Journal of Economic Entomology, 112(4), 2019, 1912–1919
doi: 10.1093/jee/toz112
Advance Access Publication Date: 14 May 2019
Research

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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Subject Editor: Brian Rector

Received 7 January 2019; Editorial decision 8 April 2019

Abstract

Calliptamus italicus L. is 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 *Pi* values. There are significant differences in Tajima's *D* and Fu's *Fs* ($P < 0.05$). The fixation index *Fst* 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

Calliptamus italicus L., commonly known as the Italian locust, belongs to *Calliptamus* Servill and is widely distributed in Eurasia and North Africa (Sergeev 1992, Stolyarov 2000, Sergeev and Van'kova 2008, Darvishzadeh and Bandani 2012). In China, it is mainly distributed on the desert and semidesert steppes of Qinghai and Northern Xinjiang (Chen 2000). *Calliptamus italicus* has high fecundity and is a polyphagous species with strong dispersal capacity. In recent years, there have been continuous *C. italicus* outbreaks along the Sino-Kazakh border, and *C. italicus* has become a dominant species in some regions. Previous work has shown that serious outbreaks of *C. italicus* are related to regional climate conditions (Li et al. 2017). In pests, the propensity to outbreak may itself play a major role in intensifying gene flow and homogenizing genetic variation (Chapuis et al. 2008, 2009). Given the frequent gene exchange among different populations, it is speculated that enhanced adaptability also leads to continuous outbreaks. We also speculated that the frequent outbreaks of *C. italicus* are related to their genetic diversity and population demography, and thus high population admixture (Chapuis et al. 2011). To verify the above assumption, we used mitochondrial DNA sequences to investigate the genetic structures and gene flow between 11 geographic populations of *C. italicus* in regions that experience frequent outbreaks. The study was designed to quantify genetic diversity and degrees of genetic differentiation of different geographic populations, which may provide a scientific basis for forecasting future outbreaks.

In this study, we selected two mitochondrial genes, cytochrome oxidase subunit I (*COI*) and cytochrome oxidase subunit II (*COII*), to examine between-population differences. Like all mitochondrial DNA, these genes have high copy numbers and, crucially, rapid evolutionary rates (Gyllensten et al. 1991, Rollins et al. 2011). As

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, Seabra 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 (Assefa 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′-CTAGAATTGCAGTC TAGAATCAT-3′, R: 5′-AGTGGTGAAGCTCCATCTTGTAATG-3′) and *COII* (F: 5′-TCTAGATTCTAATATGGCAG-3′, R: 5′-CAATA CTTACTTTCAGTCATC-3′) were used for this study. Polymerase chain reactions (PCR) were performed using a gradient thermal cycler (MastercyclerProS, Germany) at a total volume of 25 µl, containing 12.5 µl 2×Taq 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 80 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 (N_m), 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 TFPGA (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
HBH	20	Habahe, 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′	1930
BLK	20	Balikun, Xinjiang	93°38′	43°22′	2030
KZ	20	Altyn-Emel, Kazakhstan	80°20′	43°47′	500

