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How many species are there in the subgenus *Bursaphis* (Hemiptera: Sternorrhyncha: Aphididae)? CO-I evidence

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Abstract. Species-level problems in the *Aphis* (*Bursaphis*) complex are reconsidered based on the partial sequences of the mitochondrial *cox1* gene together with morphological and ecological data. This indicates that the American species *A. oenotherae* is a complex of four species (*A. oenotherae*, *A. holoenotherae*, *A. costalis* and *A. neomexicana*) and the taxonomic status of the species couples *A. varians – A. manitobensis* and *A. epilobii – A. grossulariae* require further clarification. *Aphis* sp. (USA: California, Oregon) of Blackman & Eastop (2006, p. 415) deserves the status of a species provided there is information on its host association and life cycle. Partial *cox1* sequences might be misleading when used as standard DNA barcodes of aphid species of the subgenus *Bursaphis*.

INTRODUCTION

A group of species of the genus *Aphis* Linnaeus, 1758 associated with *Ribes* spp. and/or Onagraceae, in which the ultimate rostral segment has 5 or more additional hairs was initially called "*A. oenotherae*" (Hille Ris Lambers, 1974) and later the "*A. grossulariae*" group (Eastop, 1979; Stroyan, 1984; Heie, 1986). Remaudière (1993) validated the subgenus *Bursaphis* McVicar Baker, 1934 as the formal name for this species group. For the present, *Aphis* (*Bursaphis*) comprises twelve species (Remaudière & Remaudière, 1997; Rakauskas, 1996, 2007), some of which are pests (Börner & Heinze, 1957; Shaposhnikov, 1972). There are several papers on the species level analysis (Robinson & Rojanavongse, 1976; Stroyan, 1984; Cook, 1984; Heie, 1986; Holman, 1990; Remaudière, 1993; Rakauskas, 1998, 1999, 2000, 2003) and phylogeny (Turčinavičienė et al., 2006; Coeur d'acier et al., 2007) of this subgenus, yet the exact number of species in this subgenus is unclear. Namely, the synonymy between *Aphis oenotherae* Oestlund, 1887, *Aphis neomexicana* W.P. Cockerell & T.D.A. Cockerell, 1901 and *Aphis sanborni* Patch, 1914 (Robinson & Rojanavongse, 1976; Remaudière, 1993) is based solely on morphological similarity and remains questionable (Blackman & Eastop, 2000; Rakauskas, 2000). Recently, based on a morphological analysis of aphid material in the collection of the British Museum (Natural History) it was suggested that there is possibly another two yet un-described *Aphis* (*Bursaphis*) species in the USA (California, Oregon) and New Zealand (Blackman & Eastop, 2006: p. 415).

It is common knowledge that the entire genus *Aphis* (including subgenus *Bursaphis*) is characterised by its morphological homogeneity and for a comprehensive taxonomic treatment of the genus it is necessary use information on the life cycles and host specificity of the species (Stroyan, 1984; Heie, 1986). In the case of the subgenus *Bursaphis*, life cycles and host specificity have been experimentally studied in only four of twelve species in this subgenus (Table 1). Thus it is possible that there are also cryptic species, the most recently being *Aphis holoenotherae* Rakauskas recorded in 2007 in Europe (Rakauskas, 2007). *A. holoenotherae* appeared morphologically inseparable from the Nearctic *A. oenotherae*, although the two species have different hosts and life cycles, and different distributions. For taxa that contain complexes of cryptic species and for which there is little or no information on their ecological characters, molecular data is of special interest (Coeur d'acier et al., 2007; Foottit et al., 2008; Mitchell, 2008). The aim of this paper is to reconsider species-level problems in the *Aphis* (*Bursaphis*) complex by using morphological and ecological data, and information on partial sequences of the mitochondrial *cox1* gene.

MATERIAL AND METHODS

Aphid samples

Sixty-two field-collected and clonal samples of sixteen species originating from fourteen countries were used in this study (Tables 2–3). Of the sixteen species used, 9 belong to *Aphis* (*Bursaphis*), 4 to *Aphis* s. str. and 3 were out-group species. Fifteen samples were from the USA, four from Canada and one sample each from Japan and South Korea, and the remaining samples from ten mostly Central European countries. Microscope slides were prepared for the morphological identification of the aphids. The key of R.L. Blackman & V.F. Eastop (2006) was used to identify the aphids in most cases and that of R. Rakauskas (2008) to morphological discriminate between *A. oenotherae* and *A. holoenotherae*. These slides are located at the Department of Zoology, Vilnius University. Samples asterisked in Table 2 were kindly supplied already identified by R. Foottit and A. Jensen. In this paper it is accepted that the species of *Aphis* (*Bursaphis*) are well defined if based on clear morphological specificity and at least indirect evidence on their host specificity and life cycles (asterisked in Table 1). The remaining

TABLE 1. Species of the subgenus *Bursaphis* McVicar Baker in the World (Müller, 1974; Martin, 1982; Stroyan, 1984; Cook, 1984; Heie, 1986; Holman, 1990; Remaudière, 1993; Rakauskas, 1996, 1998, 2003, 2007, 2008; Blackman & Eastop, 2006). Life cycle characteristics confirmed by experimental studies (Patch, 1927; Rakauskas, 1993, 2007) are given in bold. Asterisked species of *Aphis* (*Bursaphis*) are designated well-defined species in the text based on their specific morphology and at least indirect evidence on the specificity of their life cycles and host associations.

Species	Life - cycle	Winter hosts	Summer hosts (* indicates occasional hosts)	Distribution
* *grossulariae* Kaltenbach, 1843	**holocyclic, facultatively heteroecious**	*Ribes* spp.	*Epilobium* spp., *Chamaenerion angustifolium*, **Oenothera* spp., **Godetia* spp., **Fuchsia* spp.	Palaearctic
epilobii Kaltenbach, 1843	holocyclic, monoecious		*Epilobium montanum*, **E. lanceolatum*	Europe
oenotherae Oestlund, 1887	holocyclic, heteroecious	*Ribes* spp.	*Oenothera* spp., *Epilobium* spp.	Nearctic (other regions require confirmation)
* *varians* Patch, 1914	**holocyclic, heteroecious**	*Ribes* spp.	*Epilobium* spp., *Chamaenerion angustifolium*,	Nearctic
* *epilobiaria* Theobald, 1927	holocyclic, monoecious		*Epilobium hirsutum*, *E. palustre*	Europe
popovi Mordvilko, 1932	? holocyclic, monoecious	*Ribes* spp.		Eastern Siberia (Yakutia)
solitaria (McVicar Baker, 1934)	holocyclic, heteroecious	*Ribes* spp.	Compositae	Mexico
* *schneideri* (Börner, 1940)	**holocyclic, monoecious**	*Ribes* spp.		Palaearctic
manitobensis Robinson & Rojanavongse, 1976	holocyclic, monoecious	*Ribes* spp.		Canada
fluvialis Martin, 1982	anholocyclic, ? monoecious		*Epilobium hirsutum*	North Africa (Sudan)
* *costalis* Cook, 1984	? holocyclic, heteroecious	*Ribes* spp.	*Mimulus* spp.	USA
* *holoenotherae* Rakauskas, 2007	**holocyclic, monoecious**		*Oenothera* spp.	Europe, Korea, Japan

species in this subgenus are designated ill-defined due to the lack of information on their life cycles and host specificity.

