Genetic Variation and Geographic Differentiation Among Populations of the
Nonmigratory Agricultural Pest *Oedaleus infernalis* (Orthoptera: Acridoidea) in China

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**ABSTRACT.** The nonmigratory grasshopper *Oedaleus infernalis* Saussure (Orthoptera : Acridoidea) is an agricultural pest to crops and forage grasses over a wide natural geographical distribution in China. The genetic diversity and genetic variation among 10 geographically separated populations of *O. infernalis* was assessed using polymerase chain reaction-based molecular markers, including the intersimple sequence repeat and mitochondrial cytochrome oxidase sequences. A high level of genetic diversity was detected among these populations from the intersimple sequence repeat (*H*; 0.2628, *I*; 0.4129, *H*; 0.2130) and cytochrome oxidase analyses (*H*; 0.653). There was no obvious geographical structure based on an unweighted pair group method analysis and median-joining network. The values of FST, θ, and Gst estimated in this study are low, and the gene flow is high (*Nm* > 4). Analysis of the molecular variance suggested that most of the genetic variation occurs within populations, whereas only a small variation takes place between populations. No significant correlation was found between the genetic distance and geographical distance. Overall, our results suggest that the geographical distance plays an unimpeded role in the gene flow among *O. infernalis* populations.

**Key Words:** genetic diversity, gene flow, ISSR, COI

Outbreaks of grasshoppers cause serious economic loss to crops and forage in China (Jing and Kang 2003, Liu et al. 2008). The nonmigratory grasshopper *Oedaleus infernalis* Saussure (Orthoptera : Acridoidea), which has a wide natural geographical distribution throughout China and some east Asian countries such as, Japan, Korea, and Mongolia, is regarded as a significant pest in China. Populations of this species have the ability to rapidly grow in number and are considered to be the dominant or codominant agricultural pest of crops and forage grasses of great economic significance in Northern Steppe and North farming (Li et al. 2007).

Highly migratory species often exhibit very low geographical differentiation over their distributional ranges because of strong gene flow (Mayr 1963, Waples 1987, Pogson et al. 1995). As a nonmigratory grasshopper, the gene flow among populations of *O. infernalis* is thought to be low. Although different populations of grasshopper differ in their feeding habits, life history, and other biological characteristics, it is still not known whether such differences are caused by geographical isolation. Although several studies on *O. infernalis* have been conducted (He et al. 2006, Fartmann et al. 2008, Liang et al. 2008, Yoshioka et al. 2010, Kindler et al. 2012), few studies have focused on phylogeographic analysis. Zheng (2006) studied the genetic differentiation of three *O. infernalis* populations across Shanxi and found that most of the variation (74.9%) occurred within populations. Gao et al. (2011) estimated the genetic variation between two *O. infernalis* populations in Inner Mongolia and observed that 58.05% of the total genetic variation was within populations. However, there were some limitations to these findings due to their sampling range.

A number of developed molecular markers have been used to determine population genetic diversity and genetic variation (Abbot 2001, Avise 2004, Sato et al. 2004, Clark et al. 2007, Krumm et al. 2008), and these have become valuable tools to analyze the population genetic structure in grasshoppers (Cooper et al. 1995, Zhang et al. 2009). The intersimple sequence repeat (ISSR) approach is a polymerase chain reaction (PCR)-based technique, first reported by Zietkiewicz et al. (1994). The technique uses a single primer composed of 16–25-bp-long microsatellite sequences to amplify DNA regions between two consecutive SSRS. ISSR constitutes genetic markers that overcome many of the limitations of RAPD analyses, such as the production of more reliable bands and a higher number of polymorphic fragments (Kantety et al. 1995, Tsunura et al. 1996). ISSR has been successfully used in several population genetics studies (Esselman et al. 1999, Qian et al. 2001, Mamrique-Poyato et al. 2013). Mitochondrial DNA (mtDNA) is also a useful molecular marker for population studies because of several features, such as maternal inheritance, the absence of recombination, and a rapid rate of evolution (Moritz et al. 1987, Harrison 1989, Avise 1991, Wolstenholme 1992). The well-studied mitochondrial gene encoding subunit I of cytochrome oxidase (COI) possesses regions that evolve at different rates, possesses different evolving regions, a feature which makes this marker suitable for intra- and inter-specific studies of invertebrate taxa (Lunt et al. 1996, Zhang and Hewitt 1997), including orthopterans (Funk et al. 1995, Szymura et al. 1996, Trewick 2007).