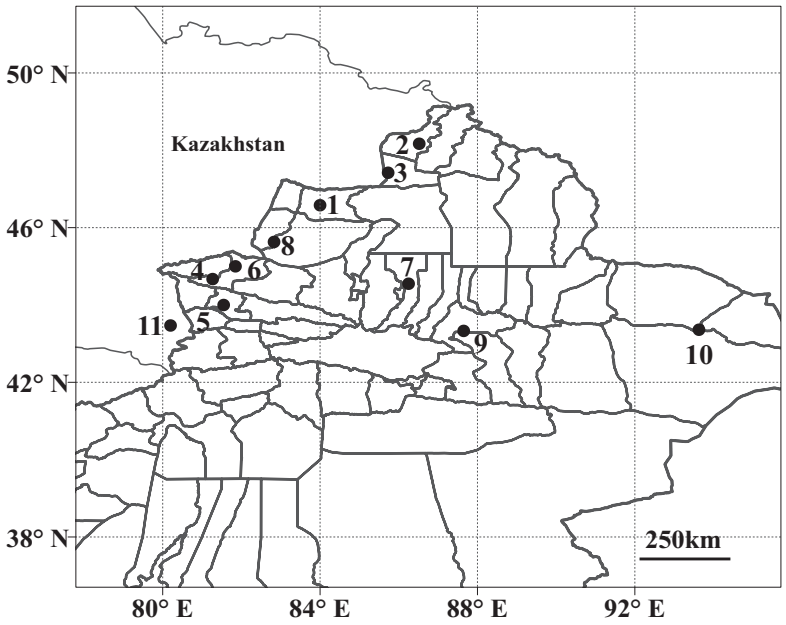

Fig. 1. Distribution map of 11 *C. italicus* populations collected across the major distributing regions in Sino-Kazakh border areas. 1 Tacheng (TC) 83°60′E, 46°35′N; 2 Habahe (HBH) 86°31′E, 48°10′N; 3 Jimunai (JMN) 85°44′E, 47°25′N; 4 Yiningshi (YNS) 81°16′E, 44°40′N; 5 Yiningxian (YNX) 81°33′E, 44°00′N; 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.

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

	Combined gene					
Population code	S	*Hd*	*Pi*	H	*D*	*Fs*
TC	32	0.985	0.00160	19	−2.3877 (*P* = 0.0000)	−23.5307***
HBH	18	0.958	0.00113	15	−1.8928 (*P* = 0.0170)	−26.5190***
JMN	21	0.916	0.00117	14	−2.1366 (*P* = 0.0060)	−26.3732***
YNS	24	0.963	0.00147	16	−1.9972 (*P* = 0.0130)	−24.5072***
YNX	36	0.989	0.00184	18	−2.3634 (*P* = 0.0020)	−21.7845***
BL	19	0.911	0.00116	13	−1.9644 (*P* = 0.0130)	−26.4280***
MNS	15	0.889	0.00087	14	−1.9998 (*P* = 0.0080)	−27.2229***
YM	29	0.984	0.00156	18	−2.2490 (*P* = 0.0020)	−23.7821***
NS	20	0.963	0.00141	13	−1.6819 (*P* = 0.0250)	−24.9186***
BLK	21	0.932	0.00162	13	−1.4987 (*P* = 0.0550)	−23.3707***
KZ	32	0.942	0.00172	15	−2.2706 (*P* = 0.0020)	−22.6444***
Total	152	0.965	0.00146	128	−2.7221 (*P* = 0.0000)	−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.

(*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 the all of 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

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 $N_m > 1$ (high gene flow), 0.25 to 0.99 (intermediate gene flow), and $N_m < 0.25$ (low gene flow; Govindajuru 1989, Low et al. 2014). Generally, gene exchange leading to low genetic differentiation between populations occurs when $N_m > 4$. The total gene flow N_m 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 96.65% 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 assumption 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). It may be a stable haplotype with high environmental adaptability. 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

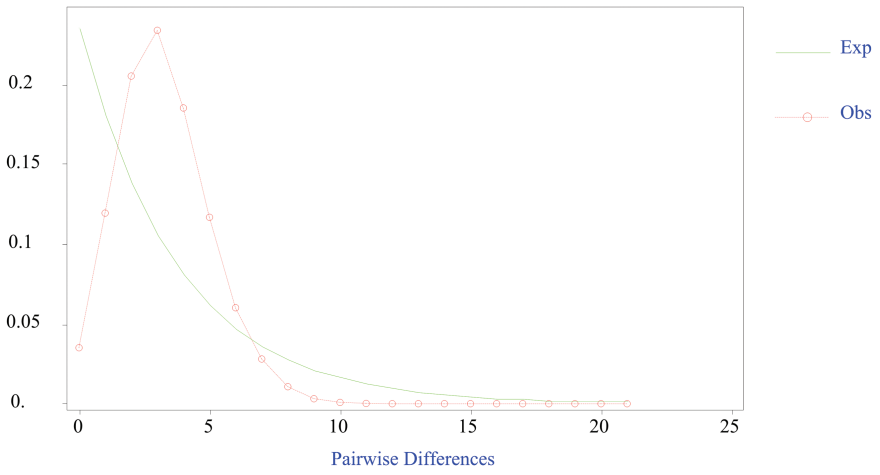

Fig. 2. Observed and expected mismatch distributions of *C. italicus* based on the combination of the *COI* and *COII* gene sequences.