When establishing aphid clones, colonies were initiated from a single parthenogenetic female and are expected to contain DNA from genetically homogenous individuals. Aphid rearing provided important information on aphid life cycles and host specificity of the aphid lineages used for DNA extraction. Detailed information on the aphid cloning methods used is already published (Rakauskas, 1993). Field samples were taken from a single colony. Aphids were either frozen at –80°C or stored in 96% ethanol.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from a single individual using the DNeasy Blood & Tissue kit (Qiagen), which involved at least a 2 h digestion of tissue with proteinase K.

The target sequence was a partial fragment from the mitochondrial gene encoding Cytochrome Oxidase subunit I (CO-I). The 620 bp region was PCR-amplified using previously published primers (Turčinavičienė et al., 2006). PCR amplification was carried out in a thermal cycler (Eppendorf) in 50 µl volumes containing 1–2 µl genomic DNA, 5 µl of each primer (10 µM), 5 µl of PCR-reaction buffer, 5 µl of dNTP mix (2 mM each), 4–8 µM of 25 mM $MgCl_2$ and 1,25 U of *Taq* polymerase (recombinant) (5U/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″, annealing at 49°C for 30″ and extension at 72°C for 30″ (32 cycles in total), and a final extension for 5 min (1 cycle). PCR products were subjected to electrophoresis on 2% Top Vision LE GQ agarose (Fermentas, Lithuania), stained with ethidium bromide and sized against a Gene Ruler 100 bp DNA ladder (Fermentas, Lithuania) under UV light. PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen). Cyclic sequencing was performed at the Institute of Biotechnology (Lithuania) using a BigDye® Terminator v3.1 Cycle Sequencing Kit and products sequenced using a 3130 × l Automated Sequencer (Applied Biosystems). 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 Bio-Edit Sequence Alignment Editor (Hall, 1999). Sequences were tested for stop codons and none were found.

DNA based species delimitation

Of the numerous methods available to delimit species boundaries (Pons et al., 2006, Vogler & Monaghan, 2007) the following were used in this study.

1. The DNA barcoding method (Hebert et al., 2003) that has been successfully used to identify Linnaean species and indicate presumably cryptic species (Footit et al., 2008). It is based on the pair wise nucleotide sequence divergences measured as distances: within-species sample divergences normally appear to be much lower than inter-specific ones. Pair wise nucleotide sequence divergences were calculated using Kimura 2-parameter (K2P) model of base substitution (Kimura, 1980), which has been evaluated as the best model for species level analysis at low distances (Hebert et al., 2003). The species Identifier tool, as implemented in TaxonDNA (Meier et al., 2006), evaluates uncorrected genetic distances and the results are similar to those obtained using the K2P model (Meier et al., 2008). The smallest inter-specific distances were used to predict cryptic species complexes in accordance with the suggestion of

TABLE 2. Field-collected aphid material studied. Morphological species identified using the keys of Blackman & Eastop (2006) and Rakauskas (2008). Morph abbreviations: apt. – apterous viviparous females; al. – winged viviparous females; ov. – oviparous females. * – identification of R. Foottit and E. Maw; ** – identification of A. Jensen.

