Study on the Genetic Differentiation of Geographic Populations of *Calliptamus italicus* (Orthoptera: Acrididae) in Sino-Kazakh Border Areas Based on Mitochondrial *COI* and *COII* Genes

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Received 7 January 2019; Editorial decision 8 April 2019

Abstract

*Calliptamus italicus* L., an important pest on the desert and semidesert steppes along the Sino-Kazakh border. To elucidate the molecular mechanism of its continuous outbreaks, we studied 11 different geographic populations of *C. italicus* to determine: 1) the complete sequences of the entire mitochondrial cytochrome oxidase subunit I (*COI*) and mitochondrial cytochrome oxidase subunit II (*COII*) genes, and 2) performed genetic diversity, differentiation, gene flow, and molecular variation analyses. Of the 11 populations, the Yining County (YNX) population had the highest haplotype diversity and *F* values. There are significant differences in Tajima’s *D* and Fu’s *F* values (*P* < 0.05). The fixation index *F* values of the total *C. italicus* population were 0.03352, and its gene flow *Nm* values of the total *C. italicus* population were 15.32. Taken together, there were five main findings: 1) the current genetic differentiation of *C. italicus* arose within populations; 2) genetic exchange levels were high between geographical populations; 3) genetic variation level was low; 4) *C. italicus* populations likely expanded in recently, and 5) there was no significant correlation between genetic distance and geographic distance for any geographic population. Findings from this study indicate that frequent gene exchange between populations may enhance the adaptability of *C. italicus* along the Sino-Kazakh border, leading to frequent outbreaks.

Key words: *Calliptamus italicus* L., *mt COI*, *mt COII*, Sino-Kazakh border areas, the Central Asia region
such, they are highly suitable for studying intraspecies genetic differences and have been used widely to study geographic populations of insects (Salvato et al. 2002, Meng et al. 2008, Seabir et al. 2015, Sekiné et al. 2017, Wu and Yan 2018). Studying genetic variation between pest populations cannot only provide information about the population structure of the species in different geographical regions, but also infer the demographic history of pests (Assessa et al. 2006, 2015, 2017). Our main objectives were to 1) analyze the genetic structure and phylogeography of C. italicus populations along steppes of the Sino-Kazakh border, 2) examine the geographical pattern of C. italicus genetic diversity and haplotypes, and 3) infer the demographic history of this species. Understanding the variation in COI and COII gene offers a potential scientific basis to monitor and control this pest species.

Materials and Methods

Insect Specimen Collection

In total, 220 individuals of C. italicus adults from 11 different geographic populations were collected in Sino-Kazakh border areas during a serious outbreak period in June to August 2017 and 2018 (Table 1). The collection sites covered longitude: 79°17′~93°38′, latitude: 43°20′~48°10′, altitudes 470~2,030 m, and desert and semidesert steppe, subalpine steppe habitat types of the Sino-Kazakh border areas (Fig. 1). The 11 collection sites were separated by a minimum distance of 47 km and a maximum distance of 1,160 km. Samples were collected randomly, immediately frozen with liquid nitrogen, and stored at -80°C. Calliptamus italicus collected in Kazakhstan was stored in a 1.5-ml centrifugal tube containing anhydrous ethanol for a short period before freezing.

DNA Extraction and Amplification

Hind femoral muscles were collected from each individual and ground into powder with liquid nitrogen. Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China) was used to extract whole-genome DNA, following the manufacturer's protocol. The concentration and purity of the extracted DNA was determined via agarose gel electrophoresis and UV/VIS spectrophotometer (NanoDropND-2000). Primers designed by using primer 5.0 software (http://www.premierbiosoft.com). Primers for mitochondrial COI (F: 5′-CTAGAATTGCAGTCCTAATCTTCA-3′ and COII (F: 5′-CATAATTGAGATGCTAATGCATG-3′) and COII (F: 5′-CTAGAATTGCAGTCCTAATCTTCA-3′) were used for this study. Polymerase chain reactions (PCR) were performed using a gradient thermal cycle (MastercyclerProS, Germany) at a total volume of 25 μl, containing 12.5 μl 2xTaq PCR MasterMix (TIANGEN, Beijing, China), 0.25 μM of both F and R primers, and 1.5 μl genomic DNA (10~30 ng/μl). PCR amplification was conducted at 94°C for 3 min, followed by 30 amplification cycles of 94°C for 30 s, primer-specific annealing temperature of 60°C (COI) or 46.9°C (COII) for 30 s, 72°C for 30 s, and then a final step at 72°C for 5 min. Amplified products were purified and sequenced by Chengdu TsingkeZixi Biological Technology Co., Ltd.

