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"DNA barcoding" is of limited value for identifying adelgids (Hemiptera: Adelgidae) but supports traditional morphological taxonomy

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Abstract. The sequence diversity in the mitochondrial cytochrome-c oxidase I (*COI*) gene was evaluated as a tool for resolving differences among species of European adelgids collected from several localities across the Czech Republic. Members of 7 genera and 16 species were examined, and as outgroups, two species of Phylloxeridae were used. Sequence divergences within species were on average less than 0.15%, whereas divergences between species ranged from 0.0 to 4.12% for congeneric and to 13.24% for intergeneric comparisons. It is concluded that DNA barcoding of Adelgidae is a powerful tool for identifying genera, but at the species level it works only in those cases where there are no species complexes. Nevertheless, it can be used as a complement to traditional, morphological taxonomy.

INTRODUCTION

Adelgids are a small group of aphids, feeding on a variety of coniferous trees. With their very complex life cycles and biology (cyclical parthenogenesis, multiple generations, host-switching) they present a great taxonomical challenge. Their larval stages are morphologically almost indistinguishable, which makes species identification by traditional methods (i.e. microscopic inspection of series of individuals from the same clone) very cumbersome and time-consuming. In fact, only a very skilled professional can distinguish adelgids with certainity, which is not very practical for every day forestry management.

The classification of adelgids both at the species and genus levels is not completely satisfactory and it is more than obvious, that besides the morphological structures and bionomical details that have been studied since the end of the 19th century, modern molecular approaches are needed.

In principle, two systems are currently used in studies of the systematics of adelgids. In the USA and UK, all the species are sorted into two genera, *Adelges* and *Pineus*. These genera are distinguished primarily by the number of abdominal spiracles in adults (Annand, 1928; Carter, 1971; Blackman & Eastop, 1994). This system is currently used by taxonomists in the New World/UK such as Havill et al. (2007) and Havill & Foottit (2007). However, the majority of systematicists in the Western and Eastern Palearctic regions distinguish 8 genera: *Adelges*, *Cholodkovskya*, *Sacchiphantes*, *Dreyfusia*, *Aphrastasia*, *Gilletteella*, *Pineus*, and *Eopineus*. This system is based on details of the morphology of first instar larvae (Börner, 1908, 1930, 1952; Inouye, 1945; Börner & Heinze, 1957; Bodenheimer & Swirski, 1957; Steffan, 1961, 1968, 1972; Heinze, 1962; Shaposhnikov, 1964; Dmitriev, 1965; Szelegiewicz, 1968; Pashchenko, 1988; Binazzi & Covassi, 1991), alatae forms (Binazzi, 2000) as well as bionomical characteristics and morphology of the galls (Cholodkovsky, 1896; Pašek, 1954; Börner & Heinze, 1957; Shaposhnikov, 1964; Lampel, 1968; Steffan, 1972).

Steffan (1968) introduced the use of chromosome numbers and appearance of endosymbionts, and suggested a modification of the subfamily grouping. He shifted the genera *Dreyfusia* and *Aphrastasia* to Pineinae, thus reducing the Adelginae group to only four genera (*Gilletteella*, *Sacchiphantes*, *Cholodkovskya*, and *Adelges*).

A first attempt to utilize molecular markers for adelgid systematics was that of Havill et al. (2007). The phylogenetic relationships based on mitochondrial cytochrome oxidase subunit I (*COI*), cytochrome oxidase subunit II (*COII*), and cytochrome b *(cytb*) genes and the nuclear elongation factor 1α (*EF-1α*) locus were reconstructed. The results support the previous morphology-based division into two genera proposed by Annad (1928) or the two subfamilies of Börner & Heinze (1957), respectively. However, this study focused on higher level phylogenetic classification and host specificity. Several species were represented by only one individual or not at all.

Recently, one approach to DNA-based species identification has quickly gained many supporters as well as opponents. The method is called "DNA barcoding" and is based on the assumption that variability found in the segment of cca 650 bp of the mitochondrial gene for cytochrome oxidase subunit I (*COI*) is sufficient for the identification of species (Hebert et al., 2004) or even for species delimitation (Tautz et al., 2003). As the literature on "DNA barcoding" grows it is clear that these generali-

zations do not apply across the whole animal kingdom (Vences et al., 2005; Shearer & Coffroth, 2008), and a more careful statistical approach should be applied (Meyer & Paulay, 2005; Meier et al., 2006, 2008; Weimers & Fiedler, 2007).

Despite its limitations, "DNA barcoding" can nevertheless help with species identification if applied correctly. However, it is first necessary to generate a database containing the "DNA barcodes" of the target taxa and then determine whether it can be used for species identification in a pilot study. Foottit et al. (2009) did this based on a set of 17 adelgid species. They concluded that "DNA barcoding" has potential for the detection of cryptic species, but can not distinguish species defined by life-cycle characteristics.

Here the goal is to establish a "DNA barcoding database" of adelgid species found in the Czech Republic, and determine its usefulness for identification compared to using morphological and ecological characters.

MATERIAL AND METHODS

Samples were collected in 2005, 2007, and 2008 from several different localities in the Czech Republic (95 samples), Lithuania (1 sample), and Serbia (1 sample) (Table 1). Specimens (several individuals per clone) were either preserved in 100% ethanol or frozen and kept at –70°C until further analysis. Several individuals of *Phylloxera coccinea* and *Viteus vitifoliae* (both Phylloxeridae) were used as an outgroup. Adelgid collections are available in the Institute of Entomology, Biology Centre ASCR, České Budějovice. Microscopic identifications were made by J. Havelka.

Total genomic DNA was extracted from 3–5 specimens per clone using two different methods. A "Quick protocol" (Frati et al., 2001) was used for the frozen samples, while the ethanol preserved samples were extracted via ZR Genomic DNA Kit (Zymoresearch Inc., Orange, CA, USA) according to the manufacturer's protocol. PCR amplification was carried out using the TaKaRa Ex *Taq* system (TaKaRa Bio Inc., Otsu, Shiga, Japan) or TopBio polymerase Unis (TopBio s.r.o., Prague, Czech Republic) and universal primers LCO1490 and HCO2198 (Folmer et al., 1994) to amplify cca. 670 bp of the 5' end of mitochondrial cytochrome oxidase I (*cox1*). Reaction volumes (50 µl) consisted of 5 µl of template DNA (not quantified), 5 µl of 10 × reaction buffer, 4 µl of dNTP mixture (2.5 mM each), 0.5 mM of each primer, and 1.25 unit of *Taq* polymerase. The PCR reactions were carried out in a Mastercycler ep gradient S thermocycler (Eppendorf AG, Hamburg, Germany) with the following profile: 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 47°C for 45 s, 72°C for 1 min, and final extension at 72°C for 3 min. PCR products were cleaned with either the DNA Clean&Concentrator-5 Kit (Zymoresearch) or enzymatically with ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) before directly sequencing.

