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Exploring the diversity of Asian *Cryptocercus* (Blattodea: Cryptocercidae): species delimitation based on chromosome numbers, morphology and molecular analysis

Qikun Bai\textsuperscript{A}, Lili Wang\textsuperscript{A}, Zongqing Wang\textsuperscript{A}, Nathan Lo\textsuperscript{B} and Yanli Che\textsuperscript{A,C}

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Abstract. Woodroaches from the genus *Cryptocercus* Scudder, 1862 are known to display low levels of morphological divergence, yet significant genetic divergence and variability in chromosome number. Compared with *Cryptocercus* taxa from North America, the diversity of the genus in Asia has received relatively little attention. We performed morphological and karyotypic examinations of multiple taxa from several previously unsampled mountainous areas of central and southwestern China, and identified nine candidate species primarily on the basis of chromosome number. We then investigated diversity across all Asian *Cryptocercus*, through phylogenetic analyses of 135 COI sequences and 74 28S rRNA sequences from individuals of 28 localities, including species delimitation analysis in General Mixed Yule Coalescent (GMYC) and Automatic Barcode Gap Discovery (ABGD). Phylogenetic results indicated that individuals from the same locality constituted well supported clades. The congruence of GMYC and ABGD results were in almost perfect accord, with 28 candidate species described on the basis of karyotypes (including the nine identified in this study). We provide evidence that each valley population in the Hengduan Mountains contains a separate evolving lineage. We conclude that the principal cause of the rich *Cryptocercus* diversity in China has been the uplift of the Qinghai-Tibet Plateau.

Additional keywords: ABGD, biogeography, DNA barcode, GMYC, Hengduan Mountains, woodroach

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Introduction

The genus *Cryptocercus* (Blattodea: Cryptocercidae) is a subsocial group consisting of ~22 described species distributed in eastern Asia and North America (Nalepa et al. 1997; Burnside et al. 1999; Grandcolas 2000; Grandcolas et al. 2001, 2005; Grandcolas et al. 2006; Fan et al. 2015). Leg armature and structure of the abdomen tip were the most commonly used characters to delineate the first three described *Cryptocercus* species (*C. punctulatus*, *C. primarius*, *C. relicitus*) (Scudder 1862; Bey-Bienko 1935, 1938; Grandcolas 2000; Grandcolas et al. 2001, 2005; Wang et al. 2015). Such DNA barcode-based approaches have been successfully used for several insect groups (Caterino et al. 2006; Hebert et al. 2003), including cockroaches (Knebelsberger and Miller 2007; Evangelista et al. 2014; Che et al. 2017). Several species delimitation methods using single-locus data have been proposed, including the General Mixed Yule-coalescent (GMYC) (Pons et al. 2006; Monaghan et al. 2009; Fujisawa and Barraclough 2013) and Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012). An advantage of GMYC is...
that it allows for statistical inference and hypothesis testing across the entire sampled clade on an ultrametric tree (Fujisawa and Barraclough 2013). ABGD divides the data into groups based on statistically inferred barcode gaps and distinguishes partitions in the genetic distances among a group of individuals. Comparison of the results from these two methods with each other and with those from traditional methods can be performed to assess their robustness.

In recent decades the number of proposed species of *Cryptocercus* from eastern Asia has increased markedly, from two to 16. These species have been described on the basis of morphological characters only, or using morphological and genetic approaches (including karyotype). To date, the use of species delineation methods such as GMYC and ABGD have not been employed. To investigate the diversity of eastern Asian *Cryptocercus*, we performed morphological and karyotypic examinations of multiple taxa from several previously unsampled mountainous areas of central and south-western China, and identified several potentially new candidate species, primarily on the basis of chromosome number. Following this, we generated new COI and 28S rRNA sequences data from a wide variety of representatives of this group, and performed phylogenetic analyses, including GMYC and ABGD. We selected numerous, biogeographically variable localities (Manchuria, Qin-Daba Mountains and Hengduan Mountains) to infer species diversity and examine the evolution of this group in China.

**Materials and methods**

**Taxon sampling**

Samples were collected from rotting logs at 28 localities in China (Fig. 1), from three main regions: Hengduan Mountains, Qin-Daba Mountains and Manchuria (Table 1). Collections were limited to single-day-trip trekking in one valley, meaning that deep valleys were difficult to reach and usually not sampled. Specimens were preserved in analytical pure ethanol and stored at −80°C until processing. All specimens are deposited in the institute of Entomology, College of Plant Protection, Southwest University, Chongqing, China.

**Morphological analyses**


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**Fig. 1.** Map of sampled localities for *Cryptocercus*. Numbers for sampling localities match those in Table 1 (red circles denote samples collected by our team, blue circles denote data obtained from GenBank). The map was generated by www.simplemappr.net using GPS coordinates.
Table 1. Samples used in species delimitation: number of location, sample collection localities, abbreviation of location, and GenBank accession numbers

