# Population Genetics of the Oriental Fruit Fly, *Bactrocera dorsalis* (Diptera: Tephritidae), in Yunnan (China) Based on Mitochondrial DNA Sequences

WEI SHI, CAROLE KERDELHUE,[1] AND HUI YE[2]

Life Science College, Yunnan University, Kunming 650091, P. R. China

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**ABSTRACT** The oriental fruit fly, *Bactrocera dorsalis* Hendel, is one of the most destructive pest insects of tropical and subtropical fruits and vegetables. It is thought to be an introduced species in Yunnan Province, China, where it causes severe damage. Depending on the latitude, the fly occurs year-round or only during the warm season. To assess the genetic diversity of the fly and to understand the relative isolation of its populations in this mountainous region, we conducted an analysis of population genetic structure using mitochondrial cytochrome oxidase (COI) gene sequences. Twenty-eight haplotypes were detected among 37 individuals with up to 13 mutations between haplotypes. Within-population diversity was high, and genetic distances between haplotypes reached 2.2%. The haplotype network showed that many haplotypes were missing in the sampled populations. Intraspecific variability in *Bactrocera dorsalis* was thus high in Yunnan. The data suggested either a longer residence of the fly in Yunnan than recognized previously or a recurrent colonization process from different origins. One population, namely Ruili, was significantly isolated from the others, probably because of geographic barriers to gene flow. This population seemed to be in a contact zone with flies originating from surrounding regions. In contrast, some populations separated by >300 km were not significantly structured. We suggest that the insects engage in long range dispersal, most probably taking advantage of prevailing air currents. The data also suggested that the region of Kunming, where the fly only occurs seasonally, is recolonized each year by migrating flies from several southern regions.

**KEY WORDS** *Bactrocera dorsalis*, mitochondrial cytochrome oxidase (COI) gene, mt DNA sequences, population genetics, oriental fruit fly

THE ORIENTAL FRUIT FLY, *Bactrocera dorsalis* Hendel (Diptera: Tephritidae), is one of the most destructive pest insects of tropical and subtropical fruits and vegetables (Vargas and Jamnes 1990). This fly, first recorded for the Asia-Pacific region in 1912 in Taiwan, expanded to most countries or regions around the Pacific Ocean area over the next 90 yr (Christenson and Foot 1960). It is thus hypothesized that the fly was introduced to mainland China from Taiwan about a century ago (Wang 1996) and could thus exhibit reduced levels of genetic diversity compared with areas within the natural range. It is highly polyphagous, being able to infest >100 host plants including many types of commercial fruits such as citrus, mango, and peach, and a wide variety of other agricultural products such as coffee, chili peppers, and watermelon (Li and Ye 2000).

The damage caused by the oriental fruit fly consists both of punctures of the host tissue during oviposition and feeding on the fruit pulp by the developing larvae. Adult *B. dorsalis* females localize their hosts by means of volatile compounds released by the plant (Prokopy et al. 1994, Shi et al. 2003). Females lay their eggs under the skin of the fruits (Andrei et al. 2001). They deposit batches of 1–20 eggs in a single fruit, and individual fruits can be infested multiple times (Li and Ye 2000, Vargas et al. 1984, Flencher 1989). The larvae have three instars that feed on the fruit pulp, which can result in its complete destruction (Arai 1975). Mature larvae drop off the fruits onto the ground, where they pupate 2–5 cm deep in the soil. Adults emerge and fly to the host to achieve a maturation feeding (Christenson and Foot 1960, Arai 1976). Reproductively immature adults are able to disperse around 50 km to find fresh food resources and breeding substrates. Adults become sexually mature in a few days. In infested areas, fruit and vegetable production may be completely lost in terms of commercial value (Li and Ye 2000).

In China, *B. dorsalis* is mostly distributed in the southern and southwestern provinces or autonomous regions (Zhang et al. 1995, Ye 2001). Yunnan is one of the major provinces where this pest causes severe damage (Ye 2001). Much of the land area (≈94%) is

[1] INRA Pierroton, UMR Biogeco, Equipe Entomologie et Biodiversité, 69 route d'Arcachon, F-33612 Cestas Cedex, France.
[2] Corresponding author, e-mail: yehui@ynu.edu.cn.

