SYSTEMATICS

On the Identity of Cereal Aphid Parasitoid Wasps *Aphidius uzbekistanicus*, *Aphidius rhopalosiphi*, and *Aphidius avenaphis* (Hymenoptera: Braconidae: Aphidiinae) by Examination of COI Mitochondrial Gene, Geometric Morphometrics, and Morphology

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ABSTRACT In this study, the relationships among and the taxonomic status of three closely related parasitic wasps that are widely used as biological control agents of cereal aphids, *Aphidius uzbekistanicus* Luzhetzki, *Aphidius rhopalosiphi* De Stefani Perez, and *Aphidius avenaphis* (Fitch), were examined. Genetic divergence at an average of 6% was recorded between *A. uzbekistanicus* and *A. rhopalosiphi* by using the mitochondrial (mt) gene cytochrome oxidase I (COI) barcoding region. Identical mtCOI gene sequences were observed in *A. uzbekistanicus* specimens that originated from Eurasia and in the North American species *A. avenaphis*. The haplotype fluctuation in *A. rhopalosiphi* specimens that originated from the west Palaearctic was an average of 1.5% (maximum, 2.4%). In contrast, specimens of *A. uzbekistanicus* from central and western parts of Eurasia were largely homogenous, with only a single mutation recorded in a specimen from eastern Europe (Serbia). The morphological and genetic diversity found in *A. rhopalosiphi* may suggest the existence of cryptic species, especially for lineages that have a large degree of mtCOI diversity and sympatric occurrence. The geometric morphometric analysis of stigma shape presented in this study demonstrated that members of *A. uzbekistanicus* have a shorter forewing r vein and a more elongated stigma, relative to those of *A. avenaphis*. Our research validates the use of stigma shape and flagellomere 1 color for morphological discrimination between wasp species.

KEY WORDS aphidiine wasps, morphological, genetic diversity, barcoding

Aphids associated with cereals are destructive pests and are a limiting factor in cereal production worldwide (Vickerman and Wratten 1979, Dixon 1987, Blackman and Eastop 2000). Among the viruses they transmit is barley yellow dwarf virus, the most widely distributed and most destructive viral pathogen that affects cereals around the world, causing significant economic losses in small grains (Fomitcheva et al. 2005). Aphidiinae parasitoids that prey upon aphids feeding on cereals contribute to aphid control and have been used as biological control agents (Starý 1972, 1981). Their impact was reported after the implementation of cereal aphid biological control programs in South America by Starý (1980). Brewer and Elliott (2004) summarized many of the biological control efforts that use aphid parasitoids to combat cereal aphids in North America.

Among a wide range of cereal aphid parasitoids, *Aphidius uzbekistanicus* Luzhetzki and *Aphidius rhopalosiphi* De Stefani Perez were determined to be the key species in the control of cereal aphid populations in the West Palaearctic (Starý 1972, 1981). Although they are considered to be different species, they are difficult to differentiate following the key provided by Eady (1969) and the several other contributions to this topic (Starý 1972, 1973, 1981; Powell 1982; Kavallieratos et al. 2005). The overlapping of morphological characteristics used for species separation even led to the synonymization of *A. uzbekistanicus* under *A. rhopalosiphi* by Pungerl (1983, 1986). Adding to the confusion is that *A. uzbekistanicus* and *A. rhopalosiphi* often occur in sympatry with a similar host range pattern. The host range of *A. uzbekistanicus* is mostly restricted to *Sitobion* spp. and to a lesser degree to *Metopolophium dirhodum* (Walker) and *Schizaphis graminum* (Rondani) (Starý 1981; Tomanović et al.

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1999, 2008), whereas *A. rhopalosiphi* has a broader host range, including *Diuraphis* spp., *Metopolophium* spp., *S. graminum, Sitobion* spp., and *Rhopalosiphum* spp. (Pennacchio 1989; Höller 1990, 1991; Pennacchio and Höller 1990; Tomanović et al. 1999, 2003, 2008; Kavallieratos et al. 2004, 2005). Due to identification problems between *A. uzbekistanicus* and *A. rhopalosiphi*, some published parasitoid-host aphid associations should be reexamined (Pungerl 1984, Pennacchio and Höller 1990).

A. uzbekistanicus and *A. rhopalosiphi* were introduced to Chile and Brazil, where they have a significant impact on the English grain aphid, *Sitobion avenae* (F.) (Zuñiga 1987). *S. avenae* and *M. dirhodum* were first reported in 1967 to attack wheat, *Triticum aestivum* L., crops in Chile. Barley yellow dwarf virus, transmitted by these aphids, caused a loss in wheat production of ≈20% in 1975 (Zuñiga 1986b, Altieri 1992). Biological control programs initiated in Chile and Brazil led to the introduction of 14 aphid parasitoid species in 1976 and 1978 (Zuñiga 1987, Altieri et al. 1989). By 1980, the total number of released parasitoids was ≈1,000,000 individuals in Brazil (Zuñiga 1986a) and 7,500,000 individuals in Chile (Zuñiga 1986b). After the successful introduction and establishment of *A. uzbekistanicus* and *A. rhopalosiphi* in Chile and Brazil, Argentina also intended to introduce four species of parasitoids from 1980 to 1982 from Brazil and France (USDA, European Parasite Laboratory) to control the cereal aphids *M. dirhodum* and *S. avenae.* However, *A. uzbekistanicus* and *A. rhopalosiphi* were not released because their populations naturally moved from Brazil to Argentina and established there (Botto et al. 1995). Furthermore, *A. uzbekistanicus* was introduced to fight cereal aphids in Idaho in 1988 (Halbert et al. 1996). Both *Aphidius* species contributed to the natural control of the newly identified cereal aphid pest, *Sitobion festucae* ssp. *cerealium* Stroyan, manifesting themselves simultaneously as biological control agents in Chile (Starý et al. 1994). In addition, *A. uzbekistanicus* and *A. rhopalosiphi* were classified as keystone species in aphid parasitoid associations on *Bacillus thuringiensis* transgenic maize, *Zea mays* L. (Pons and Starý 2003) and are now important aspects of organic farming and landscape management (Paoletti et al. 1997, Macfayden et al. 2009).