Statistical Analysis

COI (1540 bp) and COII (684 bp) sequences of C. italicus were obtained. Chromas software (Staden 1996) was used to read sequencing results and observe peak values, and the DNAstar software package was used for sequence editing and correction. After confirming that the sequences belonged to C. italicus using BLAST on the NCBI database, DNAMAN software was used to conduct multiple sequence alignment. Multiple sequences of COI and COII were concatenated to yield a total length of 2224 bp. The haplotype network of C. italicus was analyzed using a median-joining algorithm in PopART (Leigh and Bryant 2015). DnaSP 5.0 (Rozas et al. 2003) was used to analyze number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (Pi), number of polymorphic sites (S), fixation index (Fst), gene flow (Nm), Tajima's D (D) (Tajima 1989), and Fu's F statistics (Fs) (Fu 1997) in each of the populations. We assessed significance with 1,000 permutations. To examine demographic history, the distribution of pairwise differences between individual sequences was analyzed by mismatch distribution analysis using DnaSP 5.0 (Rozas et al. 2003). Analysis of molecular variance (AMOVA) of the genetic sequences was performed using Arlequin 3.5 (Schneider et al. 2000). The pairwise genetic distances were calculated by MEGA6.0 (Tamura et al. 2013) using the Kimura-2-parameter model (Kimura 1980). We also calculated the geographic distances among various collection sites based on longitude and latitude, and tested the correlation between genetic distance and geographic distance (Mantel 1967) for different populations using TFGPA (Miller 1997) with 9,999 randomizations.

Results

Analysis of Haplotype, Nucleotide Diversity, Neutrality Test, and Mismatch Distribution of COI and COII Genes

The haplotype diversity of the concatenated sequences ranged from 0.889 to 0.989 with an average of 0.948, whereas the nucleotide diversity ranged from 0.00087 to 0.00184 with an average of 0.00141 (Table 2). The YNX population had the highest haplotype diversity.

Table 1. Specimen date of difference geographic populations of C. italicus in Sino-Kazakh border areas

| Population code | Number of specimens | Collecting locality | Longitude(E) | Latitude(N) | Elevation(m) |
|-----------------|---------------------|---------------------|--------------|-------------|--------------|
| TC              | 20                  | Tacheng, Xinjiang   | 83°60′       | 46°35′      | 470          |
| HNH             | 20                  | Habuhe, Xinjiang    | 86°31′       | 48°10′      | 680          |
| JMN             | 20                  | Jimunai, Xinjiang   | 85°44′       | 47°25′      | 1070         |
| YNS             | 20                  | Yiningshi, Xinjiang | 81°16′       | 44°40′      | 1030         |
| YNX             | 20                  | Yiningxian, Xinjiang| 81°33′       | 44°00′      | 1020         |
| BL              | 20                  | Bole, Xinjiang     | 81°58′       | 45°60′      | 1010         |
| MNS             | 20                  | Manasi, Xinjiang   | 86°15′       | 43°93′      | 1292         |
| YM              | 20                  | Yumin, Xinjiang    | 82°50′       | 45°38′      | 1850         |
| NS              | 20                  | Nanshan, Xinjiang  | 87°39′       | 43°20′      | 1950         |
| BLK             | 20                  | Balkun, Xinjiang   | 93°38′       | 43°22′      | 2030         |
| KZ              | 20                  | Altyne-Emel, Kazakhstan | 80°20′     | 43°47′      | 500          |
Hd and Pi values, whereas the MNS population had the lowest. The haplotype number of different geographic populations ranged between 13 and 19 with an average of 15.3. Among the examined populations, the TC population had the largest haplotype number, with 19 haplotypes found in 20 tested individuals.

Tajima’s D values of the concatenated sequence resulted in significantly negative values of -2.7221, but were significant in most specific populations (P > 0.05 in BLK and P < 0.05 in the rest of the populations). Fu’s F statistic was significantly negative with a value of -25.9968, but was significant in all populations (P < 0.001 in all the populations; Table 2). NS and BLK populations had negative and significant Tajima’s D and Fu’s Fs values. A unimodal distribution indicates that populations have passed through a recent demographic expansion (Excoffier 2004), whereas multimodal distributions are consistent with stability (Slatkin and Hudson 1991). Distributions of pairwise differences (mismatch distributions) obtained from the overall populations were unimodal, and the distributions fitted the shape expected after a sudden demographic expansion (Fig. 2). It is suggesting that the populations of C. italicus in Sino-Kazakh border areas experienced population expansion. The significant neutral test results that were obtained from Tajima’s D and Fu’s Fs analysis further support this interpretation.

