21.60%	53.90%	16.80%	7.70%	75.50%	Ghedin et al.[25]
W. bancrofti	Onchocercinae	13,657	20.50%	54.10%	18.00%	7.40%	74.60%	McNulty et al.[17]
O. flexuosa	Onchocercinae	13,672	20.30%	53.90%	18.60%	7.20%	74.20%	McNulty et al.[17]
A. viteae	Onchocercinae	13,724	19.60%	54.00%	19.30%	7.20%	73.50%	McNulty et al.[17]
C. quiscali	Splendidofilariinae	13,757	23.00%	54.70%	15.90%	6.40%	77.70%	McNulty et al.[17]
D. immitis	Dirofilariinae	13,814	19.30%	54.90%	19.30%	6.50%	74.20%	Hu et al.[26]
S. digitata	Setariinae	13,839	19.40%	55.70%	18.20%	6.70%	75.10%	Yatawara et al.[27]

N/A = *F. flexicauda* was not fully sequenced and therefore does not have an applicable genome size.

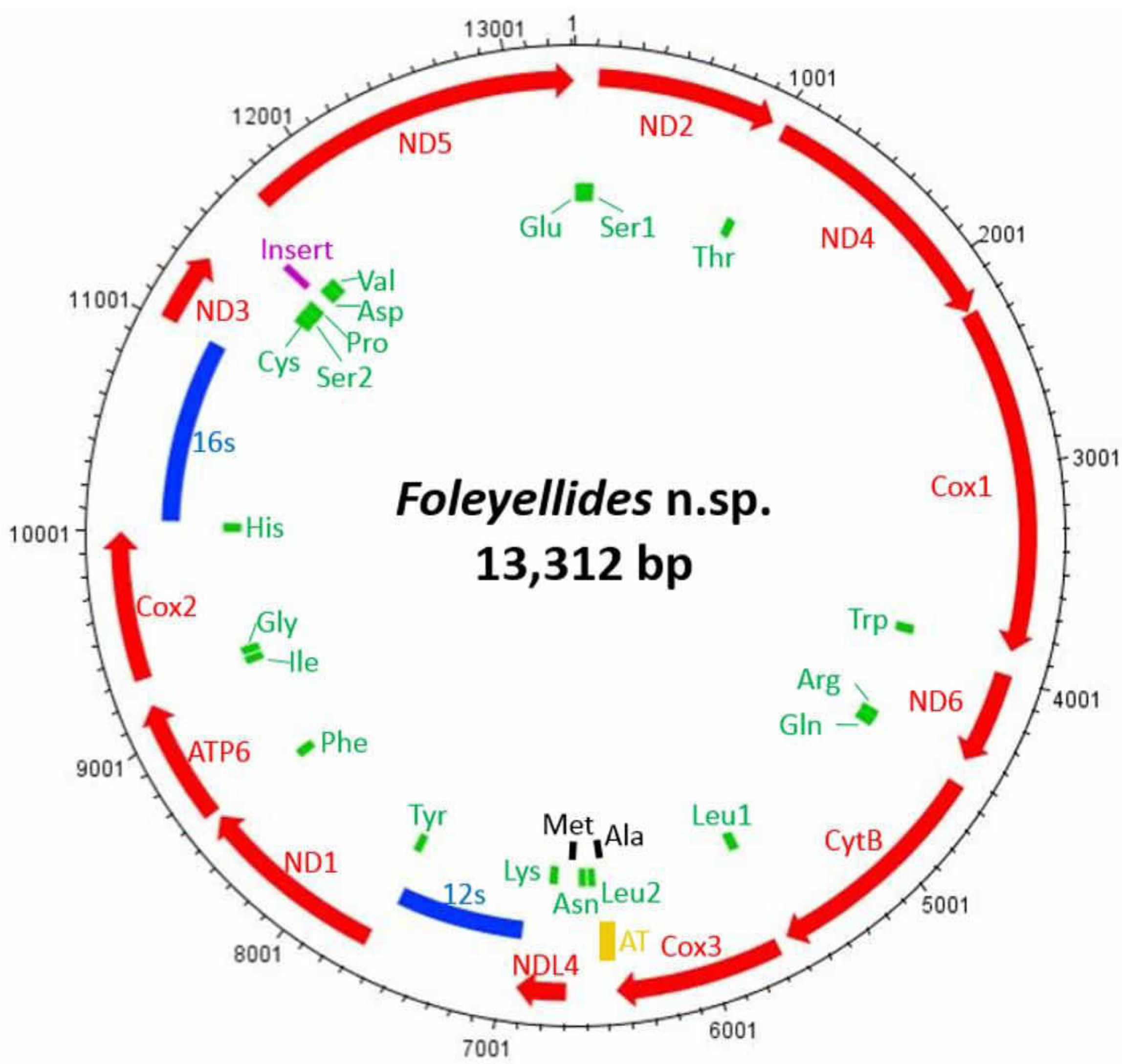

Figure 3 Gene map of the mitochondrial DNA for *Foleyellides* n. sp. Protein-coding genes are shown in **red** with the arrows indicating direction of the coding strand. rRNA genes are shown in **blue** and tRNA genes are shown in **green**. The hypothesized positions of two tRNA genes are shown in **black**. The AT rich region is shown in **yellow** (near position 6,500). The **purple** rectangle (near position 11,650) represents a small 62 bp insertion not present in other species. This map was made using DNAPlotter via Artemis v16.

Table 5. Comparison of the start/stop codons used in the 12 mitochondrial protein coding genes of the ten filarioid nematodes species.

Gene Species	ND2	ND4	Cox1	ND6	CytB	Cox3	NDL4	ND1	ATP6	Cox2	ND3	ND5
***Foleyellides* n. sp.**	ATT/TAA	TTG/TAA	ATT/T	TTT/TAA	AAT/TTT	ATA/TAA	ATT/TAA	TTG/TAT	ATG/TAA	ATT/TAA	CTT/TAA	TTT/T
O. volvulus	ATT/TAG	TTG/TAA	ATT/TAG	ATT/TAG	ATT/TAA	ATT/TAA	TTG/TAA	TTG/T	ATT/TAG	ATT/TA	CTT/TAG	TTG/TAG
L. loa	ATT/TAA	TTG/TAG	GTT/T	TAT/TAG	ATT/T	ATT/TAA	GTA/T	TTG/T	ATT/TAA	ATT/TAA	CTT/T	TTT/TAG
B. malayi	TTA/T	TTG/TAA	ATT/TAG	TAT/TAA	ATT/T	ATT/TAA	GTA/TAA	TTG/T	ATT/TAG	ATT/TAA	CTT/TAG	TTT/TAG
W. bancrofti	TTA/T	TTG/TAA	ATT/TAA	TGT/TAA	ATT/T	ATT/TAA	GTA/TAA	TTG/TAA	ATT/TAG	ATT/TAA	CTT/T	TTT/TAG
O. flexuosa	ATT/TAG	TTG/TAG	ATT/TAA	ATT/TAA	ATT/TAA	ATT/TAG	TTA/T	TTG/T	ATT/TAA	ATT/TAA	CCT/T	TTA/TAA
A. viteae	TTT/TAG	ATG/TAA	GTT/T	TAT/TAG	TCT/T	ATT/TAA	GTA/T	TTG/T	ATT/TAA	ATT/TAA	CTT/T	TTT/TAG
C. quiscali	ATT/TAG	TTG/TAA	TTG/TAA	TTG/TAG	ATT/T	ATT/TAA	GTT/TAA	TTG/T	ATT/TAG	ATT/TAA	CTT/TAG	TTT/T
D. immitis	ATT/TAG	TTG/TAG	ATT/TAG	TAT/TAG	GTT/T	ATT/TAA	GTA/TAA	TTG/T	TTG/TAA	ATT/T	CTT/T	TTG/TAG
S. digitata	TTT/TAG	ATG/TAA	ATT/TAG	TTG/TAA	GTT/T	ATA/T	TTG/T	TTG/TAA	TTT/TAG	ATT/TAG	TTT/T	TTT/TAG

Protein-coding genes

All 12 protein-coding genes were identified in *Foleyellides* n. sp. along with their corresponding start and stop codons for each gene (Table 5). Filarioid mtDNA genomes are extremely thymine (T)- and adenine (A)-rich (Table 4); therefore, it is common to bias in start/stop codons containing mostly A/T nucleotides. When compared to those filarioid species whose mitochondrial have been sequenced, *Foleyellides* n. sp. contained novel start codons, including TTT for ND6, AAT for CytB, ATT for NDL4, and ATG for ATP6 (Table 5). Novel termination codons for *Foleyellides* n. sp. included TAT for ND1 and TTT for CytB (Table 5). The incomplete stop codon T in both CoxI and ND5 is converted to TAA upon the addition of a 3' poly(A) tail; it is not possible to determine if these are conserved or novel when comparing *Foleyellides* n. sp. to previously published sequences (Table 5).

Ribosomal and transfer RNA genes

The mitochondrial genome of *Foleyellides* n. sp. encodes two rRNA genes (12s and 16s). The position of these genes corresponds to that of all other filarioids sequenced thus far with the 12s gene being positioned between the NDL4 and ND1 genes and the 16s gene positioned between the Cox2 and ND3 genes (Figure 3).

In *Foleyellides* n. sp., only 20 of the 22 tRNA genes were able to be found due to incomplete sequencing. There were small gaps left over after sequencing the mitochondrial genome because of its high AT-richness, leading to incomplete binding of primers in PCR. The two tRNA's that were unable to be sequenced were MET and ALA, and their hypothesized positions were placed on the genome based on other published

genomes [17] (Fig. 3). The remaining 20 tRNA's were found to be in similar locations based on other published genomes [17] (Fig. 3).

AT-rich region

Like previously published filarioid sequences, *Foleyellides* n. sp. has a AT-rich control region comprised of 46bp. Size of the AT-rich control region varies widely in filarioid nematodes, from 256 bp in *W. bancrofti* to 506 in *S. digitata* [17]. The smaller control region in *Foleyellides* n. sp. could be due to incomplete sequencing of the genome.

Phylogenetic analysis

Many subfamilies in the phylogeny inferred from CoxI are not monophyletic. The Onchocercinae is found in multiple clades with varying support (70% - 100% posterior probability; Fig. 5) with two other species found in a polytomy with ingroup species (*D. gracile* and *O. suzukii*). Dirofilariinae is divided between two clades, one with moderate support (86% posterior probability) and the other with no significant support (<70% posterior probability). *Piratuba scaffi* (Oswaldofilariinae) is placed within Waltonellinae, within *Ochoterenella*. The Setariinae is found to be monophyletic with strong support (100 % posterior probability). *Foleyellides* n. sp. and *F. flexicauda* are found to be sister species with 100% posterior probability, within the Waltonellinae.

