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Key characteristics of selected *Drepanaphis* Del Guercio, 1909 (Hemiptera: Aphididae) species based on various identification methods

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Abstract. The Nearctic genus *Drepanaphis* Del Guercio, 1909 currently includes 16 species with similar morphometric features, and three-dimensional structures may be important in species identification. The form (shape) of the dorsal abdominal tubercles, however, can be distorted by mounting on microscopic slides and this ultimately clouds diagnostic characters. This paper focuses on the identification of three species belonging to the genus *Drepanaphis*: *Drepanaphis acerifoliae* (Thomas, 1878), *D. kanzensis* Smith, 1941 and *D. sabrinae* Miller, 1937, to show the apparent differences of the structures of the examined individuals based on analysis of material deposited in museum collections and freshly collected material. To verify structural differences more precisely, we used Scanning Electron Microscopy to depict morphological characters accurately and DNA barcoding to analyze individuals at the molecular level.

Key words. COI, *Drepanaphis acerifoliae*, *Drepanaphis kanzensis*, *Drepanaphis sabrinae*, dorsal abdominal tubercle, SEM.

INTRODUCTION

The correct identification of species is a basis of systematic and biology because it is the starting point for most organismal research studies (Iverson 2022). When a specimen is collected in the field and becomes a museum specimen (or is included in any entomological collection), it should be properly prepared for its preservation. In addition, the appropriate conservation of collected specimens and their metadata provides an important source of information for wider taxonomic, biogeographic or molecular studies (Newbold 2010). However, morphological structures can become distorted by immersion in alcohol or after mounting on a microscope slide. Ultimately, this changes the morphology and can make species identification difficult. Comparison with type material is important because it not only confirms morphological species identifications but also provides temporal (e.g., date of collections), ecological (e.g., host plant), and geographical (e.g., collection locality) data. These data, especially locality, are fundamental for defining species as endemic / native, adventive/invasive, and sympatric/allopatric. They also aid in determining identity when compared to the known distribution of a species, or they can lead to hypothesizing a new species (Miller et al. 2018). For some species, however, it happens that the type material has been lost or is in poor condition, and the comparative analysis of morphometric features is impossible. In such cases, it is necessary to critically evaluate the species diagnostic characters and compare with as many similar museum specimens as possible, thereby clarifying its taxonomical status. When diagnostic characters are unclear or intraspecific phenotypic variation is large, the usability of DNA barcoding, i.e., sequencing a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene, has been used for additional diagnostic data in many animal groups for species identification (Hebert et al. 2003; Foottit et al. 2008; Kekkonen & Hebert 2014; Depa et al. 2012). Lastly, additional species diagnostic characters have been obtained from the comparison of micromorphological structures of closely related species using scanning electron microscopy (SEM) (Kumar et al. 2014). Thus, integrative taxonomy is a comprehensive framework to delimit and describe taxa by integrating information from different types of data and methodologies (Pante et al. 2015).

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Aphids (Hemiptera: Aphididae) are difficult to identify and taxonomically verify due to seasonal polymorphism and complicated life cycles. This is particularly the case for the largest aphid genera *Aphis* Linnaeus, 1758 and *Cinara* Curtis, 1835, where the similarities among species are so considerable, that species identification is often based on DNA sequence data (Jousselin et al. 2013; Chen et al. 2013, 2016).

Within the Aphididae, less speciose genera such as *Drepanaphis* Del Guercio, 1909 present challenges in morphological taxonomy. *Drepanaphis* is a Nearctic genus with 16 species (Favret 2023) having similar morphometric characters. A prominent feature of the dominant generation, the alate viviparous females, is the dorsal abdominal tubercles that are variably developed on abdominal tergites I–IV and often conspicuously pigmented. In addition to coloration of fore femora and wings, this feature is crucial in the determination of microscope slide-mounted specimens of *Drepanaphis* (Smith & Dillery 1968; Blackman & Eastop 2023). The dorsal abdominal tubercles are three-dimensional structures which often become distorted during microscope slide preparation. This complicates species identification using the available keys for species determination within *Drepanaphis*. Thus, species delimitation in this genus can be very challenging using only microscopic slide-mounted specimens deposited in entomological collections. In this study, based on the identification of selected species of *Drepanaphis*, we focus on: (1) describing and quantifying the differences in the appearance of the diagnostic structures by comparing microscopic slide-mounted specimens deposited in museum collections with fresh collected material and living specimens; (2) showing discrepancies in the dimensions of significant structures (e.g., size of dorsal abdominal tubercles); (3) presenting the detailed structure of the dorsal abdominal tubercles using SEM and; (4) use of mitochondrial COI for species verification at the molecular level.

MATERIALS AND METHODS

Data collection and taxon sampling

Fresh specimens of alate viviparous females of *Drepanaphis acerifoliae* (Thomas, 1878) (six individuals), *D. kanzensis* Smith, 1941 (six individuals) and *D. sabrinae* Miller, 1937 (six individuals) were preserved in 70% ethanol (Table 1). Species were initially identified based on diagnostic morphological features, including dorsal abdominal tubercles (Smith & Dillery 1968; Blackman & Eastop 2023), using a Nikon SMZ 25 stereoscopic microscope and photographed using a Nikon DS-Fi2 camera.

Additionally, we examined 61 mounted slides of alate viviparous females of the following species of the genus *Drepanaphis*: *D. acerifoliae* (45 slides), *D. kanzensis* (eight slides, including holotype from USNM) and *D. sabrinae* (eight slides) using a Nikon Ni-U light microscope and photographed with a Nikon DS-Fi2 camera.

Institutional abbreviations

CAS = Biologické Centrum AV ČR, v.v.i., České Budějovice, Czech Republic
MZLU = Lund University Biological Museum, Lund, Sweden
MNHN = Muséum national d'histoire naturelle, Paris, France
NHMUK = Natural History Museum, London, UK
USNM = U.S. National Museum of Natural History Aphidomorpha collection, located at the Henry A. Wallace Beltsville Agricultural Research Center, Beltsville, Maryland, USA
DZUS = Entomology collection of University of Silesia, Katowice, Poland
ZMPA = Zoological Institute, Polish Academy of Sciences, Warsaw, Poland

The detailed collection data are presented in Table 2.

Table 1. Collection records and GenBank accession numbers of the studied aphid species.

No	species	collection date /collector	host plant	locality	coordinates	collection	GenBank no.	reference
1	*Drepanaphis acerifoliae*	24.09.2022 Kamila Malik	*Acer rubrum*	Raleigh, North Carolina, USA	35.7822 -78.6365	DZUS 24/9.22_170	KR037245	(Gwiazdowski et al. 2015)
2	*Drepanaphis kanzensis*	21.09.2022 Kamila Malik	*A. saccharum*	Rahway, New Jersey, USA	40.6211 -74.2854	DZUS 21/9.22_171	OR573481	present study
3	*Drepanaphis sabrinae*	24.09.2022 Kamila Malik	*A. saccharum*	Raleigh, North Carolina, USA	35.7822 -78.6365	DZUS 24/9.22_172	OR575052	present study
4	*Dreapnosiphum aceris* (Walker)						KR029857	(Henry et al. 2015)

Table 2 (continued next two pages). List of analyzed species from museum collections.

