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Exotic aphid species *Brachycaudus divaricatae* in Central Europe: Distribution, host specificity and molecular diversity

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Abstract: Aphid species *Brachycaudus divaricatae* Shaposhnikov, 1956, originally described from Turkmenistan and earlier known from the Middle East and Eastern Europe only, is a successful invader to Central Europe. Its principal winter host, *Prunus cerasifera*, was originally distributed in Central Asia, Near East, and South Eastern Europe. Now it is common in Eastern and Central Europe. *Brachycaudus divaricatae* is closely related to native European aphid species *Brachycaudus lychnidis* (L., 1758). The aims of this study were: 1) to summarize and present the information on the distribution and host specificity of *B. divaricatae* from its invasive area in Europe; 2) to analyse and compare partial sequences of mitochondrial COI with those of nuclear EF-1α from samples identified as *B. divaricatae* and *B. lychnidis* to find possible hybridization or incomplete lineage sorting between these species. Since the first record from Eastern Baltic region in 2002, *B. divaricatae* changed its distribution area significantly. Now this aphid species has already reached the northern edge of its winter host distribution area. Five COI and 10 EF-1α haplotypes were detected among the analysed samples of *B. divaricatae* and *B. lychnidis*. The most abundant COI haplotype was common for both species. However, EF-1α sequences were species-specific despite their minor differences. Coalescent simulations were performed using the model assuming no gene flow after the split between species and mimicking the parameters of empirical data. The analysis of genetic distances calculated for simulated data set supported the hypothesis of possible incomplete lineage sorting.

Key words: *Brachycaudus*; exotic aphid species; distribution; Europe; mitochondrial COI; nuclear EF-1α

Introduction

Aphids (Hemiptera: Aphidoidea, Adelgoidea) (Nieto Nafría et al. 2004) are usually specialized feeders on one or several host plant species, thus changes of host plant distribution and its influence on dispersal of particular aphid species make them a favourable model for invasion studies (Lozier et al. 2009; Ahern et al. 2009).

The focus of this research is the aphid species *Brachycaudus divaricatae* Shaposhnikov, 1956, a successful invader to Central Europe. The distribution of *B. divaricatae* is associated with that of its winter host, cherry plum. This aphid species has been originally described from Turkmenistan (Shaposhnikov 1956), and was earlier known from the area of natural cherry plum distribution, namely, the Middle East (Turkmenistan, Turkey, Iran) and Eastern Europe (Northern Caucasus, Crimea) only (Blackman & Eastop 2000). In its native area *B. divaricatae* was known as holocyclic (with shortened life cycle) facultatively alternating between *Prunus cerasifera* (occasionally *P. domestica*, *P. spinosa*, *P. armeniaca*) and Caryophyllaceae (*Silene latifolia*) (Shaposhnikov 1962; Blackman & Eastop 2000; Holman 2009).

Originally, *P. cerasifera*, the winter host of *B. divaricatae*, was distributed in Central Asia and Near East, and also in submeridional and meridional zones of the South Eastern Europe; afterwards it was introduced to other regions of Europe for ornamental and fruit purposes (Meusel et al. 1965). Consequently, cherry plum is rather common in Eastern and Central Europe for use in hedgerows or as fruit crop, and it has also become established in wild stands, reaching South Karelia (Russia) in the North (Tzvelev 2000). Nowadays, it is distributed widely in Europe, from Sub Caucasus Russia to the British Isles, and from South Karelia to North Africa (Kurtto 2009). The summer host of *B. divaricatae*, *Silene alba*, naturally occupies even broader area in the boreal, temperate, submeridional and meridional zones of Europe (Meusel et al. 1965). Thus, wide distribution of both winter and summer hosts appears favourable for pan European invasion of *B. divaricatae*.

After the successful establishment of cherry plum outside its native range, the distribution range of *B. divaricatae* has also extended significantly. Starting from 2002, it appeared in the Eastern Baltic region of Europe, also Belarus and North Ukraine, and is today the most common pest on cherry plum (*P. cerasifera*) in this area (Cichocka & Lubiarz 2003; Rakauskas 2004; Rakauskas & Buga 2010). It has already invaded Czech Republic from 2011 (Bašilova et al. 2012).

