STUDIES OF PHTHIRAPTERA (INSECTA: PSOCODEA): FROM PHYLOGENY TO FOSSIL

BY

EMBER B. CLODFELTER

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Entomology
in the Graduate College of the
University of Illinois Urbana-Champaign, 2024

Urbana, Illinois

Adviser:

Dr. Kevin P. Johnson

ABSTRACT

Parasitic lice (Psocodea: Phthiraptera) are a group of obligate ectoparasites of birds and mammals that feed on the feathers, blood, and skin secretions of their hosts. Lice can be host specific, even specific to single genera or species of hosts. Understanding the coevolutionary history of lice is an important step in understanding their diversification. The work here encompasses two major aspects of louse evolutionary history. First, because lice spend their whole lives on their hosts and subsist on often specialized diets, many lice often have endosymbiotic bacteria to help supplement their nutritionally limited diets. Phylogenies of lice, their hosts, and their endosymbionts can be used to study coevolutionary relationships. Phylogenies can also hint at the duration and type of relationship between louse and their endosymbionts. The first chapter focuses on the phylogenomics of the bacterial genus *Wolbachia*, an atypical bacterial endosymbiont of insects and nematodes, in the monophyletic feather louse genus *Penenirmus* (Ischnocera). The second chapter explores the diversity of lice in deep time. Phylogenetic insights can be made using fossils to date specific nodes, however the fossil record for parasitic lice is incredibly limited. There have only been two fossils of lice described so far, one compression fossil and two individuals from Burmese amber. This work describes a new louse fossil from the Eocene.

ACKNOWLEDGMENTS

I want to start by thanking my adviser, Dr. Kevin Johnson. He has been a wealth of knowledge and support during my time at the University of Illinois. He patiently guided me through learning about phylogenetic and metagenomic techniques and I appreciate the time he has taken to help me grow as a scientist. I especially appreciate his keen eye for improving my writing.

I'd also like to thank my lab other members. To Kim Walden, thank you for all the help you have provided with my research and teaching me the techniques necessary to conduct it. I want to especially thank you for helping me troubleshoot all the processing errors! To Dr. Jorge Doña, thank you for sharing the raw read data with me and making it possible for me to continue the work you started in *Penenirmus.*

To my committee members, Dr. Dominic Evangelista and Dr. Sam Heads, thank you for guiding and mentoring me. I appreciate your insights and help shaping my research.

Lastly, I want to thank my friends: Bailey, Erinn, Jake, Joe, Josh, Morgan, Robin, Sam, and Shea. You kept me motivated with coffee and study dates. You also provided balance and encouraged me to relax when I could. I would not have completed this without your support.

To my sweet, menace of a cat, Aalaya

TABLE OF CONTENTS

CHAPTER 1: PHYLOGENOMICS OF *WOLBACHIA* IN FEATHER LICE OF THE GENUS *PENENIRMUS* (PSOCODEA: PHTHIRAPTERA: ISCHNOCERA)

Abstract

Insects that have nutritionally limited diets often harbor bacterial endosymbionts that supplement their nutritional needs. However, not all interactions between bacteria and insects are positive. *Wolbachia* is a genus of bacteria that causes cytoplasmic incompatibility and other reproductive parasitic effects on many of its arthropod hosts. In nematodes and some insects, however, *Wolbachia* is a nutritional mutualist. A lineage of *Wolbachia* closely related to mutualist strains has previously been identified in lice, including the feather feeding louse genus *Penenirmus* (Ischnocera). Lice, which feed on feathers or blood, often have bacterial endosymbionts that support their nutrient limited diet. In this study, we further examined the diversity of *Wolbachia* in the genus *Penenirmus*, with a focus on evidence of long term associations with their hosts which could indicate a mutualistic relationship. In *Penenirmus*, we assembled *Wolbachia* from three different supergroups: B, F, and V. *Wolbachia* from supergroups F and B showed evidence of potential mutualism. Supergroup V had not previously been known from lice. We also compared two different assembly methods, one reference based and one *de novo*, in their relative utility for use in *Wolbachia* studies. Lastly, we tested whether long branch attraction may be affecting the phylogenetic placement of some lineages by including each long branch one at a time in the phylogeny and determining whether the placement of this branch was different that that including all taxa.

Introduction

Insects with specialized diets often have bacterial endosymbionts that provide nutritional supplementation (McCutcheon et al., 2019). Specialized diets often do not contain all the nutrients necessary for growth and reproduction, so insects need endosymbionts to supplement the essential nutrients they need. A well-known example of this association is the aphid-*Buchnera* relationship. Aphids have a nutritionally limited diet of plant sap and their endosymbionts supplement them with amino acids and other essential nutrients (Tsuchida et al., 2005). These associations are essential for aphid survival and reproduction and can persist over millions of years (Tamas et al., 2002; Wernegreen, 2002). The diets of many hematophagous insects are nutritionally limited as well and often these insects also host endosymbionts. For example, human lice (*Pediculus humanus*) feed exclusively on blood and harbor endosymbiotic bacteria (*Riesia*) that provide these lice with vitamin B5 and, in return, receive metabolic support (Kirkness et al., 2010).

Bacterial associates of insects are not always beneficial to their hosts. For example, the bacterial genus *Wolbachia* is a ubiquitous genus of mainly vertically transmitted alphaproteobacteria that has no known free-living species. This genus is estimated to be present in 20-75% of all arthropod species (Kyei-Poku et al., 2005; Saridaki & Bourtzis, 2010). These bacteria can have diverse effects on their host. Notably, in some insects, *Wolbachia* causes reproductive parasitic effects such as cytoplasmic incompatibility, feminization of genetic males, and male-killing (Bordenstein et al., 2008; Normark, 2003; Saridaki & Bourtzis, 2010). In contrast, *Wolbachia* in nematodes is an obligate mutualist with its host (Bandi et al., 1999; Bordenstein et al., 2008; Fenn & Blaxter, 2004; Saridaki & Bourtzis, 2010). The genus *Wolbachia* is split into several clades classified into supergroups that are given letter names A-W

(but the validity of supergroups G and R have been challenged after their publication) (Baldo & Werren, 2007; Gerth, 2016; Lefoulon et al., 2020). These supergroups can be roughly characterized by their host lineage and the bacteria's effect on its host.

In certain insects, *Wolbachia* has been found to nutritionally benefit its host. For example, the bedbug (*Cimex lectularius),* has a *Wolbachia* endosymbiont that is thought to provide vitamin B supplementation to its host (Hosokawa et al., 2010a). *Wolbachia* from bedbugs are in supergroup F, which is known from both nematodes and arthropods, and is typically a mutualist with its host (Hosokawa et al., 2010b; Lo et al., 2002). This endosymbiotic *Wolbachia* possesses the vitamin B pathway for biotin, which had not previously been found in other insect associated *Wolbachia*. However, this pathway does exist in some mutualistic *Wolbachia* associated with nematodes (Nikoh et al., 2014). These are just some of the few documented cases of *Wolbachia* providing a benefit to an insect host.

Lice are one group of insects with a nutritionally limited diet, such as feeding exclusively on blood or feathers. There has been limited research on the presence of *Wolbachia* in lice, being mainly known from studies using 16S rDNA amplification and sequencing (Covacin & Barker, 2007; Kyei-Poku et al., 2005). More recently genomic approaches have been applied to *Wolbachia* in lice, documenting this bacterium in additional louse genera, including both blood and feather feeders (Mahmood et al., 2023). Importantly, Mahmood et al. (2023) showed that the *Wolbachia* from lice was closely related to the nutritionally beneficial *Wolbachia* from bedbugs. One of the lice included in Mahmood et al. (2023) was from the genus *Penenirmus* (Ischnocera), a monophyletic genus of feather lice on woodpeckers (Piciformes) and songbirds (Passeriformes) (Johnson et al., 2021; Price, 2003). Members of Ischnocera feed exclusively on feathers, which are composed of keratin, a protein rich in amino acids but lacking other nutrients. Feather-

feeding lice have been shown to harbor bacterial endosymbionts that are maternally transmitted (Fukatsu et al., 2007; Perotti et al., 2008), so we wanted to investigate the occurrence of *Wolbachia* in *Penenirmus* in more detail across the diversity of this louse genus, particularly with regards to its phylogenetic relationships.

If *Wolbachia* is a vertically transmitted beneficial endosymbiont in feather lice, we would predict that *Wolbachia* would show evidence of a long-term association with its host resulting in phylogenetic congruence between trees of *Wolbachia* and its hosts, as is the case with other supergroup F *Wolbachia* (Hosokawa et al., 2006; Mahmood et al., 2023; Nikoh et al., 2014; Toju et al., 2013). We would also predict that long-term mutualists would have reduced genome sizes (Gil et al., 2002; McCutcheon & Moran, 2012) as these bacteria lose genes that code for functions that can be taken over by the host and retain pathways related to the bacterial benefit to the host (e.g. the B vitamin pathways in bedbugs) (Nikoh et al., 2014). We would likewise expect the branches in the phylogeny of these bacteria to be longer compared to other related bacteria due to increases in underlying mutation and substitution rates (Moran, 1996; Moran et al., 1995; Woolfit & Bromham, 2003) although antagonistic coevolution might also increase rates of molecular evolution (Brockhurst & Koskella, 2013; Decaestecker et al., 2007; Papkou et al., 2019; Paterson et al., 2010).

Methods

Sequencing

The louse genomes selected for this study have been previously published in a study of the phylogeny of the louse genus *Penenirmus* (Johnson et al. 2021). Briefly, Johnson et al. (2021) used Illumina NovaSeq 6000 sequencing of whole genome extractions of entire

individual louse specimens, which would also include sequences of associated bacteria. In total, 41 samples of *Penenirmus* and 3 samples of *Turnicola* (a closely related genus) were included in our analyses (Johnson et al., 2021).

Assembling Genomic Reads

Initial analysis of the louse metagenomic data was performed with MinYS (mine your symbiont), a targeted assembly approach to assemble bacterial genomes from whole metagenomic samples (Guyomar et al., 2020). We used the *Wolbachia* from bedbugs (strain wCle) as the reference genome in the MinYS pipeline because it is closely related to the *Wolbachia* found in *Penenirmus* and has been thoroughly studied and is a high quality reference genome (Mahmood et al., 2023). This assembler was used as a first step to establishing the presence of *Wolbachia* across *Penenirmus*, which provided a basis for further investigation using a *de novo* assembler (below).

Once we confirmed that *Wolbachia* was generally widespread across *Penenirmus*, we used the metaWRAP assembly pipeline to produce *Wolbachia* assemblies for phylogenetic analysis. In this pipeline, the louse metagenomic samples were first processed through SPAdes (Bankevich et al., 2012) to prepare them for metaWRAP. Samples were then processed through metaWRAP, a *de novo* assembler, which assembled all possible bacterial genomes present in the louse metagenomic samples using different binning techniques to first separate insect reads from bacterial reads and then reassemble them (Uritskiy et al., 2018). This assembler had the capability of assembling multiple bins for different bacteria present, even those of the same genus of bacteria. This method is not restricted to using a reference genome and can assemble a broader diversity of bacteria if present.

To identify genes for phylogenomic analyses, we used MiGA (Microbial Genomes Atlas) (Rodriguez-R et al., 2020). We used the web version of this software for bacterial annotation to produce annotations of essential genes for phylogenetic reconstruction. The assemblies produced by MinYS and metaWRAP were annotated by MiGA to check for completeness, quality, and contamination. The analysis identified 106 essential genes, which consist of universally conserved genes and conserved core genes across a diversity of bacteria (Albertsen et al., 2013; Rodriguez-R et al., 2018). We then used these essential genes for the phylogenetic analysis. We calculated percent completeness of each Wolbachia assembly by dividing the number of essential genes present in each assembly, as annotated by MiGA, by the expected total, 106. Assembly sizes were calculated by a custom perl script.

Phylogenomics

In addition to assemblies generated from the metaWRAP pipeline, we downloaded a diversity of *Wolbachia* strains (ingroup) and outgroups (*Anaplasma* and *Ehrlichia* spp.) from NCBI for inclusion in the ingroup of the phylogenomic analyses (Tables 1.1 &1.2). This selection was limited to only whole genome assemblies, which we then annotated with MiGA.

Table 1.1 Strain IDs, supergroup assignments, and accession numbers for *Wolbachia* genomes used in the phylogeny.

***Wolbachia* strain**	**Supergroup**	**Host species**	**NCBI Ref #**
wUni	A	*Muscidifurax uniraptor*	GCF_000174095.1
-	A	*Philonthus cognatus*	GCF_947251755.1
-	A	*Epagoge grotiana*	GCF_947251745.1
-	A	*Eupithecia tripunctaria*	GCF_947251595.1
wGmm	A	*Glossina morsitans*	GCF_000689175.1
-	B	*Apotomis betulana*	GCF_947250475.1
-	B	*Rhopobota naevana*	GCF_947250615.1
-	B	*Ischnura elegans*	GCF_947251585.1

Table 1.1 (cont.)