No	species	country	state	locality	date collection	det.	leg.	host plant	collection
1	*Drepanaphis acerifoliae*	USA	Utah	Payson	18.06.1959	J.Holman	G.F.Knowlton	*Acer saccharinum*	CAS
2	*Drepanaphis acerifoliae*	USA	Utah	Payson	18.06.1959	J.Holman	G.F.Knowlton	*A. saccharinum*	CAS
3	*Drepanaphis acerifoliae*	USA	Utah	Payson	18.06.1959	J.Holman	G.F.Knowlton	*A. saccharinum*	CAS
4	*Drepanaphis acerifoliae*	USA	Utah	Payson	18.06.1959	J.Holman	G.F.Knowlton	*A. saccharinum*	CAS
5	*Drepanaphis acerifoliae*	USA	Utah	Payson	18.06.1959	J.Holman	G.F.Knowlton	*A. saccharinum*	CAS
6	*Drepanaphis acerifoliae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith	C.F.Smith	*A. rubrum*	CAS
7	*Drepanaphis acerifoliae*	USA	North Carolina	Raleigh	06.07.1959	C.F.Smith	C.F.Smith	*A. rubrum*	CAS
8	*Drepanaphis acerifoliae*	USA	Minnesota	St.James	03.08.1960	Dillery&Smith		*A. saccharinum*	CAS
9	*Drepanaphis acerifoliae*	USA	Minnesota	St.James	03.08.1960	Dillery&Smith		*A. saccharinum*	CAS
10	*Drepanaphis acerifoliae*	USA	Utah	Provo	21.06.1960	Pintera	G.F.Knowlton	*Acer* sp.	CAS
11	*Drepanaphis acerifoliae*	USA	North Carolina	Franklin	26,06,1970	C.F.Smith		*A. saccharinum*	CAS
12	*Drepanaphis acerifoliae*	USA	Utah	Payson	18.06.1959	J.Holman	G.F.Knowlton	*A. saccharinum*	CAS
13	*Drepanaphis acerifoliae*	USA	Utah	Provo	21.06.1960	Pintera	G.F.Knowlton	*Acer* sp.	CAS
14	*Drepanaphis acerifoliae*	USA	Utah	Provo	21.06.1960	Pintera	G.F.Knowlton	*Acer* sp.	CAS
15	*Drepanaphis acerifoliae*	USA	Utah	Salt Lake City	21.06.1960	Pintera	G.F.Knowlton	*A. saccharinum*	CAS
16	*Drepanaphis acerifoliae*	USA	Utah	Salt Lake City	22.06.1960	Pintera	G.F.Knowlton	*A. saccharinum*	CAS
17	*Drepanaphis kanzensis*	USA	Maine	Presque Isle	10.09.1956	D.H.R.L	Simpson	*A. saccharum*	Biologické Centrum AV ČR
18	*Drepanaphis kanzensis*	USA	Kansas	Hiawatha	02.09.1960	Dillery&Smith	G.F.Knowlton	*A. saccharum*	CAS
19	*Drepanaphis acerifoliae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith	C.F.Smith	*A. rubrum*	MZLU
20	*Drepanaphis acerifoliae*	Canada	Manitoba	Winnipeg	15.07.1974	A.G.Robinson		*A. saccharinum*	MZLU
21	*Drepanaphis acerifoliae*	Canada	Manitoba	Winnipeg	28.06.1961		A.G.Robinson	*A. negundo*	MZLU

Table 2 (continued).

No	species	country	state	locality	date collection	det.	leg.	host plant	collection
22	*Drepanaphis acerifoliae*	Canada	Quebec	Orsainville	20.08.1972	W.Quednau	W.Quednau		MZLU
23	*Drepanaphis acerifoliae*	USA	Florida	Gainesville	02.02.1961			*A. rubrum*	MZLU
24	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87	C.F.Smith	*A. saccharum*	MZLU
25	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
26	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
27	*Drepanaphis acerifoliae*		Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
28	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
29	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
30	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
31	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
32	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
33	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
34	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
35	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
36	*Drepanaphis acerifoliae*	USA	Maryland	Beltsville	29.06.1986	Danielsson-87		*A. saccharinum*	MZLU
37	*Drepanaphis kanzensis*	Canada	Quebec	Sainte-Foy	12.09.1970	W.Quednau	W.Quednau	*A. saccharum*	MZLU
38	*Drepanaphis acerifoliae*	USA	Maine	Orono	09.09.1976			*A. saccharinum*	MNHM
39	*Drepanaphis sabrinae*	USA	Maine	Orono	09.09.1976			*A. saccharum*	MNHM
40	*Drepanaphis acerifoliae*	USA	District of Columbia	North Carolina	22.10.1958			*A. rubrum*	USNM
41	*Drepanaphis kanzensis*	USA	Fort Scott	Kansas	17.06.1940		C.F. Smith	*A. saccharum*	USNM
42	*Drepanaphis sabrinae*	USA	North Carolina	Raleigh	10.11.1965			*A. saccharum*	USNM
43	*Drepanaphis acerifoliae*	USA	California	Lodi	04.02.1960	R.C.Dickson		*Acer* sp.	NHMUK

Table 2 (continued).

No	species	country	state	locality	date collection	det.	leg.	host plant	collection
44	*Drepanaphis acerifoliae*	USA	North Carolina	Cherokee	16.10.1961			*A. rubrum*	NHMUK
45	*Drepanaphis acerifoliae*	USA	North Carolina	Cherokee	17.10.1961			*A. rubrum*	NHMUK
46	*Drepanaphis acerifoliae*	USA	California	Berkeley	20.10.1963	D.H.R.L		*A. saccharinum*	NHMUK
47	*Drepanaphis acerifoliae*	USA	California	Berkeley	20.10.1963	D.H.R.L		*A. saccharinum*	NHMUK
48	*Drepanaphis kanzensis*	Canada	Ontario	Unionville	wrz.60	J.Sypkens		*A. rubrum*	NHMUK
49	*Drepanaphis kanzensis*	Canada	Ontario	Unionville	wrz.60	J.Sypkens		*A. rubrum*	NHMUK
50	*Drepanaphis kanzensis*	USA	Utah	Logan Canyon	03.10.1957			*A. grandidentatum*	NHMUK
51	*Drepanaphis sabrinae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A. saccharum*	NHMUK
52	*Drepanaphis sabrinae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A. saccharum*	NHMUK
53	*Drepanaphis sabrinae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A.saccharum*	NHMUK
54	*Drepanaphis sabrinae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A. saccharum*	NHMUK
55	*Drepanaphis sabrinae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A. saccharum*	NHMUK
56	*Drepanaphis sabrinae*	USA	Minnesota	Saint Paul	05.07.1959			*A. saccharum*	NHMUK
57	*Drepanaphis acerifoliae*	Canada	Ontario	London	22.06.1952	C.H.N.Smith		*A. saccharum*	ZMPA
58	*Drepanaphis acerifoliae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A. rubrum*	ZMPA
59	*Drepanaphis acerifoliae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A. rubrum*	ZMPA
60	*Drepanaphis acerifoliae*	USA	North Carolina	Raleigh	05.07.1959	C.F.Smith		*A. rubrum*	ZMPA
61	*Drepanaphis kanzensis*	Canada	Ontario	Ottawa	27.09.1952	C.H.N.Smith		*A. saccharum*	ZMPA

Measurements

The dorsal abdominal tubercles of 18 fresh individuals (six in *D. acerifoliae*, six in *D. kanzensis*, and six in *D. sabrinae*) were measured from the base of the tubercle to its tip in every pair of tubercles by a Nikon NIS Elements D 4.50.00 64-Bit software and presented in millimeters (mm). Measurement examples are illustrated in Fig. 9 G–I.

