



Evidence for co-invasion events: different chigger species (Actinotrichida, Trombidioidea: trombiculidae) share a host

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Abstract

Cases of co-invasion of various chigger species parasitizing murids and cricetids in various habitats were analysed using morphological and molecular approaches. Here we provide evidence for 25 new cases of co-parasitism of chigger mites on rodent hosts (*Myodes glareolus*, *Apodemus flavicollis*, *Apodemus agrarius*) accounting for 8.6% of all host-parasite associations observed in this study. The results confirm higher incidence of co-parasitism in vertebrate-associated Parasitengona mites compared to arthropod-associated ones. Among factors influencing the occurrence of co-parasitism in Trombiculidae the body constitution and year-round availability of hosts associated with lower host specificity of larvae should be considered.

Keywords Co-parasitism · Trombiculidae · Rodents · Host specificity · Topic preferences

Introduction

Trombiculid larvae, with few exceptions (Felska et al. 2018, and references therein) display host-parasite associations with vertebrates, mainly small mammals, as opposed to other parasitengones, which, except for few species (e.g. *Microtrombidium hirsutum* Womersley and *Johnstoniana rapax* Wendt et Eggers) which have departed from a parasitic lifestyle (Wohltmann 2000), encompass arthropod-parasitizing taxa.

The cases of simultaneous parasitism by arthropod-associated parasitengones have been reported by Southcott (1986), Key (1994), Wohltmann (2000, 2001) and Stahr (2014). Wohltmann (2001) estimated the occurrence of different ectoparasitic species on the same host at ca. 3% of observed invasions involving Parasitengona mites. Such invasions are often accompanied by distinct site preferences displayed by co-occurring species, for example, the water mites *Feltria rominji* Besseling and *Atractides nodipalpis* (Thor) parasitizing a chironomid fly, *Tanytarsus flavidulus*, or the terrestrial *Johnstoniana eximia* (Berlese) (= *J. tuberculata* Schweizer) and *Calyptostoma velutinum* (Müller) parasitizing a limoniid fly, *Limonia phragmitidis* (Efford 1963; Wohltmann 2001). According to Wohltmann

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(2000) the rarity of the phenomenon should be attributed to the fact that species inhabiting the same location generally use different host resources or display a temporal shift in using the same host resources.

Compared to arthropod-associated taxa, vertebrate-associated parasitengones (Trombiculidae s.l.) are thought to display lower selectivity toward hosts in favour of habitat selectivity (Daniel 1961; Goff 1979; Shatrov and Kudryashova 2008). The latter makes the co-occurrence of species more likely due to probably lower competitive interactions on the same host. Cases of co-parasitism of trombiculid species, widely scattered in the literature, refer mostly to the presence of 2–5 species on the same host (Mohr 1956; Daniel 1961; Vercammen-Grandjean 1963, 1975; Goff 1979; Haitlinger 1979; Kharadov and Chirov 2007; Barnard et al. 2015). Despite the close insight of some researchers into the frequency of records, the extent of the phenomenon and its ecological background remain largely unknown.

Here we present the cases of trombiculid co-invasions, discovered during our recent studies, along with the general characteristics of topic preferences displayed by larvae within the hosts' body. In order to confirm the separate identity of co-occurring species, we refer to morphological and molecular evidence.

Materials and methods

Ectoparasitic larvae were collected from murid [*Apodemus agrarius* (Pallas), *Apodemus flavicollis* (Melchior), *Apodemus sylvaticus* L.] and cricetid [*Myodes glareolus* (Schreber) and *Microtus oeconomus* (Pallas)] hosts. The hosts were captured in Sherman traps (permissions no. 66/2012, 27/2013 and 41/2013 issued by the Second Local Commission for Animal Experiments) at 23 localities in Poland, in anthropogenic [A] and natural [N] habitats, from 2012 to 2017:

