Exp Appl Acarol
https://doi.org/10.1007/s10493-018-0256-9

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*

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Received: 4 September 2017 / Accepted: 14 April 2018

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 seven species of *Tyrophagus*, including *T. curvipenis*, *T. putrescentiae*, *T. fanetzhangorum*, *T. longior*, *T. perniciosus*, and *T.* cf. *similis*. Molecular phylogenetic analyses (1) recovered two major clades corresponding to the presence or absence of eyespots, and (2) 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 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 spreading in agricultural produce.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10493-018-0256-9) contains supplementary material, which is available to authorized users.

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Published online: 26 April 2018

Keywords *Tyrophagus curvipenis* · DNA barcode · COI · *Tyrophagus* phylogeny · Genetic distances

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). *Tyrophagus 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, 2004a; Pentinsaari et al. 2016).

Several studies 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 seven 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 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 *T. 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 (UMMZ), Ann Arbor, MI, USA.

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 (TGTAAAACGACGGCCAGTTCHACWAAYCAYAARRAYA), COX1_1282R_T (CAGGAAACAGCTATGACCCCWVYTARDCCTARRAARTGTTG) (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 20-µl 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 µL 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

Table 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
	UK	Lab colony Biological Crop Protection	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 (UK) and maintained by the Crop Research Institute, Prague	AD1337	BMOC 08-1010-005	KY986251

Table 1 (continued)

Mite species	Country	Host/locality	Code	Museum (BMOC) accession number	GenBank (GB) accession number
	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

Table 1 (continued)

Mite species	Country	Host/locality	Code	Museum (BMOC) accession number	GenBank (GB) accession number
	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 (UK)			AY525572
	China	Lab culture, Shangai			HQ287793
	China	Lab culture, Shangai			HQ287795
	China	Lab culture, Shangai			HQ287796

Table 1 (continued)

Mite species	Country	Host/locality	Code	Museum (BMOC) accession number	GenBank (GB) accession number
	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

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 v.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 KY986243-80 (Table 1).

An additional 11 COI sequences of *Tyrophagus* were retrieved from the nucleotide and EST GenBank databases (NCBI) (Table 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 network 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.

Non-synonymous/Synonymous ratio (Ka/Ks) was calculated with the software HyPhy (Kosakovsky-Pond 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 in *T. putrescentiae* lineages (Murrell et al. 2015).

Phylogenetic analysis

A nucleotide substitution model and a codon-based model were tested. The best-fitting nucleotide substitution model (HKY + G + I) was selected based on the Akaike Information Criterion (AIC) as implemented in MEGA v.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. The GY94 codon-based model (Goldman and Yang 1994) was implemented in HyPhy (Kosakovsky-Pond et al. 2005) and MrBayes (Huelsenbeck and Ronquist 2001).

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. 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 fruits (Fig. 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 1).

On chayote, 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

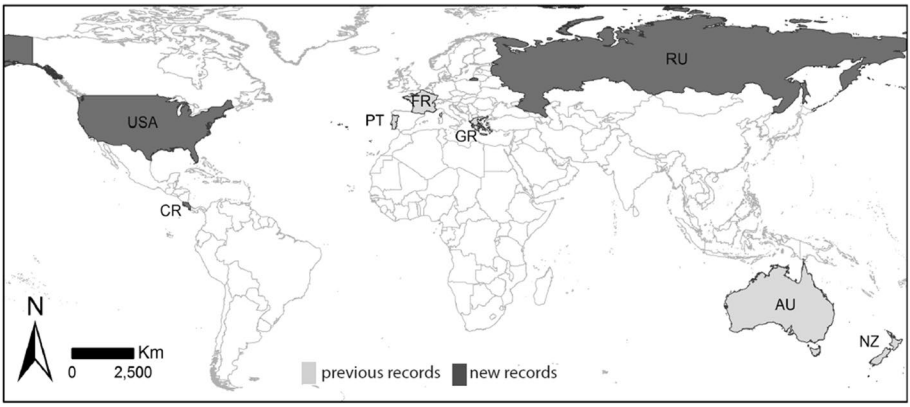

Fig. 1 Geographic distribution of *Tyrophagus curvipenis*. Light gray color indicates previous records and dark gray color indicates the new records for this species. (Color figure online)

