DNA barcoding of *Deltocephalus* Burmeister leafhoppers (Cicadellidae, Deltocephalinae, Deltocephalini) in China

Hong Zhang¹, Yalin Zhang², Yani Duan¹

¹ School of Plant Protection, Anhui Agricultural University, Hefei, Anhui Province 230036, China ² Key Laboratory of Plant Protection Resources and Pest Management of the Ministry of Education, Entomological Museum, Northwest A&F University, Yangling, Shaanxi Province 712100, China

Corresponding author: Yani Duan (duanyani@hotmail.com)

Academic editor: Mick Webb | Received 9 April 2019 | Accepted 10 July 2019 | Published 29 July 2019

Citation: Zhang H, Zhang Y, Duan Y (2019) DNA barcoding of *Deltocephalus* Burmeister leafhoppers (Cicadellidae, Deltocephalinae, Deltocephalini) in China. ZooKeys 867: 55–71. https://doi.org/10.3897/zookeys.867.35058

Abstract

We investigated the feasibility of using the DNA barcode region in identifying *Deltocephalus* from China. Sequences of the barcode region of the mitochondrial COI gene were obtained for 98 specimens (*Deltocephalus vulgaris* – 88, *Deltocephalus pulicaris* – 5, *Deltocephalus uncinatus* – 5). The average genetic distances among morphological and geographical groups of *D. vulgaris* ranged from 0.9% to 6.3% and among the three species of *Deltocephalus* ranged from 16.4% to 21.9% without overlap, which effectively reveals the existence of a “DNA barcoding gap”. It is important to assess the status of these morphological variants and explore the genetic variation among Chinese populations of *D. vulgaris* because the status of this species has led to taxonomic confusion because specimens representing two distinct morphological variants based on the form of the aedeagus are often encountered at a single locality. Forty-five haplotypes (*D. vulgaris* – 36, *D. pulicaris* – 5, *D. uncinatus* – 4) were defined to perform the phylogenetic analyses; they revealed no distinct lineages corresponding either to the two morphotypes of *D. vulgaris* or to geographical populations. Thus, there is no evidence that these variants represent genetically distinct species.

Keywords

COI, genetic distance, morphological variant
Introduction

China contains threatened biodiversity hotspots, including one spanning the Palearctic and Oriental regions and containing a high level of species diversity (Lin et al. 2010). In these regions, accurate identification of extant species is of great significance, although the taxonomic expertise is limited. Traditionally, identification of most species has been based on morphology. However, the availability of inexpensive DNA sequencing technology now provides additional tools not only for routine species identification but also for testing the validity of morphology-based species concepts. DNA barcoding is a simple, effective tool, that can identify and delimit species, including some complex taxa, rapidly and accurately using a standard short DNA sequence of the mitochondrial cytochrome c oxidase I (COI) (Hebert et al. 2003, 2004b; Ward et al. 2005; Hajibabaei et al. 2006). This method has been widely recognized and accepted in molecular phylogenetic studies (Hebert et al. 2003). The COI-based identification system has achieved remarkable success discriminating species across numerous animal groups, including birds (Hebert et al. 2004b), fishes (Hubert et al. 2008), and the insect orders Lepidoptera (Hebert et al. 2004a; Hajibabaei et al. 2006; Yang et al. 2012; Ashfaq et al. 2013), Ephemeroptera (Ball et al. 2005), and Hymenoptera (Smith et al. 2008). But this technology has also failed to identify species accurately under certain circumstances. For example, in a study of 449 species of Diptera and using 1333 COI sequences, Meier et al. (2006) obtained an identification success rate below 70% due to extensive overlap in inter- and intraspecific genetic distances. Within the dipteran family Calliphoridae, Whitworth et al. (2007) found that only 60% of species tested could be identified reliably.

Deltocephalini feed on grasses and sedges and are diverse and abundant in grassland ecosystems. This group contains 73 genera and 613 species around the world. Deltocephalus, type genus of this tribe contains 62 species distributed in the Old World and New World. Some species of this genus can transmit pathogenic diseases to economically important plants and are important economic pests; therefore, tools are needed for their rapid and accurate identification. Four species are described from China, two of them transmit pathogenic diseases. Identification of leafhopper species in most genera now requires dissection and examination of the male genitalia. However, some taxonomically problematic species apparently exhibit substantial intraspecific variation in male genital structures, and this causes confusion among taxonomists. One such practical example is D. vulgaris, which has well-documented morphological differences in the shape of the aedeagus (Figs 2, 3). Dash and Viraktamath (1998) first reported morphological variation in this species when they reviewed the genus Deltocephalus from India. Webb and Viraktamath (2009) also reported two forms of the aedeagus despite many shared morphological features in the species. Zhang and Duan (2011) redescribed D. vulgaris with detailed drawings and photos, illustrating these obvious morphological differences.
Based on DNA barcoding of leafhoppers, 63 barcodes from 45 species in Japan (15 subfamilies and 37 genera without Deltocephalini) were analysed (Kamitani 2011). DNA barcodes from 546 adult specimens of leafhoppers, planthoppers and treehoppers (Hemiptera, Auchenorrhyncha) were obtained from Barrow Island and analysed (Gopurenko et al. 2013). Species determination of members in the genus *Aphrodes* (Hemiptera, Cicadellidae) based on vibrational signals, mitochondrial DNA and morphology were performed (Bluemel et al. 2014). A total of 1482 specimens based on DNA barcodes of Nearctic Auchenorrhyncha (Insecta, Hemiptera) were studied by Foottit et al. (2014). The boundaries of seven closely related species of the evacanthine leafhopper genus *Bundera* (Cicadellidae, Evacanthinae) based on DNA barcoding, morphology and hyperspectral reflectance profiling was investigated by Wang et al. (2016), and a revision of the genus *Orosius* (Cicadellidae, Deltocephalinae, Opsiini) based on morphological and DNA barcoding was undertaken by Fletcher et al. (2017). Although, DNA barcoding research has been applied to these groups of leafhoppers, until now, a few molecular data are available for *Deltocephalus*. Therefore, a better understanding of *Deltocephalus*, and particularly the variation of *D. vulgaris* based on molecular data, is urgently needed.

