Disentangling Species Boundaries and the Evolution of Habitat Specialization for the Ecologically Diverse Mite Family Acaridae

by

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Dedication

To my husband Juan M. for his support since day one, for leaving all his life behind to join me in this journey and because you always believed in me

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Abstract

Testing habitat specialization and diversification patterns in poorly known groups such as mites contributes to our understanding of fundamental evolutionary questions. Organisms with morphological and physiological adaptations to more specialized habitats and resources are expected to have evolved from more generalized ancestors, but the opposite (i.e., generalists evolving from specialists) is not expected to occur. However, there are indications that ancestors of the mite family Acaridae had specialized habitats and that generalists lineages evolved from these specialist ancestors. Here I aimed to determine how niche breadth evolved in Acaridae and specifically evaluate whether or not habitat generalists evolved from specialist ancestors. I accomplished this objective by estimating a molecular phylogeny, reconstructing ancestral character states and transition rates for habitat use in this family. I found evidence that generalists have in fact evolved from more specialized lineages. All analyses showed that specialized habitat use is ancestral for acarid mites and confirmed that generalists evolved from specialized lineages. Ancestral character state reconstructions also revealed that specialist lineages gave rise to other specialist as well as generalist lineages.

Given the difficulties that exist in delimiting species of some genera of Acaridae such as *Tyrophagus,* I documented subtle but important morphological differences between two cosmoplolitan species, *Tyrophagus curvipenis* and *Tyrophagus putrescentiae*. I also utilized several molecular species delimitation methods based on analyses of mitochondrial and nuclear DNA to untangle the taxonomic uncertain within this genus. I first recovered much of the intra-specific molecular variation that exists within several populations of the cosmopolitan mite, *T. putrescentiae,* from different geographic regions. The results revealed a tremendous level of hidden diversity within this genus, suggesting the presence of 17 morphospecies. Moreover, there was no support for recognition of cryptic species within *T. putrescentiae* as suggested from analyses of mitochondrial DNA sequences. It also highlighted the importance of integrating several complementary methods in a taxonomic framework that incorporate different forms of evidence when delimiting species.

The work presented here advances our knowledge of the evolutionary history of a poorly known group of organisms, i.e. acarid mites, and provides important evidence regarding the diversification of this family. Even though major advances were presented in this dissertation, it will still be necessary to investigate other factors related to the genetic differences and ecological diversity that allowed this group to diversify into many habitats and develop broad distributions. On the other hand, it is not only necessary to conduct more extensive efforts for sampling in understudied regions like the Neotropics but also to develop a more extensive taxonomic study of the genus *Tyrophagus* given that I also found several undescribed morphospecies.

Chapter 1 Introduction

Background

One of the central questions of biology is the possibility to explain the different processes that generate biological diversity and how it is maintained or changed over time (Oliver, 2018). In evolutionary biology there is a widely hypothesis supporting that observed diversity nowadays is originated only from less specialized (generalist) groups (Futuyma & Moreno, 1988; Goldberg & Igić, 2008). These generalist organisms should be the only ones with capacity to give rise to other lineages of generalist or specialist groups. This hypothesis has been prevalent for many years and it assumed that as specialists depend on a particular host or habitat, they have presumably lost the functionality of many genes (Mendonça et al., 2011) or accumulate mutations (Colles et al., 2009) through time and as consequence are more vulnerable to environmental stochasticity. The concept that specialization leads to an evolutionary dead end is known as "Dollo's law" and has been a recognized hypothesis in evolutionary biology for many years (Mayr, 1963; Kelley & Farrell, 1998; Goldberg & Igić, 2008). This concept has been tested from a phylogenetic perspective checking if specialist species nodes have only recently originated and how persistent they are through the phylogeny (Futuyma & Moreno, 1988; Colles et al., 2009).

However, several works have challenge this paradigm and reached different conclusions regarding "Dollo's law" (Nosil, 2002; Morse & Farrell, 2005; Poulin, et al., 2006; Klimov & OConnor, 2013). When comparing the total number of specialist vs generalist species in mites of the family Acaridae and determining the ecological habitat of each of the species, it is possible to develop a different framework from these expectations. The underlying evolutionary history for this family remains unclear, and this research intends to take a step forward untangling the evolutionary relationships between generalist and specialist species. The approach of this research is to compare the genetic variation among acarids associated with different habitats and estimate the switches between specialists and generalists in this group through the phylogeny. More specifically, here is evaluated a different hypothesis, testing patterns of habitat specialization between generalists and specialists in the phylogenetic tree. Then, ancestral character state

reconstruction and transition rates are performed to test if different outcomes can be postulated for this system and recognize how these patterns have given rise to the diversity observed recently.

One of the most abundant and important genera within Acaridae is *Tyrophagus*. In addition, to studying the diversification in this family, in this dissertation I evaluated several methodologies for the delimitation of species within *Tyrophagus* given the difficulty that exists in separating species and the abundance of cryptic species in this genus.

Study system

Mites in the family Acaridae are globally distributed and have different levels of habitat specialization. Comprising 541 species grouped into 88 genera (Schatz et al., 2011), members of this group show various degrees of specialization on hosts and habitats (OConnor, 1982; OConnor, 2001). This habitat/host adaptation is presumably the basis for the diversification of Acaridae into many habitats during their evolutionary history. Unlike other mites, the variation in the degree of specialization has allowed this family to succeed in different habitats and diversify into different species, some of them phenotypically adapted to live in a particular host or habitat and others to be more generalized in their habitats.

On the one hand, some species appear to be complete specialists in habitat preference and host utilization (host here usually meaning the organism used by the mite for phoretic dispersal; few acarid mites, e.g. *Ewingia coenobitae*, are permanent ectosymbionts), using only the resources created by their host and dispersing solely on them. By specializing, many acarid mites have developed close relations with their carriers. For instance, the bee-associated genus *Diadasiopus* is found on bees of the genus *Diadasia* in the New World (OConnor, 2001), presumably living in habitats created by these bees and dispersing on them. Likewise, species of the genus *Naiadacarus* live in wet tree holes, co-occurring with and dispersing only on particular syrphid flies (OConnor, 1989). Other species are also habitat specialists, but instead of utilizing only one host, they are phoretic generalists, dispersing on more than one host. This is the case for species of *Schwiebea*, which live in rotting wood, but can disperse on many different insects associated with that habitat (OConnor 2001; OConnor 2009).

Acaridae also includes generalist species occurring in different habitats, consuming several resources, and being phoretic on different hosts. Mites of the widespread genus *Acarus* have been found in vertebrate nests, stored food products and house dust, utilizing a wide range of food sources, such as fungal mycelia, flour, plant parts, cheese, and others, while their dispersal is

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accomplished by different arthropod hosts (Hughes, 1976; Arlian et al., 2002; OConnor, 2009). Lastly, some species of mites such as those in the genera *Tyrophagus*, *Aleuroglyphus* and *Tyroborus* have lost the deutonymphal stage (the phoretic dispersal stage) and are habitat generalists, dispersing from one location to another as feeding stages (OConnor, 1994). In the later cases, these species are mostly associated with anthropogenic habitats and are of considerable importance in areas such as human health, veterinary medicine, and agriculture (Zhang, 2003; Kasuga & Amano, 2005; Krzysztof et al., 2011). This ecological complexity plus the difficulty to clearly distinguish between different species through morphological characters and the struggle to establish species boundaries in some genera like *Tyrophagus* make them an interesting and important study system to answer ecological and evolutionary questions.

Chapters Overview

Acaridae is the largest family within the superfamily Acaroidea and within this, the genus *Tyrophagus* is one of the most species-rich with more than 35 described species (Fan & Zhang, 2007). This genus is globally distributed and is probably the most economically and medically important genus within Acaridae. *Tyrophagus* species are frequently found in organic matter and stored food products (Hughes, 1976; Fan & Zhang, 2007) where they feed on fungi, bacteria, plants or products with high fat content. Since its description (Oudemans, 1924), this genus has been surrounded by taxonomic controversy due to morphological similarities between different species causing disagreements about the exact number of valid species (Robertson, 1959; Griffiths, 1979; Klimov & OConnor, 2009, 2010, 2015; Fan and Zhang, 2014). Although, major contributions have been made for this genus, it is still necessary to look through an integrated review including ecological, morphological and genetic data to clarify the species boundaries on this genus. During this dissertation, major advances were made on the genetic structure and species boundaries of *Tyrophagus*, uncovering hiding diversity on the genus. Like this, here was exhibited much of the intra and inter specific molecular variation that exist within several populations from different geographic regions (Chapters two, three). In Chapter two, the COI phylogeny separated the species of *Tyrophagus* into two major clades consistent with the morphological character of presence or absence of eyespots. Also, large genetic and amino acid differences were found within different populations of the species *T. putrescentiae* recognizing two major groups for it. However, it was also found that these differences did not corresponded with geographical provenance of the populations, gathering geographically distant populations in the same group. Finally, for the first

time, here was record the occurrence of *T. curvipenis* in the New World and documented the most important morphological differences between *T. curvipenis* and *T. putrescentiae,* two species that can be easily encountered in the same place (Murillo et al., 2018).

With the idea of disentangling the genetic variation found in the two groups of *T. putrescentiae* and clarifying the species boundaries for this genus chapter three of my dissertation studied the species boundaries for several populations of *Tyrophagus* coming from different parts of the world. In this chapter, I conducted several species delimitation analyses using single and multilocus data to identify the diversity within the cosmopolitan genus *Tyrophagus* and uncover the genetic and taxonomic dissimilarities of the group. Accurate delimitation of species is important to recognize the diversity in *Tyrophagus* given the high genetic differences found in chapter two, the cryptic diversity within the group and the taxonomic controversy that has been plagued this genus for many years. In this chapter, three major findings were that all species delimitation methodologies preserved the two groups of *T. putrescentiae* as a single evolutionary lineage keeping the two groups (chapter two) as a single species despite the large genetic differences shown in chapter two. Second, the results reveal hidden diversity within this group and highlight a high number of undescribed cryptic species, especially for samples that were collected in the Neotropics. Third, the different delimitation analyses conducted here contrast the species delimitation results according with the implemented methodology. On one hand, these results highlights the awareness that should be taken when using this type of tools since prior information and input data played an important role in the final conclusions. On the other hand, it shows the importance of incorporating as much information as possible for the group under study (morphology, ecology and genetics) and use of an integrative perspective when applying species delimitation methods.

Having discovered the high genetic diversity in the genus *Tyrophagus*, we proceeded to study macro-evolutionary processes that have been associated with the generation of diversity patterns within Acaridae. This family is an excellent model to study evolutionary and diversification questions given that it has a worldwide distribution and includes the highest number of species within Acaroidea. Most remarkably, Acaridae is good system to tested different hypothesis about diversification as it exhibits varying degrees of habitat and host specialization. Mapping characters on the phylogenies help in our understanding on the evolution of different traits in organisms (Huelsenbeck et al. 2003). In chapter four, I developed a phylogeny for many genera and taxa traditionally included in Acaridae using five gene sequences. Using this phylogeny, I focused on

habitat specialization patterns of Acaridae across the phylogeny, to investigate if evolutionary dynamics different from recognized hypotheses were possible in this system and tested the question of whether specialists can generate diversity and give rise to more generalist species. The results of this chapter support that generalist species can also evolve from specialist species challenging traditional hypothesis of unidirectional evolution. Phylogenetic, ancestral character states and transition rates analyses provided evidence for an ancestrally specialist lifestyle of acarid mites and found support for reversals in this system. The character changes along the phylogeny provide valuable information in our understanding of the development of diversity in this group of mites and challenge the paradigms in evolutionary biology.

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Chapter 2 Investigating species boundaries using DNA and morphology in the mite *Tyrophagus curvipenis* (Acari: Acaridae), an emerging invasive pest, with a molecular phylogeny of the genus *Tyrophagus*

Pamela Murillo, Pavel Klimov, Jan Hubert, Barry OConnor

Abstract

Mites of the genus *Tyrophagus* (Acari: Acaridae) are among the most widespread and common mites, inhabiting diverse natural and anthropogenic habitats. Some species are pests of agricultural products and stored food and/or live in house dust, causing allergies to humans. We sequenced 1.2 kb of the mitochondrial COI gene for 38 individuals belonging to 7 species of *Tyrophagus*, including *T. curvipenis*, *T. putrescentiae*, *T. fanetzhangorum*, *T. longior*, *T. perniciosus*, and *T. cf similis*. Molecular phylogenetic analyses recovered (i) two major clades corresponding to the presence or absence of eyespots and (ii) separated all included morphological species. *Tyrophagus curvipenis* and *T. putrescentiae* had the lowest between-species genetic distances (range, mean±SD): 14.20-16.30, 15.17±0.40 (K2P). The highest within-species variation was found in *T. putrescentiae* 0.00-4.33, 1.78±1.44 (K2P). In this species, we recovered two distinct groups, however, no geographical or ecological dissimilarities were observed between them. Based on results of our analyses, we document important morphological differences between *T. curvipenis* and *T. putrescentiae*. For the first time, we record the occurrence of *T. curvipenis* in the New World and suggest that it may be an emerging pest as it is currently quickly spreading in agricultural produce.

Introduction

Mites of the genus *Tyrophagus* have a worldwide distribution and are present in a varied range of natural and human-associated habitats (Robertson, 1959; Hughes, 1976). Most species are fungivorous (Hughes, 1976) and occur in a wide range of food substrates including dried meat, seeds, cheese, wheat, flour, fruits, tubers and others, reducing the nutritional value of the products through direct damage and indirectly by spreading fungi and bacteria (Hughes, 1976; Hubert et al., 2004; de Oliveira et al., 2007).

Even though only a few astigmatid mites are considered as truly phytophagous, some species of the genus *Tyrophagus*, e. g. *T. neiswanderi* and *T. similis*, are economically important plant pests that have been associated with direct damage to plants (Lange and Bacon, 1958; Hughes, 1976; Buxton, 1989; Fisher, 1993; Kasuga and Amano, 2003, 2006; Zhang, 2003; Kasuga and Honda, 2006; Khaing et al., 2014). One of the potential plant pest species, *T. curvipenis,* was described from a greenhouse in Portugal, feeding on algae and pollen of orchid flowers (Fain and Fauvel, 1993). Later, this species was reported in various Old World countries: Greece, France, New Zealand and Australia (Fan and Zhang, 2007; Badieritakis et al., 2012). *T. curvipenis* occurs in stored food products as well as on various plants and animal materials (Fan and Zhang, 2007) but unlike other species in the genus, *T. curvipenis* is more frequent on plants than in typical stored products.

Distinction among different species of *Tyrophagus* can be challenging given the morphological similarities between closely related species, the presence of cryptic species and the occurrence of several species in the same geographical region (Yang et al., 2011; Erban et al., 2016). There are disagreements about the exact number of valid species, species boundaries, and distribution (Klimov and OConnor, 2009, 2010, 2015; Fan and Zhang, 2014).

The lack of morphological differences adequate to easily distinguish between species, makes molecular tools highly valuable for precise identification. DNA barcodes are short sequences of a single gene present in a wide number of species and have been employed as a useful tool facilitating identification of closely related species and provide, most of the time, accurate species assignment (Hebert et al., 2003; Janzen et al., 2005; Meier et al., 2006; Yang et al., 2011; Puillandre et al., 2012; Pentinsaari et al., 2016).

One of the most widely employed markers is the mitochondrial Cytochrome Oxidase subunit I (COI), which has been used to investigate intra- and interspecific variation in many arthropods including various mites, such as, feather mites (Bochkov et al., 2014; Doña et al., 2015; Klimov et al., 2017), phytophagous mites (Navajas et al., 1996; Skoracka and Dabert 2010; Khaing et al., 2015) predatory mites (Li et al., 2012), and stored product mites (Webster et al., 2004; Yang et al., 2011; Khaing et al., 2014; Sun et al., 2014; Erban et al., 2016; Yang and Li 2016; Que et al., 2016). This locus possesses a high level of variability, enabling distinction between two or more groups of individuals that originally were described by morphological characters either as a single or as distinct species and helps to establish the species status of these individuals (Hebert et al., 2003; Hebert et al., 2004a; Pentinsaari et al., 2016).

Several works have used COI (Xia et al., 2007; Yang et al., 2011; Khaing et al., 2014; Erban et al., 2016) or other molecular markers (Noge et al., 2005; Yang et al., 2011; Beroiz et al., 2014) to identify some species of *Tyrophagus*: *T. putrescentiae*, *T. fanetzhangorum, T. neiswanderi, T. similis*, and *T. longior*. However, there is still much uncertainty regarding the genetic variation within and among species in *Tyrophagus*. Until now, no study has reported population genetic structure in any of the species of this genus.

Here we infer phylogenetic relationships of 7 species of *Tyrophagus* based on COI sequences (1.2 kb) including several economically important species: *T. putrescentiae*, *T. curvipenis*, *T. fanetzhangorum*, *T. longior*, *T. perniciosus*, and *T*.cf *similis*. We then use this phylogeny to validate previously defined morphospecies. For species clustering as monophyletic groups, we calculate within- and between-species genetic distances. In particular, we focus on *T. curvipenis*, a potential agricultural pest (see above), which is difficult to distinguish from *T. putrescentiae* and *T. fanetzhangorum*. Based on results of our analyses, we summarize important morphological differences between *T. curvipenis* and *T. putrescentiae* and provide new information on the distribution of the former species.

Materials and Methods

Mite samples and morphological identification

Samples were collected from 13 countries and numerous habitats (Table 2.1). Mites were manually collected with a mounting needle under a dissecting microscope and stored in ethanol (99.5%). When needed, materials containing mites were sieved. Slides were prepared in Hoyer's medium, and the specimens were identified using the most recent taxonomic keys (Fan and Zhang 2007, but see Klimov and OConnor (2009) with regard to *Tyrophagus putrescentiae*). The specimens used to sort out the relevant morphological differences between *T. putrescentiae* and *T. curvipenis* were collected in Costa Rica from chayote plants (*Sechium edule*). Voucher specimens are deposited in the University of Michigan Museum of Zoology, Ann Arbor (UMMZ).

Table 2.1 Countries, hosts, localities and DNA codes used for the different species of *Tyrophagus*. Sequences KY986243-KY986280 were generated as part of this study.

Mite species	Country	Host/Locality	Code	Museum (BMOC) accession number	GenBank (GB) accession number
Tyrophagus putrescentiae	USA: NC	Lab culture from Greer Laboratories	AD1401	BMOC 08-0801-006	KY986243
	Singapore	National University of Singapore, Allergy and Molecular Immunology Laboratory	AD1396	BMOC 08-0912-060	KY986244
	USA: OH	Larry Arlian lab culture, Montgomery Co., Dayton. Wright State University	AD1402	BMOC 08-0801-007	KY986245
	England	Lab colony Biological Crop Protection, Ltd.	AD801	BMOC 07-0223-006	KY986246
	Czechia	Lab culture from grain store maintained by the Crop Research Institute, Prague	AD1398	BMOC 08-1010-002	KY986247

USA: MI	Cricket rearing, University of Michigan	AD1695	BMOC 14-0318-002	KY986248
Brazil	On a dead scorpion (*Tityus serrulatus*)	AD1693	BMOC 13-1115-056	KY986249
Germany	Lab sample, very likely a subsample of RA Norton collection Syracuse/NY. Received via Ina Schäfer as presumed vouchers for study of Domes et al. 2007	AD1400	BMOC 08-0801-004	KY986250
Czechia	Lab cultures started from a CSL culture (England) and maintained by the Crop Research Institute, Prague	AD1337	BMOC 08-1010-005	KY986251
Brazil	Belo Horizonte, Universidade Federal de Minas Gerais, Departamento de Zoologia, Laboratório de Sistemática e Evolução de Ácaros Acariformes	AD1692, AD1721, AD1722	BMOC 13-1115-053	KY986255, KY986252, KY986253
Japan	Lab culture, Tokyo Women's University of Medicine	AD1274	BMOC 08-0801-001	KY986254
USA: OH	Dog food, Franklin Co., Columbus, OSU campus	AD1751	BMOC 14-0614-020	KY986256
Brazil	Minas Gerais, Dry fruits (with some fungus) of *Terminalia catappa* on ground	AD1974	BMOC 15-0104-002	KY986257
Brazil	Minas Gerais, Sabará *Melipona quadrifasciata* nest	AD1979	BMOC 15-0104-035	KY986259
Costa Rica	*Sechium edule.* Aged flowers attached to ripened fruit Ujarrás, Cartago.	AD1999	BMOC 15-0601-164	KY986260
Costa Rica	Polypore fungus. Parque Nacional Braulio Carrillo, Los Palmas trail	AD2004	BMOC 15-0601-208	KY986261
Costa Rica	*Partamona orizabaensis* nest, Universidad Nacional	AD2013	BMOC 15-0601-107	KY986262

Costa Rica	*Zophobas morio*, lab rearing, Museo de Insectos, Universidad de Costa Rica	AD2015	BMOC 15-0601-120	KY986263
Costa Rica	Palm seeds (Arecaceae) on the floor E.E.F.B.M. Universidad de Costa Rica	AD1912	BMOC 15-0601-181	KY986264
The Netherlands	Lab culture Koppert maintained by the Crop Research Institute, Prague	AD1924	BMOC 15-0717-012	KY986273
Czechia	Lab culture maintained by the Crop Research Institute, Prague	AD1925	BMOC 15-0717-013	KY986258
Czechia	Lab culture Phillips maintained by the Crop Research Institute, Prague	AD1926	BMOC 15-0717-014	KY986274
Italy	Lab culture Ham maintained by the Crop Research Institute, Prague	AD1927	BMOC 15-0717-015	KY986275
Czechia	Lab culture Nestlé maintained by the Crop Research Institute, Prague	AD1928	BMOC 15-0717-016	KY986276
China	GenBank sequence (no other data)			EU078968
China	GenBank sequence (no other data)			EU078969
China	GenBank sequence (no other data)			EF527826
China	Lab culture. Nanchang, Jiangxi province			NC_026079
China	Central Science Laboratory (CSL),York.			AY525572
China	Lab culture. Shangai			HQ287793
China	Lab culture. Shangai			HQ287795
China	Lab culture. Shangai			HQ287796
Singapore	EST consensus			CN766680 CN766646 CN766809 CN767131 CN766804

					CN767087
Tyrophagus fanetzhangorum	Belgium	Rotting grass	AD1691, AD1687	BMOC 06-0910-062	KY986265, KY986266
	Spain	Lab culture ALK-ABELLÓ, Madrid	AD1275	BMOC 08-0801-002	KY986267
Tyrophagus curvipenis	Costa Rica	*Sechium edule,* subcortical part of aged (discolored) stem	AD1911, AD2017, AD2018	BMOC 15-0601-167	KY986271, KY986268 KY986269
	Costa Rica	Nest of a small bird in a cow shed	AD2025	BMOC 15-0601-204	KY986270
	Russia	inside *Prunus persica* fruits (imported from Spain), Tyumenskaya Oblast'	AD1972	BMOC 14-0730-046	KY986272
Tyrophagus perniciosus	USA: MI	Grain spill	AD444	BMOC 00-1103-013	KY986277
Tyrophagus sp. (*T.* cf *similis*)	Belgium	Rotting grass	AD1686	BMOC 06-0910-062	KY986278
	South Korea	GenBank sequence from *Spinacia*			KM199641
Tyrophagus longior	USA: MI	Cheese	AD1725	BMOC 14-0602-001	KY986279
	China	GenBank sequence from flour			NC_028725
Tyrophagus sp. (close to *T. longior*)	Belgium	Rotting grass	AD1685	BMOC 06-0910-062	KY986280

DNA extraction, amplification and sequencing

   The specimens used for DNA extraction were preserved in 99.5% ethanol and stored at -80°C. Genomic DNA was extracted from a single specimens using a QIAamp DNA Micro kit (Qiagen) following the manufacturer's protocol for tissues, with some modifications (Klimov and OConnor, 2008).

   A fragment of 1257 bp of the COI gene was amplified by a nested PCR using the following primers (5´-3´): COX1_16F (TGANTWTTTTCHACWAAYCAYAA), COX1_1324R

(CDGWRTAHCGDCGDGGTAT) and COX1_25Fshort_T (<u>TGTAAAACGACGGCCAGT</u>TCHACWAAYCAYAARRAYA), COX1_1282R_T (<u>CAGGAAACAGCTATGACC</u>CCWVYTARDCCTARRAARTGTTG) (Klimov et al., 2018). For uniform sequencing, M13FORW/REV tails (underlined in the sequences above) were added to the COX1_25Fshort_T forward and COX1_1282R_T reverse primers.