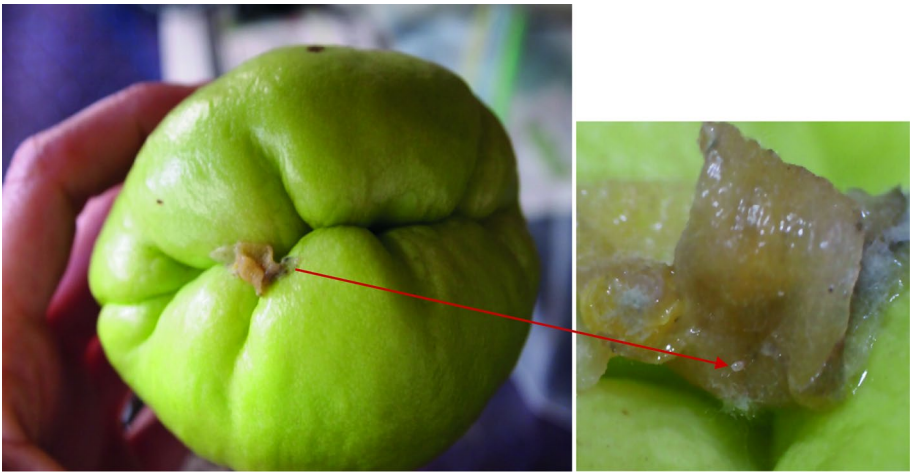

Fig. 2 Floral remains on the cavity of a chayote fruit and a close up of *Tyrophagus* sp. and fungal hyphae growing on the floral remains

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). The majority of the mites was observed in association with fungi or algae that grew on the plants or fruits (Fig. 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.

Morphological differences between *Tyrophagus 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. 3a), whereas in TP it is gradually widened (Fig. 3b); (2) coxal plate II in TC is broad and there is no distinct concavity in the posterior margin (Fig. 3c), whereas in TP, the coxal plate II is medium sized and a shallow but distinct concavity is noticeable in the posterior margin (Fig. 3d); (4) solenidia ω1 on tarsi I–II are slender in TC (Fig. 3e), whereas in TP they are wider (Fig. 3f); (4) the shaft of the supracoxal seta (scx) in TC is slender or moderately tapering from base to apex (Fig. 3g), whereas in TP it is prominently enlarged at the base of pectinations (Fig. 3h). For more details about the characters that these two species share, and differences with other species refer to Fan and Zhang (2007).

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).

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 indicated the presence of considerable within-species variation (Table 2) (range, mean ± SD): 0–4.33,