Earlier it was reported, that both apterous and alate viviparous females of *B. divaricatae* are hardly distinguishable from those of *Brachycaudus lychnidis* (L., 1758) (Shaposhnikov 1964). The latter aphid species is monoecious and holocyclic completing the

life cycle on *Lychnis* and *Silene* and is naturally distributed in Central Europe, and also eastward to west Siberia, Turkey and Caucasus (Blackman & Eastop 2000). Earlier molecular studies (Rakauskas & Turčinavičienė 2006; Coeur d'Acier et al. 2008) showed that *B. divaricatae* and *B. lychnidis* were closely related. However, the results of previous studies were based on limited numbers of samples of both species (Rakauskas & Turčinavičienė 2006; Coeur d'Acier et al. 2008; Jousselin et al. 2009, 2010). For biogeographic studies the use of both mitochondrial and nuclear DNA markers has become very important (Avise 2000), because observed incongruence between mitochondrial and nuclear gene trees can result from both introgression and ancestral polymorphisms (Funk & Omland 2003). In case of biological invasions, prediction and detection of hybridization between exotic and native species would be essential (Largiarder 2007).

In this study, to confirm the morphology-based identification as well as to investigate molecular diversity of *B. divaricatae* across the recently colonized areas throughout Central and Eastern Europe, partial sequences of both mitochondrial (COI) and nuclear (EF-1α) DNA were used. Samples were collected both from winter and summer hosts which could be shared with *B. lychnidis*. The aims of this study were: 1) to summarize and present the information on the distribution and host specificity of *B. divaricatae* from its invasive area in Europe; 2) to analyse and compare partial sequences of mitochondrial DNA with those of nuclear DNA from samples collected from winter and summer hosts of *B. divaricatae* to find possible hybridization or incomplete lineage sorting between *B. divaricatae* and *B. lychnidis*. Coalescent simulations were used to create and test the preliminary hypothesis explaining the incongruence between mitochondrial and nuclear markers (if any).

Material and methods

Collections and specimen identification

Aphid material for molecular analysis has been collected in 2003–2013 and included 155 samples from various *Prunus* species (winter hosts of *B. divaricatae*) from twelve countries and 18 samples from *Silene* spp. (summer host of *B. divaricatae* and *B. lychnidis*) from 6 countries (Supplemental data, Table 1). Microscope slides in Canada balsam were prepared according to Blackman & Eastop (2000). Ethanol-preserved and mounted specimens are stored at the Life Sciences Centre, Vilnius University. For morphology-based identification, keys of Blackman & Eastop (2000, 2006) and Rakauskas & Turčinavičienė (2006) were used. Collection data were also used to compile the host plant list of *B. divaricatae* in European countries.

DNA isolation, amplification and sequencing

For molecular analysis, a single aphid individual from one sampled plant was considered as a unique sample. Total genomic DNA was extracted from a single aphid using the DNeasy Blood & Tissue kit (Qiagen), which involved at least a 2 h digestion of tissue with proteinase K. For the amplification of COI and EF-1α fragments earlier published primers (Turčinavičienė et al. 2006) were used. PCR amplification was carried out in a thermal cycler (Eppendorf) in 50 µl volumes containing 2 µl genomic DNA, 5 µl of each primer (1 µM), 5 µl of PCR-reaction buffer, 5 µl of dNTP mix (2 mM each), 4–8 µl of 25 mM $MgCl_2$ and 1.25 U of AmpliTaq Gold 360 polymerase (5 U/µl) and ddH_2O to 50 µl. The cycling parameters were as follows: denaturizing at 95 °C for 10 min (1 cycle), denaturizing at 95 °C for 30 s, annealing at 49 °C (for COI) or 57 °C (for EF-1α) and extension at 72 °C for 30 s (32–37 cycles in total), and a final extension for 5 min (1 cycle). PCR products were subjected to electrophoresis on 2% TopVision agarose (Fermentas, Lithuania), stained with GelRed and sized against a MassRuler Low Range DNA ladder (Fermentas, Lithuania) under UV light. PCR products were purified and sequenced at Macrogen Europe (Amsterdam, the Netherlands) and Institute of Biotechnology of the Vilnius University (Vilnius, Lithuania). The amplification primers were also used as sequencing primers. DNA sequences for each specimen were confirmed with both sense and anti-sense strands and aligned in the BioEdit Sequence Alignment Editor (Hall 1999). Partial COI sequences were tested for stop codons and none were found. GenBank Accession numbers for each sample are given in Table 1 of Supplemental data.