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**Table 1. Sampling locations for the five *B. dorsalis* populations studied in Yunnan Province, China**

| Sites | Elevation (m) | Latitude (N) | Longitude (E) | Code | Sample size | Host plants | Collection date |
|---|---|---|---|---|---|---|---|
| Kunming | 1890 | 25°29′ | 102°45′ | KM | 10 | Peach | 2003.6 |
| Huanian | 1134 | 24°04′ | 102°24′ | HN | 6 | Mango | 2003.6 |
| Jinghong | 558 | 21°29′ | 100°48′ | JH | 7 | Mango | 2002.6 |
| Ruili | 907 | 24°01′ | 97°51′ | RL | 7 | Mango | 2003.6 |
| Hekou | 87 | 22°31′ | 103°57′ | HK | 7 | Mango | 2003.6 |

mountainous (Yunnan Statistical Bureau 2002). Within Yunnan Province, different infestation patterns of the pest can be observed. Based on the phenology of the fly and on geographical features, the province can be divided into two regions. (1) The southern region (south of 24° N latitude) is primarily at an elevation of <1,000 m. The climate is tropical to semitropical with a relatively mild winter, and there is abundant and diverse fruit and vegetable production. In this region, fruit fly infestations occur year round, with up to five generations per year. (2) The northern region (from 24° N to 26° N) is a plateau with an average elevation of 1,700–2,300 m above sea level. In this region *B. dorsalis* is only present seasonally, particularly from late spring to mid autumn when temperatures are warmest (Ye 2001). The fly completes two generations between May and October and disappears until the following spring (H.Y., unpublished data). However, the factors influencing the occurrence of the seasonal populations in the northern region, and the relationships of the seasonal populations to the year round populations found in the southern region, have not been examined so far.

Mitochondrial DNA is widely used in studies of population history and phylogeography because of its simple structure, maternal inheritance, and relatively rapid evolutionary rates (Simon et al. 1994, Roderick 1996, Mun et al. 1999). In this study, we used genetic analysis of mitochondrial DNA sequences to determine the population genetic structure of the oriental fruit fly in Yunnan Province. We aimed at assessing both the genetic diversity of the fly measured within and between populations and the relative isolation of the different populations to understand how gene flow occurs in this mountainous province. A secondary objective was also to determine the genetic relationships between the one population located in the seasonal occurrence zone and the year-round populations. This study will provide essential information for understanding dynamics of fruit fly populations in Yunnan Province, China.

## Materials and Methods

**Fruit Fly Sampling.** Collections of *B. dorsalis* were made in June 2003 from five sites within Yunnan Province, namely Kunming (KM), Huanian (HN), Jinghong (JH), Hekou (HK), and Ruili (RL) (Table 1). Kunming is located in the zone of seasonal occurrence of the flies, whereas the other four sites fall in the annual occurrence zone (Fig. 1).

In each region, flies were sampled from two to three fruit orchards separated by ≈10 km. The selected orchards represented the principal cultivated fruit species grown locally (Table 1). From each orchard, three to four infested fruits were collected and brought back to the Yunnan University laboratory, where they were kept individually in cages of 15 by 20 by 20 cm at room temperature. Soon after adult emergence, one adult was collected from each cage for DNA analysis. This method minimized the probability that multiple specimens from the same parents would be obtained. All of the collected specimens were immediately placed in absolute ethanol and stored for later DNA extraction.

**DNA Protocols.** DNA was extracted individually for each specimen using the commercial tissue/cell DNA Mini Kit (Watson Biotechnologies, Shanghai, China), or using methods described by Kambhampati and Rai (1991). The DNA extracted from each individual was suspended in 50 μl of Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0).