Sequencing was performed in both directions using the above primers in a BigDye v. 3.1 sequencing reaction on an ABI 310 automated sequencer (Applied Biosystems, Inc., Carlsbad, CA, USA) at the sequencing facility of the Laboratory of Genomics (Biology Centre ASCR, České Budějovice). Sequences were edited and aligned both manually and with the assistance of SeqMan (Lasergene 8 package of programs from DNASTAR, Inc., Madison, WI, USA). Morphological vouchers are kept within the collection of the Institute of Entomology, Biology Centre ASCR, České Budějovice.

Distance analysis was performed using the MEGA v.4 software (Tamura et al., 2007), which was also used to create Neighbour-Joining tree based on uncorrected *p*-distances (bootstrap analysis with 10,000 replicates) and divergence time estimates. χ^2 test as implemented in PAUP* 4.0.b10 (2003) was used to determine the homogeneity of base frequencies among sequences. The same program and Modeltest (Posada & Crandall, 1998) were used to select the best fitting model for further phylogenetic analysis.

To explore the sensitivity of our results to the choice of reconstruction method, we also conducted Bayesian analyses using the GTR – site specific rate model with MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) and maximum-likelihood (ML) analysis with PHYML (Guindon & Gascuel, 2003). In both of these analyses the GTR + I + G model selected by the Modeltest was used. In the Bayesian analysis, 1st + 2nd + 3rd codon position were treated as separate partitions. Two different settings were explored. In the first, node support was assessed as the posterior probability from five independent runs, each with one chain of 2,500,000 generations (sampled at intervals of 100 generations with a burn-in of 6250 trees). In the second, node support was assessed as the posterior probability from two independent runs each with four chains (temperature for hot chains lowered to 0.1) of 10,000,000 generations (sampled at intervals of 100 generations with a burn-in of 25000 trees). For the maximum likelihood analysis, a random initial tree, best of NNI and SPR searches for the tree topology estimate, 5 independent runs and 1000 × bootstrap, were used.

We also utilized TaxonDNA (Meier et al., 2006) to obtain a frequency distribution for intra- and interspecific congeneric genetic variability and evaluate the potential of the "DNA barcoding" for identifying species of adelgid. In short, this program evaluates the similarity of the species sequences, assigning those with the closest match and the same species name as successfully identified. If there are several equally good best matches from different species, identification is considered ambiguous, which applies also to the species represented by a single sequence.

RESULTS

The 687 bp fragment of the *COI* gene was sequenced for 97 individuals, representing 16 species of adelgids and 2 phylloxerids. Two species, *Aphrastasia pectinatae* and *Pineus pineoides*, were represented by a single sample. No insertions, deletions, or stop codons were detected. Therefore, it is concluded that it is likely that only mitochondrial loci were sequenced and there are no nuclear pseudogenes (NUMT) in our data set. Of the 195 variable sites, 181 were parsimony informative. An alignment of the sequences is available from the authors upon request. All sequences have been deposited in GenBank (Table 1).

Nucleotide composition averaged over all adelgids showed an A+T bias (A = 36.4%, T = 39.1%, C = 14.8%, G = 9.7%), a common feature of insect mitochondrial DNA (Simon et al., 1994). Base frequencies were homogeneous among all sequences (χ^2 = 37.7, df = 288, P = 1.0) and the overall transition/transversion bias was R = 1.02. According to the guidelines given in Kumar et al. (1994) this value allowed us to use the uncorrected *p*-distance in further analyses, such as, the estimates of intraspecific and interspecific divergence and phylogenetic tree construction.

TABLE 1. List of the species of Adelgidae sampled (in alphabetical order) and the Phylloxeridae outgroup species.