In this study, we studied 98 COI sequences from three species of *Deltocephalus*. DNA barcoding data were used to investigate genetic variation of Chinese populations of *D. vulgaris* and to determine whether the morphological variants previously identified in this species represent distinct lineages. Our specific aims were to test the feasibility of using DNA barcoding data for identification of species of *Deltocephalus*, to determine the levels of the genetic variation within *D. vulgaris*, and to preliminarily discuss its possible correlation with morphological variation and biogeographic patterns.

**Material and methods**

**Taxon sampling**

A total of 98 specimens of *Deltocephalus* (*D. vulgaris* – 88, *D. pulicaris* – 5, *D. uncinatus* – 5) were collected with an insect sweep net in the daytime and by a light trap at night. Specimens were all collected directly into 95% or 100% ethanol and stored in -80 °C prior to study. The sample included *D. vulgaris, D. uncinatus* and *D. pulicaris* to facilitate comparison of inter- to intraspecific genetic variation in this group. *Deltocephalus vulgaris* specimens were divided into 11 groups based on their morphological differences and different geographical distributions in China (Table 1, Figs 1–3). Voucher specimens were deposited in the Key Laboratory of Plant Protection Resources and Pest Management of Ministry of Education, Entomological Museum, Northwest A&F University, Yangling, Shaanxi Province, China (NWAFU) and the School of Plant Protection, Anhui Agricultural University, Hefei, Anhui Province, China (AAU).
Table 1. List of samples studied and their relevant information.