A portion of the mitochondrial gene COI (505 bp in length) was amplified by polymerase chain reaction (PCR) using the forward primer P1 (5′-CGTGCCTATTTCACTTCAGC-3′) and reverse primer P2 (5′-CAGCTGGAGGGGTATTTTGA-3′). These primers were designed from conserved regions based on comparisons of published mtDNA sequences of 12 species of *Bactrocera* (Genbank accession AF423102≈AF423107; AY053507≈AY053512).

PCR amplifications were carried out in a final volume of 50 μl containing 31 μl $H_2O$; 5 μl 10× Buffer (Promega, Shanghai, China); 4 μl $Mg^{2+}$ (25 mM; Promega); 1.5 μl dNTPs (25 mM; Promega); 4 μl each primer (10 pM; Boya, Shanghai, China); 2 U *Taq*DNA (5 U/μl, Promega), and 2 μl of template DNA (20≈50 ng DNA). The reaction profile was one step of initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 1 min. A final extension step of 72°C for 5 min was also added.

The purification and sequencing of PCR products was carried out by the ShenYou Biochemical Technology Co. (Shanghai, China). Sequencing reactions were carried out in both directions to increase accuracy.

**Data Analysis.** Sequences were aligned using Clustal X as implemented in Bioedit 7.0. (Hall 2004). Haplotype numbers and distribution, polymorphic sites, and Kimura two parameter distances (hereafter

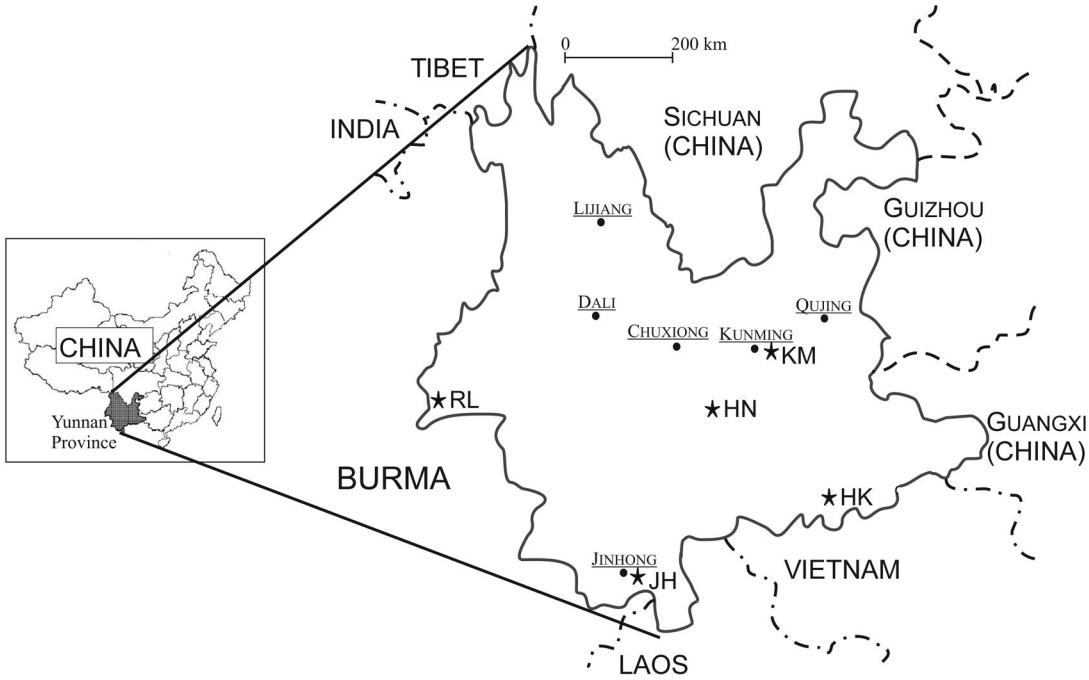


**Fig. 1.** Map showing the position of Yunnan Province within China and the five sampling sites for *B. dorsalis* in Yunnan.

K2P distances) between haplotypes were assessed using Mega 2.0 (Kumar et al. 2001). Average within- and between-population K2P distances were calculated using Mega 2.0. Haplotypes found in this study were aligned with previously published COI sequences of other *Bactrocera* species (AF423102-AF423107, AY053508) to infer interspecific distances.