Species	Place and date	Host plant	Morphs	Abbrev. on figs
	subgenus ***Bursaphis***			
A. holoenotherae	Skirgiškės, Vilnius distr., Lithuania, 3.viii.2002	*Oenothera biennis*	Apt.	Ahol0282
	Gangwon-Dunnae, South Korea, 27.vi.2003	*Oenothera lamarckiana*	Apt., al.	AholK
	Dabrowa Gornicza, Katowice distr., Poland, 18.x.2003	*Oe. rubricaulis*	Apt., nym.	Ahol125
	Puvočiai, Varena distr., Lithuania, 28.vi.2002	*Oenothera* sp.	Apt.	Ahol10
	Kalėdiškiai, Veisiejai distr., Lithuania, 1.vii.2008	*Oenothera biennis*	Apt., al.	Ahol66
	Novaja Budovka, Tolochinsk distr., Vitebsk reg., Belarus, 21.vi.2008	*Oenothera biennis*	Apt., al.	Ahol64
	Tsukuba, Japan, 16.vii.2009	*Oenothera* sp.	Apt., al.	Aoen164
	Botanical garden, Kiyv, Ukraine, 13.vi.2006	*Oenothera biennis*	Apt., al.	Ahol54
	rue du Buffon, Paris, France, 25.vi.2004	*Oenothera biennis*	Apt., al.	Ahol26
	Sn Feliz de las Lavanderas, Leon distr., Spain, 9.i.2005	*Oenothera biennis*	Apt., al.	Ahol34
	Berezovaja Gora, Smolevichi distr., Minsk reg., Belarus, 21.vi.2008	*Oenothera biennis*	Apt., al.	Ahol65
A. epilobiaria	Salaspils, Riga distr., Latvia, 4.vii.2008	*Epilobium hirsutum*	Apt., al.	Aepa94
	Botanical garden, Lublin, Poland, 3.ix.2008	*Epilobium hirsutum*	Apt., al.	Aepa95
	Skirgiškės, Vilnius distr., Lithuania, 25.ix.2002	*Epilobium palustre*	Apt., al.	Aepa101
	Prachatice, South Bohemia, Czech Rep., 16.x.2003	*Oenothera* sp.	Apt.	Aepa197
A. schneideri	Zadrach'e, Gorodok distr., Vitebsk reg., Belarus, 18.vi.2008	*Ribes nigrum*	Al, apt	Asch74
	Botanical garden, Lublin, Poland, 3.ix.2008	*Ribes rubrum*	Al, apt	Asch76
	Kanev Nature reserve, Cherkasy reg., Ukraine, 15.vi.2006	*Ribes nigrum*	Al, apt	Asch84
	Zmejinyje ostrova, Kanev distr., Cherkasy reg., Ukraine, 16.vi.2006	*Ribes nigrum*	Al, apt	Asch85
	Salaspils, Riga reg., Latvia, 4.vii.2008	*Ribes nigrum*	Al, apt	Asch75
	Vilnius, Kairėnai, 4.vii.2001	*Ribes nigrum*	Al, apt	AschS8
	Vilnius, Kairėnai, 27vii.2001	*Ribes rubrum*	Al, apt	AschS19
A. grossulariae	Pakalniškės, Vilnius distr., Lithuania, 6.vii.2008	*Epilobium montanum*	Al., apt.	Agro88
	Narva, Estonia, 25.vi.2008	*Ribes alpinum*	Al, apt	Agro105
	Staroje Selo, Gorodok distr., Vitebsk reg. Belarus, 19.vi.2008	*Epilobium nervosum*	Al, apt	Burs89
	Rabi, Modlešovice, South Bohemia, Czech Rep., 20.vi.2005	*Epilobium* sp.	Apt., al.	Agro87
	Štramberk, North Moravia, Czech Rep., 15.vi.2005	*Epilobium* sp.	Apt., al.	Agro86
	Sigulda, Latvia, 6.vii.2008	*Ribes alpinum*	Al, apt	Agro78
	Jezerishche, Gorodok distr., Vitebsk reg. Belarus, 19.vi.2008	*Ribes nigrum*	Al, apt	Agro77
	Bo, Norway, 28.vi.2003	*Epilobium* sp.	Al, apt	Agroe
	Vilnius, Kairėnai, 28.v.2002	*Ribes nigrum*	Al, apt	Agro8
	Vilnius, Kairėnai, 21.vii.2001	*Ribes rubrum*	Al, apt	Agro15
	Bo, Norway, 28.vi.2003	*Ribes alpinum*	Al, apt	Agroa
A. epilobii	Rabi, Modlešovice, South Bohemia, Czech Rep., 20.vi.2005	*Epilobium* sp.	Apt., al.	Aepi91
	Dūkštai, Vilnius distr., Lithuania, 28.vii.2005	*Epilobium palustre*	Apt., al.	Aepi90
A. epilobii-like	Kyiv, Ukraine, 13.vi.2006	*Epilobium* sp.	Apt., al.	Aepi43
A. oenotherae- like	Battle Creek, Carbon county, Wyoming, USA, 4.vii.2004	*Epilobium* sp.	Apt., al.	Aoen19
	10 miles N. of Flagstaff, Coconino county, Arizona, USA, 6.vii.2004	*Ribes* sp.	Apt.	Aneo14
	Upper Crab Creek, Lincoln county, Washington, USA, 31.v.2004	*Oenothera* sp.	Apt., al.	Aoen71
	20 Miles N. of Pecos, San Miguel county, New Mexico, USA, 6.vii.2004	*Ribes* sp.	Apt.	Aneo69
	Big Tongue Creek, Sheridan county, Wyoming, USA, 7.viii.2004	*Epilobium* sp.	Al.	Aoen68
	Potholes, Grant county, Washington, USA, 30.v.2004	*Ribes aureum*	Apt., al.	Aneo70
	20 Miles N. of Pecos, San Miguel county, New Mexico, USA, 5.vii.2004	*Oenothera biennis*	Apt., al.	Aoen16
	*Hodgson Township, Kenora distr., Canada, 18.vii.2007	*Oenothera biennis*	Nym.	Aoen97
Aphis sp. California	Road S and 9 NE, Grant county, Washington, USA, 30.vi.2004	*Epilobium paniculatum*	Apt., al.	Aepi23

TABLE 2 continued.

Species	Place and date	Host plant	Morphs	Abbrev. on figs
Aphis sp. New Zealand	Moses Lake, Grant county, Washington, USA, 26.vi.2004	*Epilobium* sp.	Apt.	Aphis83
A. manitobensis	*Upper Stoney Lake, Ontario, Canada, 28.v.1994	*Ribes cynosbati*		Aman96
A. costalis	**Potholes, Grant county, Washington, USA, 17.iv.2009	*Ribes aureum*		Burs165
	Potholes, Grant county, Washington, USA, 30.v.2004	*Mimulus* sp.	Al.	Acos80
A. varians	**Snogualmie Pass, Kittitas county, Washington, USA, 25.viii.2008	*Epilobium angustifolium*		Avar108
	*Honeymoon Bay, British Columbia, Canada, 26.viii.2007	*Epilobium angustifolium*		Avar100
	Hwy 24, Milepost 154, Eagle county, Colorado, USA, 4.vii.2004	*Epilobium angustifolium*	Al.	Avar18
	*Manibridge, Manitoba, Canada, 21.vii.2007	*Epilobium angustifolium*		Avar99
	subgenus ***Aphis*** s. str.			
A. mimuli	Hwy 24, Milepost 154, Eagle county, Colorado, USA, 4.vii.2004	*Ribes* sp.	Al.	Amim25
A. oestlundi	*Willow Slough, Morocco county, Wisconsin, USA, 23.vi.2007	*Oe. laciniata*		Aoes98
A. praeterita	Prachatice, South Bohemia, Czech Rep., 16.x.2003	*Oenothera* sp.	Apt.	Apre196
	České Vrbné, South Bohemia, Czech Rep., 27.ix.2003	*Epilobium hirsutum*	Apt., ov., males	Apre180
	genus ***Protaphis***			
P. carlinae	Būtėnai, Anykščiai distr., Lithuania, 2.viii.2006	*Carlina vulgaris*		Protaphis

Meier et al. (2008). Standardization of inter-specific sequence differences were avoided because inter-specific sequence differences appear rather variable when comparing different taxa. Pair wise nucleotide sequence divergences were calculated using MEGA version 4 (Tamura et al., 2007) and neighbour-joining (NJ) analysis (Saitou & Nei, 1987) was used to represent these distances as a tree.