The phylogeny based on the 12s gene reconstructs Onchocercinae in four clades with varying support (<70% - 100% posterior probability; Fig. 6). The Splendidofilariinae is found to be monophyletic with moderate support (89% posterior probability). The Dirofilariinae is divided into three clades; two nested within members

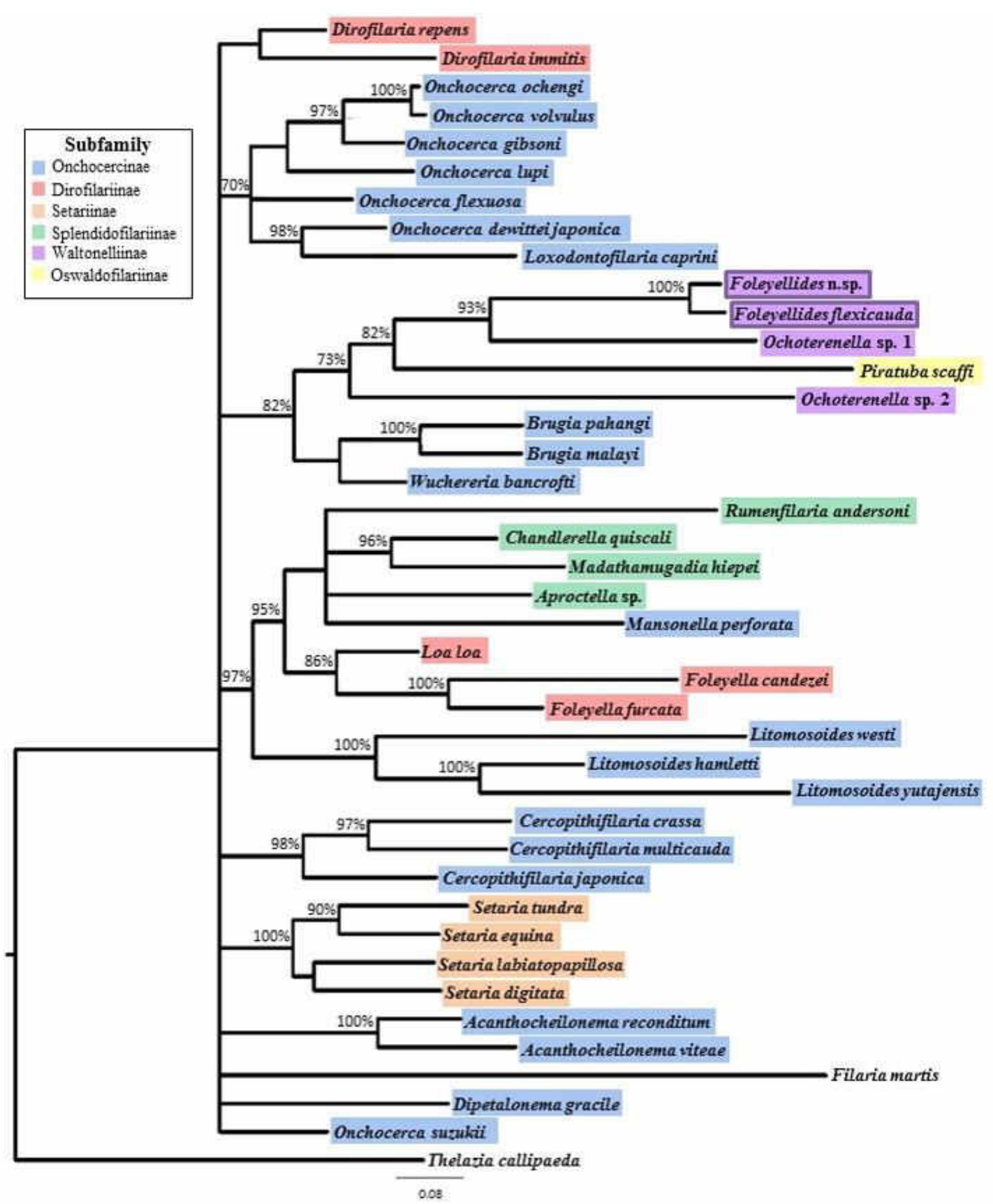

Figure 4 Phylogeny based on mitochondrial cytochrome oxidase subunit I (Cox1) sequences. Numbers at specific nodes indicate the percentages of Bayesian posterior probabilities when ≥ 70%. Scale bar indicates branch length. (-ln likelihood score = 9407.25743)

of Onchocercinae (*L. loa* and *D. immitis*), with the *Foleyella* one as an independent supported lineage (91% posterior probability; Fig. 6). The Setariinae are divided into two clades. *Setaria tundra* placed within Oswaldofilariinae and *F. martis* (a member of the outgroup); however, this relationship has no significant support (<70% posterior probability; Fig. 6). *Foleyellides* n. sp. and *F. flexicauda* are again found to be sister species with 100% posterior probability and are monophyletic with the rest of Waltonellinae (71% posterior probabilities; Fig. 6).

The phylogeny based on the combined data set with the 12 protein-coding genes in the mitochondrial genome provides information on the monophyly of the Onchocercinae and Dirofilariinae. *Foleyellides* n. sp., representing Waltonellinae, is found to be the most basal branch of the tree, but this placement lacks strong support (<70% posterior probability; Fig. 7). Onchocercinae is divided into three clades, all with high support (95% - 100% posterior probabilities). The Dirofilariinae are not monophyletic; instead *L. loa* is sister to *C. quiscali* (Splendidofilariinae) with moderate support (89% posterior probabilities; Fig. 7). *Dirofilaria immitis* is found in a clade basal to *O. volvulus* and *O. flexuosa* (Onchocercinae), with 100% posterior probability (Fig. 7).

The appendix shows phylogenies based on sequences of each of the 12 protein-coding mitochondrial genes separately (Fig. 8), (A) ND2, (B) ND4, (C) Cox1, (D) ND6, (E) CytB, (F) Cox3, (G) NDL4, (H) ND1, (I) ATP6, (J) Cox2, (K) ND3, (L) ND5. These 12 trees display highly variable structures, with only the pairings of *O. volvulus* with *O. flexuosa* and *B. malayi* with *W. bancrofti* staying constant. *Foleyellides* n. sp. is the basal branch in 5 out of 12 of the trees (Fig. 8). These trees indicate not all genes are suitable for phylogenetic analysis.

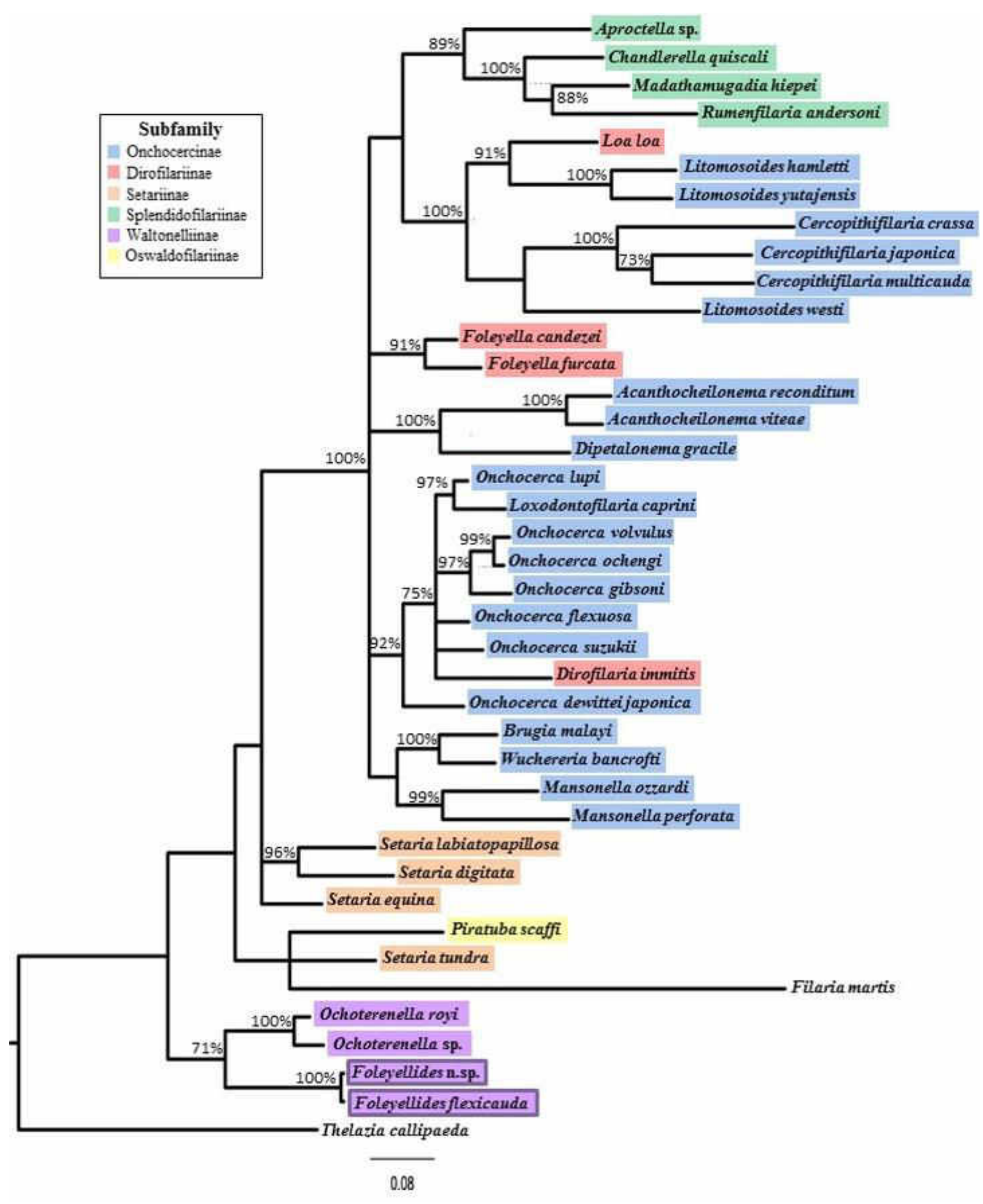

Figure 5 Phylogeny based on mitochondrial 12s rRNA gene sequences. Numbers at specific nodes indicate the percentages of Bayesian posterior probabilities when ≥ 70%. Scale bar indicates branch length. (-ln likelihood score = 6250.45146)