Nei's raw and Nei's corrected numbers of pairwise differences between populations (Nei and Li 1979) as well as pairwise *F*st estimates (Reynolds et al. 1983) were calculated using Arlequin 2.001 (Schneider et al. 2000). Statistical significance was assessed after 3,024 permutations in all cases.

A statistical parsimony network of haplotypes (Templeton et al. 1992) was constructed with the program TCS version 1.13 (Clement et al. 2000). To solve any cladogram ambiguity, we used the frequency and topological criteria (Pfenninger and Posada 2002, Duran et al. 2004) to break the loops and consistently chose the most parsimonious solutions.

Matrices of pairwise estimates of genetic differentiation (both *F*st and Nei's corrected number of differences) were compared with the matrix of geographic distances by means of a simple Mantel test (Legendre and Legendre 1998) to detect isolation by distance. The Mantel test quantifies the correlation between two distance matrices, therefore allowing determination of a relationship between the genetic and geographical distance matrices. We used 500 random permutations to test for Mantel statistical significance.

## Results

**Sequence Data and Haplotypes Characteristics.** We obtained 505-bp sequences for 37 *Bactrocera dorsalis* samples. Of the 505 characters, 30 were polymorphic, including 8 singleton polymorphic sites and 22 parsimony informative sites. Twenty-seven transitions and three transversions were observed, but there were no insertions or deletions; 44% of transitions were A-G, and 56% were C-T. Of the three transversions, two were A-T and one was T-G. Twenty-eight haplotypes were observed in the five *Bactrocera dorsalis* populations, corresponding to 37 individuals (Table 2). Only 7 haplotypes were shared by at least two individuals, and 21 haplotypes were unique. Among the seven shared haplotypes, three were found in both Kunming and Huanian populations, whereas four were shared within a single population (H1 and H4 in Kunming, H21 in Hekou and H27 in Ruili, see Table 2). Sequences obtained were deposited in GenBank (accession DQ06028–DQ060304, DQ100468, DQ100470, DQ100471).

**Genetic Relationships Within and Between Populations.** K2P genetic distances between haplotypes within single populations ranged from 0 to 0.006 in Huanian, from 0.002 to 0.016 in Kunming, Hekou, and Jinghong, and from 0.006 to 0.022 in Ruili (mean within-population, 0.0138). Mean distances between populations ranged from 0.0053 between Huanian and Kunming to 0.0139 between Ruili and Hekou (Table 3). Distances between Ruili and all other population were larger than in any other pairwise comparison.

**Table 2. Sequence variation of 28 COI haplotypes found and distribution among populations of *B. dorsalis***

| Haplotype | Sequence | | | Population (no. individuals) | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1222566789 7069302405 | 1122222233 1834559945 3560273610 | 3333444445 6789156890 5148982572 | KM (10) | HN (6) | JH (7) | HK (7) | RL (7) |
| H1 | ATACATGGAG | AACCCACAAT | TCTTATTACT | 2 | | | | |
| H2 | .........A | .......G.. | .....C.... | 1 | 2 | | | |
| H3 | ......A..A | .......G.. | .T...C.... | 1 | 1 | | | |
| H4 | G........A | .......G.. | .T...C.... | 2 | | | | |
| H5 | G......A.A | .......... | .....C..T. | 1 | | | | |
| H6 | G........A | .......G.. | .....C.... | 1 | 2 | | | |
| H7 | .....CA..A | ...T...G.. | .T...C.... | 1 | | | | |
| H8 | G..T.....A | .......G.. | .....C.... | 1 | | | | |
| H9 | .........A | .......G.. | .T...C.G.. | | 1 | | | |
| H10 | ......A... | .......G.. | .T...C.... | | | 1 | | |
| H11 | GC.......A | .......G.. | .....C.... | | | 1 | | |
| H12 | G.....A..A | .......... | .T......T. | | | 1 | | |
| H13 | .C.T.....A | .......G.. | .....C.... | | | 1 | | |
| H14 | .........A | .......G.. | .T........ | | | 1 | | |
| H15 | .........A | .......G.. | .......... | | | 1 | | |
| H16 | G.....AA.A | .......G.. | .T...C.... | | | 1 | | |
| H17 | ......A..A | .......G.. | .....C..T. | | | | 1 | |
| H18 | ..G......A | .......G.. | ..C..C.... | | | | 1 | |
| H19 | .........A | T...T..G.. | C.....G... | | | | 1 | |
| H20 | .........A | .......GG. | ..C..C.... | | | | 1 | |
| H21 | .........A | .......G.. | .....C...C | | | | 2 | |
| H22 | G.....A.GA | ....T..G.. | .....C.... | | | | 1 | |
| H23 | .........A | T......G.. | ...C...... | | | | | 1 |
| H24 | G........A | .......G.. | .T...C.GT. | | | | | 1 |
| H25 | G...G....A | .T...G.G.C | ....GC.... | | | | | 1 |
| H26 | G........A | T.T....G.. | ...C.C.... | | | | | 1 |
| H27 | G...G.A..A | ..T..GTG.C | ....GC.... | | | | | 2 |
| H28 | G.....A..A | .......... | .....C.... | | | | | 1 |