2. Character based tree building methods – maximum parsimony (MP) and maximum likelihood (ML). Different clustering methods consider monophyletic groups of specimens as distinct species. Clades were suggested to be distinct species when they received strong support (> 50%) in all phylogenetic reconstructions. MP (Swofford et al., 1996) analysis was computed using MEGA 4 (Tamura et al., 2007). Heuristic searches were carried out with ten random taxon addition replicates. Bootstrap values were generated from 1000 replicates, each with ten random-addition heuristic searches. Maximum likelihood (ML) analysis was conducted with PHYML (Guindon & Gascuel, 2003) and the best fitting model for the evolution was determined using the Akaike information criterion, as implemented in jModeltest 0.1.1 (Posada, 2008). The general time reversible model with a proportion of invariant sites and a gamma distribution (GTR + I + G) was used. Bootstrapping of the ML analysis was based on 100 replicates. Three out-group species were used in order to establish the rooting of the *Aphis* clade: *Protaphis carlinae* (Aphidini: Aphidina), which represents a closely related genus, *Schizaphis rotundiventris* (Aphidini: Rhopalosiphina, GenBank Accesion N AF220511), which represents a sister sub-tribe and *Acyrthosiphon pisum* (Macrosiphini: Macrosiphina, GenBank Accesion N AF077776), which is a more distantly related species.

3. Parsimony networks and implemented 95% parsimony connection limit (Hart & Sunday, 2007). Samples that stuck together in a single haplotype network were suggested to be con-specific. Intra-specific phylogenetic relationships among mtDNA haplotypes were inferred based on a statistical parsimony method (Templeton et al., 1992). Statistical parsimony is more convenient when investigating closely related haplotypes with a low number of substitutions. Haplotype networks were constructed using the software TCS 1.13 (Clement et al., 2000).

The sequence data for all species were submitted to the GenBank under following Accession Nos HM2 45794 to HM2 45856.

RESULTS AND DISCUSSION

The alignment of partial CO-I sequences contained 623 sites, of which 125 were variable and 91 appeared parsimony informative. The sequences were heavily biased

TABLE 3. Clonal material of the genus *Aphis* L. studied. Morph abbreviations the same as in Table 2; fx – fundatrix.

Species	Started from	Reared on	Life cycle	Sample date	Abbreviation on figures
A. holoenotherae	Karveliškės, Vilnius distr., Lithuania, 17.v.2006, *Oenothera biennis*, fx.	*Oenothera biennis*	Holocyclic, monoecious	10.vi.2006	Ahol53
A. grossulariae	Skirgiškės, Vilnius distr., Lithuania, 28.v.2002, *Ribes* sp. cult "black", fx	*Ribes* sp. cult "black", *Epilobium adenocaulon*	Holocyclic, facultatively heteroecious	26.vi.2002	AgroC
A. triglochinis	Puvočiai, Varėnos distr., Lithuania, 18.v.1995, *Ribes* sp. cult. „black"	*Ribes* sp. cult "black", *Rorippa amphibia*	Holocyclic, heteroecious	19.vi.1995	AtriT1
A. schneideri	Vilnius, Lithuania, 12.v.1997	*Ribes* sp. cult "black"	Holocyclic, monoecious	3.vii.1997	AschST

towards A and T nucleotides. The average base composition was A = 34.9%, C = 13.9%, G = 12.3% and T = 38.9%. The overall transition/transversion ratio was R = 4.113 for all sites.

The maximum parsimony (MP) analysis of partial CO-I sequences resulted in 220 equally parsimonious trees (length = 297, CI = 0.58, RI = 0.87). The strict consensus MP tree is shown in Fig. 1. A similar tree was produced by a ML analysis using model GTR + I + G. Topologies inferred in strict consensus MP and ML trees with the best likelihood scores appeared congruent and produced the same strongly supported clades. Therefore, both MP and ML bootstrap values are given at the respective nodes in Fig. 1. The neighbour joining (NJ) tree based on Kimura 2-parameter distances (Fig. 2) revealed a somewhat different topology. Noticeably, nodes, that are absent in the NJ tree had low supports in the MP and ML trees. CO-I partial sequences indicate the subgenus *Bursaphis* is a monophyletic group, although ML/MP bootstrap support values (62/78 respectively) are not very strong. Earlier studies, based on traditional (Stroyan, 1984; Heie, 1986; Remaudière, 1993) and molecular (Coeur d'acier et al., 2007; Foottit et al., 2008) data have also suggested the monophyly of *Aphis* (*Bursaphis*). The smallest pair wise inter-specific divergence was between *A. holoenotherae* and *A. oenotherae* s. str., followed by the couple *A. varians* and *A. manitobensis* (Table 4). The pair wise within-species sample distances for well-defined species of the subgenus *Aphis* (*Bursaphis*) are given in Table 5. Their minimal within-species sequence divergence ranges from 0.00 to 0.16% (*A. holoenotherae*). The maximum within-species sample distance in well-defined species reaches 0.97% in *A. varians*. Of the ill-defined species, the *A. oenotherae*-like samples are the most numerous (8 samples altogether, see Table 2). The pair wise within-group sample distances for *A. oenotherae*-like samples collected on different hosts are given in Table 6, together with the *Aphis* sp. samples collected from similar host plants. Table 6 indicates the distances between *A. oenotherae*-like samples collected on similar host plants are comparable with inter-specific distances between well-defined species. For the present, *A. oenotherae* is reported to be a holocyclic species alternating between currants and species of herbaceous plants of the family Onagraceae (Remaudière, 1993; Blackman & Eastop, 2006). This is based on the experimental study of the life cycle of *A. sanborni* (Patch, 1927), which was subsequently synonymised with *A. neomexicana* by Robinson & Rojanavongse (1976). Patch (1927) reports *A. sanborni* as a holocyclic species alternating between *Ribes* and *Epilobium* spp. (*E. adenocaulon*, *E. lineare*, *E. coloratum*). The life-cycle of American populations of *A. oenotherae*, synonymised with *A. neomexicana* by Remaudière (1993), has never been checked. In the MP/ML and NJ trees (Figs 1–2), *A. oenotherae*-like samples occur at different nodes. Four of them are located in clade A, together with samples of *A. costalis* and new unnamed species mentioned as *Aphis* sp. (New Zealand) in the key of Blackman & Eastop (2006) (Fig. 1). These samples emerge in the same nest of the haplotype parsimony network, which indicates all them belong to the same species. Of the three remaining *A. oenotherae*-like samples, one (Aneo14) collected from currants in Arizona, might be a representative of a separate species (Fig. 1). In our opinion, this justifies the restitution of *A. neomexicana* (W.P. Cockerell & T.D.A. Cockerell, 1901) as a valid species. Finally, two *A. oenotherae*-like samples emerging close to the *A. holoenotherae* node in clade D2 (Aoen19 and Aoen71, Figs 1–2) might represent one more separate species, *A. oenotherae* Oestlund, 1887 in a narrow sense. Thus, based on the partial CO-I sequences, the *A. oenotherae*-like complex (together with *A. costalis*) might include four cryptic species: *A. oenotherae*, *A. holoenotherae*, *A. costalis* and *A. neomexicana*. This supports the doubts concerning the synonymy of *A. neomexicana* and *A. oenotherae* (Blackman & Eastop, 2000, 2006; Rakauskas, 2000).