All localities are in China unless otherwise indicated. The letter ‘n’ after the voucher abbreviation means the sample is a nymph and the letter ‘f’ means the sample is female. n.a., not available.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>Location</th>
<th>Abbreviation</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. primarius</strong></td>
<td>1</td>
<td>Qujiaba (32°59’N, 105°52’E, 2000 m), Longman City, Gansu Province</td>
<td>QB1 (f)</td>
<td>KY940647 KU312257</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Dacaoping (32°54’N, 104°03’E, 2993 m), Pingwu County, Mianyang City, Sichuan Province</td>
<td>QB2 (f)</td>
<td>KY940648 KU312258</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Biaoxi (31°02’N, 101°33’E, 2582 m), Danba County, Sichuan Province</td>
<td>DCP1</td>
<td>KY940649 KU312232</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Bao’ergou (30°51’N, 102°32’E, 3022 m), Jiajinshan, Baoxing County, Ya’an City, Sichuan Province</td>
<td>DCP2 (n)</td>
<td>KY940650 KU312233</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Laojungou (31°40’N, 103°07’E, 2586 m), Lixian County, Sichuan Province</td>
<td>BEX (f)</td>
<td>KY940645 KU312255</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Danzamugou (31°24’N, 103°15’E, 2912 m), Lixian County, Sichuan Province</td>
<td>BEX (f)</td>
<td>KY940646 KU312256</td>
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<tr>
<td></td>
<td>7</td>
<td>Baishagou (32°52’N, 104°02’E, 3065 m), Pingwu County, Mianyang City, Sichuan Province</td>
<td>BEX (f)</td>
<td>KY940647 KU312257</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Mujaogou (29°55’N, 102°09’E, 2124 m), Luding County, Sichuan Province</td>
<td>MG1 (n)</td>
<td>KY940587 KY944598</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Banshanmengou (30°59’N, 102°05’E, 2666 m), Danba County, Sichuan Province</td>
<td>MG2 (f)</td>
<td>KY940588 KY944599</td>
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<tr>
<td></td>
<td>10</td>
<td>Dengsheng (30°51’N, 102°58’E, 2833 m), Wolong County, Sichuan Province</td>
<td>MG3 (f)</td>
<td>KY940589 KY944600</td>
</tr>
<tr>
<td><strong>C. shangmengensis</strong></td>
<td>5</td>
<td>Laojungou (31°40’N, 103°07’E, 2586 m), Lixian County, Sichuan Province</td>
<td>LIG1 (n)</td>
<td>KY940581 KU312224</td>
</tr>
<tr>
<td></td>
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<td>KY940582 KU312225</td>
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<td>LIG3 (f)</td>
<td>KY940583 n.a.</td>
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<tr>
<td><strong>C. zagunaoensis</strong></td>
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<td>DZMG1</td>
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<td>DZMG2 (f)</td>
<td>KY940585 KU312227</td>
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<tr>
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<td>Banshanmengou (30°59’N, 102°05’E, 2666 m), Danba County, Sichuan Province</td>
<td>DZMG3</td>
<td>KY940586 n.a.</td>
</tr>
<tr>
<td><strong>C. pingwuensis</strong></td>
<td>7</td>
<td>Baishagou (32°52’N, 104°02’E, 3065 m), Pingwu County, Mianyang City, Sichuan Province</td>
<td>BSG1</td>
<td>KY940612 KU312234</td>
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<tr>
<td><strong>C. tianbaensis, sp. nov.</strong></td>
<td>8</td>
<td>Mujaogou (29°55’N, 102°09’E, 2124 m), Luding County, Sichuan Province</td>
<td>BSG2 (f)</td>
<td>KY940613 KU312235</td>
</tr>
<tr>
<td><strong>C. baxianensis, sp. nov.</strong></td>
<td>9</td>
<td>Banshanmengou (30°59’N, 102°05’E, 2666 m), Danba County, Sichuan Province</td>
<td>MG3 (f)</td>
<td>KY940589 KY944600</td>
</tr>
<tr>
<td><strong>C. wolongensis, sp. nov.</strong></td>
<td>10</td>
<td>Dengsheng (30°51’N, 102°58’E, 2833 m), Wolong County, Sichuan Province</td>
<td>MG2 (f)</td>
<td>KY940591 KY944601</td>
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<tr>
<td><strong>C. meridianus</strong></td>
<td>11</td>
<td>Yunshanping (27°08’N, 100°14’E, 3250 m), Yulongxueshan, Lijiang City, Yunnan Province</td>
<td>YLXS1</td>
<td>KY940614 KU312236</td>
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<tr>
<td><strong>C. arcuatus</strong></td>
<td>12</td>
<td>Shikaxueshan (27°47’N, 99°36’E, 3756 m), Shangri-la, Diqing City, Yunnan Province</td>
<td>SKXS1</td>
<td>KY940609 KU312230</td>
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<tr>
<td><strong>C. baxianensis</strong></td>
<td>13</td>
<td>Habaxueshan (27°22’N, 100°08’E, 3126 m), Shangri-la, Diqing City, Yunnan Province</td>
<td>HBSX1 (f)</td>
<td>KY940578 KU312222</td>
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<tr>
<td><strong>C. pudacuensis, sp. nov.</strong></td>
<td>14</td>
<td>Pudacuo (27°48’N, 99°55’E, 3313 m), Shangri-la, Diqing City, Yunnan Province</td>
<td>HBSX2</td>
<td>KY940579 KU312223</td>
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<tr>
<td><strong>C. laojunensis, sp. nov.</strong></td>
<td>15</td>
<td>Jiushijulongtan (26°38’N, 99°45’E, 3627 m), Lijiang City, Yunnan Province</td>
<td>HBSX3 (n)</td>
<td>KY940580 n.a.</td>
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<tr>
<td><strong>C. weixiensis, sp. nov.</strong></td>
<td>16</td>
<td>Chuanadun (27°21’N, 99°18’E, 2934 m), Weixi County, Yunan Province</td>
<td>HBSX3 (n)</td>
<td>KY940581 n.a.</td>
</tr>
<tr>
<td><strong>C. relictus</strong></td>
<td>17</td>
<td>Gaolingzi (44°51’N, 128°51’E, 702 m), Shangzhi City, Heilongjiang Province</td>
<td>GLZ1 (n)</td>
<td>KY940640 KU312249</td>
</tr>
<tr>
<td><strong>C. relictus</strong></td>
<td>18</td>
<td>Shuangfenglinchang (44°32’N, 128°51’E, 1090 m), Hailin City, Heilongjiang Province</td>
<td>GLZ2</td>
<td>KY940641 KU312250</td>
</tr>
<tr>
<td><strong>C. relictus</strong></td>
<td>19</td>
<td>Mudanfeng (44°20’N, 129°53’E, 1199 m), Mudanjiang City,</td>
<td>MDF1</td>
<td>KY940642 KU312253</td>
</tr>
</tbody>
</table>