In this investigation, we evaluated the levels of gene diversity and gene variation among 10 different geographical populations of *O. infernalis* from China by using ISSR and mtDNA markers. The results obtained provide some preliminary data concerning the phylogeography of this pest.

**Materials and Methods**

**Sampling.** Specimens of the grasshopper *O. infernalis* were collected by hand from 10 geographical populations in China during their active season (August ~ October) in 2011 (Table 1, Fig. 1). The individual specimens were stored in absolute ethanol at −80°C until required.

**Total DNA Extraction.** Total genomic DNA was extracted from the muscle of the hind legs of each individual grasshopper using a CTAB
protocol. Tissue was placed into a 1.5 ml Eppendorf tube with 400 μl of 1% CTAB buffer. After being incubated at 37°C for 1 h, 0.06 mg Proteinase-K and 40 μl of 10% sodium dodecyl sulfate (SDS) were added at 65°C for 4 h. The mixture was extracted with chloroform and isoamyl alcohol and two thirds volume of ice-cold isopropanol was added. The DNA was then washed with 70% ethanol, dried, and dissolved in TE buffer. After the addition of 1 μl of per ml 10 mg RNase, the DNA concentration was determined using a NanoDrop2000 (Thermo Scientific, Waltham, MA) and then stored at −20°C.

SSR Amplification. Molecular marker simple sequence repeats (SSRs) have been employed in our previous studies, because no microsatellite loci have been developed for genetic studies on O. infernalis, primer sequences developed for the genera Locusta and Oedaleus, which belong to the same family Oedipodidae (Liu 1990), were examined in O. infernalis (Zhang et al. 2003, Chapuis et al. 2005, Yassin et al. 2006, Berthier et al. 2008, Chapuis et al. 2008). After PCR amplification, it was observed that no primer pair successfully amplified a specific product in O. infernalis. For that reason, two alternative DNA markers, ISSR and mtDNA, were used in this study.

ISSR Amplification. One hundred ISSR primers obtained from the University of British Columbia were tested in Faku (FK) and Beipiao (BP) populations to select primers that could give good amplification. The optimum annealing temperature (T_a) was detected for the primer by gradient annealing temperature screening. The PCR reaction mixture (20 μl) consisted of 10 mM Tris-HCl PH 9.0, 50 mM KCl, 2.5 mM MgCl_2, 200 μM of each dNTP, 0.6 μM primer, 40 ng DNA, and 1U Taq DNA polymerase enzyme (TaKaRa, Otsu, Japan). DNA amplification was performed using S100 thermal cycler (BIO-RAD, Richmond, CA) with an initial denaturation at 94°C for 5 min, followed by 45 s at 94°C, 1 min at the annealing temperature (T_a), and then 1 min 30 s at 72°C for 35 cycles, and 10 min at 72°C for the final extension. Negative controls were used to verify the repeatability of the results. The PCR products were electrophoresed at 80 V on a 1.5% agarose gel with ethidium bromide. The molecular weights were estimated using a 100-bp DNA ladder.

COI Amplification. The mitochondrial COI fragment, which is suited to population genetic studies (Lunt et al. 1996, Zhang and Hewitt 1997), was amplified using the primers C1-J-1763 (UEA3) and C1-N-2087 (UEA4). PCR amplification was carried out in a 50 μl volume containing 10 mM Tris-HCl PH 9.0, 50 mM KCl, 1.5 mM MgCl_2, 200 μM of each dNTP, 0.15 μM of each primer, 50 ng DNA, and 2U Taq DNA polymerase enzyme (TaKaRa, Otsu, Japan). The