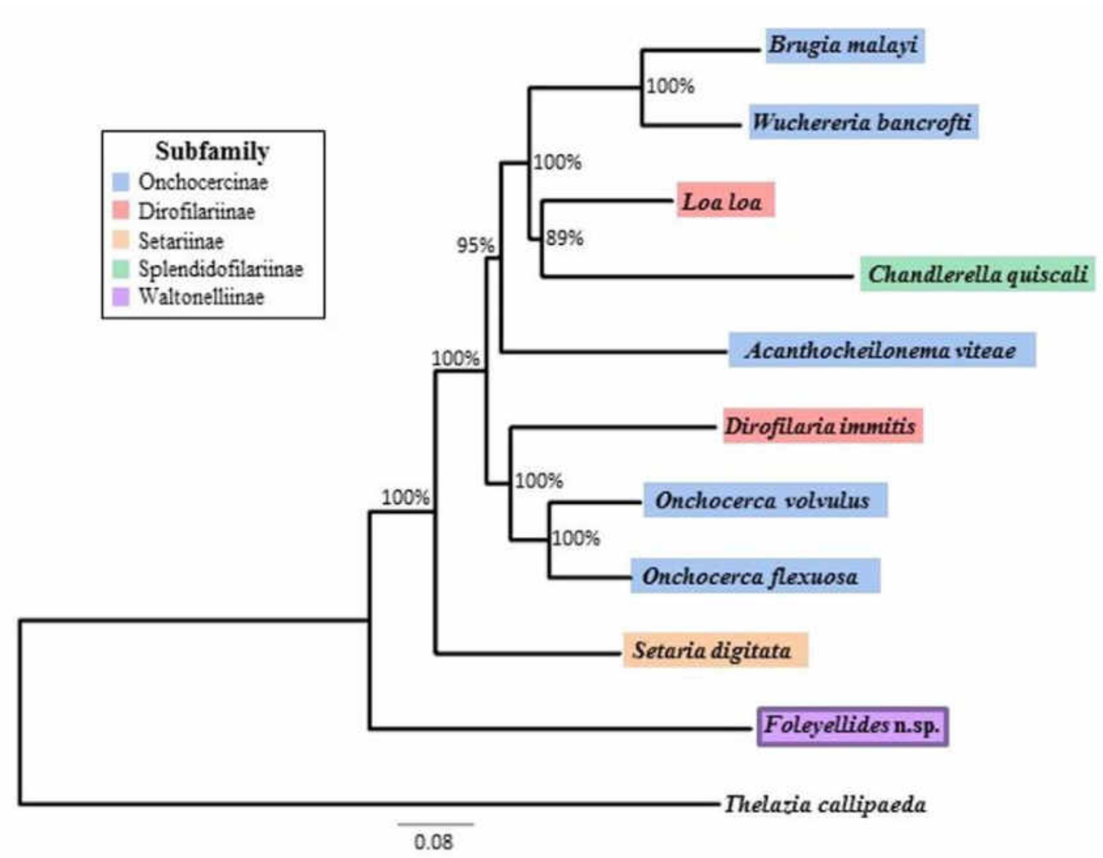

Figure 6 Phylogeny based on concatenated sequences of the 12 protein-coding mitochondrial genes. Numbers at specific nodes indicate the percentages of Bayesian posterior probabilities when ≥ 70%. Scale bar indicates branch length. (-ln likelihood score = 62816.12747)

Also in the appendix are maximum parsimony analyses based on Cox1, 12s, and combined protein-coding genes. These tree topologies did not differ from Bayesian consensus trees for well supported nodes (Figs. 9, 10, and 11; posterior probability >75%).

Absence of *Wolbachia*

There was no PCR amplification of the bacterial gene, groEL, for both *Foleyellides* n. sp. and *F. flexicauda* (Fig. 4), indicating that these two species do not harbor *Wolbachia*.

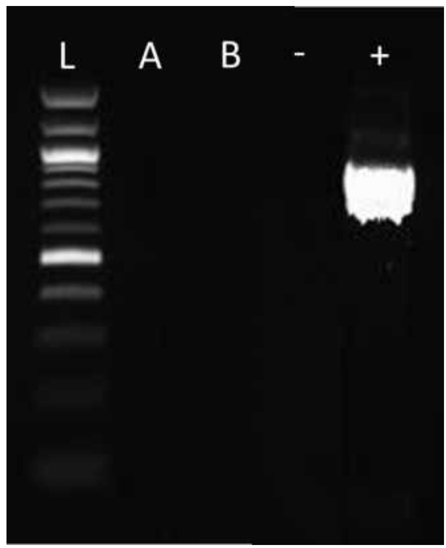

Figure 7. Gel electrophoresis results from a polymerase chain reaction (PCR) using primers to detect the presence of the groEL gene of *Wolbachia* sp. *Foleyellides* n. sp. (A), *Foleyellides flexicauda* (B), and the negative control (-) all show negative results. The positive control (+) (*Brugia pahangi* adult worm) shows the optimal band size for a positive detection of the gene. The 100 bp ladder is shown on the left (L)

CHAPTER IV

DISCUSSION

In 1974, Bain and Prod'Hon erected the subfamily Waltonellinae to group together filarioids parasitizing amphibians. Although several new species have been added to the subfamily since then, there has been very little molecular work done on these species. Not only does this study bring to light a new species of filarioid, it also adds genetic information about the subfamily Waltonellinae.

The overall size of the new *Foleyellides* sp. mitochondrial genome is the smallest in comparison to the nine other species. However, this could be due to the possibly incomplete sequencing of the A-T rich control region. This non-coding region of the mitochondrial genome in animals is called the control region because it is believed to control the transcription and replication of mitochondrial DNA (mtDNA) [18]. The control region found for the *Foleyellides* n. sp. was only 46 basepairs (bp) long, whereas the smallest recorded length of this area from filarioids to date comes from *Wuchereria bancrofti* at 256 bp [17]. Nematodes have been found to have control regions very high in A-T content and to contain multiple sections of inverted repeats [18,28]. Among other things, these features can lead to difficulty in amplifying and sequencing the DNA for this region.

The gene organization of the *Foleyellides* n. sp. was found to be very similar to eight of the nine other filarioid species whose mitochondrial genomes have been

sequenced. *Chandlerella quiscali* was the only filarioid whose mitochondrial gene organization differed [17]. However, we were unable to find the tRNA genes Met and Ala in *Foleyellides* n. sp. Again, difficulty in amplifying and sequencing portions of the mtDNA genome have led to some inconsistencies. Even though technical problems in the amplification is the most likely cause of the missing tRNA genes, it is possible that the genes do not exist within the *Foleyellides* n. sp. mitochondrial genome. It is known that in some organisms, transfer RNAs in the eukaryotic cytosol can be imported into the mitochondria and function in mitochondrial protein synthesis. This process has been described in a diversity of organisms ranging from protozoans, plants, and mammals (Rubio & Hopper, 2011). Perhaps a less likely possibility is that the two genes are split up into half genes and spread throughout the genome [30,31]. During sequencing, they may not be recognized as genes because they are fragmented and are only assembled after they have been transcribed [32].

A key result gained from this study is that the *Wolbachia* endosymbiont was absent in both *Foleyellides* n. sp. and *Foleyellides flexicauda* filarioids. This doubles the number of *Wolbachia*-free species within the Waltonellinae subfamily, suggesting that this subfamily does not harbor *Wolbachia*. Since *Wolbachia*-dependent nematodes cannot survive without the bacteria [16], there has been much speculation as to how *Wolbachia*-independent species evolutionarily lost *Wolbachia.* One notable result from this study that could be related to this theory is the finding of a small 62 bp insert in the mitochondrial genome of *Foleyellides* n. sp. This has not been documented in other filarioid species. However, *Wolbachia* fragments have been found within the mitochondrial genome of two *Wolbachia*-independent filarioids, *Onchocerca flexuosa*

and *Acanthocheilonema viteae* [33]. Both of these species are from the Onchocercinae subfamily, which currently holds the most *Wolbachia*-dependent species at 61% prevalence. Future studies could try to search for *Wolbachia* gene fragments in filarioid subfamilies that have no *Wolbachia,* such as Waltonellinae. This could help further detect the point of evolutionary loss of *Wolbachia.* Alternatively, since Waltonellinae is often found to be the most basal branch, this subfamily may have never acquired *Wolbachia*. More derived filarioid species may have acquired *Wolbachia* after they evolutionarily branched off from this point.

Since the subfamily Waltonellinae probably evolved solely in amphibians around the Mesozoic radiations of anurans [34], one would expect it to be fairly genetically different in comparison to the mammalian-parasitizing subfamily Onchocercinae. In support of this, the 12s phylogeny shows the Waltonellinae to form its own monophyletic clade and it becomes the most basal branch of this tree. Also, the 12s tree seems to separate the Onchocercinae into more distinct monophyletic clades, whereas the Cox1 tree is polyphyletic with several Onchocercinae clades spread throughout. Conflicting with this theory, the cytochrome oxidase subunit I (Cox1) phylogeny describes the amphibian- and reptilian-parasitizing Waltonellinae and Oswaldofilariinae subfamilies as monophyletic with Onchocercinae.

Another notable difference between the phylogenies Cox1 and 12s trees is that *L. loa* forms a clade with *F. candezei* and *F. furcata* at 86% posterior probability in the Cox1 tree. However in the 12s tree, *L. loa* forms a clade with *L. hamletti* and *L. yutajensis* at 92% posterior probability.

Some of these differences between the Cox1 and 12s trees may be the result of DNA sequence availability for each species in both. For example in the Cox1 phylogeny, *Dirofilaria repens* and *Dirofilaria immitis* form their own clade which is expected since they are both found in dogs and are congeneric. However, 12s sequence of *Dirofilaria repens* is lacking, therefore in the 12s tree, this Dirofilariinae is found within the Onchocercinae clades. This further exemplifies the reasoning behind adding more molecular data to lesser studied subfamilies. Even by doubling the amount of Waltonellinae species used in these phylogenies, they do not result in highly resolved trees.

Between the Cox1 and 12s trees in this type of study, we can conclude that the 12s tree yields a more robust topology for several subfamilies, especially Waltonellinae. Due to assumptions about the evolutionary history of this subfamily [34], the basal position of *Ochoterenella* and *Foleyellides* in the tree is logical because the species associated with this subfamily have been evolving solely in amphibians for millions of years [34], setting them far apart from subfamilies parasitic in mammals.

The addition of the new species to the phylogeny based on the complete mitochondrial genome leads to no major changes in topology in comparison to previous findings [17]. We do see that *Foleyellides* n. sp. becomes the most basal branch of the tree, as in the 12s tree, and appears to be most distantly related in comparison with the rest of the species included. Again, this placement could indicate the Waltonellinae subfamily (or filarioids in amphibians) as one of the first groups to exist in the filarioid nematode family. This basal placement is also seen in the 12s tree. However, due to the large polytomy in the Cox1 tree, we do not see this similarity.

Comparing the complete mitochondrial tree with the single gene Cox1 and 12s trees, it is notable that 12s gene tree shares more similarities. Of these similarities, the complete mitochondrial tree shows highest nodal support. For example, both trees group *Dirofilaria immitis* with the clade including *Onchocerca volvulus.* However, the 12s tree shows nodal support at 75%, whereas the mtDNA tree shows nodal support at 100%.

