Bulletin of Entomological Research

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DNA barcoding of pear psyllids (Hemiptera: Psylloidea: Psyllidae), a tale of continued misidentifications

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Research Paper

Cite this article: Cho G, Malenovský I, Burckhardt D, Inoue H, Lee S (2020). DNA barcoding of pear psyllids (Hemiptera: Psylloidea: Psyllidae), a tale of continued misidentifications. *Bulletin of Entomological Research* **110**, 521–534. https://doi.org/10.1017/S0007485320000012

Received: 30 October 2018
Revised: 23 June 2019
Accepted: 31 October 2019
First published online: 10 February 2020

Keywords:
Cacopsylla; jumping plant lice; molecular diagnostics; pest identification; phytoplasma vectors

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Abstract

Pear psyllids (Hemiptera: Psylloidea: Psyllidae: *Cacopsylla* spp.) belong to the most serious pests of pear (*Pyrus* spp.). They damage pear trees by excessive removal of phloem sap, by soiling the fruits with honeydew which, in turn, provides a substrate for sooty mould, and by transmission of *Candidatus* Phytoplasma spp., the causal agents of the pear decline disease. The morphological similarity, the presence of seasonal dimorphism that affects adult colour, size and wing morphology and uncritical use of species names, led to much confusion in the taxonomy of pear psyllids. As a result, pear psyllids have been frequently misidentified. Many of the entries attributed to *Cacopsylla pyricola* and other species in the GenBank are misidentifications which led to additional, unnecessary confusion. Here we analysed DNA barcodes of 11 pear psyllid species from eastern Asia, Europe and Iran using four mitochondrial gene fragments (COI 658 bp, COI 403 bp, COI-tRNA^{leu}-COII 580 bp and 16S rDNA 452 bp). The efficiency of identification was notably high and considerable barcoding gaps were observed in all markers. Our results confirm the synonymies of the seasonal forms of *Cacopsylla jukyungi* (= *C. cinereosignata*, winter form) and *C. maculatili* (= *C. qiuzili*, summer form) previously suggested based on morphology. Some previous misidentifications (*C. chinensis* from China, Japan and Korea = misidentification of *C. jukyungi*; *C. pyricola* and *C. pyrisuga* from East Asia = misidentification of *C. jukyungi* and *C. burckhardti*, respectively; *C. pyricola* from Iran = misidentification of *C. bidens*, *C. pyri* and *Cacopsylla* sp.) are also corrected. There is no evidence for the presence of European pear psyllid species in East Asia.

Introduction

DNA barcoding is a fast and efficient method for species identification using short standardized sequences. It is commonly used for identification of quarantine and agricultural pests (Hebert *et al.*, 2003; Hebert and Ratnasingham, 2007; Park *et al.*, 2010; Shin *et al.*, 2013). DNA-based methodologies are also useful for species identification in taxa where females or immatures lack diagnostic morphological characters (Boehme *et al.*, 2010).

Pear psyllids (Hemiptera: Psylloidea: Psyllidae: *Cacopsylla* spp.) are major pests of pear (*Pyrus* spp.) in the Palaearctic region and, as introductions, in the New World (Valle *et al.*, 2017). They inflict damage by excessive removal of phloem sap and by soiling the fruits with honeydew which, in turn, provides a substrate for sooty mould (Hodkinson, 1984; Burckhardt and Hodkinson, 1986; Burckhardt, 1994). Some species are known as vectors of *Candidatus* Phytoplasma spp., the causal agents of the pear decline disease (Weintraub and Jones, 2010; Seemüller *et al.*, 2011). In the past, the presence of seasonal dimorphism, controlled by temperature and photoperiod (Soroker *et al.*, 2013), that affect adult colour, size and wing morphology in some species (fig. 1), as well as uncritical use of species names led to much confusion in the taxonomy of pear psyllids (Burckhardt and Hodkinson, 1986; Cho *et al.*, 2017). Several revisions based on morphology addressed and solved many of these problems (Burckhardt and Hodkinson, 1986; Yang *et al.*, 2004; Luo *et al.*, 2012; Cho *et al.*, 2017). Currently, 34 species of *Cacopsylla* developing on *Pyrus* are considered valid but more work is needed, in particular on the psyllid fauna of the Middle East, India, Central Asia and Far East Russia, to completely untangle the confused taxonomy of the group (Cho *et al.*, 2017).

Several recent molecular studies (Kang *et al.*, 2012; Katoh *et al.*, 2013, 2014; Cho and Lee, 2015; Chen *et al.*, 2018) have addressed the problems of identity of and phylogenetic relationships between populations of pear psyllids. Judging from the respective GenBank entries, several of these sequences were attributed to the wrong species, adding to the confusion.

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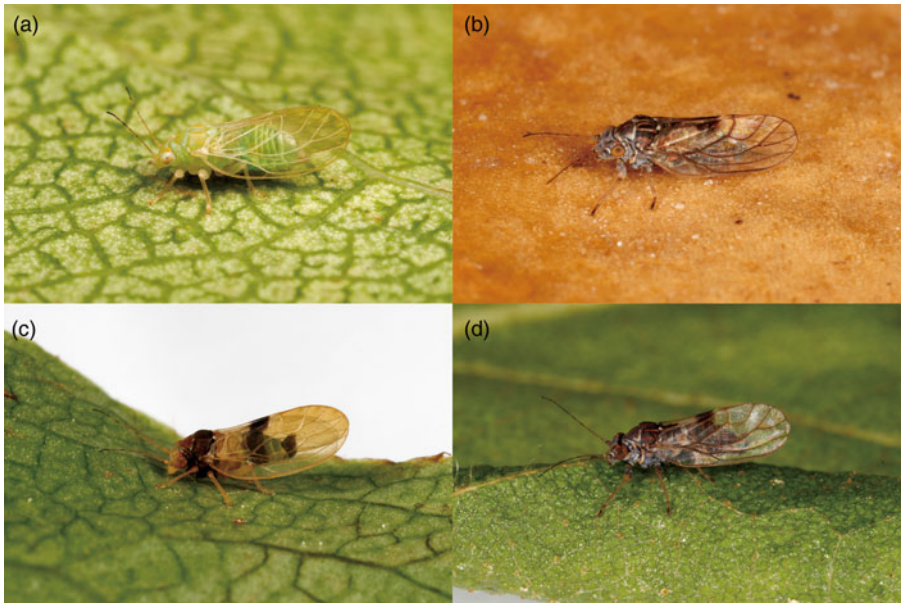

Figure 1. Habitus of seasonally dimorphic polyvoltine pear psyllids: (a) *C. jukyungi* (summer form); (b) *C. jukyungi* (winter form); (c) *C. maculatili* (summer form); (d) *C. maculatili* (winter form). Photos are from Cho *et al.* (2017), with modifications.

