Michael J. Skvarla · Ronald Ochoa ·
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Contemporary Acarology

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Editors
Michael J. Skvarla
Department of Entomology
Pennsylvania State University
Agricultural Science & Industries Building
University Park, PA, USA

Jose Carlos Verle Rodrigues
Jardin Botanico Sur
University of Puerto Rico EEA
San Juan, Puerto Rico

Ronald Ochoa
Systematic Entomology Laboratory
USDA Agricultural Research Service
Beltsville, MD, USA

H. Joel Hutcheson
National Centres for Animal Disease
Canadian Food Inspection Agency
Lethbridge, AB, Canada

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Introduction

The Acarological Society of America is the only professional group in the United States and Canada dedicated to the study of mites. The ASA currently has just shy of 100 active members and holds an annual meeting just prior to and concurrently with the Entomological Society of America annual national meeting.

Papers at the annual meeting have historically been presented at two sessions: one that served as a platform for students to showcase their work and another general session for all acarologists that was sometimes organized in honor or memoriam of influential members. However, recent interest has been such that four sessions were required in 2018 and are expected for the 2019 meeting. Presented papers have covered any and every aspect of acarology, including taxonomy, systematics, and phylogenetics; natural history; morphology; field and laboratory techniques, including specialized imaging, microscopy, and genetics processes; and applied issues such as control methods of different pest species.

This volume represents the first time papers presented at an annual ASA meeting have been collected into a print volume. The topics covered herein are diverse and include the first phylogenetic hypothesis for Cunaxidae based on morphological evidence; new field techniques for collecting soft ticks; the use of Ion Torrent Personal Genome Machine to sequence endosymbionts and pathogens directly from hard ticks; investigations into the mycobiome of tetranychoid mites; and the use of low temperature scanning electron microscopy to investigate morphological structures in Tarsonemidae.

This volume represents a collaborative effort by many people, and I thank my co-editors and the authors for their contributions. I also thank the governing board and the membership of the ASA for their support.

Pennsylvania, USA

Michael J. Skvarla
Department of Entomology
Pennsylvania State University
University Park

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Chapter 1
Use of the Ion Torrent PGM for Determining the Genomic Sequences of *Francisella* and *Coxiella*-Like Endosymbionts and *Rickettsia* Directly from Hard Ticks

Gregory A. Dasch, Arunachalam Ramaiah, Zachary C. Holmes, Maria L. Zambrano and T. Brian Shirey

Abstract Microbiome assessments based on amplification and sequencing of their 16S rRNA genes or targeted surveys of specific agents have greatly augmented our understanding of the distribution and identity of bacteria found in ticks. The most prevalent tick bacteria include approximately 10 species, such as *Coxiella*-like (CLE) and *Francisella*-like (FLE) endosymbionts, and spotted fever group *Rickettsia* (SFG). However, genomes of only a few of the many tick agents that are not cultivable have been characterized by very deep next generation sequencing (Illumina HiSeq). As an alternative approach, we have assessed use of the Ion Torrent Personal Genome Machine (IT-PGM) for direct sequencing and assembly of these agent genomes. Agents were identified and quantitated in three genera of ticks (*Amblyomma*, *Dermacentor*, *Rhipicephalus*) by qPCR assays. Geneious, CLC Genomics Workbench, BWA, and SPAdes tools were used for read mapping and assembly of the sequences obtained from size-fractionated libraries made from DNA that was extracted from single alcohol-preserved ticks on single 318 or 314 IT-PGM chips with 200 bp chemistry. Plasmid and chromosome sequences were obtained for six SFGs, four FLEs, and three CLEs. The depth and percentage of genome coverage for symbionts and *Rickettsia* were enhanced by use of Qiagen Repli-G amplification of the DNA used for the libraries. The IT-PGM is a relatively inexpensive sequencing platform for initial genomic characterization of some of the abundant bacterial agents found in hard ticks.

G. A. Dasch (✉) · A. Ramaiah · Z. C. Holmes · M. L. Zambrano
Rickettsial Zoonoses Branch, US Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30333, USA
e-mail: ged4@cdc.gov

T. B. Shirey
Enteric Diseases Laboratory Branch, US Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30333, USA

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1.1 Introduction

The intimate association of bacterial symbionts with arthropods has been recognized for decades. Buchner (1965), the influential father of this field, developed and advanced the hypothesis that nutritional deficiencies in the diet of many arthropods made such associations advantageous for them. Indeed, many such symbionts are maintained as obligate heritable associations in specialized organs called mycetomes and they are passed transovarially and transtadially. Arthropods dependent on diets consisting only of plant sap or vertebrate blood generally have symbionts, a finding consistent with the nutritional hypothesis. The advent of molecular methods for the characterization of these bacteria have led to an expanding number of symbionts whose genomes have been sequenced, thus permitting the development of molecular hypotheses about the precise beneficial physiological and immunological interactions that occur between each host and bacterium (Wernegreen 2015). The range of bacterial symbiotic associations in arthropods ranges from obligatory to facultative; besides nutritional benefits, they can confer secondary effects, including reproductive manipulation of the host, protection of the host against parasites and other enemies, and a repertoire of additional metabolic capabilities that facilitate adaptation to changing environments and variable marginal dietary resources (Moran et al. 2008; Wernegreen 2012; Corbin et al. 2017). The associations can be extremely complex, as two or more bacterial symbionts may be present and may work in concert or in competition in their interactions with their hosts and one may be obligate and the other(s) facultative. Different populations of the same host species may have vastly different complements of symbionts; indeed, the symbionts in different host populations may be genetically variable and confer different phenotypes on those hosts (Henry et al. 2015; O'Fallon 2008; Douglas and Werren 2016). However, many relatively stable associations of dominant heritable primary symbionts with their hosts ("holobionts") remain poorly understood.

One example of this paucity of detailed information is the various associations of ticks with bacterial endosymbionts. Duron et al. (2017) recently summarized information on ten maternally inherited bacteria found in ticks. Four were Gamma-Proteobacteria belonging to the genera *Coxiella*, *Francisella*, *Rickettsiella*, and *Arsenophonus*. The Alpha-Proteobacteria also had members of four genera, including *Wolbachia*, *Rickettsia*, *Midichloria*, and *Lariskella*. The remaining genera included *Spiroplasma* in the Mollicutes and *Cardinium* in the Bacteroidetes.

Direct experimental evidence for the role of tick symbionts in providing essential vitamins, cofactors, and/or amino acids is very limited. Zhong et al. (2007) demonstrated that survival and reproduction of *Amblyomma americanum* was adversely affected by reduction of its *Coxiella*-like agent with antibiotics, a classical method for inferring the essential role of a symbiont in the nutrition of its host. Since *A. amer-*

icanum lineages do not always harbor *R. amblyommatis* while its *Coxiella* is present in nearly 100% of the ticks, that agent appears to be more essential to tick survival. Zhong (2012) has reviewed the literature on this symbiosis. More recently, the value of genomic sequences in supporting a role for vitamin synthesis in 50 symbionts of insects and arachnids has been reviewed by Serbus et al. (2017). The variety of vitamin, cofactor, and amino acid pathways employed in different symbionts suggests that the symbiont-host interactions are probably not uniform in all tick-symbiont associations, so that complete genome sequencing of these agents will be an essential first step in understanding that interaction and how it may be exploited for new approaches to tick control. Furthermore, symbiont-pathogen interactions may be equally diverse (Oliva Chávez et al. 2017).

Although the widespread use of PCR assays for detecting both the symbionts and bacterial pathogens transmitted by ticks (Mixson et al. 2006) has greatly increased our understanding of the distribution of such agents across tick species and habitats, this approach is limited by sampling and sensitivity. For example, the survey of ticks by Epis et al. (2008) for the fascinating intramitochondrial symbiont, *Midichloria mitochondrii*, missed its presence in *Amblyomma americanum*. Deeper sequencing, using 16S microbiome of a larger sample of ticks from additional locations, demonstrated that three genotypes of *M. mitochondrii* were present (Williams-Newkirk et al. 2012). *Wolbachia*, which is estimated to be present in 10% of arthropods, was only recently found to be an important agent in some aphid populations. Consequently, microbiome surveys are more suited than are PCR screens to sensitive evaluation of the prevalence and relative numbers of symbionts in significant size samples of ticks (Williams-Newkirk et al. 2014). We were impressed by the large amounts of *Rickettsia* and *Coxiella* present relative to the other bacteria in the *A. americanum* microbiome. While it was possible for us to routinely isolate *R. amblyommatis*, the commonplace rickettsial agent of this tick (Mixson et al. 2006), in cell culture and to sequence its complete genome (chromosome and 3 plasmids) (strain GAT-3OV, NCBI GCA_000284055.1, Karpathy et al. 2016), the *Coxiella*-like agent (CLE) has not been cultivated. Both Smith et al. (2015) and our laboratory were able to assemble the complete sequence from shotgun metagenomics libraries sequenced on an Illumina HiSeq (Williams-Newkirk et al. 2015; Ramaiah et al. 2017). However, no complete tick genome sequences have been obtained to date, so it is not possible to filter out the reads obtained from the large tick genomes. Both de novo assembly and read mapping sequences compared with near relatives can be used as approaches for recovering the genome sequences from metagenomic data. However, the FLE from *Amblyomma maculatum* (Gerhart et al. 2016), the *Rhipicephalus turanicus* CLE (Gottlieb et al. 2015), and the *Rickettsia* endosymbiont (REIS) from *Ixodes scapularis* (Gillespie et al. 2011) were all assembled as scaffolds from this type of data. A better sequence was obtained for REIS (now *Rickettsia buchneri* sp. nov.) from tick cell culture stocks of the agent (Kurtti et al. 2015). Similarly, *Francisella persica*, the symbiont of the soft tick *Argas persicus* was cultivated and then sequenced (Larson et al. 2016). The goal of the present investigation was to determine whether a less expensive approach could be used to characterize the genomes of tick symbionts than Illumina deep sequencing. We hoped that this approach would provide new

insight into a number of these organisms when neither cell culture nor pure isolates could be obtained. We also wanted to determine if sequences could be obtained from individual ticks since pooled DNA samples had been used previously with the *A. americanum* and *R. turanicus* CLE agents.

We chose the Ion Torrent Personal Genome Machine because it can provide 200–400 bp reads and a variety of inexpensive chips were available. It has been used to assemble relatively large and complete genome sequences of bacteria and for sequencing complete 16S rRNA molecules for microbiome analysis. The Ion library technology we employed could also be applied on the new more automated Ion S5 machine which has a bigger chip and greater automation, should greater read depth and more sampling be required.

1.2 Materials and Methods

1.2.1 Tick Collection and Storage, DNA Extraction and Storage

Host-seeking adult ticks (Table 1.1) were collected by dragging a cloth over vegetation (i.e. flagging) (California, Georgia, Ohio) (Bermúdez et al. 2009; Wikswo et al. 2008). Other ticks (Table 1.1) were purchased from colonies maintained at Oklahoma State University (OSU) under an NIAID (BEI Resources) contract. Origins of the ticks used for initiating the colonies were provided by OSU. Ticks were transferred into ≥70% ethanol for storage at 4 C until their DNA was extracted. Ticks were surface disinfected with sequential washes of 10% bleach and 70% ethanol, then rinsed 3 times in sterile distilled water, frozen in a close-fitting eppdendorf 1.5 ml centrifuge tube in a LN2 bath and pulverized with a Kontes pestle prior to DNA extraction with the Promega Wizard SV Genomic DNA Kit or Qiagen DNA easy tissue kit (Williams-Newkirk et al. 2014). DNA extracts were stored at 4 °C until screening by PCR for different agents and subsequent library preparation.

1.2.2 PCR Testing of Samples for Their Content of CLE, FLE, and Spotted Fever Group Rickettsia

Rickettsia were initially detected and quantitated in all tick DNAs by SsoFastEVA-Green qPCR (BioRad) amplification of an *omp*B fragment (Eremeeva et al. 2003), *Amblyomma americanum* and *A. maculatum* were also tested for *Rickettsia*, *Ehrlichia chaffeensis*, and *E. ewingii* with the multiplex TaqMan assay using iTaq Universal Probes Supermix (BioRad) of Killmaster et al. (2014). The identity of agents in positive ticks was determined and quantitated by subsequent qPCR with an *R. parkeri*-specific TaqMan assay (Jiang et al. 2012) or by DNA sequencing of a 5′ fragment of

Table 1.1 Summary of tick samples and metagenomic sequencing results on Ion Torrent-PGM

Tick samples	USA origin	Stage	Total number of useable reads	Percentage of useable total reads (%)	Median length of reads	IT-PGM chemistry	Repli-G method
Amblyomma maculatum Amac21	Georgia	Female	4,007,976	62	113 bp	200	No
Amblyomma maculatum Amac50	Oklahoma	Female	3,810,034	43	137 bp	200	No
Dermacentor variabilis Dvar22	Georgia	Female	6,211,538	66	244 bp	200	No
Dermacentor variabilis Dvar100	Ohio	Female	3,717,748	74	218 bp	200	No
Dermacentor variabilis DvarSlov	Virginia	Adult	5,810,262	67	227 bp	200	No
Dermacentor occidentalis DoccEMCF9	California	Female	6,518,864	69	243 bp	200	No
Dermacentor andersoni DandM9	Montana	Male	4,690,550	50	220 bp	400	No
Amblyomma americanum Aam46	Georgia	Male	5,242,310	52	272 bp	200	No
Amblyomma americanum Aam10SC	Georgia	Female	3,490,856	35	138 bp	200	Single cell
Rhipicephalus sanguineus RhsangF23	Oklahoma	Female	4,490,171	54	123 bp	200	Ultrafast
Dermacentor variabilis DvarF4M4	Oklahoma	Female	2,891,366	51	80 bp	200	Mini
Dermacentor variabilis DvarF4SC4	Oklahoma	Female	72,986[a]	14.6	206 bp	200	Single cell
Dermacentor variabilis DvarF1SC1	Oklahoma	Female	19,602[a]	6.2	107 bp	200	Single cell

[a]Ion Torrent 314 chips, rest 318 chips

ompA (Bermúdez et al. 2009; Wikswo et al. 2008). *Francisella*-like endosymbionts (FLE) were detected and quantitated by EVA-Green qPCR using the 16S rRNA primers NC-Fran16S-F and NC-Fran16S-R of Dergousoff et al. (2012). *Coxiella*-like endosymbionts were detected by standard PCR with 16S primers (Scoles 2004) or detected and quantitated by the *fus*A TaqMan assay with primers AAMFUSA-F and AAMFUSA-R and probe AAMFUSA-Pr of Jasinkas et al. (2007). All EVA-Green and TaqMan assays were performed on a CFX96 Touch Real-Time PCR Detection System (BioRad).

1.2.3 DNA Sequencing of OmpA Amplicons

Amplicons were purified from amplicon bands cut from 2% ethidium bromide-stained agarose gels with the Wizard SVGel and PCR clean-up system (Promega) and sequenced with the ABI BigDye Terminator v3.1 cycle sequencing kit (ABI) according to the manufacturer's recommendations on an ABI 3130*xl* genetic analyzer (Bermúdez et al. 2009). Reads from both forward and reverse directions were analyzed and assembled with DNAStar Lasergene and the sequence identify evaluated with BLASTn on the NCBI server.

1.2.4 IT-PGM Library Preparation and Sequencing

Tick DNA extracts were quantitated with the Qubit HS-DNA assay (Life Technologies). Tick DNA (250 ng) was treated with the Ion Xpress Fragment Library kit (Life Technologies #4471269). Following treatment (37 C for 8.5 min) with the Ion Shear Plus Reagents Kit (#4471248) and purification with the DNA Clean and Concentrator-10 Kit (Zymo Research), the Ion Plus Fragment Library Kit (Life Technologies #4471252) was then used to nick-repair the DNA and ligate the DNA (25 C for 15 min, 72 C for 5 min) to IT-PGM primers. Following purification and concentration with the DNA and Concentrator-10 kit, 330 bp fragments were selected and eluted with the E-Gel Agarose Gel Electrophoresis System on Size Select 2% gels (Life Technologies). The size-selected ligated fragments were then amplified with Platinum PCR SuperMix High buffer and Library Amplification Primer Mix from the Ion Plus Fragment library kit (Denature 5 min at 95 C, 8 cycles of 15 s at 95 C, 15 s at 58 C, and 1 min at 70 C). The library was purified and concentrated again with the DNA and Concentrator-10 kit and quantified with the Qubit HS-DNA assay. Templates were generated with 100 pM of library and beads enriched on the Ion Torrent One Touch 2 system with 200 or 400 bp V2 Template kits; sequencing was performed with the Ion Torrent Personal Genome Machine, according to the manufacturer's instructions.

1.2.5 Repli-G Procedures

Qiagen Repli-G mini (M) (>10 ng, 50 ul, 16 h at 30 C), ultrafast (UF) (>10 ng, 30 ul, 1.5 h at 30 C), or single cell (SC) (10–100 pg, 50 ul, 16 h 30 C) kits were used to amplify tick DNA in 25 μl reactions prior to preparation and sequencing of IT-PGM libraries using the full fragmentation and templating procedure described above.

1.2.6 Bioinformatic Analysis of IT-PGM Sequences

Default Ion Torrent PGM parameters for exclusion of sequences (polyclonal, low quality, and adapter dimer ISPs) were used. The reads were first analyzed with Geneious 8.05 (Kearse et al. 2012) by read mapping to available genome assemblies for FLE (*A. maculatum*, *D. variabilis*, *Francisella persica*), CLE (*A. maculatum*, *Rhipicephalus turanicus*, *R. sanguineus*) and known US spotted fever group *Rickettsia* agents and their plasmids (*R. montanensis*, *R. rhipicephali* CWPP 3-7-F6, *R. bellii* OSU85-389, *R. parkeri* Portsmouth, *R. amblyommatis* GAT-3OV). The Geneious contigs were then analyzed by BLASTn and annotated by Prokka (Seemann 2014). The reads were also analyzed by use of BWA v0.7.12 (Li and Durbin 2010) for read mapping to libraries of *Francisella*, *Coxiella*, *Rickettsia* genome sequences available at NCBI or to individual genome sequences. The mapped reads were extracted using SAMtools v1.3.1 (Li et al. 2009) and subsequently these reads were assembled into contigs using SPAdes v3.10.1 (Bankevich et al. 2012) or CLC Genomics Workbench v9.5.2 (https://www.qiagenbioinformatics.com/). Assembled contigs were then analyzed by BLASTn, Prokka, and by tBLASTn using NCBI protein lists for individual genomes as well as the limited sequences available from "*Candidatus* Rickettsia andeanae".

1.3 Results

1.3.1 Library Preparation and IT-PGM Sequence Reads Obtained

Thirteen metagenome libraries were prepared using Ion Torrent shearing and ligation reagents and templating on the One Touch 2 for sequencing on the Ion Torrent Personal Genome Machine (IT-PGM) (Table 1.1). Most of the difficulties that were encountered with achieving consistency in the recovery of useable sequencing reads were related to the manual loading of the 314 and 316 Ion chips and sizing of the fragmented libraries. The small 7 μl volume used on the 314 chip and limited number of sequence reads on that chip also made accurate library preparation and quantitation more crucial; however, several later loadings of other arthropod single

cell Repli-G libraries resulted in a greater recovery of useable reads than the initial two experiments described here (178,750 useable reads, 189 bp median read length; 275,890 useable reads, 211 bp median read length). For unknown reasons, more unusable polyclonal sequences were also obtained with the smaller chip; however, this chip is convenient and less expensive for gaining experience with alternative library preparation methods, including size fractionation and amplification methods. We obtained more consistent results with library yields by cutting out specific band sizes on SYBR-Green stained standard 2% agarose gels than with the E-gel system and by using the Promega SVS gel purification method to recover the DNA from the agarose, as used for DNA sequencing of amplicons. The variable results obtained with median read lengths on the E-gels can be directly attributed to uncertainty about when to stop the gel migration and recover the correct size products. We improved this procedure by using several different DNA ladders (the BioRad EZLoad 500 bp ladder was particularly useful) to achieve greater certainty about recovery of the correct sizes of fractionated and ligated SYBR-Green stained DNA either directly from the E-gel or cut from standard 2% agarose gels on a blue light box.

1.3.2 Repli-G Enhancement of Read Depth

Although alcohol-preserved single adult males or females were used to make all of the libraries sequenced here, tick nymphs and larvae (even as pools derived from the same engorged female) contain substantially less DNA than the adults and we wished to avoid introducing more polymorphisms by pooling samples. In other studies, we initially found that even adult head lice had too little DNA for accurate quantitation and library preparation, so we evaluated the use of mini, ultrafast, and single cell Repli-G kits from Qiagen to amplify the amounts of starting DNA to have enough for library preparations. Five of the 13 samples sequenced were amplified with one or another of these kits (Table 1.1). We found the single cell kit to be the most reliable and flexible because it can be used with the least input DNA and larger volumes of the dilute DNA obtained from our conventional DNA extractions (typically 100–200 μl volumes). Mini kits provided the least amplified material and less latitude in initial DNA volume and concentration. The ultrafast kit offered little advantage other than cost over the single cell kit and did not work as well with low concentration DNA extracts (not shown). In some cases we concentrated all of the low concentration starting material (with the DNA purification and concentration kits from Zymo) to 20 μl, but adequate yields of amplified material could be obtained even with samples below the sensitivity of the Qubit HS-DNA kit by using the single-cell Repli-G kit. We did not observe any difficulties during bioinformatic analysis of the Repli-G samples when compared to the standard libraries (Tables 1.2 and 1.3).

Table 1.2 Effect of Repli-G amplification on the relative recovery of sequence reads mapping with Geneious against FLE agents

		#Reads mapping to FLE references			FLE % total reads			
Ticks (State) Sex	**# IT-PGM Reads**	**AmacFLE**	**DvarHS28 FLE**	**Fpersica**	**AmacFLE**	**DvarHS28 FLE**	**Fpersica**	**Avg**
Aam10SC (GA) F	34,90,856	55	113	151	0.0016	0.0032	0.0043	0.0030
Aam46 (GA) M	52,42,310	179	51	54	0.0034	0.0010	0.0010	0.0018
Amac21 (GA) F	40,07,976	36393	31786	33512	0.9080	0.7931	0.8361	0.8457
Amac50 (OK) F	38,10,034	5036	1448	4557	0.1322	0.0380	0.1196	0.0966
DandM9 (MT) M	46,90,550	10758	11277	86596	0.2294	0.2404	1.8462	0.7720
DoccEMCF9A (CA) F	65,18,864	26002	26242	25124	0.3989	0.4026	0.3854	0.3956
Dvar22 (GA) F	62,11,538	60304	33810	15948	0.9708	0.5443	0.2567	0.5906
Dvar100 (OH) F	37,17,748	8034	12537	12104	0.2161	0.3372	0.3256	0.2930
DvarSlov (VA) adult	58,10,262	13859	9195	14054	0.2385	0.1583	0.2419	0.2129
DvarF4M4 (OK) F	28,91,366	51567	63247	59814	1.7835	2.1874	2.0687	2.0132
DvarF1SC1 (OK) F	19,602	1316	1063	1103	6.7136	5.4229	5.6270	5.9212
DvarF4SC4 (OK) F	72,986	3171	2709	2586	4.3447	3.7117	3.5431	3.8665
RhsangF23UF6 (OK) F	44,90,171	16974	14944	53130	0.3780	0.3328	1.1833	0.6314

Green: Positive identification of FLE with wide genomic coverage (large contigs or nearly complete)
Orange: Positive presence of FLE sequences but with low and interspersed coverage (many contigs)
Pink: Positive maps of reads to FLE but with very low coverage and big gaps (Few contigs)
White: Negative for FLE, reads map to sites of low complexity

Table 1.3 Effect of Repli-G amplification on the relative recovery of sequence reads mapping with Geneious against CLE agents

		#Reads mapping to CLE references			CLE % total reads			
Ticks (State) Sex	**# IT-PGM Reads**	**AamGA-CLE**	**AamOK-CLEAA**	**CLE-Rt**	**AamGA-CLE**	**AamOK-CLEAA**	**CLE-Rt**	**Avg**
Aam10SC (GA) F	34,90,856	14668	14646	1449	0.4202	0.4196	0.0415	0.2937
Aam46 (GA) M	52,42,310	539	3508	1721	0.0103	0.0669	0.0328	0.0367
Amac21 (GA) F	40,07,976	198	198	631	0.0049	0.0049	0.0157	0.0085
Amac50 (OK) F	38,10,034	3754	3755	5765	0.0985	0.0986	0.1513	0.1161
DandM9 (MT) M	46,90,550	190	195	2773	0.0041	0.0042	0.0591	0.0224
DoccEMCF9A (CA) F	65,18,864	111	111	829	0.0017	0.0017	0.0127	0.0054
Dvar22 (GA) F	62,11,538	13746	13749	16116	0.2213	0.2213	0.2595	0.2340
Dvar100 (OH) F	37,17,748	443	443	854	0.0119	0.0119	0.0230	0.0156
DvarSlov (VA) adult	58,10,262	19	19	1299	0.0003	0.0003	0.0224	0.0077
DvarF4M4 (OK) F	28,91,366	39141	39140	64474	1.3537	1.3537	2.2299	1.6458
DvarF1SC1 (OK) F	19,602	787	788	1012	4.0149	4.0200	5.1627	4.3992
DvarF4SC4 (OK) F	72,986	2290	2298	3001	3.1376	3.1485	4.1117	3.4660
RhsangF23UF6 (OK) F	44,90,171	694425	695342	2248213	15.4654	15.4859	50.0697	27.0070

Green: Positive identification of CLE with wide genomic coverage (large contigs or nearly complete)
Orange: Positive presence of CLE sequences but with low and interspersed coverage (many contigs)
Pink: Positive maps of reads to CLE but with very low coverage and big gaps (Few contigs)
White: Negative for CLE, reads map to sites of low complexity

1.3.3 *Tick Samples with* Francisella*-Like Endosymbionts (FLE)*

An EVA-Green PCR assay detecting a fragment of 16S rRNA was used to screen samples of 6 species of ticks for the presence of *Francisella*-like agents. Some samples (4 species, 5 samples, Table 1.4) with lower Ct values (20–25 using 1/200th of DNA extracted from a tick) were selected for further analysis from a larger set of samples obtained at the same time; these 5 samples, as well as two additional samples (DvarSlov and Amac21), were found to contain FLE by IT-PGM sequence analysis. None of these samples had been amplified by Repli-G prior to sequencing. Geneious 10.0.9 was used initially to map and assemble reads using the *A. maculatum* FLE (OK), which was in 7 scaffolds (Gerhart et al. 2016); *Francisella persica* VR331, which was isolated from the soft tick *Argas persicus* and subsequently cultivated in vitro before sequencing (Larson et al. 2016); and a partial sequence we have obtained and assembled from Illumina HiSeq sequencing of a Georgia female of *Dermacentor variabilis*, obtained from the same site as Dvar22GA (Dasch et al. 2016). Read mapping of the other tick sequence libraries without FLE provided an indication of the sensitivity and background of this approach to identifying FLE-specific sequences in these metagenomic libraries (Table 1.5).

The two *A. americanum* ticks (Aam10SC, Aam46) without detectable FLE by qPCR had low numbers of sequences, coverage, and percentage of genomes covered relative to those with FLE by Geneious, but higher numbers of reads, as well as background, in negative ticks were obtained with CLC (Table 1.4). Geneious 10.0.9 mapped more reads than Geneious 8.0.5 but while the read coverage increased in most cases, the percentage of the genome mapping remained largely unchanged (not shown). Neither the FLE qPCR Ct value nor the number of useable reads had any obvious relationship to the number of reads mapping to any of the three assembled FLE that are of similar size and have significant sequence homology (Table 1.4). In general, the total number of reads and percentage coverage were also prone to be misleading because some read maps had high read coverage at specific chromosome locations (appearing as spikes in the maps) but these were not consistent between the three reference FLE used for mapping. This inconsistency can be seen clearly in Fig. 1.1, especially in Fig. 1.1f, where abundant reads were clustered but only for *F. persica* VR331. These aberrant regions did not interfere with interpreting the data. For example, Amac21GA had similar high quality read coverage for all three FLE maps (Fig. 1.1a–c), while *Dermacentor andersoni* (Fig. 1.1d–f) had many gaps and lower and less uniform coverage along the chromosome, particularly with respect to *F. persica* (Fig. 1.1f). Therefore, the percentage of the FLE sequence with read coverage was more consistent with all three FLE reference sequences than was the number of reads mapping or median read coverage; that parameter gave the best indication of how much related FLE sequence had been obtained. While sequences unique to the new FLE agents (i.e., not present in any of the reference genomes), cannot be evaluated accurately without full genome coverage and assembly, it is clear that the Amac21 FLE agent from Georgia mapped similarly to that of the *A.*

Table 1.4 Bioinformatic analysis of IT-PGM sequence libraries for the presence of CLE and FLE agent sequences

Ticks (State) Sex	# IT-PGM Reads	FLE-16S qPCR	AmacFLE (OK) (G)	AmacFLE (OK) (CLC)	DvarHS28 FLE (GA) (G)	DvarHS28 FLE (GA) (CLC)	F persica VR331 (G)	F persica VR331 (CLC)
Size of ref seq (bp)			15,56,261	15,56,261	15,75,539	15,75,539	15,16,676	15,16,676
Aam10SC (GA) F	34,90,856	neg	55 (0.0x, 0.1%)	13,392 (2.8x, 11.4%)	113 (0.0x, 0.1%)	16,385 (3.2x, 11.4%)	151 (0.0x, 0.1%)	14,381 (3.0x, 11.7%)
Aam46 (GA) M	52,42,310	neg	179 (0.0x, 0.2%)	6,681 (2.6x, 6.1%)	51 (0.0x, 0.2%)	7,871 (2.9x, 6.1%)	54 (0.0x, 0.2%)	6,849 (2.6x, 6.3%)
Amac21 (GA) F	40,07,976	nd	36,393 (2.3x, 79.4%)	28,425 (2.3x, 80.9%)	31,786 (1.8x, 74.7%)	31,042 (2.3x, 76.5%)	33,512 (2.1x, 70.6%)	27,070 (2.3x, 73.3%)
Amac50 (OK) F	38,10,034	Ct 25.1	5,036 (0.4X, 12.4%)	7,110 (1.6x, 17.3%)	1,448 (0.1x, 11.6%)	8,203 (1.7x, 16.5%)	4,557 (0.4x, 10.5%)	7,412 (1.7x, 16.1%)
DandM9 (MT) M	46,90,550	Ct 25.9	10,758 (0.8x, 25.8%)	16,092 (2.2x, 34.4%)	11,277 (0.8x, 26.0%)	18,638 (2.3x, 34.6%)	86,596 (23.5x, 23.6%)	16,315 (2.3x, 33.0%)
DoccEMCF9A (CA) F	65,18,864	Ct 25.4	26,002 (1.8x, 67.5%)	12,559 (1.9x, 69.1%)	26,242 (1.9x, 68.4%)	16,741 (2.1x, 69.9%)	25,124 (1.9x, 62.6%)	13,469 (2.0x, 65.9%)
Dvar22 (GA) F	62,11,538	Ct 22.0	60,304 (11.6x, 26.9%)	6,271 (1.4x, 29.8%)	33,810 (4.1x, 28.3%)	9,809 (1.6x, 31.0%)	15,948 (0.9x, 24.5%)	7,141 (1.6x, 28.2%)
Dvar100 (OH) F	37,17,748	Ct 21.5	8,034 (0.7x, 37.9%)	12,230 (0.8x, 38.2%)	12,537 (1.0x, 40.5%)	8,920 (1.6x, 42.0%)	12,104 (0.8x, 35.8%)	7,180 (1.6x, 38.4%)
DvarSlov (VA) adult	58,10,262	nd	13,859 (0.7x, 24.2%)	5,482 (1.4x, 26.9%)	9,195 (0.5x, 25.6%)	14,054 (0.7x, 25.7%)	14,054 (0.7x, 25.7%)	6,455 (1.5x, 25.9%)
DvarF4M4 (OK) F	28,91,366	neg	51,567 (4.4x, 2.8%)	88,370 (8.4x, 28.7%)	63,247 (5.6x, 3.0%)	97,637 (9.0x, 28.5%)	59,814 (4.7x, 3.2%)	93,438 (8.6x, 30.0%)
DvarF1SC1 (OK) F	19,602	neg	1,316 (0.2x, 14.3%)	1,066 (2.1x, 1.3%)	1,063 (0.7x, 50.0%)	1,309 (0.2x, 13.0%)	1,103 (0.7x, 51.5%)	1,357 (0.4x, 16.2%)
DvarF4SC4 (OK) F	72,986	neg	3,171 (1.0x, 34.3%)	1,903 (2.7x, 2.3%)	2,709 (1.4x, 64.2%)	3,306 (1.1x, 32.9%)	2,586 (1.3x, 61.3%)	3,263 (0.9x, 30.7%)
RhsangF23UF6 (OK) F	44,90,171	neg	16,974 (1.9x, 1.8%)	26,820 (7.1x, 14.4%)	14,944 (1.6x, 1.7%)	34,617 (8.1x, 14.2%)	53,130 (4.3x, 2,1%)	30,442 (7.8x, 15.2%)

