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Aphis pomi and *Aphis spiraecola* (Hemiptera: Sternorrhyncha: Aphididae) in Europe – new information on their distribution, molecular and morphological peculiarities

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Abstract. Aphid species *Aphis pomi* (de Geer, 1773) is oligophagous on pomoideous host plants, whilst *Aphis spiraecola* Patch, 1914 is a polyphagous species alternating between *Spiraea* spp., its primary host, and a wide variety of secondary hosts, also including pomoideous species. Despite the biological distinction, these species are difficult to separate using their morphological characters. Partial sequences of mitochondrial COI and nuclear EF-1α genes were analyzed for samples from Central and Eastern Europe, Germany, Bulgaria, Italy, Turkey, China together with available data from GenBank. Interspecific pairwise sample divergences of the COI fragment ranged from 3.1 to 4.3%. One COI haplotype of *A. pomi* was predominant (n = 24), with a pan European distribution. The most abundant COI haplotype of *A. spiraecola* (n = 16) occurred in Lithuania, Latvia, Poland, Italy, Turkey and China. Interspecific pairwise sample divergences of the EF-1α fragment ranged from 0.6 to 1.2%. Analyzed partial sequences of EF-1α were identical in *A. pomi*. The most abundant EF-1α haplotype of *A. spiraecola* (n = 14) occurred in Lithuania, Poland, Italy, Turkey and China. The length of ultimate rostral segment appeared to be the most reliable morphological character for discrimination between apple and spirea aphid species. It allowed a 100% correct identification of *A. pomi* (n = 143) and 91.5% of *A. spiraecola* (n = 94) specimens in the European samples used for the molecular analysis. The existence of *A. spiraecola* in the Eastern Baltic region of Europe is documented for the first time.

INTRODUCTION

Aphis pomi (de Geer, 1773) (apple aphid) and *Aphis spiraecola* Patch, 1914 (spirea aphid) are reported to be serious pests of horticulture all over the World (Blackman & Eastop, 2000; Holman, 2009). Their distribution, morphology, life cycles and host specificity have therefore been subjected to intensive study, together with their potential harmfulness and plant protection measures (Grasswitz & Burts, 1995; Tsai & Wang, 2001; Brown et al., 2008; Stoeckli et al., 2008; Frechette et al., 2008; Brown, 2011; Wieczorek et al., 2011; Cao et al., 2012). The apple aphid is monoecious and holocyclic on woody pomoideous hosts, with apterous males (Baker & Turner, 1916; Patch, 1923; Westigard & Madsen, 1964; Karczewska, 1965; Rakauskas & Rupais, 1983). The spirea aphid is holocyclic and heteroecious in North America, Brazil and Japan, with *Spiraea* species as its primary host and a wide variety of secondary hosts belonging to more than 20 families, including many of those of the apple aphid; anholocyclic lineages of spirea aphid occur worldwide (Patch, 1929; Komazaki, 1990; Kaakeh et al., 1993; Blackman & Eastop, 2000; Holman, 2009). Unlike the males of the apple aphid, those of the spirea aphid are winged and the oviparae have swollen tibiae (Palmer, 1952; Halbert & Voegtlin, 1992). Despite suggested morphology-based discrimination characters (Stroyan, 1985; Heie, 1986; Halbert & Voegtlin, 1992; Blackman & Eastop, 2000; Foottit et al., 2009), close morphological similarity of winged and apterous viviparous females together with overlapping of host plant lists has caused confusion over the identity of both species (Singh & Rhomberg, 1984; Stroyan, 1985; Heie, 1986; Halbert & Voegtlin, 1992; Blackman & Eastop, 2000). Namely, authors reporting winged males of the apple aphid (Davletshina, 1963; Karczewska, 1965) might actually refer to the spirea aphid. Competitive displacement of apple aphid by spirea aphid is reported as mediated by human activities in North America (Hogmire et al., 1992; Brown et al., 1995; Lowery et al., 2006; Smirle et al., 2010), making host plant based discrimination of both species even more complicated. Recently, a resolution of this discrimination problem was proposed using molecular taxonomy (Lushai et al., 2004; Foottit et al., 2009; Naaum et al., 2012).

In Europe, apple aphid is a common species, whilst spirea aphid is currently reported mostly from southern Europe, reaching British Isles, Germany and Ukraine in the north (Holman, 2009; Nieto Nafria et al., 2010). Yet morphology-based identification make some records uncertain (Jaskiewicz & Kot, 2007; Caglayan et al., 2013; Yovkova et al., 2013). Spirea aphid is reported to be the principle pest on citrus, occasionally also on Prunoidea (stone fruits), but not apple or other pomoideae in Europe (Barbagallo et al., 1997).

Recently, the spirea aphid was reported from a more northernly part of Europe, in Poland, on *Kalanchoe blossfeldiana*, *Polyscias fabiana*, *Schefflera arboricola* in a greenhouse (Labanowski, 2008) and in Belarus, on *Spi-*

raea alba outdoors (Rakauskas & Buga, 2010). *A. spiraecola*-like aphids were collected from *Spiraea* sp. outdoors also in Latvia (2008) and Lithuania (2005, 2012–2014) (R. Rakauskas, unpubl.). The aim of this study is to identify the available European samples of the *A. pomi-spiraecola* species complex using partial sequences of mitochondrial COI and nuclear EF-1α genes and test the reliability of the morphological characters used to discriminate between these two species (Blackman & Eastop, 2000; Foottit et al., 2009).

MATERIAL AND METHODS

Samples

Aphid material collected in 2004–2013 included forty nine samples from ten European countries, Turkey and China (Table 1). Microscope slides in Canada balsam were prepared according to Blackman & Eastop (2000). Ethanol-preserved and mounted specimens are stored at the Department of Zoology, Vilnius University.

DNA extraction, fragment amplification and sequencing

For molecular analysis, a single aphid from one plant was considered as a unique sample. Total genomic DNA was extracted from each 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 mitochondrial COI and nuclear EF-1α gene fragments previously published primers, Aphis-L-465/Aphis-H-1068 and Eloaphis-F/Eloaphis-R (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 (10 µM), 5 µl of PCR-reaction buffer, 5 µl of dNTP mix (2mM each), 4–8 µl of 25mM $MgCl_2$ and 1.25 U of AmpliTaq Gold 360 polymerase (5U/µl) and ddH_2O to 50 µl. The cycling parameters were as follows: denaturizing at 95°C for 10 min, denaturizing at 95°C for 30", annealing at 49°C (for COI) or 57°C (for EF-1α) for 30" and extension at 72°C for 30" (32–37 cycles in total), and a final extension for 5 min.