Amplification reactions were performed in a 20µl volume with Platinum Taq DNA Polymerase (Invitrogen) in a Mastercycler gradient, Eppendorf thermocycler. The master mix for initial PCR contained: 2.0 µl of PCR buffer (1X), 1.4 µl MgSO4 (50 mM), 1.4 uL of dNTP (10 mM each), 0.8 µl of each oligonucleotide primers COX1_16F and COX1_1324R (10 µM), 0.12 µl of Platinum Taq polymerase (1.5U) and 0.4–1 µl of genomic DNA template. The total volume was increased to 20 µl with distilled water. The thermocycler protocol was set as follows: 94 °C for 2 min, 10 cycles of 94 °C for 30 s, 40 °C for 1 min, 72 °C for 2 min, and 25 cycles of 94 ° C for 30 s, 48 °C for 35 s and 72 °C for 2 min, with a final extension step of 72 °C for 7 min.

For the second PCR, the master mix was modified with a reduced quantity of Taq Polymerase 0.08 µl (1.0 U), 0.6 µl of PCR products from the first PCR reaction and the primers COX1_25Fshort_T and COX1_1282R_T. The thermocycler protocol was set as follows: 94 °C for 2 min, 20 cycles of 94 °C for 30 s, 49 °C for 30 s, 72 °C for 2 min, and 18 cycles of 94 °C for 30 s, 52 °C for 35 s and 72 °C for 2 min, with a final extension step of 72 °C for 7 min.

PCR products were visualized by electrophoresis on a 1.5% agarose gel, 1X TA buffer, 100 V for approximately 35 min. Bands were excised under UV light and purified with QIAquick ® gel extraction kit (Qiagen). Sequencing was done in both directions using a 3730XL sequencer (Applied Biosystems) at the University of Michigan DNA sequencing Core.

Sequence editing, genetic distance and diversity indexes

Chromatograms were resolved in Sequencher ver. 5.4.6 (Sequencher, 2016); primer and low quality sequences were trimmed. The original length of the amplified COI sequences was 1257 bp. After trimming, the final COI alignment included 1227 sites for 39 sequences including the outgroup (AD513 *Sancassania* sp.). Sequences were imported to Mesquite (Maddison and Maddison, 2011) and each codon was color-coded according to its amino acid translation. No indels or stop codons, which are indicative of pseudogenes, were detected. The sequences were deposited in GenBank under the accession numbers KY986243- KY986280 (Table 2.1).

An additional eleven COI sequences of *Tyrophagus* were retrieved from the nucleotide and EST GenBank databases (NCBI) (Table 2.1). The retrieved sequences were aligned and checked for quality. Primer sequences and low quality regions were removed from the dataset.

Uncorrected and K2P (Kimura's two parameter) pairwise genetic distances were calculated in the software Sequence Matrix (Vaidya et al., 2011). The number of polymorphic sites and genetic diversity indexes (nucleotide diversity) were calculated for *T. putrescentiae* using the software DNAsp5.1 (Librado and Rozas, 2009). The results obtained in DNAsp5.1 for the haplotype diversity were used for haplotype networks analyses. We assessed the genetic structure through median-joining haplotype networks (Bandelt et al., 1999) using the software PopArt (Leigh and Bryant, 2015).

A barcoding gap analysis was performed to compare the genetic distances using the program ABGD (Puillandre et al., 2012). This program was run with the following arguments: /abgd -a -p 0.001 -P 0.17 -d 0 -X 1.5. Pairwise distances were calculated using K2P distance.

Non-synonymous/Synonymous (Ka/Ks) ratio was calculated with the software HyPhy (Kosakovsky et al., 2005) using the server Datamonkey (Delport et al., 2010). A Mixed Effects Model of Evolution (MEME) was used to detect specific sites evolving under positive selection (Murrell et al., 2012). We also used the phylogenetic partition codon model BUSTED (Branch-Site Unrestricted Statistical Test for Episodic Diversification) to test for gene-wide selection at the *T. putrescentiae* lineages (Murrell et al., 2015).

Phylogenetic analysis

The best-fitting nucleotide substitution model (HKY+G+I) was selected based on the Akaike Information Criterion (AIC) as implemented in MEGA version 7 (Kumar et al., 2016). The phylogenetic analysis was done in RAxML (Stamatakis et al., 2008) using a Maximum Likelihood (ML) framework. Statistical support for bipartitions was estimated by a bootstrap analysis with 1000 replicates.

Results

Geographic distribution of Tyrophagus curvipenis

Since its description in 1993 by Fain and Fauvel, *T. curvipenis* has been reported from several places in Europe, Australia and New Zealand. Our study records this species for the first time in the New World (Fig. 2.1). We morphologically identified *T. curvipenis* in UMMZ collected in 1977 from a *Microtus pennsylvanicus* (Rodentia: Cricetidae) nest in Maryland (USA) (BMOC 77-0510-004, no sequence data), and we found it on chayote (*Sechium edule*) fruits (Fig. 2.2) and stems from Costa Rica in 2015. Due to the commercial importance of this fruit (Barquero, 2015), these collections will be described in more detail below. In addition to these, we found *T. curvipenis* in large numbers inside a nectarine fruit (*Prunus persica*) imported from Spain to Russia, and a small population from a bird nest from Costa Rica (Table 2.1).
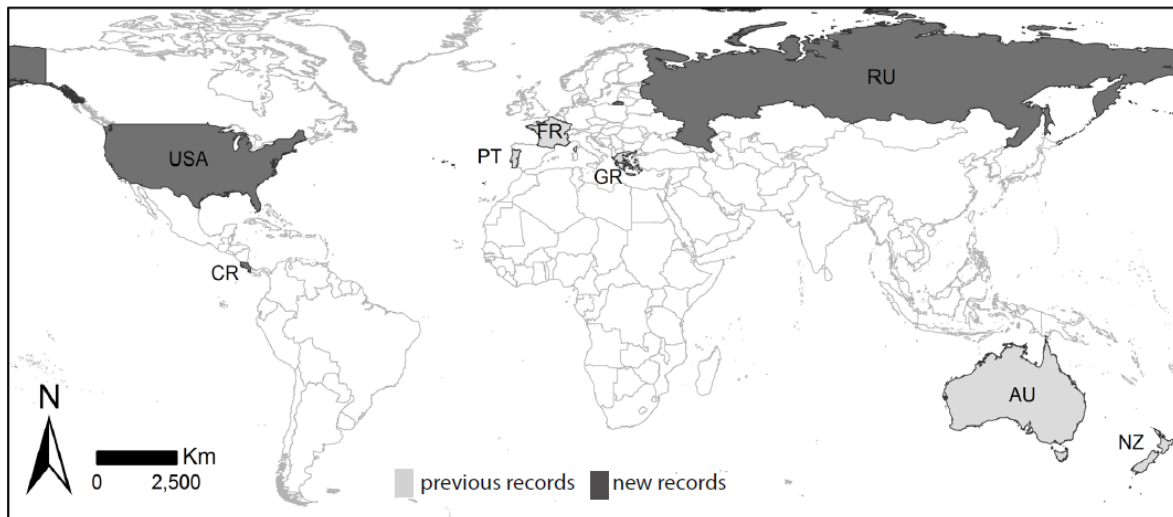
Figure 2.1 Geographic distribution of *T. curvipenis*. Light gray color indicates previous records and dark gray color indicates the new records for this species

On chayote, *Sechium edule*, we observed large numbers of mites (mixed population of *T. putrescentiae* and *T. curvipenis*) in the field. Mites initially increased their numbers on decaying fruits or leaves, and once the population increased, they migrated and colonized other chayote plants, principally those showing some level of damage or decomposition. As the leaves are very close to the fruits, it is very likely that some of the mites moved from the leaves to the fruits. Most of the mites were detected in the cavity between the flower and the fruit (where the flower is attached to the fruit) (Fig. 2.2). The majority of the mites were observed in association with fungi or algae that grew on the plants or fruits (Fig. 2.2). However, regardless of the large number of

mites found in the field, there was no (or at least we did not notice) direct damage caused by the feeding of the mites on the plants or fruits.
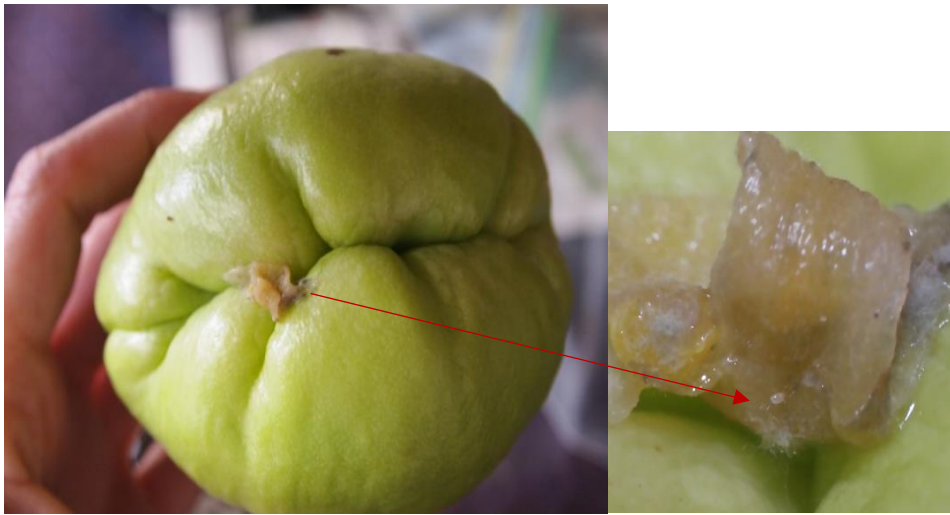
Figure 2.2 Floral remains on the cavity of the chayote fruit and a close up for *Tyrophagus* sp. and fungal hyphae growing on the floral remains

Morphological differences between T. putrescentiae and T. curvipenis

      Tyrophagus putrescentiae (TP) and *T. curvipenis* (TC) are morphologically similar, but each has distinguishing characters. We used the specimens found in chayote plants to verify the diagnostic characters that separated these two species. The major differences are: 1) proximal part of spermathecal duct in TC is slender (Fig. 2.3A), while in TP it is gradually widened (Fig. 2.3B); 2) coxal plate II in TC is broad and there is no distinct concavity in the posterior margin (Fig. 2.3C), whereas in TP, the coxal plate II is medium sized and a shallow but distinct concavity is noticeable in the posterior margin (Fig. 2.3D); 3) solenidia ω1 on tarsi I-II are slender in TC (Fig. 2.3E) while in TP they are wider (Fig. 2.3F); 4) the shaft of the supracoxal seta (scx) in TC is slender or moderately tapering from base to apex (Fig. 2.3G), while in TP it is prominently enlarged at the base of pectinations (Fig. 2.3H). For more details about the characters that these two species share, and differences with other species refer to Fan and Zhang (2007).

Character/ Species	*Tyrophagus curvipenis* Fain & Fauvel, 1993	*Tyrophagus putrescentiae* Schrank, 1781
Spermatheca		
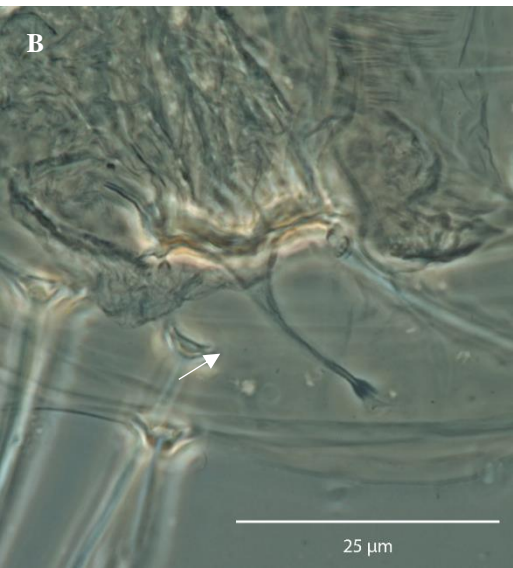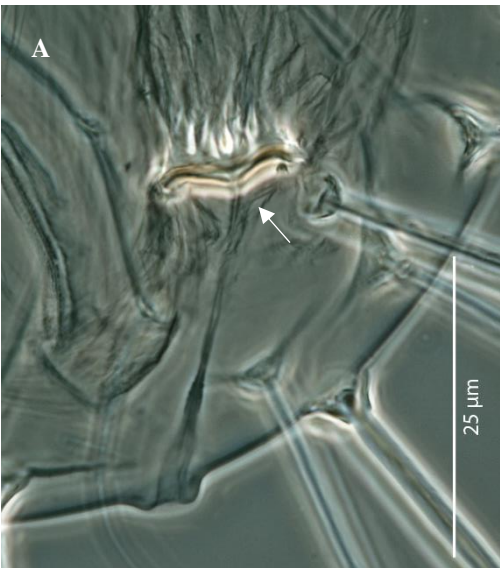
Coxal plate	
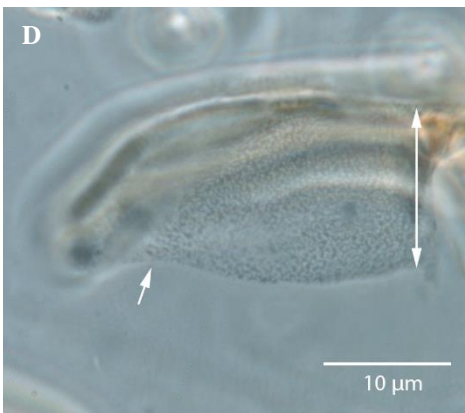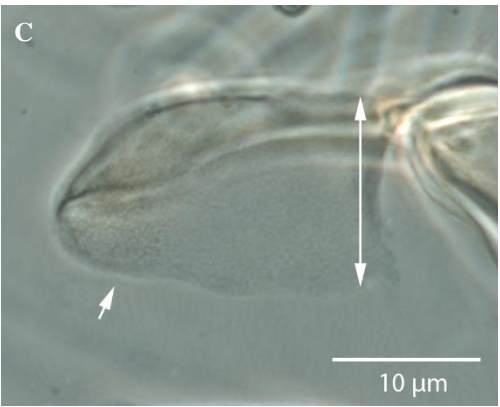
Omega
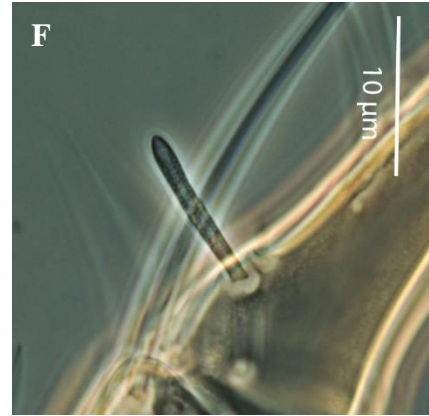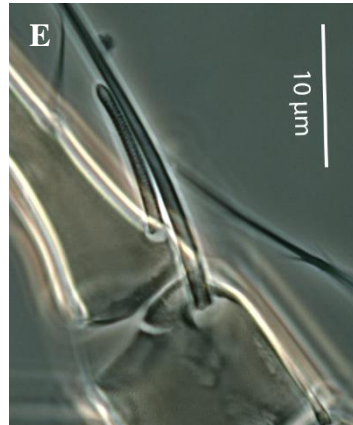
Supracoxal setae
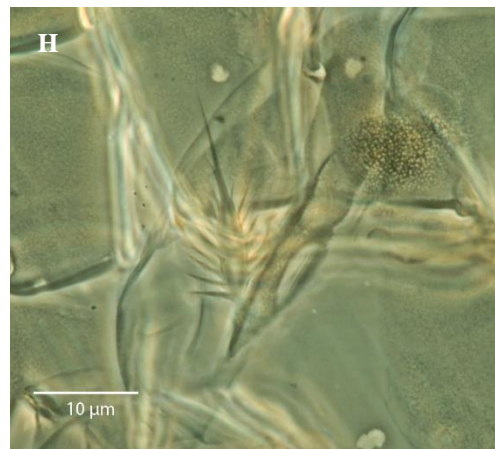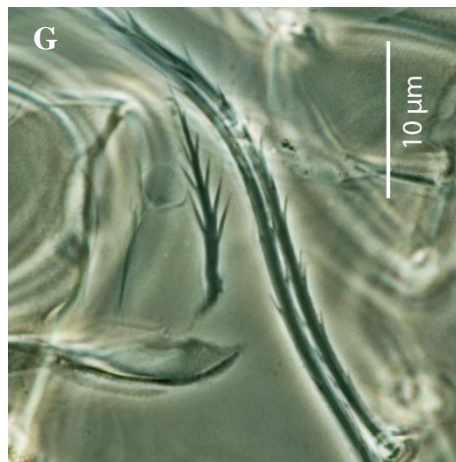
Figure 2.3 Main morphological characteristics differentiating *Tyrophagus curvipenis* and *T. putrescentiae.* **A** spermatheca *T. curvipenis* (♀)*,* **B** spermatheca *T. putrecentiae* (♀)*,* **C** coxal plate II T. *curvipenis* (♀), **D** coxal plate II *T. putrescentiae* (♀)*,* **E** omega I (ω1) *T.curvipenis* (♀)*,* **F** omega I (ω1) *T. putrescentiae* (♀)*,* **G** supracoxal seta (scx) *T. curvipenis* (♀)*,* **H** supracoxal seta *(*scx*) T. putrescentiae* (♀)

Genetic distances and barcoding gap analysis

Genetic distances among the three species, *T. putrescentiae* (TP), *T. curvipenis* (TC) and *T. fanetzhangorum* (TF), were greater than within-species distances of any of these species (Table 2.2). Genetic distances within TC (TC vs TC) and TF (TF vs TF) were lower than those exhibited by TP (TP vs TP). In TP, both K2P and the uncorrected distances showed the presence of considerable within species variation (Table 2.2) (range, mean±SD): 0-4.33, 1.78 ±1.44, K2P; and 0-3.58, 1.54± 1.34, uncorrected. Genetic distances between TP vs TF and TP vs TC were lower than those exhibited by TC vs TF (Table 2.2). The highest between-species difference was observed between TC and TF (range, mean±SD): 17.88- 19.17, 18.44±0.42, K2P; and (15.72-16.70, 16.16±SD 0.32, uncorrected).

Table 2.2 Percentages of intra- and interspecific genetic distances for *T. putrescentiae* (TP), *T. curvipenis* (TC) and *T. fanetzhangorum* (TF)

COMPARISON	K2P				UNCORRECTED			
	Average (AVG)	MAX	MIN	STD	Average (AVG)	MAX	MIN	STD
TP vs **TP**	1.78	4.33	0.00	1.44	1.54	3.58	0.00	1.34
TC vs **TC**	0.83	1.31	0.00	0.56	0.84	1.30	0.00	0.57
TF vs **TF**	0.98	1.15	0.82	0.15	0.97	1.14	0.81	0.15
TP vs **TC**	15.17	16.30	14.20	0.40	13.60	14.18	12.92	0.27
TP vs **TF**	17.03	18.11	15.89	0.43	15.12	15.97	14.32	0.29
TC vs **TF**	18.44	19.17	17.88	0.42	16.16	16.70	15.72	0.32

The barcoding gap analysis (Fig. 2.4) shows the distribution for the pairwise differences, where the left side of the histogram (low divergence) represents the intraspecific differences, and the right side of the histogram (higher divergence) represents the interspecific differences. The intraspecific distances fluctuate from 0.00 - 0.05, while the interspecific distances can vary from 0.12- 0.21. The intra-interspecies gap ranges from 0.05- 0.12 (Fig. 2.4).
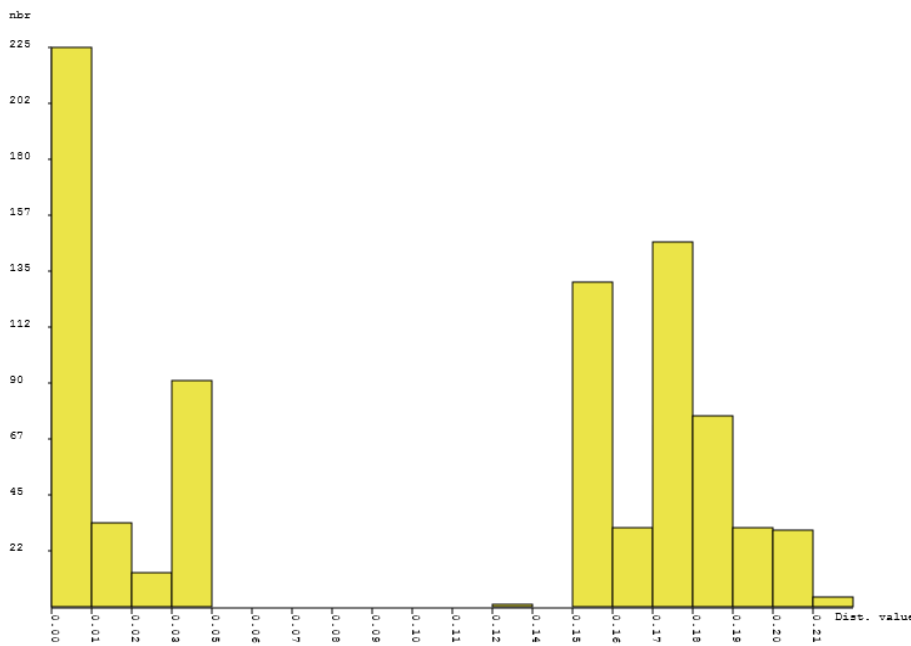
Figure 2.4 Barcoding gap analysis for the species of *Tyrophagus*. Pairwise distances for all sequences were calculated using K2P. Within- and between-species pairwise genetic distances are on left and right side of the graph, respectively.

Amino acid mutations

We looked for non-synonymous SNPs that resulted in amino acid changes. Some amino acid changes were found in the sequences within the two *T. putrescentiae* groups. Independently of the group, several samples had amino acid changing mutations (Table 2.3) while others had only synonymous mutations (not causing amino acid substitutions) when comparing to a reference (AD1398, sequenced from the population used to designate the neotype of TP).

We found a total of five non-synonymous mutations in nine of the samples; of these two mutations occurred in a single specimen belonging to group 2 (Table 2.3). It is interesting that all other sequences classified in group 2 shared the identical amino acid sequences with the reference. Several sequences from group 1 presented one amino acid change. The cluster that includes AD1912, AD1974, AD1999, AD2004, AD2013 and AD2015, showed one novel non-synonymous mutation, alanine A^{194} to serine S^{194}. Other non-synonymous mutations occurred in AD1924 (leucine L^{187} to phenylalanine F^{187}), and AD1928 (glycine G^{173} to serine S^{173}).

In comparison to *T. putrescentiae*, all sequences of *T. curvipenis* had four non-synonymous mutations (glutamic acid E^{331} to aspartic acid D^{331}, serine S^{333} to glycine G^{333}, threonine T^{402} to valine V^{402} and alanine A^{414} to valine V^{414}). *T. fanetzhangorum* had two non-synonymous mutations: isoleucine I^{361} to leucine L^{361} and valine V^{391} to isoleucine I^{391} (Table 2.3).

Table 2.3 Non-synonymous mutations in the CO1 gene of *T. putrescentiae, T. curvipenis* and *T. fanetzhangorum* (amino acid position coordinates are based on the CO1 GenBank sequence KY986247).

T. putrescentiae group 1: AD1398 (KY986247)	V81	G173	L187	A194	E331	S333	I361	V391	T402	A414
T. putrescentiae group 1: AD1398, 1401, 1396, 1402, 801, 1695, 1693, 1400, 1751, 1979, 1926, 1927. group 2: AD1337, 1692, 1721, 1722, 1274	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T. putrescentiae group 1: AD1912, 1974, 1999, 2004, 2013, 2015	✓	✓	✓	S	✓	✓	✓	✓	✓	✓
T. putrescentiae group 1: AD1924	✓	✓	F	✓	✓	✓	✓	✓	✓	✓
T. putrescentiae group 1: AD1928	✓	S	✓	✓	✓	✓	✓	✓	✓	✓
T. putrescentiae group 2: AD1925	I	✓	✓	✓	✓	✓	✓	✓	I	✓
T.curvipenis AD1911, 1972, 2017, 2018, 2025	✓	✓	✓	✓	D	G	✓	✓	V	V
T. fanetzhangorum AD1275, 1687, 1691	✓	✓	✓	✓	✓	✓	L	I	✓	✓

The ka/ks rate radio (according with MEME) for *Tyrophagus* was of 0.00384. This model found no evidence for positive/diversifying selection in all the sites. Also, BUSTED found no evidence (LRT, p-value $= 0.461 \geq .05$) of gene-wide episodic diversifying selection for the branches of *T. putrescentiae*. Therefore, there is no evidence that any sites have experienced diversifying selection.

