Population Explosion in the Yellow-Spined Bamboo Locust *Ceracris kiangsu* and Inferences for the Impact of Human Activity

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**Abstract**

Geographic distance and geographical barriers likely play a considerable role in structuring genetic variation in species, although some migratory species may have less phylogeographic structure on a smaller spatial scale. Here, genetic diversity and the phylogenetic structure among geographical populations of the yellow-spined bamboo locust, *Ceracris kiangsu*, were examined with 16S rDNA and amplified fragment length polymorphisms (AFLPs). In this study, no conspicuous phylogeographical structure was discovered from either Maximum parsimony (MP) and Neighbor-joining (NJ) phylogenetic analyses. The effect of geographical isolation was not conspicuous on a large spatial scale. At smaller spatial scales local diversity of some populations within mountainous areas were detected using Nei’s genetic distance and AMOVA. There is a high level of genetic diversity and a low genetic differentiation among populations in the *C. kiangsu* of South and Southeast China. Our analyses indicate that *C. kiangsu* is a monophyletic group. Our results also support the hypothesis that the *C. kiangsu* population is in a primary differentiation stage. Given the mismatch distribution, it is likely that a population expansion in *C. kiangsu* occurred about 0.242 Ma during the Quaternary interglaciation. Based on historical reports, we conjecture that human activities had significant impacts on the *C. kiangsu* gene flow.

**Introduction**

Highly migratory species are usually expected to have minimal population substructure over their distributional ranges because strong gene flow can counteract the isolating effects of geographical distance and physical barriers [1]. The current distribution of genetic variation within a species is a product of its demographic history as well as the interaction between mutation, genetic drift and gene flow. It is notoriously difficult to distinguish historical effects, like climate change or geological accident, from shorter-term consequences of dispersal patterns and limited population size, but the distinction is important [2]. It is the imprint of historical changes on the distribution and abundance of organisms left in current population structure that underpins phylogeography [3]. Often these spatial genetic patterns are on a much larger scale than the correlations generated by dispersal of individuals in stable populations [2].

The Quaternary glaciation period played an important role in shaping the history of global fauna and flora. In the past few decades, glacial cycles have been considered one of the most important factors in shifting population genetic structure and promoting floral and faunal diversification [4–10]. Because of climate changes species range of temperate organism is contracted during the glacial periods and is expanded during the interglacial periods [11, 12]. Glacial cycles acting on genetic diversity of grasshoppers have been well studied in Europe and North America [13–16]. The genetic legacy of Pleistocene influence remains poorly understood for Southeast Asia, where glaciation was not synchronous with the Northern Hemisphere ice sheet maxima [2,16–17]. China, especially south of China (e.g. Yunnan province), provided some key refugia for many relict rare species during the Pleistocene glaciation, due to its complex topography and numerous large mountains spanning from west to east [18, 19]. Zhang et al. [20] studied the population structure of the migratory locust *Locusta migratoria*, an infamous pest insect with exceptional migratory ability, and discovered its minimal population substructure. However, most other grasshoppers do not have excellent ability of flying to undergo such rapid climatic change. For instance, limitations due to resources like food may have prevented large-scale colonization by insects [12]. Another example in China is that the agriculture pest *Oxya hyla* intricate was separated into three deep monophyletic clades with no shared haplotype in a study by Li et al. [10]. They suggested that climatic oscillations during glacial periods in the Quaternary caused a complete demographic expansion pattern of *O. hyla* intricate populations, and that past geological events and climatic fluctuations are the most important factor in shaping the population genetic diversity and structure of the species.
The yellow-spined bamboo locust, *Ceracris kiangsu* Chai, is one of the most severe bamboo pests endemic to China [21]. It is mainly distributed in South China and is characterized by its behavior of feeding in large groups on the leaves of bamboo plants during all of its life stages [21–23]. Because of this feeding habit, their distribution range is more restricted than *L. migratoria* [20] and *O. hylae* intricata [10]. Scholars have long explored the morphological taxonomy, pesticide toxicology, physiology and development of *C. kiangsu* [24–29]. However, to our knowledge, there is only one published report of genetic studies on bamboo locust [30].

Here, we use Amplified fragment length polymorphisms (AFLPs) and 16S rDNA datasets to obtain more information about the population histories of *C. kiangsu* and infer demographic processes. AFLPs have been shown to provide phylogeographic resolution among recently and rapidly radiating groups in which sequence data have failed [31–33]. The increased resolution of AFLPs is associated with their nuclear genome-wide distribution, which overcomes problems associated with locus-specific effects, and the large number of polymorphisms that can be easily characterized. Recent population genetic analyses of multiple *Heliconius* species based on AFLPs revealed pronounced genetic structure in both *H. erato* and *H. melpomenenee* at small spatial scales [34,35], suggesting that AFLPs should be effective at distinguishing closely related and geographically proximate races in each species. Together, mtDNA and nuclear markers can provide complementary information about population structure and evolutionary patterns of *C. kiangsu*.

In this study, we compare the resolution of both marker types and address the following questions: (1) In the context of the unregularly phylogeographic structure of *C. kiangsu*, how long has geographical distance influenced the gene flow between different local populations? (2) Does geographical isolation affect the genetic structure of *C. kiangsu* in modern times? (3) If this species experienced a genetic bottleneck and/or a population explosion, did Pleistocene climate cycles play a significant role in shaping the history of *C. kiangsu*, similar to *O. hylae* intricata or other organisms? Additionally, we also estimate the minimum age of this bamboo locust based on pairwise mtDNA sequence divergence. Our results illuminate the population structure in the bamboo locust *C. kiangsu* and provide essential insights for future work focused on the forest grasshopper’s response to climate changes.