Data analysis

Alignment statistics and genetic distances (uncorrected p-distances) within and between *B. divaricatae* and *B. lychnidis* were calculated using MEGA 5 (Tamura et al. 2011). Sequences were collapsed into haplotypes and statistical parsimony networks with 95 % implemented connection limit were constructed using TCS v 1.21 (Clement et al. 2000). For further procedures, COI and EF-1α alignments were combined using FaBox 1.41 (Villesen 2007).

To investigate whether incongruence (if any) between mitochondrial and nuclear data could be explained by possible hybridization or incomplete lineage sorting between *B. divaricatae* and *B. lychnidis* coalescent simulations and their comparison with empirical data were performed. For this purpose, combined COI and EF-1α alignment was mimicked. The total of 1000 simulated data sets were produced using SimCoal2 (Laval & Excoffier 2004). Empirical data sets, including both sample size and fragment length, were mimicked, using a model where an ancestral population split 10000 generations ago (assuming that aphids have one sexual generation per year) and no gene flow occurred after this event. Sequences were generated using the same transition – transversion rate as obtained for empirical data set with jModelTest (Posada 2008). Statistical evaluation of simulated and empirical data was performed as described by Melo-Ferreira et al. (2012). The distribution of average minimum pairwise uncorrected p-distances was produced for simulated data set and descriptive statistics for this parameter were calculated using Statistica 8. If the empirical pairwise distance between two species was smaller than the 5th percentile of the simulated minimum distances, then the hypothesis of possible hybridization could be accepted, as lineage sorting could not explain the data.

Results

Distribution history of B. divaricatae in Central Europe

Starting from 2002, *B. divaricatae* was recorded in Lithuania and Poland for the first time. It was afterwards collected in Ukraine (2006), Belarus (2008),