COI 28S rRNA

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We identified the species based on five standard characters of female genitalia (anterior margin of Tergite VII, shape of the spermatheca, shape of the basivalvulae, shape and coloration of the laterosternal shelf, length of the paraprocts), which are stable and can be used to distinguish species to a certain extent (Grandcolas 2000; Grandcolas et al. 2001, 2005; Aldrich et al. 2004; Wang et al. 2015). We use abbreviations to indicate the following structures: paraprocts (pp.), first to third valve (v.I, v.II, v.III), paratergites (pt.), anterior arch (a.a.), basivalvula (bsv.), laterosternite IX (lst.IX), laterosternal shelf (lst.sh), vestibular sclerite (vst.s.), intersternal fold (inst.f.), central apodeme (c.a.) and spermatheca (sp.). Preparation of genitalia followed the standard procedure, using 10% NaOH to clear internal tissues, then all genitalia were mounted on microscope slides using a Tomic K400 stereomicroscope. Photographs of the female genitalia were made using a Leica M205A microscope with a Leica DFC camera. Adults were photographed with a digital camera (Canon 50D) with the aid of the Helicon Focus software.

Karyotype analysis
For karyotype analysis, three individuals from three different logs within each population were examined. Mitotic chromosomes from the testes of males were examined following Luykx (1983). Karyotypes are reported as the diploid complement.

DNA extraction and sequencing
Genomic DNA was extracted from legs by using the TIANamp Genomic DNA Kit (DP304, TIANGEN). Primers are provided in Table S1, available as supplementary material to this paper. All the reactions were carried out in volumes of 25 μL, containing 14.25 μL of ultrapure water, 2.5 μL of 10 × buffer (Mg²⁺ free), 2 μL of MgCl₂ (25 mM), 2 μL of dNTP mixture, 1 μL of each primer, 0.25 μL of Taq polymerase, and 2 μL of DNA template. The following steps were performed on a programmable thermal cycler. The amplification protocol setting used were 94°C for 5 min; followed by 35 cycles at 94°C for 45s, 48°C for 45s, and
72°C for 45s; and final extension at 72°C for 10 min. PCR reactions were separated by electrophoresis on a 1% agarose gel. All DNA purification and sequencing was carried out by BGI Tech (Beijing, China) using the aforementioned primers. All sequences were deposited in GenBank, at the National Center for Biotechnology Information (Table 1).

Sequence analysis
A total of 135 cytochrome c oxidase subunit I (COI) sequences were analysed, including: 86 sequences from this study; five sequences representing four species from North America and Korea downloaded from GenBank; and 44 sequences representing termite outgroups (Table S2). To provide a comparison with the results from COI, we also analysed two sequences of 28S rRNA for each species (74 total sequences) (Table 1). COI sequences were aligned using MUSCLE 3.8 (Edgar 2004) and adjusted visually and manually corrected after translation into amino acid sequences; 28S rRNA sequences were inspected visually and manually. Intraspecific and interspecific genetic divergence values were quantified based on the Kimura 2-parameter (K2P) distance model (Kimura 1980), using MEGA 6.0.6 (Tamura et al. 2013), with 1000 bootstrap replicates.

To explore phylogenetic relationships among these closely related species, phylogenetic trees were constructed on two different datasets (single COI dataset and the concatenated dataset) using Maximum Likelihood (ML) and Bayesian Inference (BI). Bayesian analysis was implemented in MrBayes 3.2 (Ronquist et al. 2012), assigning site-specific models. The COI dataset was divided into three partitions by codon position (pos1–3), and PartitionFinder 1.1.1 (Lanfear et al. 2012) was used to determine the best-fitting models for each partition. The best-fitting models were as follows: COI_pos1, TVM+G; COI_pos2, TrNef+G; COI_pos3, TrN+I+G; 28S rRNA, TrN+I+G. Two independent sets of Markov chains were run, each with one cold and three heated chains for 1 × 10^7 generations, and every 1000th generation was sampled. Convergence was inferred when a standard deviation of split frequencies <0.01 was completed. Sump and sumpt burninfrac was set to 25% and contype was set to allcomp. Maximum likelihood was implemented in RAxML 7.3.0 (Stamatakis et al. 2008); as the software does not allow different substitution models for different partitions we used the GTR+GAMMA model for analyses. Bootstrap values were performed for 1000 replicates.

All COI sequences were analysed using ABGD and GMYC. ABGD analysis was performed using a web interface (http://wwwabi.snv.jussieu.fr/public/abgd/, accessed 4 May 2017); the default parameters were used except for the relative gap width, which was set as 1.0. The GMYC method requires a fully resolved ultrametric tree as input for the analysis. We constructed a Bayesian inference tree in BEAST 1.8.1 (Drummond and Rambaut 2007) using the best-fitting models from PartitionFinder. Rate variation was modelled among branches using an uncorrelated log-normal relaxed clock model with the mean clock rate fixed to 1.0. A UPGMA starting tree was used in the analysis, and the constant size coalescent was used as a prior on divergence times. We performed two replicate MCMC runs, and sampled every 5000 steps over a total of 50 million generations. A maximum clade credibility tree was obtained using Tree Annotator within the BEAST software package with a burn-in of 1000 trees. We then applied the single-threshold GMYC method to the ultrametric gene tree generated by BEAST using the SPLITs package (Ezard et al. 2009) in R software (R Core Team 2013). The groups delimited were compared with a one-species null model using a likelihood ratio test.