**Materials and Methods**

**Ethics statement**

This species of grasshopper in this study is a famous agricultural pest in China, need to control. Collections of samples were permitted by the authority of each forestry center (Forestry Bureau of Guangning County of Guangdong Province, Forestry Department of Hunan Province, Forestry Bureau of Taoyuan County of Hunan Province, Forestry Bureau of Hengyang City of Hunan province, Vegetation Protection Central Station of Guangxi Zhuang Autonomous Region, Forestry Bureau of Rongshui County of Guangxi Zhuang Autonomous Region, Forestry Bureau of Quzhou County of Zhejiang Province, Forestry Bureau of Jianou County of Fujian Province, Forestry Bureau of Guiyang City of Jiangxi Province, Forestry Bureau of Shicheng County of Jiangxi Province, and Forestry Bureau of Pingxiang City of Jiangxi Province). For collection locations no specific permits were required for the described field studies because sample collection did not involve endangered or protected plant species or privately-owned locations.

**Sample collection and DNA extraction**

A total of 393 individuals of *C. kiangsu* were collected from 24 locations in the field covering nearly all of the species’ distribution range between 2007 and 2011 (Figure 1; Table 1). A total of 25 populations were included in our foregoing analyses. Considering the migration ability of *C. kiangsu*, most populations were collected within regions around 200 km apart, except Menglin and Mengla (90 km). Furthermore, there is a special bamboo species, *Bambusa textilis* McClure, in Guangning County, while the species in all of the other locations is the bamboo *Phyllostachys heterocycla* (Carr.) and/or others. Two individuals of the green-spined bamboo locust *C. nigrioris* collected in Shuangpai County were used as outgroups. All samples were stored in 100% ethanol upon capture.

Genomic DNA was extracted from femur samples using Wizard® Genomic DNA Purification Kit (PROMEGA, USA) following the manufacturer instructions, and stored at −30°C until needed. All DNA extracts for AFLP were run on 1% agarose gels, and samples that did not have high concentrations of high molecular weight DNA or that appeared excessively sheared were not included in AFLP analysis [36]. Sample sizes used for AFLP analysis from collected populations ranged from 2 to 10, and all individuals were used in mitochondrial DNA analysis.

**Mitochondrial DNA**

A 483 bp segment of the 16S rRNA gene was amplified in a subset of 393 individuals with primers given in Liu & Jiang (2004); LR-J-12887 (5'-CCGGTCTGAACTCAGATCACGT-3') and LR-N-13398 (5'-GGCCGTGTGTTTACAAAAACAT-3'). PCR reactions were carried out in 50 μL volumes which included 1 μL template DNA (total genomic DNA diluted 1:10 with water), 5 μL 10× buffer, 3 μL MgCl2, 4 μL dNTPs, 1 μL of each primer and 0.25 μL rTaq (TaKaRa Bio Inc,Dalian,China). PCR cycling conditions included an initial 5 min at 94°C, 30 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, plus a final extension at 72°C for 10 min.

PCR products were tested by electrophoresis on a 1% agarose gel, and all PCR products were sequenced on ABI-PRISM3730 with BigDye terminator v3.1 (Shanghai Sangon Biotech Co., Ltd.). Alignments are available on request. To confirm the identity of the mtDNA sequences, the amino acid sequences were inferred using GeneDoc 2.6.02 based on the invertebrate mitochondrial code.

**AFLPs**

We applied the original AFLP protocol of Vos et al. (1995) with a few modifications [37]. Those individuals with genomic DNA which has high consistency and purity quotients were used in this part. Because locust species have a larger genome than many other insects, we performed a longer enzyme digestion in order to obtain more polymorphic fragments. 400 ng genomic DNA was digested at 37°C with the enzymes 0.2 μL EcorI and 0.5 μL MseI (both Fermentas, with 2 μL Buffer R) for 3 h followed by 70°C for 15 min to ensure enzyme inactivation. EcoRI/MseI adapters were ligated to the enzyme digestion product using T4-DNA-Ligase (FERNETAS) at 20°C overnight. We used 16 primer combinations containing one selective base. Preselective amplification was conducted in a total volume of 30 μL including 3 μL of diluted restriction-ligation DNA, 3 μL 10× buffer, 2.4 μL MgCl2, 1.6 μL dNTPs, 0.4 μL rTaq (TAKARA) and 1 μL EcoRI+N primer and 1 μL MseI+N primer, up to 30 μL with ddH2O. The thermal cycling parameters for preselective amplification were 2 min at 94°C, 30 cycles of 30 sec at 94°C, 1 min at 56°C, 1 min at 72°C, followed by 10 min at 72°C. The ligations were diluted 1:20 and 2 μL of the diluted preselective amplification product was used in the selective amplification. Selective amplification was conducted...
in a total volume of 20 μL with 2 μL diluted preselective amplification product, 2 μL 10× buffer, 1.6 μL MgCl₂, 1.6 μL dNTPs, 0.6 μL Taq (TAKARA) and 1 μL EcoRI+3 primer (labeled with FAM) and 1 μL MseI primer, up to 20 μL with ddH₂O. Four primer combinations (E–AGG/M–CAG, E–AGG/M–CTT, E–AGC/M–CTC, E–AAG/M–CAG, where E is EcoRI, M is MseI) were used in this step. Selective amplification products were visually measured on an ABI 3700 DNA analyzer (Shanghai Sangon Biotech Co., Ltd.).

Fragment data were analyzed with GeneMarker version 2.20 (Demo). We scored fragments of size 50–500 bp as present or absent. Minimum fragment signal intensity was initially used for all fragments. The signal intensity was measured as relative fluorescent units (RFU) of 500 or 1000 depending on the primer set [36].

