Title

Integrative Insect Taxonomy of Bundera

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Integrative insect taxonomy based on morphology, mitochondrial DNA, and hyperspectral reflectance profiling

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Integrative taxonomy is considered a reliable taxonomic approach of closely related and cryptic species by integrating different sources of taxonomic data (genetic, ecological, and morphological characters). In order to infer the boundaries of seven species of the evacanthine leafhopper genus *Bundera* Distant, 1908 (Hemiptera: Cicadellidae), an integrated analysis based on morphology, mitochondrial DNA, and hyperspectral reflectance profiling (37 spectral bands from 411–870 nm) was conducted. Despite their morphological similarities, the genetic distances of the cytochrome *c* oxidase subunit I (*COI*) gene among the tested species are relatively large (5.8–17.3%). The species-specific divergence of five morphologically similar species (*Bundera pellucida* and *Bundera* spp. 1–4) was revealed in mitochondrial DNA data and reflectance profiling. A key to identifying males is provided, and their morphological characters are described. Average reflectance profiles from the dorsal side of specimens were classified based on linear discriminant analysis. Cross-validation of reflectance-based classification revealed that the seven species could be distinguished with 91.3% classification accuracy. This study verified the feasibility of using hyperspectral imaging data in insect classification, and our work provides a good example of using integrative taxonomy in studies of closely related and cryptic species.

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ADDITIONAL KEYWORDS: *16S* rDNA – classification – DNA barcoding – Hemiptera – hyperspectral imaging – leafhopper – method – species delineation.

INTRODUCTION

Given the limited availability of taxonomic expertise for many diverse and complex groups of insects, methods are needed to: (1) partially automate the initial screening and separation of insect species; and (2) develop complementary and synergistic methods to improve the performance of existing procedures for the delineation and identification of closely related and cryptic species. Regarding the latter aim, overlapping character variation within and among species is well documented (Will, Mishler & Wheeler, 2005). Integrative taxonomy consists of integrating different data types for species delineation, and it is becoming widely accepted in modern taxonomy (Glaw & Vences, 2002; Dayrat, 2005; Will *et al*., 2005; Padial *et al*., 2010; Schlick-Steiner *et al*., 2010; Yeates *et al*., 2011; Riedel *et al*., 2013; Bluemel *et al*., 2014; Miraldo *et al*., 2014). As part of integrative taxonomy, molecular techniques are widely used, including DNA barcoding (Hebert, Ratnasingham & deWaard, 2003; Hajibabaei *et al*., 2006; Rivera & Currie, 2009; Robinson *et al*., 2009; Hebert, deWaard & Landry, 2010; Park *et al*., 2011; Astrin *et al*., 2012; Alex Smith *et al*., 2013) and the use of other regions of mitochondrial DNA (Dietrich, Whitcomb & Black, 1997; Barco *et al*., 2013; Allegrucci *et al*., 2014; Yang *et al*., 2014) and nuclear genes (Rokas

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et al., 2002; Danforth, Lin & Fang, 2005; Germain *et al*., 2013; Gutierrez-Gutierrez *et al*., 2013; Taylor *et al*., 2013).

As examples of integrative molecular techniques in insect taxonomy, three cryptic species of *Anania* (Lepidoptera: Crambidae: Pyraustinae) from North America were classified based on DNA barcoding and morphology (Yang *et al*., 2012). Handoo *et al*. (2014) integrated data from nuclear ribosomal DNA genes, morphology, and morphometrics into a species classification of 18 stunt nematode species (Nematoda: Telotylenchidae). In addition, there are numerous taxonomic studies in which ecological observations are integrated into the analysis, including: host selection (Bernardo *et al*., 2008; Chesters *et al*., 2012; Knee *et al*., 2012), geographical distribution (Rius & Teske, 2013; Miraldo *et al*., 2014), biochemical characters (Falahee & Angus, 2010; Fusu, 2010; Rezac *et al*., 2014), ecological niche modelling (Raxworthy *et al*., 2007; Zhu *et al*., 2013), cross-breeding analysis (Sourassou *et al*., 2012), and sound production (Marsh, 1999; Brown *et al*., 2006; Popple, 2013). Bluemel *et al*. (2014) confirmed four cryptic species of the leafhopper genus *Aphrodes* (Hemiptera: Cicadellidae) as behaviourally, genetically, and morphologically distinct species.

In recent years, there has been a growing interest in the use of imaging technologies in species classification (Nansen & Elliot, 2016). Some applications are based on reflectance data acquired from only a few (three) wide spectral bands measured with a digital rgb camera (Arbuckle *et al*., 2001; Watson, O'Neill & Kitching, 2003; Russell *et al*., 2007; Arribas *et al*., 2011; Faria *et al*., 2014; Zhu & Zhang, 2014). Recently, Nguyen *et al*. (2014) described an imaging system that can be used to develop digitized three-dimensional models of insect species. Such digitized models of insects are easy to share and store, and may therefore reduce the need for the shipment of specimens among taxonomists and increase the availability of insect reference collections. In other taxonomic studies, detailed reflectance profiles (a series of reflectance values in narrow spectral bands) were acquired, and different data processing and classification methods were used to: (1) select only the spectral bands that contributed to the distinction/separation of species; and (2) develop accurate classification algorithms. As examples, reflectance profiling has been used successfully to classify species of stored grain insects (Singh *et al*., 2010), two species of fruit flies (*Drosophila melanogaster* Meigen, 1830 and *Drosophila simulans* Sturtevant, 1919) (Aw, Dowell & Ballard, 2012), tobacco budworm (*Heliothis virescens* Fabricius, 1777), and corn earworm [*Helicoverpa zea* (Boddie, 1850)] (Jia *et al*., 2007), and Klarica *et al*. (2011) used imaging spectroscopy to discriminate cryptic species of ants [*Tetramorium caespitum* (Linnaeus, 1758) and *Tetramorium impurum* (Foerster, 1850)]. Nansen *et al*. (2014a) demonstrated that three species of minute juvenile egg parasitoids (*Trichogramma*) developing inside moth host eggs could be accurately classified based on the reflectance profiles acquired from the host eggs. There are also studies in which reflectance profiling was used in the systematics of fossil insects (Mietchen *et al*., 2005). Finally, Luo, Wei & Nansen (2015) showed that hyperspectral imaging of forewing costae could be used to differentiate 'mute' cicadas from cicadas with tymbal sound production.

In this study, we compared and integrated three taxonomic procedures: (1) classification based on traditional insect morphology; (2) classification based on the cytochrome *c* oxidase subunit I (*COI*) mitochondrial DNA (mtDNA) gene and *16S* ribosomal DNA (rDNA) gene analyses; (3) reflectance-based classification. *Bundera* Distant, 1908 belongs to the subfamily Evacanthinae, and it is widely distributed in the Oriental Region. For the purpose of this study, we had access to specimens collected in China and Thailand. This comprehensive analysis confirmed seven distinct species of *Bundera*. With a high level of morphological similarity both in terms of external features and male genitalia (Figs 2–4) among some species of *Bundera*, this genus is considered a taxonomically challenging group and therefore highly suitable for a detailed study of the complementarity of reflectance profiling as part of an integrative taxonomy procedure. A taxonomic revision of *Bundera* leafhoppers (Wang, Nansen & Zhang, unpubl. data) was not the objective of this study, but we provide reliable species delineation and a classification key for males of the seven species. We also use this analysis to discuss the potential of reflectance profiling as part of integrative taxonomy.

