



A Formal Re-Description of the Cockroach *Hebardina concinna* Anchored on DNA Barcodes Confirms Wing Polymorphism and Identifies Morphological Characters for Field Identification

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Abstract

Background: *Hebardina concinna* is a domestic pest and potential vector of pathogens throughout East and Southeast Asia, yet identification of this species has been difficult due to a lack of diagnostic morphological characters, and to uncertainty in the relationship between macropterous (long-winged) and brachypterous (small-winged) morphotypes. In insects male genital structures are typically species-specific and are frequently used to identify species. However, male genital structures in *H. concinna* had not previously been described, in part due to difficulty in identifying conspecifics.

Methods/Principal Findings: We collected 15 putative *H. concinna* individuals, from Chinese populations, of both wing morphotypes and both sexes and then generated mitochondrial COI (the standard barcode region) and COII sequences from five of these individuals. These confirmed that both morphotypes of both sexes are the same species. We then dissected male genitalia and compared genital structures from macropterous and brachypterous individuals, which we showed to be identical, and present here for the first time a detailed description of *H. concinna* male genital structures. We also present a complete re-description of the morphological characters of this species, including both wing morphs.

Conclusions/Significance: This work describes a practical application of DNA barcoding to confirm that putatively polymorphic insects are conspecific and then to identify species-specific characters that can be used in the field to identify individuals and to obviate the delay and cost of returning samples to a laboratory for DNA sequencing.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All the new amplified sequences for *Hebardina concinna* and other species are available from Genbank (<http://www.ncbi.nlm.nih.gov/nucleotide/>) (KF640073, KF640074, KF640076, KF640077, KF640075, KF876003, KF876004, KF876006, KF876007, KF876005, KF640067, KF640069, KF640071, KF640072, KF640066, KF876000, KF640067, KF876001, KF640068, KF876002). Data are also available from the Zhongshan Entry-Exit Inspection and Quarantine Bureau study whose authors may be contacted at 2 Zhongshan 6 Road, Zhongshan 528403, Guangdong, China, or by email yueqy@zs.gdcic.gov.cn.

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Introduction

The Blattaria (cockroaches) are a diverse order of some 4000–4,500 species, the majority of them denizens of tropical forests, but about 40–50 of all the known cockroach species are important domiciliary pests or house frequenting dwellers [1,2]. They carry numerous pathogens and could potentially transmit disease to humans [2–5]. *Hebardina concinna* is one of these pests. *H. concinna* is found in human dwellings and is believed to be a primary house pest [1], and could potentially transmit disease to humans; hence monitoring populations of this cockroach and identifying individuals within human dwellings is relevant for

public health. However, due to wing-length plasticity within this species and related cockroaches, identification of individuals to the species level has been problematic.

The difficulty in describing *H. concinna* is reflected in the literature. De Haan first described this species as brachypterous [6] with the name of *Blatta (Periplaneta) concinna*. Shiraki later described females as brachypterous and males as macropterous [7]. Hebard erected a new genus of *Blattina* with *Blatta (Periplaneta) concinna* as type specimen and described the tegmina as moderately reduced, but without providing detailed descriptions of the tegmina or of male genital structures [8], although later work showed that the generic name of *Blattina* was

preoccupied by Germar in 1842 [9]. Bey-Bienko replaced *Blattina* Hebard with *Hebardina* in order to memorialize Hebard and separated *Hebardina* Bey-Bienko from *Periplaneta* Burmeister on the basis of shortened elytra and hind wings [10], while Bruijning showed that the length of tegmina and the hind-wings of *H. concinna* varied widely, and listed all the tegminal lengths of the specimens that he examined [11]. Asahina then described macropterous specimens from Thailand [1], and most recently Roth examined *H. concinna* specimens collected from Krakatau, Sumatra, Java, India, and the Philippine Islands and noted extensive wing polymorphism [12].

In sum, most authors have focused on using wing morphology to describe this species but this is plastic in many cockroaches, including—as we show below—in *H. concinna*, and therefore of poor utility for species identification. Wing polymorphism exists in many insect orders, and individuals of the same species can be brachypterous or macropterous. In some insects these differences are sex-based (termed dimorphism), but in many species, including *H. concinna*, these differences occur in both males and females and are a response to varying environmental factors during nymphal stages, such as photoperiod, temperature, nutritional status, or population density. In general, good environmental conditions allow high populations whose individuals develop large wings and disperse [13].

To date wing polymorphism in Blattodea has rarely been studied, and is made difficult by their cryptic, nocturnal habits [14] and by the difficulties in identifying species by morphological examination [15–18]. In addition, identification based on morphology has some limitations, for instance it is difficult, even for specialists, to accurately identify females and immature stages [19].

DNA barcoding was developed by Paul Hebert and colleagues in 2003 [20,21], and in the decade since this method has become an important tool for the identification of insects, including cockroaches [22–24]. “Integrated taxonomy” [25,26] or more specifically “Barcodes and morphology taxonomy” or just “B&M taxonomy” [27] combines the power of DNA barcoding with traditional taxonomic methods and the authors above, and we ourselves, believe it should be integrated into the research framework. Nevertheless, many barcoding studies are not integrated with taxonomic research [28,29]. Here we present an integrated barcoding and morphological study of a cockroach.

There is a broad consensus that barcodes contain information relevant for species delimitation, although in some cases a single mitochondrial marker is insufficient as a sole criterion [30]. Notably, successful barcoding identification depends upon genetic diversity being markedly lower within than between species [31]. The commonly adopted standard 658 bp COI segment has proven to be highly informative and useful for species-level identification [27,32], including the matching of morphotypes in species with polymorphic forms. Despite increasing use of DNA-based methods morphology remains the most commonly used method in taxonomic research despite suggestions to abolish it altogether [33], although the future role of morphology in the age of genomes is anyone’s guess [27]. The integrated B&M taxonomic method is potentially a very fruitful one [27], and our study proves its utility.

We describe, for the first time, a detailed morphology for male genital structures in *H. concinna*, compare the male genital structures of both macropterous and brachypterous specimens carefully, and compare these morphological observations with DNA barcode sequences (mitochondrial COI and COII) from males and females of *H. concinna* and related species. We also provide a complete morphological re-description of this species based on samples collected in China.

Materials and Methods

H. concinna specimens

Nine putative males and six females of *H. concinna*, as well as reference individuals from other blattotid species, were collected with a sweep net at night in the leaves and litter layer in woody habitats with the assistance of headlight. Specific permission was not required for collecting in these localities, and the GPS coordinates (latitude and longitude) were provided in Tables 1 and 2. No endangered or protected species were collected for this work.

Morphological study

General morphology. Our terminology follows McKittrick 1964 [34], Grandcolas 1996 [35], Anisyutkin 2010 [36] and Anisyutkin 2013 [37]. The genital segments of the examined specimens were macerated in 10% KOH and observed in glycerin with a Zeiss Discovery V12 stereomicroscope. Wings were floated in hot water until fully spread, embedded in neutral balsam, then mounted on slides and covered with coverslips. Drawings were made using a Zeiss Discovery V12 stereomicroscope fitted with a Canon PowerShot G1X digital camera and drawn using Adobe Illustrator CS6. All images of specimens were photographed using a Canon 60D plus a Canon EF 100 mm f/2.8L IS USM Macro lens combined with Helicon Focus software. All specimens studied were pinned in a natural posture and deposited in the medical vector collections of the Zhongshan Entry-Exit Inspection and Quarantine Bureau (ZSCIQ). The specimens we collected in China fully match other published morphological descriptions of *H. concinna* individuals from other geographic locations [6,38,39].