Data analysis

mtDNA. The mitochondrial DNA sequence data were assembled and corrected in SeqMan II version 5.00 (Lasergene, DNASTAR Inc., Madison, WI). We then carried out alignments in MEGA 5 [38] based on the ClustalW function. All the sequences were uploaded to NCBI (Accession nos: JQ799527–799883).

The number of haplotypes (h) as well as haplotype diversity (Hd) and the number of variable sites were analyzed in DnaSP v3.10 [39] with aligned sequences. Other information about geographical populations included number of segregating sites (S), and nucleotide diversity (P). The sequence sets were used to calculate FST and the p value in the AMOVA function of ARLEQUIN v3.11 [40]. We also calculated indices about gene flow between geographical groups. In genetic structure test, we divided 25 population into 18 groups according to geographical distance. Two selective neutrality tests of Tajima’s D [41,42] and Fu’s F [43] were also performed in ARLEQUIN v3.11 [39], of which index below zero indicates that the species has experienced population dynamics, and a p value in both tests indicates significance. Sum of square deviation (SSD) and the Harpending’s raggedness index (HRI) were also calculated in our analysis. Furthermore, a τ value was generated in mismatch analysis. The formula, τ = 2 ut was used to detect the population expansion time [44]. The nucleotide substitution rate in mitochondrial DNA was 2.3% per million years (MY) as suggested in Knowles et al. (2000) [45].

To represent phylogeographical structure among the haplotypes, a median-joining network was generated for all haplotypes using software NETWORK version 4.6.1.0 (Fluxus Technology Ltd.), by the Median Joining method [46]. Maximum parsimony (MP) and Neighbor-joining (NJ) phylogenetic analyses were used to identify major clades and evaluate the relationships among the haplotypes of the 16S. The TVM model (Transversional model) [47] selected by AIC in Modeltest 3.7 [48,49] was used in 16S maximum parsimony analyses with PAUP* 4.0b10 [50]. Neighbor-joining (NJ) analyses were performed in MEGA5 using Kimura 2-parameter gamma model with 1000 bootstrapping replicates for 16S [51]. The Kimura’s two-parameter gamma model corrects for multiple hits, and evolutionary rates among sites are modeled using a Gamma distribution.

AFLP analysis. Fragmentized information was exported as 0/1 popmatrix by GeneMarker and transformed in format in the AFLPDAT program [52], a collection of R functions which facilitates the handling of dominant genotypic data and can convert data from tables into input formats of several programs including ARLEQUIN, AFLP-SURV, STRUCTURE [53] POPGENE and others [52]. ARLEQUIN v3.11 was used in evaluating genetic variation among 25 geographic populations. Population pairwise FST (Pairwise genetic distance) and p value (the significance of FST value) with 1000 bootstrapping replicates
Using AFLP-SURV [54], we estimated allelic frequencies by applying the Bayesian method with non-uniform prior distribution of allele frequencies [55]. When we use the Bayesian method, two important indexes were considered: sample size and the number of

Table 1. Sampling site, geographical coordinates, elevation (metres), sample size (Nind) of sampled Ceracris kiangsu populations, and sequence information of each geographical population calculated from DnaSP software.

| NO | Sampling site | Latitude | Longitude | Elevation | Nind | S | h | Variance of Hd | Std.Dev | PI | Sampling variance | St.Dev |
|----|---------------|----------|-----------|-----------|------|---|---|----------------|---------|----|------------------|--------|
| 1  | Guangde, Anhui | 30°47’19”N | 119°28’51”E | 216 | 15 | 3 | 3 | 0.448 | 0.018 | 0.134 | 0.002 | 0.000 | 0.001 |
| 2  | Shucheng, Anhui | 31°22’05”N | 116°59’13”E | 42 | 15 | 49 | 3 | 0.362 | 0.021 | 0.145 | 0.014 | 0.000 | 0.011 |
| 3  | Jinyunshan, Chongqing | 29°50’14”N | 106°23’46”E | 600 | 25 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 4  | Jianou, Fujian | 27°02’08”N | 118°14’14”E | 194 | 14 | 24 | 3 | 0.275 | 0.022 | 0.148 | 0.013 | 0.000 | 0.007 |
| 5  | Nanjing, Fujian | 24°29’18”N | 117°21’01”E | 345 | 2 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 6  | Guangning, Guangdong | 23°36’17”N | 112°23’18”E | 52 | 34 | 21 | 3 | 0.266 | 0.008 | 0.092 | 0.010 | 0.000 | 0.004 |
| 7  | Guilin, Guangxi | 25°18’25”N | 110°23’40”E | 277 | 10 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 8  | Quanzhou, Fujian | 25°55’43”N | 111°09’44”E | 286 | 20 | 1 | 2 | 0.100 | 0.008 | 0.088 | 0.000 | 0.000 | 0.000 |
| 9  | Rongan, Guangxi | 25°12’29”N | 109°23’45”E | 160 | 18 | 9 | 2 | 0.111 | 0.009 | 0.096 | 0.002 | 0.000 | 0.002 |
| 10 | Jinping, Guizhou | 26°43’04”N | 109°10’52”E | 552 | 6 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 11 | Mayanghe, Guizhou | 28°41’47”N | 108°16’16”E | 662 | 2 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 12 | Wuhan, Hebei | 31°05’30”N | 114°21’01”E | 100 | 20 | 2 | 3 | 0.279 | 0.015 | 0.123 | 0.001 | 0.000 | 0.000 |
| 13 | Hengyang, Hunan | 27°07’01”N | 112°41’36”E | 140 | 4 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 14 | Huarong, Hunan | 29°35’23”N | 112°32’17”E | 32 | 20 | 2 | 3 | 0.563 | 0.004 | 0.063 | 0.001 | 0.000 | 0.000 |
| 15 | Shuangpai, Hunan | 26°06’30”N | 111°49’28”E | 460 | 18 | 24 | 2 | 0.111 | 0.009 | 0.096 | 0.006 | 0.000 | 0.005 |
| 16 | Taoyiang, Hunan | 28°30’09”N | 112°09’08”E | 55 | 16 | 13 | 10 | 0.917 | 0.002 | 0.049 | 0.006 | 0.000 | 0.002 |
| 17 | Taoyuan, Hunan | 28°54’09”N | 111°29’20”E | 183 | 46 | 4 | 5 | 0.572 | 0.001 | 0.037 | 0.001 | 0.000 | 0.000 |
| 18 | Changsha, Hunan | 28°10’11”N | 112°40’06”E | 65 | 19 | 1 | 2 | 0.526 | 0.001 | 0.040 | 0.001 | 0.000 | 0.000 |
| 19 | Zijinshan, Jiangsu | 32°04’14”N | 118°50’57”E | 170 | 20 | 2 | 3 | 0.595 | 0.005 | 0.073 | 0.001 | 0.000 | 0.001 |
| 20 | Shicheng, Jiangxi | 26°19’35”N | 116°20’36”E | 408 | 19 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 21 | Changning, Sichuan | 28°29’57”N | 104°55’58”E | 309 | 19 | 19 | 3 | 0.368 | 0.0158 | 0.016 | 0.005 | 0.000 | 0.004 |
| 22 | Ziyang, Sichuan | 30°07’45”N | 104°37’44”E | 355 | 5 | 0 | 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 23 | Mengla, Yunnan | 21°29’18”N | 101°33’23”E | 771 | 5 | 68 | 5 | 1.000 | 0.016 | 0.126 | 0.073 | 0.000 | 0.014 |
| 24 | Menglun, Yunnan | 21°55’25”N | 101°15’56”E | 549 | 7 | 44 | 6 | 0.952 | 0.009 | 0.096 | 0.425 | 0.000 | 0.010 |
| 25 | Quzhou, Zhejiang | 30°19’03”N | 119°25’57”E | 191 | 14 | 1 | 2 | 0.143 | 0.014 | 0.119 | 0.003 | 0.000 | 0.000 |