MATERIAL AND METHODS

Insect sampling

Leafhoppers were collected using a sweep net, light trap, or Malaise trap. Some specimens (24-7, 24-8, 51-1, 51-2, 23-1, 23-2, 23-4, 23-5, 23-6, 40-7, 40-8, 40-10, 40-11, 51-3, and 51-4) were directly mounted on small pieces of triangular paper after sampling, whereas other specimens were previously preserved in alcohol (99%) and subsequently mounted on triangular pieces of paper. In total, 52 specimens from seven species were included in this study (Table 1).

DNA extraction, amplification, sequencing, and molecular analysis

Genomic DNA was extracted from the whole abdomens of single specimens by the EasyPure Genomic DNA Kit (EE101; Transgen, Beijing, China). We followed the manufacturer's protocol, but with some modifications: the entire abdomen was incubated at 55 °C

Table 1. Sample information for the *Bundera* specimens included in the sequence

Species/specimen ID	Collection locality	Collection date	GenBank accession no. *COI*	GenBank accession no. *16S*
***Bundera pellucida* Li & Wang, 2001**				
24-1	China Guizhou Leigongshan Datangwan	2012/07/21	KT183631	KT183670
24-2	China Guizhou Leigongshan Xiannvtang	2012/07/21	KT183632	KT183671
24-3	China Guizhou Leigongshan Xiannvtang	2012/07/21	KT183633	KT183672
24-4	China Guizhou Leigongshan Xiannvtang	2012/07/21	KT183634	KT183673
24-5	China Guizhou Leigongshan	2012/07/20	KT183635	KT183674
24-6	China Guizhou Leigongshan	2012/07/19	–	–
24-7	China Hubei Wufeng Houhe	2006/07/13	KT183636	KT183675
24-8	China Hubei Wufeng Houhe	2006/07/13	–	–
51-1	China Guangxi Huaping Ankouping	2006/08/01	–	–
51-2	China Guangxi Huaping Ankouping	2006/08/01	KT183656	KT183693
***Bundera* sp. 4**				
53-1	China Hubei Shennongjia Xujiazhuang	2013/07/19	KT183659	KT183696
53-2	China Hubei Shennongjia Xujiazhuang	2013/07/19	KT183660	KT183697
53-3	China Hubei Shennongjia Xujiazhuang	2013/07/19	KT183661	KT183698
53-4	China Hubei Shennongjia Xujiazhuang	2013/07/19	KT183662	KT183699
53-5	China Hubei Shennongjia Xujiazhuang	2013/07/19	KT183663	KT183700
53-6	China Hubei Shennongjia Xujiazhuang	2013/07/19	KT183664	KT183701
53-7	China Hubei Shennongjia Xujiazhuang	2013/07/19	KT183665	KT183702
***Bundera* sp. 1**				
42-1	Thailand Chiang Mai Doi Phu Kha NP office 14	2007/12/15	–	–
42-2	Thailand Chiang Mai Doi Chiang Dao WS Nature Trail	2007/08/28–09/04	KT183644	KT183683
42-3	Thailand Chiang Mai Doi Chiang Dao WS Nature Trail	2007/08/28–09/04	KT183645	KT183684
42-4	Thailand Chiang Mai Doi Phahompck NP Headquarter	2008/02/07–14	KT183646	KT183685
42-5	Thailand Chiang Mai Doi Chiang Dao WS Nature Trail	2007/08/28–09/04	KT183647	KT183686
42-7	Thailand Chiang Mai Doi Chiang Dao WS Nature Trail	2007/08/28–09/04	KT183648	KT183687
42-8	Thailand Chiang Mai Doi Phahompok NP Headquarter	2007/08/01–07	KT183649	–
***Bundera* sp. 2**				
43-1	China Guizhou Kuangkuoshui Fenshuiling	2012/08/15	KT183650	KT183688
43-2	China Guizhou Kuangkuoshui Fenshuiling	2012/08/15	KT183651	–
43-3	China Guizhou Kuangkuoshui Fenshuiling	2012/08/15	KT183652	KT183689
43-4	China Guizhou Kuangkuoshui Fenshuiling	2012/08/15	–	–
51-3	China Guangxi Napo Fude	2013/08/07	KT183657	KT183694
51-4	China Guangxi Napo Fude	2013/08/07	KT183658	KT183695
***Bundera heichiana* Li & Wang, 1991**				
40-1	China Guizhou kuankuoshui	2012/08/11	KT183637	KT183676
40-2	China Guizhou Duyun Doupengshan	2012/07/24	–	–
40-3	China Guizhou Leigongshan	2012/07/20	KT183640	KT183679
40-4	China Guozhou Kuangkuoshui Fenshuiling	2012/08/15	–	–
40-5	China Guozhou Kuangkuoshui	2012/08/10	–	–
40-6	China Guozhou Kuangkuoshui Fenshuiling	2012/08/15	KT183641	KT183680
40-7	China Hunan Sangzhi Tianpingshan	2001/08/13	KT183642	KT183681
40-8	China Hunan Hupingshan Quanpingcun	2006/07/17	–	–
40-9	China Guizhou Duyun Doupengshan	2012/07/23	KT183643	KT183682
40-10	China Hunan Hupingshan Quanpingcun	2006/07/17	KT183638	KT183677
40-11	China Hunan Hupingshan Dadongping	2006/07/20	KT183639	KT183678
***Bundera emeiana* Li & Wang, 1994**				
23-1	China Sichuan Luding Gonggashan	2009/08/01	KT183627	KT183666
23-2	China Sichuan Emeishan	2006/08/07	KT183628	KT183667
23-3	China Sichuan Luding Gonggashan	2009/08/06	KT183629	KT183668
23-4	China Sichuan Emeishan	1957/08/20	–	–
23-5	China Sichuan Emeishan	1957/08/25	–	–
23-6	China Sichuan Luding Gonggashan	2009/08/01	KT183630	KT183669
***Bundera* sp. 3**				
50-1	China Xizang Motuo Dexing	2013/07/23	KT183653	KT183690
50-2	China Xizang Motuo Dexing	2013/07/23	KT183654	KT183691
50-3	China Xizang Motuo Dexing	2013/07/23	–	–
50-4	China Xizang Motuo Dexing	2013/07/23	–	–
50-5	China Xizang Motuo Dexing	2013/07/23	KT183655	KT183692

Table 2. Polymerase chain reaction (PCR) primers used in this study

Primer Name	Primer sequence (5′ → 3′)	Primer source
LCO1490	GGTCAACAAATCATAAAGATATTGG	(Hebert *et al.*, 2004)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	(Hebert *et al.*, 2004)
tRWF1_t1*	AAACTAATARCCTTCAAAG	(Park *et al.*, 2011)
tRWF2_t1*	AAACTAATAATYTTCAAAATTA	(Park *et al.*, 2011)
16SF1	CCGGTYTGAACTCARATCAWGT	(Dietrich *et al.*, 1997)
16SR1	CTGTTTAWCAAAAACATTTC	(Dietrich *et al.*, 1997)
16SF2	CCGGTCTGAACTCAGATCA	(Yeh, Yang & Hui, 2005)
16SR2	GCCTGTTTATCAAAAACAT	(Yeh *et al.*, 2005)

*Two primers combined in cocktail primer, C-tRWF.

for 15–17 h, and DNA extractions were performed without destruction of the specimens, to allow for the subsequent examination of morphology. Extracted nucleic acids were diluted in TE (pH 8.0) to a final volume of 100 µl and stored at −20 °C.