Here, we test the suitability of DNA barcoding for the identification of pear psyllid species with a particular focus on the East Asian fauna. East Asia (China, Japan, Taiwan and Korea) constitutes one of the largest and most important pear producing regions of the world (FAO, 2016). We also rectify some of the wrong entries in the GenBank.

Materials and methods

Sampling, identification and data acquisition

Samples were collected by G. Cho in Korea in 2014–2015, H. Inoue in Japan in 2014 and I. Malenovský, L. Štarhová Serbina and P. Lauterer in central Europe (Czech Republic, Poland and Slovenia) in 2008–2018. They were stored in 95–99% ethanol at −20°C for genomic DNA extraction. DNA-extracted samples were permanently mounted on microscopic slides in Canada balsam as voucher specimens. The vouchers are deposited at the College of Agriculture and Life Sciences (CALS), Seoul National University (SNU), the Republic of Korea. Specimens were identified based on morphology using the keys by Burckhardt and Hodkinson (1986) and Cho *et al.* (2017). Additional sequences of several pear psyllid species from China, Japan and Taiwan published by Lee *et al.* (2007, 2008), Katoh *et al.* (2013) and Chen *et al.* (2018) and unpublished sequences of '*Cacopsylla pyricola*' from France and Iran were downloaded from the GenBank (table 1).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from single individuals using a DNeasy Blood and Tissue kit (QIAGEN, Germany). To make voucher specimens, we used a non-destructive DNA extraction protocol by Kim *et al.* (2010) modified by leaving samples in the lysis buffer with proteinase K solution at 56°C overnight instead of 24 h. Polymerase chain reaction (PCR) was performed with the PTC-100 thermocycler (MJ Research Inc., USA) using AccuPower PCR premix (BIONEER, Korea). A reaction mixture (20 µl) contained 1 unit of *Top* DNA polymerase (BIONEER, Korea), 250 µM of dNTP, 10 mM of Tris-HCl, 30 mM of KCl and 1.5 mM of $MgCl_2$, 1 µl of each primer (10 µM) and 5–20 ng of template DNA. The primers used for amplification and PCR conditions are listed in table 2. PCR products were cleaned using a QIAquick PCR purification kit (QIAGEN, Inc.) and directly sequenced using an automated sequencer (ABI PrismH 3730 XL DNA Analyzer) at Macrogen, Inc.

Sequence alignment and phylogenetic analysis

All sequences were initially assembled and examined using SeqMan Pro ver. 7.1.0 (DNASTAR, Inc., USA). Alignment of the DNA sequences was conducted online using the MAFFT ver. 7 package (Katoh and Toh, 2008; Katoh *et al.*, 2002, 2005) on the server (http://mafft.cbrc.jp/alignment/software/). The COI and COI-tRNA^{leu}-COII sequences were aligned using the FFT-NS-I strategy implemented in MAFFT with the default settings. The Q-INS-I strategy was chosen for the 16S rDNA gene using the default setting which considers RNA secondary structure and small data sets (Katoh *et al.*, 2002). COI and COII sequences translation was checked in MEGA 6 (Tamura *et al.*, 2013) for the presence of in-frame stop codons and indels, which can indicate nuclear mitochondrial pseudogenes (NUMTs), generally known to be an impediment to DNA barcoding (Song *et al.*, 2008; Leite, 2012). For the aligned data set, phylogenetic trees were constructed using the neighbour-joining (NJ) algorithm with bootstrap support analysis (1000 replicates) in MEGA 6 based on a Kimura 2-Parameter (K2P) model. This has been the most widely used method for DNA barcoding analyses (e.g. Yeh *et al.*, 1997; Shin *et al.*, 2013; Gwiazdowski *et al.*, 2015; Wu *et al.*, 2016; Amouroux *et al.*, 2017; Kanturski *et al.*, 2018; Song *et al.*, 2018), including previous studies on pear psyllids (Lee *et al.*, 2008; Kang *et al.*, 2012; Katoh *et al.*, 2013, 2014; Chen *et al.*, 2018). To compare our results with those papers, we preferred to use the same methodology despite some potential limitations, such as a poor fit of the K2P model at the species level (Srivathsan and Meier, 2012; Collins *et al.*, 2012). Eleven pear psyllid species of the genus *Cacopsylla* (Psyllidae: Psyllinae) were included into the analyses and two *Acizzia* species (Psyllidae: Acizzinae) were

https://doi.org/10.1017/S0007485320000012 Published online by Cambridge University Press

Table 1. Pear psyllid sequences of COI-tRNA^{leu}-COII, COI658, COI403, and 16S rDNA used in this study.

Species	Abbreviation	Form	Country	GenBank number				Name in GenBank	Ref.
				COI-tRNA^{leu}-COII	COI658	COI403	16S		
C. bidens	162-1	Adult	Slovenia	MK039659	N/A	N/A	MK039579		
	162-2	Adult	Slovenia	MK039660	N/A	MK039595	MK039580		
	Z20		Iran	N/A	N/A	KT258661	N/A	*C. pyricola*	Unpublished
	Z8		Iran	N/A	N/A	KT258660	N/A	*C. pyricola*	Unpublished
	Z		Iran	N/A	N/A	KT258659	N/A	*C. pyricola*	Unpublished
	T22		Iran	N/A	N/A	KT258658	N/A	*C. pyricola*	Unpublished
	T12		Iran	N/A	N/A	KT258657	N/A	*C. pyricola*	Unpublished
	T8		Iran	N/A	N/A	KT258656	N/A	*C. pyricola*	Unpublished
	SH22		Iran	N/A	N/A	KT258655	N/A	*C. pyricola*	Unpublished
	SH20		Iran	N/A	N/A	KT258654	N/A	*C. pyricola*	Unpublished
	SH12		Iran	N/A	N/A	KT258653	N/A	*C. pyricola*	Unpublished
	SH		Iran	N/A	N/A	KT258652	N/A	*C. pyricola*	Unpublished
	O22		Iran	N/A	N/A	KT258651	N/A	*C. pyricola*	Unpublished
	O20		Iran	N/A	N/A	KT258650	N/A	*C. pyricola*	Unpublished
	K22		Iran	N/A	N/A	KT258647	N/A	*C. pyricola*	Unpublished
	K20		Iran	N/A	N/A	KT258646	N/A	*C. pyricola*	Unpublished
	K8		Iran	N/A	N/A	KT258645	N/A	*C. pyricola*	Unpublished
	G23		Iran	N/A	N/A	KT258644	N/A	*C. pyricola*	Unpublished
	G22		Iran	N/A	N/A	KT258643	N/A	*C. pyricola*	Unpublished
	G13		Iran	N/A	N/A	KT258642	N/A	*C. pyricola*	Unpublished
	G3		Iran	N/A	N/A	KT258641	N/A	*C. pyricola*	Unpublished
	F22		Iran	N/A	N/A	KT258640	N/A	*C. pyricola*	Unpublished
	F20		Iran	N/A	N/A	KT258639	N/A	*C. pyricola*	Unpublished
	F8		Iran	N/A	N/A	KT258638	N/A	*C. pyricola*	Unpublished
	F1		Iran	N/A	N/A	KT258637	N/A	*C. pyricola*	Unpublished
	A22		Iran	N/A	N/A	KT258636	N/A	*C. pyricola*	Unpublished
	A12		Iran	N/A	N/A	KT258635	N/A	*C. pyricola*	Unpublished
C. burckhardti	Cp-2fs	Adult	Japan	AB721007	N/A	N/A	AB721003	*C. pyrisuga*	Katoh *et al.* (2013)
	Cp-3ms	Adult	Japan	AB721008	N/A	N/A	AB721004	*C. pyrisuga*	Katoh *et al.* (2013)
	Cp-5fs	Adult	Japan	AB721009	N/A	N/A	AB721005	*C. pyrisuga*	Katoh *et al.* (2013)

