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Journal of Stored Products Research 48 (2012) 120–125

Contents lists available at SciVerse ScienceDirect

Journal of Stored Products Research

journal homepage: www.elsevier.com/locate/jspr

Diagnosis of *Liposcelis entomophila* (Insecta: Psocodea: Liposcelididae) based on morphological characteristics and DNA barcodes

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A R T I C L E I N F O

Article history:
Accepted 25 October 2011

Keywords:
Storage pests
Electron microscopy
mtDNA COI
Diagnosis
Psocoptera

A B S T R A C T

Liposcelis entomophila infests stored grain and is one of the most important psocid species worldwide. Six geographically isolated strains of *L. entomophila* from Asia, Europe, and United States of America (USA) were compared based on morphological attributes and by molecular methods. Decisive characters of morphological diagnosis were studied using body size measurements and Scanning Electron Microscopy (SEM). Molecular identification of the six strains was performed via identification of DNA sequence similarities and phylogenetic analyses based on a 655-bp fragment from the 5′ end of the standard mitochondrial gene cytochrome *c* oxidase I (COI) barcode region. The results showed that both morphological and molecular approaches were able to accurately identify this species. Kimura-2-Parameter (K2P) divergence between geographically isolated strains was on average 1.75% for the COI sequence. Phylogenetic analyses revealed that sequences of *L. entomophila* strains' COI barcodes formed clusters with tight cohesion that were clearly distinct from those of allied species.

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1. Introduction

Psocids, which include a number of domestic and stored products pests, pose an increasing threat to stored products worldwide (Rees, 2004) and are commonly found in a wide range of synanthropic habitats, such as processed and unprocessed dry foods in households, granaries, and product warehouses (Rees, 2004; Turner, 1994). In the last two decades, psocids have become an increasingly serious problem not only in grain storages, warehouses with bagged commodities but also in collection centres and export terminals (Opit and Throne, 2008). In addition, psocids have become stored product pests of considerable economic importance, contaminating foods and negatively affecting the quality and safety of food in various parts of the world (Nayak, 2006). Additionally, these pests are a potential cause of respiratory and dermatological allergies (Musken et al., 1998; Patil et al., 2001; Turner, 1994), transferring microorganisms to humans. They can transmit bacteria and fungi both on the surfaces of their bodies and also internally (Kalinović et al., 2006). In recent years, the importance of studying psocids has been enhanced by the fact that they develop rapid resistance to insecticides, phosphine and controlled atmosphere treatments (Cao et al., 2003; Ding et al., 2002; Nayak and Daglish, 2007).

Traditional diagnosis of psocids from the genus *Liposcelis* (Liposcelididae) through identification of specific morphological characters is difficult, especially in the case of juvenile stages; this method requires specialized taxonomic knowledge and microscopy techniques (Kučerová et al., 2009; Lienhard, 1990). DNA-based approaches offer an effective complement to traditional taxonomic methods and are currently widely employed for insect species identification. Molecular diagnostics have been applied to the identification of the *Liposcelis* species; for example, PCR-RFLP (Restriction Fragment Length Polymorphism) analysis was used for rapid discrimination of four common species of catalogued *Liposcelis* from China and the Czech Republic (Qin et al., 2008), and 16S rDNA has been proven to be effective in defining intra-species diversity of *Liposcelis bostrychophila* Badonnel (Li et al., 2011).

DNA barcoding is a DNA-based species identification system which offers a promising supplemental technique with standardized portions of the genome (Hebert et al., 2003). The most

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doi:10.1016/j.jspr.2011.10.007

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commonly used barcode gene, mitochondrial (mt) DNA cytochrome *c* oxidase I (COI), has been shown to be a reliable, quick and cost-effective tool for the identification of organisms of various taxa in all life stages (Augot et al., 2010; Cywinska et al., 2010; Hebert et al., 2003; Rach et al., 2008). A threshold of 2–3% mtDNA COI sequence divergence was recommended to define separate species for insects and mammals (Hebert et al., 2003). In studies of butterflies and ants, DNA barcoding has been successful in defining species boundaries by genetic distance thresholds (Hebert et al., 2004; Smith et al., 2005); however, there is no established universal distance threshold value to distinguish between taxonomic groups (Rach et al., 2008).

