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Molecular Characterization of Mitogenome of *Cacopsylla picta* and *Cacopsylla melanoneura*, Two Vector Species of '*Candidatus* Phytoplasma mali'

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Abstract: The mitochondrial genomes of two vector psyllids of the '*Candidatus* Phytoplasma mali', *Cacopsylla picta* and *C. melanoneura*, were sequenced using high-throughput sequencing on the Illumina platform. The main objective of the study was to describe their mitogenome and characterize their genetic variability and the potential changes in the context of the observed global warming. The four complete sequences for *C. picta*, 14,801 bp and 14,802 bp in length, two complete and one partial sequence for *C. melanoneura*, ranging from 14,879 bp to 14,881 bp in length, were obtained for the first time for these European apple psyllids. The detected intraspecies mtDNA identity was highly similar (99.85–99.98%), the identity's similarity with other *Cacopsylla* species varied between 79.79 and 86.64%. The mitogenomes showed a typical mitochondrial DNA structure with 13 protein-coding genes, 2 rRNA genes and 22 tRNA genes; the presence of CGGA motif in the ND1-trnS2 junction was detected in both species. Phylogenetic analysis placed both species in close relationship with *C. burckhardti* within the Cacopsylla clade-I O group. The analysis of complete mitogenomes and of partial COI sequences of fifty-two *Cacopsylla* individuals showed a high homogeneity of genotypes over 15 years and among the different localities in the Czech Republic.

Keywords: apple pests; high-throughput sequencing complete mitochondrial DNA; phylogenetic analysis; fruit tree phytoplasma

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1. Introduction

Psyllids, or jumping plant lice, are phytophagous insects that comprise about 4000 species with a worldwide distribution, the majority of which are found in tropical and subtropical areas, but only 100 which are considered to be economically important pests in agriculture or forestry. They are hemimetabolous phloem feeders with stylets that penetrate intercellularly into phloem sieve elements [1–3]. Some species of this group are vectors of serious bacterial pathogens. Among them, one of the most important are phytoplasmas ('*Candidatus* Phytoplasma sp.'), which are the causal agents of serious diseases of more than 1000, mainly dicotyledonous, herbaceous plants and trees [4,5].

The genus *Cacopsylla* includes the univoltine or polyvoltine psyllids that are vectors of fruit phytoplasmas [6,7]. Among them, *Cacopsylla picta* and *Cacopsylla melanoneura* are univoltine apple psyllids found in Europe. Both species are vectors of '*Ca*. Phytoplasma mali' (apple proliferation phytoplasma) [8–10], the causal agent of apple proliferation

disease with typical symptoms such as witches' broom, enlarged stipules, tasteless, and dwarf fruits [4,7,9]. Both species complete their life cycle on different plants of the *Rosaceae* family. The overwintering adults (re-migrants) migrate in early spring to the host feeding plants where they complete their life cycle, and the new generation migrates in early summer to the sheltering conifers in the highlands [6,11–13].

C. picta (Föerster, 1848) is considered to be a monophagous species feeding on the *Malus* plants [2,14], but recent studies based on high-throughput sequencing (HTS) analysis have shown that it can also feed on herbaceous plants such as squash (*Cucurbita*) and dandelion (*Taraxacum* sp.), and other trees such as birch (*Betula* sp.), oak (*Quercus* sp.), ash (*Fraxinus* sp.) or even spruce (*Picea* sp.), pine (*Pinus* sp.) or cedar (*Cedrus* sp.) [15]. This psyllid is the main vector of '*Ca*. Phytoplasma mali' in Europe, in terms of abundance and the relatively high infectivity of the psyllids [16–18]. *C. picta* is able to transmit the phytoplasma at all stages of its life cycle, including vertical and transovarial transmission [17,19]. The spring migration of *C. picta* begins in mid-March or early April, depending on the country and year, and peaks in the second half of April–early May with adults of the new generation gradually emigrating to shelter plants during July [6,12,20].

C. melanoneura (Förster, 1848) is an oligophagous species feeding on the hawthorn (*Crataegus* sp.), apple (*Malus* sp.), mosses (*Mespilus* sp.) and pear (*Pyrus* sp.) [2,9]. The life cycle is shifted about 3 weeks earlier compared to *C. picta*; the re-migrants begin to infest feeding plants in mid-February and the emigrants move to the overwintering area in the late May [11,20,21]. However, its efficacy in phytoplasma transmission and its importance in the spread of apple proliferation disease is still ambiguous. It seems that although it has been reported from orchards in almost all of Europe, its efficiency in spreading phytoplasma depends on the combination of the local biotic and abiotic conditions. As the main vector of apple proliferation phytoplasma has been detected only in the northwestern Italy [8,11,22], its importance in other European countries is probably low in terms of psyllid abundance and infectivity [6,12,16,21].

The determination of psyllids to date is mainly based on morphological observation, which is difficult in the case of females and almost impossible for the larval stages. For this reason, the methods of molecular determination and DNA barcoding based mainly on the analysis of the mitochondrial cytochrome oxidase subunit I gene have been repeatedly applied for their determination [22–28]. The evolution of psyllids has also been intensively studied in recent years, suggesting a polyphyletic origin of this taxonomic group [29]. Surprisingly, however, only a few complete mitochondrial genomes of these agronomically important psyllids are publicly available. To date, six mitogenomes have been characterized, mostly from pear or Asian psyllids—*C. burckhardti*, *C. jukyungi*, *C. pyri*, *C. coccinae*, *C. citrisuga*, and recently *C. fuscicella* [29–34].

The main objective of this work was to characterize the mitogenome of important apple psyllids, vectors of '*Ca*. Phytoplasma mali', *C. picta* and *C. melanoneura*, in order to increase the knowledge of these agronomically important pests and their evolution, and to contribute to a better understanding of the epidemiology of the apple proliferation phytoplasma. The hypothesis that led to this study was that the currently observed global warming and the increasing average temperature as well as the changing character of the year seasons, i.e., mild winters and warm springs and summers generally reported in Central Europe [35,36], could lead to the expansion of different psyllid genotypes. These could then have a different/higher ability to spread the phytoplasma that causes severe apple proliferation disease in susceptible apple cultivars. The different genetic structure of *C. melanoneura* populations could be a reason for the ambiguous and still discussed vector status and the importance of this psyllid in the effective phytoplasma spread in Central Europe.

2. Materials and Methods

2.1. Biological Material

Adult psyllids were collected via sweep netting from localities in southern Moravia in 2005, 2006 and 2022, and in eastern Bohemia in 2022 (See the list in Table 1, Figure 1), Czech Republic. Collected samples were kept in 96% ethanol, at −20 °C, and they were determined based on their morphological characteristics.

Table 1. List of *Cacopsylla* samples used in the analyses.