Quantitative morphology. Length measurements were taken from the specimens using vernier calipers. Three measurements were taken for each of the 15 specimens: tegmen length, pronotum length, and body length (excluding tegmen length). These are reported in Table 1 and plotted (tegmen vs. pronotum and tegmen vs. body length) in Figure 1. The plots suggested that these three measures do not vary independently, and their relationships were tested using a means-independent T-test (SPSS 19.0) on pronotum/tegmen length and body length/tegmen length between sexes and within morphotypes.

Molecular methods

Sampled specimens. The sampled individuals were preserved in 8 mL 95% ethanol immediately after capture, this was replaced with fresh 95% ethanol twice the next day. Macropterous and brachypterous males and females were used for genomic DNA purification. Various species of *Periplaneta*, *Blattella* and *Rhabdoblatta* were also studied as references. Sampled species are summarized in Table 2.

Genomic DNA extraction. A single hind tibia and tarsus were removed from each specimen for DNA extraction. All instruments used to remove leg tissues were cleaned with 70% ethanol and flame sterilized between each specimen. Genomic DNA was purified with a TIANamp Genomic DNA Kit (DP304, TIANGEN). Voucher specimens were labeled uniquely and deposited in the Medical vector collections of the Zhongshan Entry-Exit Inspection and Quarantine Bureau.

PCR amplification. We amplified a 658 bp segment of the mitochondrial COI gene using the standard arthropod DNA barcoding primers [40] and a 601 bp segment across the mitochondrial COI and COII genes, also using previously published and widely used primers [41] from five of the 15 collected *H. concinna*. Five macropterous and brachypterous male

Table 1. *Hebardina concinna* specimens collected for morphological study.

Tegmena type	Sex	Collection locality within Guangdong	Collection location (N/E)	Collection date	Collector	Specimen accession number	Pronotum length (mm)	Tegmen length (mm)	Body length (mm)
Macropterous	♂	Huizhou	23°11'10.80"/114°30'41.36"	12-Oct-2010	HUANG YW	HC-16a*	4.5	15.7	16.9
	♂	Huizhou	23°11'10.80"/114°30'41.36"	06-May-2012	HUANG YW	HC-16b	4.8	15.8	16.2
	♂	Zhongshan	22°31'10.15"/113°25'02.10"	06-Apr-2013	Liu DX	HC-126a*	4.7	14.5	20.4
	♂	Zhongshan	22°30'15.95"/113°24'37.36"	02-Apr-2013	YUE QY	HC-126b	4.3	15.8	15.3
	♂	Zhongshan	22°31'10.15"/113°25'02.10"	06-Apr-2013	WU KL	HC-126C	4.5	14.5	15.4
	♀	Zhongshan	22°31'10.15"/113°25'02.10"	22-Oct-2012	YUE QY	HC-188a*	4.3	15.5	15.4
	♀	Zhongshan	22°31'10.15"/113°25'02.10"	06-Apr-2013	WU KL	HC-188b	5.3	16.9	21.2
	♀	Zhongshan	22°31'10.15"/113°25'02.10"	06-Apr-2013	WU KL	HC-188C	4.5	14.4	15.1
Brachypterous	♂	Zhongshan	22°31'10.15"/113°25'02.10"	06-Apr-2013	WU KL	HC-305a	4.9	10.1	15.4
	♂	Zhongshan	22°31'10.15"/113°25'02.10"	06-Apr-2013	Liu DX	HC-305b*	4.7	9.9	14.5
	♂	Zhongshan	22°31'10.15"/113°25'02.10"	06-Apr-2013	WU KL	HC-305c	4.9	10.7	16.9
	♂	Zhongshan	22°56'31.51"/112°03'38.32"	06-Apr-2013	WU KL	HC-305d	4.3	9.9	15.1
	♀	Zhongshan	22°31'10.15"/113°25'02.10"	22-Oct-2012	YUE QY	HC-187a*	5.3	10.6	14.9
	♀	Zhongshan	23°11'10.80"/114°30'41.36"	22-Oct-2012	YUE QY	HC-187b	5.1	10.4	15.8
	♀	Zhongshan	23°11'10.80"/114°30'41.36"	06-Apr-2013	Liu DX	HC-187c	4.7	10.2	16.5

Macropterous is defined as tegmen fully developed and extending well beyond the end of abdomen; brachypterous is defined as tegmen just about reaching to the hind margin of the second abdominal tergum. Note that there is no overlap in tegmen length between the two size classes. Individuals marked with an asterisk (*) were selected for DNA barcoding.
doi:10.1371/journal.pone.0106789.t001

Table 2. Sequences used for DNA Barcode analysis.

Family	Species	Individual descriptors (H. concinna only)	Accession No. for COI	Accession No. for COII	Collection location (N/E)		
Blattidae	<i>Hebardina concinna</i>	Macropterous ♂ HC6a	KF640073	KF876003	23°11'10.80"/114°30'41.36"		
		Macropterous ♂ HC126a	KF640074	KF876004	22°31'10.15"/113°25'02.10"		
		Macropterous ♀ HC188a	KF640076	KF876006	22°31'10.15"/113°25'02.10"		
		Brachypterous ♂ HC305b	KF640077	KF876007	22°31'10.15"/113°25'02.10"		
		Brachypterous ♀ HC187a	KF640075	KF876005	22°31'10.15"/113°25'02.10"		
	<i>Periplaneta americana</i>		KC617846				
				KF640070		22°34'14.10"/113°32'06.94"	
				JQ350707			
				JN900479			
					DQ181546		
	<i>Periplaneta australasiae</i>			KF640069		22°34'43.17"/113°26'17.78"	
				AM114928			
		<i>Periplaneta fuliginosa</i>			JQ350729		
					AB126004	AB126004	
						AB014067	
Ectobiidae	<i>Blattella germanica</i>		JQ267496				
				KF640071		22°29'39.54"/113°24'32.46"	
				KF640072		22°29'49.54"/113°25'02.10"	
					DQ874268		
					EF363216		
	<i>Blattella bisignata</i>			NC018549	NC018549		
				JX233805	JX233805		
		<i>Rhabdoblatta atra</i>			KF640066	KF876000	22°09'43.36"/108°27'34.36"
					KF640067	KF876001	22°08'59.45"/108°11'50.38"
					KF640068	KF876002	22°12'38.17"/108°09'19.28"

Sequences with accession numbers starting with KF (in bold) are newly reported in this paper, and collection locations are reported. Other sequences were retrieved from NCBI GenBank: for collection locations see the GenBank accession. COI and COII accession numbers in the same row indicate sequences from the same individual; otherwise only a COI or a COII sequence was available for that individual. Note that within the Blattidae COI and COII sequences from the same individual are available only for individuals newly reported in this paper, from a single *P. fuliginosa*, and from a single *B. bisignata*: a combined COI/COII dataset with only these 10 individuals from within the Blattidae would not have been useful for analyses aimed at determining the relationship between *H. concinna* and other Blattidae, so separate COI and COII datasets were compiled and analyzed independently.
doi:10.1371/journal.pone.0106789.t002

and female individuals were selected for DNA barcoding (Table 1). COI primers were LCO1490 (GGTCAACAAATCA-TAAAGATATTGG) and HCO2198 (TAAACTTCAGGGT-GACCAAAAAATCA). The COII primers were CO1DL (CCWCGWCGWTAYTCWGAYTAYCCWGA) and CO2DL (WGAATARRCATAWSWTCARTATCATTTG).

Reaction volumes were 10× Taq polymerase buffer 5 µL, dNTP (2.5 mM each) 2 µL, primer (20 µM) 1 µL each, Taq polymerase (5 U/µL) 0.5 µL, DNA template 70–100 ng, and ddH₂O to a total volume of 50 µL.