Two mtDNA fragments were amplified by polymerase chain reaction (PCR): a 660- or 800-bp fragment of the *COI* gene, and a 550-bp fragment of the *16S* gene. Reactions were performed in a total volume of 25 µl of reaction buffer, containing 12.5 µl of 2X Taq PCR Master Mix, 9.5 µl of sterile water, 0.5 µl of each oligonucleotide primer (10 µM), and 2 µl of genomic DNA solution.

The *COI* fragment was amplified by primers LCO1490 and HCO2198 (Table 2) with the following thermal cycling protocol: 2 minutes at 95 °C; five cycles of 40 seconds at 94 °C, 40 seconds at 45 °C, and 1 minute at 72 °C; 35 cycles of 40 seconds at 94 °C, 40 seconds at 51 °C, and 1 minute at 72 °C; 5 minutes at 72 °C; and finally held at 4 °C (Park *et al.*, 2011). When these primers were not successful, the primer cocktail C-tRWF_t1 (Table 2) enabled the amplification of the standard 658-bp barcode region, together with a short upstream sequence, in an additional 15% of the specimens (Park *et al.*, 2011).

Amplification of the *16S* fragment was accomplished by primers 16SF1 and 16SR1 (Table 2) and the following thermal cycling protocol: 5 minutes at 95 °C; 11 cycles of 1 minute at 92 °C, 1 minute at 48 °C, and 1.5 minutes at 72 °C; 33 cycles of 1 minute at 92 °C, 35 seconds at 54 °C, and 1.5 minutes at 72 °C; and a final extension of 7 minutes at 72 °C (Dietrich *et al.*, 1997). This protocol worked well for most specimens; however, we used primers 16SF2 and 16SR2 (Table 2) for a few species. After verification via gel electrophoresis, the PCR templates were purified and then sequenced in both directions using the same primer pairs by Sangon Biotech Company (Shanghai, China). All sequences gathered in this study have been submitted to GenBank (see Table 1).

Multiple sequences were aligned using CLUSTALW, and genetic distances within and among lineages were estimated using the Kimura two-parameter (K2P) model algorithm in MEGA 6.06 (Kimura, 1980; Tamura *et al.*, 2013), under K2P and Tamura three-parameter (T3P) substitution models, respectively. The branch support values of each clade were estimated by bootstraping 1000 replicates (Felsenstein, 1985; Yang *et al.*, 2012; Bluemel *et al.*, 2014), and neighbour-joining (NJ) and minimum-evolution (ME) trees based on distance were constructed in MEGA. To obtain results directly comparable with the existing *COI* barcode literature for Hemiptera (Kamitani, 2011; Park *et al.*, 2011; Foottit, Maw & Hebert, 2014), the genetic distances within and among lineages were estimated based only on *COI* data. Trees were constructed based on *COI* data alone and combined *COI* + *16S* data, respectively.

Hyperspectral imaging

We used a hyperspectral spectral camera (PIKA II; Resonon Inc., Bozeman, MT, USA) with the lens mounted 15 cm above the *Bundera* specimens, and reflectance data were acquired with a spatial resolution of 15 × 10 pixels per mm^2. The main specifications of the spectral camera are as follows: interface, Firewire (IEEE 1394b); output, digital (12 bit); angular field of view, 7°. The objective lens had a 35-mm focal length (maximum aperture of F1.4), optimized for the near-infrared and visible near-infrared spectra. Hyperspectral images were collected with artificial lighting from 15-W, 12-V LED light bulbs mounted on either side of the lens. A piece of white teflon (K-Mac Plastics, Grand Rapids, MI, USA) was used for white calibration, and 'relative reflectance' referred to the proportional reflectance compared with the reflectance obtained from Teflon, and ranged between 0 (complete darkness) and 1 (white).

Reflectance data processing and analysis

In total, we acquired average reflectance profiles from 52 male specimens, with between five and 11 profiles taken from each species. Similar to previously pub-

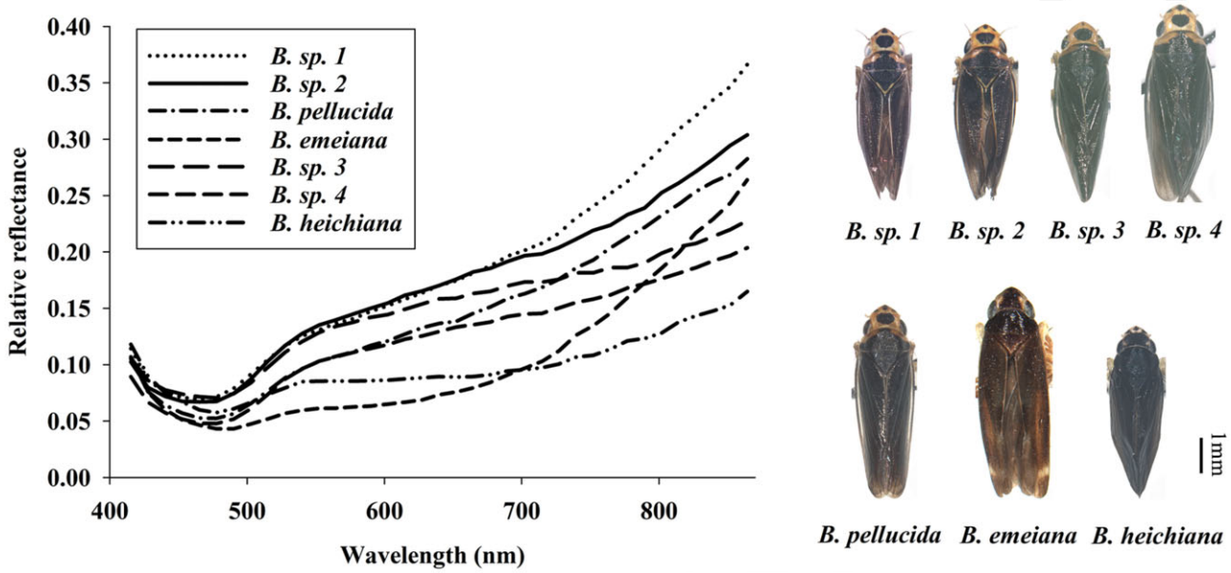


Figure 1. Average reflectance profiles and dorsal habitus of the seven species included in this study.

lished studies (Nansen *et al*., 2013, 2014a,b), the pixels from each specimen were selected based on a radiometric filter, so that a pixel was excluded unless it met the following criteria: $R_{461} > 0.20$, $R_{650} > 0.30$, and $R_{878} < 0.28$.

Using this radiometric filter, we obtained average reflectance profiles (about 450 pixels from the dorsal side of each specimen; Fig. 1). The original spectral data consisted of 240 spectral bands from 392 to 889 nm (with a spectral resolution of 2.1 nm); however, ten bands in each end were eliminated because of concerns about radiometric stochasticity, so we only used data from 220 narrow spectral bands from 411 to 870 nm, and these were spectrally binned (averaged across six adjacent spectral bands) to create 37 spectral bands. The main reason for the spectral binning was to use fewer spectral bands, and therefore avoid any concerns about over-fitting the classification model (Nansen *et al*., 2013; Zhang *et al*., 2015), as happens with 'Hughes phenomenon' (Guo *et al*., 2008; Lu *et al*., 2011), or violation of 'the principle of parsimony' (Hawkins, 2004). With 37 spectral bands as potential explanatory variables and 52 average reflectance profiles, the risk of model overfitting was considered to be negligible. In PC-SAS 9.4 (SAS, Cary, NC, USA) we used forward linear discriminant analysis (LDA; Fisher, 1936) (PROC STEPWISE) to only select the spectral bands that contributed significantly to the separation of species. In total, 17 of the 37 spectral bands were selected and included in an LDA with jackknife cross validation (PROC DICRIM option = crossvalidate). In the cross validation, a single specimen is excluded from the training data set and used for independent validation. This procedure is repeated for all specimens, and the average classification accuracy is calculated based on these independent validations.