- (1) Pyrzycko-Stargardzka Plain: Małkocin [A], N53°24' E15°01', leg. Hanna Moniuszko (HM);
- (2) Dolna Narew Valley: Kaszewiec [N], N52°52' E21°26', leg. HM;
- (3) Bielska Plain: Białowieża [N], N52°41' E23°52', leg. HM;
- (4) Wielkopolskie Lake District: Murowana Goślina [A], N52°33' E17°06', leg. HM, Paula Zajkowska (PZ);
- (5) Kujawskie Lake District: Babiak [N], N52°03' E18°23', leg. HM;
- (6) Twardogórskie Hills: Antonin [A], N51°33' E17°30', leg. HM;
- (7) Milicka Basin: Ruda Milicka [N], N51°31' E18°20', leg. Joanna Mąkol (JM), Grzegorz Zaleśny (GZ);
- (8) Milicka Basin: Milicz [N], N51°30' E17°15', leg. GZ;
- (9) Twardogórskie Hills: Syców [N], N51°17' E17°42', leg. HM;
- (10) Wieluńska Upland: Szachownica cave [N], N51°03' E18°48', leg. HM, PZ;
- (11) Ślęża Massif: Sobótka [N], N50°54' E16°44', leg. HM;
- (12) Karkonosze Mts: Szklarska Poręba [N], N50°50' E15°35', leg. HM, JM;
- (13) Jeleniogórska Basin: Karpacz [N], N50°46' E15°44', leg. HM;
- (14) Częstochowska Upland: Dobrogoszczyce [N], N50°36' E19°33', leg. HM;
- (15) Częstochowska Upland: Morsko [A], N50°31' E19°29', leg. HM, JM, PZ;
- (16) Stołowe Mts: Ostra Góra [N], N50°29' E16°18', leg. JM, GZ;
- (17) Orlickie Mts: Niemojów [N], N50°09' E16°33', leg. HM, JM;

- (18) Katowicka Upland: Łaziska [A], N50°08' E18°50', leg. HM;
- (19) Sanocko-Turczańskie Mts: Olchowiec [N], N49°26' E22°31', leg. PZ;
- (20) Western Tatra Mts: Zakopane [N], N49°16' E20°05', leg. Jan Cichocki (JC);
- (21) Western Bieszczady Mts: Lutowska [N], N49°15' E22°41', leg. HM;
- (22) Western Tatra Mts: at Zielony Staw [N], N49°14' E19°59', leg. JC;
- (23) Western Bieszczady Mts: Stuposiany [N], N49°11' E22°41', leg. HM.

Data on collection time (month) are provided in Table 1. Various habitats—including primeval forest, mountain river banks, deciduous mountain forest, mixed forest in a municipal park, ruderal surroundings of an artificial water reservoir and mountain clearing—were represented among the collection sites. Sampling at regular, 2- to 4-week intervals was carried out from September 2012 to September 2014 at locality no. 9, whereas for the remaining localities incidental collecting was applied.

Larvae were preserved in 96% ethanol. Specimens intended for experimental rearing were transferred to rearing vials, filled to 1/3 with a mixture of plaster-of-Paris and charcoal. The vials were placed in a climatic chamber with 80% humidity, L12/D12 photoperiod and 22 °C in light period/15 °C in the dark, set to imitate those recorded in the natural environment in the period when larvae develop into further instars. The experimental rearing aimed at obtaining subsequent instars from field-collected specimens, aiding also in species identification. The latter is especially important in case of taxa whose knowledge is limited to larvae, as it enables further comparisons with species known also from active post-larval forms, whereas in case of loss or damage of larval exuvia, it constitutes the basis for identification. The morphological description of instars, being beyond the scope of the present paper, will be dealt with separately.

