Inter- and intraspecific variability of morphological and molecular characters in *Allothrombium* species, with special reference to *Allothrombium fuliginosum*

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Abstract

Morphology-based identification of *Allothrombium* spp., in view of the limited knowledge of intraspecific variation, hinders the recognition of species borders and affects the views on the actual distribution of species. Therefore, identification will benefit from reference to molecular methods. The separate species identity of specimens putatively representing *Allothrombium fuliginosum* and *A. pulvinum*, both reported as widely distributed in the Palaearctic region and considered as potential biological control agents, was checked using morphological and molecular analyses. The representatives of various *Allothrombium* spp. collected in the Palaearctic were included in the analysis in order to ascertain the distance between species. The results of the morphological examination, supported by statistical inference, along with the comparison of COI and/or ITS2 sequences, weaken the hypothesis of synoccurrence of both species in the Palaearctic region. Hence, we hypothesize that *A. fuliginosum* is widely distributed in the Palaearctic, whereas *A. pulvinum* should be regarded a Nearctic species.

Keywords *Allothrombium* · Palaearctic · Morphology · COI · ITS2 · *Allothrombium pulvinum*

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Introduction

Among Trombidioidea (Acariformes: Parasitengona), the representatives of *Allothrombium* have been indicated the most effective agents in biological control of pests of crops and orchards (e.g. Welbourn 1983; Zhang 1992, 1998), with high potential in integrated pest management. Accurate assessment of their success as control agents is difficult, however, in view of insufficient knowledge of the taxonomy, biology and ecology of species (Muñoz-Cárdenas et al. 2015). Limited knowledge of intraspecific variation of morphological (qualitative and quantitative) traits in active developmental stages of *Allothrombium*, hinders the inference on actual species limits and, in consequence, affects the views on distribution patterns. A reference to molecular methods would contribute to verification of the status of presently distinguished, nominal taxa. Several members of *Allothrombium* have been known from single or few specimens representing only one instar. The description of heteromorphic instars (both larvae and active post-larval forms) has been provided for seven species, i.e. as few as 10% of all nominal taxa assigned to the genus (Moss 1962a, b; Robaux 1974; Robaux and Aeschlimann 1987; Zhang and Xin 1989b; Saboori and Zhang 1997; Saboori and Kamali 2000; Wohltmann and Małol 2009; Małol and Wohltmann 2012), whereas only for *Allothrombium fuliginosum* (Hermann), *Allothrombium ovatum* Zhang and Xin (known exclusively from larvae) and *Allothrombium pulvinum* Ewing substantial material was examined, encompassing long series of specimens (e.g. Małol 2005; Zhang 2010).

Both *A. fuliginosum* and *A. pulvinum* are among the most often listed members of the genus, associated with crops and orchards (e.g. Chen and Zhang 1991; Zhang and Chen 1993; Zhang et al. 1993; Zhang and Faraji 1994; Zhang 1998). *Allothrombium fuliginosum*, with the type locality in Poland (Małol 2005), was subject to detailed analyses, including data on morphology and life cycle (Henking 1882; Robaux 1974; Małol 2005) and is considered as widely distributed in the Western Palaearctic (Małol and Wohltmann 2012). A comprehensive treatise of *A. pulvinum*, supplemented with information on its biology and distribution, was provided by Zhang and Xin (1989a), Zhang and Faraji (1994), Saboori and Zhang (1996) and Zhang (1996). The data on occurrence of *A. pulvinum* are widely scattered in the Holarctic. The species, originally described from Marshall, Illinois, USA (Ewing 1917), has been recorded also from several countries in the Palaearctic region (Małol and Wohltmann 2012; Šundić and Pajović 2012). The actual distribution of both *A. fuliginosum* and *A. pulvinum*, however, may depart from the one reported in the literature, due to the still unresolved limits between the species.

The problem of separate identity of *A. pulvinum* and *A. fuliginosum*, already depicted by Zhang and Xin (1992), Małol (2005) and Wohltmann and Małol (2009), is especially crucial in view of their reported overlapping distribution in the Palaearctic and is further intensified by the recurrent records on *A. pulvinum* from the area in question. For the relatively well studied countries, with respect to fauna of *Allothrombium*, such as China, Iran and Poland (e.g. Zhang 1988; Zhang and Faraji 1994; Małol 2005; Khalili Mahani et al. 2009), the synoccurrence of both species has not been confirmed. The latter may suggest the limits of latitudinal distribution of both species falling on boundaries between the Western and Eastern Palaearctic or between the Palaearctic and Nearctic Region. On the other hand, given the overall scarcity of morphological differences between *A. fuliginosum* and *A. pulvinum* (for details see Zhang and Xin 1989a, b; Zhang and Faraji 1994), which pertain predominantly to larvae, the question of the synoccurrence of both species in the Palaearctic and of their overall separate identity, entails the necessity to appeal to other evidence.

The present study aims to discover character traits that differentiate between specimens assigned to *A. fuliginosum* and *A. pulvinum* originating from the Palearctic region, based on morphological and molecular data [cytochrome c oxidase subunit I (COI) and nuclear internal transcribed spacer of rDNA (ITS2)].

Materials and methods

The material included representatives of active life instars (larvae and post-larval forms) of *Allothrombium* spp. collected at various localities within the recorded distribution of *A. fuliginosum* and *A. pulvinum* in the Palearctic (for purpose of the present study, the name '*A. pulvinum*' is given in single quotation marks, in order to stress the Palearctic origin of specimens assigned to *A. pulvinum* based on preliminary identification of the material originating from Europe and Asia, and to distinguish them from North American representatives of the species). For specification and affiliation of the material see Table 1.

Larvae were also obtained from cultures in climatic chambers (material from Poland: 80% RH, L12 (22 °C):D12 (15 °C) photo-thermoperiod; material from Iran: 80% RH, L16 (27 °C):D8 (22 °C) photo-thermoperiod) started with field-collected, ovigerous females preliminarily assigned to *A. fuliginosum* (specimens from Poland) and '*A. pulvinum*' (specimens from Iran).