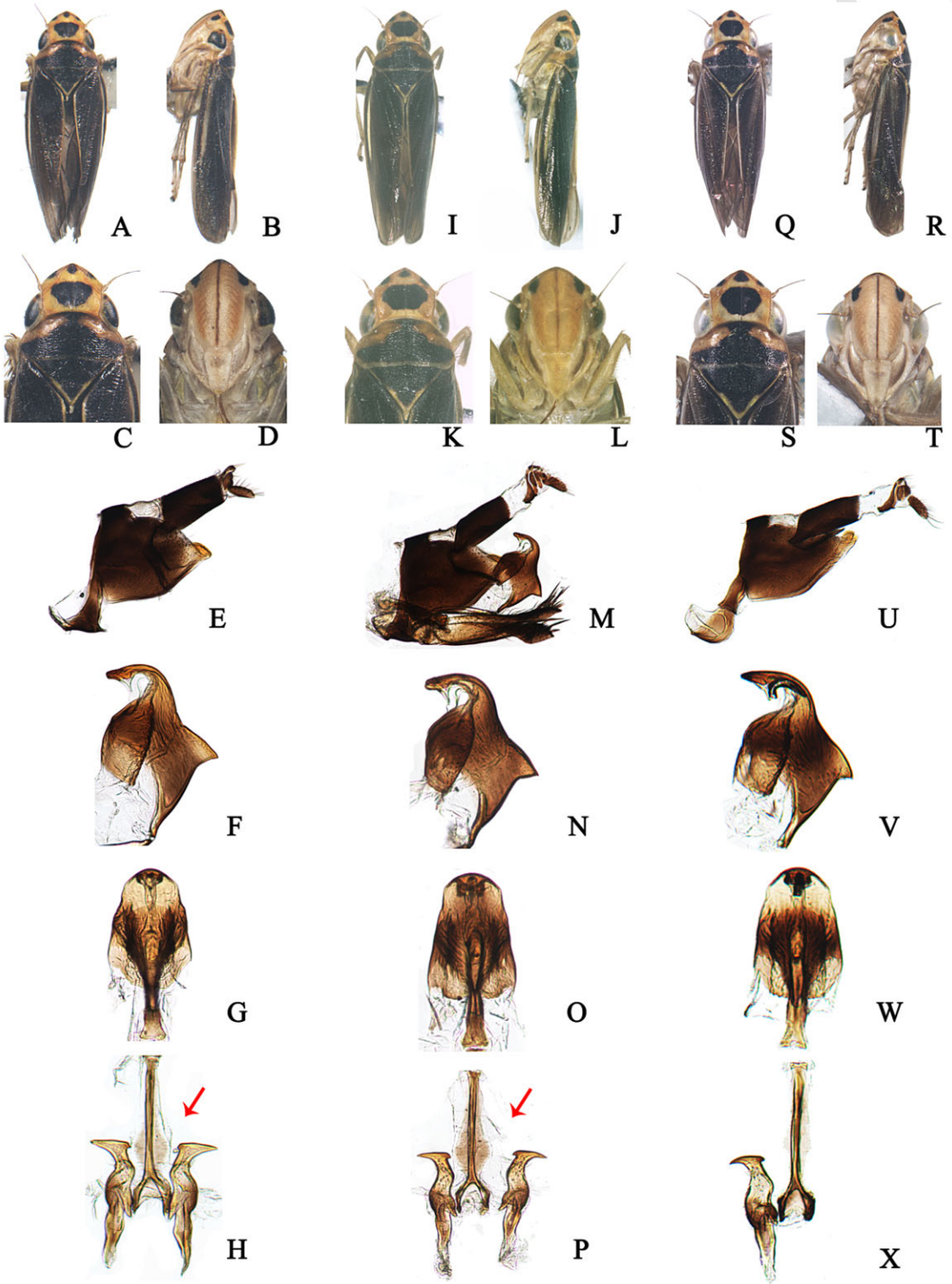
Morphometric imaging and description

External morphology was observed using a Leica MZ 125 microscope. The male genitalia were rinsed with water after DNA extraction, immersed in a droplet of glycerol, and dissected following standard procedures. They were observed under a compound light microscope (Nikon Eclipse 50i). Photos were taken using a Scientific Digital Micrography System equipped with an auto-montage imaging system and a QIMAGING Retiga 4000R digital camera (CCD). The morphological terminology follows Dietrich (2004, 2005).