PCR products were purified and sequenced at the Institute of Biotechnology, 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. The sequence data have been submitted to GenBank, Accession numbers are given in Table 1.

DNA sequence data analysis

In addition, available partial sequences of mitochondrial COI (1 of *A. pomi* and 11 of *A. spiraecola*) and nuclear EF-1α (7 of *A. spiraecola*) were downloaded from GenBank (Table 2). To avoid any discrepancies when analyzing data, sequences of both fragments were aligned and those matching partial sequences obtained from samples collected during this study were selected for further procedures. For sequences from GenBank geographic origin of samples and their host plants were obtained from publications (Table 2).

Phylogenetic analyses with a sequence of *Nasonovia ribisnigri* (Mosley, 1841) (tribe Macrosiphini, family Aphididae) as outgroup species, included Neighbour joining (NJ), Maximum parsimony (MP), Maximum likelihood (ML) and Bayesian inference in phylogeny (BI). NJ, MP and ML analyses were performed using MEGA 5 (Tamura et al., 2011). For NJ and distance analyses Kimura 2-parameter (K2P) model of base substitution was used. ML analysis was performed using Tamura 3-parameter model with invariable sites (T92+I) for COI and Tamura 3-parameter model (T92) for EF-1α, which were selected by MEGA 5 model selection option (Tamura et al., 2011). Bootstrap values for NJ, MP and ML trees were generated from 1000 replicates. Bayesian analysis was conducted in MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003) using Hasegawa-Kishino-Yano model with Invariable sites (HKY+I) for COI and Felsenstein model with Invariable sites and Gamma distribution (F81+I+G) for EF-1α, which were selected by jModeltest (Posada, 2008). One run for 1,000,000 generations with tree sampling every 1,000 generations was performed using the uniform model of the molecular clock.

Statistical parsimony networks with 95% implemented connection limit were constructed using TCS v 1.21 (Clement et al., 2000). For analysis of partial COI sequences gaps were treated as missing data, while EF-1α fragment gaps were treated as a 5th state.

Morphometrics

Samples representing different clades in the molecular tree and haplotype network were used to verify the characters commonly used in the morphology-based keys discriminating both species (Halbert & Voegtlin, 1992; Blackman & Eastop, 2000; Foottit et al., 2009). The following characters were selected: URS – ultimate rostral segment length; SIPHON – siphunculus length; CAUDA – length of cauda (apical part); MT2-4(5) – numbers of marginal tubercles on abdominal tergites II–IV(V); HCAUDA – numbers of caudal hairs; SIPHON/CAUDA – ratio of siphuncular length to caudal (apical part) length. Measurements of slide-mounted apterous viviparous females were made using the interactive measurement system Micro-Image (Olympus Optical Co. GmbH).

RESULTS

The alignment of COI fragment contained 621 sites, of which 68 were variable and 26 parsimony informative. Average nucleotide composition was T – 37.7%, C – 14.6%, A – 35.2%, G – 12.5%. The overall transition/transversion bias was R = 3.075. The range in the intraspecific pairwise sample divergences (K2P model) was 0.0–0.8% (average 0.1%) for *A. pomi* and 0.0–0.5% (average 0.2%) for *A. spiraecola*. Interspecific pairwise sample divergences between these two species ranged from 3.1 to 4.3% (average 3.6%).

After the construction of networks based on statistical parsimony (Fig. 1) 31 partial COI sequences of *A. pomi* and 30 sequences of *A. spiraecola* were collapsed into seven haplotypes each. The number of COI haplotypes, sequence length and sample or sequence numbers are given in Table 3; details for each sample (country, host plant and collection date) are given in Tables 1–2.

Most of the *A. pomi* samples (n = 24) had the same COI haplotype (No. 1) and were collected in Latvia (n = 7), Lithuania (n = 5), Poland (n = 4), Estonia (n = 2), Czech Republic (n = 2), Belarus (n = 1), Ukraine (n = 1), Bulgaria (n = 1) and Germany (n = 1). This haplotype seems to be the most common in Europe. COI haplotype No. 5 was detected only in samples from Lithuania (n = 1) and Latvia (n = 1). Remaining 5 haplotypes were represented by single samples from Estonia, Ukraine, Czech Republic and China (Tables 1–3, Fig. 1).

Table 1. Samples of *Aphis pomi* and *Aphis spiraecola* examined in this study. COI – Mitochondrial cytochrome oxidase subunit I, EF-1α – nuclear elongation factor 1 alpha.