| Species | Group code | Sample size | Individual code | Haplotype | Locality | GenBank accession |
|---------|------------|-------------|-----------------|-----------|----------|------------------|
| D. vulgaris | YNA | 8 | YNA1 | Hap1 | Banhong Town, Yunnan Province | MK764780 |
| | | | YNA2 | Hap2 | Banhong Town, Yunnan Province | MK764781 |
| | | | YNA3 | Hap3 | Banhong Town, Yunnan Province | MK764782 |
| | | | YNA4 | Hap2 | Banhong Town, Yunnan Province | MK764783 |
| | | | YNA5 | Hap4 | Banhong Town, Yunnan Province | MK764784 |
| | | | YNA6 | Hap2 | Banhong Town, Yunnan Province | MK764785 |
| | | | YNA7 | Hap1 | Banhong Town, Yunnan Province | MK764786 |
| | | | YNA8 | Hap2 | Banhong Town, Yunnan Province | MK764787 |
| | YNB | 13 | YNB1 | Hap5 | Banhong Town, Yunnan Province | MK764788 |
| | | | YNB2 | Hap1 | Banhong Town, Yunnan Province | MK764789 |
| | | | YNB3 | Hap5 | Banhong Town, Yunnan Province | MK764790 |
| | | | YNB4 | Hap5 | Banhong Town, Yunnan Province | MK764791 |
| | | | YNB5 | Hap5 | Banhong Town, Yunnan Province | MK764792 |
| | | | YNB6 | Hap5 | Banhong Town, Yunnan Province | MK764793 |
| | | | YNB7 | Hap5 | Banhong Town, Yunnan Province | MK764794 |
| | | | YNB8 | Hap6 | Banhong Town, Yunnan Province | MK764795 |
| | | | YNB9 | Hap7 | Banhong Town, Yunnan Province | MK764796 |
| | | | YNB10 | Hap8 | Banhong Town, Yunnan Province | MK764797 |
| | | | YNB11 | Hap5 | Banhong Town, Yunnan Province | MK764798 |
| | | | YNB12 | Hap5 | Banhong Town, Yunnan Province | MK764799 |
| | | | YNB13 | Hap5 | Banhong Town, Yunnan Province | MK764800 |
| | ZJA | 7 | ZJA1 | Hap9 | Lin'an County, Zhejiang Province | MK764801 |
| | | | ZJA2 | Hap10 | Lin'an County, Zhejiang Province | MK764802 |
| | | | ZJA3 | Hap11 | Lin'an County, Zhejiang Province | MK764803 |
| | | | ZJA4 | Hap12 | Lin'an County, Zhejiang Province | MK764804 |
| | | | ZJA5 | Hap13 | Lin'an County, Zhejiang Province | MK764805 |
| | | | ZJA6 | Hap12 | Lin'an County, Zhejiang Province | MK764806 |
| | | | ZJA7 | Hap12 | Lin'an County, Zhejiang Province | MK764807 |
| | ZJB | 8 | ZJB1 | Hap14 | Kowloon Mountain, Zhejiang Province | MK764808 |
| | | | ZJB2 | Hap10 | Kowloon Mountain, Zhejiang Province | MK764809 |
| | | | ZJB3 | Hap15 | Kowloon Mountain, Zhejiang Province | MK764810 |
| | | | ZJB4 | Hap12 | Kowloon Mountain, Zhejiang Province | MK764811 |
| | | | ZJB5 | Hap16 | Kowloon Mountain, Zhejiang Province | MK764812 |
| | | | ZJB6 | Hap17 | Kowloon Mountain, Zhejiang Province | MK764813 |
| | | | ZJB7 | Hap18 | Kowloon Mountain, Zhejiang Province | MK764814 |
| | | | ZJB8 | Hap19 | Kowloon Mountain, Zhejiang Province | MK764815 |
| | FJA | 7 | FJA1 | Hap20 | Shajian Town, Fujian Province | MK764816 |
| | | | FJA2 | Hap20 | Shajian Town, Fujian Province | MK764817 |
| | | | FJA3 | Hap5 | Shajian Town, Fujian Province | MK764818 |
| | | | FJA4 | Hap21 | Shajian Town, Fujian Province | MK764819 |
| | | | FJA5 | Hap5 | Shajian Town, Fujian Province | MK764820 |
| | | | FJA6 | Hap20 | Shajian Town, Fujian Province | MK764821 |
| | | | FJA7 | Hap5 | Shajian Town, Fujian Province | MK764822 |
| | FJB | 7 | FJB1 | Hap22 | Shajian Town, Fujian Province | MK764823 |
| | | | FJB2 | Hap20 | Shajian Town, Fujian Province | MK764824 |
| | | | FJB3 | Hap20 | Shajian Town, Fujian Province | MK764825 |
| | | | FJB4 | Hap20 | Shajian Town, Fujian Province | MK764826 |
| | | | FJB5 | Hap20 | Shajian Town, Fujian Province | MK764827 |
| | | | FJB6 | Hap8 | Shajian Town, Fujian Province | MK764828 |
| | | | FJB7 | Hap23 | Shajian Town, Fujian Province | MK764829 |
| Species | Group code | Sample size | Individual code | Haplotype | Locality | GenBank accession |
|---------|------------|-------------|-----------------|-----------|-----------|------------------|
| **D. vulgaris** | HNA | 9 | HNA1 | Hap24 | Jianfeng Mountain, Hainan Province | MK764830 |
| | | | HNA2 | Hap8 | Jianfeng Mountain, Hainan Province | MK764831 |
| | | | HNA3 | Hap8 | Jianfeng Mountain, Hainan Province | MK764832 |
| | | | HNA4 | Hap8 | Jianfeng Mountain, Hainan Province | MK764833 |
| | | | HNA5 | Hap8 | Jianfeng Mountain, Hainan Province | MK764834 |
| | | | HNA6 | Hap25 | Jianfeng Mountain, Hainan Province | MK764835 |
| | | | HNA7 | Hap8 | Jianfeng Mountain, Hainan Province | MK764836 |
| | | | HNA8 | Hap26 | Jianfeng Mountain, Hainan Province | MK764837 |
| | | HNA9 | Hap27 | Jianfeng Mountain, Hainan Province | MK764838 |
| | HNB | 8 | HNB1 | Hap20 | Jianfeng Mountain, Hainan Province | MK764839 |
| | | | HNB2 | Hap38 | Jianfeng Mountain, Hainan Province | MK764840 |
| | | | HNB3 | Hap29 | Jianfeng Mountain, Hainan Province | MK764841 |
| | | | HNB4 | Hap8 | Jianfeng Mountain, Hainan Province | MK764842 |
| | | | HNB5 | Hap8 | Jianfeng Mountain, Hainan Province | MK764843 |
| | | | HNB6 | Hap31 | Jianfeng Mountain, Hainan Province | MK764844 |
| | | | HNB7 | Hap8 | Jianfeng Mountain, Hainan Province | MK764845 |
| | | | HNB8 | Hap8 | Jianfeng Mountain, Hainan Province | MK764846 |
| | GDB | 9 | GDB1 | Hap32 | Patio Hill, Guangdong Province | MK764847 |
| | | | GDB2 | Hap8 | Patio Hill, Guangdong Province | MK764848 |
| | | | GDB3 | Hap8 | Patio Hill, Guangdong Province | MK764849 |
| | | | GDB4 | Hap8 | Patio Hill, Guangdong Province | MK764850 |
| | | | GDB5 | Hap8 | Patio Hill, Guangdong Province | MK764851 |
| | | | GDB6 | Hap20 | Patio Hill, Guangdong Province | MK764852 |
| | | | GDB7 | Hap8 | Patio Hill, Guangdong Province | MK764853 |
| | | | GDB8 | Hap8 | Patio Hill, Guangdong Province | MK764854 |
| | | | GDB9 | Hap8 | Patio Hill, Guangdong Province | MK764855 |
| | GXA | 4 | GXA1 | Hap33 | Lingyun County, Guangxi Province | MK764856 |
| | | | GXA2 | Hap1 | Lingyun County, Guangxi Province | MK764857 |
| | | | GXA3 | Hap34 | Lingyun County, Guangxi Province | MK764858 |
| | | | GXA4 | Hap20 | Lingyun County, Guangxi Province | MK764859 |
| | GXB | 8 | GXB1 | Hap35 | Shangsi County, Guangxi Province | MK764860 |
| | | | GXB2 | Hap20 | Shangsi County, Guangxi Province | MK764861 |
| | | | GXB3 | Hap32 | Shangsi County, Guangxi Province | MK764862 |
| | | | GXB4 | Hap1 | Shangsi County, Guangxi Province | MK764863 |
| | | | GXB5 | Hap5 | Shangsi County, Guangxi Province | MK764864 |
| | | | GXB6 | Hap5 | Shangsi County, Guangxi Province | MK648065 |
| | | | GXB7 | Hap20 | Shangsi County, Guangxi Province | MK764866 |
| | | | GXB8 | Hap36 | Shangsi County, Guangxi Province | MK764867 |
| **D. pulicaris** | XJ | 5 | XJ1 | Hap37 | Altay City, Xinjiang Province | MK764868 |
| | | | XJ2 | Hap38 | Altay City, Xinjiang Province | MK764869 |
| | | | XJ3 | Hap39 | Altay City, Xinjiang Province | MK764870 |
| | | | XJ4 | Hap40 | Altay City, Xinjiang Province | MK764871 |
| | | | XJ5 | Hap41 | Altay City, Xinjiang Province | MK764872 |
| **D. uncinatus** | YN | 5 | YN1 | Hap42 | Menglong Town, Yunnan Province | MK764873 |
| | | | YN2 | Hap43 | Menglong Town, Yunnan Province | MK764874 |
| | | | YN3 | Hap43 | Menglong Town, Yunnan Province | MK764875 |
| | | | YN4 | Hap44 | Menglong Town, Yunnan Province | MK764876 |
| | | | YN5 | Hap45 | Menglong Town, Yunnan Province | MK764877 |