Phylogenetic analysis

Our phylogenetic analysis recovered two distinct groups of *Tyrophagus* that coincide with the presence or absence of eyespots (Fig. 2.5). The first clade corresponds to the group with eyespots, including *T. putrescentiae*, *T. curvipenis* and *T. fanetzhangorum,* and the second, to the group without eyespots including *T. longior*, *T. perniciosus* and *T*.cf *similis*. Two different groups were identified among *T. putrescentiae* samples (Fig. 2.5). The first group includes the majority of the samples and was widely distributed, being present in several countries and substrates/hosts. No geographical or food preferences were apparent for the second group. Both the GenBank EST sequence data (Table 2.1) and the sequence AD1398 originated from the population used to designate the neotype by Klimov and OConnor (2009), fall within the group 1 of *T. putrescentiae* (Fig. 2.5).

Some inconsistences were found in the sequences previously deposited in GenBank, and for this reason, these samples were not used in the final phylogenetic analyses (Supplementary Fig. S1). The inconsistences include sample NC_028725.1 (*T. longior* from China), which did not cluster with the group of mites without eyespots, and sample NC_026079.1 (*T. putrescentiae* from China), which did not cluster with either of the two groups of *T. putrescentiae*, but instead formed a basal branch of *T. putrescentiae* (Supplementary Fig. S1).
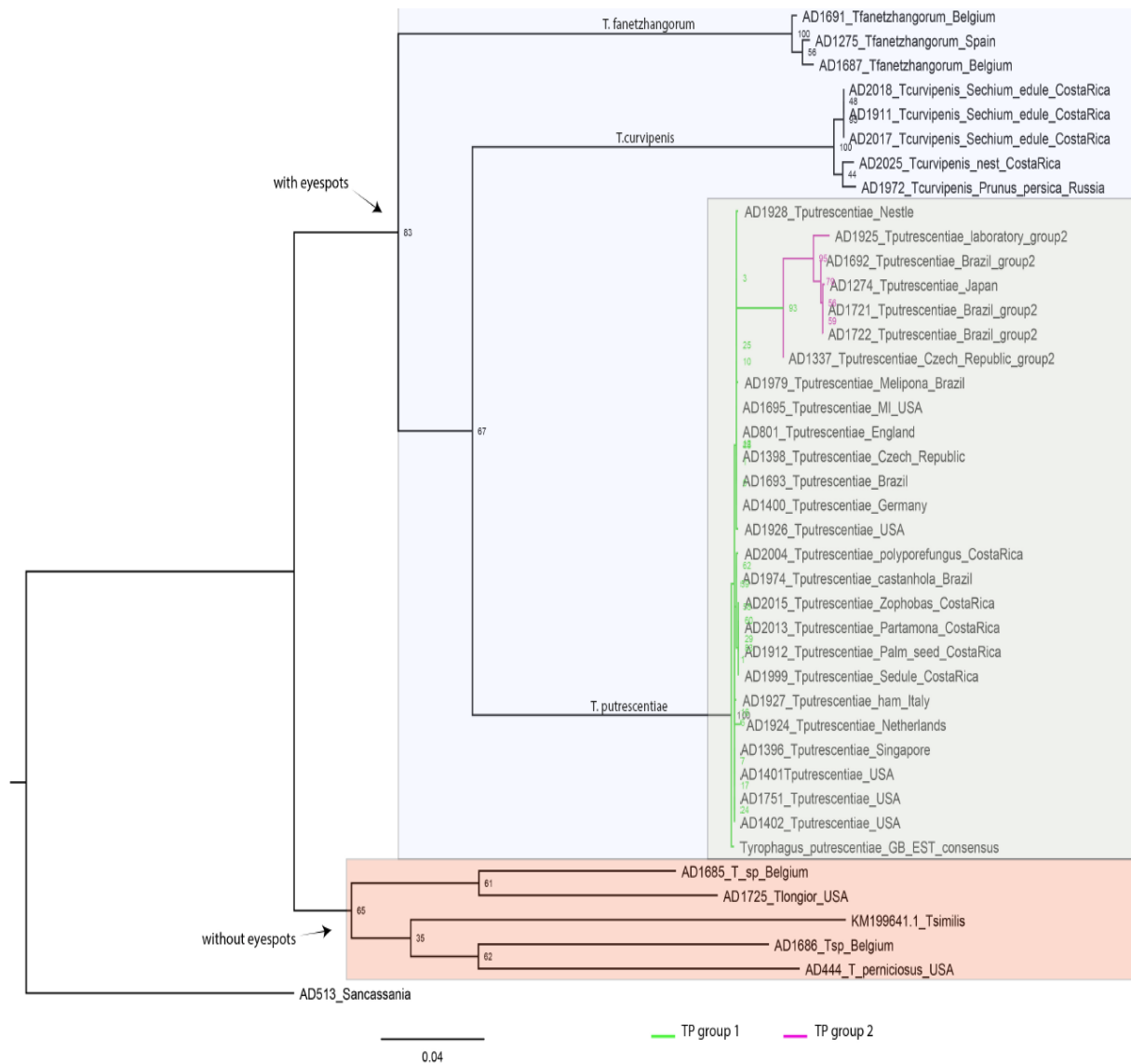
Figure 2.5 Maximum likelihood gene tree for species of *Tyrophagus* inferred from COI. Bootstrap support are indicated next to each branch.

Genetic diversity indexes and haplotype network analysis of T. putrescentiae

Genetic diversity indexes were calculated for *T. putrescentiae* (Table 2.2). A total of 1165 sites were monomorphic, out of 1227 nucleotides sequenced. From the 62 polymorphic sites, 57 included synonymous and 5 non-synonymous mutations.

As it was mentioned above (phylogenetic analysis), two different groups were identified within *T. putrescentiae*. Our analyses here, showed 16 different haplotypes for the two groups of *T. putrescentiae* which are separated from each other by 25 mutational steps (Fig. 2.6). Haplotype diversity was 0.934 and the average number of nucleotide differences (K) was 16.667. The network (Fig. 2.6) shows closely related haplotypes connected by lines and indicates the number of mutations (in parenthesis) between the most similar ones. For example, sample AD1979 (group 1) had more variation with respect to the other haplotypes that belong to the same group. Furthermore, sample AD1925 is the most distinct from the rest of the haplotypes from the group 2 (Fig. 2.6). This sample has two unique non-synonymous mutations (Table 2.3). According with this network, AD1979 and AD1925, are the most dissimilar haplotypes from each other.
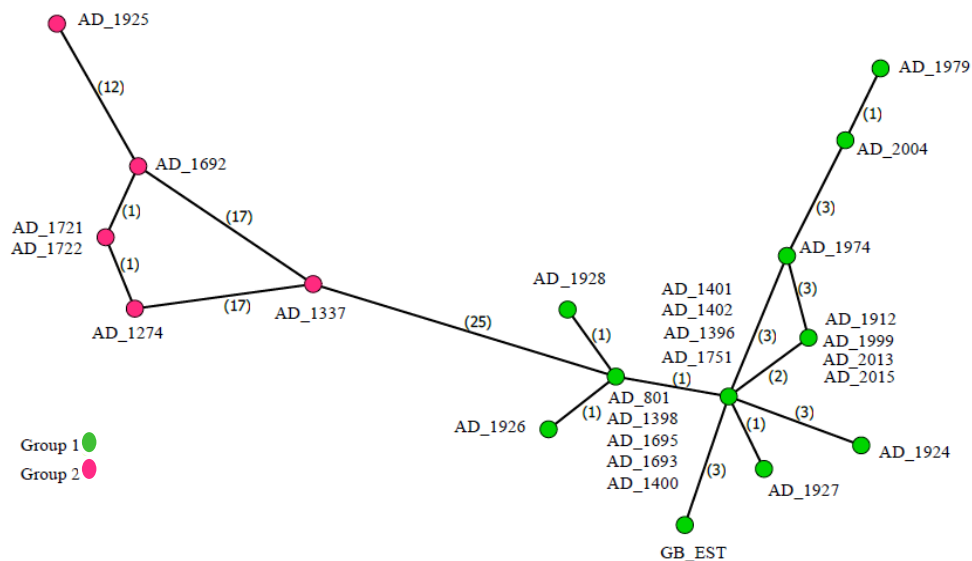
Figure 2.6 Haplotypes network for *T. putrescentiae*. Numbers in parenthesis correspond to the mutational steps. Green color corresponds to group 1 and pink color to group 2.

Discussion

Tyrophagus curvipenis: species boundaries, distribution and pest status

Even though *T. curvipenis* has been previously reported from several places around the world (Fig. 2.1), it is very likely that the actual distribution of this mite is much broader. The limited number of records of *T. curvipenis* could be due to difficulties is distinguishing *T. curvipenis* from other closely related species, in particular the widespread species *T. putrescentiae*. *T. curvipenis* is usually found in field situations rather than in stored products. It prefers habitats or hosts with irregular surfaces, such as the natural openings of chayote fruits (Fig. 2.2) or crevices formed on other plants. The preference of *T. curvipenis* for these areas is probably due to the high humidity retained there and the proliferation of fungi and algae that are available as a source of food. While *T. longior*, *T. putrescentiae*, *T. similis* and *T. neiswanderi* have been reported as agricultural pests causing direct damage to plants (Buxton, 1989; Fisher, 1993; Kasuga and Amano, 2003, 2006; Zhang, 2003; Kasuga and Honda, 2006; de Oliveira et al., 2007), there are no reports correlating the presence of *T. curvipenis* with any particular direct damage to plants or fruits. However, the possibility that *T. curvipenis* can feed on young leaves, flowering buds or fruits should then not be ignored. On the other hand, one can expect fungal transmission into the wounds on the leaves surface caused by the feeding of mites. An analogous situation is documented for *Rhizoglyphus robini* feeding on onions (Hanuny et al., 2008).

It is known that *T. curvipenis*, like other species of *Tyrophagus,* is an omnivorus generalist and can consume different food types with different nutrient contents. Ye and Zhang (2014) evaluated the effect caused by three different diets on the development of *T. curvipenis* and concluded that even when the food type affects the body length and width of the adults, this species survived and developed normally regardless of different food sources. Even though *T. curvipenis* has not been associated with any particular damage, it is regularly intercepted at international ports of entry by quarantine inspectors in natural cavities or crevices of fruits and leaves, causing substantial disruptions in international trade (Barquero, 2015; Ronald Ochoa, personal communication 2017).

Phylogeny of the genus Tyrophagus

Our topology (Fig. 2.5) fully agrees with the current morphology-based taxonomy where the different species cluster separately. The tree clusters the species of *Tyrophagus* into two main lineages coinciding with the presence or absence of eyespots. In the lineage with eyespots (*T. putrescentiae, T. curvipenis* and *T. fanetzhangorum*), the three species were clearly separated from each other (Fig. 2.5). Our tree agrees with other phylogenetic works, separating different species of *Tyrophagus* (Beroiz et al., 2014; Khaing et al., 2014; Erban et al., 2016). However, our topology also showed some differences with respect to some of these studies. For example, Beroiz et al. (2014) inferred a tree for several species of *Tyrophagus* where this genus formed a paraphyletic group, while our tree found a monophyletic group for all the species under study. This previous result, however, probably simply represents a rooting artifact (an ingroup root was used) rather than true phylogenetic relationships.

Our phylogenetic tree (Fig. 2.5) detected two distinct groups within the clade of *T. putrescentiae*. The first group is widely distributed and is found more commonly than the second. Even though COI sequences separate these two groups of *T. putrescentiae,* this locus did not show any differences among geographical populations nor indicate any correlation between the populations and their habitats. This implies that gene flow may be limited between the two groups. Given these results, it is necessary to conduct additional studies to elucidate the genetic composition and morphological differences between these groups.

It is important to emphasize that a single-locus does not provide enough evidence to absolutely delimit species, and it is better to use an integrative approach combining this data source along with other information (Padial et al., 2010; Schlick-Steiner et al., 2010; Yeates et al., 2011; Carstens et al., 2013; Pante et al., 2015). In particular, analyzing multiple loci under the multispecies coalescent model is clearly more advantageous than single-locus estimates. A multispecies coalescent framework can provide more accurate assessments of the process of allele coalescence for a given species history (DeSalle, 2005; Felsenstein, 2006; Heled and Drummond, 2010; O'Meara, 2010; Rannala and Yang, 2013; Zhang et al., 2014).

COI genetic distances and aminoacid changes in three closely related species

Among *T. curvipenis*, *T. putrescentiae*, and *T. fanetzhangorum*, the highest intraspecific variation was found within the species *T. putrescentiae* (Table 2.2). This species also showed one or two aminoacid changes for some of the populations sequenced here (Table 2.3). In the

circumstances where amino acid changes occur, it is possible the protein structure could change in different species. In this case, these amino acid changes do not represent a different species but might be an indication of divergence of these populations from other *T. putrescentiae* populations.

These results are not surprising given its worldwide distribution and the extraordinary capacity that this species has to live in diverse habitats and consume different kinds of food (Hughes, 1976; Duek et al., 2001; Zhang, 2003; Fan and Zhang, 2007; Klimov and OConnor, 2009; OConnor 2009; Khaing et al., 2014). Additionally, international trade (mainly agricultural products and processed food) has allowed this species to move from one geographical region to another with few constraints (Dhooria, 2016). The results obtained here contrast with those of Yang et al. (2011) who did not find any intraspecific variation within *T. putrescentiae* using the COI, probably due to the small size of the amplified region (377bp) and the relatively few sequenced populations. Khaing et al. (2014) reported a small genetic variation (0.1 and 0.2% K2P) in their *T. putrescentiae* sample (370bp for COI) with respect to other samples deposited in the GenBank database. Other studies (Beroiz et al., 2014; Erban et al., 2016) suggest a much larger variation within *T. putrescentiae*, as indicated by the large distances between terminals on the tree, but these did not report exact values.

The average interspecific K2P genetic distances were higher than 15% for *T. curvipenis, T. putrescentiae* and *T. fanetzhangorum* (Table 2), suggesting that they are likely independent evolutionary lineages with no gene flow among them. Our results agree with previous findings (Beroiz et al., 2014; Erban et al., 2016) suggesting that *T. putrescentiae* and *T. fanetzhangorum* are valid species, despite the small number of morphological differences between them.

Similarly, our results agree with crossing experiments demonstrating complete reproductive isolation between *T. curvipenis* and *T. putrescentiae* (=*T. communis*) (Fan and Zhang, 2007). Although the mites were able to copulate with each other, females did not lay eggs (Fan and Zhang, 2007), indicating that these two populations are reproductively isolated and could represent two separate species under the Biological Species Concept. Thus, results obtained in those breading experiments do support the general utility of DNA barcoding approach for reliable species delimitation. However, special attention is needed when species with high genetic variability are under study, as is the case of *T. putrescentiae*. Our results (Table 2.2) showed a high intraspecific variability reaching 4.33% (K2P distances), whereas the difference for the other species did not exceed 1.31% (*T. curvipenis*, K2P distances). Considering these results, one might interpret the

data for the populations with the highest genetic distances (4.33%) and place them as separate species, however according with our barcode gap analysis (Fig. 2.4) the intraspecific variation for these mites can range from 0% to 5% and still belong to the same species but to different haplotypes.

The within-species distance of *T. putrescentiae* (4.33% or 5.75%, full dataset vs the Folmer fragment only), is relatively high and broadly overlaps with between-species distances reported in the literature, e.g., 4% (Dowton et al., 2014), 3.14% (Doña et al., 2015), 3% (Hebert et al., 2004; Smith et al., 2005), 2% (Rossini et al., 2016; Smith et al., 2005), or lower (Hebert et al., 2004a). However, species having compatible or higher within-species genetic distances are known as well: 10.1% in the human follicle mite, *Demodex folliculorum* (Demodecidae) (conservatively recalculated from Palopoli et al., 2015), 5.7-6.8% in the common blue butterfly, *Polyommatus icarus* (Lycaenidae) (Wiemers and Fiedler, 2007), and about 6% in the sea snail, *Echinolittorina vidua* (Littorinidae) (Williams and Reid, 2004).

Our haplotype analysis separated *T. putrescentiae* into 16 haplotypes and 2 groups (Fig.2. 6), however there was no strong geographic or habitat structure in the networks. Other studies found a similar pattern, where populations geographically separated were genetically more similar than geographically close populations (Noge et al., 2005; Beroiz et al., 2014).

Quality of GenBank data

The standardization of DNA barcodes enables using data from many studies (Pentinsaari et al., 2016), however, it is known that many sequences in GenBank are misidentified or still have attached primer/vector sequences. This makes it difficult to use those sequences in other studies and forces the user to be very careful in the analysis of the data when using these (Meier et al., 2006). Misidentification and primer joined to the sequences were two of the principal problems encountered here. Misidentification was suspected for *T. longior* sequence NC-028725 (Yang and Li, 2016) as it clusters with other sequences of *T.putrescentiae* (Supplementary Fig. S1). Furthermore, we detected primer sequence contamination (oligonucleotide primer sequences which were not removed from the finished sequence) in several *T. putrescentiae* sequences EU078968, EU078969, EF527826, AY525572, HQ287793, HQ287795, HQ287796 (Webster et al., 2004; Xia et al., 2007; Wu et al., 2007; Yang et al., 2011). On the other hand, the sequences of those studies have in general a length of 377 bp (trimmed to 334bp) corresponding to the central part of the COI region. Comparing these sequences with our trimmed fragments (1227bp), the

difference in sizes created inconsistencies in the tree changing its final topology (Supplementary Fig. S1).

Conclusions

In this work, our barcode analysis confirmed the species status of *T. curvipenis* and other closely related species of *Tyrophagus*, which are difficult to separate by morphological characters and revealed a high genetic variability and complexity within *T. putrescentiae.* The large genetic distances and amino acid changes found for some *T. putrescentiae* populations could be explained by the worldwide distribution and large effective population size. Future studies, including species delimitation analyses, are not only necessary to disentangle the status for all the species of *Tyrophagus* but also to better determine the genetic structure of the two groups of *T. putrescentiae* among various populations. Important morphological differences between *T. curvipenis* and *T. putrescentiae* are presented, and we recorded for the first time the occurrence of *T. curvipenis* in the New World.

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Supplementary material from Chapter II

Figure S1. Maximum likelihood COI topology based on the dataset generated as part of this study plus GeneBank sequences.

Chapter 3 Insights from the genus *Tyrophagus* (Acari: Acaridae): molecular species delimitation analyses to disentangle the complexity of the *Tyrophagus putrescentiae* species complex

Abstract

Difficulties in the ability to delimit species of *Tyrophagus* has led to controversy concerning the number of species in this genus. In the past few years there has been an increase in the use of molecular species delimitation approaches to separate and discover new species. In this study we use sequences from five loci (four nuclear and one mitochondrial) and utilize several coalescent-based species delimitation methods to disentangle the species status of populations of the cosmopolitan mite, *Tyrophagus putrescentiae* collected from different geographical regions, substrates and hosts. We also evaluate the accuracy of these delimitation methods to test species boundaries for additional lineages of *Tyrophagus*. Our analyses identified between 23 and 27 putative species of *Tyrophagus.* The multilocus approaches provide more conservative interpretations and agree with morphological diagnoses. All but one method shows that *T. putrescentiae* represents a single species irrespective of whether or not some populations are highly genetically divergent from others at mitochondrial gene regions. Given that changes in the priors can affect the performance of molecular species delimitation, it is necessary to include all possible known information in the analyses. Our results reveal the hidden diversity in this group and the importance of integrating several complementary methods in a taxonomic framework at incorporating different forms of evidence when delimiting species.

Introduction

The species represents the central element of investigation and is broadly defined as groups of organisms that evolve and diverge separately from other lineages (De Queiroz, 2007; Fujisawa and Barraclough, 2013). The accurate separation and identification of species is crucial in the biological sciences, as this has a direct impact in fields such as pest management, agriculture, conservation and disease control (De Queiroz, 2007; Yang & Rannala, 2010; Yu et al., 2017; Pavón-Vázquez et al., 2018). Therefore, delimitation of species is necessary and consists of the challenge to place a group of individuals into one or other species (De Queiroz, 2007; Jones, 2017).

Assessment of species boundaries can be carried out by integrating evidence from different data types (morphological, ecological, genetic, etc.) with several statistical approaches and methodologies (De Queiroz, 2007; Fujita et al., 2012; Puillandre et al., 2012; Zhang et al., 2013; Edwards & Knowles, 2014; Pante et al., 2015). Several methods to test distinct genetic species hypotheses have been developed in the past few years and have received considerable attention (Fujita et al., 2012; Carstens et al., 2013; Yang & Rannala, 2010, 2017). However, the outcomes of the species boundaries may fluctuate in accord with changes in assumptions or algorithms employed by the different methodologies (Olave et al., 2014). In most cases, these methods use the multispecies coalescent model (MSCM). This model is based on a population genetics context, and take in consideration priors related with species population sizes and divergence times, while reflecting ancestral polymorphisms and incomplete lineage sorting (Rannala & Yang, 2003; Yang and Rannala, 2010, 2014, 2017; Leaché et al., 2018). Approaches that utilize the MSCM are convenient as they can detect recently diverged taxa and help in the separation and identification of cryptic and closely related species or species complexes (Zhang et al., 2011; Yu et al., 2017).

The application of different data provides important information about the species under study; however, in many cases the lack of morphological, ecological or geographical differences to distinguish closely related species, makes the identification of some groups very challenging. Diverse and poorly-known groups such as mites have the capacity to include many cryptic species that occur in the same geographical region, or poorly known species that can be found in the same habitat-host as other well-known and related species, which make them difficult to separate (Coimbra-Dores et al., 2016; Laska et al., 2018; Skoracka et al., 2015). Even though recognition

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of this diversity is challenging, it is crucial to distinguish the species that inhabit a particular place to understand how these species are connected and how this diversity affects ecological interactions (Bickford et al., 2007).

The mite genus *Tyrophagus* is a diverse and complex group within the family Acaridae and a noteworthy example of these taxonomic problems. Around 60 species names have been proposed to members of this genus but, currently only about 35 species are recognized (Fan & Zhang, 2007). Members of this group exhibit extensively overlapping distributions with no evident geographic, ecological nor climatic barriers between many of them. Moreover, as populations of *Tyrophagus* tend to have high population sizes and exist in many different habitats, the group is likely to contain a number of cryptic species complexes. For example, Fan & Zhang (2007) discovered five new species of *Tyrophagus* in their revision of Asian-Pacific species, which suggests that hidden diversity might be present here as well as in other understudied geographical regions like the Neotropics.

The challenge for *Tyrophagus* is even much greater due to the taxonomic uncertainty surrounding several species, as they have inconspicuous morphological differences between them, especially at recent divergence levels where morphological characters have presumably not had enough time to evolve fixed differences (Klimov & OConnor, 2009, 2010, 2015; Yang et al., 2011; Erban et al., 2016; Murillo et al., 2018). The separation of closely related species might be much more problematic than in other mite groups because the variation is prone to be biased by human discernment of morphological characters (Skoracka et al., 2015). These issues have led to possible failure in the ability to detect diagnostic characters and an underestimation of the real species diversity of this genus. Consequently the distinction of species of *Tyrophagus* requires incorporation of other tools and data including examination of multi-locus genetic information for precise identification, as these approaches provide potential evidence of divergence for species that have undergone recent speciation events (Knowles & Carstens, 2007).