| Locality name            | Abbreviation of locality name | Date        | Number of individuals for ISSR | Number of individuals for COI |
|--------------------------|-------------------------------|-------------|-------------------------------|-------------------------------|
| Faku, Liaoning           | FK                            | August 2011 | 15                            | 15                            |
| Beipiao, Liaoning        | BP                            | October 2011| 15                            | 13                            |
| Nong’an, Jilin           | NA                            | August 2011 | 15                            | 15                            |
| Tongliao, Inner Mongolia | TL                            | August 2011 | 15                            | 15                            |
| Gerichaolu, Inner Mongolia | GR                         | August 2011 | 15                            | 15                            |
| Xilinhaote, Inner Mongolia | XL                         | September 2011 | 15                          | 13                            |
| Cangzhou, Hebei          | CZ                            | September 2011 | 15                        | 15                            |
| Helan, Ningxia           | HL                            | September 2011 | 15                        | 14                            |
| Qingxu, Shanxi           | QX                            | September 2011 | 15                        | 15                            |
| Wuqi, Shaanxi            | WQ                            | September 2011 | 15                        | 14                            |
amplification was performed in the same thermal cycler as in the ISSR analysis; the conditions were as follows: 94°C for 4 min followed by 40 s at 94°C, 1 min at 43°C, and then 1 min 20 s at 72°C for 35 cycles, and 10 min at 72°C for the final extension. The PCR product was purified using the DNA Fragment Quick purification kit (Ding Guo Chang Sheng, Beijing, China) and sequenced using the BigDye Terminator v. 3.1 sequencing kit (Applied Biosystems, Carlsbad, CA) and an ABI 3730xl automated DNA sequencer (Applied Biosystems).

Data Analysis. The amplified bands from ISSR analysis were treated as dominant genetic markers. For each primer, amplified fragments were scored as present (1) or absent (0) in a binary matrix. The POPGEN 1.32 software (Endersby et al. 2005) was used to analyze the genetic parameters of the observed number of alleles ($N_a$), effective number of alleles ($N_e$), percentage of polymorphic bands (PPB), Nei’s gene diversity ($H$) (Nei 1973), Shannon information index ($I$), genetic similarity, genetic distance (Nei 1972), coefficient of genetic differentiation ($G_{st}$) and the gene flow ($N_{m}$). The cluster analysis was based on the genetic distance using the unweighted pair group method analysis (UPGMA). The $f$-free model was implemented because of unreasonable estimates of $f$ calculated with the full model. Default sampling parameters were used (nBurnin = 5,000, nSample = 25,000, thin = 5).

A Mantel test was performed to compare the matrix genetic distance with the matrix of geographical distance among the populations using GenALEx 6.41 (Peakall and Smouse 2006).

| Table 2. Primers for ISSR analysis |
|-----------------------------------|
| **Primer name** | **Sequence (5’–3’)** | **Annealing temperature (°C)** | **No. of bands scored** | **No. of polymorphic bands** | **PPB (%)** |
|-----------------|----------------------|-------------------------------|------------------------|-----------------------------|------------|
| UBC 809         | (AG)_{10}G           | 49                            | 10                     | 10                          | 100        |
| UBC 812         | (GA)_{12}A           | 47                            | 9                      | 8                           | 88.9       |
| UBC 815         | (CT)_{10}G           | 49                            | 10                     | 10                          | 100        |
| UBC 823         | (TG)_{12}G           | 49                            | 9                      | 9                           | 100        |
| UBC 840         | (GAT)_{16}Y          | 49                            | 10                     | 10                          | 100        |
| UBC 843         | (CT)_{10}R           | 49                            | 9                      | 6                           | 66.7       |
| UBC 844         | (CT)_{10}C           | 51                            | 13                     | 13                          | 130        |
| UBC 845         | (CT)_{10}G           | 51                            | 9                      | 8                           | 88.9       |
| UBC 848         | (CA)_{10}G           | 51                            | 8                      | 8                           | 100        |
| UBC 854         | (GCT)_{12}G          | 51                            | 6                      | 6                           | 100        |
| UBC 866         | (CTC)_{16}           | 56                            | 10                     | 10                          | 100        |
| UBC 900         | ACT TCC CCA CAG      | 53                            | 8                      | 8                           | 100        |
| **Total**       |                      |                               | 111                    | 106                         | 95.5       |