Interspecific K2P distances ranged from 0.093 between *Bactrocera dorsalis* and *B. correcta* to 0.185 between *B. diversa* and *B. latifrons.*

Nei's raw average pairwise number of differences between populations and pairwise *F*st estimates are given in Table 4. Ruili and Hekou populations differed significantly from all other populations but Huanian (except a significant *F*st value between HN and RL). In contrast no significant differentiation appeared between Kunming, Huanian, and Jinghong populations. Again, both *F*st estimates and Nei's average number of differences were higher between Ruili and any other populations than in all other pairwise comparisons.

The haplotype network is shown in Fig. 2. The haplotypes were found along six branches that evolved from a central haplotype shared by only three individuals (two from Kunming and one from Huanian). Three branches clustered halopypes found in four different populations (branches 1–3; see Fig. 2). These branches grouped the vast majority of the haplotypes (20 of 28). Branch 4 grouped three haplotypes from Kunming and Jinhong, whereas branches 5 and 6 were specific for divergent haplotypes found only in Ruili. There were as many as 25 missing haplotypes. The most divergent haplotypes were distant by 13 mutation steps.

Mantel tests showed significant correlations between geographic distances and both pairwise *F*st (standardized Mantel statistics $r_M = 0.72$, $P < 0.01$) and corrected Nei's number of pairwise differences between populations ($r_M = 0.73$, $P < 0.01$). However, the tests performed without the individuals from Ruili were not significant.

**Table 3. Average K2P distances between populations (below diagonal) and within populations (diagonal elements)**

| | KM | HN | JH | HK | RL |
|---|---|---|---|---|---|
| KM | 0.0072 | | | | |
| HN | 0.0053 | 0.0034 | | | |
| JH | 0.0074 | 0.0061 | 0.0084 | | |
| HK | 0.0088 | 0.0065 | 0.0095 | 0.0089 | |
| RL | 0.0123 | 0.0106 | 0.0131 | 0.0139 | 0.0138 |

## Discussion

Twenty-eight haplotypes were found in the 37 individuals of *B. dorsalis* sampled from five populations. Many of these haplotypes were unique, and only three were shared between two populations. These results show that the oriental fruit fly is highly polymorphic

**Table 4. Average number of pairwise differences between populations (below diagonal), within population (diagonal elements), and Fst values (above diagonal) for five populations of *B. dorsalis* from Yunnan Province, China**

| | KM | HN | JH | HK | RL |
|---|---|---|---|---|---|
| KM | 3.65546 | −0.04013 | −0.05356 | 0.08255** | 0.16486** |
| HN | 2.65208 | 1.74131 | 0.01645 | 0.02856 | 0.16886* |
| JH | 3.73212 | 3.06983 | 4.23006 | 0.08946* | 0.15546** |
| HK | 4.42681** | 3.26126 | 4.80360* | 4.51767 | 0.18254** |
| RL | 6.21295** | 5.34851 | 6.62301** | 7.01834** | 6.95677 |

*: $p < 0.10$; **: $p < 0.05$.