The remaining samples tend to form a sister group to clade A and include European, East Palaearctic and North American material (Fig. 1). European species form two well supported separate clades consisting of *A. grossulariae*, *A. schneideri* and *A. epilobii* samples (clade B, 68/90% bootstrap support for ML/MP respectively), and *A. epilobiaria* samples (clade C, 93/97%). Generally, CO-I partial sequences correspond well with the available information on the ecological and morphological features of the European species of the subgenus *Bursaphis* (Stroyan, 1984; Heie, 1986). However, the European *A. epilobii* samples remain poorly resolved in the MP/ML and NJ trees (Figs 1–2). Noticeably, these samples emerge together with those of *A. grossulariae* and *A. schneideri* in the same nest of the haplotype parsimony network, which suggests they all belong to the same species. *A. epilobii* is reported as a holocyclic species, oligophagous on *Epilobium* herbs, mostly *E. montanum* (Stroyan, 1984; Heie, 1986; Holman, 2009). Some of the samples collected on *Epilobium* spp. for this study were difficult to identify using the morphological characters available in the recent widely accepted key (Blackman & Eastop, 2006), because of a mixture of or intermediate characters. For example, the sample of *Aphis* (*Bursaphis*) (Aepi43) collected in Kyiv (Ukraine) from *Epilobium* sp. had the key morphological characters closer to *A. epilobii*, but the body colour (light green) of *A. grossulariae*. This sample appeared inside the well supported *A. grossulariae* node (Figs 1–2). Sample (Aepi90) collected in Dūkštai (Lithuania) had the morphology of *A. epilobii*, but appeared in the *A. grossulariae* node. Sample (Aepi91) from Rabi (Czech Republic) also had morphological characters of *A. epilobii*, although appeared closer to the *A. schneideri* node. This indicates the need for a rigorous study of host specificity and life cycles, and a subsequent morphological and molecular analysis of *A. epilobii* in Europe.

The present data, together with that published by Turčinavičienė et al. (2006), confirm that the European species *A. grossulariae* and *A. schneideri* are very similar. The *A. grossulariae* and *A. schneideri* samples used in this study

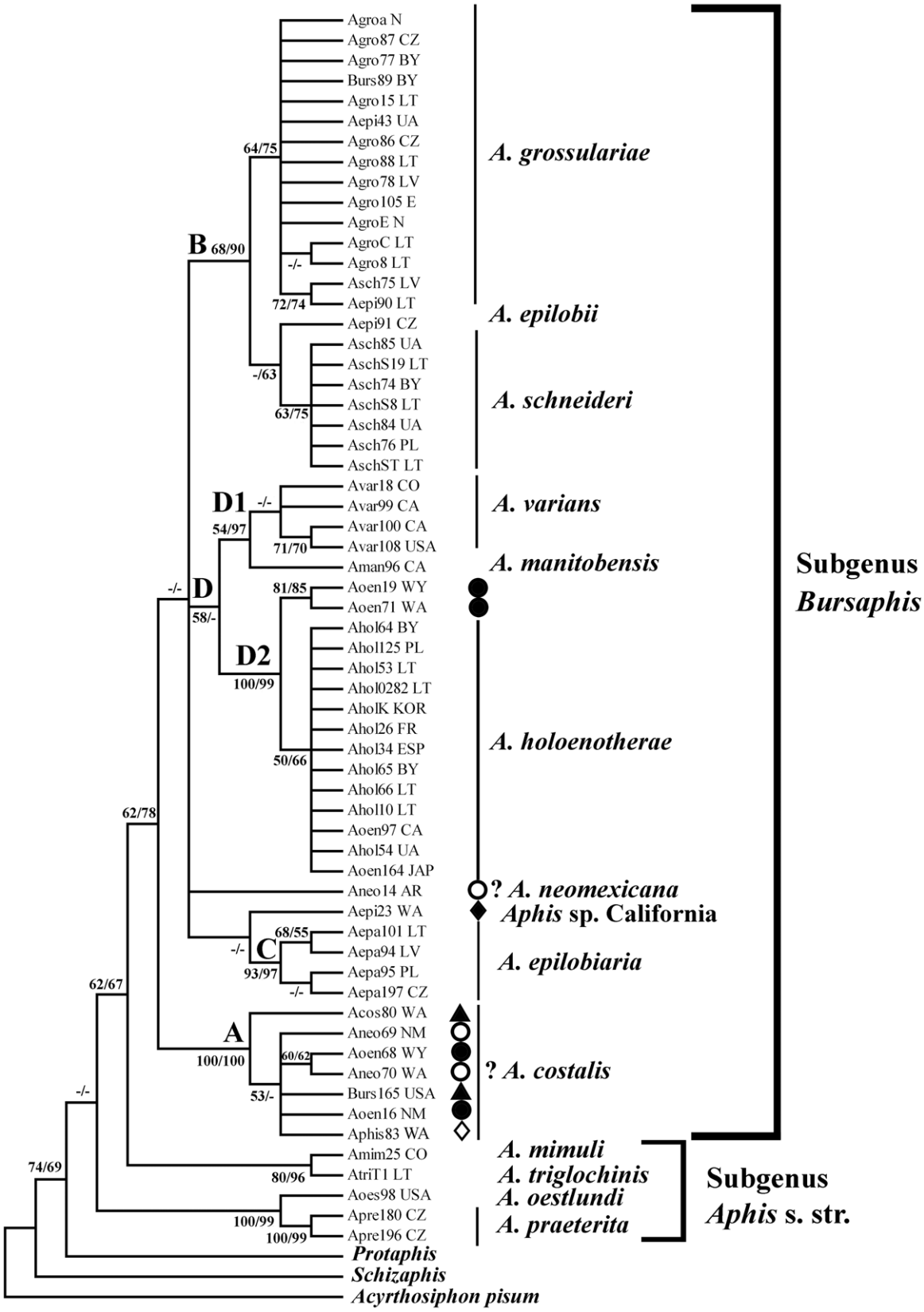

Fig. 1. Phylogeny of the genus *Aphis* based on a region of mtCO-I. The maximum parsimony strict consensus topology is shown. Bootstrap values at branches are ML (left) and MP (right). Only those exceeding 50% are indicated. (–) indicates nodes that collapsed in ML consensus due to low support (bootstrap values less than 50%). Sample abbreviations as in Tables 2–3. ◊ – new, the yet undescribed species *Aphis* sp. New Zealand in Blackman & Eastop (2006: p. 415); ♦ – new, the yet undescribed species *Aphis* sp. California in Blackman & Eastop (2006: p. 415); ● – *A. oenotherae*-like samples collected on *Epilobium* or *Oenothera*; ○ – *A. oenotherae*-like samples collected on *Ribes*; ▲ – *A. costalis*.