$R = (A, G); Y = (C, T).$

Table 3. Genetic variation statistics among populations

| Population | $N_a$ | $N_e$ | No. of polymorphic bands | PPB (%) | $H$ | $I$ | $H_e$ |
|------------|-------|-------|--------------------------|---------|-----|-----|-------|
| BP         | 1.7027 ± 0.4591 | 1.4342 ± 0.3553 | 78 | 70.27 | 0.2550 ± 0.1962 | 0.3787 ± 0.2790 | 0.2174 ± 0.0152 |
| CZ         | 1.7117 ± 0.4550 | 1.4672 ± 0.3565 | 79 | 71.17 | 0.2563 ± 0.1966 | 0.3805 ± 0.2790 | 0.2138 ± 0.0150 |
| NA         | 1.6396 ± 0.4823 | 1.3664 ± 0.3578 | 71 | 63.96 | 0.2317 ± 0.1983 | 0.3257 ± 0.2827 | 0.2122 ± 0.0146 |
| GR         | 1.6396 ± 0.4823 | 1.4015 ± 0.3570 | 71 | 63.96 | 0.2364 ± 0.1998 | 0.3508 ± 0.2867 | 0.2141 ± 0.0149 |
| HL         | 1.6577 ± 0.4766 | 1.3782 ± 0.3454 | 73 | 65.77 | 0.2261 ± 0.1957 | 0.3390 ± 0.2805 | 0.2065 ± 0.0153 |
| FK         | 1.6757 ± 0.4702 | 1.3850 ± 0.3355 | 75 | 67.57 | 0.2321 ± 0.1920 | 0.3487 ± 0.2759 | 0.2090 ± 0.0150 |
| QX         | 1.6396 ± 0.4823 | 1.4117 ± 0.3629 | 71 | 63.96 | 0.2405 ± 0.2020 | 0.3555 ± 0.2896 | 0.2110 ± 0.0158 |
| TL         | 1.6216 ± 0.4872 | 1.3829 ± 0.3570 | 69 | 62.16 | 0.2261 ± 0.1999 | 0.3364 ± 0.2872 | 0.2154 ± 0.0149 |
| XL         | 1.6396 ± 0.4823 | 1.4208 ± 0.3838 | 71 | 63.96 | 0.2408 ± 0.2086 | 0.3538 ± 0.2960 | 0.2150 ± 0.0151 |
| WQ         | 1.6486 ± 0.4796 | 1.3991 ± 0.3465 | 72 | 64.86 | 0.2372 ± 0.1971 | 0.3927 ± 0.2842 | 0.2161 ± 0.0146 |
| **Total**  | 1.9550 ± 0.2083 | 1.4066 ± 0.2636 | 111 | 95.5  | 0.2682 ± 0.1432 | 0.4129 ± 0.1933 | 0.2130 ± 0.0135 |

$N_a$: genetic parameter of observed number of alleles; $N_e$: effective number of alleles; $H$: Nei’s gene diversity; $I$: Shannon information index. $H_e$: genetic diversity using a Bayesian approach.
populations ranged from 4 to 8, with an average of 4.9 per population. The Bayesian estimate ($I_H$) across all populations was 0.2130 and ranged from 0.2065 to 0.2174.

Table 4 lists the genetic similarity and genetic distance among populations of *O. infernalis*. An UPGMA dendrogram was constructed based on the genetic distance of the ISSR data and is shown in Figure 2. The dendrogram did not show any major geographic structure for these populations. The Mantel test (Fig. 3) revealed that there was no significant correlation between genetic distance and geographical distance ($r = -0.109$, $P = 0.240$). The $Gst$ value was 0.099, and the result showed that the great majority of the genetic variation (90.1%) resided within populations, whereas only 9.9% resided between populations. A similar result was obtained from the Bayesian approach, where the average Bayesian estimate of $\theta^I$ was 0.039 for the $f$ free model. The estimate of the $N_m$ value was 4.55. These results indicated that genetic differentiation of these populations of *O. infernalis* is impeded by a high gene flow.