One of the most important stored product psocids worldwide is *Liposcelis entomophila* (Enderlein) (Psocodea, formerly Psocoptera; Yoshizawa and Lienhard, 2010). The objectives of research presented here were the following: (1) to identify the six different strains of *L. entomophila* from geographically different locales of Europe, Asia and USA using morphological trait identification through optical and scanning electron microscopy and COI gene sequencing diagnostic methods and (2) to enrich the COI barcode identification system while evaluating the proper sequence divergence threshold within *L. entomophila*. This work builds on preliminary results published in Yang et al. (2011).

2. Materials and methods

2.1. Insects

The six geographical strains of *L. entomophila* used for the morphological and molecular study were initially collected in grain stores from China (Shandong and Yunnan), the Czech Republic (Central Bohemia), Croatia (Baranja), the United Kingdom (Wales) and the United States of America (USA - Kansas). The laboratory cultures of these stocks (maintained by the following: Crop Research Institute, Prague; China Agricultural University, Beijing; University of Josip Juraj Strossmayer, Osijek; University of Wales Bangor, Bangor; Oklahoma State University, Stillwater) were reared at 27 ± 1 °C, 75 ± 5% relative humidity (r.h.), on a scotoperiod of 24 h in glass containers (125 ml) and on a wheat based diet (Ding et al., 2001; Opit and Throne, 2008; Kučerová et al., 2009).

2.2. Morphological identification

2.2.1. Optical microscope

Decisive diagnostic characters and size measurements were used for species identification. A minimum of 50 individuals (females) were examined and measured for each geographic strain. Head width (W) measurements were taken using a light microscope equipped with an objective micrometer. Differences in size among specimens of the geographic strains were displayed in box plots (developed using the software STATISTICA 8). Other morphological characters examined were numbers of ommatidia contained in the compound eye and numbers of the following setae: pronotal setae (PNS) on the lateral lobe, and prothoracic and mesothoracic sternite setae.

2.2.2. Scanning electron microscopy (SEM)

Morphological details (ommatidia, vertex surface sculpturing and prothoracic setae) were imaged with SEM micrographs. A minimum of 10 females was examined for each geographic population. The specimens were sputter-coated with platinum in the Sputter Coater SDC 050 (4 nm thick platinum layer). The head and thorax were then studied with the JEOL JSM-6400 SEM at magnifications of 650–2500×.

2.3. Molecular identification

2.3.1. DNA extraction, mtDNA COI amplification and sequencing

Total genomic DNA was isolated from the whole body of *L. entomophila* specimens using cetyl trimethyl ammonium bromide (CTAB; Qin et al., 2008); three individuals from each geographical population were used. PCR was performed with a pair of universal primers, LCO1490 (fw) 5′ GGT CAA CAA ATC ATA AAG ATA TTG G 3′ and HCO2198 (rev) 5′ TAA ACT TCA GGG TGA CCA AAA AAT CA 3′, amplifying an approximately 710 bp fragment of the standard mtDNA COI-5 barcode (Folmer et al., 1994). PCR products were separated on a 1.0% (w/v) agarose gel (1× TAE), stained with ethidium bromide, and visualised under UV light. The agarose gel slice containing the PCR amplicon of interest was excised and placed in a centrifuge tube. The agarose gel slice containing the PCR amplicon of interest was excised and the DNA was gel extracted. Bidirectional sequencing reactions were carried out from a single individual of each geographical isolate (Beijing Aoke Biotechnology Co., Ltd.).

2.3.2. Genetic distance and phylogenetic analysis

Pairwise genetic distances for COI were calculated using the Kimura-2-Parameter (K2P) distance model implemented in the software Molecular Evolutionary Genetics Analysis 5 (MEGA 5; Tamura et al., 2011). All phylogenetic analyses were carried out using the program PAUP 4.0 (Swofford, 2002). Two different types of phylogenetic trees, neighbour-joining (NJ) and maximum parsimony (MP), were graphically displayed and compared. A heuristic search was employed using tree bisection and reconnection (TBR) branch swapping and random addition for 100 replicates, and bootstrapping was performed using 1000 replications (Felsenstein, 1985). In the phylogenetic analysis, two geographical stocks were introduced: one additional closely related *Liposcelis* species and one more distantly related Psocoptera species. Collection localities and the GenBank accession numbers are available in Table 1.