Uncertainty in the ability to recognize new species can be considerable for lineages that diverged recently (Knowles & Carstens, 2007; Leaché & Fujita, 2010). In the case of recently evolved species such as *Tyrophagus putrescentiae*, it is possible that individuals might erroneously cluster as a single species when they should belong to separate species. Previous examination of COI sequences exposed two distinct groups within the species *T. putrescentiae* and uncovered large genetic distances and amino acid changes among individuals of this species (Murillo et al.,

2018). However, it is known that genetic distance approaches do not test for species boundaries and cannot infer speciation events nor population genetic processes related to them (Hambäck et al., 2013). Because of this high genetic variability, morphological similarity, and complexity observed in several species, especially *T. putrescentiae,* the genus *Tyrophagus* provides an intriguing system to study. Species delimitation analyses are suitable methods to disentangle the genetic structure among the two groups for *T. putrescentiae* and define potential boundaries within this species. Also, these types of analyses can corroborate the status of other closely related species that had been previously separated by morphology and facilitate the identification of species with no obvious morphological differences between them. Here, we analyze single and multi-locus approaches and assess the performance of five species delimitation methods to i) disentangle the species status of different populations of *T. putrescentiae* from diverse geographical regions, ii) compare the performance of species delimitation analyses in recently diverged species iii) find hidden diversity within this genus and iv) corroborate the status of other known lineages of *Tyrophagus*. We take a conservative approach in which analyses showing similar results will be considered as the more reliable species delimitation interpretations.

Material and Methods

Mite samples

Samples of *Tyrophagus* were collected from 17 countries and various habitats (Supplementary Table S1). Specimens were manually collected from substrates with a mounting needle under a dissecting microscope and stored in ethanol (99.5%). When needed, additional materials containing mites were sampled by Berlese-funnel extraction or sieved. Slides were prepared in Hoyer's medium, and mites were identified using the most recent taxonomic keys (Fan & Zhang, 2007 but see Klimov & OConnor, 2009 with regard to *Tyrophagus putrescentiae*). Voucher specimens were deposited in the University of Michigan Museum of Zoology, Ann Arbor (UMMZ).

DNA extraction and amplification

The mites used for DNA extraction were preserved in 99.5% ethanol and kept at -80°C. Genomic DNA was extracted from single specimens using a QIAamp DNA Micro kit (Qiagen)

following the manufacturer's protocol for tissues, with some modifications (Klimov & OConnor, 2008).

A total of 71 individuals of *Tyrophagus* (Supplementary Table A-S1) and two outgroup species (AD613_*Aleuroglyphus* sp., AD513_*Sancassania* sp.) were sequenced using nested PCR, consisting of two PCR reactions (first PCR product is used in the second reaction) with different primers (Supplementary Table A-S2). These amplification reactions were performed in a 20µl volume with Platinum Taq DNA Polymerase (Invitrogen) in a Mastercycler gradient, Eppendorf thermocycler. The master mix of each gene for the first PCR (nested PCR) contained: 2.0 µl of PCR buffer (final 1X), 1.4 µl $MgSO_4$ (3.5 mM), 1.4 µL of dNTP (0.7 mM each), 0.8 µl of each oligonucleotide primers (0.4 µM), 0.12 µl of Platinum Taq polymerase (1.5U) and 0.4-1 µl of genomic DNA template. For the second PCR, the master mix was modified with a reduced quantity of Taq Polymerase 0.08 µl (1.0 U), and 0.6 µl of PCR product from the first PCR reaction. The total volume of each reaction was increased to 20 µl with distilled water and the thermocycler protocol was set for each oligonucleotide primer pair (Supplementary Table A-S2).

In total five gene regions were amplified: one mitochondrial protein-coding gene (COI), two nuclear protein-coding genes (EF1α, HSP70) and two encoding structural ribosomal RNA genes (18S, 28S). PCR products were electrophoresed on a 1.5% agarose gel in 1X TA buffer at 100 V for approximately 35 min. Bands were visualized and excised under UV light and purified with a QIAquick® gel extraction kit (Qiagen). Sequencing was done in both directions using a 3730XL sequencer (Applied Biosystems) at the University of Michigan DNA sequencing Core.

Sequence editing

Chromatograms were resolved in Sequencher ver. 5.4.6 (Sequencher, 2016); primer and low quality sequences were trimmed. Sequences were imported to Mesquite (Maddison and Maddison, 2011), and in the case of protein-coding genes, each codon was color-coded according to its amino acid translation to verify that no stop codons were present. In the cases were insertions or deletions were present (unalignable due to the lack of common secondary structure), these regions were excluded from analyses, resulting in a very conservative alignment containing no gaps.

Phylogenetic analyses

The best-fitting nucleotide substitution models were selected for each gene region based on corrected Akaike information criterion (AICc) in the program jModelTest 2.0 (Darriba et al.,

2012). The best substitution models were as follows: GTR+I+G for 18S and 28S, HKY+I+G for COI, TRN+I+G for EF1A and SYM+I+G for HSP70.

The phylogenetic analyses for the species tree were performed with Maximum Likelihood (ML) and Bayesian frameworks using the programs RAxML (Stamatakis, 2014) and *BEAST v2.5.1 (Bouckaert et al., 2014) respectively. Convergence and burn-in were checked for Bayesian analyses using the program Tracer v1.7 (Rambaut et al., 2018). As the Bayesian tree was similar to the ML tree, we report the results from the ML tree with the Bayesian posterior probabilities for key divergences additionally indicated here.

Species delimitation analyses

To assess the congruence of molecular species delimitation methods, we performed a series of analyses to demarcate species boundaries of individuals identified as *T. putrescentiae,* as well as the *Tyrophagus* species included in our tree. For this, we used five statistical methods: PTP (Poisson Tree Processes), GMYC (Generalized Mixed Yule Coalescent), bGMYC (Bayesian Generalized Mixed Yule Coalescent) STACEY (Species Tree And Classification Estimation Yarely) and BPP (Bayesian Phylogenetic and Phylogeography).

For the first analysis, a RAxML (Stamatakis, 2014) tree was analyzed through the program PTP using the PTP webmask (Zhang et al., 2013); both the heuristic ML and Bayesian implementations algorithms were used. The MCMC was applied with the following parameters: 200000 generations, 100 thinning, 0.1of burnin and 123 for seed. This program, differs from others software's like GMYC as it takes the number of substitutions on the branches instead of time intervals.

Analysis using the program GMYC (Pons et al., 2006) was conducted using the COI data for the purpose of comparison with the results obtained by PTP. Here, we used an ultrametric tree generated by *BEAST v2.5.1 (Bouckaert et al., 2014), in which a single threshold was carry out through the package "splits" in R 3.5.1(Fujisawa & Barraclough, 2013). This method uses a stochastic birth-death process that analyzes the time intervals on branches and relies on the independent evolution until the appearance of distinct clusters (Fujisawa & Barraclough, 2013).

Both, GMYC and PTP methods were run on single COI gene trees and species trees (all genes). This was generated with the aim of evaluating potential differences in results. However, these programs are intended to be used for single gene trees, and the usage on species trees represents a violation of the assumptions for both models. However, in both cases we found a

general consistency in the results. To corroborate the results obtained with these two programs, we performed the next analysis, which was developed for multiple trees.

A Bayesian implementation for the general mixed yule-coalescent model was used through the program bGMYC (Reid & Carstens, 2012). The analysis was executed through the packages "apes" and "bGMYC" in R 3.5.1 (Reid & Carstens, 2012). We ran the multiple-threshold model with the following parameters: multiphylo trs3, mcmc = 10000, burnin = 2500, thinning = 10, py2 = 1.2, t1 =2, t2 =50, start = c (1,0.5,5), scale = c (1,40,0.5).

We also used two multilocus Bayesian methods (BPP and STACEY) to validate and better infer the evolutionary history of the delimited species, especially for the lineage *T. putrescentiae,* given that the phylogenetic tree analyses (see Results) showed two dissimilar groups and the same outcome was obtained in previous barcoding analysis (Murillo et al., 2018).

Four species delimitation scenarios were tested with the program BPP v4.0 (Yang, 2015; Flouri et al., 2018) for *T. putrescentiae*. We also included *T. fanetzhangorum* in the analysis as it is the sister species of *T. putrescentiae*. BPP considers the population parameters ancestral population size (θ) and species divergence times (τ) as priors since these factors can affect the posterior probabilities of the models (Yang & Rannala, 2010). Here, the hypotheses were conducted with analysis (A10) that allows for delimitation of species while using a fixed guide tree. In this analysis it is also possible to obtain posterior probabilities for other probable species trees. We evaluated three different combinations of inverse-gamma distribution priors and the default priors given by the program ($\theta \sim$ G (3, 0.002) and $\tau \sim$ G (3, 0.004)), to determine how choice of priors impacted our results.

The first combination of inverse-gamma priors assumes large ancestral population sizes and deep divergences: $\theta \sim$ G (3, 0.56) and $\tau \sim$ G (3, 0.6). The second combination of priors uses small ancestral population sizes and shallow divergence times: $\theta \sim$ G (3, 0.0056) and $\tau \sim$ G (3, 0.006). The third tested prior combination is more conservative and assumes large ancestral populations sizes $\theta \sim$ G (3, 0.56) and relatively shallow divergences times $\tau \sim$ G (3, 0.006) among species. This last combination is expected to favor fewer species. Three replicate runs were conducted for each prior combination.

We also performed likelihood-ratio tests (LRT) in BPP to compare the best fit of two possible models for *T. putrescentiae*. The first model tested the likelihood for the occurrence of a single

species in all populations of *T. putrescentiae,* while the second model evaluated the possibility of two species.

Lastly, a species delimitation analysis was performed using STACEY implemented in the program BEAST 2 (Jones, 2017). This method incorporates population sizes for branches and maximizes the tree likelihood using a MCMC model. According with Jones (2014), one of the differences between this program and BPP is that it does not require a prior assignment for the species or a guide tree. After running the analysis in STACEY, a species delimitation analyzer (speciesDA) (Jones, 2017) was used to process the log files and find out the species assignment. Finally, an R script (Jones, 2014) was performed to obtain the species delimitation matrix.

Results

Sequences

Sequences for the five loci had on average a length of 1257 bp for COI, 1704 for HSP70, 1222 for EF1α, 1739 for 28S and 1812 for 18S. Loci that did not amplified for each individual are shown in supplementary material Table A-S1. In total, the original length for all amplified sequences was of 7734 aligned nucleotides before translation and character exclusion, and 7527 nucleotides for the final alignment used during the analyses.

Phylogenetic analyses

The species tree derived from this study separated two major clades of *Tyrophagus* in which clade membership corresponds with the presence or absence of eyespots (Fig. 3.1). Individuals of *Tyrophagus* occur in 23 distinct clades with high support values for most of these clades (i.e, bootstrap and posterior probability > 70% for most tip clades and internal nodes). The phylogeny also confirmed the monophyly of six previously described species: *T. putrescentiae*, *T. curvipenis* and *T. fanetzhangorum*, *T. longior*, *T. perniciosus* and *T*. cf. *similis*. It also showed 17 separated morpho species (Fig. 3.1 with asterisk mark) which are in most of the cases closely related to previous known species. For example, specimens AD1958 and AD 1960 are related to the well-known species *T. curvipenis*, however they cluster separated from each other and from individuals of *T. curvipenis*. In this case some morphological differences between these individuals were noticed. The same was observed for specimens AD1727 (same as AD2001), AD2016, AD2021 (same as AD2023, AD2024), AD1993 (same as 2081) which are related to *T. roberstsonae* (Fig.

3.1). Specimen AD701 (same as AD1685, AD1688) is morphologically similar to AD 1725 *T. longior* but does not group with individuals of this taxon. A number of other individuals did not cluster with any other well-defined clades including AD 2011 (same as AD2078), AD1714 (same as AD1715), AD2008, AD1973, AD2020, AD1975, AD471, AD1731, and AD1976 (Fig. 3.1).

In the case of the species *T. putrescentiae* two groups were identified within this clade. The first group which is paraphyletic with respect to the second, includes most populations that are widely distributed and showed shallow divergence. The second group includes individuals from different localities and have deeper divergence than the first group (Fig. 3.1).

All gene trees for nuclear loci showed variation between the different populations of *T. putrescentiae* (individual gene trees not showed here). However, neither one cluster all populations in the exact same two groups as the species tree and populations from group 1 and 2 cluster in different groups depending on the gene used.
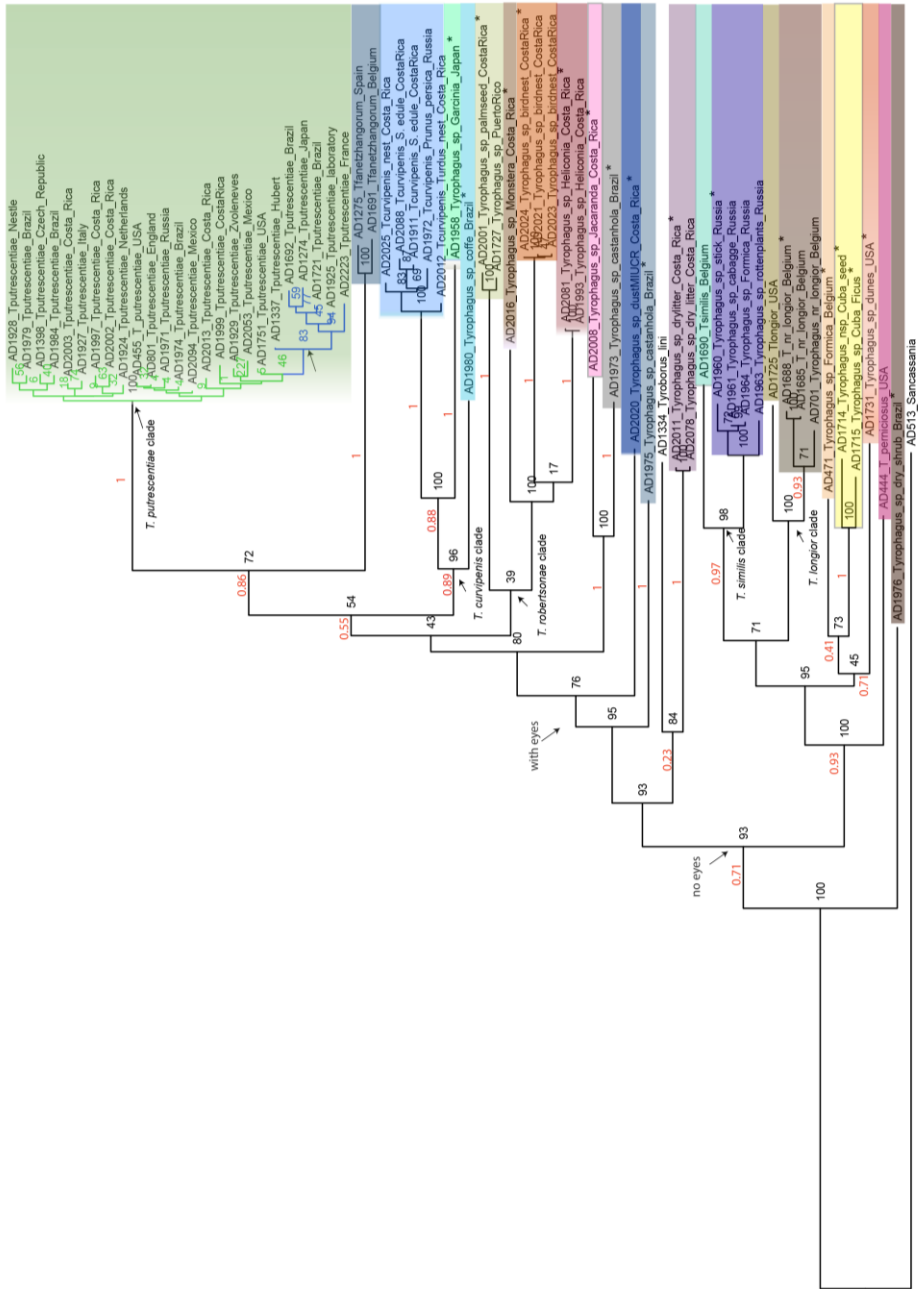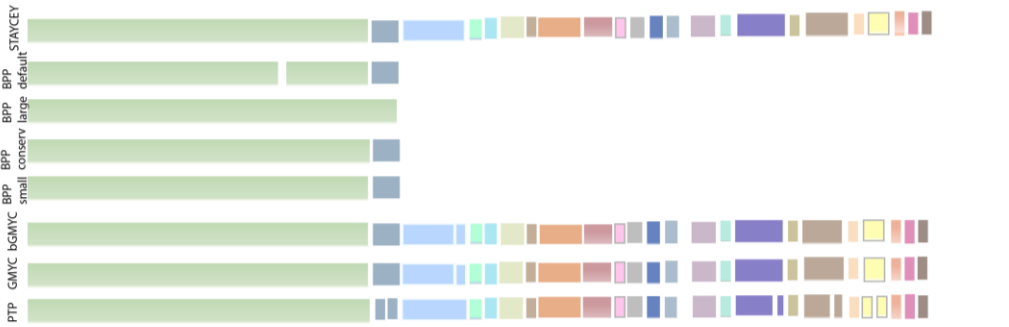
Figure 3.1 Species tree for *Tyrophagus* inferred from COI, 18S, 28S, EF1A, HSP70 genes. Bootstrap (black) and posterior probabilities (red) values obtained under a Maximum Likelihood (RAXML) and Bayesian (*BEAST) approach, respectively. Branches for populations of T. *putrescentiae* for group 1 are highlighted in green and for group 2 in blue. Colored bars to the right of the phylogeny represent hypothesized species delimitations based on different analyses and priors.

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Species delimitation analyses

Species boundaries were tested using multiple approaches. Results from the PTP analysis suggested additional species than the number of well delivered clades in the phylogenetic tree (Fig. 3.1). This analysis identified a total of 27 lineages, and separated specimens AD1714 from AD1715 *Tyrophagus*_n.sp. (Cuba) (support= 0.99); AD701 from AD1685, 1688 *Tyrophagus* nr. *longior* (Belgium) (support= 0.97); AD1275 from AD1691 *T. fanetzhangorum* (support= 0.798); AD 1963 *Tyrophagus* sp. from the rest of the Russian samples AD 1960, 1961, 1964 (support= 0.77). Also, PTP kept all populations of *T. putrescentiae* as a single species (support= 0.988) (Fig. 3.1).

On the other hand, GMYC separated the lineages into 25 species. However the lineages that were separated by GMYC were different from PTP (Fig. 3.1). In this case, the individual AD 2012 was separated from AD 2025, 2088, 1911, 1972, all identified by morphological characters as *T. curvipenis*. In accordance with PTP, the program GMYC preserved the species *T. putrescentiae* as a single group (Fig. 3.1).

The sequence by sequence matrix conducted in the program bGMYC (Fig. 3.2) produces a tree by tree matrix, with each cell containing the probability that the two corresponding terminals are members of the same species. Here, similar results for the species boundaries through the program GMYC were obtained and individual AD 2012 was separated (support= 0.54) from the rest of the samples identified as *T. curvipenis* (Fig. 3.1). In addition, the matrix clustered all individuals of *T. putrescentiae* as the same lineage with posterior probabilities higher than 0.95.
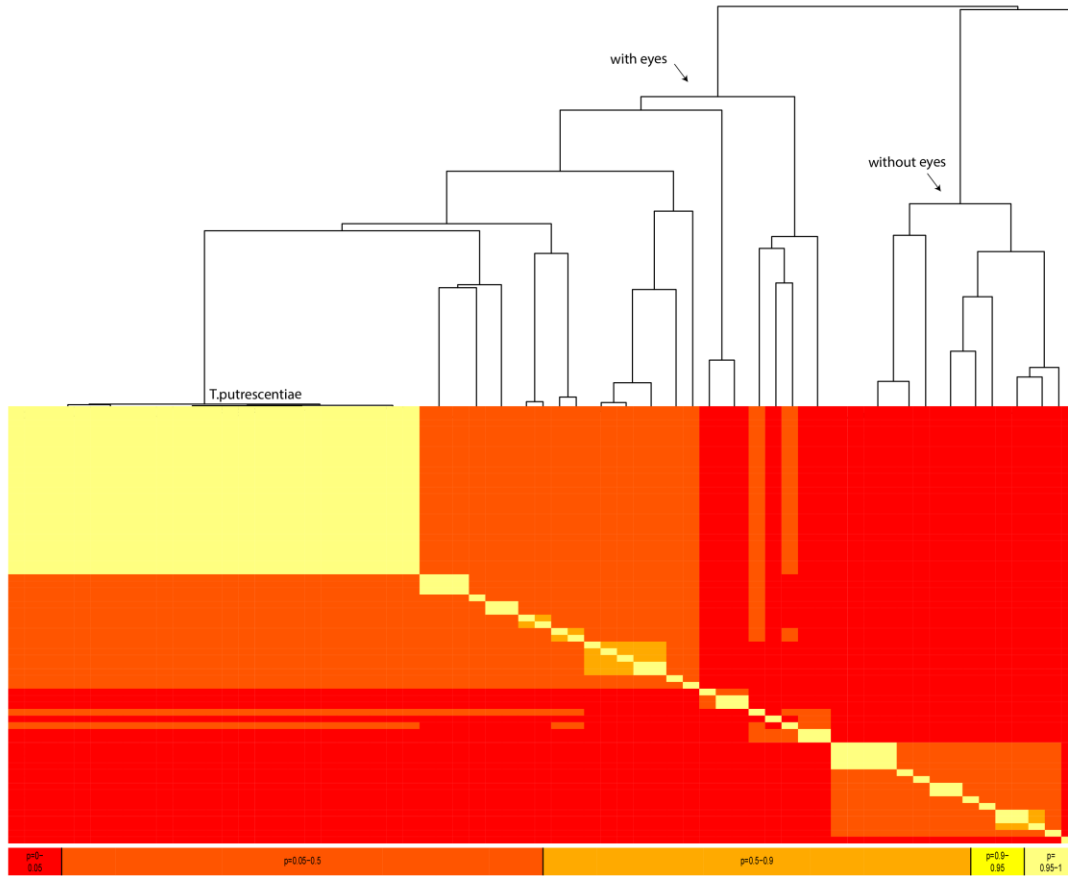
Figure 3.2 Sequence by sequence matrix for species delimitation of *Tyrophagus* conducted through the program bGMYC. Cells are colored by the posterior probability value (yellow-high, red-low) corresponding to the conspecific sequences.

Subsequently, four hypotheses were tested with the program BPP to evaluate the effect of different population parameters in the species *T. putrescentiae* and corroborate results obtained with the other species delimitation methods. BPP supported the existence of only one lineage for *T. putrescentiae* when the model considers small ancestral population sizes and shallow divergence times as priors (Table 3.1); this model obtained a posterior probability value of 0.99 and separated *T. putrescentiae* from *T. fanetzhangorum* (Fig. 3.1). The same results were found when large ancestral population sizes and relatively shallow divergences times (conservative approach) were taken into consideration (Table 3.1, Fig. 3.1). Here the mean posterior probability was 0.82. However, results changed when priors assumed a large ancestral population size and deep divergences. With this set of priors, BPP was not able to separate any of the lineages, and retained all individuals as one species, including individuals of *T. fanetzhangorum* (Fig. 3.1); the mean posterior probability recovered here was 1 (Table 3.1). Similarly, when BPP was tested with

default assumptions (those given by the program), it not only split *T. fanetzhangorum* from *T. putrescentiae* but also separated *T. putrescentiae* into two independent lineages, resulting in three species (Fig. 3.1) with a mean posterior probability of 0.87 (Table 3.1).

Table 3.1 . Bayesian species delimitation employing BPP for the species *T. putrescentiae* (sp1=*T.putrescentiae* group1, sp2= *T. putrescentiae* group 2) and *T. fanetzhangorum* (sp3) with four prior combinations (θ population size, τ divergence time). Posterior probability, number of species and delimited species in parenthesis are showed in order for each of the three runs.