It is clear from all of these trees that the subfamily Onchocercinae is non-monophyletic and is split among several clades across the phylogeny. In many instances, certain species consistently group with each other, for example *Brugia malayi*/*Wuchereria bancrofi*; *Onchocera volvulus*/*O. flexuosa*. However, these smaller clades do not cluster together with the rest of the subfamily. This indicates that Onchocercinae is a large, genetically diverse subfamily. It is difficult to bring up a topic about changing the arraignment of this subfamily when they have been grouped based on morphology. Instead, we can look at how intriguing it is to have a subfamily be so genetically diverse, yet share very similar morphology. One thing that could be useful is to split Onchocercinae into smaller subfamilies (or construct several tribes within the subfamily) to account for this diversity. Perhaps the divisions can be based upon the vector. For example, with the 12s phylogeny, the clade that includes *Litomosoides* and *Cercopithifilaria* are transmitted by ticks and mites. In contrast, the members of the Onchocercinae clade that includes *Onchocerca*, *Brugia*, and *Mansonella* are transmitted by bloodsucking insects. Dirofilariinae also shows such differences between morphological identity and phylogenetic comparison. All other subfamilies have stayed fairly consistent throughout the phylogenetic analysis.

Conclusions

In summary, I sequenced the mitochondrial genome of two species of filarial nematodes (subfamily Waltonellinae) recovered from the peritoneal cavity of leopard frogs. Phylogenetic analyses of multiple mitochondrial genes support the hypothesis that these nematodes belong to the basal lineage within the family Onchocercidae. Neither species contained the bacterial endosymbiont *Wolbachia.* This suggests that the ancestral state for filarioid nematodes is not dependent on *Wolbachia* mutualism and that the acquisition of *Wolbachia* occurred later during the evolution of filarioid nematodes.

APPENDIX

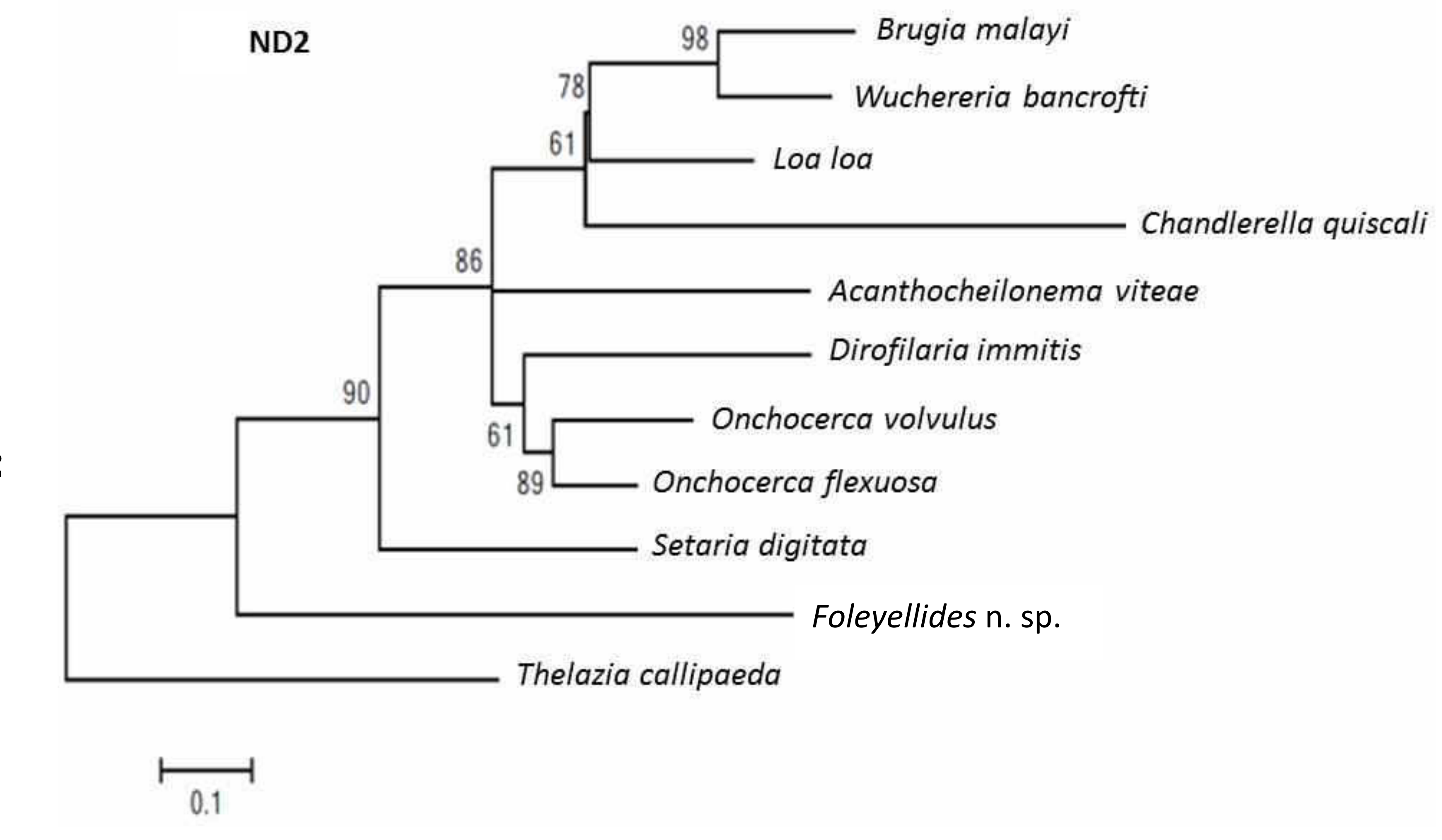

Figure 4 Phylogeny based on mitochondrial encoded NADH dehydrogenase 2 gene (ND2).

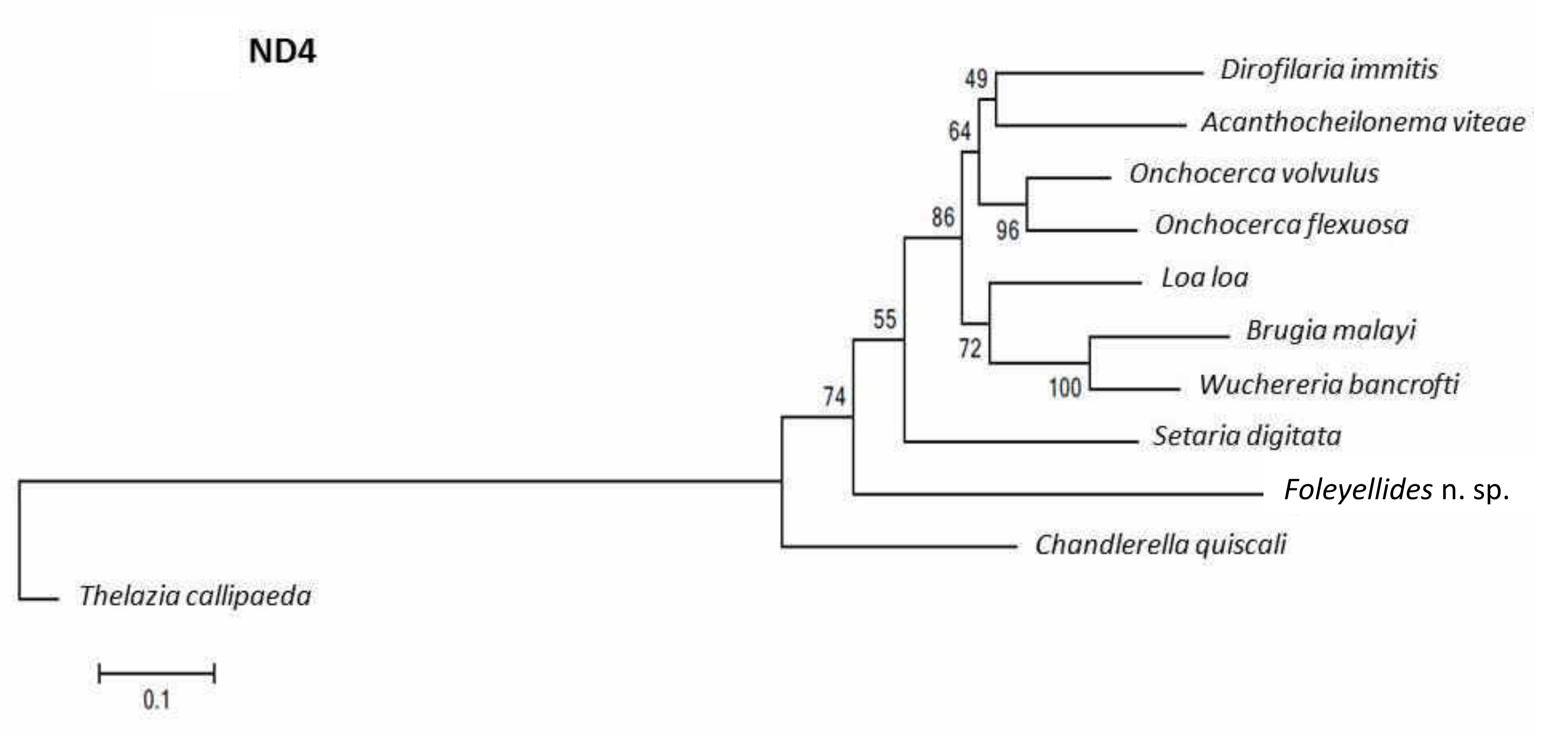

Figure 9 Phylogeny based on mitochondrial encoded NADH dehydrogenase 4 gene (ND4).