(*Continued*)

Table 1. (*Continued.*)

Species	Abbreviation	Form	Country	GenBank number				Name in GenBank	Ref.
				COI-tRNA^{leu}-COII	COI658	COI403	16S		
	Cp-6ms	Adult	Japan	AB721010	N/A	N/A	AB721006	*C. pyrisuga*	Katoh *et al.* (2013)
	10-1	Adult	South Korea	N/A	MK039639	MK039614	MK039560		
	10-2	Immature	South Korea	MK039675	MK039640	MK039615	N/A		
	25-1	Adult	South Korea	MK039673	MK039638	MK039616	MK039570		
	25-2	Adult	South Korea	MK039674	N/A	MK039617	MK039571		
C. chinensis	JA-1	Summer	Taiwan	AB364024	N/A	N/A	AB363708	*C. chinensis*	Lee *et al.* (2007)
	JA-2	Summer	Taiwan	AB364025	N/A	N/A	AB363709	*C. chinensis*	Lee *et al.* (2007)
	JA-3	Summer	Taiwan	AB364026	N/A	N/A	AB363710	*C. chinensis*	Lee *et al.* (2007)
	JB	Summer	Taiwan	AB364027	N/A	N/A	AB363711	*C. chinensis*	Lee *et al.* (2007)
	CL-A1	Summer	Taiwan	AB364009	N/A	N/A	AB363693	*C. chinensis*	Lee *et al.* (2007)
	CL-A2	Summer	Taiwan	AB364010	N/A	N/A	AB363694	*C. chinensis*	Lee *et al.* (2007)
	CL-B1	Summer	Taiwan	AB364011	N/A	N/A	AB363695	*C. chinensis*	Lee *et al.* (2007)
	CL-B2	Summer	Taiwan	AB364012	N/A	N/A	AB363696	*C. chinensis*	Lee *et al.* (2007)
	CL-C1	Summer	Taiwan	AB364013	N/A	N/A	AB363697	*C. chinensis*	Lee *et al.* (2007)
	CL-C2	Summer	Taiwan	AB364014	N/A	N/A	AB363698	*C. chinensis*	Lee *et al.* (2007)
	LB	Summer	Taiwan	AB364030	N/A	N/A	AB363714	*C. chinensis*	Lee *et al.* (2007)
	LC-F	Winter	Taiwan	AB364031	N/A	N/A	AB363715	*C. chinensis*	Lee *et al.* (2007)
	LC-M	Winter	Taiwan	AB364032	N/A	N/A	AB363716	*C. chinensis*	Lee *et al.* (2007, 2008)
	TA	Winter	Taiwan	AB364039	N/A	N/A	AB363723	*C. chinensis*	Lee *et al.* (2007)
	TA-F	Winter	Taiwan	AB364040	N/A	N/A	AB363724	*C. chinensis*	Lee *et al.* (2007)
	TA-M	Winter	Taiwan	AB364041	N/A	N/A	AB363725	*C. chinensis*	Lee *et al.* (2007)
	TC-M	Winter	Taiwan	AB364042	N/A	N/A	AB363726	*C. chinensis*	Lee *et al.* (2007)
	TD-A	Summer	Taiwan	AB364043	N/A	N/A	AB363727	*C. chinensis*	Lee *et al.* (2007)
	TD-N	Immature	Taiwan	AB364044	N/A	N/A	AB363728	*C. chinensis*	Lee *et al.* (2007, 2008)
	HD	summer	Taiwan	AB364018	N/A	N/A	AB363702	*C. chinensis*	Lee *et al.* (2007)
	HE-1	Summer	Taiwan	AB364019	N/A	N/A	AB363703	*C. chinensis*	Lee *et al.* (2007)
	HE-2	Winter	Taiwan	AB364020	N/A	N/A	AB363704	*C. chinensis*	Lee *et al.* (2007)
	HG-1	Winter	Taiwan	AB364021	N/A	N/A	AB363705	*C. chinensis*	Lee *et al.* (2007)
	HG-2	Winter	Taiwan	AB364022	N/A	N/A	AB363706	*C. chinensis*	Lee *et al.* (2007, 2008)
	HG-3	Winter	Taiwan	AB364023	N/A	N/A	AB363707	*C. chinensis*	Lee *et al.* (2007)
	KA-F	Summer	Taiwan	AB364028	N/A	N/A	AB363712	*C. chinensis*	Lee *et al.* (2007)

	KA-M	Summer	Taiwan	AB364029	N/A	N/A	AB363713	*C. chinensis*	Lee *et al.* (2007)
	MC-CD	Winter	Taiwan	AB364038	N/A	N/A	AB363722	*C. chinensis*	Lee *et al.* (2007)
	MB-CL	Winter	Taiwan	AB364037	N/A	N/A	AB363721	*C. chinensis*	Lee *et al.* (2007)
	BA-A	Summer	China	AB364007	N/A	N/A	AB361072	*C. chinensis*	Lee *et al.* (2007)
	BA-N	Immature	China	AB364008	N/A	N/A	AB361073	*C. chinensis*	Lee *et al.* (2007)
	CuA	Summer	China	AB364015	N/A	N/A	AB363699	*C. chinensis*	Lee *et al.* (2007)
C. jukyungi	IC-1	Summer	South Korea	MK039649	N/A	N/A	MK039564		
	IC-2	Summer	South Korea	MK039650	N/A	N/A	MK039565		
	SW-1	Summer	South Korea	MK039651	N/A	N/A	MK039566		
	SW-2	Summer	South Korea	MK039652	N/A	N/A	MK039567		
	22-1	Summer	South Korea	MK039647	MK039623	MK039600	MK039556		
	22-2	Summer	South Korea	MK039648	MK039622	MK039601	MK039557		
	I-1mc	Summer	Japan	AB720877	N/A	N/A	AB720878	*C. chinensis*	Katoh *et al.* (2013)
	113-1	Winter	South Korea	MK039645	MK039624	MK039603	MK039568		
	113-2	Winter	South Korea	MK039646	MK039625	MK039602	MK039569		
	CP2067		South Korea	N/A	JF327670	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2070		South Korea	N/A	JF327671	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2071		South Korea	N/A	JF327672	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2072		South Korea	N/A	JF327673	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2073		South Korea	N/A	JF327674	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2074		South Korea	N/A	JF327675	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2158		South Korea	N/A	JF327676	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2159		South Korea	N/A	JF327677	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2160		South Korea	N/A	JF327678	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2162		South Korea	N/A	JF327679	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2163		South Korea	N/A	JF327680	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2164		South Korea	N/A	JF327681	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2273		South Korea	N/A	JF327682	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2274		South Korea	N/A	JF327683	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2276		South Korea	N/A	JF327684	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2277		South Korea	N/A	JF327685	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2280		South Korea	N/A	JF327686	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2281		South Korea	N/A	JF327687	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2282		South Korea	N/A	JF327688	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)