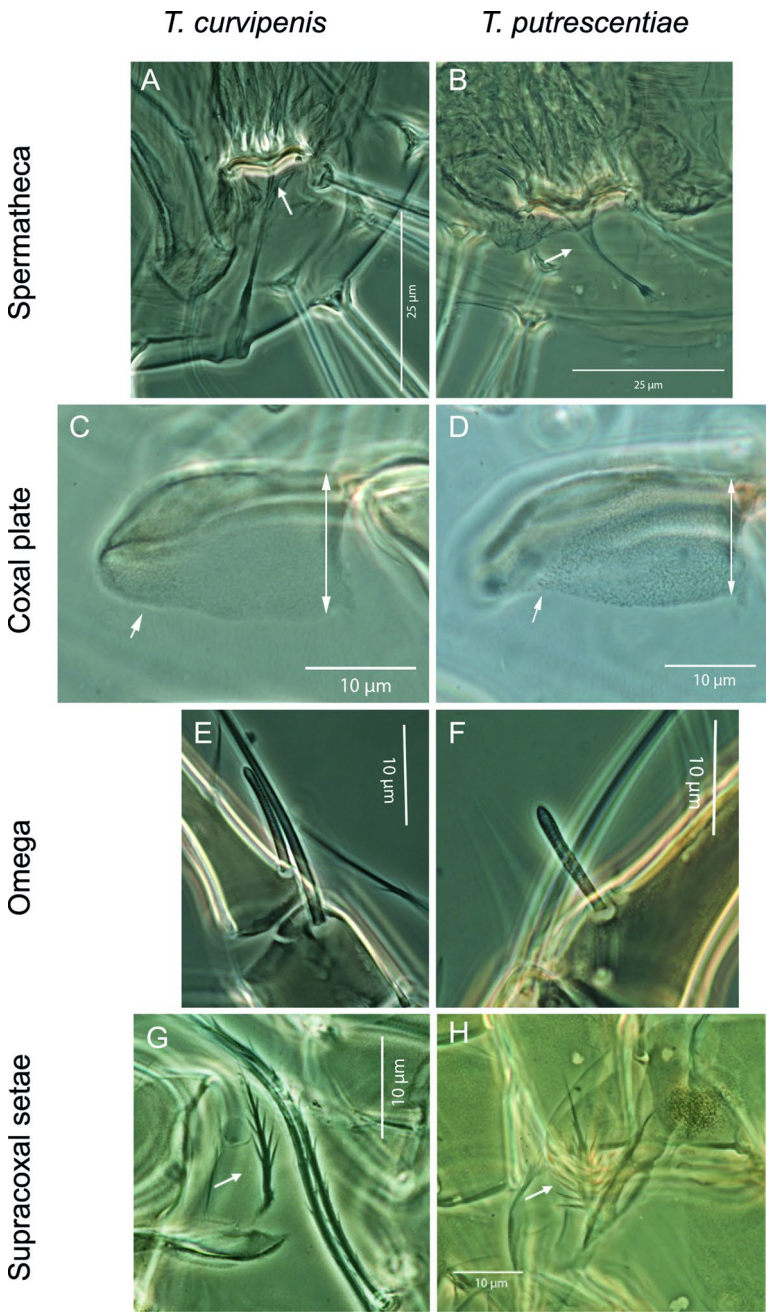

Fig. 3 Main morphological characteristics differentiating *Tyrophagus curvipenis* and *T. putrescentiae*. **a** spermatheca *T. curvipenis* (♀), **b** spermatheca *T. putrecentiae* (♀)**, c** coxal plate II *T. curvipenis* (♀), **d** coxal plate II *T. putrescentiae* (♀), **e** omega I (ω1) *T. curvipenis* (♀), **f** omega I (ω1) *T. putrescentiae* (♀), **g** supracoxal seta (*scx*) *T. curvipenis* (♀), **h** supracoxal seta (*scx*) *T. putrescentiae* (♀)

Table 2 Uncorrected and K2P (Kimura's two parameter) intra- and interspecific genetic distances (%) of *Tyrophagus putrescentiae* (TP), *T. curvipenis* (TC) and *T. fanetzhangorum* (TF)

Comparison	K2P		Uncorrected	
	Mean ± SD	Range	Mean ± SD	Range
TP versus TP	1.78 ± 1.44	0.00–4.33	1.54 ± 1.34	0.00–3.58
TC versus TC	0.83 ± 0.56	0.00–1.31	0.84 ± 0.57	0.00–1.30
TF versus TF	0.98 ± 0.15	0.82–1.15	0.97 ± 0.15	0.81–1.14
TP versus TC	15.17 ± 0.40	14.20–16.30	13.60 ± 0.27	12.92–14.18
TP versus TF	17.03 ± 0.43	15.89–18.11	15.12 ± 0.29	14.32–15.97
TC versus TF	18.44 ± 0.42	17.88–19.17	16.16 ± 0.32	15.72–16.70

1.78 ± 1.44 (K2P) and 0–3.58, 1.54 ± 1.34 (uncorrected). Genetic distances between TP versus TF and TP versus TC were lower than those exhibited by TC versus TF (Table 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 ± 0.32 (uncorrected).

