



HAL
open science

DNA barcoding and the Associated PhylAphidB@se Website for the identification of European Aphids (Insecta: Hemiptera: Aphididae)

Armelle Coeur d'Acier, Astrid Cruaud, Emmanuelle Artige, Guénaëlle Genson, Anne Laure Clamens, Eric Pierre, Sylvie Hudaverdian, Jean-Christophe Simon, Emmanuelle Jouselin, Jean Yves Rasplus

► To cite this version:

Armelle Coeur d'Acier, Astrid Cruaud, Emmanuelle Artige, Guénaëlle Genson, Anne Laure Clamens, et al.. DNA barcoding and the Associated PhylAphidB@se Website for the identification of European Aphids (Insecta: Hemiptera: Aphididae). PLoS ONE, 2014, 9 (6), pp.e97620. 10.1371/journal.pone.0097620 . hal-01190033

HAL Id: hal-01190033

<https://hal.science/hal-01190033>

Submitted on 1 Sep 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



DNA Barcoding and the Associated PhylAphidB@se Website for the Identification of European Aphids (Insecta: Hemiptera: Aphididae)

Armelle Coeur d'acier^{1*}, Astrid Cruaud¹, Emmanuelle Artige¹, Gwenaëlle Genson¹, Anne-Laure Clamens¹, Eric Pierre¹, Sylvie Hudaverdian², Jean-Christophe Simon², Emmanuelle Jousselin¹, Jean-Yves Rasplus¹

1 INRA, UMR 1062 CBGP (Centre de Biologie pour la Gestion des Populations), Montferrier-sur-Lez, France, **2** INRA, UMR IGEPP (Institute of Genetics, Environnement and Plant Protection), Le Rheu, France

Abstract

Aphids constitute a diverse group of plant-feeding insects and are among the most important crop pests in temperate regions. Their morphological identification is time-consuming and requires specific knowledge, training and skills that may take years to acquire. We assessed the advantages and limits of DNA barcoding with the standard *COI* barcode fragment for the identification of European aphids. We constructed a large reference dataset of barcodes from 1020 specimens belonging to 274 species and 87 genera sampled throughout Europe and set up a database-driven website allowing species identification from query sequences.

Results: In this unbiased sampling of the taxonomic diversity of European aphids, intraspecific divergence ranged from 0.0% to 3.9%, with a mean value of 0.29%, whereas mean congeneric divergence was 6.4%, ranging from 0.0% to 15%. Neighbor-joining analysis generated a tree in which most species clustered in distinct genetic units. Most of the species with undifferentiated or overlapping barcodes belonged to the genus *Aphis* or, to a lesser extent, the genera *Brachycaudus*, *Dysaphis* and *Macrosiphum*. The taxa involved were always morphologically similar or closely related and belonged to species groups known to present taxonomic difficulties.

Conclusions: These data confirm that *COI* barcoding is a useful identification tool for aphids. Barcode identification is straightforward and reliable for 80% of species, including some difficult to distinguish on the basis of morphological characters alone. Unsurprisingly, barcodes often failed to distinguish between species from groups for which classical taxonomy has also reached its limits, leading to endless revisions and discussions about species and subspecies definitions. In such cases, the development of an effective procedure for the accurate identification of aphid specimens continues to pose a difficult challenge.

Citation: Coeur d'acier A, Cruaud A, Artige E, Genson G, Clamens A-L, et al. (2014) DNA Barcoding and the Associated PhylAphidB@se Website for the Identification of European Aphids (Insecta: Hemiptera: Aphididae). PLoS ONE 9(6): e97620. doi:10.1371/journal.pone.0097620

Editor: Carlos López-Vaamonde, Institut National de la Recherche Agronomique (INRA), France

Received: December 18, 2013; **Accepted:** April 23, 2014; **Published:** June 4, 2014

Copyright: © 2014 Coeur d'acier et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was based on financial supports received from the BIOFIS project (number 1001-001) allocated by the French Agropolis Fondation (RTRA – Montpellier) to JYR, the project @-Speed-ID included in the network “Bibliothèque du Vivant” funded by the CNRS, the Muséum National d'Histoire Naturelle, the INRA and the CEA (Genoscope), the SDIPS project allocated by INRA to EJ and the ANR JJC-05 (Brachycaudus) grant awarded to EJ and ACDA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: coeur@supagro.inra.fr

Introduction

Aphids (Hemiptera: Aphididae) constitute a diverse group (about 4800 species [1]) of plant-feeding insects. They occur mostly in temperate regions and European aphids account for one third of the world's fauna, with approximately 1400 species [2]. The intricate life cycles of aphids and their close association with their host plants, polyphenism and ability to reproduce both asexually and sexually make these insects interesting systems for studying many issues in evolution and ecology [3], but they also make species identification challenging.

Furthermore, aphids are among the most serious agricultural pests of temperate regions [4]. In addition to causing direct damage by feeding on phloem, they also act as vectors of many plant viruses [5],[6]. Aphids are small insects that are often

transported around the globe, constituting an invasive threat to native and cultivated plants [2],[7]. The Aphididae is the insect family containing the largest number of invasive alien species introduced into Europe [8]. Aphids can cause very severe direct and indirect damage to crops. For example, introductions of *Aphis glycines* Matsumura, 1917, *Toxoptera citricidus* (Kirkaldy, 1907) and *Diuraphis noxia* (Kurdjumov, 1913) into North America have resulted in crop losses amounting to millions of dollars [7].

The reliable identification, to species level, of all developmental stages of aphids is critical for improvements in border controls and biomonitoring and for the success of integrated pest management strategies. However, the routine morphological identification of aphids is time-consuming and requires specific knowledge, training and skills that may take years to acquire. The accurate identification of aphids is difficult, because many species are

morphologically similar and complexes of ecologically contrasting taxa frequently occur [9],[10],[11],[12]. Morphological identification is also hampered by the high level of intraspecific variation. Indeed, the range of continuous morphological variation is probably wider in aphids than in any other insect group [13]. The presence of different morphs on different host plants and at different periods of the year further complicates species identification [1]. Finally, for several genera (e.g. *Aphis*, *Dysaphis*), identification on the basis of morphological characters alone is often impossible and a knowledge of host-plant association is required for accurate species identification [14],[15]. For the genus *Aphis*, no taxonomist has yet succeeded in writing a comprehensive dichotomous morphological key that effectively separates all the species of a local fauna [15]. In this genus, some species can be identified on the basis of one easily distinguishable morphological character, but many are grouped within morphological entities known as “species groups”. These “species groups”, which have no taxonomic validity, bring together species that are difficult to tell apart morphologically [16]. In practice, there are two ways to identify these difficult taxa: i) the use of morphological characters to identify the “species group” to which the specimen belongs, followed by the use of host-plant association criteria to define the nominal species, ii) initial identification of the host plant, followed by the checking of morphological criteria against a list of associated aphid species (if available) to identify the specimen [15],[17]. With this approach, only specimens for which an accurate host-plant association is available can be correctly identified. The morphological identification of winged morphs is reliable for only a fraction of the specimens caught in traps [15]. Furthermore, correct identification requires the taxonomist to have expertise in both entomology and botany.

The development of a reliable molecular tool based on sound taxonomic knowledge would therefore facilitate aphid identification by non-specialists (i.e. non-taxonomists) using aphids as model systems for their studies. This tool would also be useful for biomonitoring programs (such as that based on suction trap networks operating in Europe, see EXAMINE <http://www.rothamsted.ac.uk/examine>), for which the fast and accurate identification of large numbers of aphid individuals is required and in which winged morphs are captured.

DNA barcoding with the 5'-terminal fragment of the mitochondrial cytochrome C oxidase subunit I gene (*COI*) [18] has proved to be an effective standardized approach for the characterization of diverse organisms [19], including insects [20]. Most DNA barcoding-based studies in aphids have involved comparisons of small numbers of economically important species [21],[22],[23],[24]. Only a few recent studies have included relatively large numbers of aphid species. Wang *et al.* [25] focused on subtribe Aphidina, a difficult group, and two studies have demonstrated the utility of DNA barcoding for the identification of specimens from the large regional aphid fauna of North America [1],[26] and Korea [27]. However, the accuracy of DNA barcoding for the identification of European aphids has never before been assessed.

Here (i) we present the first European aphid barcode database including a large number of species (274), (ii) we discuss the usefulness, accuracy and limitations of this database for identifying European aphids and (iii) we introduce a database-driven website including taxonomic and biological data and images and allowing the identification of species through BLAST sequence comparisons with a query sequence.

Materials and Methods

Ethics Statement

No permission was required for sampling at the sites studied. This study involved no endangered or protected species.

Taxonomic Sampling

Specimens were collected between 1997 and 2008. They were killed and preserved in 70% ethanol, at 2°C. The DNA extraction process was destructive, so we selected vouchers from other specimens from the same colony (i.e. sampled on the same host plant at the same time). Voucher specimens were mounted on microscope slides and deposited in the Aphididae collection of the Center for Biology and Management of Populations (CBGP) at Montferrier-sur-Lez, France. Specimens were identified to species level by the first author. Taxonomy and nomenclature were as described by Remaudière and Remaudière [28], Nieto Nafria *et al.* [29], Eastop and Blackman [30] and Favret [31]. For nine samples, identification to species level was not possible, although the morphological characters of these specimens clearly indicated that they belonged to different species. In these cases, species names were replaced by “sp.”, followed by the sample code.

Comprehensive lists of all the specimens included in the study, with voucher numbers, sampling and taxonomic data, are provided in Supporting Information Table S1 and Table S2 and are available in the ACEA project in BOLD (<http://www.barcodinglife.org>).

DNA Extraction, Amplification and Sequencing

DNA was isolated from single individuals with the Qjagen DNeasy or ZyGem extraction kit, according to the standard protocol recommended by the manufacturer. DNA was recovered in 50 µl of purified H₂O. The cytochrome c oxidase I gene was amplified with either LepF (5'-ATTCAACCAATCATAAAGA-TATTGG-3') (forward) and LepR (5'-TAAACTTCTG-GATGTCCAAAAATCA-3') (reverse) [32] primers or with LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (forward) [33] and a degenerate reverse primer HCO2198-puc (5'-TAAACTTCWGGRTGWCCAAARAATC-3') [34] if amplification with the first primer pair failed. The 25 µl PCR mixtures contained 1 X Qjagen® enzyme buffer (containing 1.5 mM MgCl₂), 1 unit of *Taq* polymerase, 17.5 pmol of each primer, 25 nM of each dNTP and 2 µl of DNA extract. Samples were subjected to initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 30 s at 94°C, 1 minute at 48°C and 1 minute at 72°C, before a final elongation for 10 minutes at 72°C.

PCR products were purified by treatment with exonuclease I and phosphatase and sequenced directly with the Big Dye Terminator V3.1 kit (Applied Biosystems) and an ABI3730XL sequencer at Genoscope, Evry, France. Contigs were assembled from forward and reverse reads and corrected with GENEIOUS V3.7 sequence editing software [35].

The same software was used to align the sequences, and the alignment was translated into an amino-acid sequence with MEGA ve.5 software [36], which was used to detect frameshift mutations and premature stop codons potentially indicative of the presence of pseudogenes.

We tried to obtain complete sequences (658 bp) with no ambiguous nucleotides for any specimen, to establish a valuable reference database. We therefore repeated PCR and sequencing for all sequences that were incomplete or contained ambiguous base pairs. All sequences were deposited in GenBank (KF638720 to KF639739) and are also available from the PhylAphidB@se

website (<http://aphiddb.supagro.inra.fr>) and from BOLD (<http://www.barcodinglife.org>).