Results
Karyotypes and taxonomy of novel Cryptocercus species
Through examination of the morphology and chromosome numbers of cockroaches from previously unsampled areas, we identified nine new candidate Cryptocercus species: C. changbaiensis, sp. nov.; C. weixiensis, sp. nov.; C. luanchuansensis, sp. nov. (2n = 39) (Fig. 2H}; C. chengkouensis, sp. nov. (2n = 29) (Fig. 2A); C. wolongensis, sp. nov. (2n = 43) (Fig. 2B); C. banxianmensensis, sp. nov. (2n = 29) (Fig. 2C); C. tianbaensis, sp. nov. (2n = 35) (Fig. 2D); C. laojunensis, sp. nov. (2n = 21) (Fig. 2E); C. pudacuoensis, sp. nov. (2n = 21) (Fig. 2F). All species of Chinese Cryptocercus examined in this study were highly similar in external morphology, including female genitalia (see Table 2, e.g. C. luanchuansensis, sp. nov. and C. neixiangensis). All candidate new species are named after their type localities.

COI and 28S rRNA sequence variation across Asian taxa
We next obtained molecular sequence data from mitochondrial COI and 28S rRNA from multiple Asian taxa. The sequenced lengths of COI and 28S rRNA, excluding primers, were ~658 bp and 635 bp respectively. All new sequences have been deposited in GenBank with accession numbers KY940572 to KY940657 for COI (accession numbers for 28S in Table 1). Among aligned COI sequences 232 sites were variable, of which 217 were parsimony informative. Among aligned 28S rRNA sequences, 106 sites were variable, of which 103 were parsimony informative.

Intraspecific COI genetic divergence (K2P) ranged from 0.00 to 0.61%, with an average of 0.14%. The greatest intraspecific COI genetic divergence (0.61%) occurred in C. darwini. All intraspecific COI genetic divergence values were less than 0.5%, and many intraspecific COI genetic divergence values were 0.00% (Table S3). Interspecific COI genetic divergence ranged from 2.18% (C. neixiangensis and C. luanchuansensis, sp. nov.) to 20.36% (C. chengkouensis, sp. nov. and C. darwini), with an average of 11.81% (Table S3). The 28S sequences of Cryptocercus had low levels of genetic divergence of 96.27% and lacked sufficient variation to resolve some species (Table S4). 28 species were identified through the use of the barcoding gap, of which nine were new (Fig. 38).

GMYC, ABGD, and other phylogenetic analyses
The likelihoods of the null and GMYC models from COI analysis were 239.49 and 268.42 respectively. The GMYC was an improvement over the null model, and was clustered into 72 (confidence interval: 72–73) entities (likelihood ratio = 57.87) including 28 Cryptocercus and 44 termite species. ABGD
based on the same dataset also detected 72 species with a range of prior intraspecific values ($P = 0.0046–0.0215$) (Fig. 3C).

Phylogenetic analyses performed using ML and BI recovered similar tree topologies in COI analysis with some differences in branching patterns at deeper nodes. Support values from ML analysis were much higher than those from BI analysis (Figs 3, S1). Representatives from candidate species (described primarily on the basis of karyotype number) were found to form monophyletic groups. Analyses based on concatenated COI and 28S sequences revealed similar topologies to those inferred from COI only, with some minor differences.

Asian and American lineages ($a$ and $b$) formed monophyletic groups, as recovered in BI and ML analyses for COI and combined datasets (Figs 3, S1, S2). For COI analysis (Fig. 3), all species from Qin-Dada Mountains were clustered together with strong support (Clade $g$). Species from Manchuria and the Hengduan Mountains were clustered together (Clade $d$) with one exception ($C. pudacuoensis$, sp. nov. (PDC)), although this grouping was not well supported.

**Taxonomy**

**Genus Cryptocercus** Scudder


**Cryptocercus chengkouensis**, sp. nov.

(Figs 4a1–4a3, 5)


**Material examined**

Holotype. ♀, China: Chongqing City, Chengkou County, Xinglongcun, 1621 m, 30.x.2014, coll. Xinran Li and Yan Shi.

Paratypes. 2♂, 1♀, same data as holotype.

**Measurements**

Male and female – pronotum, length × width: 6.5 × 8.5 mm; body length: 25.5–27.5 mm.

**Description**

Tergite VII with anterior margin slightly arched and posterior margin rounded (Fig. 5D). Sternum VII slightly produced at apex, posterior margin truncate (Fig. 5C). Tergite X rounded at apex; paraprocts (pp.) developed, triangular, with apices extending to the posterior margin of Tergite X (this sample was broken at apex) (Fig. 5A).

**Female genitalia**

Basivalvulae (bsv.) well-developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins obscure and dark brown, anterior area dark brown with a distinctive prominence (Fig. 5E). Spermatheca sitting behind the basivalvulae and with a large and oval apical ampulla, and an oval basal ampulla on a short duct. Laterosternal shelf (lst.sh.) large and translucent, nearly oval and brown on both sides, with dense spinules at apical half (Fig. 5F).

**Cryptocercus changbaiensis**, sp. nov.

(Figs 4b1–4b3, 6)

http://zoobank.org/urn:lsid:zoobank.org:act:DEA5D9D6-FB94-4C8F-B9A4-18C454C09C4C

**Material examined**

Holotype. ♀, China: Chongqing City, Chengkou County, Xinglongcun, 1621 m, 30.x.2014, coll. Xinran Li and Yan Shi.

Paratypes. 2♂, 1♀, same data as holotype.

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Male and female – pronotum, length × width: 6.5 × 8.5 mm; body length: 25.5–27.5 mm.