**Haplotypes Analysis of Different Geographical Populations**

All populations displayed large numbers of mitochondrial haplotypes, with a total 92 haplotypes obtained for COI, 49 haplotypes for COII, of which 16 and 13 were common haplotypes, respectively. Of

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**Table 2. Parameters of genetic diversity and the neutral test based on mitochondrial sequence data of 11 populations of C. italicus**

| Population code | S     | Hd     | Pi     | H    | D       | Fs      |
|-----------------|-------|--------|--------|------|---------|---------|
| TC              | 32    | 0.985  | 0.00160| 19   | -2.3877 | -23.5307*** |
| HBH             | 18    | 0.958  | 0.00113| 15   | -1.8928 | -26.5190*** |
| JMN             | 21    | 0.916  | 0.00117| 14   | -2.1366 | -26.3732*** |
| YNS             | 24    | 0.963  | 0.00147| 16   | -1.9972 | -24.5072*** |
| YNX             | 36    | 0.989  | 0.00184| 18   | -2.3634 | -21.7845*** |
| BL              | 19    | 0.911  | 0.00116| 13   | -1.9644 | -26.4280*** |
| MNS             | 15    | 0.889  | 0.00087| 14   | -1.9998 | -27.2229*** |
| YM              | 29    | 0.984  | 0.00156| 18   | -2.2490 | -23.7821*** |
| NS              | 20    | 0.963  | 0.00141| 13   | -1.6819 | -24.9186*** |
| BLK             | 21    | 0.932  | 0.00162| 13   | -1.4987 | -23.3707*** |
| KZ              | 32    | 0.942  | 0.00172| 15   | -2.2706 | -22.6444*** |
| Total           | 152   | 0.965  | 0.00146| 128  | -2.7221 | -25.9968*** |

This table includes population code, number of polymorphic sites (S), Haplotype diversity (Hd), Nucleotide diversity (Pi), number of haplotypes (H), Tajima’s D (D), and Fu’s Fs (Fs).

*** P < 0.001, according to significance tests with 1,000 permutations.

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Fig. 1. Distribution map of 11 C. italicus populations collected across the major distributing regions in Sino-Kazakh border areas. 1) Tacheng (TC) 83°60’ E, 46°35’ N; 2) Habohe (HBH) 86°31’ E, 48°10’ N; 3) Jimunai (JMN) 85°44’ E, 47°25’ N; 4) Yiningxian (YNX) 81°33’ E, 45°38’ N; 5) Yining (YM) 82°50’ E, 45°38’ N; 6) Bole (BL) 81°58’ E, 45°60’ N; 7) Manasi (MNS) 86°15’ E, 43°93’ N; 8) Yumin (YM) 82°50’ E, 45°38’ N; 9) Nanshan (NS) 87°39’ E, 43°20’ N; 10) Balikun (BLK) 93°38’ E, 43°22’ N; 11) Kazakhstan Altyn-Emel (KZ) 80°20’ E, 43°47’ N.
the 128 examined haplotypes found in the concatenated sequences, 109 were unique. The COI and COII median joining network displayed a genealogy with one main haplotype separated by one mutational step (Figs. 3 and 4). The haplotype network suggested that the most common haplotype (H1) might be the ancestral haplotype, as this haplotype had an internal position in the network, had many lineages that arose from it, and appeared at high frequencies.

Analysis on the Genetic Diversity, Gene Flow, Genetic Differentiation, and Genetic Variation of Different Geographic Populations

The values of pairwise Fst ranged from −0.00904 to 0.12507 with an average of 0.03179. Of the 55 comparisons, 10 showed moderate genetic differentiation. Referring to the criterion for genetic differentiation by Wright (Wright 1978), we defined genetic differentiation as low for Fst < 0.05, moderate for 0.05 < Fst < 0.15, high for 0.15 < Fst < 0.25, and very high for Fst > 0.25 (Govindajuru 1989). The pairwise Fst values of all the populations, except in BLK, were less than 0.05, which indicated low genetic differentiation (Table 3). The levels of gene flow was categorized as Nm < 0.25, intermediate gene flow, and Nm > 0.25 (Goveindajuru 1989). Generally, gene exchange leading to low genetic differentiation between populations occurs when Nm > 4. The total gene flow Nm was 15.32, indicating that there was sufficient gene flow between populations. The values of pairwise genetic distance range from 0.001 to 0.002, further supporting that there was low genetic differentiation between populations.

The AMOVA test showed that 96.65% of the genetic variation was within population, whereas 3.35% was among population. Exact tests showed a significant genetic variance on all two levels (P < 0.001; Table 4), and 96.63% of the genetic variations of C. italicus were explained by intralocality variation. The remaining 3.35% was explained by variation among localities.