Aphidius avenaphis (Fitch) is North American cereal aphid parasitoid (Marsh 1979; Pike et al. 1997, 2000). It was described as *Praon avenaphis* Fitch 1861, and was reported to occur in United States and Canada (Fitch 1861, Fletcher 1899, Muesebeck and Walkley 1951). However, there are no reliable taxonomic characteristics to separate *A. avenaphis* from the Palaearctic species *A. uzbekistanicus* (Pike et al. 1997). Watanabe (1941) noted that *Aphidius granarius* Marshall (synonymized under *A. uzbekistanicus*) (Starý 1973) from Europe is probably a synonym of *A. avenaphis*, which is a parasitoid of the same aphids in North America.

In this study, we explore the relationships among three closely related cereal aphid parasitoids: *A. rhopalosiphi* and *A. uzbekistanicus* from the Palaearctic and *A. avenaphis* from North America. DNA sequence variation from the mitochondrial (mt) gene cytochrome oxidase I (COI) was used to characterize the evolutionary divergences among them. We also use geometric morphometrics to characterize *A. avenaphis* and *A. uzbekistanicus,* which was necessary due to the lack of morphological characters to distinguish them. The aim of our study is to evaluate the taxonomic status of the three important cereal aphid parasitoids that are commonly used as biological control agents and to test the validity of morphological characteristics previously used for their identification.

Materials and Methods

Study Area and Materials for DNA Study. *Aphidius* specimens were collected from at least 12 countries throughout the Central and West Palaearctic and from North America. For genetic variation study, 50 *Aphidius* specimens in total were collected from known aphids and host plants. When parasitoids were collected from mixed populations of different hosts, all aphid species were listed as a possible host source. All specimens were collected after emerging from mummies, preserved in 96% ethanol, and maintained at 4°C. Sampling localities and data related to the collected specimens are summarized in Table 1. Our approach was based on DNA sequencing of the specimens that were accepted a priori as well-identified individuals (Meyer and Paulay 2005).

DNA Extraction, Polymerase Chain Reaction (PCR) Amplification, and Sequencing. Total genomic DNA was extracted from each individual adult wasp using the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. The barcoding region of the mitochondrial COI gene was amplified and sequenced from a total of 50 individuals representing three species: *A. uzbekistanicus, A. rhopalosiphi,* and *A. avenaphis.* A fragment of ≈710 bp of the barcoding region of the mtCOI gene was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGCTGACCAAAAAATCA-3′) (Folmer et al. 1994). DNA amplification was performed in 20 μl of total reaction volume. As template, the reaction mixture contained 1 μl of the extracted DNA, 3.75 mM $MgCl_2$, 0.6 mM each dNTP, 0.5 μM each primer, and 2 U of *Taq* polymerase (MBI Fermentas, Hanover, MD). PCR cycles were carried out in a Mastercyclerep (Eppendorf, Hamburg, Germany) using the following thermal profile: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 54°C for 1 min, 72°C for 90 s, and a final extension step at 72°C for 7 min. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN) according to manufacturer's instructions. DNA sequencing was performed by BMR Service (Padova, Italy), and nucleotide sequence data were deposited in GenBank.

Genetic Analysis. Sequences were edited using FinchTV (www.geospiza.com) and then aligned by CLUSTALW integrated in MEGA4 software (Tamura et al. 2007). Kimura's two-parameter method (K2P) of

Table 1. Sampling data for the *A. rhopalosiphi*, *A. uzbekistanicus*, and *A. avenaphis* specimens used in this study