Green: Positive identification of FLE with wide genomic coverage (large contigs or nearly complete)
Orange: Positive maps of reads to FLE but with very low coverage and big gaps (Few contigs)
Pink: Positive maps of reads to FLE but with very low coverage and big gaps (Few contigs)
White: Negative for FLE, reads map to sites of low complexity
(G) reads mapping with Geneious 10.0.9
(CLC) reads mapping with CLC Workbench 8
Boxes: number of reads mapping (median read coverage per base, percentage of reference genome mapped)
nd: not done

Table 1.5 Bioinformatic analysis of IT-PGM sequence libraries for the presence of CLE agent sequences

Ticks (State) Sex	# IT-PGM Reads	AamGA-CLE (G)	CLEAA (OK) (G)	CLEAA (OK) (CLC)	CLE-RsangF23 Final (G)	CLE-Rturanicus (G)	CLE-Rturanicus (CLC)
Size of ref seq (bp)		656,933	656,901	656,901	1,115,981	1,733,840	1,733,840
Aam10SC (GA) F	3,490,856	14,668 (2.0x, 35.9%)	14,646 (2.0x, 35.8%)	8,485 (1.9x, 43.6%)	13,015 (1.3x, 1.3%)	1,449 (0.2x, 1.0%)	15,074 (3.3x, 12.4%)
Aam46 (GA) M	5,242,310	539 (0.1x, 3.6%)	3,508 (0.5x, 3.6%)	3,338 (2.3x, 9.7%)	1,081 (0.1x, 0.5%)	1,721 (0.6X, 0.3%)	7,449 (3.7x, 5.4%)
Amac21 (GA) F	4,007,976	198 (0.0x, 0.8%)	198 (0.0x, 0.8%)	3,742 (3.5x, 7.8%)	750 (0.1x, 0.5%)	631 (0.1x, 0.4%)	7,568 (4.0x, 5.4%)
Amac50 (OK) F	3,810,034	3,754 (0.9x, 0.2%)	3,755 (0.9x, 0.2%)	2,990 (2.9x, 6.5%)	7,067 (2.5x, 0.2%)	5,765 (0.7x, 0.2%)	8,296 (6.1x, 4.8%)
DandM9 (MT) M	4,690,550	190 (0.1x, 0.2%)	195 (0.1x, 0.2%)	6,923 (3.3x, 12.1%)	5,577 (3.8x, 0.2%)	2,773 (0.5x, 0.2%)	17,180 (5.6x, 9.5%)
DoccEMCF9A (CA) F	6,518,864	111 (0.0x, 0.6%)	111 (0.0x, 0.6%)	2,704 (4.5x, 4.7%)	18,822 (1.5x, 0.4%)	829 (0.1x, 0.3%)	7,166 (8.2x, 3.3%)
Dvar22 (GA) F	6,211,538	13,746 (3.3x, 0.3%)	13,749 (3.3x, 0.3%)	2,310 (2.9x, 4.4%)	15,254 (1.5X, 0.2%)	16,116 (2.2x, 0.2%)	6,706 (7.6x, 3.2%)
Dvar100 (OH) F	3,717,748	443 (0.1x, 0.8%)	443 (0.1x, 0.8%)	1,838 (6.2x, 3.7%)	613 (0.1x, 0.5%)	854 (0.1X, 0.3%)	4,578 (9.3x, 2.2%)
DvarSlov (VA) adult	5,810,262	19 (0.0x, 0.3%)	19 (0.0x, 0.3%)	1,962 (2.9x, 3.8%)	831 (0.2x, 0.2%)	1,299 (0.1x, 0.2%)	5,969 (7.7x, 2.8%)
DvarF4M4 (OK) F	2,891,366	39,141 (6.0x, 10.0%)	39,140 (6.0x, 10.0%)	43,190 (8.4x, 37.1%)	54,919 (4.7x, 70.0%)	64,474 (3.6x, 62.5%)	96,820 (4.5x, 69.3%)
DvarF1SC1 (OK) F	19,602	787 (0.6x, 30.6%)	788 (0.6x, 29.7%)	482 (2.3x, 1.5%)	860 (0.4x, 14.0%)	1012 (0.3x, 14.1%)	662 (1.7x, 1.0%)
DvarF4SC4 (OK) F	72,986	2,290 (1.4x, 38.6%)	2,298 (1.5x, 41.4%)	996 (2.9x, 3.7%)	2,569 (0.7x, 24.0%)	3,001 (0.7x, 23.3%)	1,921 (1.6x, 5.1%)
RhsangF23UF6 (OK) F	4,490,171	694,425 (170.8, 66.1%)	695,342 (170.6x, 66.1%)	254,141 (88.4x, 60.5%)	1,675,572 (219.5X, 100%)	2,248,213 (191.0, 97.2%)	2,366,498 (192.8x, 95.6%)

Green: Positive identification of CLE with wide genomic coverage (large contigs or nearly complete)
Orange: Positive presence of CLE sequences but with low and interspersed coverage (many contigs)
Pink: Positive maps of reads to CLE but with very low coverage and big gaps (few contigs)
White: Negative for CLE, reads map to sites of low complexity
(G) reads mapping with Geneious 10.0.9
(CLC) reads mapping with CLC Workbench 8
boxes: number of reads mapping (median read coverage per base, percentage of reference genome mapped)
nd: not done

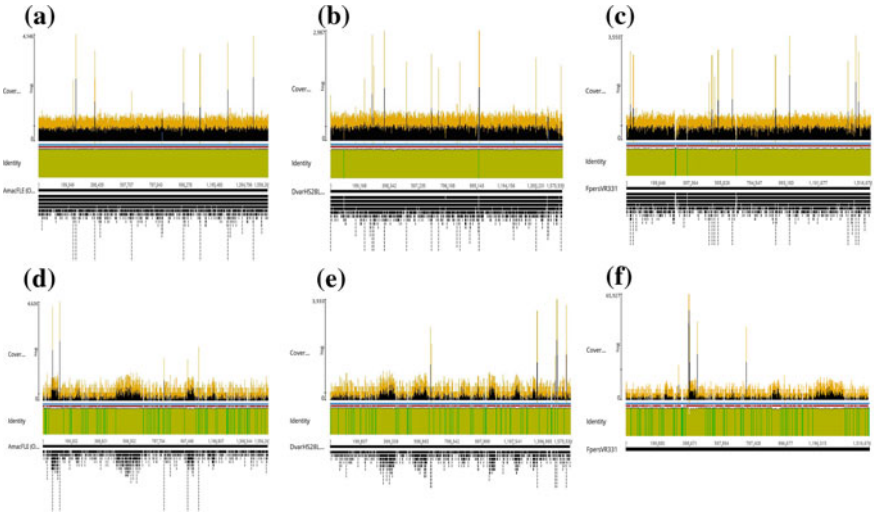

Fig. 1.1 Geneious read mapping of Amac21 and DandM9 IT-PGM sequences against *Francisella*—like endosymbiont (FLE) reference genomes. **a**–**c** Amac21, **d**–**f** DandM9; **a**, **d** *Amblyomma maculatum* OK FLE; **b**, **e** *Dermacentor variabilis* HS28 FLE; **c**, **f** *Francisella persica* VR331. Identity bar over coverage at that coordinate: greeny-brown (100%), green (low or no coverage). Coverage: log scale, black mean coverage, orange maximum coverage, blue bar minimum coverage greater than 5, red bar, contiguous coverage, black bars-reads mapped (**f** not displayed because of very high coverage spikes)

maculatum FLE agent from the Oklahoma tick colony and slightly less well to the *D. variabilis* HS28 FLE or *F. persica*. This difference was confirmed by Prokka analysis of the predicted ORFs from their consensus sequences. However, while all of the positive libraries had reads distributed along the reference target FLE sequences, only 10–80% of the reference sequence was obtained in the assemblies. CLC generally gave comparable positive coverage of read maps to Geneious with the same specific individual reference FLE sequences but the reads and contigs recovered had no predictable trend to each. This inconsistency is probably due to the relatively low genome coverage obtained because the majority of the sequence reads obtained were actually from the tick's large genome. However, the *D. andersoni* and *D. occidentalis* FLE appear to have genomes similar in size to the three reference FLE agents.

A higher percentage of the target FLE genomes was recovered by mapping IT-PGM sequences with a database comprised of all complete *Francisella* genomes and the FLE sequences by use of BWA, followed by SPAdes or CLC workbench assembly (not shown). This approach increased the expected genome coverage of novel FLE by Prokka annotation. While identification of much of the expected complement of core genes had been obtained, many protein sequences were fragmented, so it was difficult to discern if those proteins were pseudogenes undergoing degradation or if this breakage was solely an artefact of insufficient read depth.

Four libraries (RhsangF23, DvarF4M4, DvarF4SC4, DvarF1SC1) from three ticks from the Oklahoma tick colony provided surprising data from the FLE read mapping. All were negative by FLE 16S qPCR. Their DNAs had been Repli-G amplified, in the hope that their bacterial sequences would be selectively amplified relative to the host tick DNA, as well as to obtain more DNA for library preparation. This selective amplification appeared to have occurred, since the average of the percentage of FLE-mapped reads compared to the total number of reads obtained was higher with those samples treated with the Repli-G kits (Table 1.2). The RhsangF23 sequences mapped to the FLE reference sequences but in greatest abundance at specific locations across the chromosome (Fig. 1.2a, b), in contrast to the broader coverage seen with homologous mapping of Amac21 (GA) to its highly related *A. maculatum* OK FLE agent. This abundance and site restriction suggested that the RhsangF23 bacterium was not a FLE agent and that another organism with sequence similarity at the core genome level was being detected. This conclusion is consistent with the FLE qPCR results. A similar result was also obtained for the DvarF4M4 sequences, with even higher FLE coverage (Fig. 1.3a, b). These results are also consistent with the idea that these sequences are not spurious artefacts due to the Repli-G amplification.

Despite the incomplete genome coverage, several features of these previously uncharacterized FLE genomes in *D. andersoni*, *D. variabilis*, and *D. occidentalis* could be discerned. (1) The genome sizes of the novel FLE, as well as for the one from Georgia *A. maculatum*, clearly approached that of the few FLE sequences now available. (2) The sequences obtained were accurate and permitted identification of SNPs and INDELs in comparison to the three assembled FLE available. (3) The base composition and contiguity of a number of adjacent genes could be confirmed as well as intergenic sequences between contiguous genes. (4) tRNAs and much of the rRNA sequences could be extracted. (5) Housekeeping genes being analyzed for a FLE-specific endosymbiont Multiple Locus Sequence Typing (MLST) protocol could be compared to identify conserved primer sites for amplifying these genes. On the other hand, the IT-PGM data was insufficient for completing the assembly of the 7 contigs for *A. maculatum* OK FLE or for improved scaffolding of the partial *D. variabilis* (Dvar28) FLE agent assembly we had obtained previously from Illumina HiSeq data.

1.3.4 Tick Samples with Coxiella*-Like Endosymbionts (CLE)*

Three CLE sequences were available to us for read mapping the IT-PGM sequences (Table 1.5). Two sequences were the highly genome-reduced CLE agent from *Amblyomma americanum*, one from a pool of Oklahoma State University colony ticks (CLEAA-OK) (Smith et al. 2015) and the other a highly related sequence from our own work: AamGA-CLE was assembled from Illumina Hi-Seq 2500 sequences obtained from a single female tick from GA (Williams-Newkirk et al. 2015). The two sequences are nearly the same length but differ in 360 sites (39 INDELS, 321 SNPs) scattered over the chromosome (Ramaiah et al. 2017). The other, much larger CLE

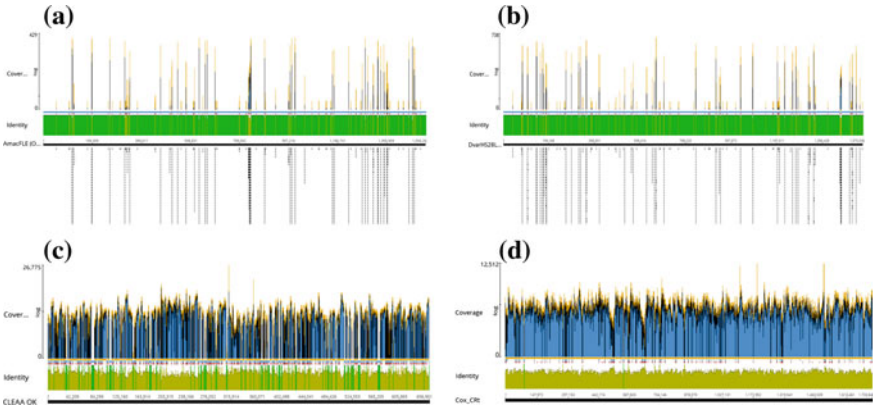

Fig. 1.2 Geneious read mapping of RhsangF23 IT-PGM sequences against *Francisella*—like endosymbiont (FLE) and *Coxiella*-like endosymbiont (CLE) reference genomes. **a** *Amblyomma maculatum* OK FLE; **b** *Dermacentor variabilis* HS28 FLE, *Francisella persica* VR331; **c** *Amblyomma americanum* OK CLE (CLEAA); **d** *Rhipicephalus turanicus* FLE. See Fig. 1.1 for other details

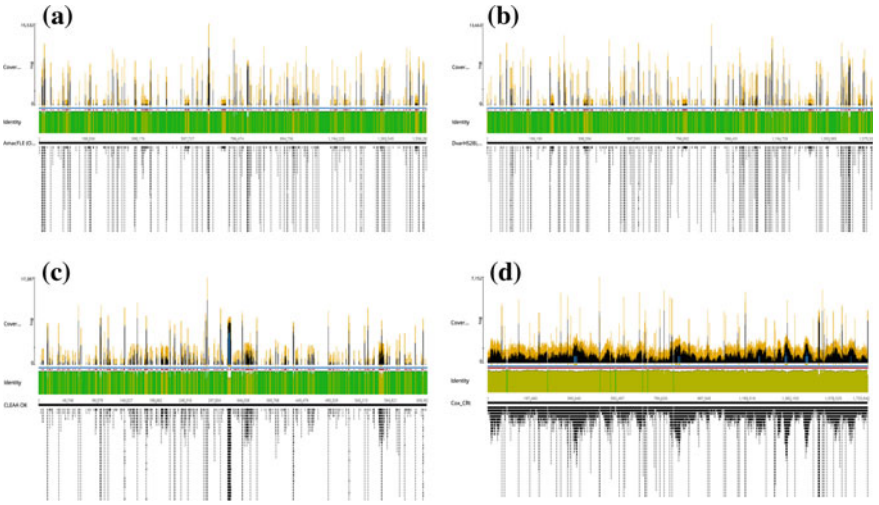

Fig. 1.3 Geneious read mapping of DvarF4M4 IT-PGM sequences against *Francisella*—like endosymbiont (FLE) and *Coxiella*-like endosymbiont (CLE) reference genomes. **a** *Amblyomma maculatum* OK FLE; **b** *Dermacentor variabilis* HS28 FLE, *Francisella persica* VR331; **c** *Amblyomma americanum* OK CLE (CLEAA); **d** *Rhipicephalus turanicus* FLE. See Fig. 1.1 for other details

sequence is from a pool of tissues from nine *Rhipicephalus turanicus* from Israel and sequence polymorphisms were detected between CLE in individual field collected ticks (Gottlieb et al. 2015). It is also smaller than the genome of the pathogen *Coxiella burnetii*. We could detect a large portion of the CLE agent in both of our samples of *A. americanum* [one from GA obtained at the same time as the sample used for Illumina sequencing, another from the same Oklahoma State University colony used for CLEAA by Smith et al. (2015)] as well as a larger but partial CLE sequence assembled from RhsangF23 (Ramaiah et al. 2017). We used the CLC workbench assembly we got for CLE-Rs from the latter tick to do read mapping of all the other samples with Geneious. As was expected, the CLE-Rs assembly remapped in Geneious to a mean coverage of 216.2 and 100% of this reference sequence (Table 1.5). CLE has not been reported previously from *Dermacentor variabilis* and, while we detected it in both of the Oklahoma tick colony samples, we also detected it in the 3 *D. variabilis* from Virginia, Ohio, and Georgia (Table 1.5). The CLE in both *Rhipicephalus sanguineus* and the two Oklahoma samples of *D. variabilis* (F4M4/F4SC4 and F1SC1) also mapped to the 3 FLE sequences as previously noted (Table 1.5), which is unsurprising, given that both agents are in the gamma proteobacteria. Similarly, the FLE agents found in eastern *D. variabilis* from Virginia, Ohio, and Georgia and in *D. andersoni*, *D. occidentalis*, and *Amblyomma maculatum* were also detected by read mapping with CLE but at low levels. In some cases, the relative numbers of reads mapped to CLE and FLE for a tick permitted direct determination of the correct agent but it was obvious from the Geneious read mapping that the FLE agent sequences were more closely related to the sequences obtained for both RhsangF23 (Fig. 1.2c, d) and for DvarF4M4 (Fig. 1.3c, d). Indeed, both of these CLE agents were much closer to the *Rhipicephalus turanicus* lineage than the smaller 657 Kb *A. maculatum* CLE and they both had reads mapping to much of the 2.5 times larger CRt genome sequence. However, while highly related, these two new CLE agents do not appear to be identical because the RhsangF23 agent mapped more closely to the A. *americanum* CLE agent (Fig. 1.2c) as did DvarF4M4 (Fig. 1.3c). It is possible that the greater read coverage for RhsangF23 may account for this difference, since DvarF4M4 mapped to 70% of CLE-Rsanguineus (Table 1.5). PROKKA, BLASTn, and tBLASTn analyses of the two assembled contigs clearly confirmed their correct assignments to *Coxiella* and not to *Francisella* (not shown).

The CLE Geneious read mapping data again demonstrated an apparent selective enhancement of the number of bacterial reads following Repli-G amplification (Table 1.3).

1.3.5 Amblyomma americanum *Tick Samples Containing* Rickettsia amblyommatis *and AamGA-CLE*

The genome sequence of the reference strain *Rickettsia amblyommii* GAT-30V [recently renamed as *R. amblyommatis*—(Karpathy et al. 2016)] was obtained from DNA purified from purified rickettsiae obtained from a cell cultivated isolate from

the ovaries of a female tick (by G. Dasch) from Georgia (Panola Mountain State Park) by Roche 454 and Sanger sequencing (NCBI Assembly GCA_000284055.1). It contains a 1,407,796 bp chromosome and, atypically for species of *Rickettsia*, 3 plasmids (pRam1-18,263 bp, pRam2-22,851 bp, and pRam3-31,974 bp) (Table 1.6). Two other GA tick samples from Barnesville (Midland Piedmont), GA were obtained in June 2014; one adult female (Aam10) underwent Repli-G single cell amplification and the other adult male (Aam46) was not amplified before IT-PGM sequencing (Tables 1.1 and 1.6). Neither sample had high levels of *Rickettsia amblyommatis* (Ct 25.3 and 24.3, respectively) when compared to many GA ticks (Ct of 20 is common) but good assemblies of both the chromosome and all three plasmids were obtained with both the Geneious 10.0.9 and the BWA-SPAdes/CLC assembly pipelines (Table 1.6). The Geneious maps for the chromosome and plasmids are shown in Fig. 1.4 for Aam46, which had greater read depth, more useable reads, and a larger median sequence read length (Table 1.1). While amplification with Repli-G did not appear to improve the final results in this case (because the library preparation and chip loading was suboptimal for Aam10), much less of the original tick DNA extracted was needed for the analysis so it was still available for other PCR analyses or additional deep sequencing. The coverage of all 3 plasmids was 100% for both ticks and nearly identical with that of the reference isolate GAT-30V (Table 1.7). The plasmids read mapped much the same with either Geneious or CLC analysis but the lower *R. amblyommatis* coverage was better analyzed by CLC. The same bias was observed for the CLE agent found in each tick, but the relative amount detected was substantially higher in Aam10 than in Aam46 (Table 1.7). The relative amounts of *R. amblyommatis* and CLE have been found to vary in different adult ticks by both quantitative PCR and by microbiome analysis (Zhong et al. 2007; Williams-Newkirk et al. 2014). In Oklahoma *Amblyomma americanum*, the males often lack detectable *R. amblyommatis* and they are lower in abundance in general than the CLE (Zhong et al. 2007). Georgia ticks generally have consistently high amounts (Williams-Newkirk et al. 2014); the Aam46 Georgia male used here clearly had abundant *Rickettsia*. Whether the relative abundance of *R. amblyommatis* and CLE can also affect the relative sequence read coverage of each agent obtained on the IT-PGM is difficult to know without more testing of samples with known relative amounts of each agent. Since both agents are so abundant in most ticks, that could be evaluated on the IT-PGM by barcoding of samples so they can be pooled and assayed on the same sequencing chip.

1.3.6 Amblyomma maculatum *Tick Samples Containing* Rickettsia parkeri *or* R. andeanae *and Amac-FLE*

The genome sequence of the human reference isolate *Rickettsia parkeri* Portsmouth was obtained from a human case of spotted fever rickettsiosis that occurred in the Tidewater area of Virginia (Whitman et al. 2007). It has a genome size similar to those of other core (classic) spotted fever rickettsiae, including *R. rickettsii*, *R. peacockii*,

Table 1.6 Comparison of the chromosome and plasmid assemblies obtained with Geneious (G) and CLC genomics workbench (CLC) from IT-PGM sequence libraries obtained from two Georgia *Amblyomma americanum* female ticks with those of the reference Georgia isolate *Rickettsia amblyommatis* GAT-3OV and the CLEAA agent from the Oklahoma *A. americanum* colony tick

R. amblyommatis domain	GAT-3OV size	Aam10 (G)	Aam10 (CLC)	Aam46 (G)	Aam46 (CLC)
Chromosome	1,407,796 bp	263,648 bp	399,706 bp	988,360 bp	1,017,362 bp
pRam1	18,263 bp	18,263 bp	18,263 bp	18,267 bp	18,263 bp
pRam2	22,851 bp	22,851 bp	22,844 bp	22,851 bp	22,855 bp
pRam3	31,974 bp	31,930 bp	31,965 bp	31,974 bp	32,005 bp
	AamOK CLEAA	Aam10 (G)	Aam10 (CLC)	Aam46 (G)	Aam46 (CLC)
CLEAA-AamOK domain	656,901 bp	235,285 bp	286,832 bp	23,779 bp	64,192 bp
Total IT-PGM reads		**3,490,856**		**5,242,310**	

Table 1.7 Geneious 10.0.9 bioinformatic analysis of IT-PGM sequence libraries for the presence of Rickettsia agent chromosome and plasmid sequences

Ticks	# IT-PGM Reads	*R. ambylommatis* GAT-3OV	pRam1	pRam2	pRam3	*R. peacockii* Rustic	pRpr1
Size of ref		14,07,796	18,263	22,851	31,974	12,88,492	26,406
Amac21 (GA)	40,07,976	24,697 (1.3x, 54.2%)	8 (0.0x, 4.5%)	0 (0.0x, 0%)	1 (0.0x, 0.3%)	17,880 (1.2x, 57.9%)	1 (0.0x, 0.4%)
Amac50 (OK)	38,10,034	14,270 (1.3x, 60.4%)	1,389 (10.6x, 87.0%)	597 (3.6x, 38.4%)	786 (3.4x, 46.7%)	13,167 (1.2x, 60.4%)	165 (0.9x, 16.3%)
DandM9	46,90,550	7,706 (0.5x, 0.1%)	0 (0.0x, 0%)	1 (0.0x, 0.1%)	1 (0.0x, 0.1%)	43 (0.0x, 0.1%)	0 (0.0x, 0.0%)
DoccEMCF9A(CA)	65,18,864	28,491 (3.1x 74.3%)	20,134 (94.7x, 82.2%)	593 (6.4x, 21.3%)	18,008 (38.5x, 15.7%)	27,993 (2.8x, 76.5%)	94 (0.9x, 3.3%)
Aam10 (GA)	34,90,856	20,938 (2.2x, 18.7%)	172,806 (1519.7x, 100.0%)	97,707 (740.6x, 100.0%)	60,630 (491.0x, 99.9%)	28,507 (4.1x, 18.1%)	22,997 (232.3x, 22.9%)
Aam46 (GA)	52,42,310	25,943 (7.0x, 70.2%)	322,013 (4352.8x, 100.0%)	278,577 (3110.8x, 100.0%)	138,142 (1553.1x, 100.0%)	40,377 (12.3x, 65.6%)	59,287 (979.6x, 25.7%)
RhsangF23 UF6(OSU)	44,90,171	107,536 (9.2x, 87.0%)	1 (0.0x, 0.8%)	19 (0.1x, 1.4%)	21 (0.1x, 1.3%)	103,933 (10.0x, 90.9%)	32,840 (155.8, 0.3%)
Dvar22 (GA)	62,11,538	13,249 (1.1x, 0.1%)	1 (0.0x, 0.1%)	0 (0.0x, 0%)	0 (0.0x, 0%)	9 (0.0x, 0.1%)	0 (0.0x, 0%)
Dvar100 (OH)	37,17,748	65,754 (9.1x, 68.4%)	1,090 (14.5x, 14.4%)	1,845 (18.4x, 33.3%)	3,038 (22.0x, 24.0%)	63,901 (9.7x, 73.2%)	1,556 (13.7x, 24.7%)
DvarSlov(VA)	58,10,262	12 (0.0x, 0.1%)	0 (0.0x, 0%)	0 (0.0x, 0%)	0 (0.0x, 0%)	9 (0.0x, 0.1%)	0 (0.0x, 0%)
DvarF4 M4(OK)	28,91,366	1,884,592 (137.3x, 88.9%)	152 (0.8x, 47.5%)	977 (5.4x, 8.1%)	2,464 (8.8x, 9.1%)	1,807,884 (142.4x, 93.2%)	30 (0.1x, 0.8%)
DvarF1 SC1 (OK)	19,602	17,223 (1.7x, 71.9%)	277 (2.9x, 72.1%)	206 (1.0x, 32.7%)	226 (2.1x, 42.1%)	16,583 (1.8x, 73.3%)	294 (3.0x, 54.2%)
DvarF4 SC4 (OK)	72,986	61,536 (8.5x, 90.0%)	11 (2.2x, 62.5%)	277 (5.4x, 87.3%)	460 (4.3x, 52.2%)	59,263 (9.0x, 95.0%)	572 (7.7x, 69.8%)

Green: Positive identification of *Rickettsia* with wide genomic coverage (large contigs or nearly complete)
Orange: Positive presence of *Rickettsia* sequences but with low and interspersed coverage (many contigs)
Pink: Positive maps of reads to *Rickettsia* but with very low coverage and big gaps (few contigs)
White: Negative for *Rickettsia* , reads map to sites of low complexity
boxes: number of reads mapping (median read coverage per base, percentage of reference genome mapped)

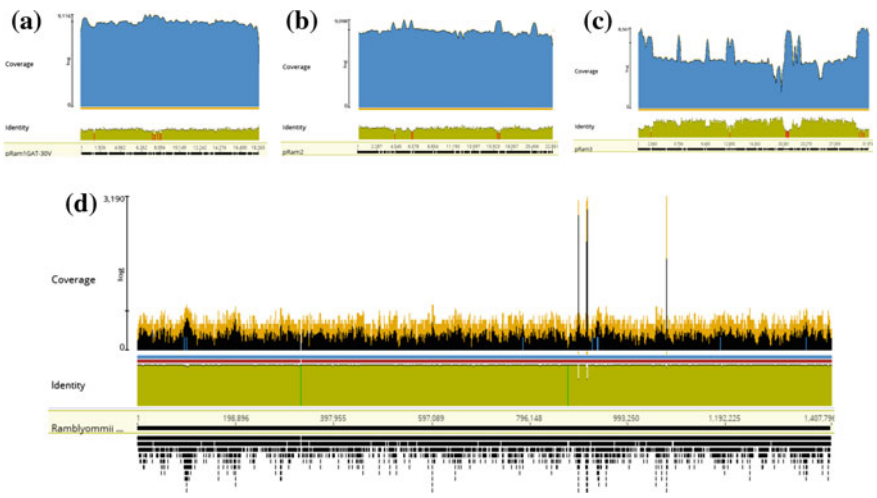

Fig. 1.4 Geneious read mapping of Aam46 IT-PGM sequences against *Rickettsia amblyommatis* GAT-30V reference genome. **a** Plasmid *pRam1;* **b** plasmid *pRam2*; **c** plasmid *pRam3*; **d** chromosome. See Fig. 1.1 for other details

and *R. philipii* in the USA, *R. sibirica* in Europe and Asia, and *R. conorii* in Europe, Africa, and the Indian subcontinent, and it lacks a plasmid (Table 1.8). *Amblyomma maculatum* are widely infected with *R. parkeri* at high rates of infection (Pagac et al. 2014). We analyzed a female tick (Amac21) collected in June 2014 from Barnesville (Midland Piedmont), GA (Table 1.1), which was shown to be infected with *R. parkeri* and the *A. maculatum* FLE agent by qPCR. A slightly better assembly was obtained with CLC and the total number of reads mapping to Portsmouth was greater than observed with other spotted fever group rickettsiae (Tables 1.8, 1.9 and 1.10). As found for *R. amblyommatis* in *A. americanum* ticks, the IT-PGM read coverage was not sufficient for full genome assembly but BLAST analysis of protein contigs identified in Prokka confirmed the tick contained *R. parkeri*. The second *A. maculatum* female tick (Amac50), obtained from the Oklahoma State University Tick Colony, contained a spotted fever *Rickettsia* that was not detected by the *R. parkeri* specific assay and was presumed to be "*Candidatus* R. andeanae" (hereafter *R. andeanae*), which is common in ticks from Oklahoma and present with varying abundance in other populations of *A. maculatum* (Fornadel et al. 2011; Jiang et al. 2012; Paddock et al. 2015). Because no genome sequence has been obtained yet for *R. andeanae*, in part because it has proven difficult to obtain an isolate in sustained cell culture (Luce-Fedrow et al. 2012), we employed Geneious and CLC read mapping of the IT-PGM Amac50 sequences against a number of other spotted fever rickettsiae with available genome sequences in order to extract its reads and to assemble its genome. We confirmed our hypothesis about the identity of its *Rickettsia* by comparing our partial sequence to several gene fragments available for *R. andeanae* (*sca*4-GU395298, *omp*B-GU395297, AY652981, *omp*A-GU395296, AY590796) and affirmed their ori-

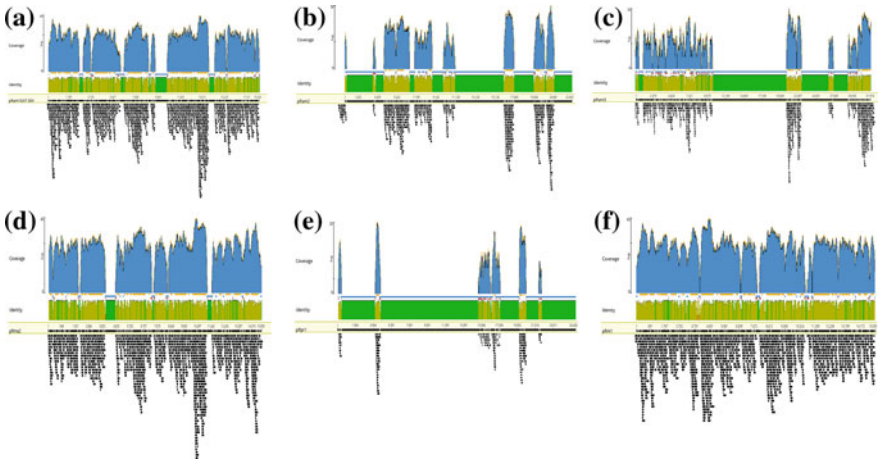

Fig. 1.5 Geneious read mapping of Amac50 IT-PGM sequences against selected *Rickettsia* plasmids. **a–c** *R. amblyommatis* GAT-30V pRam1, pRam2, pRam3; **d** R. massiliae AZT80 pMa2; **e** *R. peacockii* Rustic pRpr1; **f** *R. rhipicephali* 3-7-F6 CWPP pRrh1. See Fig. 1.1 for other details

gin. The prior phylogenetic placement of *R. andeanae* in a clade with *R. montanensis*, *R. rhipicephali*, *R. massiliae*, and *R. amblyommatis* was confirmed as well. Since all of these species except *R. montanensis* contain a plasmid, we also mapped the Amac50 sequence reads against six plasmid sequences from this clade and to the plasmid of *R. peacockii* Rustic with Geneious 10.0.9 (Fig. 1.5). This analysis confirmed that *R. andeanae* has a plasmid that is most related to those of the similar plasmids from *R. rhipicephalus* 3-7-F6 (pRrh1) and *R. massiliae* AZT80 (pRma2) (Fig. 1.5d, f; Tables 1.7, 1.9 and 1.10); pRam1, the smallest *R. amblyommatis* plasmid and one most similar to pRh1 and pRma2, also mapped to many Amac50 reads (Fig. 1.5a), while both pRam2 and pRam3 had regions of high homology (Fig. 1.5b, c) to the plasmid (name proposed here as pRan1) of *R. andeanae* but it had very incomplete coverage. Similarly, *R. peacockii* plasmid pRpR1 had only a few domains with high similarity (Fig. 1.5e). Because pRrh1, pRma2, and pRam1 all had domains with no homology to *R. andeanae* reads, it is likely that pRan1 has a unique sequence. It is probably at least 15–16 Kb in size (98.2% coverage of the 15 Kb plasmid pRrh1, whereas the 18 Kb plasmid pRam1 had 87.0% coverage) and belongs to this widespread small rickettsial plasmid family.