-	B	*Melanostoma melinum*	GCF_947251465.1
-	B	*Euphydryas aurinia*	GCF_947250535.1
wPip	B	*Culex quinquefasciatus*	GCF_000073005.1
wFur	B	*Ostrinia furnaclis*	GCF_023559125.1
wSca	B	*Ostrinia scapulalis*	GCF_023559145.1
wMau	B	*Drosophila mauritiana*	GCF_004795975.1
wAnM	B	*Anopheles moucheti*	GCF_018491625.2
wVulC	B	*Armadillidarium vulagre*	GCF_001027565.1
wOo	C	*Onchocerca ochengi*	GCF_000306885.1
wOv	C	*Onchocerca voluvulus*	GCF_000530755.1
-	C	*Dirofilaria immitis*	GCF_013365455.1
wLsig	D	*Litmosoides sigmodontis*	GCF_013365435.1
wLbra	D	*Litmosoides brasiliensis*	GCF_013366805.1
wWb	D	*Wucheraria bancrofti*	GCF_002204235.2
wFol	E	*Folsomia candida*	GCF_001931755.2
wPaur	F	*Penenirmus auritus*	GCA_029784575.1
wMelo	F	*Melophagus ovinus*	GCA_023661065.1
wMeur1	F	*Menacanthus eurysternus*	GCA_029715105.1
wMeur2	F	*Menacanthus eurysternus*	GCA_029784615.1
wMmer	F	*Meromenopon meropis*	GCA_029784595.1
wMoz2	F	*Mansonella ozzardi*	GCF_020278625.1
wCle	F	*Cimex lectularius*	GCF_000829315.1
wMhi	F	*Madathamugadi heipei*	GCF_013366855.1
wCfeF	F	*Ctenocephalides felis*	GCA_028571325.1
wCtub	J	*Cruorifilaria tuberocauda*	GCF_013365475.1
wDcau	J	*Dipetalonema caudispina*	GCF_013365495.1
wCfeJ	V	*Ctenocephalides felis*	GCF_012277315.1
wCfeT	W	*Ctenocephalides felis*	GCF_012277295.1

Table 1.2 Outgroup species and their NCBI Reference #s

Species	**NCBI Ref #**
Anaplasma marginale	GCF_000020305.1
Anplasma phagocytophilum	GCF_000439775.1
Ehrlichia chafeensis	GCF_000632965.1
Ehrlichia ruminantium	GCF_013460375.1

Initially we aligned individual gene sequences for the 106 essential gene set. First, a custom python script (Johnson et al., 2021) was run to convert the nucleotide sequences into amino acid sequences. These sequences were then aligned using MAFFT (Katoh et al., 2002)

with the following parameters (-auto -preservecase -adjustdirection -amino). The sequences were then converted back into nucleotide sequences using the same custom python script (Johnson et al., 2021) and then trimmed using trimAl (-gt 0.4) (Capella-Gutiérrez et al., 2009). The individual gene alignments were then used to create individual gene trees using IQTree2 (Minh et al., 2020). We used these individual gene trees to detect and remove contamination in the assemblies and verify that binning was consistent and did not produce chimeric assemblies in cases where a single louse sample produced multiple *Wolbachia* assemblies. Specifically, we examined individual gene trees for positions of taxa that were not consistent with other gene trees. In the case, we found only 8 gene trees in which the position of a taxon was markedly different that for other genes. In these cases we removed that taxon for that gene only. After the individual gene trees were examined and potentially contaminating sequences removed, the individual gene files were concatenated using AMAS.py (Borowiec, 2016).

This concatenated file was then used to construct a phylogeny using IQTree2 of all samples. To assess branch support, we performed 1,000 bootstrap replicates using the model for general time reversal DNA base substitutions that have unequal rates and unequal base frequencies with the discrete Gamma model and four rate categories across site heterogeneity. We noted that the concatenated phylogenetic tree had several long branches, some of which clustered together (see Results). To assess whether this clustering might be an artifact of long branch attraction, we repeated the analyses, but this time including only one of the long branch taxa at a time. For these trees we assessed whether a long-branch taxon was in the same position relative to the remaining taxa, if it was the only long-branch taxon in the tree, and contrasted this to its position when all long-branch taxa were included. This allowed us to identify samples that changed dramatically in position when other long branch taxa were excluded.

Finally, to understand the coevolutionary relationship between the *Wolbachia* and their louse hosts, we constructed a cophylogenetic tanglegram in R (version 4.3.2) using the cophylo function in R (Paradis et al., 2004; Revell, 2024).

Results

Reference guided assembly of the 41 samples of *Penenirmus* using MinYS yielded 12 samples positive for *Wolbachia* and 29 that did not detect *Wolbachia*. The *de novo* assembly pipeline, metaWRAP, yielded more assemblies of *Wolbachia* for these samples, overall tending to have more resolving power. Of the 41 samples of *Penenirmus*, from 24 we were able to assemble at least one strain of *Wolbachia*, from five of which we assembled two strains. We were not able to assemble *Wolbachia* from the other 17 samples. Six of these *Penenirmus* samples did not successfully complete the metaWRAP pipeline and thus did not yield any results and could not be confirmed to be either positive or negative for any bacterial genera in this study. Of the three samples of the closely related louse genus *Turnicola*, we were only able to assemble *Wolbachia* from one of the samples (the other two did not successfully complete the metaWRAP pipeline). The MinYS samples were excluded from the final analysis because of their redundancy with metaWRAP samples (see Discussion for details). In total, we obtained 30 different *Wolbachia* assemblies to be included in phylogenetic analyses.

The only other genus of bacteria assembled by the metaWRAP pipeline in the *Penenirmus* samples was identified by NCBI BLASTn and MiGA as *Burkholderia*. This genus contains both free-living and symbiotic lineages, some of which are pathogenic to plants or animals, including humans (Coenye & Vandamme, 2003; Mahenthiralingam et al., 2005). A

genome of *Burkholderia* was assembled in 28 of our samples, 18 of which also produced an assembly of *Wolbachia* from the same host.

Table 1.3 Louse samples and associated bird hosts with results of metaWRAP assembly and MiGA identification. Number of *Wolbachia* strains found per sample is indicated, with supergroup indicated in parentheses. N/A indicates samples did not complete the metaWRAP pipeline. An asterisk indicates *Burkholderia* was also detected from this louse.

Louse species	**Bird host species**	**# of *Wolbachia* detected (supergroup)**
Penenirmus auritus	*Chloropicus goertae*	0*
Penenirmus auritus	*Colaptes puntigula*	1 (F)*
Penenirmus auritus	*Dendrocopos major*	1 (F)
Penenirmus auritus	*Dryobates pubescens*	2 (F)*
Penenirmus auritus	*Dryocopus pileatus*	2 (F)*
Penenirmus auritus	*Melanerpes aurifrons*	1 (F)*
Penenirmus auritus	*Melanerpes candidus*	1 (F)*
Penenirmus auritus	*Melanerpes cruentatus*	1 (F)
Penenirmus auritus	*Melanerpes erythrocephalus*	1 (F)*
Penenirmus auritus	*Picoides tridactylus*	N/A
Penenirmus auritus	*Piculus flavigula*	N/A
Penenirmus auritus	*Picumnus aurifrons*	N/A
Penenirmus auritus	*Sphyrapicus varius*	1 (F)*
Penenirmus guineensis	*Lybius dubius*	2 (B,F)*
Penenirmus jungens	*Colaptes auratus*	2 (F)*
Penenirmus marginatus	*Indicator indicator*	1 (F)*
Penenirmus pici	*Picus canus*	1 (V)
Penenirmus sp.	*Anthus lineiventris*	0*
Penenirmus sp.	*Bradypterus baboecala*	0*
Penenirmus sp.	*Campylorhynchus turdinus*	0*
Penenirmus sp.	*Capito auratus*	N/A*
Penenirmus sp.	*Capito aurovirens*	N/A
Penenirmus sp.	*Capito brunneipectus*	0*
Penenirmus sp.	*Certhia americana*	0*
Penenirmus sp.	*Chloropicus griseocephalus*	1 (B)*
Penenirmus sp.	*Cisticola rufilatus*	1 (F)*
Penenirmus sp.	*Dryobates nigriceps*	0
Penenirmus sp.	*Eubucco richardsoni*	1 (F)*
Penenirmus sp.	*Eubucco versicolor*	0
Penenirmus sp.	*Gymnobucco calvus*	N/A

Table 1.3 (cont.)

Penenirmus sp.	*Gymnobucco peli*	0*
Penenirmus sp.	*Indicator minor*	1 (F)*
Penenirmus sp.	*Indicator variegatus*	2 (F)*
Penenirmus sp.	*Indicator willcocksi*	0*
Penenirmus sp.	*Melanerpes rubricapillus*	2 (F)
Penenirmus sp.	*Pogoniulis bilineatus*	1 (B)*
Penenirmus sp.	*Psaltriparus minimus*	0*
Penenirmus sp.	*Psilopogon chrysopogon*	1 (V)*
Penenirmus sp.	*Sphyrapicus nuchalis*	1 (F)*
Penenirmus sp.	*Tricholaema leucomelas*	0*
Penenirmus zumpti	*Lybius torquatus*	1 (F)*
Turnicola angustissimus	*Turnix nigricollis*	N/A
Turnicola sp.	*Turnix pyrrothorax*	1 (F)
Turnicola sp.	*Turnix varius*	N/A

Phylogeny

The maximum likelihood phylogeny for *Wolbachia* and related genera using DNA sequences of 106 essential bacterial genes, for 66 ingroup taxa (30 newly generated in this study) and 4 outgroup taxa, is generally well resolved (Figure 1.1) with most nodes having 100% bootstrap support, and only five nodes below a support value of 95%. The *Wolbachia* from *Penenirmus* fell into three distinct groups within *Wolbachia*. The majority of *Wolbachia* from *Penenirmus* fell within the *Wolbachia* supergroup F clade. Two samples fell within supergroup V and three samples with comparatively long branches were within supergroup B.

For samples of *Penenirmus* that possessed two strains of *Wolbachia*, they generally had one strain on a long terminal branch and the other on a short terminal branch. Most of these were pairs of long and short branches within the same supergroup (F). However, one sample (*Penenirmus guineensis* ex *Lybius dubius*) produced long and short branch strains from different supergroups.

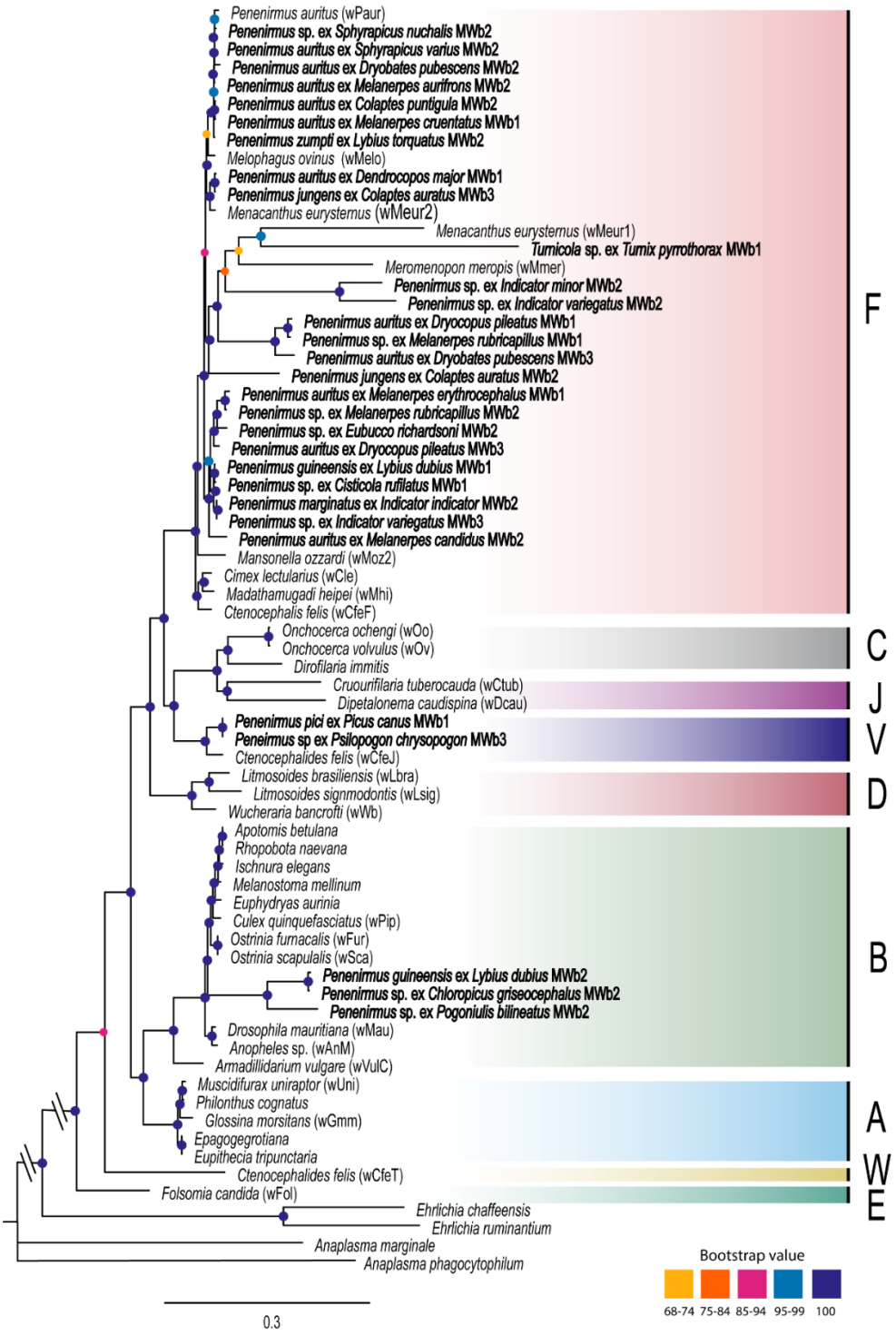

Figure 1.1 Phylogeny of *Wolbachia* based on maximum likelihood concatenated analyses of DNA sequence from the 106 essential genes annotated by MiGA. Branch lengths proportional to subsitutions per site. *Wolbachia* samples are named after their host (*Wolbachia* from lice also have their bird host); strain name is included where possible. Assemblies newly generated by this study are in bold. Outgroups include representatives of *Ehrlichia* and *Anaplasma*, with rooting on *Anaplasma*. Supergroups of *Wolbachia* are indicated in letters on the right.

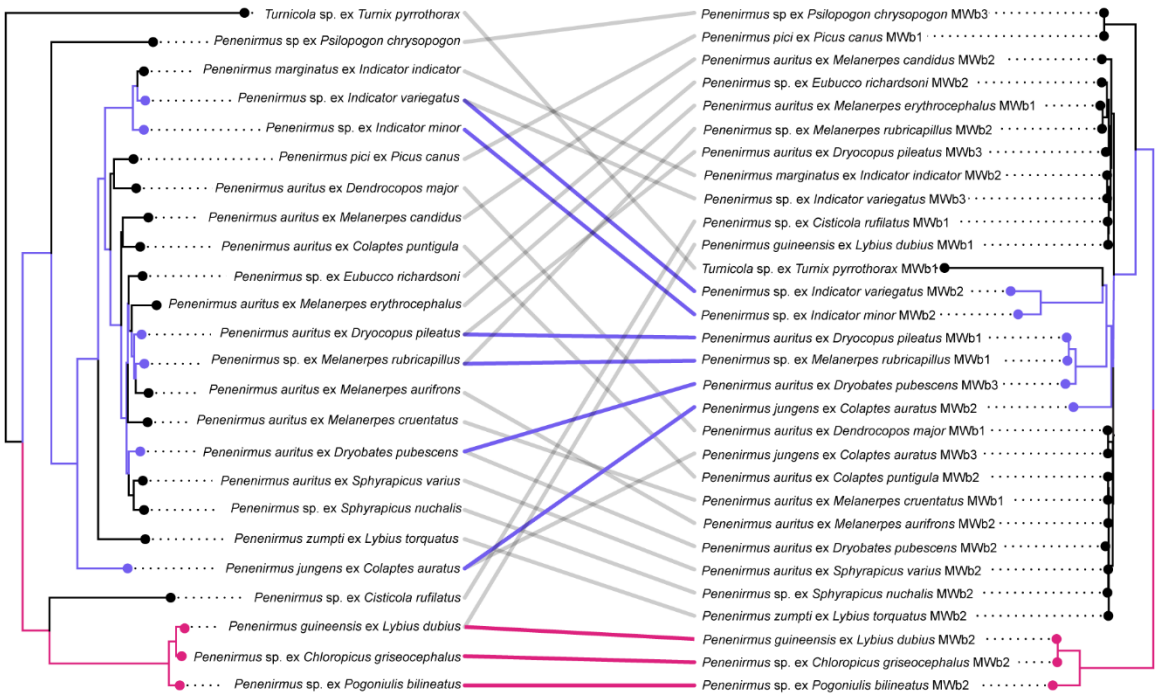

Figure 1.2 Tanglegram of the original louse phylogeny and *Wolbachia* phylogeny. In blue are samples from supergroup F that show congruent branch structure between the phylogenies. In pink are samples from supergroup B that showed congruent branch structures between phylogenies.