ID	Locality (GPS Location)	Date of Collection	Determined by
C. picta			
499-2	Velké Němčice 1 (49°0′10.538″ N, 16°39′10.617″ E)	16 May 2006	P. Lauterer
457-1	Podhoří (49°33′43.939″ N, 17°37′1.056″ E)	26 April 2013	P. Lauterer
S7	Lednice (48°47′24.188″ N, 16°48′5.808″ E)	28 April 2022	M. Starý
AO353	Holovousy (50°22′48.503″ N, 15°34′28.442″ E)	29 April 2022	J. Ouředníčková
51-4,6,10,11,20	Brno (49°9′27.367″ N, 16°34′20.229″ E)	5 April 2005	P. Lauterer
61-1,4,8,12	Velké Bílovice (48°51′19.028″ N, 16°54′46.743″ E)	27 April 2005	P. Lauterer
357-1,2,3,4,5,6	Drahanská vrchovina (49°15′40.686″ N, 16°50′40.728″ E)	10 February 2012	P. Lauterer
359-1,2,3,4	Drahanská vrchovina (49°15′40.686″ N, 16°50′40.728″ E)	1 November 2012	P. Lauterer
491-13,14,15,16	Kozov (49°42′9.601″ N, 16°51′8.130″ E)	6 May 2015	P. Lauterer
498-7,8	Lednice (48°47′24.188″ N, 16°48′5.808″ E)	28 April 2022	M. Starý
AO283, AO286	Holovousy (50°22′48.503″ N, 15°34′28.442″ E)	22 March 2022	J. Ouředníčková
AO350-352,354-356	Holovousy (50°22′48.503″ N, 15°34′28.442″ E)	29 April 2022	J. Ouředníčková
C. melanoneura			
S3 (male)	Lednice (48°47′24.188″ N, 16°48′5.808″ E)	13 June 2006	P. Lauterer
498-2	Lednice (48°47′24.188″ N, 16°48′5.808″ E)	28 April 2022	M. Starý
AO282	Holovousy (50°22′41.76″ N, 15°34′33.405″ E)	22 March 2022	J. Ouředníčková
495-1,3	Velké Němčice 2 (48°58′47.055″ N, 16°42′2.957″ E)	29 May 2006	P. Lauterer
495-2	Velké Bílovice (48°51′19.028″ N, 16°54′46.743″ E)	18 April 2006	P. Lauterer
495-4; 496-7,10	Lednice (48°47′24.188″ N, 16°48′5.808″ E)	13 June 2006	P. Lauterer
496-1,5,6	Velké Němčice 2 (48°58′47.055″ N, 16°42′2.957″ E)	29 May 2006	P. Lauterer
497-3,4,6,7	Lednice (48°47′24.188″ N, 16°48′5.808″ E)	28 April 2022	M. Starý
498-3,4,7,8	Lednice (48°47′24.188″ N, 16°48′5.808″ E)	28 April 2022	M. Starý

Figure 1. Map showing sampling localities distribution. Blue—*C. picta*; green—*C. melanoneura*.

2.2. DNA Isolation

Individual adult psyllids were homogenized in ATL buffer in 1.5 mL microtube using a micropestle homogenizer and total DNA was isolated using the QIAmp DNA Blood mini kit (Qiagen, Hilden, Germany, Cat. No. 51106) and eluted in 100 µL of deionized water. Purified DNA was stored at a temperature of −20 °C until used in HTS or PCR assays.

2.3. PCR Amplification and Sanger Sequencing

For the species confirmation and to study of the variability of the larger sample set, the partial COI gene was amplified using the universal primer pair CPF4 (5′-TAAGAACTAACC ATAAGATTATCGG-3′) and CPR4 (5′-CACTTCAGGGTGTCCAAAGAATC-3′) according to the work of Kang et al. [24]. The PCR reaction mix consisted of MyTaq polymerase buffer (1×), MyTaq DNA polymerase (1U) (both Bioline, London, UK, Cat. No. BIO-21105) and 0.2 mM of each primer, and 2 µL of isolated DNA in a concentration of 50–200 ng, in total volume of 25 µL. The PCR reaction was conducted under the following conditions: 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, and a final extension 72 °C for 5 min. Amplicons were separated in 1% agarose gel in TAE buffer and visualized using GelRed nucleic acid stain (Biotium, Fremont, CA, USA, Cat. No. 41002-0.5).

The amplicons were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Dueren, Germany, Cat. No. 740609.50) and Zymo-Spin III columns (Zymo Research, Irvine, CA, USA, Cat. No. C1005-250) and directly sequenced via bi-directional Sanger sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems-Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. 4337455) and Genetic Analyzer ABI Prism 3730 at the Sequencing Centre of the Institute of Experimental Botany of the Czech Academy of Sciences, v. v. i. and Palacký University, Olomouc (CRH-UPOL), Czech Republic. The obtained Sanger sequences were analyzed and assembled into final contigs using Geneious Prime assembler 2023.1.2.

2.4. High-Throughput Sequencing

The 50 ng of DNA was fragmented in 50 µL of Sonication buffer [TE buffer (10 mM Tris, 1 mM EDTA), pH 7.5–8.0] using the Bioruptor Plus (Diagenode, Denville, NJ, USA) five times for 30 s at the HIGH setting. The sheared DNA was used to prepare a sequencing library using the NEBNext Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA, Cat. No. E7645L) with a mean fragment size of 500 bp. The library was sequenced on an Illumina NovaSeq 6000 to generate 2 × 160 or 2 × 250 bp paired-end reads to achieve at least 100-fold mitochondrial genome coverage. The samples were sequenced in multiple runs using the NovaSeq6000 at CATRIN-UPOL, Czech Republic.

2.5. Assembly of HTS Reads and the Genome Annotation

The high-throughput reads were paired by name, trimmed using BBDuk, merged and de novo assembled using Spades assembler, under standard conditions, all algorithms implemented in Geneious Prime version 2023.1.2. The resulting contigs were screened using the BLASTN algorithm [37] against the NCBI *Cacopsylla* mitochondrion sequences and circularized based on the identical 5′- and 3′- overhangs. The derived mtDNAs were subjected to mitogenome analysis using the MITOS Web server (http://mitos.bioinf.uni-leipzig.de, accessed on 22 June 2023) [38] under the invertebrate genetic code (code 5), and the annotation files and tRNA structure analyses were generated. The position of the identified genes was manually verified based on the additional comparisons with the available *Cacopsylla* mitogenomes using MEGA11 version 11.0.9 [39] and Geneious Prime 2023.1.2 software. Geneious Prime 2023.1.2 was also used for the visualization of the mitogenome map.

2.6. Phylogenetic Analysis

Multiple alignments of the obtained HTS mtDNA sequences and complete mtDNA sequences of *Cacopsylla* sp. available in the GenBank were performed using ClustalW algorithm. The obtained nt matrices were used for the estimation of genetic variability and phylogenetic relationships: (1) genetic variability was calculated using p-distance algorithm; (2) frequency of synonymous and nonsynonymous codons was calculated using distance analysis and the Nei–Gojobori model; (3) the phylogenetic trees were constructed on nucleotide sequences of complete mitogenome and discrete genes using the neighbor-joining method and the Tajima–Nei (G+I) model (selected using the model algorithm). The 1000 bootstrap replicates were used for all analyses, and trees were rooted using the appropriate *Psylla alni* sequences. The phylogenetic tree was visualized using Tree Explorer. All of the above analyses were performed using MEGA11 version 11.0.9 [39]. A similar strategy was used for the analysis of partial *COI* sequences except for the selection of the Tamura–Nei three-parametric model (G+I) used in the phylogenetic analysis.