(*Continued*)

Table 1. (*Continued.*)

Species	Abbreviation	Form	Country	GenBank number				Name in GenBank	Ref.
				COI-tRNA^{leu}-COII	COI658	COI403	16S		
	CP2283		South Korea	N/A	JF327689	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2284		South Korea	N/A	JF327690	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2326		South Korea	N/A	JF327691	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2327		South Korea	N/A	JF327692	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2328		South Korea	N/A	JF327693	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2331		South Korea	N/A	JF327694	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2332		South Korea	N/A	JF327695	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2347		South Korea	N/A	JF327696	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2348		South Korea	N/A	JF327697	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2373		South Korea	N/A	JF327698	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2374		South Korea	N/A	JF327699	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2375		South Korea	N/A	JF327700	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2379		South Korea	N/A	JF327701	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2380		South Korea	N/A	JF327702	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2381		South Korea	N/A	JF327703	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2382		South Korea	N/A	JF327704	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2383		South Korea	N/A	JF327705	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2384		South Korea	N/A	JF327706	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2386		South Korea	N/A	JF327707	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2387		South Korea	N/A	JF327708	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	CP2388		South Korea	N/A	JF327709	N/A	N/A	*C. pyricola*	Kang *et al.* (2012)
	JLHL	Summer	China	MG905761	N/A	N/A	MG905759	*C. chinensis*	Chen *et al.* (2018)
	JLYL2	summer	China	MG905762	N/A	N/A	MG905759	*C. chinensis*	Chen *et al.* (2018)
C. maculatili	26-1	Summer	South Korea	MK039653	N/A	MK039604	MK039572		
	26-2	Summer	South Korea	MK039655	MK039626	MK039605	MK039573		
	27-1	Winter	South Korea	MK039654	MK039627	MK039606	MK039574		
	27-2	Winter	South Korea	MK039656	MK039628	MK039607	MK039575		
	160-1	Summer	Japan	MK039657	MK039629	MK039608	MK039576		
	160-2	Summer	Japan	MK039658	MK039630	MK039609	MK039577		
C. pyri	163-1	Adult	Czech Republic	MK039661	MK039631	MK039596	MK039581		
	163-2	Adult	Czech Republic	MK039664	MK039634	MK039597	MK039582		

	163-3	Adult	Czech Republic	MK039662	MK039632	MK039598	MK039583		
	163-4	Adult	Czech Republic	MK039663	MK039633	MK039599	MK039584		
	O15		Iran	N/A	N/A	KT258649	N/A	*C. pyricola*	Unpublished
	O8		Iran	N/A	N/A	KT258648	N/A	*C. pyricola*	Unpublished
C. pyricola	164-1	Adult	Poland	MK039669	MK039635	MK039590	MK039585		
	164-2	Adult	Poland	MK039666	N/A	N/A	MK039586		
	164-3	Adult	Czech Republic	MK039667	N/A	MK039592	MK039587		
	164-4	Adult	Czech Republic	MK039670	N/A	MK039591	MK039588		
	164-5	Adult	Czech Republic	MK039665	N/A	MK039593	MK039589		
	164-6	Adult	Czech Republic	MK039668	N/A	MK039594	MK039578		
		N/A	France	N/A	N/A	AF493566	N/A	*C. pyricola*	Unpublished
		N/A	N/A	N/A	N/A	MG988673	N/A	*C. pyricola*	Percy *et al.* (2018)
C. pyrisuga	165-1	Adult	Czech Republic	N/A	N/A	MK039612	N/A		
	165-2	Adult	Czech Republic	N/A	N/A	MK039613	N/A		
C. sandolbaea	24-1	Adult	South Korea	MK039671	MK039636	MK039610	MK039558		
	24-2	Adult	South Korea	MK039672	MK039637	MK039611	MK039559		
C. qianli	MA	Adult	Taiwan	AB364033	N/A	N/A	AB363717	*C. qianli*	Lee *et al.* (2008)
	MA-Q	Adult	Taiwan	AB364034	N/A	N/A	AB363718	*C. qianli*	Lee *et al.* (2008)
	MB-Q	Adult	Taiwan	AB364035	N/A	N/A	AB363719	*C. qianli*	Lee *et al.* (2008)
	MC-Q	Adult	Taiwan	AB364036	N/A	N/A	AB363720	*C. qianli*	Lee *et al.* (2008)
Cacopsylla sp.	527	N/A	Iran	N/A	N/A	KP192848	N/A	*C. pyricola*	Unpublished
	525	N/A	Iran	N/A	N/A	KP843860	N/A	*C. pyricola*	Unpublished
Acizzia jamatonica	1-1	Adult	South Korea	N/A	MK039641	MK039620	MK039561		
	1-2	Adult	South Korea	MK039676	MK039642	MK039621	N/A		
Acizzia sasakii	2-1	Adult	South Korea	MK039677	MK039643	MK039618	MK039562		
	2-2	Adult	South Korea	MK039678	MK039644	MK039619	MK039563		

Table 2. Primers and PCR conditions used in this study.