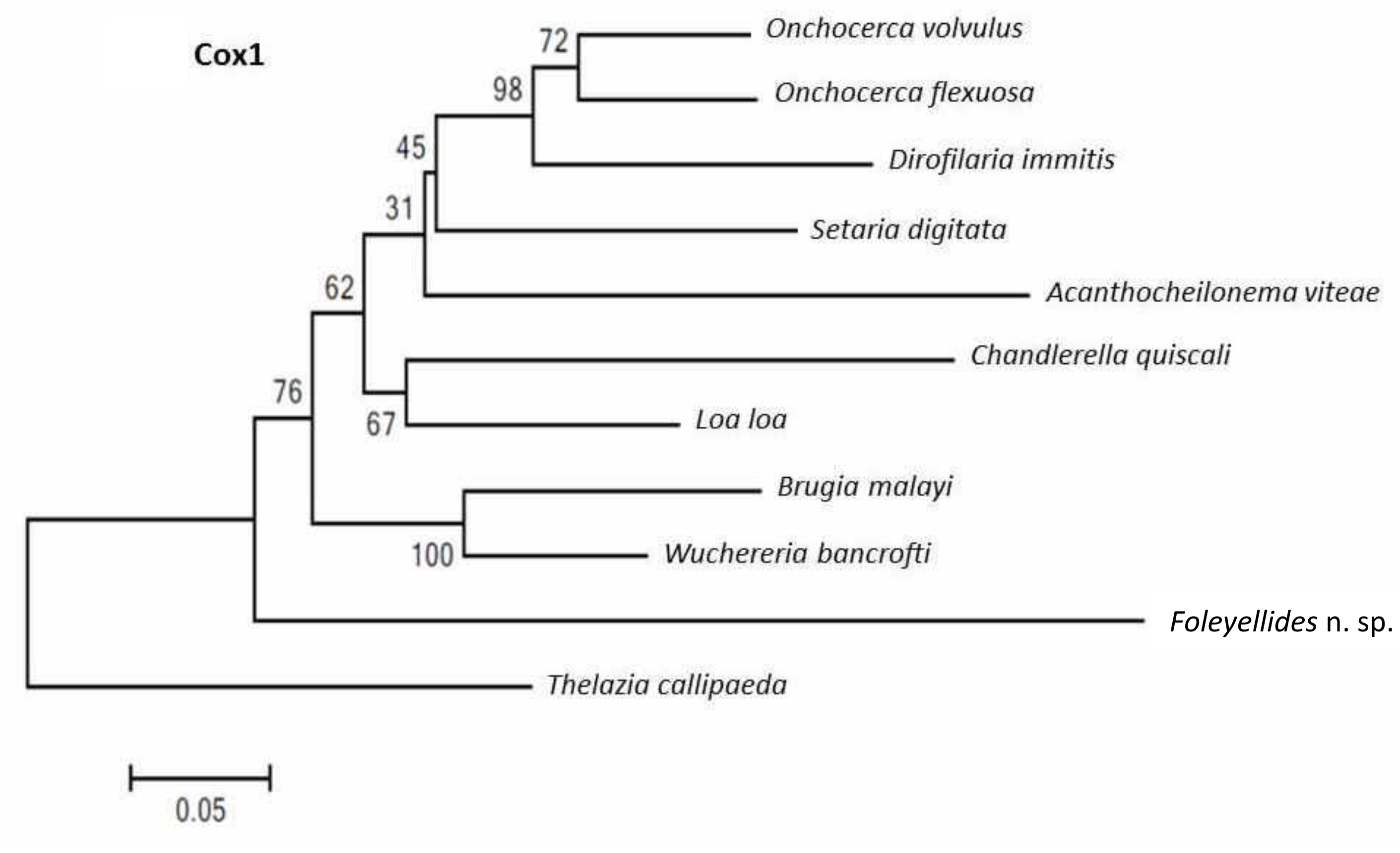

Figure 10 Phylogeny based on mitochondrial encoded cytochrome c oxidase I gene (Cox1).

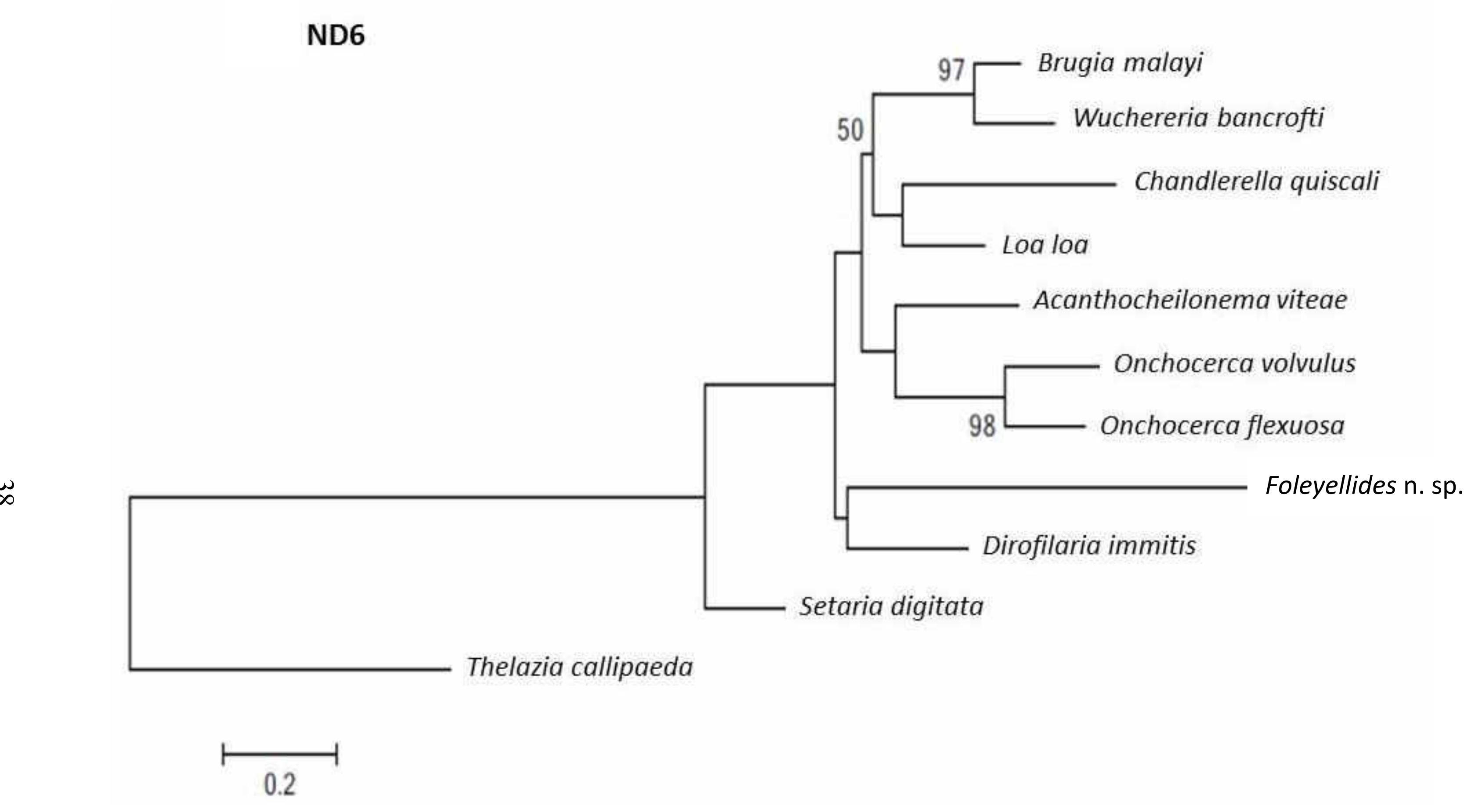

Figure 11 Phylogeny based on mitochondrial encoded NADH dehydrogenase 6 gene (ND6).

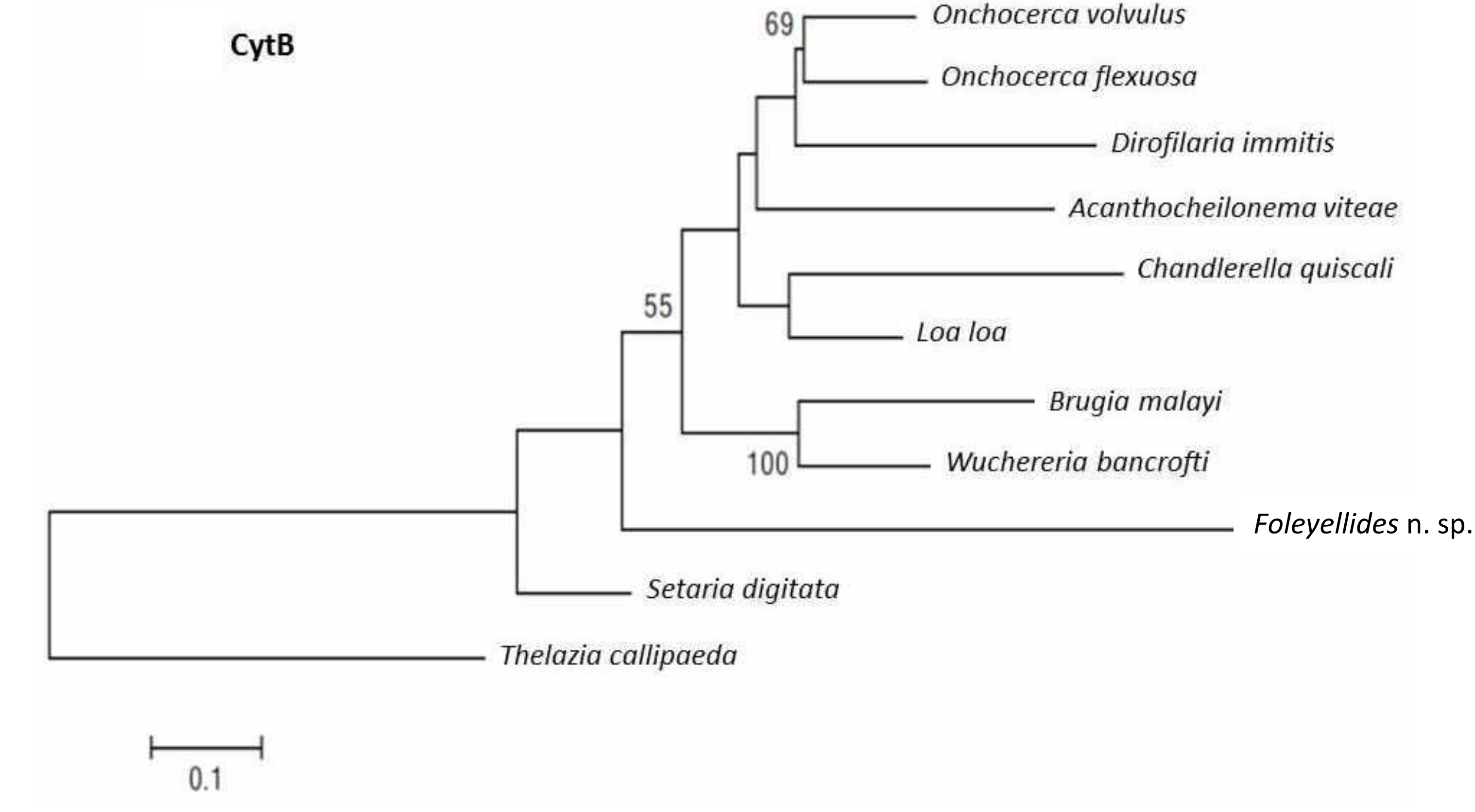

Figure 12 Phylogeny based on mitochondrial encoded cytochrome b gene (CytB).

Figure 13 Phylogeny based on mitochondrial encoded cytochrome c oxidase III gene (Cox3).