3. Results

In order to gain a broader insight into the structure of the mitogenome of the '*Candidatus* Phytoplasma mali' vector species, *Cacopsylla picta* and *Cacopsylla melanoneura*, four individuals of *C. picta* and three of *C. melanoneura* collected in 2005, 2006 and 2022 in the southern Moravia and the eastern Bohemia of the Czech Republic were subjected to high-throughput sequencing.

3.1. HTS Analysis

The four complete mtDNAs of *C. picta*, and two complete and one partial mtDNAs of *C. melanoneura* were obtained after de novo assembly of the HTS reads (Table 2); one overlapping contig was obtained for each sample. The sequences were deposited in the GenBank under Acc. Nos. OR346833-OR346839, OR351141-OR351187.

The length of the *C. picta* mtDNA varied between 14,801 and 14,802 bp, with an indel -/A present at position 1314. These mtDNA sequences showed 99.85–99.98% similarity with each other and 79.79–86.51% similarity with complete mitochondrial sequences of other *Cacopsylla* species available in GenBank, namely *C. burckhardti*, *C. pyri*, *C. coccinea*, *C. yukiungi*, *C. citrinella*, *C. fuscicella*. The correct morphological determination of the analyzed individuals was confirmed via BLASTN analysis, in which 99.32–100% similarity was found with the different partial *COI* sequences of *C. picta* available in the GenBank database.

Two complete mitochondrial sequences (gt S3 and 498-2) and one partial sequence (gt AO282), lacking the complete A+T rich control region, measuring 14,879–14,881 bp in length were obtained for *C. melanoneura* samples. The sequences differ according to the presence of specific indels in non-coding regions, with A/- at position 6034, AT/-- at position 14,400, T/- at position 14,608; and they showed 99.86–99.98% similarity with each other, 80.02–86.64%

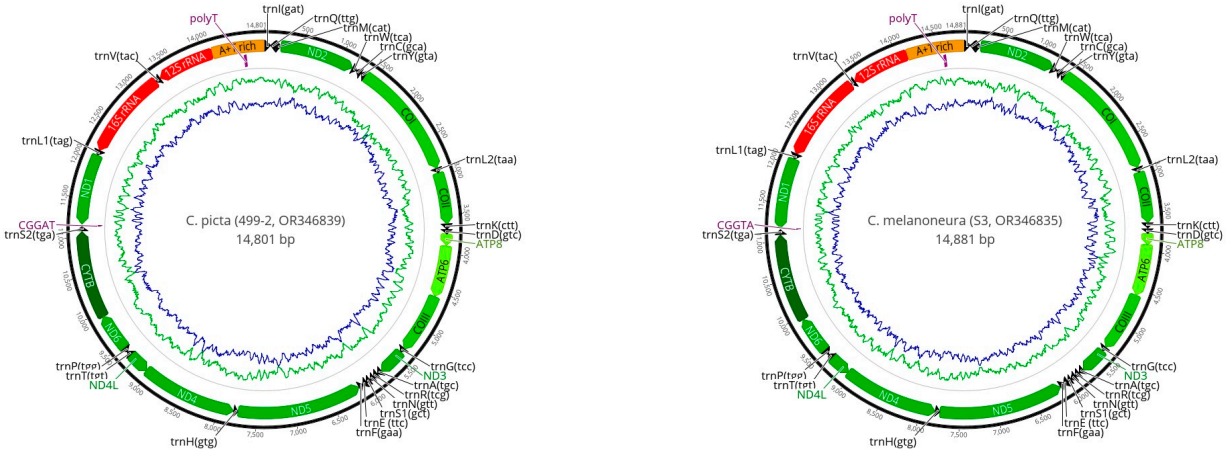
with the complete mitochondrial sequences of the other *Cacopsylla* sp. and 99.73–100% similarity with various *C. melanoneura* sequences available in GenBank, respectively.

Table 2. Number of HTS reads and coverage of obtained mtDNA sequences.

Sample (Acc. No.)	mtDNA Length	Reads Coverage (Average ± stdev)	HTS/mtDNA-Specific Reads
C. picta			
499-2 (OR346839)	14,801	1202 ± 272	14,439,480/83,388
AO353 (OR346838)	14,801	644.6 ± 209.9	8,074,058/43,722
457-1 (OR346836)	14,802	476.3 ± 122.7	7,902,966/31,389
S7 (OR346837)	14,802	754.9 ± 256.6	15,544,208/43,722
C. melanoneura			
S3 (OR346835)	14,881	1243.3 ± 196.1	19,038,072/118,977
498-2 (OR346833)	14,879	513.1 ± 76.4	5,920,406/32,409
AO282 (OR346834)	14,880	56.8 ± 17.2	3,732,814/3662

3.2. Mitogenome Structure of *C. picta* and *C. melanoneura*

All molecules showed the standard organization of the mitogenome consisting of 36 typical genes, 13 protein-coding genes, 2 ribosomal (rRNA) RNA genes and 22 transfer RNA (tRNA) genes (Figure 2, Table 3 and Table S1). All mtDNAs obtained showed high frequency of A and T bases, with 39.1% of A, 35% of T, 16.3% of C, 9.6% of G and a GC content of 25.8% for the complete sequence and slightly higher, 26.9%, for protein-coding genes for the *C. picta*. A similar situation was found for the *C. melanoneura* with the frequencies 38.8% of A, 35.1 of T, 16.1% of C, 10.1 of G, and 26.2% of GC in the complete sequence and 27.5% of GC, in protein-coding genes.



(**a**) (**b**)

Figure 2. Mitogenome map of (**a**) *Cacopsylla picta* (499-2, Acc. No. OR346839) and (**b**) *Cacopsylla melanoneura* (S3, Acc. No. OR346835). Protein-coding genes are shown in green, rRNA genes in red, tRNA genes in black, A+T rich control region in orange, and specific motifs in purple.

Table 3. Characterization of the mitogenome of selected genotypes of *C. picta* and *C. melanoneura*.

Name		*C. picta* (499-2, AO353)			*C. melanoneura* (S3)		
		Start	End	Length	Start	End	Length
trnI(gat)	Fw	1	66	66	1	66	66
trnQ(ttg)	Rev	71	136	66	71	136	66
trnM(cat)	Fw	142	205	64	142	207	66
ND2	Fw	206	1177	972	208	1179	972
trnW(tca)	Fw	1176	1237	62	1178	1240	63