Total: 393 122 33 0.436 0.001 0.029 0.060 0.000 0.001

4, 3, 2, 1

S, number of polymorphic/indel/missing sites; h, number of haplotypes; Hd, haplotype diversity; sampling variance of Hd; St.Dev, standard deviation of haplotype diversity; PI, nucleotide diversity; sampling variance of PI, and standard deviation of PI.

doi:10.1371/journal.pone.0089873.t001

were produced using AMOVA analysis in ARLEQUIN. And we also run a genetic structure test with the same groups we shown in 16S rRNA.
individuals lacking AFLP fragment. The frequency of the null (–) allele at each locus was computed as well as the distribution of allele frequencies based on the variation over loci frequencies. Using the 100000 bootstrapped matrices of Nei’s genetic distance estimated in AFLP, a Neighbor-joining tree was constructed using the Neighbor and Consense programs in Phylip 3.6 [56]. The percentage of polymorphic loci (P), observed number of alleles (Na), effective number of alleles (Ne), gene diversity (He) [57], Shannon’s Information index (I), and Nei’s genetic distance (SSD) = 0.0387, P = 0.188] were from 0.179 (Guangning) to 0.019 (Nanjing) (Table 4). Observed/effective number of alleles (Na/Ne) in each population is above 1.0 (Table 4). Nei’s gene diversity (H) values in five populations are above 0.1, which are Guangning, Guilin, Huarong, Mengla and Shucheng (Table 4). The highest Nei’s gene diversity is observed in Guangning (0.115), and the 2nd highest H is observed in Mengla (0.111) (Table 4). Shannon’s Information indexes (I) were from 0.179 (Guangning) to 0.019 (Nanjing) (Table 4). In the population pairwise analysis, the Fst values between seven population pairs (Changsha – Menghu/Nanjing, Guilin – Menghu/Nanjing, Huarong – Menghu, Jingpin – Mayanghe and Nanjing – Wuhan) are all 0, and the respective p values are all high (p>0.5), except Huarong – Menghu (p = 0.188) (see Table S2).

In the neighbor-joining tree based on Nei’s genetic distance shown in the Table S3, all individuals form three main branches (Figure 4). The Guilin, Rongan and Taoyuan groups remain at the center of the whole tree, while the Nanjing, Guangning and Guanxu groups are the extremities of the three branches, respectively. The individuals of C. nigricornis, the outgroup, are not separated from C. kiangu individuals but instead share the same branch with Guangde (Figure 4).

For the purpose of testing for possible gene introgression among different populations, we used a Structure analysis based on a Bayesian model to infer the population admixture of the C. nigricornis populations (Figure 5a). Testing K (the number of distinct groups ranging from 2 to 16) only the Jianou and Quzhou populations are in a monophyletic group (K = 2 to 4), while the
Population structure

Discussion

Population structure
Our study, despite some contradictions between AFLP and mitochondrial DNA, reveals that there is a high level of genetic diversity and a low genetic distance among the individuals in the C. kiangsu population as a whole. The disparity of the genetic diversity and a low genetic distance among the individuals in the groups.

| Population | 16S rRNA |
|------------|----------|
| Changning  | 0.224    |
| Changsha   | 0.213    |
| Guangde    | 0.225    |
| Guangning  | 0.250    |
| Guilin     | 0.290    |
| Hengyang   | 0.290    |
| Huarong    | 0.207    |
| Jianou     | 0.250    |
| Jiping     | 0.290    |
| Jinyunshan | 0.290    |
| Mayanghe   | 0.290    |
| Mengla     | 0.166    |
| Menglun    | 0.164    |
| Nanjing    | 0.290    |
| Quanzhou   | 0.275    |
| Quzhou     | 0.269    |
| Rongan     | 0.273    |
| Shicheng   | 0.290    |
| Shuangpai  | 0.273    |
| Shucheng   | 0.238    |
| Taoyuan    | 0.157    |
| Wuhan      | 0.249    |
| Zijinshan  | 0.203    |
| Ziyang     | 0.290    |

Assumed all group have a common ancestral population, these population-specific coefficients would represent the degree of evolution of particular populations from a common ancestral population.