RESULTS

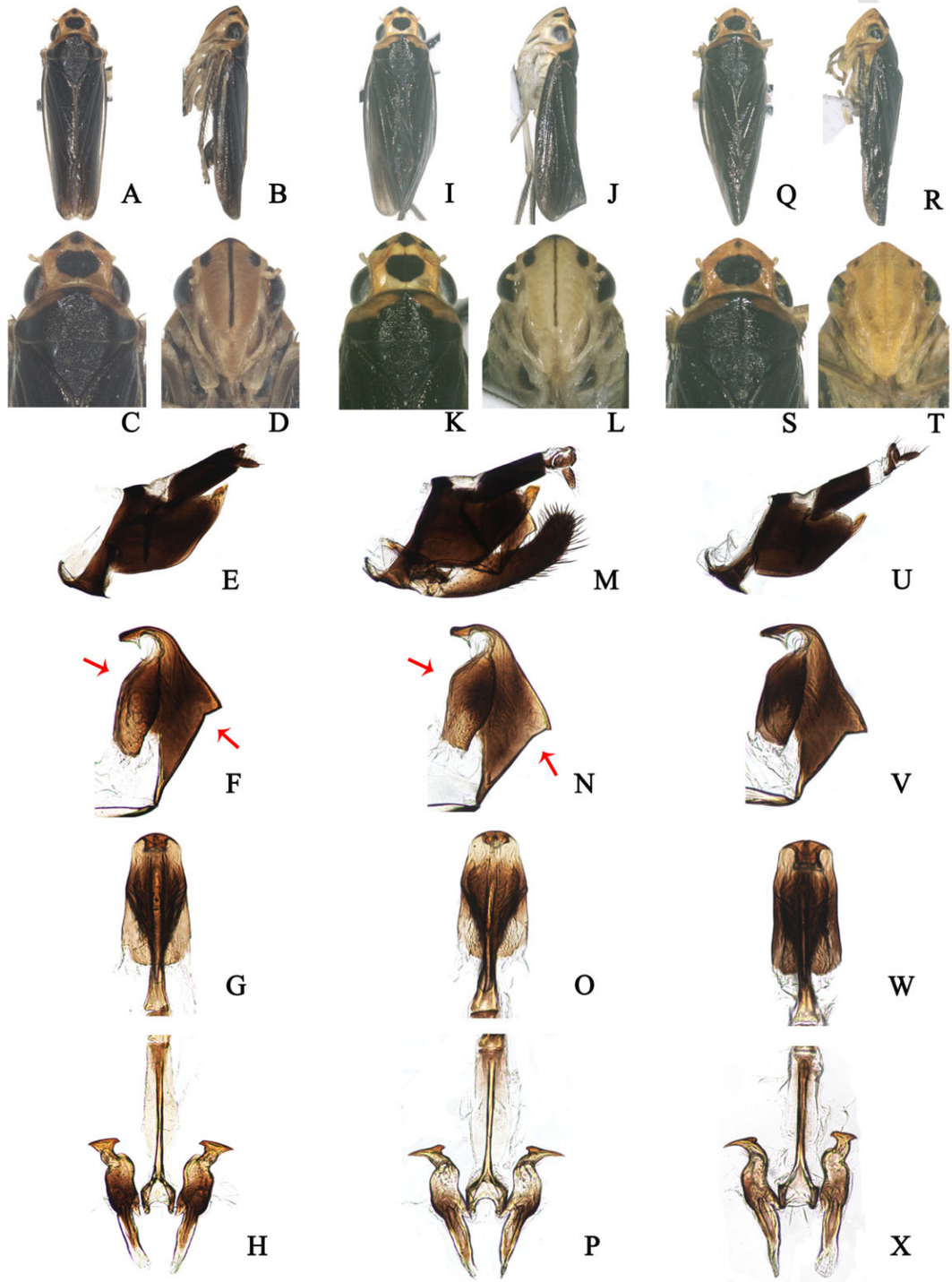
Morphological comparison

Comparisons of morphological characters showed seven groups and species from the 52 tested *Bundera* specimens (Figs 2–4). *Bundera emeiana* Li & Wang, 1994 and *Bundera heichiana* Li & Wang, 1992 could fairly easily be delimited from the others, but the taxon status of the other five groups was considered ambiguous because of low morphological variation. The overlapping morphology was present in both habitus and male genitalia: crown with discal large black spot, and anterior median and one lateral smaller black spot on anterior margin; pronotum with one transverse lateral yellowish-brown stripe on anterior margin; forewing black with a partially transparent stripe along the costal



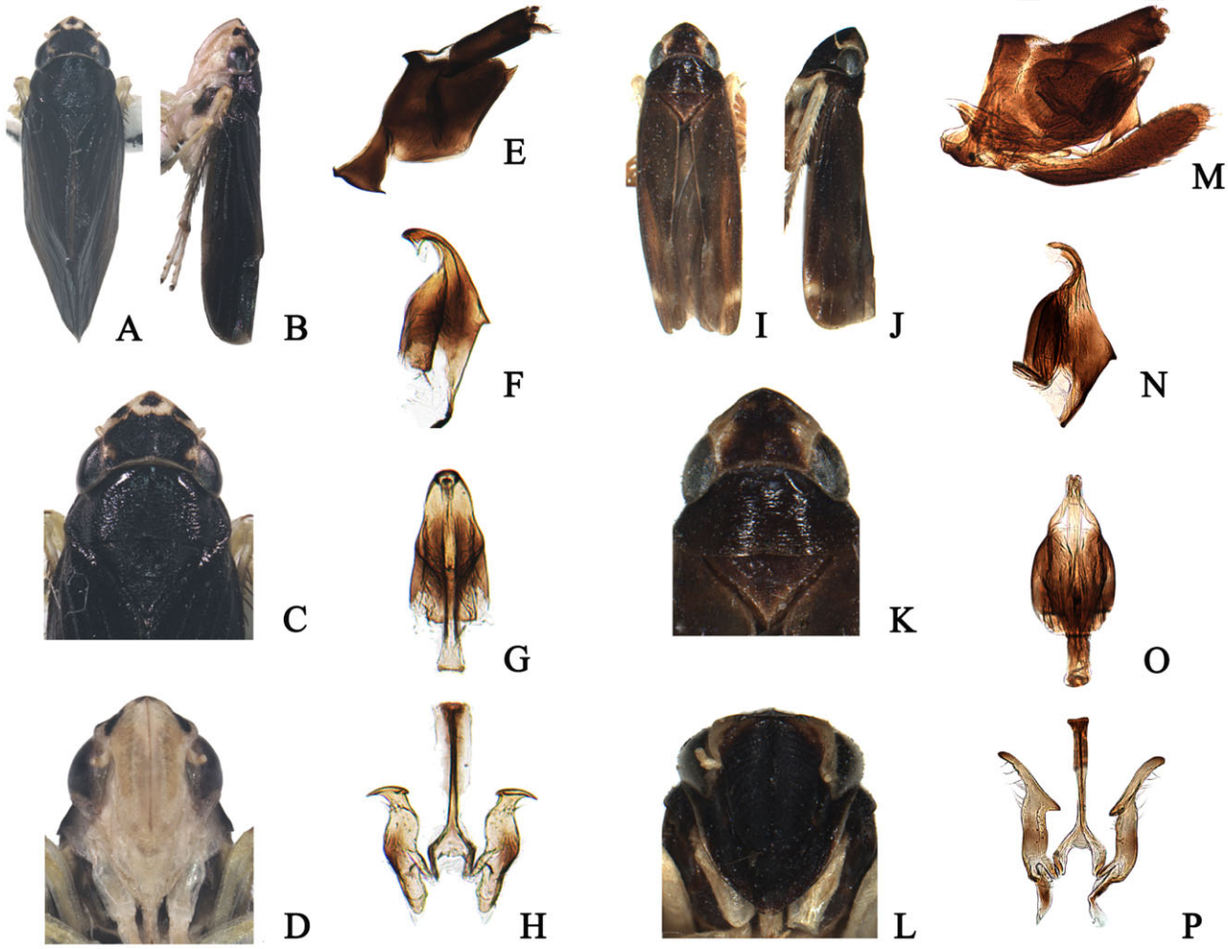
Colour

Figure 2. Male: A–P, *Bundera* sp. 2; Q–X, *Bundera* sp. 1. A, I, Q, habitus, dorsal view; B, J, R, habitus, lateral view; C, K, S, head, dorsal view; D, L, T, face; E, M, U, pygofer, lateral view; F, N, V, aedeagal, lateral view; G, O, W, aedeagal, ventral view; H, P, X, connective and style, ventral view.



Colour

Figure 3. Male: A–H, *Bundera pellucida* Li & Wang, 2001; I–P, *Bundera* sp. 4; Q–X, *Bundera* sp. 3. A, I, Q, habitus, dorsal view; B, J, R, habitus, lateral view; C, K, S, head, dorsal view; D, L, T, face; E, M, U, pygofer, lateral view; F, N, V, aedeagal, lateral view; G, O, W, aedeagal, ventral view; H, P, X, connective and style, ventral view.



Colour

Figure 4. Male: A–H, *Bundera heichiana* Li & Wang, 1991; I–P, *Bundera emeiana* Li & Wang, 1994. A, I, habitus, dorsal view; B, J, habitus, lateral view; C, K, head, dorsal view; D, L, face; E, M, pygofer, lateral view; F, N, aedeagal, lateral view; G, O, aedeagal, ventral view; H, P, connective and style, ventral view.

vein; aedeagus with bilobed, lamellate apodeme arising from atrium and extended dorsolaterad; aedeagus with lamellate triangular ventral apophysis; aedeagus shaft short, recurved dorsally, gonopore apical on dorsal surface. Among these five groups, it is difficult to determine whether the subtle variation in morphological characters is intra- or interspecific. We assumed that they are five different species here, and a key to these species is provided below.