Note: individual code with province initials and A or B and number; A and B are representative of two different morphological variants of *D. vulgaris* respectively.
Morphology

Morphological observations were made using an Olympus SZX10 stereoscopic microscope (Olympus Corporation, Tokyo, Japan). All photographs and drawings were modified with Adobe Photoshop CS.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from the whole abdomen of each leafhopper using the EasyPure Genomic DNA Kit (EE101; Transgen, Beijing, China) following the manufacturer’s instructions with the following modifications: abdomen incubated at 55 °C for about 20 hours, and with a nondestructive DNA extraction procedure to allow subsequent morphological observation. Genomic DNA extracts were stored in a freezer at -20 °C.

The barcode region (630bp) of the COI gene was amplified using primer combination (Folmer et al. 1994), LCO1490 (5’–GGT CAA ATC ATA AAG ATA TTG G–3’) and HCO2198 (5’–TAA ACT TCA GGG TGA CCA AAA AAT CA–3’) by the standard polymerase chain reaction (PCR) method. Total reaction volume was 25 μl, containing 12.5 μl of 2×Taq MasterMix, 8.5 μl of double distilled water (ddH₂O), 2 μl of forward and reverse primer (1 μl, respectively), and 2 μl of DNA template solution. The following thermal cycling protocol was used: an initial denaturation step at 94 °C
for 3 min, followed by 5 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1.5 min and extension at 72 °C for 1.5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 53.5 °C for 1 min and extension at 72 °C for 1 min, with a final extension of at 72 °C for 5 min, and ending with incubation at 12 °C.

The PCR products were examined using 1% agarose gel electrophoresis with ethidium bromide stain to check for successful amplification. The successful PCR products were sent to Beijing Tsingke Biotechnology Co., Ltd (China) for sequencing of both strands using the original PCR primers. All sequences collected in this study have been submitted to GenBank and accession numbers are shown in Table 1.

Molecular data analysis

The forward and reverse chromatograms were proofread and then assembled and edited using DNASTAR software (DNASTAR, Madison, Wisconsin, USA). Multiple sequence alignments were performed by CLUSTAL X 2.0.21 (Thompson et al. 1997; Jeannotteau et al. 1998). Primer sequences were manually deleted with BIOEDIT 7.0.9.0 (Hall 1999). To ensure that the correct target gene fragment was obtained, all sequences were checked in NCBI by Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). To ensure nonexistence of stop codons and pseudogenes, the nucleotide sequences were translated to amino acids by MEGA 7 (Kumar et al. 2016). Sequence composition analyses were performed in MEGA 7. Pairwise genetic distances were calculated using the Kimura 2-parameter (K2P) model in MEGA 7 (Kimura 1980). Haplotypes were defined by DNASP 5.0 (Librado and Rozas 2009). The detailed statistics for haplotypes are shown in Table 1. The substitution saturation tests of 45 haplotype sequences segments were conducted in DAMBE 5.3.74 (Xia 2013) by comparing the index of substitution saturation (Iss) with critical values (Iss.c). To construct phylogenetic trees, neighbor joining (NJ), minimum evolution (ME), Bayesian inference (BI) and maximum likelihood (ML) analyses were performed. NJ and ME analyses (Saitou and Nei 1987) were performed in MEGA 7 under K2P substitution model. Branch support was measured using 1000 replicates in each analysis (Felsenstein 1985). Results were summarized as 50% majority consensus trees in MEGA 7. BI analysis was performed in MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fit nucleotide evolution substitution model was selected by JMODELTEST 2.1.7 (Darriba et al. 2012). The Bayesian information criterion (BIC) was used to compare substitution models. The HKY+G model of nucleotide evolution was used. Two replicate runs with four independent Markov chain Monte Carlo (MCMC) chains (one cold chain and three hot chains) to conduct for 2 million generations, with trees sampled every 1000 generations with default parameter values. The average standard deviation of split frequency was lower than 0.01, indicating that the sampling of posterior distribution was adequate. The average standard deviation of split frequencies and Potential Scale Reduction Factor (PSRF) were used for examining convergence.
Figure 2. Morphological variant marked with A for *D. vulgaris* A habitus in dorsal view B subgenital plate C subgenital plate D style E aedeagus and connective, dorsal view F aedeagus and connective, lateral view (after Zhang and Duan 2011).