Evidence of long term associations

There are three metrics we used to examine the relationship between the lice and their *Wolbachia* to determine if there is evidence of a long term association. We performed a cophylogenetic analysis to compare the structure of the two phylogenies. We also analyzed genome size and compared our assemblies to the average *Wolbachia* genome size. Lastly, we used branch length to examine the substitution rates of each sample, longer branches being associated with higher substitution rates and endosymbiosis.

Two groups of *Wolbachia* and their lice hosts show congruent patterns of speciation between the trees (Figure 1.2). The first group, from supergroup F, consists of the *Wolbachia*

samples *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus auritus* ex *Dryobates pileatus* MWb1, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, *Penenirmus auritus* ex *Dryobates pubescens* MWb3, and *Pennirmus jungens* ex *Colaptes auratus* MWb2 (blue in Figure 1.2). The second group, from supergroup B, contains the *Wolbachia* samples *Penenirmus guineensis* ex *Lybius dubius* MWb2, *Penenirmus* sp. ex *Chloropicus griseocephalus* MWb2, and *Penenirmus* sp. ex *Pogoniulis bilineatus* MWb2 (pink in Figure 1.2).

We examined genome size as one line of evidence to identify potential long term associations. We assembled 14 *Wolbachia* genomes under 1Mbp, below average for *Wolbachia*. Three of these samples were over 90% complete (as determined by MiGA and their essential gene count): *Penenirmus* sp. ex *Chloropicus griseocephalus* MWb2, *Penenirmus auritus* ex *Melanerpes candidus* MWb2, and *Penenirmus* sp. ex *Indicator variegatus* MWb2 at 92%, 94%, and 95% completeness respectively (Table 1.4). Of note is *Penenirmus* sp. ex *Indicator variegatus* MWb2, which was 95% complete and had an assembly size of only 622,001 bp.

Table 1.4 Assembly sizes for each *Wolbachia* assembly. Wolbachia assemblies from the same louse are differentiated by their metaWRAP bin #. Completeness is calculated based on the percentage of essential genes present (out of 106).

Louse species	Bird Host	meta WRAP bin #	Assembly size (bp)	Completeness of assembly (from MiGA)	Predicted completeness of full genome (bp)
Penenirmus auritus	*Colaptes puntigula*	2	1,185,736	93.4%	1,269,525
Penenirmus auritus	*Dendrocopos major*	1	1,130,816	95.3%	1,186,586
Penenirmus auritus	*Dryobates pubescens*	2	1,205,052	54.7%	2,203,020
Penenirmus auritus	*Dryobates pubescens*	3	800,774	53.8%	1,488,428
Penenirmus auritus	*Dryocopus pileatus*	1	1,050,799	73.6%	1,427,716
Penenirmus auritus	*Dryocopus pileatus*	3	718,980	64.2%	1,119,907
Penenirmus auritus	*Melanerpes aurifrons*	2	1,128,089	93.4%	1,207,804
Penenirmus auritus	*Melanerpes candidus*	2	963,750	94.3%	1,022,004
Penenirmus auritus	*Melanerpes cruentatus*	1	1,184,646	94.3%	1,256,252

Table 1.4 (cont.)

Penenirmus auritus	*Melanerpes erythrocephalus*	1	806,735	51%	1,581,833
Penenirmus auritus	*Sphyrapicus varius*	2	1,227,052	94.3%	1,301,222
Penenirmus guineensis	*Lybius dubius*	1	1,039,967	95.3%	1,091,256
Penenirmus guineensis	*Lybius dubius*	2	646,999	51.9%	1,246,626
Penenirmus jungens	*Colaptes auratus*	2	801,960	83.9%	955,852
Penenirmus jungens	*Colaptes auratus*	3	1,156,203	95.3%	1,213,225
Penenirmus marginatus	*Indicator indicator*	2	991,507	86.7%	1,143,607
Penenirmus pici	*Picus canus*	1	1,278,024	96.2%	1,337,863
Penenirmus sp.	*Chloropicus griseocephalus*	2	962,774	92.4%	1,041,963
Penenirmus sp.	*Cisticola rufilatus*	1	1,267,835	94.3%	1,344,470
Penenirmus sp.	*Eubucco richardsoni*	2	694,613	67%	1,036,736
Penenirmus sp.	*Indicator minor*	2	941,610	86.7%	1,086,055
Penenirmus sp.	*Indicator variegatus*	2	622,001	95.4%	651,993
Penenirmus sp.	*Indicator variegatus*	3	1,205,982	95.3%	1,265,459
Penenirmus sp.	*Melanerpes rubricapillus*	1	795,956	78.3%	1,016,558
Penenirmus sp.	*Melanerpes rubricapillus*	2	1,032,233	90.6%	1,139,330
Penenirmus sp.	*Pogoniulis bilineatus*	2	900,814	86.7%	1,039,001
Penenirmus sp.	*Psilopogon chrysopogon*	3	1,231,589	96.2%	1,280,238
Penenirmus sp.	*Sphyrapicus nuchalis*	2	1,141,994	88.7%	1,287,479
Penenirmus zumpti	*Lybius torquatus*	2	1,200,571	94.3%	1,273,140
Turnicola sp.	*Turnix pyrrothorax*	1	670,916	63.2%	1,061,576

There are two groups of *Wolbachia* from *Penenirmus* lice that have relatively long branches on the phylogeny, in supergroups B and F. Samples on long branches from supergroup F are *Turnicola* sp. ex *Turnix pyrrothorax* MWb1, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus auritus* ex *Drocopus pileatus* MWb1, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, *Penenirmus auritus* ex *Dryobates pubescens* MWb3, and *Penenirmus jungens* ex *Colaptes auratus*. Samples on long branches from supergroup B are *Penenirmus guineensis* ex *Lybius dubius* MWb2, *Penenirmus* sp. ex *Chloropicus grisecephalus* MWb2, and *Penenirmus* sp. ex *Pogoniulis bilineatus* MWb2.

Long branch attraction

We tested for long branch attraction by retaining one long branch sample that clumped together on the phylogeny at a time and removing the other eight samples in the cluster. The samples we tested were *Menacanthus eurysternus* (wMeur1), *Turnicola* sp. ex *Turnix pyrrothorax* MWb1, *Meromenopon meropis* (wMmer), *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, *Penenirmus auritus* ex *Dryobates pubescens* MWb3, and *Penenirmus jungens* ex *Colaptes auratus* MWb2. Of the nine samples we analyzed, seven samples showed no change between when isolated and when in the full phylogeny (Figures A.1-A.7). The eigth and ninth samples, *Mencanthus eurysternus* (wMeur1) and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1, moved slighlty when isolated (Figures A.8 & A.9).

Discussion

Phylogenomic analysis of *Wolbachia* bacteria from 44 samples of feather lice in the genera *Penenirmus* and *Turnicola* revealed several new insights into the diversity of these bacteria in lice. The *Wolbachia* assemblies generated in this study fall into three different supergroups: F, V, and B. Supergroup F is often a mutualist with its host (Hosokawa et al., 2010a; Lo et al., 2002; Mahmood et al., 2023; Nikoh et al., 2014), while V and B are characterized as parasitic (Driscoll et al., 2020; Lo et al., 2002). We did not find any *Wolbachia* from supergroup A, despite this group being found in previous work on *Wolbachia* in lice (Covacin & Barker, 2007; Kyei-Poku et al., 2005). Lineages of *Wolbachia* in supergroups F and B show evidence of long-term associations with their hosts through their branching pattern,

genome size, and branch length. *Wolbachia* from supergroup B showing evidence of a long-term association is somewhat unexpected because the other known *Wolbachia* from this group are parasitic (Lo et al., 2002). Among the long branch samples, there is evidence of long branch attraction for two of the samples, but most show stability of their placement in the phylogeny.

Sample distribution on the phylogeny

All the *Wolbachia* supergroups that were included in our tree and represented with multiple samples were recovered as monophyletic. The *Wolbachia* assembled from samples of the louse genus *Penenirmus* do not form a monophyletic group, but rather are distributed across the phylogenetic tree in three different groups. Most samples of *Wolbachia* from *Penenirmus* are in supergroup F, which is known from both arthropods and nematodes and is mutualistic with its host (Mahmood et al., 2023; Nikoh et al., 2014). For example, a member of this group found in bed bugs (*Cimex lectularius*) has been shown to provide B-vitamins to its insect host, which feeds exclusively on blood (Nikoh et al., 2014). This supergroup has been previously found in lice (Covacin & Barker, 2007; Mahmood et al., 2023), but it is unknown whether the *Wolbachia* in lice are beneficial to their hosts. Given that the lice with *Wolbachia* in this clade are dietary specialists, consuming exclusively feathers (*Penenirmus*) or blood (*Menacanthus*), *Wolbachia* in these lice could also function in a nutritionally provisioning role.

Two samples of *Wolbachia* from *Penenirmus* form a monophyletic group sister to the cat flea *Wolbachia* (wCfeJ) in supergroup V. This supergroup has not previously been documented in lice and has only been recently described (Sharma & Som, 2023). This *Wolbachia* from cat fleas has been found to be parasitic to its host and causes cytoplasmic incompatibility (Driscoll et al., 2020). Additionally, while the gene sequences of the *Wolbachia* from these two louse

samples are nearly identical, their hosts are not closely related within the phylogeny of *Penenirmus* (Johnson et al., 2021).

Another group of three *Wolbachia* from *Penenirmus* fall within supergroup B. These are on relatively long branches compared to the other members of this supergroup. This group is known from arthropods and is typically a reproductive parasite (Lo et al., 2002). This group has been previously found in lice (Covacin & Barker, 2007; Kyei-Poku et al., 2005), but the function is yet unknown. These *Wolbachia* may be causing sex ratios to skew either by feminization of genetic males or by cytoplasmic incompatibility. However, there is evidence that these specific *Wolbachia* may have more mutualist roles (discussed below). These three samples are from lice with African distributions (Johnson et al., 2021), so there could potentially be a biogeographic component to the existence of these bacteria where like bacteria come from a similar geographic region.

From 16S rDNA sequences, the presence of *Wolbachia* supergroup A has also been found in feather lice (Covacin & Barker, 2007; Kyei-Poku et al., 2005). However, we did not find evidence of this in any of the samples of *Penenirmus*. Supergroup A is typically recovered in phylogenetic studies as sister to supergroup B (Czarnetzki & Tebbe, 2004; Mahmood et al., 2023; Rowley et al., 2004; Vandekerckhove et al., 1999), as we found here. Members of both supergroups A and B are reproductive parasites in arthropods (Lo et al., 2002).

The diversity of supergroups of *Wolbachia* found in just the single louse genus *Penenirmus* suggests a broader diversity of *Wolbachia* may exist in lice in general. While the finding of supergroups B and F reflects previous work on lice, the occurrence of *Wolbachia* from supergroup V is novel (Sharma & Som, 2023). There appears to not only be a diversity of *Wolbachia* across species of lice, but also multiple *Wolbachia* strains within a single louse

species. Given the intimate coevolutionary histories of lice with their vertebrate hosts, this group of ectoparasites may be an untapped resource to understanding the diversity of *Wolbachia* and its function as a mutualist in arthropod hosts, a phenomenon that is still being uncovered in more detail.

The previous work with *Wolbachia* in lice using PCR and sequencing of the 16S rDNA gene found the presence of multiple strains of *Wolbachia* (superinfections) in several samples, indicating this phenomenon may be widespread for *Wolbachia* in lice (Covacin & Barker, 2007; Kyei-Poku et al., 2005). Five of our samples were superinfected with *Wolbachia* (see Table 1.3). These five paired samples each contain one lineage on a long branch and one lineage on a short branch. This could potentially mean the strains were acquired at different times and could perform different functions in their hosts. Alternatively, these strains may be subject to different mutational and selection constraints resulting in differences in the rates of DNA substitution (Moran, 1996; Moran et al., 1995; Woolfit & Bromham, 2003). Four of the five lice with *Wolbachia* superinfections yielded strains within the same supergroup (F), while one louse yielded strains from different supergroups, B and F. The majority of *Wolbachia* supergroups, except A and B are less prone to superinfection (Bordenstein et al., 2008), making this combination of bacteria from groups F and B in the same louse notable. Also notable with the presence of these two supergroups is the increased potential for a diversity of functions of these *Wolbachia* within their host.

Evidence of long-term associations

We used three lines of evidence to infer the existence of long-term associations between *Penenirmus* and *Turnicola* sp. lice and their *Wolbachia*. These are not mutually exclusive and we

may conclude that long-term associations exist if we see evidence of one or more of these three metrics for a given sample. We explain each of these separately below.

One indicator of long-term association is the codivergence of *Wolbachia* with their insect hosts, visible in the congruence of branching patterns between the louse and *Wolbachia* trees (Hafner & Page, 1995; Johnson & Clayton, 2003; Leonardi et al., 2019). Both groups of *Wolbachia* with long branches (see below) have some evidence of perfect congruence with their louse host phylogeny (Figure 1.2). Mirroring of the branching structure between the two trees could indicate that these *Wolbachia* were acquired quite a long time ago and have evolved with their hosts, speciating alongside them.