GenBank accession No.		Sample information and No.
COI	EF-1α	
		Aphis pomi
KM017584	KM017457	Štramberk, North Moravia, Czech Rep., 2005.vi.15, *Cotoneaster* sp., 05-22
KM017583	KM017458	České Budějovice, South Bohemia, Czech Rep., 2005.vi.17, *Crataegus* sp. 05-34
KM017589	KM017459	České Budějovice, South Bohemia, Czech Rep., 2005.vi.17, *Spiraea* sp. 05-35
KM017591	KM017460	České Budějovice, South Bohemia, Czech Rep., 2005.vi.18, *Malus domestica*, 05-42
KM017582	KM017461	Skirgiškės, Vilnius distr., Lithuania, 2005.vii.12, *Cotoneaster* sp., 05-68
KM017579	KM017462	Kyiv, Ukraine, 2006.vi.12, *Spiraea salicifolia*, 06-32
KM017590	KM017463	Kyiv, Ukraine, 2006.vi.13, *Crataegus* sp., 06-43
KM017580	KM017464	Zadrachje, Gorodok distr., Belarus, 2008.vi.18, *Malus domestica*, 08-23
KM017581	KM017465	Riga, Latvia, 2008.vii.03, *Malus domestica*, 08-72
KM017592	KM017466	Salaspils, Latvia, 2008.vii.04, *Aronia melanocarpa*, 08-91
KM017577	KM017467	Lublin, Poland, 2008.ix.01, *Malus domestica*, 08-112
KM017578	KM017468	Lublin, Poland, 2008.ix.02, *Crataegus* sp., 08-117
KM033438	KM017469	Przewiez, Podlasie, Poland, 2009.ix.29, *Malus* sp. cult., 09-45
KM017567	KM017470	Blagojevgrad, Bulgaria, 2012.vi.26, *Malus* sp., 12-87
KM017568	KM017471	Frankfurt/Maine, Germany, 2012.vi.30, *Malus domestica*, 12-105
KM017593	KM017472	Kraujaliai, Molėtai distr., Lithuania, 2012.vii.10, *Pyrus* sp., 12-112
KM017569	KM017473	Kraujaliai, Molėtai distr., Lithuania, 2012.vii.10, *Malus domestica*, 12-115
KM017570	KM017474	Stirniai, Molėtai distr., Lithuania, 2012.vii.12, *Cotoneaster* sp., 12-125
KM017571	KM017475	Vidugiris, Molėtai distr., Lithuania, 2012.vii.13, *Sorbus aucuparia*, 12-133
KM017572	KM017476	Kegums, Latvia, 2012.vii.30, *Pyrus communis*, 12-144
KM017573	KM017477	Kegums, Latvia, 2012.vii.30, *Malus domestica*, 12-145
KM017574	KM017478	Skriveri, Latvia, 2012.viii.01, *Crataegus* sp., 12-152
KM017575	KM017479	Sangaste, Valga county, Estonia, 2012.viii.01, *Malus domestica*, 12-159
KM033439	KM017480	Tartu, Estonia, 2012.viii.03, *Cotoneaster* sp., 12-160
KM017588	KM017481	Tartu, Estonia, 2012.viii.03, *Crataegus* sp., 12-161
KM017576	KM017482	Juodkrantė, Neringa, Lithuania, 2012.viii.10, *Crataegus* sp., 12-183
KM017585	KM017483	Wojslawice, Lower Silesia, Poland, 2013.vi.20, *Amelanchier lamarckii*, 13-100
KM033441	KM017484	Rundale, Latvia, 2013.vii.02, *Cydonia* sp., 13-106
KM017586	KM017485	Rēzekne, Latvia, 2013.vii.16, *Cotoneaster* sp., 13-127
KM017587	KM017486	Karsava, Latvia, 2013.vii.17, *Cotoneaster* sp., 13-134
		Aphis spiraecola
KM017600	KM017487	Catania, Sicily, Italy, 2004.vi.26, *Pittosporum* sp., 04-39
KM017599	KM017488	Skirgiškės, Vilnius distr., Lithuania, 2005.vii.12, *Spiraea* sp., 05-70
KM017595	KM017489	Salaspils, Latvia, 2008.vii.04, *Spiraea* sp., 08-78
KM017597	KM017490	Sigulda, Latvia, 2008.vii.06, *Spiraea* sp., 08-97
KM017596	KM017492	Lublin, Poland, 2008.ix.02, *Cotoneaster* sp., 08-115
KM017610	KM017491	Randazzo, Sicily, Italy, 2009.vi.10, *Cotoneaster* sp., 09-18
KM017611	KM017493	Karamanmarash, Turkey, 2011.v.28, *Spiraea* sp., 11-16
KM017598	KM017494	Karamanmarash, Turkey, 2011.v.28, *Armeniaca vulgaris*, 11-17
KM017594	KM017495	Šalčininkai, Lithuania, 2012.vi.13, *Spiraea* sp., 12-52
KM017609	KM017496	Preila, Neringa, Lithuania, 2012.viii.13, *Prunus cerasifera*, 12-200
KM017601	KM017497	Bagnolo Mella, Brescia prov., Italy, 2013.iv.28, *Chaenomeles* sp., 13-17
KM017602	KM017498	Poncarale, Brescia prov., Italy, 2013.v.02, *Chaenomeles* sp., 13-34
KM017604	KM017499	Beijing, China, 2013.vi.03, *Malus* sp., 13-68
KM017605	KM017500	Muntianyu, Huairou county, China, 2013.vi.04, *Malus* sp., 13-69
KM017606	KM017501	Beijing, China, 2013.vi.06, *Chaenomeles* sp., 13-71
KM017607	KM017502	Beijing, China, 2013.vi.06, *Prunus cerasifera Pissardii*, 13-72
KM017608	KM017503	Beijing, China, 2013.vi.03, *Malus* sp., 13-74
KM033440	KM017504	Pawłowice, Lower Silesia, Poland, 2013.vi.21, *Prunus cerasifera*, 13-101
KM017603	KM017505	Pawłowice, Lower Silesia, Poland, 2013.vi.21, *Prunus mahaleb*, 13-102