Due to the different condition of the slide mounted samples, dorsal abdominal tubercles of 36 individuals (16 in *D. acerifoliae*, eight in *D. kanzensis*, and 12 in *D. sabrinae*) were measured from the base of the tubercle to its tip in every pair of tubercles by a Nikon NIS Elements D 4.50.00 64-Bit software and presented in millimetres (mm).

Scanning electron microscopy

We prepared two individuals of *D. acerifoliae*, *D. kanzensis* and *D. sabrinae* for SEM analysis. Each specimen was stored in 70% ethanol prior to imaging. Dehydration was provided by ethanol series of 80%, 90%, 96% and two changes of absolute as follows: 20 minutes in 80% ethanol, 15 minutes in 90% ethanol, 10 minutes in 96% ethanol, and two baths in absolute ethanol 10 minutes. The dehydrated samples were dried, stuck on the tables, sprayed with 30 nm of gold, and imaged with the Phenom XL field emission scanning electron microscope (Phenom-World B.V., Eindhoven, The Netherlands).

DNA extraction, PCR amplification, sequencing and alignment

Sampled alate viviparous females (five individuals of *D. kanzensis* and *D. sabrinae*) were preserved in 96% ethanol and then used for genomic DNA extraction. All extraction procedure followed the A&A Biotechnology protocol provided by the manufacturer, using Sherlock AX extraction kit. The 5-prime end of the mitochondrial COI barcode region was amplified using the primers LCO1490 (Forward: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (Reverse: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994). The PCR reaction included Color OptiTaq PCR Master Mix (EURx) and was performed on a Biometra TProfessional Basic Gradient thermocycler. The thermal profile of the PCR reaction was: initial denaturation 94°C – 60 s; denaturation 95°C – 40 s, annealing 45°C – 45 s, elongation 72°C – 60 s, extension 72°C – 180 s, number of cycles: 35. The amplicons were sequenced in both directions at GenoMed Warsaw. Raw chromatograms were viewed using Chromas ver. 2.6.6 software (Technelysium Pty Ltd. 2004) and edited with MEGA11 (Tamura et al. 2021). Alignments were made for each gene using Clustal W (Larkin et al. 2007) using default settings. From obtained sequences, the best were selected for further analysis. The resulting sequences were deposited in the GenBank (Table 1).

RESULTS

(1) Differences in appearance of diagnostic structures by comparing microscopic slides deposited in museum collections with freshly collected material and living specimens.

Drepanaphis acerifoliae is characterized by long, finger-like dorsal abdominal tubercles (d.a.t.) I and III. *D. kanzensis* has large, black d.a.t. III, while *D. sabrinae* is distinguished by d.a.t. II and III being large and sub-equal. These are the most important morphological differences of the species used for identification (Smith & Dillery 1968; Blackman & Eastop 2023). However, initial verification of these species based on type specimens may not provide key information on diagnostic characteristics. For example, in the holotype of *D. kanzensis*, d.a.t. are invisible (Fig. 1A). Among the remaining analyzed material from museum collections d.a.t. III are distinct (Fig. 1B). Some, however, are deformed. For example, the tubercles may overlap, making the whole d.a.t asymmetrical (Fig. 1C), whereas d.a.t. II are short or weakly visible and d.a.t. I and IV are inconspicuous (Fig. 1C–E). *D. acerifoliae* with d.a.t. I–IV conspicuous and d.a.t. I, III long and finger-like (Fig. 2A), seems to be the most characteristic and easiest species to identify in the genus *Drepanaphis* (Smith & Dillery 1968). However, a comparative analysis of museum material shows that it is sometimes confused with *D. sabrinae* (Fig. 3A). In particular, d.a.t. of *D. acerifoliae* are sometimes deformed (Fig. 2B) or only one pair of four is visible (Fig. 2C). In the case of *D. sabrinae*, mostly all d.a.t. overlap (Fig. 3B–C) and it is difficult to determine which are largest or of equal length, as is noted in the key of Smith & Dillery (1968). Therefore, species determination may be difficult in the examination of traditional microscope slides for *Drepanaphis* species where three-dimensional structures in the form of dorsal processes are key features. A solution for more accurate species identification may be mounting the specimen in a lateral orientation. However, few examples of such preserved material have been found in museum collections (Fig. 4A–C).

The study of species based on the fresh, not slide-mounted material, allows for a more detailed analysis of the indicated structures. In specimens of *D. kanzensis* d.a.t. I–IV are all clearly visible (Fig. 5A–C), whereas d.a.t. II and IV were practically not visible on any specimens from museum collections studied. The comparison of the fresh materials of *D. acerifoliae* and *D. sabrinae* underscores the usefulness of the features of three-dimensional dorsal tubercles in distinguishing these species. In *D. acerifoliae*, all d.a.t. are visible, with clear proportion between each pair and reflecting the size included in the Smith & Dillery (1968) key: I and III the largest pair, II and IV smaller (Fig. 6A–C). In *D. sabrinae*, all d.a.t. are conspicuous, symmetric, and clearly proportional to each other (Fig. 7A–C). The arrangement of the dorsal tubercles is visible in every projection of the body and is best observed when the insect is in the lateral view (Figs 5A, 6A, 7A).

Live images further emphasize species differences. In the photographed individual of *D. acerifoliae* (Fig. 8A–C), color features such as red eyes, head and thorax reddish brown, light-colored abdomen and distinctly dark-bordered wing veins, are a unique combination of characters for this species. Morphological features, such as size and arrangement of dorsal tubercles, and wax markings are also visible. Macrophotography is, therefore, a good tool for identification and documenting the