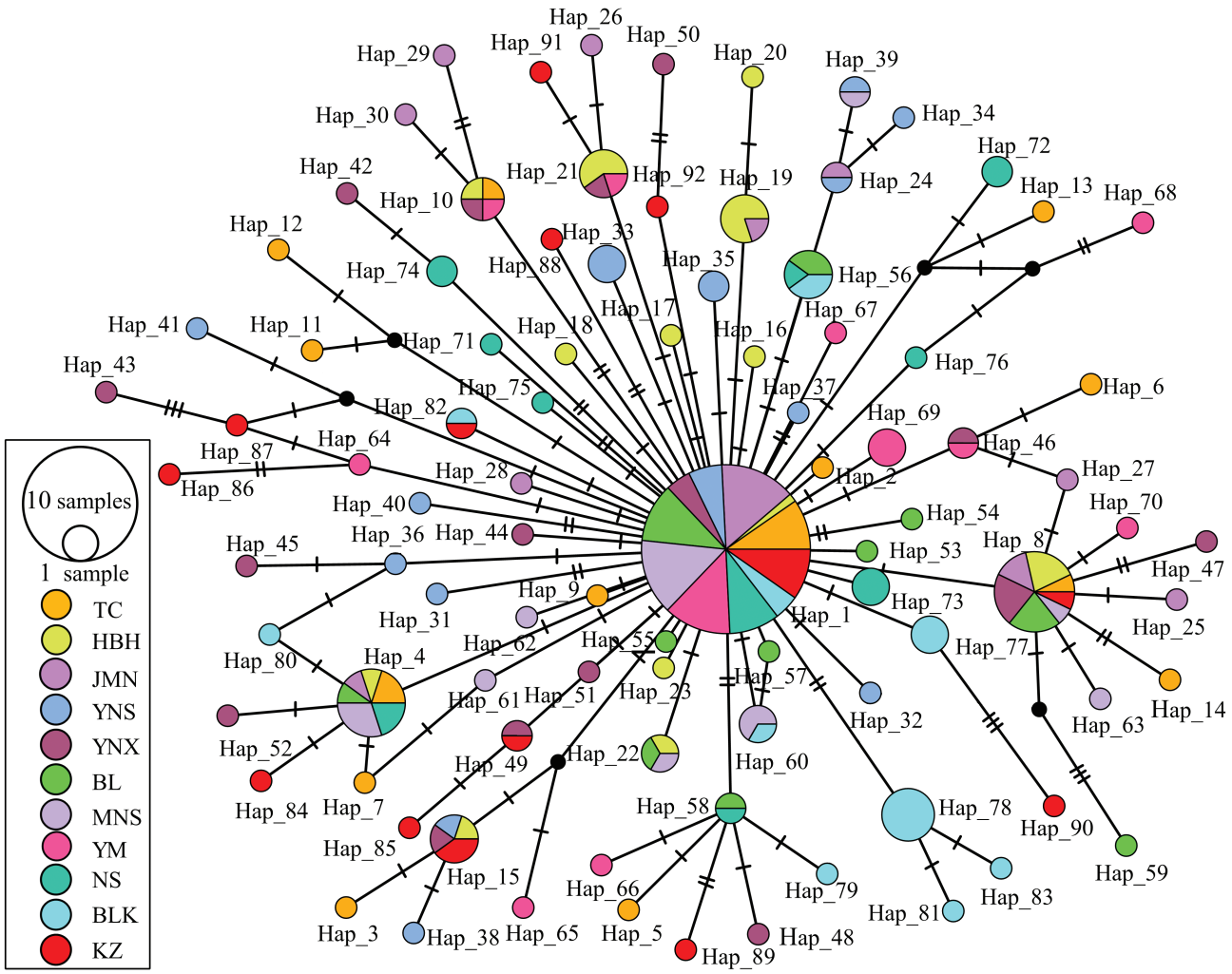

Fig. 3. Median-joining network based on the single genes of *COI* haplotypes. Each circle represents a haplotype, and the area of a circle is proportional to the number of observed individual. Colors within the nodes refer to the *C. italicus* sampling regions.