Gene (16S). Furthermore, the variation tendency in both AMOVA and mismatch analysis were consistent with each other. The phylogenetic analysis highly supported monophyly of C. kiangsu (bootstrap value = 1000). However, when using AFLP and 16S as markers, there is no clear phylogeographic structure in either the neighbor-joining or the maximum parsimony tree. Instead, some populations from distant geographic areas appeared on the same branch. Despite the possibility of some basic structure in the NJ and MP trees, there is currently little support for the branches in either tree. The results in our study, taken together, support the hypothesis that the C. kiangsu population is in a primary differentiation stage.

Jiang et al. (2011) amplified 506 bp of 16S mtDNA from 5 populations of Rammeacris kiangsu Tsai (i.e. C. kiangsu). Four of their sampling locations were the same as ours, with the exception of Yongchang, which is not far from Jinyun Mountain. Their findings support our conclusion that no apparent population differentiations exist among these five geographical populations. Their NJ tree was constructed using 5 populations and O. chinensis as outgroup. Only the populations in Changning and Taojiang had some divergence from each other [61]. However, the genetic distance in their study was only 0.004, while our pairwise distance calculated in MEGA5 between Taojiang and Changning is 0.008. This difference between these two studies may be due to the different sampling number. We also found that C. nigricornis is not a suitable outgroup candidate due to the two C. nigricornis were not separated well from C. kiangsu.

There are several possible factors responsible for genetic differentiation of the populations in phytophagous insects, from low dispersal ability and geographical barriers, to habitat fragmentation and host plant availability [62]. Generally, geographic distance plays an important role in gene flow and diversity. But migratory species are often deemed to have less phylogeographic structure in their distribution range, as strong gene flow effects the homogenization of genetic variation as well as counteracts random drift, selection and mutation [63–66]. High-present populations from distant geographic areas appeared on the same branch. Instead, some markers, there is no clear phylogeographic structure in either the neighbor-joining or the maximum parsimony tree. Therefore, geographic isolation might still be the preeminent factor in the population differentiation of the locust, while on a smaller spatial scale, the effects of geographical isolation might decrease and the effects of migration might increase [68]. But in our study, although our sampling range covered the all range of C. kiangsu from east to southeast of China. Most of our samples were assembled and clustered into one big clade. Weak differentiation signs can be found in the whole C. kiangsu population. Besides, the result of STRUCTURE (Fig. 5)

| Table 2. Analysis of molecular variance (AMOVA) of C. kiangsu based on geographical distances. |
|---------------------------------------------------------------|
| Source of variation | Df | Sum of squares | Variance components | Percentage of variation | Ψ-statistics | P value |
|---------------------|----|----------------|---------------------|------------------------|-------------|--------|
| Among groups        | 19 | 21.229         | 0.031 Va            | 13.98                  | ΨST = 0.140 | 0.000  |
| Among population within groups | 5  | 2.855         | 0.023 Vb            | 10.42                  | ΨSC = 0.121 | 0.022  |
| Within populations  | 368| 62.643         | 0.170 Vc            | 75.59                  | ΨST = 0.244 | 0.152  |
| Total               | 392| 86.728         | 0.224               |                        |             |        |

doi:10.1371/journal.pone.0089873.t002

doi:10.1371/journal.pone.0089873.t003
shows poorer regularity than previous studies [10]. It means although *C. kiangsu* may have experienced population declines, the following events may be more important, like human activity which creates strong signal of introgression between different geographical populations.

It is important to note that there are potential limitations in using AFLPs for phylogenetic reconstruction [69,70], some of which are due to the sharing of absences present in the calculation of distances [34]. The degree of homoplasy in the data and suitable distance measures for tree-building also seem to be important in this process [71]. Mitochondrial DNA is a marker among the most frequently used in analyses due to its maternal inheritance, haploid status, high rate of evolution and practicability [72–77,2]. However, mtDNA may not be ideal for characterizing populations with a high frequency of a common haplotype [78]. Our study may have too small of a sampling number - for different geographical populations’ shared same haplotypes the individuals from some locations are less than 10. Thus many local haplotypes may not have been included in our analyses. And the abnormal condition of Mayanghe population shown in AFLP analysis (far genetic distance and long NJ branch) were also attributed to the small sampling size. Intensive sampling must be employed in our future work. Furthermore, the incomplete nuclear lineage sorting or gene flow [79] may be responsible for the lack of AFLP structure between well supported mtDNA results [80], like the position of the Guilin population. Because the collection number of specimens is too low at several points, it is hard to determine the degree of diversity at Menghu, Nanjing and Mayanghe. We plan on continuing to increase the sample size and make use of 4-base selective primer pairs in AFLP analysis as part of our future work.