Figure 3. Morphological variant marked with B for *D. vulgaris* A habitus in dorsal view B subgenital plate C subgenital plate D style E aedeagus and connective, dorsal view F aedeagus and connective, lateral view (after Zhang and Duan 2011).
The stationarity was determined in TRACER 1.5 (Rambaut and Drummond 2009) by plotting the log-likelihood values versus generation number and the effective sample sizes >200 for all parameters. After stationarity had been reached, the first 25% trees were discarded as burn-in and a 50% majority-rule consensus tree with the posterior probability considered as node support values was constructed by summarizing the remaining trees. ML analysis was performed in RAXMLGUI 1.3.1, a graphical front-end for RAXML (Silvestro and Michalak 2012). All ML analyses with thorough bootstrap were run 10 times starting from random seeds under the GTR+GAMMA model. The bootstrap support value (BS) was evaluated by analysis with 1000 replicates. All tree topologies were displayed in FIGTREE 1.4 (Rambaut 2009).

**Results**

**Morphological variation of *D. vulgaris***

Our specimens from China included representatives of both previously reported morphotypes of the aedeagus of *D. vulgaris*. They also exhibited a range of more subtle variation in the curvature of the aedeagal shaft in lateral view. Under the current morphology-based concept, this species can nevertheless be identified by the colour pattern and the presence of a shallow apical notch on the aedeagus in posterior view.

**Sequence composition**

The COI sequences are 630bp in length after alignment and trimming. Details of nucleotide composition are listed in Table 2. As is typical for insect mtDNA, the gene is AT-rich (Liu et al. 2012).

**Table 2.** The average nucleotide composition of the COI sequences of *Deltocephalus*.

| Group/Species | T (%) | C (%) | A (%) | G (%) | A+T (%) |
|---------------|-------|-------|-------|-------|---------|
| YNA           | 32.8  | 18.8  | 34.0  | 14.1  | 56.8    |
| YNB           | 33.1  | 18.3  | 33.5  | 15.1  | 66.6    |
| ZJA           | 32.8  | 19.0  | 34.2  | 14.4  | 67.0    |
| ZJB           | 32.9  | 18.9  | 34.1  | 14.1  | 67.0    |
| FJA           | 33.0  | 18.3  | 33.8  | 14.9  | 66.8    |
| FJB           | 33.0  | 18.4  | 34.1  | 14.5  | 67.1    |
| HNA           | 33.1  | 18.4  | 33.9  | 14.6  | 67.0    |
| HNB           | 33.0  | 18.3  | 34.0  | 14.7  | 67.0    |
| GDB           | 33.0  | 18.3  | 34.0  | 14.7  | 67.0    |
| GXA           | 33.0  | 18.4  | 33.9  | 14.7  | 66.9    |
| GXB           | 33.0  | 18.3  | 33.9  | 14.8  | 66.9    |
| A total of A  | 33.0  | 18.6  | 34.0  | 14.5  | 67.0    |
| A total of B  | 33.0  | 18.4  | 33.9  | 14.7  | 66.9    |
| A total of A and B | 33.0 | 18.5 | 33.9 | 14.6 | 66.9 |
| *D. pulicaris* | 33.7  | 20.9  | 30.6  | 14.8  | 64.3    |
| *D. uncinatus* | 35.2  | 18.0  | 32.0  | 14.9  | 57.2    |
Substitution saturation test

The results of haplotype sequences for the substitution saturation test indicate the value of Iss is smaller than Iss.c; namely, little substitutional saturation was detected, which is strongly informative for constructing phylogenetic trees.

Analysis of the genetic distance and phylogenetic trees

The average genetic distances among morphological and geographical groups of *D. vulgaris* ranged from 0.9% to 6.3% and among species of *Deltocephalus* ranged from 16.4% to 21.9% without overlap (Table 3). This effectively reveals the existence of “DNA barcoding gap” and indicates the variation among morphological and geographical groups of *D. vulgaris* have not reached species level. Forty-five haplotypes (*D. vulgaris* – 36, *D. pulicaris* – 5, *D. uncinatus* – 4) were defined to perform phylogenetic analyses. The phylogenetic analyses based on NJ, ME, BI and ML methods nearly yielded identical trees except for the slight change of the position of a few individuals of *D. vulgaris* and bootstrap values (Figs 4, 5). *Deltocephalus vulgaris* haplotypes grouped into several distinct clades. However, these groups included individuals of both morphotypes and formed a distinct monophyletic clade with strong support value (BS(NJ) = 100, BS(ME) = 100, PP = 1, BS(ML) = 97) with no obvious biogeographic structure. Furthermore, different morphotypes of *D. vulgaris* share the same haplotype (Table 1). Thus, the COI sequence data suggest that previous authors were correct in treating the two morphotypes of *D. vulgaris* as belonging to the same species.