The sequence reads mapping to the FLE agent present in both the Amac21 GA and Amac50 OK ticks confirmed that both of these agents were similar to the AmacFLE agent assembled from Illumina HiSeq (7 contigs) by Gerhart et al. (2016). Although the number of *Rickettsia* reads mapping to chromosomes of *R. parkeri* and *R. andeanae*, respectively, were similar in the two ticks, 4.7–6.4 times as many FLE reads mapped from Amac21 data than from Amac50 (Table 1.4). Whether the presence of the plasmid pRan1 reduced FLE detection in Amac50 is not known but that

Table 1.8 Comparison of the chromosome assemblies obtained with Geneious (G) and CLC genomics workbench (CLC) from IT-PGM sequence libraries obtained from two *Amblyomma maculatum* female ticks with those of reference isolates of spotted fever group *Rickettsiae* and the CLEAA agent from the Oklahoma *A. americanum* colony tick. *R. parkeri* identified in tick (Green). Candidatus R. andeanae, closest relative, identified in tick (orange)

Rickettsia domain	Size	Amac21GA (G)	Amac21GA (CLC)	Amac50-OK (G)	Amac50-OK (CLC)
R. parkeri Portsmouth	1,300,386 bp	802,742 bp	837,142 bp	807,327 bp	
R. rhipicephali 3-7-F6 CWPP	1,290,368 bp	743,661 bp		812,157 bp	846,610 bp
R. montanensis OSU 85-930	1,279,798 bp	738,210 bp		811,465 bp	845,102 bp
R. massiliae AZT80	1,263,719 bp	732,041 bp		792,829 bp	827,566 bp
R. amblyommatis GAT-3OV	1,407,796 bp	763,164 bp		850,057 bp	888,178 bp
		Amac21 (G)	Amac21 (CLC)	Amac50 (G)	Amac50 (CLC)
FLE-AmacOK domain	1,556,261 bp	1,236,324 bp	1,259,409 bp	192,960 bp	269,992 bp
Total IT-PGM reads		**4,007,976**		**3,810,034**	

Table 1.9 Geneious 10.0.9 bioinformatic analysis of IT-PGM sequence libraries for the presence of *Rickettsia* agent chromosome and plasmid sequences

Ticks	# IT-PGM Reads	*R. parkeri* Portsmouth	*R. rhipicephali* 3-7-F6 CWPP	pRrh1	*R. massiliae* AZT80	pRma2
Size of ref		13,00,386	12,90,368	15,099	12,73,719	15,000
Amac21 (GA)	40,07,976	26,442 (2.2x, 61.7%)	25,639 (1.8x, 57.6%)	5 (0.0x, 3.6%)	24,871 (1.7X, 57.9%)	6 (0.0X, 4.2%)
Amac50 (OK)	38,10,034	13,771 (1.4x, 62.1%)	13,843 (1.4x, 62.9%)	15,979 (11.9x, 98.2%)	13,676 (1.5x, 62.7%)	1,200 (11.2x, 91.4%)
DandM9	46,90,550	38 (0.0x, 0.1%)	42 (0.0x, 0.1%)	0 (0.0x, 0%)	46 (0.0x, 0.1%)	0 (0.0x, 0%)
DoccEMCF9A(CA)	65,18,864	28,433 (2.9x, 79.1%)	28,826 (2.8X, 83.2%)	18,203 (110.8x, 98.8%)	28408 (2.7x, 81.3%)	2,600 (44.4x, 96.5%)
Aam10 (GA)	34,90,856	20,689 (2.5x, 17.4%)	21,957 (2.8x, 17.2%)	136,383 (1371.2 (99.3%)	15,845 (1.3x, 17.1%)	123,975(1348.8, 98.3%)
Aam46 (GA)	52,42,310	23,948 (7.1x, 66.6%)	32,257 (10.5x, 66.0%)	243,649 (3783.6, 99.3%)	13,710 (2.9x, 66.0%)	245,441 (3774.0x, 98.4%)
RhsangF23 UF6(OSU)	44,90,171	82,212 (8.6x, 92.7%)	100,535 (9.3x, 93.2%)	3 (0.0x, 3.0%)	106,231 (10.4x, 94.9%)	4 (0.0x, 2.5%)
Dvar22 (GA)	62,11,538	8 (0.0x, 0.1%)	7 (0.0x, 0.1%)	0 (0.0x, 0%)	7 (0.0x, 0.1%)	0 (0.0x, 0%)
Dvar100 (OH)	37,17,748	63,770 (9.4x, 71.7%)	64,035 (9.6x, 72.8%)	654 (10.7x, 10.5%)	63,058 (10.0x, 73.1%)	647 (10.6x, 10.5%)
DvarSlov(VA)	58,10,262	9 (0.0x, 0.1%)	0 (0.0x, 0%)	0 (0.0x, 0%)	8 (0.0x, 0.1%)	0 (0.0x, 0%)
DvarF4 M4(OK)	28,91,366	1,865,192 (144.5, 94.3%)	1,795,425 (144.9x, 95.2%)	222 (1.4x, 62.0%)	1,851,895 (146.8x, 96.7%)	139 (1.0x, 58.7%)
DvarF1 SC1 (OK)	19,602	16,997 (1.8x, 74.3%)	16,891 (1.8x, 75.0%)	220 (1.7x, 17.9%)	16,898 (1.8x, 76.2%)	77 (1.3x, 23.0%)
DvarF4 SC4 (OK)	72,986	60,813 (9.0x, 94.6%)	60,229 (9.0x, 95.8%)	34 (1.5x, 75.2%)	60,530 (9.2x, 97.3%)	95 (4.7x, 70.2%)

Green: Positive identification of *Rickettsia* with wide genomic coverage (large contigs or nearly complete)
Orange: Positive presence of *Rickettsia* sequences but with low and interspersed coverage (many contigs)
Pink: Positive maps of reads to *Rickettsia* but with very low coverage and big gaps (few contigs)
White: Negative for *Rickettsia* , reads map to sites of low complexity
Boxes: number of reads mapping (median read coverage per base, percentage of reference genome mapped)

Table 1.10 Geneious 10.0.9 bioinformatic analysis of IT-PGM sequence libraries for the presence of *Rickettsia* agent chromosome and plasmid sequences

Ticks	# IT-PGM Reads	*R. montanensis OSU85-930*	*R. bellii* OSU85-389	pRbe1	*R. rickettsii* SSmith
Size of ref		12,79,798	15,28,980	48,775	12,57,710
Amac21 (GA)	40,07,976	25,275 (2.0x, 57.7%)	18,921 (1.1X, 19.9%)	0 (0.0x, 0%)	24,523 (1.6x, 60.1%)
Amac50 (OK)	38,10,034	13,765 (1.3x, 63.4%)	11,148 (1.4x, 23.9%)	425 (1.2x, 17.2%)	13,586 (1.4x, 62.9%)
DandM9	46,90,550	45 (0.0x, 0.1%)	327 (0.1x, 0.2%)	1 (0.0x, 0.1%)	41 (0.0x, 0.1%)
DoccEMCF9A(CA)	65,18,864	28,270 (2.9x, 78.7%)	23,708 (1.6x, 36.2%)	288 (1.3x, 7.4%)	28,279 (2.8x, 80.2%)
Aam10 (GA)	34,90,856	20,632 (2.5x, 17.4%)	30,515 (4.4x, 5.1%)	53,180 (311.4, 31.2%)	20,326 (2.5x, 17.2%)
Aam46 (GA)	52,42,310	23,498 (6.8x, 66.3%)	55,922 (18.0x, 28.4%)	126,218 (1128.9x, 33.4%)	23,257 (7.1x, 66.2%)
RhsangF23 UF6(OSU)	44,90,171	109,892 (11.5x, 97.1%)	82,974 (5.8x, 55.2%)	1 (0.0x, 0.1%)	101,051 (9.3x, 94.7%)
Dvar22 (GA)	62,11,538	8 (0.0x, 0.1%)	13,234 (1.3x, 0.1%)	0 (0.0x, 0%)	9 (0.0x, 0.1%)
Dvar100 (OH)	37,17,748	65,136 (9.7x, 74.7%)	98,731 (12.9x, 99.1%)	18,926 (82.4x, 100.0%)	61,925 (9.5x, 92.4%)
DvarSlov(VA)	58,10,262	11 (0.0x, 0.1%)	13 (0.0x, 0.1%)	1 (0.0x, 0.0%)	9 (0.0x, 0.1%)
DvarF4 M4(OK)	28,91,366	1,925,309 (151.1x, 97.8%)	1,491,092 (106.2x, 69.3%)	2,345 (6.4x, 5.5%)	1,831,678 (145.6x, 96.5%)
DvarF1 SC1 (OK)	19,602	17,561 (1.9x, 78.4%)	12,571 (1.2x, 51.5%)	234 (1.7x, 34.7%)	16,742 (1.8x, 76.3%)
DvarF4 SC4 (OK)	72,986	62,416 (9.4x, 97.7%)	51,493 (7.2x, 88.6%)	658 (4.5x, 34.2%)	59,920 (9.2x, 96.5%)

Green: Positive identification of *Rickettsia* with wide genomic coverage (large contigs or nearly complete)
Orange: Positive presence of *Rickettsia* sequences but with low and interspersed coverage (many contigs)
Pink: Positive maps of reads to *Rickettsia* but with very low coverage and big gaps (few contigs)
White: Negative for *Rickettsia* , reads map to sites of low complexity
Boxes: number of reads mapping (median read coverage per base, percentage of reference genome mapped)

might have occurred since the *R. rhipicephali* chromosome and plasmid had similar read coverage in the Amac50 IT-PGM library despite their 85-fold difference in size.

1.3.7 *Analysis of Bacterial Sequences Present in the Ticks* Dermacentor andersoni *and* D. occidentalis

Dermacentor andersoni is a vector for the agent of Rocky Mountain spotted fever, *R. rickettsii*, and commonly harbors the so-called East Side agent, *R. peacockii*, which is thought to block transovarial maintenance of *R. rickettsii* (Niebylski et al. 1997). Our adult male sample from Montana from the Oklahoma State University tick colony (DandM9) had no detectable *Rickettsia* DNA by qPCR and thus served as an excellent negative control for the read mapping procedure as only minimal background reads mapped to a few sites in both *Rickettsia* chromosomal and plasmid targets (Tables 1.7, 1.9 and 1.10) and to CLE agents (Table 1.5). The library and sequencing worked because its FLE agent (Scoles 2004) was readily detected (Table 1.4). This library was the only one sequenced with 400 bp chemistry, but that difference did not result in a significantly longer median read length (220 bp). CLC mapping was better than Geneious for assembling its FLE agent (Table 1.4).

Dermacentor occidentalis is the vector of *Rickettsia philipii*, the etiologic agent of Pacific Coast Tick fever on the West Coast of the USA (Padgett et al. 2016). It also more commonly harbors *Rickettsia rhipicephali* (Wikswo et al. 2008; Stephenson et al. 2017). This latter agent is not identical to the *R. rhipicephali* from *Rhipicephalus sanguineus* (Wikswo et al. 2008), whose genome sequence was obtained from the type strain 3-7-F6 CWPP, which was isolated from a *Rhipicephalus sanguineus* tick from Mississippi in 1973 (Burgdorfer et al. 1975). Our female adult *D. occidentalis* sample (EMCF9) was collected on February 14, 2014 at Crystal Cove State Park, El Moro Canyon, California. *Rickettsia rhipicephali* was present, as shown by qPCR and DNA sequencing. 83% of the chromosome and 98.8% of the pRh1 plasmid sequence could be mapped and assembled with Geneious (Table 1.9); CLC analysis provided similar results (not shown).

1.3.8 *Analysis of* Dermacentor variabilis *from Virginia, Georgia, and Ohio for* Rickettsia *and FLE*

Dermacentor variabilis is commonly infected with *R. montanensis* and *R. bellii* (Pagac et al. 2014; Stephenson et al. 2017; Wood et al. 2016; Yunik et al. 2015) and is the major vector of sporadic cases of Rocky Mountain spotted fever in two thirds of the USA (Anderson et al. 1986). It can also be infected with *R. rhipicephali* in populations on the West Coast of the USA (Wikswo et al. 2008). Much like the *D. andersoni* tick DandM9 from Montana, two samples of *D. variabilis* adults from

Georgia (Dvar22, female) and Virginia (DvarSlov) gave insignificant numbers of sequence reads mapping to *Rickettsia* chromosomes or plasmids (Tables 1.7, 1.9 and 1.10), further confirming the accuracy of the mapping methodology. However, another qPCR negative *D. variabilis* female tick, obtained from The Wildlands Park in Ohio in August 2014 (Dvar100), contained many sequence reads, mapping to both the chromosome and plasmid of *R. bellii* OSU85-389 (Fig. 1.6); this reference isolate had originally been obtained from an adult female collected in Franklin County, Ohio 5/13/1989 (Fuerst et al. 1990), a location about 50 miles west of Wildlands Park. *R. bellii* is not detected with the rickettsia qPCR assays employed (Eremeeva et al. 2003). Remarkably, this quality sequence assembly had 99.1% coverage of the *R. bellii* chromosome and 100.0% of the 48,775 bp plasmid (Fig. 1.6). None of the other plasmids in US ticks had more than patchy similarity to this sequence (only the large *Rickettsia felis* plasmid pRF1 is shown-Fig. 1.6b).

All three eastern *D. variabilis* ticks had sequences reads that each mapped to the three FLE agents at comparable levels, but at much lower levels than to *R. bellii* OSU85-389 in Dvar100 (Tables 1.2 and 1.10).

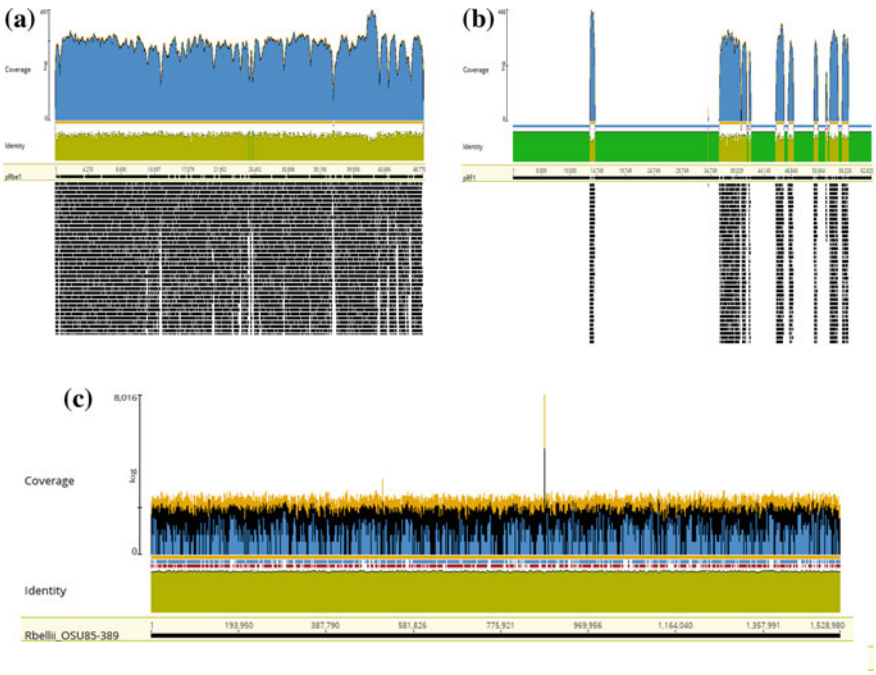

Fig. 1.6 Geneious read mapping of Dvar100 IT-PGM sequences against selected *Rickettsia* sequences. **a** *R. bellii* OSU85-389 plasmid pRbe1; **b** *R. felis* URRWXCal2 plasmid pRF1; **c** *R. bellii* OSU 85-389 chromosome. See Fig. 1.1 for other details

1.3.9 *Detection of* Rickettsia montanensis *in* Dermacentor variabilis *and* Rhipicephalus sanguineus *from Oklahoma*

Two *D. variabilis* females (DvarF4 and DvarF1) from the Oklahoma State University Tick colony were analyzed following Repli-G amplification of their DNA. The mini-Repli-G amplified library (DvarF4M4) was sequenced on the standard Ion 318 chip while the other two libraries from single cell (SC) amplification (DvarF4SC4, DvarF1SC1) were analyzed on the small Ion 314 chips to mimic the results to be expected from individual barcoded library samples. Because of problems occurring during loading the small chip and fractionation of the two SC libraries, this experiment was imperfect but it provides a sense of the lower boundaries of results that might be expected by the library input reductions required by mixing barcoded samples at equivalent loadings. A *Rickettsia* agent, mapping most strongly to the reference strain *R. montanensis* OSU-85-930, was obtained with all three libraries (Fig. 1.7c; Table 1.10) at 97.8, 97.7 and 78.4% coverage for DvarF4M4 (1,914,722 reads mapping), DvarF4SC4, and DvarF1 SC1 libraries, respectively. However, unexpectedly, since the reference isolate of *R. montanensis* has no plasmid, these samples also contained sequences that mapped against rickettsial plasmids, particularly pRrh1 (Fig. 1.7a) and pRma2 (not shown) and their abundance decreased with the total number of sequences obtained (Table 1.9). In comparison, while a *Rhipicephalus sanguineus* female (RhsangF23) from the same Oklahoma State University tick colony had about twice the coverage (109,892 reads) as DvarF4SC4 of the *R. montanensis* chromosome (62,416 reads, 97.7% coverage) (Fig. 1.7d; Table 1.10); it had only 3 reads to pRrh1 (Fig. 1.7b) while DvarF4SC4 had 34 reads mapping. The low plasmid coverage in RhsangF23 suggests that if this plasmid is indeed from *R. montanensis*, it has a very low copy number. Possibly of more significance is the fact the *R.*

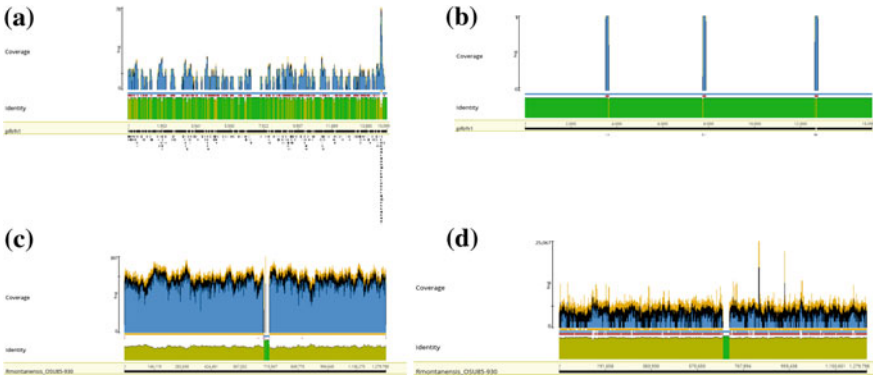

Fig. 1.7 Geneious read mapping of DvarF4M4 (**a**, **c**) and RhsangF23 (**b**, **d**) IT-PGM sequences against selected *Rickettsia* sequences. **a**, **b** *R. rhipicephali* 3-7-F6 CWPP plasmid pRrh1; **c**, **d** *R. montanensis* OSU 85-930 chromosome. See Fig. 1.1 for other details

montanensis-like agents in both DvarF4M4 and RhsangF23 contained no sequences mapping to the same site of 28.7 Kb located close to coordinate 700 Kb in *R. montanensis* OSU85-930 (Fig. 1.7c, d). This variant type of *R. montanensis* may in fact have been exchanged by horizontal transmission at some point, possibly originating from *D. variabilis*, since wild *Rhipicephalus sanguineus* have been reported only rarely to contain *R. montanensis* (Trout-Fryxell et al. 2015), while it is commonplace in *D. variabilis* (Fuerst et al. 1990; Stromdahl et al. 2011). Because *omp*A detection was inefficient relative to *omp*B assays in the Trout-Fryxell paper, surveillance with the former target may account for its low detection rate; however, the *omp*A region used in the Eremeeva et al. (2003) EvaGreen assay was present in the *R. montanensis* assembled from both the DvarF4M4 and RhsangF23 sequences, so other factors may account for its low detection rate rather than its absence in some agents.

1.4 Discussion and Conclusions

1.4.1 Library Preparation and Sequencing

The most variable aspects of sequencing on the IT-PGM were obtaining consistent mean read lengths and recovery of sufficient amounts of the library for loading. In part, this variability was due to the fact that tick samples with different histories and yields of DNA were used, as well as different approaches to fractionation of the DNA. Manual loading of the chips improved greatly with experience and this step can now be automated with the Ion Chef system. The use of the Repli-G single cell procedure did not appear to affect adversely read mapping or genome assembly. Indeed, it had significant advantages in stretching limited single sample DNAs and what appears to be preferential amplification of the microbial and mitochondrial (not shown) sequences over the tick chromosomal reads. This result may be due to preferential fragmentation of the tick DNA relative to the microbial and mitochondrial DNA, so that amplification of the preferred latter longer more intact DNA occurs. This effect was most spectacular with the 20 mitochondrial minicircles from human lice (not shown) and varied more among the tick samples. Because the *Rickettsia* plasmids detected here are relatively large, no consistent or marked effect was seen relative to the chromosomal reads. No plasmid was detected in the CLE or FLE agents.

1.4.2 Genome Assembly and Annotation

Geneious proved to be a very efficient tool for mapping the amount of IT-PGM reads on a desk top computer (usually less than 10 min). The de novo assembly did not provide significantly better results than the contigs obtained by read mapping.

This similarity is probably due to the low read depth, but it could be improved by removing short reads and aberrant read pileup spikes. The key to successful read mapping assembly is having reference sequences that are very closest to that of the agent being sequenced. The biggest advantage in using CLC genomics workbench on a high performance cluster (and with the other command line tools available) was being able to map against a database containing all of the related genome sequences available, a task that Geneious cannot perform on a laptop. However, the quality of the final CLC assemblies was again only marginally better than that obtained with Geneious. This program is also very suitable for rapid screening of the quality and usefulness of IT-PGM data by non-bioinformaticians. It can also be used for annotations using GenBank reference sequences. In general, we were pleasantly surprised at the amount of useful data that was obtained on the platform.

1.4.3 Limitations of Approach and Possible Remedies

The limitations of read depth for assembling symbiont genomes from metagenomics samples could be overcome only partially with Repli-G amplification. The larger Ion S chip will help but it also increases cost. It is likely that successful implementation of the 400 bp chemistry and improvements in accurate library size fractionation would also help but these aspects were not tested enough to confirm any advantages. One of our goals was to obtain enough symbiont genome coverage that long range PCR could be employed to make baits for enrichment of the target symbiont DNAs (Dunning Hotopp et al. 2017; Kent et al. 2011; Jones and Good 2016) and to design primers suitable for multiple locus sequence typing (MLST) of the symbionts, for population studies of mitochondria evolution in the ticks (Ketchum et al. 2009). We confirmed that primers for MLST target genes could be recovered for FLE, CLE and *Rickettsia* (not shown). The bait enrichment strategy should also work since reads were mapped over much of each of these chromosomes for multiple agents and for the *Rickettsia* plasmids. The tick mitochondrial assemblies (14.5–15.0 Kb) also provided enough assembled data to permit PCR amplification of target regions using specific primers rather than relying solely on conserved or degenerate tick primers and long-range PCR (not shown).

1.4.4 Value of Partial Symbiont Genome Sequences from the IT-PGM

Besides facilitating direct approaches to obtaining partial or complete genome sequences for symbionts and non-cultivable *Rickettsia*, we have demonstrated that substantial unknown information could be extracted from the DNA of single alcohol-preserved ticks. In many cases involving outbreaks of disease or tick samples that

must be shipped from other countries, alcohol-preserved ticks may be the only available material.

It was possible to determine if each agent had been previously sequenced and how similar it was to available sequences. This comparison was demonstrated unequivocally with the *Rickettsia* sequences, wherein *R. parkeri*, *R. amblyommatis*, and *R. bellii* could be compared with genome sequences from isolates; a significant variant of *R. montanensis* was identified in both *D. variabilis* and *R. sanguineus*; a plasmid was confirmed to be present in *R. andeanae* and *R. bellii*; and a large amount of new sequence was obtained for *R. andeanae.* Sufficient DNA sequence was obtained to determine the %GC content and minimal genome sizes for the *R. sanguineus* CLE and *D. variabilis* FLE agents and the genome sizes of the *A. maculatum* FLE and *A. americanum* CLE could be confirmed. For some tick bacterial agents, the read depth was sufficient to identify SNPs and INDELs in the assembled genomes relative to the available reference sequences. Finally, phylogenetic trees could also be constructed with concatenated sequences to further confirm agent identity. These features will permit routine evaluation and selection of specific tick DNA samples, which can warrant the cost of deeper metagenomic sequencing both from the standpoint of DNA quality and relative agent abundance and the uniqueness of the target agent. However, one confounding factor in metagenome-derived assemblies may be the presence of symbiont sequences in the host genome that have been inserted following endosymbiont-host lateral gene transfers (Dunning Hotopp et al. 2017).

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Data Submission The Ion Torrent PGM sequence data for each sample (Table 1.1) is available at the NCBI SRA resources site under Bioproject numbers PRJNA408163 (*Rhipicephalus sanguineus* F23UF), PRJNA413819 (*Dermacentor andersoni* M9), PRJNA413914 (*Dermacentor occidentalis* EMCF9), PRJNA413920 (*Amblyomma americanum* Aam10 and Aam46), PRJNA413923 (*Amblyomma maculatum* Amac21, Amac50), and PRJNA413929 (*Dermacentor variabilis* Dvar22, Dvar200, DvarSlov, DvarF4M4, DvarFvSC4, and DvarF1SC1).

Conflict of Interest Statement The authors declare they have no conflicts of interest affecting this work.

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Chapter 2
Field Studies in Acarology: Joint Base San Antonio, TX: 2015–2017

David Nielsen and Walter Roachell

Abstract Public Health Command-Central is responsible for entomological surveys, vector surveillance programs, and diagnostic testing for vector-borne pathogens for the army throughout a 20 state region. This responsibility affords civilian entomologists and military entomology officers assigned to PHC-Central the opportunity to conduct field studies for emerging pathogens, range expansions of medically important arthropods, and novel mitigation and control strategies. Two on-going studies at Joint Base San Antonio are diagnostic testing of mite parasites of reduviid bugs for *Trypanosoma cruzi*, the causative agent of Chagas disease, and evaluating trapping methods for soft ticks with the long-term goal of determining their potential role in vectoring the tick-borne relapsing fever historically known as "Camp Bullis Fever".

Keywords Camp Bullis Fever · Chagas · *Ornithodorus* · Erythraeidae

2.1 Introduction

Joint Base San Antonio (JBSA) is composed of four Air Force and Army installations located around San Antonio, Texas: Fort Sam Houston, Lackland Air Force Base (AFB), Randolph AFB, and Camp Bullis training site. Public Health Command-Central (PHC-Central) conducts field surveys and studies to better characterize the public health threat of multiple vector-borne diseases at JBSA in order to understand these diseases and protect service members from contracting them. This paper focuses on our work related to Chagas disease and mite parasites of reduviid bugs; a possible

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D. Nielsen (✉) · W. Roachell
Public Health Command-Central, 2899 Schofield Rd, Ft Sam Houston, San Antonio, TX 78234, USA
e-mail: david.nielsen@nebraska.gov

D. Nielsen
Nebraska Department of Agriculture, 301 Centennial Mall South, Lincoln, NE 68509-4756, USA

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tick-borne relapsing fever (TBRF) historically known as "Camp Bullis Fever"; and the TBRF caused by *Borellia turicatae.*

Chagas disease is an emerging pathogen in Texas and other states in the Southwestern United States. Typically considered a disease restricted to Latin America, Chagas is also native to the southern-most United States where it has a well-established enzootic cycle that consists of multiple reduviid vectors and mammalian hosts (Bern et al. 2011). In 2011, Air Force Trainee Health requested PHC-Central conduct surveillance for reduviid bugs (commonly called kissing bugs) at Medina Annex, Lackland AFB after a Military Working Dog (MWD) died from what was believed to be Chagas. This request has led to a long-standing relationship between PHC-Central and Air Force Trainee Health to provide continued surveillance for kissing bugs in order to mitigate the risk of Chagas for both MWDs and trainees at JBSA.

During our surveillance over the past few years, PHC-Central has collected a number of immature kissing bugs that had larval mites of the family Erythraeidae parasitizing them. Larval erythraeids parasitize insects and other arthropods and have been associated with reduviids in North, Central and South America (Anderson 1968 and Newell & Ryckman 1966). Recently, there has been interest in determining if these mites play a role in inter-population maintenance or spread of *Trypanasoma cruzi*, the causative agent of Chagas disease. Since research related to reduviid parasites is limited, there is little known about what role, if any, erythraeid larvae play in the transmission of Chagas disease. PHC-Central Entomology and collaborators at Harris County Texas are planning to collect these mites and begin laboratory analysis for *T. cruzi*. If mites are positive for this pathogen, further work will be conducted to determine if these mites are a viable host for the *T. cruzi* and if they can successfully transmit it to uninfected bugs.

In 2016, Dr. Job Lopez at the Baylor School of Medicine-Houston contacted PHC-Central Entomology to see if we would be interested in collecting soft ticks in the genus *Ornithodorus* at JBSA for his work with *Borellia turicate* prevalence in Texas. *Borellia turicatae*, vectored by the relapsing fever soft tick (*Ornithodoros turicata*), is the principal causative agent of TBRF in Texas (Goubau 1984). Despite this, *B. turicatae* is understudied compared to other TBRF agents. Trainee Health at Lackland AFB approved access of PHC-Central Entomology on all unrestricted training areas on JBSA and PHC-Central Entomology set out to determine the role *Ornithodorus* soft ticks play in the transmission of *B. turicatae* at training sites at Camp Bullis and other JBSA installations.

"Camp Bullis Fever" was first documented from Camp Bullis in the early 1940s (Murray and Dooley 2004). It is considered a tick-borne relapsing fever and thought to be vectored by the lone star tick (*Amblyomma americanum*), although the actual causative agent and potential vector/s remain unknown. The last case of "Camp Bullis Fever" was recorded in 1947 (Woodland and Richards 1943; Murray and Dooley 2004). Murray and Dooley (2004) speculated that "Camp Bullis Fever" has vanished over the years due to disuse of older training areas and/or due to alterations to the habitat. However, Camp Bullis is still an active training site at JBSA that hosts field training for the Air Force's Survival Evasion Resistance and Escape (SERE) school, Basic Officer Training for Army Medical Officers, and various other trainings

that require soldiers, airmen, and sailors to be in the field for extended periods of time, often overnight.

Due to the request to conduct soft tick surveys and potential vector role of Lonestar ticks for "Camp Bullis Fever", PHC-Central Entomology surveyed for both hard and soft ticks for the past two years across the approximately 28,000 acres of training areas at Camp Bullis.

2.2 Materials and Methods

2.2.1 Erythraeidae Collection

Kissing bugs were collected on JBSA through physical deconstruction of wood rat (*Neotoma* sp.) nests, which is the methodical removal of individual sticks and wood debris that have been built up around the burrow entrance by the wood rat. As each piece is removed it is examined for kissing bugs of all life stages, which in Central Texas can be collected throughout most of the year. Bugs collected from a single site are placed in 5 mL vials for return to PHC-Central for identification and diagnostic testing for *T. cruzi*. During the summer months through mid-autumn in Central Texas, we found erythraeid larvae parasitizing some of the kissing bugs that were collected. Initially, these mites were removed from the bugs, analyzed under a dissecting scope, photographed, and disposed of. However, as increasing numbers of mites were collected with the bugs, we questioned if the mites might act as a bridge vector of *T. cruzi* between the bugs. Specimen collected were placed in 2 mL safety lock micro-centrifuge tubes and stored in −20 °C freezer until testing.