Data Analyses

We first evaluated the extent to which our database was representative of the known European fauna [37]. We then compared the number of haplotypes obtained with the number of specimens sequenced per species. We also constructed frequency histograms of pairwise genetic distance values at three levels: between specimens from the same species (intraspecific), between species from the same genus (congeneric) and between species from different genera (intergeneric). The distribution of pairwise distances and associated statistical values may be biased by the uneven sampling of different taxa (e.g. intensive sampling of a few species and the overrepresentation of a few haplotypes), so we repeated our analyses, taking into account a maximum of two specimens per haplotype for each species, which is equivalent to considering haplotype diversity instead of haplotype frequency.

For each species, we also plotted maximum within-species divergence (Max-WSD i.e. maximum intraspecific divergence) against minimum between-species divergence (Min-BSD, i.e. minimum interspecific divergence) to detect incidences of misleading barcode-based assignment (Max-WSD \geq Min-BSD). Pairwise nucleotide sequence divergences were calculated with a Kimura two-parameter model of base substitution [38], using the “pairwise-deletion” option. This distance is commonly used in DNA barcoding studies, making it possible to compare our results with those of many other published studies, including previous studies on aphids. The R (v.2.15.0) packages *ape* 3.0 [39] and *Spider* 1.1–2 [40] were used for all analyses and for the creation of graphical illustrations.

Finally, neighbor-joining (NJ) trees were reconstructed on the basis of the same evolutionary model, to provide a graphical representation of the phenetic distance matrix. We performed a bootstrap test of node support, with 500 replications, with MEGA version 5 [36]. Trees were edited with *TreeDyn* (v.1.98.3) software [41].

Database and Website

A database was constructed with *BioMICS* Software (www.Bio-Aware.com) [42], to manage all arthropod specimens, including aphids, hosted by the Center for the Biology and Management of Populations (CBGP, France). This database includes taxonomic and collection information, the DNA sequences available for each specimen, photographs and host-plant associations, when relevant. The *BioMICS* Net Module was used to create the *PhylAphid* database (*PhylAphid@base*, available from <http://aphiddb.supagro.inra.fr>) dedicated to aphid specimens. The pairwise sequence alignment function embedded in *BioMICS* Software is implemented in *PhylAphid@base* as an identification tool. The *Fauna Europaea* [37] and *Aphid Species Files* V.5.0 [31] were used as references for aphid species names. The ISO 3166 standard published by the International Organization for Standardization (ISO) was chosen as the reference for geographic information. ISO 3166-1 defines codes for the names of countries, dependent territories and special areas of geographical interest. ISO 3166-2 defines codes for identifying the principal subdivisions (e.g., provinces or states) of all countries represented in ISO 3166-1. Plant nomenclature was as in *The Plant List* V.1 [43].

Results

Representativeness of the Dataset

Our complete dataset included 1020 samples, from 274 species (20% of all European species), 87 genera (38.5% of the genera present in Europe) and 11 subfamilies (Table 1). All European subfamilies with more than five species were represented, with the exception of *Saltusaphidinae*. The number of species sampled per genus was significantly correlated with the number of species from the aphid genus concerned known to be present in Europe (Figure 1; $R^2 = 0.9562$, $t = 48.9198$, $df = 224$, $p\text{-value} < 0.001$). Our dataset may therefore be considered to correspond to an unbiased sample of the taxonomic diversity of European aphids. *Aphis*, the genus with the largest number of species in Europe, was slightly oversampled, but only five genera containing more than 10 European species were not represented in our dataset: *Microsiphum* (10 European species), *Xerobion* (12), *Eulachmus* (13), *Coloradoa* (21) and *Schizaphis* (27). Most specimens were sampled in France (730), Greece (148) and Italy (112). A few were collected in the United Kingdom (24) and Serbia (6) (Table S1).

After several rounds of PCR amplification and sequencing of *COI*, only four of the 1020 barcodes still contained ambiguous bases at either the 5'- (specimens ACOE1772, ACOE1982, ACOE1007) or 3'- (specimen ACOE1586) end. As these specimens were singletons, their incompleteness had little impact on the analysis and we left them in the dataset. Alignment was straightforward, due to a lack of sequence length variation and an absence of stop codons and frameshifts, suggesting that our dataset contained no NUMTs.

We obtained a mean of 3.7 barcode sequences per species, with 58% of the species represented by at least two barcodes and 40% represented by at least three barcodes (Figure 2 A, Table S3). Three species were densely sampled: *Aphis fabae* (96 specimens), *Brachycaudus helichrysi* (42) and *Aphis craccivora* (23) (Table S3).

We found 457 haplotypes among the 1020 barcodes. Some species had the same haplotype and a given haplotype could be common to two to eight species (see species with a min-BSD of 0 in Table S3). If we excluded species represented by a single specimen, the number of haplotypes per species ranged from 1 to 13 (Figure 2 B, Table S3), with a mean of 2.3 haplotypes per species. This mean number increased with the number of specimens sampled (Figure 2 C), although haplotype accumulation curves never reached the asymptote, even for the three most heavily sampled species (Figure 3). Haplotype numbers increased rapidly with the number of specimens sampled per species ($R = 0.7149$, $t = 12.7304$, $DF = 155$, $p\text{-value} = ***$), but they increased less rapidly with mean and maximum intraspecific distances ($R = 0.36$, $t = 4.78$, $df = 155$, $p\text{-value} = ***$ and 0.51 , $t = 7.45$, $df = 155$, $p\text{-value} = ***$ respectively). Thus, greater intraspecific sampling results in greater haplotype diversity but has no major effect on intraspecific genetic distances.

Intra- and Interspecific Divergences

Frequency histograms of pairwise genetic distances (Figure 4 A) showed that there were (i) no gaps between congeneric and intergeneric distances, (ii) a gap between intraspecific and intergeneric distances, (iii) a slight overlap between intraspecific and congeneric distances.

Intraspecific divergences (8205 pairwise comparisons) ranged from 0.0% to 3.9%, with a mean value of 0.29%, a median of 0.15% and a 95% confidence interval (CI) of 0.0–0.7% (Figure 4 B).

The average divergence in the 515571 interspecific comparisons was 9.5% (median = 9.2%, ranged = 0.0% to 20.7%). These

Table 1. Representativeness of the sampling analyzed in our study: Numbers of genera and species included in our dataset, known in Europe and occurring worldwide are reported for each aphid subfamily.

Subfamilies	N taxa in dataset*		N taxa in Europe		N taxa worldwide	
	Genera	Species	Genera	Species	Genera	Species
Anoeciinae	1	1	1	14	1	24
Aphidinae	51	198	124	946	337	2860
Calaphidinae	15	23	29	92	91	356
Chaitophorinae	3	14	7	62	11	178
Drepanosiphinae	1	2	3	7	5	37
Eriosomatinae	8	10	24	84	60	369
Greenideinae	1	1	1	1	16	173
Lachninae	4	19	12	98	18	397
Phyllaphidinae	1	1	2	2	2	14
Pterocommatinae	1	3	4	14	5	57
Thelaxinae	1	2	2	6	4	19
Subfamilies not sampled						
Hormaphidinae	0	0	3	5	41	197
Israelaphidinae	0	0	1	4	1	4
Lizerinae	0	0	1	1	3	34
Mindarinae	0	0	1	2	1	9
Neophyllaphidinae	0	0	1	1	1	12
Phloeomyzinae	0	0	1	1	1	2
Phyllaphidinae	0	0	2	2	2	14
Saltusaphidinae	0	0	10	33	12	71

*A full list of the materials analyzed and associated data are available in Supporting Information Table S1 and Table S2. Classification is as for Remaudière and Remaudière [28] and Nieto Nafria *et al.* [29]. European data were provided by Fauna Europea (<http://www.faunaeur.org/>) [37], and world data were provided by Footitt *et al.* [1].

doi:10.1371/journal.pone.0097620.t001

comparisons included 73473 congeneric pairwise comparisons with a mean divergence of 6.4% (median = 6.7% range = 0.0% to 15% with a 95% CI = 3.1–10.5% (Figure 4 B)). The remaining

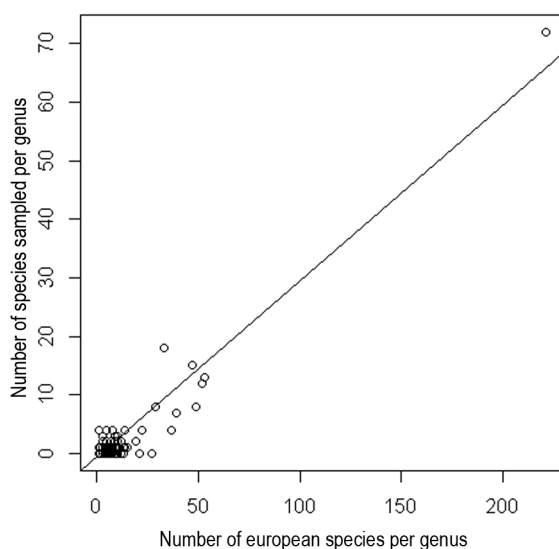


Figure 1. Taxonomic representativeness of our dataset. Linear regression of the number of species per genus sampled in this study against the number of known species per genus in Europe. doi:10.1371/journal.pone.0097620.g001

442098 intergeneric pairwise comparisons had a mean divergence of 9.8% (range = 2.6% to 20.7% with a 95% CI = 2.6 to 18.9% (Figure 4 B)). Exclusion of the outliers from the pairwise distance distribution (dots on Figure 4 B) resulted in a gap between intraspecific and interspecific (intergeneric + congeneric) genetic distances, with no overlap of their distribution curves between 0.7% and 2.6%.

If we considered a maximum of two specimens per haplotype, the mean intraspecific divergence (421 pairwise comparisons) was 0.45% (median = 0.3% with a 95% CI of 0.0–1.7% (Figure 4 C)) and the mean interspecific divergence (87594 comparisons) was 10.37% (median = 10.27% with a CI of 0.46–20.3%). The mean congeneric divergence was 6.5% (median = 6.9% with a 95% CI of 1.5–12.1% for 8756 pairwise comparisons) and the mean intergeneric divergence was 10.8% (median = 10.6% with a 95% CI of 2.6 to 19.6% for 78838 pairwise comparisons).

Following the exclusion of outlier values (dots on Figure 4 C), the intraspecific divergence distribution overlapped with the congeneric divergence distribution between 1.5% and 1.7%, whereas the gap between intraspecific and intergeneric divergence remained.