3. Results

3.1. Morphological identification

Diagnosis of the species *L. entomophila* by decisive and secondary morphological characters, as put forth by Lienhard (1990); (Key – Section I, Group IA): The basic body colour is yellowish, and abdominal terga 3–4 and 6–9 are usually marked with a brown transverse band and often broadly interrupted medially. The dorsal side of the body appears as follows: the vertex of the head has spindle-shaped areas lacking tubercles or bear only indistinct tubercles of medium size; the average distance between

Table 1
Origin of species and populations used in this study.

Population	Location collected	GenBank accession nos.
L. entomophila SD-P.R. China	Shandong, P.R. China	HQ018872
L. entomophila YN-P.R. China	Yunnan, P.R. China	JF910135
L. entomophila P-CZ	Central Bohemia, Czech Republic	HQ018874
L. entomophila CRO	Bobota, Croatia	HQ658137
L. entomophila UK	Wales, UK	JF910133
L. entomophila USA	U.S.A	HQ018873
L. brunnea USA	U.S.A	JF910139
L. brunnea P-CZ	Central Bohemia, Czech Republic	JF910140
Psocoptera sp.	Arizona, U.S.A	HQ582230

Populations were coded by combining species names with acronyms of collection countries and sites.

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small fine hairs on the vertex is approximately equal to their length or slightly less. Compound eyes consist of 6–8 (most often 8, occasionally 7 and very rarely 6) ommatidia. The lateral lobe of the pronotum has a strong and long shoulder bristle (SI) and a transverse row of 3–5 other bristles. In addition, some small fine hairs originate behind the SI. The abdomen is compact; terga 3 and 4 lack posterior delimitation by intersegmental membranes. The ventral side of the body has 4–7 setae on the anterior half of the prosternum, the posterior prosternum is without setae and the mesosternal region has 6–11 setae.