2.2.2 Hard Tick Collection

Hard tick collection was conducted using standard dragging and flagging techniques on days when temperatures were above 50 F and with no visible moisture on the ground or on vegetation. Drags were used over tall grass and forest floor areas and were checked for ticks at 20 step intervals. Flags were used in brushy areas and checked intermittently. Collection numbers for drags and flags were recorded every 15 min. Collection notes were maintained for each training site and collector.

2.2.3 Ornithodorus Collection

Ornithodorus turicata are often difficult to collect because they are associated with rodent burrows, so we developed a number of methods to collect them, which are described below.

Initial soft tick collection was conducted with use of a 50 mL tube with a cap. An opening was cut in the center of the cap so that CO_2 could easily vent from the tube and also serve to direct the flow of CO_2. A single vial filled with dry ice was placed at the entrance of a small mammal burrow. Vials were left in place for 20–60 min. After the minimum time had passed, we used a flashlight to examine the area around the vial and burrow entrance for the presence of soft ticks. Any soft ticks that were present were collected by hand or forceps and placed in a 5 mL tube for delivery back to PHC-Central. This method was slow and typically drew out only a few ticks per hour, which made collection difficult and inefficient

After employing this method for most of 2016, we next used a ladle and sieve method in conjunction with hand collecting. For this method, we again placed a 50 mL tube filled with dry ice at the entrance of a burrow to lure out ticks. Once individual ticks were identified, we hand collected those near the entrance and then used the ladle to scoop out the length of burrow we could reach. The soil and nesting material obtained with the ladle was dumped onto the sieve to separate the soil aggregates from the soft ticks. This method was effective for collecting 4th and 5th instar nymphs and adults; however, earlier instars would typically fall through the openings in the sieve plate. The smaller ticks that were not separated with the use of the sieve plate were collected by forceps from the soil accumulated after each scoop of material was sieved. This method enabled us to collect more ticks per collection on average but was more labor intensive and caused damage to the rodent burrow through the removal of loose soil and materials lining the entrance.

In October of 2017, we developed a modified pitfall trap to collect soft ticks (Fig. 2.1). The trap consisted of a 500 mL sample bottle with cap, a 3 inch diameter funnel, ¼ inch plastic tubing, and plastic string. The bottom of the bottle was removed with a saw so that the funnel could be set in its place. Two holes were drilled in the side of the 500 mL sample bottle, one near the bottom and one near the top that went through the funnel as well. The ¼ inch tubing was threaded through the two openings and served as a friction point to hold the funnel on top. The tubing will later serve as a vent for excess CO_2 inside the 500 mL sample bottle to prevent damage to the cap or funnel. A piece of plastic string was used to tie the opposite side of the funnel from the vent tube to the 500 mL sample bottle to properly secure the funnel in place.

For field deployment, we would first excavate a hole into the soil at the front of the rodent burrow using a hand drill with a 3 × 12 inch drill bit. This created an almost perfect sized hole for the pitfall trap (Fig. 2.2). After prepping the hole, dry ice was placed inside the 500 mL sample bottle and the cap was then secured (Fig. 2.3). Loose dirt was used to create a level surface with the funnel edge (Fig. 2.4). Once in place, the trap would remain in place for 3–4 h before being collected with the potential of being left overnight if needed. Once the minimum time had passed the traps were removed and taken back to the lab to determine how many ticks, if any, were present (Figs. 2.5 and 2.6).

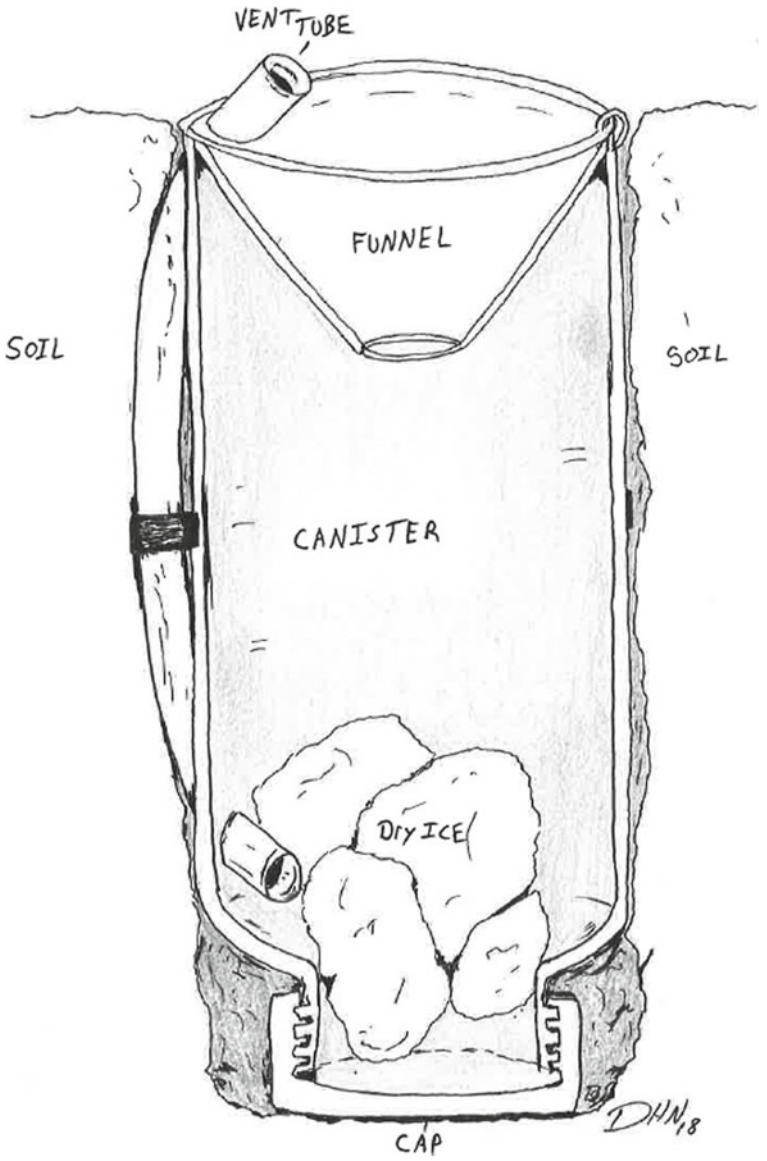

Fig. 2.1 Illustration shows the outline of the trap placed in the ground

Fig. 2.2 Hole being drilled into entrance to rodent burrow

2.3 Results

2.3.1 Erythraeidae

To date no testing has been conducted on the erythraeid mites collected from the kissing bugs. Collections are ongoing at JBSA as well as with collaborators at Harris County, TX. We intend to begin testing in 2018.

Fig. 2.3 Dry ice is placed in the bottom of the trap and cap is secured

Fig. 2.4 Trap is placed in pre-drilled hole soil is moved around the edge of the funnel

Fig. 2.5 Two ticks (one adult and one blood-fed nymph) observed crawling into the trap

Fig. 2.6 One collection from Salado Creek containing 18 ticks

2.3.2 Tick Collection

After multiple collections, only two adult and one nymph lone star tick were collected. These limited collections of hard ticks cast some doubt on the potential of these ticks presenting a TBRF risk to service members currently training at Camp Bullis.

Overall collections of soft ticks were similar regardless of method used, with one exception at Salado Creek in November of 2018 that used the modified pitfall trap

Table 2.1 Collection data for *Ornithodorus sp.* using modified pitfall trap at Medina Annex, Lackland AFB; Salado Creek Park, Ft Sam Houston; and Camp Bullis

	Medina Annex	Salado Creek	Camp Bullis
October			
Site 1	2	N/A	N/A
Site 2	1	N/A	N/A
Site 3	0	N/A	N/A
Site 4	N/A	N/A	N/A
November			
Site 1	6	0	0
Site 2	0	2	0
Site 3	N/A	18	0
Site 4	N/A	2	0
December			
Site 1	N/A	0	0
Site 2	N/A	1	0
Site 3	N/A	6	0
Site 4	N/A	2	0

(Table 2.1). The primary difference in collection methods is that ticks collected in the pitfall trap do not require extra effort to collect specimen since they are contained in the trap. This advantage also allows field staff to conduct other field surveys without having to check rodent burrows at regular intervals to collect ticks prior to the dry ice completely sublimating. Collection results specific to the modified pitfall trap are reflected in Table 2.1.

2.4 Discussion

Diagnostic testing for the *Erythraeidae* is planned for 2018. If mites test positive for *T. cruzi*, then a long-term study to determine vector competency will be conducted, with the goals of determining if these mites can serve as a reservoir for *T. cruzi*, maintain the pathogen transtadially and transovarially, and if the mites transmit *T. cruzi* from infected to uninfected bugs.

Tick collection with modified pitfall traps are planned to continue in 2018. Research will focus on expanding the use of pitfall traps to other military installations in PHC-Central's region; conduct a more thorough side by side comparison with other collection methods to determine if there is a quantifiable benefit to using the modified pitfall trap versus other collection methods; and if the traps can be used to collect both hard and soft ticks or other arthropod vectors attracted to CO_2.

The most notable advantages of the modified pitfall trap are related to the storage of dry ice. First, due to the design of the trap, the collected ticks are exposed directly to the dry ice. The exposure generally kills the ticks, although we have found viable tick specimens when we collected the traps. Further study into the amount of dry ice

within the trap and modifications to the trap (e.g., inclusion of a mesh screen) need to be studied if live specimen are desired. The other advantage is the fact that the dry ice is well insulated both within the trap and the surrounding soil, which limits additional heating from sunlight and slows the release of CO_2, which increases the amount of time that the trap can be left in the field for tick collection. Conventional CO_2 traps with open pans tend to have a 2-h limit in full sun and our design enables the trap to stay in the field for almost twice the time, which may attract more ticks.

Acknowledgements We would like to thank Dr. Job Lopez for his guidance on field soft tick surveillance methods for PHC-C staff in 2016 and his continued support for soft tick work at JBSA. We also would like to thank Dr. Leo Cropper, director Air Force Trainee Health for his continued support and approval of our field studies on Air Force Installations. We also want to thank the vector borne disease program staff at Harris County for their work in kissing bug and parasitic mite collection in 2016–2017. We are also grateful for the support of Public Health Command-Central allowing us time to conduct field studies of military medical importance on JBSA and within our region.

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Chapter 3
New Findings on Tarsonemid Mites (Prostigmata: Tarsonemidae) Under the LT-SEM (Low Temperature Scanning Electron Microscopy)—The Case of Genera *Daidalotarsonemus* and *Excelsotarsonemus*

J. M. Rezende, G. R. Bauchan, A. C. Lofego, W. C. Welbourn and Ronald Ochoa

Abstract *Daidalotarsonemus* De Leon and *Excelsotarsonemus* Ochoa and Naskręcki are tarsonemids considered to be plant inhabiting genera. Both present complex structured bodies which are very difficult to be interpreted by traditional light microscopy techniques. In light microscopy slide mounting distorts the individual by flattening the specimen between the slide and the coverslip. In recent years Low Temperature Scanning Electron Microscopy (LT-SEM) has been incorporated to acarological studies, improving the understanding about the external morphology of mites in general. The impact of LT-SEM analysis on studies of the tarsonemid genera is discussed here. The use of this technology allows the recognition of small details e.g. palpal setae, protuberances on palpal tibiotarsus, cheliceral tips, dorsal ornamentation and chaetotaxy among others. This information is important not only for diagnosis but also to understand their ecology and behaviour.

J. M. Rezende (✉) · A. C. Lofego
Department of Zoology and Botany, Institute of Biosciences, Humanities and Exact Sciences (IBILCE), São Paulo State University (UNESP), Campus São José do Rio Preto, São José do Rio Preto, SP 15054-000, Brazil
e-mail: jmrezende@live.com

J. M. Rezende
Acari Collection, Department of Entomology, NHNM, Smithsonian Institution, Washington, DC 20560, USA

G. R. Bauchan
Electron and Confocal Microscopy Unit, United States Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705, USA

W. C. Welbourn
Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL 32614-7100, USA

R. Ochoa
Systematic Entomology Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705, USA

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3.1 Introduction

There are close to 45 genera and more than 900 described species currently placed in the family Tarsonemidae (Lin and Zhang 2002). The family includes phytophagous, fungivores, omnivorous, predaceous, parasitic and insectivorous mites (Lindquist 1986). It contains species considered to be tropical and subtropical, however this is unclear as no extensive studies have been carried out from the Antarctica continent, Arctic regions and Pacific islands.

Daidalotarsonemus De Leon is a tarsonemid genus consisted of 39 described species (De Leon 1956; Lin and Zhang 2002; Lofego et al. 2005; Sousa et al. 2014; Rezende et al. 2015a, b, c). *Excelsotarsonemus* Ochoa and Naskręcki is a smaller taxon, with six known species (Ochoa et al. 1995; Ochoa and OConnor 1998; Rezende et al. 2015a; Sousa et al. 2018). Both genera are plant inhabiting taxa with preferences for hosts located in humid areas. They are distinguished from other tarsonemids by their complex dorsal ornamental patterns and the shape of the dorsal opisthosomal setae, which makes them especially difficult to study using light microscopy techniques.

One of most effective techniques that has been integrated into the Acarology is Low Temperature Scanning Electron Microscopy (LT-SEM). With this method a sample is instantly frozen with liquid nitrogen creating a frozen snap-shot of the specimen as it occurs in nature (Bolton et al. 2014a). Rezende et al. (2015a) described one *Daidalotarsonemus* and two *Excelsotarsonemus* species using LT-SEM micrographs. The impact of these LT-SEM analysis on the understanding of the morphology for these two genera is discussed here.

3.2 Materials and Methods

Several individuals of the species *Daidalotarsonemus oliveirai* Rezende, Lofego and Ochoa, *D. somalatus* Attiah, *Excelsotarsonemus caravelis* Rezende, Lofego and Ochoa and *E. tupi* Rezende, Lofego and Ochoa were analysed under the LT-SEM. With this technique, live specimens were secured to 15 mm × 30 mm copper plates using ultra smooth, round (12 mm diameter) carbon adhesive tabs (Electron Microscopy Sciences, Inc., Hatfield, PA). The specimens were frozen in a styrofoam box by placing the plates on the surface of a pre-cooled (−196 °C) brass bar while the lower half was submerged in liquid nitrogen (LN2). After 20–30 s, the holders containing the frozen samples were transferred the Quorum PP2000 cryo-prep chamber (Quorum Technologies, East Sussex, UK) attached to an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Dallas, TX). The specimens were etched inside the cryotransfer system to remove any surface contamination (condensed water vapour) by raising the temperature of the stage to −90 °C for 10–15 min. Following etching, the temperature inside the

chamber was lowered below −130 °C, and the specimens were coated with a 10 nm layer of platinum using a magnetron sputter head equipped with a platinum target. The specimens were transferred to a pre-cooled (−130 °C) cryostage in the SEM for observation. An accelerating voltage of 5 kV was used to view the specimens. Images were captured using a 4pi Analysis System (Durham, NC) (see Bolton et al. 2014a for additional details).

3.3 Results and Discussion

Characteristics of the gnathosoma, stigmata and leg chaetotaxy are important for diagnosis as well as understanding their ecology and behaviour. Use of LT-SEM allowed the recognition of details of palpal setae, protuberances and the cheliceral tips (Figs. 3.1 and 3.2). With light microscopy minute palpal structures, apart from their degree of elongation and orientation, are usually not used taxonomically due to difficulty in interpreting the images (Lindquist 1986). Three pairs of palpal setae for *D. somalatus* (Fig. 3.1) and *E. caravelis* (Fig. 3.2) were observed under LT-SEM. Lindquist (1986) noted the difficulty in observing these structures but also remarked on their potential usefulness in distinguishing and classifying tarsonemids. In this context, palpal protuberances on tibiotarsus appear to be useful phylogenetic characters because they have unique patterns and forms for each species in this study. The cheliceral tips are visibly cuspidate (Fig. 3.2), which is used by these mites to puncture structures e.g. spores and/or plant epidermal tissues.

The most obvious advantage of LT-SEM is the ability to study mites in 3D. Slide mounting for light microscopy distorts specimens by flattening them between the slide and the coverslip. This often pushes the gnathosoma forward and squeezes prodorsal shielding structures, giving the specimen an unnatural appearance. Using LT-SEM we observed the gnathosoma is capable of protracting and retracting under the prodorsum and between legs I. This ability is complete for *Excelsotarsonemus* (Fig. 3.3) and partial for *Daidalotarsonemus* (Fig. 3.4). There was also noticeably different forms, positions and even diameters for the stigmata (Figs. 3.5, 3.6 and 3.7), despite the close phylogenetic relationship between these genera (Ochoa et al. 1995).

The dorsal ornamentation and chaetotaxy are key characters for both *Daidalotarsonemus* and *Excelsotarsonemus*. These structures are especially difficult to discern under light microscopy. The micrographs presented here show intricate folding patterns on the opisthosoma and its setae (Figs. 3.8. 3.9, 3.10, 3.11, 3.12, 3.13 and 3.14). Rezende et al. (2015a) used LT-SEM images to draw these setae which helped to unfold their morphology and perhaps their role in the species behaviour. According to the results, sail-like setae might allow these mites to glide within the canopies (Ochoa and OConnor 1998; Rezende et al. 2015a). Some of these setae have complex patterns and asymmetry (Figs. 3.13 and 3.14). Due to their position and the manner they lay above tergite H, these setae are probably related to protection, entrapping fungal spores or possibly aerodynamic for gliding (Rezende et al. 2015a).

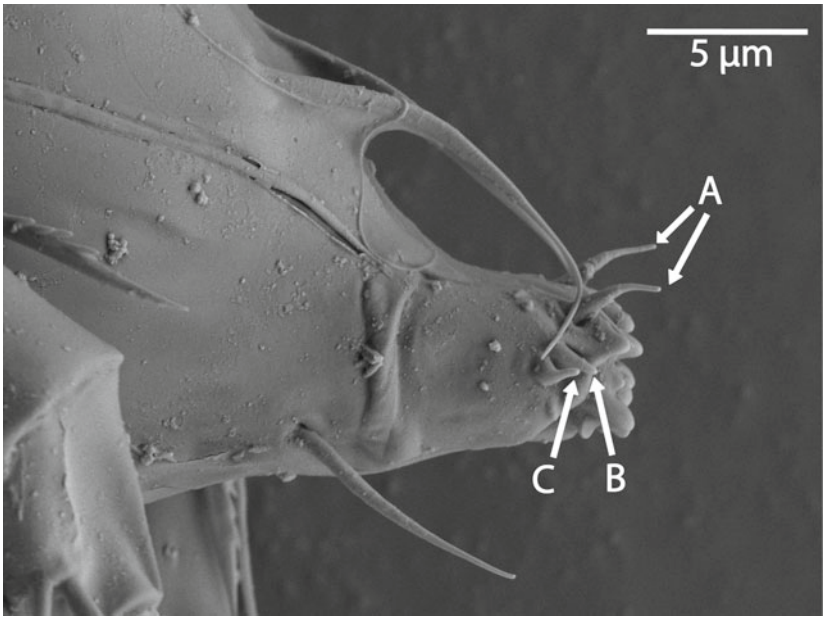

Fig. 3.1 Details of the three pairs of palpal setae (A–C) of *Daidalotarsonemus somalatus*

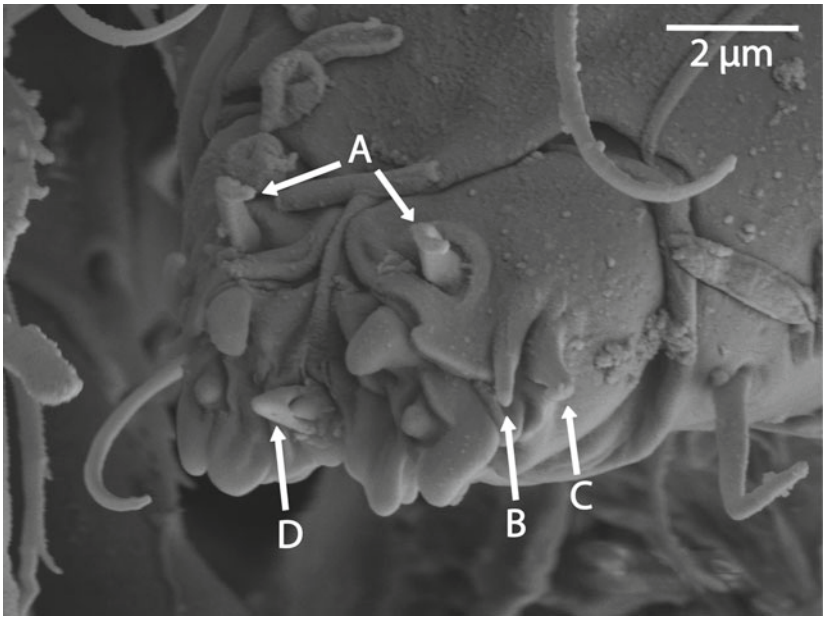

Fig. 3.2 Details of the three pairs of palpal setae (A–C) and cheliceral tip (D) of *Excelsotarsonemus caravelis*

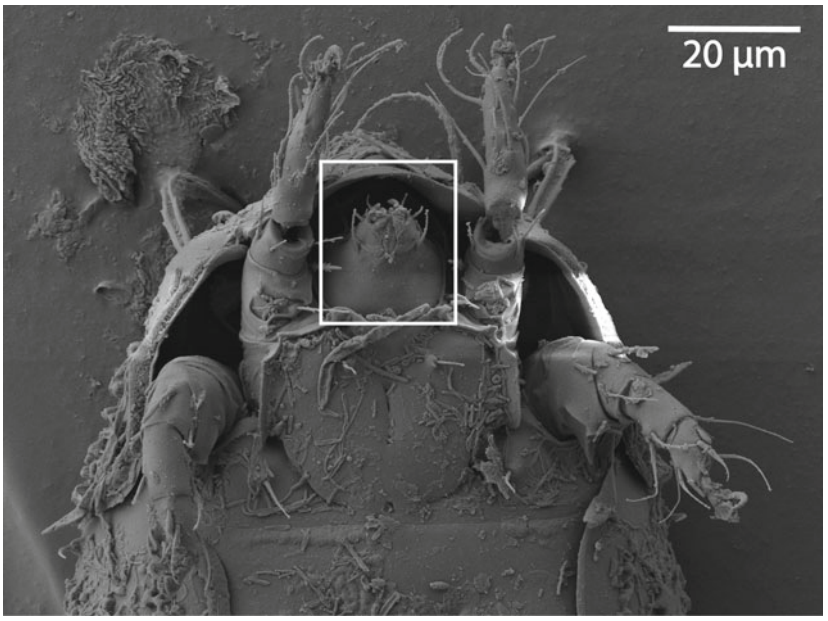

Fig. 3.3 Detail of the gnathosoma fully covered by the prodorsum for *Excelsotarsonemus caravelis*

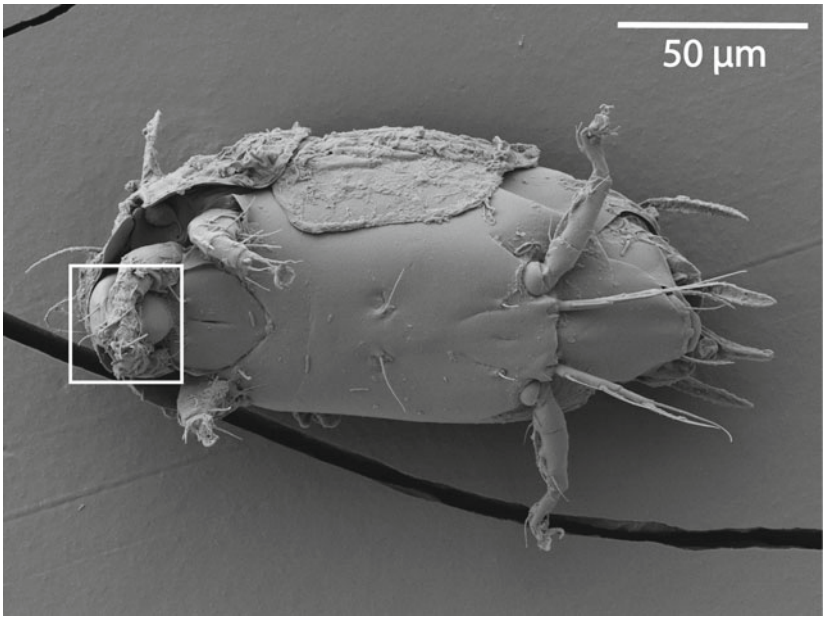

Fig. 3.4 Detail of the gnathosoma partially covered by the prodorsum for *Daidalotarsonemus oliveirai*