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 (Millar and Libby 1991, Boivin et al. 2004). The total values of N_m 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

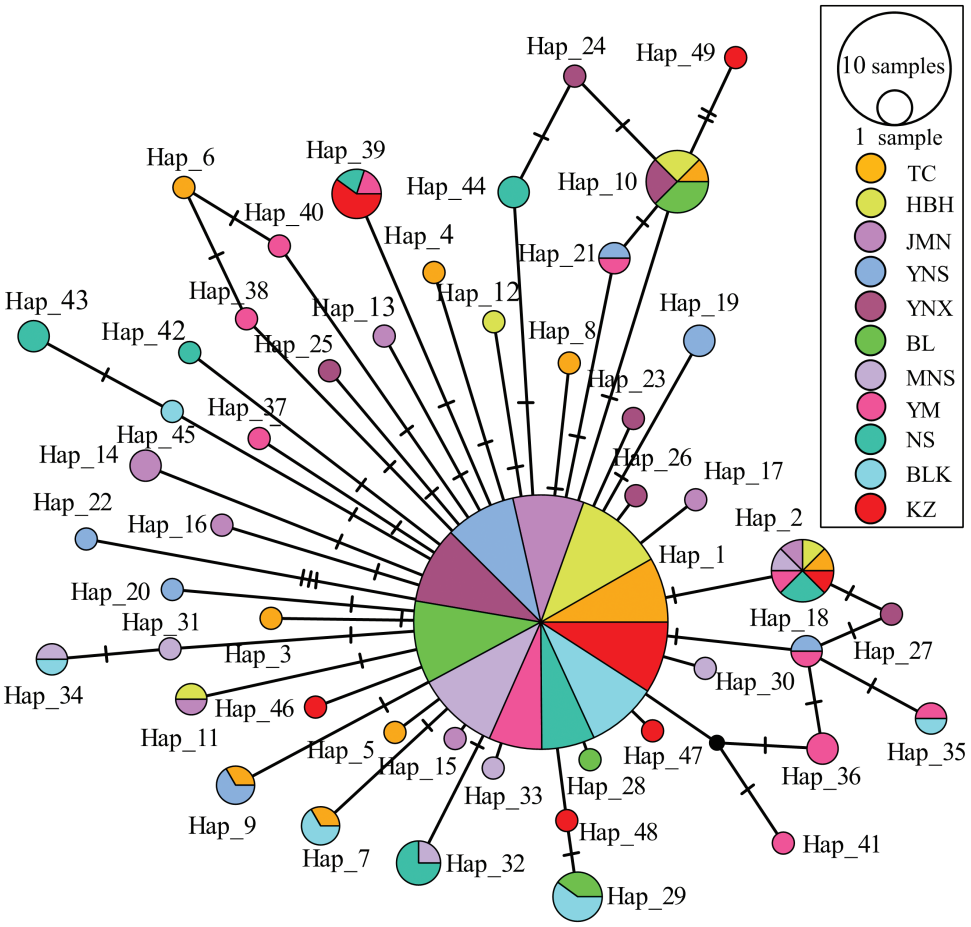

Fig. 4. Median-joining network based on the single genes of *COII* haplotypes. Each circle represents a haplotype, and the area of a circle is proportional to the number of observed individual. Colors within the nodes refer to the *C. italicus* sampling regions.

Table 3. Pairwise *Fst* (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	0.002	0.002	0.002	0.002
HBH	0.01166		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
JMN	−0.00369	0.00072		0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
YNS	0.01795	0.04393	0.03321		0.002	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	0.002	0.002	0.002	0.002
BL	0.00146	0.02024	0.00976	0.03920	−0.00884		0.001	0.001	0.001	0.002	0.001
MNS	−0.00628	0.02878	0.00610	0.02985	0.00304	0.01301		0.001	0.001	0.001	0.001
YM	0.00622	0.03154	0.01794	0.02787	0.00612	0.02854	0.02863		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
BLK	0.08785	0.12507	0.11302	0.10588	0.08892	0.09221	0.11068	0.09726	0.10414		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	0.05450Va	3.35	<0.001
Within populations	209	328.400	1.57130Vb	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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