**Figure 2. Most parsimonious median-joining (MJ) network for *C. kiangsu* 16S haplotypes.** The size of the circles is proportional to the frequency of represented haplotypes. Geographical population indicated by colour as below. Nodes with small white circles are median vectors that represent hypothetical missing links or unsampled haplotypes. doi:10.1371/journal.pone.0089873.g002

**Geographical isolation**

The Hengduan Mountains have been reported as refugia for *O. hyla intricata*, and during the Quaternary, *O. hyla intricata* was distributed along the altitudinal gradient of the mountains. Following free retreats and expansions, frequent gene exchanges may have occurred among populations [10]. In our sampling range, Changning, Jinyunshan and Ziyang are near Hengduan Mountains and separated by Emei Mountain, so genetic distance among the locust groups from these locations were quite far. However, there are no mountains as high as the Hengduan Mountains in the southeast of China. Wuyi Mountains and Naling Mountains are the main mountains in the southeast of China, and their height above sea level is about 350 m and 1000 m, receptively, and prominent peaks only as high as 1,800 m and 2,200 m, respectively. But among some sampling locations around Wuyi Mountains and Nanling Mountains, like Jianou, Shicheng and Nanjing, which are located on both sides of Wuyi Mountains, FST between Shicheng and Nanjing and between Shicheng and Jianou is above 0.1. Wuyi Mountains may cut off the migration path between these locations. At the same time, gene flow likely exists between Taoyuan and Huarong, and the Qinlin Mountains and Daba Mountains do not obstruct the gene flow between the two populations. We took this phenomenon as the evidence of preliminary geographical isolation, although the whole population structure of *C. kiangsu* is still vague. However higher variation were found within population than among geographical group (Table 2), suggesting that this geographical isolation is still weak.

**Effect of the Quaternary glaciations events**

Species divergence induced by the effects of genetic drift has often been linked to divergence that took part during displacements into glacial refugia and recolonization of previously
glaciated areas [15], and the Pleistocene glacial cycles have been shown to have played a direct role in species divergence [81–83,15]. For example, *L. migratoria*, although it is a highly migratory species deriving from different ancestral refugial populations following postglacial expansions, form three groups within China: the Tibetan group, the South China group and the North China group [20]. Zhang et al. (2009) attributed high haplotype diversity in populations of a mountain frog *Leptobrachium ailaonicum* to climatic oscillations during glacial periods in the Quaternary which allowed for population expansions while others remained stable [17].

In view of Tajima’s D and Fu’s Fs identified in the 16S, which were surprisingly below zero [86,87], a population explosion of *C. kiangsu* likely occurred between 0.242 Ma. This range is within the Quaternary, between Stage II and Stage III of Penultimate Glaciation [88]. Thus, the Quaternary glaciation events appear to be linked to population explosion. Most likely the *C. kiangsu* species migrated to one refugium where frequent gene flow occurred among different ancestral populations, followed by postglacial

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**Figure 3. Maximum parsimony tree based on 16S rRNA haplotypes of 393 C. kiangsu individuals.**
doi:10.1371/journal.pone.0089873.g003
proposed by Chen et al. [2].

AFLPs NJ tree (Fig. 4) [89], supporting the refugial region Yunnan phylogenetic placement in both the 16S haplotype and nucleotide diversity (Table 1) as well as its the main refugium, due to the fewer sampling number and its high haplotype diversity, we consider Mengla of Yunnan province as Taojiang, Taoyuan and Mengla all have a high degree of Taojiang were more primitive. Although Changning, Guangning, (Table 2). This suggests the populations in Menglun, Mengla and in Guangning. However, it is hard to say whether different kinds of host plants have real influence on genetic differences, because the individuals from Guangning are not different from other individuals in either the NJ or MP tree. Furthermore, identification of genetic structure depends on the marker system [92]. Our AMOVA analysis did not reveal any noticeable differences of mtDNA, FST or Ne for the Guangning individuals when compared to other sites, while in the AFLPs, these same individuals have an outstanding percentage of polymorphic loci, observed number of alleles (Na), effective number of alleles (Nei’s (1973) gene diversity (H), Shannon’s Information index [Lewontin (1972)](l) and standard deviation of all index (St. Dev), the number of polymorphic loci in each population, and the percentage of polymorphic loci.