Gene region	Length	Primer		Reference	Condition	Reference
COI-tRNA^{leu}-COII	580	UEA9	GTAAACCTAACATTTTTTCCTCAACA	Lunt *et al.* (1996)	An initial denaturation at 95°C for 10 min, 35 amplification cycles (95°C for 50 s, 45°C for 1 min, and 72°C for 1 min), a final extension at 72°C for 10 min	Katoh *et al.* (2013)
		C2-N-3389	TCATAAGTTCARTATCATTG	Simon *et al.* (1994)		Katoh *et al.* (2013)
		DP1	GTTAGTAGTGGGTTATTAAGTTCRTC	Percy (2003)		Katoh *et al.* (2013)
		DP2	CGATAATTTTAATTGTTAGTAGYGG	Percy (2003)		Katoh *et al.* (2013)
		UEA9-MOD	GGTATGCCTCGTCGTTATTCTAAYTAYC	Percy (2003)		Katoh *et al.* (2013)
COI	658	CPF4	TAAGAACTAACCATAAGATTATCGG	Kang *et al.* (2012)	An initial denaturation at 94°C for 7 min, 35 amplification cycles (94°C for 1 min, 50–60°C for 1 min, and 72°C for 1 min), a final extension at 72°C for 7 min	Kang *et al.* (2012)
		CPR4	CACTTCAGGGTGTCCAAAGAATC	Kang *et al.* (2012)		Kang *et al.* (2012)
COI	403	C1-J1709	AATTGGWGGWTTYGGAAAYTG	Simon *et al.* (2006)	An initial denaturation at 94°C for 5 min, 40 amplification cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 1 min), a final extension at 72°C for 7 min	Martoni *et al.* (2018)
		HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* (1994)		Martoni *et al.* (2018)
16S	452	16SR21	GCGTGTTTATCAAAAACAT	Yeh *et al.* (1997)	35 amplification cycles (95°C for 50 s, 50°C for 1 min, and 72°C for 2 min)	Katoh *et al.* (2013)
		16S22	CCGGTCTGAACTCAGATCA	Yeh *et al.* (1997)		Katoh *et al.* (2013)

used as outgroups (table 1). Pairwise distances were also computed using MEGA 6 (Tamura *et al.*, 2013).

Results

A total of 375 aligned sequences (COI 403–658 bp, COI-tRNA^{leu}-COII 580 bp and 16S rDNA 452 bp sequence fragments; table 1) of 11 pear psyllid species and two outgroup species were analysed (table 1). All species are characterized by a distinctive set of COI, COI-tRNA^{leu}-COII and 16S rDNA sequences that form well-supported clusters in the NJ-trees (bootstrap values of 94–100%; fig. 2). No internal stop codons or frame shifts were detected in the aligned COI and COII sequences, suggesting that none derive from pseudogenes (NUMTs).

Korean '*Cacopsylla pyricola*' sequences from the GenBank (Kang *et al.*, 2012) show no significant divergence from *C. jukyungi* in the COI gene fragment (fig. 2) but significantly differ from European specimens of *C. pyricola*; this indicates that the material of Kang *et al.* (2012) was misidentified, as was suggested by Cho *et al.* (2017). Also, all GenBank sequences under '*Cacopsylla pyricola*' from Iran by Zohdi and Hossini and Zendehdel *et al.* (data deposited in the GenBank without reference to a published article) are misidentified; based on our analysis, these specimens belong to three different species, viz. *C. pyri*, *C. bidens* and *Cacopsylla* sp. (fig. 2, table 1). The last taxon is close to *C. pyri* but distinct with a mean of 16.6% genetic difference (range 12.4–20.3%). This species may be *C. permixta* though the corresponding material was not available for morphological identification. Another previous misidentification concerns the sequences of '*C. pyrisuga*' by Katoh *et al.* (2013) from Japan. Based on our analysis, they are conspecific with samples of *C. burckhardti* from Korea (fig. 2).

Chinese and Taiwanese *Cacopsylla chinensis* sequences (Lee *et al.*, 2007, 2008) together form well-supported clusters in the NJ trees based on COI-tRNA^{leu}-COII and 16S rDNA gene fragments (fig. 2). The sequences of COI-tRNA^{leu}-COII and 16S

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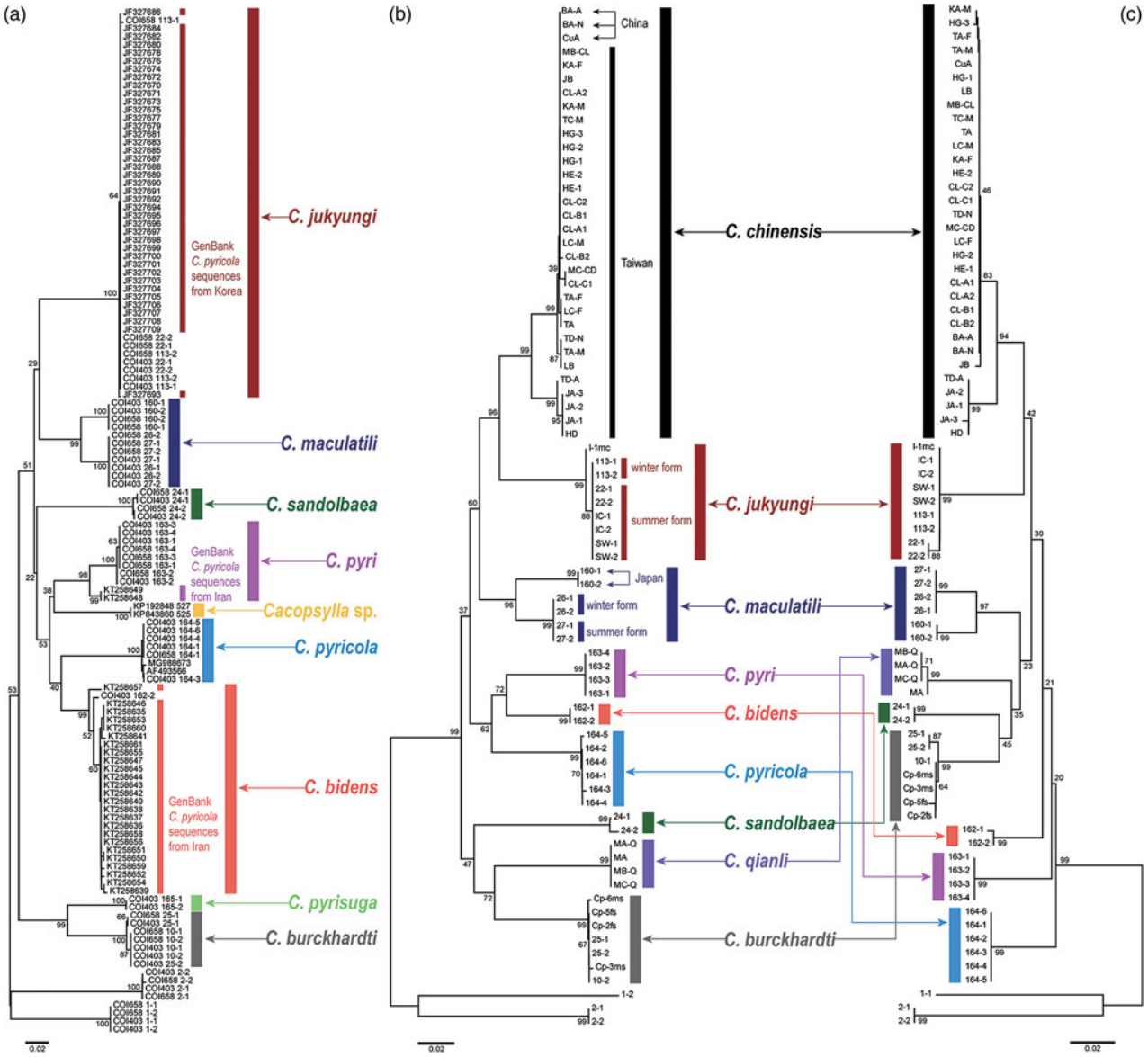