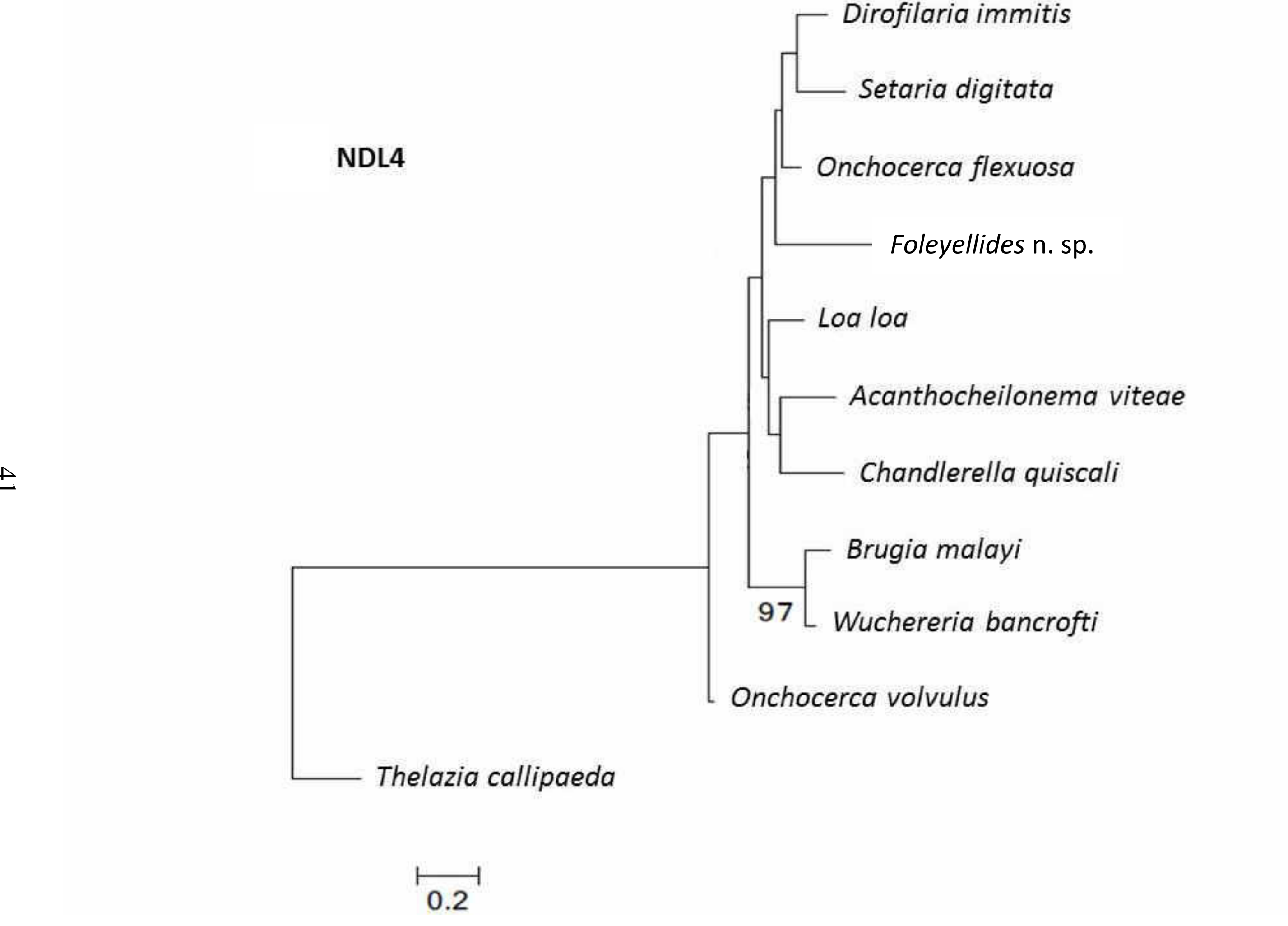

Figure 14 Phylogeny based on mitochondrial encoded NADH dehydrogenase subunit gene (NDL4).

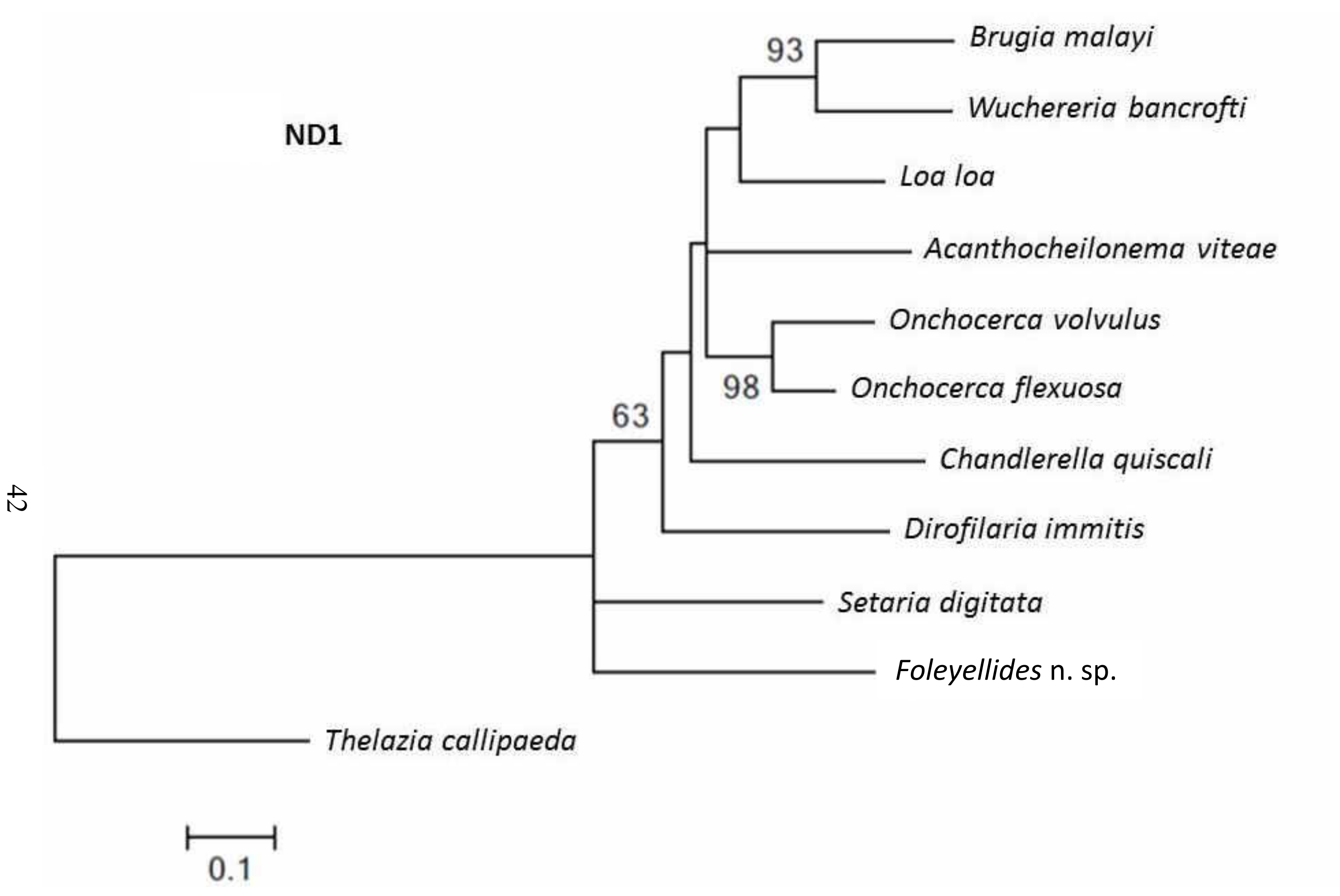

Figure 15 Phylogeny based on mitochondrial encoded NADH dehydrogenase 1 gene (ND1).

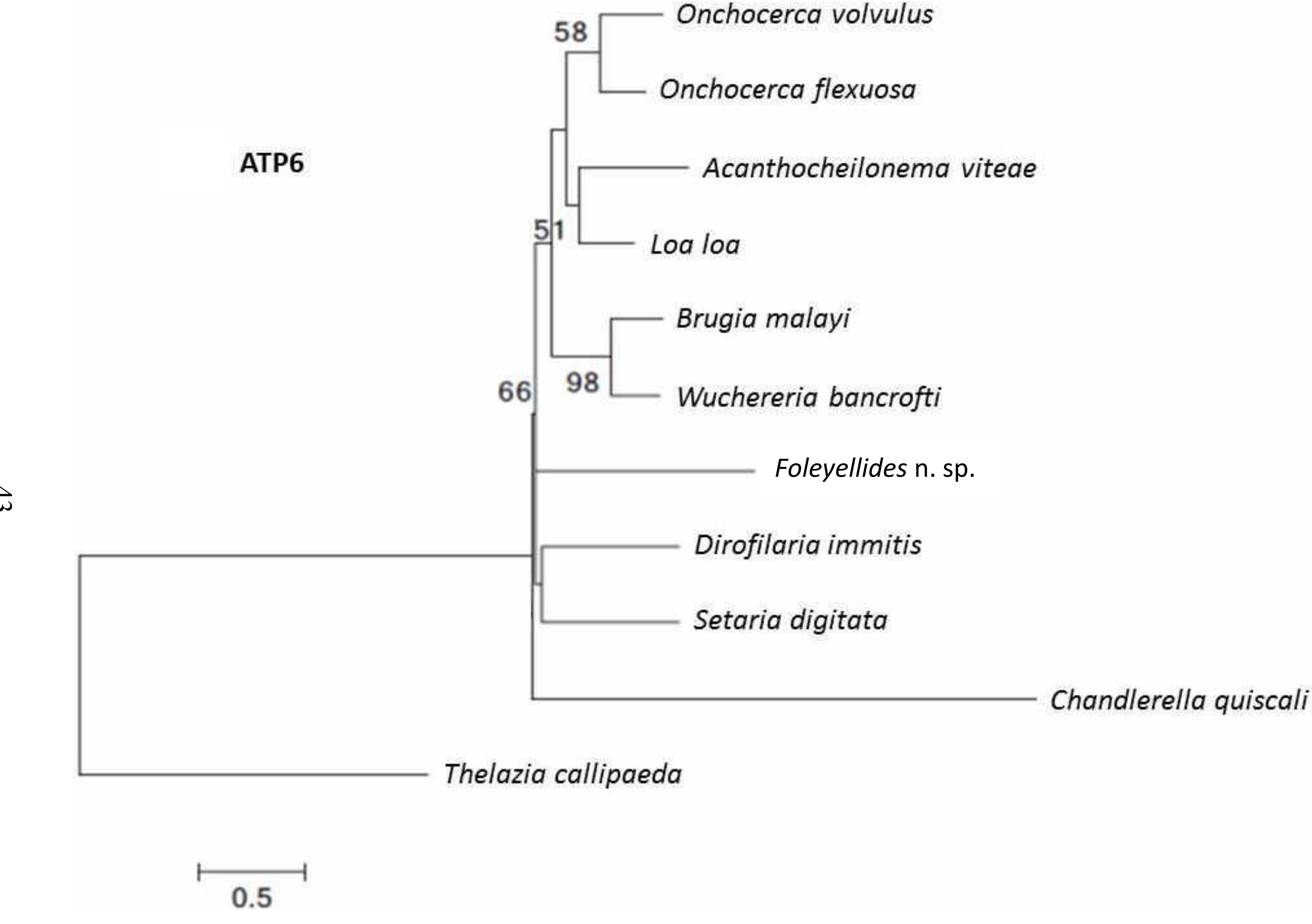

Figure 16 Phylogeny based on mitochondrial encoded ATP synthase 6 gene (ATP6).