Table 3. Kimura 2-parameter genetic distances between groups/species of *Deltocephalus*.

|          | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| YNA      |       |       |       |       |       |       |       |       |       |       |       |       |
| YNB      | 0.047 |       |       |       |       |       |       |       |       |       |       |       |
| ZJA      | 0.041 | 0.063 |       |       |       |       |       |       |       |       |       |       |
| ZJB      | 0.041 | 0.057 | 0.017 |       |       |       |       |       |       |       |       |       |
| FJA      | 0.045 | 0.011 | 0.063 | 0.056 |       |       |       |       |       |       |       |       |
| FJB      | 0.043 | 0.029 | 0.047 | 0.043 | 0.023 |       |       |       |       |       |       |       |
| HNA      | 0.042 | 0.031 | 0.049 | 0.046 | 0.026 | 0.031 |       |       |       |       |       |       |
| HNB      | 0.044 | 0.014 | 0.062 | 0.056 | 0.007 | 0.022 | 0.023 |       |       |       |       |       |
| GDB      | 0.043 | 0.019 | 0.057 | 0.052 | 0.012 | 0.023 | 0.024 | 0.009 |       |       |       |       |
| GXA      | 0.045 | 0.023 | 0.050 | 0.046 | 0.021 | 0.030 | 0.032 | 0.021 | 0.023 | 0.028 |       |       |
| GXB      | 0.045 | 0.022 | 0.055 | 0.052 | 0.019 | 0.031 | 0.032 | 0.020 | 0.023 | 0.028 |       |       |
| *D. pulicaris* | 0.207 | 0.219 | 0.206 | 0.204 | 0.212 | 0.206 | 0.210 | 0.210 | 0.209 | 0.212 | 0.213 |       |
| *D. uncinatus* | 0.171 | 0.171 | 0.169 | 0.168 | 0.166 | 0.164 | 0.165 | 0.164 | 0.168 | 0.168 | 0.168 | 0.219 |

Note: the values indicate average intergroup and interspecific distances.
Figure 4. NJ/ME tree of 45 COI haplotypes. The node support: NJ/ME bootstrap values. Bootstrap values of less than 50 are not displayed.
Figure 5. BI/ML tree of 45 COI haplotypes. The node support: BI posterior probabilities/ML bootstrap values. Posterior probabilities and bootstrap values under 0.5 and 50 are shown “_”. “?” means the positions of the different individual of *D. vulgaris* in ML tree is slightly different from those in BI tree.

**Discussion**

DNA barcoding as a standardised method to provide rapid and accurate species demarcation and has been widely applied in identifying and delimiting taxa since it was first reported by Hebert et al. (2003). Two standard criteria have generally been accepted in delimiting species using COI-based DNA barcodes. Based on the existence of a DNA barcoding gap, the feasibility of COI-based DNA barcoding depends on the fact that genetic distances among species are usually much higher than distances within species, without overlap. Different numbers of single species always form an independent clade in a phylogenetic tree (Wiens and Penkrot 2002; Hebert et al. 2003). Our analysis of COI sequences of *Deltocephalus* suggests a low level of genetic variation among morphotypes and geographical populations of *D. vulgaris*, and even different morphotypes of *D. vulgaris* share the same haplotype (e.g., YNA1 and YNB2; FJA1 and HNB1). The intergroup average genetic distances (0.9%–6.3%) of *D. vulgaris* among morphotypes and geographical populations is distinctly lower than that among species of *Deltocephalus* (16.4%–21.9%), without overlap. The phylogenetic tree (Figs 4, 5) recovered three independent lineages representing each of the three species with moderate to high support val-
ues. The genetic distances among a few morphotypes and geographical populations of *D. vulgaris* exceeded the 3% standard threshold (e.g., ZJA and HNB; YNA and HNA). The more detailed genetic distances between groups/species of *Deltocephalus* are summarized in Table 3. However, all individuals of *D. vulgaris* grouped into a single clade with strong support comprising several subordinate clades but with no obvious correspondence to morphological or geographic groups. Furthermore, different morphotypes from the same and different geographical distributions of *D. vulgaris* share the same haplotype (Table 1).

We consider that the intraspecific genetic distance of a 3% standard threshold can be an inconsistent standard in different groups and maternal inheritance of mitochondrial genes can be affected in the process of evolution by the same mode of inheritance as Wolbachia infection, which also may result in a higher divergence in host mtDNA (Frezal and Leblois 2008; Muñoz et al. 2011). The low level of variation among morphotypes and geographical populations of *D. vulgaris* supports the notion that they represent a single species.