Reaction conditions were 95°C 3 min; 95°C 45 s, 50°C 45 s, 72°C 1 min, 34 cycles; 72°C 10 min. PCR products are stored at

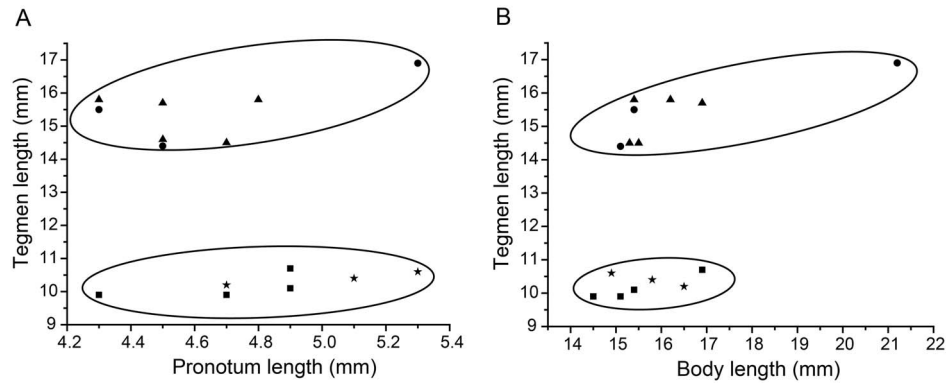


Figure 1. Dotplots of Tegmen length vs Pronotum length and body length in *Hebardina concinna*. These correlated characters form statistically non-overlapping groups in macropterous vs. brachypterous individuals. A. Tegmen length vs pronotum length; B. Tegmen length vs body length. Remarks: Triangle for male macropterous; circle for female macropterous; box for male brachypterous; star for female brachypterous. doi:10.1371/journal.pone.0106789.g001

−20°C at the Zhongshan Entry-Exit Inspection and Quarantine Bureau.

PCR product purification and sequencing. PCR products were purified with a TIANGel Midi purification Kit (DP209-02), linked to a T-vector with the TIANGen pGEM-T ligation kit, at 16°C overnight, then transformed into DH5 α competent cells (TakaRa Biotechnology (Dalian) Co., Ltd.) for white/blue selection. White clones on the LB-agar selection medium plate with Ampicillin (100 μ g/mL), IPTG (1 mM) and X-Gal (20 μ g/mL) were selected for PCR Screening with a TIANGen pGEM-T recombinant colony identification Kit, then 3–5 randomly chosen positive colonies were cultured in LB medium with Ampicillin (100 μ g/mL) at 37°C overnight, plasmid DNA was purified with TIANGen Mini Plasmid Kit and then sequenced commercially (Life Technologies Corporation). Plasmid DNA is stored at −20°C, and colonies in 20% glycerol at −80°C. Mutation rates are higher in sequencing directly from PCR products than sequencing colonies from a cloned PCR product sequencing [42,43], so we chose to sequence clones for this work. For each PCR product 3–5 sequenced clones were used for analysis: the maximum differences among different clones were 3 bp out of the 658 (0.46%). This is much lower than the species limitation proposed by Hebert in 2003 [21], so a consensus sequence for each clone was used for all analyses.

Phylogenetic analysis. Sequences from *H. concinna* and other species were submitted to the International Nucleotide Sequence Database Collaboration via NCBI GenBank. As noted above we used two primer sets: one amplified a section of the mitochondrial COI gene and a second amplified a short stretch of the 3' terminus of the COI gene, the tRNA-leu gene, and the 5' 306 bp of the COII gene. For this second amplicon the tRNA-leu and 3' COI sequences have little variation, and we used only the COII sequences from this amplicon for our analysis.

We also identified nine additional cockroaches in GenBank for which either or both the COI and COII regions were available and created separate COI and COII data sets for phylogenetic analysis (Table 2). Unfortunately there were not enough individuals for which both regions had been sequenced to assemble a combined dataset using both genes so we analyzed the two data sets separately.

We estimated maximum likelihood and neighbor-joining phylogenetic trees for both the COI and COII data sets using Mega 5.2 [44], and tested robustness of the results using non-parametric bootstrapping. For both data sets the most appropriate maximum likelihood model (TN+I) was identified using the model

testing function of Mega and this model was used to estimate the ML tree for each data set. Support for each branch was assessed using the same model for 1000 bootstrap replicates. Neighbor joining trees were constructed for each data set using the same Tamura-Nei model and also tested using 1000 non-parametric bootstrap replicates.

Results

Taxonomic description

Hebardina Bey-Bienko, 1938 [10].

Blattina Hebard, 1929: 84 [8].

Hebardina Bey-Bienko, 1938: 234 [10].

Type species: *Blatta (Periplaneta) concinna* de Haan, 1842 [6].

Generic diagnosis

Middle sized and uniformly dark colored cockroaches. Sexual dimorphism inconspicuous. Tegmen and wings fully developed or reduced. Front femur Type A. Tarsus with 2 rows of spines along lower margin; pulvilli and arolium present; post-tarsus claws symmetrical, unspecialized. First abdominal tergum of male specialized, with a densely setose medial tergal gland. Supra-anal plate and paraprocts symmetrical. Hypandrium slightly asymmetrical. Left phallomere with L2d large, occupied upper margin. L3d with a hook in the terminal. L2v elongated, plate-like and additional with a spiniform curved process. Right phallomere with caudal part of sclerite R1 plate-like, processes; sclerite R2 with groove at cranial part and right side; R4 plate-like and inset in the groove of sclerite R2.

This species has also been described with the following Names [9]

***Hebardina concinna* (de Haan, 1842).**

Blatta (Periplaneta) concinna Haan, 1842: 50.

Periplaneta borrei Saussure, 1873: 113. Synonymized by Princis, 1966: 467.

Stylopyga concinna, Krauss, 1902: 747.

Methana concinna, Kirby, 1904: 136.

Stylopyga concinna, Shiraki, 1906: 17, 30.

Blatta concinna, Shelford, 1910: 15.

Blattina concinna, Hebard, 1929: 12, 84.

Blatta concinna, Hanitsch, 1932: 5.

Blattina concinna, Chopard, 1934: 728.

Hebardina concinna, Bey-Bienko, 1938: 234.