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Table 2. Comparison of female genitalia of 21 Chinese Cryptocercus species

<table>
<thead>
<tr>
<th>Species</th>
<th>Anterior margin of Tergite VII</th>
<th>Standard characters of female genitalia that were used in this study</th>
<th>Length of paraprocts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manchuria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. changbaiensis</em>, sp. nov.</td>
<td>Slightly arched</td>
<td>Anterior area narrow and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. relictus</em></td>
<td>Slightly truncate</td>
<td>Anterior area wide and dark brown</td>
<td>Extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td>Qin-Daba Mountains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. hirtus</em></td>
<td>Slightly truncate</td>
<td>Anterior area dark brown with a distinctive prominence</td>
<td>Extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. ningshanensis</em></td>
<td>Slightly arched</td>
<td>Anterior area dark brown with a distinctive prominence</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. neixiangensis</em></td>
<td>Slightly arched</td>
<td>Anterior area dark brown with a distinctive prominence</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. luanchuanensis</em>, sp. nov.</td>
<td>Truncate and weakly concave at middle</td>
<td>Anterior area dark brown with a distinctive prominence</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. shennongjiaensis</em></td>
<td>Slightly arched</td>
<td>Anterior area dark brown with a distinctive prominence</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. wuxiensis</em></td>
<td>Slightly arched</td>
<td>Anterior area dark brown with a distinctive prominence</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. chengkouensis</em>, sp. nov.</td>
<td>Slightly arched</td>
<td>Anterior area dark brown with a distinctive prominence</td>
<td>Extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td>Hengduan Mountains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. meridianus</em></td>
<td>Distinctly truncate</td>
<td>Anterior area narrow and dark brown</td>
<td>Extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. arcuatus</em></td>
<td>Slightly truncate</td>
<td>Anterior area wide and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. pudacuoensis</em>, sp. nov.</td>
<td>Distinctly truncate</td>
<td>Anterior area wide and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. habaensis</em></td>
<td>Slightly truncate</td>
<td>Anterior area narrow and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. laojunensis</em>, sp. nov.</td>
<td>Truncate and weakly concave at middle</td>
<td>Anterior area narrow and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. weixiensis</em>, sp. nov.</td>
<td>Slightly arched</td>
<td>Anterior area narrow and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. primarius</em></td>
<td>Distinctly truncate</td>
<td>Anterior area narrow and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. pingwuensis</em></td>
<td>Slightly truncate</td>
<td>Anterior area narrow and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. tianbaensis</em>, sp. nov.</td>
<td>Slightly truncate</td>
<td>Anterior area wide and dark brown</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
</tbody>
</table>

(continued next page)
Table 2. (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Anterior margin of Tergite VII</th>
<th>Shape of spermatheca</th>
<th>Shape of basivalvulae</th>
<th>Shape and coloration of laterosternal shelf</th>
<th>Length of paraprocts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. wolongensis</em>, sp. nov.</td>
<td>Slightly truncate</td>
<td>Water-drop</td>
<td>Anterior area narrow and dark brown</td>
<td>Nearly oval and light brown at middle</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. convexus</em></td>
<td>Truncate and weakly concave at middle</td>
<td>Round</td>
<td>Anterior area narrow and dark brown</td>
<td>Nearly oval and light brown at middle</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
<tr>
<td><em>C. banshanmenensis</em>, sp. nov.</td>
<td>Slightly truncate and concave</td>
<td>Water-drop</td>
<td>Anterior area narrow and dark brown</td>
<td>Nearly oblong</td>
<td>Do not extend to the posterior margin of Tergite X</td>
</tr>
</tbody>
</table>

Description

Tergite VII with anterior margin slightly truncate and posterior margin truncate (Fig. 7D). Sternum VII produced at apex, posterior margin truncate (Fig. 7C); Tergite X rounded at apex; paraprocts (pp.) developed, triangular, with apices not extending to the posterior margin of Tergite X (Fig. 7A).

Female genitalia

Basivalvulae (bsv.) well-developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins obscure and dark brown, anterior area narrow and dark brown (Fig. 6F). Spermatheca sitting behind the basivalvulae and with a large and water-drop apical ampulla, and a water-drop basal ampulla on a short duct. Laterosternal shelf (ltst.sh.) large and translucent, nearly oval, with dense spinules at apex (Fig. 6F).

Cryptocercus weixiensis, sp. nov.

(Figs 4d1–4d3, 8)

http://zoobank.org/urn:lsid:zoobank.org:act:F1F83000-09D1-4529-9C13-7A633208FB34

Material examined

Holotype. ♀, China: Yunnan Prov., Weixi County, Pantiange, 2934 m, 21.viii.2015, coll. Qikun Bai and Lu Qiu.

Paratypes. 1♂, 2♀♀, same data as holotype.

Measurements

Male and female – pronotum, length × width: 5.5 × 7.5 mm; body length: 21–22.5 mm.

Description

Tergite VII with anterior margin slightly arched and posterior margin truncate (Fig. 8D). Sternum VII produced at apex, posterior margin truncate (Fig. 8C). Tergite X rounded at apex; paraprocts (pp.) developed, triangular, with apices not extending to the posterior margin of Tergite X, with the gap between paraprocts narrow (Fig. 8A).

Material examined

Holotype. ♀, China: Jilin Prov., Mt Changbai, 750 m, 7.viii.2015, coll. Yejie Lin.

Paratypes. 4♂, 3♀♀, same data as holotype.