Code	Parasitoid	Aphid	Plant	Locality	Haplotype code	Accession
A. rhop - SWE K50	*A. rhopalosiphi*	*R. padi*	*Hordeum vulgare* L.	Sweden (Fru Alstad)	R1	JN164770
A. rhop - SWE K51	*A. rhopalosiphi*	*R. padi*	*H. vulgare*	Sweden (Borgeby)	R1	JN164774
A. rhop - GER K3	*A. rhopalosiphi*	*R. padi*	*Triticum aestivum* L.	Germany (Bodensee)	R1	JN164753
A. rhop - GER K5	*A. rhopalosiphi*	*R. padi*	*T. aestivum*	Germany (Waake)	R1	JN164755
A. rhop - GER K6	*A. rhopalosiphi*	*M. dirhodum*	*T. aestivum*	Germany (Reinshof)	R1	JN164756
A. rhop - GER K7	*A. rhopalosiphi*	*R. padi*	*T. aestivum*	Germany (Grossenrode)	R1	JN164757
A. rhop - GER K58	*A. rhopalosiphi*	*M. dirhodum*	*T. aestivum*	Germany (Jena)	R1	JN164760
A. rhop - CZE K26	*A. rhopalosiphi*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	R1	JN164761
A. rhop - CZE K27	*A. rhopalosiphi*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	R1	JN164762
A. rhop - POL K42	*A. rhopalosiphi*	*S. avenae*	*T. aestivum*	Poland (Kopana)	R1	JN164767
A. rhop - SLO K12	*A. rhopalosiphi*	*S. avenae*	*H. vulgare*	Slovenia (Olimje)	R1	JN164773
A. rhop - GER K4	*A. rhopalosiphi*	*R. padi*	*T. aestivum*	Germany (Grossenbrode)	R2	JN164754
A. rhop - GER K8	*A. rhopalosiphi*	*M. dirhodum*	*T. aestivum*	Germany (Benniehausen)	R2	JN164758
A. rhop - GER K57	*A. rhopalosiphi*	*M. dirhodum*	*T. aestivum*	Germany (Jena)	R2	JN164759
A. rhop - SLO K10	*A. rhopalosiphi*	*S. avenae*	*H. vulgare*	Slovenia (Koper) R3	R3	JN164771
A. rhop - SLO K11	*A. rhopalosiphi*	*S. avenae*	*H. vulgare*	Slovenia (Koper)	R3	JN164772
A. rhop - SER K14	*A. rhopalosiphi*	*S. avenae*	*T. aestivum*	Serbia (Padinska skela)	R3	JN164775
A. rhop - SER K25	*A. rhopalosiphi*	*S. avenae*	*T. aestivum*	Serbia (Ovča)	R3	JN164776
A. rhop - CZE K37	*A. rhopalosiphi*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	R3	JN164763
A. rhop - CZE K38	*A. rhopalosiphi*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	R3	JN164764
A. rhop - CZE K41	*A. rhopalosiphi*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	R3	JN164766
A. rhop - SWE K45	*A. rhopalosiphi*	*R. padi*	*H. vulgare*	Sweden (Oderup)	R4,R5	JN164769
A. rhop - CZE K40	*A. rhopalosiphi*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	R4,R5	JN164765
A. rhop - POL K53	*A. rhopalosiphi*	*M. dirhodum*	*T. aestivum*	Poland (Górna Wieś)	R4,R5	JN164768
A. rhop - SER K54	*A. rhopalosiphi*	*S. scirpi*	*Typha* sp.	Serbia (Padinska skela)	R6	JN164778
A. rhop - SER K55	*A. rhopalosiphi*	*S. scirpi*	*Typha* sp.	Serbia (Padinska skela)	R6	JN164779
A. rhop - SER K52	*A. rhopalosiphi*	*S. avenae*	*T. aestivum*	Serbia (Padinska skela)	R7	JN164777
A. uzbe - GER K1	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Germany (Ebergoetzen)	U1	JN164741
A. uzbe - SLO K9	*A. uzbekistanicus*	*S. avenae*	*H. vulgare*	Slovenia (Kranj)	U1	JN164745
A. uzbe - IRN K16	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Iran (Mashad)	U1	JN164735
A. uzbe - IRN K17	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Iran (Ram Hormoz)	U1	JN164736
A. uzbe - IRN K18	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Iran (Tehran)	U1	JN164737
A. uzbe - CZE K28	*A. uzbekistanicus*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	U1	JN164749
A. uzbe - PAK K31	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Pakistan	U1	JN164738
A. uzbe - PAK K32	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Pakistan	U1	JN164739
A. uzbe - PAK K33	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Pakistan	U1	JN164740
A. uzbe - CZE K36	*A. uzbekistanicus*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic	U1	JN164750
A. uzbe - POL K39	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Poland (Pogroszew)	U1	JN164743
A. uzbe - CZE K43	*A. uzbekistanicus*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic (J. Moravia)	U1	JN164751
A. uzbe - CZE K44	*A. uzbekistanicus*	*S. avenae, R. padi, D. noxia*	*H. vulgare*	Czech Republic (J. Moravia)	U1	JN164744
A. uzbe - POL K46	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Poland (Duninopol)	U1	JN164742
A. uzbe - SER K47	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Serbia (Kovilovo)	U1	JN164747
A. uzbe - SER K48	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Serbia (Kovilovo)	U1	JN164748
A. uzbe - SWE K49	*A. uzbekistanicus*	*S. avenae*	*H. vulgare*	Sweden (Bessinge)	U1	JN164752
A. uzbe - SER K13	*A. uzbekistanicus*	*S. avenae*	*T. aestivum*	Serbia (Ovča)	U2	JN164746
A. avenaphis- USA K62	*A. avenaphis*	*D. noxia, S. avenae*	*T. aestivum*	USA (Benton)	A1	JN164780
A. avenaphis - USA K64	*A. avenaphis*	*D.noxia, S.avenae*	*T. aestivum*	USA (Benton)	A1	JN164781
A. avenaphis - USA K65	*A. avenaphis*	*S. avenae*	*T. aestivum*	USA (Benton)	A1	JN164782
A. avenaphis - USA K67	*A. avenaphis*	*S. avenae*	*T. aestivum*	USA (Benton)	A1	JN164783
A. avenaphis - USA K68	*A. avenaphis*	*S. avenae*	*T. aestivum*	USA (Benton)	A1	JN164784
A. avenae - GER A2	*A. avenae*	*S. avenae*	*T. aestivum*	Germany (Jena)		JN164785
Ephedrus blattnyi E3	*E. blattnyi*	*Pterocomma rufipes* (Hartig)	*Salix retusa* L.	Montenegro (Durmitor)		JN164786

base substitution was used to calculate average genetic distances between sequences within each group and between groups of species by the bootstrap method (500 replicates). Neighbor joining (NJ) and maximum parsimony (MP) trees also were obtained using MEGA4 software. Five hundred bootstrap replicates were performed to assess branch support in the resulting trees. *Aphidius avenae* Haliday and *Ephedrus blattnyi* Starý were used as outgroups taxa for molecular phylogenetic analyses. In addition, to infer *A.*

Table 2. Percentages of correct identification based on stigma shape

Species assignment	Host aphid	Population	*N*	% correct
A. uzbekistanicus	*S. avenae*	Iran, Tehran	4	0
A. uzbekistanicus	*S. avenae*	Pakistan	11	81
A. uzbekistanicus	*S. avenae*	Serbia	34	85
A. uzbekistanicus	*S. avenae*	Czech Republic	6	33
A. uzbekistanicus	*S. avenae*	Iran, Mashad	21	52
A. avenaphis	*S. avenae*	USA	19	95
Total			95	73

rhopalosiphi haplotype networks using statistical parsimony (Templeton et al. 1992), TCS version 1.21 (Clement et al. 2000) was used with a confidence limit of 95%.

Geometric Morphometrics. Wings of insect parasitoids have important adaptive and functional roles in the environment regarding host location, oviposition behavior, and mating (Godfray 1994, Wootton 2001). To confirm the native status of the *A. avenaphis* population, we compared the wing shapes of *A. avenaphis* and *A. uzbekistanicus.* The latter was introduced from the Palaearctic to North America several times.

The geometric morphometric approach (Zelditch et al. 2004) was applied to explore and quantify variation in the shape of the pterostigma. Ninety-five female specimens of five *A. uzbekistanicus* populations and one *A. avenaphis* population all of which emerged from the aphid host *Sitobion avenae* (F.) were investigated (the number of specimens per population for each species are given in Table 2). *A. uzbekistanicus* material was collected from four countries in Central and West Palaearctic, whereas *A. avenaphis* material originated from North America. Specimens were collected between 1995 and 2009. The left wing of each specimen was photographed using a Canon G10 digital camera, connected to a Leica microscope at 50× magnification. To describe pterostigma shape, we used a combination of landmarks and semilandmarks. In total, four landmarks and 11 semilandmarks were chosen to describe pterostigma shape (their positions and definitions are given in Fig. 1). All landmarks and semilandmarks were digitized using TpsDig software (Rohlf 2005) by the same person. Before digitizing, semilandmarks were defined using MakeFan6. By placing "fans" on the images, we ensured the consistent placement of semilandmarks at equal angular displacements along the curves superimposed by the Generalized Procrustes Analysis, which eliminates variation due to the scale, position and orientation of landmark configurations (Rohlf and Slice 1990, Bookstein 1991). Semilandmarks were superimposed by allowing them to slide along curves bounded by landmarks to minimize the Procrustes distances among individuals (Bookstein 1997). Superimposition of