We can also use genome size to assess if there is evidence of a long term association, or in this case the assembly size. Bacteria that are obligate endosymbionts typically have reduced genome sizes (Gil et al., 2002; Mahmood et al., 2023; McCutcheon & Moran, 2012). The typical size of the *Wolbachia* genome is 1-2 Mbp (Lo et al., 2002). Fourteen of our assemblies of *Wolbachia* were less than 1 Mbp. *Penenirmus* sp. ex *Indicator variegatus* MWb2 is 95% complete and only 622,001 bp, which could mean the complete genome is around the size or smaller than the smallest published *Wolbachia* genome wMeur1 (733,850 bp) from *Menacanthus eurysternus* (another avian louse) (Mahmood et al., 2023). While a small genome is one indicator of long term association and may signal the loss of genes for functions that have been taken on by the host, a larger genome size does not exclude the possibility of mutualism. The *Wolbachia* from *Cimex lectularius* (wCle) is well within the typical size range for other *Wolbachia* at 1,250,060 bp (Mahmood et al., 2023; Nikoh et al., 2014). Although wCle contains a beneficial vitamin pathway for its host, its relatively larger genome could indicate a more recent acquisition of the bacteria as compared to wMeur1 (Nikoh et al., 2014).

Lastly, branch length can also be an indicator of long term associations. Longer branches can be indicative of higher mutation or substitution rates that occur when bacteria become isolated inside a host. There are two groups of long branches in the phylogeny within supergroups F and B. Supergroup F is known to contain mutualists, like wCle, which incidentally has a relatively short branch possibly due to recent acquisition by the bedbug. Finding long branches in this groups is not unexpected, and in fact several of the NCBI reference genomes are on long branches next to assemblies generated in this study. The presence of long branches in supergroup B is less common, and all other samples included in the phylogeny within supergroup B have short branches.

Members of supergroup B are mainly known to be reproductive parasites in arthropods. On the other hand, the *Wolbachia* of *Penenirmus* in this supergroup show potential evidence of a different relationship. In particular, the branching structure of these three samples is congruent with the louse phylogeny. However, the limited number of samples in this clade is a consideration, and this pattern may change with more *Wolbachia* added to the phylogeny. These three assemblies are also all under 1Mbp, though completeness ranges from 51.9-92.4%. Finally, these samples are all on long branches. These three lines of evidence taken together could indicate that these samples do not follow the trend for this supergroup and are instead potential mutualists with their hosts. Future investigations, including gene annotation would provide additional insights by assessing the presence of genes responsible for reproductive parasitic effects or those responsible for nutrient supplementation.

Long branch attraction

Because several of the *Wolbachia* lineages in lice were on long terminal branches, the clustering of these long branches in the phylogeny could be an artifact of long branch attraction (Bergsten, 2005; Felsenstein, 1985; Susko & Roger, 2021). If the placement of the long branch is stable to the removal of the other long branch taxa, then its placement in the tree is unlikely to be an artifact of long branch attraction. We tested nine samples that had noticeably long terminal branches by including them singly in a phylogenetic analysis. For seven of the nine samples, the position of the long branch did not change. However, the *Wolbachia* from *Turnicola* sp. ex *Turnix pyrrothorax and Menacanthus eurysternus* moved from being close to the position of other long branch *Wolbachia* in supergroup F to being sister to and outside of supergroup F when they were the only long branch taxon in the phylogeny (Figures A.8 & A.9). Thus, long branch attraction is likely to play a role in the position of these *Wolbachia* when all taxa are in the tree together.

MinYS and final sample selection

The *Wolbachia* assemblies generated by the MinYS pipeline were excluded from the final analysis for several reasons. First, this assembly method only detected and assembled a limited number of *Wolbachia* samples. While MinYS assembled *Wolbachia* from all three supergroups, it did not assemble all the *Wolbachia* from *Penenirmus* that metaWRAP assembled from supergroup F, despite using a reference genome from that supergroup. Of the *Wolbachia* MinYS assembled, only one was on a long branch. All other samples it assembled were short branch samples. This is likely because MinYS uses a reference sequence, and the more distant the reference is from the target genome, the more difficult the assembly becomes (Guyomar et

al., 2020). This is a significant bias, especially in studies focused on endosymbiont *Wolbachia* that may have long branches. MinYS was also unable to separate and assemble multiple strains of *Wolbachia* from the same louse. However, the MinYS generated assemblies appeared to be single strains and not chimeric assemblies, based on careful examination of individual gene trees. In the initial phylogeny, each MinYS sample paired on the phylogeny with a metaWRAP sample from the same louse. Thus, the MinYS samples were redundant and excluded from the final analysis. We recommend future studies dealing with *Wolbachia* pay attention to the method of *Wolbachia* genome assembly and its limitations.

Conclusion

The diversity and distribution of *Wolbachia* in lice is more complex than previously thought. In this paper, we confirm the presence of three different supergroup lineages of *Wolbachia* in a single genus of louse (*Penenirmus*). Of note is the fact that while *Penenirmus* is a monophyletic genus of lice, its *Wolbachia* are not. We also found that some louse samples harbored two different strains of *Wolbachia*. The function of these bacteria in their hosts is still unknown and comparing the different lineages could shed light on their effects on their hosts.

CHAPTER 2: A NEW COMPRESSION FOSSIL OF A LOUSE (INSECTA: PSOCODEA: PHTHIRAPTERA) FROM THE EOCENE

Abstract

The fossil record for the various insect orders is unevenly distributed with some orders being represented by few or single specimens and others well represented in the fossil record. The fossil record of parasitic lice (Insecta: Psocodea: Phthiraptera) is particularly limited, with few fossils described. In this paper, we present a new fossil louse, *Archiphthirus eidolon*, from the Piceance Basin of the Green River Formation in Colorado, USA. This exceptionally well-preserved fossil represents the second compression fossil of a louse ever described and is an important addition to the fossil record of this group.

Introduction

The fossil record for parasitic lice (Psocodea: Phthiraptera) is very limited. Parasitic lice complete their entire life cycles on their hosts (Price, 2003). This parasitic lifestyle has likely contributed to their sparse representation in the fossil records (Wappler et al., 2004), as they are not often separate from their host, making preservation unlikely. While there have been several papers that have described fossils as lice (Kumar & Kumar, 1999, 2001; Rasnitsyn & Zherikhin, 1999), these taxonomic placements have been called into question since their publication (Dalgleish et al., 2006; Price, 2003), and are likely fossils of other arthropod lineages. Until this paper, there have only been two confirmed incidents of fossil parasitic louse, one compression fossil (Wappler et al., 2004) and two specimens from Burmese amber (Zhang et al., 2024).

The first louse fossil described was an exceptionally well-preserved specimen, *Megamenopon rasnitsyni,* that even contained traces of feathers in the crop, making it an incredible discovery, and first of its kind (Wappler et al., 2004). The amber preservation of parasitic lice is almost equally sparse. Recently, stem chewing lice, *Archimenopon myanmarensis*, have been described from Burmese amber (Zhang et al., 2024). There is also evidence in Baltic amber of eggs preserved on mammal hair that may belong to ancient sucking lice (Anoplura) (Voigt, 1952).

Our specimen was preserved in the Parachute Creek Member of the Piceance Basin in the Green River Formation in northwestern Colorado, USA. This is a Konservat-Lagerstätte from the Lower-Middle Eocene (48-53 Ma.). There have been many fish, plant, and insect fossils discovered from this site and it is well known for its excellent preservation of minute details, such as fossilized tympana (Plotnick & Smith, 2012) and even a Strepsiptera specimen (Antell & Kathirithamby, 2016).

In this paper we present the second louse compression fossil to be described. We include both a high-resolution image of the specimen as well as an illustrative interpretation to clarify key details. Although the exact placement of this specimen within Phthiraptera cannot be conclusively determined, this fossil provides further evidence for the existence of parasitic lice over 40 million years ago.

Methods

We took images of the specimen on a Keyence VHX-7000 digital microscope. We then edited the images in Adobe Photoshop to increase the visibility of desired features. This included adjusting contrast, brightness, and some minor color correction. We used the final image to

create the illustration in Adobe Illustrator. Only the larger setae are included in the illustration. Microsetae are not illustrated but are visible in the original image. We used ImageJ to take measurements of the specimen. All measurements are in mm. Abbreviations are as follows: HL, head length; TW, temple width; PW, prothorax width at midline; PL, prothorax length; MW, metathorax width at midline; ML, metathorax length; AW, abdominal width at level of segment III; AL, abdominal length, TL, total length (Sychra & Palma, 2021).

The specimen is deposited in the Illinois Natural History Survey Fossil insect Collection (INHS-P) and the PRI Center for Paleontology, University of Illinois, USA.

The morphology of the fossil was compared to the key to Phthiraptera suborders and chewing louse families from Price et al. (2003).

Results

Class Insecta Linnaeus, 1758

Order Psocodea Hennig, 1966

Suborder Troctomorpha Roesler, 1944

Infraorder Phthiraptera Haekel, 1896

Phthiraptera *incertae sedis*

Archiphthirus gen. nov.

Type species. *Archiphthirus eidolon* sp. nov.

Etymology. The generic name is based upon the Greek prefix *arche-* (ἀρχή) meaning "ancient" or "old" and the Greek word for "louse", *phthirus* (φθείρ), which was originally used to name the parasitic louse order.

Diagnosis. In the absence of morphological characters used to diagnose extant parasitic lice, the present diagnosis is descriptive: clypeal region expanded, temples lobed, mesothoracic and metathoracic segments fused, abdominal setae arranged in parallel series along posterior margins of tergites.

Archiphthirus eidolon sp. nov.

See Figure 2.1

Holotype. INHS-P2020-16, a small phthirapteran preserved in dorsal aspect on a roughly diamond-shaped piece of shale approximately 115 mm long by 70 mm wide. Other fossils present include: two scuttle flies (Diptera: Phoridae) [INHS-P2020-2, INHS-P2020-10]; a chalcid wasp (Hymenoptera: Chalcidoidea) [INHS-P2020-12]; two indeterminate small wasps (Hymenoptera) [INHS-P2020-1, INHS-P2020-9]; a katydid (Orthoptera: Tettigoniidae) [INHS-P2020-11]; and an isolated beetle elytron (Coleoptera) [INHS-P2020-13]. Various small pieces of comminuted plant debris are also present across the slab.

Type locality and horizon. Rio Blanco County, Colorado, USA. Parachute Creek Member, Green River Formation (Eocene).

Etymology. The specific epithet *eidolon* (εἴδωλον) is Greek for the "image of" or "copy of" a living being which appears several times in the Iliad and Homer's Odyssey.

Diagnosis. As for the genus.

Description. Life stage and sex could not be determined. The specimen is visible entirely in dorsal aspect and appears to be tilted to one side slightly.

Head

Expanded clypeus distinguishable, temples narrowly lobed. Head slightly wider than thorax. No eyes visible on the specimen (dark spot on head is rock pigmentation). Head setose. One appendage visible extending from the middle of the head and down (length 0.198). Origin of appendage unclear, making determination of whether the appendage is a maxillary palp or an antenna difficult.

Thorax

Two distinct segments of the thorax are visible. Thorax appears to have fused segments (mesothorax and metathorax) characteristic of extant lice, suture not distinguishable. Thorax setose. Two legs are visible below the main body of the specimen though not connected, also setose. Anterior leg is represented by femur and tibia, (length 0.404) posterior by femur alone (length 0.192). Eighteen setae present on posterior edge of thorax. This section of the specimen appears somewhat stretched, perhaps by the fossilization process.

Abdomen

Abdomen appears relatively intact in its preservation. Several distinct segments are visible, though the terminal segment appears rather large and perhaps lacks the definition of additional segmentation. There are many large setae visible on the abdomen in addition to the microsetae that cover the whole body. Tergal setae: I, 25; II, 26; III, 28; IV, 29. 39 setae around posterior edge of abdomen. Spiracles not visible on any segments. Without visible genitalia, determination of sex is not possible.

Table 2.1 Measurements of Archiphthirus eidolon in milimeters.

Measurements: (in mm)		
Total length	TL	1.87
Head length	HL	0.473
Temple width	TW	0.440
Prothorax width at midline	PW	0.479
Prothorax length	PL	0.475
Metathorax width at midline	MW	0.628
Metathorax length	ML	0.506
Abdominal width at level of segment III	AW	0.786
Abdominal length	AL	1.104

Remarks. Initial inspection of the fossil confirmed it was an insect based on the three main body segments and appearance of jointed legs. We were able to rule out several orders of insects based on the lack of certain characters (e.g. wings). Characters that confirmed the specimen is a louse in addition to the general body plan are the expanded clypeal region as opposed to a narrow and insignificant clypeus, lobed temples instead of a completely narrow head, fused thoracic segments instead of three distinct segments, absence of eyes, absence of wings, and the position of setae on the abdomen congruent with extant lice.

Since there are few fossil lice to compare this to, we feel certain that our specimen is a different species, as the body shapes between the specimens are very different. While the parvorder and family placement of this fossil remain elusive, we believe it is either in Amblycera or Ischnocera. Without visible antennae, we do not feel comfortable ascribing to one or the other. However, we felt it important to give the specimen a name for ease of reference.