Table 3. *Cont.*

Name		*C. picta* (499-2, AO353)			*C. melanoneura* (S3)		
		Start	End	Length	Start	End	Length
trnC(gca)	Rev	1240	1302	63	1243	1305	63
trnY(gta)	Rev	1303	1364	62	1306	1367	62
COI	Fw	1366	2895	1530	1369	2901	1533
trnL2(taa)	Fw	2895	2958	64	2901	2965	65
COII	Fw	2959	3622	664	2966	3629	664
trnK(ctt)	Fw	3623	3692	70	3630	3699	70
trnD(gtc)	Fw	3691	3752	62	3701	3761	61
ATP8	Fw	3753	3902	150	3762	3914	153
ATP6	Fw	3902	4573	672	3911	4582	672
COIII	Fw	4573	5350	778	4582	5359	778
trnG(tcc)	Fw	5351	5408	58	5360	5419	60
ND3	Fw	5409	5759	351	5420	5770	351
trnA(tgc)	Fw	5761	5821	61	5770	5829	60
trnR(tcg)	Fw	5826	5886	61	5840	5900	61
trnN(gtt)	Fw	5886	5951	66	5900	5964	65
trnS1(gct)	Fw	5952	6005	54	5965	6018	54
trnE(ttc)	Fw	6006	6066	61	6019	6078	60
trnF(gaa)	Rev	6055	6117	63	6068	6130	63
ND5	Rev	6118	7735	1618	6131	7748	1618
trnH(gtg)	Rev	7736	7796	61	7749	7809	61
ND4	Rev	7797	9045	1249	7810	9055	1246
ND4l	Rev	9039	9326	288	9049	9336	288
trnT(tgt)	Fw	9328	9388	61	9338	9397	60
trnP(tgg)	Rev	9389	9451	63	9398	9463	66
ND6	Fw	9454	9939	486	9466	9951	486
CYTB	Fw	9939	11,075	1137	9951	11,087	1137
trnS2(tga)	Fw	11,077	11,14	64	11,091	11,153	63
GCCTA motif	Rev	11,152	11,156	5	11,165	11,169	5
ND1	Rev	11,168	12,082	915	11,181	12,095	915
trnL1(tag)	Rev	12,083	12,146	64	12,096	12,158	63
16S rRNA	Rev	12,147	13,303	1157	12,159	13,31	1152
trnV(tac)	Rev	13,304	13,365	62	13,311	13,373	63
12S rRNA	Rev	13,366	14,112	747	13,375	14,122	748
A+T rich	--	14,113	14,801	689	14,123	14,881	759
polyT motif	--	14,531	14,550	20	14,608	14,630	23

Abbreviations used as follows: ND2—NADH dehydrogenase subunit 2; COI—cytochrome c oxidase subunit I; COII—cytochrome c oxidase subunit II; ATP8—ATP synthase membrane subunit 8; ATP6—ATP synthase membrane subunit 6; COIII—cytochrome c oxidase subunit III; ND3—NADH dehydrogenase subunit 3; ND5—NADH dehydrogenase subunit 5; ND4—NADH dehydrogenase subunit 4; ND4L—NADH dehydrogenase subunit 4L; ND6—NADH dehydrogenase subunit 6; ND1—NADH dehydrogenase subunit 1; CYTB—cytochrom b; A+T rich—A+T rich control region; trn—tRNA (amino acids marked by IUPAC code); Fw—forward orientation; Rev—reverse orientation.

The mtDNA of both psyllid studies is similar to those described to date, namely for *Cacopsylla* species, i.e., *C. burckhardti*, *C. pyri*, *C. coccinea*, *C. yukiungi*, *C. citrinella*, *C. fuscicella* psyllids shows a standard structure typical for the *Cacopsylla* mitogenome. The 13 protein-coding genes have been identified there, of which 9 (ND2, COI, COII, APT6, ATP8, COIII, ND3, ND6 and CYTB) are localized on the H strand and 4 (ND5, ND4, ND4L, ND1) on the L strand. The genes are flanked by AT- start codons, mainly ATG, followed by ATA and ATT (specific TTG as a start codon has been detected for the ND5 gene, at the 5′ end) and usually by TAA and less frequently TAG termination codons at the 3′ end. The incomplete T- termination, which is thought to be converted to TAA after specific adenylation is present in the COII, COIII, ND4 and ND5 genes (Table 4). The coding region consists of 3604 codons, and the most frequent codons were ATT (9.40 and 9.49%), TTA (8.70% and 8.46%), TTT (8.34% and 8.10%) and ATA (5.55% and 5.55%) for *C. picta* and *C. melanoneura*, respectively.

The analysis of the overall codon usage shows a clear preference for codons with A or T in the third position compared to G or C (Figure S1). As expected, the shortest gene is ATP8 with a length of 153 bp for both species, and the longest genes are ND5 with length of 1618 bp, followed by the COI gene, which varies in length from 1530 bp in *C. picta* to 1533 bp in *C. melanoneura*. The length of the protein coding genes was identical for the both species, except for the COI gene mentioned above and the ND4L gene (1249 and 1246 bp). Minimal nucleotide variability was found among the genes within each *Cacopsylla* species. The interspecies comparisons reflected the species differences. The lowest diversity (12.3%) was found for the COI gene, while the most variable genes were ND4L (21.7–24.1% diversity), ATP8 (27.2%), and ND6 (28.9–28.7%). The analysis of the frequency of synonymous and nonsynonymous mutations showed the more frequent presence of synonymous codons at intra-species and inter-species levels, with intra-species dN-dS values close to zero and inter-species dN-dS values ranging from −0.225 to −1.175 (Table 4), implying the effect of negative selection in all genes, except for ND6 in *C. melanoneura*.

Table 4. Characterization of protein-coding genes of *C. picta* and *C. melanoneura*.

Gene	*C. picta*				*C. melanoneura*				Both Species	
	Start/Stop Codons	Protein Mw [kDa]	Distance [%]	dN-dS	Start/Stop Codons	Protein Mw [kDa]	Distance [%]	dN-dS	Distance [%]	dN-dS
ND2	ATA/TAA	37.68	0–0.1	0	ATA/TAA	37.5	0–0.2	0	17.1–17.3	−0.760
COI	ATG/TAA	57.02	0–0.3	−0.005	ATG/TAA	57.14	0–0.1	−0.002	12.3–12.4	−0.760
COII	ATA/T	25.53	0–0.3	−0.010	ATA/T	25.61	0	0	12.8–13.1	−0.942
ATP6	ATG/TAA	25.37	0–0,3	0	ATG/TAA	25.37	0–0.3	0	15.6–15.7	−0.933
ATP8	ATC/TAA	6.03	0	−0.010	ATC/TAA	5.89	0	0	27.2	−0.810
COIII	ATG/T	30.65	0–0.1	−0.010	ATG/T	30.56	0.1–0.3	0	13.8–14.0	−0.701
ND3	ATA/TAA	13.65	0–0.3	−0.009	ATT/TAA	13.58	0	0	17.4–17.7	−1.197
ND5	TTG/T	60.29	0–0.2	−0.005	TTG/T	60.27	0–0.1	−0.002	15.2	−0.271
ND4	ATG/T	47.83	0–0.1	0.000	ATG/T	47.44	0–0.2	−0.004	16.1–16.3	−0.513
ND4L	TTG/TAG	11.12	0	0	TTG/TAG	11.01	0–0.3	−0.010	21.7–24.1	−0.225
ND6	ATA/TAA	18.66	0–0.6	−0.006	ATA/TAA	18.64	0–0.2	0.002	20.4–20.6	−0.705
CYTB	ATG/TAA	43.42	0–0.2	−0.010	ATG/TAA	43.3	0–0.2	0	14.6–14.8	−0.959
ND1	ATA/TAA	34.96	0	0	ATA/TAG	34.92	0–0.4	−0.008	13.2–13.4	−0.500

Abbreviations used as follows: ND2—NADH dehydrogenase subunit 2; COI—cytochrome c oxidase subunit I; COII—cytochrome c oxidase subunit II; ATP8—ATP synthase membrane subunit 8; ATP6—ATP synthase membrane subunit 6; COIII—cytochrome c oxidase subunit III; ND3—NADH dehydrogenase subunit 3; ND5—NADH dehydrogenase subunit 5; ND4—NADH dehydrogenase subunit 4; ND4L—NADH dehydrogenase subunit 4L; ND6—NADH dehydrogenase subunit 6; ND1—NADH dehydrogenase subunit 1; dN- nonsynonymous distance; dS—synonymous distance.