In order to confirm the separate status of species distinguished based on morphological traits, the genetic distance between COI sequences of *Neotrombicula inopinata* (Oudemans), *Miyatrombicula muris* (Oudemans) sp. A and sp. B was estimated. The DNA was extracted from four specimens (larvae representing *N. inopinata* and *M. muris* and adults of tentatively representing spp. A and B). For DNA extraction and isolation protocol see Moniuszko et al. (2015). Amplification of the DNA barcode region (COI) was done using degenerate primers bcdF01 (5'-CATTTCCTACTAAYCATAARGAT ATTGG-3') and bcdR04 (5'-TATAAACYTCDGGATGNCCAAAAA-3') (Dabert et al. 2010). Multiple alignment was done using the CLUSTALW algorithm, implemented in the MEGA7 package (Kumar et al. 2016), with the default parameters (gap opening penalty—15, gap extension penalty—6.66, both for pairwise and multiple alignment). Pairwise sequence alignment and cutting resulted in a compact 518 bp data block. Distance calculation between sequences was performed using Kimura's two parameter model (K2P) (Kimura 1980) implemented in MEGA7. The sequences of *N. inopinata* and *M. muris* were deposited in the GenBank under accession numbers MH607466 and MH622154. For the purpose of comparison, we used sequences of *Hirsutiella zachvatkini* (Schluger) (acc. no. KR071845) and *Neotrombicula vulgaris* (Schluger) (acc. no. KY888693), earlier deposited in the GenBank (Moniuszko et al. 2015, 2017).

Specimens which served for morphological identification, including also the exoskeletons remaining after DNA extraction, were mounted on microscopic slides in Hoyer's medium. Photos were taken under Nikon Eclipse E600 coupled with DS-Fi1 camera and Nikon Eclipse 80i coupled with DS-Fi3 systems. The morphological identification of species based on larvae was based on the criteria provided by Kudryashova (1998).

Table 1 Co-invasions of trombiculid species

Co-habiting species	Number of co-habiting specimens representing two species, respectively	Host	Locality/collection time	Location of larvae on host
1 <i>Hirsutiella zachvatkini</i> / <i>Miyatrombicula muris</i>	13/3	<i>Myodes glareolus</i>	Loc. No. 3 August 2013	Ears
2 <i>H. zachvatkini</i> / <i>M. muris</i>	4/10	<i>M. glareolus</i>	Loc. No. 3 August 2013	Ears
3 <i>H. zachvatkini</i> / <i>M. muris</i>	4/1	<i>M. glareolus</i>	Loc. No. 3 August 2013	Ears
4 <i>H. zachvatkini</i> / <i>M. muris</i>	1/1	<i>Apodemus flavicollis</i>	Loc. No. 3 August 2013	Ears
5 <i>H. zachvatkini</i> / <i>M. muris</i>	4/12	<i>A. flavicollis</i>	Loc. No. 3 August 2013	Ears
6 <i>H. zachvatkini</i> / <i>M. muris</i>	6/4	<i>A. flavicollis</i>	Loc. No. 3 August 2013	Ears
7 <i>H. zachvatkini</i> / <i>M. muris</i>	7/3	<i>A. flavicollis</i>	Loc. No. 3 August 2013	Ears
8 <i>H. zachvatkini</i> / <i>Neotrombicula inopinata</i>	1/1	<i>A. flavicollis</i>	Loc. No. 3 August 2013	Ears
9 <i>H. zachvatkini</i> / <i>N. vulgaris</i>	26/15	<i>M. glareolus</i>	Loc. No. 4 October 2015	Ears
10 <i>H. zachvatkini</i> / <i>N. vulgaris</i>	2/6	<i>A. agrarius</i>	Loc. No. 4 October 2015	Ears
11 <i>H. zachvatkini</i> / <i>N. vulgaris</i>	4/9	<i>A. agrarius</i>	Loc. No. 4 October 2015	Ears
12 <i>H. zachvatkini</i> / <i>N. vulgaris</i>	4/1	<i>A. agrarius</i>	Loc. No. 4 October 2015	Ears
13 <i>H. zachvatkini</i> / <i>N. vulgaris</i>	4/1	<i>A. agrarius</i>	Loc. No. 4 October 2015	Ears
14 <i>H. zachvatkini</i> / <i>N. vulgaris</i>	4/2	<i>A. agrarius</i>	Loc. No. 4 October 2015	Ears

Table 1 (continued)