Thirty larvae and 15 specimens representing active post-larval forms of *A. fuliginosum* and of '*A. pulvinum*' were subjected to morphometric analysis. Damaged or obscure structures were excluded from the analysis. Due to the presence of three pairs of genital acetabula in the representatives of active post-larval forms of *Allothrombium* spp. (contrary to two pairs in deutonymphs and three in adults of most Trombidoidea), and in order to avoid misinterpretation of instars, all individuals representing active post-larval stages were pooled together. Morphological analysis covered 54 measurable traits of larvae and 52 of active post-larval forms. Additionally, the meristic characters of larvae were also considered in order to ascertain the constancy of character states. Measurements (in µm) were made under Nikon Eclipse E600 and 80i with differential interference contrast and NIS-Elements BR software. Shapiro–Wilk and Mann–Whitney U tests to check for normality and to compare sample means, as well as cluster analysis (K-means clustering), aiming at the discovery of groups representing potentially distinct species, were made using STATISTICA v.13.1 (Dell Software, Round Rock, TX, USA). The terminology and abbreviations follow Mąkol (2005).

In morphology-based identification of specimens assigned to *A. pulvinum* we refer to Ewing (1917), Zhang and Xin (1989b, 1992) and Zhang and Faraji (1994). Due to some incongruence in characteristics of the larva of *A. fuliginosum* provided by various authors (e.g. Henking 1882; Hirst 1926; Feider 1951), as already pointed at by Zhang and Xin (1992) and confirmed during our recent studies, we refer to the detailed description of the larva provided by Robaux (1974) and of larval and active post-larval forms by Mąkol (2005). The identification of other *Allothrombium* spp. was based on original descriptions (for references see Mąkol and Wohltmann 2012).

Eighteen specimens of *Allothrombium* spp. (Table 1) were designed for molecular examination. Altogether, 25 sequences were obtained, including 18 of COI and seven of ITS2 (including one partial sequence, forward strain only). For DNA extraction and isolation protocol see Moniuszko et al. (2015). Amplification of the COI barcode region was performed using degenerate primers: bcdF04 (5'-CATTTTCHACTAAYCATAARGAT

Table 1 Representatives of *Allothrombium* spp. used for morphological and molecular analyses

Locality/sampling date/collector(s)	Instar (number of specimens)	Sample ID	Molecular markers	Acc. no. (GenBank)	Species ^a
1. Belgium, Sint-Truiden, Limburg province, pear orchard, 50°48'N, 5°11'E; 28 October 2014; leg. N. Brenard	POSTLV (1)	DISE135	COI	MK962142	<i>A. fuliginosum</i>
2. Belgium, Flemish Brabant province, Bierbeek, pear orchard, 50°49'N, 4°45'E; 29 July 2015; leg. N. Brenard, M. Felska	POSTLV (1)	DISE156	COI, ITS2	MK962143, MK995465	<i>A. fuliginosum</i>
3. Belgium, Flemish Brabant province, Bierbeek, pear orchard, 50°49'N, 4°45'E; 23 July 2015; leg. N. Brenard, M. Felska	POSTLV (1)	DISE157	COI, ITS2	MK962144, MK995466	<i>A. fuliginosum</i>
4. China, Noah Organic Farm, Pinggu near Beijing, on aphid from the eggplant, 40°14'N, 116°08'E; 18 June 2016; leg. M. Felska	LV (2)	DISE172	COI	MK962145	<i>A. ovatum</i>
5. Croatia, Orahovica, side of the asphalt road, close to the beech forest, 45°32'N, 17°51'E, 263 m a.s.l.; 18 September 2017; leg. J. Pročków	POSTLV (1)	DISE226	COI, ITS2	MK962146, MK995468	<i>A. fuliginosum</i>
6. Denmark, Faroe Islands, Kol-lafjørður, 62°06'N, 6°54'W; 15 August 2011; leg. K. Poulsen	POSTLV (1)	DISE220	COI	MK962147	<i>A. fuliginosum</i>
7. Georgia, W Caucasus, Sukhumi, university campus, 42°58'N, 41°04'E, c. 50 m a.s.l., litter, <i>Eucapytus</i> , etc., 7 December 2003; leg. Yu. M. Marusik, G.N. Antipova	POSTLV (1)	DISE232	COI	MK962148	<i>Allothrombium</i> sp. 1

Table 1 (continued)

	Locality/sampling date/collector(s)	Instar (number of specimens)	Sample ID	Molecular markers	Acc. no. (GenBank)	Species ^a
8.	Iran, Nowshahr (Mazandaran province), garden close to the Caspian Sea coast, 36°39'N, 51°29'E; 10 April 2016; leg. A. Saboori	POSTLV (15), LV (30)	–	–	n/a	' <i>A. pulvinum</i> '
9.	Iran (locality as in row 8)	POSTLV (1)	DISE125	COI, ITS2	MN019009, MK995469	' <i>A. pulvinum</i> '
10.	Italy, South Tyrol, Egna, 46°18'N, 11°16'E; 1 May 2002; leg. H. Schatz	POSTLV (1)	DISE230	COI	MK962149	<i>A. fuliginosum</i>
11.	Poland, Wrocław, university campus, 51°05'N, 17°05'E; 21 April 2014; leg. M. Konikiewicz	POSTLV (5)	H7073, H7074, H7075, H7077, H7082	–	n/a	<i>A. fuliginosum</i>
12.	Poland, Wrocław, university campus, 51°05'N, 17°05'E; 11 October 2013; leg. M. Felska	POSTLV (10)	H7123a-j	–	n/a	<i>A. fuliginosum</i>
13.	Poland, Wrocław, university campus, 51°05'N, 17°05'E; 18 April 2013 (ovigerous female); leg. M. Felska; larvae obtained by experimental rearing (21 May 2013)	LV (9)	H5515	–	n/a	<i>A. fuliginosum</i>
14.	Poland, Wrocław, university campus, 51°05'N, 17°05'E; 10 April 2015 (ovigerous female); leg. B. Beszterda; larvae obtained by experimental rearing (18 May 2015)	LV (7)	BB119	–	n/a	<i>A. fuliginosum</i>
15.	Poland, Wrocław, university campus, 51°05'N, 17°05'E; 15 April 2013; leg. M. Felska	POSTLV (1)	DISE077	COI	MK962150	<i>A. fuliginosum</i>