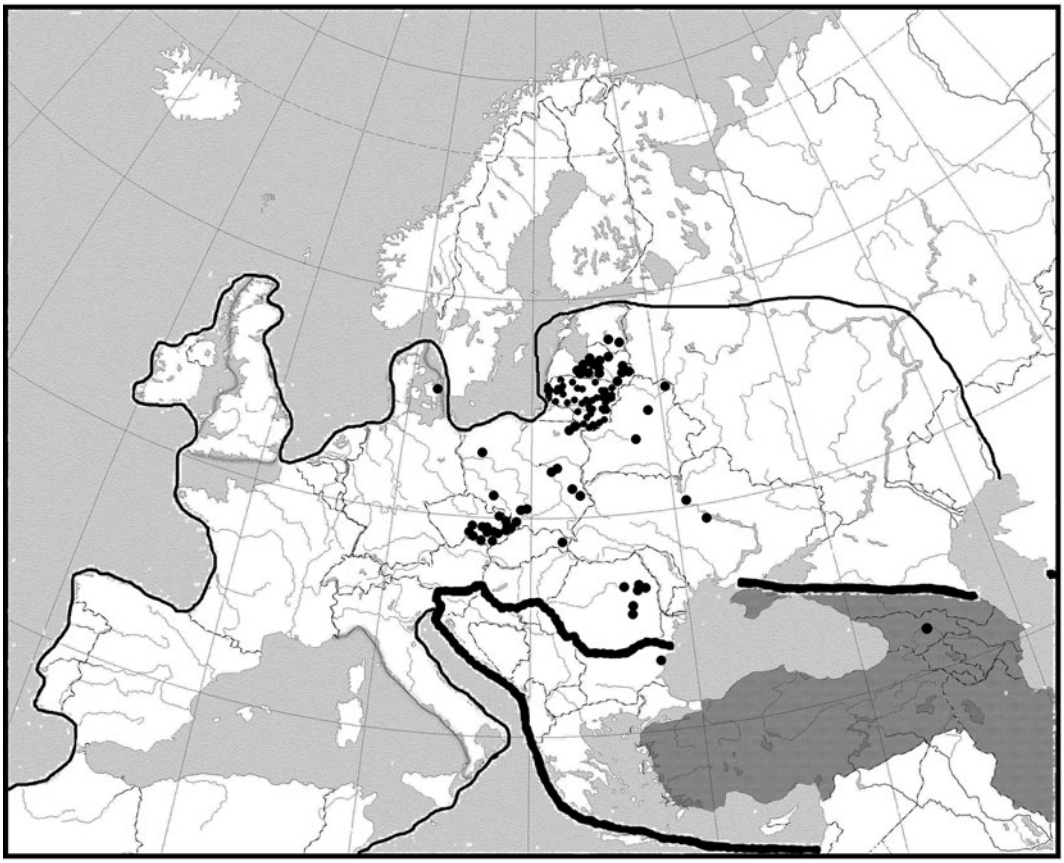

Fig. 1. Sample collection sites (dots). Grey area – native distribution range of *B. divaricatae* after Blackman & Eastop (2000) and Nieto Nafría et al. (2004). Bold line – area of the original distribution and thin line – area of subsequent anthropogenic introduction of *P. cerasifera* after Meusel et al. (1965), Tzvelev (2000) and Kurtto (2009).

Latvia (2008), Estonia (2012), Slovakia (2012), Romania (2012), Bulgaria (2012) and Denmark (2013). From 2011, when *B. divaricatae* was recorded in Czech Republic for the first time, it has spread significantly and was observed in Northern Bohemia in 2015 (P. Starý, personal communication), although during the field sampling in 2005 this species was not detected. Denmark and Estonia are on the northernmost board, where cherry plums were introduced. Bulgaria and Georgia, where single samples of *B. divaricatae* were collected, are in the natural range of the cherry plum distribution (Fig. 1). It is important to note that *B. divaricatae* has not been recorded (at least for now) from Southern and Western Europe, despite the availability of its principal host plants. This aphid species can hardly be overlooked for two main reasons. First, numerous colonies of *B. divaricatae* are formed on cherry plum trees. Second, highly experienced long lasting aphid research traditions are attributable to France, Germany, Great Britain, Italy and Spain. In addition, we have performed special (although unsuccessful) research efforts for this aphid species in Italy, France, Germany and Austria.

Host specificity of B. divaricatae

The majority of *B. divaricatae* samples analysed during this study were collected from *P. cerasifera*, some were from *P. domestica*, or *P. salicina* var *skoroplodnaya* (Table 1). Samples from *Silene* spp. were identified as morphospecies *B. lychnidis*, therefore, it seems that *B. divaricatae* does not host alternate in the invasive part of its distribution area.

Table 1. Host plants of *B. divaricatae* and *B. lychnidis* samples used for molecular studies.

	Number of samples	Percentage of samples
Winter host plant (n = 155)		
P. cerasifera	142	91.6
P. domestica	1	0.6
P. cerasus	1	0.6
P. salicina var. *skoroplodnaya*	7	4.5
Prunus sp.	1	0.6
P. americana	3	1.9
Summer host plant (n = 18)		
Silene sp.	18	100

Molecular diversity of B. divaricatae and B. lychnidis

COI fragment

The alignment of COI fragment contained 581 sites, out of them 6 variable sites, including 1 parsimony informa-

Table 2. Uncorrected p-distances of partial sequences of mitochondrial and nuclear DNA.

Parameter	*B. divaricatae* (n = 155)	*B. lychnidis* (n = 18)
	COI	
Within-species, range (average)	0.00–0.52% (0.08%)	0.00–0.52% (0.06%)
Between species, range (average)	0.00–0.86% (0.09%)	
Number of haplotypes	3	3
	EF-1α	
Within-species, range (average)	0.00–0.89% (0.02%)	0.00–0.66 (0.20%)
Between species, range (average)	0.22–1.55% (0.42%)	
Number of haplotypes	5	5