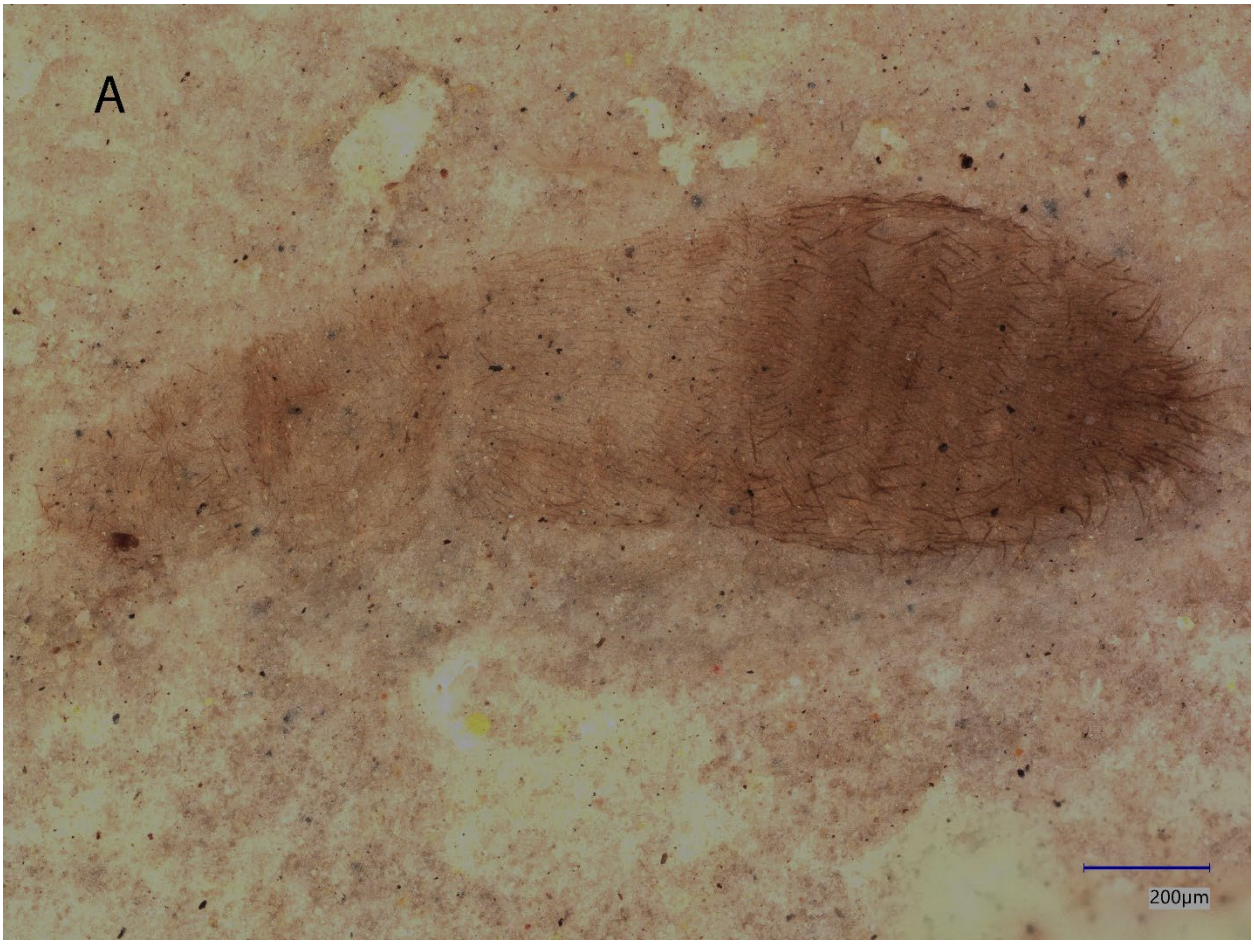

Figure 2.1 A: Keyence VHX-7000 digital microscope image of the fossil (INHS-P2020-16) *Archiphthirus eidolon* sp. nov. at 150x magnification.

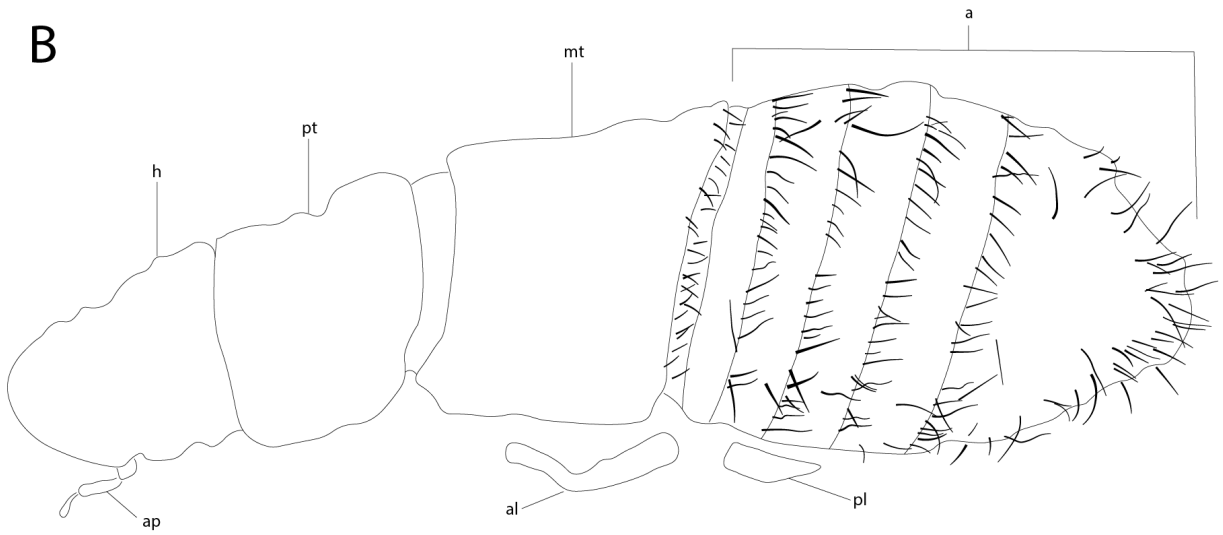

Figure 2.1 (cont.) B: an illustration of key characters of and structures of the fossil *Archipthirus eidolon* (made in Adobe Illustrator), h-head; pt- prothorax; mt-fused mesothorax and metathorax; a- abdomen; pl- posterior leg; al- anterior leg; ap- unidentified appendage

Discussion

Although this specimen cannot be definitively placed in either Amblycera or Ischnocera, the fossil exhibits clear characters that place it within Phthiraptera. This well-preserved specimen is an important addition to the otherwise sparse number of fossilized parasitic lice.

The size of the specimen is within the expected range for extant lice (Price, 2003) – smaller than *Megamenopon rasnitsyni* (Wappler et al., 2004) and slightly larger than *Archimenopon myanmarensis* (Zhang et al., 2024). The preservation of the specimen is remarkable with even small setae distinguishable on most segments of the body. This detailed level of preservation is not uncommon in the Green River formation where the specimen was found and its propensity to preserve the minutiae of specimens.

Speculation for potential hosts for this louse remains broad given the uncertain placement of the specimen in either Amblycera (which feed on birds and mammals) or Ischnocera (which

feed exclusively on birds). It is clear from the fossil record that there was a large diversity of mammals and birds in the area that could be candidate hosts for lice (Lockley & Smith, 2021; Olson, 1987; Van Houten, 1945). There is of course, the prospect that ancient lice also parasitized feathered dinosaurs, though conclusive proof remains elusive, and these would have been extinct at the time of this fossil preservation.

This specimen is from the Lower-Middle Eocene, which agrees with Wappler et al. (2004) and places the origin of parasitic lice at least at this time period. The definitive earliest age of the appearance of Phthiraptera may be earlier, because the stem chewing lice from Burmese amber date to 55 million years ago from the Paleogene (Zhang et al., 2024).

REFERENCES

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K. L., Tyson, G. W., & Nielsen, P. H. (2013). Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. *Nature Biotechnology*, *31*(6), 533–538. https://doi.org/10.1038/nbt.2579

Antell, G. S., & Kathirithamby, J. (2016). The First Twisted-Wing Parasitoids (Insecta: Strepsiptera) from the Early Eocene Green River Formation of Colorado. *Bulletin of the Peabody Museum of Natural History*, *57*(2), 165–174. https://doi.org/10.3374/014.057.0204

Baldo, L., & Werren, J. H. (2007). Revisiting Wolbachia supergroup Typing Based on WSP: Spurious Lineages and Discordance with MLST. *Current Microbiology*, *55*(1), 81–87. https://doi.org/10.1007/s00284-007-0055-8

Bandi, C., John, W. M., Genchi, C., Corona, S., Venco, L., & Sacchi, L. (1999). Effects of tetracycline on the filarial worms Brugia pahangi and Dirofilaria immitis and their bacterial endosymbionts Wolbachia. *International Journal for Parasitology*, *29*, 357–364.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *Journal of Computational Biology*, *19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

Bergsten, J. (2005). A review of long-branch attraction. In *Cladistics* (Vol. 21, Issue 2, pp. 163–193). https://doi.org/10.1111/j.1096-0031.2005.00059.x

Bordenstein, S. R., Paraskevopoulos, C., Dunning Hotopp, J. C., Sapountzis, P., Lo, N., Bandi, C., Tettelin, H., Werren, J. H., & Bourtzisà, K. (2008). Parasitism and Mutualism in

Wolbachia: What the Phylogenomic Trees Can and Cannot Say. *Molecular Biology and Evolution*, *26*(1), 231–241. https://doi.org/10.1093/molbev/msn243

Borowiec, M. L. (2016). AMAS: A fast tool for alignment manipulation and computing of summary statistics. *PeerJ*, *2016*(1). https://doi.org/10.7717/peerj.1660

Brockhurst, M. A., & Koskella, B. (2013). Experimental coevolution of species interactions. In *Trends in Ecology and Evolution* (Vol. 28, Issue 6, pp. 367–375). https://doi.org/10.1016/j.tree.2013.02.009

Capella-Gutiérrez, S., Silla-Martínez, J. M., & Gabaldón, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics*, *25*(15), 1972–1973. https://doi.org/10.1093/bioinformatics/btp348

Coenye, T., & Vandamme, P. (2003). Diversity and significance of Burkholderia species occupying diverse ecological niches. In *Environmental Microbiology* (Vol. 5, Issue 9, pp. 719–729). https://doi.org/10.1046/j.1462-2920.2003.00471.x

Covacin, C., & Barker, S. C. (2007). Supergroup F Wolbachia bacteria parasitise lice (Insecta: Phthiraptera). *Parasitology Research*, *100*, 479–485. https://doi.org/10.1007/s00436-006-0309-6

Czarnetzki, A. B., & Tebbe, C. C. (2004). Detection and phylogenetic analysis of Wolbachia in Collembola. *Environmental Microbiology*, *6*(1), 35–44. https://doi.org/10.1046/J.1462-2920.2003.00537.X

Dalgleish, R. C., Palma, R. L., Price, R. D., & Smith, V. S. (2006). Fossil lice (Insecta: Phthiraptera) reconsidered. In *Systematic Entomology*. https://doi.org/10.1111/j.1365-3113.2006.00342.x

Decaestecker, E., Gaba, S., Raeymaekers, J. A. M., Stoks, R., Van Kerckhoven, L., Ebert, D., & De Meester, L. (2007). Host-parasite “Red Queen” dynamics archived in pond sediment. *Nature*, *450*(7171), 870–873. https://doi.org/10.1038/nature06291

Driscoll, T. P., Verhoeve, V. I., Brockway, C., Shrewsberry, D. L., Plumer, M., Sevdalis, S. E., Beckmann, J. F., Krueger, L. M., Macaluso, K. R., Azad, A. F., & Gillespie, J. J. (2020). Evolution of Wolbachia mutualism and reproductive parasitism: insight from two novel strains that co-infect cat fleas. *PeerJ*. https://doi.org/10.7717/peerj.10646

Felsenstein, J. (1985). Phylogenies and the Comparative Method. In *Source: The American Naturalist* (Vol. 125, Issue 1). https://www.jstor.org/stable/2461605

Fenn, K., & Blaxter, M. (2004). Are filarial nematode Wolbachia obligate mutualist symbionts? *Trends in Ecology and Evolution*, *19*(4), 163–166. www.sciencedirect.com

Fukatsu, T., Koga, R., Smith, W. A., Tanaka, K., Nikoh, N., Sasaki-Fukatsu, K., Yoshizawa, K., Dale, C., & Clayton, D. H. (2007). Bacterial Endosymbiont of the Slender Pigeon Louse, Columbicola columbae, Allied to Endosymbionts of Grain Weevils and Tsetse Flies. *Applied and Environmental Microbiology*, *73*(20), 6660–6668. https://doi.org/10.1128/AEM.01131-07

Gerth, M. (2016). *Classification of Wolbachia (Alphaproteobacteria, Rickettsiales): No evidence for a distinct supergroup in cave spiders*. https://doi.org/10.1101/046169

Gil, R., Sabater-Muñoz, B., Latorre, A., Silva, F. J., & Moya, A. (2002). Extreme genome reduction in Buchnera spp.: Toward the minimal genome needed for symbiotic life. *PNAS*, *99*(7), 4454–4458. https://www.pnas.org

Guyomar, C., Delage, W., Legeai, F., Mougel, C., Simon, J. C., & Lemaitre, C. (2020). MinYS: mine your symbiont by targeted genome assembly in symbiotic communities. *NAR Genomics and Bioinformatics*, *2*(3). https://doi.org/10.1093/nargab/lqaa047

Hafner, M. S., & Page, R. D. M. (1995). Molecular Phylogenies and Host-Parasite Cospeciation: Gophers and Lice as a Model System. *Source: Philosophical Transactions: Biological Sciences*, *349*(1327), 77–83.

Hosokawa, T., Kikuchi, Y., Nikoh, N., Shimada, M., & Fukatsu, T. (2006). Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. *PLoS Biology*, *4*(10), 1841–1851. https://doi.org/10.1371/journal.pbio.0040337

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X. Y., & Fukatsu, T. (2010a). Wolbachia as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(2), 769–774. https://doi.org/10.1073/pnas.0911476107

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X. Y., & Fukatsu, T. (2010b). Wolbachia as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(2), 769–774. https://doi.org/10.1073/pnas.0911476107

Johnson, K. P., & Clayton, D. H. (2003). Coevolutionary History of Ecological Replicates: Comparing Phylogenies of Wing and Body Lice to Columbiform Hosts. In R. D. M. Page (Ed.), *Tangled Trees: Phylogeny, Cospeciation, and Coevolution* (pp. 262–286). University of Chicago Press.

Johnson, K. P., Weckstein, J. D., Virrueta Herrera, S., & Doña, J. (2021). The interplay between host biogeography and phylogeny in structuring diversification of the feather louse genus

Penenirmus. *Molecular Phylogenetics and Evolution*, *165*. https://doi.org/10.1016/j.ympev.2021.107297

Katoh, K., Misawa, K., Kuma, K.-I., & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, *30*(14), 3059–3066.

Kirkness, E. F., Haas, B. J., Sun, W., Braig, H. R., Perotti, M. A., Clark, J. M., Lee, S. H., Robertson, H. M., Kennedy, R. C., Elhaik, E., Gerlach, D., Kriventseva, E. V., Elsik, C. G., Graur, D., Hill, C. A., Veenstra, J. A., Walenz, B., Tubío, J. M. C., Ribeiro, J. M. C., … Pittendrigh, B. R. (2010). Genome sequences of the human body louse and its primary endosymbiont provide insights into the permanent parasitic lifestyle. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(27), 12168–12173. https://doi.org/10.1073/pnas.1003379107

Kumar, P., & Kumar, P. (1999). Insect remains from Upper Triassic sediments of Satpura Basin, India. *Current Science*, *76*(12), 1539–1541.