The Mantel test for 11 populations revealed no correlation between genetic distances and geographic distances (r = 0.2799, P = 0.927 > 0.05), suggesting that isolation by distance did not limit gene flow.

Discussion

The adaptability of a species to environmental change depends largely on genetic diversity and genetic structure of its populations (Zu et al. 1999). Results from this study indicate that the genetic differentiation level of C. italicus was low both between and within the 11 geographic populations that were sampled. This suggests that the frequent gene flow between populations likely increased in adaptability of this species to environmental changes. The low genetic differentiation of C. italicus may also underlie how this species progressed from a companion species into a dominant harmful species in some regions of Sino-Kazakh border areas; on the other hand, it constituted an intrinsic genetic factor of the continuous serious outbreaks of C. italicus in Sino-Kazakh border areas, and further verified the hypothesis proposed in this paper. What is more, the lower level of genetic differentiation and the more intensive gene flow can be observed among outbreaking populations from outbreaking areas. Gene flow is substantially larger among outbreaking populations than among nonoutbreaking populations. The more intensive gene flow among populations of outbreaking areas may be the result of demographic or behavioral factors (Chapuis et al. 2009). In this study, the C. italicus population has an intensive gene flow, which may also be the result of demographic or behavioral factors.

The median-joining network demonstrated that C. italicus populations along the Sino-Kazakh border had high genetic diversity. In the median-joining network, the most common haplotype (H1) had strong support as the ancestral haplotype due to its representation in a significant proportion of individuals in all populations and its central location in the network (Posada and Crandall 2001). The high values of haplotype diversity suggest the existence of small populations that have suffered recent population growth. This situation is observed for YNX population, whereas haplotype diversity is relatively high. Neutrality tests, conducted through the Tajima’s D and Fu’s Fs indices, support the hypotheses of a recent expansion from a relatively small population. Distributions of pairwise differences obtained using concatenated COI and COII sequences from the overall populations were unimodal, further supporting that the population of C. italicus experienced population expansion. However, the unique haplotypes that exist independently in different geographical populations indicate that there is a certain degree of genetic differentiation, as well as gene flow in different geographical populations. The haplotype network did not reveal any obvious geographical structure in the different clades and most haplotypes had a relatively mixed distribution pattern.

Calliptamus italicus showed rich intrapopulation haplotype diversity, suggesting that it has strong adaptability to different
environments. However, different geographic populations had different haplotype and nucleotide diversities. This can be attributed to the wide distribution range and different eco-environmental conditions of *C. italicus*. Among the 11 geographic populations of *C. italicus*, the YNX population had the highest genetic diversity level. Environmental homogeneity leads to low genetic diversity levels, whereas environmental heterogeneity, in terms of geographic environment, climate, vegetation, and so forth, results in a high genetic diversity level (Sun et al. 2011). YNX is located in the Ili River Valley and is characterized by its diverse and rich vegetation (Yan et al. 2017). As such, there is high spatial heterogeneity and high genetic diversity level. The study results also coincide with the actual outbreaks of *C. italicus*. Each year, the mountain front steppes of YNX experience serious outbreaks of *C. italicus*.

Gene flow cannot only reveal the possible genetic infiltration and genetic differentiation among populations, but also weaken the genetic differences among populations (Miallar and Libby 1991, Boivin et al. 2004). The total values of *N*∞ were more than 4, indicating a high level of gene flow and a low or medium genetic differentiation among some populations of *C. italicus*. The level of gene exchange among populations may be determined by the flight capacity. *Calliptamus italicus* is generally regarded as a migratory species as it has high flight capacity, with a sphere of activity extending over 200–300 km (Huang and Zhu 2001). The strong flight capacity of *C. italicus* can increase gene flow among populations. Similar trends have been observed in many migratory species, such as *Oedaleus asiaticus* (Bienko) (Gao et al. 2011) and *Locusta migratoria manilensis* (Meyen) (Cheng 2005). The same result was reported by Liu et al. (2018).