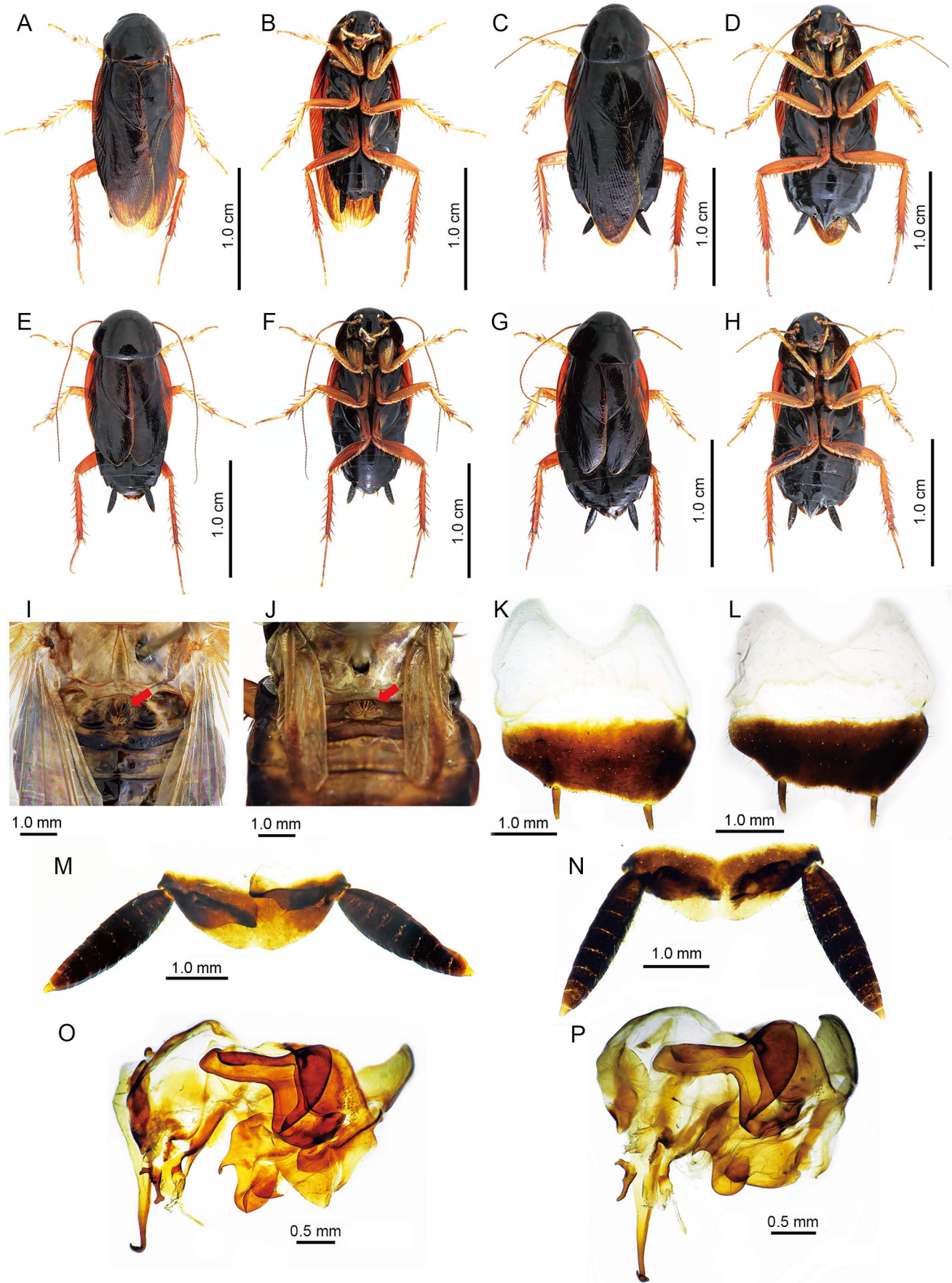


Figure 2. Overview of *Hebardina concinna* morphology clearly showing differences in wing length. A–D. Macropterous. A. ♂, dorsal view. B. ♂, ventral view. C. ♀, dorsal view. D. ♀, ventral view. E–H Brachypterous. E. ♂, dorsal view. F. ♂, ventral view. G. ♀, dorsal view. H. ♀, ventral view. I–J. First abdominal tergum. I. ♂, macropterous. J. ♂, brachypterous. K–L. hypandrium, dorsal view. K. ♂, macropterous. L. ♂, brachypterous. M–N. supraanal plate, ventral view. M. ♂, macropterous. N. ♂, brachypterous. O–P. male genitalia, dorsal view. O. macropterous, P. brachypterous. Remarks: Arrow (←) indicate the medial tergal gland on the first abdominal tergum. doi:10.1371/journal.pone.0106789.g002

Blatta concinna, Dammerman, 1948: 484, 555.

Blattina concinna, Bruijning, 1948: 39, 114.

Hebardina concinna, Princis, 1950: 204, 210.

General Description

Sizes of the examined *H. concinna* specimens are summarized in Table 1.

Except for the differences in the length and the vein of the tegmen and hindwings, the morphology and structure of the male genitalia of the macropterous and brachypterous specimens are identical as illustrated in Figure 2, Figure 3 and Figure 4.

Body reddish brown (Figs. 1. A–H). Head reddish brown (Figs. 1. B, D, F, H). Antennae yellowish brown (Figs. 1. A–H). Eyes black (Figs. 1. B, D, F, H). Ocelli yellowish (Figs. 1. B, D, F, H). Maxillary palpi yellowish (Figs. 1. B, D, F, H). Coxa reddish brown, and other leg segments yellowish brown (Figs. 1. B, D, F, H). Vertex with interocular space equal to the distance between antennal sockets (Figs. 1. B, D, F, H). Third, fourth, and fifth maxillary palp the same length (Figs. 1. B, D, F, H). Front femur Type A2 (Figs. 1. B, D, F, H). All tarsi with 2 rows of spines along lower margin; pulvilli and arolium present; post-tarsus claws symmetrical, unspecialized (Figs. 2. E–F). The tegmina and wings in this species occur in two types, macropterous and brachypterous (Figs. 1. A–H; 2. A–D). The former is fully developed extending well beyond the end of abdomen (Figs. 1. A–D). And the later is smaller, just about reaching to the hind margin of the second abdominal tergum (Figs. 1. E–H). Tegmen with subcostal veins strongly oblique and without branches. The branches of the radial veins and cubital veins of macropterous individuals are more numerous than in the brachypterous individuals (Figs. 2. A–B). The hind wing of macropterous individuals has thickened costal veins, particularly in the apical area. Medial vein simple, without branches or with one branch. Cubital vein with 3 complete branches to the apex and some branches behind them not reaching the apex, triangular apical area moderate in size. Hind wing of brachypterous individuals reduced and smaller than in macropterous individuals, and without triangular apical area (Figs. 2. C–D).

First abdominal tergum of male specialized, with a densely setose medial tergal gland (Figs. 1. I–J). Male genitalia with supraanal plate symmetrical, hind margin convex with a weak medial indentation, paraprocts nearly symmetrical. Cercus coned (Figs. 1. M–N; 2. G). Hind margin of hypandrium slight asymmetrical, left side slightly more convex than right side; styli small, cylindroid (Figs. 1. K–L; 2. H). Left phallomere with L2d large, caudal slightly widened and with hooklike terminus, cranial section bent; Elongated L3d occupying ventral part of phallomere, with a hook in the terminus; Sclerite L1 simple, large, plate-like, slightly squared; Small L3v elongated, with one side enlarged, the other side plated; L2v large, occupying dorsal and right side of phallomere, elongated, plate-like and additionally with a spiniform curved process; Sclerite L4, L5, L7 triangular, weakly sclerotized, situated on middle part of phallomere; Sclerite L6 elongated, middle part strongly sclerotized; Sclerite L8 small, with three round apices, located at the left side of L3d. (Figs. 1. O–P; 3. A–B). Right phallomere sclerite R1 trilateral, apex round and with a round small process on the left basal part; sclerite R2 is in the

shape of a numeral "7" with a groove at the cranial end on the right, there is an acute process in the corner of sclerite R2; the upper part of sclerite R4 widened, inset in the groove of sclerite R2, cranial margin slightly curly and with a rounded apophysis in lower margin, connected with the acute process at the corner of sclerite R2, basal part bifurcate, branch sheeted; sclerite R3, R5, R6 sheeted, situated on right side and left side of phallomere; sclerite R7 sheeted, left side widened and rounded, with a pointed apex in lower margin, right side slender. (Figs. 1. O–P; 3. C–D).

Male genital structures of the macropterous and brachypterous specimens are the same.