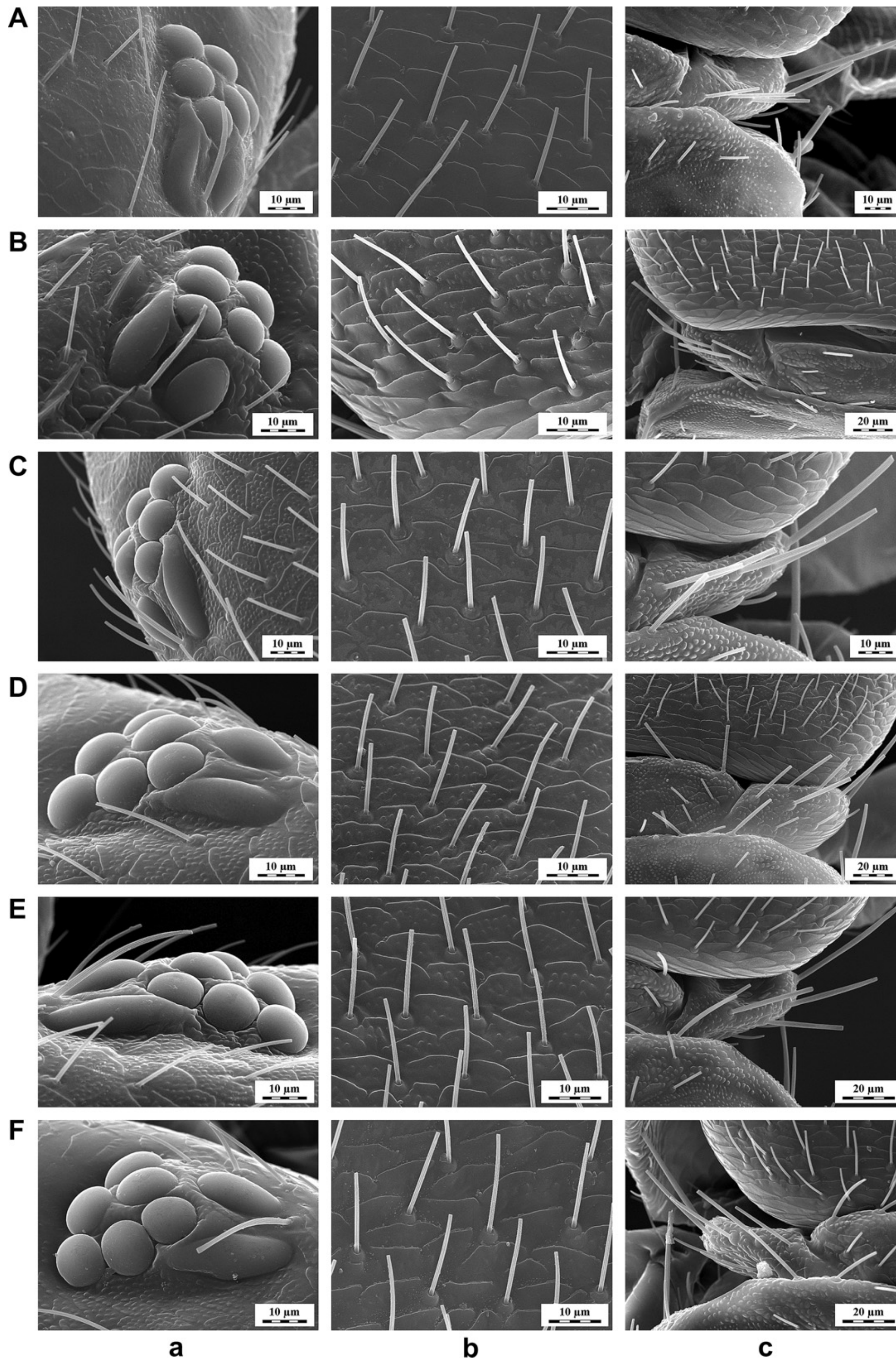

Fig. 1. *Liposcelis entomophila* (female) – morphological comparison of the compound eye (a), vertex sculpture and setae (b) and lateral lobe of pronotum (c) in the six geographical strains (A – China - Shandong, B – Czech Republic, C – Croatia, D – USA, E – UK, F – China - Yunnan).

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All six geographic strains exhibit the decisive diagnostic morphological characteristics of *L. entomophila* described above. SEM micrographs of compound eyes, the vertex surfaces and lateral lobes of the pronotum are shown in Fig. 1. Tubercles on spindle-shaped areas of the vertex are slightly more pronounced in Croatia, USA and UK strains than in the other strains. No significantly distinguishable differences among strains were found in other morphological characters, including the number of ommatidia in the compound eye, distance between setae of the vertex and the number of thoracic setae. Box plots of head size measurements (head width) for these six strains are shown in Fig. 2. Based on numerical differences among head measurements alone, the Croatia, two China, Czech, USA, and UK strains showed a decreasing order of head size. The median head size of the six strains differed, but the 25–75% confidence intervals or the minimal and maximal values overlapped. This means that statistically the six strains do not differ in head size.

3.2. Molecular identification

The mtDNA COI sequences of six *L. entomophila* strains obtained in this study have been deposited in GenBank available under accession numbers listed in Table 1.

The sequences were all trimmed to a 655 bp core region that could be unambiguously aligned to one another. No sequences contained indels or nonsense codons, allowing for easy alignment and supporting their origin in the mitochondrial gene.

All geographic isolates possessed a distinctive COI sequence, yet most showed low intra-species divergence. Conspecific Kimura-2-Parameter (K2P) divergence of *L. entomophila* averaged 1.75% (ranging from 0.15 to 3.45%). *Liposcelis entomophila* from Croatia (CRO) showed the highest divergence (K2P: 3.13–3.45%) from the other conspecific stocks. All other *L. entomophila* strains showed less than 2% K2P divergence. In contrast, COI sequences diverged widely between the two different *Liposcelis* species (*L. entomophila* and *Liposcelis brunnea*), generating K2P scores of 44.59–45.44%, an average of 45.12% (Table 2). In summary, the interspecies average divergence between closely related species was 25-fold higher than that of the intra-species divergence.

Both the neighbour-joining (NJ) and maximum parsimony (MP) phylogenetic analysis of the COI gene generated the same tree topology; distance value results were consistent, and grouped the six geographical isolates, along with the closely and distantly related species, into three clades (Fig. 3). The resulting trees showed a clear clade comprising of *L. entomophila* stocks, distinct from *L. brunnea* species and the out-group clades. There was high bootstrap support (100%) for the terminal branches at the species level.