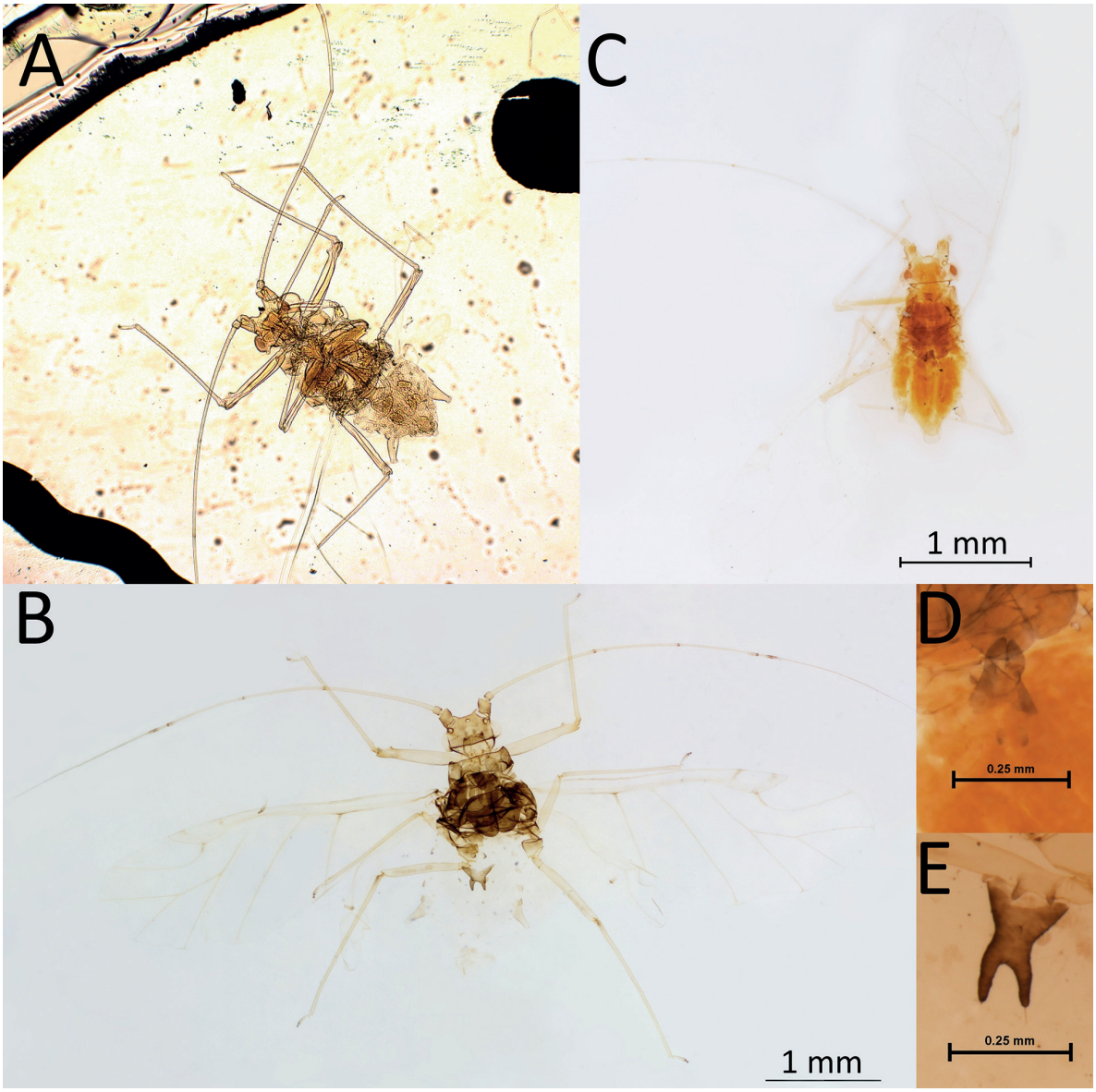

Fig 1. *Drepanaphis kanzensis* Smith, 1941. **A**. Holotype. **B**. Slide-mounted specimens with clearly visible dorsal abdominal tubercles. **C–E**. Slide-mounted specimens with deformed dorsal abdominal tubercles.

characteristics of species belonging to the genus *Drepanaphis*. It is, however, rarely used. Conversely, for some species such as *D. kanzensis* and *D. idahoensis*, which are entirely covered with white wax, this may not be a sufficient method to distinguish species (Fig. 8D–E).

(2) Discrepancies in the dimensions of key diagnostic structures – the size of dorsal abdominal tubercles

Smith and Dillery (1968) emphasized the importance of the size of the arrangement of d.a.t., as key features in the identification of aphids of the genus *Drepanaphis* (Fig. 9A–C). Often when analyzing individual museum specimens, it is difficult to measure the lengths of each d.a.t. because (I) the tubercles overlap and their placement is not visible, (II) the tubercles are not visible at all, (III) the tubercles are deformed by the microscope slide and it is difficult to determine size range (Fig. 9D–F).

In specimens of *D. kanzensis* from museum collections, only the third pair of tubercles is visible, while the second and third pairs are indiscernible (Fig. 9D). Measurements performed on fresh specimens in a lateral position make it possible to additionally measure the second and fourth pair of tubercles and in the case of this species they will be appropriate: II pair 0.07–0.11 mm, III pair 0.19–0.28 mm, IV pair 0.04–0.07 mm (Fig. 9G). In measurements carried out on eight individuals of this species from museum collections, it was possible to measure only the third pair of tubercles, the size of which was 0.2–0.28 mm (Table 3).

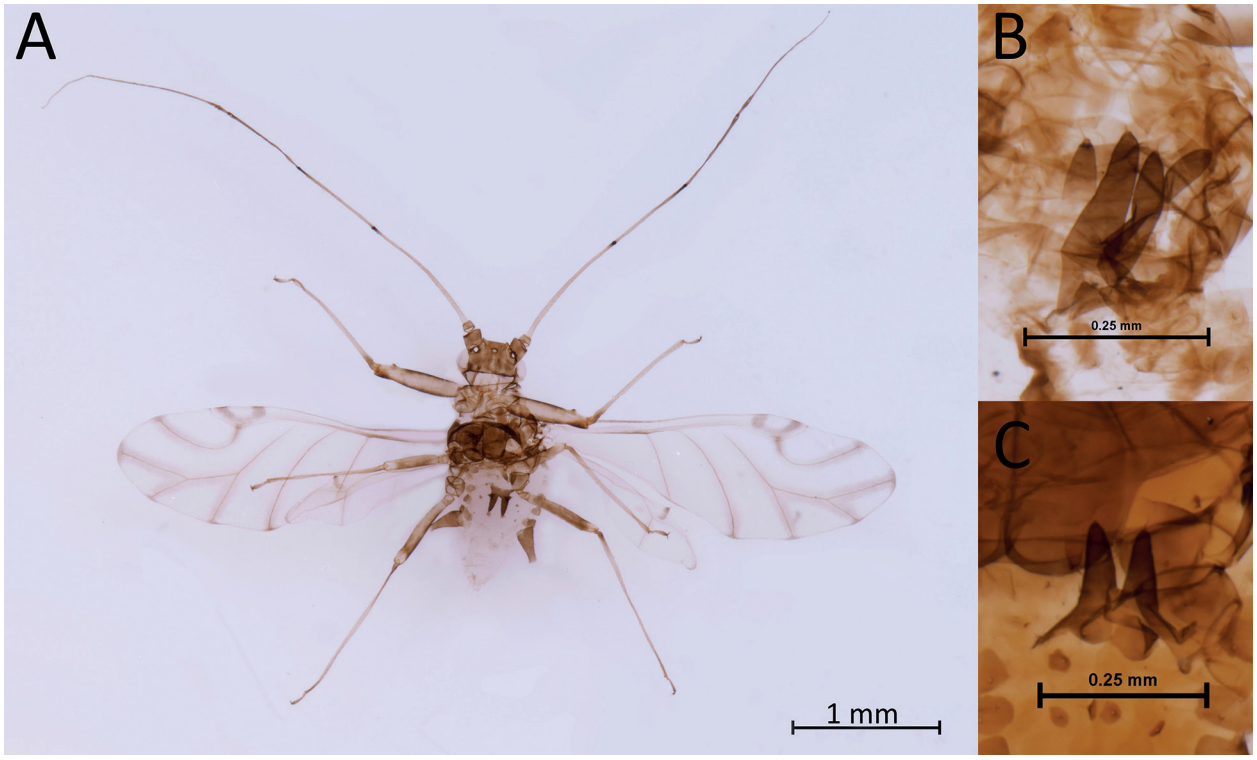

Fig 2. *Drepanaphis acerifoliae* (Thomas, 1878). **A**. Slide-mounted specimens with clearly visible dorsal abdominal tubercles (LM). **B–C**. Slide-mounted specimens with deformed dorsal abdominal tubercles (LM).

Fig 3. *Drepanaphis sabrinae* Miller, 1937. **A**. Slide-mounted specimens with clearly visible dorsal abdominal tubercles. **B–C**. Slide-mounted specimens with deformed dorsal abdominal tubercles.