The barcoding gap analysis (Fig. 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 to 0.05, whereas the

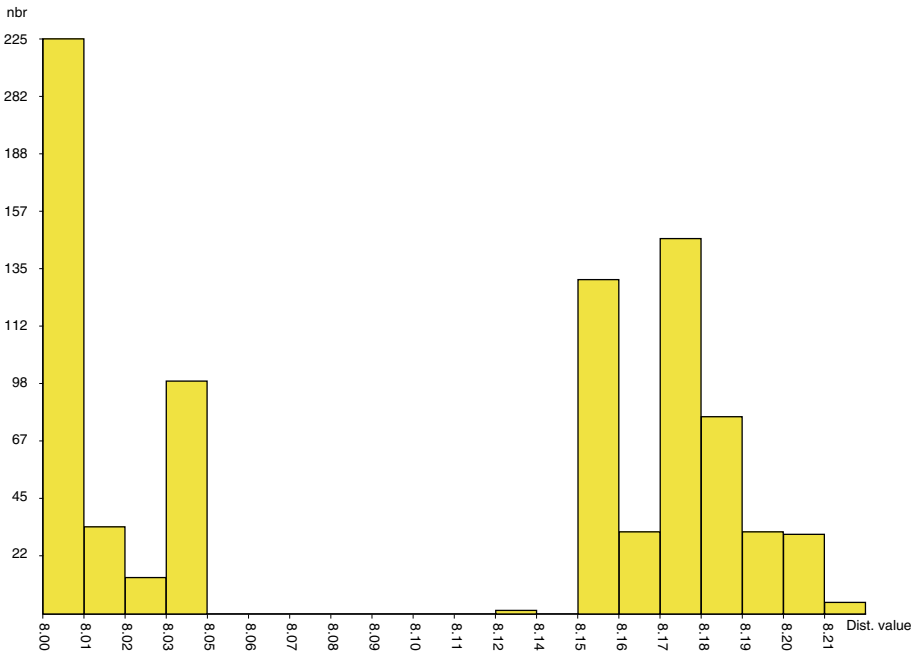

Fig. 4 Barcoding gap analysis for the species of *Tyrophagus*. Pairwise distances for all sequences were calculated using K2P. Within- and between-species pairwise genetic distances are on left and right side of the graph, respectively

interspecific distances can vary from 0.12 to 0.21. The intra-interspecies gap ranges from 0.05 to 0.12 (Fig. 4).

Amino acid mutations

We looked for non-synonymous mutations that resulted in amino acid changes. In the circumstances where no amino acid changes occur, it is more likely that we are dealing with the same species as mutations at the DNA level do not result in phenotypical changes. In addition, departure from neutral evolution, which is the common assumption of many phylogenetics programs, can be detected by comparing synonymous versus non-synonymous mutations.

Some amino acid changes were found in the sequences within the two *T. putrescentiae* (TP) groups. Independently of the group, several samples had amino acid changing mutations (Table 3) whereas 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 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 TP, 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}) and *T. fanetzhangorum* had two non-synonymous mutations: isoleucine I^{361} to leucine L^{361} and valine V^{391} to isoleucine I^{391} (Table 3).