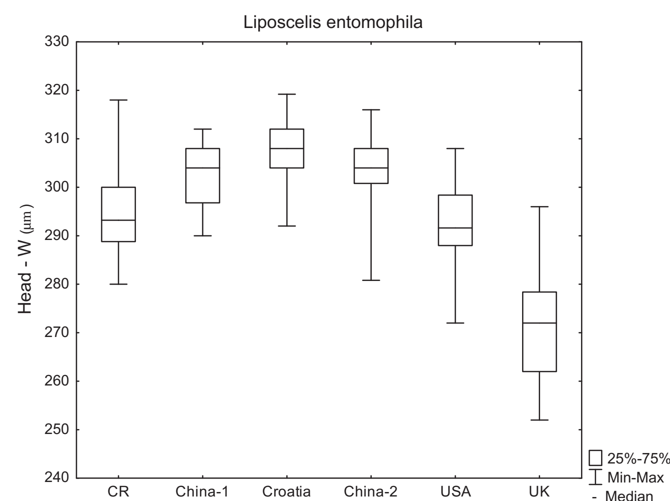

Fig. 2. Head size measurements of female *L. entomophila* from geographic strains from China - Shandong, Czech Republic, Croatia, U.S.A, UK, and China - Yunnan.

Table 2
Pairwise sequence divergence (%) based on the Kimura-2-parameter of COI of the six population stocks of *L. entomophila* and two *L. brunnea* population stocks that were investigated.

		1	2	3	4	5	6	7	8
1	*L. entomophila* SD-P.R. China								
2	*L. entomophila* YN-P.R. China	0.15							
3	*L. entomophila* P-CZ	0.77	0.92						
4	*L. entomophila* CRO	3.29	3.45	3.13					
5	*L. entomophila* UK	0. 61	0.77	0.61	3.13				
6	*L. entomophila* USA	1.55	1.70	1.39	3.45	1.39			
7	*L. brunnea* USA	45.44	45.44	45.17	44.91	45.17	44.59		
8	*L. brunnea* P-CZ	45.44	45.44	45.17	44.91	45.17	44.59	0.00	

4. Discussion

This work shows a comprehensive diagnostic method for *L. entomophila* through detailed morphological observation and molecular methods. The combination of decisive morphological characters and COI DNA barcoding analyses is a very constructive means of species identification.

All geographical stocks were unambiguously diagnosed as *L. entomophila* species based on identification of decisive morphological characteristics (Lienhard, 1990). Previous studies have shown that certain morphological characteristics such as number of ommatidia, number of prosternal and pronotal setae, head size, colouration and surface structures (Lienhard, 1990) have some distinct intra-specific variability based on data from the *Liposcelis* strains examined. Slight variability of head size and vertex structures revealed by the present study of *L. entomophila* geographical strains does not support consistent discrimination of these isolates based on morphological traits.

Intra-specific divergence of the mitochondrial COI gene within *L. entomophila* geographical isolates from different sites all over the world was not significant enough to be considered deep divergence. Inter-specific divergence between *L. entomophila* and *L. brunnea* was 25-fold higher than the observed intra-specific divergence of *L. entomophila*, despite the fact that *L. brunnea* is a closely related species belonging to the same taxonomic group (IA) (Lienhard, 1990). These results hold potential for use of DNA barcodes in phylogenetic delineation and association studies of other *Liposcelis* species. Very few psocid species are present in the DNA barcode databases, and our submission of *Liposcelis* COI sequences will enlarge this universal and accessible identification system.

Published studies show that approximately 98% of lepidopteran species have been identified through molecular taxonomy within a 3% threshold (Hebert et al., 2003), while 90% of 260 bird species from North America have been identified within a 2.7% threshold (Hebert et al., 2004). It is difficult to define a general threshold of genetic distance in distinguishing taxonomic groups, as divergence rates between taxa are a dynamic process, and species boundaries change with the time and population considered (Rubinoff et al., 2006). In our particular investigated stocks of *L. entomophila* from Asian, European and American populations, we determined that