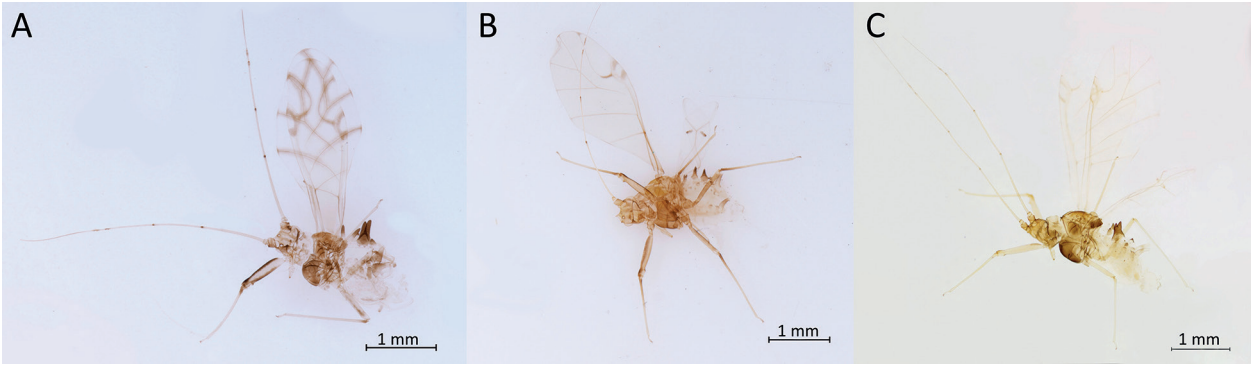

Fig 4. Lateral view of slide-mounted specimens. **A**. *Drepanaphis kanzensis* Smith, 1941. **B**. *Drepanaphis acerifoliae* (Thomas, 1878). **C**. *Drepanaphis sabrinae* Miller, 1937.

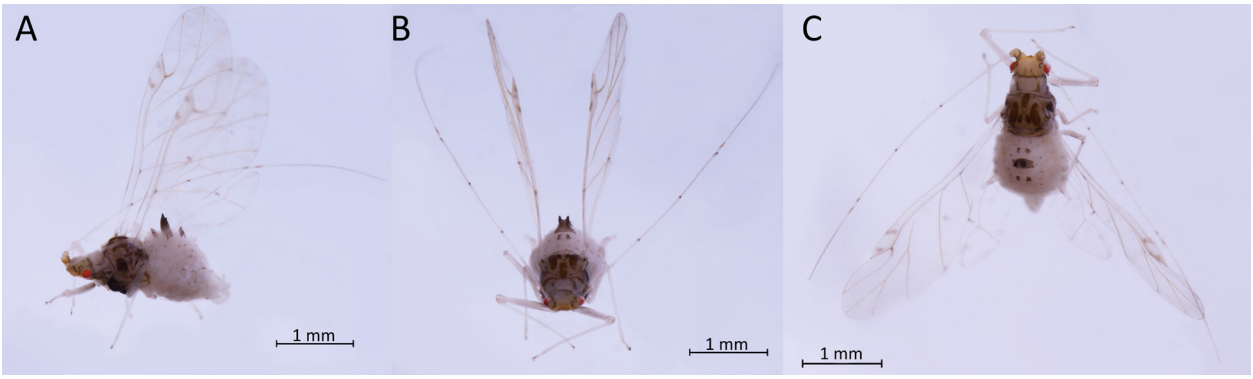

Fig 5. Fresh specimen of *Drepanaphis kanzensis* Smith, 1941. **A**. Lateral view. **B**. Frontal view. **C**. Dorsal view.

In the case of museum specimens of *D. acerifoliae*, it is difficult to measure the length of the second pair of tubercles for two reasons: (I) the tubercles of the second pair are lighter than the others, (II) the tubercles overlap. In half of the 16 specimens from museum collections measured we found it difficult to measure the second pair (Fig. 9E). When measuring fresh material, the second d.a.t. is clearly visible and an accurate measurement of 0.14 mm is possible (Fig. 9H). Good positioning of individuals on a microscope slide, including lateral position also gives similar results for tubercle measurements obtained from measurements of fresh material. Lengths of the tubercles of *D. acerifoliae* as follows: I pair 0.18–0.28 mm, II pair 0.07–0.14 mm, III pair 0.27–0.37 mm, IV pair 0.07– 0.15 mm (Table 3).

Measurements carried out on fresh *D. sabrinae* material make it possible, above all, to observe the proportions between selected tubercles. Because *D. sabrinae* is the only species in the genus *Drepanaphis* that has a second and third pair of equal length, it is necessary to carefully measure these structures. When measuring the material from museum collections, in four out of 12 individuals the second and third pairs of tubercles completely overlapped and they were impossible to measure (Fig. 9F).

Table 3. Measurements of the dorsal abdominal tubercles of *D. acerifoliae* (Thomas, 1878), *D. kanzensis* Smith, 1941 and *D. sabrinae* Miller, 1937 based on slide-mounted specimens and the fresh material.

No	species and number of individuals mounted/fresh	I pair of d.a.t		II pair of d.a.t		III pair of d.a.t		IV pair of d.a.t	
		mounted specimens	fresh specimens	mounted specimens	fresh specimens	mounted specimens	fresh specimens	mounted specimens	fresh specimens
1	*D. acerifoliae* n = 16/6	0.14–0.28	0.18–0.28	0.07–0.14	0.07–0.14	0.23–0.37	0.27–0.37	0.04–0.06	0.07–0.15
2	*D. kanzensis* n = 8/6	–	–	–	0.07–0.11	0.2–0.28	0.19–0.28	–	0.04–0.07
3	*D. sabrinae* n = 12/6	0.09–0.15	0.09–0.15	0.12–0.23	0.13–0.23	0.12–0.23	0.13–0.23	0.03–0.07	0.04–0.07

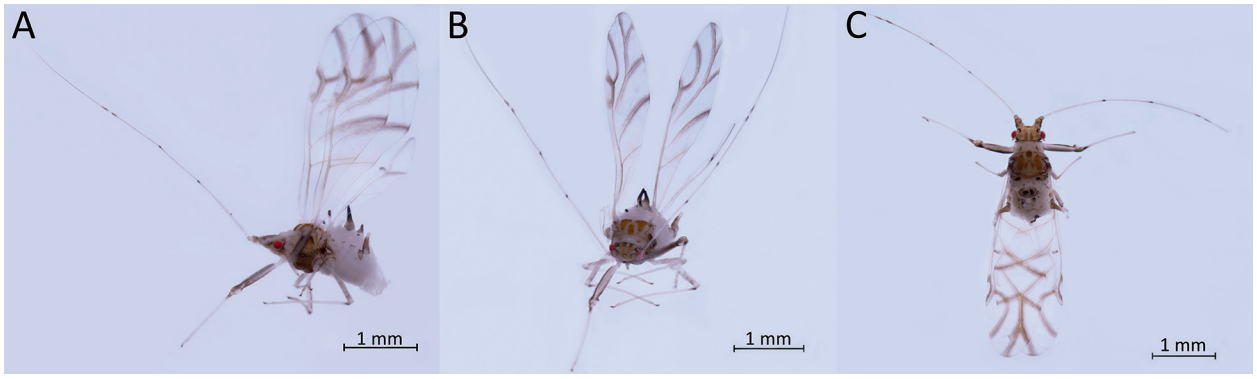

Fig 6. Fresh specimen of *Drepanaphis acerifoliae* (Thomas, 1878). **A**. Lateral view. **B**. Frontal view. **C**. Dorsal view.