Co-habiting species	Number of co-habiting specimens representing two species, respectively	Host	Locality/collection time	Location of larvae on host
15 <i>H. zachvatkini</i> / <i>M. muris</i>	7/4	<i>A. flavicollis</i>	Loc. No. 9 May 2013	Ears
16 <i>H. zachvatkini</i> /species A	6/1	<i>A. flavicollis</i>	Loc. No. 16 August 2012	Ears
17 <i>H. zachvatkini</i> / <i>N. inopinata</i>	7/40	<i>M. glareolus</i>	Loc. No. 17 August 2015	Ears
18 <i>H. zachvatkini</i> / <i>N. inopinata</i>	10/6	<i>M. glareolus</i>	Loc. No. 17 August 2015	Ears
19 <i>H. zachvatkini</i> / <i>N. inopinata</i>	1/117	<i>M. glareolus</i>	Loc. No. 17 September 2013	Ears
20 <i>N. inopinata</i> /species A	17/1	<i>M. glareolus</i>	Loc. No. 20 September 2012	Ears
21 <i>N. inopinata</i> /species B	18/1	<i>M. glareolus</i>	Loc. No. 20 September 2012	Ears
22 <i>H. zachvatkini</i> / <i>N. inopinata</i>	3/3	<i>M. glareolus</i>	Loc. No. 20 September 2012	Ears
23 <i>H. zachvatkini</i> /species A	3/1	<i>M. glareolus</i>	Loc. No. 20 September 2012	Ears
24 <i>H. zachvatkini</i> / <i>N. inopinata</i>	151/11	<i>M. glareolus</i>	Loc. No. 23 March 2014	Ears (<i>H. zachvatkini</i>)/genitals (<i>N. inopinata</i>)
25 <i>H. zachvatkini</i> / <i>N. inopinata</i>	131/1	<i>M. glareolus</i>	Loc. No. 23 March 2014	Ears (<i>H. zachvatkini</i>)/genitals (<i>N. inopinata</i>)

Detailed locality data are provided in the “Materials and methods” section

Results

Of 291 rodents collected in the course of our studies and infested with Trombiculidae, 25 specimens (8.6%) carried more than one trombiculid species. Co-parasitizing chigger species (Table 1) were found in three out of five host species, at seven out of 23 localities examined. The hosts with co-occurring chigger species were captured in both disturbed and undisturbed habitats.

Altogether, six pairwise combinations of various sets of species were recorded. Co-occurring mites were assigned to four nominal species: *H. zachvatkini*, *Neotrombicula inopinata*, *Neotrombicula vulgaris* and *Miyatrombicula muris*. Of those, only *M. muris* remains known exclusively from larvae. In the course of laboratory rearing we obtained also deutonymphs and adults representing two distinct species, here referred to as sp. A and sp. B, due to the loss of larval exuvia that would allow comparison followed by correlation of instars for species hitherto known exclusively from larvae or would serve for new taxa description. Conspecificity of spp. A and B with *H. zachvatkini*, *N. inopinata* and *N. vulgaris* was excluded based on morphological and molecular evidence (for habitus of spp. A and B, *H. zachvatkini* and *N. inopinata* see Fig. 1), whereas the value of genetic distance between sp. A, sp. B (forward and reverse strand) and *M. muris* (reverse strand only) served as evidence for separate status of the latter three taxa. The genetic distance (K2P) between sequences obtained from species involved in multiple infestations ranged from 21.1 to 41.3%. The distance between congeneric species *N. inopinata*