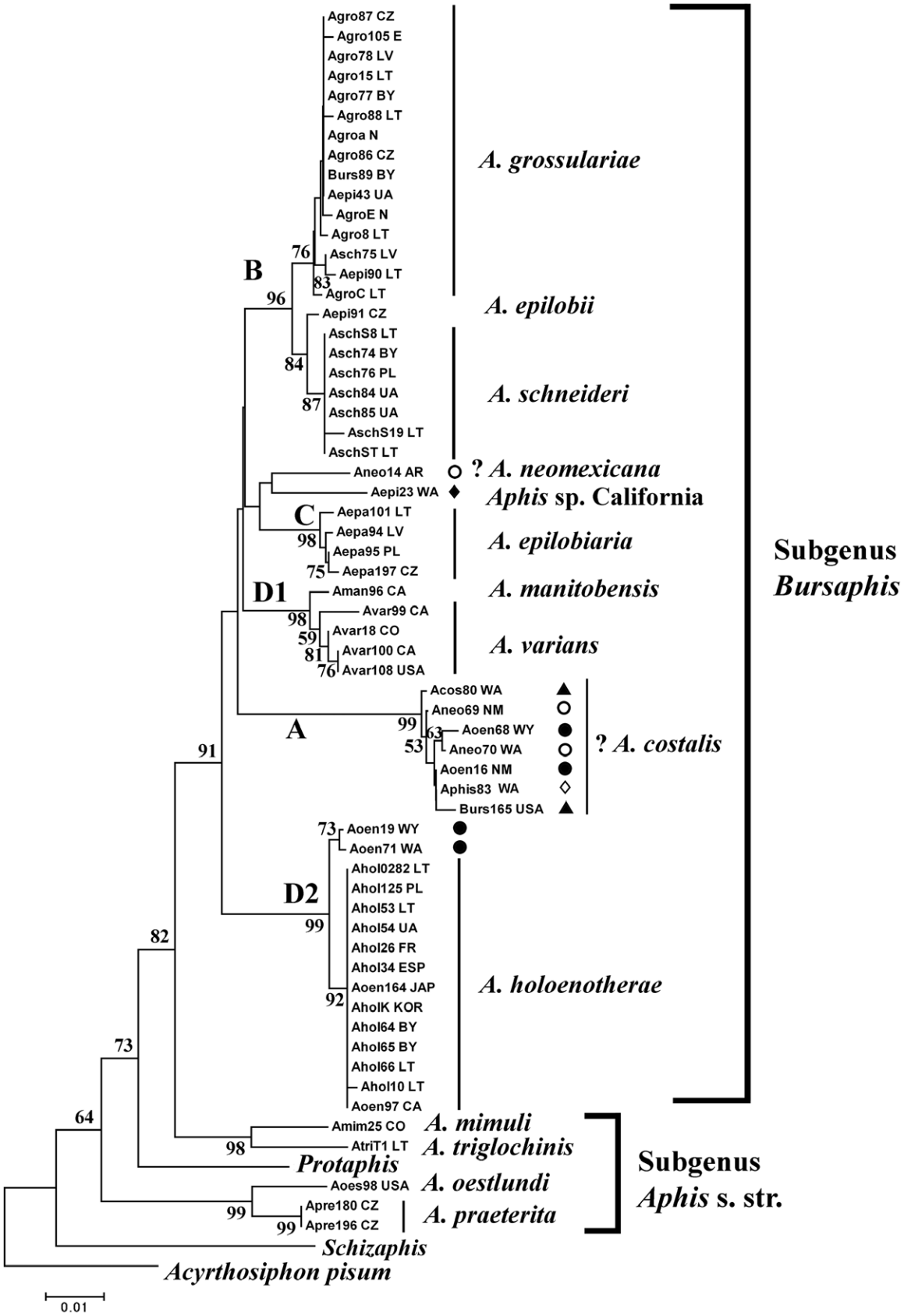

Fig. 2. Neighbour-joining tree based on the Kimura 2 parameter model. Sample abbreviations as in Tables 2–3. Bootstrap values exceeding 50% are indicated. ◊ – new, the yet undescribed species *Aphis* sp. New Zealand in Blackman & Eastop (2006: p. 415); ♦ – new, the yet undescribed species *Aphis* sp. California in Blackman & Eastop (2006: p. 415); ● – *A. oenotherae*-like samples collected on *Epilobium* or *Oenothera*; ○ – *A. oenotherae*-like samples collected on *Ribes*; ▲ – *A. costalis*.

TABLE 4. Range of pair wise inter-specific sample divergences (Kimura 2-parameter model) for well-defined *Aphis* (*Bursaphis*) species (number of samples in parentheses).