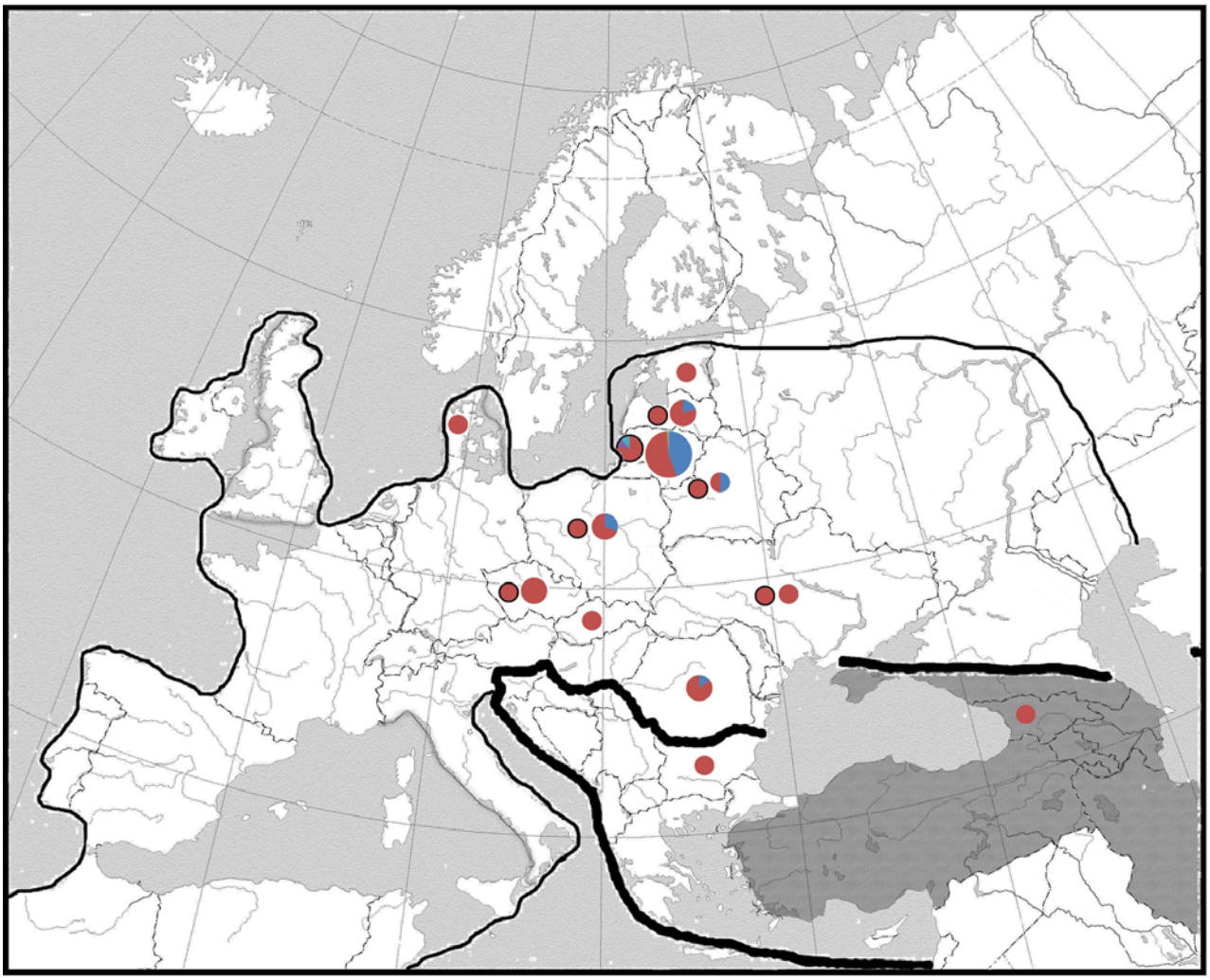

Fig. 2. Distribution and frequency of *Brachycaudus divaricatae* (from *Prunus* sp.) and *Brachycaudus lychnidis* (from *Silene* sp.) COI haplotypes in Europe. Grey area – native distribution range of *B. divaricatae* after Blackman & Eastop (2000) and Nieto Nafría et al. (2004). Bold line – area of the original distribution and thin line – area of subsequent anthropogenic introduction of *P. cerasifera* after Meusel et al. (1965), Tzvelev (2000) and Kurtto (2009). COI haplotypes: haplotype 1 ■; haplotype 2 ■; haplotype 3 ■; haplotype 4 ■; haplotype 5 ■ *B. divaricatae* – no outline; *B. lychnidis* – outlined circles. Circle size: small – less than 5 samples; medium – from 5 to 20 samples; large – more than 20 samples.

tive. Average nucleotide composition was T – 40.3%, C – 13.6%, A – 34.4%, G – 11.8%. Intra- and interspecific uncorrected p-distances are presented in Table 2.