The outliers in the intraspecific divergence distribution, with exceptionally high intraspecific divergences, included nine species: *Tuberculatus annulatus*, *Myzocallis coryli*, *Brachycaudus helichrysi*, *Chaitophorus leucomelas*, *Siphia maydis*, *Lachmus roboris*, *Thelaxes suberi*, *Brachyunguis tamaricis* and *Uroleucon hypocheridis* (Figure 5). The outliers in the congeneric divergence distribution with exception-

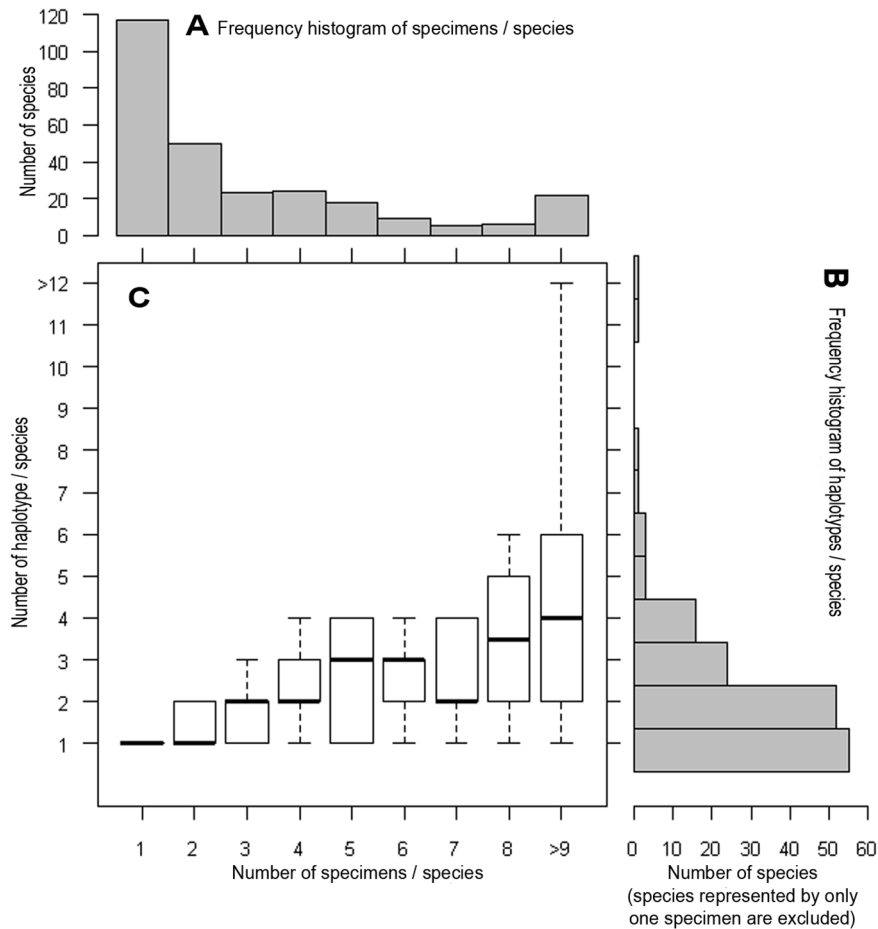


Figure 2. Intraspecific representativeness of our dataset. Frequency histograms of specimen numbers (A), number of haplotypes per species (B) and changes in the number of haplotypes with respect to the number of specimens sampled per species (C; box and whisker plot with the bottom and top of the boxes representing the 25th and 75th percentiles, respectively, bands near the middle of the boxes representing the medians and the ends of the whiskers representing the 10th and 90th percentiles). doi:10.1371/journal.pone.0097620.g002

ally low levels of interspecific divergence included 73 species (species with Min-BSD <1.5% in Table S3).

Species Identification through the Exploration of Genetic Variation within and between Species

Species assignment was correct for 85% of the 274 species included in our dataset. Species represented by a single specimen ($n = 119$) had distinct haplotypes, and those represented by more than one specimen (155) had a Max-WSD value that was smaller than min-BSD (Figure 5, Table S3). NJ analysis generated a tree (Figure S4) in which most species formed distinct genetic units; 77.8% of the species represented by several specimens were recovered as monophyletic units, 95% of which were supported by a bootstrap value (BP) >80%.

A misleading barcode-based assignment to a particular species could occur when the maximum sequence divergence among individuals belonging to one species (max-WSD) equals or exceeds the minimum sequence divergence with another species (min-BSD) (Hajibabaei *et al.*, 2006). In our dataset, this situation was encountered for 41 species (dots below the diagonal on Figure 5, species shown in bold in Table S3). Two of these species, *Brachycaudus helichrysi* and *Myzocallis coryli* (green dots in Figure 5) were previously identified as species with exceptionally high levels of intraspecific divergence. In the NJ tree (Figure 6.4), specimens

of *B. helichrysi* were segregated into two well supported clades (BP = 100) (containing 16 and 26 specimens, respectively). *B. helichrysi* was rendered paraphyletic by one specimen of *B. spiraeae* (Figure 6.4) branching with a high BP value (88) as a sister group to one of the clades. The high degree of intraspecific divergence observed for *Myzocallis coryli* (Figure 6.1) was due to a single specimen, which diverged strongly from the other representatives of the species. Species paraphyly was due to a single specimen of *Myzocallis carpini* branching within one clade of *M. coryli* with a low BP value (BP < 50).

The other 39 species, with a max-WSD \geq min-BSD (Table S3, red dots in Figure 5), had previously been identified as species with exceptionally low levels of interspecific genetic divergence and low levels of intraspecific divergence, but within the normal distribution for aphid species. Most of these species with undifferentiated or overlapping barcodes belonged to the genus *Aphis* (26 species), or, to a lesser extent, the genera *Brachycaudus* ($n = 7$), *Dysaphis* ($n = 3$) and *Macrosiphum* ($n = 3$). Twenty-eight of these species had a haplotype in common with another species (see Min-BSD value = 0 in Table S3), always from the same genus. In the NJ tree, these 39 species belonged to 14 clades encompassing a total of 50 species (Table 2) and were characterized by short internal branches, low levels of internal node resolution and, except for one species, high BP values (BP > 80) (Figure 6.2 to Figure 6.3 and

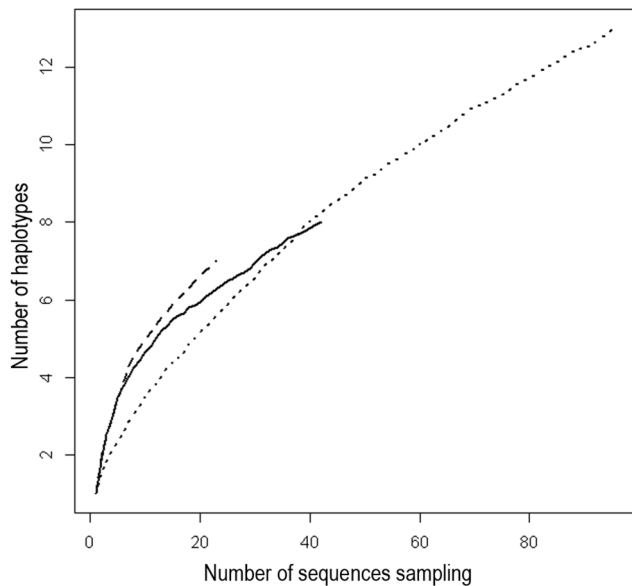


Figure 3. Haplotype accumulation curves. The curves represent the mean number of haplotypes accumulated through random permutations (subsampling of sequences) for *Aphis fabae* (dotted line), *A. craccivora* (dashed line) and *Brachycaudus helichrysi* (solid line). doi:10.1371/journal.pone.0097620.g003

Figure 6.5; Figure 7, Figure 8). The min-BSD values within each clade (mean: 0–0.69%, range: 0–1.23%) were within the range of intraspecific divergence for aphids and, for each clade (except for cluster N), these values were clearly below the min-BSD with the sister species of the clade (Table 2).

More than half these clades (8/14) consisted exclusively of species from the genus *Aphis*. The clade with the highest species richness (A) included nine species (Table 2, Figure 8.9). Eight of these species – *Aphis crepidis*, *A. confusa*, *A. leontodontis*, *A. longisrostrata*, *A. plantaginis*, *A. sedi*, *A. taraxacicola* and *A. gossypii* – had the same haplotype. The single specimen of *Aphis chloris* was nested within this clade. It did not share a haplotype with any other species of the clade, but there was less of a difference between its barcode and those of some other species of the clade (Min-BSD = 0.15%) than between *A. gossypii* haplotypes (Max-WSD = 0.61). Clade A was closely related to another *Aphis* clade including five species (cluster F, Table 2, Figure 8.9): *Aphis mamonthovae*, *A. parietariae*, *A. punicea*, *A. frangulae-like* and *A. teucii*, all with some identical barcodes in common. A similar pattern was observed in cluster D (Table 2, Figure 8.10), for *Aphis fabae*, *A. hederæ* and *A. viburni*. Two other species – *A. lambersi* and *A. newtoni* – were nested within this clade. Most specimens of each of these species formed a clade, but all included a specimen with a slightly divergent haplotype (ACOE2018 for *A. lambersi* and ACOE678 for *A. newtoni*) more closely related to *A. fabae* than to any other conspecific specimen. *Aphis galiiscabri* and *A. spiraeophaga* also included specimens with the same haplotype, clustering in clade E (Table 2, Figure 7.7). In all the other four *Aphis* clades (Table 2, Figure 7.7, 7.8 and Figure 8.9., Clade B–C, G–H), there were four polyphyletic species: *A. craccivora* (in Clade B), *A. cytisorum* (in Clade C), *A. serpylli* (in Clade G) and *A. nasturtii* (in Clade H). Monophyly of the remaining species was generally poorly supported (BP < 80%), with the exception of *Aphis intybi* (Clade B) and *A. origani* (Clade G). *Aphis intybi* had a relatively high min-BSD value for an *Aphis* species (0.61). Its placement in clade B resulted from a single specimen of

Aphis craccivora (ACOE1410) branching at its root. The two specimens of *A. origani* were nested within *A. serpylli* specimens.

Some *Brachycaudus* species also displayed very little, if any interspecific divergence. They were grouped into three clades (I, J, K, on Figure 6.2 and Figure 6.5, Table 2). Barcode sequences did not distinguish *B. cardui* from *B. lateralis* (Clade I), *B. bychnidis* from *B. populi* (Clade J) and *B. tragopogonis*, *B. schwartzi* and *B. prunicola* (Clade K) from one another.

More than half (6/11) the *Dysaphis* species included in our dataset were grouped into a single clade displaying little differentiation (Clade L Table 2, Figure 6.3). Three of these species – *D. apiifolia*, *D. crataegi* and *D. crithmi* – were represented by specimens sharing one haplotype. The single specimen of *D. angelicae* was nested within these polyphyletic species, like *D. tulipae* and *D. lauberti* specimens, although these two species formed monophyletic groups that were either highly (BP = 87 for *D. lauberti*) or poorly (BP = 63 for *D. tulipae*) supported. *Macrosiphum cerinthiacum* and *M. stellariae* could not be distinguished by barcoding, because they share a common haplotype (Clade M, Figure 7.6). Finally, the single specimen of *Macrosiphum cholodkovskiyi* was nested within the clade including all haplotypes of *M. euphorbiae*, making this species paraphyletic (Clade N, Table 2, Figure 7.6). Some haplotypes of *M. euphorbiae* diverged considerably from the others (Max-WSD = 1.23, Table S3) and the genetic distance between clade N and its closest relatives (specimens from *Macrosiphum albifrons*) remained low (min-BSD = 0.92, Table 2).

Database and Website Use

Data for the specimens included in this study have been uploaded to the PhylAphidB@se database and can be accessed online via the following website: <http://aphidb.supagro.inra.fr/>.

PhylAphidB@ase queries can be carried out easily with basic or advanced search tools. Detailed information about the records (species, specimens, geography, pictures, taxonomy, molecular data, etc.) are automatically displayed. The locations at which specimens were collected can be visualized with Google Earth maps.

The PhylAphidB@se pairwise sequence alignment tool allows users to run an algorithm similar to Blastn, to align unknown *COI* DNA sequences with the reference sequence in the database. Several pairwise alignment parameters can be modified by the user (e.g. minimum similarity, minimum overlap). The results can be presented as a list of blast hits of decreasing similarity, or as a phenetic tree (several algorithms are available e.g. UPGMA, neighbor-joining). Detailed information about the reference specimens can be obtained by clicking on their IDs either in the list of blast hits or on the leaf of the tree.