|                | n  | Na  | St.Dev | Ne  | St.Dev | H   | St.Dev | I   | St.Dev | loci | percentage |
|----------------|----|-----|--------|-----|--------|-----|--------|-----|--------|------|------------|
| Changning      | 10 | 1.366 | 0.488 | 1.129 | 0.230 | 0.087 | 0.136 | 0.144 | 0.207 | 139  | 38.61%     |
| Changsha       | 10 | 1.456 | 0.499 | 1.126 | 0.192 | 0.091 | 0.124 | 0.155 | 0.195 | 164  | 45.56%     |
| Guangde        | 9  | 1.264 | 0.441 | 1.113 | 0.245 | 0.072 | 0.140 | 0.113 | 0.209 | 95   | 26.39%     |
| Guangning      | 10 | 1.419 | 0.494 | 1.175 | 0.276 | 0.113 | 0.159 | 0.179 | 0.236 | 151  | 41.94%     |
| Guilin         | 10 | 1.544 | 0.499 | 1.137 | 0.197 | 0.100 | 0.121 | 0.174 | 0.189 | 196  | 54.44%     |
| Hengyang       | 4  | 1.143 | 0.360 | 1.070 | 0.195 | 0.047 | 0.117 | 0.074 | 0.179 | 55   | 15.28%     |
| Huorang        | 10 | 1.494 | 0.501 | 1.147 | 0.230 | 0.102 | 0.136 | 0.171 | 0.207 | 178  | 49.44%     |
| Jianou         | 10 | 1.103 | 0.304 | 1.040 | 0.152 | 0.026 | 0.089 | 0.041 | 0.135 | 37   | 10.28%     |
| Jingxiong      | 6  | 1.322 | 0.468 | 1.125 | 0.233 | 0.083 | 0.139 | 0.134 | 0.211 | 116  | 32.22%     |
| Jinyunshan     | 10 | 1.381 | 0.486 | 1.135 | 0.234 | 0.091 | 0.140 | 0.149 | 0.213 | 137  | 38.06%     |
| Mayanghe       | 2  | 1.078 | 0.268 | 1.055 | 0.190 | 0.032 | 0.111 | 0.047 | 0.162 | 28   | 7.78%      |
| Mengla         | 3  | 1.319 | 0.467 | 1.181 | 0.294 | 0.111 | 0.170 | 0.169 | 0.253 | 115  | 31.94%     |
| Menglu         | 2  | 1.128 | 0.334 | 1.090 | 0.236 | 0.053 | 0.139 | 0.077 | 0.202 | 46   | 12.78%     |
| Nanjing        | 2  | 1.031 | 0.172 | 1.022 | 0.122 | 0.013 | 0.071 | 0.019 | 0.104 | 11   | 3.06%      |
| Quanzhou       | 10 | 1.378 | 0.486 | 1.140 | 0.256 | 0.091 | 0.145 | 0.147 | 0.217 | 136  | 37.78%     |
| Quzhou         | 10 | 1.200 | 0.401 | 1.066 | 0.183 | 0.044 | 0.108 | 0.072 | 0.164 | 82   | 20.00%     |
| Rongan         | 10 | 1.389 | 0.488 | 1.127 | 0.228 | 0.086 | 0.135 | 0.143 | 0.205 | 140  | 38.89%     |
| Shicheng       | 10 | 1.239 | 0.427 | 1.081 | 0.191 | 0.055 | 0.116 | 0.091 | 0.179 | 86   | 23.89%     |
| Shuangpi       | 10 | 1.392 | 0.489 | 1.104 | 0.193 | 0.074 | 0.118 | 0.127 | 0.184 | 141  | 39.17%     |
| Shucheng       | 10 | 1.394 | 0.489 | 1.159 | 0.271 | 0.101 | 0.154 | 0.162 | 0.229 | 142  | 39.44%     |
| Taojiang       | 10 | 1.269 | 0.444 | 1.077 | 0.180 | 0.054 | 0.110 | 0.091 | 0.171 | 97   | 26.94%     |
| Taoyuan        | 10 | 1.297 | 0.458 | 1.099 | 0.204 | 0.068 | 0.124 | 0.111 | 0.192 | 107  | 29.72%     |
| Wuhan          | 10 | 1.350 | 0.478 | 1.079 | 0.144 | 0.061 | 0.099 | 0.108 | 0.163 | 126  | 35.00%     |
| Zijinshan      | 10 | 1.442 | 0.487 | 1.129 | 0.221 | 0.009 | 0.130 | 0.152 | 0.199 | 159  | 44.17%     |
| Ziyang         | 5  | 1.319 | 0.467 | 1.138 | 0.231 | 0.089 | 0.146 | 0.142 | 0.221 | 115  | 31.94%     |

doi:10.1371/journal.pone.0089873.t004

Host plants

Some parasite species may have host-based genetic structure, like Nomuraea rileyi [90], and some insects with a strong ability of migration also depend on their host plants [36]. This even holds true for some vertebrate animals like certain bird species [91]. As we mentioned above, there is a unique bamboo species, *B. textilis*, in Guangning. However, in the purpose of further clarifying the problem, high density sample data aimed at Guangning and typical locations of other host plants is specially needed in the future.

Human activity

Some insecticide treatments may lead to niche-targeting selected and modified the genetic structure of injurious insect [93]. In our sampling sites, some individuals were from nature parks or landscapes, like the populations in Mengla county and Purple landscapes, like the populations in Mengla county and Purple

expansions. Because of a lack of geographical isolation, gene flow between the geographical populations may have been quite frequent. This also explains why there are so many haplotypes shared among different geographical populations and so many assemblages in one phylogeographic branch. As we assumed all *C. kiangsu* has a common ancestral population, Menglu population, Mengla population and Taojiang population have smallest degree of differentiation based on the population specific FST indices (Table 2). This suggests the populations in Menglu, Mengla and Taojiang were more primitive. Although Changning, Guangning, Taojiang, Taoyuan and Mengla all have a high degree of haplotype diversity, we consider Mengla of Yunnan province as the main refugium, due to the fewer sampling number and its high haplotype and nucleotide diversity (Table 1) as well as its phylogenetic placement in both the 16S MP tree (Fig. 3) and AFLPs NJ tree (Fig. 4) [89], supporting the refugial region Yunnan proposed by Chen et al. [2].
Mountain of Nanjing, and some were from an artificial forest, as the population in Wuhan. Thus, we propose that the C. kiangsu individuals from these places had experience a recent selective pressures resulting from human activities and conduct genetic variation in population.