Measurements

Male and female – pronotum, length × width: 7.1 × 8.9 mm; body length: 29.9–30.5 mm.
Fig. 3. Maximum-likelihood tree based on COI genes (BPP: Bayesian Posterior Probabilities; MLB: Maximum Likelihood Bootstrap Values). A square (■) at a node indicates that both BPP and MLB > 95; a circle (●) indicates that only MLB > 95. Numbers after the species name are chromosome numbers, numbers in red are results from this study. Coloured bars indicate different species delimitation by different methods: A, morphology; B, DNA barcoding gap; C, GMYC model; D, ABGD result. We selected 86 samples of 28 Cryptocercus species. The topology shown was very similar to that produced by the BI tree, with some differences (see Fig. S1).
Fig. 4. a1–a3: Cryptocercus chengkouensis, sp. nov., holotype: a1, dorsal view; a2, ventral view; a3, lateral view. b1–b3: Cryptocercus changbaiensis, sp. nov., holotype: b1, dorsal view; b2, ventral view; b3, lateral view. c1–c3: Cryptocercus wolongensis, sp. nov., holotype: c1, dorsal view; c2, ventral view; c3, lateral view. d1–d3: Cryptocercus weixiensis, sp. nov., holotype: d1, dorsal view; d2, ventral view; d3, lateral view. e1–e3: Cryptocercus pudacuoensis, sp. nov., holotype: e1, dorsal view; e2, ventral view; e3, lateral view. f1–f3: Cryptocercus luanchuanensis, sp. nov., holotype: f1, dorsal view; f2, ventral view; f3, lateral view. g1–g3: Cryptocercus laojunensis, sp. nov., holotype: g1, dorsal view; g2, ventral view; g3, lateral view. h1–h3: Cryptocercus banshanmenensis, sp. nov., paratype: h1, dorsal view; h2, ventral view; h3, lateral view. i1–i3: Cryptocercus tianbaensis, sp. nov., paratype: i1, dorsal view; i2, ventral view; i3, lateral view. Scale bars: 1.0 cm.
Female genitalia
Basivalvulae (bsv.) well-developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins obscure and dark brown, anterior area narrow and dark brown (Fig. 8E). Spermatheca sitting behind the basivalvulae and with a large and oval apical ampulla, and an oval basal ampulla on a short duct. Laterosternal shelf (lst.sh.) large and translucent, nearly oval and light brown on both sides, with dense spinules at apical half (Fig. 8F).

Cryptocercus pudacuoensis, sp. nov.  
(Figs 4e1–4e3, 9)

Material examined
Holotype. ♀, China: Yunnan Prov., Diqing City, Shangri-La Pudacuo, 3313 m, 14.x.2014, coll. Qikun Bai and Yan Shi.  
Paratypes. 4♂, 2♀, same data as holotype.

Measurements
Male and female – pronotum, length × width: 6.5 × 8.5 mm; body length: 26.5–27.5 mm.

Description
Tergite VII with anterior margin truncate and posterior margin rounded (Fig. 9D). Sternum VII slightly produced at apex, posterior margin truncate (Fig. 9C). Tergite X rounded at apex; paraprocts (pp.) developed, triangular, with apices not
extending to the posterior margin of Tergite X, with the gap between paraprocts narrow (Fig. 9A).

**Female genitalia**

Basivalvulae (bsv.) well-developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins obscure and dark brown, anterior area wide and dark brown (Fig. 9E). Spermatheca sitting behind the basivalvulae and with a large and round apical ampulla, and a round basal ampulla on a short duct. Laterosternal shelf (ltst.sh.) large and translucent, nearly oval, with dense spinules at apex (Fig. 9F).

**Cryptocercus luanchuanensis**, sp. nov.

(Figs 4f–4j, 10)

http://zoobank.org/urn:lsid:zoobank.org:act:FF00A947-0C54-47B9-90B3-0A39479E7BA5

**Material examined**

Holotype. ♂, China: Henan Prov., Luanchuan County, Longyuwan, 1718 m, 26.x.2014, coll. Xinran Li and Yan Shi.

Paratypes. 4♂, 4♀, same data as holotype.

**Measurements**

Male and female – pronotum, length × width: 5.5 × 8.2 mm; body length: 26.5–27.5 mm.

**Description**

Tergite VII with anterior margin slightly arched and posterior margin truncate (Fig. 10D). Sternum VII slightly produced at apex, posterior margin truncate and weakly concave at middle (Fig. 10C). Tergite X rounded at apex; paraprocts (pp.) developed, triangular, with apices not extending to the posterior margin of Tergite X, with the gap between paraprocts narrow (Fig. 10J).
Female genitalia

Basivalvulae (bsv.) well-developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins obscure and dark brown, anterior area dark brown with a distinctive prominence (Fig. 10E). Spermatheca sitting behind the basivalvulae and with a large and oblong apical ampulla, and an oblong basal ampulla on a short duct. Laterosternal shelf (ltst.sh.) large and translucent, nearly oval and brown on both sides, with dense spinules at apical half (Fig. 10F).

Cryptocercus laojunensis, sp. nov.

(Figs 4g1–4g3, 11)


Material examined

Holotype. ♀, China: Yunnan Prov., Lijiang City, Mt Laojunshan, Jiushijulongtan, 3627 m, 12.x.2014, coll. Qikun Bai and Yan Shi. Paratypes. 3♂, 6♀♀, same data as holotype.

Measurements

Male and female – pronotum, length × width: 6.1 × 8.0 mm; body length: 23.6 mm.

Description

Tergite VII with anterior margin slightly truncate and posterior margin truncate (Fig. 11D). Sternum VII slightly produced at apex, posterior margin truncate and weakly concave at middle. Tergite X rounded at apex (Fig. 11C); paraprocts (pp.) developed, triangular, with apices not extending to the posterior margin of Tergite X, with the gap between paraprocts narrow (Fig. 11D).
Female genitalia

Basivalvulae (bsv.) well-developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins obscure and dark brown, anterior area narrow and dark brown (Fig. 11E). Spermatheca sitting behind the basivalvulae and with a large and round apical ampulla, and a very small basal ampulla on a short duct. Laterosternal shelf (lst.sh.) large and translucent, nearly oblong and brown over much of both sides, with dense spinules at apical half (Fig. 11F).

Cryptocerus banshanmenensis, sp. nov.

(Figs 4h1–4h2, 12)


Material examined

Holotype. ♀, China: Sichuan Prov., Danba County, Banshanmengou, 2666 m, 6.x.2014, coll. Qikun Bai, Zhiwei Qiu and Xinran Li.
Paratypes. 2♂, 2♀, same data as holotype.

Measurements

Male and female – pronotum, length × width: 6.5 × 9.0 mm; body length: 28.5 mm.