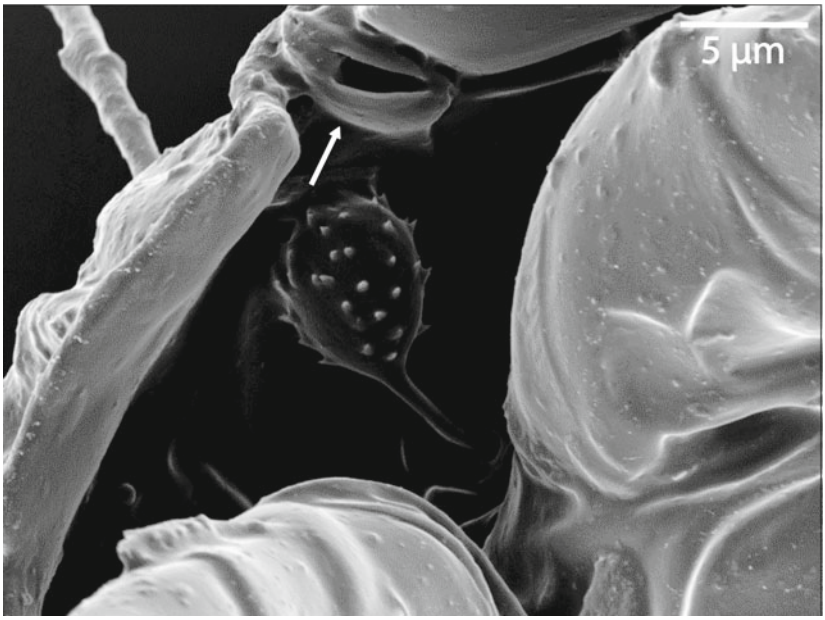

Fig. 3.5 Seta indicating the stigma of *Daidalotarsonemus somalatus*

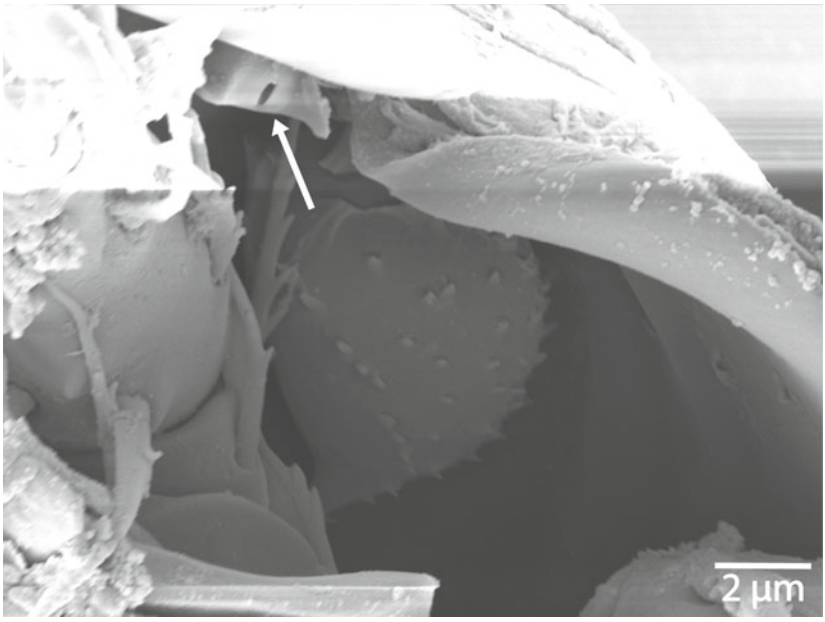

Fig. 3.6 Seta indicating the stigma of *Excelsotarsonemus caravelis*

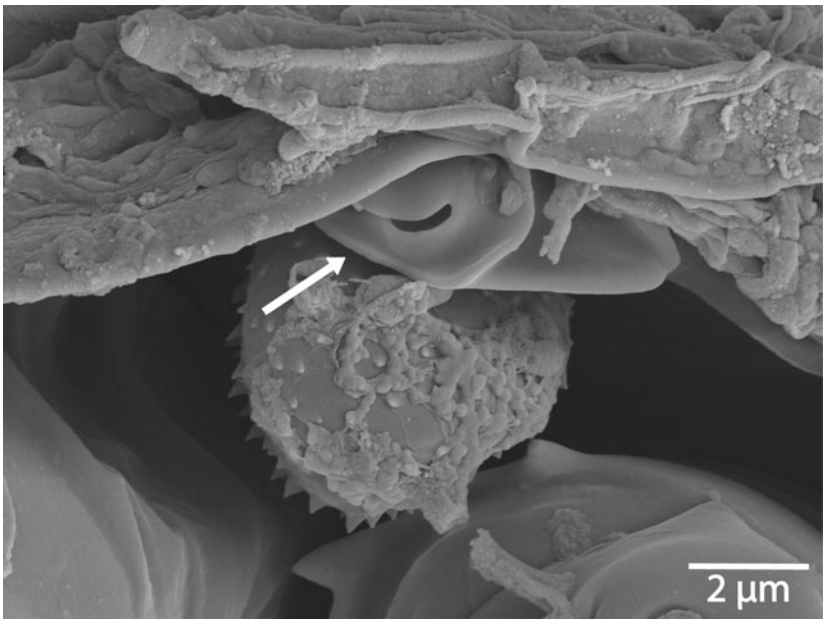

Fig. 3.7 Seta indicating the stigma of *Excelsotarsonemus tupi*

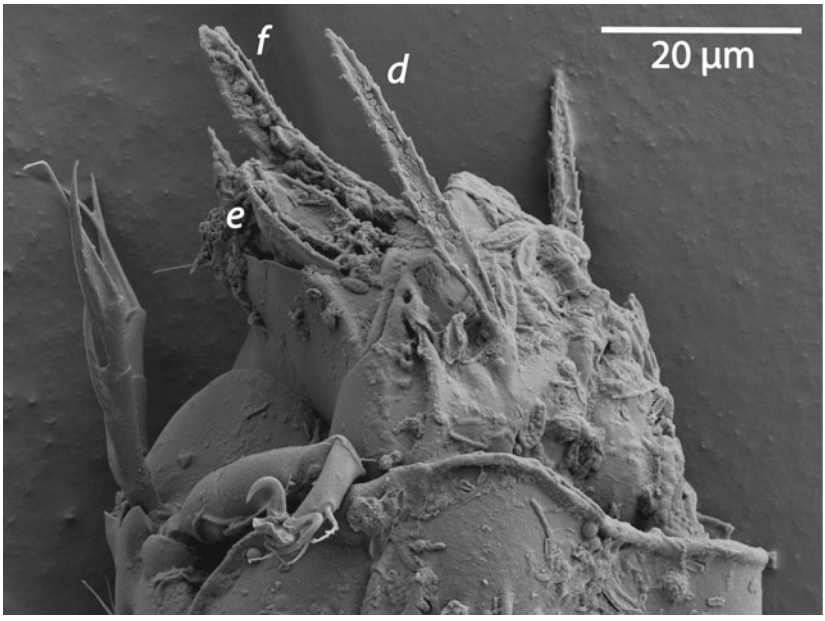

Fig. 3.8 Posterior dorsal setae of *Daidalotarsonemus oliveirai*

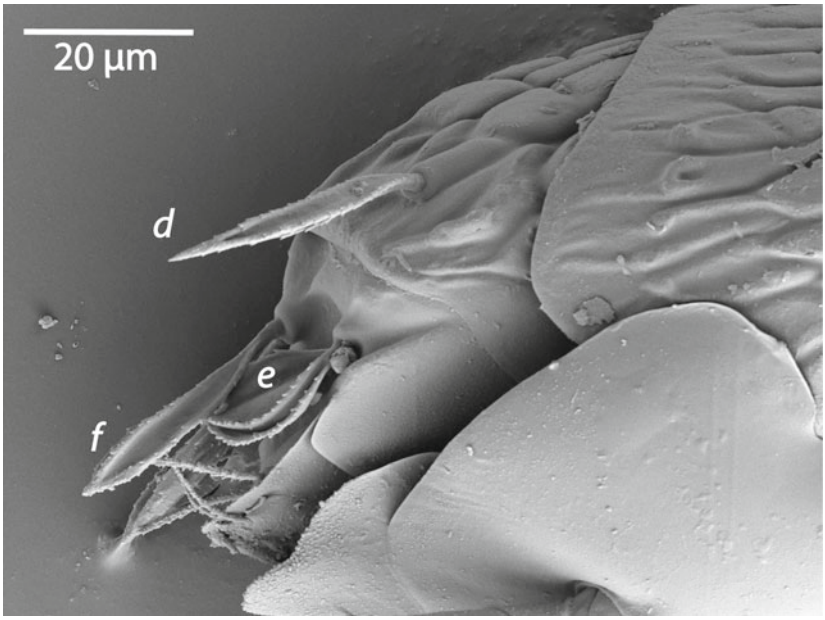

Fig. 3.9 Posterior dorsal setae of *Daidalotarsonemus somalatus*

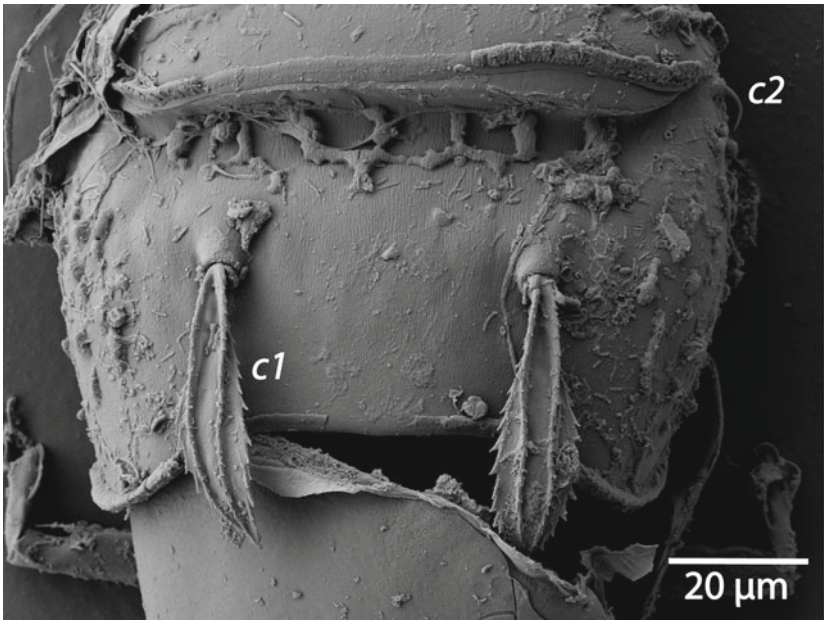

Fig. 3.10 Setae of the tergite C of *Excelsotarsonemus caravelis*

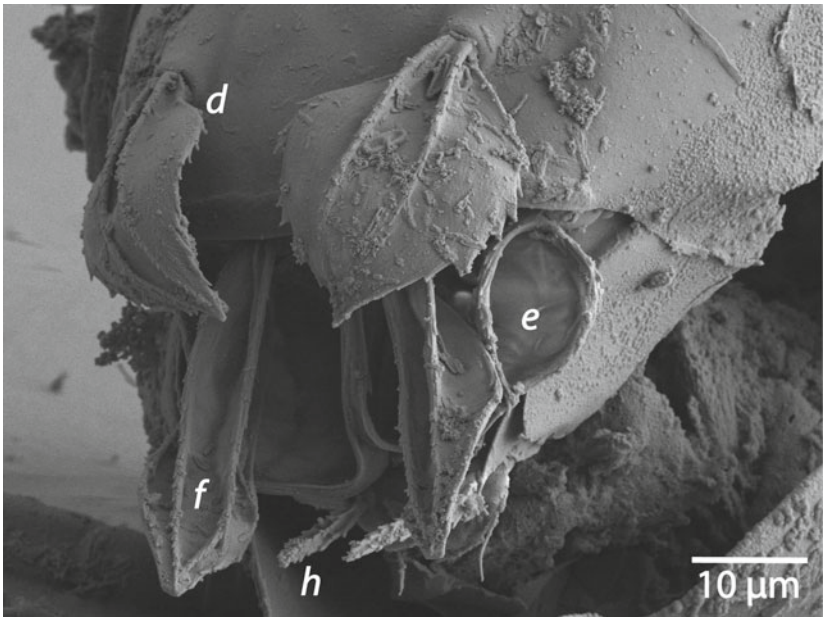

Fig. 3.11 Posterior dorsal setae of *Excelsotarsonemus caravelis*

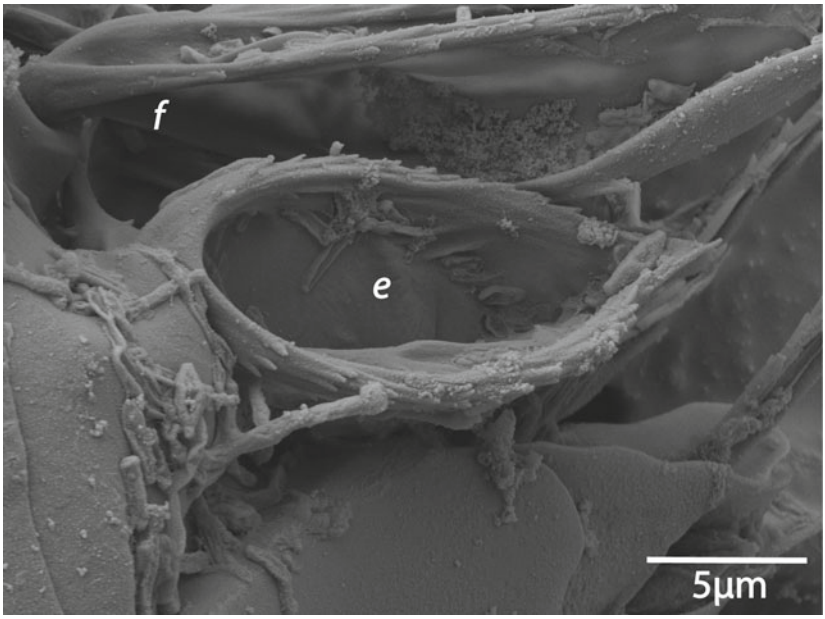

Fig. 3.12 Detail of seta *e* of *Excelsotarsonemus caravelis*

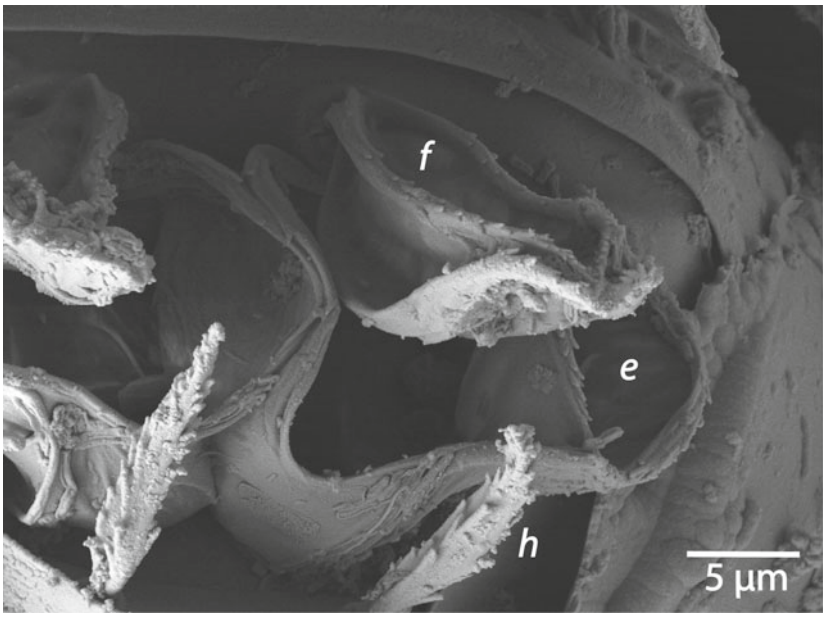

Fig. 3.13 Detail of dorsal setae *e, f* and *h* for *Excelsotarsonemus caravelis*

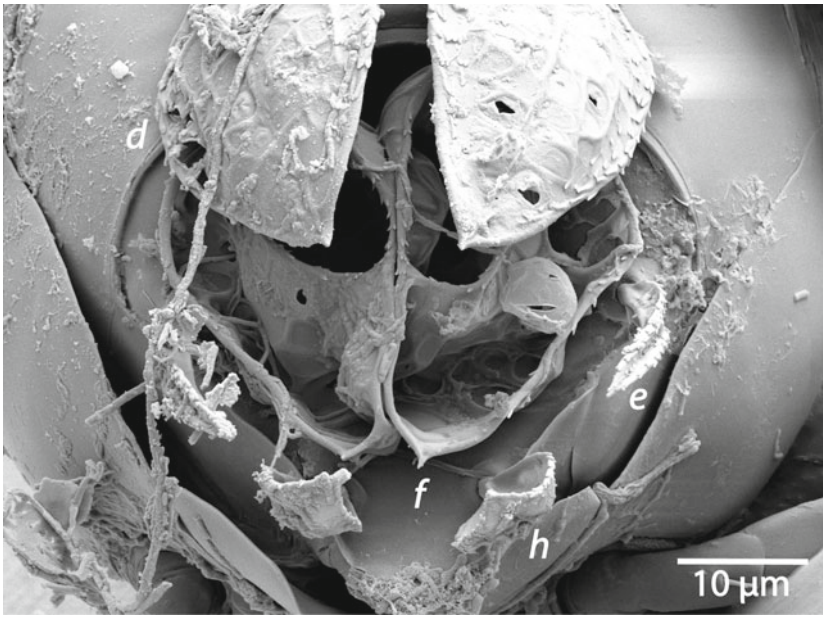

Fig. 3.14 Detail of dorsal setae *d, e, f* and *h* for *Excelsotarsonemus tupi*

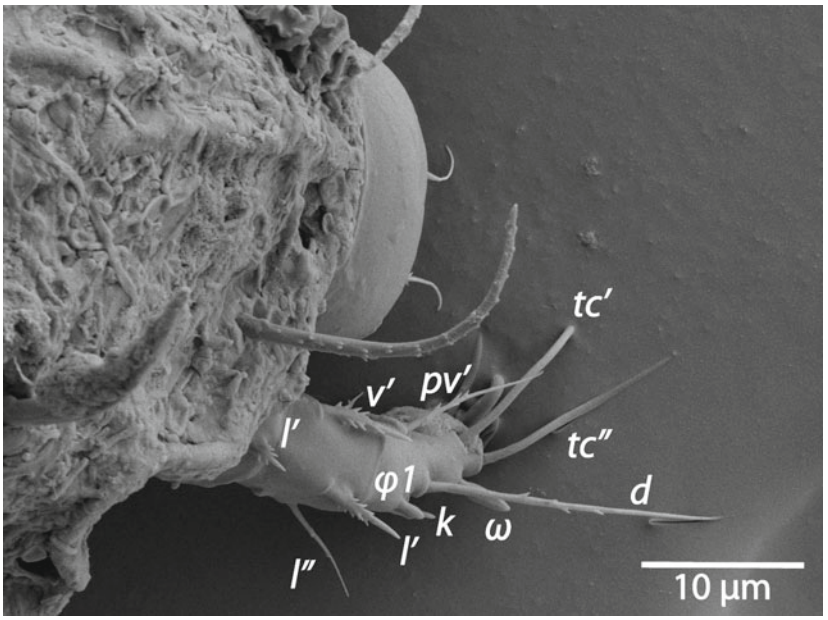

Fig. 3.15 Detail of leg I of *Daidalotarsonemus oliveirai*

Unlike the opisthosomal characteristics discussed, leg chaetotaxy has been important since the beginning of tarsonemid descriptions (Canestrini and Fanzago 1877). However, use of LT-SEM micrographs enable these characters be examined and interpretated in greater detail. Each leg seta has an unique shape and number of barbs (Figs. 3.15, 3.16, 3.17 and 3.18). Solenidia and eupathidia have small grooves and furrows over their surface (Figs. 3.19, 3.20, 3.21 and 3.22) which may be used in taxonomic and phylogenetic studies.

LT-SEM images are also important to understanding the role of mites in the environment (Bolton et al. 2014b; Ochoa et al. 2011). It has been observed that many tarsonemids have the capacity to carry fungi using their sporothecae (Krantz 2009; Moser 1985). In addition to it, there is a wax-like substance called cerotegument (Rezende et al. 2015a), which extends over the body of both genera and can have fungi, lichens and bacteria attached. The primary function of the cerotegument seems to be protection against pathogens, but the cerotegument also allows these species to carry debris over the bodies (Figs. 3.23, 3.24 and 3.25). When dispersing through the canopy these mites may act as reservoirs of microorganisms, suggesting a ecological association between fungi, bacteria, mites and the canopies. Cerotegument also provides a phylogenetic character due to the singular shape it presents (Rezende et al. 2015a). Common treatments for alcohol-preserved specimens such as lactophenol, Nesbitt's fluid or KOH (Walter and Krantz 2009) may remove the cerotegument and cause misidentification or inaccurate descriptions.

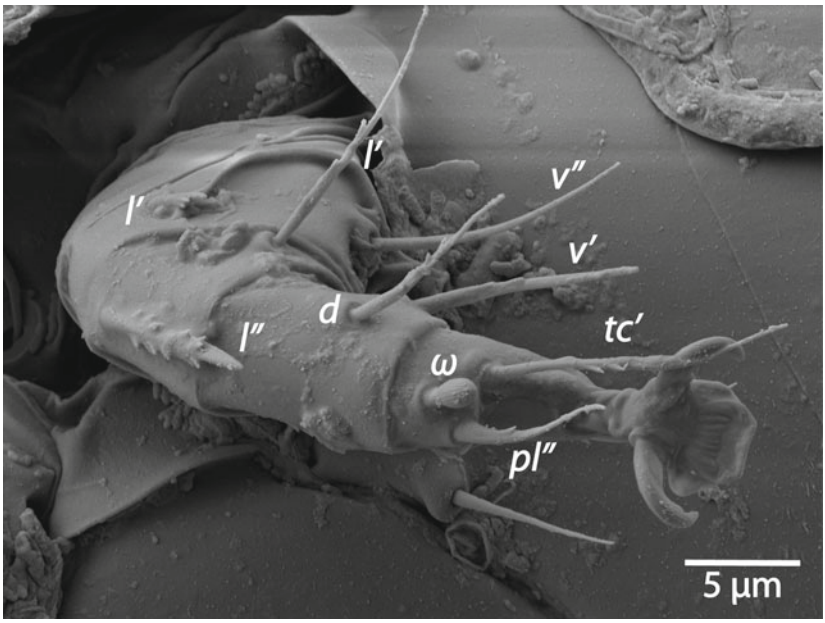

Fig. 3.16 Detail of leg II of *Daidalotarsonemus oliveirai*

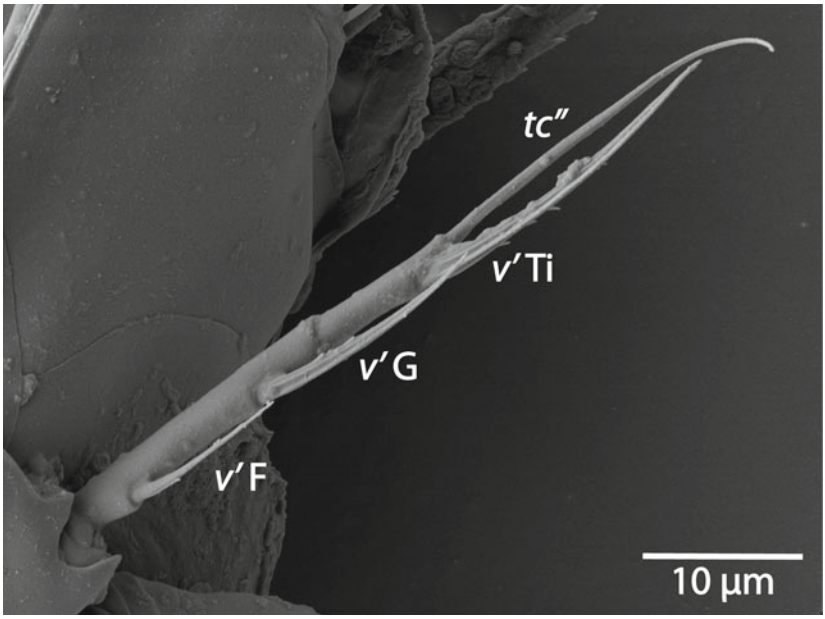

Fig. 3.17 Detail of leg IV of *Daidalotarsonemus oliveirai*

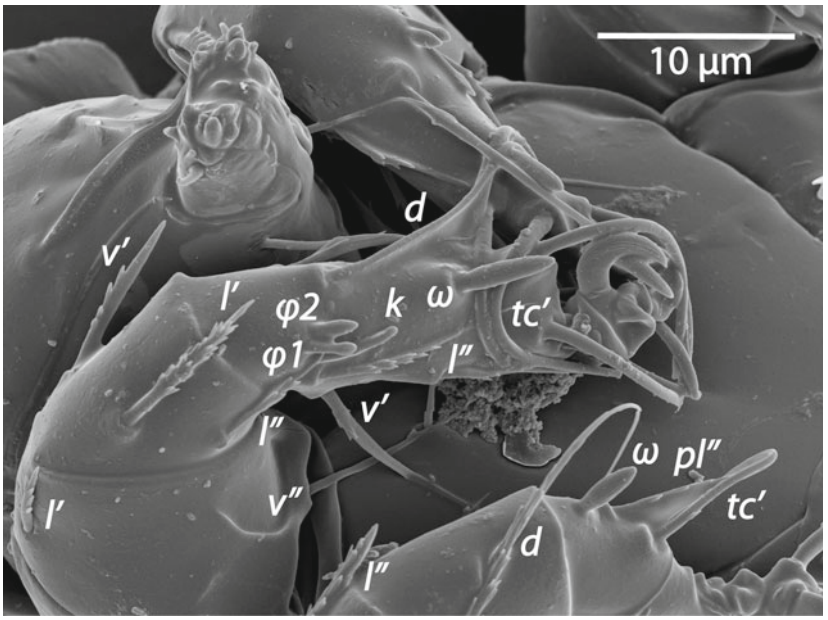

Fig. 3.18 Detail of legs I and II of *Daidalotarsonemus somalatus*

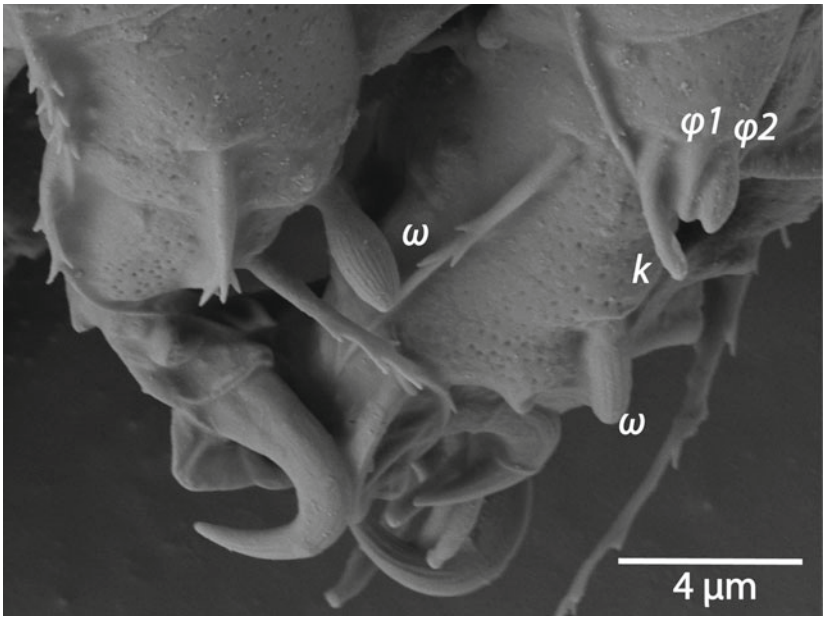

Fig. 3.19 Detail of the solenidia of legs I and II of *Daidalotarsonemus oliveirai*

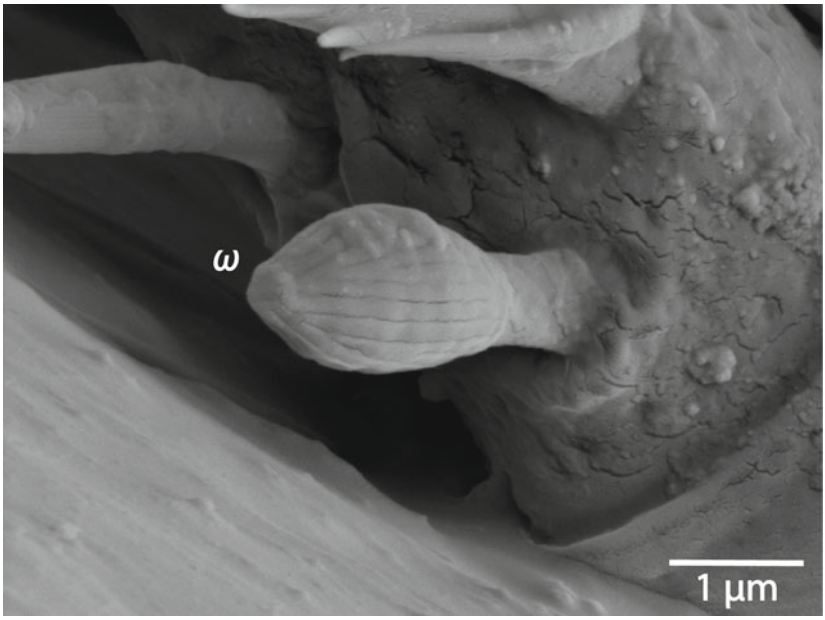

Fig. 3.20 Detail of the solenidium *ω* of leg II of *Daidalotarsonemus oliveirai*

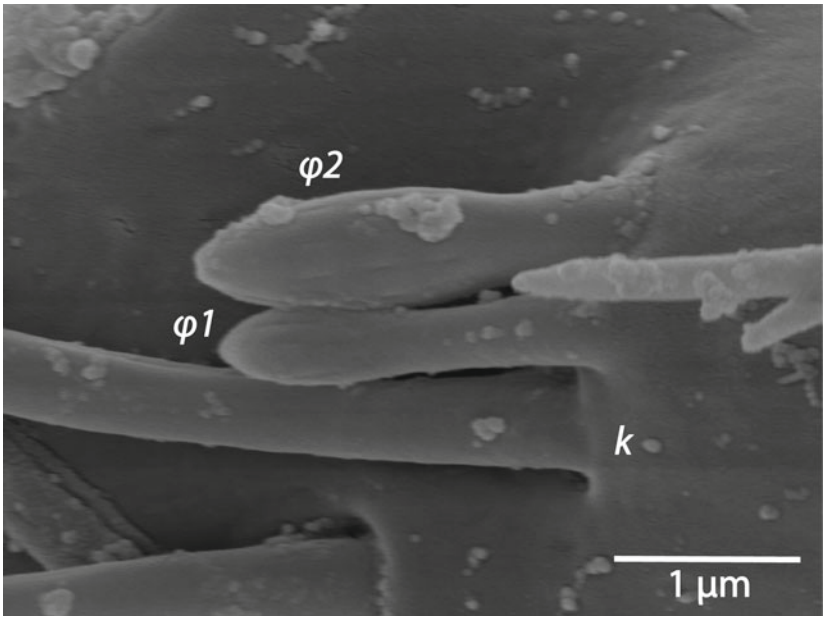

Fig. 3.21 Detail of the sensorial cluster of leg I of *Excelsotarsonemus caravelis*

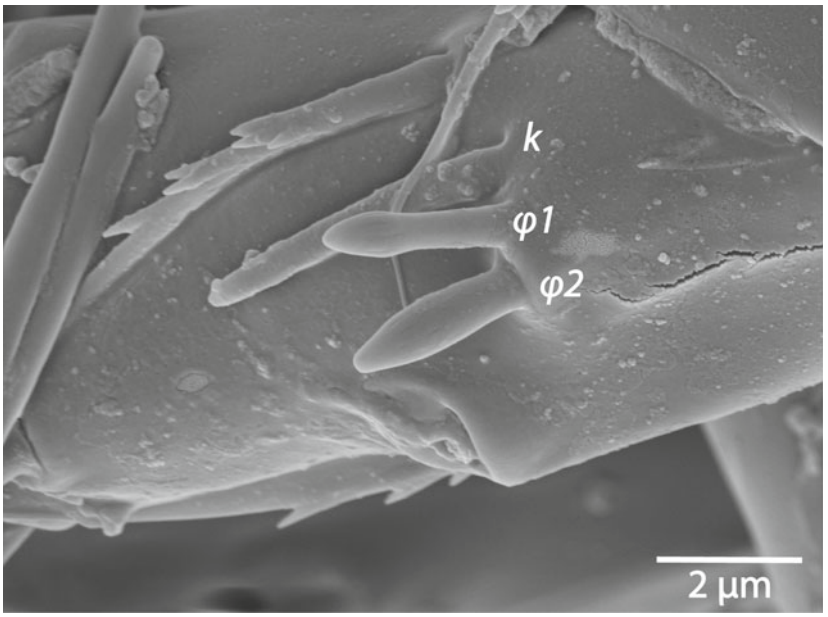

Fig. 3.22 Detail of the sensorial cluster of leg I of *Daidalotarsonemus somalatus*

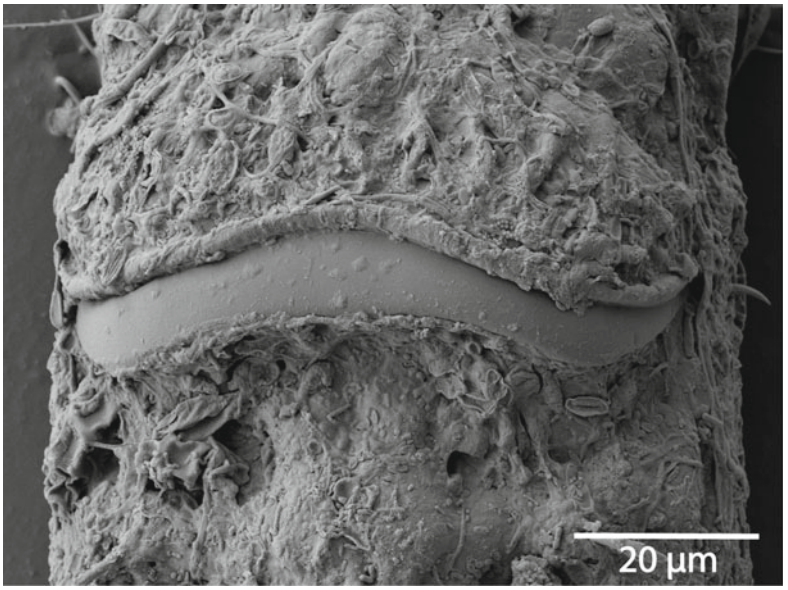

Fig. 3.23 Detail of the dorsum of *Daidalotarsonemus oliveirai* covered with the wax-like substance and debris attached to it

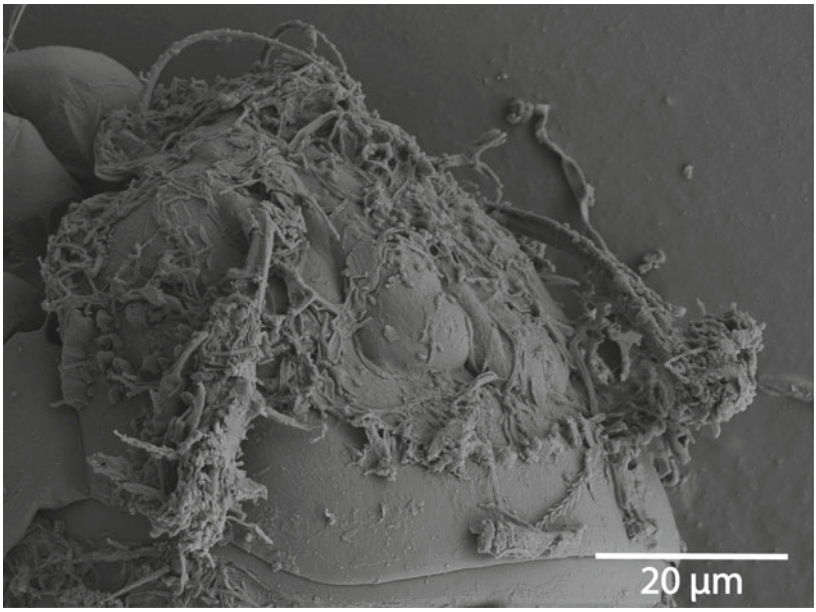

Fig. 3.24 Lateral view of the prodorsum of *Excelsotarsonemus caravelis* covered with the wax-like substance and debris attached to it

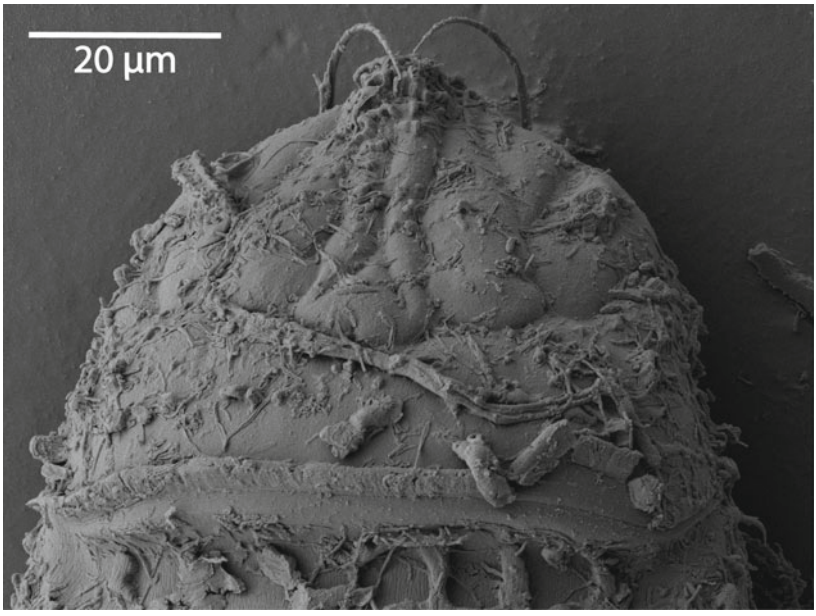

Fig. 3.25 Dorsal view of the prodorsum of *Excelsotarsonemus caravelis* covered with the wax-like substance and debris attached to it

Scanning electron microscopes enable the observation of fine details impossible to study using traditional techniques. Other tarsonemid genera also present complex patterns e.g. *Ceratotarsonemus* De Leon; *Kaliszewskia* Lofego, Demite and Moraes; and *Ochoanemus* Lofego, Pitton and Rezende (De Leon 1956; Lofego et al. 2015, 2016) and need to be studied using new technologies. Undoubtedly the family Tarsonemidae has many more secrets to be revealed under the LT-SEM.

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Chapter 4
A Preliminary Phylogenetic Hypothesis for Cunaxidae (Acariformes: Trombidiformes: Prostigmata: Eupodina)

Michael J. Skvarla and Ashley P. G. Dowling

Abstract We present the first character-based phylogenetic hypothesis for cunaxid genera and subfamilies based on 47 morphological characters scored from adult females. The phylogeny suggests that two of the subfamilies are not monophyletic, and although bootstrap support was not high enough to confidently redefine the subfamilies, it presents a testable hypothesis that will hopefully spur phylogenetic investigations of Cunaxidae using molecular techniques.

Keywords Acariformes · Cunaxidae · Phylogeny · Phylogenetic hypothesis · Tree · Morphology

Cunaxidae (Acariformes: Trombidiformes: Prostigmata: Eupodina: Bdelloidea) (Fig. 4.1a–d) are cosmopolitan predatory mites that are commonly encountered in terrestrial habitats, including forest leaf litter and soil, tree holes, grasslands, agriculture fields, and anthropogenically disturbed areas such as house dust and stored food products (Skvarla et al. 2014). They are generalist predators that employ active and ambush hunting techniques to feed on a variety of active and sessile prey, including, Psocoptera, phytophagous mites, nematodes, scale insects, and arthropod eggs (Ewing and Webster 1912; Walter and Kaplan 1991; Smiley 1992; Walter and Proctor 1999; Castro and Moraes 2010). Cunaxids fail to survive when offered only plant material, and although one report of honeydew feeding exists, it is unknown if they can survive solely on honeydew (Zaher et al. 1975; Walter and Proctor 1999; Skvarla et al. 2014). Smiley (1992) and Skvarla et al. (2014) reviewed the biology, biogeography, morphology, and taxonomy and systematics of the family.