Run	Basic θ ~ G (3, 0.002) τ ~ G (3, 0.004)	Small θ ~ G (3, 0.0056) τ ~ G (3, 0.006)	Conservative θ ~ G (3, 0.56) τ ~ G (3, 0.006)	Large θ ~ G (3, 0.56) τ ~ G (3, 0.6)
1	0.925840 3 (sp1, sp2, sp3) 0.074160 2 (sp1sp2, sp3)	0.999340 2 (sp1sp2, sp3) 0.000660 3 (sp1, sp2, sp3)	0.896920 2 (sp1sp2, sp3) 0.103080 3 (sp1 sp2, sp3)	1.000000 1 (sp1sp2sp3)
2	0.899990 3 (sp1, sp2, sp3) 0.100010 2 (sp1sp2 ,sp3)	0.999320 2 (sp1sp2, sp3) 0.000680 3 (sp1, sp2, sp3)	0.797170 2 (sp1sp2, sp3) 0.202830 3 (sp1, sp2, sp3)	1.000000 1 (sp1sp2sp3)
3	0.844810 3 (sp1, sp2, sp3) 0.155190 2 (sp1sp2, sp3)	0.999780 2 (sp1sp2, sp3) 0.000220 3 (sp1, sp2, sp3)	0.793040 2 (sp1sp2, sp3) 0.206960 3 (sp1, sp2, sp3)	1.000000 1 (sp1sp2sp3)

To compare the best fit of the two possible hypotheses for *T. putrescentiae* (one or two species) a Likelihood radio-test (LRT) was also evaluated in BPP. According to the LRT, it is more likely that one species of *T. putrescentiae* (lnL= -65298.413) is the correct hypothesis for this lineage instead of the two separate species hypothesis (lnL= -65323.6567).

Lastly, results from STACEY identified 23 different species (Fig. 3.1, Fig. 3.3) and showed some probability that AD2008_*Tyrophagus*_Jacaranda_CR belongs to the same species as AD1973_*Tyrophagus*_castanhola_Brazil, however still kept them as separate species. Likewise, results showed a small probability that AD1725 belongs to the same species as AD1685, AD1688 and AD701_*Tyrophagus*_nr_*longior,* but kept them separate. On the other hand, (consistent with BPP conservative, small, and large hypotheses), results from STACEY placed all populations of *T. putrescentiae* as a single species but recognized some differences between the populations (Fig. 3.3).
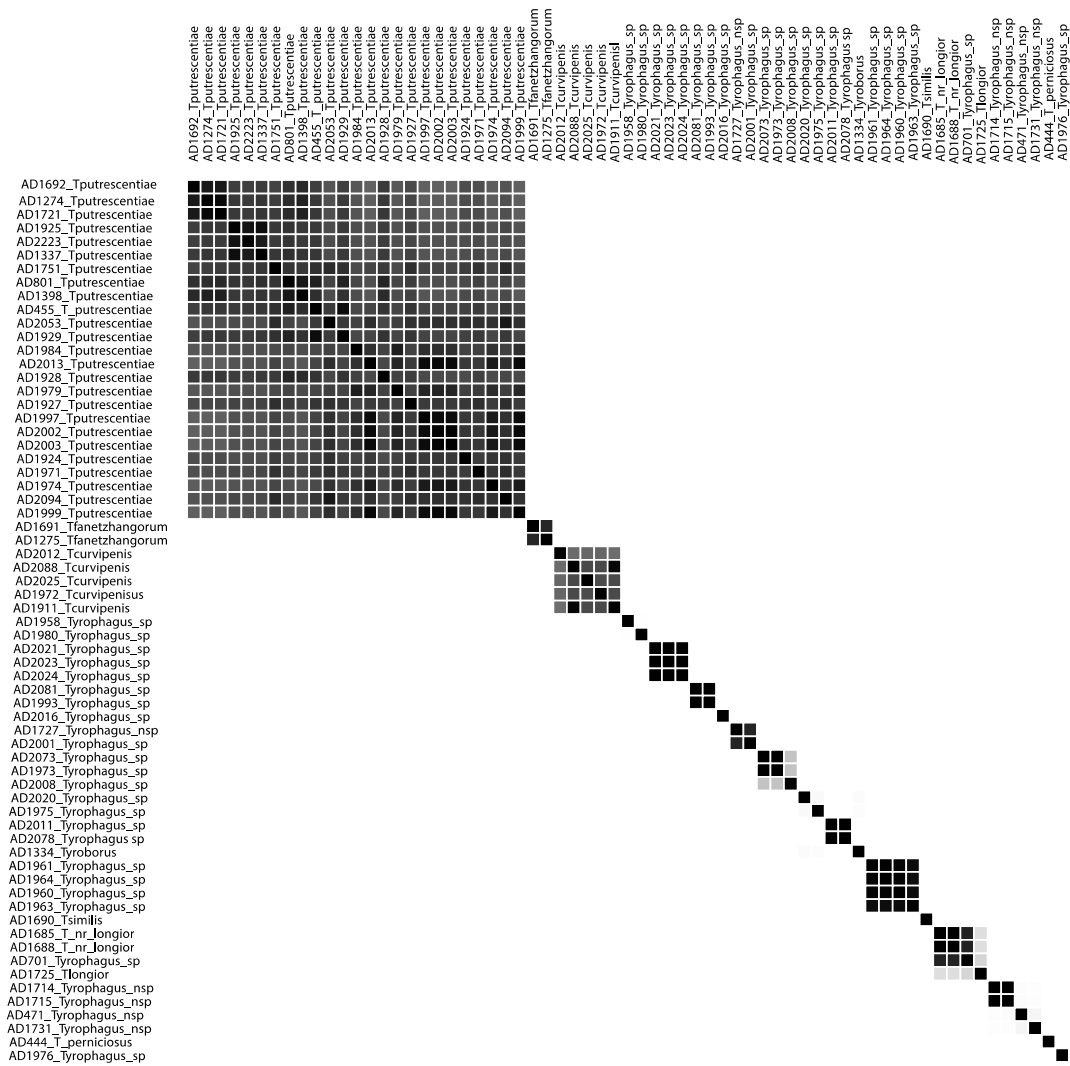
Figure 3.3 Species delimitation matrix for *Tyrophagus* assembled through the program STACEY (BEAST 2). Cells are colored code according with their posterior probabilities to belong to the same species (darker-high, lighter-low).

Discussion

Status of Tyrophagus species and performance of the species delimitation methods

Molecular species delimitation serves as a useful tool in modern taxonomy to distinguish species in complex groups like *Tyrophagus*, where it is difficult to recognize and separate closely related species (Pante et al., 2015). Our analyses identified between 23 and 27 putative species of *Tyrophagus*, depending on the type of analysis used. From these, 17 morphospecies were exposed (Fig. 3.1 with asterisk mark) which were in the majority of the cases, genetically and morphologically similar to other well-known and closely related species. However some inconspicuous differences were found in morphology that appear to be diagnostic. Because of this, a comprehensive morphological study should be performed to determine if these lineages are in fact new species or if they belong to already described species.

Results from single locus methods provide taxonomic interpretations that are similar to traditional views in most cases; however, these approaches still tend to further subdivide species with high levels of intraspecific genetic variation into more than one species. For example, analysis with PTP gave the highest number of proposed species within *Tyrophagus* (27 species) and split species that are traditionally considered to represent "good species" such as *T. fanetzhangorum* (Fig. 3.1). This particular program tends to find different species delimitation results that depend on the dataset that is used and overestimates the intraspecific variations. This has a direct impact on our interpretations as it has in other studies (Kapli et al., 2017; Vitecek et al., 2017; Luo et al., 2018), and reflect the reduced applicability of this approach for groups such as *Tyrophagus* that have high levels of genetic variation and evolved very recently.

Considerable similarities were found in the performances of the methods GMYC and bGMYC (Fig. 3.1, Fig. 3.2). Both programs separated the individual *T. curvipenis* AD 2012 from the rest of the clade, although the support for the separation of these lineages was not too high (0.54). Reid & Carstens (2012) mention that when using GMYC it is necessary to be aware of clades that diverged recently and experience rapid radiation, as is the case for *Tyrophagus*, because this approach might unreliably separate species with these characteristics. To avoid this, it is necessary to include other kinds of data or compare results of this method with other approaches and consider an integrative taxonomy point of view. In this case we do not consider this population to be a separate species because we have not noticed any morphological distinctiveness, and the genetic distances for this clade were very low.

In general, GMYC (Fujisawa & Barraclough, 2013) and PTP (Zhang et al., 2013) are similar approaches because they utilize distance-based methods and consider the evolutionary relationships inferred from single loci (Kapli et al., 2017). However, their outcomes may not be optimal as they tend to postulate too many species (Pentinsaari et al., 2016; Kapli et al., 2017). Our findings for single locus methods (PTP and GMYC) showed similar results as Vitecek et al. (2017), who did get a higher number of species for Trichoptera when using these methods in comparison with proposed number of species by morphological data, as these methods tend to overestimate the total number of species. Similarly, Luo et al. (2018) obtained distinct species delimitation results across a range of PTP strategies. One of the biggest shortcomings for the use of these approaches is that they assume gene trees are good estimate of species trees (true phylogeny), bringing the problems of the phylogeny to the delimitation of the species (Yang & Rannala, 2017). Because these methods depend on the input tree, it is necessary to compare and corroborate results with other analyses that employ multilocus species delimitations and do not rely on the accuracy of the tree, especially if using a gene tree.

The multiple locus method STACEY has the advantage of not requiring an input tree and can condense and rewrite subtrees while modeling different population parameters (Vitecek et al., 2017). This approach tends to be more conservative and splits the taxa to fewer lineages while showing the intraspecific variation within the individuals. In our results, STACEY tended to be a more conservative method than single locus analyses and predicts species numbers closer to what is inferred from morphological taxonomy. STACEY distinguished 23 species with high posterior probabilities (Fig. 3.3), recovering the same clades than the phylogenetic tree (Fig. 3.1). This approach was in general more robust to the influence of intraspecific variation and mutation rate heterogeneity, and outperforms PTP, GMYC and bGMYC analyses in distinguishing species boundaries.

Additional gene sequences, when they are available, should be used to validate interpretations from single gene approaches (Fujisawa & Barraclough, 2013). Our results strongly support the implementation of a multi-locus algorithm, such as the one used by STACEY, for cryptic and highly diverse lineages such as *Tyrophagus*. Furthermore it is important to be cautious regarding results when a single locus analysis is used to delimit species given the dissimilar results obtained here.

The Tyrophagus putrescentiae species complex

Large genetic distances have been found in several populations of *T. putrescentiae* using single gene analyses (Murillo et al., 2018); however, when applying single and multiple gene programs to delimit species the models showed different outcomes.

Contrary to the section above for the other clades of *Tyrophagus*, the three single locus analyses (PTP, GMYC and bGMYC) showed comparable performances and clustered all individuals of *T. putrescentiae* as a single species (Fig. 3.1, Fig. 3.2), even though some populations within this species have smaller branches and larger divergence times on the tree (Fig. 3.1). Similarly, the multilocus approach utilized by STACEY was robust to the influence of intraspecific differences and kept all populations as one, confirming the morphological expectations that all *T. putrescentiae* belong to the same species. STACEY was able to show some level of intraspecific variation within the populations in the similarity matrix (Fig. 3.3). These dissimilarities within populations were more evident for "group 2" when compared with "group 1" as it was observed in a previous work (Murillo et al., 2018) where large genetic distances between these two groups were found.

In contrast, results from the multi-locus Bayesian method BPP differed depending on the population genetic priors applied during the analyses (Table 3.1, Fig. 3.1). BPP supported the existence of only one species, including the distinct and previously recognized species, *T. fanetzhangorum*, when large population size (θ) and long divergence time (τ) were applied as priors. In this scenario, the probability of speciation is smaller, which is an unrealistic scenario given the small divergence time that is expected for *Tyrophagus*, yielding the potentially misleading results obtained here.

On the other hand, the small (small θ and small τ) and conservative (large θ and small τ) scenarios support the two species hypothesis; for these, BPP split *T. fanetzhangorum* from *T. putrescentiae* and kept the former as a single lineage. Here the chances for speciation are higher given the small divergence time of the analyses, which is more realistic for *Tyrophagus*; nevertheless, it seems that population size (large or small) did not play an important role as different values did not impact the outcomes between the two analyses. Similarly to what was found by Leaché & Fujita (2010), our results tend to vary with the set of priors used during the analyses; however, in the case of the former authors their results were affected by the size of the population and not by the divergence time. Equally, Luo et al. (2018) found that the main factor

influencing the performance of these methods was the ratio of population size and divergence time, with limited impacts concerning the number of loci and sample size per species.

Lastly, the default condition of the program split the three groups (TP_group1, TP_group2 and TF) into three different species (Table 3.1). However, we consider that this scenario is even less probable given the results obtained in the other BPP scenarios tested here, as well as the results recovered in STACEY and the single locus analyses where all consider *T. putrescentiae* as a single species. According to Luo et al. (2018), using proper priors in BPP is necessary to avoid overestimation (our case using BPP default priors) or underestimation (our case using BPP large priors) of species, which seems to be the case for some of our analyses. In general, it is important to interpret these analyses while considering previous information and knowledge of the group under study, such as generation time, habitat or host, geographical distribution, morphology, etc., because the results in the delimitation of the species are subject to change according to the applied priors, which may lead to erroneous interpretations of the results and false conclusions for the designated species (Leaché & Fujita, 2010; Olave et al., 2014). The geographically distinct populations of *T. putrescentiae* collapsed as a single species by the different approaches applied here. This could be an indication that these populations are still positioned within the speciation continuum (Leaché & Fujita, 2010). Alternatively genetic differences detected in the two groups of *T. putrescentiae* (Fig. 3.1) may not be due to reproductive isolation, but reflect population structure (Sukumaran & Knowles, 2017).

To test for the reproductive isolation of the two groups we arranged some controlled crosses (data not shown here) between geographically distant populations of *T. putrescentiae* from "group 1" and "group 2". From this, no indication of reproductive incompatibility between the two groups was found as the crosses produced fertile offspring (crosses made until F2). We also did not observe any particular morphological or ecological differences between these two lineages.

It is recognized that recently isolated populations, as the case for *T. putrescentiae* (Fig.3.1) are not going to instantaneously give rise new species. According to Huang and Knowles (2016), the perception associated with the biological species concept is that allopatric populations are expected to be reproductively isolated, however, if these species prosper in similar conditions it is very likely that they show genetic differences, but not many morphological dissimilarities.

Nevertheless, this isolation may begin to surge genetic differences between populations, where other characters (like morphology) have not had enough time to accumulate differences (De

Queiroz, 2007; Sukumaran & Knowles, 2017). It might also be possible that if these populations are separated from each other for enough time (i.e. no gene flow), a speciation event will eventually occur (Heled et al., 2013; Sukumaran & Knowles, 2017).

Given the similar results obtained in the majority of the programs here (GMYC, bGMYC, PTP, STACEY, two of the BPP analyses), and the lack of other evidence to separate those two groups, we follow a conservative approach and maintain the populations of *T. putrescentiae* from this study as a single species.

Conclusions

Our genetic data highlight the variation that is within *Tyrophagus* and suggest that the genus needs an integrative taxonomic review including further morphological descriptions of the new independent lineages revealed here. The different molecular species delimitation methods used in this study are of taxonomic significance in the recognition of species boundaries within *Tyrophagus* and can accelerate the identification of new species. However, multilocus analyses gave more consistent results than single locus methods. STACEY (multilocus analysis) was the method that most reliably delineated the molecular species status for *Tyrophagus* corresponding with the prior morphological expectations; on the other hand, our results suggest being cautious when implementing a single locus model to delimit species given the dissimilar results obtained here. In general, all single and multilocus approaches agreed with the hypothesis of a single lineage for the cosmopolitan species *T. putrescentiae*. The importance of applying different strategies for the delimitation of species is highlighted by our findings that the approaches can yield false positives in complex species such as *T. putrescentiae*.

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Appendix B

Supplementary material from Chapter III

Table A-S1. Countries, hosts, localities and DNA codes used for the different species of *Tyrophagus.*

Mite species	Country	Host/Locality	Code	Museum (BMOC) accession number	Non amplified regions
Tyrophagus putrescentiae	USA: NC	Lab culture from Greer Laboratories	AD1401	BMOC 08-0801-006	
	Singapore	National University of Singapore,Allergy and Molecular Immunology Lab.	AD1396	BMOC 08-0912-060	
	USA: OH	Larry Arlian lab culture, Montgomery Co., Dayton. Wright State University	AD1402	BMOC 08-0801-007	
	USA:MI	Tulip bulbs	AD455	BMOC 02-1113-001	HSP70, EF1α
	England	Lab colony Biological Crop Protection, Ltd.	AD801	BMOC 07-0223-006	HSP70
	Czechia	Lab culture from grain store maintained by the Crop Research Institute, Prague	AD1398	BMOC 08-1010-002	
	USA: MI	Cricket rearing, Univ. of Michigan	AD1695	BMOC 14-0318-002	
	Brazil	On a dead scorpion (*Tityus serrulatus*)	AD1693	BMOC 13-1115-056	
	Germany	Lab sample, very likely a subsample of RA Norton collection Syracuse/NY. Received via Ina Schäfer as presumed vouchers for study of Domes et al. 2007	AD1400	BMOC 08-0801-004	
	Czechia	Lab cultures started from a CSL culture (England) and maintained by the Crop Research Institute, Prague	AD1337	BMOC 08-1010-005	
	Brazil	Belo Horizonte, Univ. Federal de Minas Gerais, Departamento de Zoologia, Laboratório de Sistemática e Evolução de Ácaros Acariformes	AD1692, AD1721,	BMOC 13-1115-053	
	Japan	Lab culture, Tokyo Women's University of Medicine	AD1274	BMOC 08-0801-001	EF1α
	USA: OH	Dog food, Franklin Co., Columbus, OSU campus	AD1751	BMOC 14-0614-020	
	Brazil	Minas Gerais, Dry fruits (with some fungus) of *Terminalia catappa* on ground	AD1974	BMOC 15-0104-002	
	Brazil	Minas Gerais, Sabará *Melipona quadrifasciata* nest	AD1979	BMOC 15-0104-035	
	Brazil	*Melipona quadrifasciata* Escola Superior de Agricultura Luiz de Queiroz, Piracicaba, São Paulo	AD1984	BMOC 15-0104-062	EF1α
	Costa Rica	*Sechium edule.* Aged flowers attached to ripened fruit Ujarrás, Cartago.	AD1999	BMOC 15-0601-164	
	Costa Rica	Palm seeds (Arecaceae) on the floor E.E.F.B.M. Universidad de Costa Rica	AD2002	BMOC 15-0601-181	
	Costa Rica	Polypore fungus. Parque Nacional Braulio Carrillo, Los Palmas trail	AD2003	BMOC 15-0601-208	
	Costa Rica	*Partamona orizabaensis* nest, Universidad Nacional	AD2013	BMOC 15-0601-107	
	Costa Rica	*Zophobas morio*, lab rearing, Museo de Insectos, Universidad de Costa Rica	AD2015	BMOC 15-0601-120	
	Costa Rica	Inflorescence of *Monstera deliciosa* Central campus, Universidad de Costa Rica	AD1997	BMOC 15-0601-122	
	Russia	*Allium sativum* stored in a fridge, Tyumen.	AD1971	BMOC 14-0730-041	
	The Netherlands	Lab culture Koppert maintained by the Crop Research Institute, Prague	AD1924	BMOC 15-0717-012	
	Czechia	Lab culture maintained by the Crop Research Institute, Prague	AD1925	BMOC 15-0717-013	28S, EF1α, HSP70
	Czechia	Lab culture Phillips maintained by the Crop Research Institute, Prague	AD1926	BMOC 15-0717-014	
	Italy	Lab culture Ham maintained by the Crop Research Institute, Prague	AD1927	BMOC 15-0717-015	EF1α
	Czechia	Lab culture Nestlé maintained by the Crop Research Institute, Prague	AD1928	BMOC 15-0717-016	EF1α
	Czechia	Lab culture, Zvoleněves	AD1929	BMOC 15-0717-017	EF1α
	Mexico	*Typha* sp.	AD2094	BMOC 17-0108-02	

	Mexico		AD2053	BMOC 17-0108-02	
	France		AD2223		18S
Tyrophagus fanetzhangorum	Belgium	Rotting grass	AD1691,	BMOC 06-0910-062	
	Spain	Lab culture ALK-ABELLÓ, Madrid	AD1275	BMOC 08-0801-002	
Tyrophagus curvipenis	Costa Rica	*Sechium edule,* subcortical part of aged (discolored) stem	AD1911, AD2088	BMOC 15-0601-167	HSP70
	Costa Rica	*Turdus grayi* nest	AD2012	BMOC 15-0601-106	HSP70, EF1α
	Costa Rica	Nest of a small bird in a cow shed	AD2025	BMOC 15-0601-204	
	Russia	inside *Prunus persica* fruits (imported from Spain), Tyumenskaya Oblast'	AD1972	BMOC 14-0730-046	
Tyrophagus sp. (close to *T. curvipenis*)	Japan	Fruits of *Garcinia subelliptica*	AD1958	BMOC 14-0730-006	
Tyrophagus sp. (close to *T. curvipenis*)	Brazil	*Coffea arabica*, drupes of ornamental plant on ground, Bello Horizonte, Minas Gerais	AD1980	BMOC 15-0104-037	
Tyrophagus robertsonae	Costa Rica	Palm seeds (Arecaceae) on the floor E.E.F.B.M. Universidad de Costa Rica	AD2001	BMOC 15-0601-181	COI
	Puerto Rico	Bracket fungi, San Juan Botanical Garden, Monnet Garden	AD1727	BMOC 08-1227-002	
Tyrophagus sp.	Costa Rica	Inflorescence of *Monstera deliciosa* Central campus, Universidad de Costa Rica	AD2016	BMOC 15-0601-122	
Tyrophagus sp.	Costa Rica	Small bird nest build in cow shed, Tapanti, Cartago	AD2021	BMOC 15-0601-201	
	Costa Rica	Small bird nest build in cow shed, Tapanti, Cartago	AD2023	BMOC 15-0601-203	
	Costa Rica	Small bird nest build in cow shed, Tapanti, Cartago	AD2024	BMOC 15-0601-204	
Tyrophagus sp. (close to *T. savasi*)	Costa Rica	Heliconia, Central campus, Universidad de Costa Rica	AD1993	BMOC 15-0601-112	
	Costa Rica	Heliconia, Central campus, Universidad de Costa Rica	AD2081	BMOC 15-0601-113	
Tyrophagus sp.	Costa Rica	*Crescentia alata* fruit, Santa Rosa National Park, Guanacaste	AD2008	BMOC 15-0601-82	HSP70
Tyrophagus sp.	Costa Rica	dust under floor outside Museo de Insectos, Universidad de Costa Rica	AD2020	BMOC 15-0601-193	
Tyrophagus sp.	Brazil	*Terminalia Catappa* with some fungus, Arraial do Cabo, Rio de Janeiro	AD1973	BMOC 15-0104-001	
Tyrophagus sp.	Brazil	*Terminalia Catappa* with some fungus, Arraial do Cabo, Rio de Janeiro	AD1975	BMOC 15-0104-003	
Tyrophagus sp.	Costa Rica	Dry litter, Santa Rosa National Park, Guanacaste	AD2011 AD2078	BMOC 15-0601-105	
Tyrophagus perniciosus	USA: MI	Grain spill	AD444	BMOC 00-1103-013	
Tyrophagus sp. (*T.* cf *similis*)	Belgium	Rotting grass	AD1690	BMOC 06-0910-062	28S
Tyrophagus sp. (close to *T.* cf *silvester*)	Russia	Stick in ground with some mold, Bratsk	AD1960	BMOC 14-0730-008	
	Russia	Rotten cabbage leaves on ground, Bratsk	AD1961	BMOC 14-0730-009	
	Russia	Soil under pile of rotten plants, Bratsk	AD1963	BMOC 14-0730-012	
	Russia	*Formica rufa* anthill, Bratsk	AD1964	BMOC 14-0730-018	
Tyrophagus longior	USA: MI	Cheese	AD1725	BMOC 14-0602-001	
Tyrophagus sp. (close to *T. longior*)	Belgium	Rotting grass	AD1685 AD1688 AD701	BMOC 06-0910-062	28S COI
Tyrophagus nsp.	USA:MI	Formica obscuripes	AD471	BMOC 04-1209-001	

Tyrophagus nsp.	Cuba	spilled of bird seeds, La Habana	AD1714	BMOC 14-0515-006	28S
	Cuba	*Ficus* sp., fruit on ground, La Habana	AD1715	BMOC 14-0515-003	
Tyrophagus nsp.	USA:IN	Sand dunes, Lake Co., Gary, Marquette Park	AD1731	BMOC 11-0524-002	HSP70, EF1α
	Brazil	Dry shrub, Arraial do Cabo, Rio de Janeiro	AD1976	BMOC 15-0104-006	
Aleuroglyphus sp.			AD613		
Sancassania sp.			AD513		

Table A-S2. Species-specific oligonucleotide primers used in nested amplification and thermocycler reaction protocols. *For uniform sequencing, M13FORW/REV tails (underlined in the sequences below) were added to the primers for the second PCR.