The analysis of the variability of the mtDNAs of both species did not show any significant differences between the psyllids collected in 2005, 2006 and 2022, and between the samples collected in the Czech Republic in the regions of Eastern Bohemia and Southern Moravia.

For the both *Cacopsylla* species, 22 tRNA genes were identified in the each mitogenome, 17 tRNAs carrying anticodon specific to unique amino acid, and 2 tRNAs carrying specific Leu anticodon (L1, L2) and 2 carrying Ser anticodon (S1, S2).

The mitogenome harbors two ribosomal RNA genes, both in the reverse orientation positioned between the *ND1* and A+T rich Control region, the 16S rRNA gene of 1157 bp in length for *C. picta* and 1154 bp in length for *C. melanoneura*, and the 12S rRNA gene of 747 bp and 748 bp in length, respectively. Both species differ in the length of the A+T rich control regions, which are 689 bp and 757–758 bp in length, respectively, and contain a 20 bp and 23 bp long poly-T motif.

Phylogenetic analysis of the complete mitochondrial sequences using neighbor-joining analysis allowed the construction of a phylogenetic tree, whose topology placed *C. picta* and *C. melanoneura* sequences in relationship to *C. burckhardti* (Figure 3). Both species

formed a homogeneous branch that differed significantly from the other sequences analyzed. Phylogenetic trees of similar topology with the strong support for the *C. picta* and *C. melanoneura* branches were obtained not only for the COI, 16S rRNA and CYTB genes used for taxonomic classification, but also for all other protein-coding genes.

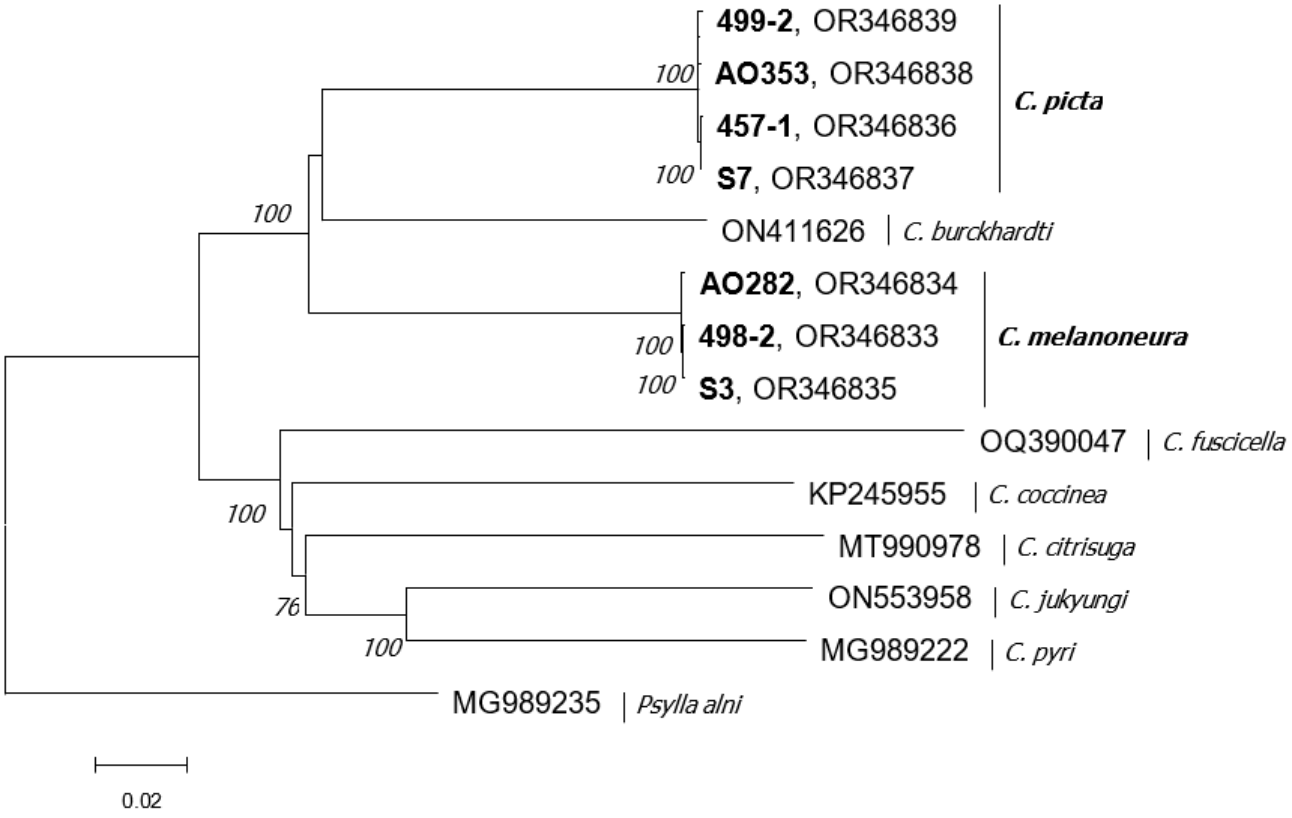
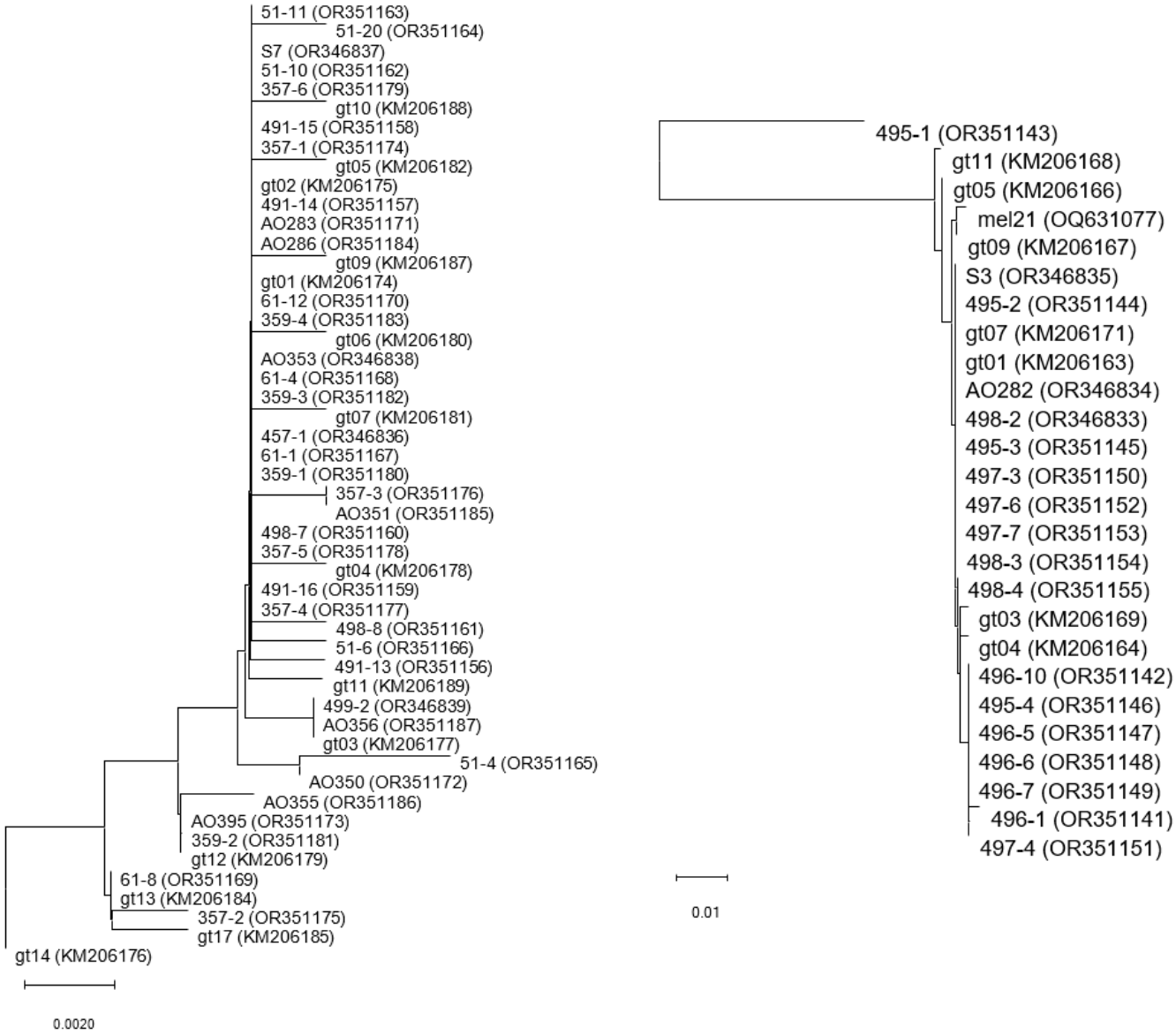