Quantitative morphology

Tegmen length was significantly different between the macropterous and brachypterous *H. concinna* morphotypes (t-test, $P < < 0.01$); tegmen lengths of the macropterous individuals were greater than the brachypterous ones without any overlap. Moreover, the ratios of pronotum/tegmen length and body/tegmen length were significantly different (t-test, $P < < 0.01$, Table 3). This relationship is visually apparent in Figure 1, where macropterous individuals and brachypterous individuals cluster separately when tegmen length is plotted against body length or pronotum length. There were no significant differences between the sexes in any characters (Table 3). Tegmen length, tegmen/pronotum length, and tegmen/body length were significantly different between the macropterous and brachypterous *H. concinna* morphotypes, but were not significantly different between males and females within the same morphotype (Table 3).

Distribution

China (Fujian, Guangdong, Guangxi, Hainan, Yunnan, Guizhou, Sichuan, Xizang, Beijing); Vietnam; Thailand; Burma; Malaysia; Indonesia; Australia; Japan [38,45].

DNA barcoding

PCR products of *H. concinna*. Amplified COI sequences (not including primers) for all individuals were 658 bp, with no stop codons, insertions or deletions, and could be translated into 219 amino acids without any interruption, mean nucleotide content of COI sequences was A (31.9%), T (37.4%), G (15.7%) and C (15.0%). As reported for other insect mitochondrial sequences [46–48], A + T (69.4%) was in higher proportion than G + C (30.7%), and were comparable to those typical of insects in general for this COI gene region [46]. The 601 bp COII amplicon (after removing primer sequences) included 199 bp of the 3' terminus of the COI gene, the entire 96 bp tRNA-Leu gene, and the 306 5' bases of the COII gene. The mean nucleotide content of this amplicon was A (40.4%), T (36.0%), G (10.5%) and C (13.1%), again A + T (76.4%) was in much higher proportion than G + C (23.6%), as is usual for insects.

Phylogenetic analysis. Sequences of 30 individuals belonging to 9 species, 4 genera, and 3 families were analyzed. Phylogenetic trees were estimated using aligned COI or COII data sets as described in the methods and presented in Figures 5 and 6. The COI data set included 21 taxa with 658 nucleotides. Of the 658 nucleotides 41 of 219 first positions, 5 of 219 second positions, and 170 of 220 third positions were variable, but this

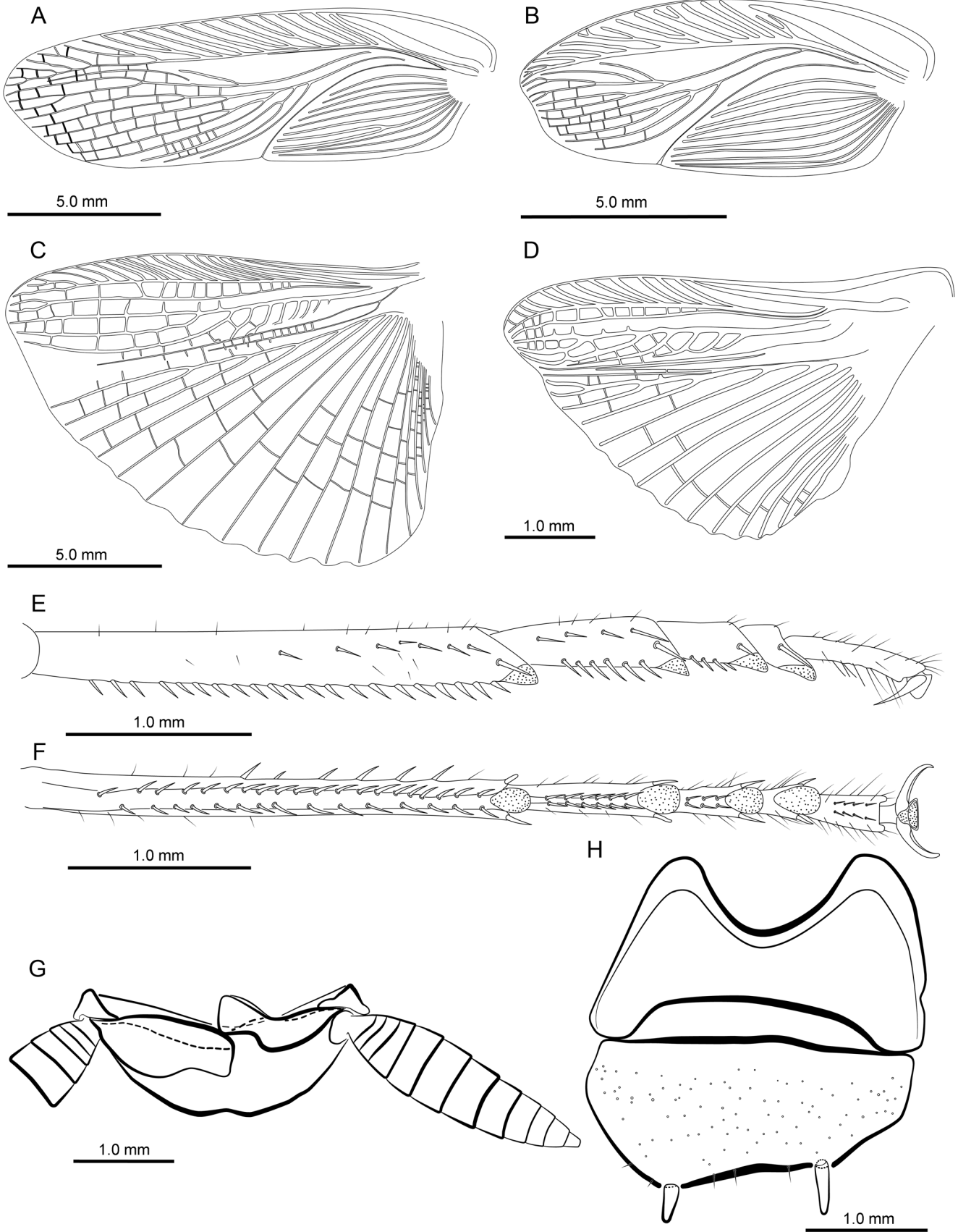


Figure 3. *Hebardina concinna*: primary morphological characters and macropterous vs. brachypterous wings and tegmina. Wings and tegmina differ in macropterous and brachypterous *Hebardina concinna* whereas tarsi and abdominal morphology do not. A. macropterous, tegmen. B. brachypterous, tegmen. C. macropterous, wings. D. brachypterous, wings. E. hind tarsus from outside. F. hind tarsus from below. G. supra-anal plate, ventral view. H. hypandrium, ventral view.
doi:10.1371/journal.pone.0106789.g003

variation resulted in only 14 of 220 amino acid changes. Two of the variable sites at first positions and 12 at third positions occurred only in one taxon. The COII data set included 21 taxa with 306 nucleotide. Of the 306 nucleotides 33 of 102 first positions, 12 of 102 second positions, and 67 of 102 third positions were variable, with 28 of 102 amino acids varying. Three of the variable sites at first positions and ten at third positions occurred only in one taxon.

The aim of the phylogenetic analysis was to assess the relatedness of macropterous and brachypterous *H. concinna* individuals. The short mitochondrial sequences used for DNA barcoding are appropriate for this purpose. For deeper phylogenetic analyses such short mitochondrial DNA barcoding region(s) may be uninformative. Nevertheless, the data sets we have assembled do strongly support the three cockroach families represented: the Blattellidae, Blaberidae, and Ectobiidae. This result

is completely concordant with the morphological classification. Within each family each named genus; *Hebardina*, *Periplaneta*, *Rhabdoblatta*, and *Blattella* are also supported by at least 90% of bootstrap replicates.