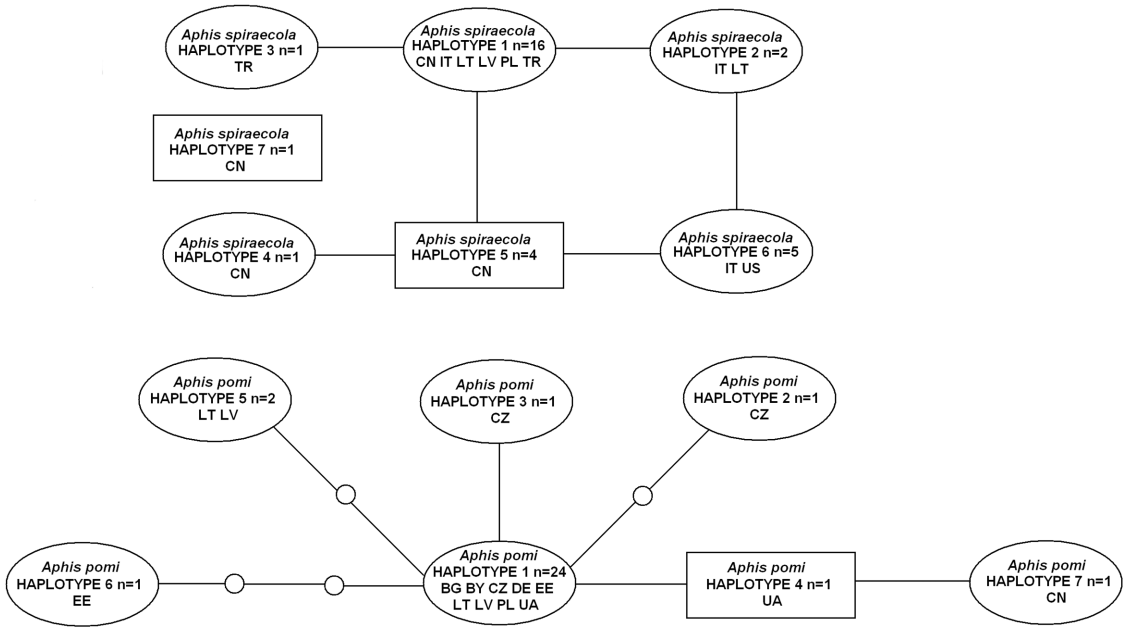

Fig. 1. Haplotype networks for COI fragment (621 positions in final set, 95% connection limit, gaps treated as missing data) haplotypes of *A. pomi* and *A. spiraecola*. The haplotype with the highest outgroup probability is displayed as a square, while others are displayed as ovals. For sample information, see Tables 1–3. BG – Bulgaria, BY – Belarus, CN – China, CZ – Czech Republic, DE – Germany, EE – Estonia, IT – Italy, LV – Latvia, LT – Lithuania, PL – Poland, TR – Turkey, UA – Ukraine, US – United States of America.

The most common COI haplotype of *A. spiraecola* (No. 1, n = 16) occurred in samples from China (n = 5), Italy (n = 3), Poland (n = 3), Latvia (n = 2), Lithuania (n = 2) and Turkey (n = 1). (Tables 1–3, Fig. 1). Haplotype No. 6 (n = 5) was collected in Italy (n = 3) and USA (n = 2) and haplotype No. 5 (n = 4) only in China. Haplotype No. 2 (n = 2) was collected in Italy and Lithuania. Unique COI haplotypes represented by a single sequence were detected in material collected in Turkey (No. 3) and China (No. 4). Our data do not indicate any clear geographical background in the distribution of the COI haplotypes of apple and spirea aphids analyzed.

Out of 31 partial COI sequences of *A. pomi*, host plant information was available for 30 of them (Tables 1–2). The most abundant COI haplotype No. 1 (n = 24) occurred in samples collected from *Malus*, *Pyrus*, *Cotoneaster*, *Crataegus*, *Spiraea*, *Sorbus*, *Cydonia* and *Amelanchier*, and is clearly not host specific. Unique COI haplotypes were also not host specific as they were collected from the same hosts (*Malus*, *Spiraea* and *Crataegus*) as the most common

Table 2. Partial sequences of COI and EF-1α from GenBank that were used in the present study for comparison.

GenBank Accession No. [Reference]		Place, date, host plant, collection No. (when available)
COI	EF-1α	
Aphis pomi		
FJ965738[1]		China
Aphis spiraecola		
FJ965690, FJ965733–FJ965737[1]		China
KC897200–KC897201[2]	KC897309–KC897310[2]	University of Missouri at Columbia, Boone Co., Missouri, 06/02/08, *Spiraea* spp., 510386
KC897197–KC897199[2]	KC897307–KC897308[2]	Botanical Garden, Maneace, Sicily, Italy, 06/10/09, *Viburnum tinus,* 512834–512835
	KC897306[2]	3103 Amy Dr., Champaign Co., Illinois, 06/27/08, *Spiraea* spp., 510384–510385
	EU358925[3]	GG, Anyang, 03-Jun-05, *Spiraea thunbergii* (Rosaceae), 050603HJ6
	AY219725[4]	USA: UT, Cache Co., Logan, 3Jul 96, *Malus* sp. (Rosaceae), 96-18
Outgroup species *Nasonovia ribis-nigri* (Macrosiphini)		
DQ153169		Taiwan, Republic of China
	DQ005158[5]	NZ: South Island, Christchurch, 30 Apr 2002, 02-52, *Lactuca* sp.

[1]Wang et al., 2011; [2]Lagos et al., 2014; [3]Kim & Lee, 2008; [4]von Dohlen & Teulon, 2003; [5]von Dohlen et al., 2006.

Table 3. COI haplotypes of *Aphis pomi* and *Aphis spiraecola* revealed by the haplotype network analysis. Sample numbers are the same as in Tables 1–2.

Haplotype no.	No. of sequences	Length of haplotype (bp) without gaps	Sequences belonging to haplotype
Aphis pomi			
1	24	621	05-22; 05-34; 05-68; 06-32; 08-23; 08-72; 08-112; 08-117; 09-45; 12-87; 12-105; 12-115; 12-125; 12-133; 12-144; 12-145; 12-152; 12-159; 12-160; 12-183; 13-100; 13-106; 13-127; 13-134
2	1	621	05-35
3	1	621	05-42
4	1	621	06-43
5	2	621	08-91; 12-112
6	1	621	12-161
7	1	621	FJ965738
Aphis spiraecola			
1	16	621	04-39; 05-70; 08-78; 08-97; 08-115; 11-17; 12-52; 13-17; 13-34; 13-68; 13-69; 13-71; 13-72; 13-74; 13-101; 13-102
2	2	621	09-18; 12-200
3	1	621	11-16
4	1	621	FJ965737
5	4	621	FJ965736; FJ965735; FJ965734; FJ965733
6	5	621	KC897201; KC897200; KC897199; KC897198; KC897197
7	1	621	FJ965690

haplotype. This is also the case for the rare haplotype No. 5, which was recorded in samples from *Aronia* and *Pyrus*.

Of the 30 partial COI sequences of spirea aphid there is host plant information for 24. As in case of the apple aphid, the most common haplotype of spirea aphid appeared to be polyphagous, as it was collected from a broad spectrum of hosts, including those of the apple aphid: *Spiraea* (n = 4), *Chaenomeles* (n = 3), *Prunus* (n = 3), *Malus* (n = 3), *Cotoneaster* (n = 1), *Pittosporum* (n = 1) and *Armeniaca* (n = 1). Remaining haplotypes of spirea aphid shared the same hosts with the commonest haplotype, except three samples of haplotype No. 6, collected from *Viburnum* in Italy (Sicily).