Kumar, P., & Kumar, P. (2001). Phthirapteran insect and larval Acanthocephala from the Late Triassic sediments of the Satpura basin, India. *Journal of the Palaeontological Society of India*, *46*, 141–146.

Kyei-Poku, G. K., Colwell, D. D., Coghlin, P., Benkel, B., & Floate, K. D. (2005). On the ubiquity and phylogeny of Wolbachia in lice. *Molecular Ecology*, *14*, 285–294. https://doi.org/10.1111/j.1365-294X.2004.02409.x

Lefoulon, E., Clark, T., Borveto, F., Perriat-Sanguinet, M., Moulia, C., Slatko, B. E., & Gavotte, L. (2020). Pseudoscorpion Wolbachia symbionts: diversity and evidence for a new supergroup S. *BMC Microbiology*, *20*(188). https://doi.org/10.1186/s12866-020-01863-y

Leonardi, M. S., Virrueta Herrera, S., Sweet, A., Negrete, J., & Johnson, K. P. (2019). Phylogenomic analysis of seal lice reveals codivergence with their hosts. *Systematic Entomology*, *44*, 699–708. https://doi.org/10.1111/syen.12350

Lo, N., Casiraghi, M., Salati, E., Bazzocchi, C., & Bandi, C. (2002). How Many Wolbachia Supergroups Exist? *Mol. Biol. Evol*, *19*(3), 341–346. https://academic.oup.com/mbe/article/19/3/341/981063

Lockley, M., & Smith, J. (2021). Bird Tracks From the Uinta Formation, Eocene, Colorado. *Fossil Record*, *82*, 185–193.

Mahenthiralingam, E., Urban, T. A., & Goldberg, J. B. (2005). The Multifarious, Multireplicon Burkholderia cepacia Complex. In *Nature Reviews Microbiology* (Vol. 3, pp. 144–156). https://doi.org/10.1038/nrmicro1085

Mahmood, S., Nováková, E., Martinů, J., Sychra, O., & Hypša, V. (2023). Supergroup F Wolbachia with extremely reduced genome: transition to obligate insect symbionts. *Microbiome*, *11*(22). https://doi.org/10.1186/s40168-023-01462-9

McCutcheon, J. P., Boyd, B. M., & Dale, C. (2019). The Life of an Insect Endosymbiont from the Cradle to the Grave. In *Current Biology* (Vol. 29, Issue 11, pp. R485–R495). Cell Press. https://doi.org/10.1016/j.cub.2019.03.032

McCutcheon, J. P., & Moran, N. A. (2012). Extreme genome reduction in symbiotic bacteria. In *Nature Reviews Microbiology* (Vol. 10, pp. 13–26). https://doi.org/10.1038/nrmicro2670

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., Von Haeseler, A., & Lanfear, R. (2020). IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution*, *37*(5), 1530–1534. https://doi.org/10.1093/molbev/msaa015

Moran, N. A. (1996). Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proceedings of the National Academy of Sciences*, *93*, 2873–2878. https://www.pnas.org

Moran, N. A., Yon Dohlen, C. D., & Baumann, P. (1995). Faster Evolutionary Rates in Endosymbiotic Bacteria Than in Cospeciating Insect Hosts. *J Mol Evol*, *41*, 727–731.

Nikoh, N., Hosokawa, T., Moriyama, M., Oshima, K., Hattori, M., & Fukatsu, T. (2014). Evolutionary origin of insect-Wolbachia nutritional mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(28), 10257–10262. https://doi.org/10.1073/pnas.1409284111

Normark, B. B. (2003). The Evolution of Alternative Genetic Systems in Insects. *Annual Review of Entomology*, *48*, 397–423. https://doi.org/10.1146/annurev.ento.48.091801.112703

Olson, S. L. (1987). An Early Eocene Oilbird from the Green River Formation of Wyoming (Caprimulgiformes: Steatornithidae). *Documents Des Laboratoires de Géologie Lyon*, *99*, 57–69.

Papkou, A., Guzella, T., Yang, W., Koepper, S., Pees, B., Schalkowski, R., Barg, M. C., Rosenstiel, P. C., Teotónio, H., & Schulenburg, H. (2019). The genomic basis of red queen dynamics during rapid reciprocal host–pathogen coevolution. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(3), 923–928. https://doi.org/10.1073/pnas.1810402116

Paradis, E., Claude, J., & Strimmer, K. (2004). APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics*, *20*(2), 289–290. https://doi.org/10.1093/bioinformatics/btg412

Paterson, S., Vogwill, T., Buckling, A., Benmayor, R., Spiers, A. J., Thomson, N. R., Quail, M., Smith, F., Walker, D., Libberton, B., Fenton, A., Hall, N., & Brockhurst, M. A. (2010).

Antagonistic coevolution accelerates molecular evolution. *Nature*, *464*(7286), 275–278. https://doi.org/10.1038/nature08798

Perotti, M. A., Kirkness, E. F., Reed, D. L., & Braig, H. R. (2008). Endosymbionts of lice. In *Insect Symbiosis* (Vol. 3, pp. 205–219). https://doi.org/10.1201/9781420064117.ch9

Plotnick, R. E., & Smith, D. M. (2012). Exceptionally Preserved Fossil Insect Ears from the Eocene Green River Formation of Colorado. *Journal of Paleontology*, *86*(1), 19–24. https://doi.org/10.1666/11-072.1

Price, R. D. (2003). *The chewing lice: world checklist and biological overview*. Illinois Natural History Survey.

Rasnitsyn, A. P., & Zherikhin, V. V. (1999). First fossil chewing louse from the lower Cretaceous of Baissa, Transbaikalia (Insecta, Pediculida- Phthiriaptera, Saurodectidae fam. n.). *Russian Entomological Journal*, *8*(4), 253–255.

Revell, L. J. (2024). phytools 2.0: an updated R ecosystem for phylogenetic comparative methods (and other things). *PeerJ*, *12*. https://doi.org/10.7717/peerj.16505

Rodriguez-R, L. M., Gunturu, S., Harvey, W. T., Rosselló-Mora, R., Tiedje, J. M., Cole, J. R., & Konstantinidis, K. T. (2018). The Microbial Genomes Atlas (MiGA) webserver: Taxonomic and gene diversity analysis of Archaea and Bacteria at the whole genome level. *Nucleic Acids Research*, *46*(W1), W282–W288. https://doi.org/10.1093/nar/gky467

Rodriguez-R, L. M., Harvey, W. T., Rosselló-Mora, R., Tiedje, J. M., Cole, J. R., & Konstantinidis, K. T. (2020). Classifying prokaryotic genomes using the Microbial Genomes Atlas (MiGA) webserver. In *Bergey's Manual of Systematics of Archaea and Bacteria* (pp. 1–11). Wiley. https://doi.org/10.1002/9781118960608.bm00042

Rowley, S. M., Raven, R. J., & McGraw, E. A. (2004). Wolbachia pipientis in Australian Spiders. *Current Microbiology 2004 49:3*, *49*, 208–214. https://doi.org/10.1007/S00284-004-4346-Z

Saridaki, A., & Bourtzis, K. (2010). Wolbachia: more than just a bug in insects genitals. In *Current Opinion in Microbiology* (Vol. 13, pp. 67–72). https://doi.org/10.1016/j.mib.2009.11.005

Sharma, A. K., & Som, A. (2023). Assigning new supergroups V and W to the Wolbachia diversity. *Bioinformation*, *19*(3), 336–340. https://doi.org/10.6026/97320630019336

Susko, E., & Roger, A. J. (2021). Long Branch Attraction Biases in Phylogenetics. *Systematic Biology*, *70*(4), 838–843. https://doi.org/10.1093/sysbio/syab001

Sychra, O., & Palma, R. L. (2021). A new species of Myrsidea (Insecta: Phthiraptera: Menoponidae) from Chile. *Zootaxa*, *5016*(3), 441–447. https://doi.org/10.11646/zootaxa.5016.3.9

Tamas, I., Klasson, L., Canback, B., Naslund, A. K., Eirksson, A.-S., Wernegreen, J. J., Sandstrom, J. P., Moran, N. A., & Andersson, S. G. E. (2002). 50 Million Years of Genomic Stasis in Endosymbiotic Bacteria. *Science*, *296*, 2376–2379. https://www.science.org

Toju, H., Tanabe, A. S., Notsu, Y., Sota, T., & Fukatsu, T. (2013). Diversification of endosymbiosis: Replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils. *ISME Journal*, *7*(7), 1378–1390. https://doi.org/10.1038/ismej.2013.27

Tsuchida, T., Koga, R., Meng, X. Y., Matsumoto, T., & Fukatsu, T. (2005). Characterization of a Facultative Endosymbiotic Bacterium of the Pea Aphid Acyrthosiphon pisum. *Microbial Ecology*, *49*, 126–133. https://doi.org/10.1007/s00248-004-0216-2

Uritskiy, G. V., DiRuggiero, J., & Taylor, J. (2018). MetaWRAP - A flexible pipeline for genome-resolved metagenomic data analysis. *Microbiome*, *6*(158). https://doi.org/10.1186/s40168-018-0541-1

Van Houten, F. B. (1945). Review of Latest Paleocene and Early Eocene Mammalian Faunas. *Source: Journal of Paleontology*, *19*(5), 421–461. https://www.jstor.org/stable/1299000

Vandekerckhove, T. T. M., Watteyne, S., Willems, A., Swings, J. G., Mertens, J., & Gillis, M. (1999). Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium Wolbachia from the novel host Folsomia candida (Hexapoda, Collembola) and its implications for wolbachial taxonomy. *FEMS Microbiology Letters*, *180*, 279–286. https://doi.org/10.1111/j.1574-6968.1999.tb08807.x

Voigt, E. (1952). *Ein Haareinschluss mit Phthirapteren-Eiern im Bernstein*. Verlag nicht ermittelbar.

Wappler, T., Smith, V. S., & Dalgleish, R. C. (2004). Scratching an ancient itch: An Eocene bird louse fossil. *Proceedings of the Royal Society B: Biological Sciences*, *271*, S255–S258. https://doi.org/10.1098/rsbl.2003.0158

Wernegreen, J. J. (2002). Genome Evolution in Bacterial Endosymbionts of Insects. *Nature Reviews Genetics*, *3*, 850–861. https://doi.org/10.1038/nrg931

Woolfit, M., & Bromham, L. (2003). Increased Rates of Sequence Evolution in Endosymbiotic Bacteria and Fungi with Small Effective Population Sizes. *Molecular Biology and Evolution*, *20*(9), 1545–1555. https://doi.org/10.1093/molbev/msg167

Zhang, Y., Rasnitsyn, A. P., Zhang, W., Song, F., Shih, C., Ren, D., Wang, Y., Li, H., & Gao, T. (2024). Stem chewing lice on Cretaceous feathers preserved in amber. *Current Biology*, *34*, 916-922.e1. https://doi.org/10.1016/j.cub.2024.01.027

APPENDIX A: SUPPLEMENTARY PHYLOGENIES

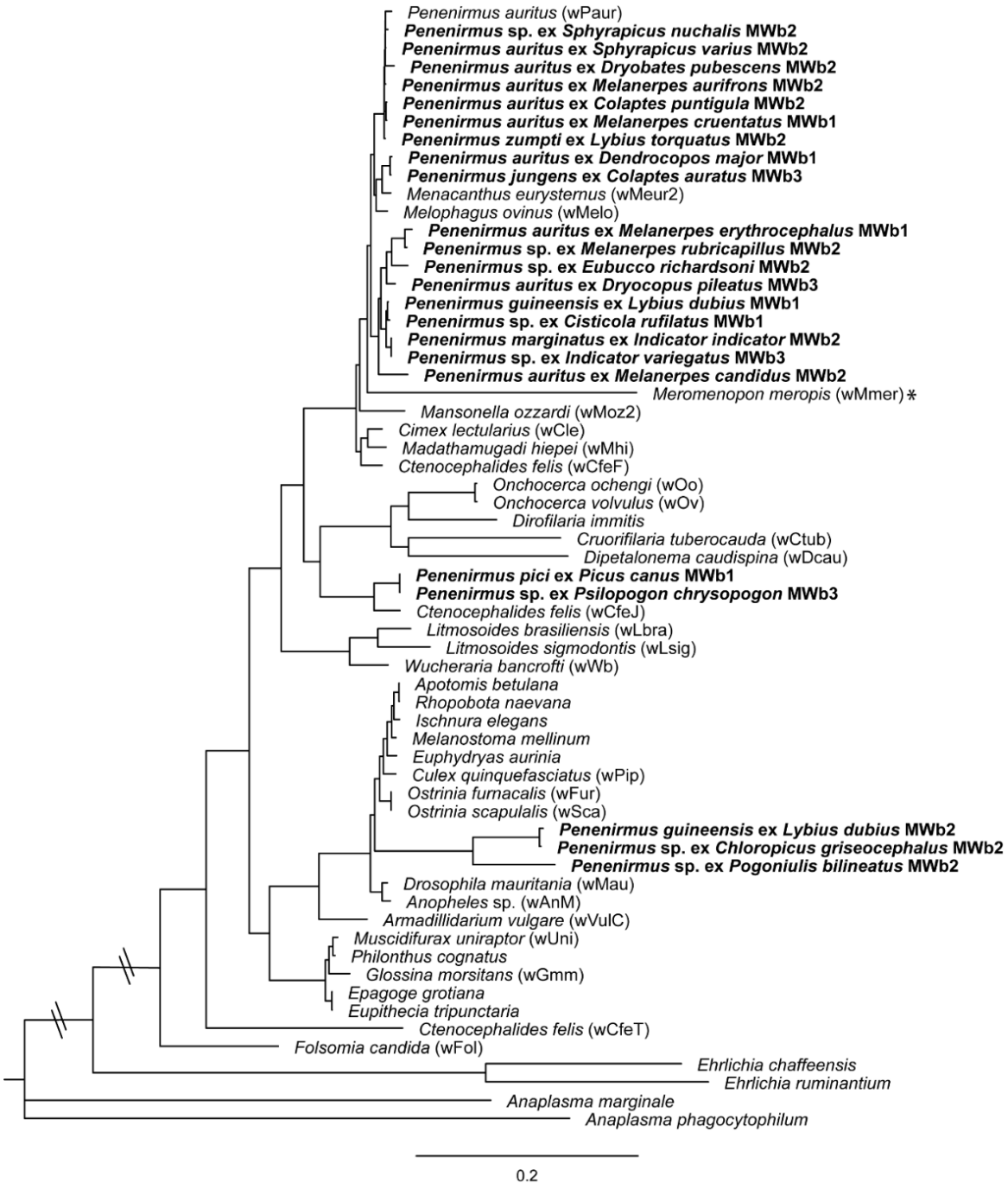

Figure A.1 Phylogeny of the long branch attraction assay of *Meromenopon meropis* (wMmer), sample location is consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1