Description

Tergite VII with anterior margin slightly truncate and concave, posterior margin truncate (Fig. 12D). Sternum VII strongly produced at apex, posterior margin truncate (Fig. 12C). Tergite X slightly acute at apex; paraprocts (pp.) developed, triangular, with apices not extending to the posterior margin of Tergite X (Fig. 12D).
Female genitalia
Basivalvulae (bsv.) well-developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins obscure and dark brown, anterior area narrow and dark brown (Fig. 12E). Spermatheca sitting behind the basivalvulae and with a large and water-drop apical ampulla, and a water-drop basal ampulla on a short duct. Laterosternal shelf (lst.sh.) large and translucent, nearly oblong, with dense spinules at apical half (Fig. 12F).

Cryptocercus tianbaensis, sp. nov.
(Figs 4i–4i3, 13)

Material examined
Holotype, ♀, China: Sichuan Prov., Luding County, Tianba, Muijiaogou, 2124 m, 8.x.2014, coll. Qikun Bai, Zhiwei Qiu and Xinran Li.
Paratypes, 1♂, 3♀, same data as holotype.

Measurements
Male and female – pronotum, length × width: 5.5 × 8.0 mm; body length: 24.5 mm.

Description
Tergite VII with anterior margin slightly truncate, posterior margin truncate (Fig. 13D). Sternum VII produced at apex, posterior margin truncate (Fig. 13C). Tergite X rounded at apex; paraprocts (pp.) developed, triangular, with apices not extending to the posterior margin of Tergite X (Fig. 134).
Female genitalia

Basivalvulae (bsv.) well developed and divided into two symmetrical parts; basivalvulae with anterior and posterior margins clearly delimited, posterior margins dark brown, anterior area wide and dark brown (Fig. 13E). Spermatheca sitting behind the basivalvulae and with a large and oblong apical ampulla, and an oblong basal ampulla on a short duct. Laterosternal shelf (ltst.sh.) large and translucent, nearly oblong and narrow, brown at middle, with dense spinules at apical half (Fig. 13F).

Remarks

All nine new species of Cryptocercus were extremely similar in external morphology. We chose five standard characters of female genitalia that have been used to distinguish species in previous studies (Grandcolas 2000; Grandcolas et al. 2001, 2005; Aldrich et al. 2004; Wang et al. 2015). Most species can be identified on the basis of these characters, e.g. interspecific character variation of characters between C. chengkouensis, sp. nov. and C. changbaiensis, sp. nov. (Table 2): C. chengkouensis, sp. nov. has a distinctive prominence on the anterior area of the basivalvulae (Fig. 5E) but the latter does not (Fig. 6E); two sides of the laterosternal shelf in the former are brown (Fig. 5F), but not in the latter (Fig. 6F); the spermatheca of C. chengkouensis, sp. nov. is oval (Fig. 5E), but nearly round in the latter (Fig. 6E). Unfortunately, identification of similar Cryptocercus species using just these five characters would be challenging for a large-scale biodiversity study, because there is a considerable morphological overlap between the related species (e.g. C. luanchuanensis sp. nov. (Fig. 10) and C. neixiangensis). DNA-based tools have been shown to be a
valuable and effective approach to identify Cryptocercus species (Kambhampati 1996; Burnside et al. 1999), so we combined DNA barcoding and morphology with chromosome number to delimit species. Until now, molecular techniques have been the most rapid and convenient approach for studying these species of woodroaches.

**Discussion**

**Species delimitation**

The nine candidate species we identified, primarily on the basis of chromosome numbers, are in addition to the 15 identified previously by Grandcolas (2000), Grandcolas et al. (2005), Wang et al. (2015), and Che et al. (2016). Molecular species delimitation analysis corroborated the existence of each of these species. Our results indicate that DNA-based species-delimitation methods using COI perform well for these subsocial and xylophagous cockroaches, and are likely to be of utility given the lack of defining morphological characters among males, females and nymphs of these organisms. Our study is the first attempt to delimit Cryptocercus species on a large scale, including males, females and nymphs.

As the genus Cryptocercus comprises mainly alpine cockroaches, we were able to obtain only a small number of samples from each locality. This may have contributed to the limited genetic variability within each species. Barcode gap (COI) analysis showed that the maximum intraspecific distance (0.61%) was distinctly lower than the interspecific distance (2.18–20.36%), even for those species (C. neixiangensis and C. luanchuanensis, sp. nov.) with the lowest interspecific divergence (2.18%). Hebert et al. (2004) proposed that the genetic divergence cutoff for species identification should be at least 10 times greater than within species. Our study (average intraspecific distance: 0.14%, average interspecific distance: 11.81%) showed that there is a distinct gap between intraspecific and interspecific distances. DNA-based analyses

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**Fig. 11.** Cryptocercus laojunensis, sp. nov.: (A) supraanal plate, ventral view; (B) supraanal plate, dorsal view; (C) subgenital plate, dorsal view; (D) Tergite VII, dorsal view; (E) basivalvula and spermatheca, ventral view; (F) laterosternal shelf, dorsal view. Scale bars: 1.0 mm.
resolved most Cryptocercus samples from Asia to a putative species. GMYC, ABGD and chromosome numbers enabled their differentiation of the two geographically proximate species C. neixiangensis (BTM) ($2n=37$) and C. luanchuanensis, sp. nov. (LYW) ($2n=39$) (Fig. 2) (2.18% in COI, 1% in 28S), although they had highly similar morphologies. Some other species pairs were particularly well differentiated despite being geographically proximate to each other (e.g. C. primarius (DCP) and C. pingwuensis (BSG) – 4 km apart).