The five *H. concinna* individuals shared identical COI sequences and differed only by one or two bases in the COII region: this phylogeny shows that these individuals are clearly distinct from other species within the Blattellidae. There is no genetic distinction between macropterous and brachypterous individuals in either sex. This agrees with our observation that male genital morphology is identical in cockroaches with both wing morphs and we conclude, as hypothesized, that macroptery and brachyptery in both males and females of *H. concinna* are different ecotypes of the same species.

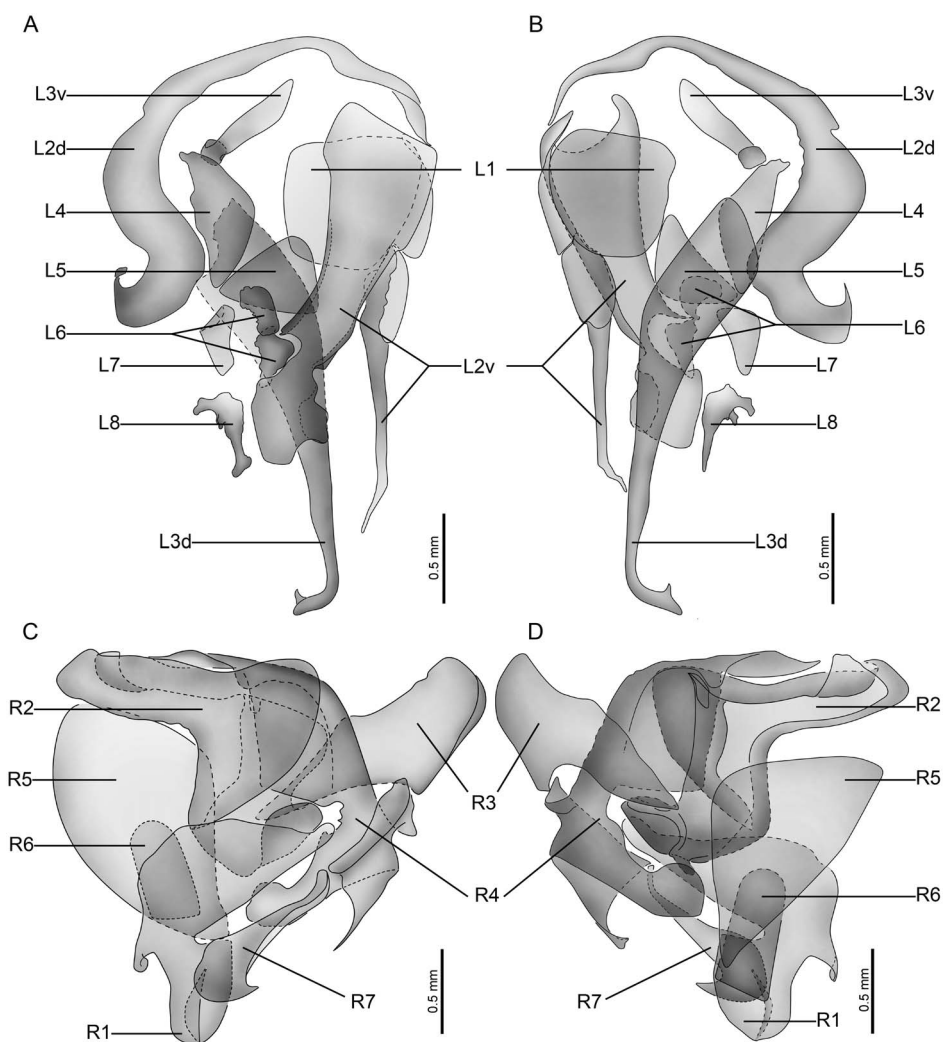


Figure 4. Male genital structures of *Hebardina concinna*. A. left phallomere, dorsal view. B. left phallomere, ventral view. C. right phallomere, dorsal view. D. right phallomere, ventral view. (R for right, L for left, v for ventral, d for dorsal).
doi:10.1371/journal.pone.0106789.g004

Table 3. Results of t-tests for quantitative morphology.

Morphotype	No. of individuals (males+females)	Items	P value	Significance
M & B	15 (8+7)	TL	7.46E-08 << 0.01	significantly different
		PL/TL	9.18E-09 << 0.01	significantly different
		BL/TL	4.41E-08 << 0.01	significantly different
		PL	0.193>0.5	Insignificant
		BL	0.36>0.5	Insignificant
Mm & Mf	8 (5+3)	TL	0.631>0.5	Insignificant
		PL/TL	0.29>0.5	Insignificant
		BL/TL	0.56>0.5	Insignificant
		PL	0.598>0.5	Insignificant
		BL	0.562>0.5	Insignificant
Bm & Bf	7 (4+3)	TL	0.352>0.5	Insignificant
		PL/TL	0.297>0.5	Insignificant
		BL/TL	0.924>0.5	Insignificant
		PL	0.195>0.5	Insignificant
		BL	0.734>0.5	Insignificant

There Are Clear and Significant Correlations between Tegmen Length (TL), Pronotum Length (PL), and Body Length (BL). M, Macropterous; B, Brachypterous; Mm, Macropterous Male; Mf, Macropterous Female; Bm, Brachypterous Male; Bf, Brachypterous Female.
doi:10.1371/journal.pone.0106789.t003

Discussion

Wing polymorphism exists in many insects, including the Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera,

Lepidoptera, and Thysanoptera. The reasons for this polymorphism may relate to both population size and selection pressure. For instance, when population densities are high the proportion of macropterous brown plant hoppers *Nilaparvata lugens* is higher

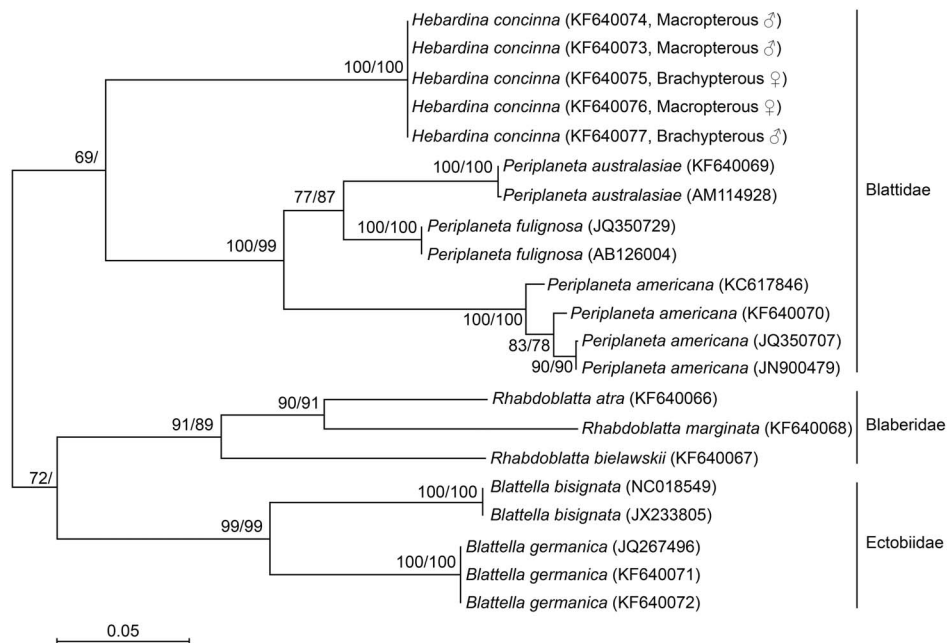


Figure 5. Distance matrix/neighbor joining phylogenetic tree based on 658 bp of aligned cockroach COI nucleotide sequences. The maximum likelihood tree was topologically identical, although with differing branch lengths. Numbers on branches represent support from 1000 non-parametric bootstrap replicates for distance matrix-NJ analysis and maximum likelihood analysis, respectively. Missing numbers indicate branches with less than 50% support. This analysis clearly supports grouping of the five *H. concinna* individuals as a single species.
doi:10.1371/journal.pone.0106789.g005