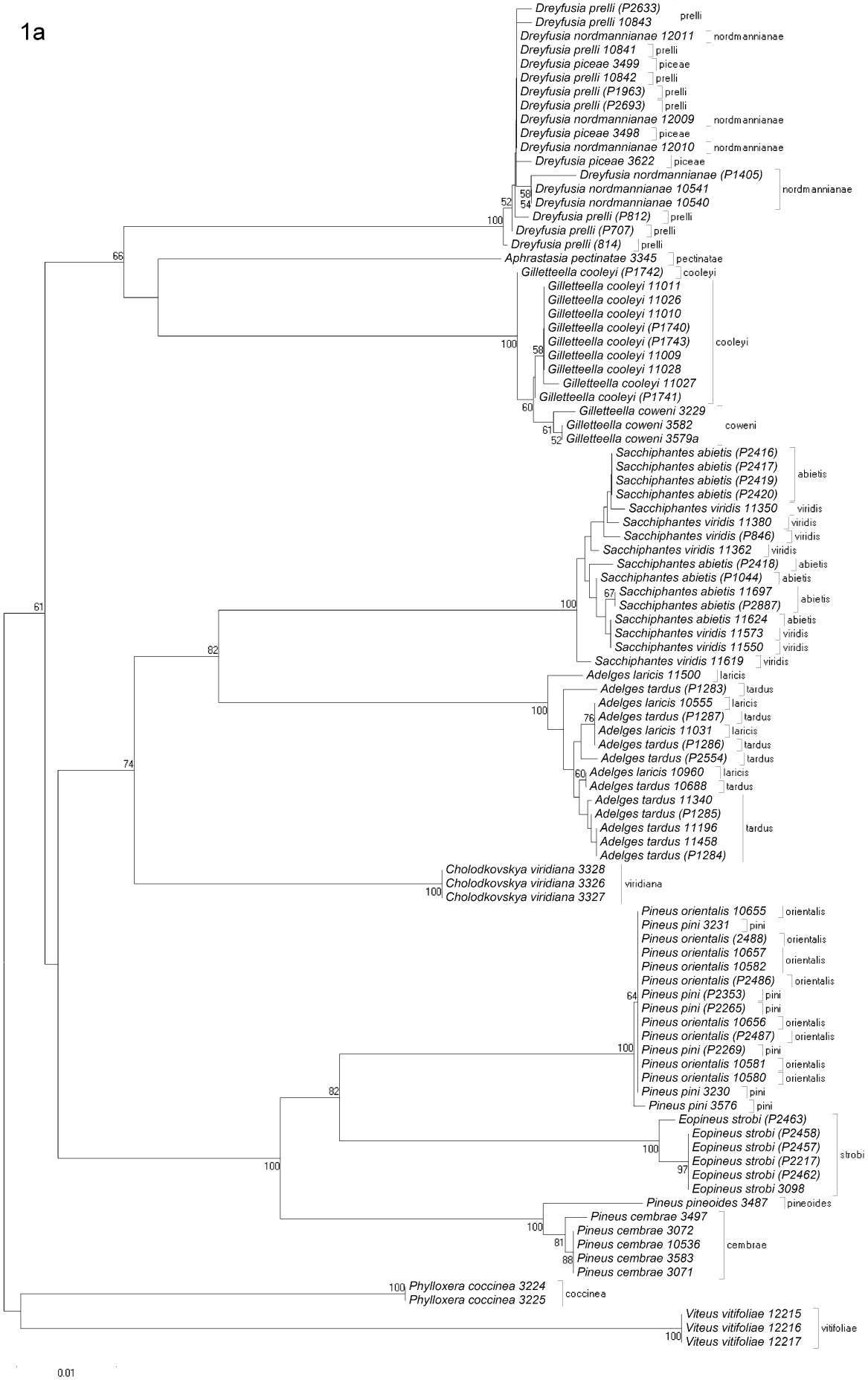
Species	Sample No.	GenBank accession no.	Collection host plant	Locality	Date	Conservation
Adelges laricis (Vallot, 1836)	10555	GU570998	*Picea abies* (L.) H.Karst.	Buchlovice	5.6.2008	kept at –70°C
Adelges laricis (Vallot, 1836)	11500	GU570999	*Picea abies* (L.) H.Karst.	České Budějovice, Borek	13.8.2008	ditto
Adelges laricis (Vallot, 1836)	10960	GU571000	*Picea abies* (L.) H.Karst.	Nový Dvůr	4.6.2008	ditto
Adelges laricis (Vallot, 1836)	11031	GU571001	*Picea abies* (L.) H.Karst.	Nový Dvůr	4.6.2008	ditto
Adelges tardus (Dreyfus, 1888)	P2554	GU571002	*Picea abies* (L.) H.Karst.	Nový Dvůr	7.6.2007	pure ethanol
Adelges tardus (Dreyfus, 1888)	P1283–P1287	GU571003–GU571007	*Picea abies* (L.) H.Karst.	České Budějovice	8.6.2007	ditto
Adelges tardus (Dreyfus, 1888)	10688	GU571008	*Picea abies* (L.) H.Karst.	Nový Dvůr	4.6.2008	kept at –70°C
Adelges tardus (Dreyfus, 1888)	11458	GU571009	*Picea abies* (L.) H.Karst.	České Budějovice, Stromovka	3.8.2008	ditto
Adelges tardus (Dreyfus, 1888)	11196	GU571010	*Picea abies* (L.) H.Karst.	České Budějovice, Stromovka	3.8.2008	ditto
Adelges tardus (Dreyfus, 1888)	11340	GU571011	*Picea abies* (L.) H.Karst.	Praha, Břevnov	10.8.2008	ditto
Aphrastasia pectinatae (Cholodkovsky, 1888)	3345	GU571012	*Abies concolor* Lindl. & Gord.	Vilnius (Lithuania)	31.7.2008	pure ethanol
Cholodkovskya viridana (Cholodkovsky, 1896)	3326–3328	GU571013–GU571015	*Larix kaempferi* Fortune	Nový Dvůr	16.7.2008	ditto
Dreyfusia nordmannianae (Eckstein, 1890)	P1405	GU571016	*Abies alba* Mill.	Chvalčov	13.4.2007	ditto
Dreyfusia nordmannianae (Eckstein, 1890)	10540–10541	GU571017–GU571018	*Abies alba* Mill.	Libín	11.3.2008	kept at –70°C
Dreyfusia nordmannianae (Eckstein, 1890)	12009–12011	GU571019–GU571021	*Picea orientalis* (L.) Peterm.	Buchlovice	15.8.2008	ditto
Dreyfusia piceae (Ratzeburg, 1844)	3498–3499	GU571022–GU571023	*Abies alba* Mill.	Bystřice pod Hostýnem	17.9.2008	pure ethanol
Dreyfusia piceae (Ratzeburg, 1844)	3622	GU571024	*Abies alba* Mill.	České Budějovice, Branišov	9.11.2008	ditto
Dreyfusia prelli (Grossmann, 1935)	P812, P814	GU571025, GU571026	*Picea orientalis* (L.) Peterm.	Buchlovice	28.6.2005	ditto
Dreyfusia prelli (Grossmann, 1935)	P707	GU571027	*Picea orientalis* (L.) Peterm.	Nový Dvůr	29.6.2005	ditto
Dreyfusia prelli (Grossmann, 1935)	P1963, P2693	GU571028, GU571029	*Picea orientalis* (L.) Peterm.	Buchlovice	17.7.2007	ditto
Dreyfusia prelli (Grossmann, 1935)	P2633	GU571030	*Picea orientalis* (L.) Peterm.	Lednice	28.6.2007	ditto
Dreyfusia prelli (Grossmann, 1935)	10841–10843	GU571031–GU571033	*Picea orientalis* (L.) Peterm.	Buchlovice	20.6.2008	kept at –70°C
Eopineus strobi (Hartig, 1837)	P2462–P2463	GU571034–GU571035	*Pinus strobus* L.	Nový Dvůr	24.5.2007	pure ethanol
Eopineus strobi (Hartig, 1837)	P2217	GU571036	*Pinus strobus* L.	České Budějovice	22.2.2007	ditto
Eopineus strobi (Hartig, 1837)	P2457–P2458	GU571037–GU571038	*Pinus strobus* L.	České Budějovice	22.5.2007	ditto
Eopineus strobi (Hartig, 1837)	3098	GU571039	*Pinus strobus* L.	Nový Dvůr	21.5.2008	ditto
Gilletteella cooleyi (Gillette, 1907)	P1740–P1743	GU571040–GU571043	*Pseudotsuga menziesii* (Mirb.) Franco	České Budějovice	26.3.2007	ditto
Gilletteella cooleyi (Gillette, 1907)	11009–11011	GU571044–GU571046	*Picea pungens* Engelm.	České Budějovice	30.6.2008	kept at –70°C
Gilletteella cooleyi (Gillette, 1907)	11026–11028	GU571047–GU571049	*Picea pungens* Engelm.	Praha	21.6.2008	ditto
Gilletteella coweni (Gillette, 1907)	3229	GU571050	*Pseudotsuga menziesii* (Mirb.) Franco	Praha, Břevnov	22.6.2008	pure ethanol
Gilletteella coweni (Gillette, 1907)	3579a	GU571051	*Pseudotsuga menziesii* (Mirb.) Franco	Stráž nad Nežárkou	12.2.2008	ditto

TABLE 1 (continued).