**COI Analysis.** Sequences for the 305 bp of the COI fragment were analyzed from 144 individuals from 10 geographical populations. Fifteen sites were polymorphic, of which eight were parsimony informative. Sixteen substitutions were found, of which there were 10 transitions, 4 transversions, and 1 parallel mutation. The ratio of transition and transversion was 2.9. The average base composition was $A$ (36.19%), $T$ (31.72%), $C$ (18.98%), and $G$ (13.09%). The average $A + T$ content was high (67.91%), which is in agreement with values for insects in general (Liu and Beckenbach 1992, Simon et al. 1994).

Twenty-one haplotypes labeled based on the COI sequences were deposited in GenBank with the accession number KC297197~KC297217. The genetic distance of the different haplotypes ranged from 0.003 to 0.027, with an average of 0.010 per haplotype. Haplotype H1, which was found in 83 individuals among all the populations, was the most common shared haplotype. The second most common was H2, which was found in 14 individuals in 6 populations. The third most common was H3, which was found in 10 individuals in two populations. Figure 4 describes the haplotype network produced from the Bayesian approach, where the average Bayesian estimate of $\theta^I$ was 0.039 for the $f$ free model. The estimate of the $N_m$ value was 4.55. These results indicated that genetic differentiation of these populations of *O. infernalis* is impeded by a high gene flow.

**Discussion**

The rapid development of molecular techniques for analyzing the genetic differentiation of a species has enabled direct measurement of the level of genetic diversity and genetic differentiation among populations (Mutun and Borst 2004). To date, comprehensive genetic studies on species within the Orthoptera group have not been carried out, but many phylogeographic characteristics of the grasshopper have been resolved by using molecular markers. Zhang et al. (2009) used multiloci microsatellite genotyping analysis to show that migratory locusts in China formed three unexpected groups as the Tibetan group, the South China group, and the North China group. Ortego et al. (2009, 2010) investigated the phylogeography of the Iberian populations of *Moscirtus Wagneri* (Kittay) by using mitochondrial genes and microsatellite markers and found that the grasshopper exhibits a marked phylogeographic structure, forming three main clades. In this study, the genetic diversity and genetic differentiation of *O. infernalis* have been examined in an attempt to characterize the population structure. Two promising and effective DNA markers, ISSR and mtDNA, were used in this study, as it was considered that using markers for two different techniques might provide a more accurate and complementary information to understand population structure.

In the analysis shown, Haplotype H1, identified by COI analysis, which was observed in all the sample sites, appeared predominantly from ancestral haplotype, with a frequency of 57.6%, while the limited geographical distribution of some of the other haplotypes suggest the existence of a genetic differentiation among the populations. The distribution of COI haplotypes based on networks did not show a clear pattern corresponding to the geographic distribution.

Genetic diversity is assumed to be important for the environmental adaptation, evolutionary potential, and viability of populations (Hughes et al. 2008, Kearney et al. 2011, Wenzel et al. 2012). Here, a
Fig. 3. Mantel test between geographical distance and genetic distance among different populations of *O. infernalis* based on ISSR data.

Fig. 4. Median-joining haplotype network of *O. infernalis* based on COI gene of mtDNA. The circle areas are proportional to haplotypes frequencies, while the color portions represent the proportions of the same haplotype occurring in each sampling region.