The original version of this chapter was revised: Sub-heading numbering has now been incorporated. The correction to this chapter is available at https://doi.org/10.1007/978-3-030-17265-7_7

M. J. Skvarla (✉)
Department of Entomology, Pennsylvania State University, Agricultural Science & Industries Building, University Park, PA, USA
e-mail: mxs1578@psu.edu

A. P. G. Dowling
Department of Entomology, University of Arkansas, Fayetteville, AR, USA

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Cunaxidae is a relatively small family consisting of approximately 436 species, which are organized in 27 genera and 6 subfamilies (Table 1). Most authors consider Cunaxidae and Bdellidae (Fig. 4.1e, f) to be sister taxa that comprise Bdelloidea based on a number of derived characters, including the presence of trichobothria on leg segments, a solenidia present on the palp tibiotarsi, and unique leg segmentation consisting of a divided femur and fused tibiotarsus (Smiley 1992; Skvarla et al. 2014), although this classification is not universally accepted (Lindquist, pers. comm.). Bdelloidea are sometimes considered to be sister to the aquatic and mostly marine Halacaroidea, and are generally placed in the cohort Eupodina within the suborder Prostigmata (e.g., Norton et al. 1993; Lindquist 1996).

Cunaxid subfamilies have traditionally been proposed with little or no cladistic backing, which creates two major problems. First, basal taxa may be grouped together because they have fewer derived characters or share pleisomorphic characters lost in derived taxa. Second, highly derived taxa that stem from within established groupings may be classified separate from those groups, leading to paraphyly of the larger group. Both of these situations lead to classifications that do not reflect evolutionary history and are thus misleading.

Testing the validity of cunaxid subfamilies is compounded by the fact that no rigorous phylogenetic hypotheses of higher classifications (i.e., Prostigmata, Eupodina, Bdelloidea) exist, which makes outgroup selection difficult. For example, Norton et al. (1993) and Lindquist (1996) provided cladograms of Prostigmata, but the relationships were based on unpublished analyses and data (Proctor 1998). Numerous studies (morphological: O'Connor 1984; molecular: Dabert et al. 2010; Pepato et al. 2010; Pepato and Klimov 2015; Dabert et al. 2016; Xue et al. 2017) have recovered a monophyletic Prostigmata, but none have focused on the group and suffer from limited taxon sampling; additionally, recent molecular studies have recovered various phylogenetic hypotheses, some of which do not support recognized or hypothesized groups or relationships, including a monophyletic Euopdina (Dabert et al. 2010; Pepato and Klimov 2015; Dabert et al. 2016; Xue et al 2017), a sister-group relationship between Halacaroidea and Bdelloidea (Pepato and Klimov 2015; Dabert et al. 2016), or monophyletic Bdelloidea (Pepato and Klimov 2015).

In addition to testing the current subfamilial classification scheme, well-supported phylogenetic hypotheses allow stories to be told about evolutionary trends and character evolution. For example, Smiley (1992) considered *Parabonzia* the most basal cunaxid genus as they share a number of characteristics with Bdellidae, including 6–9 pairs of setae on the subcapitulum and "a five segmented palpus which resembles the palpi of the Bdellidae". He also considered cunaxids with 3–segmented palps the most derived, having "body sizes [that] are smaller and…adaptations to exploit different habitats and smaller prey." However, without a well-supported phylogenetic hypothesis, these opinions cannot be corroborated.

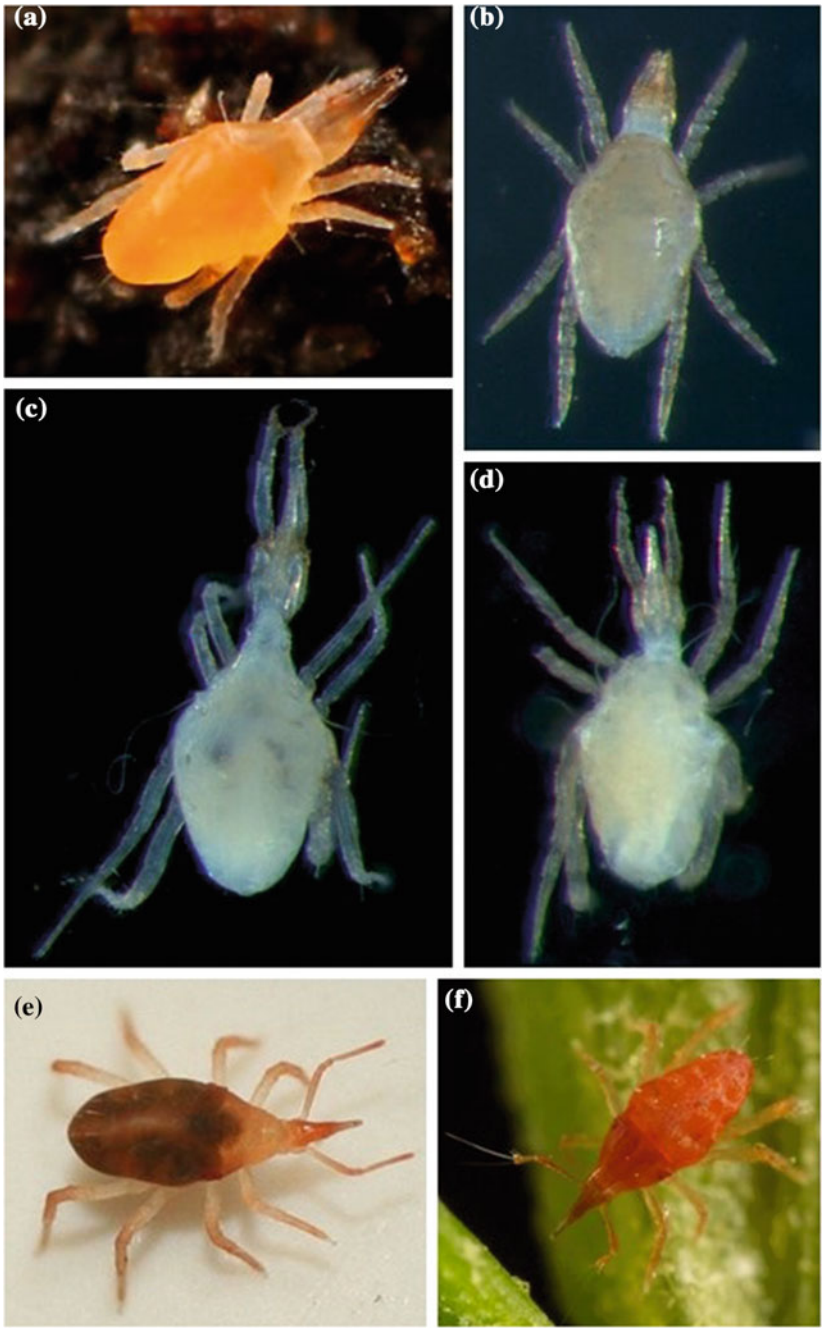

Fig. 4.1 Cunaxidae and Bdellidae. **a** Cunaxoidinae. **b** *Pulaeus*. **c** *Armascirus*. **d** *Rubroscirus*. **e**, **f** Bdellidae. (**a**) © Scott Justis, (**b**–**d**) originally published in Skvarla et al. (2014), (**e**) © Graham Montgomery, (**f**) © Alice Abela. All photos used with permission

Table 1 Table showing 27 genera and 6 subfamilies

Subfamily	Genus	Number of species
Bonziinae	*Bonzia*	7
Bonziinae	*Parabonzia*	7
Coleoscirinae	*Coleoscirus*	32
Coleoscirinae	*Neobonzia*	25
Coleoscirinae	*Neoscirula*	29
Coleoscirinae	*Pseudobonzia*	10
Coleoscirinae	*Scutascirus*	8
Cunaxinae	*Allocunaxa*	1
Cunaxinae	*Armascirus*	47
Cunaxinae	*Cunaxa*	68
Cunaxinae	*Cunaxatricha*	1
Cunaxinae	*Dactyloscirus*	33
Cunaxinae	*Riscus*	4
Cunaxinae	*Rubroscirus*	24
Cunaxoidinae	*Bunaxella*	3
Cunaxoidinae	*Cunaxoides*	26
Cunaxoidinae	*Denheyernaxoides*	3
Cunaxoidinae	*Dunaxeus*	3
Cunaxoidinae	*Funaxopsis*	3
Cunaxoidinae	*Lupaeus*	28
Cunaxoidinae	*Neocunaxoides*	19
Cunaxoidinae	*Paracunaxoides*	1
Cunaxoidinae	*Pulaeus*	33
Cunaxoidinae	*Qunaxella*	1
Cunaxoidinae	*Scutopalus*	15
Orangescirulinae	*Orangescirula*	3
Scirulinae	*Scirula*	2
Total		436

4.1 Materials and Methods

This manuscript is modified from Chapter VI of Skvarla's (2011) unpublished MS thesis. Terminology has been updated and follows Skvarla et al. (2014).

4.1.1 Cladistic Analysis

The analysis included every genus of Cunaxidae (27 genera) and Bdellidae (13 genera) as ingroup taxa. Anystidae, Eupodidae, Tydeidae, and Labidostommatidae were chosen as outgroup taxa as they represent many of the larger groups (e.g., Anystina, Eupodina) and morphological diversity within Prostigmata. Halacarioidea, which has been suggested as the sister group of Bdelloidea, was excluded from the analysis as their many adaptations to an aquatic lifestyle make determining homologies difficult or impossible. 47 characters were scored from adult females; males and non-adult stages were excluded as they are unknown from most species of Cunaxidae.

Characters states were obtained from literature and, when possible, confirmed with specimens. Characters present in at least two taxa, and thus potentially synapomorphic, were included. Characters were equally weighted and unordered. Characters were coded as polymorphic when more than one character state existed within a single taxa. In some instances, an apparently polymorphic character (e.g., tarsal lobes absent; present and small; present and large) was divided into two characters (character 1: tarsal lobes absent or present; character 2: if present, tarsal lobes small or large) as an underlying relationship appeared to exist between the characters.

Character states that could not be determined were coded with a question mark. The uncertainty of a character was due to the complete absence of the character in the taxa in question (such as the number of setae on the palp femurogenu when the femora and genua of a particular taxa are not fused) or to uncertainty as to the homology of the character across taxa. A question mark was also scored when the state of a character could not be determined from the literature.

A heuristic search for 1000 most parsimonious cladograms was carried out using Mesquite 2.74 (Maddison and Maddison 2010). Trees were rearranged by subtree pruning and regrafting. A strict consensus tree was calculated using the 1000 most parsimonious cladograms. Bootstap values, consistency index (C.I.) and retention index (R.I.) were calculated using WinClada 1.00.08 (Nixon 2002).

4.1.2 Characters and Character States

Gnathasoma

I. *Shape of gnathasoma*: normal (0); elongated (1).
II. *Setae* hg_1: not geniculate (0); geniculate (1).
III. *Number of subcapitular setae*: 2 (0); 4 (1); 5 or more (2).
IV. *Pedipalps extend beyond distal end of subcapitulum by at least the last two segments*: no (0); yes (1).
V. *Pedipalp ends in a claw*: no (0); yes (1).
VI. *Pedipalp fixed digit*: absent (0); present (1).
VII. *Pedipalp femora divided*: no (0); yes (1).

VIII. *Pedipalp femora and genua fused*: no (0); yes (1).
IX. *Pedipalp tibiae and tarsi fused*: no (0); yes (1).
X. *Femoral apophysis*: absent (0); present (1).
XI. *Apophysis between genua and tibiae*: absent (0); present (1).
XII. *Number of setae on femurogenua*: 5 (5); 6 (6).
XIII. *Number of setae on basifemora*: 1 (0); more than 1 (1).
XIV. *Shape of basifemoral seta if only 1 seta present*: simple (0); spine-like (1).
XV. *Shape of telofemoral seta*: simple (0); spine-like (1); multi-branched (2).
XVI. *Cheliceral fixed digit*: absent (0); present (1).
XVII. *Number of cheliceral seta(e)*: 0 (0); 1(1), more than 1 (2).
XVIII. *Number of adoral setae*: 0 (0); 1 (1); 2 (2).

Dorsal idiosoma

XIX. *Eyes*: absent (0); present (1).
XX. *Naso*: absent (0); present (1).
XXI. *Number of dorsal trichobothria*. 0 (0); 1 (1); 2(2).
XXII. *Hysterosomal median plate*: absent (0); present (1).
XXIII. *Hysterosomal median plate fused to protersomal shield if median plate present*: no (0); yes (1).
XXIV. *Idiosomal plates and shields patterned with reticulations*: no (0); yes (1).
XXV. *Cupule* ia: absent (0); present (1).
XXVI. *Cupule* im: absent (0); present (1).
XXVII. *Cupule* ip: absent (0); present (1).
XXVIII. *Setae* f_2: absent (0); present (1).

Ventral idiosoma

XXIX. *Coxae I-II fused into sternal shield*: no (0); yes (1).
XXX. *Number of setae on coxae I*: 3 or fewer (0); more than 3 (1).
XXXI. *Number of setae on coxae II*: 3 or fewer (0); more than 3 (1).
XXXII. *Number of setae on coxae III*: 3 or fewer (0); more than 3 (1).
XXXIII. *Number of setae on coxae IVI*: 3 or fewer (0); more than 3 (1).
XXXIV. *Internal genital setae*: absent (0); present (1).
XXXV. *Cupule* ih: absent (0); present (1).
XXXVI. *Number of setae on genital plates*: 4 (0); more than 4 (1).

Legs

XXXVII. *Tibiae I trichoborhtium*: absent (0); present (1).
XXXVIII. *Tibiae II trichoborhtium*: absent (0); present (1).
XXXIX. *Tarsus III trichoborhtium*: absent (0); present (1).
XL. *Tibiae IV trichoborhtium*: absent (0); present (1).
XLI. *Tarsus IV trichoborhtium*: absent (0); present (1).
XLII. *Tasri constricted distally, forming lobes*: no (0); yes (1).
XLIII. *Tarsal lobes, if present*: small (0); large (1).
XLIV. *Leg tibiae divided into basi- and telofemora*: no (0); yes (1).
XLV. *Shape of empodium*: pad-like (0); 4-rayed (1).

XLVI. *Ambulacral claw sculpturing*: smooth (0); rippled (1).
XLVII. *Number of setae complementing anal plates*: 1 (1); 2 (2); more than 2 (3).

The coded matrix run in this analysis is presented in Fig. 4.2.

4.2 Results and Discussion

The heuristic search resulted in 1000 cladograms with a length of 131 (CI = 52; RI = 86). The strict consensus of these cladograms is given in Fig. 4.3. Bootstrap values >50 are presented over each branch.

4.2.1 Monophyly of Bdelloidea and Cunaxidae

The strict consensus cladogram suggests that Bdelloidea is a monophyletic lingeage and that Cunaxidae and Bdellidae are sister clades, rather than a single clade in which one family grades into the other. However, Halacaroidea was excluded from the analysis and the number of outgroups was extremely limited. Inclusion of additional Prostigmata outgroups or Halacaroidea within a molecular phylogeny may change one or both of these conclusions.

4.2.2 Validity of Subfamilies

Parabonzia was recovered as the most basal cunaxid genus and sister to the rest of the family. This is not surprising as *Parabonzia* shares many characteristics with Bdellidae (e.g., non-raptorial pedipalps). The grouping of *Parabonzia* with *Bonzia* in Bonziinae was not recovered. This subfamily is identified primarily by the presence of a multi-branced seta on the palp telofemora, which suggests that the multi-branched seta is plesiomorphic or evolved independently.

The subfamily Cunaxinae was recovered as a monophyletic lineage within a larger clade formed by the addition of three genera (*Pseudobonzia*, *Neoscirula*, and *Neobonzia*) currently classified within Coleoscirinae. This suggests that Cunaxinae is a valid subfamily, but should be redefined to accommodate the coleoscirine genera. The defining character of the larger clade is the absence of fusion between a hysterosomal plate (if it is present) with the protersomal plate and presence of 5-segmented palps (excluding *Allocunaxa*).

The second major clade recovered contains an unresolved basal polytomy formed by the remaining coleoscirine genera (*Coleoscirus* and *Scutascirus*), and a grade formed by the monobasic Orangescirulinae and Scirulinae into an unresolved Cunax-

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV	XXV	XXVI	XXVII	XXVIII	XXIX	XXX
Anystidae	0	0	1&2	0&1	1	0	1	0	0&1	0	0	?	?	?	0	0	?	?	1	1	2	0	?	0	1	0&1	0&1	0&1	1	1
Eupodidae	0	0	1	0	0	0	0	0	1	0	0	?	?	?	0	1	1	0&1	1	0	0	2	0	?	0	0&1	0&1	0&1	0	0
Tydeidae	0	0	?	0	0	0	0	0	1	0	0	?	?	?	0	?	?	?	0	0	1	?	?	?	0	1	1	0	?	?	?
Labidostomatid	0	0	?	0	?	0	0	0	0	0	0	?	?	?	0	1	2	0	1	0	2	1	1	0	0	0	0	1	1	1
Parabonzia	1	0	2	0	0	1	0	1	0	1	0	?	1	0	2	0	1	2	0	0	2	1	0	2	1	0	0	1	1	1	1
Bonzia	1	1	1	0	1	0	0	1	0	1	0	?	1	0	2	0	0&1	2	0	0	2	1	0	0	0	0	1	0	1	0	0
Cokeostirus	1	0	1	0	1	0	0	1	0	1	0	?	1	0	0	0	1	2	0	0	2	1	1	0&1	0	1	0	1	0	0	0
Scutascirus	1	0	1	0	1	0	0	1	0	1	0	?	1	0	?	0	1	2	0	0	2	1	1	0&1	0	1	0	1	0	0	0
Neoscirula	1	0	1	0	1	0	0	1	0	1	0	?	1	0&1	?	0	0&1	2	0	0	2	0	?	0&1	0	1	0	0&1	0	0	0
Pseudobonzia	1	0	1	0	1	0	0	1	0	1	0	?	1	0	?	0	1	2	0	0	2	0	?	0	0	1	0	0	0	0	0
Neobonzia	1	0	1	0	1	0	0	1	0	1	0	?	1	0	0	0	1	2	0	0	2	0	?	1	0	1	0	0	0	0	0
Armascirus	1	0	1	1	1	0	1	1	0	1	1	?	1	0	1	0	1	2	0	0	2	0&1	0	1	0	1	0	0	0	0	0
Dactyloscirus	1	0	1	1	1	0	1	1	0	1	1	?	1	1	1	0	1	2	0	0	2	0&1	0	1	0	1	0	0	0	0	0
Cunaxa	1	0	1	1	1	0	1	1	0	1	0	?	1	0	0	0	1	2	0	0	2	0&1	0	2	0&1	0	0	0	0	0	0
Riscus	1	0	1	1	1	0	0	1	0	1	0	?	1	0	0	0	1	2	0	0	2	0	?	1	0	1	0	0	0	0	0
Allocunaxa	1	0	1	1	1	0	0	1	0	1	0	?	1	0	0	0	1	2	0	0	2	1	0	0	0	1	0	0	0	0	0
Cunaxatricha	1	0	1	1	1	0	0	1	0	1	0	?	1	0	0	0	1	2	0	0	2	0	?	1	0	1	0	0	0	0	0
Rubroscirus	?	?	?	?	?	?	?	?	0	1	0	?	1	0	0	0	1	2	0	0	2	0&1	0	1	0	1	0	0	0	0	0
Pulaeus	1	0	1	0	1	0	0	0	1	1	0	5	?	?	?	0	1	2	0	0	2	1	1	0	0	1	0	0	0&1	0	0
Lupaeus	1	0	1	0	1	0	0	0	1	1	0	5	?	?	?	0	1	2	0	0	2	1	1	0	0	1	0	0	0&1	0	0
Cunaxoides	1	0	1	0	1	0	0	1	0	1	0	5	?	?	?	0	0	2	0	0	2	1	0&1	0	0	1	0	0	0&1	0	0
Neocunaxoides	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	1	2	0	0	2	1	1	0	0	1	0	0	0&1	0	0
Denheyenaxoides	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	?	0	0	0	2	0	?	0	0	1	0	0	0	0	0
Scutopalpus	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	?	2	0	0	2	1	1	0	0	1	0	0	0&1	0	0
Bunaxella	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	0	2	0	0	2	0	?	0	0	1	0	0	0	0	0
Dunaxeus	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	0	2	0	0	2	0	?	0	0	1	0	0	0	0	0
Qunaxella	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	0	2	0	0	2	0	?	0	0	1	0	0	0	0	0
Funaxopsis	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	0	2	0	0	2	0	?	0	0	1	0	1	0	0	0
Paracunaxoides	1	0	1	0	1	0	0	0	1	1	0	6	?	?	?	0	?	0	0	0	2	0	?	0	0	1	0	0	0	0	0
Orangescirula	1	1	1	0	1	0	0	0	1	1	0	?	1	1	1	0	0	2	0	0	2	1	1	1	0	1	0	?	0	0	0
Scirula	1	0	1	0	1	0	0	0	0	1	0	?	1	1	1	0	1	2	0	0	2	1	1	0	0	1	0	1	0	0	0
Polytrichus	1	0	2	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	?	0	0
Biscirus	1	0	0	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	0	1	0
Monotrichobdella	1	0	0	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	0	1	1
Tetrabdella	1	0	0	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	0	1	1
Spinabdella	1	0	0	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	0	1	1
Rigibdella	1	0	0	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	1	0	0	?	?	?	?	?	1	1
Cyta	1	0	0	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	1	1	1	?	?	1	1
Trachymolgus	1	0	0	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	1	0	0	1	1	1	1	1	1	1
Hexabdella	1	0	2	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	?	1	1
Bdella	1	0	2	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	?	1	1
Necmolgus	1	0	2	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	?	1	1
Bdellodes	1	0	2	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	?	1	1
Odontoscirus	1	0	2	0	0	0	0	1	0	1	0	?	2	?	0	1	?	2	1	0	2	0	?	0	?	?	?	?	?	1	1

Fig. 4.2 Morphological character matrix

	XXXI	XXXII	XXXIII	XXXIV	XXXV	XXXVI	XXXVII	XXXVIII	XXXIX	XL	XLI	XLII	XLIII	XLIV	XLV	XLVI	XLVII
Arystidae	1	0&1	?	0&1	1	1	0	0	0	0	0	0	?	0	0	?	?
Eupodidae	0	0	1	0&1	1	1	0	0	0	0	0	0	?	0&1	0	?	?
Tydeidae	?	?	1	1	1	1	0	0	0	0	0	0	?	?	0	?	?
Labidostomatid	1	1	?	0	1	1	0	0	0	0	0	0	?	0	0	?	3
Parabonzia	1	1	1	1	1	1	0	0	0	1	0	0	?	1	1	?	3
Bonzia	0	0	0	1	1	0	0	0	0	1	0	0	?	1	1	?	2
Coleoscirus	0	0	0	1	1	0	0	0	0	1	0	0	?	1	1	?	2
Scutascirus	0	0	0	1	1	0	0	0	0	1	0	0	?	1	1	?	2
Neoscirula	0	0	0	1	0	0	0	0	0	1	0	0	?	1	1	0	2
Pseudobonzia	0	0	0	1	1	0	0	0	0	1	0	0	?	1	1	1	2
Neobonzia	0	0	0	1	0	0	0	0	0	1	0	0	?	1	1	1	2
Armoscirus	0	0	0	1	0	0	0	0	0	1	0	1	1	1	1	?	2
Dactyloscirus	0	0	0	1	0	0	0	0	0	1	0	1	1	1	1	?	2
Cunaxa	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	?	1
Riscus	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	?	1
Allocunaxa	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	?	1
Cunaxatricha	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	?	1
Rubroscirus	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	?	1
Pulaeus	0	0	0	1	1	0	0	0	0	1	0	0	?	1	1	?	1
Lupaeus	0	0	0	1	1	0	0	0	0	1	0	0	?	1	1	?	1
Cunaxoides	0	0	0	1	0	0	0	0	0	1	0	0	?	1	1	?	1
Neocunaxoides	0	0	0	1	0	0	0	0	0	1	0	0	?	1	1	?	1
Denheyernaxoides	0	0	0	1	1	0	0	0	0	1	0	0	?	1	1	?	1
Scutopalpus	0	0	0	1	0	0	0	0	0	1	0	0	?	1	1	?	1
Bunaxella	0	0	0	1	0	0	0	0	0	0	0	0	?	1	1	?	1
Dunaxeus	0	0	0	1	0	0	0	0	0	0	0	0	?	1	1	?	1
Qunaxella	0	0	0	1	0	0	0	0	0	0	0	0	?	1	1	?	1
Funaxopsis	0	0	0	1	0	0	0	0	0	0	0	0	?	1	1	?	1
Paracunaxoides	0	0	0	1	0	0	0	0	0	1	0	0	?	1	1	?	1
Orangescirula	0	0	0	1	0	0	0	0	0	0	0	0	?	1	1	?	2
Scirula	0	0	0	1	0	0	0	0	0	1	0	0	?	1	1	?	?
Polytrichus	0	0	1	1	1	1	1	1	1	1	1	0	?	1	0	?	?
Biscirus	1	1	1	?	1	1	1	0	0	1	1	0	?	1	0	?	?
Monotrichobdella	1	1	1	?	1	1	1	0	1	1	1	0	?	1	0	?	?
Tertabdella	1	1	1	?	1	1	0	0	1	0	1	0	?	1	0	?	?
Spinabdella	1	1	1	?	1	1	1	0	1	1	1	0	?	1	0	?	?
Rigibdella	1	1	1	?	1	1	1	0	1	1	0	0	?	1	0	?	?
Cyta	1	1	1	0	1	1	1	0	1	1	0	0	?	1	0	?	?
Trachymolgus	1	1	1	0	1	1	1	0	1	1	0	0	?	1	0	?	?
Hexabdella	1	1	1	?	1	1	1	0	1	1	0	0	?	1	0	?	?
Bdella	1	1	1	?	1	1	1	0	1	1	1	0	?	1	0	?	?
Neomolgus	1	1	1	?	1	1	1	1	1	1	1	0	?	1	0	?	?
Bdellodes	1	1	1	?	1	1	1	1	1	1	1	0	?	1	0	?	?
Odontoscirus	1	1	1	?	1	1	1	1	1	1	1	0	?	1	0	?	?

Fig. 4.2 (continued)

oidinae. This larger clade is defined by the expansion of the hysterosomal plate and fusion with the protersomal shield.

The lack of resolution and support in the phylogenetic hypothesis is problematic. There are trends that suggest the need for changes in classification, but resolution and support are too weak to confidently make those changes. For example, as previously mentioned, *Bonzia* is not recovered with *Parabonzia*, thus prompting the dissolution of Bonziinae. However, the placement of *Bonzia* within Cunaxidae is uncertain. With more data it may be recovered with *Parabonzia* as a monophyletic clade, as an independent lineage (as is suggested by this analysis), or within one of the two major clades.

The classification of the clade containing Cunaxoidinae depends on better resolution. If the basal polytomy is resolved and *Coleoscirus* and *Scutascirus* form a monophyletic lineage the clade could be broken into two subfamilies: *Coleoscirus* + *Scutascirus* and Orangescirulinae + Scirulinae + Cunaxoidinae. Alternately, it could

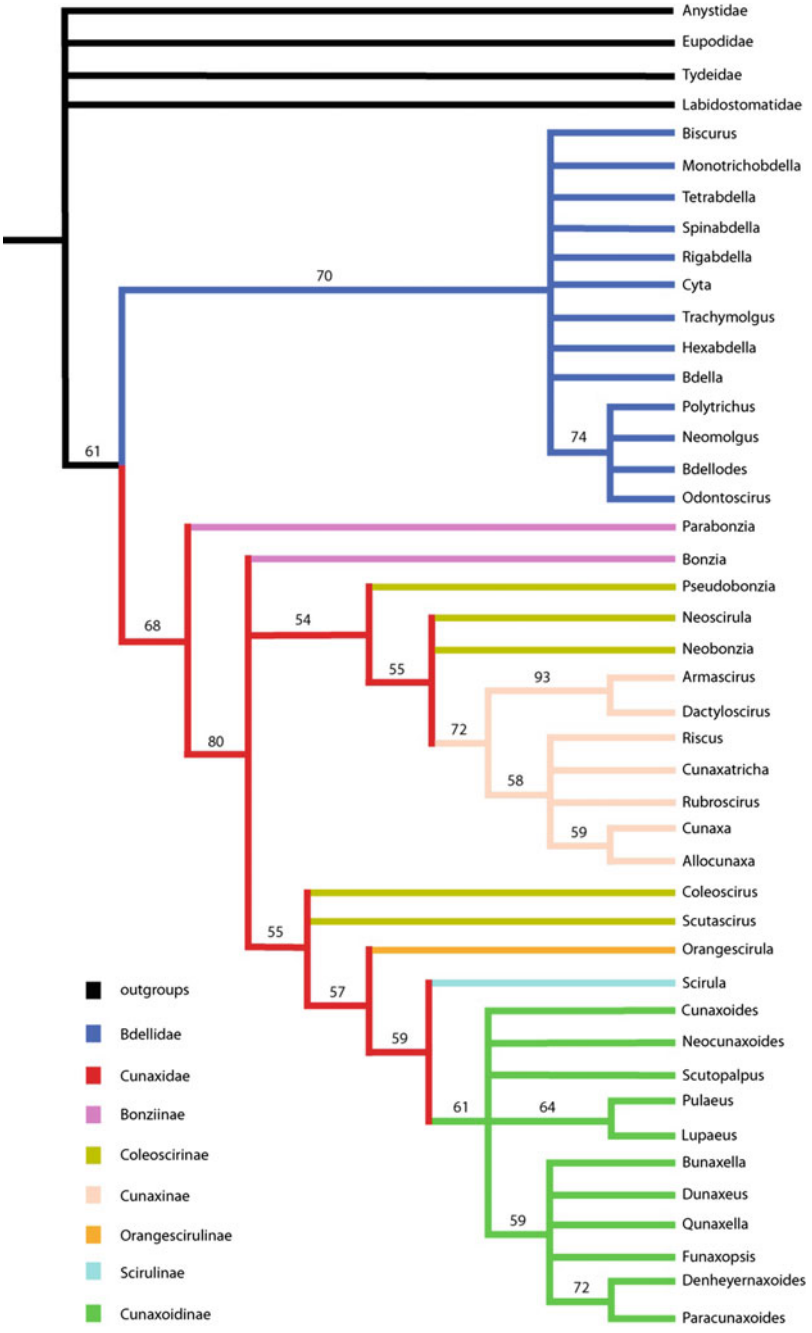

Fig. 4.3 Strict consensus tree of 1000 most parsimonious trees (length = 131; CI = 52; RI = 86) obtained using 47 characters. Bootstrap values >50 are presented over each branch

be broken into four subfamilies, *Coleoscirus* + *Scutascirus*, Orangescirulinae, Scirulinae, Cunaxoidinae. If the basal polytomy is resolved and the coleoscirine genera are not monophyletic, that is form grade with Orangescirulinae and Scirulinae into Cunaxoidinae, Cunaxoidinae could redefined to include them, thus forming a single diverse subfamily, or they could be broken into five subfamilies (i.e., Coleoscirinae, Scutascirinae, Orangescirulinae, Scirulinae, and Cunaxoidinae), four of which would be monobasic.

4.2.3 Evolutionary Trends

While the strict consensus cladogram is not resolved enough to be a basis for classification changes, it does suggest directions in evolutionary trends. The most derived taxa in both larger clades (*Allocunaxa* and Cunaxoidinae) have palps in which the basifemora, telofemora, and genu have fused into a femurogenu, resulting in a 3-segmented pedipalp. This fusion in Cunaxinae may be more evolutionarily recent evolutionarily as species with fused pedipalpal segments still retains dark lines that indicate where the sutures are. Conversely, the palp segmentation of Cunaxoidinae can only be inferred through muscle attachment and setal placement as they lack sutures any external indication of the fused segments.

4.3 Conculsions

The morphological phylogenetic hypothesis presented here illustrates possible inconsistencies between current classification schemes and evolutionary history. However, a phylogenetic hypothesis with better support must be elucidated before such changes can be made with any kind of confidence. As the morphological characters presented in this study did not provide the needed resolution and support, molecular characters are the next logical step.