Species	Sample No.	GenBank accession no.	Collection host plant	Locality	Date	Conservation
Gilletteella coweni (Gillette, 1907)	3582	GU571052	*Pseudotsuga menziesii* (Mirb.) Franco	Stráž nad Nežárkou	28.10.2008	ditto
Sacchiphantes abietis (Ratzeburg, 1843	P1044	GU571053	*Picea abies* (L.) H.Karst.	Zdiměřice	24.8.2005	ditto
Sacchiphantes abietis (Linnaeus, 1758)	P2416–P2420	GU571054–GU571058	*Picea abies* (L.) H.Karst.	Troják	27.4.2007	ditto
Sacchiphantes abietis (Linnaeus, 1758)	P2887	GU571059	*Picea abies* (L.) H.Karst.	Čertovo jezero	20.8.2007	ditto
Sacchiphantes abietis (Linnaeus, 1758)	11624	GU571060	*Picea abies* (L.) H.Karst.	Šumava Mts, Jenišov	24.8.2008	kept at –70°C
Sacchiphantes abietis (Linnaeus, 1758)	11697	GU571061	*Picea abies* (L.) H.Karst.	Šumava Mts, Vítkův Kámen	27.8.2008	ditto
Sacchiphantes viridis (Ratzeburg, 1843)	P846	GU571062	*Picea abies* (L.) H.Karst.	Český Krumlov	25.7.2005	pure ethanol
Sacchiphantes viridis (Ratzeburg, 1843)	11380	GU571063	*Picea abies* (L.) H.Karst.	České Budějovice, Haklovy Dvory	8.8.2008	kept at –70°C
Sacchiphantes viridis (Linnaeus, 1758)	11350	GU571064	*Picea abies* (L.) H.Karst.	Kapaonik Natl. Park (Serbia)	8.8.2008	ditto
Sacchiphantes viridis (Ratzeburg, 1843)	11362	GU571065	*Picea abies* (L.) H.Karst.	Lednice	13.8.2008	ditto
Sacchiphantes viridis (Ratzeburg, 1843)	11550	GU571066	*Picea abies* (L.) H.Karst.	Strmilov	13.8.2008	ditto
Sacchiphantes viridis (Ratzeburg, 1843)	11573	GU571067	*Picea abies* (L.) H.Karst.	Želetava	13.8.2008	ditto
Sacchiphantes viridis (Ratzeburg, 1843)	11619	GU571068	*Picea abies* (L.) H.Karst.	Fulnek	14.8.2008	ditto
Pineus cembrae (Cholodkovsky, 1888)	10536	GU571069	*Pinus cembra* L.	Těchobuz u Pacova	22.4.2008	pure ethanol
Pineus cembrae (Cholodkovsky, 1888)	3071–3072	GU571070–GU571071	*Pinus cembra* L.	Těchobuz u Pacova	22.4.2008	ditto
Pineus cembrae (Cholodkovsky, 1888)	3497	GU571072	*Pinus cembra* L.	Těchobuz u Pacova	7.9.2008	ditto
Pineus cembrae (Cholodkovsky, 1888)	3583	GU571073	*Pinus cembra* L.	Buchlovice	30.10.2008	ditto
Pineus orientalis (Dreyfus, 1889)	P2486–2488	GU571074–GU571076	*Picea orientalis* (L.) Peterm.	Buchlovice	23.5.2007	ditto
Pineus orientalis (Dreyfus, 1889)	10580–10582	GU571077–GU571079	*Picea orientalis* (L.) Peterm.	Lednice	3.6.2008	kept at –70°C
Pineus orientalis (Dreyfus, 1889)	10655–10657	GU571080–GU571082	*Picea orientalis* (L.) Peterm.	Buchlovice	5.6.2008	ditto
Pineus pineoides (Cholodkovsky, 1903)	3487	GU571083	*Picea abies* (L.) H.Karst.	Šumava Mts, Jenišov	24.8.2008	pure ethanol
Pineus pini (Macquart, 1819)	P2269	GU571084	*Pinus sylvestris* L.	Praha, Břevnov	4.4.2007	ditto
Pineus pini (Macquart, 1819)	P2265	GU571085	*Pinus sylvestris* L.	Praha, Břevnov	4.4.2007	ditto
Pineus pini (Macquart, 1819)	P2353	GU571086	*Pinus sylvestris* L.	Bystřice pod Hostýnem	25.4.2007	ditto
Pineus pini (Macquart, 1819)	3230–3231	GU571087–GU571088	*Pinus sylvestris* L.	Praha, Břevnov	22.6.2008	ditto
Pineus pini (Macquart, 1819)	3576	GU571089	*Pinus sylvestris* L.	České Budějovice	10.11.2008	ditto
Viteus vitifoliae (Fitch, 1855)	12215–12217	GU571090–GU571091	*Vitis vinifera* L.	Polešovice	17.9.2008	kept at –70°C
Phylloxera coccinea (von Heyden, 1837)	3205, 3224–3225	GU571092 GU571093 GU571094	*Quercus robur* L.	Lednice	17.6.2008	pure ethanol

The phylogenetic analysis showed that all genera formed monophyletic groups. Since the topology of the trees generated by different methods was similar, only the NJ and Bayesian trees are presented (Fig. 1). The only difference between the NJ and the Bayesian trees is the position of the *Pineus/Eopineus* cluster. While in the NJ