By using these online tools (Blast and/or tree reconstruction), users can assign a species name to an unknown *COI* sequence.

Discussion

DNA barcoding aims to identify species, as accurately as, and faster than a taxonomist. It requires the use of an appropriate DNA marker with an adequate rate of evolution, and the availability of a reference dataset representative of the taxonomic diversity of the group studied. We present here the first large barcoding dataset for European aphids, providing records for 1020 individuals from 274 species. We show that this dataset corresponds to an unbiased sample of the taxonomic diversity of European aphids and provides a useful tool for species identification, at least as useful as an aphid taxonomist, who would not conduct thorough and time-consuming comprehensive studies on each problematic taxon.

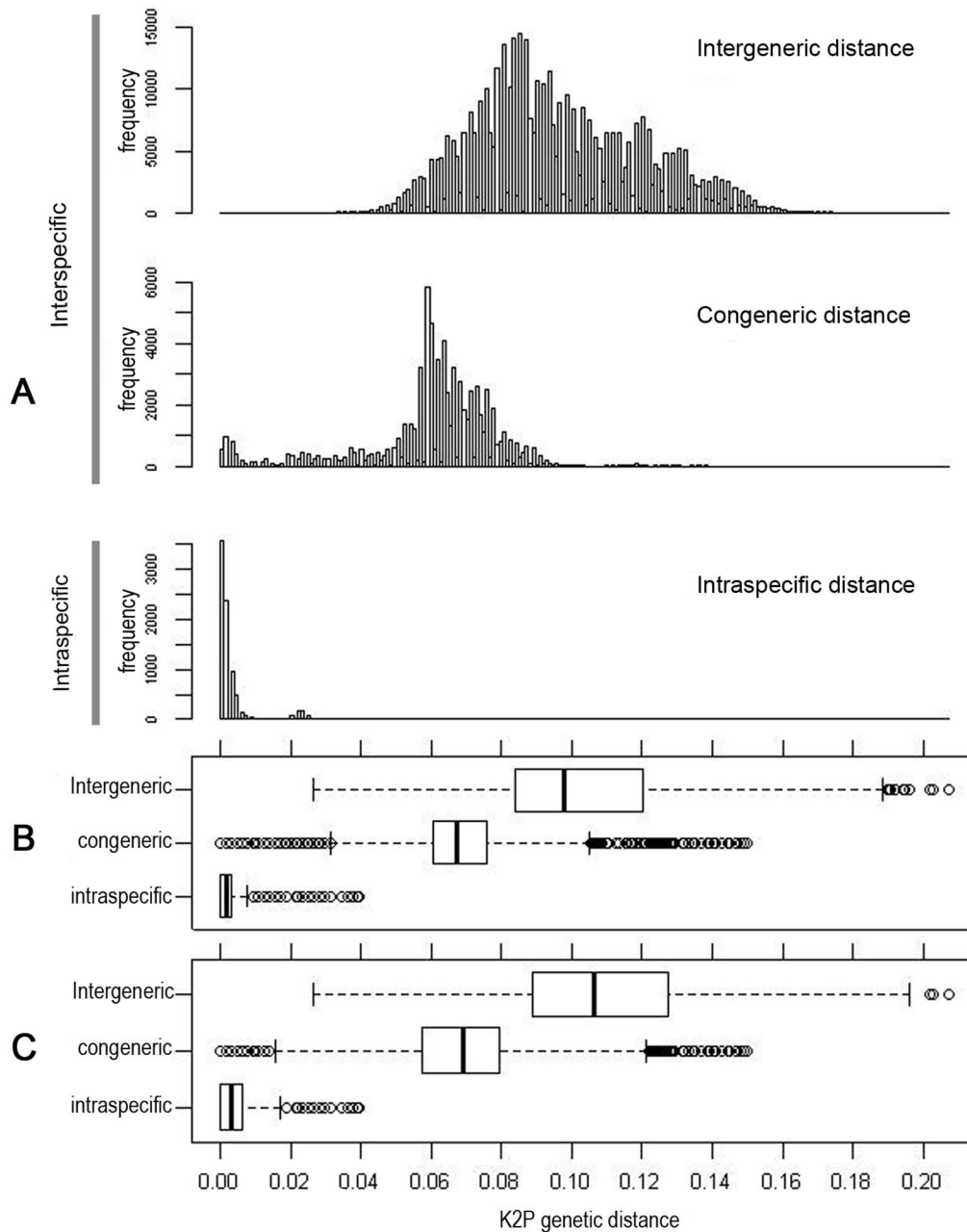


Figure 4. Distribution of pairwise K2P distances among 1020 specimens of aphids, based on COI sequences. Graphs A and B include all pairwise comparisons, graph C includes each pair of haplotypes only once. On the box and whisker plots in B and C, the bottom and top of the boxes represent the 25th and 75th percentiles, respectively, the bands near the middle of the boxes represent the median, the ends of the whiskers represent the 2.5th and 97.5th percentiles and dots represent the outliers beyond 95% of the distribution.
doi:10.1371/journal.pone.0097620.g004

COI Variation and its Use for Barcoding in Aphids

The intraspecific and interspecific COI divergences obtained for our dataset were of a similar order of magnitude to those for the North American and Korean aphid fauna [1],[27]. However, the mean intraspecific divergence (0.29%, range: 0–3.9%) was slightly higher than the values obtained for the North American (mean: 0.201%, SE 0.004) and Korean (mean: 0.05%; range: 0.00–1.00%) fauna, possibly reflecting differences in the magnitude of sampling efforts rather than differences between the fauna. Indeed, the number of specimens per species was higher in our dataset (1020 specimens/274 species; ratio: 3.72) than for the North

American (690/335; ratio: 2.06) and Korean (249/154; ratio: 1.61) datasets. This increase in intraspecific divergence with the number of specimens sampled per species has already been highlighted by several studies [44],[45], although exceptions have been reported [46]. Even for highly sampled species (96 specimens of *Aphis fabae*), haplotype accumulation curves never reached an asymptote. This is consistent with the results of Zhang *et al.* [47] for neotropical butterflies, showing that a sample size of 32 to 618 specimens per species was required to unravel most of the genetic diversity (80%) in simulated cases, and that a sample size of 9.5–216.6 was required for the actual species they were studying.

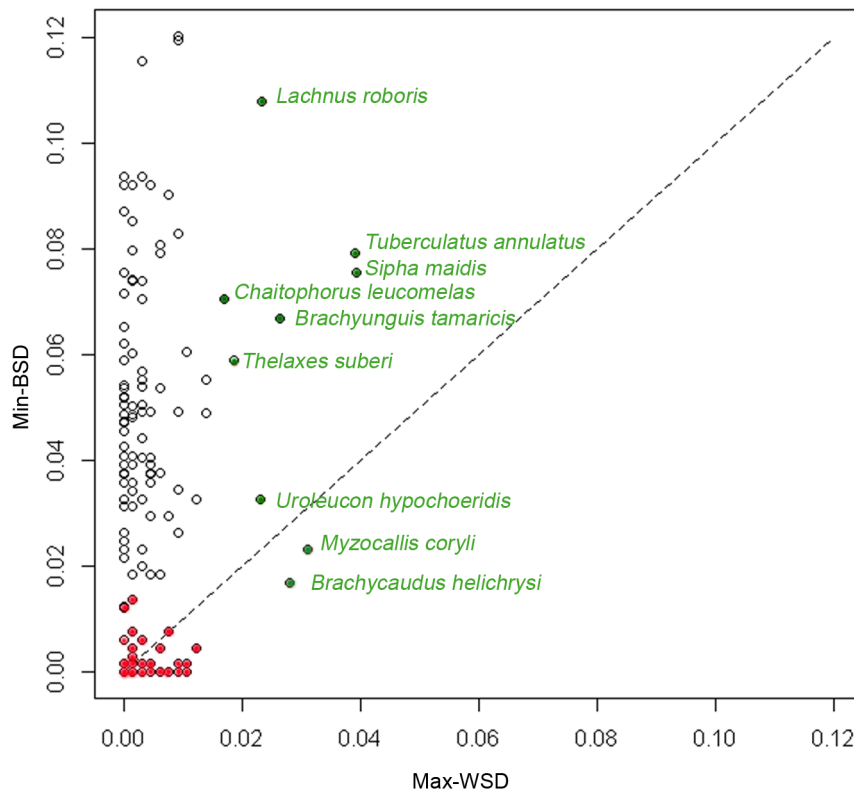


Figure 5. Patterns of *COI* divergence for 155 species represented by at least two individuals. For each nominal species, minimum between-species divergence (Min-BSD) is plotted against maximum within-species divergence (Max-WSD). Points above the diagonal correspond to cases in which species identification is straightforward. Colored dots represent nominal species detected as outliers in the species divergence distribution. Green dots represent the species with high levels of intraspecific divergence; red dots represent species with exceptionally low levels of interspecific genetic divergence. Distances are calculated with a K2P model of base substitution. doi:10.1371/journal.pone.0097620.g005

However, increasing levels of genetic diversity does not necessarily affect deeply intraspecific divergence values if haplotypes differ at only a few autapomorphic positions, as appears to be the case in our dataset. If we increased our intraspecific sampling effort, the mean intraspecific divergence would probably increase a little, but would probably remain low.

Aphids are at the lower end of the range of intraspecific divergence found in insect species (0 to 7.64%) [20]. Our values are very close to those recorded for other well studied phytophagous, species-rich groups and families, such as Hesperidae (mean intraspecific K2P divergence 0.17%), Sphingidae (0.43%) and Saturniidae (0.46%) [32].

Interspecific divergences for European congeneric species (mean 6.4%, range 0–15%) were intermediate between those for the Korean fauna (mean 5.84% range 0–14.04%) [27] and for the North American fauna (mean 7.25%, range 0.46–13.1%) [1]. These values are again at the lower end of the distribution of interspecific divergences obtained for congeneric species of insects (the means of 95% of which fall between 2.47 and 21% [20]), and approach those obtained for lepidopteran families (Hesperidae 4.58%, Sphingidae 4.41% Saturniidae 6.02%) [32].

The interspecific divergence distribution overlaps the intraspecific divergence distribution, resulting in the absence of a perfect gap between the two, making it impossible to define a species distance threshold. However, we detected an “imperfect gap” in the distribution (between 0.7% and 2.6%) in our dataset. This made it possible to define an optimal threshold minimizing assignment errors, between these values. The usefulness of this gap

is debatable, but its presence, by contrast to the continuous distribution observed for congeneric and intergeneric divergences, suggests that levels of *COI* variation can be used for species delimitation, but not for genus delimitation. This may be due to the rate of evolution of *COI* and/or the fact that species delimitations are more consistent than the definitions of genera in aphids.