Figure 3. Phylogenetic tree of *Cacopsylla* sp. constructed based on the complete mtDNA sequences using the neighbor-joining algorithm. Sequences marked by genotype and/or species name and GenBank Acc. No, and the new studied sequences are marked in bold. Sequence of *Psylla alni* was used to root the tree. Only bootstrap values ≥70 are shown. The bar represents 2% nucleotide variability.

With the aim of mapping the variability of the studied psyllids and the change in the Czech population structure in the context of global warming and the possible spread of different genotypes in the area over more than 15 years, the partial COI sequence analysis was performed on the set of *C. picta* and *C. melanoneura* psyllids collected in the 2005–2022 period from different localities in the Czech Republic. The species-specific sequences were almost identical, showing 99.7–100% similarity between each other and compared to Italian samples available in GenBank for *C. picta*, and 99.3–100% for *C. melanoneura*, respectively. The only exception was the sample adult genotype 495-1, which was determined to be *C. melanoneura* and showed only 91.5–92.0% similarity to other *C. melanoneura* sequences.

In the phylogenetic analysis using neighbor-joining analysis (Figure 4), samples of *C. picta* collected from different localities in Moravia and eastern Bohemia formed one major and four minor clusters, but without the statistical support of bootstrap values, among which the sequences were distributed together with the Italian sequences available in GenBank without correlating with their origin or the date of their collection. The *C. melanoneura* psyllids formed a homogeneous cluster covering all analyzed sequences without any correlation with the origin or date of collection. The specific situation of adult male 495-1 was also confirmed in this analysis, when this sequence formed a significant single branch (bootstrap value 97) that was distinct from the other *C. melanoneura* and *C. picta* psyllids.



(**a**) (**b**)

Figure 4. Phylogenetic tree of (**a**) *C. picta* and (**b**) *C. melanoneura* constructed based on the partial COI sequence using the neighbor-joining method, Tamura three-parametric model, with bootstrap replicates 1000×. Sequence of *Psylla alni* (NC_038139.1) was used to root the tree /not shown/). Sequences marked by genotype name and/or GenBank Acc. No. Only bootstrap values ≥ 70 are shown. The bar represents 0.05% and 0.01% nucleotide variability.

4. Discussion

In our study, we have obtained the first complete mitochondrial genomes of two apple psyllids *Cacopsylla picta* and *Cacopsylla melanoneura*. The length of the mitogenomes, 14,801–14,802 bp and 14,879–14,881 bp is similar to the mitogenomes of *Cacopsylla* species described so far, with the detected length of around 14.8–14.9 kb [29–31,33,40]. The phylogenetic analysis of the complete mtDNA places the two species characterized here in a close relationship with *C. burckhardti*. Despite the lack of complex data publicly available for further comparisons, it could be deduced that both of them belong to the *Cacopsylla* clade I of the O-group of psyllids, psyllids of the Old and New World [29], which is in agreement with the expectation based on their distribution limited to the Europe and the

variability of the partial cytochrome c oxidase subunit I gene used for the DNA barcoding and molecular identification of psyllid species [11,22,25–28,41].

The A/T content is the same for mitochondrial DNA from other members of the *Cacopsylla* species, typically ranging from 72.04 for *C. citrisuga* to 73.85% for *C. pyri,* and 73.69% for *C. burckhardti*, respectively. The codon bias, with a preference for A/T in the third position of the codon, also reflects this situation. TTA, ATT, TTT and ATA codons are the most frequently observed codons in their mitogenomes, and their frequency in *C. picta* (31.99%) and *C. melanoneura* (31.6%) belongs to the upper limit of their frequency, showing again a high similarity with the *C. burckhardti* (31.89–32% frequency of the mentioned codons) and the use of these codons in the range of 28.91–31.69% by the other *Cacopsylla* psyllid species [33].

The variability of all protein-coding genes and the observed identical topology of the phylogenetic tree constructed for each of them support the previous suggestion that all of them and not only *COI* could be used for the species discrimination and their molecular identification. Thus, the most variable genes, ND6 and ATP8, could be efficient in the study of population variability at least for the *C. picta* and *C. melanoneura*. On the other hand, the conservative character and low variability of the COI gene, also confirmed in our study, regardless of whether we discuss the complete or partial sequences, shows that this gene is not so suitable for intrapopulation variability studies, although it is still used for them. The combination of chromosomal and mitochondrial markers might be more useful [24,26,28,42]. In addition to this gene, the mitochondrial genes, such as ND5, COIII or ND4L, are recommended for the study of interspecies variability [33]; however, their different variability among the species or higher taxonomic groups shows that the further studies of the mitogenome structure of the other *Cacopsylla* species are necessary for a better understanding of their evolution and their adaptations to the environment and life strategies. Their study is still under progress.

The finding of genetic and phylogenetic distance of sample genotype 495-1, male determined as *Cacopsylla melanoneura* (Pavel Lauterer, personal communication), which shows almost 8.5% distance from any other *C. melanoneura* plant lice and even higher with the other representatives of the genus *Cacopsylla*, is surprising. The significant phylogenetic position between *C. melanoneura* and *C. burckhardti* and its distance from them raises questions about the identity of this insect. The two distinct lineages have been previously observed for the *Cacopsylla chinensis* in Japan, and China and Taiwan [27,42], and one of them has recently been described as a new species, *Cacopsylla jukyungi* [33]. Given our high respect for the morphological determination by Dr. P. Lauterer, the possible existence of the two cryptic *C. melanoneura* species could not be excluded, similar to the existence of two cryptic species of *C. pruni* and the existence of A and B genotypes [43,44]. However, further efforts to clarify the situation could be complicated by the age of the sample analyzed and by the fact that individuals could occur quite randomly in the area, in minimal abundance, due to their random transfer by air currents [13] during the re-emigration from their from their wintering grounds and that their natural population occurs in the other area. Their different ability to transmit phytoplasma '*Ca*. Phytoplasma mali' could not be excluded.