1a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Dreyfusia nordmannianae 12011
Dreyfusia prelli 10841
Dreyfusia piceae 3499
Dreyfusia prelli 10842
Dreyfusia prelli (P1963)
Dreyfusia prelli (P2693)
Dreyfusia nordmannianae 12009
Dreyfusia piceae 3498
Dreyfusia nordmannianae 12010
Dreyfusia piceae 3622
Dreyfusia nordmannianae (P1405)
Dreyfusia nordmannianae 10541
Dreyfusia nordmannianae 10540
Dreyfusia prelli (P812)
Dreyfusia prelli (P707)
Dreyfusia prelli (814)
Aphrastasia pectinatae 3345
Gilletteella cooleyi (P1742)
Gilletteella cooleyi 11011
Gilletteella cooleyi 11026
Gilletteella cooleyi 11010
Gilletteella cooleyi (P1740)
Gilletteella cooleyi (P1743)
Gilletteella cooleyi 11009
Gilletteella cooleyi 11028
Gilletteella cooleyi 11027
Gilletteella cooleyi (P1741)
Gilletteella coweni 3229
Gilletteella coweni 3582
Gilletteella coweni 3579a
Sacchiphantes abietis (P2416)
Sacchiphantes abietis (P2417)
Sacchiphantes abietis (P2419)
Sacchiphantes abietis (P2420)
Sacchiphantes viridis 11350
Sacchiphantes viridis 11380
Sacchiphantes viridis (P846)
Sacchiphantes viridis 11362
Sacchiphantes abietis (P2418)
Sacchiphantes abietis (P1044)
Sacchiphantes abietis 11697
Sacchiphantes abietis (P2887)
Sacchiphantes abietis 11624
Sacchiphantes viridis 11573
Sacchiphantes viridis 11550
Sacchiphantes viridis 11619
Adelges laricis 11500
Adelges tardus (P1283)
Adelges laricis 10555
Adelges tardus (P1287)
Adelges laricis 11031
Adelges tardus (P1286)
Adelges tardus (P2554)
Adelges laricis 10960
Adelges tardus 10688
Adelges tardus 11340
Adelges tardus (P1285)
Adelges tardus 11196
Adelges tardus 11458
Adelges tardus (P1284)
Cholodkovskya viridiana 3328
Cholodkovskya viridiana 3326
Cholodkovskya viridiana 3327
Pineus orientalis 10655
Pineus pini 3231
Pineus orientalis (2488)
Pineus orientalis 10657
Pineus orientalis 10582
Pineus orientalis (P2486)
Pineus pini (P2353)
Pineus pini (P2265)
Pineus orientalis 10656
Pineus orientalis (P2487)
Pineus pini (P2269)
Pineus orientalis 10581
Pineus orientalis 10580
Pineus pini 3230
Pineus pini 3576
Eopineus strobi (P2463)
Eopineus strobi (P2458)
Eopineus strobi (P2457)
Eopineus strobi (P2217)
Eopineus strobi (P2462)
Eopineus strobi 3098
Pineus pineoides 3487
Pineus cembrae 3497
Pineus cembrae 3072
Pineus cembrae 10536
Pineus cembrae 3583
Pineus cembrae 3071
Phylloxera coccinea 3224
Phylloxera coccinea 3225
Viteus vitifoliae 12215
Viteus vitifoliae 12216
Viteus vitifoliae 12217
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0.01

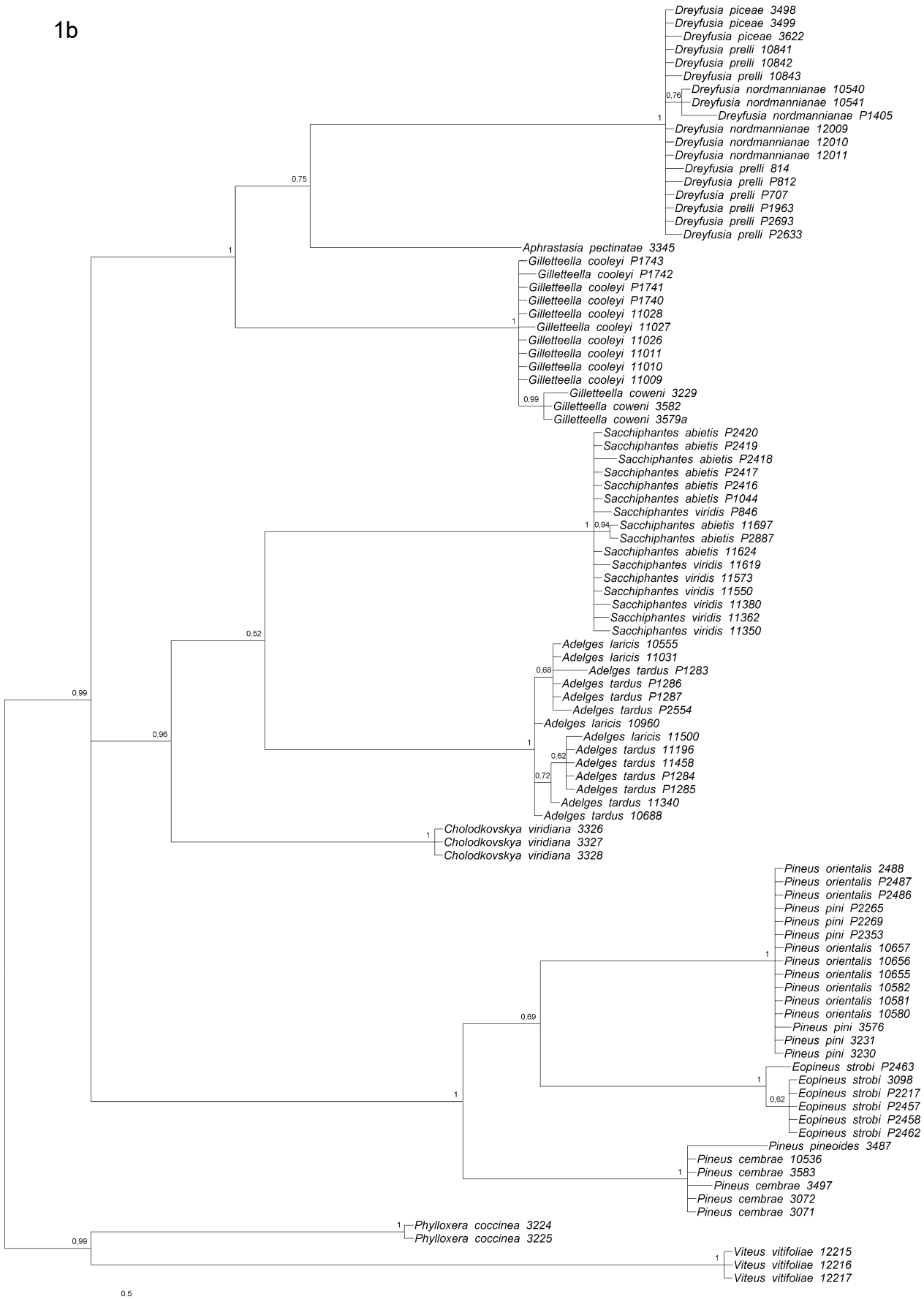

Fig. 1. Evolutionary relationships of 97 specimens representing 16 species of adelgids and 2 phylloxerids. 1a – Neighbour-Joining tree, the bootstrap values (10000 replicates) are shown next to the branches; 1b – Phylogram from the MrBayes analysis. Numbers above each node represent the posterior probability support.