Figure 2. NJ trees based on Kimura 2-parameter genetic distance: (a) COI; (b) COI-tRNA^{leu}-COII; (c) 16S rDNA. *Bootstrap support values are shown at the branch points and are based on 1000 replications.

rDNA of *C. chinensis* lineage III from Northeast China (Jilin and Heilongjang, near North Korea) by Chen *et al.* (2018) and *C. jukyungi* from Korea are identical suggesting that the two taxa are conspecific and that the former constitutes a misidentification. Japanese (Katoh *et al.*, 2014) and Korean *C. jukyungi* (of both summer and winter forms) sequences are monophyletic, supported by 99% bootstrap values in COI-tRNA^{leu}-COII and 16S rDNA NJ trees (fig. 2). The conspecificity of the summer and winter forms of *C. maculatili* is also highly supported by bootstrap values of 99% in COI-tRNA^{leu}-COII and 16S rDNA NJ trees as well. *Cacopsylla sandolbaea* and *C. qianli* form distinct clades recognized in all NJ trees (fig. 2).

The mean intraspecific K2P distance is 0.1% in COI 658 bp (range 0–5.9%), 0.7% in COI 403 bp (range 0–5.5%), 1% in COI-tRNA^{leu}-COII (range 0–5.9%) and 0.6% in 16S rDNA (range 0–3.8%), with a maximum observed value of 5.9% for *C. maculatili* (fig. 3, table 3). The interspecific divergences (K2P distance) between the examined *Cacopsylla* species average 15.7% (range 12.6–20.8%) in COI 658 bp, 15.7% (range 8.0–22.5%) in COI 403 bp, 12.4% (range 7.6–18.3%) in COI-tRNA^{leu}-COII and 7.5% (range 4.4–10.3%) in 16S rDNA (table 3). Significant barcoding gaps are thus observed between the intra- and interspecific K2P distance divergences of congeneric species (fig. 3, table 3). The barcoding gaps average 4% for all gene fragments. Intra- and inter-specific variations for the examined pear psyllids are shown in table 3.

In most analyses, the western Palaearctic species *C. pyri*, *C. pyricola* and *C. bidens* form a monophyletic clade (including *Cacopsylla* sp. from Iran in the COI tree), albeit with only a moderate support, while *C. chinensis* and *C. jukyungi* are strongly to moderately supported as sister species. *Cacopsylla pyrisuga* and *C. burckhardti* constitute sister species in the COI tree (fig. 2).

Discussion

The present study shows that DNA barcoding can correctly identify the species of pear psyllids (*Cacopsylla* spp.). Overall, the

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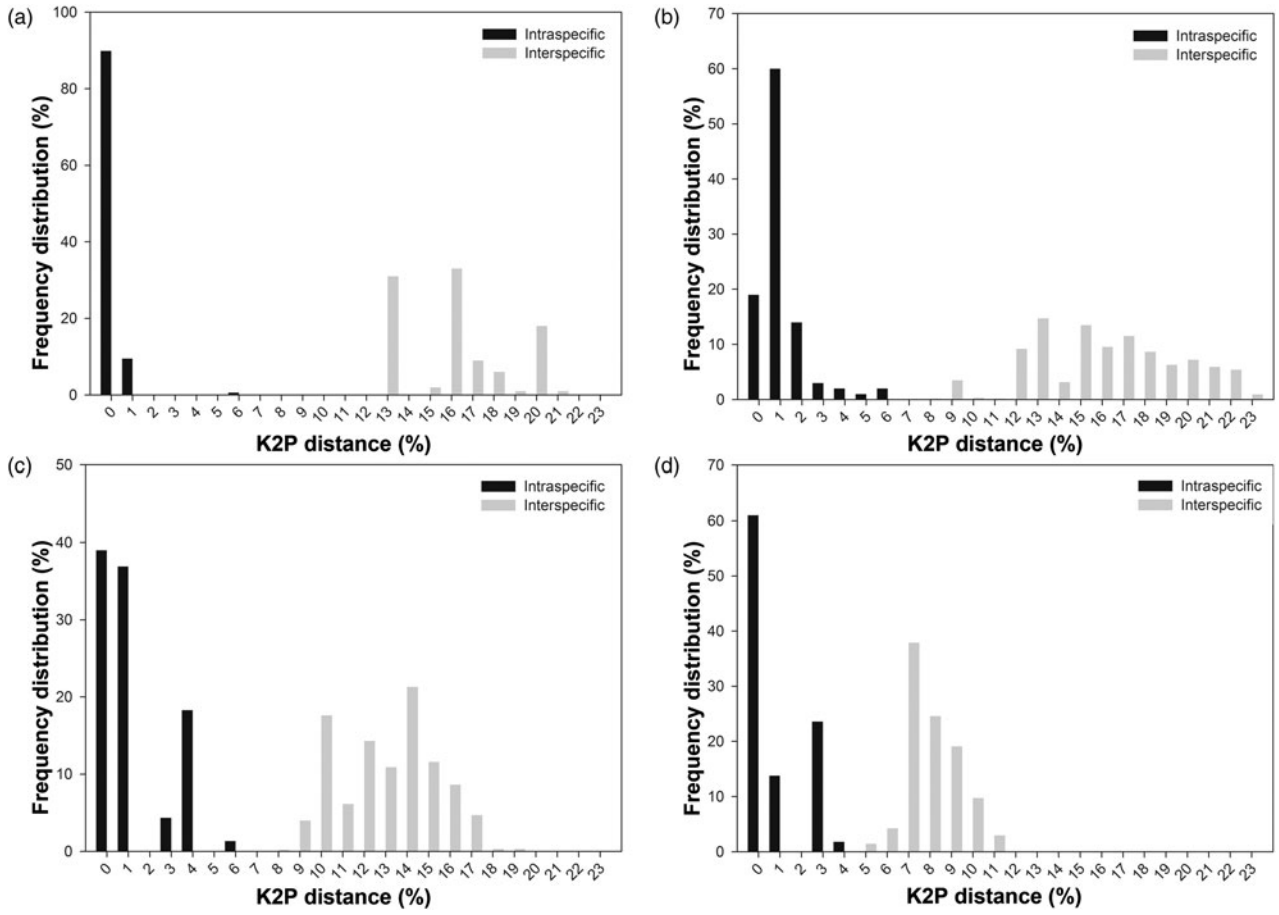