5. Conclusions

The complete mitogenome of two apple plant lice, *C. picta* and *C. melanoneura*, is characterized for the first time for these species. It brings comprehensive information about the mitogenome of European members of the genus *Cacopsylla*, and its stability on the set of samples collected more than seventeen years apart, at least in the center of Europe, Czech Republic. Both species share common characteristics with other members of the genus described to date, such as length of mtDNA, its molecular and genetic structure, including codon bias, structure of the A+T rich control region, and carry the CGGTA motif in the non-coding region between ND1 and tRNA-S2 (- orientation). On the other hand, the observed variability in the length of this region between the individuals within the same species of *C. picta* implies that the length and conservation of this repetitive region might

not be so strict. The obtained results show a high genetic homogeneity of the mtDNA of both psyllid species occurring in the studied Czech localities and clear differences over a 15-year period were not detected.

Increasing the knowledge about the structure of the mitogenome of other European psyllids of the genus *Cacopsylla*, not only *C. picta* and *C. melanoneura* described in our study, could be beneficial in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13092210/s1, Figure S1: Characterization of codon usage by *C. picta* and *C. melanoneura*. The frequencies calculated for each amino acid; Table S1: Characterization of the mitogenome of selected genotypes of *C. picta* (457-1, S7) and *C. melanoneura* (498-2, AO282).

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References

1. Moreno, A.; Miranda, M.P.; Fereres, A. Psyllids as major vectors of plant pathogens. *Entomol. Gen.* **2021**, *41*, 419–438. [CrossRef]
2. Ouvrard, D.; Chalise, P.; Percy, D.M. Host-plant leaps versus host-plant shuffle: A global survey reveals contrasting patterns in an oligophagous insect group (Hemiptera, Psylloidea). *Syst. Biodivers.* **2015**, *13*, 434–454. [CrossRef]
3. Burckhardt, D.; Ouvrard, D.; Percy, D.M. An updated classification of the jumping plant-lice (Hemiptera: Psylloidea) integrating molecular and morphological evidence. *Eur. J. Taxon.* **2021**, *736*, 137–182. [CrossRef]
4. Bertaccini, A. Plants and Phytoplasmas: When bacteria modify plants. *Plants* **2022**, *11*, 1425. [CrossRef] [PubMed]
5. Namba, S. Molecular and biological properties of phytoplasmas. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **2019**, *95*, 401–418. [CrossRef] [PubMed]
6. Jarausch, B.; Tedeschi, R.; Sauvion, N.; Gross, J.; Jarausch, W. Psyllid Vectors. In *Phytoplasmas: Plant Pathogenic Bacteria—II: Transmission and Management of Phytoplasma—Associated Diseases*; Bertaccini, A., Weintraub, P.G., Rao, G.P., Mori, N., Eds.; Springer: Singapore, 2019; pp. 53–78.
7. Bertaccini, A.; Duduk, B.; Paltrinieri, S.; Contaldo, N. Phytoplasmas and phytoplasma diseases: A severe threat to agriculture. *Am. J. Plant Sci.* **2014**, *5*, 1763–1788. [CrossRef]
8. Tedeschi, R.; Alma, A. Transmission of apple proliferation phytoplasma by *Cacopsylla melanoneura* (Homoptera: Psyllidae). *J. Econ. Entomol.* **2004**, *97*, 8–13. [CrossRef]
9. Janik, K.; Barthel, D.; Oppedisano, T.; Anfora, G.; Schuler, H. *Apple Proliferation—A Joint Review*; Laimburg Research Centre: Stadio-Laimburg, Italy, 2020.
10. Fischnaller, S.; Parth, M.; Messner, M.; Stocker, R.; Kerschbamer, C.; Janik, K. Surveying potential vectors of apple proliferation phytoplasma: Faunistic analysis and infection status of selected Auchenorrhyncha species. *Insects* **2021**, *12*, 12. [CrossRef]
11. Tedeschi, R.; Bosco, D.; Alma, A. Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation phytoplasma in northwestern Italy. *J. Econ. Entomol.* **2002**, *95*, 544–551. [CrossRef]
12. Safarova, D.; Stary, M.; Valova, P.; Opatikova, M.; Bilkova, L.; Navratil, M. Impact of insecticides treatment on phytoplasma infection risk in apple orchards. *Hortic. Sci.* **2016**, *43*, 112–116. [CrossRef]
13. Cermak, V.; Lauterer, P. Overwintering of psyllids in South Moravia (Czech Republic) with respect to the vectors of the apple proliferation cluster phytoplasmas. *Bull. Insectol.* **2008**, *61*, 147–148.
14. Malenovsky, I.; Lauterer, P. Jumping plant-lice (Hemiptera: Psylloidea) of the Bílé Karpaty Protected Landscape Area and Biosphere Reserve (Czech Republic). *Acta Musei Moraviae Sci. Biol.* **2012**, *96*, 105–154.
15. Barthel, D.; Schuler, H.; Galli, J.; Borruso, L.; Geier, J.; Heer, K.; Burckhardt, D.; Janik, K. Identification of plant DNA in adults of the phytoplasma vector *Cacopsylla picta* helps understanding its feeding behavior. *Insects* **2020**, *11*, 835. [CrossRef] [PubMed]
16. Jarausch, B.; Fuchs, A.; Schwind, N.; Krczal, G.; Jarausch, W. *Cacopsylla picta* as most important vector for '*Candidatus* Phytoplasma mali' in Germany and neighbouring regions. *Bull. Insectol.* **2007**, *60*, 189–190.