TABLE 2. Intraspecific and interspecific congeneric distances (uncorrected *p*-distance) for 16 species of adelgids.

Species	No. of specimens	Intraspecific divergence (%)		Interspecific congeneric divergence (%)	
		Mean	Range	Mean	Range
Dreyfusia prelli	9	0.14	0.0–0.32		
Dreyfusia nordmannianae	6	0.24	0.0–0.61	*Dreyfusia*	
Dreyfusia piceae	3	0.10	0.0–0.15	0.18	0.0–0.76
Aphrastasia pectinatae	1	n/c			
Gilletteella coweni	3	0.21	0.0–0.31	*Gilletteela*	
Gilletteella cooleyi	10	0.06	0.0–0.32	0.20	0.0–0.76
Sacchiphantes abietis	9	0.21	0.0–0.46	*Sacchiphantes*	
Sacchiphantes viridis	7	0.35	0.0–0.48	0.28	0.0–0.62
Adelges laricis	4	0.43	0.0–0.76	*Adelges*	
Adelges tardus	10	0.38	0.0–0.94	0.38	0.0–1.10
Cholodkovskya viridiana	3	0.00	0.00		
Pineus orientalis	9	0.00	0.00		
Pineus pini	6	0.05	0.0–0.15		
Eopineus strobi	6	0.15	0.0–0.46		
Pineus pineoides	1	n/c		*Pineus*	
Pineus cembrae	5	0.12	0.0–0.30	4.12	0.0–7.61
Phylloxera coccinea	2	0.00	0.00		
Viteus vitifoliae	3	0.00	0.00		

n/c – evolutionary distances not computed

tree *Pineus/Eopineus* stands as a sister group to the *Sacchiphantes*/*Adelges*/*Cholodkovskya* cluster (Fig. 1a), in the Bayesian tree it forms a polytomy with the branches leading to the *Giletteella*/*Dreyfusia* and *Sacchiphantes*/*Adelges*/*Cholodkovskya* clusters (Fig. 1b). Note that the bootstrap support for such clustering on the NJ tree is very low (21%).

Most clusters were strongly supported and formed either by single species (e.g. *Cholodkovskya*) or by species complexes (*Adelges*, *Sacchiphantes*, *Dreyfusia*, *Gilletteella*, and *Pineus pini*/*orientalis*). *Eopineus* is nested within *Pineus* and should thus either be synonymized or three genera have to be recognized in this cluster. On the other hand, nodes representing the subfamilies sensu Annand (1928), Börner & Heinze (1957) or Steffan (1968) were not monophyletic.

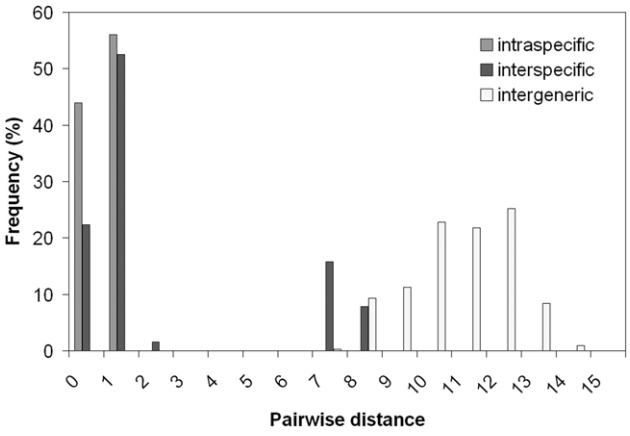

Fig. 2. Overlap of the intraspecific, interspecific congeneric, and intergeneric genetic variability (*p*-distance uncorrected).

The mean intraspecific divergence for all adelgid species was 0.15% (range 0.00–0.76%). Mean intraspecific divergence was 0.38 (range 0.00–1.10%) for the *Adelges* complex (*laricis*/*tardus*), 0.3 (range 0.00–0.62%) for the *Sacchiphantes* complex (*abietis*/*viridis*), and 0.02 (range 0.00–0.15) for the *Pineus* complex (*orientalis*/*pini*). The mean interspecific divergence was 8.23% (range 0.00–13.24%) for all adelgid species. However, the interspecific congeneric divergence was much lower, ranging from 0.0 to 4.12 % (Table 2). There was a considerable overlap of the intraspecific and interspecific congeneric divergencies (Table 2), as can be seen also on the histogram in Fig. 2. On the other hand, there is no overlap with the divergencies at the generic level (Table 3 and Fig. 2), with the mean value being 10.26% (range 7.00–13.24%).

The threshold for species identification evaluated by TaxonDNA was 0.6%, nevertheless, only 41 (44.56%) of the sequences were correctly identified according to the "Best Match" criteria; 42 sequences were ambiguous (45.65%) and 9 (9.78%) were incorrectly identified (including 2 species represented by only a single specimen). The higher threshold values (at 1%, 2% and 3% levels) were also tested as recommended by Meier et al. (2008) and Ratnasingham & Hebert (2007). As expected, these values did not increase the identification rate, which remained the same as at the 0.6% level in all these tests.

DISCUSSION AND CONCLUSIONS

Our results indicate that "DNA barcoding" using the *COI* gene can successfully identify adelgid species that are clearly delineated by classical taxonomy. Molecular data produced monophyletic groups representing single

TABLE 3. Estimates of evolutionary divergence (uncorrected *p*-distance) among genera.