Species 1	Species 2	Range of divergence, %
A. grossulariae (15)	*A. schneideri* (7)	0.81–1.63
A. grossulariae (15)	*A. varians* (4)	2.80–3.65
A. grossulariae (15)	*A. manitobensis* (1)	2.46–2.80
A. manitobensis (1)	*A. varians* (4)	0.48–1.30
A. schneideri (7)	*A. manitobensis* (1)	3.15–3.49
A. epilobiaria (4)	*A. manitobensis* (1)	3.32–3.45
A. epilobiaria (4)	*A. grossulariae* (15)	2.80–3.14
A. epilobiaria (4)	*A. schneideri* (7)	2.47–2.98
A. epilobiaria (4)	*A. varians* (4)	3.15–3.65
A. epilobiaria (4)	*A. costalis* (2)	4.83–5.52
A. epilobiaria (4)	*A. triglochinis* (1)	5.37–5.72
A. manitobensis (1)	*A. costalis* (2)	4.83–5.35
A. grossulariae (15)	*A. triglochinis* (1)	5.72–5.89
A. holoenotherae (13)	*A. grossulariae* (15)	3.48–4.52
A. holoenotherae (13)	*A. schneideri* (7)	3.82–4.51
A. holoenotherae (13)	*A. varians* (4)	2.97–3.81
A. holoenotherae (13)	*A. manitobensis* (1)	3.48–3.81
A. holoenotherae (13)	*A. costalis* (2)	5.88–6.59
A. holoenotherae (13)	*A. epilobiaria* (4)	3.82–4.34
A. holoenotherae (13)	*A. oenotherae* s. str. (2)	0.48–0.81

form separate clades in MP/ML and NJ trees, but they appear in the same nest of the haplotype parsimony network. This supports an earlier hypothesis that *A. grossulariae* and *A. schneideri* hybridize naturally (Rakauskas, 1999, 2003). Experimental crosses between these species resulted in hybrid clones that differ in their morphology, host specificity and life cycle characteristics, namely, some hybrid clones have the morphology of *A. schneideri* but the life cycle of *A. grossulariae* (Rakauskas, 1999). Subsequently, hybrid morphotypes were found in field samples (Rakauskas, 2003). In this study, a sample (Asch75) collected from black currants at Salaspils (Latvia) had the morphology of *A. schneideri*, but appeared in the *A. grossulariae* clade (Figs 1–2). This might be due to the hybrid origin of the sample and certain maternal effects. Noticeably, *A. epilobii* samples are located inside the *A. grossulariae* + *A. schneideri* clade. *A. epilobii* sample Aepi90 groups together with the above mentioned Asch75 sample of *A. schneideri* in the *A. grossulariae* cluster (Figs 1–2). This suggests that some of the *A. epilobii* samples were also of hybrid origin (*A. grossulariae* × *A. schneideri*). Life-cycle and host specificity data (Stroyan, 1984; Rakauskas, 1993, 1998), however, indicate that *A. grossulariae* and *A. schneideri* are species.

Due to the lack of material, it is still unclear whether *A. popovi* is a separate species. *A. popovi* was described from aphid material collected on currants at Yakutia (Russian Siberia). It is morphologically similar to *A. schneideri* (Rakauskas, 1996), but its life cycle and host specificity are unknown.

Several North American (*A. manitobensis*, *A. varians, A. oenotherae* s. str.) and Palaearctic (*A. holoenotherae*) samples tend to form a separate clade in the MP and ML trees, but it has low bootstrap support (D, 58/42% for ML/MP, respectively, Fig. 1) and is not apparent in the NJ tree (Fig. 2). In this group, the *A. varians* and *A. manitobensis* samples nest together in the haplotype parsimony network and form clearly separated clades in MP/ML and NJ trees (Figs 1–2, D1, 54/97 and 98%, respectively). Foottit et al. (2008) report that *A. manitobensis* samples differ from all *A. varians* haplotypes, although the distance between *A. manitobensis* and *A. varians* samples is about the same as that between the two *A. varians* clusters. When these authors were asked to enlarge on this (Foottit & Maw, 2009; pers. comm.) they replied that "given the relative uniformity of the transcontinental *A. varians* cluster, the observed difference between that group and the few *A. manitobensis* samples available may prove to be constant despite the small distance". This is in accord with the results of this study. The case of *A. manitobensis* and *A. varians* is very similar to that of *A. grossulariae* and *A. schneideri* (see above), with the ranges of pair wise inter-specific sample divergences comparable for both couples of species (0.48–1.30 and 0.81–1.63, respectively; Table 4). In addition the morphological and ecological evidence indicates that the four above mentioned species are good species **(**Remaudière, 1993; Remaudière & Remaudière, 1997). In contrast to the case of *A. grossulariae-A. schneideri*, proper evidence of the principal ecological niche characteristics (host specificity and life cycles) is lacking for *A. manitobensis*. Life cycle and host specificity of *A. varians* is holocyclic, with the species host alternating between currants and *Epilobium* herbs (Patch, 1927), and only indirect information indicating that *A. manitobensis* is monoecious on currants (Robinson & Rojanavongse, 1976). Therefore, it is necessary to rear both species and carry out morphological and molecular analyses of the lineages in order to resolve this problem.

A highly supported clade D2 (100/99 and 99% for ML/MP and NJ respectively, Figs 1–2) contains both Palaearctic and American samples. Palaearctic samples of *A. holoenotherae* form a uniform group, nearly all iden-

TABLE 5. Range of pair wise within-species sample divergences (Kimura 2-parameter model) for well-defined *Aphis* (*Bursaphis*) species (number of samples in parentheses).

Species	Range of divergence, %	Geographical origin of samples (n)
A. grossulariae	0.00–0.65	LT(5), EE(1), UA(1), BL(2), CZ(2), LV(2), N(2)
A. schneideri	0.00–0.32	LT(3), UA(2), BL(1), PL(2)
A. varians	0.00–0.97	CA(BC-1, MA-1), USA(CO-1, WA-1)
A. epilobiaria	0.16–0.65	LT(1), LV(1), PL(1), CZ(1)
A. holoenotherae	0.00–0.16	LT(4), PL(1), FR(1), ESP(1), UA(1), BL(2), KOR(1), JAP(1), CA(1)
A. costalis	0.48	USA (WA-2)

TABLE 6. Range of pair wise within-group sample divergences (Kimura 2-parameter model) for *A. oenotherae*-like and *Aphis* sp. samples collected from different host plants (number of samples in parentheses).