Table 1 (continued)

	Locality/sampling date/collector(s)	Instar (number of specimens)	Sample ID	Molecular markers	Acc. no. (GenBank)	Species ^a
16.	Poland, Wrocław, university campus, 51°05'N, 17°05'E; 11 October 2013; leg. M. Felska	POSTLV (1)	DISE109	COI	MK962151	<i>A. fuliginosum</i>
17.	Poland, Gębzyce near Strzelin, 50°43'N, 17°03'E; 27 April 2013 (ovigerous female); leg. K. Gardziejewska; larvae obtained by experimental rearing (2 June 2013)	LV (14)	H5532	–	n/a	<i>A. fuliginosum</i>
18.	Poland, Gębzyce near Strzelin, 50°43'N, 17°03'E; 31 August 2013; leg. K. Gardziejewska	POSTLV (1)	DISE114	COI	MK995461	<i>A. fuliginosum</i>
19.	Poland, Maniów Wielki, 50°95'N, 16°69'E; 18 August 2013; leg. M. Felska	POSTLV (1)	DISE108	COI, ITS2	MK962152, MK995464	<i>A. fuliginosum</i>
20.	Poland, Wrzozowska Cedyńskie nature reserve near Osinów Dolny, 52°51'N, 14°10'E; 8 April 2011; leg. M. Wanat	POSTLV (1)	DISE158	COI	MK995462	<i>A. fuliginosum</i>
21.	Poland, Raduń near Chojna, slope with oak-elm forest, 52°57'N, 14°25'E; 7 April 2011; leg. M. Wanat	POSTLV (1)	DISE160	COI	MK962153	<i>A. fuliginosum</i>
22.	Poland, Zagorze Śl., Wałbrzyskie Mts, 50°45'N, 16°23'E; 10 September 2011; leg. E. Kowalska	POSTLV (1)	DISE161	COI, ITS2	MK995463, MK995467	<i>A. fuliginosum</i>
23.	Ukraine, Crimea, between Saky and Eupatoria, 45°11'N, 33°16'E, 1 m a.s.l.; 18 March 2002, leg. Y.M. Marusik	POSTLV (1)	DISE159	COI, ITS2 ^b	MK962154, MK995470	<i>Allothrombium</i> sp. 2

Table 1 (continued)

Locality/sampling date/collector(s)	Instar (number of specimens)	Sample ID	Molecular markers	Acc. no. (GenBank)	Species ^a
24. USA, North Carolina, Transylvania Co., Nantahala National Forest, low vegetation on trail to White-water Falls observation point; 35°2'N, 83°1'W; 16 June 2016; leg. W.C. Welbourn	POSTLV (1)	DISE185	COI	MK962155	<i>A. mitchelli</i>

LV larva, *POSTLV* active post-larval form (deutonymph or adult)

^aPreliminary identification based on morphological traits, supported with data on distribution and later (in case of specimens designed for molecular identification—columns 'Molecular analysis' and 'Acc. no.') confirmed by molecular data

^bForward sequence (524 bp) only

ATTGG-3') and bcdR04 (5'-TATAAACYTCDGGATGNCCAAAAA-3') (Dabert et al. 2010) in the following thermocycling conditions: 95 °C for 3 min—initial denaturation; 95 °C for 20 s, 46 °C for 20 s, 72 °C for 45 s—40 cycles; 72 °C for 7 min—final extending. Amplification of the ITS2 was performed using the following primers: LC1 (5'-CGAGTA TCGATGAAGAACGCAGC-3') and HC2 (5'-ATATGCTTAAGTTCAGCGGG-3') located between the 5.8S and 28S rDNA flanking regions, respectively (Navajas et al. 1992, 1994; Khalili Mahani et al. 2009). PCR conditions for the ITS2 fragment were as follows: 95 °C for 3 min—initial denaturation; 95 °C for 45 s, 53 °C for 45 s, 72 °C for 45 s—35 cycles; 72 °C for 7 min—final extending. The sequences are deposited in the GenBank under the following accession numbers: MK962142–MK962155, MK995461–MK995463, MN019009 and MK995464–MK995470 for COI and ITS2, respectively (for sample identification see Table 1). Voucher specimens are stored in the Department of Invertebrate Systematics and Ecology, Wrocław University of Environmental and Life Sciences (Wrocław, Poland). In case of single specimens originating from Crimea and Georgia, the identification was limited to genus level; however, the separate specific affiliation of both specimens, temporarily assigned to *Allothrombium* sp. 1 and *Allothrombium* sp. 2, as well as their distinctness to other *Allothrombium* spp. considered in the survey, could be confirmed by molecular analyses.

In addition to the sequences obtained in the present study and for purpose of comparison, the ITS2 sequences (FJ263064.1–FJ263089.1) of specimens assigned to *A. pulvinum* by Khalili Mahani et al. (2009) were retrieved from the GenBank. Multiple alignment was done with the ClustalW algorithm, implemented in MEGA7 package (Kumar et al. 2016), using the default parameters (gap opening penalty: 15, gap extension penalty: 6.66, both for pairwise and multiple alignment). All the sequences of the same gene region were trimmed to the same length to allow comparisons within each dataset. Distance between sequences was calculated using the Kimura 2-parameter model (K2P) (Kimura 1980). Standard error estimates were obtained by a bootstrap procedure (1000 replicates). Phylogenetic trees were constructed using maximum likelihood (MLE) and Bayesian (BE) estimates. MLE was based on the Kimura 2-parameter model and bootstrap analysis with 1000 replicates in MEGA7. Information on haplotype data for ITS2 sequences was estimated using the DnaSP software package v.6.12.01 (Rozas et al. 2017). Bayesian analyses were performed with MrBayes (Huelsenbeck and Ronquist 2001) using the GTR substitution model. Markov Chain Monte Carlo (MCMC) analysis was run for 1,000,000 generations and trees were sampled every 100 generations. The outgroup sequences (COI) were obtained from *Trombidium holosericeum* (Trombidiidae) (acc. no. MK995460), *Podothrombium* sp. (Podothrombiidae) (acc. no. MK995458), and *Platytrombidium fasciatum* (Microtrombidiidae) (acc. no. MK995459) in the present study, and from *Milandaniella intermedia* (Microtrombidiidae) (acc. no. KR633048.1; Felska and Małkol 2015).