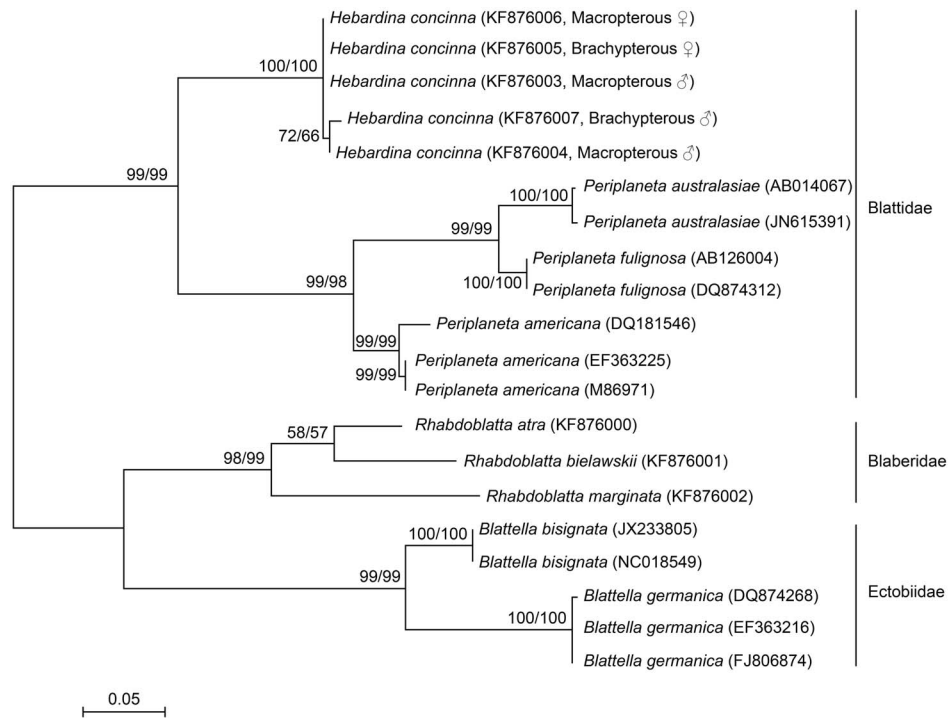


Figure 6. Distance matrix/neighbor joining phylogenetic tree based on 306 bp of aligned cockroach COII nucleotide sequences. The maximum likelihood tree was topologically identical, although with differing branch lengths. Numbers on branches represent support from 1000 non-parametric bootstrap replicates for distance matrix-NJ analysis and maximum likelihood analysis, respectively. Missing numbers indicate branches with less than 50% support. This analysis clearly supports grouping of the five *H. concinna* individuals as a single species. doi:10.1371/journal.pone.0106789.g006

than when it is low [49]. This same phenomenon has also been described in Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera, Lepidoptera, and Thysanoptera [50]. When populations are low, nymphs develop into solitary brachypterous adults, the energy saved by not building large wings and flight muscles is instead directed into reproduction, thus allowing populations to increase rapidly [51]. Conversely, when populations are high, individuals develop into macropterous gregarious adults that are strong flyers who disperse to other habitats with lower population densities. There is a resource allocation trade-off relationship between the development and maintenance of flight muscles and reproductive capacity. Our results clearly demonstrate wing polymorphism in cockroaches that is not sex-based, and therefore is likely to have an environmental trigger. Further research using *H. concinna* or other Blattodea might reveal these triggers in cockroaches.

Wing polymorphism is rarely reported in Blattodea. This is likely due to their secretive, nocturnal habits, to the lack of molecular data, and the lack of description of male genital morphology: these factors together mean that collecting is difficult and identification of collected individuals is problematic. Male genital structures are the most important characters used for species identification in many insects, especially for species that exhibit polymorphism in other characters. In particular, McKittrick [34] first used male genital characters to describe cockroach species. Following this work male genital characters have been widely used by many taxonomists such as Anisyutkin, Roth, and Grandcolas, to distinguish different species of Blattodea [35,36,39,52]. Our results show that male genital structures are also useful for identification of *H. concinna*, but also demonstrate that it may be necessary to use other methods, such as DNA

barcoding, to identify a cohort of conspecific animals in order to develop morphological keys to the genitalia.

Our results showed that, for population in China, both *H. concinna* males and females have macropterous and brachypterous morphotypes and allowed us to develop morphologically based criteria for identification of (males of) this species. This means that entomologists, and in this case pest control agents and public health officials, can be trained to identify *H. concinna* quickly in the field by picking up individuals and looking at their abdomens without the need to bring samples back to a laboratory for costly and time consuming DNA barcoding. We have no doubt that similar efforts will allow leveraging of additional up front investments in laboratory barcoding to develop field-friendly keys for morphological identification of previously problematic species across the Metazoa.

H. concinna is distributed throughout East and Southeast Asia, and the type location is in Malaysia. Our work is based on individuals collected in China and our results therefore remain to be confirmed for the rest of this species' range. In particular, our work represents the only report of mitochondrial sequences from this species, so further work is necessary to understand genetic variation in "*H. concinna*" across the rest of its range. However, our experience with numerous cockroach species strongly suggests that our observations on genital morphology will be confirmed across the entire range of the species, and we hope our work will stimulate further research on this topic.

Author Contributions

Conceived and designed the experiments: QY DQ. Performed the experiments: QY KW DQ JH DL XW JC. Analyzed the data: QY KW DQ JH JC CEC. Contributed reagents/materials/analysis tools: CEC. Contributed to the writing of the manuscript: QY KW CEC.