Human project often generates habitat fragmentation, and this can influence the ecology and genetic diversities of organisms. Habitat fragmentation can especially influence mimicry in species in an endangered habitat, for example Heliconius butterflies in Brazil’s Atlantic Forest [94]. Highly fragmented distributions were considered to cause deep genetic structure and strong differentiation [95]. Some continuous human activities such as destruction of forest for reclamation, grazing, mine exploitation, and cutting of firewood, herbicide application and sometimes even certain types of afforestation play important roles in wild species population declines [95–99]. Even some short-term and localized programs considered to be uninjurious could cause negatively affect in insects that are not very good at migration, like Palingenia longicauda, and decrease genetic diversity [100]. Grasshopper control in agricultural practices reduces the population size and genetic diversity in populations [101]. The consequences of population size reductions include particularly low variability and marked genetic signatures [95]. Although many species have suffered population declines, increased population fragmentation, or even extinction in connection with these human impacts, others seem to have benefitted from human modification of their habitat,

Figure 4. Arbitrarily rooted neighbour-joining tree based on Nei’s genetic distances of AFLPs between 25 geographic populations.
doi:10.1371/journal.pone.0089873.g004
such as an insectivorous bat (*Tadarida brasiliensis mexicana*) [102]. In our study, the close genetic distance and low number of haplotypes among the Hengyang, Shuangpai, Quanzhou and Guilin populations, which exist despite the high elevation of Xuefeng Mountain, is easily linked to a series of inter-provincial highways flowing along the area, as well as some large-scale project like South-to-North Water Diversion, Three Gorges Dam, etc. These projects reduced some geographical barriers and provided potential new migration path to *C. kiangsu*. But at the same time, human trade activities and plant breeding activities also cause a high genetic exchange of agricultural pest [103], even cause invasive alien species [104]. In 1960s, central government launched a great project of bamboo transplanting, called South Bamboo Transfer to North, and the purpose of this project is transplanting well-grown bamboo species into north appropriate areas to afforest north cities. In consideration of the graph shown in Figure 5, human activity may be the most fundamental reason of current geographical population structure, which may be the most fundamental reason of the recent colonization and population expansion. And our future work will investigate the sources of the main host plant of *C. kiangsu* and the migration path of *C. kiangsu*.

**Conclusions**

Generally, geographic distance and geographical barriers probably play a considerable role in structuring genetic variation within species, although some migratory species may have less phylogeographic structure on smaller spatial scales [111]. In this study, 393 individuals collected from South and Southeast China were markered with mitochondrial DNA (16S rRNA), and 203 individuals were examined using AFLPs. Our results indicate that there is a high level of genetic diversity and a low genetic distance among the *C. kiangsu* populations. Although the broad geographic range in this study covers a large part of the habitat of *C. kiangsu*, our study did not reveals a clear geography-related population structure in *C. kiangsu*.

According to our analyses, *C. kiangsu* should be considered a monophyletic group. All our results supported the hypothesis that *C. kiangsu* populations are in a primary divergent stage. There are several potential reasons for this result. The most likely factor is that there is little geographical isolation along most parts of our sampling range. Additionally, human activity, host plant selection and sexuality relative factors also potentially affect the species’ phylogeographic structure. More research is required to determine the strength of these three factors.

The Tajima’s D and Fu’s Fs values identified in the 16S rRNA, which were unexpectedly below zero, reveal that there was a population expansion during the species history of *C. kiangsu*, and our mismatch analysis indicated that the population explosion likely occurred about 0.242 Ma. The Quaternary glaciation events may have been responsible for this population explosion. According to the phylogenetic trees and haplotype distribution, Mengla of Yunnan province, Changhai of Sichuan province and Guangning of Guangdong province are possible refugia of *C. kiangsu*. Furthermore, those populations colonizationed in Mengla may have been separated from the other two refugia after the Quaternary glaciation. Because of the subsequent lack of physical barriers in East and South China [19], the main group was then free to migrate to most parts of the distribution range of *C. kiangsu*. And in our study, we first discovered human activity may have a significant impact on *C. kiangsu* population structure, which may be the most fundamental reason of current geographical population pattern. Our future work may focus on finding ancestor of different geographical populations.

**Supporting Information**

**Figure S1**: NJ phylogenetic tree based on 16S rRNA gene sequences of 25 geographic populations of *C. kiangsu*. (TIF)

**Table S1**: Computing conventional F-Statistics from haplotype frequencies, population pairwise FST values (below diagonal) and p values (above diagonal) based on mtDNA data. (DOCX)
Table S2 Population pairwise $F_{ST}$ values (below diagonal) and $p$ value (above diagonal) based on AFLP data calculated from Arlequin software.

(DoCX)

Table S3 Nei’s genetic distance after Lynch & Milligan method calculated from AFLPsurv software.

(DoCX)

Acknowledgments

Thanks to the following people for their invaluable help in locust collection: Yue-ming Cheng and Xiu-jin Chen (Forestry Bureau of Guangning County of Guangdong Province), Li-xia Dai (Forestry Department of Hunan Province), Li-jun Dong (Forestry Bureau of Taoyuan County of Hunan Province), Zhao-hui Tang and Ye-cheng Wang (Forestry Bureau of Hengyang City of Hunan Province), Dai-lin Wang, Shu-lin Guo, Jian-wen Liu (Vegetational Protection Central Station of Guangxi Zhuang Autonomous Region), Jian-ming Luo (Forestry Bureau of Rongshui County of Guangxi Zhuang Autonomous Region), Ru-ming Liu (Forestry Bureau of Quzhou County of Zhejiang Province), Dan-yang Shi (Forestry Bureau of Jianyou County of Fujian Province), Gan-jian Qi (Forestry Bureau of Guigang City of Jiangxi Province), Heng-qing Xie (Forestry Bureau of Shichang County of Jiangxi Province), and Wei-ning Liu (Forestry Bureau of Pingxiang City of Jiangxi Province). Thanks to L. Lacey Knowles and members of the Knowles’ laboratory for their suggestions, especially Carlos Munoz Ramirez for his help in the cartography and, Huotong Huang for her detail suggestions. We also thank Sir Zhi-xiang Pan to help make a graphic.

Author Contributions

Conceived and designed the experiments: GFJ. Performed the experiments: ZF YXL. Analyzed the data: ZF QXH. Contributed reagents/materials/analysis tools: GEJ QXH. Wrote the manuscript: ZF GFJ. Performed the molecular analyses: ZF YXL.

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