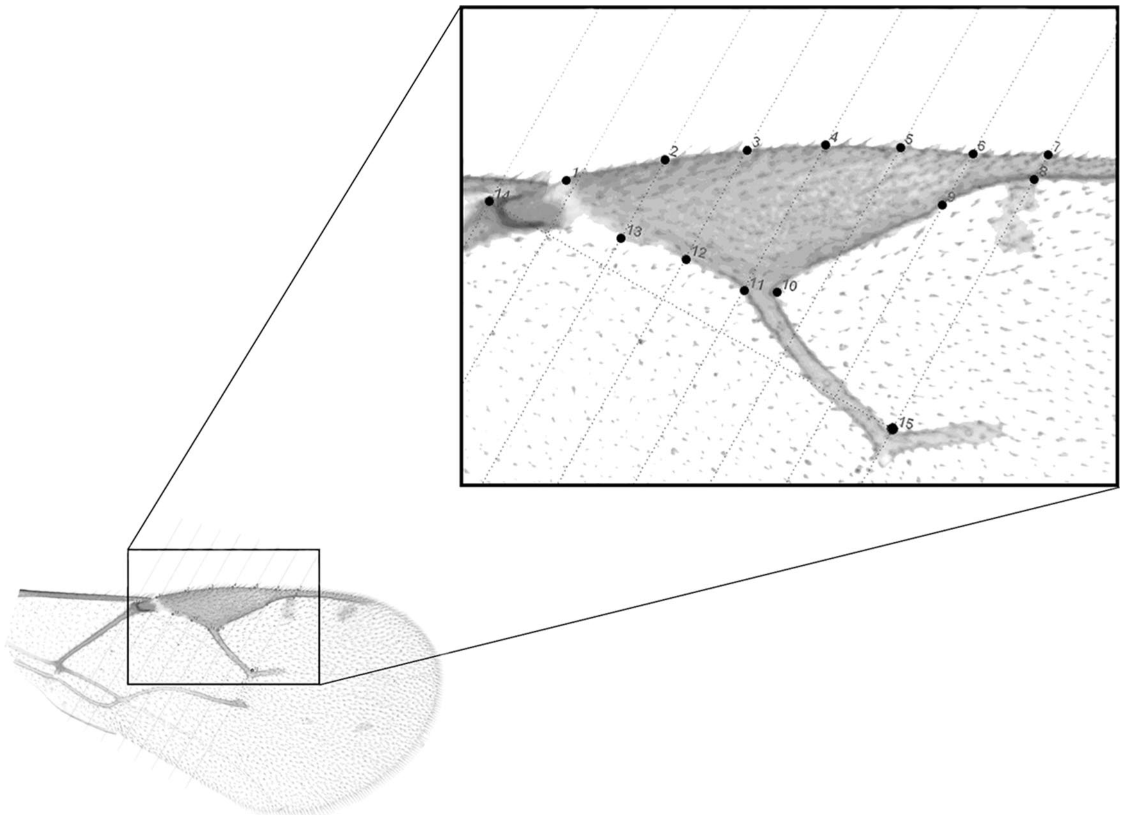

Fig. 1. Forewing stigma of *A. avenaphis* with marked landmarks (1, 10, 14, and 15) and semilandmarks (2–9 and 11–13).

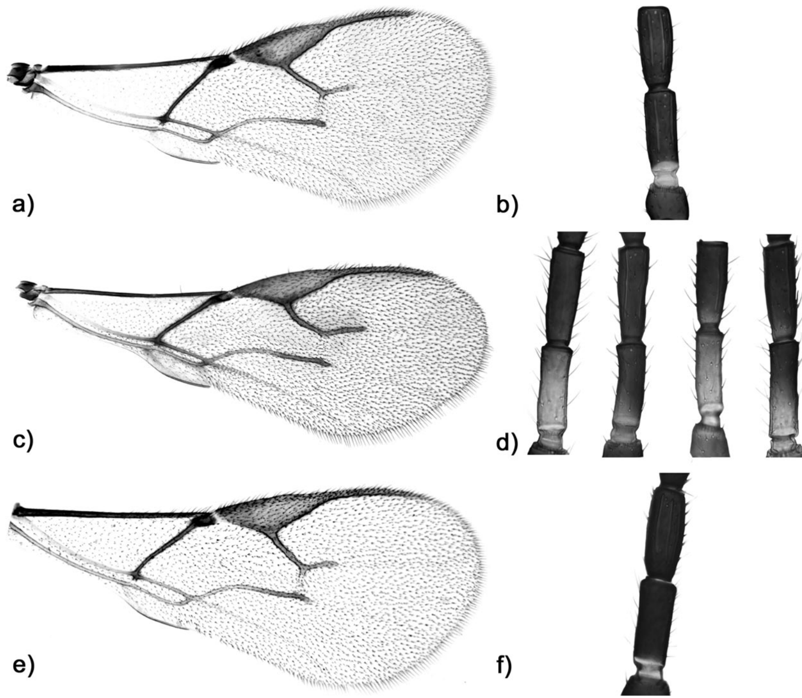

Fig. 2. The *A. uzbekistanicus*, *A. rhopalosiphi*, and *A. avenaphis* forewing (a, c, and e) and F1 and two (b, d, and f), respectively.

semilandmarks was done in SemiLand6. Programs MakeFan6 and Semiland6 belong to the IMP series (Sheets 2003). To analyze variation in the shape of pterostigma, we used the full set of shape variables (partial warps, including both nonuniform and uniform shape components).

Multivariate analysis of variance was performed to test differences in the pterostigma shape between populations. To explore differences in shape between populations, we performed canonical variate analysis. The program MorphoJ was used to analyze and visualize shape changes described by canonical axis (Klingenberg 2008). All standard statistical analyzes were performed in SAS (SAS Institute 2009).