Problematic Species for which Further Taxonomic Studies are Required

The high levels of intraspecific divergences displayed by some nominal species (*Brachycaudus helichrysi*, *Brachyunguis tamaricis*, *Chaitophorus leucomelas*, *Lachnus roboris*, *Myzocallis coryli*, *Siphia maydis*, *Thelaxes suberi*, *Tuberculatus annulatus*, *Uroleucon hypochoeridis*) may reflect geographical or biological history (i.e. merged phylogeographic variants or retained ancestral polymorphism) or the existence of sibling taxa that have not yet been described. Even in a group for which extensive taxonomic studies have been carried out, such as aphids [48], there are probably undescribed species and DNA barcoding, allowing the rapid detection of deep intraspecific barcode divergences, may facilitate the choice of interesting species for future taxonomic works [19]. The presence of several sibling taxa has already been suggested for some species displaying large intraspecific divergences in our study. Recent studies on *Brachycaudus helichrysi* with several mitochondrial, nuclear and *Buchnera* symbiont genes and microsatellite markers have highlighted the existence of two specific taxa that have not yet been formally described [12], [49]. The presence of several sibling

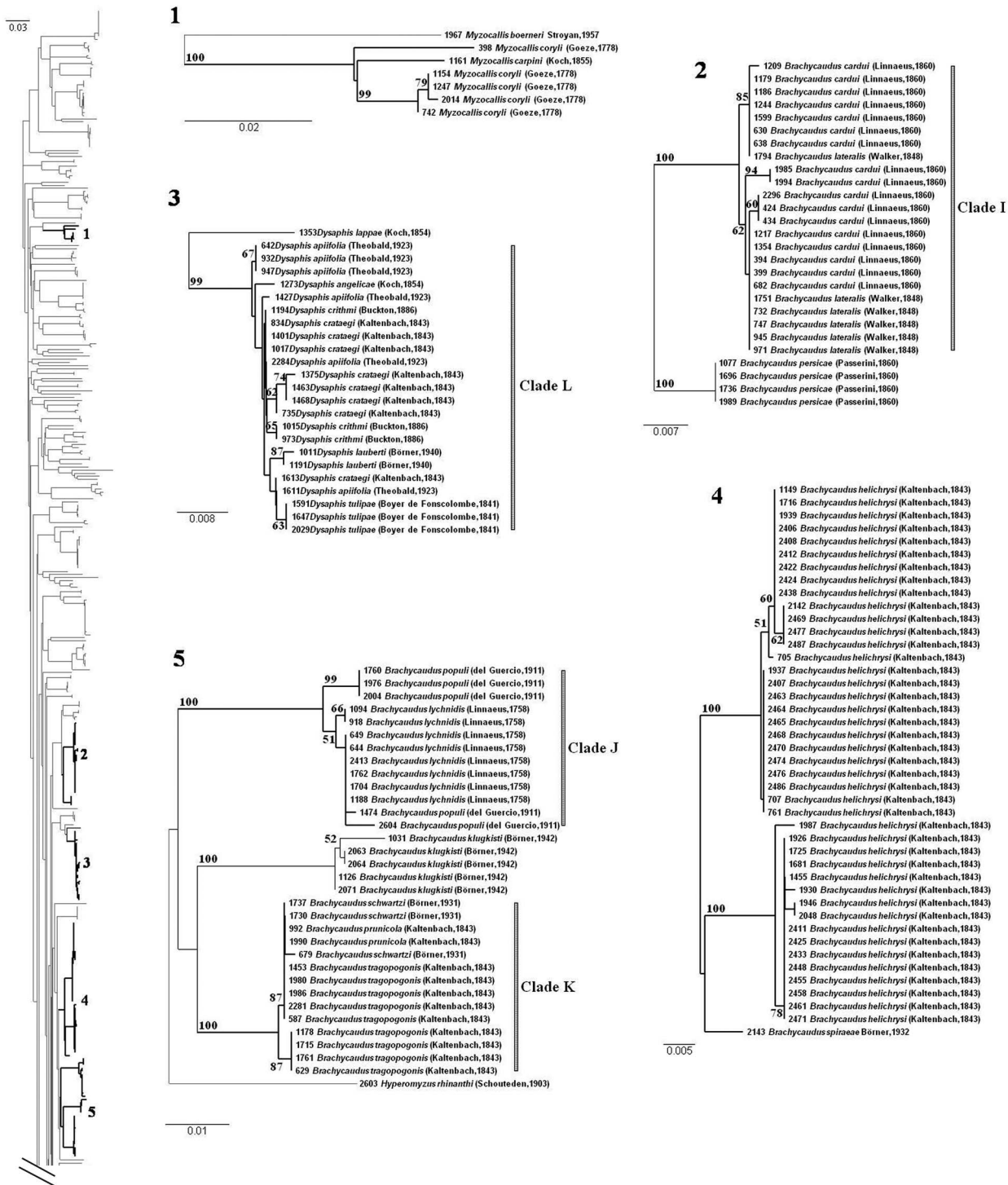


Figure 6. Focus on some problematic clades for barcode assignment. See Figure S4 for the complete NJ tree. Identification numbers of each clade are reported on the tree silhouette. Bootstrap support values >50 are provided. Note that the scale of genetic K2P divergence differs between subtrees.

doi:10.1371/journal.pone.0097620.g006

species within *Lachnus roboris* and *Chaitophorus leucomelas* has also been discussed before. Hille Ris Lambers [50] grouped together several *Lachnus* from various *Quercus* species under the name *L. roboris*, considering morphological variation to be environmentally induced. However, other authors [51], [52] have suggested that *L. roboris* may be a complex of species associated with different host

plants and with different karyotypes. One of these species, *L. iliciphilus* (del Guercio, 1909) is considered to be valid [53], although it differs from *L. roboris* mostly in terms of its size, and further confirmation is required [51], [52]. Some of our specimens may belong to this species. Indeed, in the absence of diagnostic morphological or ecological characters, we have adopted a

Table 2. Clusters of nominal species poorly discriminated by *COI* barcodes.

Clade	Species (number of specimens)	Min-BSD (%)		BP-Value	Min-BSD (%) with closest species
		Range	Mean		
A	<i>A. chloris</i> (1), <i>Aphis confusa</i> (5), <i>A. crepidis</i> (2), <i>A. gossypii</i> (15), <i>A. leontodontis</i> (1), <i>A. longiristris</i> (1), <i>A. plantaginis</i> (3), <i>A. sedi</i> (2), <i>A. taraxacicola</i> (4)	0–0.61	0.09	90	1.70
B	<i>A. coronillae</i> (5), <i>Aphis craccivora</i> (23), [<i>A. intybi</i> (4)], <i>A. tirucallis</i> (1)	0.15–1.23	0.64	97	2
C	<i>Aphis cytsorum</i> (8), <i>A. ulicis</i> (3)	0.15–0.76	0.32	100	3.61
D	<i>Aphis fabae</i> (96), <i>A. hederæ</i> (7) <i>A. lamberti</i> (4), <i>A. newtoni</i> (5), <i>A. viburni</i> (2)	0–1.07	0.27	77	1.23
E	<i>Aphis galiiscabri</i> (2), <i>Aphis spiraeophaga</i> (1)	0–0.15	0.1	100	1.38
F	<i>Aphis mamonthovae</i> (2), <i>A. parietariae</i> (1), <i>A. punicae</i> (2), <i>A. frangulae-like</i> (8), <i>A. teucrii</i> (1)	0–0.46	0.11	82	1.23
G	<i>Aphis serpylli</i> (4), [<i>A. origani</i> (2)]	0.15–1.07	0.61	98	1.70
H	<i>Aphis althaeae</i> (1), <i>A. nasturtii</i> (2), <i>A. sp.rostellum-like</i> (4), <i>A. umbrella</i> (3)	0.15–0.76	0.41	100	5.2
I	<i>Brachycaudus cardui</i> (17), <i>B. lateralis</i> (6)	0–0.77	0.22	100	3.6
J	<i>Brachycaudus lychnidis</i> (8), <i>B. populi</i> (5)	0.15–0.92	0.69	100	4
K	<i>Brachycaudus prunicola</i> (2), <i>B.tragopogonis</i> (9), <i>B. schwartzi</i> (3)	0–0.46	0.15	100	2.96
L	<i>Dysaphis angelicae</i> (1), <i>D. apiifolia</i> (6), <i>D. crataegi</i> (8), <i>D. crithmi</i> (3), [<i>D. lauberti</i> (2)], <i>D. tulipae</i> (3)	0–1.07	0.41	99	2.64
M	<i>Macrosiphum cerinthiacum</i> (1), <i>M. stellariae</i> (5)	0–0	0	100	0.92
N	<i>Macrosiphum euphorbiae</i> (18), <i>M. cholodkovskyi</i> (1)	0.46–0.92	0.65	71	0.92

Means and ranges of genetic distances between the species included in each cluster (BSD) and between the cluster and its closest relative are reported. Bootstrap support (BP) values for each cluster are given. Square brackets indicate monophyletic species in the NJ tree.
doi:10.1371/journal.pone.0097620.t002

“lumping” approach, grouping our specimens together under the name *L. roboris*. *Chaetophorus leucomelas* is a species with a large geographic distribution that presents different numbers of chromosomes according to its origin. This suggests that there may be sibling species within this taxon [51], [52]. Our results confirm that further investigations, including morphological and genetic studies, are required for these species. However, if we exclude *Brachycaudus helichrysi* and *Myzocallis coryli*, both of which are paraphyletic on our NJ trees, the use of DNA barcodes leads to the correct assignment of query sequences to current species names.

Disentangling Species Groups in Aphid: Barcoding and Morphology are Subject to the Same Limitations

Overall, 77.8% of the species represented by multiple specimens clustered into distinct clades on the NJ trees for *COI*. These clades were separated from their nearest neighbors, indicating that specimen assignment to species by DNA barcoding should be correct. About 19% of the nominal species appeared to be polyphyletic or paraphyletic with respect to other recognized species (including *B. helichrysi* and *M. coryli*, as previously discussed). Situations in which the distances between congeneric species are extremely small are problematic. We detected 14 polyphyletic clades of nominal species poorly discriminated by *COI* barcodes. These clades included a total of 50 aphid species belonging to four

(*Aphis*, *Dysaphis*, *Brachycaudus*, and *Macrosiphum*) of the 87 genera represented in our dataset. These genera are known to contain taxonomically problematic species groups formed by species that are morphologically very similar. A detailed taxonomic discussion of each of these species groups and the match between taxonomic divisions and our DNA barcoding data is provided in the supplementary material (Text S1). In summary, eight of the 14 problematic clades cluster specimens from the genus *Aphis* and almost half the *Aphis* species appear to be problematic for identification to the species level by barcoding. This is not surprising given the findings of taxonomic studies on *Aphis*. *Aphis* is the largest aphid genus [54] and contains several of the most damaging aphid pests. It is also the genus most recalcitrant to any comprehensive taxonomic treatment [15]. Most species can easily be classified into species groups forming morphologically well-defined entities, but many of the species within these groups are difficult to tell apart morphologically and identification keys remain ambiguous and are mostly based on host-plant associations [55]. In a few cases, DNA barcode sequences are useful for differentiating between species that are often confused because of their morphological similarities, such as *Aphis pomi* and *A. spiraeicola*. Our findings confirm previous reports [21],[56] that specimens from these two distinct clades are separated from each other by considerable *COI* gene divergence. However, barcodes mostly display the same limitations as morphological characters and



Figure 7. Focus on some problematic clades for barcode assignment (following on from Figure 6). See Figure S1 for the complete NJ tree. The identification numbers of each clade are reported on the tree silhouette. Bootstrap support values >50 are indicated. The scale of genetic K2P divergence differs between subtrees.
doi:10.1371/journal.pone.0097620.g007