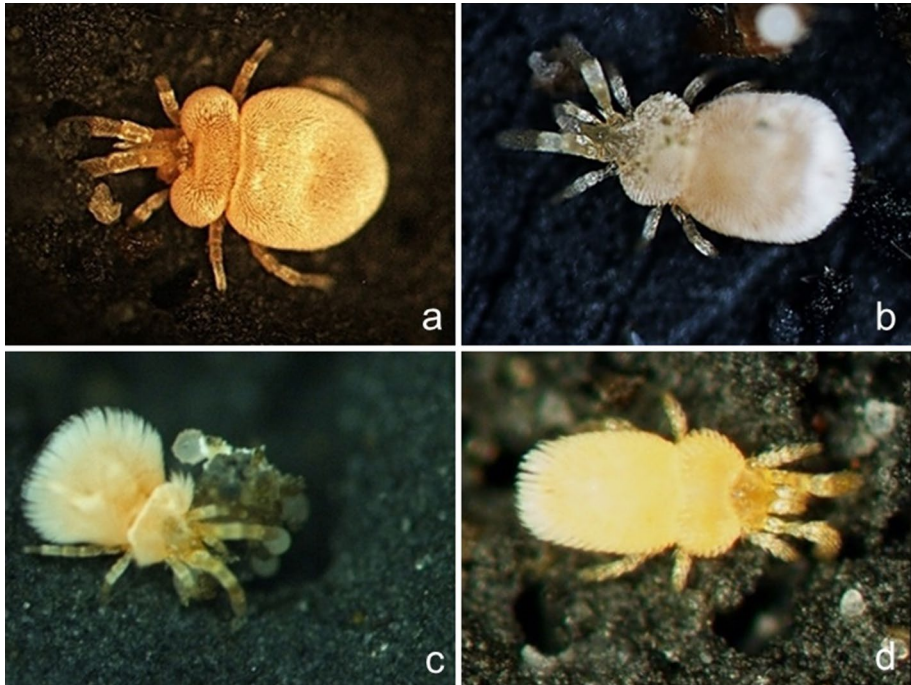


Fig. 1 Active postlarval forms experimentally reared from co-parasitizing larvae: **a** species A (adult), **b** species B (adult), **c** *Hirsutiella zachvatkini* (adult), **d** *Neotrombicula inopinata* (deutonymph) (not to scale)

and *N. vulgaris* was 24.2%. The most remote sequence from the others (34.3–41.3%) was sp. A. The pairwise differences are specified in Table 2.

The distinct specific affiliation of larvae of *H. zachvatkini*, *M. muris*, *N. inopinata* and *N. vulgaris* was confirmed based on slide-mounted specimens (for structure and chaetotaxy of prodorsal sclerite, being among the main diagnostic traits see Fig. 2), whereas the differences between *Neotrombicula* spp. and engorged specimens of *H. zachvatkini* could be also preliminarily inferred from the different coloration of idiosoma of live specimens (larvae dark orange in *N. inopinata* and in *N. vulgaris*, pale yellow in engorged *H. zachvatkini*).

The proportion of specimens representing different chigger species co-occurring on one host varied widely—from equal (1:1 on *A. flavicollis*) to vast predominance of one species (e.g., 131:1 on *M. glareolus*). The chiggers tended to form clusters, irrespective of the host and parasite species. For the majority of cases (23 out of 25) the larvae were attached to the ears of rodents, whereas only in two cases the parasitism within the genital area was stated. Both cases, however, refer to pairwise association that was recorded also within another part of the body (ears) in other host specimens and/or species, and in both of them the spatial segregation of parasitic larvae was observed.

Discussion

Cases of co-invasions were recorded in our study in various habitats and on three out of the five host species examined, which confirms the fairly widespread phenomenon. The varying number of samples collected both in anthropogenic and in undisturbed habitats as well as the season-dependent abundance of larvae do not allow to regard the absence of larvae on *A. sylvaticus* and on *M. oeconomus* as reflecting host- or habitat-dependent trends in the occurrence of multi-species invasions. The frequency of co-invasions, however, is likely to be higher in habitats influenced by anthropopressure. In his research carried out in Papua

Table 2 Pairwise genetic distance calculation (K2P) [%] for co-parasitizing trombiculid species