Primer name	Primer sequence (5' to 3')	Thermocycler protocol:
COI:		
COX1_16F	TGANTWTTTTCHACWAAYCAYAA	94 °C: 2 min, 10 cycles of 94 °C: 30 s, 40 °C: 1 min, 72 °C: 2 min, and 25 cycles of 94 ° C: 30 s, 48 °C: 35 s and 72 °C:2 min, with a final extension step of 72 °C:7 min.
COX1_1324R	CDGWRTAHCGDCGDGGTAT	
COX1_25Fshort_T*	<u>TGTAAAACGACGGCCAGT</u>TCHACWAAYCAYAARRAYA	94 °C: 2 min, 20 cycles of 94 °C: 30 s, 49 °C: 30 s, 72 °C:2 min, and 18 cycles of 94 °C: 30 s, 52 °C: 35 s and 72 °C: 2 min, with a final extension step of 72 °C: 7 min.
COX1_1282R_T*	<u>CAGGAAACAGCTATGACC</u>CCWVYTARDCCTARRAARTGTTG	
28S:		
28S_f1_4F	GACCTCAGATCARGCgAGAH	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 2:15 min, with a final extension step of 72 °C:7 min.
28S_P1_3R	GCTGTTCACATGRAACCCTTC	
28Sa_DD_T*	<u>TGTAAAACGACGGCCAGT</u>GACCCGTCTTGAAACACGGA	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 53 °C: 25s, 72 °C: 1:20 min, with a final extension step of 72 °C:7 min.
28SIR_acar2_T*	<u>CAGGAAACAGCTATGACC</u>TGCTACTACCACCAAGATCTGC	
28S_1F_Arach3_T*	<u>TGTAAAACGACGGCCAGT</u>ACCCGCTGAATTTAAGCAT	94 °C: 2 min, 20 cycles of 94 °C: 30 s, 51 °C: 20 s, 72 °C: 1:40 min, and 15 cycles of 94 °C: 30 s, 57 °C: 20 s and 72 °C: 1:40 min, with a final extension step of 72 °C: 7 min
28S_1661R_T*	<u>CAGGAAACAGCTATGACC</u>AGTTCACCATCTTTCGGGTA	
18S:		
18S_1F_G	TACCTGGTTGATCCTGCCAGTAG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 2 min, with a final extension step of 72 °C:7 min.
18S_8r_MH	TAATGATCCTTCCGCAGGTTCACCT	
18S_1F_sh_T*	<u>TGTAAAACGACGGCCAGT</u>CCTGCCAGTAGTCATATGCTTG	94 °C: 2 min, 15 cycles of 94 °C: 30 s, 51 °C: 23s, 72 °C: 1:10 min, and 15 cycles of 94 °C: 30 s, 57 °C: 25 s and 72 °C: 1:10 min, with a final extension step of 72 °C: 7 min
18SR_719R_T*	<u>CAGGAAACAGCTATGACC</u>AATATACGCTATTGGAGCTGGA	
18S_521F_T*	<u>TGTAAAACGACGGCCAGT</u>ACATCCAAGGAAGGCAGCAG	94 °C: 2 min, 15 cycles of 94 °C: 30 s, 51 °C: 23s, 72 °C: 1:10 min, and 15 cycles of 94 °C: 30 s, 57 °C: 25 s and 72 °C: 1:10 min, with a final extension step of 72 °C: 7 min
18S_1587R_T*	<u>CAGGAAACAGCTATGACC</u>AACTAAGAACGGCCATGCAC	
EF1A:		
EF1A_1F	ATGGGMAARGARAAGRYBCA	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min.
EF1A_1638R	TTRATVACDCCVACRGCVAC	
EF1a_19Fm	CAYATYAAYATyGTbGTIATHGG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 1:30 min, with a final extension step of 72 °C:7 min.
EF1a_52R	CCDATYTTRTANACRTCYTG	
EF1A_45.71F	GTNGSNGTIAAYAARATGGA	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 52.5 °C: 20s, 72 °C: 1:40 min, with a final extension step of 72 °C:7 min
EF1a_41.21R	TGYCTCATRTCDCGVACRGCRAA	
EF1A_102F_Tyr	CAAGCGAACCATCGAGAAGT	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 58 °C: 30s, 72 °C: 1:45 min, with a final extension step of 72 °C:7 min
EF1A_1244R_Tyr	AGGGGWGGGAAGTCVGTG	
EF1a115F_115Tyr_T	<u>TGTAAAACGACGGCCAGT</u>CGAGAAGTTCGARAAGGARGC	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 62 °C: 30s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min
EF1a1232R_Tyr_T	<u>CAGGAAACAGCTATGACC</u>GGAAGTCVGTGAAGGACTCGAC	
HSP70:		
HSP70_9B_129F	TGYGTDGCHRTNATGGARG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 35s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min
HSP70_9B_1896R	GCCATYTTYTTRTANGCCATYTC	
HSP70_162F_Tyr	GTRATYGARAAYGCNGARGG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 54 °C: 35s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min
HSP70_1636R_Tyr	GHARRTCRTTGCAVACYTCCTT	
HSP70_251F_Tyr_T	<u>TGTAAAACGACGGCCAGT</u>GCNGCMAACACYTTYTAYGC	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 56 °C: 30s, 72 °C: 1:40 min, with a final extension step of 72 °C:7 min
HSP70_1609R_Tyr_T	<u>CAGGAAACAGCTATGACC</u>TCVGGHGTYTCRTTDTCYTT	

In primer names: F – forward; R – reverse; T– hasT tail (underlined in primer sequence)

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Chapter 4 Investigating evolutionary patterns of habitat specialization in the mite family Acaridae. Can generalist species evolve from specialists?

Abstract

Testing patterns of evolution of habitat specialization in understudied groups such as mites contributes to the understanding of fundamental questions in evolutionary biology. According to hypotheses on the evolution of specialization and generalization, organisms with morphological and physiological adaptations to specialized habitats and resources are thought to have evolved from more generalized ancestors. However, indications that ancestors of the mite family Acaridae were habitat specialists and from which a number of generalist lineages evolved, suggests different evolutionary dynamics from expectations. We defined specialist taxa as those restricted to a particular habitat and generalist as those taxa that thrive in more than one type of different habitat. To evaluate the hypothesis that generalist species evolved from specialist ancestors, we estimated a molecular phylogeny for Acaridae and reconstructed ancestral character states and estimated transition rates across the tree through different methodologies. We found that generalists also evolved from more specialized lineages. Phylogenetic analysis, ancestral character states and transition rates provide evidence of an ancestrally specialist lifestyle for acarid mites and confirmed that reversals have occurred. The ancestral character state reconstructions exposed ancestral specialist nodes that gave rise to other specialist as well as generalist lineages. The specialist and generalist species also showed a structured habitat preference across the phylogeny. According to our results and previous hypothesized ecological data for Acaridae, inherited ancestral features like tolerance to humidity and host access to newer habitats played an important role in the diversification of this family.

Introduction

The evolution of specialization in habitat or resource use occurs in practically every taxon where a number of ecological, morphological and physiological characteristics differ between specialists and generalists species (Börschig et al., 2013; Kamath & Losos, 2017). Specialization can be described from an ecological perspective as limitations in the types of resources used by an organism (Futuyma & Moreno, 1988; Devictor et al., 2010; Poisot et al., 2011; Pandit et al., 2016). Specialist taxa persistently use a narrow range of resources, are more constrained to a particular habitat, and normally take advantage of stable environments, and hence are less likely to colonize new locations (Devictor et al., 2008; Devictor et al., 2010, Li et al., 2014). The tradeoff here is that specialize species in habitat or host, increase their efficiency to use one resource, and lower their ability to use multiple resources (Levins, 1968; Devictor et al., 2010; Colles et al., 2009). On the other hand, if specialists accumulates mutations in their current stable environment, it is likely that these taxa are not going to be affected by these mutations here; however, if the environmental conditions change these might bring a selective disadvantage in the new habitat (Colles et al., 2009). Consequently, the potential for specialists to give rise to new lineages should be limited (Börschig et al., 2013). Generalists, on the other hand, are less sensitive to stochastic fluctuations in the environment and subsist on a larger variety of resources, which enables them to persist in many habitats. They are also able to replace one scarce resource for another which may increase opportunities for survival and consequently increase their chances of speciation (Devictor et al., 2008, 2010; Li et al., 2014).

Species with adaptations to specialized habitats are generally thought to have evolved from ancestors with generalized habitat use (Schluter et al. 1997). Consequently, more specialized species tend to be derived. Taxa then would exhibit higher transition rates from generalists to specialists than the reverse, or reversals should be rare (Mayr, 1963; Futuyma & Moreno, 1988). Specialization is normally presumed to lead to an "evolutionary dead end", where highly specialized organisms do not give rise to new lineages (Mayr, 1963; Futuyma & Moreno, 1988). In other words, specialists should evolve from generalists unidirectionally, and once a feature is lost in the course of evolution (i.e. the capacity for generalized resource use), the probability of it re-evolving is low (Golberg & Ignic 2008).

A classic example of this concept is the overwhelming abundance of specialist herbivore insects compared to generalists where host plant specialization is the derived evolutionary form of generalism (Schoonhoven, 2005; Groot et al., 2011). Similarly, in bark beetles of the genus *Dendroctonus*, specialists arose from generalists in at least six separate lineages (Kelley & Farrell, 1998). However, the evolution for specialist-generalist strategies can fluctuate and this scenario may change for other taxa. One important aspect for the evolution of specialization is the question of whether or not a generalist can evolve from a specialist. In fact, previous studies of different animal groups challenge the paradigm that the evolution of specialization is both unidirectional and irreversible. For instance, Desdevises et al. (2002) constructed a molecular phylogeny for ectoparasites of fishes to test the host specificity of these parasites and found that the most highly host-specific species were not necessarily the most derived in the group. Similarly, Nosil (2002) as well as Morse & Farrell (2005) found the same pattern among groups of butterflies, bark and seed beetles where transitions between specialists to generalists were frequent. In addition, work on house dust mites by Klimov & OConnor, (2013) presented evidence that these mites evolved from permanent parasites of vertebrates, and then diversified into a number of lineages that occupy diverse habitats. This evidence suggests that something similar is happening in other groups of mites even though they show very different traits than those present in house dust mites.

The family Acaridae represents a good system to determine patterns between generalist and specialist resource use modes because the diversity of habitats; degree of association with one, various or no host species (ranging from highly host-specific to host-opportunistic); the presence or absence of a specialized, non-feeding, dispersing stage (deutonymph) in many species; and globally distribution. This model system is also interesting because the majority of acarids are specialists (although collectively occupying a wide range of habitats), but only relatively few taxa are generalists. The evolution of traits associated with resource use may form the basis for the radiation of Acaridae into many habitats during their evolutionary history, including, colonization of anthropogenic habitats.

As mentioned above, the diverse modes of specialization in Acaridae can take many forms. For the propose of this research, however, we focused on the specialization of these mites with respect to habitat. We emphasize this question of habitat specialization, as previous studies (OConnor 2001) have hypothesized that Acaridae evolved from ancestors living in vertebrate nests (a specialist habitat) and then evolved to occupy other habitats (more generalist habitats). The aim

of this research was to determine the direction and frequency of transitions between generalist and specialist habitat use. Despite the fact that species of Acaridae (especially generalist species) are of considerable importance in areas such as human health, veterinary medicine, and agriculture (Zhang, 2003; Kasuga & Amano, 2005; Krzysztof et al., 2011), our knowledge about the evolutionary history of this group is scant and poorly understood. OConnor, (2001) presented a tree of some acarid taxa using traditional "Hennigian argumentation" with morphological characters, but due to space constraints, the data upon which the phylogeny was developed were not presented. Prior to this, no phylogenetic studies of the Acaridae family were attempted, and to date, there has been no broadly inclusive phylogeny developed for it. The known taxonomy of the family has been developed piecemeal using traditional methods and a number of suprageneric names have been proposed (see supplementary text in appendix C for details about suprageneric names and taxonomy) which we will be using in the tree to name clades.

Determining patterns of evolution of certain characters on the phylogenies provide information about the diversification and the evolutionary changes that give rise to existing species (Bollback, 2006; Schluter, 2016; Li et al. 2018; Title & Rabosky, 2019). Understanding the relationships within acarid mites will clarify trends on the evolution of resource use and may provide for information on how they have been so successful in colonizing new habitats. In addition to establishing a phylogeny for the family and clarifying the status of various taxa, the main goal here is to determine the frequency of transitions between generalist and specialist resource use. For this, the phylogenetic relationships among Acaridae must first be inferred and then habitat use analyzed based on how character states are distributed in the tree. The prevailing hypothesis posits that transition rates from generalists to specialists should be higher than vice versa. The approach here tests this potential scenario for the family Acaridae through phylogenetically explicit statistical tests. This research takes advantage of the ecological diversity of this family and provides a rigorous inference of evolution of generalized and specialized to habitat use.

Material and Methods

Mite samples

Members of the family Acaridae were collected from a number of countries, habitats and hosts (Supplementary Table B-S1) including samples from temperate and tropical climates and various groups of plant and animal hosts. Mites were manually collected from substrates or hosts with a mounting needle under a dissecting microscope and stored in ethanol (99.5%). When needed, additional materials containing mites were sampled by Berlese-funnel extraction or sieved. In cases where mites were collected from unidentified hosts (usually other arthropods), these were preserved separately in a vial with ethanol (99.5%) or in a dry paper towel. Identification of hosts was subsequently carried out by different taxonomic specialists on the groups of interest when possible. Slides of mites were prepared in Hoyer's medium, and their identification was accomplished using available taxonomic keys and original descriptions. Voucher specimens are deposited in the Museum of Zoology, in the Research Museums Center, University of Michigan, Ann Arbor, Michigan (UMMZ).

DNA extraction and amplification

Genomic DNA was extracted from single specimens using a QIAamp DNA Micro kit (Qiagen) following the manufacturer's protocol for tissues, with some modifications (Klimov and OConnor, 2008). A total of 101 specimens of Acaridae were used for this study (Supplementary Table B-S1). Also, three members of the superfamily Acaroidea were used as outgroups (AD983 *Lardoglyphus zacheri (*Lardoglyphidae*)*, AD317 *Suidassia pontifica* and AD560 *Tortonia* sp. (Suidasiidae)). Sequences were obtained using nested PCR which consisted of two consecutive PCR reactions (first PCR product is used as template in the second reaction) with different primers and thermocycler protocols (Supplementary Table B-S2). Amplification reactions were performed in 20µl volumes with Platinum Taq DNA Polymerase (Invitrogen) in a Mastercycler gradient, Eppendorf thermocycler. The master mix of each gene for the first PCR (nested PCR) contained 2.0 µl of PCR buffer (final 1X), 1.4 µl MgSO$_4$ (3.5 mM), 1.4 µL of dNTP (0.7 mM each), 0.8 µl of each oligonucleotide primers (0.4 µM), 0.12 µl of Platinum Taq polymerase (1.5U) and 0.4-1 µl of genomic DNA template. For the second PCR, the master mix was modified with a reduced quantity of Taq Polymerase 0.08 µl (1.0 U) and 0.6 µl of PCR product from the first PCR reaction.

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In total five gene regions were amplified: one mitochondrial protein-coding gene (Cytochrome c Oxidase I (COI) with an amplified length of 1257 bp), two nuclear protein-coding genes (Elongation Factor 1-alpha (EF1α) with an amplified length of 1222 bp, and Heat Shock Proteins (HSP70) with an amplified length of 1704 bp) and two encoding structural ribosomal RNA genes (18S with an amplified length of 1812 bp, and 28S with an amplified length of 1739 bp). PCR products were electrophoresed on a 1.5% agarose gel in 1X TA buffer at 100 V for approximately 35 min. Bands were excised under UV light and purified with QIAquick® gel extraction kit (Qiagen). Sequencing was done in both directions using a 3730XL sequencer (Applied Biosystems) at the University of Michigan DNA Sequencing Core.

Sequence editing

Chromatograms were resolved in Sequencher ver. 5.4.6 (Sequencher 2016); primer and low quality sequences were trimmed. Sequences were imported to Mesquite (Maddison & Maddison, 2011) where they were automatically aligned with the pairwaise aligment tool, and then checked by eye for inconsistencies. In cases where indels were present (unalignable due to the lack of common secondary structure), these were excluded from the analyses.

Phylogenetic tree estimation for the family Acaridae

The best-fitting nucleotide substitution models were selected based on corrected Akaike information criterion (AICc) in the program jModelTest 2.0 (Darriba et al., 2012). The best substitution models for each gene were as follows: GTR +I+G for COI and HSP70 and SYM+I+G for EF1A, 18S and 28S.

Phylogenetic analyses across the family Acaridae were estimated in a Maximum Likelihood framework using the program RaxML (Stamatakis, 2014). Nodal support was calculated for the best scoring ML tree by bootstraping with 1000 replicates. The phylogeny was then used to calculate a time calibrated tree through the penalized likelihood function chronopl (APE package) implemented in R (Paradis & Schliep, 2019).

Ancestral character state reconstruction

Based on the ultrametric phylogenetic tree, an ancestral character state reconstruction was performed. Each terminal node on the tree was categorized as Generalist ("G") or Specialist ("S"), in accordance with available data on its habitat-ecology. Species associated in one particular type

of habitat were treated as specialists while species occurring in more than one habitat as generalists. For example, the genus *Australhypopus* spp. is a specialist mite associated with termites, with most species known only from a single hosts. Other species like *Thyreophagus* spp. are considered generalist as these can occur in many different habitats.

We inferred the phylogenetic history of specialization strategy for these mites using stochastic character mapping (Bollback, 2006; Huelsenbeck et al., 2003) through the package phytools implemented in R (Revell, 2012). This method allows for the inference of discrete character changes while adjusting uncertainty for topology and branch lengths. In this case we obtained a sample of histories for our discrete character evolution (habitat specialization) on the tree. For the first analysis, a sample of 100 stochastic maps were simulated from our dataset. The total changes of each state in every particular node, and their corresponding posterior probabilities were then estimated and summarized in the tree (Revell, 2012). We also performed some simulations of this analysis but changing all character states of outgroups to generalist, to evaluate the effect of the outgroups in the outcome of our hypothesis. For the second analysis, we used the method densityMap in phytools to observe the changes that occurred across all the tree. Here we also obtained the posterior probability of being in each state throughout the tree (Revell, 2012).

Most of the models used for discrete characters assumed that rates of evolution across the phylogeny are homogenous (constant through time and among lineages). However, methods for discrete characters have been developed to accommodate rate heterogeneity among lineages in phylogenies (King & Lee, 2015; Joy et al., 2016; Davis-Rabosky et al., 2016). To assess the robustness of the reconstructions of Acaridae through the methods mentioned above, an ancestral character state reconstruction implementing a parsimony model was used (Davis-Rabosky et al., 2016). This method was implemented through the R package phangorn (Schliep, 2011) to accommodate rate heterogeneity across all clades of the tree.

Transition Rates

In addition to evaluating the distribution of specialists and generalists and frequencies of transitions between these states on the tree, different transition rates were modeled to test the directionality of changes across the whole phylogeny and trace the evolutionary history of these mites. Five scenarios to trace the evolutionary history were evaluated with respect to generalist (G) or specialist (S) designations. For this approach, two character transition rates (G to S, S to

G) and five different scenarios were tested depending on the prevalence of one rate or the other: A) the first corresponded to symmetrical evolution, where rates of transitions are the same (Fig. 4.1A); here each state has the same chance of being the ancestral. B) Unidirectional evolution, where one transition in one direction occur but not in the other (Fig. 4.1B). C) Asymmetrical evolution where transitions in one direction are more frequent than the other (Fig. 4.1C); here the transitions from generalist to specialist are more frequent than the back transitions or vice versa. If the rate of transitions from generalist to specialist is inferred as unidirectional, then this suggests that the classical hypothesis of generalist/specialist evolution holds in this system. On the other hand, a higher transition rate from specialist to generalist will disprove this hypothesis.

The different hypotheses for transition rates were tested using Markov models for discrete character evolution through the software Diversitree (Fitzjohn, 2012). The different transition rates from state generalist (G) to state specialist (S) and vice versa, were simulated on the tree for binary characters. Likelihood scores (lnL) for transition rates were obtained for each scenario (q_{GS}, q_{SG}). Then, this model was compared with a constrained model (Mk1). The purpose was to determine if constrained models provided a better fit than full models (Mk2). AIC scores determine which model provided the best fit to our data.
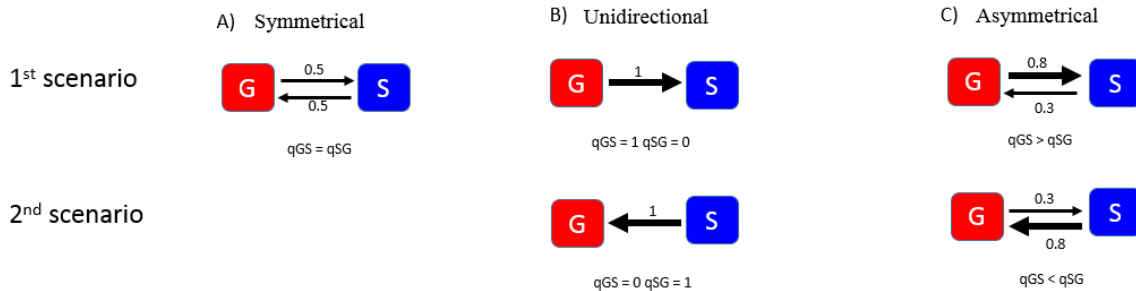
Figure 4.1 Models of transition rates for **A)** symmetrical evolution, **B)** unidirectional evolution, and **C)** asymmetrical evolution (two scenarios). Arrows show the directionality and the numbers on the arrows correspond to the frequency of occurrence for each transitions (0= never occur, 1=always occur). qGS correspond to transition rates from generalist to specialist and qSG from specialist to generalist.

Results

Sequence editing and phylogenetic tree estimation

Sequences of the five loci had an average length of 1227 bp for COI, 1641 for HSP70, 1198 for EF1α, 1667 for 28S and 1794 for 18S. Regions that did not amplified for a specific individual are shown in supplementary material Table B-S1. In total, the average length for all amplified sequences was 7734 aligned nucleotides before translation and character exclusion, and 7527 nucleotides for the final alignment used during the analyses.