Results

Larvae obtained by experimental rearing from females collected in Poland (*A. fuliginosum*) and in Iran ('*A. pulvinum*') displayed high constancy of qualitative traits. No characters that would allow to assign specimens to separate taxa were discovered. The constancy of character states (values) was revealed also during comparison of quantitative (meristic) traits (fPp: 0-0-0-BNN₂-BBNNNω; fD: 2-2-6-4-4-2=20; fV: 2-2-2u-2=8; leg I: Tr (1n)–Fe (5n)–Ge (4n, 2σ, 1κ)–Ti (5n, 2φ, 1κ)–Ta (16–17n, 2ζ, 1ω, 1ε); leg II:

Tr (1n)–Fe (4n)–Ge (3n, 2σ, 1κ)–Ti (5n, 2φ)–Ta (13–14n, 1ω, 1ε); leg III: Tr (1n)–Fe (4n)–Ge (3n, 2σ)–Ti (5n)–Ta (13n). For one larva collected in Iran the presence of five setae on one side of the symmetry axis on femur II, and in another specimen from the same area the presence of three solenidia on one side of symmetry axis was stated. The consistency of morphological traits was observed also in relation to active post-larval forms.

The analysis of metric data obtained for larvae and active post-larval forms of *A. fuliginosum* and '*A. pulvinum*' with the application of K-means algorithm did not result in the discovery of data groups, thus did not reveal the difference between the groups of specimens representing two potentially distinct species. However, the ranges of metric characters overlapped, both for larvae (Table 2) and active post-larval forms (Table 3) and statistically significant differences in several metric traits obtained from two potentially different species were observed (Tables 2 and 3).

The molecular analyses resulted in a positive response in case of 18 specimens representing *Allothrombium* spp., out of 37 designed for examination. For COI (18 sequences), the alignment resulted in obtaining sequences of 409 bp (531 bp before inclusion of the shortest sequence). In case of ITS2 (seven sequences), after aligning with the 26 sequences (920–925 bp) of '*A. pulvinum*' obtained from GenBank (Khalili Mahani et al. 2009), the data block length was 514 bp (849 bp before final inclusion of one partial sequence).

General topologies of the phylogenetic trees inferred by MLE and BE analyses of the nucleotide COI and ITS2 datasets (consisting in 18 and seven sequences, respectively) were similar and both estimators consistently revealed the same structure of *Allothrombium* spp. populations. Thus, only the ML trees are presented here (Figs. 1 and 2).

The tree based on COI sequences (Fig. 1) confirmed the separate identity of specimens preliminarily assigned to distinct species based on morphology. Members of *Allothrombium* spp. formed an ingroup clade which was strongly supported by bootstrap value (96%). The within-clade relationships showed an outlying sequence of clearly distinct *Allothrombium ovatum* (China), but also of *Allothrombium mitchelli* Davis (USA) + *Allothrombium* sp. 1 (Georgia) and of *Allothrombium* sp. 2 (Crimea). The clade encompassing *A. fuliginosum* from Belgium, Croatia, Denmark (Faroe Islands), Italy and Poland, but also '*A. pulvinum*' from Iran, was nested within *Allothrombium* spp. clade with 94% bootstrap support.

Eight haplotypes were detected for 33 ITS2 sequences of *Allothrombium* spp. (Supplementary Table S1, Fig. 2) with the overall haplotype diversity (Hd) amounting at 0.6742. Sequences obtained for '*A. pulvinum*' from Iran (Khalili Mahani et al. 2009 and present study) were assigned to haplotypes 1–5, sequences of *A. fuliginosum* from Poland, Belgium and Croatia to haplotypes 6 and 8, and sequence of *Allothrombium* sp. 2 from Crimea to haplotype 7 (Supplementary Table S1). All haplotypes, except no. 7, formed a clade, in which haplotypes 6 and 8 received the strongest bootstrap support (97%) (Fig. 2).

The genetic distance (K2P) in the COI region for specimens assigned to *A. fuliginosum* and to '*A. pulvinum*' was 0–5.3%; however, lower divergence of nucleotides between haplotypes (0–3.8%) was observed when the most departing sequence of *A. fuliginosum* from Belgium (DISE135) was excluded (Supplementary Table S2). The respective value of K2P calculated for '*A. pulvinum*' by Khalili Mahani et al. (2009) was 0.2–2.8% (Supplementary Table S3). The 'interspecific' distance between *A. fuliginosum* and '*A. pulvinum*' in the COI region, calculated as a distance between sequences of *A. fuliginosum* from Europe and the sequence of '*A. pulvinum*' from Iran (DISE125), was 1.7–4.8% (1.7–3.8% when the DISE135 was excluded from analysis).

The genetic distance in the COI region between clades formed by *A. fuliginosum* + '*A. pulvinum*' and sequences of congeneric taxa was 13.3–14.7% in relation to *Allothrombium*

Table 2 Morphometric data on larvae of *Allothrombium fuliginosum* from Poland, ‘*A. pulvinum*’ from Iran (present study) and ‘*A. pulvinum*’ from Spain, Iran and China. The character abbreviations are explained in Makol (2005)