	Species A [DISE152]	Species B [DISE153]	<i>Neotrom- bicula inopinata</i> [MH607466]	<i>Miyatrom- bicula muris</i> [MH622154]	<i>Hirsutiella zachvatkini</i> [KR071845]	<i>N. vulgaris</i> [KY888693]
Species A [DISE152]						
Species B [DISE153]	36.7					
<i>Neotrombicula inopinata</i> [MH607466]	40.6	22.9				
<i>Miyatrombicula muris</i> [MH622154]	34.3	28.7	24.8			
<i>Hirsutiella zachvat- kini</i> [KR071845]	41.3	27.4	21.1	23.7		
<i>N. vulgaris</i> [KY888693]	41	22.4	24.1	26.8	26.4	

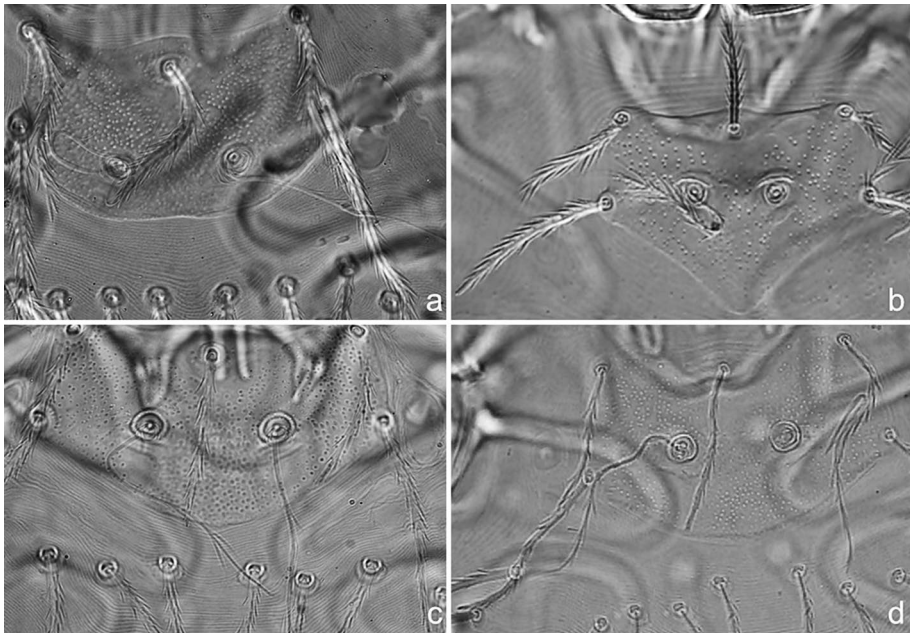


Fig. 2 Scutum of co-parasitizing species (larvae): **a** *Hirsutiella zachvatkini*, **b** *Miyatrombicula muris*, **c** *Neotrombicula inopinata*, **d** *N. vulgaris* (not to scale)

New Guinea, Goff (1979) reported 34.8% cases of co-parasitism at the generic level and 46% at the specific level for trombiculids associated with *Rattus ruber* in disturbed areas. The respective values calculated for chiggers associated with *Rattus niobe* in undisturbed habitats were 0.6 and 12.5%. The latter trend, considering the sample size, was slightly reflected also in our study, with the 100% incidence of co-invasions in the highly human-affected collection site in Murowana Goślina. The high incidence of co-invasions (up to 46%) in Papua New Guinea (Goff 1979), may be related to the much higher diversity and abundance of chigger species in the tropical compared to the temperate zone. As many as 114 nominal species of chiggers were reported from New Guinea (primarily from Papua New Guinea) (Goff 1982), whereas only 19 nominal species were recorded in Poland (Moniuszko and Małkol 2014; Małkol and Korniluk 2017).