**Uplifting of the Qinghai–Tibetan plateau caused rich diversity of Cryptocercus in the Hengduan Mountains**

To date 22 Cryptocercus species have been described worldwide, of which 16 are from China (Bey-Bienko 1938; Grandcolas 2000; Grandcolas et al. 2005; Wang et al. 2015; Che et al. 2016). Another nine new Cryptocercus species, mainly from the Hengduan Mountains (six species), are described in the current study. There are now 25 Cryptocercus species distributed in the mountain forests from 702 m (Gaolingzi, Heilongjiang Province) to 3756 m (Shikaxueshan, Yunnan Province) in China, most of which are in the Hengduan Mountains.

The Hengduan Mountains comprise a large mountainous region in south-western China with an average elevation of more than 4000 m (Spicer et al. 2003). Fifteen Cryptocercus species were discovered here at an altitude of ~3000 m. The Himalayan and Hengduan Mountains are known to encompass global biodiversity hotspots with high levels of plant and animal biodiversity and endemism (Myers et al. 2000; Wu et al. 2013; Liu et al. 2014). The region is thought to harbour at least 15,000 land plant species, of which more than 29% are endemic, and at least 1141 vertebrate species, of which 15% are
endemic (Myers et al. 2000). The rapid and extensive uplift of the Himalaya–Hengduan Mountains is considered to be the major driving force in shaping such high species diversity (Liu et al. 2013; Wu et al. 2013; Wen et al. 2014). The Hengduan Mountains can be considered to be one of the hotspots of Cryptocercus diversity in China.

Since the early Miocene, extensive uplifting of the Qinghai–Tibetan plateau occurred over at least four major periods: 25–17, 15–13, 8–7, and 3.5–1.6 million years ago (Allégre et al. 1984; Spicer et al. 2003; Royden et al. 2008). The Cryptocercus lineages in the Hengduan Mountains began to diversify 20.7–11.7 million years ago, and the most recent diversification between C. arcuatus (SKXS) and C. habaensis (HBXS) occurred ~5.82–1.58 million years ago (Che et al. 2016), which corresponds well with the major uplifts of the Qinghai–Tibetan plateau. This region is characterised by a series of parallel mountain ranges (Yin and Harrison 2000), but all members of Cryptocercus are wingless and lack the ability to migrate over long distances. The physical isolation caused by these mountain ranges is likely to have contributed to interspecific and intraspecific divergences. The localities of two close geographic species, C. habaensis (Fig. 14E) and C. meridianus (Fig. 14D), are found just 28 km apart, but they are separated by two snow mountains (Haba Snow Mountain, 5396 m; Yulong Snow Mountain, 5596 m) and one gorge (Tiger Leaping Gorge) (Fig. 14A). Tiger Leaping Gorge passes between the two Snow Mountains in a dramatic vertical drop of 3500 m, through which a rapid stream flows and xeric shrubland exists on both banks along the Jinsha River (Qikun Bai, pers. obs.). These natural barriers prevent gene flow among Cryptocercus species. Significant genetic differences (9.50% in COI) can be detected between these two species. Similarly, there is no vegetation on the

Fig. 13. Cryptocercus tianbaensis, sp. nov.: (A) supraanal plate, ventral view; (B) supraanal plate, dorsal view; (C) subgenital plate, dorsal view; (D) Tergite VII, dorsal view; (E) basivalvula and spermatheca, ventral view; (F) laterosternal shelf, dorsal view. Scale bars: 1.0 mm.
peaks of ridges at altitudes of 4000–5000 m (Wang et al. 2004), which would prevent migration by Cryptocercus from one peak to another (Fig. 14B,1C).

Compared with the lineages from the Hengduan Mountains (2124–3756 m, average 2986 m), the lineages from the Qin-Daba Mountains (1470–2600 m, average 1814 m) and Manchuria (702–1199 m, average 935 m) occur at lower elevations. Relatively poor Cryptocercus diversity has been found in these two regions (Qin-Daba Mountains lineages: 7 species; Manchuria lineages: 2 species). Manchuria is a large north-eastern Asian geographic region of ~130 × 10^6 km^2 which mostly consists of vast plains (Elliott 2000). C. relictus has been collected by us at Gaolingzi, Shuangfenglinchang, and Mudanfeng as well as from Russia (Bey-Bienko 1935) and South Korea (Park et al. 2004). This species therefore appears to be widely distributed in north-eastern Asia. The relatively simple geography of this region compared with the Hengduan Mountains is likely the primary cause of the low diversity in this area, because there are no major barriers to hinder the gene flow among different populations. Changbai Mountain, the highest in the Manchuria region, is an active volcano situated on the boundary between China and North Korea, and was formed within a short period between 2.77 and 0.31 million years ago (Wan 2012; Wei et al. 2007). It is therefore unlikely to have influenced the evolution of Cryptocercus in the way that the Hengduan Mountains have.

Fig. 14. Collection sites for Cryptocercus at Mt Habaxueshan and Mt Yulongxueshan: (A) the whole topographical map of Mt Habaxueshan and Mt Yulongxueshan, map data: Google, Copernicus; (B) photograph of Mt Yulongxueshan; (C) photograph of Mt Habaxueshan; (D) habitat of C. meridianus; (E) habitat of C. habaensis. All photographs by Qikun Bai.
Conclusion
Our results show that molecular methods generate species hypotheses for woodroaches that are highly consistent with those based on morphological and chromosome-based hypotheses. The DNA-based technique shows promise as a rapid, precise, independent identification approach to discriminate woodroaches of different life stages and sexes. Because of its high performance, COI can be recommended as a useful DNA barcode for Cryptocercus. The approaches we used may also help to increase our understanding of the rich diversity of Cryptocercus. The wide diversity of Cryptocercus in the Hengduan Mountain indicates that more detailed ecological data and multiregion populations are needed to further clarify the phylogenetic relationships within Asian Cryptocercus. The high endemism typically displayed by Cryptocercus species in China makes them particularly vulnerable to habitat destruction. Our work therefore highlights the importance of protecting the alpine forests that these taxa inhabit.

Conflicts of interest
The authors declare no conflicts of interest.

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