References

- Asahina S (1983) Domiciliary cockroach species in Thailand. *Promotion of Provincial Health Services* 5: 1–12.
- Cochran DG (1999) Cockroaches: Their biology, distribution and control. World Health Organization pp. 1–83.
- Corsaro D, Thomas V, Goy G, Venditti D, Radek R, et al. (2006) 153 *Rhabdochlamydia crassificans*, sp. nov., comb. nov., an intracellular bacterial pathogen of the cockroach *Blatta orientalis*. *International Journal of Infectious Diseases* 10: S85. doi:10.1016/S1201-9712(1006)80149-80149.
- Corsaro D, Thomas V, Goy G, Venditti D, Radek R, et al. (2007) '*Candidatus Rhabdochlamydia crassificans*', an intracellular bacterial pathogen of the cockroach *Blatta orientalis* (Insecta: Blattodea). *Systematic and Applied Microbiology* 30: 221–228.
- Stek JM (1982) Cockroaches and enteric pathogens. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 76: 566–567.
- de Haan W (1842) Bijdragen tot de kennis der Orthoptera. In: Temminck C, editor. *Verhandlingen over natuurlijke Geschiedenis der Nederlandsche Overzeesche Bezittingen*. Leiden: de leden de Natuurkundige Commissie in Indie en andere Schrijvers. pp. 50.
- Shiraki T (1906) Die Blattiden Japans. *The Zoological Society of Japan* 6: 17–35.
- Hebard M (1929) Studies in Malayan Blattidae (Orthoptera). *Proceedings of the Academy of Natural Sciences of Philadelphia* 81: 1–109.
- Princis K (1966) Blattariae: Suborbo [sic] Blattoidea. Fam.: Blattidae, Nocticolidae. In: Beier, M, editors *Orthopterorum Catalogus. Pars 8*. W. Junk, 's-Gravenhage PP402–614.
- Bey-Bienko GY (1938) Some new or interesting Asiatic Blattodea. *The Annals and Magazine of Natural History* 11: 230–238.
- Bruijning CFA (1948) Studies on Malayan Blattidae. *Zoologische Mededelingen* 29: 1–174.
- Roth LM (1990) Cockroaches from the Krakatau Islands (Dictyoptera: Blattaria). *Memoirs of the Museum of Victoria* 50: 357–378.
- Gullan PJ, Cranston PS (2005) *The insects: An outline of Entomology*; 3, editor: Blackwell Publishing 505 p.
- Bell WJ, Roth LM, Nalepa CA (2007) Habitats. In: Bell WJ, editor. *Cockroaches: Ecology, Behavior, and Natural History*. Maryland: The Johns Hopkins University press. pp. 37–60.
- Harrison RG (1980) Dispersal polymorphisms in insects. *Annual Review of Ecology and Systematics* 11: 95–118.
- Roff DA (1994) Habitat persistence and the evolution of wing dimorphism in insects. *The American Naturalist* 144: 772–798.
- Roff DA, Fairbairn DJ (1991) Wing dimorphisms and the evolution of migratory polymorphisms among the insects. *American Zoologist* 31: 243–251.
- Zera AJ, Denno RF (1997) Physiology and ecology of dispersal polymorphism in insects. *Annual Review of Entomology* 42: 207–230.
- Wallman JF, Donnellan SC (2001) The utility of mitochondrial DNA sequences for the identification of forensically important blowflies (Diptera: Calliphoridae) in southeastern Australia. *Forensic Science International* 120: 60–67.
- Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* 270: 313–322.
- Hebert PDN, Ratnasingham S, de Waard JR (2003) Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences (Suppl)* 270: S96–S99.
- Evangelista D, Buss L, Ware JL (2013) Using DNA barcodes to confirm the presence of a new invasive cockroach pest in New York City. *Entomological Society of America* 106: 2267–2684.
- Hamilton CA, Hendrixson BE, Brewer MS, Bonda JE (2014) An evaluation of sampling effects on multiple DNA barcoding methods leads to an integrative approach for delimiting species: A case study of the North American tarantula genus *Aphonopelma* (Araneae, Mygalomorphae, Theraphosidae). *Molecular Phylogenetics and Evolution* 71: 79–93.
- Hausmann A, Haszprunar G, Hebert PDN (2011) DNA barcoding the geometrid fauna of Bavaria (Lepidoptera): Successes, Surprises, and Questions. *PLoS ONE* 6: e17134. doi:10.1371/journal.pone.0017134.
- Dayrat B (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society* 85: 407–415.
- Padial JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. *Frontier in Zoology* 7: 16.
- Bergsten J, Brilmyer G, Crampton-Platt A, Nilsson AN (2012) Sympatry and colour variation disguised well-differentiated sister species: *Suphrodytes* revised with integrative taxonomy including 5 kbp of housekeeping genes (Coleoptera: Dytiscidae). *DNA Barcodes* 1: 1–18.
- Miller SE (2007) DNA barcoding and the renaissance of taxonomy. *Proceeding of the National Academy of Sciences of the United States of America* 104: 4775–4776.
- Smith MA, Wood DM, Janzen DH, Hallwachs W, Hebert PDN (2007) DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *Proceeding of the National Academy of Sciences of the United States of America* 104: 4967–4972.
- Hajibabaei M, Singer GAC, Clare EL, Hebert PDN (2007) Design and applicability of DNA arrays and DNA barcodes in biodiversity monitoring. *BMC biology* 5: 24. doi:10.1186/1741-7007-1185-1124.
- Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. *PLoS Biology* 2: 1657–1663.
- Yang ZH, Rannala B (2010) Bayesian species delimitation using multilocus sequence data. *Proceeding of the National Academy of Sciences of the United States of America* 107: 9264–9269.
- Tautz D, Arctander P, Minelli A, Thomas RH, Vogler AP (2003) A plea for DNA taxonomy. *Trends in Ecology & Evolution* 18: 70–74.
- McKittrick FA (1964) *Evolutionary studies of cockroaches*. New York: Cornell University Agricultural Experiment Station Memoir. 197 p.
- Grandcolas P (1996) The phylogeny of cockroach families: a cladistic appraisal of morpho-anatomical data. *Canadian Journal of Zoology* 74: 508–527.
- Anisutkin LN (2010) New data on the genus *Neostylopyga* Shelford, 1911 (Dictyoptera, Blattidae) with description of a new species from Laos. *Entomological Review* 90: 871–876.
- Anisutkin LN (2013) *Macrostylopyga* gen. nov., a new genus of cockroaches (Dictyoptera: Blattidae), with descriptions of two new species. *Zootaxa* 3635: 520–532.
- Feng PZ, Guo YY, Wu FZ (1997) *China cockroach species and prevention*: China Science and Technology Press.
- Roth LM (1998) The cockroach genera *Chorisoneura* Brunner, *Sorimeuchora* Caudell, *Chorisoneurodes* Princis, and *Chorisoserrata* gen. nov. (Blattaria: Blattellidae: Pseudophyllodromiinae). *Oriental Insects* 32: 1–33.
- 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: 294–299.
- Cook CE, Yue QY, Akam M (2005) Mitochondrial genomes suggest that hexapods and crustaceans are mutually paraphyletic. *Proceedings of the Royal Society B: Biological Sciences* 272: 1295–1304.
- Ding T, Yue L, Yu B, Zhou H, Yuan J, et al. (2011) A Comparison between direct sequencing and clone sequencing in detection for methylation imprinting of H19 gene in infertile males. *Journal of Molecular Diagnosis and Therapy* 3: 249–254.
- Zeng J, Zhang L, Fan X, Zhang C, Zhang HQ, et al. (2006) Comparison Analysis on Direct Sequencing of PCR Products and Sequencing of Cloning PCR Products of ITS Sequence of Nuclear rDNA in *Kengyilia* (Poaceae: Triticeae). *Journal of Sichuan Agricultural University* 24: 369–374.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731–2739.
- Beccaloni G (2007) Blattodea Species File Online. Version 5.0/5.0. World Wide Web electronic publication.
- Renaud AK, Savage J, Adamowicz SJ (2012) DNA barcoding of Northern Nearctic Muscidae (Diptera) reveals high correspondence between morphological and molecular species limits. *BMC Ecology* 12: doi:10.1186/1472-6785-1112-1124.
- Rivera J, Currie D (2009) Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). *Molecular Ecology Resources* 9: 224–236.
- Schuehli GSE, de Carvalho CJB, Wiegmann BM (2007) Molecular phylogenetics of the Muscidae (Diptera: Calyptratae): new ideas in a congruence context. *Invertebrate Systematics* 21: 263–278.
- Iwanaga K, Tojo S, Nagata T (1985) Immigration of the brown planthopper *Nilaparvata lugens*, exhibiting various responses to density in relation to wing morphism. *Entomologia Experimentalis et Applicata* 38: 101–108.
- Zhu DH (2009) Regulatory mechanism and evolution of insect wing polymorphism. *Chinese Bulletin of Entomology* 46: 11–16.
- Saglam IK, Roff DA, Fairbairn DJ (2008) Male sand crickets trade-off flight capability for reproductive potential. *Journal of Evolutionary Biology* 21: 997–1004.
- Anisutkin LN (2000) New cockroaches of the subfamily Epilamprinae, with description of a new genus *Rhabdoblattella* (Diptoptera: Blaberidae). *Zoosystematica Rossica* 8: 251–255.