Character	‘ <i>A. pulvinum</i> ’ Spain (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996) Mean (min.–max.)	‘ <i>A. pulvinum</i> ’ Iran (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996) Mean (min.–max.)	‘ <i>A. pulvinum</i> ’ China (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996) Mean (min.–max.)	<i>A. fuliginosum</i> Poland (n = 30) present study Mean ± SD (min.–max.)	‘ <i>A. pulvinum</i> ’ Iran (n = 30) present study Mean ± SD (min.–max.)
LB	–	–	–	399 ± 16.59a (377–435)	413 ± 23.30b (356–450)
WB	–	–	–	243 ± 10.79a (225–267)	261 ± 14.84b (213–284)
LB/WB	–	–	–	1.6 ± 0.07 (1.5–1.8)	1.6 ± 0.06 (1.5–1.8)
LS (=L)	102 (97–146)	138 (123–150)	133 (128–139)	141 ± 5.10 (130–150)	140 ± 6.65 (126–149)
WS (=W)	–	112 (103–119)	112 (108–115)	114 ± 3.58 (105–120)	114 ± 4.19 (103–121)
AM	47 (43–66)	49 (38–55)	45 (40–50)	48 ± 3.60a (41–55)	50 ± 2.98b (43–56)
AA	57 (54–69)	61 (56–65)	56 (54–88)	58 ± 2.75 (53–65)	59 ± 4.20 (51–68)
AL	63 (51–67)	61 (50–78)	63 (59–70)	57 ± 3.76 (51–65)	57 ± 3.20 (51–63)
AW	85 (75–86)	81 (75–88)	80 (79–81)	80 ± 3.72 (74–86)	80 ± 4.74 (71–88)
PL	75 (69–79)	73 (68–78)	76 (75–78)	73 ± 3.42 (66–78)	71 ± 2.49 (67–77)
PW	89 (72–105)	91 (83–100)	81 (79–85)	87 ± 3.39 (78–92)	86 ± 4.83 (76–96)
S	63 (56–67)	67 (58–88)	67 (62–70)	65 ± 3.75 (59–71)	65 ± 3.54 (60–73)
SB	58 (51–60)	53 (50–57)	51 (45–55)	54 ± 3.36 (47–60)	55 ± 3.54 (47–63)
ASB	76 (60–94)	87 (82–93)	84 (80–88)	91 ± 3.52a (83–98)	94 ± 5.07b (82–104)
PSB	46 (41–67)	51 (40–60)	49 (45–48) sic!	49 ± 2.96 (42–55)	48 ± 3.60 (40–55)
AP	32 (30–39)	35 (30–40)	33 (30–35)	37 ± 2.96 (33–44)	38 ± 3.39 (29–43)
MA	35 (30–49)	45 (43–47)	51 (48–54)	49 ± 2.55a (44–55)	51 ± 3.13b (46–57)
HS	40 (32–49)	40 (35–45)	47 (45–48)	45 ± 2.97 (40–49)	45 ± 2.10 (43–50)
LSS	85 (60–97)	76 (63–91)	88 (76–98)	93 ± 7.20 (79–104)	93 ± 5.43 (83–104)
SL	67 (66–77)	69 (53–78)	73 (68–83)	71 ± 3.60a (65–79)	78 ± 5.43b (69–89)
SS	40 (32–45)	38 (35–43)	37 (30–43)	37 ± 3.27a (31–46)	40 ± 3.15b (33–46)
pDS mean	–	–	–	66 ± 2.81 (59–71)	65 ± 3.59 (59–71)

Table 2 (continued)

Character	' <i>A. pulvinum</i> ' Spain (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996)	' <i>A. pulvinum</i> ' Iran (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996)	' <i>A. pulvinum</i> ' China (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996)	<i>A. fuliginosum</i> Poland (n = 30) present study	' <i>A. pulvinum</i> ' Iran (n = 30) present study
	Mean (min.–max.)	Mean (min.–max.)	Mean (min.–max.)	Mean ± SD (min.–max.)	Mean ± SD (min.–max.)
pDS min.	–	–	–	60 ± 2.67 (54–65)	59 ± 3.59 (52–65)
pDS max.	–	–	–	72 ± 3.54 (64–78)	72 ± 3.42 (64–79)
PaFe	–	–	–	33 ± 2.37 (27–38)	33 ± 2.38 (28–38)
PaGe	–	–	–	13 ± 1.17 (11–16)	13 ± 0.95 (11–15)
PaTi	–	–	–	17 ± 1.20 (14–19)	17 ± 1.12 (14–19)
PaTa	–	–	–	9 ± 0.72a (8–11)	10 ± 0.90b (8–11)
Odo	–	–	–	10 ± 1.55 (8–14)	10 ± 1.13 (8–13)
Ch base	–	–	–	55 ± 4.43a (47–62)	58 ± 3.97b (50–65)
ChCl	–	–	–	16 ± 1.32 (14–19)	16 ± 1.19 (14–19)
OS	–	–	–	30 ± 1.29 (27–33)	29 ± 1.00 (28–31)
Cx I	73 (69–90)	75 (70–80)	77 (75–78)	76 ± 3.33a (70–83)	78 ± 3.74b (71–86)
Tr I	47 (43–51)	52 (50–53)	48 (45–50)	49 ± 1.80 (45–52)	48 ± 2.32 (43–53)
Fe I	75 (73–94)	83 (78–88)	78 (70–83)	84 ± 2.69 (77–88)	83 ± 2.80 (76–88)
Ge I	44 (39–52)	51 (48–53)	44 (40–46)	47 ± 2.85 (41–52)	47 ± 1.83 (44–51)
Ti I	70 (66–84)	81 (78–87)	70 (63–75)	76 ± 5.64a (66–86)	79 ± 4.75b (71–88)
Ta I	97 (80–103)	92 (85–97)	93 (88–98)	96 ± 4.48 (88–102)	98 ± 2.81 (90–103)
Leg I	407 (387–446)	437 (416–460)	409 (389–419)	427 ± 12.01 (404–451)	432 ± 11.56 (410–451)
Cx II	80 (77–84)	83 (78–88)	84 (83–85)	81 ± 3.39 (74–86)	82 ± 3.29 (75–87)
Tr II	47 (39–51)	50 (50–50)	47 (40–50)	48 ± 2.34 (44–52)	47 ± 3.34 (41–53)
Fe II	82 (62–82)	76 (73–78)	71 (59–75)	75 ± 2.73 (69–81)	75 ± 3.9 (64–81)
Ge II	38 (34–47)	43 (40–48)	40 (33–45)	42 ± 3.02a (37–48)	44 ± 2.06b (38–47)

Table 2 (continued)