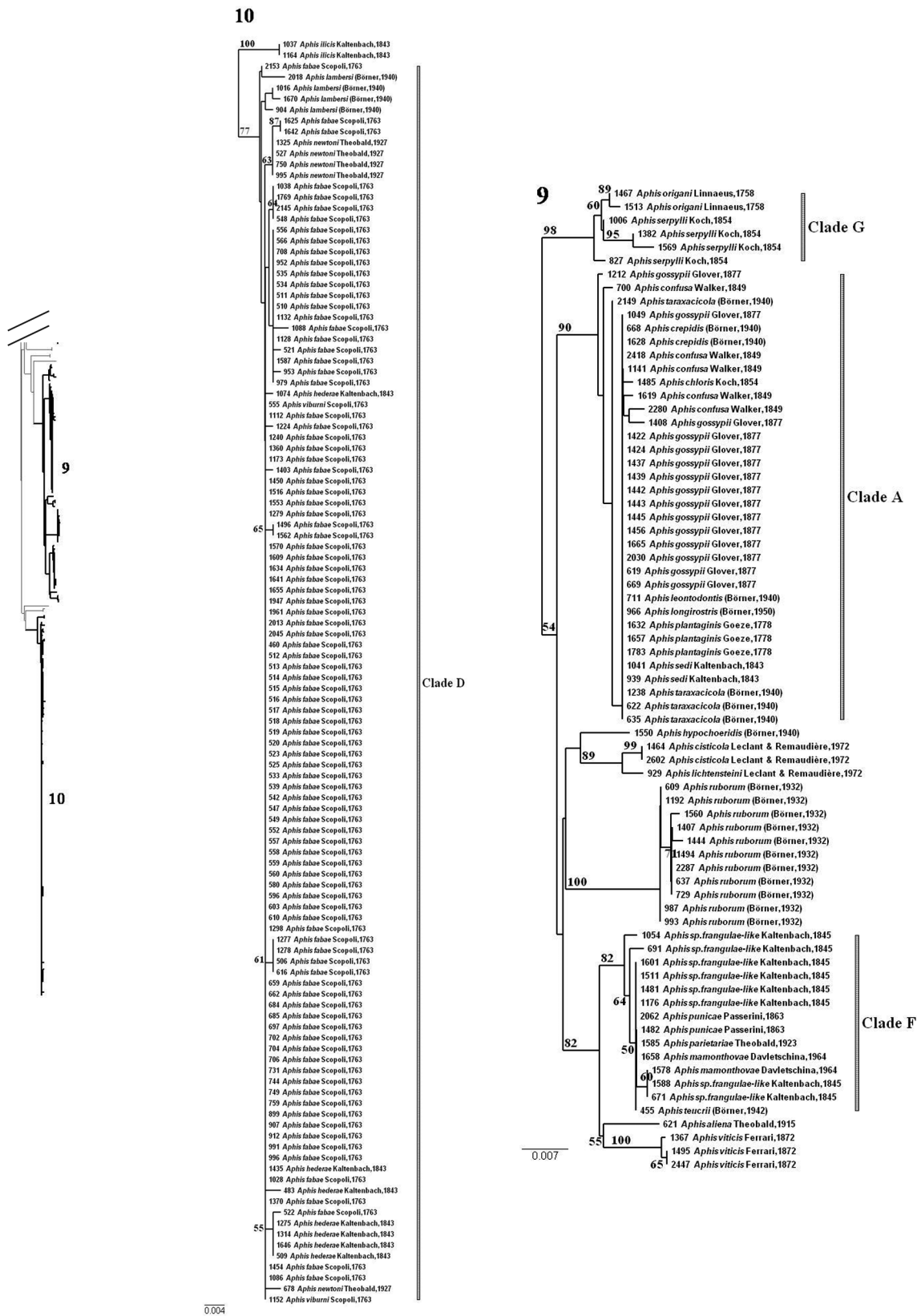


Figure 8. Focus on some problematic clades for barcode assignment (following on from Figure 7). See Figure S1 for the complete NJ tree. The identification numbers of each clade are reported on the tree silhouette. Bootstrap support values >50 are indicated. The scale of genetic K2P divergence differs between subtrees. doi:10.1371/journal.pone.0097620.g008

cannot differentiate between species that are difficult to identify by traditional approaches. Four major morphological *Aphis* species groups have been reported in recent European studies: [14],[15] *frangulae*-like, black backed aphid (= *A. craccivora* group), black aphid (= *A. fabae* group) and *nasturtii*-like aphids. Most of the specimens from the species belonging to one of these groups were recovered in one of the problematic clades highlighted in our study. The genus *Brachycaudus* has been the subject of recent molecular phylogenetic studies [57],[58], based on several genes, including the *COI* barcode fragment. Our results confirm the taxonomic issues identified in these papers. Three clades of poorly discriminated *Brachycaudus* species are found, each displaying some haplotype diversity. However, the observed structure does not match morphological species delineation. In the supplementary material (Text S1), we present a short historical review highlighting the difficulty, within each of these species groups, encountered in the delimitation of taxa, specification of their taxonomic rank and the description of their biological features. The *Dysaphis* (*Dysaphis*) subgenus is traditionally divided into several clearly defined species groups, together with a number of isolated species of uncertain taxonomic position [55]. Only one of these groups, the *D. crataegi* group, is represented by several species in our dataset. Unsurprisingly, all these species were grouped together to form a clade with species displaying an overlapping barcode. This group has been studied in detail in Western Europe, first by Börner [59] and then by Stroyan [55],[60],[61]. It remains a matter of debate whether these taxa should be treated as species or subspecies of *D. crataegi*: this classification is somewhat arbitrary, as it is not based on valid biological criteria [51]. The last genus containing poorly discriminated species is *Macrosiphum*. Four of the seven species present in our sample form two pairs of species, *M. cerinthiacum/stellariae* and *M. cholodkovskiy/euphorbiae*. With the exception of the little studied *M. cerinthiacum*, these species have been recognized as belonging to the morphologically similar *M. euphorbiae* species group [62],[63].

There are several possible explanations for the overlapping barcodes in the 14 clades. First, some of these clades may represent recently diverged taxa. These may be relatively young species in which the *COI* sequence has not yet accumulated mutations. These aphid species groups may have undergone recent adaptive radiation [64]. Two evolutionary scenarios have often been put forward. In the first, an ancestral polyphagous species is thought to have colonized herbaceous plants during their diversification, leading to rapid and extensive speciation through a gradual restriction of host range [65],[66] (but see [67] for an alternative scenario in *Brachycaudus*). This rapid diversification is probably still underway [68], potentially accounting for the homogeneity of these species groups. A non-exclusive second scenario would involve recent speciation through host shifts, with populations colonizing a new plant species and diverging from their population of origin [69]. Such cases of recent speciation accompanied by very small number of *COI* mutations and/or incomplete lineage sorting represent the ultimate limit for barcoding, as they result in non-monophyletic clades. In such cases, it has generally been suggested that more extensive sequence data would improve resolution [46]. Other genes have been tested for aphid barcoding or phylogeny [70],[71]. The use of more variable DNA fragments from the endosymbiotic bacterium *Buchnera aphidicola* currently seems to be a promising way to resolve the problematic cases encountered with *COI* barcoding [72]. However, within *Brachycaudus*, the use of highly variable *Buchnera* DNA fragments has been shown to result in the same conclusions for species delimitation as *COI* barcoding [58]. Even more variable markers, such as those used for population studies, including microsatellites, might be

useful for studying relationships between taxa within these species groups [73]. However, they are too variable and too specific for use as a routine identification tool.

Alternatively, the lack of correspondence between sequence variants and existing Linnean binomials may reflect failings of the procedures used for species delimitation in traditional taxonomy or an inconsistent application of the species concept [45],[74]. Imperfect taxonomy can cause non-monophyly when different morphotypes or ecotypes are inappropriately recognized as species. The species concept in aphids has been the subject of considerable debate [62],[75],[76],[77],[78]. Information about life cycle, host specificity and morphology are essential for the delimitation of aphid species [75]. Host plant association is a major driver of reproductive isolation and speciation in aphids [9],[62],[79],[80],[81],[82]. The ecological species concept has thus been intensively used in some species-rich genera, such as *Aphis* and *Dysaphis*. Due to the considerable overlap in morphological characters, all attempts to correlate morphology and host-plant association in the black aphid species group [15] have been unsuccessful. Multivariate morphometric methods have facilitated morphological separation in some cases (e.g. the *Brachycaudus punicola* species group [83] or the *D. crataegi* species group [60]). However, Shaposhnikov [84] reported that within a single clone of *Dysaphis foeniculus* (Theobald), the allometry of some parts of the aphid body may change in response to different host-plant associations. This led him to conclude that new species have probably been described erroneously. Intensive host-plant transfers have also been conducted in the *Aphis* [85],[86],[87],[88],[89], *Dysaphis* [90],[91],[92], *Macrosiphum* [63],[93],[94] and *Brachycaudus* [95],[96] species groups. Conflicting results have been obtained between different investigations, suggesting that host plant associations may be inconsistent over both time and space. These inconsistencies are probably intrinsic features of the structure of these species complexes, rather than reflecting experimental shortcomings [15].

DNA barcode database users must accept that species definitions are established on the basis of traditional taxonomy, which may be imperfect. It must, therefore, be borne in mind that many formal species are not monophyletic. In addition, due to morphological homogeneity, incorrect species identification may occur more frequently in some species, contributing to the high frequency of polyphyletic species. Misidentifications with the use of GenBank as a barcode database have been reported [97], but the rate of misidentification in the construction of barcode databases has never been evaluated. The use of barcode databases built in collaboration with a taxonomist decreases the risk of misidentification, although mistakes may still occur, particularly for challenging taxonomical groups. All these factors increase the error rates for barcode-based identification and it is thus the traditional way of delimiting and describing species that requires re-evaluation. In this context, trying to identify the perfect gene for barcoding may be pointless. Furthermore, even if aphid taxonomists are, by necessity, also “amateur” botanists, they are not specialists in plant systematics. In situations in which the identification of the aphid is dependent on correct host-plant identification, the frequency of misidentification may be increased further. Even the most recognized aphid taxonomists acknowledge that there has been confusion between species in the past (e.g. [98]). Taxonomists can make identification mistakes that can be traced back with voucher specimens. In some cases, the lack of morphological characters for diagnosis make aphid vouchers useless for future identification. We therefore suggest the establishment of a host-plant herbarium linked to the aphid

voucher specimens, to allow the checking of aphid species identification, when issues are highlighted.

Finally, species may share haplotypes due to mitochondrial introgression. These species may lie in the indeterminate zone between differentiated populations and distinct species [99] or formed species that are losing their genetic identity due to secondary contact and hybridization. Most of the aphid species sharing *COI* barcodes hybridize at least occasionally and can produce fertile hybrid offspring. This has been demonstrated experimentally for black aphids [86],[88],[100],[101], *frangulae*-like aphids [102], the *Brachycaudus prunicola* species group [95], the *Dysaphis devectora* species group [103] and the *Macrosiphum euphorbiae* group [63],[104]. These hybrids, which were obtained experimentally, are frequently considered not to occur in natural conditions due to prezygotic (mating on different host plants, phenology shifts etc.) or postzygotic (hybrid sterility, hybrid weakness or F2 breakdown) barriers [62]. However, the co-existence of potential “parental” taxa on shared host plants may help to remove some of these constraints in natural conditions [15]. Natural hybridization may break down isolation and delay the divergence of species within aphid species groups [60].

Conclusion

Our study contributes to the assembly of a DNA barcode library for the world aphid fauna. The addition of our dataset to those from North America [1] and Korea [27] results in the coverage of only 15% of the described species with published barcodes, this percentage being only slightly increased by the inclusion of recent taxonomic studies (i.e. [25],[105]). More efforts are therefore required for the barcoding of this group of economically important families and model systems for evolutionary biologists.

The geographic scale of the available samples and the relatively well known taxonomy of this group of insects make aphids ideal for the testing of several issues relating to DNA barcoding, such as the impact of geography or taxon coverage on the accuracy of species assignment.