	Dreyfusia	*Aphrastasia*	*Gilletteella*	*Sacchiphantes*	*Cholodkovskya*	*Adelges*	*Pineus/Eopineus*
Dreyfusia		0.078	0.083	0.107	0.088	0.104	0.109
Aphrastasia	0.010		0.071	0.092	0.091	0.083	0.104
Gilletteella	0.010	0.010		0.095	0.081	0.097	0.119
Sacchiphantes	0.012	0.011	0.011		0.077	0.079	0.120
Cholodkovskya	0.011	0.011	0.010	0.010		0.078	0.097
Adelges	0.011	0.011	0.011	0.010	0.010		0.103
Pineus/Eopineus	0.011	0.011	0.012	0.011	0.010	0.010	

The number of base differences per site obtained by averaging over all sequence pairs between genera is shown above diagonal. All results are based on the pairwise analysis of 97 sequences. Standard error estimates are shown in the lower-left part of the matrix and were obtained by a bootstrap procedure (1000 replicates).

genera. However, this marker was not sufficient to distinguish morphologically identical species in species complexes, whose description is based on ecology.

Mean values of overall intraspecific (0.15%) and interspecific (8.23%) divergence are comparable to those of other insects, such as 0.46% and 4.41–6.02% reported for tropical Lepidoptera (Hajibabaei et al., 2006) or 0.17% and 5.78% for parasitoid flies (Smith et al., 2006). They are also comparable to the results obtained by Havill et al. (2007) and Foottit et al. (2009), although direct comparison is difficult since these authors use a different generic taxonomy and the species sampled only partially overlapped. In addition, figures in the Foottit et al. (2009) study are also higher because the extensive sampling of two pest species suggested the existence of two or three cryptic species.

According to the guidelines proposed by Hebert et al. (2004), successful species identification is possible if mean interspecific divergence equals 10 × the mean intraspecific divergence, and in an ideal case, there should be an observable "barcoding gap", that is, a separation between mean intra- and interspecific congeneric *COI* sequences (Meyer & Paulay, 2005). However, it is argued by Meier et al. (2006) that instead of the mean value, only the smallest interspecific distance should be used. Although the observed difference in the mean values for our data set fulfills the first criterion, it is not so for the other two. The biggest issue is the large overlap of intra- and interspecific divergence, which is most likely caused by the inclusion of species complexes. But there are examples of interspecific congeneric distances as low as 0.001 for the well defined species *D. prelli* and *D. piceae*, which makes the use of "DNA barcoding" doubtful. Since the evolution of the species complexes is recent, it is likely that the *COI* locus is not the best marker for individual species identification.

Our analysis supports the recognition of eight genera as proposed by Börner & Heinze (1957) or Steffan (1968) rather then the two genera system of Annand (1928). Phylogenetic trees distinguished most genera very clearly. However, our results differ from Havill et al. (2007). The first difference is the placement of the *Pineus* cluster, which is not distinctly separated from all the other adelgids. In our NJ tree and Bayesian analysis, it is positioned closer to the *Adelges* and *Sacchiphantes* groups. Second, both trees support sister group relationships between *Gilletteella* and *Dreyfusia*, while Havill et al. (2007) placed *Gilletteella* closer to the *Sacchiphantes*/*Adelges* group. Taken together, our data best fit the system presented by Börner & Heinze (1957).

The most problematic group appears to be *Dreyfusia*. The status of its species has been disputed. Mantovani et al. (2001) conclude, based on mitochondrial DNA sequences, that at least three of the five *Dreyfusia* species known from conifers in Italy are doubtful. Our results are similar. Although some species form a species complex (*nordmannianae*/*piceae*), the observed pattern is striking. *D. prelli* is very distinct, based on morphology and life cycle characteristics. It is therefore quite surprising that the DNA marker did not reveal any differences with respect to the other species.

On the other hand, two sister species, *G. cooleyi* and *G. coweni*, can be identified based on the 3rd codon position difference (one silent substitution). Adding more sympatric samples from different localities is in this case highly desirable, as well as using other, more quickly evolving markers.

In conclusion, Adelgidae are another group for which the "DNA barcoding"is not the tool of first choice for species identification, although it can provide helpful suggestions for the identification of species at the genus level. The main problem is that the intraspecific and interspecific congeneric variability do not form two separate intervals with a distinct "barcoding gap". In addition, several species share the same haplotypes, thus the identification of these species is impossible. Furthermore, it is suggested that the eight genus system (with revision of the *Pineus* genus), previously proposed based on morphological studies, should continue to be used. Species complexes still remain an interesting puzzle both at the ecological and genetic level, and as suggested by Foottit et al. (2009) further studies are needed to resolve their species status.

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REFERENCES

ANNAND P.N. 1928: *A Contribution Toward a Monograph of the Adelginae (Phylloxeridae) of North America*. Stanford Univ. Press, Palo Alto, CA, 146 pp.

BINAZZI A. 2000: Notes on and key to winged forms of adelgids recorded from Italy (Homoptera, Aphidoidea, Adelgidae). *Redia* **83**: 187–215.

BINAZZI A. & COVASSI M. 1991: Contributions to the knowledge of the conifer aphid fauna XII, a review of the species of Dreyfusia Boerner occurring in Italy with description of Dreyfusia nebrodensis n. sp. (Homoptera: Adelgidae). *Redia* **74**: 233–288.

BLACKMAN R.L. & EASTOP V.F. 1994: *Aphids on the World's Trees: An Identification and Information Guide.* CABI, Wallingford, 987 pp.

BODENHEIMER F.S. & SWIRSKI E. 1957: *Aphidoidea of the Middle East*. The Weizmann Science Press of Israel, Jerusalem, 378 pp.

BÖRNER C.V. 1908: Eine monographische Studie über die Chermiden. *Arb. Kais. Biol. Anst. Land. Forstw.* **6**: 81–320.

BÖRNER C.V. 1930: Beiträge zur einem neuen System der Blattläuse. *Arch. Klassif. Phylogen. Entomol.* **50**: 115–194.

BÖRNER C. 1952: Europae centralis Aphides, die Blattläuse Mitteleuropas. *Mitt. Thurin. Bot. Ges.* **3**: 1–484.

BÖRNER C.V. & HEINZE K. 1957: Aphidina – Aphidoidea. In Blunck H. (ed.): *Tierische Schädlinge an Nutzpflanzen. Homoptera. 2. Teil.* Paul Parey, Berlin, pp. 323–355.

CARTER C.I. 1971: *Conifer Woolly Aphids (Adelgidae) in Britain*. Forestry Commission Bulletin No. 42, London, 51 pp.

CHOLODKOVSKY N. 1896: Zur Biologie der Lärchen-Chermes-Arten. *Zool. Anz.* **19**: 37–40.

DMITRIEV G.V. 1965: Chermesinae – pests of coniferous trees of Ukraine. In: *Nature Conservation.* Urozhai, Kiev, pp. 157–203.