The maximum parsimony (MP) analysis of partial COI sequences resulted in 618 equally parsimonious trees (length = 105, CI = 0.79, RI = 0.98). ML tree (T92+I model) had a similar topology to the NJ (K2P distances) and BI (HKY+I model) analyses. NJ, MP and ML bootstrap values over 40% together with BI posterior probabilities over 0.50 are given at the respective nodes of the same tree in Fig. 2. The two *Aphis* species form distinct strongly supported clusters. The apple aphid clade is highly homogenous. Only four specimens from Lithuania, Latvia, Ukraine and China do not group with the remaining samples (Fig. 2). The clade of the spirea aphid appears more complex, comprising four moderately supported branches, one of them being represented only by Chinese samples (n = 5). Noticeably, GenBank sequence No. FJ965690 from China deposited as *A. spiraecola*, grouped outside the spirea aphid clade, both in the haplotype network (Fig. 1) and phylogenetic tree (Fig. 2). It appeared closer to the outgroup sequence of *Nasonovia ribis-nigri*, belonging to the tribe Macrosiphini of the aphid subfamily Aphidinae (Aphididae). This indicates an incorrect identification of the sequenced aphid specimen, because the genus *Aphis* belongs to the tribe Aphidini.

The analyzed region of EF-1α consisted of two parts of three exons and two introns, which were not removed before further analysis. The alignment of this fragment contained 510 sites, 6 of which were variable and parsimony informative. The average nucleotide composition was: T – 30.3%, C – 18.2%, A – 31.4% and G – 20.1%. The overall transition/transversion bias was R = 1.335. Interspecific pairwise sample divergences between spirea and apple aphid species ranged from 0.6 to 1.2% (average 0.9%). The range of the intraspecific pairwise sample divergences (K2P model) for the spirea aphid was 0–0.6% (average 0.2%), whilst all sequences of the apple aphid appeared identical. Noticeably, EF-1α sequences of the apple aphid differed from the closest haplotype of the spirea aphid in terms of only three base changes (Fig. 3), which is usually the characteristic of closely related haplotypes, attributable to the same species in the haplotype network.

All partial EF-1α sequences of the apple aphid (n = 30) from 9 European countries were identical, thus, no correlation between haplotypes and geographic origin could be detected. Five haplotypes were identified among the 26 partial EF-1α sequences of spirea aphid, (Tables 1, 2, 4, Fig. 3). Most of the samples (n = 14 altogether) from China (n = 5), Italy (n = 4), Poland (n = 2), Lithuania (n = 1) and Turkey (n = 2) included EF-1α haplotype No. 1. EF-1α haplotype No. 2 (n = 5) was recorded in samples from Lithuania (n = 2), Latvia (n = 2) and Poland (n = 1), and EF-1α haplotype No. 3 in samples from Italy (n = 3) and USA (n = 2). There is one more haplotype (No. 5, n = 1) reported in the USA. Haplotype (No. 4) was represented by single sequence from Korea. As for the COI haplotypes, our data do not indicate any geographical background in