Morphology. The morphology of all specimens was determined before they were used in DNA study. We defined specimen morphology on the basis of taxonomic characters that are commonly used in their characterization and separation (Starý 1981, Pennacchio and Höller 1990, Höller 1991, Tomanović et al. 1999). The *A. rhopalosiphi, A. uzbekistanicus* and *A. avenaphis* material was morphologically compared using the color pattern of the flagellomeres (=F) 1, 2; by the number of palpomeres of the maxillary and labial palps; and by shape of stigma (Fig. 2). The terminology used in this paper regarding taxonomic characters of the aphidiines follows Sharkey and Wharton (1997). All material used in this study is deposited in the

Table 3. Evolutionary divergence between haplotypes based on the pairwise analysis of 627-bp mtCOI sequences conducted using Kimura two-parameter method in Mega4 (R1–R7 = *A. rhopalosiphi;* U = *A. uzbekistanicus;* AA = *A. avenaphis*)

	1	2	3	4	5	6	7	8	9	10	11	12
1. R1[a]												
2. R2	0.005											
3. R3	0.006	0.008										
4. R4	0.018	0.016	0.015									
5. R5	0.019	0.018	0.016	0.002								
6. R6	0.008	0.010	0.011	0.023	0.024							
7. R7	0.023	0.021	0.019	0.018	0.019	0.024						
8. U1[b]	0.062	0.062	0.062	0.058	0.060	0.063	0.058					
9. U2	0.063	0.063	0.063	0.060	0.062	0.065	0.060	0.002				
10. AA1[c]	0.062	0.062	0.062	0.058	0.060	0.063	0.058	0.000	0.002			
11. *A. avenae*	0.086	0.088	0.086	0.079	0.081	0.086	0.081	0.092	0.093	0.092		
12. *E. blattnyi*	0.190	0.186	0.184	0.188	0.190	0.188	0.186	0.213	0.215	0.213	0.209	

[a] R, *A. rhopalosiphi.*
[b] U, *A. uzbekistanicus.*
[c] AA, *A. avenaphis.*

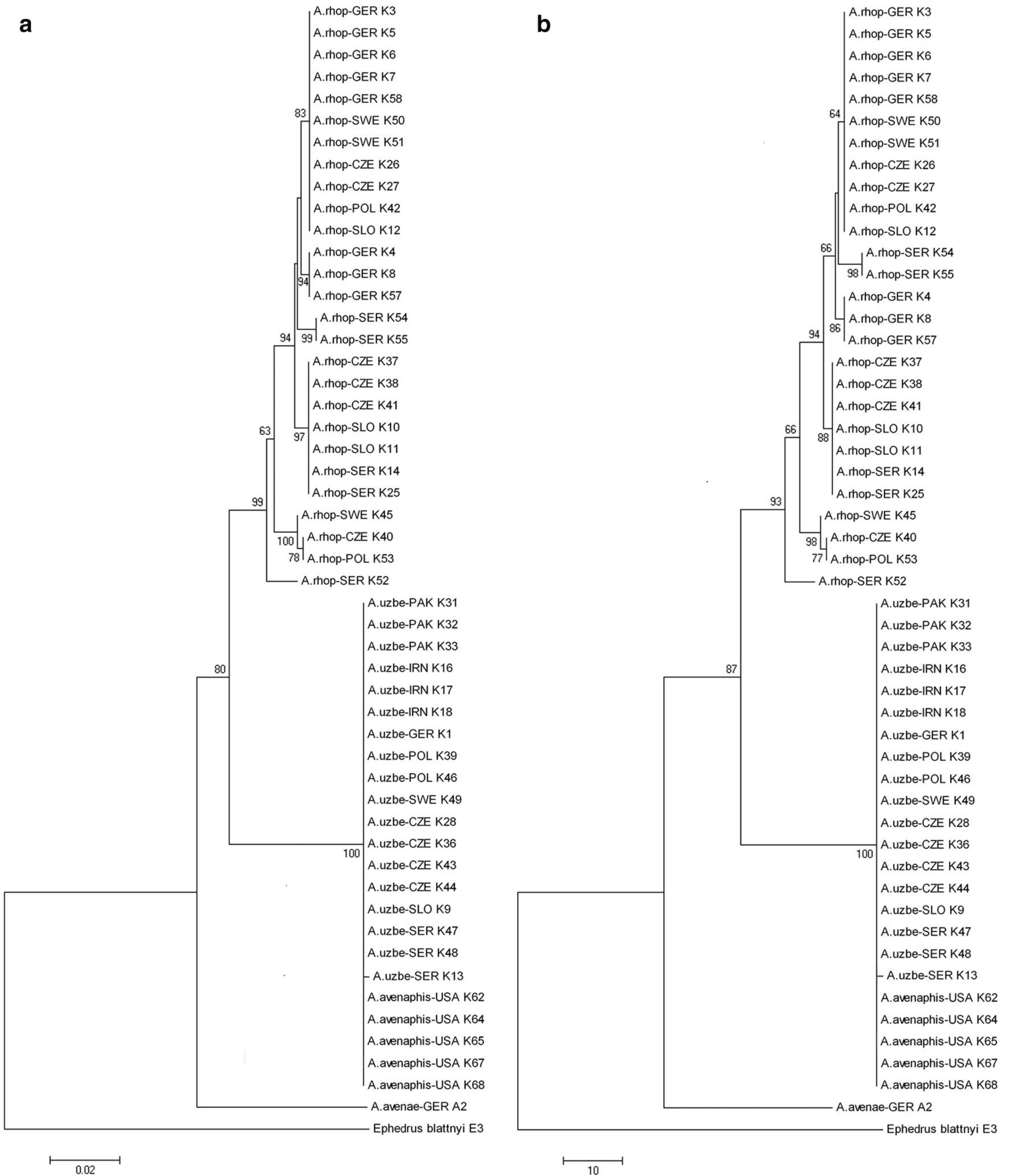

Fig. 3. (a) NJ trees based on K2P distances for COI of a priori determined specimens of *A. uzbekistanicus*, *A. rhopalosiphi*, and *A. avenaphis.* (b) The first of the 675 most parsimonious trees (length = 186) having a consistency index of 0.78. *A. avenae* and *Ephedrus blattnyi* were used for the outgroup species. Numbers above/below the branches represent the bootstrap values (percentages). A voucher code representing the DNA isolate and geographic origin is given for each individual taxa. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

collection of the Institute of Zoology, Faculty of Biology, University of Belgrade and Laboratory of Aphidology, Department of Experimental Ecology, Institute of Entomology, Biology Centre, Academy of Sciences of the Czech Republic.