The data presented here confirm that *COI* barcodes are a potentially useful tool for aphid identification. This approach simplifies identification for 80% of the species, including some species that are difficult to identify on the basis of morphological characters only. However, our work also highlights identification difficulties in *Aphis*, *Brachycaudus*, *Dysaphis* and *Macrosiphum*, genera including a large number of pest species. This may be the stumbling block for the actual use of the aphid barcoding tool, particularly in agricultural management programs, which are likely to be the principal users of this tool. These problematic groups of species have been studied by taxonomists for a very long time. Barcoding cannot replace a comprehensive taxonomic analysis. Detailed genetic, morphological and ecological investigations are required to define species boundaries, and this is the job of taxonomists. However, systematics studies take much longer

References

1. Footit RG, Maw HEL, von Dohlen CD, Hebert PDN (2008) Species identification of aphids (Insecta: Hemiptera: Aphididae) through DNA barcodes. *Molecular Ecology Resources* 8: 1189–1201.
2. Coeur d'acier A, Pérez-Hidalgo N, Petrovic-Obradovic O (2010) Aphids (Hemiptera, Aphididae). In: Roques A, Kenis M, Lees D, Lopez-Vaamonde C, Rabitsch W et al., editors. *Alien terrestrial arthropods of Europe: Biorisk*. 435–474.
3. Dixon AFG (1998) *Aphid ecology*. Glasgow & London United Kingdom: Blackie and Son Ltd. 300 p.
4. Minks AK, Harrewijn P (1987) *Aphids: Their biology, natural enemies and control*. Amsterdam (Netherlands): Elsevier. 450 p.

than barcoding [106]. Such long-term work is incompatible with the urgency of societal demands for a powerful, user-friendly identification tool. One possible pragmatic solution would be to mimic the procedure used by aphid systematics specialists: assigning specimens to a group of species, returning their names with information about the host plants of the different nominal species included in the group, and then allowing the user of the system to identify the specimen on the basis of the available information.

This procedure, together with the different assignment methods, will require evaluation in future studies before the use of aphid barcoding databases as accurate identification tools for applications in pest management and plant quarantine.

Supporting Information

Figure S1 Neighbor-joining tree. Neighbor-joining tree (K2P model) obtained from the analysis of the 1020 *COI* sequences from the 274 species of European aphid included in the study. Bootstrap values >50 are indicated at nodes. (PDF)

Table S1 Data collection. Data collected for the 1020 specimens used in the study. (DOCX)

Table S2 Identification and taxonomic data. Identification and taxonomic data for the 1020 specimens used in the study. (DOCX)

Table S3 List of species included in the study. List of species included in the study, with a summary of the results obtained in barcoding analysis. (DOCX)

Text S1 Detailed discussion on aphid species groups. Detailed discussion and references for each aphid species groups encountered in the study and the match to our DNA barcoding data. (DOCX)

Acknowledgments

We thank all the aphid collectors for their assistance. We also thank two anonymous reviewers for helpful criticisms and suggestions on earlier versions of this manuscript and Alex Edelman Translation services for proof reading the paper. Many thank to Vincent Robert for his work and for solving the problems encountered in using the BLAST tools.

Author Contributions

Conceived and designed the experiments: ACd. Performed the experiments: ACd GG ALC SH. Analyzed the data: ACd. Contributed reagents/materials/analysis tools: AC EA EP. Wrote the paper: ACd AC JCS EJ JYR.

9. Carletto J, Blin A, Vanlerberghe-Masutti F (2009) DNA-based discrimination between the sibling species *Aphis gossypii* Glover and *Aphis frangulae* Kaltenbach. *Systematic Entomology* 34: 307–314.
10. Peccoud J, Ollivier A, Planegenest M, Simon JC (2009) A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. *Proceedings of the National Academy of Sciences* 106: 7495–7500.
11. Peccoud J, Simon J-C, von Dohlen C, Coeur d'acier A, Planegenest M, et al. (2010) Evolutionary history of aphid-plant associations and their role in aphid diversification - Histoire évolutive des associations plantes-pucerons et leur rôle dans la diversification des pucerons. *Comptes Rendus Biologies* 333: 474–487.
12. Piffaretti J, Rossi J-P, Vanlerberghe-Masutti F, Genson G, Coeur d'acier A, et al. (2013) Molecular identification and ecological characteristics of two cryptic lineages within a cosmopolitan aphid pest, *Brachycaudus helichrysi* (Hemiptera: Aphididae). *Applied Entomology and Zoology* 48: 155–164.
13. Blackman RL, Eastop VF (2000) *Aphids on the World's Crops - An identification and information guide*. Chichester: John Wiley & Sons, Ltd. 466 p.
14. Heie OE (1986) The Aphidoidea (Hemiptera) of Fennoscandia and Denmark. III. Family Aphididae: subfamily Pterocommatinae & tribe Aphidini of subfamily Aphidinae. *Fauna Entomologica Scandinavica* 17: 1–314.
15. Stroyan HLG (1984) *Aphids- Pterocommatinae and Aphidinae (Aphidini) Homoptera, Aphididae*. London: The Royal Entomological Society of London. 232 p.
16. Coeur d'acier A, Joussetin E, Martin J-F, Raplus J-Y (2007) Phylogeny of the genus *Aphis* Linnaeus, 1758 (Homoptera: Aphididae) inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 42: 598–611.
17. Nieto Nafria JM, Mier Durante MP, Garcia Prieto F, Perez Hidalgo N (2005) *Hemiptera Aphididae III*. Madrid: Museo Nacional de Ciencias Naturales, CSIC. 362 p.
18. Herbert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* 270: 313–322.
19. Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics* 23: 167–172.
20. Virgilio M, Backeljau T, Nevado B, De Meyer M (2010) Comparative performances of DNA barcoding across insect orders. *BMC Bioinformatics* 11: 1–10.
21. Footitt RG, Lowery DT, Maw HEL, Smirle MJ, Lushai G (2009) Identification, distribution, and molecular characterization of the apple aphids *Aphis pomi* and *Aphis spiraeola* (Hemiptera: Aphididae: Aphidinae). *Canadian Entomologist* 141: 478–495.
22. Kim H, Hoelmer KA, Lee W, Kwon YD, Lee S (2010) Molecular and morphological identification of the soybean aphid and other *Aphis* species on the primary host *Rhamnus davurica* in Asia. *Annals of the Entomological Society of America* 103: 532–543.
23. Shufran KA, Puterka GJ (2011) DNA barcoding to identify all life stages of holoecyclic cereal aphids (Hemiptera: Aphididae) on wheat and other Poaceae. *Annals of the Entomological Society of America* 104: 39–42.
24. Wang JF, Qiao GX (2009) DNA barcoding of genus *Toxoptera* Koch (Hemiptera: Aphididae): Identification and molecular phylogeny inferred from mitochondrial COI sequences. *Insect Science* 16: 475–484.
25. Wang JF, Jiang LY, Qia GX (2011) Use of mitochondrial COI sequence to identify species of the subtribe Aphidina. *ZooKeys* 122: 1–17.
26. Footitt RG, Maw E, Pike KS (2009) DNA barcodes to explore diversity in aphids (Hemiptera, Aphididae and Adelgidae). *Redia* 92: 87–91.
27. Lee W, Kim H, Lim J, Choi HR, Kim Y, et al. (2011) Barcoding aphids (Hemiptera: Aphididae) of the Korean Peninsula: updating the global data set. *Molecular Ecology Resources* 11: 32–37.
28. Remaudière G, Remaudière M (1997) *Catalogue des Aphididae du monde - Catalogue of the world's Aphididae [Homoptera, Aphidoidea]*. Paris: INRA 376 p.
29. Nieto Nafria JM, Mier Durante MP, Remaudière G (1998) Les noms des taxa du groupe famille chez les Aphididae (Hemiptera). *Revue Française d'Entomologie* 19: 77–92.
30. Eastop VF, Blackman RL (2005) Some new synonyms in Aphididae [Homoptera: Sternorrhyncha]. *Zootaxa* 1089: 1–36.
31. Favret C (2013) *Aphid Species File*. Version 5.0/5.0. Available: <http://Aphid.SpeciesFile.org>. Accessed 2013 April 18.
32. Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences* 103: 968–971.
33. 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. *Molecular Marine Biology and Biotechnology* 3: 294–297.
34. Cruaud A, Jabbour-Zahab R, Genson G, Cruaud C, Couloux A, et al. (2010) Laying the foundations for a new classification of Agaonidae (Hymenoptera: Chalcidoidea), a multilocus phylogenetic approach. *Cladistics* 26: 359–387.
35. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, et al. (2010) *Geneious v5.5*. Available: <http://www.geneious.com>. Accessed 2013 October 5.
36. Tamura K, Peterson D, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731–2739.
37. Nieto Nafria JM (2013) *Fauna Europaea: Aphididae*. *Fauna Europaea* version 2.6 Available: <http://www.fauna-eu.org>. Accessed 2013 April 8.
38. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.
39. Popescu AA, Huber KT, Paradis E (2012) Ape 3.0: New tools for distance-based phylogenetics and evolutionary analysis in R. *Bioinformatics* 28: 1536–1537.
40. Brown SDJ, Collins RA, Boyer S, Malumbres-Olarte J, Lefort M, et al. (2012) Spider: an R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. *Molecular Ecology Resources* 12: 532–565.
41. Chevenet F, Brun C, Banuls A-L, Jacq B, Christen R (2006) TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics* 7: 1–9.
42. Robert V, Szoke S, Jabas B, Vu D, Chouchen O, et al. (2011) *BioMICS* Software: Biological data management, identification, classification and statistics. *The Open Applied Informatics Journal* 5: 87–98.
43. The Plant List (2010) Version 1. Published on the Internet; Available: <http://www.theplantlist.org/>. Accessed: 2013 November 8.
44. Bergsten J, Bilton DT, Fujisawa T, Elliott M, Monaghan MT, et al. (2012) The effect of geographical scale of sampling on DNA barcoding. *Systematic Biology* 61: 851–869.
45. Meyer CP, Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology* 3: 2229–2238.
46. Kerr KCR, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, et al. (2007) Comprehensive DNA barcode coverage of North American birds. *Molecular Ecology Notes* 7: 535–543.
47. Zhang AB, He LJ, Crozier RH, Muster C, Zhu CD (2010) Estimating sample sizes for DNA barcoding. *Molecular Phylogenetics and Evolution* 54: 1035–1039.
48. Miller GL, Footitt RG (2009) The taxonomy of crop pests: The aphids. In: Footitt RG, Adler PH, editors. *Insect Biodiversity: Science and Society*. United Kingdom: Wiley-Blackwell Publishing. 463–474.
49. Piffaretti J, Vanlerberghe-Masutti F, Tayeh A, Clamens A-L, Coeur d'acier A, et al. (2012) Molecular phylogeny reveals the existence of two sibling species in the aphid pest *Brachycaudus helichrysi* (Hemiptera: Aphididae). *Zoologica Scripta* 41: 266–280.
50. Hille Ris Lambers D (1956) Lachnids from Elaeagnaceae. *Zeitschrift für Angewandte Entomologie* 39: 467–473.
51. Blackman RL, Eastop VF (1994) *Aphids on the world's trees: an identification and information guide*. Wallingford, Oxon: C.A.B. International. 987 p.
52. Blackman RL, Eastop VF (2013) *Aphids on the world's plants: An online identification and information guide*. Available: <http://www.aphidsonworldsplants.info>. Accessed: 2013 November 15.
53. Barbagallo S, Binazzi A, Bolchi Serini G, Conchi C, Longo S, et al. (1995) *Homoptera Sternorrhyncha*. Bologna: Calderini. 57 p.
54. Blackman RL, Eastop VF (2006) *Aphids on the world's herbaceous plants and shrubs*. Chichester: John Wiley & Sons Ltd. 1439 p.
55. Stroyan HLG (1985) Recent developments in the taxonomic study of the genus *Dysaphis* Börner. In: Szelegiewicz H, editor; Jablona. *Polska Akademia Nauk, Instytut Zoologii*. 347–391.
56. Naam AM, Footitt RG, Maw HEL, Hanner R (2012) Differentiation between *Aphis pomi* and *Aphis spiraeola* using multiplex real-time PCR based on DNA barcode sequences. *Journal of Applied Entomology* 136: 704–710.
57. Coeur d'acier A, Cocuzza G, Joussetin E, Cavalieri V, Barbagallo S (2008) Molecular phylogeny and systematics in the genus *Brachycaudus* (Homoptera: Aphididae): insights from a combined analysis of nuclear and mitochondrial genes. *Zoologica Scripta* 37: 175–193.
58. Joussetin E, Desdèvises Y, Coeur d'acier A (2009) Fine scale cospeciation between species of the aphid genus *Brachycaudus* and their obligatory endosymbiont *Buchnera aphidicola*: can bacterial genome help refining species delimitation and evolutionary relationships in aphids? *Proceedings of the Royal Society B: Biological Sciences* 276: 187–196.
59. Börner C (1950) *Neue europäische Blattlausarten*. Naumburg: Privately published 19 p.
60. Stroyan HLG (1958) A contribution to the taxonomy of some British species of *Sappaphis* Matsumura, 1918 (Homoptera, Aphidoidea). *The Journal of Linnean Society of London* 43: 643–713.
61. Stroyan HLG (1963) A revision of the British species of *Dysaphis* Börner: Part.II - The Subgenus *Dysaphis* sensu stricto; Ministry of Agriculture FaF, editor. London: Her Majesty's Stationery Office. 119 p.
62. Müller FP (1985) Biotype formation and sympatric speciation in aphids (Homoptera: Aphidinea). *Entomologia Generalis* 10: 161–181.
63. Möller FW (1972) Überwinterung des Artenkomplexes der Grünstreifigen Kartoffelblattlaus *Macrosiphum euphorbiae* (Thomas) und Polyözic der Fundatrizen. *Archiv Pflanzenschutz* 8: 305–312.
64. Kim H, Lee W, Lee S (2010) Morphometric relationship, phylogenetic correlation, and character evolution in the species-rich genus *Aphis* (Hemiptera: Aphididae). *PLoS One* 5: e11608 doi:10.1371/journal.pone.0011608.