Five haplotypes were revealed after the analysis of partial sequences of mitochondrial COI obtained for 172 samples collected from *Prunus* and *Silene* in 12 European countries (Fig. 2). There were six segregating sites among those five haplotypes. Haplotype No. 2 was the most frequent: it was characteristic for 105 out of 155 sampled specimens identified as *B. divaricatae*

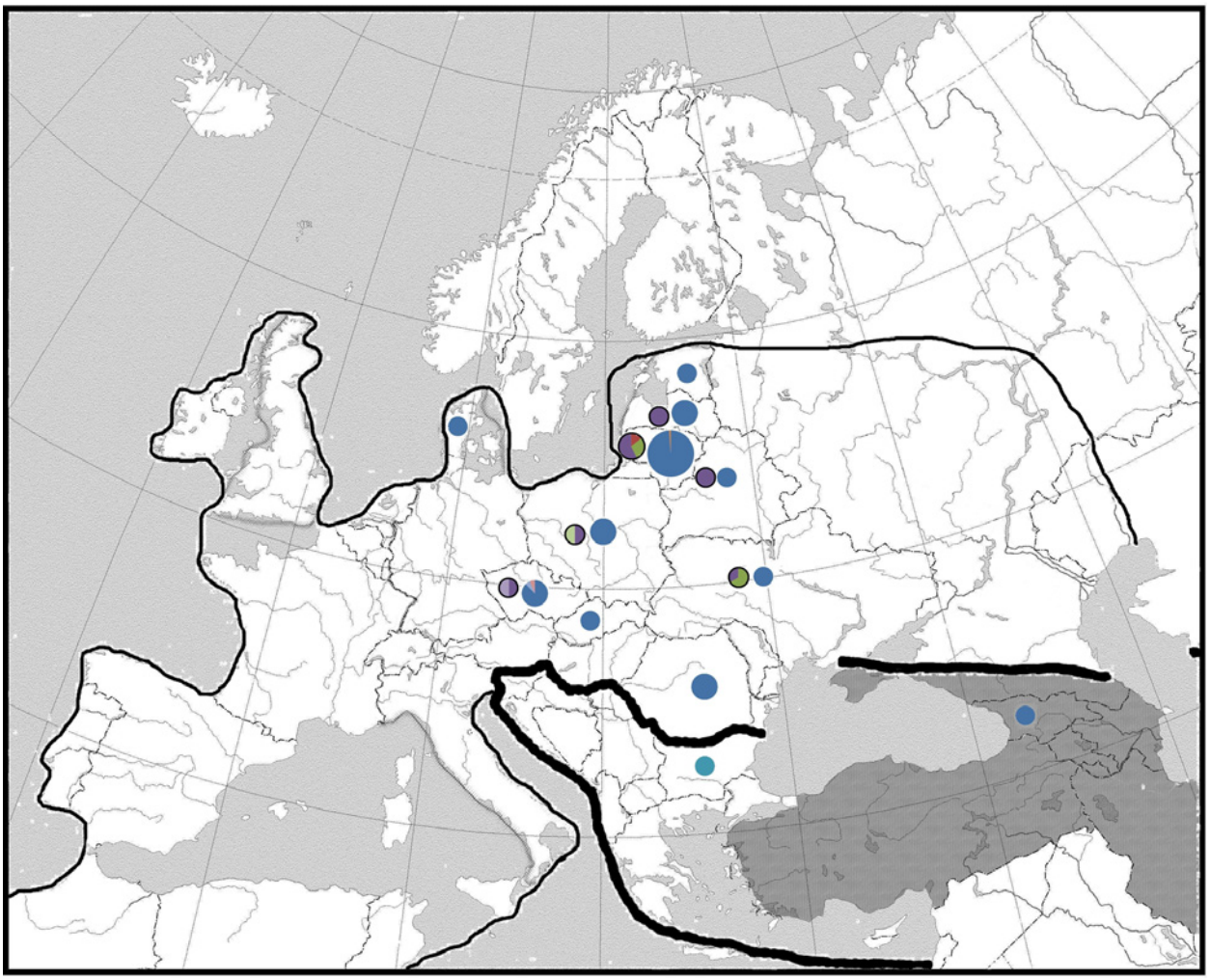

Fig. 3. Distribution and frequency of *Brachycaudus divaricatae* (from *Prunus* sp.) and *Brachycaudus lychnidis* (from *Silene* sp.) EF-1α haplotypes in Europe. Grey area – native distribution range of *B. divaricatae* after Blackman & Eastop (2000) and Nieto Nafría et al. (2004). Bold line – area of the original distribution and thin line – area of subsequent anthropogenic introduction of *P. cerasifera* after Meusel et al. (1965), Tzvelev (2000) and Kurtto (2009). EF-1α haplotypes: haplotype 1 ■; haplotype 2 ■; haplotype 3 ■; haplotype 4 ■; haplotype 5 ■; haplotype 6 ■; haplotype 7 ■; haplotype 8 ■; haplotype 9 ■; haplotype 10 ■. *B. divaricatae* – no outline; *B. lychnidis* – outlined circles. Circle size: small – less than 5 samples; medium – from 5 to 20 samples; large – more than 20 samples.

from 12 countries. Noticeably, it was also most frequent in specimens identified as *B. lychnidis* (n = 15 out of 17 sampled individuals). Haplotype No. 1 was found in 31.21% (n = 49) of the individuals of *B. divaricatae*, sampled in Lithuania, Latvia, Belarus, Poland and Romania. Haplotype No. 3 was detected in a single sample of *B. divaricatae*, haplotypes No. 4 and 5 – in single samples of *B. lychnidis*.

EF-1α fragment

The analysed region of EF-1α consisted of two parts of three exons and two introns, which were not removed before the further analysis. The alignment of this fragment contained 452 sites, 9 out of them were variable, including 4 parsimony informative. Average nucleotide composition: T – 31.4%, C – 17.7%, A – 31.2%, G – 19.7%. Intra- and interspecific uncorrected p-distances are presented in Table 2.