Character	' <i>A. pulvinum</i> ' Spain (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996)	' <i>A. pulvinum</i> ' Iran (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996)	' <i>A. pulvinum</i> ' China (n = 11) data combined after Zhang and Faraji (1994), Gol- darazena and Zhang (1996)	<i>A. fuliginosum</i> Poland (n = 30) present study	' <i>A. pulvinum</i> ' Iran (n = 30) present study
	Mean (min.–max.)	Mean (min.–max.)	Mean (min.–max.)	Mean ± SD (min.–max.)	Mean ± SD (min.–max.)
Ti II	77 (66–77)	77 (75–82)	67 (60–73)	72 ± 5.43a (63–83)	76 ± 3.29b (68–81)
Ta II	84 (76–97)	87 (83–90)	88 (83–90)	92 ± 4.07 (84–99)	93 ± 3.22 (86–100)
Leg II	407 (388–414)	414 (403–431)	397 (377–410)	409 ± 12.96a (389–431)	417 ± 10.14b (394–433)
Cx III	76 (71–79)	78 (75–83)	76 (75–78)	76 ± 2.75a (70–81)	78 ± 2.85b (74–84)
Tr III	57 (51–71)	62 (55–67)	55 (53–58)	54 ± 1.96 (50–57)	54 ± 2.46 (50–60)
Fe III	72 (66–82)	76 (73–78)	72 (65–80)	78 ± 4.43 (71–86)	80 ± 4.07 (71–87)
Ge III	43 (39–52)	50 (45–55)	43 (39–45)	45 ± 2.54a (40–50)	47 ± 2.84b (41–52)
Ti III	84 (75–96)	96 (88–105)	83 (78–88)	88 ± 5.05a (80–99)	92 ± 4.68b (84–101)
Ta III	98 (94–107)	102 (95–110)	96 (93–100)	101 ± 4.84 (92–110)	102 ± 3.10 (97–108)
Leg III	430 (425–452)	464 (436–486)	425 (412–440)	442 ± 11.62a (415–462)	454 ± 13.11b (426–474)
IP	1244 (1203–1296)	1316 (1255–1365)	1234 (1178–1269)	1278 ± 33.43a (1210–1332)	1304 ± 31.05b (1233–1350)

LS length of scutum, WS width of scutum (SD and W, respectively, of Zhang and Faraji (1994) and Goldarazena and Zhang (1996))

Values for specimens from Spain rounded to integers. OS ocular sclerite

Different letters following SD indicate significant difference of character values between *A. fuliginosum* from Poland and '*A. pulvinum*' from Iran (Mann–Whitney U tests; $p < 0.05$)

Table 3 Morphometric data on active post-larval forms of *Allothrombium fuliginosum* from Poland and ‘*A. pulvinum*’ from Iran (present study). The character abbreviations are explained in Małol (2005)

Character	n	<i>A. fuliginosum</i> Mean $\pm$ SD (min.–max.)	n	‘ <i>A. pulvinum</i> ’ Mean $\pm$ SD (min.–max.)
LB	15	2608 $\pm$ 326.62 (2174–3127)	13	2727 $\pm$ 338.63 (2160–3172)
WB	15	1967 $\pm$ 277.6 (1644–2435)	13	1789 $\pm$ 236.47 (1427–2131)
LB/WB	15	1.29 $\pm$ 0.13a (1.1–1.6)	13	1.53 $\pm$ 0.17b (1.3–2.0)
PaTa	15	209 $\pm$ 17.01a (180–236)	15	194 $\pm$ 12.80b (170–210)
Odo	15	120 $\pm$ 11.68a (101–139)	13	111 $\pm$ 6.72b (98–121)
ChCl	15	91 $\pm$ 8.32 (80–103)	15	88 $\pm$ 6.27 (76–96)
CML	15	461 $\pm$ 33.48 (420–535)	15	436 $\pm$ 18.41 (403–465)
S	11	236 $\pm$ 23.43a (206–267)	13	211 $\pm$ 11.15b (191–228)
SB	15	100 $\pm$ 12.52 (82–116)	15	97 $\pm$ 7.97 (85–111)
aPr	15	163 $\pm$ 24.71 (122–198)	15	155 $\pm$ 13.95 (132–176)
pPr	15	97 $\pm$ 8.79 (85–114)	15	100 $\pm$ 9.24 (86–111)
pDS min.	15	75 $\pm$ 3.78a (69–82)	15	68 $\pm$ 8.77b (51–79)
pDS max.	15	100 $\pm$ 2.64a (97–105)	15	94 $\pm$ 5.80b (83–104)
OP	15	124 $\pm$ 8.41a (113–141)	15	116 $\pm$ 7.35b (105–128)
pVS	15	57 $\pm$ 4.62 (52–68)	15	60 $\pm$ 4.55 (54–68)
GOP (L)	15	346 $\pm$ 26.63 (290–393)	15	339 $\pm$ 16.08 (313–362)
GOP (W)	15	214 $\pm$ 30.70a (150–256)	14	192 $\pm$ 20.93b (161–229)
GOP (L/W)	15	1.65 $\pm$ 0.29 (1.1–2.3)	14	1.8 $\pm$ 0.21 (1.5–2.1)
Cx I	15	450 $\pm$ 24.01 (400–475)	14	455 $\pm$ 21.19 (407–487)
Tr I	15	265 $\pm$ 15.94a (239–290)	15	229 $\pm$ 18.94b (210–264)
bFe I	15	316 $\pm$ 20.49a (285–350)	15	283 $\pm$ 16.51b (256–306)
tFe I	15	362 $\pm$ 21.76a (322–392)	15	321 $\pm$ 23.49b (287–351)
Ge I	15	482 $\pm$ 33.90a (423–520)	15	430 $\pm$ 28.16b (385–483)
Ti I	15	481 $\pm$ 33.79a (435–528)	14	452 $\pm$ 23.59b (415–492)
Ta I (L)	15	508 $\pm$ 38.57a (445–554)	13	478 $\pm$ 20.44b (449–515)
Ta I (W)	15	182 $\pm$ 18.88 (155–225)	13	174 $\pm$ 13.05 (148–187)
Leg I	15	2865 $\pm$ 163.46 (2582–3050)	13	2833 $\pm$ 115.82 (2604–2969)
Cx II	15	340 $\pm$ 18.18a (312–370)	13	297 $\pm$ 10.40b (282–313)
Tr II	15	184 $\pm$ 12.54a (170–205)	15	153 $\pm$ 10.36b (140–172)
bFe II	15	202 $\pm$ 12a (185–220)	15	191 $\pm$ 12.76b (167–210)
tFe II	15	195 $\pm$ 14.37a (172–215)	15	173 $\pm$ 15.16b (148–198)
Ge II	15	258 $\pm$ 16.42a (230–290)	15	234 $\pm$ 16.24b (204–266)
Ti II	15	293 $\pm$ 22.69a (262–335)	15	274 $\pm$ 15.29b (251–298)
Ta II (L)	15	386 $\pm$ 21.32a (340–414)	15	368 $\pm$ 15.70b (335–387)
Leg II	15	1859 $\pm$ 94.85a (1702–2027)	14	1686 $\pm$ 53.86b (1596–1788)
Cx III	15	274 $\pm$ 21.94a (233–307)	14	252 $\pm$ 11.42b (235–277)
Tr III	15	169 $\pm$ 15.03a (142–190)	15	145 $\pm$ 10.01b (131–160)
bFe III	15	194 $\pm$ 13.36a (165–220)	15	170 $\pm$ 11.51b (155–187)
tFe III	15	176 $\pm$ 12.98a (150–196)	14	159 $\pm$ 9.98b (145–176)
Ge III	15	242 $\pm$ 17.15a (217–278)	14	225 $\pm$ 15.41b (199–253)
Ti III	15	280 $\pm$ 20.77 (245–315)	14	266 $\pm$ 18.82 (236–289)
Ta III (L)	15	354 $\pm$ 21.95 (320–385)	14	346 $\pm$ 15.56 (311–376)
Leg III	15	1689 $\pm$ 97.53a (1503–1853)	14	1563 $\pm$ 64.85b (1426–1665)