Molecular analysis

In total, we obtained 39 *COI* gene sequences and 37 *16S* gene sequences. Except for samples 24-7 (637 bp) and 40-10 (643 bp), the alignments of all the *COI* sequences are 658 nucleotide positions in length. The alignment of the *16S* sequences is about 510 nucleotide positions in length. Two neighbour-joining trees were constructed under the K2P model, one based on 39 *COI* gene sequences (Fig. 5), and another based on 37 sequences combining the *COI* and *16S* genes (Fig. 5). ME yielded similar results to those of NJ based on the K2P model, and the same NJ tree topology was produced under K2P and T3P substitution models (Figs 5, 6). Seven distinct lineages were revealed from these trees, and they are hereafter treated as seven putative species.

Based on the *COI* gene, the pairwise genetic distances within and between lineages are shown in Table 3. The mean genetic distance between lineages ranged from 5.8 to 17.3%, whereas the maximum intralineage variation ranged from 1.0 to 4.5%. The minimum genetic distance among lineages was between *B.* sp. 4 and *B. pellucida* (5.8%), whereas the maximum genetic distance within lineages was between sample 51-4 and 43-1 (4.5%). Geographically separated lineages of *B.* sp. 2 from Guangxi Province (51-3 and

KEY TO SPECIES OF *BUNDERA* DISTANT, 1908 IN THIS STUDY (MALES)

1. Face entirely black (Fig. 4L); style apophysis lateral prolongation about one-half or more as long as style (Fig. 4P) .....*Bundera emeiana* Li & Wang, 1994
- Face not black; style apophysis lateral prolongation about one-quarter or less as long as style 2
2. Pronotum entirely black (Fig. 4C); forewing black without any stripe (Fig. 4A, B) .....*Bundera heichiana* Li & Wang, 1992
- Pronotum with one transverse lateral yellowish-brown stripe on anterior margin; forewing black with a partially transparent stripe along the costal vein 3
3. Scutellum entirely black (Fig. 3C, K, S).....4
- Scutellum black with apical margin yellowish-brown (Fig. 2C, K, S).....6
4. Median longitudinal carina of frontoclypeus not blackish ochreous (Fig. 3T); three small black spots on anterior margin of head, of diameter less than 0.13 mm (Fig. 3S, T).....*Bundera* sp. 3
- Median longitudinal carina of frontoclypeus distinct blackish ochreous (Fig. 3D, L,); three small black spots on anterior margin of head, of diameter larger than 0.22 mm (Fig. 3C, D, K, L).....5
5. Posterior margin of aedeagal ventral apophysis in lateral view slightly concave (Fig. 3N); dorsal margin of aedeagal dorsal apodeme arched (Fig. 3N).....*Bundera* sp. 4
- Posterior margin of aedeagal ventral apophysis in lateral view distinctly concave, forming obtuse angle (Fig. 2F); the dorsal margin of aedeagal dorsal apodeme smooth (Fig. 3F).....*Bundera pellucida* Li & Wang, 2001
6. Connective stem in ventral view with lateral membranous structure at base (Fig. 2H, P); male pygofer narrowed along with the dorsal and ventral margin in lateral view (Fig. 2E, M).....*Bundera* sp. 2
- Connective stem without lateral membranous structure (Fig. 2X); male pygofer only narrowed along with the ventral margin in lateral view (Fig. 2U).....*Bundera* sp. 1

51-5) and Guizhou Province (43-1, 43-2, and 43-3) showed a 4.3% divergence, suggesting the need for more intensive sampling of this species; they were considered as one species. Except for the lineage of *B.* sp. 2, the divergence within lineages, regardless of geographic area, is less than 2.4%. In contrast, the divergence between any pair of lineages exceeded 5.8%. Sequence variation was distinctly smaller within than among species, revealing the existence of a barcoding gap.

HYPERSPECTRAL IMAGES

Based on the classification derived from molecular data and morphological observations, average hyperspectral reflectance data were acquired from the same specimens and used to classify the leafhopper specimens. Average reflectance profiles from the seven species are presented in Figure 1. It is seen that from about 700–867 nm, there was considerable among-species variation. It was also seen that *B. heichiana* and *B.* sp. 4 were associated with reflectance profiles with slightly different features than those from other species. The independent cross-validation suggested that the specimens could be classified with 91.3% accuracy across all seven species; however, some important species-specific variation was observed (Table 4): (1) specimens from four species (*B. heichiana*, *B.* sp. 2, *B.* sp. 3, and *B.* sp. 4) were classified with 100% accuracy; (2) *B.* sp. 1 specimens were partially misclassified (17%) as *B.* sp. 2; (3) *B. emeiana* specimens were partially misclassified (20%) as *B. pellucida*; (4) *B. pellucida* specimens were only classified with 70% classification accuracy.

DISCUSSION

Seven distinct species from the leafhopper genus *Bundera* were delineated based on integrative analysis of morphology, mitochondrial DNA, and reflectance profiling. The divergence of five morphologically similar species (*B. pellucida*, *B.* sp. 1, *B.* sp. 2, *B.* sp. 3, and *B.* sp. 4) was revealed in mitochondrial DNA data and reflectance profiling. It is confirmed that morphological differences among these species are, nevertheless, still relatively small.

We proposed morphospecies based on subtle divergences of morphological features, and then clarified their specific status by mtDNA and hyperspectral reflectance profiling. Our analyses of mtDNA sequences showed low levels of intraspecific genetic variation in all seven species. The existence of a 'barcoding gap' between intraspecific and interspecific genetic distances supports the notion that they are seven distinct species. The mean sequence divergences obtained in the *COI* gene among the seven species were in the range of 5.8–17.3% (Table 3), which corresponded to the interspecies divergence levels found in other insect taxa (Hebert *et al.*, 2003; Ballman *et al.*, 2011; Han *et al.*, 2012; Miraldo *et al.*, 2014). In addition, Park *et al.* (2011) reported that congeneric species in Heteroptera showed