The drainage basin of Lake Balkhash in South-East Kazakhstan is one of the largest breeding areas of locusts in the region of Central Asia. The reed grass-covered area of the River Ili delta represents the perfect habitat for locust oviposition and development. Since *C. italicus* can aggregate, outbreaks and even plagues are frequently observed. These occur at more or less regular intervals, depending on favorable climate conditions (Stolyarov 2000). During the 20th century, the number of ascents and outbreaks of *C. italicus* in Kazakhstan occurred 9 times (1909–1912; 1924–1927; 1931–1933; 1944–1947; 1953–1956; 1967–1970; 1977–1982; 1988–1991; 1997–2003). In such years, the locusts cover great distances without borders. Cross-border flights occur mainly between the West, North, and East Kazakhstan and neighboring regions of the Russian Federation, between South Kazakhstan and Kyrgyzstan, between East Kazakhstan and China (Azhbenov et al. 2015). In recent years, there have been no reports of large-scale cross-border migration of *C. italicus*, suggesting that the population of *C. italicus* collected in

![Median-joining network based on the single genes of COI haplotypes.](image-url)
Table 3. Pairwise $F$s (below diagonal) and genetic distance (above diagonal) based on mitochondrial sequence data of 11 populations of *C. italicus*

|        | TC     | HBH    | JMN    | YNS     | YNX     | BL     | MNS     | YM     | NS     | BLK   | KZ    |
|--------|--------|--------|--------|---------|---------|--------|---------|--------|--------|-------|-------|
| TC     | 0.001  | 0.001  | 0.002  | 0.002   | 0.001   | 0.001  | 0.001   | 0.002  | 0.002  | 0.002 | 0.002 |
| HBH    | 0.01166| 0.001  | 0.001  | 0.001   | 0.001   | 0.001  | 0.001   | 0.002  | 0.001  | 0.002 | 0.002 |
| JMN    | -0.00369| 0.00072| 0.001  | 0.001   | 0.001   | 0.001  | 0.001   | 0.002  | 0.001  | 0.002 | 0.002 |
| YNS    | 0.01795| 0.04393| 0.03321| 0.002   | 0.001   | 0.001  | 0.001   | 0.002  | 0.002  | 0.002 | 0.002 |
| YNX    | -0.01354| 0.00064| -0.00904| 0.02091| 0.001   | 0.001  | 0.001   | 0.002  | 0.002  | 0.002 | 0.002 |
| BL     | 0.00146| 0.02024| 0.00976| 0.03920| -0.00884| 0.001  | 0.001   | 0.002  | 0.002  | 0.002 | 0.002 |
| MNS    | -0.00628| 0.02878| 0.00610| 0.02985| 0.00304| 0.01301| 0.001   | 0.001  | 0.002  | 0.001 | 0.002 |
| YM     | 0.00622| 0.03154| 0.01794| 0.02787| 0.00612| 0.02854| 0.02863| 0.002  | 0.002  | 0.002 | 0.002 |
| NS     | 0.01335| 0.04981| 0.03568| 0.04399| 0.01673| 0.03775| 0.02140| 0.02994| 0.002  | 0.002 | 0.002 |
| BLK    | 0.08785| 0.12507| 0.11302| 0.10588| 0.08892| 0.09221| 0.11068| 0.09726| 0.10414| 0.002 | 0.002 |
| KZ     | -0.00179| 0.01906| 0.01518| 0.01750| -0.00515| 0.01397| 0.01496| 0.01160| 0.01863| 0.08512|        |

The genetic distances (above diagonal) were used the Kimura-2-parameter model.

Table 4. Analysis of molecular variance (AMOVA) of populations of *C. italicus*

| Source of variation | d.f. | Sum of squares | Variation components | Percentage of variation | $P$   |
|---------------------|------|----------------|----------------------|------------------------|-------|
| Among populations   | 10   | 26.614         |          0.05430Va    | 3.35                  | <0.001|
| Within populations  | 209  | 328.400        | 1.75130Vb            | 96.65                 | <0.001|
| Total variance      | 219  | 355.014        | 1.62580              |                       |       |
Xinjiang was not from Kazakhstan. Calliptamus italicus is widely distributed on the desert and semidesert steppes of Central Asia and peripheral regions (Huang and Cheng 1999). In recent years, serious outbreaks of C. italicus have occurred in western Russia (Azhbenov et al. 2015) and southeastern Kazakhstan, and the border regions adjacent to Xinjiang of China have seen frequent migrations of C. italicus (Baybussenov et al. 2014, 2015). In this study, the collection sites of C. italicus have covered the Sino-Kazakh border areas; however, in-depth studies are still needed. More insects from over a broader geographical range and use of molecular markers suitable for faster evolutionary time scales will further elucidate the relationships between the populations of C. italicus in Xinjiang of China and those in Kazakhstan and Russia, and whether genetic differences underlie the occurrence of outbreaks.

Acknowledgments

This study was supported by the International S&T Cooperation Project of China (ISTCP) (Grant 2016YFE0203100, 2015DFR30290) and the University Innovation Team Project of Xinjiang Uygur Autonomous Region (Grant No. XJEDU2017T007).

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