For the majority of the recorded cases of parasitism, involving different host and parasite species, we could observe larvae to aggregate around the ear area. Only in the case of *N. inopinata* larvae were found also within the genital area. The latter observation, however, coincided with a relatively high level of infestation (Table 1). It cannot be excluded that larvae of particular species display a tendency to occupy alternative sites on the host's body at high levels of infestation in order to avoid competition with co-invading species. This may explain the differences in feeding sites observed in *Neotrombicula sympatricus* Stekolnikov in summer versus autumn, but also the reduced preferences for attachment sites observed in *H. zachvatkini* at maximum level of infestation in winter (Kharadov and Chirov 2007; Moniuszko and Małkol 2016). Nonetheless, the observed parasitopes are among those listed by Goff (1979) and Kharadov and Chirov (2007). According to Goff (1979) the topic preferences of chiggers may be genus-dependent. Traub and Wisseman (1968) claimed that topic preferences of larvae were

host species-dependent. Barnard et al. (2015) pointed out that different chigger species displayed similar, non-random preferences for attachment to certain areas on the host's body and the knowledge of spatial segregation of parasites on host, translated into particular assemblage of parasites (sub-infra-community), was largely neglected. The tendency to form multiple individuals and species clusters by larvae may be targeted at reduction of moisture loss, and thus underlines the role of microclimatic conditions for the survival of larvae (Traub and Wisseman 1974; Barnard et al. 2015).

The relatively high proportion of hitherto observed co-invasions points to the need of separate identification of each individual parasite. Especially at high level of infestation, the selective identification of larvae may lead to false conclusions. The higher proportion of co-invasions reported for trombiculid mites (8.6% in our study) compared to arthropod-associated terrestrial parasitengones may result from several factors. In case of chiggers, co-invasion may be facilitated by the constitution of the host body, physiological condition and availability of the hosts throughout the year, but also the potentially lower selectivity towards hosts in favour of habitat selectivity. According to Wohltmann (2000) parasitizing vertebrates, contrary to arthropod-associated taxa, reduced the selection pressures and made it possible for several species to exploit the same host synchronously. Xing-Yuan et al. (2007) revealed a positive correlation of both abundance and species richness of chigger mites versus the body weight of the host in *Apodemus chevrieri* (Milne-Edwards). For some trombiculids, for example *H. zachvatkini*, a prolonged contact with the host was described, extending beyond the parasitic phase; it also favours co-parasitic events (Moniuszko and Małkol 2016). The fact that widely distributed chiggers usually exploit more hosts (Wrenn and Loomis 1984) should also be considered in the context of possible, increased numbers of co-invasion.

The genetic distance between the six compared sequences from representatives of possibly five genera varied between 21.1 and 41.3%. Molecular intraspecific variation of trombiculid species is poorly known. Three COI sequences for *Neotrombicula microti* (Ewing) deposited in NCBI databases (Young et al. 2012) indicate that the intraspecific distance of this species is 0–3%, whereas the distance to another member of the same genus (*Neotrombicula* sp.) is 10–11.5%. Hebert et al. (2003, 2004) defined the 'barcoding gap' as the existence of average interspecific distance at least 10× greater than the average intraspecific genetic distance, but there are no guidelines for determination of higher systematic ranks (genera and families). Among other invertebrates K2P distances at COI within species, within genus and within family varied within 0.17–0.36, 5.8–9.1 and 10–12.5, respectively, for Lepidoptera (Hebert et al. 2003); within 0–7.6, 19–27 and 22.3–35.6 for Thysanoptera (Iftikhar et al. 2016); they were 0–7.7, up to 24.8 and up to 35.8 for Hemiptera (Park et al. 2011), up to 26.2, up to 37 and even up to 40.3 for molluscs (Sun et al. 2016). It is clear that all the analysed trombiculid specimens represent distinct species, whether they all belong to separate genera requires further analysis. Specimens assigned in our study to spp. A and B may actually represent one of the seven rodent-associated trombiculid species hitherto recorded from Poland (Moniuszko and Małkol 2014) and known exclusively from larvae.

Despite all the hitherto records, the actual prevalence of co-parasitism, its ecological background and topic segregation of parasites still remain largely obscure due to various factors. Of those, difficulties in proper processing and time-consuming counts of the mites from particular parts of the host body as pointed by Barnard et al. (2015) play a crucial role, but also the unrecognized intra- and interspecific variation of parasites, the selective sampling of mites at high intensity of infestation as well as the side-effect conclusions drawn by the way of projects focusing either on host or on particular species of parasite.

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