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Chapter 5
Mycobiome of *Brevipalpus* Mite Strains and Insights on Metabolic Function in the Bacteriome of the Tetranychoidea Mites

Jose Carlos Verle Rodrigues, Oscar E. Ospina and Steven E. Massey

Abstract Studies of arthropod microbiota and arthropod-microbe interactions are helping elucidate the strategies adopted by arthropods to colonize and succeed in complex environments, as well as leading to the development of unique pest management approaches. Tetranychoidea (Acariformes: Trombidiformes) are important pests of several crops due to their feeding habits and transmission of pathogens. In tetranychoid mites, the endosymbiont bacterium *Cardinium* represents a tantalizing first target for pest management research because it affects mite reproduction. In this study, we used previously published 16S ribosomal RNA sequence data of the microbiome bacteria in *Brevipalpus yothersi* (Baker 1949), *Raoiella indica* Hirst, 1924, and *Oligonychus* sp. and the PICRUSt pipeline to predict the content of genes with metabolic function in the bacteriome of the mites. Our results indicate that the bacteriomes of *B. yothersi* and *Oligonychus* sp. (which harbor *Cardinium*) contain significantly more genes involved in the metabolism of indole-alkaloids, glutamine, and biotin when compared with *R. indica* (which has no *Cardinium*). The genes for metabolism of biotin and nicotinate are also more abundant in adult *B. yothersi* and *Oligonychus* sp. than in their eggs, which is associated with lower abundance of *Cardinium* in the eggs. The metabolic specialization of *Cardinium*-dominated bacteriomes could also lead to lack of resistance to β-lactam antibiotics and DDT. While these results are predictive, they highlight the necessity of testing these variations in laboratory. We also present initial data on fungal microbial diversity associated with four different strains of the phytophagous mite vector *Brevipalpus*, which showed significant variation between strains, while all are dominated by the skin- and surface-specialist genus *Malassezia*.

J. C. V. Rodrigues (✉) · O. E. Ospina
Agro-Environmental Sciences Department, Center for Excellence in Quarantine & Invasive Species, Agricultural Experimental Station - Río Piedras, University of Puerto Rico, 1193, Calle Guayacan, San Juan, PR 00926, USA
e-mail: jose_carlos@mac.com

S. E. Massey
Bioinformatics Laboratory, Department of Biology, University of Puerto Rico – Rio Piedras, San Juan, PR 00931-3360, USA

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Keywords False-spider mite · Red-palm mite · *Malassezia* · Acari metabolism · *Cardinium* · Antibiotic resistance · Xenobiotics · Amino acid metabolism · Vitamin supplementation

5.1 Introduction

New pest management tools are being developed with the aid of state-of-the-art molecular techniques such as Next Generation Sequencing (NGS) (Busby et al. 2017). In particular, attention has been directed to the study of arthropod endosymbionts because of their potential for practical applications to immediate pest problems (Crotti et al. 2012), such as the use of *Wolbachia* in mosquito control (Iturbe-Ormaetxe et al. 2011).

Several crops (e.g., beans, citrus, tea, coconut, tomato, pepper, and passion vine) are damaged by mites in the superfamily Tetranychoidea (e.g., *Brevipalpus* Donnadieu, 1875 and *Raoiella* Hirst, 1924 (Tenuipalpidae Berlese, 1913) and *Oligonychus* Berlese, 1886 (Tetranychidae Donnadieu, 1875)), either through direct feeding or the ability of the mites to transmit plant pathogens (Kitajima et al. 2003; Maoz et al. 2011; Rodrigues and Childers 2013; Rodrigues et al. 2007). Members of the endosymbiotic bacterial 'Candidatus *Cardinium*' are commonly found in mites, including some Tetranychoidea (Weeks et al. 2003; Zchori-Fein and Perlman 2004). Consequently, *Cardinium* is a prime target for pest control applications because it not only effects the sexuality of mites, including feminization of males (Weeks et al. 2001), but could also affect traits related to mite survival and fitness, which has been demonstrated in other arthropods (Wang et al. 2008).

In *Brevipalpus yothersi* (Baker 1949), an important virus vector pest of citrus and other crops, the bacteriome is dominated by the presence of *Cardinium* (Ospina et al. 2016; Weeks et al. 2003). The endosymbiont is highly abundant regardless of the immediate ecological conditions of the mite. Nonetheless, the bacteria can be up to 10 times more abundant in the adults when compared with its eggs (Ospina et al. 2016). Such abrupt proliferation results in a decrease of overall bacterial diversity, which in turn can influence functional and metabolic specialization of the bacteriome. With the use of bioinformatic methods, it is possible to predict which functional or metabolic traits are present in a bacterial community. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline allows the inference of gene families present in a bacterial community. It does so based on Operational Taxonomical Units (OTUs), generated by sequencing of the 16S rRNA gene (Langille et al. 2013). The pipeline uses a reconstruction of gene content across the bacterial taxa in a community based on known genomes. The gene families that are predicted in the bacteria are identified in reference to the KEGG Orthology Database.

At least equally important as the bacteriome, but comparatively little studied, is the presence and role of fungi within the microbial community of mites. Internal Transcribed Spacer (ITS) of the rRNA locus has been routinely used as a standard

'barcode' marker for fungal species detection and delineation (Schoch et al. 2012), and the efficacy of the approach promises to reveal insights as substantial as those generated from NGS analysis of the bacteriome. For example, recent fungal microbiomes ('mycobiomes') from around the world reveal unexpectedly high diversity that greatly surpasses counts from morphological studies and culturing (Hawksworth 2012), thus confirming the utility of NGS based approaches for the mycobiome. However, studies elucidating the mycobiomes in arthropods are still in their infancy, with examples in fruit flies (Quan and Eisen 2018; Malacrinò et al. 2017) and ants (Duarte et al. 2016).

Herein, we used the bacterial OTUs reported by Ospina et al. (2016) in *B. yothersi*, *Raoiella indica* Hirst, 1924, and *Oligonychus* sp. to infer metabolic distinctions in their bacteriomes, while considering the presence or absence of *Cardinium*. We predicted the content and abundance of gene families related to the metabolism of amino acids, vitamins, and xenobiotics present in the bacteriomes by using the PICRUSt pipeline. Although predictive, this study delineates new and promising research leading to the development of potential applications for pest management of these mites. In addition, we present the initial determination by NGS of fungal communities associated with four *Brevipalpu*s strains originally obtained from four different plant hosts, finding inter-strain mycobiome variation and novel fungal-mite associations.

5.2 Materials and Methods

5.2.1 Genomic DNA Extraction and Pyrosequencing

Bacterial metabolic predictions—The PICRUSt 1.1.0 pipeline (Langille et al. 2013) was used to generate predictions regarding the functional diversity in the bacteriomes. The pipeline takes as input the number and abundance of OTUs in each sample as well as the taxonomy assigned to each OTU. In a previous study, Ospina et al. (2016) generated data on the OTUs present in mites of the superfamily Tetranychoidea. These OTUs were used in the present study to produce our predictions on functional diversity. In the next section, we summarize the methods used by Ospina et al. (2016) to handle NGS data and identify the OTUs in these mites.

Mycobiome—Total genomic DNA was extracted from 20 eggs and 20 mites per colony following the CTAB method used in our previous study (Ospina et al 2016). The *fITS7-ITS4* primer pair (Ihrmark et al. 2012) was used to amplify a $\approx$ 300 bp fragment of the gene coding for fungal ITS2 rDNA Ribosomal Subunit with the HotStarTaq Plus Master Mix Kit (Qiagen). The fragments were sequenced by MrDNA (Shallowater, TX) using a 454 GS FLX System on a 7075 GS Picotiter Plate (Roche, Branford, CT, USA). Sequences were filtered (Q-value > 25), classified by barcode,

with removal of chimeric sequences and low abundance reads. Clustering was based on 97% similarity and OTUs were assigned based on the UNITE reference Sequence Dataset (v7.0) and alpha and beta diversity were calculated.

5.2.2 *Study Organisms, Quality Control of Data and Taxonomic Assignations*

Brevipalpus yothersi were originally collected from sweet orange (*Citrus* × *sinensis* (L.) Osbeck), Tahiti lime (*Citrus* × *latifolia* (Yu. Tanaka) Tanaka), and glorybower (*Clerodendrum thomsoniae* Balf.) in Puerto Rico. The colonies were reared on "Valencia" sweet oranges from a single egg for 10 generations under a constant temperature of 25 °C and 75% relative humidity (Fig. 5.1). We also collected other Tetranychoidea from the field for comparative purposes: *Raoiella indica* from *Cocos nucifera* L., and *Oligonychus* sp. from *Phaseolus vulgaris* L. Specimens from all species were slide mounted and barcoded (Rodrigues et al. 2004). Accession numbers for the barcodes are KP180424 to KP180429. Amplification of ~250 bp fragments of genomic DNA from the V4 region of the 16S rRNA was performed by using primers bac515F and bac806R (Ospina et al 2016). Sequencing of the 16S rRNA amplicons was carried out by on a 454 GS FLX System using a 7075 GS Picotiter Plate (Roche, Branford, CT, USA).

Bacterial OTUs were assigned to samples by using unique 8 bp barcodes (demultiplexing). This process was carried out by the script "split_libraries.py" included in the pipeline Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al. 2010a). During de-multiplexing, we also removed the primer, linkers, and barcodes from the sequences. Quality filtering included the removal of the sequences with a Phred-score lower than 25 and elimination of 454-characteristic noise signals with QIIME's script "denoiser.py". Sequences were aligned using PyNAST v1.2 against the Greengenes reference alignment (Caporaso et al. 2010b). Identification and elimination of chimeric sequences was performed with ChimeraSlayer v4.29.2010 as implemented in QIIME and using the template alignment provided by PyNAST. For each sequence, taxonomy was assigned by clustering the sequences based on 97% similarity to generate OTUs. OTUs represented by a single sequence were removed from the dataset. Taxonomy was indicated for each OTU by using the Ribosomal Database Project (RDP) Classifier v2.11 (Wang et al. 2007) and the Greengenes Reference Database included in QIIME. The OTU set was rarefied 10 times to estimate average Chao1 and Shannon diversity indexes. Additionally, estimations of the UniFrac distances and the Bray-Curtis dissimilarity index were performed.

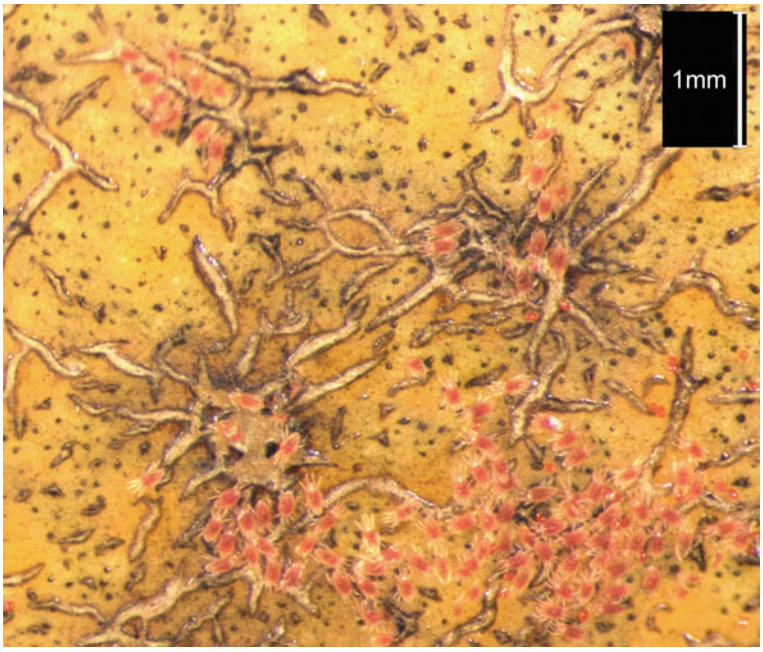

Fig. 5.1 Appearance of typical female haploid *Brevipalpus yothersi*, colony reared on sweet orange arena. San Juan, Puerto Rico

5.2.3 Analysis of Metabolic Function

We generated predictions of the metabolic profiles of these bacteriomes with the PICRUSt pipeline, which allows the inference of gene families from bacterial taxa in a host's metagenome as detected by the sequencing of the 16S rRNA. Specifically, we used PICRUSt to identify metabolic pathways potentially contributed by the bacteria in the mites. Since the number of 16S rRNA gene copies varies among bacterial taxa, the first step in this pipeline was the normalization of the OTUs by the predicted 16S rRNA copy number abundance. Secondly, using PICRUSt's "predict_metagenomes.py" we predicted the gene families with functional/metabolic importance that was contributed by each normalized OTU. The identification of these gene families was based on the KEGG Orthology Database (http://www.genome.jp/kegg/ko.html).

Because PICRUSt analyses yield a large number of predictions, we focused on gene families related to the metabolism of amino acids, vitamins/cofactors, and xenobiotics. To visualize differences in abundance of these gene families, we performed Welch's t-tests to detect significant differences in the mean proportion of gene families, using the STAMP v2.1.3 package (Parks et al. 2014).

5.3 Results and Discussion

5.3.1 Bacteriome, and Metabolic Predictions

The PICRUSt analysis predicted 328 gene families with metabolic/functional importance in the bacteriome of the mites in this study. We narrowed this predicted dataset to 91 gene families related to metabolism and degradation of amino acids, vitamins/cofactors, and xenobiotics. The analysis yielded 10 gene families that were significantly different in abundance among mites with and without *Cardinium* (Fig. 5.2). Genes involved in the metabolism of indole-alkaloids, Glutamine, and Biotin were significantly more abundant in the bacteriomes of mites with *Cardinium*. Several examples have arisen indicating the potential of endosymbionts as providers of nutrients to the host (Aksoy 2000; Feldhaar et al. 2007; Sabree et al. 2009). *Cardinium* may be supplementing the diet of *B. yotherisi* and *Oligonychus* sp., resulting in possible host mite selection in favor of the endosymbiont.

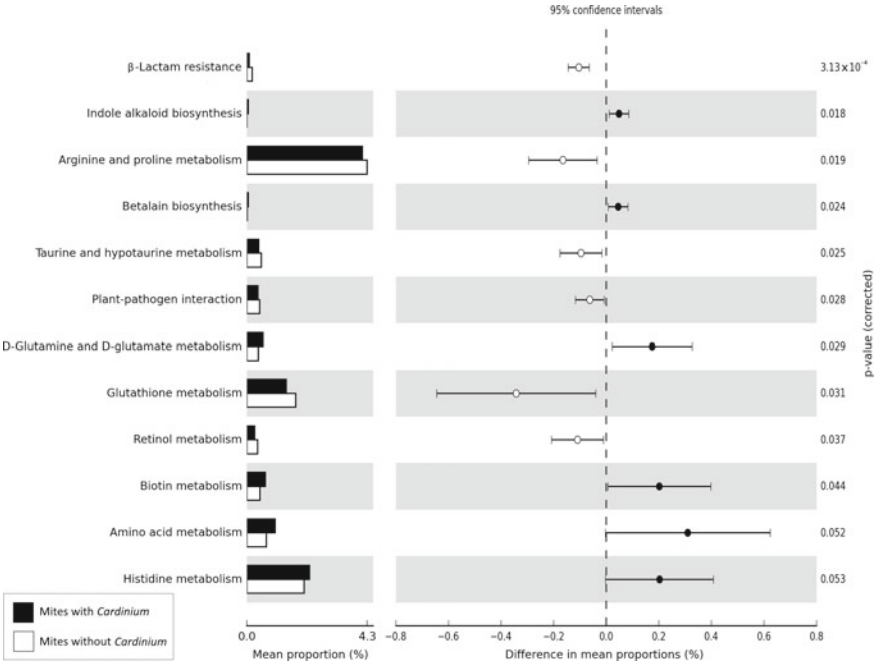

Fig. 5.2 Mean proportion of gene families associated with inferred metabolic/functional traits in mite bacteriomes with the endosymbiont *Cardinium* (black) and without it (white). Confidence intervals for the means are provided as well as the corrected p-value for the comparisons. Gene families correspond to those in the KEGG Orthology Database (http://www.genome.jp/kegg/ko.html)

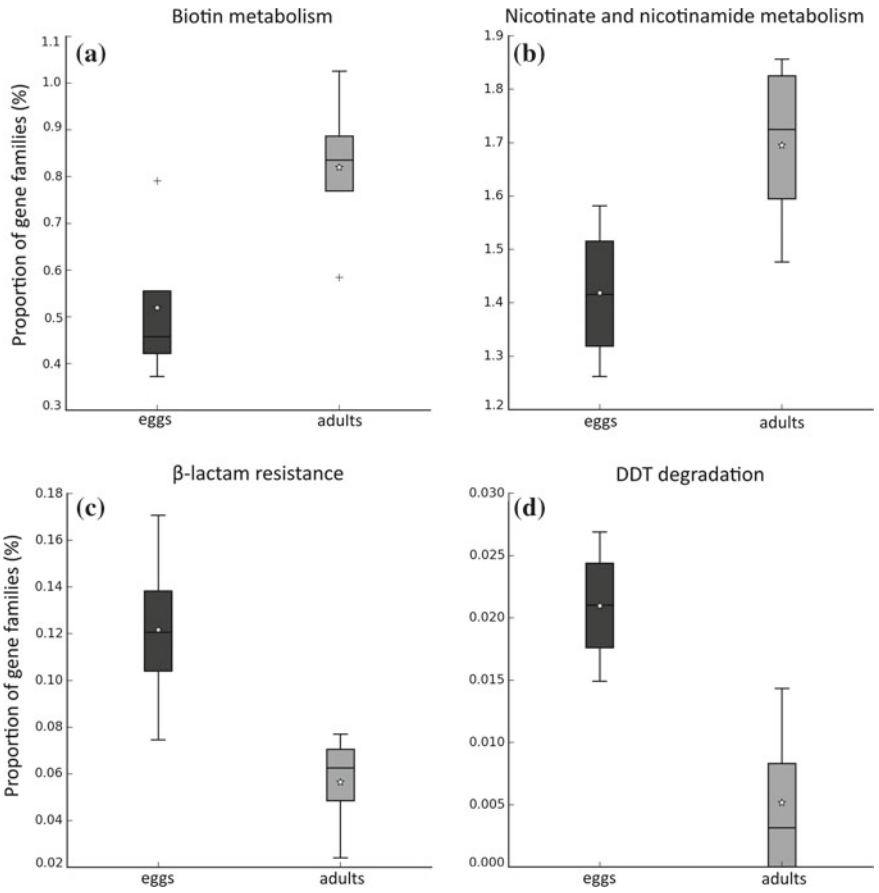

Fig. 5.3 Proportion of inferred gene families involved in the Biotin metabolism (**a**), Nicotinate metabolism (**b**), β-lactam resistance (**c**), and DDT degradation (**d**). The comparison here involves only mite eggs and adults that contain 'Candidatus *Cardinium*' in their bacteriome. The mean is indicated with a star. Outliers are shown as crosses

The contribution of favorable traits to the host is a plausible reason for the increase in abundance of *Cardinium* when comparing eggs to adult mites (Ospina et al. 2016). Our analyses showed that egg bacteriomes had a lower abundance of genes related to the metabolism of biotin in comparison to adult bacteriomes (Fig. 5.3a). Similarly, our results suggest that genes related to the metabolism of nicotinate are present at higher abundance in egg bacterial communities if compared to those in adult mites (Fig. 5.3b). Having a bacterial endosymbiont with the ability to contribute vitamins confers to the host an advantage when dietary conditions are not optimal (Nakabachi and Ishikawa 1999; Smith et al. 2015).

Despite these positive contributions from the endosymbiont, we also observed that such functional specialization of the bacteriome could be detrimental for the

system. Genes involved in the resistance to β-lactam antibiotics were more abundant in eggs in comparison to adults (Fig. 5.3c). Although the use of antibiotics is not recommended for commercial or environmental use, the reduction of resistance to antibiotics in adults opens a possibility for pest management provided a suitable delivery method is available (Douglas 2007). We also observed that genes involved in the metabolism of the DDT were also relatively reduced in adults (Fig. 5.3d). The microbial response and plasticity to the exposition of these compounds is still to be verified.

5.3.2 NGS Fungal Diversity

We observed a high diversity of fungal OTUs associated with the four different strains of *Brevipalpus* that originated from distinct host plants, even though mite colonies had been reared for 10 generations in the same sweet orange fruit arenas. However, the core mycobiome shared among the four strains analyzed was reduced (n = 19) (Figs. 5.4 and 5.5). We also observed a slight increase in diversity from eggs to adults, which is an inverse pattern to that observed in the bacteriome (eggs > adults) (Ospina et al. 2016).

The predominant genus identified was *Malassezia*, with OTUs indicating *M. restricta, M. globosa and M. dermatis* as frequent species (Table 5.1). This yeast-like basidiomycetous genus is usually associated with human skin and epidermis of animals from different environments, where, jointly with other microbes, the fungi play a major role in microbial community stability (Human Microbiome Project Consortium 2012). To our knowledge, this is the first time that this genus has been associated with the tenuipalpid family. *Brevipalpus* mites present a complex multilayer epidermis (Rodrigues 2000), providing abundant opportunities for a sustainable association with skin-epidermis specialists such as *Malassezia*. This discovery opens up new

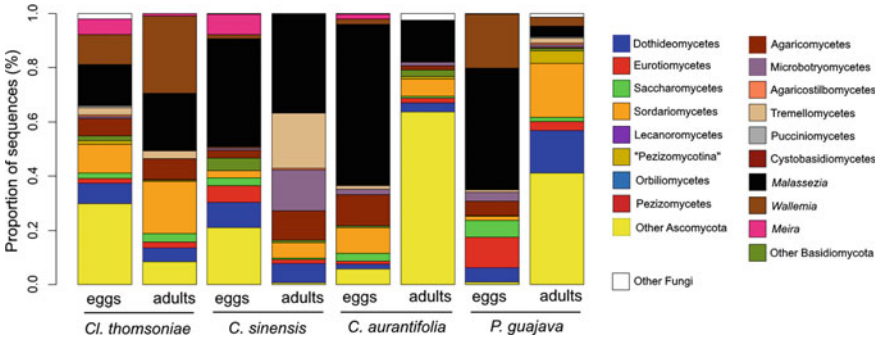

Fig. 5.4 Relative abundance of fungal classes in the mycobiome of egg and adult *Brevipalpus yothersi*. Each portion of the bar indicates the percentage of sequences assigned to a taxon. The relative abundance of genera *Malassezia*, *Wallemia*, and *Meira* are also shown

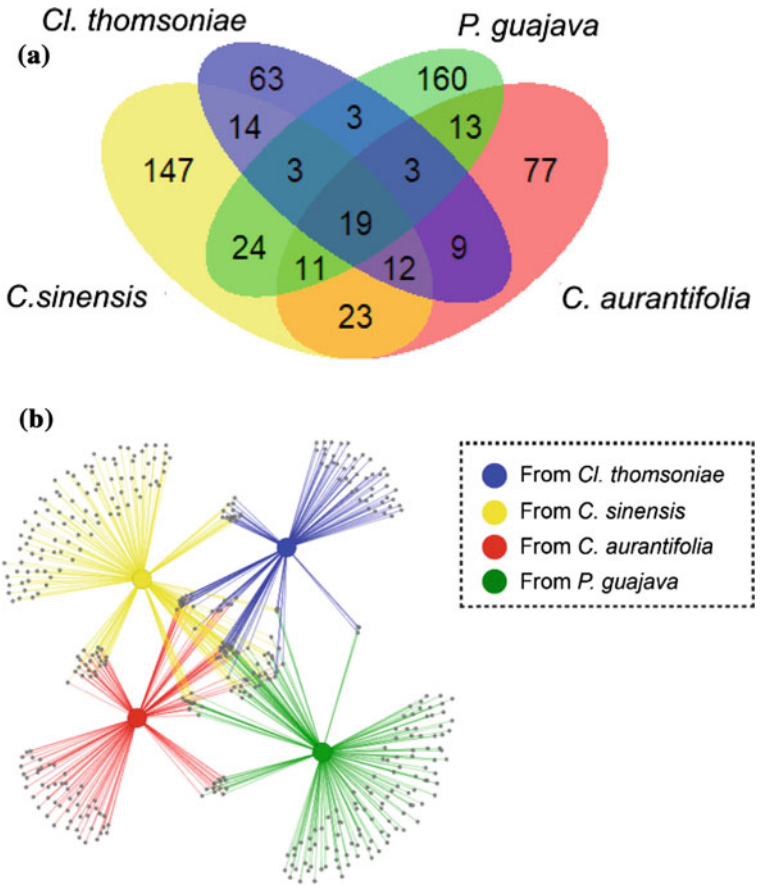

Fig. 5.5 Venn diagram **a** showing the number of shared fungal taxa amongst adult *Brevipalpus yothersi*, regarding their original host plant. **b** Network indicating shared OTUs among adult samples of *B. yothersi*

opportunities for studies of the role of the fungi on multiple interactions of the mite with chemical products and other biological agents. However poorly understood, new mycobiome studies are showing the importance of fungi-arthropod interactions. For example, Angleró-Rodríguez et al. (2016) suggested that the mycobiome can have an important impact on pathogen transmission because fungi produce antimicrobial compounds and influence vector immunity, affecting vector competence. This understanding and the consequent possibilities of mycobiota manipulation could lead to new control strategies for pathogen vectors, as suggested by Saldaña et al. (2017).

Other species found in the mite mycobiome were the phytofagous genera *Diaporthe*, *Giberella* (anamorph = *Colletotrichum*), and the free-living *Aspergillus.* They are likely ingested by the mites during the feeding on sweet orange fruit skins that serve as rearing arenas.

Table 5.1 Relative abundance (%) of most common species-level inferred OTUs in the microbiomes of *Brevipalpus yothersi*, with regard to the mite's life stage and original host plant

Fungi spp.	*Clerodendrum thomsoniae*		*Citrus sinensis*		*Citrus aurantifolia*		*Psidium guajava*	
	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
Malassezia restricta	50.3	41.5	35.5	13.0	32.3	17.7	13.1	3.5
Malassezia globosa	7.8	2.2	3.2	2.2	4.3	3.3	1.7	0.3
Malassezia dermatis	1.1	1.6	0.0	0.0	25.5	0.3	0.0	0.0
Diaporthe citri	8.1	0.0	0.0	1.6	0.0	0.5	0.0	2.2
Wallemia ichthyophaga	2.0	19.6	1.5	11.1	0.0	28.6	0.0	3.2
Meira argovae	1.9	0.0	6.2	5.6	0.0	0.9	0.2	0.1
Aspergillus aff. niger	0.9	9.8	2.7	0.2	0.6	1.4	0.0	0.6
Gibberella sp.	0.0	0.5	0.0	3.1	2.4	13.3	0.2	9.3
Microcera larvarum	0.0	0.0	0.0	0.0	0.0	0.1	6.1	2.2
Colletotrichum chrysanthemi	0.0	0.2	0.4	3.8	0.0	0.1	0.0	0.0
Sporobolomyces koalae	0.0	0.8	0.0	0.0	11.9	0.0	0.0	0.0

The genus *Wallemia* was found associated with both eggs and adults of *Brevipalpus*, and comprise xerophilic fungi species, which are an exception in the basidiomycetes. These fungi have been previously reported from air, soil, dried food, and salt (Zalar et al. 2005). Here we report for first time that they are associated with *Brevipalpus* mites. This phytophagous mite is particularly successful in colonizing plant species in drier environmental conditions (Rodrigues et al. 2016). Xerophilic fungi have been isolated from the digestive tract of house-dust mites, (*Dermatophagoides* sp.) and the stimulation or reduction of populations is dependent on the environmental conditions (Hart and Douglas 1991; Lustgraaf 1978).

Other OTUs of basidiomycetes commonly found associated with *Brevipalpus* in this study included the yeast-like fungus *Meira argovae*, which has been previously reported from the corpses of dead mites in Israel and bamboo shoots in Japan (Tanaka et al. 2008). In addition, this species had been suggested as a potential biological control agent for mites (Boekhout et al. 2003).

5.4 Conclusion

The bacteriomes of *B. yothersi* and *Oligonychus* sp. show metabolic specialization towards the supplementation of diet with vitamins. Such specialization is associated with the abundant endosymbiont *Cardinium*, as observed when comparing eggs with adults. The dominance of *Cardinium* in these mites also can bring trade-offs related to vulnerability to antibiotics and acaricides. Although this study is based on bioin-

formatic predictions, it suggests potential metabolic pathways that can be tested in a laboratory setting. Provided these results are tested, they may generate tools that target *Cardinium* as a proxy for control of mite populations.

The four *Brevipalpus* strains exhibited higher mycobiome diversity, with limited core fungal diversity. *Malassezia*, which is reported for the first time on tenuiplapid mites, was the predominant fungal genus found. A slight increase of fungal diversity from eggs to adults was observed, which is an inverse pattern to the observed at bacteriome. Further studies are necessary to elucidate the role of fungi complex and their interaction with mites and their other microbial communities.

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Chapter 6
Biodiversity of Phytoseiidae (Acari: Mesostigmata) of Annual Specialty Crop Systems: The Current State of Knowledge Worldwide and the Need for Study in North America

Monica A. Farfan and Rebecca A. Schmidt-Jeffris

Abstract Predatory mites in the family Phytoseiidae (Acari: Mesostigmata) are known to be important natural enemies of common plant pests, including tetranychids, tarsonemids, eriophyids, whiteflies, and thrips. While a great deal is known about the diversity and abundances of phytoseiid species in perennial agricultural systems in the United States, such as orchards and vineyards, very little is known regarding the phytoseiid community in annual specialty crop systems. Most information regarding endemic phytoseiid diversity comes from studies in other parts of the world, which host phytoseiid species specific to those areas and where common annual specialty crops tend to differ from those in North America, especially the U.S. The aims of this review are to present an argument for further research in this area, present what is known worldwide regarding the presence of phytoseiids on annual specialty crops, and discuss patterns of diversity and life-style classification of phytoseiid species related to presence on crop species. Though different species are found in other parts of the world, comparisons in their life-style specificity and general theories of food web ecology can be used to predict patterns in endemic species composition in U.S. annual vegetable agroecosystems.

Phytoseiids are a group of predatory mites that can be utilized as natural enemies and biological control agents of pests in a variety of perennial crops and in greenhouses as a part of integrated mite management (IMM) strategies (Hoy 2011a, b, c). Tetranychids are the most common target of phytoseiid releases or conservation, but other mites (e.g., Tydeidae and Eriophyidae) and insect pests (e.g., thrips

M. A. Farfan (✉)
Clemson University, Coastal Research and Education Center,
2700 Savannah Hwy., Charleston, SC 29414, USA
e-mail: mfarfan@clemson.edu

R. A. Schmidt-Jeffris
USDA-ARS, Temperate Tree Fruit and Vegetable Research Unit,
5230 Konnowac Pass Rd., Wapato, WA 98951, USA

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(Thysanoptera) and whitefly (Hemiptera: Aleyrodidae) (McMurtry et al. 1992) are also control targets.

Phytoseiid species display varying levels of prey specificity and degrees of phytophagy (typically pollen) for survival and oviposition. Previous research has suggested that generalist species are common while specialists on particular prey items are rare (Croft et al. 2004). Initially, the most common phytoseiid released for biocontrol was *Phytoseiulus persimilis* Athias-Henriot, 1957, a specialist on the twospotted spider mite, *Tetranychus urticae* Koch, 1836. Many other endemic mite species found in agroecosystems have been investigated for efficacy as natural enemies of *T. urticae* with varying results, such as *Neoseiulus californicus*, *Neoseiulus longispinosus*, and *Metaseiulus* (now *Galendromus*) *occidentalis* (McMurtry and Scriven 1964; Gilstrap and Friese 1985; Morris et al. 1999; Skirvin and Fenlon 2001; Komi et al. 2008a; Song et al. 2016).

While much is known about commercially available predators deployed for biocontrol from lab and microcosm experiments, and some work has been done on phytoseiid species found in annual specialty crops elsewhere in the world, very little is known regarding endemic phytoseiid diversity within U.S. annual specialty crop systems, including their life-style classification compositions (McMurtry et al. 1971; Tuttle and Muma 1973; Hadam et al. 1986). This gap in knowledge exists for multiple reasons, the largest of these being the annual destruction of the agroecosystem. Due to regular disturbance at these sites, other vegetation (mostly agricultural weeds) establishes and likely plays a major role in the presence and establishment of both phytoseiids and pest species. Additionally, over-wintering sites and the process of phytoseiid immigration to crop plants remain largely unknown.