The analysis of the nuclear EF-1α fragment obtained for 170 samples collected from *Prunus* and *Silene* in 12 European countries revealed ten different haplotypes, five species-specific haplotypes per every species (Fig. 3). There were eleven segregating sites among those ten haplotypes. Haplotype No. 1 was found in 97.39% of the sampled *B. divaricatae* individuals, collected in all countries except for Bulgaria, where single haplotype No. 5 was detected. Remaining haplotypes of *B. divaricatae* were represented by single sequences detected in Lithuania (No. 6) and Czech Republic (No. 7–8). Haplotype No. 4 was found in ten out of 17 sequences of *B. lychnidis* collected in 6 countries. Haplotype No. 3 was detected in 4 individuals of *B. lychnidis* from Lithuania and Ukraine. Three haplotypes were represented by the single sequences sampled from Lithuania (No. 2), Poland (No. 9) and Czech Republic (No. 10).

Coalescent simulations and their comparison with empirical data

Shared COI haplotype and minor (although stable) differences of analysed EF-1α fragment required testing the hypothesis of possible hybridisation or incomplete lineage sorting between *B. divaricatae* and *B. lychnidis*.

For coalescent simulations and their comparison with empirical data, combined COI and EF-1α alignment was mimicked. For empirical combined data set within-species uncorrected p-distances ranged from 0 to 0.58% (average 0.05%) for *B. divaricatae* and from 0 to 0.39% (average 0.12%) for *B. lychnidis*, while between-species p-distances were 0.10–0.68% (average 0.24%). The values of within-species uncorrected p-distances calculated for simulated sequences were 0.01–0.81% (average 0.19%) for *B. divaricatae* and 0.01–0.87% (average 0.19%) for *B. lychnidis*. For the evaluation of between-species divergences, descriptive statistics of minimum uncorrected p-distances from 1000 simulated data sets were calculated. Their range was from 0.001 to 1.24% (average 0.20%). The value of 5^{th} percentile and its comparison with empirical data was used as boundary for supporting or rejecting the hypothesis of possible hybridization as described by Melo-Ferreira et al. (2012). In case of simulated combined data set of *B. divaricatae* and *B. lychnidis* the value of 5^{th} percentile was 0.03%. It was lower than the range of between-species uncorrected p-distances for empirical data set (0.10–0.68%), therefore, the hypothesis of possible incomplete lineage sorting should be accepted.