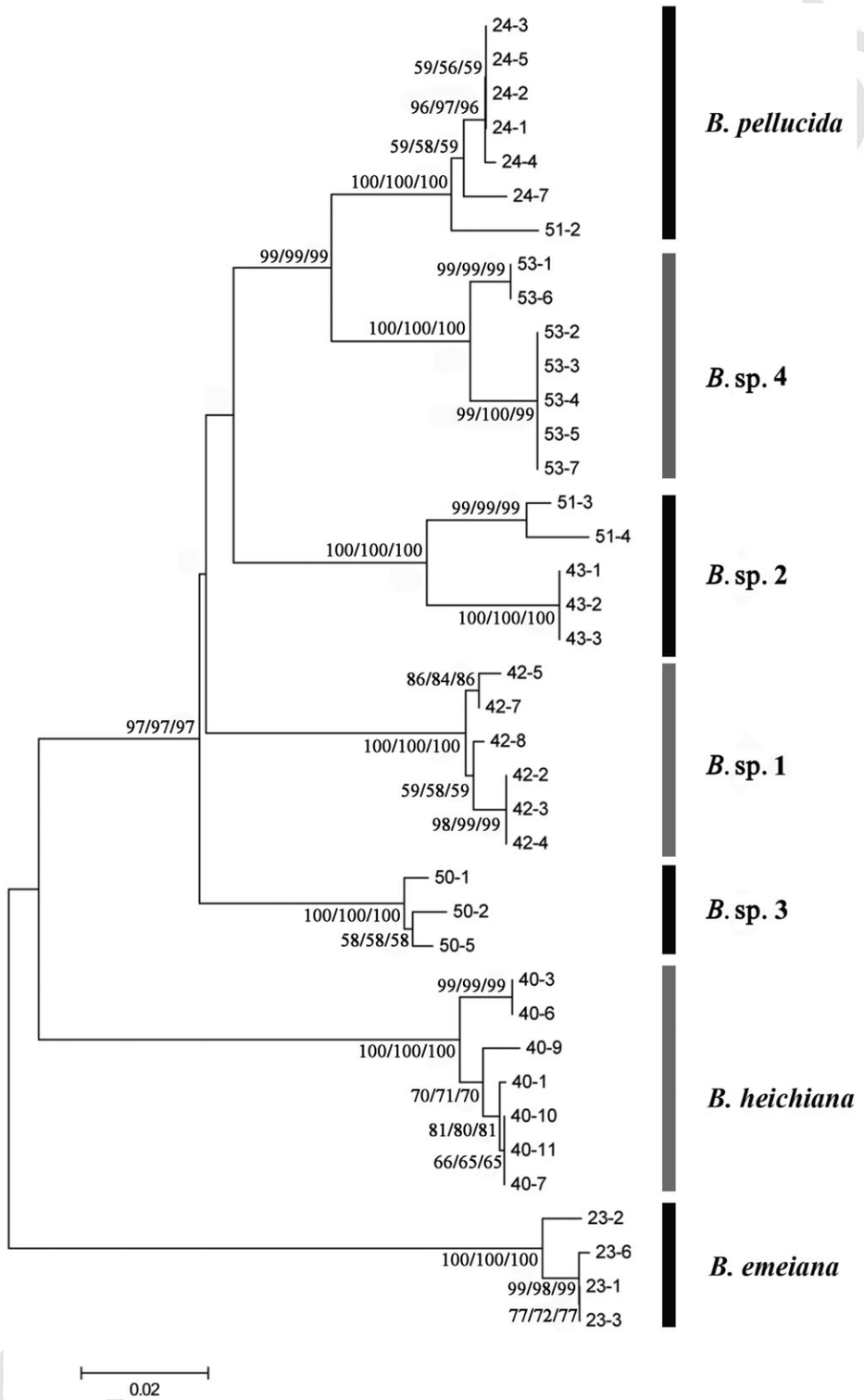


Figure 5. Neighbour-joining tree (Kimura two-parameter, K2P) for 39 barcode cytochrome *c* oxidase subunit I (*COI*) sequences. The node support: bootstrap neighbour-joining (NJ) (K2P)/NJ (Tamura three-parameter, T3P)/minimum evolution (ME) (K2P). Single values on the MB tree correspond to Bayesian posterior probabilities. Question marks represent bootstrap values of less than 50.

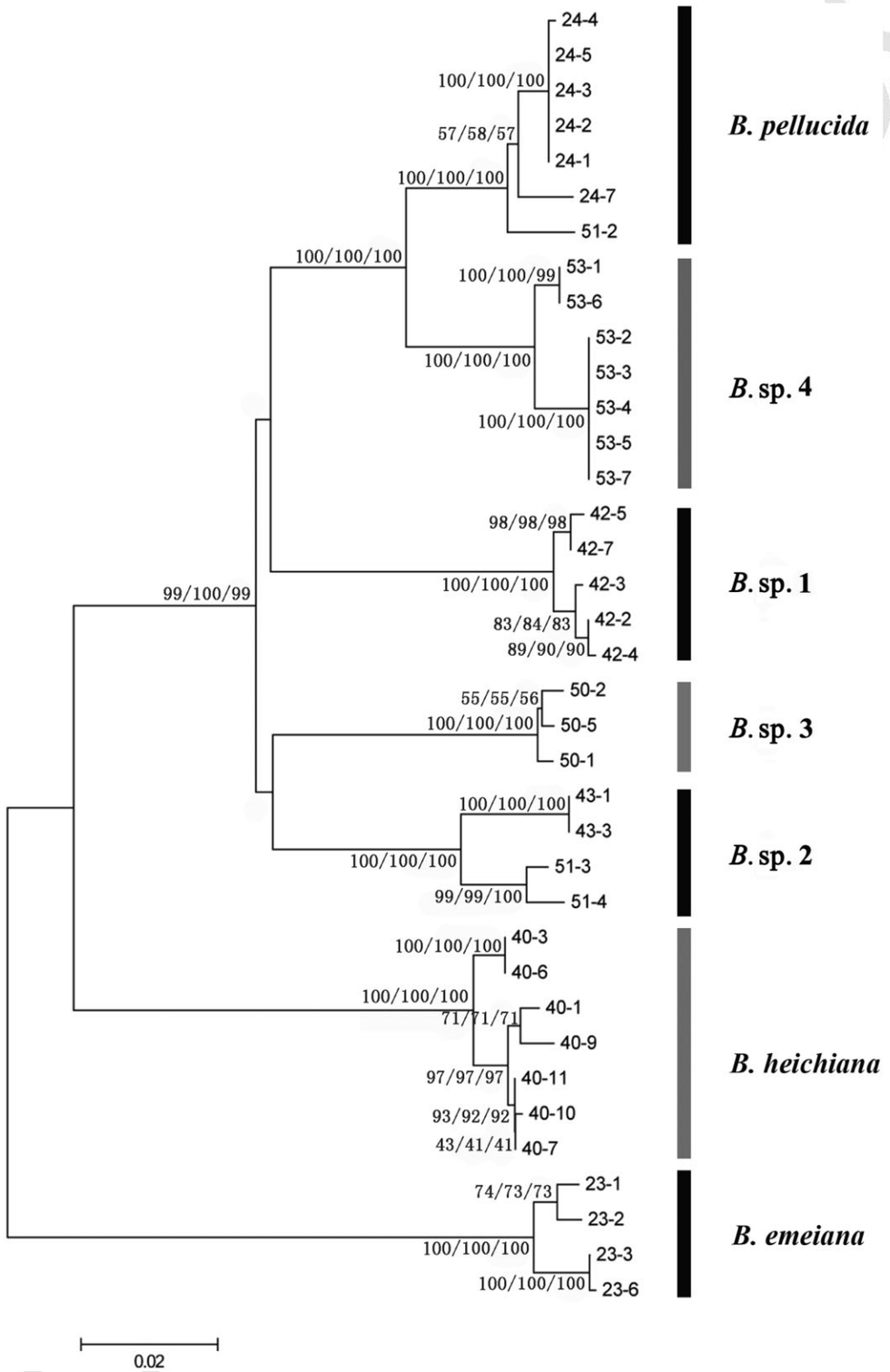


Figure 6. Neighbour-joining (NJ) tree (Kimura two-parameter, K2P) for 37 sequences of combined cytochrome *c* oxidase subunit I (*COI*) and *16S*. The node support: bootstrap NJ (K2P)/NJ (Tamura three-parameter, T3P)/minimum evolution (ME) (K2P). Single values on the MB tree correspond to Bayesian posterior probabilities. Question marks represent bootstrap values of less than 50.