At the same time, there is an increasing need to fill knowledge gaps regarding the abundance and diversity of native natural enemies, including the endemic phytoseiid community, as past over-reliance on acaricides and other pesticides has exacerbated infestations of some common agricultural pests. This is due to the fact that pesticide applications reduce populations of the endemic natural enemies; and populations of some pests, such as *T. urticae*, which has developed resistance to over 95 active ingredients (Van Leeuwen et al. 2010; Arthropod Pesticide Resistance Database 2017), rebound faster than natural enemies and experience secondary outbreaks. Therefore, understanding 1) the species composition, 2) the range of food resources, including prey and non-prey, being utilized by phytoseiids in endemic communities, and 3) the factors affecting the diversity in these communities is important to developing alternate management strategies to pesticide application.

Developing strategies to manage for phytoseiid communities in agroecosystems can lead to decreased costs for growers by reducing pesticide applications and increasing sustainability by keeping pest populations at low levels. Understanding the behavior and preferences of phytoseiids of differing life-style types in laboratory or microcosm experiments and linking this to empirical studies of complex, endemic communities and environmental factors in real-world annual cropping systems provides a pathway to these sustainable IMM strategies.

With these predictions and challenges in mind, herein we (1) summarized what is known regarding endemic phytoseiid communities in annual crop agroecosys-

tems world-wide, (2) discussed patterns in diversity and life-style across the crops surveyed, and, with this background, (3) provided predictions for what patterns are likely to exist in annual cropping systems in the U.S.

6.1 Methods

Literature was collected electronically using the search terms 'phytoseiidae' or 'phytoseiid' in combination with 'diversity', 'biodiversity' 'annual crop', and 'vegetable crop' in the Web of Science literature database and Google Scholar internet browser. A total of 18 scholarly articles presenting both the annual crop(s) name(s) and a list of phytoseiid species collected from the crop(s) were found.

We categorized phytoseiids according to the life-style type classification system developed by McMurtry and colleagues (McMurtry and Croft 1997; McMurtry et al. 2013), which groups phytoseiids by prey and habitat specificity in order to better manage agroecosystems for more effective biological control by these predators. Table 6.1 provides a synopsis of the lifestyle category traits suggested by McMurtry et al. (2013) and example species or genera, if pertinent. This system relies, in part, on the degree of specialization on *Tetranychus* spp. due to the extreme economic importance of spider mite pests. However, specializations on other mite groups, like eriophyoids and tydeoids, are also important factors in categorizing the life-style diversity of these communities. Trends in dispersal and habitat preferences also separate phytoseiids into generalist and specialist categories (Croft et al. 2004). In evaluating the structures of communities present in annual crop systems in this review, these characteristics are the ones we can most easily assess.

The life-style classifications given to species in Table 6.2 come from a combination of sources (McMurtry and Croft 1997; Croft et al. 2004. McMurtry et al. 2013), most of which are review literature. If a specific classification from McMurtry et al. (2013) could not be arrived at, a less specific classification from McMurtry and Croft (1997) was used, as in the case of many *Amblyseius* species. Species were not given a classification if no mention of their feeding or habitat preferences was found.

6.2 Results

6.2.1 Diversity of Phytoseiids in Annual Specialty Crops Worldwide

Most knowledge regarding diversity of the phytoseiid community in annual vegetable agroecosystems comes from countries other than the U.S. Table 6.2 details phytoseiid species found in entire community surveys of annual specialty crops around the world and their level of prey specialization when known. Ninety species of phyto-

Table 6.1 Life-style classifications of phytoseiids

Classification type	Description	Examples
Type I. a	Specialists on *Tetranychus* sp.	*Phytoseiulus persimilis* (Athias-Henriot) *Phytoseiulus fragariae* Denmark and Schicha *Phytoseiulus macropilis* (Banks)
Type I. b	Specialists on web-producing mites (Tetranychidae)	*Typhlodromus* (*A.*) *bambusae* (Ehara)
Type I. c	Specialists on Tydeoidea	*Typhlodromina eharai* Muma and Denmark *Proprioseiopsis cabonus* (Schicha and Elshafie)
Type II	Selective predators of Tetranychidae	*Neoseiulus* spp., *Galendromus* spp. *Typhlodromus* (*Anthoseius*) spp.
Type III. a	Generalists living on pubescent leaves	*Eharius chergui* (Athias-Henriot) *Kampimodromus aberrans* Oudemans *Amblydromella caudiglans* Schuster *Typhlodrmus (T.) pyri* Scheuten Some *Paraphytoseius* spp., *Phytoseius* spp., *Kampimodromus* spp., *Typhlodromalus* spp.
Type III. b	Generalists living on glabrous leaves	*Amblyseius swirskii* (Athias-Henriot) *Amblyseius limonicus* Garman and McGregor *Amblyseius andersoni* (Chant)
Type III. c	Generalists living on dicotyledonous plants in confined spaces	The *desertus* group of *Neoseiulus* Species found only in domatia: *Amblyseius herbicolus* (Chant) *Euseius hibisci* (Chant) *Galendromus longipilus* (Nesbitt) *Phytoseius hawaiiensis* Prasad *Typhlodromus* (*A.*) *haramotoi* Prasad
Type III. d	Generalists living on monocotyledonous plants	*Neoseiulus baraki* (Athias-Henriot) *Neoseiulus neobaraki* (Zannou, Moraes and Oliveira) *Neoseiulus paspalivorus* (DeLeon)
Type III. e	Generalists from soil and litter habitats	*Neoseiulus barkeri* (Hughes) *Neoseiulus cucumeris* (Oudemans) Many *Proprioseiopsis*, *Chelaseius* and *Graminaseius* spp.
Type IV	Generalists that are also pollen feeders	*Euseius* spp., *Iphiseius* spp., *Iphiseiodes* spp.

The most recent life-style classification system developed by McMurtry et al. (2013) detailing food resource and plant morphological preferences by phytoseiids, with example genera and species

Table 6.2 Species of phytoseiidae collected from annual specialty crop systems

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
Amblydromalus limonicus	III. a	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Amblydromella himalayensis Gupta	?	India	Eggplant (*Solanum melongena* L.)	Singh and Chauhan (2014)
Amblydromella mori Gupta	?	India	Eggplant	Singh and Chauhan (2014)
Amblydromella ndibu (Pritchard and Baker)	?	Nigeria	Muskmelon (*Cucumis melo* L.)	Matthysse and Denmark (1981)
Amblyseilla setosa (Muma)	?	Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Amblyseiuella amanoi Ehara	III	Japan	Eggplant	Komi et al. (2008b)
Amblyseius aegyptiacus Denmark and Matthysse	III	Nigeria	*Luffa aegyptica* (Mill.)	Matthysse and Denmark (1981)
Amblyseius aerialis (Muma)	III	India	Elephant Foot Yam *(Amorphophallus campanulatus* (Blume))	Haneef and Sadanandan (2013)
Amblyseius alstoniae	III	India	Okra *(Abelmoschus esculentus* (L.))	Naga et al. (2017)
Amblyseius andersoni (Chant)	III. b	Turkey	Cucumber	Özsisli and Çobanoğlu (2011)
		Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)
		Oregon, U.S.A.	Strawberry (*Fragaria* × *ananassa* Duchesne)	Hadam et al. (1986)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Amblyseius aurescens Athias-Henriot	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
Amblyseius brevispinus (Kennett)	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
		Oregon, U.S.A.	Strawberry	Hadam et al. (1986)
Amblyseius californicus (McGregor)	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
		Valencia, Spain	Strawberry	Garcia-Mari and Gonzalez-Zamora (1999)
Amblyseius channabasavannai Gupta and Daniel	III	India	Okra	Haneef and Sadanandan (2013)
Amblyseius cucumeris (Oudemans)	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
Amblyseius eharai Amitai and Swirski	III	Japan	Green pepper, Eggplant	Komi et al. (2008b)
Amblyseius fallacis (Garman)		Oregon, U.S.A.	Strawberry	Hadam et al. (1986)
Amblyseius herbicolus Chant	III. c	India	Eggplant, Peas (*Pisum sativum* L.)	Haneef and Sadanandan (2013)
		India	Cucumber	Singh and Chauhan (2014)
Amblyseius sp. nr. *herbicolus*		India	Cucumber	Singh and Chauhan (2014)
Amblyseius hibisci (Chant)	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
Amblyseius indirae Gupta	III	India	Winter squash (*Cucurbita maxima* Duchesne), Long pepper (*Piper longum* L.)	Haneef and Sadanandan (2013)
Amblyseius kulini Gupta	III	India	Chili pepper (*Capsicum annuum* L.)	Haneef and Sadanandan (2013)
Amblyseius largoensis (Muma)	III	India	Eggplant, *Coccinia* sp., Cowpea (*Vigna unguiculata* (L.) Walp.)	Maheswary et al. (2015)
		India	Chili pepper	Mandape and Shukla (2017)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
		India	Cucumber	Singh and Chauhan (2014)
		Vietnam	African eggplant (*Solanum macrocarpon* L.)	Nguyen et al. (2016)
Amblyseius lindquisti Schuster and Pritchard	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
Amblyseius orientalis Ehara	III	India	Hyacinth/Dolichos bean (*Dolichos lablab* (L.) Sweet))	Haneef and Sadanandan (2013)
		India	Tomato (*Solanum lycopersicum* L.)	Mandape and Shukla (2017)
Amblyseius paraaerialis Muma	III	India	Chili pepper	Binisha and Bhaskar (2013)
		India	Bitter gourd (*Momordica charantia* L.), Eggplant, Chili pepper, *Coccinia* sp., Cowpea, Cucumber, Okra, Snake gourd (*Trichosanthes cucumerina* L.), Snap melon (*Cucumis melo* L.)	Maheswary et al. (2015)
Amblyseius rademacheri Dosse	III	Japan	Green pepper, Eggplant	Komi et al. (2008b)
Amblyseius reticulatus (Oudemans)	III	Oregon, U.S.A.	Strawberry	Hadam et al. (1986)
Amblyseius raoiellus Denmark and Muma	III	India	Okra	Mandape and Shukla (2017)
Amblyseius similoides Buchelos and Pritchard	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
Amblyseius solus Denmark and Matthysse	III	Nigeria	Common bean (*Phaseolus vulgaris* L.), Winged bean (*Psophocarpus tetragonolobus* (L.))	Matthysse and Denmark (1981)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
Amblyseius sundi Prtichard and Baker	III	Nigeria	Sweet potato (*Ipomoea batatas* (L.)), Chili pepper	Matthysse and Denmark (1981)
Amblyseius tamatavensis Bloomers	III	Vietnam	Winter melon (*Benincasa hispida* (Thunb.)), Cucumber, Luffa (*Luffa acutangula* (L.) Roxb.)	Nguyen et al. (2016)
Amblyseius tsugawai Ehara	III	Japan	Green pepper, Eggplant	Komi et al. (2008b)
Amblyseius umbraticus (Chant)	III	Oregon, U.S.A.	Strawberry	Hadam et al. (1986)
Amblyseius nr. zwoelferi		Oregon, U.S.A.	Strawberry	Hadam et al. (1986)
Amblyseius sp.	III	India	Cowpea, Eggplant, Okra	Binisha and Bhaskar (2013)
		India	Chili pepper	Kaur and Sangha (2016)
		India	Cucumber	Sanjta and Chauhan (2015)
		India	Cucumber	Singh and Chauhan (2014)
Aristadrompis masseei	?	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Euseius bwende (Pritchard and Baker)	IV	Nigeria	*Solanum* sp.	Matthysse and Denmark (1981)
Euseius chitradurgae (Gupta)	IV	India	Eggplant	Singh and Chauhan (2016)
Euseius coccineae Gupta	IV	India	Dolichos bean, Luffa	Haneef and Sadanandan (2013)
Euseius distinctus Denmark and Matthysse	IV	Nigeria	*Dioscorea* sp.	Matthysse and Denmark (1981)
Euseius eucalypti (Ghai and Menon)	IV	India	Eggplant	Singh and Chauhan (2016)
Euseius finlandicus (Oudemans)	IV	India	Eggplant, Beans, Cucurbits, Chili pepper	Mandape and Shukla (2017)
		Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Euseius fustis (Pritchard and Baker)	IV	Nigeria	Winged bean	Matthysse and Denmark (1981)
Euseius macrospatulatus Gupta	IV	India	Bitter gourd	Binisha and Bhaskar (2013)
		India	Bitter gourd, Eggplant, Chili pepper, *Coccinia* sp., Cowpea, Cucumber, Okra, Snake gourd, Snap melon	Maheswary et al. (2015)
Euseius neococcinaea Gupta	III	India	Cucumber	Singh and Chauhan (2014)
Euseius ovalis (Evans)	IV	India	Beans	Mandape and Shukla (2017)
Euseius sp. nr. *prasadi*	IV	India	Bitter gourd, Cowpea, Okra	Maheswary et al. (2015)
Euseius saltus Denmark and Matthysse	IV	Nigeria	Thorn apple (*Solanum incanum* L.), *Dioscorea* sp.	Matthysse and Denmark (1981)
Euseius sojaensis (Ehara)	IV	Japan	Green pepper, Eggplant	Komi et al. (2008b)
Euseius stipulatus	IV	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Euseius sp.	IV	India	Chili pepper, Eggplant	Binisha and Bhaskar (2013)
Graminaseius polisensis (Schicha and Corpus-Raros)	III. e	Vietnam	African eggplant	Nguyen et al. (2016)
Gynaeseius liturivorus (Ehara)	?	Japan	Green pepper, Eggplant	Komi et al. (2008b)
Indoseiulus ricini (Ghai and Menon)	?	India	Beans	Mandape and Shukla (2017)
Kampimodromus aberrans	III. a	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Neoseiulus barkeri Hughes	III. e	Japan	Green pepper, Eggplant, Cucumber	Komi et al. (2008b)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
		Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Neoseiulus bicaudus (Wainstein)	?	Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Neoseiulus californicus (McGregor)	II	Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Neoseiulus longispinosus (Evans)	II	India	Cowpea, Eggplant, Okra	Binisha and Bhaskar (2013)
		India	Eggplant	Haneef and Sadanandan (2013)
		India	Bitter gourd, Eggplant Chili pepper, *Coccinia* sp., Cowpea, Cucumber, Snake Gourd, Snap melon	Maheswary et al. (2015)
		India	Egglant, Tomato, Beans, Cucurbits	Mandape and Shukla (2017)
		Vietnam	African eggplant	Nguyen et al. (2016)
			Winter melon	
			Field pumpkin (*Cucurbita pepo* L.)	
			Cucumber	
			Luffa	
Neoseiulus makuwa (Ehara)	I or II	Japan	Eggplant	Komi et al. (2008b)
Neoseiulus setulus (Fox)	I or II	Arizona, U.S.A.	Carrot	Tuttle and Muma (1973)
Neoseiulus sp. nr. *neoghanii*	I or II	India	Eggplant	Singh and Chauhan (2016)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
Neoseiulus oahuensis Prasad	I or II	India	Cucumber	Singh and Chauhan (2014)
Paraphytoseius multidentatus Swirski and Schechter	III. a	India	Eggplant	Mandape and Shukla (2017)
		India	Eggplant	Singh and Chauhan (2014)
		India	Eggplant	Singh and Chauhan (2016)
		Vietnam	Eggplant	Nguyen et al. (2016)
Paraphytoseius orientalis Narayanan	III. a	India	Eggplant	Binisha and Bhaskar (2013)
		India	Eggplant	Maheswary et al. (2015)
Paraphytoseiulus scleroticus Gupta and Ray	III. a	India	Okra	Haneef and Sadanandan (2013)
Pennaseiulus amba Pritchard and Baker	?	Nigeria	Sweet potato	Matthysse and Denmark (1981)
Pennaceius kapuri Gupta	?	India	Eggplant	Singh and Chauhan (2014)
Phytoseiulus macropolis (Banks)	I. a	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Phytoseiulus persimilis (Athias-Henriot)	I	Valencia, Spain	Strawberry	Garcia-Mari and Gonzalez-Zamora (1999)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Phytoseius corniger Wainstein	III	India	Eggplant	Singh and Chauhan (2016)
Phytoseius crinitus Swirski and Shechter	III	India	Eggplant	Singh and Chauhan (2016)
	III	India	Eggplant	Singh and Chauhan (2014)
Phytoseius finitimus (Ribaga)	III. a	Turkey	Eggplant	Özsisli and Çobanoğlu (2011)
		Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
Phytoseius intermedius (Evans and MacFarlane)	III	India	Eggplant	Maheswary et al. (2015)
Phytoseius kapuri (Gupta)	III	India	Eggplant	Singh and Chauhan (2016)
Phytoseius sp. nr. *maldhaensis*	III	India	Eggplant	Singh and Chauhan (2016)
Phytoseius sp.		India	Eggplant	Binisha and Bhaskar (2013)
Proprioseiulus dahonagnas (Schicha and Corpus-Raros)	?	Vietnam	Cucumber	Nguyen et al. (2016)
Proprioseiopsis messor	?	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Proprioseiopsis nemotoi (Ehara and Amano)	III. e	Japan	Green pepper, Eggplant	Komi et al. (2008b)
Scapulaseius asiaticus (Evans)	III. b	Vietnam	Eggplant	Nguyen et al. (2016)
Scapulaseius sp.	III. b	India	Eggplant	Maheswary et al. (2015)
Typhlodromalus chitradurgae Gupta	III. a	India	Eggplant	Singh and Chauhan (2014)
Typhlodromina conspicua	?	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Typhlodromips guajavae Gupta	III	India	Cucumber	Singh and Chauhan (2014)
Typhlodromips rykei (Pritchard and Baker)	III	Nigeria	Peanut (*Arachis hypogaea* L.), Sweet potato, Eggplant, Cowpea, Dolichos bean, *Xanthosoma* sp	Matthysse and Denmark (1981)
Typhlodromips shi (Pritchard and Baker)	III	Nigeria	*Fluted pumpkin (Telfairia occidentalis* Hook.f.)	Matthysse and Denmark (1981)

(continued)

Table 6.2 (continued)

Phytoseiidae species	Type	Country	Specialty crop	Source(s)
Typhlodromips syzygii (Gupta)	III	India	Bitter gourd, Eggplant, Chili pepper, *Coccinia* sp., Cowpea, Cucumber, Snake gourd, Snap melon	Maheswary et al. (2015)
Typhlodromips tetranychivorous (Gupta)	III	India	Eggplant	Mandape and Shukla (2017)
Typhlodromips sp.	III	India	Bitter gourd, Chili pepper, Cowpea, Eggplant, Okra	Binisha and Bhaskar (2013)
Typhlodromus arboreus Chant	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
Typhlodromus (*A.*) *athiasae* (Porath and Swirski)	III	Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)
Typhlodromus (*A.*) *himalayensis* (Gupta)	III	India	Eggplant	Singh and Chauhan (2016)
Typhlodromus kerkirae	III	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Typhlodromus occidentalis Nesbitt	III	Orange County, California, U.S.A.	Strawberry	McMurtry et al. (1971)
		Oregon, U.S.A.	Strawberry	Hadam et al. (1986)
Typhlodromus phialatus	III	Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Typhlodromus (*T.*) *pyri* Scheuten	III. a	Oregon, U.S.A.	Strawberry	Hadam et al. (1986)
		Turkey	Tomato (*Solanum lycopersicum* L.)	Chorąży et al. (2016)
Typhlodromus (*T.*) *recki* Wainstein	III	Turkey	Chili pepper (*Capsicum annuum* L.)	Çobanoğlu and Kumral (2016)
Typhlodromus sp.	III	Vietnam	Winter melon	Nguyen et al. (2016)

Species of predatory phytoseiids and relationships to different annual vegetable crops worldwide and their known life-style type (McMurtry et al. 2013). In cases where a crop species is perennial in the country of the study but an annual crop in parts of the U.S., the crop is included here. Names of mite and plant species are listed by common or scientific name, as presented in their respective source, with describing author(s) following species name when given

seiids were definitively identified. Twenty-eight of these were reported as species of *Amblyseius*, which was the most speciose genus in this review. *Amblyseius*, *Euseius*, and *Neoseiulus* were each present on over half of the crop plants surveyed in the literature. Most of these species are generalists of classification Types III and IV, which include non-*Tetranychus* prey in their diet, or are specialists on pollen or fungi with occasional non-*Tetranychus* prey consumed (McMurtry and Croft 1997; Croft et al. 1998).

6.2.2 Vegetable Specialty Crop Species

A total of 31 crop species were surveyed for phytoseiid diversity (Table 6.3) in the studies included herein. Most of the crops were in the families Cucurbitaceae (12 crops), Fabaceae (8 crops), or Solanaceae (5 crops). The most surveyed crop was eggplant, *Solanum melongena* L., also known as brinjal in other areas of the world.

6.2.3 Patterns in Phytoseiid Presence in Annual Specialty Crop Agroecosystems

Some patterns were apparent when we compared the phytoseiid species present to crop plants. Four genera, *Amblyseius*, *Euseius*, *Neoseiulus*, and *Typhlodromips*, showed little preference for any particular crop plant as they were present in over one-third of the specialty crops and across all three of the three main crop families surveyed. *Neoseiulus longispinosus* (Evans), which is a specialist of *Tetranychus* sp., was found on leaves of 12–14 crop plants surveyed (two crops were mentioned only by common name and each could represent two different species). Phytoseiid species considered generalists in their prey preferences were the most numerous found.

6.3 Discussion

The majority of the phytoseiid species identified were predatory generalists in lifestyle classification Types III and IV. If this pattern holds true for phytoseiid communities in annual specialty crop systems in general, communities are comprised of many generalist species with only a few specialists. This agrees with community ecology theory that a strong connection between a consumer and resource will cause greater abundance oscillations of these specialist populations (McCann et al. 1998). Classical omnivory (feeding from more than one trophic level) weakens individual interaction strengths between species in a community (McCann et al. 1998) and leads to more interactions between species, creating a web-like set of trophic connections

Table 6.3 Species of phytoseiid mites in annual specialty crop systems

Specialty crop	*Amblydromalus*	*Amblydromella*	*Amblyseilia*	*Amblyseiuella*	*Amblyseius*	*Aristadrompis*	*Euseius*	*Graminaseius*
Cucurbitaceae								
Winter melon					1			
Bitter gourd					1		2	
Coccinia sp.					2		1	
Muskmelon		1						
Cucumber					5		2	
Winter squash					1			
Field pumpkin								
Luffa acutangula					1		1	
Luffa aegyptica								
Snake gourd					1		1	
Snap melon					1		1	
Fluted pumpkin								
Fabaceae								
Peanut							2	
Cowpea					2		2	
Dolichos bean					1		1	
Common bean					1			
Peas					1			
Winged bean					1		1	

(continued)

Table 6.3 (continued)

Specialty crop	*Amblydromalus*	*Amblydromella*	*Amblyseilia*	*Amblyseiuella*	*Amblyseius*	*Aristadrompis*	*Euseius*	*Graminaseius*
Solanaceae								
Eggplant		2		1	6		3	
Green pepper, Chili pepper			1		9		3	
Thorn apple							1	
African eggplant					1			1
Tomato	1		1		2	1	2	
Other								
Okra					3		2	
Elephant Foot Yam					1			
Carrot								
Discorea sp.							2	
Sweet potato					1			
Long pepper					1			
Strawberry					12			
Xanthosoma sp.								
Specialty crop	*Gynaeseius*	*Indoseiulus*	*Kampimodromus*	*Neoseiulus*	*Paraphytoseius*	*Pennaseius*	*Pennaseiulus*	*Phytoseiulus*
Cucurbitaceae								
Winter melon				1				
Bitter gourd				1				

(continued)

Table 6.3 (continued)

Specialty crop	*Gynaeseius*	*Indoseiulus*	*Kampimodromus*	*Neoseiulus*	*Paraphytoseius*	*Pennaseius*	*Pennaseiulus*	*Phytoseiulus*
Coccinia sp.				1				
Muskmelon								
Cucumber				3				
Winter squash								
Field pumpkin				1				
Luffa acutangula				1				
Luffa aegyptica								
Snake gourd				1				
Snap melon				1				
Fluted pumpkin								
Fabaceae								
Peanut				1				
Cowpea				1				
Dolichos bean								
Common bean								
Peas								
Winged bean								
Solanaceae								
Eggplant	1			4	2	1	1	
Green pepper, Chili pepper	1			2				

(continued)

Table 6.3 (continued)

Specialty crop	*Gynaeseius*	*Indoseiulus*	*Kampimodromus*	*Neoseiulus*	*Paraphytoseius*	*Pennaseius*	*Pennaseiulus*	*Phytoseiulus*
Thorn apple								
African eggplant				1				
Tomato			1	2				
Other								
Okra		1		1	1			
Elephant Foot Yam								
Carrot				1				
Discorea sp.								
Sweet potato						1		
Long pepper								
Strawberry								1
Xanthosoma sp.								
Specialty crop	*Phytoseius*	*Proprioseiopsis*	*Proprioseiulus*	*Scapulaseius*	*Typhlodromalus*	*Typhlodromina*	*Typhlodromips*	*Typhlodromus*
Cucurbitaceae								
Winter melon								
Bitter gourd							1	
Coccinia sp.							1	
Muskmelon								
Cucumber			1				2	

(continued)

Table 6.3 (continued)

Specialty crop	*Phytoseius*	*Proprioseiopsis*	*Proprioseiulus*	*Scapulaseius*	*Typhlodromalus*	*Typhlodromina*	*Typhlodromips*	*Typhlodromus*
Winter squash								
Field pumpkin								
Luffa acutangula								
Luffa aegyptica								
Snake gourd							1	
Snap melon							1	
Fluted pumpkin							1	
Fabaceae								
Peanut							1	
Cowpea							2	
Dolichos bean							1	
Common bean								
Peas								
Winged bean								
Solanaceae								
Eggplant	6	1			1		4	2
Green pepper, Chili pepper		1					1	2
Thorn apple								

(continued)

Table 6.3 (continued)

Specialty crop	*Phytoseius*	*Proprioseiopsis*	*Proprioseiulus*	*Scapulaseius*	*Typhlodromalus*	*Typhlodromina*	*Typhlodromips*	*Typhlodromus*
African eggplant				1				
Tomato		1				1		3
Other								
Okra								
Elephant Foot Yam								
Carrot								
Discorea sp.								
Sweet potato							1	
Long pepper								
Strawberry								3
Xanthosoma sp.							1	

Species richness of phytoseiids present on annual specialty crops in this review. Crop species were attempted to be matched with common name. In cases of uncertain species, common name was kept as a separate category. This table does not include mite taxonomic designations from authors to the genus level only or when genus was not specified

(Strong 1992). This results in less chance of extinction of any one species, or a whole life-style type, and a level of complexity characteristic of real-world communities (as opposed to models) (Polis 1991).

We found specialists to be rare overall, except in the case of *Neoseiulus longispinosus*. This species was found in a wide number of annual cropping systems in India and Vietnam (Binisha and Bhaskar 2013; Haneef and Sadanandan 2013; Maheswary et al. 2015; Nguyen et al. 2016; Mandape and Shukla 2017). Maheswary et al. 2015 reported that *N. longispinosus* was the predominant predatory mite in communities collected from vegetables and found it associated with both *Tetranychus* spp. and *Polyphagotarsonemus latus* (Banks). Mandape and Shukla (2017) mentioned the largest proportion of species collected in their survey were of *N. longispinosus*. The seeming ubiquity of this species could be because of particularly acute spider mite infestations. Croft et al. (2004) suggested that Type I and II specialists may be the "oddities" in phytoseiid communities and are only found in places where their preferred prey item(s) are found in abundance. The majority of Type I and II species were found in conjunction with species of *Tetranychus*. It is likely that these species are present specifically for the prey provided by the system with little preference being given to plant anatomy (McMurtry and Croft 1997).

The greatest species richness found in annual specialty crop surveys was in India (Table 6.2). In the last 20 years, investigations into phytoseiid diversity in agroecosystems in India have greatly increased, and the high described-species richness is likely the result of this increased sampling effort.

While the crops most surveyed in literature belong to the families Cucurbitaceae, Fabaceae, and Solanaceae, we found no surveys of endemic phytoseiid communities from these crops in the U.S., which makes comparisons to specialty crops here impossible. Considering the importance of these crop families in U.S. agriculture, this lack of survey effort is surprising.

Given the results from this literature review, we expect that annual specialty crops in the U.S. will be dominated by generalist phytoseiids. This has several implications for integrated mite management in these systems. First, these communities may be highly sensitive to pesticide applications, making conservation difficult. Generalist phytoseiids tend to be more susceptible to non-target effects of pesticide applications that specialists (Downing and Moilliet 1972; Argolo et al. 2013; Schmidt-Jeffris and Beers 2015; Schmidt-Jeffris et al. 2015). This has been attributed to the close association of specialists with pest species, which increases their opportunities for resistance development (Croft and Brown 1975). Additionally, because they do not aggregate with pest populations (Sabelis and Van de Baan 1983; Nyrop 1988) or have high host specificity, generalists are also better at maintaining low populations of pests, as opposed to stopping outbreak populations. This indicates that early season conservation of generalists could be critical in annual specialty crops, so that pest populations do not reach outbreak levels. Because of their tendency to consume pollen, generalists are also likely to benefit from agricultural practices like cover cropping, companion planting, wildflower plantings, and minimally managed weedy margins, which could provide a diversity of pollen resources (Alston 1994; Mailloux et al. 2010). Finally, because some generalist phytoseiids have high host-plant specificity

and significant cultivar preferences (Camporese and Duso 1996; McMurtry and Croft 1997), future surveys will have to examine many different host crops and cultivars to fully understand the available species diversity.

One difficulty in comparing diversity and composition of species and life-styles from annual crops surveys worldwide in order to determine what trends may occur in the U.S. is that crops that are perennial in other parts of the world, such as *Capsicum annuum* L., are considered annual in many parts of the North America due to differing climate conditions. It should be noted that being part of a perennial system likely provides an opportunity for species succession in phytoseiid communities over the long term, which leads to high complexity in community structure, different species composition, and higher species diversity. However, these communities might be used as measure of "high" diversity in a phytoseiid community, and, therefore, a target "reference" condition.

6.4 Conclusions and Future Work

We found that endemic phytoseiid communities in annual specialty crop systems tend to be dominated by generalists that are found across many crop species in the three most common annual vegetable families, Cucurbitaceae, Fabaceae, and Solanaceae. Second, specialists are present but uncommon in these studies. The number of life-style-generalist and classically omnivorous species compared with the number of specialists found agrees with theories of food web ecology, i.e., that omnivory is common and characteristic of complex, real-world communities. Finally, most of the studies published on endemic phytoseiid communities are from India, making the ability to generalize to other parts of the world challenging, if not dubious. However, it seems very likely that endemic phytoseiid communities in annual crops are likely to be composed of many generalist species intermixed with a few specialist species.

Croft et al. (2004) suggested that the best way to know how useful life-style classifications are in making decisions about biological control is to survey endemic communities of phytoseiids in natural and open-field agroecosystems. This has clearly been done in some parts of the world, but we found only three studies from the U.S., and none from the rest of North America, that examined phytoseiid biodiversity in annual specialty crop systems. Polis (1991) mentioned that the most difficult part of evaluating patterns in empirical systems is that our knowledge of diversity of "real" systems and exactly what those collected species are consuming is very poor, which makes our understanding of these webs inadequate. This is especially challenging, yet critical, in these annual systems, where phytoseiid communities must, by definition, be subject to extreme disturbance annually (at least!). We also found that, for the majority of phytoseiid species collected, exact knowledge of their use of food resources is lacking or absent. This forced many of our designations to be broader in scope than those proposed by McMurtry et al. (2013).

Considering this, there is a continuing need for investigation into the diversity and composition of species in endemic phytoseiid communities in annual specialty crop-

ping systems and further for studies on the food resource usage of specific species within these communities. From a pest management and an ecological standpoint, these predator communities represent a unique opportunity, and best hope, for developing and investigating long-term management practices to sustain complex annual specialty agroecosystem.

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Correction to: Contemporary Acarology

Michael J. Skvarla, Ronald Ochoa, Jose Carlos Verle Rodrigues and H. Joel Hutcheson

Correction to:
M. J. Skvarla et al. (eds.), *Contemporary Acarology*, https://doi.org/10.1007/978-3-030-17265-7

The original version of the book was inadvertently published with the following errors in Chapters 2, 4 and Front Matter and the same have been updated.

In Chapter 2 "Field Studies in Acarology: Joint Base San Antonio, TX: 2015–2017", the sub-heading numbering has been incorporated for "Materials and Methods" and "Results" as Section 2.2 and Section 2.3, respectively.

In Chapter 4 "A Preliminary Phylogenetic Hypothesis for Cunaxidae (Acariformes: Trombidiformes: Prostigmata: Eupodina)", the sub-heading numbering has been incorporated for "Materials and Methods" and "Results and Discussion" as Section 4.1 and Section 4.2, respectively.

In FM, "Agricultural Science & Industries Building" has now been added to the affiliation of Editor Michael J. Skvarla.

The erratum book has been updated with these changes.

The updated versions of these chapters can be found at
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