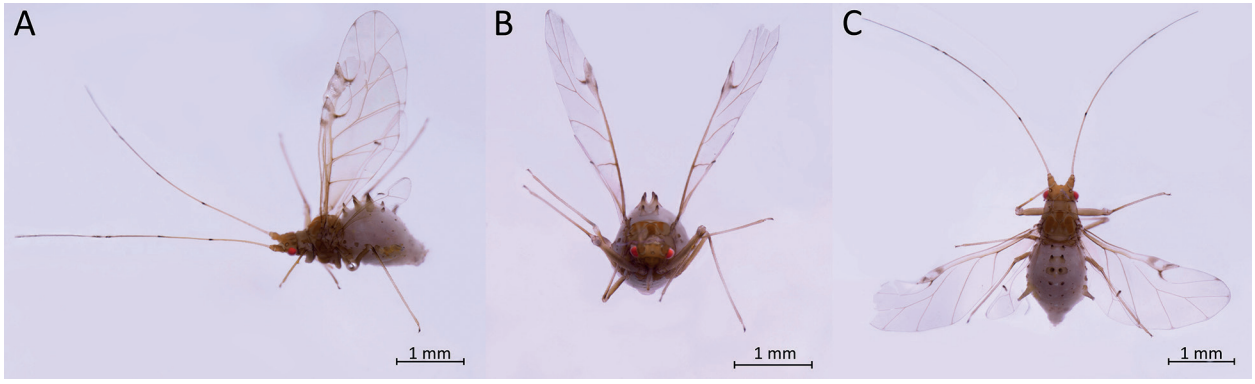

Fig 7. Fresh specimen of *Drepanaphis sabrinae* Miller, 1937. **A**. Lateral view. **B**. Frontal view. **C**. Dorsal view.

In the remaining individuals, the measurements were appropriate: I pair 0.09–0.15 mm, II pair 0.13–0.23 mm, III pair 0.13–0.23 mm, IV pair 0.04–0.07 mm (Fig. 9I). This also coincides with the dimensions of the tubercles obtained from measurements of fresh material of *D. sabrinae* (Table 3).

(3) Detailed structure of the dorsal abdominal tubercles using SEM

Verification of species using SEM techniques primarily allows for high magnification/resolution examination of the shape of the tubercles and their tips. On slide-mounted museum specimens only the general shape of the tubercles is visible. Even on the highest magnification, it is difficult to accurately identify the structures at their tips (Fig. 10A–C). Using SEM, we were able to measure the sensilla on the tips of tubercles in detail, whose size is 20–25µm. In addition, SEM images precisely illustrate the position of the tubercles on the abdomen, because the tubercles of each pair may grow on the abdomen independently or be connected at the base and branch at the tips. In *D. acerifoliae*, pairs of d.a.t. I, II and IV arise from the abdomen independently of each other. The third pair of d.a.t. is elongated and each of the tubercles branches less than half the length of the process (Fig. 10D). *D. kanzensis* also has a third pair of d.a.t. fused at the base and fork-branched at the ends (Fig. 10E). D.a.t. I and IV of *D. sabrinae* are independent and not fused basally; d.a.t. II and III are fused at the base (Fig. 10F).

(4) Mitochondrial COI as species verification at the molecular level

Our mitochondrial COI analysis further supported each of the three *Drepanaphis* species examined in this study as distinct species based on DNA sequence data. The interspecific pairwise genetic distances ranged from 7.14% to 10.55% (Table 4). These sequence divergences reflect the species differentiation sequence divergences of greater than 3% which are applicable for 96% of the Aphididae (Foottit et al. 2008).

DISCUSSION

Natural history collections are amazing resources that document the world's biodiversity in space and time (Miller et al. 2018). They are also an invaluable source for comparative research. *Drepanaphis* is an example of a genus characterized by few morphological features useful for species discrimination. Therefore, Smith and Dillery (1968) divided species described in this genus into five groups. The dorsal abdominal tubercles are a key diagnostic feature of the entire genus *Drepanaphis*. These structures are so unique that they are an import-

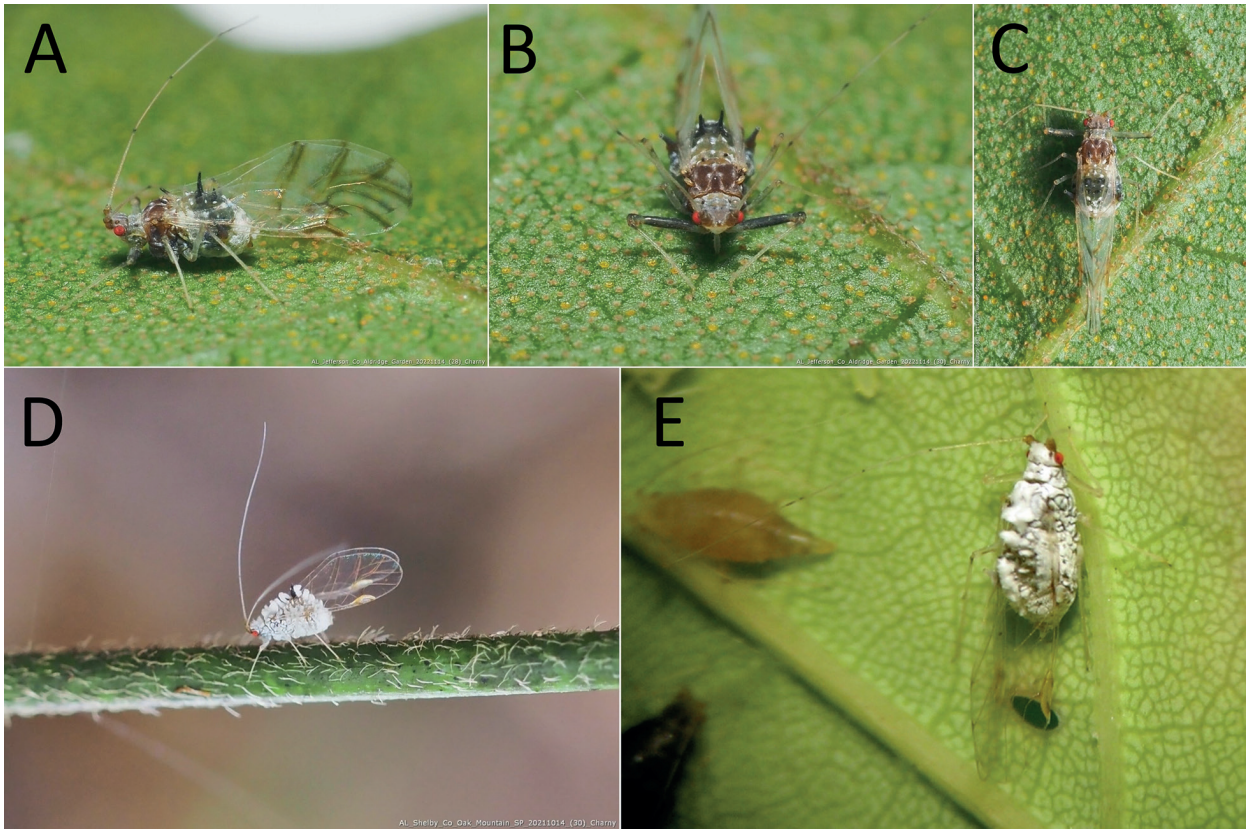

Fig 8. Live specimens. **A–C**. *Drepanaphis acerifoliae* (Thomas, 1878). **A**. Lateral view. **B**. Frontal view. **C**. Dorsal view. **D**. *Drepanaphis kanzensis* Smith, 1941, lateral view. **E**. *Drepanaphis idahoensis*, dorsal view. A–D. Image copyright V. Charny, under a Creative Commons 3.0 License.