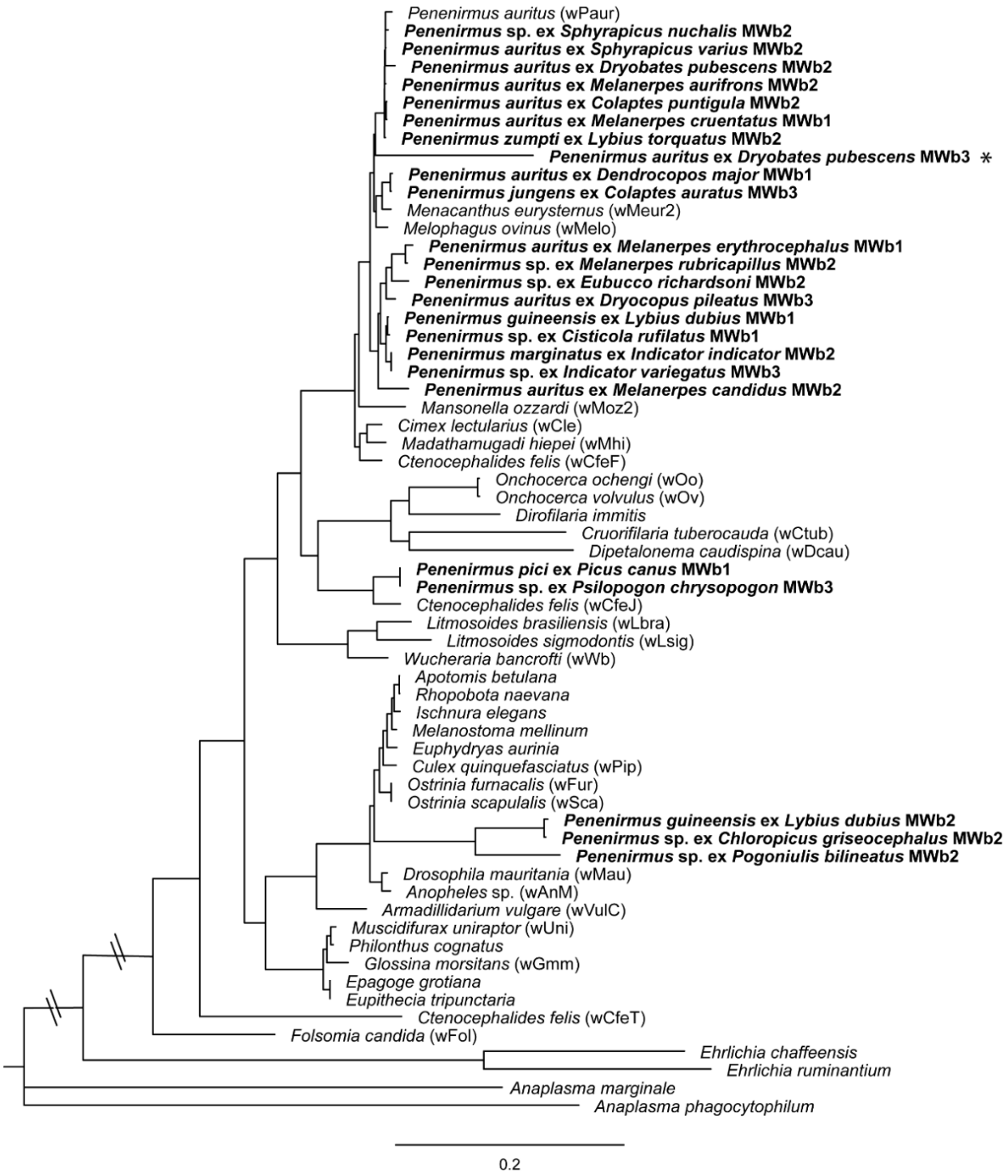

Figure A.2 Phylogeny of the long branch attraction assay of *Penenirmus auritus* ex *Dryobates pubescens* MWb3, sample location is consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1), *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1

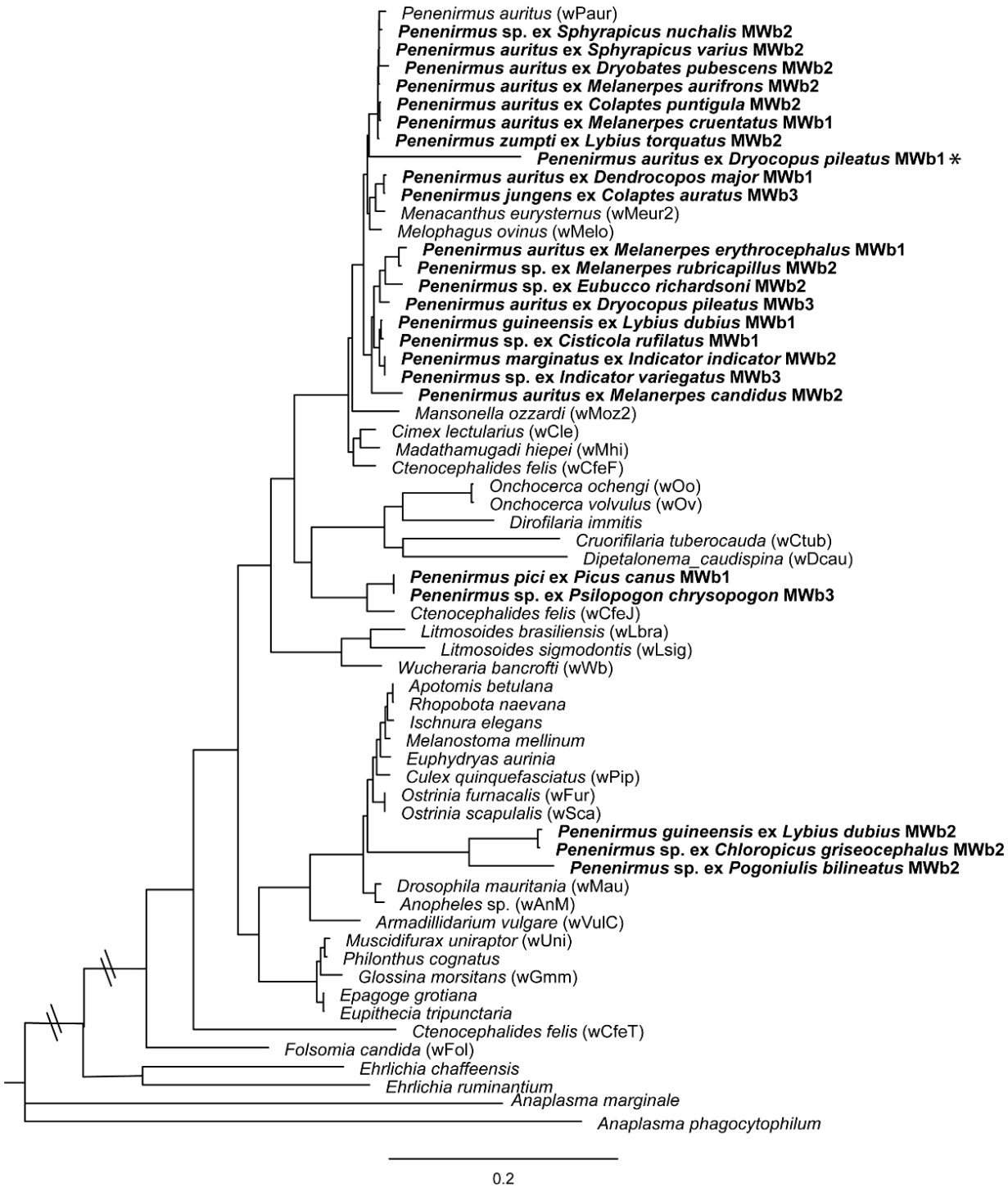

Figure A.3 Phylogeny of the long branch attraction assay of *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, sample location is consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1), *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1

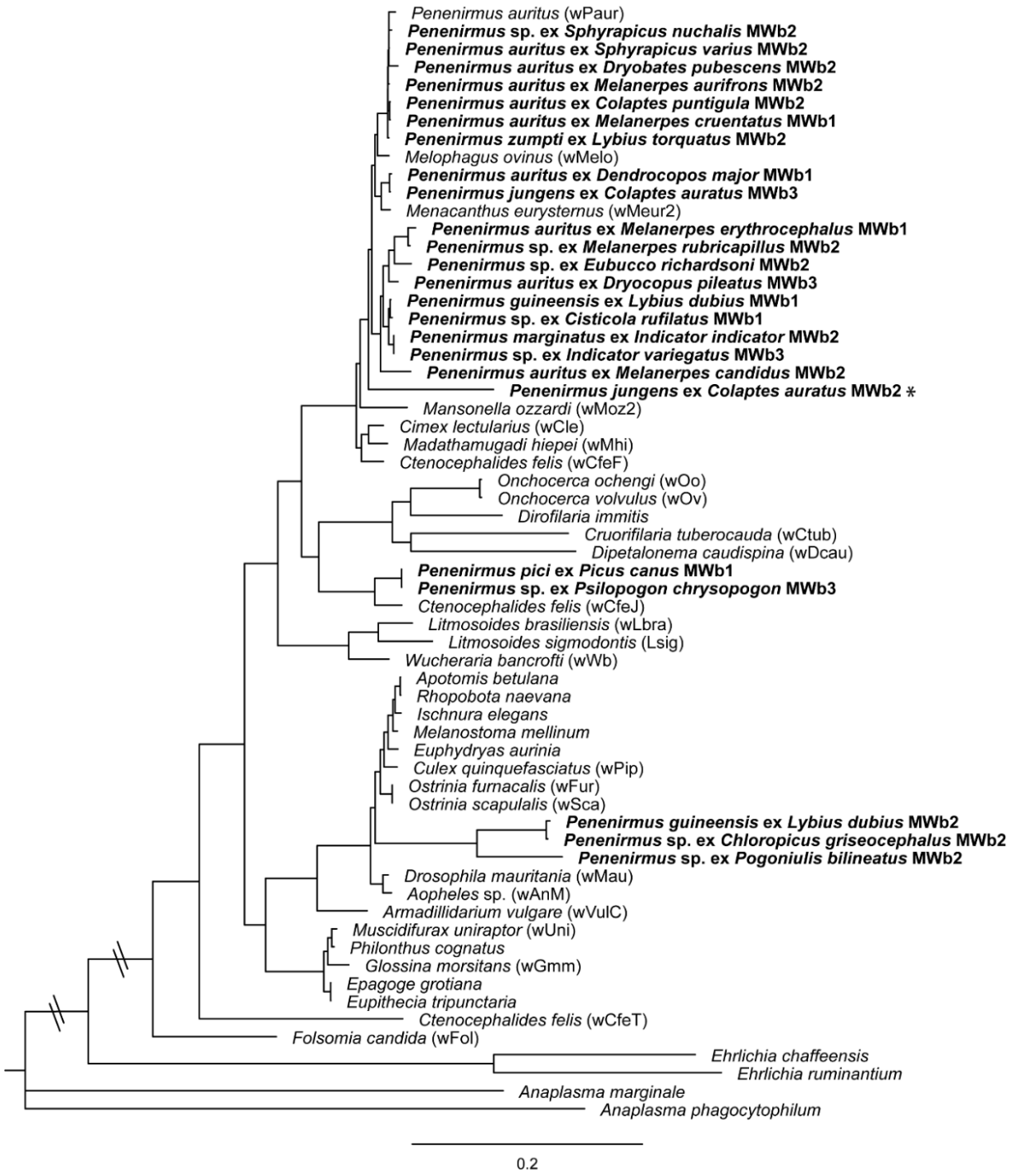

Figure A.4 Phylogeny of the long branch attraction assay of *Penenirmus jungens* ex *Colaptes auratus* MWb2, sample location is consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1), *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1

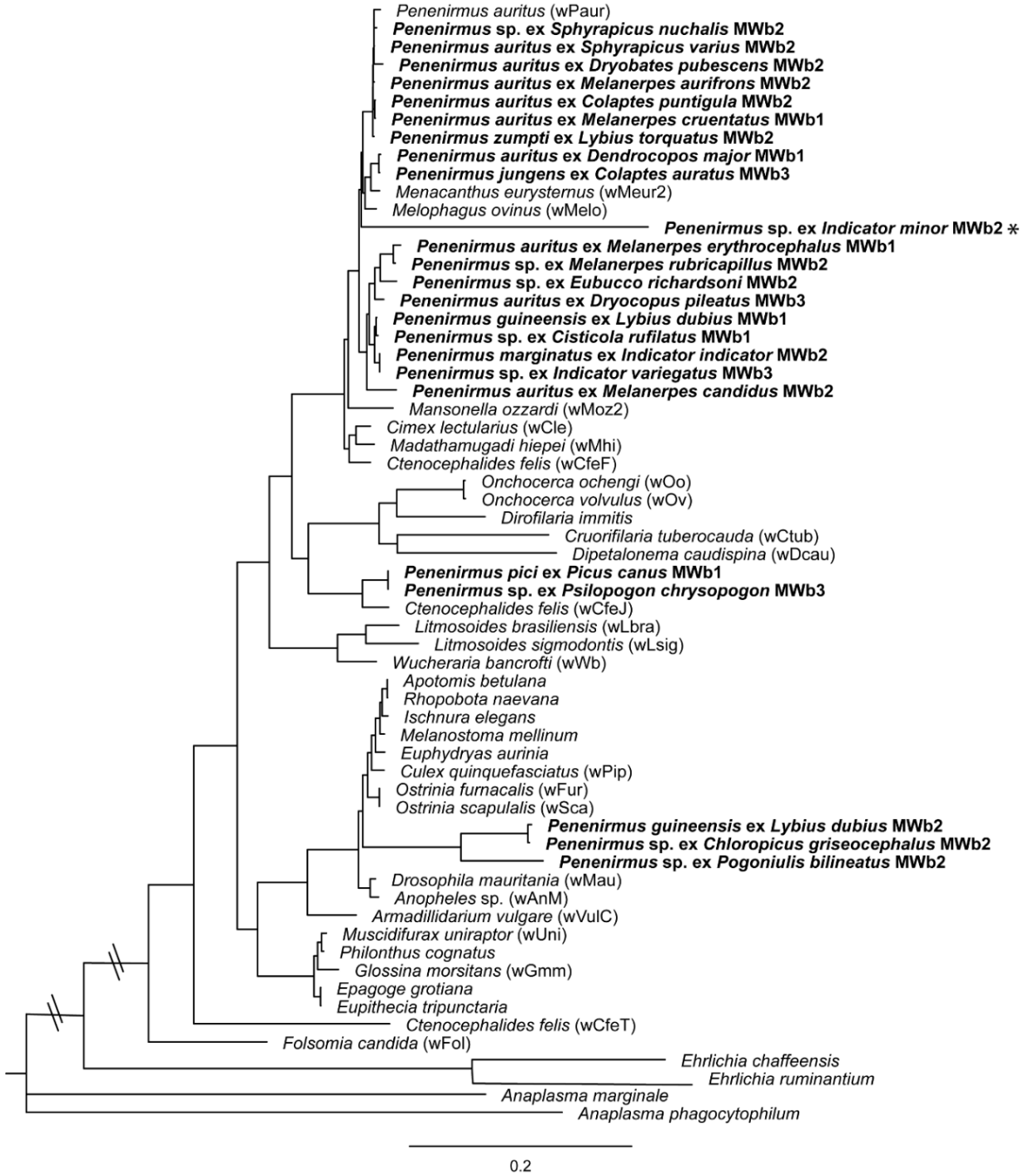

Figure A.5 Phylogeny of the long branch attraction assay of *Penenirmus* sp. ex *Indicator minor* MWb2, sample location is consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1), *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1