Table 3 (continued)

Character	n	<i>A. fuliginosum</i> Mean $\pm$ SD (min.–max.)	n	<i>'A. pulvinum'</i> Mean $\pm$ SD (min.–max.)
Cx IV	15	355 $\pm$ 26.69a (312–398)	14	322 $\pm$ 13.05b (290–338)
Tr IV	15	213 $\pm$ 18.12a (183–245)	15	175 $\pm$ 10.22b (159–194)
bFe IV	15	239 $\pm$ 16.53a (210–276)	15	216 $\pm$ 15.28b (178–237)
tFe IV	15	251 $\pm$ 16.47a (225–288)	15	238 $\pm$ 15.39b (216–262)
Ge IV	15	336 $\pm$ 23.70 (305–390)	15	324 $\pm$ 20.84 (287–357)
Ti IV	15	381 $\pm$ 31.73 (345–462)	15	378 $\pm$ 23.93 (323–412)
Ta IV (L)	15	424 $\pm$ 30.04 (375–484)	15	412 $\pm$ 19.88 (374–437)
Leg IV	15	2200 $\pm$ 136.85a (1984–2524)	15	2065 $\pm$ 92.53b (1839–2191)
IP	15	8613 $\pm$ 472.13a (7833–9421)	15	8167 $\pm$ 257.06b (7655–8520)

OP eye peduncle

Different letters following SD indicate significant difference of character values between *A. fuliginosum* from Poland and *'A. pulvinum'* from Iran (Mann–Whitney U tests: $p < 0.05$)

Fig. 1 COI bootstrap original tree, analysis by ML method, sequences 409 bp long

sp. 2 collected in Crimea, 16.1–17.3% in relation to *A. mitchelli*, 19.1–20.5% in relation to *Allothrombium* sp. 1 collected in Georgia and 22.4–24.5% in relation to *A. ovatum* (Supplementary Table S2).

Fig. 2 ITS2 bootstrap original tree, analysis by ML method, sequences 514 bp long. Hap=haplotype

The genetic distance (K2P) in the ITS2 region for specimens representing *A. fuliginosum* and '*A. pulvinum*' was 0–0.2% (Supplementary Table S4), whereas for '*A. pulvinum*' (after Khalili Mahani et al. 2009) it attained the range 0–0.5% (Supplementary Table S5). The distance between the European *A. fuliginosum* and Iranian '*A. pulvinum*' (sequence DISE125) amounting at 1.7–1.9%, was about 10× lower than the distance (17.9–18.9%) between *A. fuliginosum* + '*A. pulvinum*' clade and *Allothrombium* sp. 2 collected in Crimea (Supplementary Table 4). The putative interspecific variation between specimens preliminarily assigned to both nominal species (*A. fuliginosum*, '*A. pulvinum*') was slightly higher than the magnitude of intraspecific variation, calculated independently for each 'species'.

The overall low value of genetic distance between the nucleotide sequences obtained from *A. fuliginosum* and from specimens preliminarily assigned to '*A. pulvinum*' originating from the Palaearctic (COI: 1.7–3.8%; ITS2: 1.7–1.9%), along with the lack of well supported differences in morphological traits, militate in favor of recognizing the common species identity of both.

Discussion

The complex taxonomic situation within the genus *Allothrombium*, with special reference to taxa inhabiting the same geographic units, has resulted mainly from insufficiency of reliable morphological characters that allow species discrimination. The limited knowledge of intraspecific variation constitutes the main obstacle in studies on the biology of species and their use in biological control.

Several authors referred to the problem related to morphology-based discrimination of *Allothrombium* spp., with special reference to *A. fuliginosum* and *A. pulvinum*. The first description of *A. pulvinum* larvae was provided by Howard (1918), based on material collected in Minnesota, USA. Zhang and Xin (1989b), when listing detailed characteristics of larvae collected in China, referred to the presence of four setae on femur II, contrary to five setae reported earlier by Robaux (1974) for *A. fuliginosum*. It is noteworthy that Robaux (1974) pointed to the presence of five setae on femur II in *A. fuliginosum*, whereas in the drawing accompanying the description (Fig. 12 in Robaux 1974) four setae are shown. Nevertheless, the difference in the character state (5 vs. 4 setae on femur II) was followed also by subsequent authors (Zhang and Xin 1992; Zhang and Faraji 1994; Zhang

and Norbakhsh 1995) as a criterion to distinguish between larvae of *A. fuliginosum* and *A. pulvinum*. At the same time, due to much less attention paid to active post-larval forms, no differences between deutonymphs and adults of potentially distinct species have been depicted.