Even though the scope of this research does not include a taxonomic revision of the family, because this study included genera that are types for available suprageneric names, we will apply those names to clades on the tree where those genera are included, to facilitate discussion of the clades (see supplementary text in appendix C for more details). The phylogenetic tree separated the subfamilies Acarinae, Pontoppidaniinae, Horstiinae, Thyreophaginae and Rhizoglyphinae (Fig. 4.2). Most of the recognized genera for all the subfamilies identified here were recovered with strong support across the phylogeny.
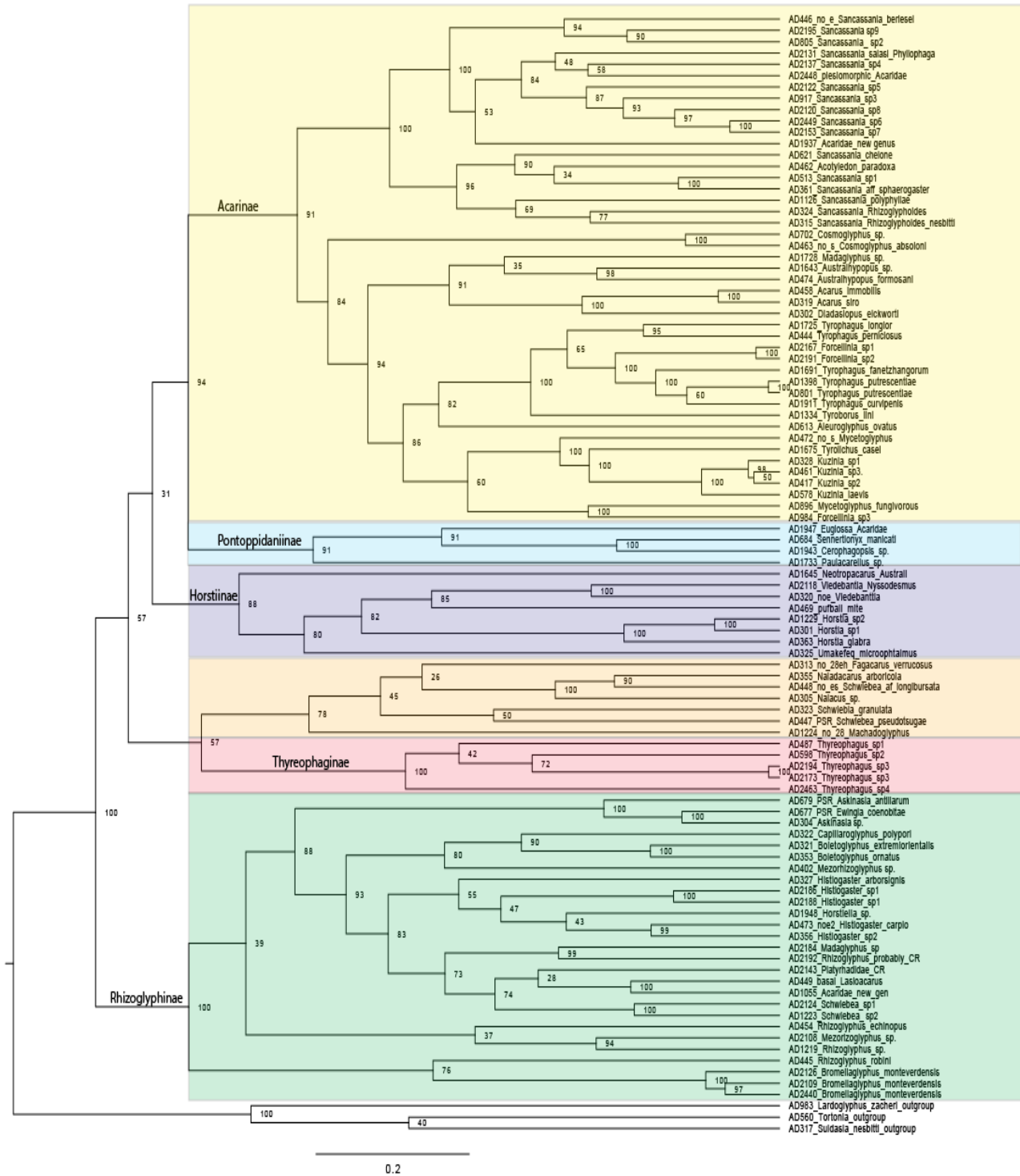
Figure 4.2 Phylogeny of the family Acaridae based on analyses of one mitochondrial and four nuclear and gene regions. Maximum likelihood reconstruction and respective bootstrapped node support are shown for the phylogenetic relationships of 104 OTU's

Ancestral character state reconstruction

Mapping characters states for habitat specialization (specialist or generalist) reveals that most generalist and specialist species are intermixed throughout the phylogeny (Fig. 4.3, Fig. 4.4, Fig. 4.5). For example, the subfamily Acarinae is mostly composed of generalist species while other subfamilies primarily include specialist species. However, specific individual cases of specialists or generalist species were also found within clades dominated by generalists or specialists respectively. For instance, genera *Histiogaster* (AD327, AD356, AD2186, and AD2188) and *Madaglyphus* (AD2184) are generalist that occurs within a clade that contains other specialist species genera that represents the subfamily Rhizoglyphinae. On the other hand, specialist genera *Kuzinia* (AD328, AD417 and AD461), *Forcellinia* (AD2167 and AD2191), *Australhypopus* (AD474 and AD1643), *Madaglyphus* (AD1728), and *Diadiasiopus eickworti* (AD302) occur within a clade that mostly contains by generalist taxa.

These analyses present the history of character evolution and revealed ancestral specialist nodes that have led to the development of generalist lineages (Fig. 4.3, Fig. 4.4, Fig. 4.5). All analyses of ancestral character states reconstruction, including the analysis where all outgroups were set as generalist (Supplementary Fig. S2), show that the specialist condition is ancestral for acarid mites and for deeper nodes in the phylogeny. They also reveal that generalist lineages arose from specialist ancestors as well as showed on the tree in at least three separate places (Fig.3, Fig.4, Fig.5): once in the ancestor for the subfamily Acarinae, and another two times deeply within into clades composed mostly by specialists (Thyreophaginae and a specific branch for Rhizoglyphinae). At the same time some reversals occurred in several lineages of the subfamily Acarinae, where ancestral specialist species nodes gave rise to generalists and then reversing to specialists in subsequently derived lineages. This was observed in the nodes for *Kuzinia* (AD328, AD417 and AD461), *Forcellinia* (AD2167 and AD2191), *Australhypopus* (AD474 and AD1643), *Madaglyphus* (AD1728) and *Sancassania* (AD2137 and AD2448) (Fig.4.3, Fig. 4.4, Fig. 4.5).
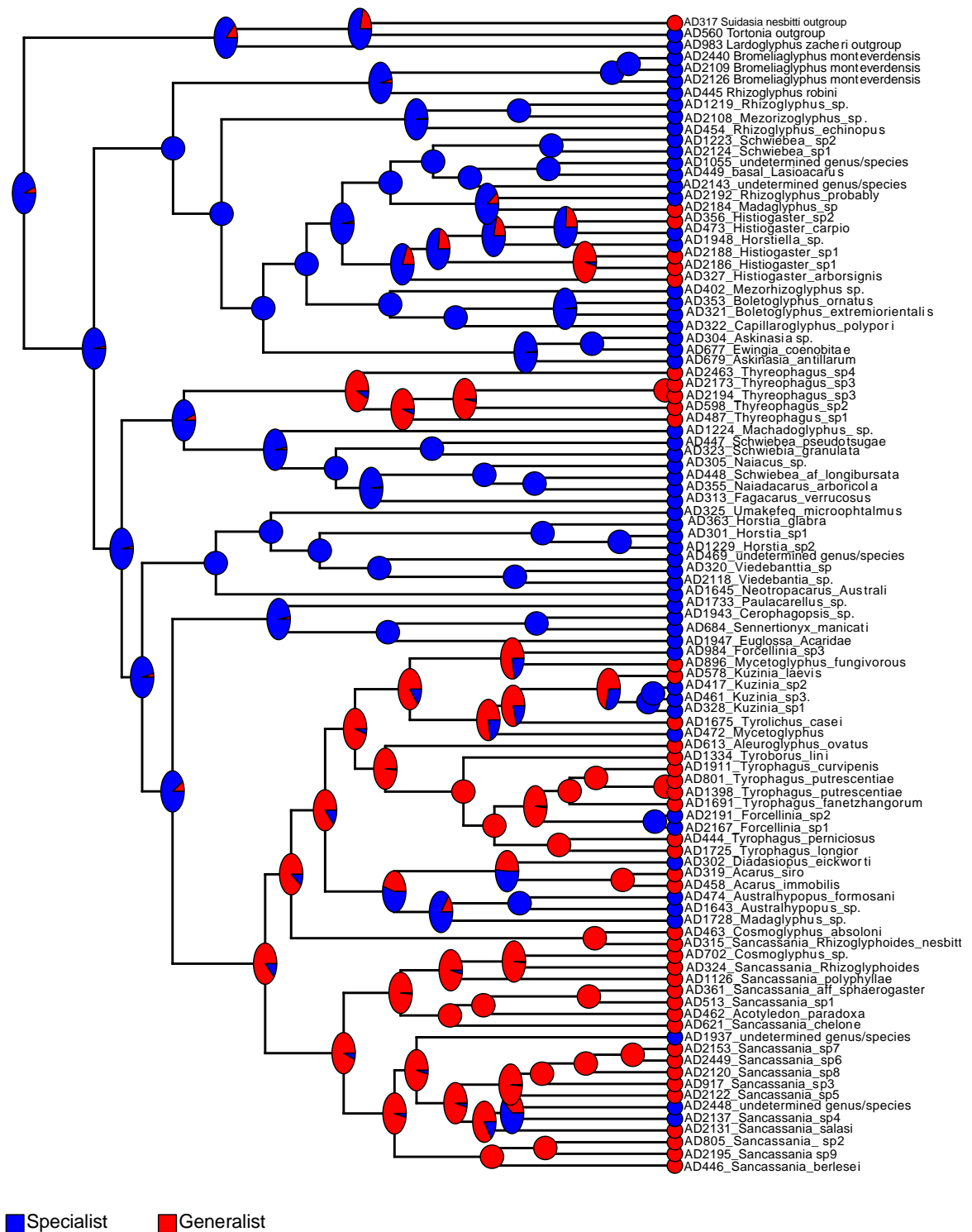
Figure 4.3 Ancestral character state reconstruction employing a stochastic map. The area of pie slices indicates the posterior probability of being generalist or specialist indicated with red and blue colors respectively.
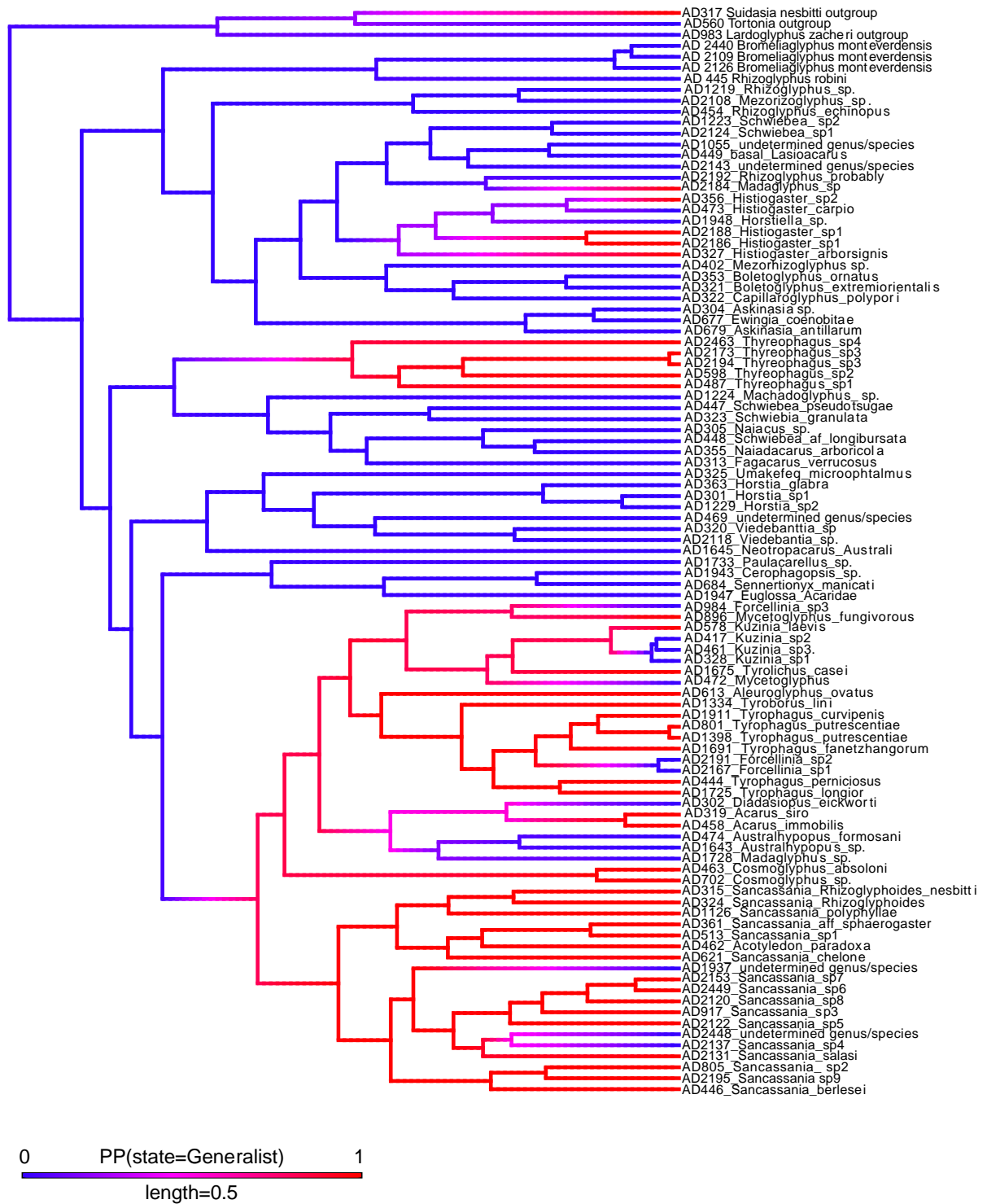
Figure 4.4 Ancestral character state reconstruction employing the method DensityMap. Posterior probability for each state across all branches and nodes of the tree. Blue colors show probabilities of being specialist and red of being generalist.
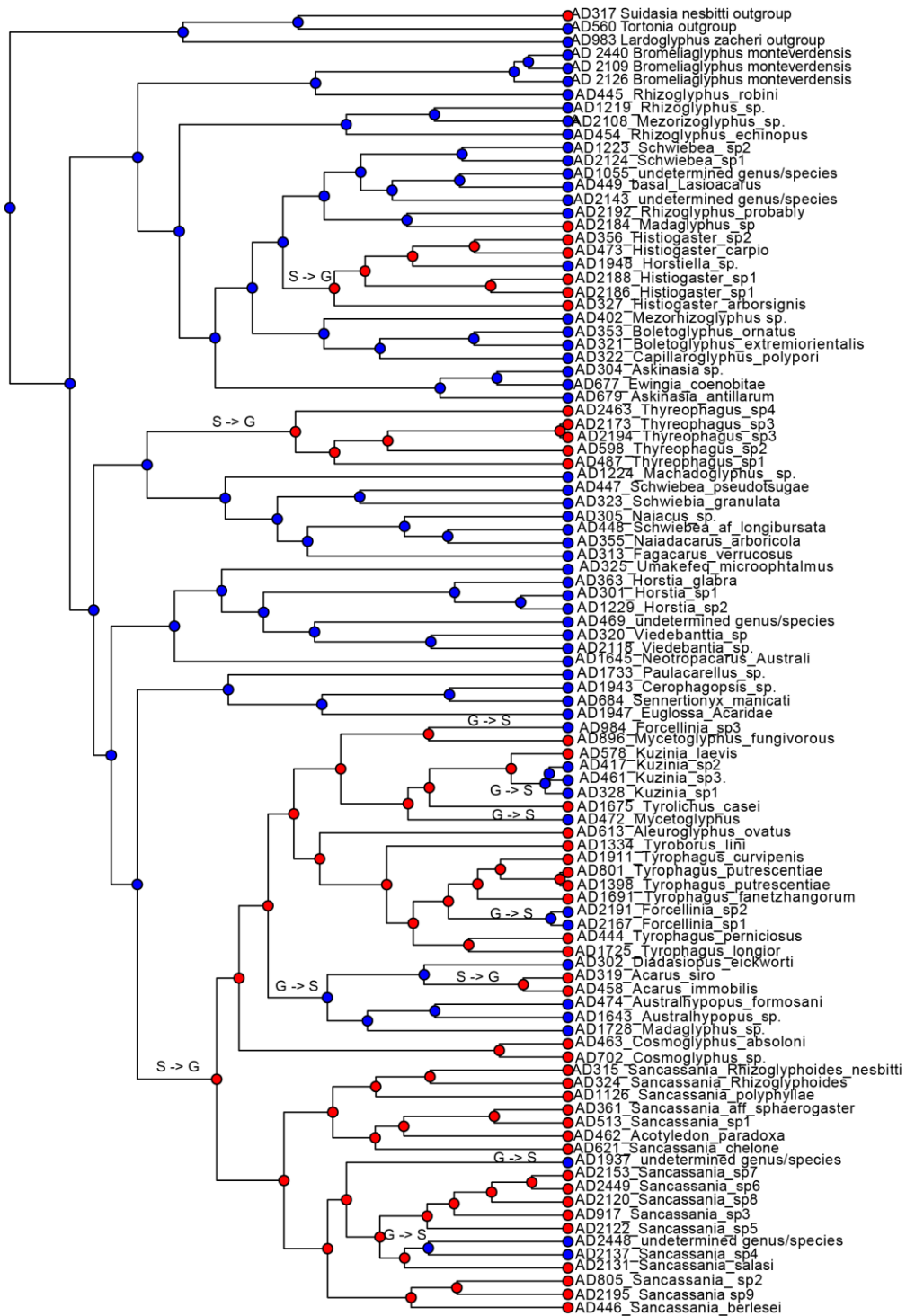
Figure 4.5 Ancestral character state reconstruction incorporating rate of heterogeneity on the phylogeny through parsimony analysis. Blue and red colors indicate specialist and generalist nodes.

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Transition rate data

According to the coefficients generated in diversitree, transition rates show differences in the directionality of the evolutionary rates according with the scenario evaluated (Table 4.1). The scenarios for symmetrical evolution (scenario 1), unidirectional evolution from specialist to generalist (scenario 3) and asymmetrical evolution from generalist to specialist (scenario 4), favored transitions going from specialist to generalist species. However, according to AIC, scenario 3 which assumes unidirectional evolution from specialist to generalist (assuming a specialist root) was significantly inferior to the constrained model, rejecting the unidirectionality of transitions.

On the other hand, unidirectional evolution from generalist to specialist (scenario 2) and asymmetrical evolution from specialist to generalist (scenario 5) favor transitions from generalist to specialist. However, and more crucial for propose of this study, the AIC of scenario 2 (assuming a generalist root) was significantly poorer than the constrained model, rejecting the 'specialist as dead end' hypothesis.

Likewise, differences in transitions rates from specialist to generalist were in all cases much higher than transitions from generalist to specialist (Table 4.1). When comparing scenarios (scenario 1, scenario 4, scenario 5) with their respective constrained models (Mk1), the AIC favors in all cases the full models tested here, however the differences between these models were not significant (Table 4.1).

Table 4.1 Transition rates scenarios for 1) symmetrical evolution, 2-3) unidirectional evolution (two scenarios), and 4-5) asymmetrical evolution (two scenarios). Transition rates from Generalist to Specialist (q01), and from Specialist to Generalist (q10). Degree of freedon (DF), likelihood scores (lnL), Akaike Information Criterion (AIC), Chi square (ChiSq), and p-value

Scenario	q01	q10	Df	lnL	AIC	ChiSq	p-value
1 Symmetrical	0.2195	4.1465	Full 2	-18.125	40.249		
			MK1	-18.773	39.545	1.2964	0.2549
2 Unidirectional G to S (root G)	8.33E-01	5.59E-10	Full 2	-29.307	62.613		
			MK1	-36.435	74.871	14.257	0.00015
3 Unidirectional S to G (root S)	0.0001	1.0599	Full 2	-4.8697	13.739		
			MK1	-8.5711	19.142	7.4027	0.006512
4 Asymmetrical G to S	0.2202	4.6632	Full 2	-16.433	36.867		
			MK1	-17.113	36.226	1.3593	0.2437
5 Asymmetrical S to G	0.5555	0.3307	Full 2	-25.722	55.444		
			MK1	-25.91	53.821	0.37685	0.5393

Discussion

Organisms with adaptations to specialized habitats and resources are thought to have evolved from more generalized ancestors by unidirectional evolution (Futuyma & Moreno, 1988; Goldberg & Igić, 2008). However, results from our study on acarid mites are in contrast to these expectations and suggest that shifts from specialist to generalist also occur and played an important role in the diversification of this family. We found widespread signal across the phylogeny over long and short time scales where several transitions from specialist to generalist arose. Similarly, the majority of transition rate scenarios showed a preference for specialists giving rise to generalists (Table 4.1). Analogous to Nosil & Mooers (2005), our analyses also highlighted that both scenarios of unidirectional evolution from generalist or specialist species are very unlikely. These authors found transition from and to specialized phytophagous insects, but as in our system, the mean ratio was predisposed to higher transitions to specialist lineages.

According to the ancestral states reconstructions, the common ancestor of Acaridae was a specialist that gave rise to new lineages and a number of specialized clades. Likewise, other lineages represented mostly by generalist species originated from this specialist ancestor as in the case of the subfamily Acarinae (Fig. 4.3, Fig. 4.4, Fig. 4.5). As well, evidence of specialists giving rise to generalists was provided by generalist species deeply nested within specialist lineages on the tree (Fig. 4.3, Fig. 4.4, Fig. 4.5). For example, species in the subfamily Tyrophaginae (generalist) were nested within the subfamilies Rhizoglyphinae and Horstiinae, which consist mostly of specialist species. These sets of evidence follow the same pattern shown by Klimov & OConnor (2013) for house dust mites, where permanent parasites (specialists) gave rise to free living mites (generalists) and then radiated to new lineages. Similarly, Poulin et al. (2006), showed that the degree of specificity of fleas for particular hosts decreased in many lineages. They reported that transitions from specialist to generalist strategies occurred more frequently and proposed that by doing so, species could gain advantage using new resources provided by their new hosts. Thus, as demonstrated in these works, specialized organisms can adapt to and colonize new habitats, but how is it possible that acarids shifted to generalist habitat use, and what advantages would that have brought for them?

According to morphological, ecological data, and fossil records (OConnor, 1982), the ancestors of Acaridae possibly lived in association with vertebrate nests and evolved from there

using nidicolous insects for dispersal to new habitats (OConnor, 2001). This suggests that acarid mites probably switched to hosts that were not necessarily specialists and established connections with different habitats. This eventually allowed them abandon a specialist lifestyle, switching to broader habitats and then radiating as generalist species. In this manner, as proposed by Klimov & Oconnor (2013) it is probable that an arrangement of ancestral characteristics played a fundamental role in switching habitat use modes. In this case, ancestral characteristics like tolerance to humidity, low fat content diets and frequent access to unrelated hosts, probably played an important role facilitating the diversification of this group.

Likewise, phoretic interactions with different hosts (or not using a host for dispersal) increased the contact of these mites with different environments, decreased the dependence on a specific habitat, and increase opportunities for generalization (Poisot et al., 2011). For example, many modern species of *Sancassania* have expanded their habitat range by switching between hosts and, as a consequence, their habitat. By contrast, if organisms are continuously exposed to and have preference for a particular environment, this can reinforce the dependence on that habitat and thus favor specialization (Poisot et al., 2011). For example, some modern mites associated with bees, genus *Diadasiopus*, depend on the habitat created by their host (bees from the genus *Diadasia)* and disperse on them (OConnor, 2001), increasing the correlation between their genotype and the surrounding environment and enhancing specialization via the niche created by their host.

Because Acaridae is the largest and most species-rich family within Acaroidea, with more than 88 genera and 400 species (Schatz et al., 2011), it is very difficult for any study to include a total representation of all species taxa in the family. However, it is possible to have a good indication of the general diversity for it. For example, in our analyses we were able to include common generalist genera, as most of them are relatively easy to find due to their worldwide distribution and lack of restriction to particular hosts. On the other hand, most specialist species are restricted to certain geographic regions and/or hosts making them more difficult to be sampled. Despite the fact that we included just a fraction of the total number of specialist genera of this family, our results were able to show transitions occurring from specialist to generalist. Besides, it is very likely that if more samples are added in our phylogeny, these will probably be specialist taxa. Therefore, it is unlikely that our current hypothesis for specialization and generalization would be disproved with addition data. Nonetheless, adding more genera of Acaridae in future

studies will let us recognize and clarify the phylogenetic relationships and evolutionary history of this family.

Similarly, in the simulations evaluating the effect of outgroups (changing all outgroups as generalist) in the outcome of our hypothesis (Supplementary Fig. S2), we confirmed that even when all outgroups were set as generalists, this did not overturn the results obtained in our analyses and preserves the ancestral state as specialist. Even though in our case, we consider that adding more taxa will not change the results for the transitions of Acaridae, it is necessary to be aware that incomplete taxon sampling and asymmetries of characters on the tree are considered by many authors (Schluter et al., 1997; Salisbury & Kim, 2001; Stadler & Bokma, 2013; Rabosky & Goldberg, 2015; Olave et al., 2019) as a source of error when calculating transitions rates and can give incorrect interpretations on the traits under analysis.

Certainly, the ecological diversification of this group is likely to be far more complicated than our two-rate scenario, as Acaridae possesses much variation in specialization in terms of habitat preference and host utilization. Here we do not suggest that these results represent the whole ecological complexity of this family, however, this research is a good starting point for study the diversification, dynamics and evolutionary traits that give rise to existing species in this family and supports the notion that ecological shifts different from known hypotheses on the evolution of specialization and generalization are possible.