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, $p=0.46$) of gene-wide episodic diversifying selection for the branches of TP. 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. 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. 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 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. 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

Table 3 Non-synonymous mutations in the CO1 gene of *Tyrophagus putrescentiae*, *T. curvipenis* and *T. fanetzhangorum* (amino acid position coordinates are based on the CO1 GenBank sequence KY986247)

T. putrescentiae Group 1: AD1398 (KY986247)	V^{81}	G^{173}	L^{187}	A^{194}	E^{331}	S^{333}	I^{361}	V^{391}	T^{402}	A^{414}
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	✓	✓

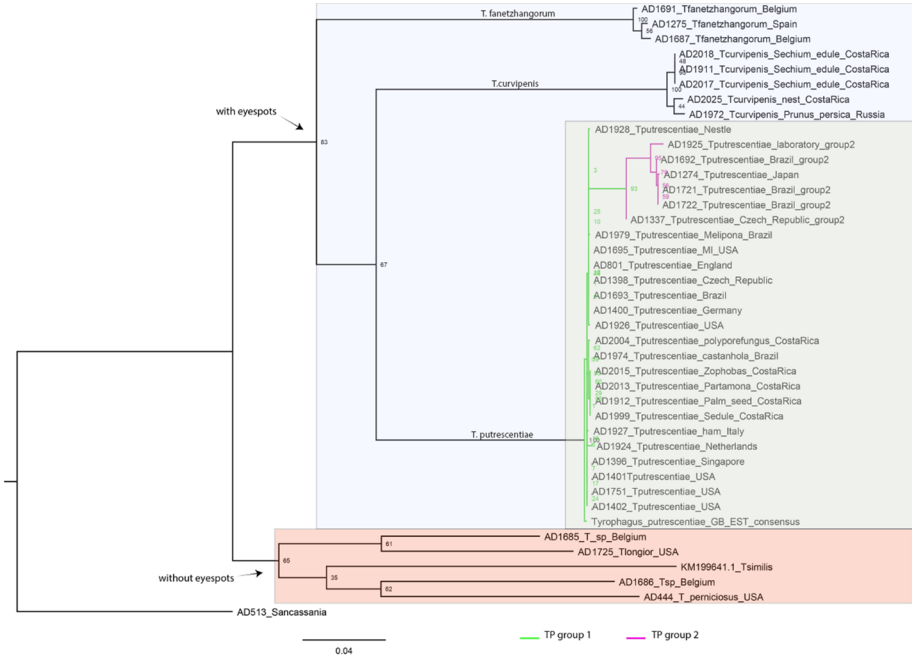

Fig. 5 Maximum likelihood tree for species of *Tyrophagus* inferred from COI sequences. Bootstrap support is indicated next to each branch

(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).

Genetic diversity indexes and haplotype network analysis of *Tyrophagus putrescentiae*

Genetic diversity indexes were calculated for *T. putrescentiae* (Table 2). A total of 1165 sites were monomorphic, out of 1227 nucleotides sequenced. From the 62 polymorphic sites, 57 included synonymous and five 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. 6). Haplotype diversity was 0.934 and the average number of nucleotide differences (K) was 16.667. The network (Fig. 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. 6). This sample has two unique non-synonymous mutations (Table 3). According with this network, AD1979 and AD1925, are the most dissimilar haplotypes from each other.