Results

mtCOI gene sequences were obtained from 50 specimens, i.e., *A. uzbekistanicus* (18), *A. rhopalosiphi* (27) and *A. avenaphis* (5). After multiple DNA sequence alignment using ClustalW, sequences showed no in-

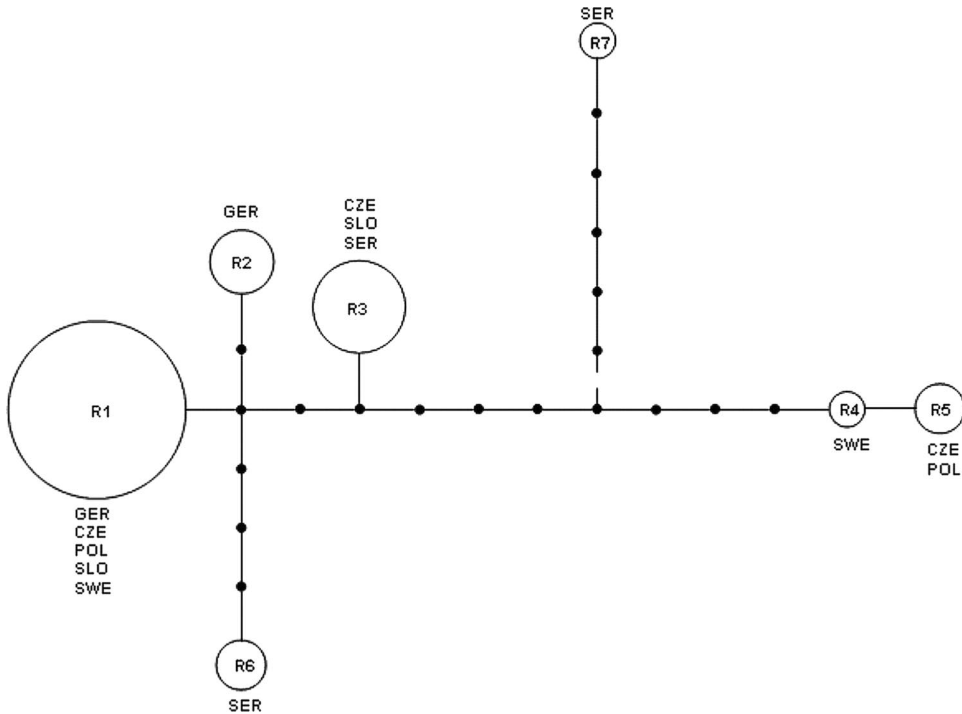

Fig. 4. Haplotype network obtained from *A. rhopalosiphi* mtCOI gene sequences using statistical parsimony (TCS version 1.21). Cycle sizes are proportional to the haplotype frequency, correspond to the mitochondrial COI lineages. The broken line representing unconnected haplotype R7 with a confidence limit of 95% and its connection with a confidence limit of 94%. Geographical distribution of the sequenced specimens is abbreviated below/above cycles.

sertion/deletion (indels) and were trimmed to 627 bp. Sequences of that length were used for all further genetic analyzes. Haplotypes were numerically determined according to the first letter of the species name, i.e., R, *A. rhopalosiphi;* U, *A. uzbekistanicus;* and A, *A. avenaphis* (Table 3). All sequences, including those obtained from *A. avenae* and *Ephedrus blattnyi* (outgroups) are deposited under accessions JN164735–JN164786 in GenBank.

COI gene sequences of *A. uzbekistanicus* from the Central and West Palaearctic and the North American species *A. avenaphis* were identical (U1 = A1), with only a single nucleotide mutation recorded in one *A. uzbekistanicus*-SER K13 (U2) specimen from Serbia. In total, seven haplotypes were recorded in specimens determined to be *A. rhopalosiphi.* The mean nucleotide distance (K2P) between *A. rhopalosiphi* haplotypes was 1.5% (SE = 0.3%; range, 0.5−2.4%), whereas the mean nucleotide distance between *A. rhopalosiphi* and *A. uzbekistanicus* (including *A. avenaphis*) was 6.1% (SE = 0.9%; range, 5.8–6.3%). The distance matrix for all identified haplotypes of analyzed species is presented in Table 3.

The topology of both NJ and MP trees significantly supported differences between *A. rhopalosiphi* and *A. uzbekistanicus,* with high bootstrap values of >90% (Fig. 3). In contrast to the uniform COI gene sequences found for *A. uzbekistanicus* and *A. avenaphis,* several subclusters were recorded for *A. rhopalosiphi,* and most nodes received substantial bootstrap support of >80% (NJ) and 64% (MP). Thus, excluding the a singleton mutation between haplotype R4 and R5 (0.02% divergence), subclustering of *A. rhopalosiphi* was characterized as lacking haplotype diversity within particular mitochondrial lineages, even for specimens that originated from widely separated geographic areas (e.g., R1, R3) (Figs. 4 and 5). In addition, an inferred haplotype network using statistical parsimony showed no ambiguous connections between the mitochondrial lineages of *A. rhopalosiphi* (Fig. 4), but haplotype R7 was not connected to the main network at the 95% parsimony connection limit. Connection of the R7 haplotype was established when the limit was reduced to 94%.

All *A. uzbekistanicus* and *A. avenaphis* specimens had a very uniform color pattern of flagellomeres 1 and 2 (possessing only one narrow yellow ring at the base of F1), maxillary palp with four palpomeres and labial palp with three palpomeres and wide triangular stigma. *A. rhopalosiphi* specimens had a more variable color pattern of F1 and F2 (F1 from one third to completely yellow or light brown and F2 from one third to entirely yellow (Fig. 2). Most specimens of *A. rhopalosiphi* specimens had four maxillary palpomeres and three labial palpomeres, but in specimens reared from *Typha* sp./*Schizaphis scirpi* (Passerini) association (R6 haplotype), the terminal labial palpomere was undivided, resulting in a labial palp with two palpomeres. We found that haplotype R6 was characterized by yellow F1 and prevailingly yellow F2. It