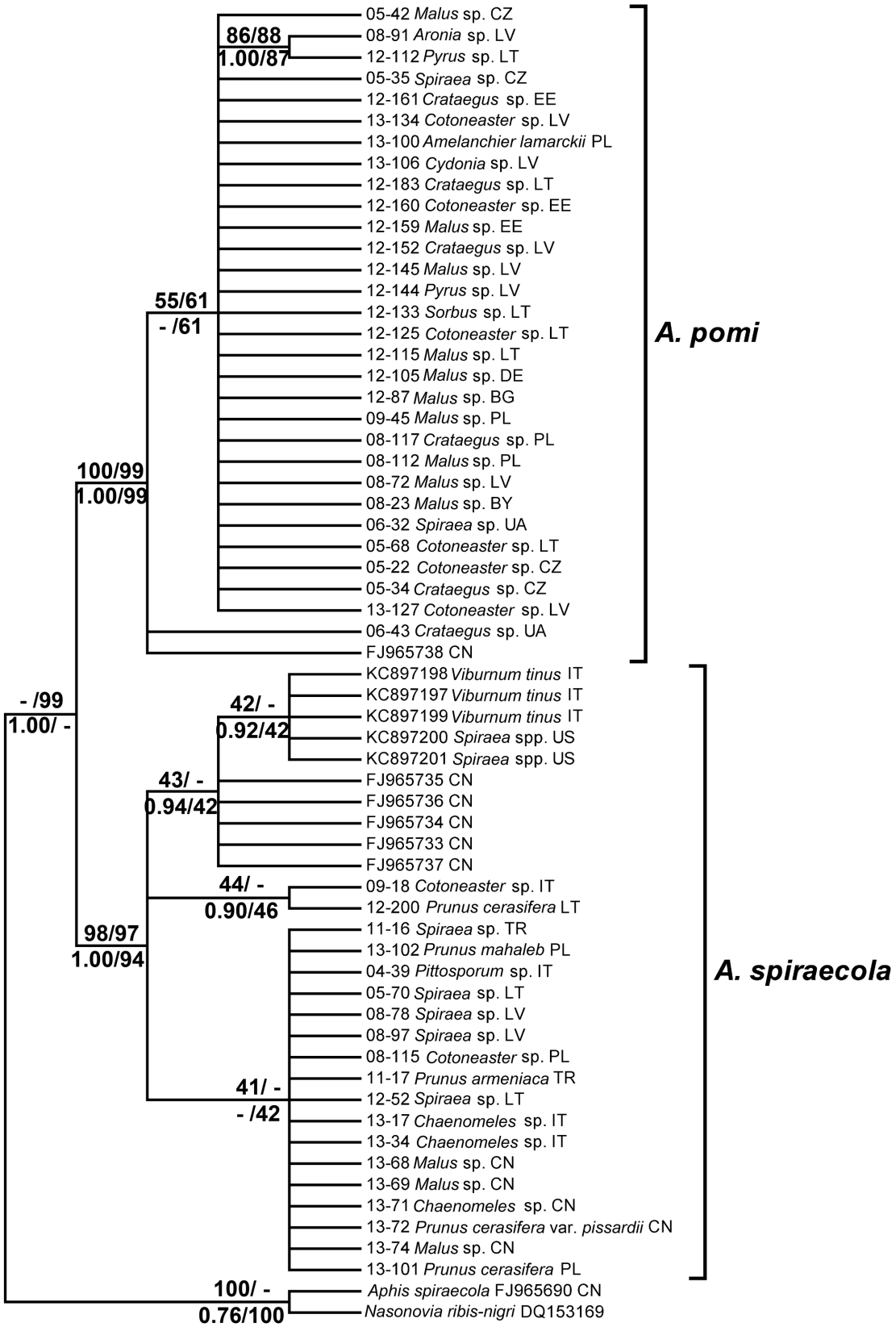

Fig. 2. Maximum Likelihood (ML) tree showing phylogenetic relationships among *A. pomi* and *A. spiraecola* based on partial sequences of mitochondrial COI (621 positions in final set). Numbers above branches indicate support of NJ (left, > 40%) and MP (right, > 40%) based on bootstrap test with 1000 replicates, and numbers below branches indicate support of ML (right, > 40%) bootstrap test with 1000 replicates and posterior probabilities of BI analysis (left, > 0.50). Sample numbers are the same as in Tables 1–2, together with the abbreviated symbol of the relevant country BG – Bulgaria, BY – Belarus, CN – China, CZ – Czech Republic, DE – Germany, EE – Estonia, IT – Italy, LV – Latvia, LT – Lithuania, PL – Poland, TR – Turkey, UA – Ukraine, US – United States of America.

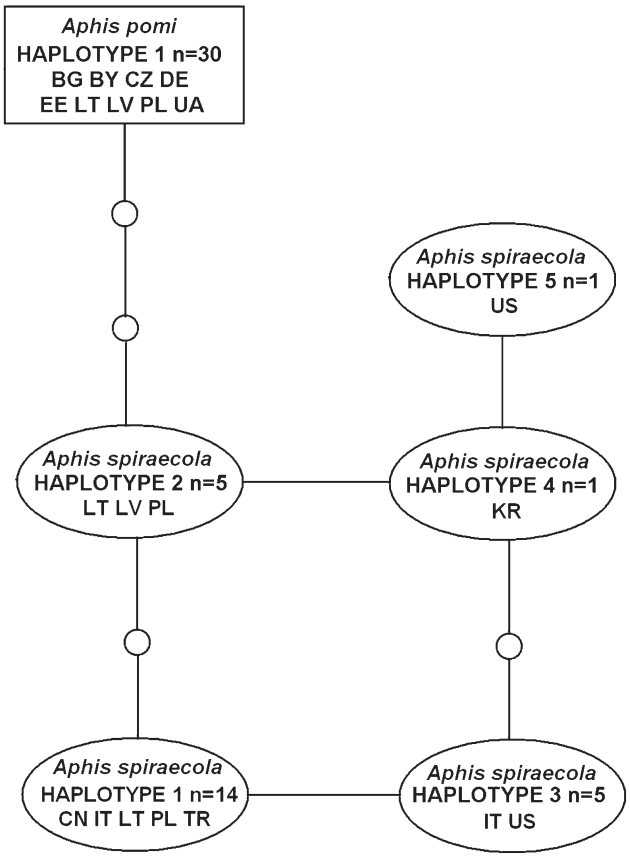

Fig. 3. Haplotype network for EF-1α fragment (508 positions in final set, 95% connection limit, gaps treated as 5th state) haplotypes of *A. pomi* and *A. spiraecola*. The haplotype with the highest outgroup probability is displayed as a square, while others are displayed as ovals. For sample information, see Tables 1–3. BG – Bulgaria, BY – Belarus, CN – China, CZ – Czech Republic, DE – Germany, EE – Estonia, IT – Italy, KR – Korea, LV – Latvia, LT – Lithuania, PL – Poland, TR – Turkey, UA – Ukraine, US – United States of America.

the haplotype distribution of the spirea aphid partial EF-1α sequences.

All partial EF-1α sequences for the apple aphid (n = 30) from 9 genera of pomoideous hosts were identical, thus, no correlation between haplotypes and host plant could be detected. Out of 26 partial EF-1α sequences for the spirea aphid, host plant information was available for 20 and the commonest haplotype was associated with host plants of 6 genera: *Prunus* (n = 4), *Malus* (n = 3), *Chaenomeles* (n = 3), *Cotoneaster* (n = 2), *Spiraea* (n = 1) and *Pittosporum* (n = 1). Haplotype No. 2 was collected predominantly from *Spiraea* (n = 4), with just one sample from *Prunus*. Samples from *Spiraea* (n = 3) and *Viburnum* (n = 2) were of haplotype No. 3. Two remaining unique haplotypes were not unique in terms of their host plant associations, being collected from *Spiraea* (haplotype No. 4) and *Malus* (No. 5). As for COI haplotypes, our data do not indicate any host based background in the haplotype distribution of the spirea aphid partial EF-1α sequences.

The maximum parsimony (MP) analysis of partial EF-1α sequences resulted in 1010 equally parsimonious trees (length = 56, CI = 0.80, RI = 0.98). ML tree (T92 model) had a similar topology to the NJ (K2P distances) and BI (F81+I+G) analyses. NJ, MP and ML bootstrap values over 40% together with BI posterior probabilities over 0.50 are given at the respective nodes of the same tree in Fig. 4. The apple and spirea aphids form distinct clusters. The apple aphid clade is homogenous because the sequences are all identical. The clade of the spirea aphid appears more complex and includes three moderately supported branches, none of them with geographic or host plant specificity. Noticeably, spirea aphids from Italy and USA collected from *Viburnum* and *Spiraea* respectively, were grouped together both by their COI and EF-1α partial sequences (COI haplotype No. 6 and EF-1α haplotype No. 3, respectively, Tables 2–4, Figs 1–4). Of the 14 specimens of spirea aphid with identical sequence of EF-1α (haplotype No. 1, Table 4) 12 also had identical COI sequences (haplotype No. 1, Table 3). Such congruence might indicate evolutionary specificity of certain lineages of spirea aphid.

Halbert & Voegtlin (1992), followed by Blackman & Eastop (2000), suggest three morphological characters can be used to discriminate between apterous viviparous females of apple and spirea aphids: numbers of marginal tubercles on abdominal tergites II–IV (present in apple aphid, absent in spirea aphid); numbers of caudal hairs (10–19 hairs

Table 4. EF-1α haplotypes of *Aphis pomi* and *Aphis spiraecola* revealed by the haplotype network analysis. Sample numbers are the same as in Tables 1–2.