FOLMER O., BLACK M., HOEH W., LUTZ R. & VRIJENHOEK R. 1994: DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Marine Biol. Biotechnol.* **3**: 294–299.

FOOTTIT R.G., MAW E.L., HAVILL N.P., AHERN R.G. & MONTGOMERY M.E. 2009: DNA barcodes to identify species and explore diversity in the Adelgidae (Insecta: Hemiptera: Aphidoidea). *Mol. Ecol. Res.* **9**: 188–195.

FRATI F., SPINSANTI G. & DALLAI R. 2001: Genetic variation of mt COII gene in the collembolan Isotoma klovstadi from Victoria land, Antarctica: evidence for population differentiation. *Polar Biol.* **24**: 934–940.

GUINDON S. & GASCUEL O. 2003: A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**: 696–704.

HAJIBABAEI M., JANZEN D.H., BURNS J.M., HALLWACHS W. & HEBERT P.D.N. 2006: DNA barcodes distinguish species of tropical Lepidoptera. *PNAS* **103**: 968–977.

HAVILL N.P. & FOOTTIT R.G. 2007: Biology and evolution of Adelgidae. *Annu. Rev. Entomol*. **52**: 325–349.

HAVILL N.P., FOOTTIT R.G. & VON DOHLEN C.D. 2007: Evolution of host specialization in the Adelgidae (Insecta: Hemiptera) inferred from molecular phylogenetics. *Mol. Phylog. Evol*. **44**: 357–370.

HEBERT P.D.N., PENTON E.H., BURNS J.M., JANZEN D.H. & HALLWACHS W. 2004: Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. *PNAS* **101**: 14812–14817.

HEINZE K. 1962: Pflanzenschädliche Blattläusarten der Familien Lachnidae, Adelgidae und Phylloxeridae, eine systematisch-faunistische Studie. *Dt. Entomol. Z.* **9**: 143–227.

INOUYE M. 1945: Monographische Studie über die japanischen Koniferen-Gallenläuse (Adelgidae). *Bull. Sapporo Bran. Gov. For. Exp. Stn*. **15**: 1–91.

KUMAR S., TAMURA K. & NEI M. 1994: MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *Comput. Appl. Biosci.* **10**: 189–191.

LAMPEL G. 1968: *Die Biologie des Blattlaus-Generationswechsels*. Gustav Fischer, Jena, 264 pp.

MANTOVANI B., FRANCARDI V., BINAZZI A. & LECCESE A. 2001: A molecular approach to differentiate the species of Dreyfusia Börner occurring in Italy (Aphidoidea, Adelgidae). *Redia* **84**: 151–159.

MEIER R., KWONG S., VAIDYA G. & NG P.K.L. 2006: DNA Barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Syst. Biol.* **55**: 715–728.

MEIER R., ZHANG G. & FARHAN A. 2008: The use of mean instead of smallest interspecific distances exaggerates the size of the "barcoding gap" and leads to misidentification. *Syst. Biol.* **57**: 809–813.

MEYER C.P. & PAULAY G. 2005: DNA barcoding: error rates based on comprehensive sampling. *PLoS Biol.* **3**: 2229–2238.

PASHCHENKO N.F. 1988: Suborder Aphidinea, Aphids. In Leer P.A. (ed.): *Keys to Insects of the Far East of USSR*. Nauka, Leningrad, pp. 546–686 [in Russian].

PAŠEK V. 1954: *Vošky našich lesných drevín. [Adelgids of our Coniferous Trees.]* Veda, Bratislava, 319 pp.

POSADA D. & CRANDALL K.A. 1998: Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

RATNASINGHAM S. & HEBERT P.D.N. 2007: BOLD: the Barcode of Life Data System. *Mol. Ecol. Notes* **7**: 355–364.

RONQUIST F. & HUELSENBECK J.P. 2003: MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

SHAPOSNIKOV G.CH. 1964: Aphidinea. In Bej-Bienko G.Ja. (ed.): *Insects of the European Part of the USSR: Identification Key.* Nauka, Moscow, pp. 489–616 [in Russian].

SHEARER T.L. & COFFROTH M.A. 2008: Barcoding corals: limited by interspecific divergence, not intraspecific variation. *Mol. Ecol. Res.* **8**: 247–255.

SIMON S., FRATI F., BECKENBACH A., CRESPI B., LIU H. & FLOOK P. 1994: Evolution, weighting, phylogenetic utility of mitochondrial gene sequences and compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* **87**: 651–701.

SMITH M.A., WOODLEY N.E., JANZEN D.H., HALLWACHS W. & HEBERT P.D.N. 2006: DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *PNAS* **103**: 3657–3662.

STEFFAN A.W. 1961: Die Stammes- und Siedlungsgeschichte des Artenkreises Sacchiphantes viridis (Ratzeburg, 1843) (Adelgidae, Aphidoidea). *Zoologica* **109**: 1–113.

STEFFAN A.W. 1968: Evolution und Systematik der Adelgidae (Homoptera: Aphidina): Eine Verwandtschaftsanalyse auf vorwiegend ethologischer, zytologischer und karyologischer Grundlage. *Zoologica* **115**: 1–139.

STEFFAN A.W. 1972: Unterordnung Aphidina, Blattläuse. In Schwenke W. (ed.): *Die Forstschädlinge Europas. Ein Handbuch in fünf Bänden*. Parey, Hamburg, pp. 162–386.

SWOFFORD D.L. 2003: *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4*. Sinauer Associates, Sunderland, Massachusetts.

SZELEGIEWICZ H. 1968: Mszyce, Aphidoidea. *Katalog Fauny Polski* **21**: 1–316 [in Polish].

TAMURA K., DUDLEY J., NEI M. & KUMAR S. 2007: MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. **24**: 1596–1599.

TAUTZ D., ARCTANDER P., MINELLI A., THOMAS R.H. & VOGLER A.P. 2003: A plea for DNA taxonomy. *Trends Ecol. Evol.* **18**: 70–74.

VENCES M., THOMAS M., BONETT R.M. & VIEITES D.R. 2005: Deciphering amphibian diversity through DNA barcoding: chances and challenges. *Phil. Trans. R. Soc. (B)* **360**: 1859–1868.

WIEMERS M. & FIEDLER K. 2007: Does the DNA barcoding gap exist? – a case study in blue butterflies (Lepidoptera: Lycaenidae). *Front. Zool.* **4**: 8.

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