Differences in morphological characteristics, especially in male genitalia, have been the most reliable standard for discriminating among complex groups for many years. However, some cases of intraspecific variation in genital structures have been reported and these have led to uncertainty in the status of species and morphotypes. Mutanen et al. (2007) reported the male genital features that are most accepted and widely used standards to delimit species have been doubted in comparative study on male genital variation in *Pammene luedersiana* (Lepidoptera, Tortricidae). Yang et al. (2014) found 31 morphological variants in six species of *Mogannia* (Cicadidae, Cicadinae), but analysis of molecular data revealed low levels of intraspecific variation, although these morphological features have routinely been used to delimit species in this group. On the other hand, Wang et al. (2016) delimited seven different species of *Bundera* (Cicadelidae, Evacanthinae) based on molecular data, but only very minor morphological differences were found among six of these species. We are gradually becoming aware that similar morphological variation may have a different significance in different groups of leafhoppers and morphology-based species concepts may require confirmation using other kinds of data. DNA barcoding can efficiently complement morphology-based taxonomy and improve accuracy and rapidity in species identification.

*Deltocephalus vulgaris*, including 88 individuals in this study, and mainly representing two different forms of the aedeagus, were confirmed to be a single species grouped into a single clade with strongly support value in its phylogenetic trees (Figs 4, 5). Individuals collected both at the same place and time and different times and places have the same two forms of the aedeagus (e.g., FJA2 and FJB2; YNB10 and HNA2), which indicates forms are not related to temperature, humidity, precipitation, day length, altitude or latitude.

Our study shows a low intraspecific genetic distance between Guangdong and Hainan populations of *D. vulgaris* in southern China, suggesting that the Qiongzhou Strait (Fig. 1), a well-known biogeographic barrier has not significantly restricted gene flow for this species and they even share the same haplotype (Table 1). One logical assumption to explain this discovery is that Hainan and Guangdong arose earlier than the Qiongzhou Strait historically. Therefore, *D. vulgaris* freely exchanged genes when Guangdong and Hainan had been connected.
In the present study, lack of apparent correlation between morphology and COI haplotype is consistent with the hypothesis that the observed morphological variation is intraspecific. Nevertheless, we acknowledge the possibility that two different leaf-hopper species may share the same, or similar, COI haplotype. Thus, study of other genes may, in the future, reveal higher levels of divergence between the two forms and support recognition of some morphological variants as separate species.

Acknowledgements

We express our sincere thanks to M. D. Webb, the Natural History Museum, London, UK and Dr C.H. Dietrich, Department of Entomology, University of Illinois, USA for reading the manuscript and making some suggestions. We also thank Dr J. R. Schrock, Emporia State University, USA for revising the manuscript. This study is supported by the National Natural Science Foundation of China (31000968), Anhui Provincial Natural Science Foundation (1608085MC55) and Anhui Provincial Colleges and Universities Natural Science Foundation (KJ2015A006).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215(3): 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Ashfaq M, Akhtar S, Khan AM, Adamowicz SJ, Hebert PDN (2013) DNA barcode analysis of butterfly species from Pakistan points towards regional endemism. Molecular Ecology Resources 13(5): 832–843. https://doi.org/10.1111/1755-0998.12131

Ball SL, Hebert PDN, Burian SK, Webb JM (2005) Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. Journal of the North American Benthological Society 24(3): 508–524. https://doi.org/10.1899/04-142.1

Bluemel JK, Derlink M, Pavlovič P, Russo I-RM, King RA, Corbett E, Sherrard-Smith E, Blejč A, Wilson MR, Stewart AJA, Symondson WOC, Virant-Doberlet M (2014) Integrating vibrational signals, mitochondrial DNA and morphology for species determination in the genus Aphrodes (Hemiptera, Cicadellidae). Systematic Entomology 39: 304–324. https://doi.org/10.1111/syen.12056

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9(8): 772. https://doi.org/10.1038/nmeth.2109

Dash PC, Viraktamath CA (1998) A review of the Indian and Nepalese grass feeding leaf-hopper genus Deltocephalus (Homoptera, Cicadellidae) with description of new species. Hexapoda 10(1–2): 1–59.

Felsenstein J (1985) Confidence limits on phylogenies an approach using the bootstrap. Evolution 39(4): 783–791. https://doi.org/10.2307/2408678
DNA barcoding of Deltocephalus Burmeister leafhoppers in China

Fletcher M, Löcker H, Mitchell A, Gopurenko D (2017) A revision of the genus Orosius Distant (Hemiptera, Cicadellidae) based on male genitalia and DNA barcoding. Austral Entomology 56: 198–217. https://doi.org/10.1111/aen.12224

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3(5): 294–299.

Footitt RG, Maw E, Hebert PDN (2014) DNA barcodes for Nearctic Auchenorrhyncha (Insecta, Hemiptera). PLoS ONE 9: e101385. https://doi.org/10.1371/journal.pone.0101385

Frezal L, Leblois R (2008) Four years of DNA barcoding: current advances and prospects. Infection Genetics and Evolution 8(5): 727–736. https://doi.org/10.1016/j.meegid.2008.05.005

Gopurenko D, Fletcher M, Löcker H, Mitchell A (2013) Morphological and DNA barcode species identifications of leafhoppers, planthoppers and treehoppers (Hemiptera, Auchenorrhyncha) at Barrow Island. Records of the Western Australian Museum Supplement 83(1): 253–285. https://doi.org/10.18195/issn.0313-122x.83.2013.253-285

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences of the United States of America 103(4): 968–971. https://doi.org/10.1073/pnas.0510466103

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B 270(1512): 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004a) Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly Astraptes fulgerator. Proceedings of the National Academy of Sciences of the United States of America 101(41): 14812–14817. https://doi.org/10.1073/pnas.0406166101