17. Mattedi, L.; Forno, F.; Cainelli, C.; Grando, M.S.; Jarausch, W. Research on Candidatus phytoplasma mali transmission by insect vectors in Trentino. *Acta Hortic.* **2008**, *781*, 369–374. [CrossRef]
18. Jarausch, B.; Schwind, N.; Jarausch, W.; Krczal, G.; Dickler, E.; Seemuller, E. First report of *Cacopsylla picta* as a vector of apple proliferation phytoplasma in Germany. *Plant Dis.* **2003**, *87*, 101. [CrossRef]
19. Mittelberger, C.; Obkircher, L.; Oettl, S.; Oppedisano, T.; Pedrazzoli, F.; Panassiti, B.; Kerschbamer, C.; Anfora, G.; Janik, K. The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny. *Plant Pathol.* **2017**, *66*, 1015–1021. [CrossRef]
20. Mayer, C.J.; Vilcinskas, A.; Gross, J. Chemically mediated multitrophic interactions in a plant-insect vector-phytoplasma system compared with a partially nonvector species. *Agric. Forest Entomol.* **2011**, *13*, 25–35. [CrossRef]
21. Fialova, R.; Navratil, M.; Valova, P.; Lauterer, P. Molecular tests to determine apple proliferation phytoplasma presence in psyllid vectors from apple tree orchards in the Czech Republic. *Acta Hortic.* **2008**, *781*, 471–476. [CrossRef]
22. Tedeschi, R.; Nardi, F. DNA-based discrimination and frequency of phytoplasma infection in the two hawthorn-feeding species, *Cacopsylla melanoneura* and *Cacopsylla affinis*, in northwestern Italy. *Bull. Entomol. Res.* **2010**, *100*, 741–747. [CrossRef]
23. Nokkala, C.; Kuznetsova, V.G.; Rinne, V.; Nokkala, S. Description of two new species of the genus *Cacopsylla* Ossiannilsson, 1970 (Hemiptera, Psylloidea) from northern Fennoscandia recognized by morphology, cytogenetic characters and COI barcode sequence. *Comp. Cytogenet.* **2019**, *13*, 367–382. [CrossRef]
24. Kang, A.R.; Baek, J.Y.; Lee, S.H.; Cho, Y.S.; Kim, W.S.; Han, Y.S.; Kim, I. Geographic homogeneity and high gene flow of the pear psylla, *Cacopsylla pyricola* (Hemiptera: Psyllidae), detected by mitochondrial COI gene and nuclear ribosomal internal transcribed spacer 2. *Anim. Cells Syst.* **2012**, *16*, 145–153. [CrossRef]
25. Cho, G.; Malenovsky, I.; Burckhardt, D.; Inoue, H.; Lee, S. DNA barcoding of pear psyllids (Hemiptera: Psylloidea: Psyllidae), a tale of continued misidentifications. *Bull. Entomol. Res.* **2020**, *110*, 521–534. [CrossRef] [PubMed]
26. Chen, P.; Liu, Q.Z.; Qiao, X.F.; Wang, J.W.; Zhang, T. Identification and phylogenetic analysis of pear psyllids (Hemiptera: Psyllidae) in Chinese pear orchards. *J. Econ. Entomol.* **2018**, *111*, 2908–2913. [CrossRef] [PubMed]
27. Katoh, H.; Inoue, H.; Kuchiki, F.; Ide, Y.; Uechi, N.; Iwanami, T. Identification of a distinct lineage of *Cacopsylla chinensis* (Hemiptera: Psyllidae) in Japan on the basis of two mitochondrial DNA sequences. *J. Econ. Entomol.* **2013**, *106*, 536–542. [CrossRef]
28. Oettl, S.; Schlink, K. Molecular identification of two vector species, *Cacopsylla melanoneura* and *Cacopsylla picta* (Hemiptera: Psyllidae), of apple proliferation disease and further common psyllids of Northern Italy. *J. Econ. Entomol.* **2015**, *108*, 2174–2183. [CrossRef]
29. Percy, D.M.; Crampton-Platt, A.; Sveinsson, S.; Lemmon, A.R.; Lemmon, E.M.; Ouvrard, D.; Burckhardt, D. Resolving the psyllid tree of life: Phylogenomic analyses of the superfamily Psylloidea (Hemiptera). *Syst. Entomol.* **2018**, *43*, 762–776. [CrossRef]
30. Wang, Y.; Cen, Y.; He, Y.; Wu, Y.; Huang, S.; Lu, J. The first complete mitochondrial genome sequence of *Cacopsylla citrisuga* (Yang & Li), a new insect vector of Huanglongbing in Yunnan Province, China. *Mitochondrial DNA B Resour.* **2021**, *6*, 575–577. [CrossRef]
31. Zhao, Y.S.; Luo, X.Y.; Liu, Y.Q.; Shi, A.X.; Cai, W.Z.; Song, F. *Cacopsylla fuscicella* Sp. Nov. (Hemiptera, Psyllidae), a New Loquat Pest in China dagger. *Insects* **2023**, *14*, 414. [CrossRef]
32. Que, S.Q.; Yu, L.P.; Xin, T.R.; Zou, Z.W.; Hu, L.X.; Xia, B. Complete mitochondrial genome of *Cacopsylla coccinae* (Hemiptera: Psyllidae). *Mitochondrial DNA Part A* **2016**, *27*, 3169–3170. [CrossRef]
33. Kang, A.R.; Kim, M.J.; Park, J.S.; Seo, H.J.; Song, J.H.; Won, K.H.; Choi, E.D.; Kim, I. Comparative analysis of two pear pests, *Cacopsylla jukyungi* and *Cacopsylla burckhardti* (Hemiptera: Psyllidae), based on complete mitochondrial genomes and comparison to confamilial species. *Agronomy* **2022**, *12*, 37. [CrossRef]
34. Jo, E.; Cho, G. The complete mitochondrial genome of *Cacopsylla burckhardti* (Hemiptera, Psylloidea, Psyllidae). *Biodivers. Data J.* **2022**, *10*, e85094. [CrossRef]
35. Zahradnicek, P.; Brazdil, R.; Stepanek, P.; Trnka, M. Reflections of global warming in trends of temperature characteristics in the Czech Republic, 1961–2019. *Int. J. Climatol.* **2021**, *41*, 1211–1229. [CrossRef]
36. Rousi, E.; Fink, A.H.; Andersen, L.S.; Becker, F.N.; Beobide-Arsuaga, G.; Breil, M.; Cozzi, G.; Heinke, J.; Jach, L.; Niermann, D.; et al. The extremely hot and dry 2018 summer in central and northern Europe from a multi-faceted weather and climate perspective. *Nat. Hazard. Earth Syst.* **2023**, *23*, 1699–1718. [CrossRef]
37. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [CrossRef]
38. Bernt, M.; Donath, A.; Juhling, F.; Externbrink, F.; Florentz, C.; Fritzsch, G.; Putz, J.; Middendorf, M.; Stadler, P.F. MITOS: Improved de novo metazoan mitochondrial genome annotation. *Mol. Phylogenet. Evol.* **2013**, *69*, 313–319. [CrossRef]
39. Tamura, K.; Stecher, G.; Kumar, S. MEGA11 Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef]
40. The World Psylloidea Database (from Psyl'list). Available online: https://data.nhm.ac.uk/dataset/psyl-list/resource/8746ceec-4846-4899-b607-9ba603002033 (accessed on 12 July 2023).
41. Tedeschi, R.; Lauterer, P.; Brusetti, L.; Tota, F.; Alma, A. Composition, abundance and phytoplasma infection in the hawthorn psyllid fauna of northwestern Italy. *Eur. J. Plant Pathol.* **2009**, *123*, 301–310. [CrossRef]

42. Tsai, C.L.; Lee, H.C.; Cho, G.; Liao, Y.C.; Yang, M.M.; Yeh, W.B. Invasive and quarantine risks of *Cacopsylla chinensis* (Hemiptera: Psyllidae) in East Asia: Hybridization or gene flow between differentiated lineages. *J. Econ. Entomol.* **2020**, *113*, 2890–2899. [CrossRef]
43. Sauvion, N.; Peccoud, J.; Meynard, C.N.; Ouvrard, D. Occurrence data for the two cryptic species of *Cacopsylla pruni* (Hemiptera: Psylloidea). *Biodivers. Data J.* **2021**, *9*, e68860. [CrossRef]
44. Peccoud, J.; Labonne, G.; Sauvion, N. Molecular test to assign individuals within the *Cacopsylla pruni* complex. *PLoS ONE* **2013**, *8*, e72454. [CrossRef]