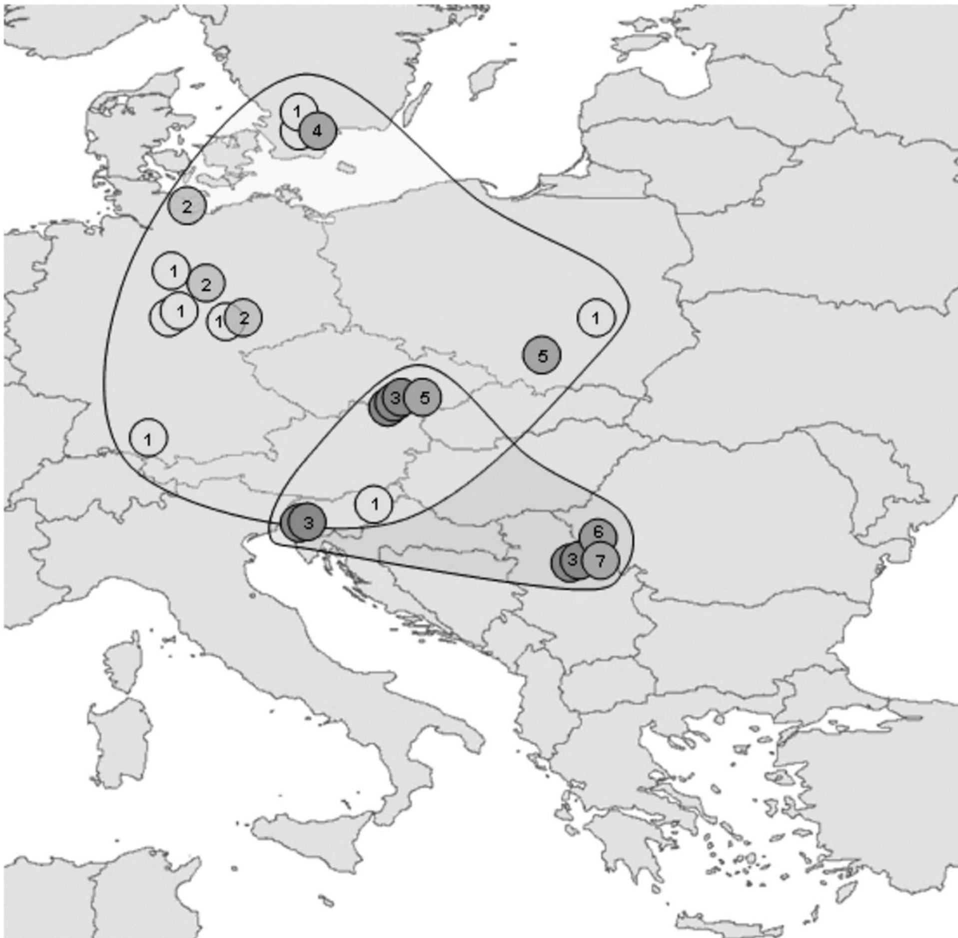

Fig. 5. Distribution of *A. rhopalosiphi* haplotypes (R1–R7). Circles in different shades and numbers indicate the mitochondrial COI gene lineages.

is interesting to note that the R3 haplotype showed almost uniform yellow color pattern of F1. The pattern of F1 and F2 coloration in haplotypes R1, R2, R4 and R5 is variable, ranging from F1 one third yellow to entirely yellow and F2 partially yellow.

We compared the forewing stigma shape of six populations of *A. uzbekistanicus* and *A. avenaphis.* The analyzed populations significantly differed with regard to stigma shape (Wilks' Lambda = 0.0239, $F = 2.75$, df1 = 130, df2 = 315.38, $P < 0.0001$). Canonical variate analysis showed clear discrimination between the analyzed samples (Fig. 6). The first two canonical variates (CVs) accounted for 83.16% of the total differences in the stigma shape. In the morphospace defined by these two axes, *A. uzbekistanicus* populations clustered together, whereas *A. avenaphis* was clearly discriminated along the first coefficient of variation axis. *A. avenaphis* differed from *A. uzbekistanicus* in having longer forewing r (landmarks 10 and 15) and a much narrower distal part of the stigma (semilandmarks 4, 5, 6, 7, 8, 9, 10). In addition, specimens of the population of *A. uzbekistanicus* from Iran had an intermediate position between *A. avenaphis* and other *A. uzbekistanicus* specimens. Using these CVs, 73% of the specimens were correctly classified into their *a priori* groups based on stigma shape (Table 2).

Discussion

The taxonomic status of the *rhopalosiphi-uzbekistanicus* complex was defined using host range and several morphological characters (Starý 1972, 1973; Powell 1982; Pungerl 1983, 1986; Pennacchio 1989; Pennacchio and Höller 1990; Höller 1991; Tomanović et al. 1999, 2003; Kavallieratos et al. 2005). Starý (1981) summarized the morphological differences between *A. uzbekistanicus* and *A. rhopalosiphi* and pointed out the tentorial index (tentorio-ocular line/intertentorial line), the wing venation ratio pattern, and a color pattern that is generally darker for *A. uzbekistanicus* than *A. rhopalosiphi* specimens. Powell (1982) categorized *A. uzbekistanicus* and *A. rhopalosiphi* in the "*uzbekistanicus*" group, based on the costulate sculpture of their petioles.

Using host ranges, morphological characters, isoelectric focusing banding patterns, cross-breeding experiments, and sex pheromone specificities, Höller