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004b) Identification of birds through DNA barcodes. PLoS Biology 2(10): 1657–1663. https://doi.org/10.1371/journal.pbio.0020312

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, Watkinson D, Dumont P, Curry A, Bentzen P, Zhang JB, April J, Bernatchez L (2008) Identifying Canadian freshwater fishes through DNA barcodes. PLoS ONE 3: e2490. https://doi.org/10.1371/journal.pone.0002490

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trend in Biochemical Sciences 23: 403–405. https://doi.org/10.1016/S0968-0004(98)01285-7

Kamitani S (2011) DNA barcodes of Japanese leafhoppers. Esakia 50: 81–88.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16(2): 111–120. https://doi.org/10.1007/BF01731581
Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Librado PJR, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25(11): 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Lin LH, Ji X, Dione CH, Du Y, Lin CX (2010) Phylogeography and population structure of the Reeves's Butterfly Lizard (Leiolepis reevesi) inferred from mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 56(2): 601–607. https://doi.org/10.1016/j.ympev.2010.04.032

Liu S, Li MF, Ye J, Wang Y, Wang CL (2012) The molecular phylogenetic analysis of Penaeus based on 16S rRNA and COI sequences. Journal of Biology 29(5): 37–42.

Meier R, Shiyang K, Vaidya G, Peter KLN (2006) DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. Systematic Biology 55(5): 715–728. https://doi.org/10.1080/10635150600969864

Muñoz AG, Baxter SW, Linares M, Jiggins CD (2011) Deep mitochondrial divergence within a Heliconius butterfly species is not explained by cryptic speciation or endosymbiotic bacteria. BMC Evolutionary Biology 11(1): 358. https://doi.org/10.1186/1471-2148-11-358

Mutangen M, Rytkönen S, Lindén J, Sinkkonen J (2007) Male genital variation in a moth Pammene luedersiana (Lepidoptera, Tortricidae). European Journal of Entomology 104(2): 259–265. https://doi.org/10.14411/eje.2007.040

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12): 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4): 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Silvestro D, Michalak I (2012) raxmlGUI: a graphical front-end for RAxML. Organisms Diversity & Evolution 12: 335–337. https://doi.org/10.1007/s13127-011-0056-0

Smith MA, Rodriguez JJ, Whitfield JB, Deans AR, Janzen DH, Hallwachs W, Hebert PDN (2008) Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology and collections. Proceedings of the National Academy of Sciences of the United States of America 105(34): 12359–12364. https://doi.org/10.1073/pnas.0805319105

Rambaut A (2009) FigTree Version 1.3.1. http://tree.bio.ed.ac.uk/software/figtree/

Rambaut A, Drummond AJ (2009) Tracer Version 1.5.0. http://beast.bio.ed.ac.uk/software/tracer/ [Accessed on: 2015-3-10]

Thompson JD, Gibson TJ, Plewnia F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25(24): 4876–4882. https://doi.org/10.1093/nar/25.24.4876
Wang Y, Nansen C, Zhang YL (2016) Integrative insect taxonomy based on morphology, mitochondrial DNA and hyperspectral reflectance profiling. Zoological Journal of the Linnean Society 177: 378–394. https://doi.org/10.1111/zoj.12367
Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia’s fish species. Philosophical Transactions of the Royal Society B 360(1462): 1847–1857. https://doi.org/10.1098/rstb.2005.1716
Webb MD, Viraktamath CA (2009) Annotated check-list, generic key and new species of Old World Deltocephalini leafhoppers with nomenclatorial changes in the Deltocephalus group and other Deltocephalinae (Hemiptera, Auchenorrhyncha, Cicadellidae). Zootaxa 2163: 1–64.
Whitworth TL, Dawson RD, Magalon H, Baudry E (2007) DNA barcoding cannot reliably identify species of the blowfly genus Protocalliphora (Diptera, Calliphoridae). Proceedings of the Royal Society B 274(1619): 1731–1739. https://doi.org/10.1098/rspb.2007.0062
Wiens JJ, Penkrot TA (2002) Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (Sceloporus). Systematic Biology 51(1): 69–91. https://doi.org/10.1080/106351502753475880
Xia X (2013) DAMBE5: a comprehensive software package for data analysis in molecular biology and evolution. Molecular Biology and Evolution 30(7): 1720–1728. https://doi.org/10.1093/molbev/mst064
Yang MS, Chen X, Huo WX, Wei C (2014) Morphological variation versus genetic divergence: a taxonomic implication for Mogannia species (Cicadidae, Cicadinae). Systematics and Biodiversity 12(4): 456–472. https://doi.org/10.1080/14772000.2014.946980
Yang ZF, Landry JF, Handfield L, Zhang YL, Solis MA, Handfield D, Scholtens B, Mutanen M, Nuss M, Hebert PDN (2012) DNA barcoding and morphology reveal three cryptic species of Anania (Lepidoptera, Crambidae, Pyraustinae) in North America, all distinct from their European counterpart. Systematic Entomology 37: 686–705. https://doi.org/10.1111/j.1365-3113.2012.00637.x
Zhang YL, Duan YN (2011) Review of the Deltocephalus group of leafhoppers (Hemiptera, Cicadellidae, Deltocephalinae) in China. Zootaxa 2870: 1–47. https://doi.org/10.11646/zootaxa.2870.1.1