ant starting point for identification. In this study, our data have shown that deformation of these structures can lead to misidentification of the species. In the sampled museum material from NHMUK, four slides (eight individuals) of *D. acerifoliae* were mistakenly labeled as *D. sabrinae*, and the latter confused with *D. carolinensis* Smith. *D. sabrinae* has pairs of tubercles II and III equal, whereas *D. acerifoliae* has the third pair largest. The latter arrangement occurred on these individuals which indicated wrong determinations. These three species were placed in a group named *acerifoliae*-group (Smith & Dillery 1968). Since host plants and morphometric features overlap in these species, the most important diagnostic feature is the size of the dorsal abdominal tubercles, which are usually distorted in microscope slides. In the case of species in which morphometric features fail, analysis of fresh material is required. Even though *D. kanzensis* has not been classified by Smith and Dillery (1968) to any of the species groups, some museum specimens (e.g., from NHMUK) were confused with *D. idahoensis*. In the field, *D. kanzensis* is all white (waxy) with clear wings, pale legs, and a large, black d.a.t. III. None of the other white species has clear wings with pale legs except *D. idahoensis*, which is western in its distribution in North America and has blunt rather than pointed d.a.t. setae (Smith & Dillery 1968). Correct identification of these two species, therefore, benefits from use of a combination of features resulting from macro- and microscopic observations. Preparation of lateral microscope slide mounts for *Drepanaphis* should also be used to avoid dorso-ventral distortion of abdominal tubercles. This would be espe-

Table 4. p-distance and nucleotide divergence (expressed as percentage) of studied species of the genus *Drepanaphis* Del Guercio, 1909.

	1	2	3
1 *Drepanaphis sabrinae*			
2 *Drepanaphis kanzensis*	7.14		
3 *Drepanaphis acerifoliae*	9.35	10.55	
4 *Drepanosiphum aceris*	15.69	13.83	13.28

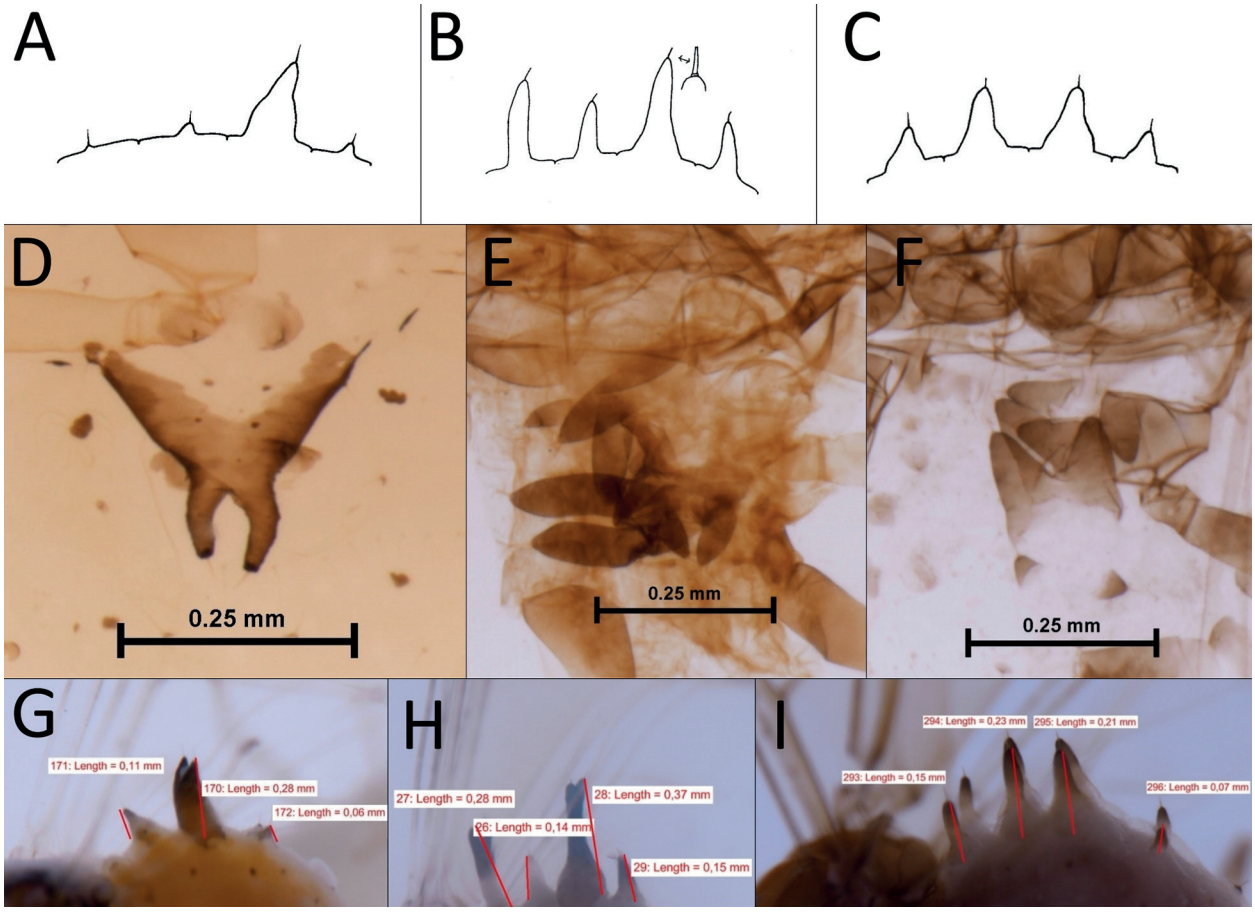

Fig 9. Dorsal abdominal tubercles. **A, D, G**. *Drepanaphis kanzensis* Smith, 1941. **B, E, H**. *Drepanaphis acerifoliae* (Thomas, 1878). **C, F, I**. *Drepanaphis sabrinae* Miller, 1937. **A–C**. Redrawn from the original description Smith & Dillery 1968. **D–F**. Slide-mounted specimens. **G–I**. Measured on the fresh specimens.

cially prudent for long series of specimens from a single sample.

Accurate morphometric measurements can also be useful in analyzing many groups of insects. Studies conducted by Seifert (2002) clearly indicate that careful measurements can distinguish even very similar species of ants. However, basic measuring errors occurring during stereomicroscope research may increase the percentage of individuals outside the range of interspecies overlap and correct identification (Seifert 2002). Comparisons of morphological characters, morphometric variables and morphometric ratios of type specimens and individuals from various localities were also successfully carried out for aphids. In particular, multivariate morphometric analyses (i.e., multiple discriminant analysis and the use of canonical variates) have demonstrated differences between closely related taxa or samples from clearly defined populations (Foottit et al. 2010; Wieczorek et al. 2017; Skvarla et al. 2020; Namgung et al. 2022). Due to the time-consuming process of taxonomic verification by morphological measurements, the interest in automatic identification of species has increased (Gaston & O'Neill 2004). For example, Yang et al. (2015) designed an identification system using insect wing outlines (DAIIS). The method provides a very high level of correct verification and a simple taxonomic tool especially for users who do not have extensive knowledge of algorithms and programing (Yang et al. 2015). However, the experiments were carried out only on individuals from the group Neuroptera and it is difficult to verify the effectiveness of the method on other winged insects. Automatic species verification is costly, which significantly limits its implementation in species classification (Gaston & O'Neill 2004). Such novel taxonomic approaches may not provide sufficient results on all groups, but it is promising in the case of insects represented only by the generation of winged viviparous fermales, as in the case of the genus *Drepanaphis*. Classical methods of species verification such a conventional light microscopy can be strengthened by detailed observations of body structures in SEM images. Evaluation of morphometric variation at the macro- and micro-morphological levels using SEM for taxonomic differentiation of closely related taxa of insects (including aphids) is increasingly used (Kanturski et al. 2015, 2018a, 2020, 2023; Mittné et al. 2022). Our SEM work with of *Drepanaphis* has resulted in morphological discernment and reveals new diagnostic features, such as the