Figure 3. Frequency distributions of the intra- and interspecific K2P distances for congeneric sequences: (a) COI 658 bp; (b) COI 403 bp; (c) COI-tRNA^{leu}-COII; (d) 16S rDNA.

identification efficiency using DNA barcoding is extremely high (100%) and all morphologically recognized species are clearly separated in all NJ trees (fig. 2). All markers (COI, COI-tRNA^{leu}-COII and 16S rDNA) with criteria (K2P distance) performed well and no overlap between intra- and inter-specific divergences is observed in any of the analyses (fig. 3). The COI and COI-tRNA^{leu}-COII fragments are more effective for comparison of relatively closely related (congeneric) species than 16S rDNA because of wider gaps and divergences (fig. 3). A DNA study of west Palaearctic species of *Arytaina*, *Arytinnis* and *Livilla* (Psyllinae) associated with Fabaceae showed maximum intraspecific variation generally of less than 3% in case of widespread continental species; the intraspecific divergence was higher within some species occurring on different islands (Percy, 2003). The threshold of 3% mostly holds also for the Palaearctic pear psyllids though it is slightly higher in *C. chinensis* and *C. maculatili* (3.8 and 5.9% maximum intraspecific divergence, respectively) which were sampled in this study from continental eastern Asia and the islands of Taiwan and Japan, respectively (table 3). Percy (2003) explained this pattern as a result either from greater gene flow on the continent than between islands or by the establishment of recent continental distributions.

In the past, seasonal dimorphism within some of the pear psyllid species led to misidentifications and taxonomic chaos but several studies using morphological evidence solved the puzzle (Burckhardt and Hodkinson, 1986; Yang *et al.*, 2004; Luo *et al.*, 2012; Cho *et al.*, 2017). Here, we confirm that *C. cinereosignata* is the winter form of *C. jukyungi*, and *C. maculatili* that of *C. qiuzili*; the names have been formally synonymized by Cho *et al.* (2017).

Previously, the two east Palaearctic species *C. burckhardti* and *C. jukyungi* were misidentified as the west Palaearctic *C. pyrisuga* and *C. pyricola*, respectively (Paik, 1963; Kwon, 1983; Park, 1996; Kim *et al.*, 2000, 2007; Inoue, 2010; Kang *et al.*, 2012; Park *et al.*, 2013, 2016; Kwon *et al.*, 2016; Cho *et al.*, 2017). By including *C. pyrisuga* samples from central Europe in our analyses we show that the records and sequences of '*C. pyrisuga*' from Japan (Katoh *et al.*, 2013) belong, in fact, to *C. burckhardti* which is also known from Korea. Our study also shows that '*C. pyricola*' reported from Korea by Kang *et al.* (2012) is a misidentification of *C. jukyungi*. There is no evidence for the presence of west Palaearctic pear psyllids in East Asia, confirming the conclusions by Cho *et al.* (2017) based on morphological evidence. Furthermore, Inoue *et al.* (2012) and Katoh *et al.* (2013, 2014) misidentified *C. jukyungi* from Japan as *C. chinensis*. They detected exceptionally high genetic differences from *C. chinensis* from Taiwan and concluded that the Japanese populations belong to a distinct lineage of the same species. Later, Cho and Lee (2015) followed this interpretation and misidentified *C. jukyungi* from South Korea as *C. chinensis*.

The same misidentified *C. chinensis* sequences from China deposited in the GenBank were also used uncritically by Chen *et al.* (2018). Their sequences from Northeast China (Harbin, Heilongjiang; Helong, Jilin; Yanlong, Jilin) named '*C. chinensis*

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Table 3. Intraspecific and interspecific K2P distances of tested species (COI-tRNA^{leu}-COII/COI658/COI403/16S rDNA).

Species	Mean (%)	Min (%)	Max (%)
Intraspecific K2P distances			
C. bidens	0.0/–/0.6/0.3	0.0/–/0.0/0.3	0.0/–/2.5/0.3
C. burckhardti	0.1/0.3/0.7/0.3	0.0/0.2/0.0/0.0	0.4/0.5/1.3/0.8
C. chinensis	1.1/–/–/0.6	0.0/–/–/0.0	3.8/–/–/3.1
C. jukyungi	0.1/0.0/0.2/0.2	0.0/0.0/0.0/0.0	0.4/0.3/0.2/0.5
C. maculatili	3.2/3.6/2.9/2.0	0.0/0.0/0.0/0.0	5.9/5.9/5.5/3.8
C. pyri	0.0/0.1/2.1/0.0	0.0/0.0/0.0/0.0	0.0/0.2/4.1/0.0
C. pyricola	0.1/0.0/0.5/0.0	0.0/0.0/0.0/0.0	0.4/0.0/1.0/0.0
C. pyrisuga	–/–/0.0/–	–/–/0.0/–	–/–/0.0/–
C. sandolbaea	0.5/0.5/0.8/0.0	0.5/0.5/0.8/0.0	0.5/0.5/0.8/0.0
C. qianli	0.0/–/–/0.1	0.0/–/–/0.0	0.0/–/–/0.3
Interspecific K2P distances			
C. bidens–C. burckhardti	14.8/–/21.2/8.3	14.8/–/19.7/8.0	15.0/–/22.5/8.6
C. bidens–C. chinensis	11.1/–/–/5.5	10.5/–/–/4.4	11.5/–/–/7.4
C. bidens–C. jukyungi	13.5/–/14.5/8.6	13.1/–/13.8/8.3	13.6/–/15.0/9.2
C. bidens–C. maculatili	11.3/–/15.7/8.1	10.6/–/14.1/7.7	12.7/–/17.0/8.6
C. bidens–C. pyri	8.4/–/11.2/6.7	8.4/–/8.0/6.6	8.4/–/13.5/6.8
C. bidens–C. pyricola	10.3/–/12.0/6.4	10.2/–/11.0/6.3	10.4/–/12.8/6.6
C. bidens–C. pyrisuga	–/–/19.8/–	–/–/19.0/–	–/–/20.7/–
C. bidens–C. sandolbaea	16.1/–/17.5/7.9	15.8/–/16.8/7.7	16.5/–/18.1/8.0
C. bidens–C. qianli	15.0/–/–/8.9	15.0/–/–/8.6	15.0/–/–/9.2
C. burckhardti–C. chinensis	13.4/–/–/7.3	11.6/–/–/6.3	14.1/–/–/9.1
C. burckhardti–C. jukyungi	16.4/19.7/20.4/9.6	16.0/19.4/20.0/9.5	16.7/20.0/21.0/9.8
C. burckhardti–C. maculatili	13.2/17.7/17.1/9.2	13.0/16.0/14.8/9.2	13.7/18.8/18.7/9.5
C. burckhardti–C. pyri	14.1/16.1/18.4/8.2	14.1/15.8/17.3/8.0	14.3/16.5/19.6/8.6
C. burckhardti–C. pyricola	13.8/19.7/20.3/9.9	13.7/19.6/19.6/9.8	14.1/19.8/21.3/10.1
C. burckhardti–C. pyrisuga	–/–/11.7/–	–/–/10.9/–	–/–/12.4/–
C. burckhardti–C. sandolbaea	15.3/20.7/20.7/7.1	15.2/20.4/19.7/6.9	15.6/20.8/21.4/7.4
C. burckhardti–C. qianli	12.6/–/–/7.5	12.5/–/–/7.1	12.8/–/–/7.7
C. chinensis–C. jukyungi	9.0/–/–/6.3	8.4/–/–/4.9	9.4/–/–/7.2
C. chinensis–C. maculatili	9.6/–/–/7.1	7.6/–/–/6.6	11.0/–/–/8.2
C. chinensis–C. pyri	12.3/–/–/7.2	11.4/–/–/5.8	12.9/–/–/7.8
C. chinensis–C. pyricola	11.9/–/–/6.9	11.5/–/–/5.8	12.4/–/–/7.5
C. chinensis–C. pyrisuga	–/–/–/–	–/–/–/–	–/–/–/–
C. chinensis–C. sandolbaea	15.0/–/–/8.3	14.0/–/–/7.7	15.6/–/–/9.6
C. chinensis–C. qianli	14.9/–/–/7.7	14.6/–/–/6.8	15.4/–/–/9.1
C. jukyungi–C. maculatili	11.4/12.7/14.0/8.5	10.7/12.6/13.5/8.0	12.0/13.0/14.8/8.9
C. jukyungi–C. pyri	13.8/15.6/15.4/8.3	13.3/15.5/14.7/8.3	13.8/15.9/15.9/8.3
C. jukyungi–C. pyricola	15.1/17.0/18.7/7.6	14.4/17.0/18.4/7.5	15.4/17.2/19.4/8.1
C. jukyungi–C. pyrisuga	–/–/19.6/–	–/–/19.3/–	–/–/19.7/–
C. jukyungi–C. sandolbaea	16.3/16.1/17.7/8.7	15.6/15.9/17.2/8.7	16.6/16.5/18.2/8.7
C. jukyungi–C. qianli	15.0/–/–/9.4	14.6/–/–/9.2	15.0/–/–/9.5
C. maculatili–C. pyri	11.6/14.1/14.5/8.6	11.3/12.7/12.5/8.6	11.7/15.2/16.0/8.6