Zhang (1996), in his comprehensive study on parasitism of aphids by larvae of *Allothrombium*, stated that *A. pulvinum* from USA and UK, both examined by the author (Zhang 1991, 1996), differed in some morphological measurements and that their biology and host relations might also differ. Zhang and Faraji (1994) compared the specimens of *A. pulvinum* from China with those collected for the first time in Iran and attributed the metric differences in some quantitative traits to intraspecific geographical variation. Zhang and Xin (1992) and Zhang (1996) claimed that what was reported as *A. fuliginosum* may actually be a mixture of several species, whereas Wohltmann and Małol (2009) considered the larvae of *A. pulvinum* and of yet another species, *Allothrombium reinholdi* Haitlinger, as falling within the variability range of *A. fuliginosum*.

The question of separate identity of *A. fuliginosum* and *A. pulvinum* became the subject of long-lasting dispute. Having in mind the necessity to refer to type material of *A. pulvinum*, originating from the Nearctic for purpose of possible synonymisation, another question has been posed of likely misidentification of specimens actually representing *A. fuliginosum*, recorded in the Palaearctic.

During our examination of *A. fuliginosum* from the Western Palaearctic we have not come across larval specimens with five setae on femur II and only in one specimen originating from Iran and preliminarily assigned to *A. pulvinum*, the asymmetrical state (5n/4n) in chaetotaxy of femur II was observed, most likely caused by some developmental malformation. The larvae of *A. fuliginosum* revealed high consistency of meristic traits with larvae assigned to *A. pulvinum*, examined by Zhang and Faraji (1994).

The reference to morphology-based identification of species, at limited knowledge of intraspecific variation, but also in view of possible misidentifications caused by the paucity of diagnostic traits, has blurred the inference on species borders and has stimulated the application of molecular methods. Moreover, the molecular evidence allows to avoid false inference related to characters which display phenotypic plasticity. However, despite the recently intensified efforts made at ascertaining species limits by means of molecular methods, identification still largely depends on morphology.

COI and ITS2 markers have been commonly applied as DNA barcodes (e.g. Navajas et al. 1992, 1994, 1996; Ben-David et al. 2007; Xie et al. 2008; Yao et al. 2010) to assess genetic variation and to find differences between species and strains that cannot be distinguished on the basis of morphological features. The method has been hitherto applied to a different extent in relation to various systematic groups and, at the same time, the diversified criteria for taxa discrimination have been adopted. Hebert et al. (2004) proposed a 10× rule, being the 10× average intraspecific difference between COI sequences that can sanction provisional species distinction. Smith et al. (2005) considered the COI sequence divergence threshold of 3% for Molecular Operational Taxonomic Unit (MOTU) as adequate for morphologically defined species.

Yang et al. (2011) reported an intraspecific distance up to 3% in COI and up to 7.8% in ITS2 at identification of six species of astigmatid mites in China. Ben-David et al. (2007) used a 2% threshold in ITS2 sequence to confirm the separate identity of species assigned to *Tetranychus* spp. and claimed that nucleotide divergence of 1–2% should be treated carefully and involve the confirmation of species identity by morphological criteria. The low nucleotide divergence of 1.7% (Gotoh et al. 1998), 2.5 and 1.2% (Navajas et al. 1997) between other morphologically similar *Tetranychus* spp., supported

by detailed morphological analyses and crossing experiments, confirmed the separate identity of species. Despite several attempts made to establish a standard limit between intra- and interspecific difference, none of the limits can be generalized to many groups of organisms (Puillandre et al. 2012).

A survey of Khalili Mahani et al. (2009), who investigated genetic variation of *A. pulvinum* in Iran by COI and ITS2 sequences, revealed that at least two geographically distinct populations or even more than one species are represented in the material. No corresponding studies on genetic variation of *A. fuliginosum* have been carried out until present. Due to the fact that different primers for COI amplification were used by Khalili Mahani et al. (2009) and in the present study, only a direct comparison of ITS2 sequences between *A. fuliginosum* and '*A. pulvinum*' was possible. In our study, with the application of phylogenetic tree method for COI sequences, a specimen of '*A. pulvinum*' (DISE125, Iran) was located in the clade containing specimens of *A. fuliginosum*. The intraspecific nucleotide divergence for *A. fuliginosum* + '*A. pulvinum*' based on ITS2 sequences obtained from geographically distinct populations ranged from 1.7 to 1.9%.

The data on two or more nominal species which, based on molecular evidence, have overlapping variability ranges and thus their names become synonyms, are still scarce, contrary to the increasing research effort devoted to cryptic species in mites (e.g. Skoracka and Dabert 2010; Skoracka et al. 2012, 2015; Tixier et al. 2006, 2017).

Our study corroborates the variously developed and interpreted criteria and, again, illustrates the necessity of an integrative taxonomic approach. Two hypotheses could be proposed here: (i) specimens representing the Palaearctic populations of *A. fuliginosum* and '*A. pulvinum*' belong to the same species which is characterised by relatively high molecular polymorphisms, and (ii) specimens assigned to *A. fuliginosum* and '*A. pulvinum*' represent distinct, however closely related species. In case of (ii), the common identity of *A. pulvinum* from the Nearctic and of Palaearctic members of '*A. pulvinum*' should be confirmed in future studies, based on molecular methods. The present survey does not allow an answer to the question whether both *A. fuliginosum* and *A. pulvinum* are present in Iran. However, the common species identity of specimens collected in Europe and in Asia (Iran), is also supported by inference made by Zhang and Faraji (1994) on conspecificity of material ('*A. pulvinum*') originating from China and Iran.

In conclusion, we hypothesize that *A. fuliginosum* is a species widely distributed in the Palaearctic. The wide host spectrum reported for larvae of *A. fuliginosum* (Zhang 1996 for *A. pulvinum* collected in England; Felska et al. 2018) allows to place the species among generalists. Similarly, an even wider array of hosts has been hitherto reported for *A. pulvinum* (Felska et al. 2018); however, due to the questioned occurrence of the species in the Palaearctic, the records from the eastern hemisphere may actually expand the list of hosts known for *A. fuliginosum*. The differences in metric data (already pointed to by Zhang and Faraji 1994), but also the slight shift in phenology of specimens originating from different populations of *A. fuliginosum* collected within the Palaearctic realm, should be attributed to geographical variation.

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