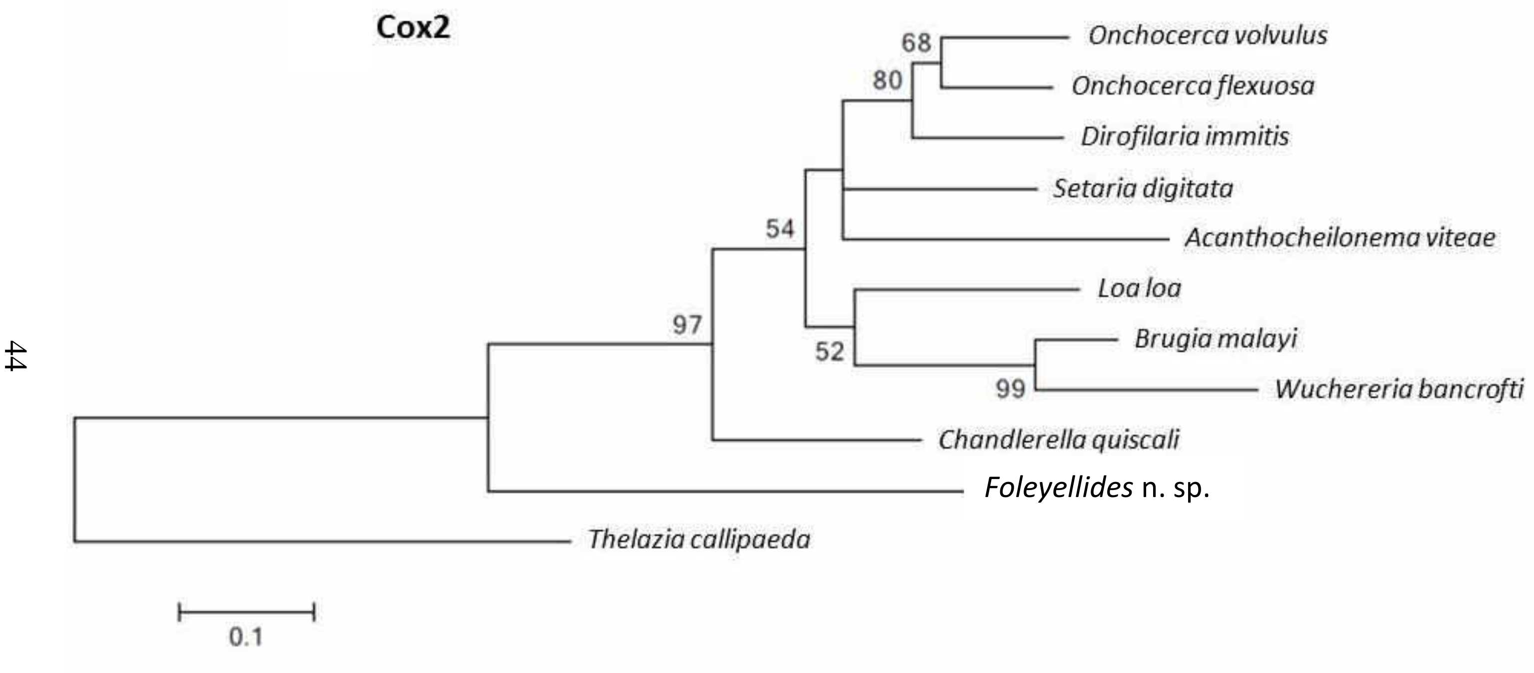

Figure 17 Phylogeny based on mitochondrial encoded cytochrome c oxidase II gene (Cox2).

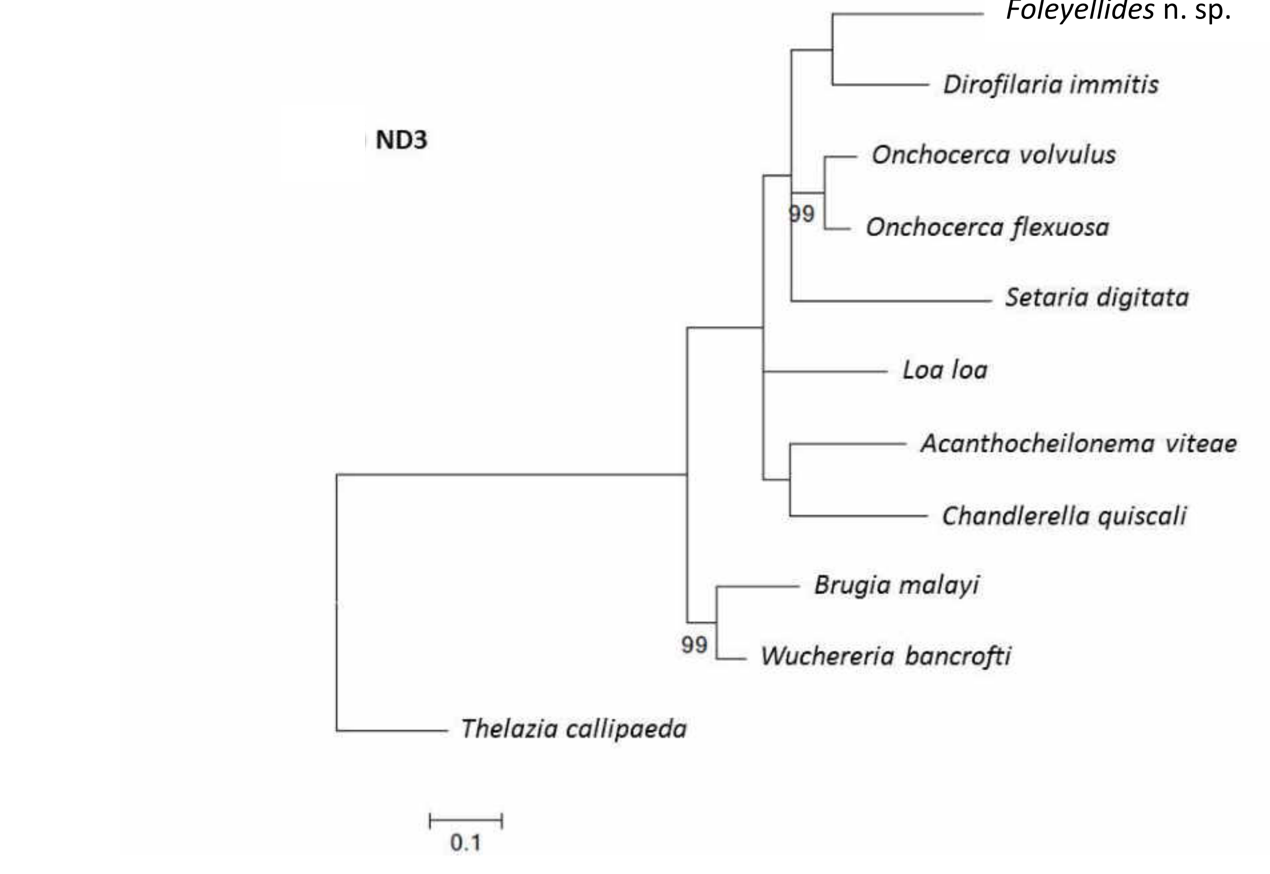

Figure 18 Phylogeny based on mitochondrial encoded NADH dehydrogenase 3 gene (ND3).

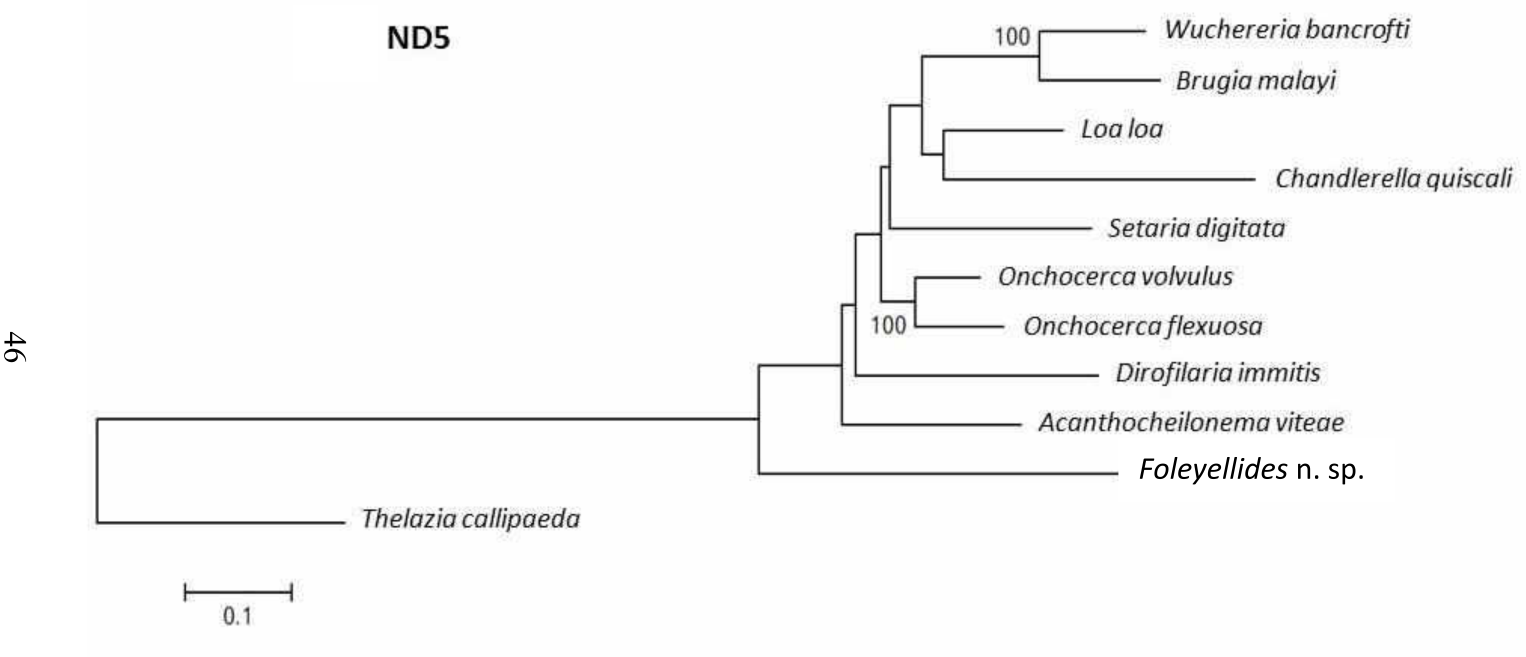

Figure 19 Phylogeny based on mitochondrial encoded NADH dehydrogenase 5 gene (ND5).