65. Börner C (1949) Die erblichen Grundlagen von Befall und Nichtbefall der Pflanzen durch tierische Parasiten. Nachrichtenblatt für den Deutschen Pflanzenschutzdienst 3: 121.
66. Börner C (1952) Europae centralis Aphides. Die Blattläuse Mitteleuropas. Namen, Synonyme, Wirtspflanzen, Generationszyklen. Mitteilungen der Thüringischen Botanischen Gesellschaft 3: 1–488.
67. Jousselin E, Genson G, Coeur d'acier A (2010) Evolutionary lability of a complex life cycle in the aphid genus *Brachycaudus*. BMC Evolutionary Biology 10: 295.
68. Heie OE (1967) Studies on fossil aphids (Homoptera: Aphidoidea). Spolia Zoologica Musei Hauniensis 26: 1–273.
69. Shaposhnikov GC (1985) The main features of the evolution of aphids. In: Szelegiewicz H, editor. Evolution and biosystematics of aphids: Proceedings of the International Aphidological Symposium at Jablonna, 5–11 April, 1981. Jablonna: Polska Akademia Nauk, Instytut Zoologii. 19–99.
70. Novakova E, Hyspa V, Klein J, Foottit RG, von Dohlen CD, et al. (2013) Reconstructing the phylogeny of aphids (Hemiptera: Aphididae) using DNA of the obligate symbiont *Buchnera aphidicola*. Molecular Phylogenetics and Evolution 68: 42–54.
71. Ortiz-Rivas B, Martinez-Torres D (2010) Combination of molecular data support the existence of three main lineages in the phylogeny of aphids (Hemiptera: Aphididae) and the basal position of the subfamily Lachninae. Molecular Phylogenetics and Evolution 55: 305–317.
72. Chen R, Jiang L-Y, Liu L, Liu Q-H, Wen J, et al. (2013) The *gnd* gene of *Buchnera* as a new, effective DNA barcode for aphid identification. Systematic Entomology 38: 615–625.
73. Gauffre B, Coeur d'acier A (2006) New polymorphic microsatellite loci, cross-species amplification and PCR multiplexing in the black aphid, *Aphis fabae* Scopoli. Molecular Ecology Notes 6: 440–442.
74. Wiens JJ, Penkrot TA (2002) Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Systematic Biology 51: 69–91.
75. Rakauskas R (1998) What is the (aphid) species? In: Nieto Nafria JM, Dixon AFG, editors. Aphids in natural and managed ecosystems. Léon: Universidad, Secretariado de Publicaciones. 451–455.
76. Shaposhnikov GC (1981) Populations and species in aphids and the need for a universal species concept. Ottawa: Agriculture Canada, Direction générale de la recherche. 61 p.
77. Shaposhnikov GC. Defining the notion 'species' in aphids. In: Szelegiewicz H, editor; 1985; Jablonna. Polska Akademia Nauk, Instytut Zoologii. 167–169.
78. Shaposhnikov GC (1987) Organization (structure) of populations and species, and speciation. In: P MAKH, editor. Aphids: Their Biology, Natural enemies and Control. Amsterdam: Elsevier. 415–429.
79. Caillaud MC, Via S (2000) Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. American Naturalist 156: 606–621.
80. Ferrari J, Godfray HCJ, Faulconbridge AS, Prior K, Via S (2006) Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. Evolution 60: 1574–1584.
81. Via S (1999) Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. Evolution 53: 1446–1457.
82. Via S, Bouck AC, Skillman S (2000) Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. Evolution 54: 1626–1637.
83. Cocuzza G, Cavalieri V, Jousselin E, Coeur d'acier A, Barbagallo S (2007) Morphological and molecular analysis of *Brachycaudus*, subgenus *Appelia* complex (Rhynchotha, Aphididae). Redia 90: 33–49.
84. Shaposhnikov GC (1986) New species of the genus *Dysaphis* Börner (Homoptera, Aphidinea) and peculiarities of the taxonomic work with aphids. Entomologicheskoe Obozrenie 65: 535–550.
85. Jorg E, Lampel G (1995) Morphological studies on the *Aphis fabae* group (Homoptera, Aphididae). Mitteilungen der Schweizerischen Entomologischen Gesellschaft 68: 387–412.
86. Iglisch I (1968) Über die Entstehung der Rassen der "Schwarzen Blattläuse" (*Aphis fabae* Scop. und verwandte Arten), über ihre phytopathologische Bedeutung und über die Aussichten für erfolgversprechende Bekämpfungsmassnahmen (Homoptera: Aphididae). Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft 131: 5–34.
87. Janisch R (1926) Lebensweise und Systematik der "Schwarzen Blattläuse". Arbeiten aus der Biologischen Reichsanstalt für Land- und Forstwirtschaft 14: 291–366.
88. Müller FP (1982) Das Problem *Aphis fabae*. Zeitschrift fuer Angewandte Entomologie 94: 432–446.
89. Thieme T (1987) Members of the complex of *Aphis fabae* Scop. and their host plants. In: Holman J, Pelikan J, Dixon AFG, Weismann L, editors. Population structure, genetics and taxonomy of aphids and thysanoptera. The Hague: SPB Academic Publishing. 314–323.
90. Mordvilko A (1907) Beiträge zur biologie der pflanzenläuse, Aphididae Passerini. Die zyklische fortpflanzung der pflanzenläuse. II. Die migrationen der pflanzenläuse, ihre ursachen und ihre entstehung. Biologisches Centralblatt 27: 769–816.
91. Shaposhnikov GC (1965) Morphological divergence and convergence in experiments with aphids (Homoptera, Aphididae). Entomologicheskoe Obozrenie 44: 3–25.
92. Shaposhnikov GC (1961) Specificity and appearance of adaptations to new hosts in aphids during the process of natural selection. Entomologicheskoe Obozrenie 40: 739–762.
93. Möller FW (1971) *Macrosiphum stellariae* (Theobald) - eine bisher nicht von der Grünstreifigen Kartoffelblattlaus *Macrosiphum euphorbiae* (Thomas) abgegrenzte Art (Homoptera: Aphididae). Deutsche Entomologische Zeitschrift 18: 207–215.
94. Müller FP (1968) Weitere ergänzungen zur blattlausfauna von mitteleuropa (Homoptera, Aphidina). Faunistische Abhandlungen 2: 101–106.
95. Thomas KH (1962) Die Blattläuse des Formenkreises *Brachycaudus prunicola* (Kalt.). Wissenschaftliche Zeitschrift der Universität Rostock 11: 325–342.
96. Hille Ris Lambers D (1947) On some mainly western European aphids. Zoologische Mededelingen 28: 291–333.
97. Shen YY, Chen X, Murphy RW (2013) Assessing DNA barcoding as a tool for species identification and data quality control. PloS One 8: e57125 doi:10.1371/journal.pone.0057125.
98. Garcia Prieto F, Pérez Hidalgo N, Mier Durante MP, Nieto Nafria JM (2004) Update checklist of Iberian-Balearic Aphidini (Hemiptera: Aphididae). Graellsia 60: 197–214.
99. De Queiroz K (2007) Species concepts and species delimitation. Systematic Biology 56: 879–886.
100. Tosh CR, Vamvatsikos PG, Hardie J (2004) A highly viable cross between *Aphis fabae* (Homoptera: Aphididae) clones with different plant preference. Environmental Entomology 33: 1081–1087.
101. Raymond B (2001) On the processes shaping reproductive isolation in aphids of the *Aphis fabae* (Scop.) complex (Aphididae: Homoptera). Biological Journal of the Linnean Society 74: 205–215.
102. Thomas KH (1968) Die Blattläuse aus der engeren Verwandtschaft von *Aphis gossypii* Glover und *A. frangulae* Kaltenbach unter besonderer Berücksichtigung ihres Vorkommens an Kartoffel. Entomologische Abhandlungen 35: 337–389.
103. Stekolshchikov AV (2004) The species complex of aphids closely related to *Dysaphis devectora* (Walker) (Homoptera, Aphididae): Possible ways of its formation. In: Simon J-C, Dedryver C-A, Rispé C, Hulle M, editors. Aphids in a new millennium. Proceedings of the Sixth International Symposium on Aphids, September, 2001. Rennes (France): INRA editions. 183–188.
104. Möller FW (1970) Die erste gelungene bisexuelle fortpflanzung mit europäischen herkünften von *Macrosiphum euphorbiae* (Thomas) (Homoptera: Aphididae). Zoologischer Anzeiger 184: 107–119.
105. Chen R, Jiang L-Y, Qiao G-X (2012) The effectiveness of three regions in mitochondrial genome for aphid DNA barcoding: A case in Lachninae. PloS One 7: e46190. doi:10.1371/journal.pone.0046190.
106. Huang X, Qiao G-X, Favret C (2013) The high cost of overspecialization. Nature 497: 40.