| Population | Number of haplotypes (h) | Haplotype Diversity ($H_d$) | Nucleotide diversity ($P_i$) | Average number of nucleotide differences ($K$) |
|------------|--------------------------|-----------------------------|-----------------------------|---------------------------------------------|
| BP         | 4                        | $0.423 \pm 0.164$           | $0.0043 \pm 0.0023$         | 1.3333                                       |
| CZ         | 5                        | $0.733 \pm 0.089$           | $0.0030 \pm 0.0006$         | 0.9333                                       |
| NA         | 5                        | $0.752 \pm 0.076$           | $0.0037 \pm 0.0007$         | 1.1428                                       |
| GR         | 8                        | $0.790 \pm 0.105$           | $0.0055 \pm 0.0011$         | 1.6952                                       |
| HL         | 4                        | $0.491 \pm 0.175$           | $0.0017 \pm 0.0007$         | 0.5454                                       |
| FK         | 6                        | $0.758 \pm 0.079$           | $0.0035 \pm 0.0006$         | 1.0719                                       |
| QX         | 5                        | $0.562 \pm 0.143$           | $0.0035 \pm 0.0011$         | 1.0666                                       |
| TL         | 4                        | $0.543 \pm 0.133$           | $0.0028 \pm 0.0010$         | 0.8571                                       |
| XL         | 4                        | $0.423 \pm 0.164$           | $0.0020 \pm 0.0009$         | 0.6153                                       |
| WQ         | 4                        | $0.648 \pm 0.116$           | $0.0037 \pm 0.0008$         | 1.1318                                       |
| Total population | 21                      | $0.653 \pm 0.044$           | $0.0036 \pm 0.0004$         | 1.1097                                       |
high degree of genetic diversity was observed within populations of O. infernalis from both the ISSR and COI analyses. However, this may be explained by the behavioral factors of this grasshopper. First, O. infernalis disperse to different ecosystems due to habitat choice and food, which may result in an increased degree of genetic diversity. Second, the ecological suitability and diversity of their life history may be another contributing factor. Similar results were detected in an analysis of the genetic diversity of the South American grasshopper Dichroplus elongatus Giglio Toss, which is an agricultural pest of crops and forage in Argentina (Rosetti and Remis 2012). The high genetic diversity can be attributed to the strong adaptability of O. infernalis. To reveal the biological characteristics of grassland grasshoppers, species such as Calliptamus abbreviatus Ikonn, O. decorus asiaticus Bienko, Bryodemella tuberculatum dilutum (Stoll), and Dasyhippus peipingenesis (Chang) were collected in Gerichaolu from June to September 2011. Samples were transferred to the laboratory and reared in a box (35 by 35 by 40 cm) with fresh grass at 28°C [photoperiod of 14:10 (L:D) h]. However, both nymphal and adult subjects of these species almost completely died within 3 d, except for O. infernalis (Sun 2013). This grasshopper showed high adaptability against the changing environmental conditions.

Migratory locusts are expected to have a minimal population substructure because the strong migratory ability should minimize genetic variation over their distributional ranges (Zhang et al. 2009). Given that migration plays an important role in gene flow, nonmigratory grasshoppers are generally assumed to have a high value of genetic variation. Geographical isolation is considered to be one of the important factors for the population differentiation. Li et al. (2011) studied the population genetic structure of the rice grasshopper, Oxya hyla intricata (Stal), which has a poor dispersal capability, across Southeast Asia using both DNA sequences and AFLP technology, and found that the distances between ranges impeded gene flow among populations. In this study, the Fst, θPT, and Gst values calculated in the analyses were lower, and the gene flow was higher among O. infernalis populations than expected, based on the two markers. A high gene flow is expected to lower the genetic differentiation of populations if Nm > 4. Zheng (2006) pointed out that although the gene flow of O. infernalis populations is lower than migratory locusts, the value is higher than for other nonmigratory grasshoppers in the survey. The reasons for this are likely to be a consequence of the grasshopper O. infernalis having a high dispersal capability compared with low mobility grasshoppers such as Oedaleus chinensis, as both nymphs and adults of O. infernalis are good jumpers, and adults have wings and are good fliers. The third-stage nymph have been shown to move up to 10 m in 2 h when there is a food shortage, and the adults often perform long-range movement from grassland to farming due to the seasonal changes. However, it is possible that the high dispersal ability of this species results in a high gene flow that weakens the genetic differentiation over the widely distributed geographical populations. Furthermore, other possibilities such as natural intervention may also be contributing factors for gene flow among populations.

The AMOVA based on FST showed that most of the genetic variation resided within populations. Also, no correlations between genetic distance and geographical distance were found by the Mantel tests. Taken together, a high degree of genetic diversity and a low level of population differentiation were observed among populations of O. infernalis, and the gene flow was not affected by geographical distance. This study therefore provides valuable data on the phylogeography for O. infernalis. However, further studies with additional populations and different genetic markers are necessary before final conclusions can be reached.

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