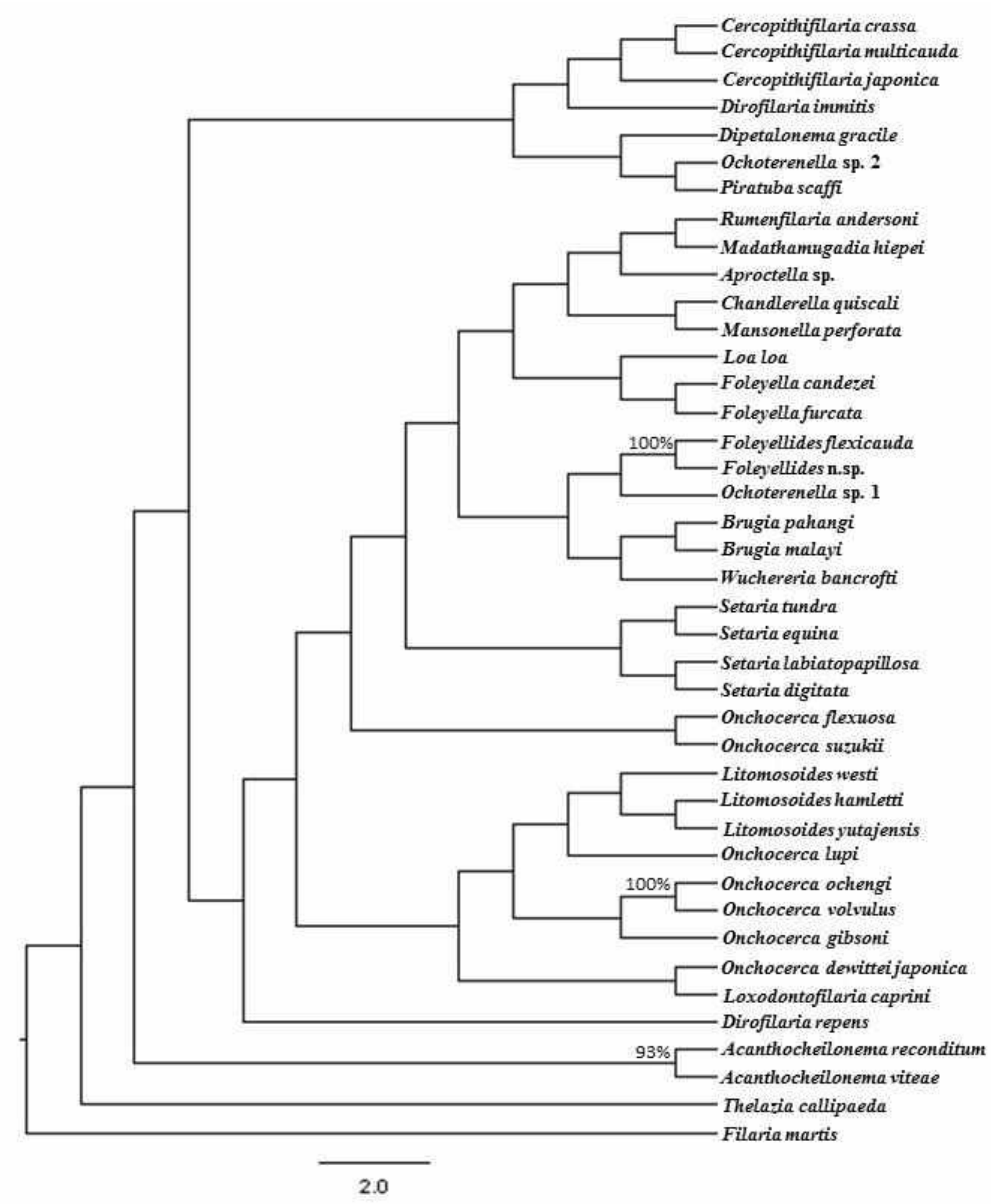

Figure 20 Maximum parsimony heuristic search in PAUP based on mitochondrial cytochrome oxidase subunit I (Cox1) sequences. Numbers at specific nodes indicate the percentages of bootstrap support (>75%). Consensus of 5 trees produced (length: 1748 steps; consistency index: 0.2946; retention index: 0.3575).

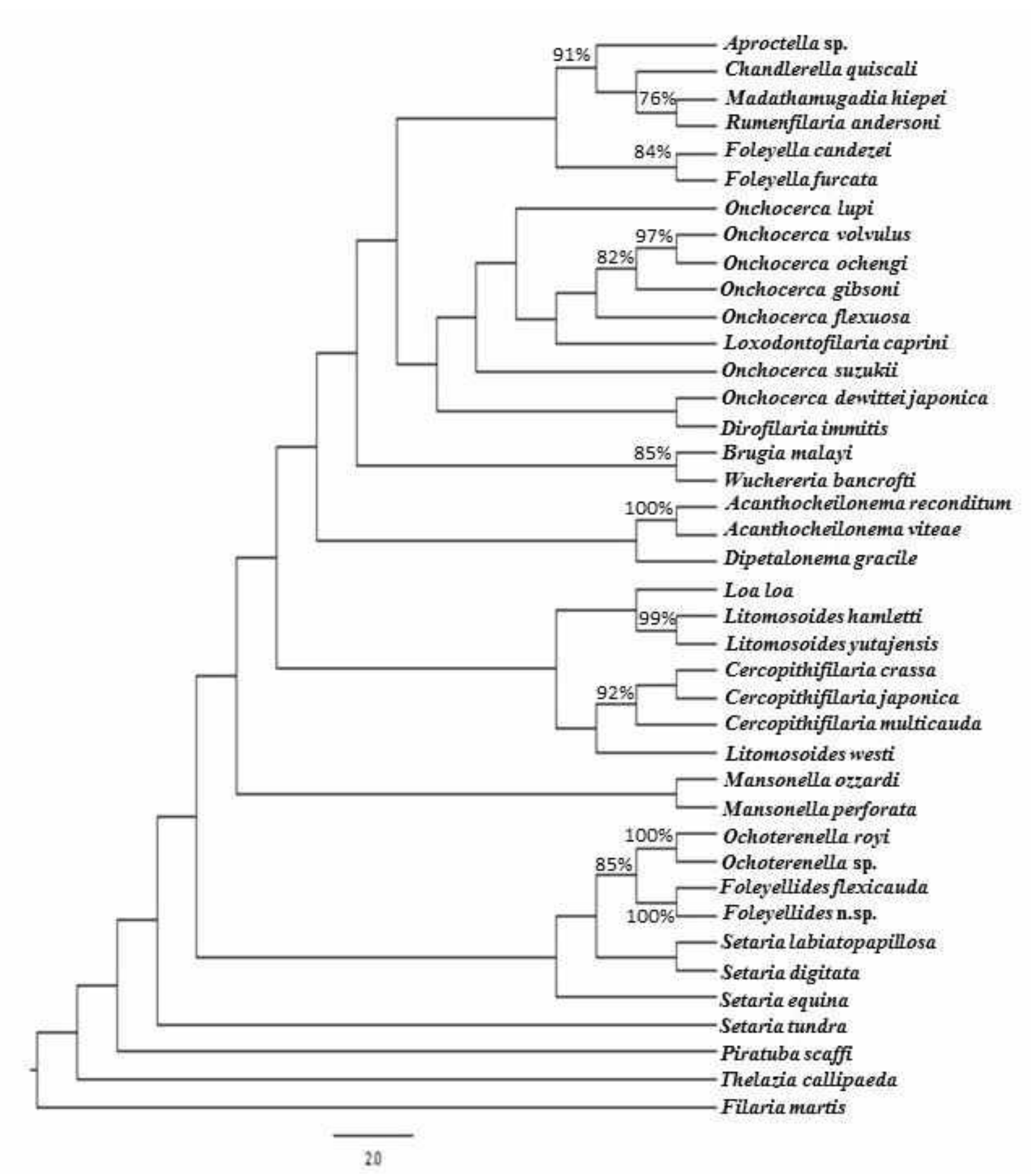

Figure 21 Maximum parsimony heuristic search in PAUP based on mitochondrial 12s rRNA gene sequences. Numbers at specific nodes indicate the percentages of bootstrap support (>75%). Consensus of 62 trees produced (length: 1139 steps; consistency index: 0.4056; retention index: 0.4871).

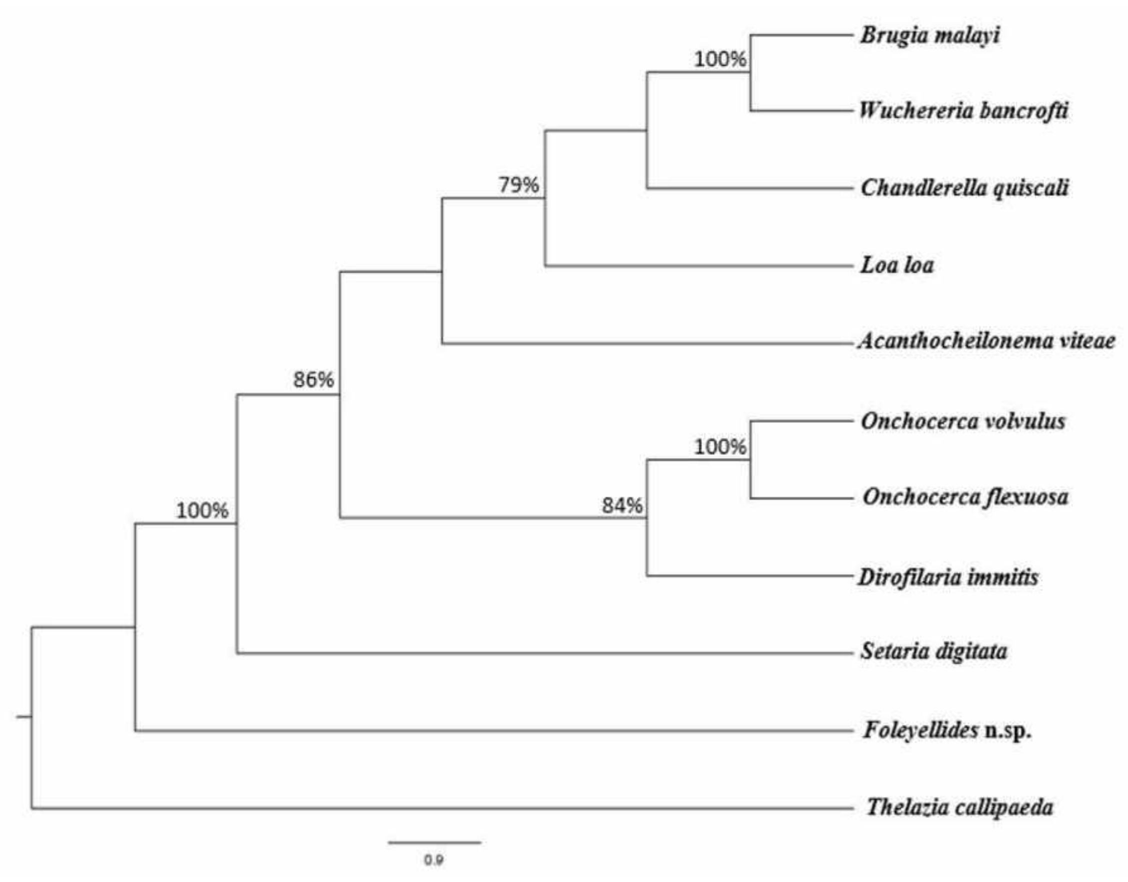

Figure 22 Maximum parsimony heuristic search in PAUP based on concatenated sequences of the 12 protein-coding mitochondrial genes. Numbers at specific nodes indicate the percentages of bootstrap support (>75%). One tree produced (length: 11553 steps; consistency index: 0.6121; retention index: 0.3038).

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