Haplotype no.	No. of sequences	Length of haplotype (bp) without gaps	Sequences belonging to haplotype
			Aphis pomi
1	30	507	05-22; 05-34; 05-35; 05-42; 05-68; 06-32; 06-43; 08-23; 08-72; 08-91; 08-112; 08-117; 09-45; 12-87; 12-105; 12-112; 12-115; 12-125; 12-133; 12-144; 12-145; 12-152; 12-159; 12-160; 12-161; 12-183; 13-100; 13-106; 13-127; 13-134
			Aphis spiraecola
1	14	507	04-39; 09-18; 08-115; 11-16; 11-17; 12-200; 13-17; 13-34; 13-68; 13-69; 13-71; 13-72; 13-74; 13-102
2	5	507	05-70; 08-78; 08-97; 12-52; 13-101
3	5	507	KC897310; KC897309; KC897308; KC897307; KC897306
4	1	507	EU358925
5	1	508	AY219725

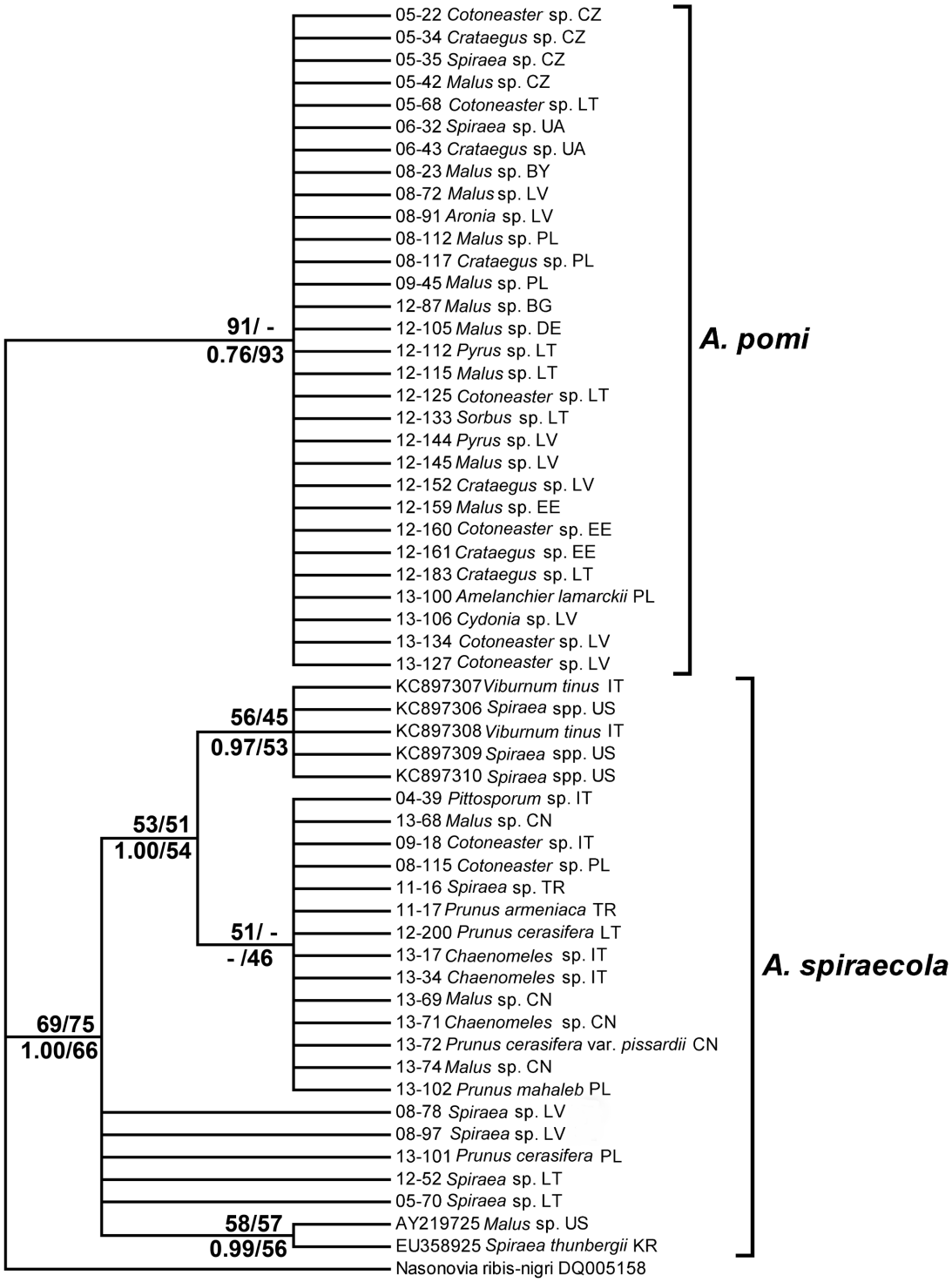

Fig. 4. Maximum Likelihood (ML) tree showing phylogenetic relationships among *A. pomi* and *A. spiraecola* based on partial sequences of nuclear EF-1α (510 positions in final set). Numbers above branches indicate support of NJ (left, > 40%) and MP (right, > 40%) based on bootstrap test with 1000 replicates, and numbers below branches indicate support of ML (right, > 40%) bootstrap test with 1000 replicates and posterior probabilities of BI analysis (left, > 0.50). Sample numbers are the same as in Tables 1–2, together with the abbreviated symbol of the relevant country BG – Bulgaria, BY – Belarus, CN – China, CZ – Czech Republic, DE – Germany, EE – Estonia, IT – Italy, KR – Korea, LV – Latvia, LT – Lithuania, PL – Poland, TR – Turkey, UA – Ukraine, US – United States of America.

in apple aphid, 7–15 in spirea aphid); length of ultimate rostral segment (exceeding 120 µm in apple aphid, less than 120 µm in spirea aphid). Of these three, the length of ultimate rostral segment appeared the most reliable when applied to our material: all individuals of apple aphid (n = 143, Table 5) had ultimate rostral segment lengths greater than 120 µm (mean 155.44; range 127–182). Of 94 individuals of spirea aphid collected in Europe, 86 (91.5%) had an ultimate rostral segment length ranging from 76 to 120 µm. Of 61 individuals of spirea aphid collected in China,

TABLE 5. Summary statistics for the key morphological characters of apterae of *Aphis pomi* and *A. spiraecola*. For comparison the same data from Foottit et al. (2009) are given. URS – ultimate rostral segment length (all lengths in μm), SIPHON – siphunculus length, CAUDA – length of cauda (apical part), MT2-4(5) – numbers of marginal tubercles on abdominal tergites II–IV(V), HCAUDA – numbers of caudal hairs, Siph/cauda – ratio of siphuncular length to caudal (apical part) length.