Host plant genus	Range of divergence, %	Geographical origin of samples (n)
Ribes	0.32–5.30	USA (AR-1, NM-1, WA-1)
Epilobium	0.48–5.94	USA (WA-2, WY-2)
Oenothera	6.26	USA (WA-1, NM-1)

tical in their sequences; with only one differing in one nucleotide. The *A. holoenotherae* node appears in sister-group relationships with two *A. oenotherae* s. str. samples from Wyoming and Washington in the ML/MP and NJ trees (Figs 1–2). The range of pair wise inter-specific sample divergences between *A. holoenotherae* and *A. oenotherae* s. str. is the lowest among the couples of well-defined species analysed (0.48–0.81, Table 4). American samples differ from the rest of the *A. holoenotherae* node in three (Aoen71) and four (Aoen19) substitutions and therefore nest together in the haplotype parsimony network. Moreover, the partial CO-I sequences of the Canadian sample, Aoen97, are identical with most of the Palaearctic *A. holoenotherae* samples. It was not possible to morphologically identify this sample, because there were only immature nymphs in the subsample that R. Foottit kindly supplied. In reply to our request for further information R. Foottit & E. Maw (2010, pers. comm.) stated that "there is only a single adult aptera in their collection … Terminal process lengths (left/right) are 0.216/0.221 mm. Measurements (left/right) for 3 specimens of instar 4 alates: 0.187/0.187, 0.187/0.171, 0.210/0.200". The key of Rakauskas (2008) places apterae with these characteristics in *A. oenotherae*, but the lengths of nymphs are more similar to those of *A. holoenotherae*. The case of *A. oenotherae* s. str. and *A. holoenotherae* is comparable to that of *A. manitobensis* and *A. varians* (see above). *A. holoenotherae* is holocyclic and monoecious on *Oenothera* spp. in Lithuania and Poland (Rakauskas, 2007), but there is no experimentally based information on the life cycle and host specificity of *A. oenotherae* s. str. in North America. In addition, both of these presumed species seem to have separate distributions (Palaearctic versus Nearctic for *A. holoenotherae* and *A. oenotherae* s. str. respectively). In order to resolve this problem it is necessary to determine the life cycle and host specificity of *A. oenotherae* s. str. and subject it to morphological and molecular analyses.

Sample Aepi23, collected from *Epilobium* in Washington State, which ran to *Aphis* sp. California in the key of Blackman & Eastop (2006) is not included in any grouping in MP and ML cladograms (Fig. 1), but forms a separate node together with the *A. oenotherae*-like sample Aneo14 in the NJ tree (Fig. 2). This tends to confirm the presupposition of Blackman & Eastop (2006) that there is a new species inhabiting *Epilobium* in western USA. In contrast, sample Aphis83 collected from *Epilobium* in Washington State (Moses Lake), which ran to the *Aphis* sp. (New Zealand) in the same key appears to be a member of clade A (Figs 1–2), which consists of *A. costalis* and *A. oenotherae*-like samples. The sequences of sample Aphis83 are identical with those of *A. oenotherae*-like sample Aoen16 from New Mexico.

In conclusion, the exact number of species in the subgenus *Aphis* (*Bursaphis*) still needs further clarification. This is due to the lack of reliable information on the host specificity and life cycles of eight of the twelve species in this subgenus. Based on the results of the present study, *cox1* gene partial sequences indicate that the following *Aphis* (*Bursaphis*) species need to be revised. The *A. oenotherae*-like complex seems to include four or more species. Rigorous life cycle and host specificity studies on the species in this complex, together with morphological and molecular analysis of the same material, might lead to the resurrection of *A. neomexicana*. The same also applies to the *A. varians – A. manitobensis* and *A. epilobii – A. grossulariae* species couples. *Aphis* sp. California in the key of Blackman & Eastop (2006), which corresponds morphologically to our sample Aepi23 collected from *Epilobium* in Washington State, awaits a proper definition based on a combined ecological, morphological and molecular study. Three out of twelve currently recognized species in the subgenus *Aphis* (*Bursaphis*) (*A. solitaria*, *A. fluvialis*, *A. popovi*) were not included in the present study due to the lack of material. *A. popovi* might be of special interest due to its similarity to *A. schneideri* (see Rakauskas, 1996 for details).

The data presented here support the statement of Coeur d'acier et al. (2007) that "mitochondrial DNA does not allow the differentiation of species that are difficult to identify morphologically", but clearly indicates the existence of complexes of cryptic species and the need to synonymize morphologically distinct "species" (see above). Distance and tree-building methods of species delimitation appeared congruent when dealing with closely related species of the subgenus *Bursaphis*. Designation of any threshold for distance based methods can be accepted only in cases when ecological data (life cycle and host specificity) for the specimens are also available, because even the smallest inter-specific distances might correctly reflect different species (e.g. the case of *A. grossulariae – A. schneideri*, see above). Once a species is assigned to a monophyletic group, only those that are well supported clades deserve the nomination of distinct species (Meier et al., 2006). Congruence of topologies produced by different clustering methods is deemed to be important evidence when delimiting species, despite the small inter-specific pair wise nucleotide sequence divergences. Recently, mitochondrial DNA analysis was used to indicate the possible existence of cryptic species in the genus *Toxoptera* (Wang & Qiao, 2009). We have reservations about the claim that partial sequence of *cox1* gene (658-bp fragment from the 5' region) can be "adopted as

the standard DNA barcode region for animal life" (Foottit et al., 2008). Apart from the problem of whether it can be used for all animal life, the use of partial CO-I sequences as the standard barcode for aphids is limited. *A. manitobensis* and *A. varians* are good species (see above), so partial CO-I sequences might appear misleading when applied as standard barcodes in this case. Aphid species *Macrosiphum rosae* (Linnaeus, 1758) and *Macrosiphum knautiae* Holman, 1972 differ in their ecology and morphology, but their *cox1* gene partial sequences are identical [see Turčinavičienė & Rakauskas (2009) for details]. It is likely that molecular data has the same limitations as any other character used in classification, including the most popular morphological ones. Therefore, ecological specificity is the most important feature when trying to classify living things, followed by morphological and molecular analyses [see Rakauskas (2009) for wider discussion]. The experience of the authors leads them to support the opinion that DNA barcoding is not a replacement for morphology- (and ecology!-) based taxonomy (Coeur d'acier et al., 2007; Mitchell, 2008; Žurovcová et al., 2010; Tan et al., 2010).

CONCLUSIONS

Summarising, the results of the present study, based on an analysis of partial sequences of the mitochondrial *cox1* gene:

1. Partial CO-I sequences might be misleading when used as standard DNA barcodes for aphid species of the subgenus *Bursaphis*. In addition to DNA studies, it is necessary to undertake ecological, morphological and molecular studies in order to determine the number of species in this subgenus.

2. *A. oenotherae* is likely to be a complex of cryptic species. In addition to *A. oenotherae*; *A. holoenotherae*, *A. costalis* and *A. neomexicana* should be validated.

3. *Aphis* sp. (USA: California, Oregon) of Blackman & Eastop (2006, p. 415) deserves the status of a good species once there is information on its host association and life cycle.

4. Taxonomic status of *A. varians-A. manitobensis* and *A. epilobii-A. grossulariae* demands further clarification.

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