Table 3. Percentage of divergence in the cytochrome *c* oxidase subunit I (*COI*) gene sequences

	1	2	3	4	5	6	7
1 *Bundera emeiana*	**1.4**						
2 *Bundera pellucida*	17.3	**2.4**					
3 *Bundera heichiana*	16.9	14.5	**2.1**				
4 *Bundera* sp. 1	17.2	8.8	14.2	**1.4**			
5 *Bundera* sp. 2	16.0	9.6	16.0	11.0	**4.5**		
6 *Bundera* sp. 3	15.5	8.1	13.8	8.2	8.8	**1.0**	
7 *Bundera* sp. 4	16.9	5.8	15.2	10.0	9.6	9.3	**1.7**

All genetic mean distances (%) were corrected with the Kimura two-parameter (K2P) substitution model using MEGA 6; the numbers in bold are the maximum intraspecific distances.

an average of 10.7% divergence (range 0–24.8%), with minimum interspecific distances exceeding 3% for more than three-quarters of the species pairs. Bluemel *et al*. (2014) reported divergence ranges from 4.21 to 7.0% between four closely related species of the genus *Aphrodes* (Hemiptera: Cicadellidae).

Based on morphology, *B. pellucida* and *B.* sp. 4 are very similar, but with the following differences: (1) median longitudinal carina of frontoclypeus marked with wide stripe (Fig. 3L), the stripe in *B.* sp. 4 is thin (Fig. 3D); (2) aedeagal (Fig. 3N) ventral apophysis in lateral view, posterior margin distinctly concave, at an angle of 130°, but only slightly concave in *B.* sp. 4 (Fig. 3F). *Bundera pellucida* and *B.* sp. 4 are the most closely related pair of genetic lineages in the phylogenetic trees (Figs 5, 6), with 5.8% divergence. Interestingly, *B. pellucida* specimens were only classified with 70% classification accuracy in reflectance analysis, but no one specimen was misclassified as *B.* sp. 4 (Table 4). Consequently, we argue that *B. pellucida* and *B.* sp. 4 should be two separate species.

Bundera sp. 2 has two geographically separated lineages that showed 4.3% divergence (a species-specific level), but there was less morphological divergence between the two geographical groups (Fig. 2A–P). They shared a particular morphological character distinguishing them from other species: a connective stem with a lateral membranous structure at base (Fig. 2H, P). At the same time, *B.* sp. 2 was classified with 100% classification accuracy in the reflectance analysis (Table 4), indicating low divergence within *B.* sp. 2. Furthermore, mitochondrial inheritance in arthropods can be affected by symbiont *Wolbachia* infections with resultant high divergence in host mtDNA (Hurst & Jiggins, 2005; Frezal & Leblois, 2008; Munoz *et al*., 2011). Considering the low number of specimens (only two or three individuals for each geographical group), we treated them as one species here, but more sampling is certainly warranted.

The aedeagus of *B. pellucida*, *B.* sp. 4, and *B.* sp. 3 (Fig. 3F, N, V) were very similar, and may even be a clinal variation. By contrast, *B.* sp. 3 presented a distinctly independent lineage, and distant from *B. pellucida* and *B.* sp. 4 in the phylogenetic trees (Figs 5, 6). At the same time, *B.* sp. 3 specimens were classified with 100% accuracy based on hyperspectral reflectance data. Although the differences in genital morphology are considered the most reliable characters to delimitate related species, and aedeagal morphology has been a prominent taxonomic character used for leafhoppers for about 80 years, the male genital morphology as an adaptation to prevent hybridization between syntopic species may not be the only factor (Eberhard, 1985, 2010; Bluemel *et al*., 2014). *Bundera* sp. 3 was collected from Tibet, which is geographically isolated from the others. Thus, *B.* sp. 3 is likely to be a distinct species from *B. pellucida* and *B.* sp. 4.

Insect systematics has a long tradition for integrative congruence, which promotes taxonomic stability. Considering that characters do not change at all levels and the rates of character change are different, this runs the risk of underestimating species numbers (Padial & De La Riva, 2010). Several recent studies of insects (Padial & De La Riva, 2010; Yang *et al*., 2012; Bluemel *et al*., 2014) have discussed or illustrated the power of 'integrative taxonomy'. A comprehensive analysis of leafhoppers of the genus *Bundera* using multiple criteria delimited seven distinct species: the five morphologically similar species (*B. pellucida*, *B.* sp. 1, *B.* sp. 2, *B.* sp. 3, and *B.* sp. 4) were clearly discriminated. Furthermore, morphological differences among species were validated by molecular analysis and allowed us to confirm reliable morphological characters to distinguish species (see the key to species).

Based on integrative taxonomy, taxonomists can propose hypothetical morphospecies based on the variation of morphological features among individuals. Hypotheses about such morphospecies could be tested and clarified by other approaches and additional data (Dayrat, 2005). The suitability of the *COI* gene for DNA barcoding in species identification and delineation is widely acknowledged for animal groups (Hebert *et al*.,

Table 4. Number of observation and percentage classified into species, and error count estimates for species

Classified into from	1		2		3		4		5		6		7		Error	
species	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
1 *Bundera emeiana*	**5**	**83.33**	1	16.67	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	16.67
2 *Bundera pellucida*	0	0.00	**7**	**70.00**	1	10.00	1	10.00	1	10.00	0	0.00	0	0.00	3	30.00
3 *Bundera heichiana*	0	0.00	0	0.00	**11**	**100.00**	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
4 *Bundera* sp. 1	0	0.00	0	0.00	0	0.00	**6**	**85.71**	1	14.29	0	0.00	0	0.00	1	14.29
5 *Bundera* sp. 2	0	0.00	0	0.00	0	0.00	0	0.00	**6**	**100.00**	0	0.00	0	0.00	0	0.00
6 *Bundera* sp. 3	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	**5**	**100.00**	0	0.00	0	0.00
7 *Bundera* sp. 4	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	**7**	**100.00**	0	0.00
Total	5	9.62	8	15.38	12	23.08	7	13.46	8	15.38	5	9.62	7	13.46	5	8.71
Priors	0.1429		0.1429		0.1429		0.1429		0.1429		0.1429		0.1429			

N, number of specimens; %, percentage classified into species; the numbers in bold are the numbers and percentages of specimens that are classified accurately.

2004; Foottit *et al*., 2014). Interestingly, Rodriguez-Fernandez *et al*. (2011) suggested that spectroscopy may actually fit that metaphor (species barcoding) much better, and may be a true 'barcoding of life', interacting with morphological, genomic, or geographic data. Our reflectance-based profiling of *Bundera* species probably reflects the species-specific variation in cuticular composition. Hyperspectral reflectance profiling, which represents a powerful tool for the rapid and non-destructive identification of closely related insect species, should be an important component of integrative taxonomy.

Taxonomic misidentification of the specimens used to obtain DNA sequences is a growing problem, which threatens the utility of the deposited sequences in public DNA databases and increases the confusion of taxa (Vilgalys, 2003; de Mendonca *et al*., 2011; Auger *et al*., 2013). The reliable DNA data should be extracted from the holotype, but often the specimens are old, precious, and potentially contaminated with DNA from other organisms. In such cases, hyperspectral reflectance profiling may provide a complement or substitute to other taxonomic approaches.

Many character systems (e.g. DNA barcoding, morphology, and ecological niche profiling) are applied in taxonomy, but characters do not change at all levels and the rates of character change are heterogeneous (Padial & De La Riva, 2010). In contrast to the results analysed in this study, Yang *et al*. (2014) reported that some species of the cicada genus *Mogannia* (Cicadidae: Cicadinae) show considerable morphological variation but less genetic divergence. The use of integrative taxonomic analysis will greatly accelerate the assessment of biodiversity and the discovery of characters, new species, and relationships between species (Yang *et al*., 2012; Rajaei Sh *et al*., 2013). Our work showed that when dealing with genera such as *Bundera*, with low morphological differentiation between syntopic congeners, the integration of reflectance profiling and molecular data enables the clarification of morphological characters and of species differentiation.

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