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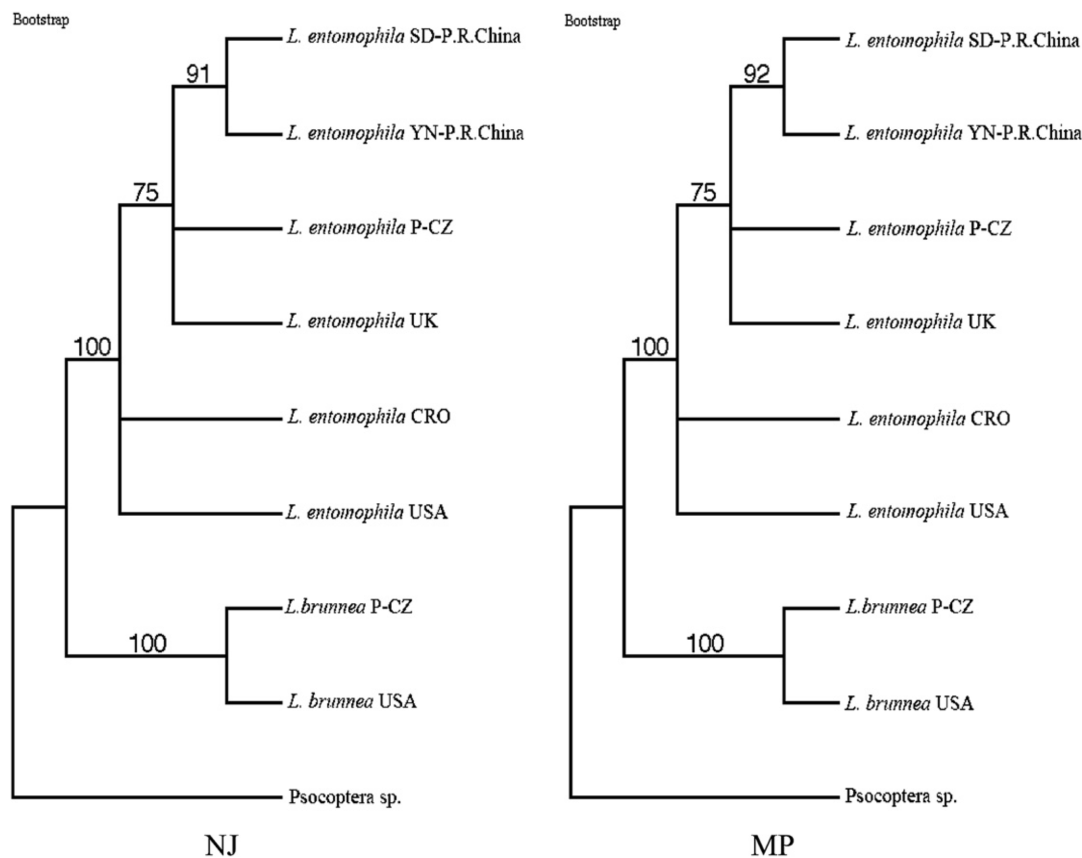

Fig. 3. Neighbour-joining (NJ) and maximum parsimony (MP) phylogenetic trees developed from COI barcodes analysis. The number at each branch point is the percentage supported by bootstrap. Psocoptera sp. (HQ582230) is the out-group.

the tested sequence threshold value might be appropriate at 3–3.5%.

In phylogenetic analyses, both the NJ and MP phylogenetic trees yielded an identical topology, and their congruence revealed that the geographical isolates from the two locations in China, the Czech Republic and the UK were of the closest phylogenetic relationship and members of the same clade. The Croatian and American strains were relatively distantly related to the other four isolates and were thus placed as sister cluster relative to them. All six populations were distinct from the close related species *L. brunnea* and the out-group.

The correlation between morphological and molecular identifications in our study indicated that COI-based DNA barcode data were useful in species discrimination for the genus *Liposcelis*. It has already been shown that the 16S rDNA gene is effective for distinguishing intra-specific diversity (Li et al., 2011). However, the COI gene seems more suitable for distinguishing separate species. The integrated approaches of morphological and molecular analyses provide useful support in the monitoring and rational control of insect pest species (Bertin et al., 2010). From this perspective, the present study serves as a useful contribution in the development of a rapid, practical and more accurate diagnostic molecular method of pest species identification, allowing confirmation in quarantine during international trade and other pest control efforts. The utility of the COI barcode system as a BLASTing tool for all known psocids species, as well as a taxonomic tool for unknown species, will become possible when a comprehensive psocid molecular reference library has been constructed, including reference barcodes for all recognised psocid species from wide geographic areas.

Acknowledgements

We thank J. Hromádková (IMCH Academy of Sciences) for processing SEM micrographs and M.A. Perotti and H.R. Braig (University of Wales Bangor, UK) for supplying biological material from the UK. This study was supported by the program of international cooperation No. 38-46 of the Chinese–Czech cooperation project (China), No. 2009-1-49 of the Chinese Universities Scientific Fund (China), No. ME 09080 – KONTAKT (Czech Republic), No. MZOŠ 079-0427 (Croatia) and by the Oklahoma Agricultural Experiment Station (Project No. OKL02695).

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