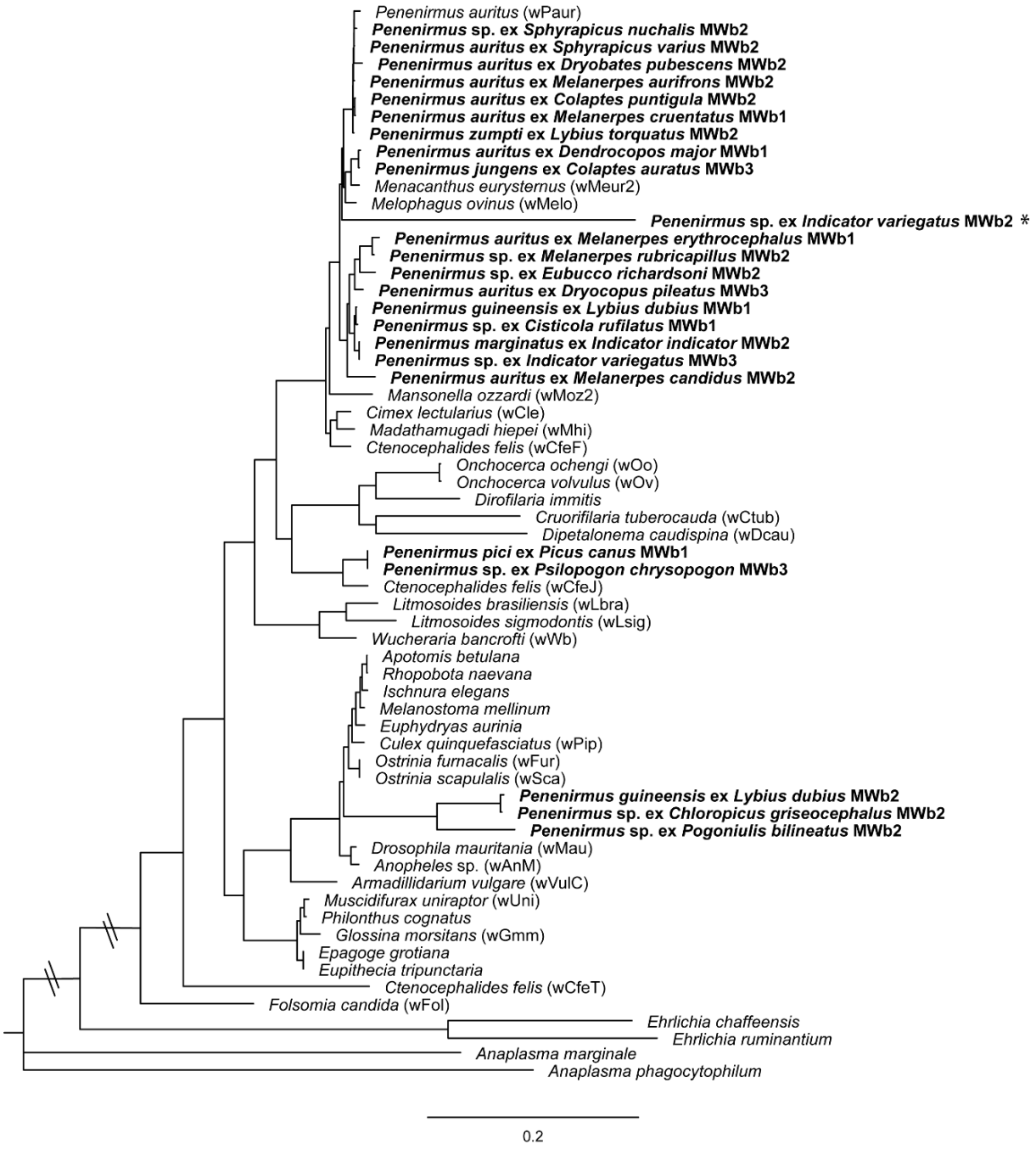

Figure A.6 Phylogeny of the long branch attraction assay of *Penenirmus* sp. ex *Indicator variegatus* MWb2, sample location is consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1), *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1

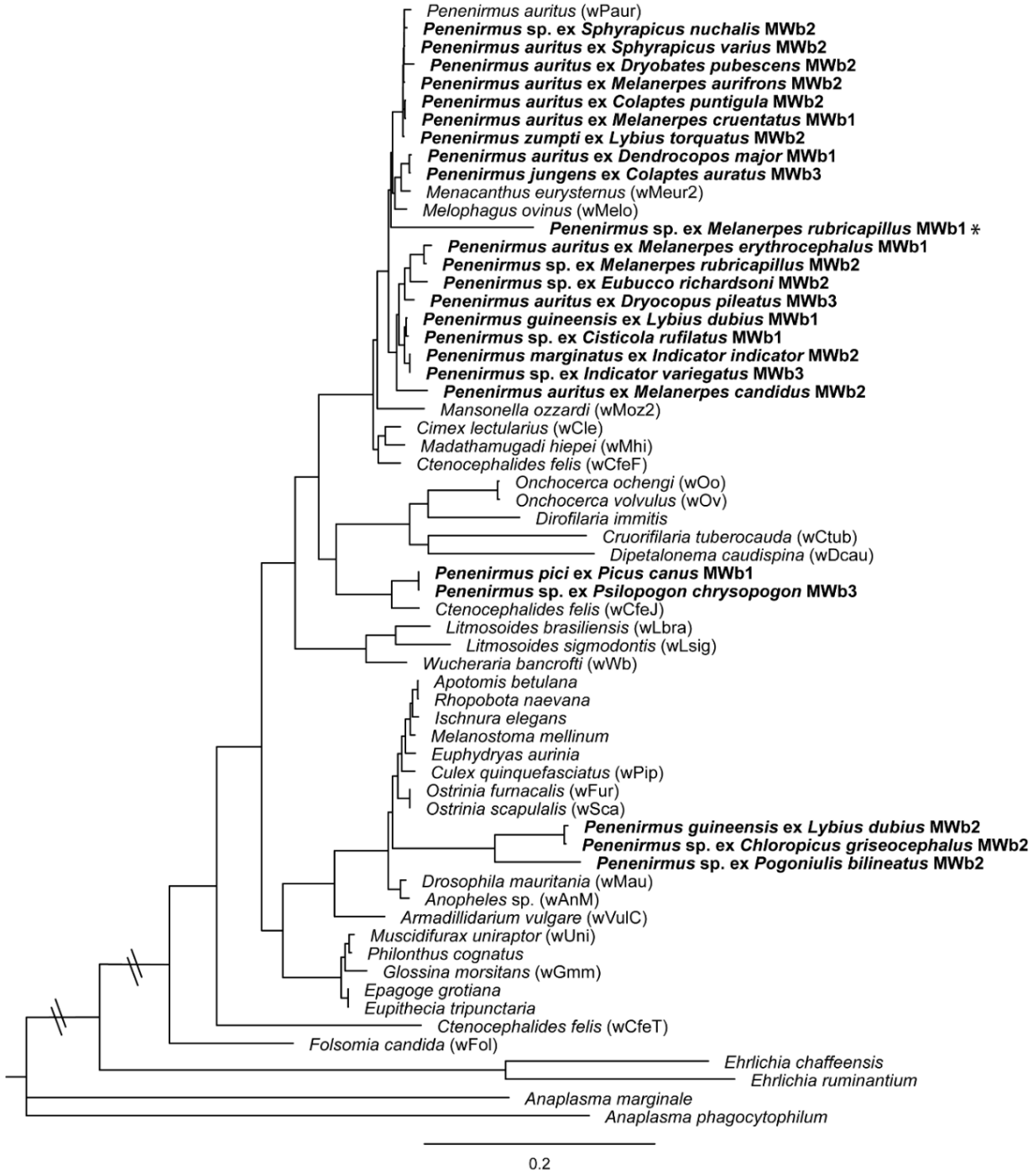

Figure A.7 Phylogeny of the long branch attraction assay of *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, sample location is consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1), *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1

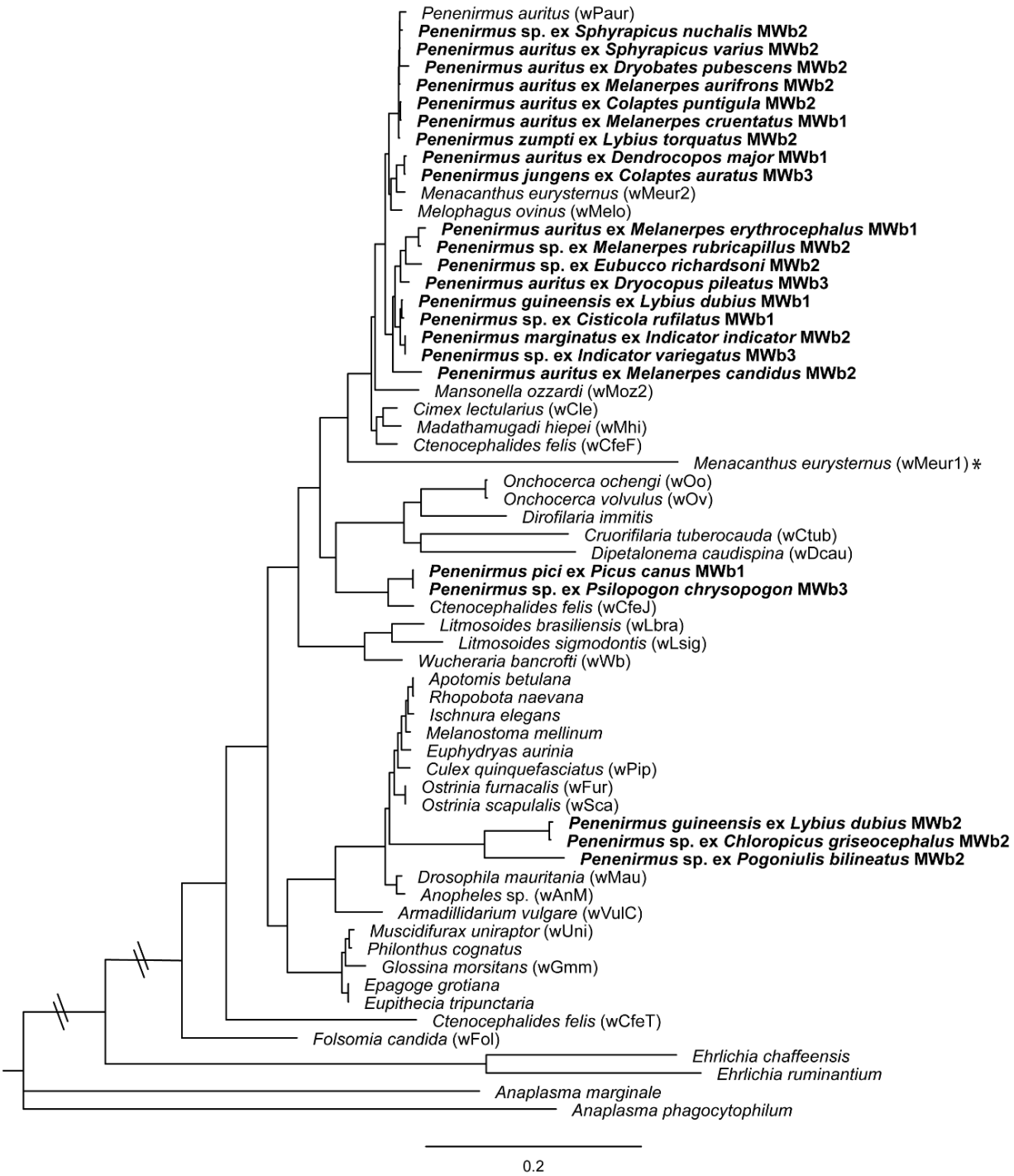

Figure A.8 Phylogeny of the long branch attraction assay of *Menacanthus eurysternus* (wMeur1), sample location is not consistent. *Wolbachia* removed for assay: *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1, and *Turnicola* sp. ex *Turnix pyrrothorax* MWb1.

Figure A.9 Phylogeny of the long branch attraction assay of *Turnicola* sp. ex *Turnix pyrrothorax* MWb1, sample location is not consistent. *Wolbachia* removed for assay: *Menacanthus eurysternus* (wMeur1) *Meromenopon meropis* (wMmer), *Penenirmus auritus* ex *Dryobates pubescens* MWb3, *Penenirmus auritus* ex *Dryocopus pileatus* MWb1, *Penenirmus jungens* ex *Colaptes auratus* MWb2, *Penenirmus* sp. ex *Indicator minor* MWb2, *Penenirmus* sp. ex *Indicator variegatus* MWb2, and *Penenirmus* sp. ex *Melanerpes rubricapillus* MWb1.