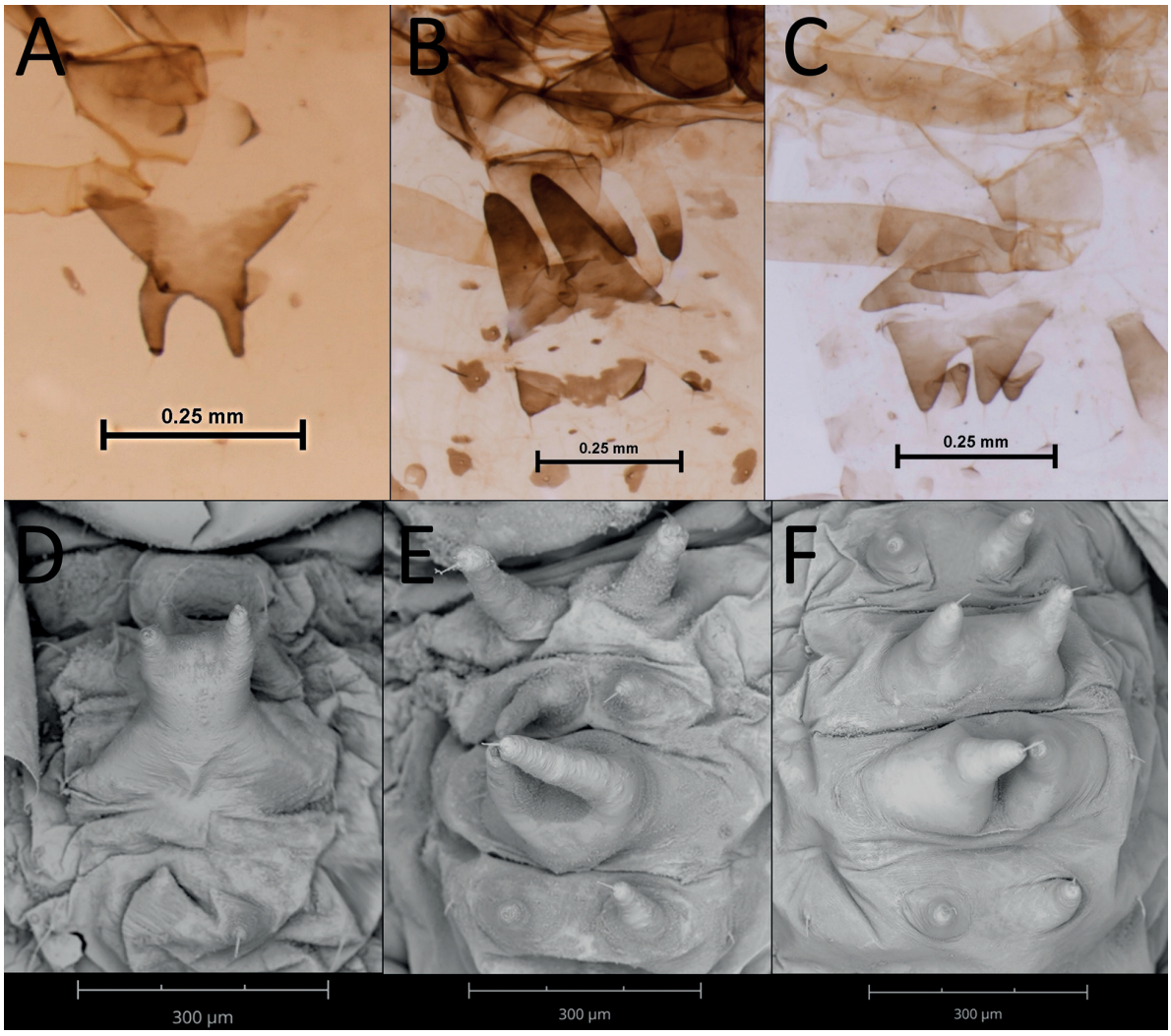

Fig 10. Dorsal abdominal tubercles. **A, D**. *Drepanaphis kanzensis* Smith, 1941. **B, E**. *Drepanaphis acerifoliae* (Thomas, 1878). **C, F**. *Drepanaphis sabrinae* Miller, 1937. **A–C**. Slide-mounted specimens in light microscopy. **D–F**. Specimens from scanning electron microscopy (SEM).

size of d.a.t sensilla and the position of each pair of the tubercles on abdomen.

The combination of precision morphological comparisons at the macro- and microscopic levels along with DNA sequence data add to our understanding of species delimitation. The most commonly used gene for species delimitation in animals is cytochrome c oxidase subunit I (COI) (Machida et al. 2017). COI species profile allows for 100% identification success even in one of the most taxonomically diverse groups of Lepidoptera, which show low sequence divergences (Hebert et al. 2003). Hebert et al. (2003) found that the distance between conspecific individuals of lepidopterans were always small, with an average intraspecific genetic distance of 0.25%. Later, the intraspecific genetic variation in other insect groups was mainly deduced from the DNA barcoding studies of various taxa (reviewed by Zhang & Bu 2022). In aphids, this effective tool was also widely used for species diagnosis, describing cryptic species or closely related species delimitation (Foottit et al. 2008; Lozier et al. 2008; Lee et al. 2017; Kanturski et al. 2018b; Théry et al. 2018; Barjadze et al. 2022; Massimino Cocuzza et al. 2022; Wieczorek & Sawka-Gądek 2023). On the other hand, genera morphologically similar or closely related and belonged to species groups known to present taxonomic difficulties (e.g., *Aphis*, *Brachycaudus*, *Dysaphis* or *Macrosiphum*) also characterize undifferentiated or overlapping barcodes, which limits of the standard COI barcode fragment for their identification (Coeur d'acier et al. 2014). COI has not been the only gene marker used for aphid DNA barcoding, other genes from the mitochondrial genome (e.g., COII, Cytb) and from endosymbionts have been used for various aphid groups (Lozier et al. 2008; Wang et al. 2011; Chen et al. 2012, 2013; Zhu et al. 2017).

Aphids are significantly economically important invasive pests throughout the world. Their correct identification is complicated by similarity among species, polymorphism, complex life cycles and host plant relationships. Relative 'size' of structures can also present some challenges that require statistical transformations for some measurements to be useful (Skavarla et al. 2020). Spectroscopic methods using the chemical composition of the body, heretofore little applied to aphids, may also be a means to achieve objective and quick identifications of an insect (Barbosa et al. 2018; Durak et al. 2022).

Species identification is essential for integrated management of pest aphids and for early detection and risk analysis of adventive species (Miller & Foottit 2017). Traditional morphological study can be inadequate with some aphid species determination. Therefore, integrated information from different sources like molecular data, morphological or biochemical characters, and ecological data can contribute substantially to effective identification of relatively homogeneous groups of species such as aphids of the genus *Drepanaphis*.

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