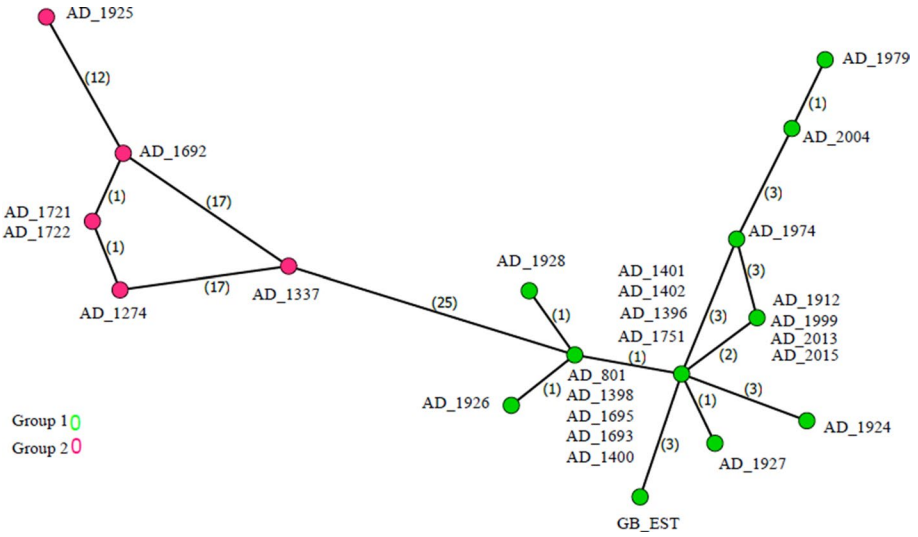

Fig. 6 Haplotypes network for *Tyrophagus putrescentiae*. Numbers in parenthesis correspond to the mutational steps. Green dots correspond to group 1 and pink dots to group 2. (Color figure online)

Discussion

Tyrophagus curvipenis: species boundaries, distribution and pest status

Even though *T. curvipenis* has been previously reported from several places around the world (Fig. 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*. *Tyrophagus 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) 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. Whereas *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 omnivorous 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, pers. comm. 2017).

Phylogeny of the genus *Tyrophagus*

Our topology (Fig. 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. 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, whereas our tree found a monophyletic group for all the species under study. This previous result, however, probably simply represents a rooting artifact (an in-group root was used) rather than true phylogenetic relationships.

Our phylogenetic tree (Fig. 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; Hamilton et al. 2014; 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 et al. 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). This species also showed one or two aminoacid changes for some of the populations sequenced here (Table 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 (377 bp) 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 (370 bp 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) showed a high intraspecific variability reaching 4.3% (K2P distances), whereas the difference for the other species did not exceed 1.3% (*T. curvipenis*, K2P distances). Considering these results, one might interpret the data for the populations with the highest genetic distances (4.3%) and place them as separate species; however, according with our barcode gap analysis (Fig. 4) the intraspecific variation for these mites can range from 0 to 5%.

The within-species distance of *T. putrescentiae* (4.3 or 5.8%, 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), 3.1% (Doña et al. 2015), 3% (Hebert et al. 2004b; 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) (conseravtively 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. 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 334 bp) corresponding to the central part of the COI region. Comparing these sequences with our trimmed fragments (1227 bp), 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.

Acknowledgements We thank Dr. Hugo Aguilar and Ing. Humberto Lezama from the University of Costa Rica for helping us arrange the visit and join us to the field. Greg Schneider (University of Michigan) and Ing. Humberto Lezama (University of Costa Rica) provided advice and practical help with sending samples from Costa Rica. Dr. Raquel Marchan (University of Michigan) helped with the elaboration of the map, and Dr. Abdolazim Mortazavi (Tarbiat Modares University) helped with some of the sequences used here. Maribel Beltrán for help with the illustrations. Martin Markovic and two anonymous reviewers provided important comments and feedback on the manuscript. PM was supported by Block grants from the Department of Ecology and Evolutionary Biology and a Rackham Graduate Student Research Grant (University of Michigan), University of Costa Rica (OAICE-08-CAB-147-2013), Consejo Nacional para Investigaciones Científicas y Tecnológicas (CONICIT-Costa Rica, FI-0161-13). PBK was supported by the Russian Foundation for Basic Research (No 15-04-05185-a and 15-04-02706 A to Sergey G. Ermilov), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Ciência sem Fronteiras (Brazil; PVE 88881.064989/2014-01), the Russian Science Foundation (Project No. 16-14-10109 to A. A. Khaustov), and the Ministry of Education and Science of the Russian Federation (No 6.1933.2014/K project code 1933). JH was supported by the Ministry of Agriculture of the Czech Republic (Project Number RO0417). The molecular work for this study was conducted in the Genomic Diversity Laboratory (GDL) of the Museum of Zoology of the University of Michigan.

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