Discussion

When applying the definition of an invasive species as "… a set of individuals that has been introduced into a new area, in which these individuals have established themselves, increased in numbers and spread geographically" (Estoup & Guillemaud 2010), *B. divaricatae* appears to be a good example. Generally, aphids are particularly good models for invasion studies because they possess many biological characteristics favouring their success in establishing new populations, such as parthenogenetic reproduction, short generation times, and high dispersal capacity (Dixon 1998). High availability of winter host plant together with low abundance of other aphid species on cherry plums (Rakauskas et al. 2015) enabled *B. divaricatae* to reach the northern edge of the invasive distribution area of *P. cerasifera*, its winter host, in Europe. Moreover, there are more opportunities for invasive species to establish successfully, when competition with native related species is reduced through niche divergence (Diez et al. 2008). In present case, *B. divaricatae* does not host alternate in the invasive part of its distribution area, and it does not compete with closely related *B. lychnidis*. Phylogenetic patterns of invasion may provide interesting insights into the organizations of ecological communities and could partly predict success of invasion (Diez et al. 2008). For this purpose, as well as for interpreting the pattern of invasion, resolving phylogenetic relationships of closely related invasive and native species could be useful.

Genetic diversity of aphids is usually low comparing with other insects (Virgilio et al. 2010). Based on global data set, the average genetic divergence of COI barcode sequences between the aphid species within the same genus was reported to be 5.84% (0–14.04%) for Korean, 6.4% (0–15%) for European and 7.25% (0.46–13.01%) for North American aphid faunas (Coeur d'acier et al. 2014). The range of within species divergences was 0.05% (0.00–1.00%), 0.29% (0–3.9%) and 0.201%, respectively (Coeur d'acier et al. 2014). The level of between species COI fragment divergence was reported to be lower than the average for *Bursaphis* species (Rakauskas et al. 2011), *Macrosiphum rosae* (L., 1758) and *M. knautiae* Holman, 1972 (Turčinavičienė & Rakauskas 2009), several species groups in the genera *Aphis*, *Brachycaudus* and *Dysaphis* (Coeur d'acier et al. 2014) and adelgids (Žurovcová et al. 2010). All this supports the earlier opinion (Coeur d'Acier et al. 2008) on the limited value of COI sequence data when solving the species-level taxonomy issues in some aphid groups.

Available molecular data (Coeur d'Acier et al. 2008; Jousselin et al. 2010), including the present study revealed high similarity between the alien aphid species *B. divaricatae* and native *B. lychnidis*, a monoeciuos aphid species native to Central Europe. According to Coeur d'Acier et al. (2008) these aphid species together with *Brachycaudus lychnicola* Hille Ris Lambers, 1966, *Brachycaudus klugkisti* (Börner, 1942), *Brachycaudus populi* (del Guercio, 1911) and *Brachycaudus pallidus* Andreev, 1990 belong to the subgenus *Acaudus* van der Goot, 1913. Within-group genetic distances (mean ± standard deviation) of barcoding COI fragment for all species of the subgenus *Acaudus* ranged from 1.9 to 2.6% and from 0.6 to 1.1% when *B. klugkisti* was excluded (Coeur d'Acier et al. 2008).These values are closer to maximum interspecific distances calculated for fragment analysed in current study (see Table 1 for details).

The analysis of partial COI sequences showed that the most abundant haplotype is common for *B. divaricatae* and *B. lychnidis*. In such a case, one might suggest that it was the same species with slightly different morphs collected from summer and winter hosts. Therefore, the analysis of at least one more independent marker was crucial. Partial sequences of EF-1α appeared to be more species-specific and more congruent with available host specificity and life cycle data, indicating that *B. lychnidis* and *B. divaricatae* are not the same species despite their close morphological similarity (Rakauskas & Turčinavičienė 2006).

Incongruence among genes might result due to introgression (Shaw 2002; Bossu & Near 2009) or incomplete lineage sorting and ancestral polymorphisms (Avise 2004). In this study, coalescent simulations followed by the analysis of genetic distances calculated for simulated data set and its comparison to empirical data supported the preliminary hypothesis of possible incomplete lineage sorting between in *B. divaricatae* and *B. lychnidis*. It is important to note, that independent loci, such as nuclear and mitochondrial genes, are important for estimating species boundaries between closely related species. Sequences of many individuals of the same species helped revealing clearer picture of relationships between closely related and morphologi-

cally similar species. Even in case of incomplete lineage sorting, sufficient signal for reconstructing species boundaries of recently evolved species remains.

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