Characters	*Aphis pomi*				*Aphis spiraecola*			
	n	Range	Mean	SD	*n*	Range	Mean	SD
				Europe				
URS	143	127–182	155.44	10.00	94	76–130	104.98	14.00
SIPHON	144	156–614	417.30	80.00	94	94–470	257.20	88.00
CAUDA	143	78–179	144.22	18.00	90	88–206	141.19	21.95
MT2-4	144	2–6	4.72	1.00	94	0–3	0.33	0.66
HCAUDA	144	8–23	14.77	3.00	92	7–19	12.19	2.83
Siph/cauda	143	2.00–3.86	2.88	0.32	90	0.67–2.77	1.79	0.50
				China				
URS					61	80–125	100.40	13.14
SIPHON					62	180–438	284.29	48.11
CAUDA					63	96–177	129.35	20.40
MT2-4					63	0–2	0.32	0.53
HCAUDA					63	7–15	10.21	1.76
Siph/cauda					62	1.74–2.95	2.21	0.29
				Canada and USA (Foottit et al., 2009)				
URS	155	120–156	139	7	54	94–123	110	8
SIPHON	155	167–562	386	86	54	134–377	266	58
CAUDA	153	68–201	128	19	54	91–177	141	21
MT2-5	154	1–7	5.25	1.06	50	0–2	0.24	0.65
HCAUDA	155	10–21	15.6	2.1	54	7–14	10.4	1.8
Siph/cauda		> 2.45				< 2.45		

60 (98.4%) had an ultimate rostral segment ranging from 80–120 μm. The numbers of marginal tubercles on abdominal tergites II–IV ranged from 2–6 in the apple aphid, compared to 0–3 in European collected spirea aphid (Table 5), which might result in the misidentification of 24.5% of the individuals of spirea aphid from Europe included in the present study. Of the 63 individuals of spirea aphid from China, 45 (71.4%) lacked marginal tubercles on their abdominal tergites II–IV. Numbers of caudal hairs in both species showed much more overlap (Table 5). In addition to these three characters, Foottit et al. (2009) suggest the ratio of siphuncular length to caudal (apical part) length, with the threshold discriminating value being 2.45. In our case, this ratio ranged from 2.00 to 3.86 in the apple aphid, and for the spirea aphid it was 0.67–2.77 and 1.74–2.95 for samples from Europe and China, respectively (Table 5).

DISCUSSION

Molecular markers are widely used to reveal cryptic insect species, including aphids (Rakauskas et al., 2011). Partial COI sequences used for DNA barcoding were analyzed for *A. pomi* (n = 76) and *A. spiraecola* (n = 56) by Foottit et al. (2009). The values of interspecific pairwise sample divergences were higher (mean 5.0%, range 4.8–5.1%) than those obtained for the COI fragments used in this study (mean 3.6%, range 3.1–4.3%). The majority of the spirea aphids (50 out of 56) from North America, Australia, Guam, Palau and Marshall Islands, also have identical COI barcode sequences (Foottit et al., 2009). The remaining individuals from New Zealand (n = 1), New York (n = 2) and British Columbia (n = 3) differed from the most abundant COI haplotype by one to three base changes, giving a maximum pairwise within-species divergence of 0.6%. In our study, maximum pairwise within-species divergence of the spirea aphid was 0.5% (average 0.2%, range 0.0–0.5%). This is in accordance with the conclusion of Foottit et al. (2009) that the variation in biological characteristics among populations of spirea aphid was greater than in those of apple aphid.

Analysis of COI barcode fragments indicate that sequences for the apple aphid collected in North America are identical (Foottit et al., 2009). In our study we recorded a greater diversity in COI fragments from European specimens of the apple aphid. Six haplotypes were detected in 30 samples. This might be because of the presumed Palaearctic origin of the apple aphid, which is reported to be a non-native species in the Nearctic, where it was first noted in North America in 1844 (Foottit et al., 2006). This fact could account for the homogeneity of apple aphid COI sequences from North American populations (Foottit et al., 2009). Unlike the partial COI sequences, our study indicates that partial sequences of the nuclear EF-1α from European samples of the apple aphid are very homogeneous (1 haplotype, Table 4). Such homogeneity might be attributed to the isolated mode of reproduction of this species: gynoparae and males are apterous resulting in a high incidence of intraclonal inbreeding. Genetic consequences of such a reproductive system appear similar to those of anholocyclic populations resulting in a few predominant

clones that appear to be considerably different from one another (Kanbe & Akimoto, 2009).

In general, species level identification of apple and spirea aphids by means of COI and EF-1α partial sequences coincided with that based on commonly used morphological characters (Halbert & Voegtlin, 1992; Blackman & Eastop, 2000; Foottit et al., 2009). However, none of the above mentioned morphological characters (Table 5) on their own can ensure a 100% correct discrimination between apple and spirea aphids. All four characters should be used to determine the identity of series of individual aphids in each sample. The situation might be even more complicated due to the presence of mixed colonies of both species. For example, apterous viviparous females (n = 5) collected from *Cotoneaster* in Lublin, Poland (sample 08–115) had clear morphological characters typical of the apple aphid: ultimate rostral segment length 148–156 µm; numbers of marginal tubercles on abdominal tergites 4–6; ratio of siphuncular length to caudal length 2.40–2.80. For DNA extraction one winged individual aphid was used and its sequence grouped together with those of the spirea aphid. When the morphology of the voucher specimen was checked, it resembled the spirea aphid. This case demonstrates that mixed colonies might also complicate identification by means of DNA sequences, because several individuals per colony should be subject to DNA analysis.

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