(*Continued*)

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Table 3. (*Continued.*)

Species	Mean (%)	Min (%)	Max (%)
C. maculatili–C. pyricola	13.3/14.7/16.9/6.9	13.2/13.0/15.5/6.9	13.5/15.8/18.2/6.9
C. maculatili–C. pyrisuga	–/–/18.8/–	–/–/18.0/–	–/–/19.3/–
C. maculatili–C. sandolbaea	14.6/16.1/16.5/8.1	14.1/15.0/16.0/7.7	14.9/16.8/16.9/8.7
C. maculatili–C. qianli	15.2/–/–/8.4	14.6/–/–/8.0	16.5/–/–/8.6
C. pyri–C. pyricola	10.5/14.3/13.5/6.9	10.4/14.2/12.3/6.9	10.7/14.4/14.7/6.9
C. pyri–C. pyrisuga	–/–/19.0/–	–/–/18.2/–	–/–/19.9/–
C. pyri–C. sandolbaea	15.7/15.4/16.4/8.9	15.5/15.1/15.2/8.9	15.8/15.7/18.2/8.9
C. pyri–C. qianli	13.5/–/–/10.3	13.5/–/–/10.0	13.5/–/–/10.3
C. pyricola–C. pyrisuga	–/–/18.4/–	–/–/18.2/–	–/–/18.5/–
C. pyricola–C. sandolbaea	17.8/16.2/16.4/8.6	17.3/15.9/15.8/8.6	18.3/16.5/16.8/8.6
C. pyricola–C. qianli	13.2/–/–/10.0	13.0/–/–/9.8	13.4/–/–/10.1
C. pyrisuga–C. sandolbaea	–/–/18.1/–	–/–/17.6/–	–/–/18.6/–
C. pyrisuga–C. qianli	–/–/–/–	–/–/–/–	–/–/–/–
C. sandolbaea–C. qianli	16.3/–/–/9.2	16.1/–/–/8.9	16.4/–/–/9.3

lineage III' grouped with '*C. chinensis*' from Japan, a misidentification of *C. jukyungi*. Their intraspecific divergences of 'lineage III' showed 12.4 and 9% sequence divergence in 16S rDNA and COI-tRNA^{leu}-COII from *C. chinensis* from the Chinese mainland and Taiwan, respectively, confirming the first being *C. jukyungi*.

Our molecular analyses indicate that, except for two sequences from Europe, all the '*C. pyricola*' sequences in the GenBank are misidentified. These misidentifications concern, in addition to *C. jukyungi* mentioned above, three species from Iran: *C. bidens*, *C. pyri* and *Cacopsylla* sp. (probably *C. permixta*, a species associated with pears and recorded from Iran by Burckhardt and Lauterer, 1993). *C. pyricola* was reported from Iran by Ossiannilsson (1992), by Burckhardt and Lauterer (1993) with doubts on the basis of two females and by Zendedel *et al.* (2016) on the basis of GenBank information but without morphological identification (D. Burckhardt, pers. information). Cho *et al.* (2017) suggested that the records of *C. pyricola* from Iran concern *C. bidens*, which is partly supported here.

DNA barcoding is a useful identification tool for insects in general and, as shown here, also for pear psyllids. A major problem in some molecular studies is the misidentification of the specimen subjected to DNA sequencing and the difficulty to check later its identity by other researchers. For developing a reliable library of DNA barcodes of pear psyllids, it is crucial that new entries are correctly identified and existing mistakes weeded out. Misidentifications of pear psyllid species may result in serious problems in the quarantine and control measures regarding these pests. Our study also confirms that morphological characters reliably diagnose species of *Cacopsylla* associated with pear.

Acknowledgements. We are grateful to David Ouvrard (Natural History Museum, London), Valerie Dennis (Editorial board, *Bulletin of Entomological Research*) and an anonymous reviewer for comments on earlier manuscript drafts and Liliya Štarhová Serbina (Masaryk University, Brno) for the donation of material.

Financial support. This work was partly supported by a grant from the Korean National Arboretum 'Development of an integrated identification system of Korean insects' (Project No. KNA 1-1-20, 16-1) and a grant from the Rural Development Administration, Republic of Korea 'Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01257202)'.

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