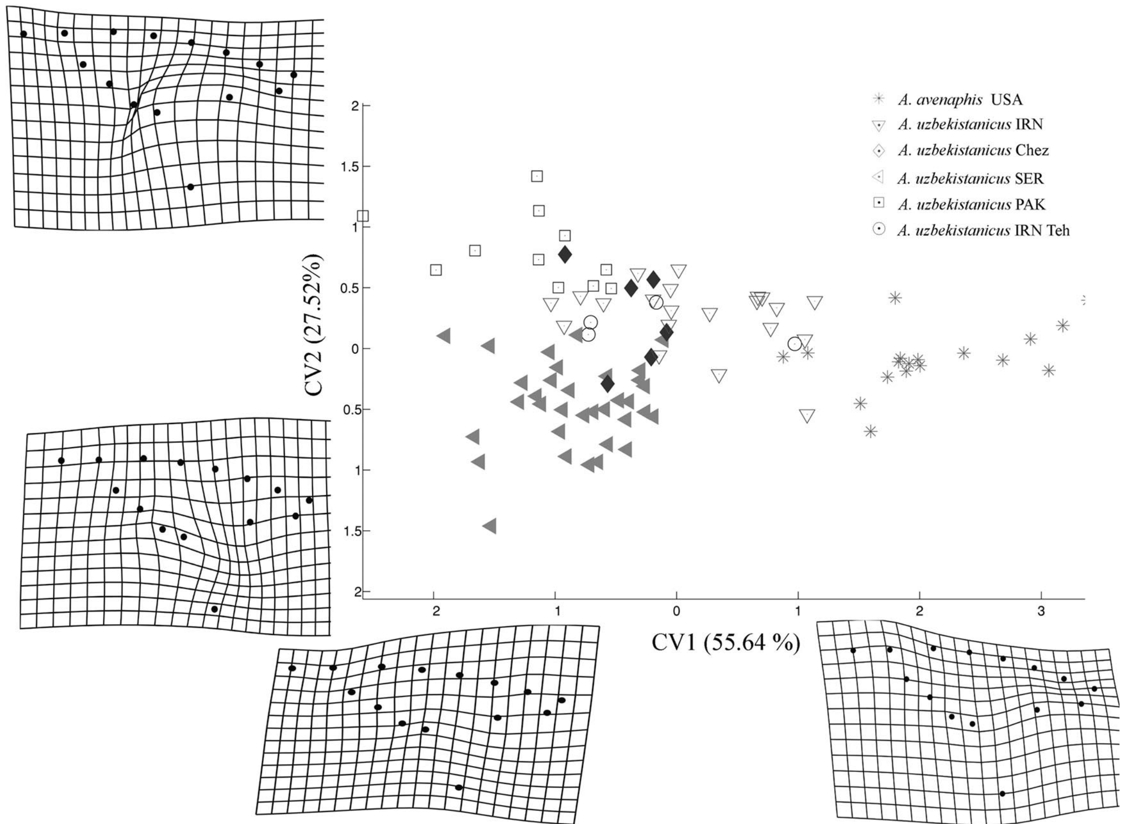

Fig. 6. Ordination of the *A. uzbekistanicus* and *A. avenaphis* specimens in the space of the first two canonical axes. The thin-plate spline deformation grids that illustrate stigma shape changes correlated with the first and the second axis.

(1991) confirmed the existence of two *Aphidius* species on cereal aphids: *A. uzbekistanicus* and *A. rhopalosiphi.* Pennacchio and Höller (1990) on the basis of stepwise discriminate analysis, proposed three morphological characteristics (length/width ratio of F1, stigma length/R1 [metacarpus] length ratio, and stigma length/width ratio) to be reliable for the separation of *A. rhopalosiphi* and *A. uzbekistanicus.* Tomanović et al. (1999) showed that *Aphidius* cereal aphid parasitoids had different patterns of morphological variability depending on geographical area.

A. uzbekistanicus is a specialized parasitoid of several cereal aphid species throughout the Palaearctic. We expected that a species with such a broad distribution would have geographically structured populations. However, mtCOI was surprisingly conservative, with negligible genetic distance (only a single mutation in one specimen from Serbia was found). All analyzed specimens had a uniform color pattern, possessing only one narrow yellow ring at the base of F1 and maxillary and labial palps with four and three palpomeres, respectively.

Pike et al. (1997) stated that *Aphidius avenaphis* (Fitch) is a native species of North America recorded from *Diuraphis noxia* (Kurdjumov), *Rhopalosiphum padi* (L.), and *S. avenae* that is morphologically similar to the introduced European species *A. uzbekistanicus.* On the basis of the shape of the forewing stigma and the length of the forewing r vein, *A. avenaphis* phenotypes can be morphologically discriminated from *A. uzbekistanicus.* Unexpectedly, on the basis of analysis of the COI mitochondrial gene, our results showed that *A. avenaphis* and *A. uzbekistanicus* have identical sequences and that *A. avenaphis* and *A. uzbekistanicus* are probably conspecific. If this is the case, *A. uzbekistanicus* has a Holarctic distribution, and on the basis of field evidence, host range patterns are somewhat more diversified in North America, including the prevailing parasitization of *S. avenae* and, less frequently, of *R. padi* and *D. noxia* (a newly introduced aphid pest from central Asia) (Pike et al. 1997, 2000).

The divergence of ≈6% in the DNA sequences of the COI region between *A. uzbekistanicus* and *A. rhopalosiphi* clearly suggests that populations of both species have been isolated for a sufficiently long time and are probably reproductively isolated (Fig. 3). In addition, the aphid hosts of *A. uzbekistanicus* are mainly restricted to *Sitobion* spp. on cereals (Starý 1981; Höller 1991; Tomanović et al. 1999, 2008), although some references suggest a broader host range (Gruber et al. 1994). In contrast, the aphid host range for *A. rhopalosiphi* is much wider and includes *Diuraphis* spp., *Metopolophium* spp., *Schizaphis* spp., *Rhopalosi-*

phum spp., and *Sitobion* spp. (Pennacchio and Höller 1990, Kavallieratos et al. 2004).

A. rhopalosiphi has the same distribution and shares the same aphid hosts as *A. uzbekistanicus.* However, the sampled *A. rhopalosiphi* specimens had a more variable color pattern (from one third to completely yellow flagellomere 1 and occasionally a partial or even completely yellow flagellomere 2; Fig. 2). Usually, *A. rhopalosiphi* specimens have four-segmented maxillary palps and three-segmented labial palps, but occasionally the terminal labial palpomere is undivided, resulting in two-segmented labial palps. The *A. rhopalosiphi* identity problem was obscured after *Aphidius frumentarius* was described by Latteur and Rassel (1980). A study of a series of *A. frumentarius*-like specimens, showed that their color patterns are very close to those of *A. rhopalosiphi* (i.e., one third to completely yellow flagellomere one and narrow forewing stigma), which suggests that no known morphological characteristics can distinguish *A. frumentarius* from *A. rhopalosiphi.* However, the existence of distinct mitochondrial lineages in the material analyzed here indicates that the taxonomic position of all specimens that are close morphologically to the *A. rhopalosiphi* group should be carefully revised, including previously synonymized taxa.

Although the host range of *A. rhopalosiphi* includes a wide spectrum of cereal aphids, it seems that some host specialization occurs within the *A. rhopalosiphi* group. Our results support host specialization in at least two haplotypes, i.e., R3 with *S. avenae* as the preferable host and R6 with *S. scirpi* in association with *Typha* sp. as the host plant (Table 1). Morphological characteristics are correlated with these two haplotypes. The R6 haplotype has four-segmented maxillary and two-segmented labial palps, as well as F1 yellow and F2 prevailingly yellow. In addition, the R3 haplotype is characterized by F1 being uniformly completely yellow.

The morphological and genetic diversity found in *A. rhopalosiphi* may suggest the existence of cryptic species within the COI mitochondrial lineages, especially for lineages that have a large amount of mtCOI diversity and sympatric occurrence, for example, between R6 and R7, or R1 and R4/R5. A more elaborate molecular and morphological study is needed to determine the potential correlation between this group of very important parasitoids and their aphid hosts.

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