Conclusions

Our approach provides a framework to investigate broader ecological questions associated with the diversification of the mite family Acaridae related to their habitat specialization. Overall, phylogenetic analysis, ancestral character state reconstructions and estimates of transition rates provide evidence for an ancestrally specialist lifestyle for acarids mites and found support that several changes from specialist to generalist and then again to specialist state (reversals) are possible here. This research demonstrated that generalist species can also evolve from specialist species challenging traditional hypotheses of unidirectional evolution of specialization. In general, three major points support this evidence. First, unidirectional evolution was always rejected and not supported in both cases (transitions from specialist to generalist and vice versa). Also, estimated transitions rates from specialist to generalist, were in all cases higher than transitions from generalist to specialist. Second, ancestral character state reconstructions showed the most recent common ancestor for the family as a specialist node diversifying into different specialization modes from there through the evolutionary history. Third, specialist and generalist taxa were deeply nested in groups containing mostly with generalists and specialists respectively. For future studies, it would be helpful to include more samples, and incorporate additional ecological data in the parameters evaluated, as this family includes greater ecological complexity than the two matrix parameters assessed here.

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Appendix C

Supplementary material from Chapter IV

Supplementary Text: Taxonomy of the family Acaridae (details about suprageneric names)

A number of suprageneric names have been proposed for Acaridae, many of which are monogeneric and are based on perceived degree of morphological differences from other taxa. To date, there have been 16 suprageneric (i.e. "family-group") names proposed. Two of these, Hypopidae Murray, 1877, and Tyroglyphidae Donnadieu, 1868, have not been used in recent times due to either ambiguity (Hypopidae) or synonymy. The name "Tyroglyphidae", based on the genus *Tyroglyphus*, was used in many early works, but when the name *Tyroglyphus* was shown to be a junior objective synonym of *Acarus* (i.e. based on the same type genus/species), Acaridae, which was properly attributed to Latreille, 1802 by OConnor (1984), became standard.

In addition to coining the name Rhizoglyphidae in 1923, Oudemans named four other families that include species we now consider acarids: Caloglyphidae (Oudemans, 1932), Forcelliniidae (Oudemans, 1927), Pontoppidaniidae (Oudemans, 1927), and Tyrophagidae (Oudemans, 1924). Zachvatkin (1941) recognized two subfamilies under "Tyroglyphidae", and synonymized the other Oudemans names. He used the names Tyroglyphinae (now Acarinae) and Rhizoglyphinae for these. OConnor (1982) formally included the genus Ewingiia into Acaridae (former family Ewingiidae) and Klimov (1998) grouped several existing genera under the name Thyreophagini.

After that, various authors coined new subfamily or tribe names for the single genera they described: Naiadacarinae (Fashing, 1974), Fagacarinae (Fain & Norton, 1979), Horstiinae (Fain, 1984), Lasioacarini (Fain & Chmielewski, 1987), and Megachilopodini (Fain & Pauly, 2001). Finally, Mahunka (1979) coined the name Pinoglyphidae for a single ant-associated genus. However, it has not been formally included in Acaridae.

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Table B-S1 Countries, hosts, localities, DNA codes and UMMZ accession numbers used for the different species of Acaridae

Mite species	Country	Host/Locality	Code	Museum (BMOC) accession number	Non amplified regions
Acarus immobilis	USA	dry straw	AD458	BMOC 00-1103-002	COI
Acarus siro	USA	cracked corn	AD319	BMOC 01-0903-003	
Acotyledon paradoxa	USA	grain spill	AD462	BMOC 04-1205-003	
"Acotyledon" formosani	N/A	lab culture	AD474	N/A	
Aleuroglyphus ovatus	USA	medium wet grain spill, with animal feces	AD613	BMOC 05-0918-003	
Askinasia sp.	Mexico	*Coenobita compressus*	AD304	BMOC 04-0612-008	
Askinasia antillarum	USA	*Coenobita clypeatus*	AD679	BMOC 05-0516-001	COI
Australhypopus sp.	Australia	*Mastotermes darwinensis* (Isoptera) in hollowed tree	AD1643	BMOC 08-0520-006	28S, EF1α, HSP70
Boletoglyphus_extremiorientalis	Russia	polypore fungi inside tubes of the fruiting body	AD321	BMOC 01-0922-001	
Boletoglyphus_ornatus	USA	*Ganoderma applanatum*	AD353	BMOC 04-0924-009	
Bromeliaglyphus monteverdensis DN	Costa Rica	Scarabaeidae *Anomala* sp.	AD2126	BMOC 15-0601-029	
Bromeliaglyphus monteverdensis	Costa Rica	*Xanthosoma*	AD2440	BMOC 15-0601-002	
Bromeliaglyphus monteverdensis	Costa Rica	*Xanthosoma sp.*	AD2109	BMOC 15-0601-005	
Capillaroglyphus_polypori	Russia	polypore fungus	AD322	BMOC 01-1021-005	
Cerophagus nearcticus			AD667		
Cerophagopsis sp.	Brazil	*Tetragonisca angustula*	AD1943	BMOC 15-0104-060	
Cosmoglyphus absoloni	N/A	lab culture	AD463	N/A	COI
Cosmoglyphus sp.	Belgium	rotting grass	AD702	BMOC 06-0910-062	
Diadasiopus eickworti	USA	Apidae: *Diadasia*	AD302	BMOC 04-0524-055	COI
Ewingia coenobitae	US Virgin islands	*Coenobita clypeatus*	AD677	BMOC 05-0516-001	COI
Euglossa_Acaridae	Brazil	*Euglossa ignita*	AD1947	BMOC 15-0104-017	28S, EF1α, HSP70
Fagacarus verrucosus	USA	decayed log, under bark	AD313	BMOC 00-1001-004	COI, EF1α, HSP70
Forcellinia sp._1	Costa Rica	Apidae: *Partamona sp.*	AD2167	BMOC 15-0601-107	
Forcellinia sp._2	Costa Rica	Apidae: *Plebeia sp.*	AD2191	BMOC 15-0601-147	
Forcellinia sp._3	USA	*Lasius umbratus* (nest in dead tree)	AD984	BMOC 07-0621-001	
Histiogaster carpio	USA		AD473	N/A	COI
Histiogaster sp._1	USA	*Acer* bark	AD_356	BMOC 04-0924-019	
Histiogaster sp. arborsignis	USA	*Quercus sp.*	AD327	BMOC 02-0523-049	
Histiogaster sp._2	Costa Rica	Scarabaeidae: *Cyclocephala kaszabi*	AD2186	BMOC 15-0601-131	
Horstia glabra	Tanzania	*Xylocopa nigrita*	AD363	BMOC 03-1005-001	COI
Horstia sp._1	USA	*Xylocopa californica arizonensis*	AD301	BMOC 03-0904-004	COI
Horstia sp._2	Mexico	*Xylocopa sp.*	AD1229	BMOC 08-0515-138	COI
Horstiella_sp.	Brazil	*Epicharis sp*	AD1948	BMOC 15-0104-016	28S, EF1α, HSP70
Kuzinia laevis	Italy	Apidae: *Bombus agrorum*	AD578	BMOC 06-0422-004	

Kuzinia sp._1	USA	*Bombus morrisoni*	AD328	BMOC 03-0820-001	
Kuzinia sp._2	Mexico	Apidae: *Bombus*	AD417	BMOC 04-0503-006	COI
Kuzinia sp._3	USA	*Bombus sp.*	AD461	APJD 067	
Machadoglyphus sp.	Mexico	*Nasutitermes* sp. (Isoptera: Termitidae) in fallen log	AD1224 BMOC 08-0515-001		COI
Madaglyphus sp._1	Puerto Rico	Polipore fungi	AD1728	BMOC 08-1227-002	28S, EF1α
Madaglyphus sp._2	Costa Rica	palm inflorescence	AD2184	BMOC 15-0601-128	
Mezorhizoglyphus sp._1	USA	polypore fungi	AD402	BMOC 04-1005-001	COI
Mezorhizoglyphus sp._2	Costa Rica	*Russula*	AD2108	BMOC 15-0601-003	
Mycetoglyphus sp.		*Formica obscuripes*	AD472	BMOC 04-1209-001	COI
Mycetoglyphus fungivorous	USA	hay on barn floor	AD896	BMOC 07-0607-012	
Naiacus sp.	Mexico	Bromeliacea: *Liliopsida*	AD305 BMOC 04-0612-001		
Naiadacarus arboricola	USA	*Fagus grandifolia* tree hole	AD355 BMOC 04-0924-014		
Neotropacarus sp.	Australia	*Lophostemon confertus*	AD1645 BMOC 08-0516-001		COI,28S
Paulacarellus sp.	USA	beach wrack	AD1733	BMOC 13-1002-002	
Rhizoglyphus echinopus	USA	tulip bulbs	AD454	BMOC 02-1113-001	
Rhizoglyphus robini	USA	NY Orange Co (culture)	AD445 BMOC 04-1127-001		COI, EF1α, HSP70
Rhizoglyphus sp	Mexico	rain forest litter, under fallen log	AD1219 BMOC 08-0515-049		COI
Rhizoglyphus sp	Costa Rica	rotten leaves	AD2192	BMOC 15-0601-149	
*Sancassania*_aff_*sphaerogaster*	USA	Insect culture	AD361	BMOC 04-0913-001	
Sancassania berlesei	USA	nematode culture	AD446	BMOC 02-0405-001	
Sancassania_chelone	Italy	Scarabaeidae: *Pentodon bidens*	AD621	BMOC 05-1206-001	
Sancassania salasi	Costa Rica	Scarabaeidae: *Phyllophaga sp.*	AD2131	BMOC 15-0601-032	
Sancassania rhizoglyphoides	Russia	*Panellus serotinus*	AD324	BMOC 01-1021-003	
Sancassania rhizoglyphoides	USA	*Lactarius sp.*	AD315	BMOC 00-0929-005	
Sancassania polyphyllae	Turkey	Undetermined Scarabaeidae	AD1126	BMOC 08-0318-001	
Sancassania sp._1	USA	*Allium sativum* rotting bulb	AD513	BMOC 05-0827-001	
Sancassania sp._2	England	Lab colony	AD805	BMOC 07-0223-004	
Sancassania sp._3	USA	Scarabaeidae: *Osmoderma eremicola*	AD917	BMOC 07-0630-005	
Sancassania sp._4	Costa Rica	Undetermined Cerambycidae	AD2137	BMOC 15-0601-041	
Sancassania sp._5	Costa Rica	Scarabaeidae: *Spodochlamys cupreola*	AD2122	BMOC 15-0601-026	
Sancassania sp._6	Costa Rica	Scarabaeidae: *Spodochlamys cupreola*	AD2449	BMOC 15-0601-023	
Sancassania sp._7	Costa Rica	Undetermined Scarabaeidae	AD2153	BMOC 15-0601-072	
Sancassania sp._8	Costa Rica	Scarabaeidae: *Spodochlamys cupreola* Bates	AD2120	BMOC 15-0601-023	
Sancassania sp._9	Costa Rica	*Sechium edule* steem base	AD2195	BMOC 15-0601-166	
Schwiebea_pseudotsugae	USA	bark ex branch	AD447	BMOC 02-0523-007	COI

*Schwiebea*_aff._*longibursata*	USA	Chilopoda on legs & pleura	AD448	BMOC 02-0523-074	COI
Schwiebea sp._1.	Mexico	*Nasutitermes*	AD1223	BMOC 08-0515-001	COI
Schwiebea sp._2	Costa Rica	Undetermined Cerambycidae	AD2124	BMOC 15-0601-028	
Schwiebia granulata	Russia	under bark of fallen deciduous tree	AD323	BMOC 01-1021-001	
Sennertionyx manicati	Italy	*Anthidium manicatum*	AD684	BMOC 05-1111-002	COI
Thyreophagus sp._1	USA	*Euglosa dilema*	AD487	BMOC 04-1223-001	
Thyreophagus sp._2	New Zealand	*Nothofagus solandri*	AD598	BMOC 03-0701-001	COI
Thyreophagus sp._3	Costa Rica	*Sechium edule*	AD2194	BMOC 15-0601-165	
Thyreophagus sp._3	Costa Rica	Apidae: *Partamona*	AD2173	BMOC 15-0601-107	
Thyreophagus sp._4	Costa Rica	*Dieffenbachia sp.*	AD2463	BMOC 15-0601-153	
Tyroborus_lini	Czechia	Lab colony_Culture started from a CSL culture (England)	AD1334	BMOC 08-1010-001	28S, HSP70
Tyrolichus casei	USA	Mimolette cheese	AD1675	BMOC 13-0903-002	28S, EF1α
Tyrophagus curvipenis	Costa Rica	*Sechium edule,* subcortical part of aged (discolored) stem	AD1911	BMOC 15-0601-167	HSP70
Tyrophagus fanetzhangorum	Belgium	Rotting grass	AD1691	BMOC 06-0910-062	
Tyrophagus longior	USA: MI	Cheese	AD1725	BMOC 14-0602-001	
Tyrophagus perniciosus	USA: MI	Grain spill	AD444	BMOC 00-1103-013	
Tyrophagus putrescentiae	England	Lab colony Biological Crop Protection, Ltd.	AD801	BMOC 07-0223-006	HSP70
Tyrophagus putrescentiae	Czechia	Lab culture from grain store maintained by the Crop Research Institute, Prague	AD1398	BMOC 08-1010-002	
undetermined genus/species	Costa Rica	*Nyssodesmus*	AD2143	BMOC 15-0601-058	
undetermined genus/species	Chile	*Ceroglossus magellanicus*	AD1937	BMOC 15-0721-001	HSP70
undetermined genus/species near *Reckicarus*	USA	*Pinus banksiana* branch on ground	AD1055	BMOC 07-0615-001	COI
undetermined genus/species near *Reckicarus*	USA	*Clinidium* under bark	AD449	BMOC 02-0523-079	COI
undetermined genus/species	USA	*Lycoperdon pyriforme*	AD469	BMOC 04-1209-003	
undetermined genus/species	Costa Rica	*Nyssodesmus sp.*	AD2448	BMOC 15-0601-014	
Umakefeq microophtalmus	Russia	Ciidae beetle in white polypore fungus	AD325	BMOC 01-0922-008	COI
Viedebanttia sp.	Russia	black, lichen-like fungus	AD320	BMOC 01-1021-002	COI, EF1α
Viedebantia sp.	Costa Rica	*Nyssodesmus sp.*	AD2118	BMOC 15-0601-014	

Table B-S2 Species-specific oligonucleotide primers used in nested amplification and thermocycler reaction protocols. *For uniform sequencing, M13FORW/REV tails (underlined in the sequences below) were added to the primers for the second PCR

Primer name	Primer sequence (5' to 3')	Thermocycler protocol:
COI:		
COX1_16F	TGANTWTTTTCHACWAAYCAYAA	94 °C: 2 min, 10 cycles of 94 °C: 30 s, 40 °C: 1 min, 72 °C: 2 min, and 25 cycles of 94 ° C: 30 s, 48 °C: 35 s and 72 °C:2 min, with a final extension step of 72 °C:7 min.
COX1_1324R	CDGWRTAHCGDCGDGGTAT	
COX1_25Fshort_T*	<u>TGTAAAACGACGGCCAGT</u>TCHACWAAYCAYAARRAYA	94 °C: 2 min, 20 cycles of 94 °C: 30 s, 49 °C: 30 s, 72 °C:2 min, and 18 cycles of 94 °C: 30 s, 52 °C: 35 s and 72 °C: 2 min, with a final extension step of 72 °C: 7 min.
COX1_1282R_T*	<u>CAGGAAACAGCTATGACC</u>CCWVYTARDCCTARRAARTGTTG	
28S:		
28S_f1_4F	GACCTCAGATCARGCgAGAH	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 2:15 min, with a final extension step of 72 °C:7 min.
28S_P1_3R	GCTGTTCACATGRAACCCTTC	
28Sa_DD_T*	<u>TGTAAAACGACGGCCAGT</u>GACCCGTCTTGAAACACGGA	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 53 °C: 25s, 72 °C: 1:20 min, with a final extension step of 72 °C:7 min.
28SIR_acar2_T*	<u>CAGGAAACAGCTATGACC</u>TGCTACTACCACCAAGATCTGC	
28S_1F_Arach3_T*	<u>TGTAAAACGACGGCCAGT</u>ACCCGCTGAATTTAAGCAT	94 °C: 2 min, 20 cycles of 94 °C: 30 s, 51 °C: 20 s, 72 °C: 1:40 min, and 15 cycles of 94 °C: 30 s, 57 °C: 20 s and 72 °C: 1:40 min, with a final extension step of 72 °C: 7 min
28S_1661R_T*	<u>CAGGAAACAGCTATGACC</u>AGTTCACCATCTTTCGGGTA	
18S:		
18S_1F_G	TACCTGGTTGATCCTGCCAGTAG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 2 min, with a final extension step of 72 °C:7 min.
18S_8r_MH	TAATGATCCTTCCGCAGGTTCACCT	
18S_1F_sh_T*	<u>TGTAAAACGACGGCCAGT</u>CCTGCCAGTAGTCATATGCTTG	94 °C: 2 min, 15 cycles of 94 °C: 30 s, 51 °C: 23s, 72 °C: 1:10 min, and 15 cycles of 94 °C: 30 s, 57 °C: 25 s and 72 °C: 1:10 min, with a final extension step of 72 °C: 7 min
18SR_719R_T*	<u>CAGGAAACAGCTATGACC</u>AATATACGCTATTGGAGCTGGA	
18S_521F_T*	<u>TGTAAAACGACGGCCAGT</u>ACATCCAAGGAAGGCAGCAG	94 °C: 2 min, 15 cycles of 94 °C: 30 s, 51 °C: 23s, 72 °C: 1:10 min, and 15 cycles of 94 °C: 30 s, 57 °C: 25 s and 72 °C: 1:10 min, with a final extension step of 72 °C: 7 min
18S_1587R_T*	<u>CAGGAAACAGCTATGACC</u>AACTAAGAACGGCCATGCAC	
EF1A:		
EF1A_1F	ATGGGMAARGARAAGRYBCA	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min.
EF1A_1638R	TTRATVACDCCVACRGCVAC	
EF1a_19Fm	CAYATYAAYATyGTbGTIATHGG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 30s, 72 °C: 1:30 min, with a final extension step of 72 °C:7 min.
EF1a_52R	CCDATYTTRTANACRTCYTG	
EF1A_45.71F	GTNGSNGTIAAYAARATGGA	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 52.5 °C: 20s, 72 °C: 1:40 min, with a final extension step of 72 °C:7 min
EF1a_41.21R	TGYCTCATRTCDCGVACRGCRAA	
EF1A_102F_Tyr	CAAGCGAACCATCGAGAAGT	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 58 °C: 30s, 72 °C: 1:45 min, with a final extension step of 72 °C:7 min
EF1A_1244R_Tyr	AGGGGWGGGAAGTCVGTG	
EF1a115F_115Tyr_T	<u>TGTAAAACGACGGCCAGT</u>CGAGAAGTTCGARAAGGARGC	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 62 °C: 30s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min
EF1a1232R_Tyr_T	<u>CAGGAAACAGCTATGACC</u>GGAAGTCVGTGAAGGACTCGAC	
HSP70:		
HSP70_9B_129F	TGYGTDGCHRTNATGGARG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 50 °C: 35s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min
HSP70_9B_1896R	GCCATYTTYTTRTANGCCATYTC	
HSP70_162F_Tyr	GTRATYGARAAYGCNGARGG	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 54 °C: 35s, 72 °C: 2:20 min, with a final extension step of 72 °C:7 min
HSP70_1636R_Tyr	GHARRTCRTTGCAVACYTCCTT	
HSP70_251F_Tyr_T	<u>TGTAAAACGACGGCCAGT</u>GCNGCMAACACYTTYTAYGC	94 °C: 2 min, 35 cycles of 94 °C: 30 s, 56 °C: 30s, 72 °C: 1:40 min, with a final extension step of 72 °C:7 min
HSP70_1609R_Tyr_T	<u>CAGGAAACAGCTATGACC</u>TCVGGHGTYTCRTTDTCYTT	

Figure S2. Ancestral character state reconstruction employing a stochastic map with all outgroups set as generalist. The area of pie slices indicates the posterior probability of being generalist or specialist indicated with red and blue colors respectively.

These authors are going to be included when submitted this chapter for publication: Pamela Murillo, Pavel Klimov, Barry OConnor.

Chapter 5 Conclusions

This work utilizes the remarkable diversity and worldwide distribution of the mite family Acaridae to investigate questions on ecological patterns and diversification. More specifically, this dissertation focuses on how habitat specialization switches have influenced the diversification of this family. It also shows the importance to study micro-evolutionary changes to recognize the general diversity that occurs at the species level and from there predict macro-evolutionary processes involved in the diversification of the group. The genetic diversity found in our results for the genus *Tyrophagus* highlights the necessity of future studies on population genetics and ecological speciation.

This study provides documentation of the enormous variability and hidden diversity that exists both between and within species of *Tyrophagus* at the genetic level, especially for those populations occurring in the Neotropical region. The findings in chapters two and three indicate extraordinary intraspecific genetic variability in the widespread species *T. putrescentiae*. The results also point out that geographic origin of the populations does not correlate with the existing genetic variability as no population structure was recovered. For example, some populations of *T. putrescentiae* from Brazil were closely related with other population from Japan but not with other populations from Brazil or nearby countries. These strong genetic differences presented in these populations might be an indication of an early stage of speciation between these two groups.

Similarly, results from chapter two are of broader significance not only in systematics and population genetics but also on the economic impact of acarid mites in agricultural systems. For example, during the fieldwork for this chapter, several farmers expressed their concerns associated with the interception of different mite species by quarantine inspectors in the natural cavities of fruits and leaves, causing substantial disruption in the international trade of their products. For this particular case it was imperative to determinate the species found in the fields to determine if these had quarantine restrictions or not. To assist with this, as part of chapter two the major morphological differences between the two most common species (*T. curvipenis*

and *T. putrescentiae*) encountered in the agricultural fields in Costa Rica were summarized. Later, the genetic distances for these species were calculated and major genetic differences were found for *T. putrescentiae*.

In this dissertation it was also possible to disentangle the genetic complexity between populations of *T. putrescentiae,* as the different species delimitation methods tested here agreed with the hypothesis of a single lineage for this cosmopolitan species. The results were consistent even though some populations showed higher genetic diversity than others. On the other hand, not all the programs reliably delineated the other species of *Tyrophagus* and depending on the implemented method, these tended to over split and yield false positives. Consequently, is necessary to be cautious when using species delimitation analyses, and together with other information such as morphology or ecological data, to reach a more educated conclusion for the system under study.

Besides, given that *T. putrescentiae* is the most widespread species in the genus, a future direction from this investigation would be to increase the sample size (include more populations) and include additional species in order to have a better indication of the real biodiversity that surrounds this genus. The results from this dissertation revealed 17 new morphospecies when applying the species delimitation methods. Even though I was expecting to find new species at the beginning of this research, I never anticipated finding so many new morphospecies in such a short period of time and in such a small area of the neotropics. These results demonstrate that the diversity of this mites are underestimated and lead us to the conclusion that major efforts still have to be made to approach the real diversity in this and other genera within Acaridae.

Perhaps more importantly, the results for this dissertation support a hypotheses that the ancestor of Acaridae was habitat specialist. In that study, OConnor proposed that the ancestors of these mites lived in association with vertebrate nests and from there, were able to disperse to new habitats promoting the dispersion and divergence of this group (OConnor, 1982). I found widespread evidence for this hypothesis of ancestral adaptation to specialized habitats across the Acaridae phylogeny over long and short time scales confirming that several changes occurred from specialist to generalist as well as the opposite. Although it was beyond the scope of this project to address issues of the classification of the Acaridae, the results are similar to the tree first presented by Klimov and OConnor (2013) in suggesting the monophyly and

traditional division of Acaridae should be questioned. Here I also challenged the notion of unidirectionality in the evolution of organisms, showing that different transitions between generalist and specialist preferences could have taken place in the diversification of this family. This research provided a good example that evolutionary patterns are more complex than originally thought, and organisms do not always follow prevalent theories in evolutionary biology.

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