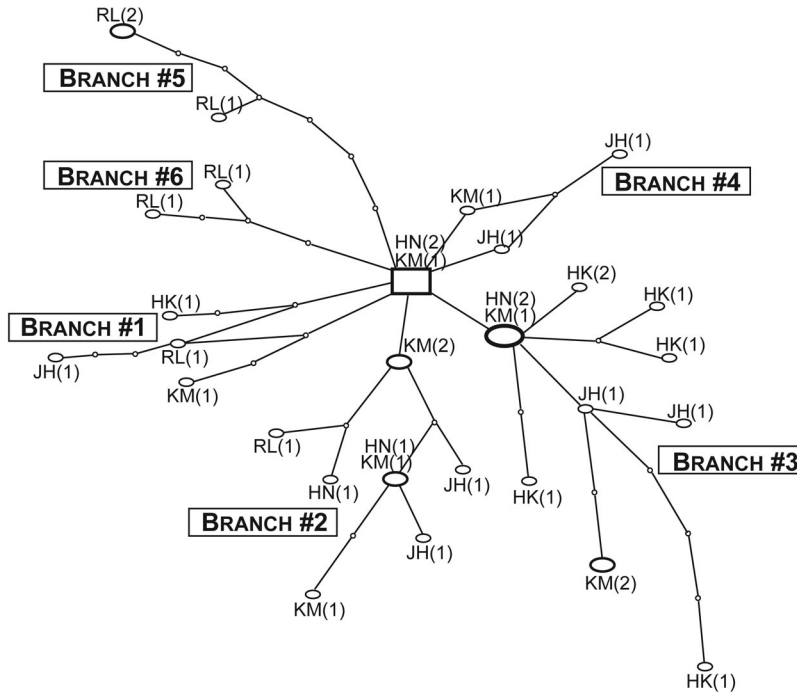


**Fig. 2.** Haplotypes from the 37 individuals sampled in this study. The sizes of ellipses are proportional to the number of individuals having that haplotype. The empty circles correspond to missing intermediate haplotypes. Rectangle indicates probable ancestral haplotype. The origin of individuals having each haplotype is shown above the corresponding ellipse; numbers in parentheses correspond to the number of individuals from each locality.

for this mtDNA gene. High intraspecific polymorphisms were previously reported within the genus *Bactrocera* (Ochando and Reyes 2000, Mun et al. 2003). Moreover, haplotype variation within Yunnan was quite high, with distances reaching 0.022 (mean number of differences >7). However, haplotype variability is fully consistent with intraspecific variability in this genus, being very similar to that found in *B. depressa* (Mun et al. 2003). Furthermore, it is much lower than the interspecific distances we measured and the distances previously found in *Bactrocera* complexes (Jamnongluk et al. 2003a, b).

Finding such a high number of haplotypes in Yunnan Province was quite unexpected, especially because the fly was supposed to have been recently introduced there (less than a century ago). However, it can be difficult to distinguish between a recent invasion and a historically widespread distribution and thus to determine which species are native and which are introduced (Carlton 1996, Mun et al. 1999). Genetic diversity is usually much reduced in introduced populations, even in the highly polymorphic Tephritid species *Ceratitis capitata* (Meixner et al. 2002). *B. dorsalis* was introduced in Hawaii in 1945 and rapidly spread to the whole island (Van Zwaluvenburg 1947). Genetic investigations found a reduced number of haplotypes in Hawaii compared with natural populations of Thailand (He and Haymer 1997). Our results suggest either that the fly has been present in Yunnan for a longer period of time than is recognized but remained undetected until it started to cause damage in orchards or that it has been repeatedly introduced from different locations.

The expected genealogy of a recently introduced population that has expanded in size from a low number of founders would be a common haplotype shared by a majority of individuals and many much rarer haplotypes connected to the main one by a few independent mutations (Slatkin and Hudson 1991, Avise 2000). Most haplotypes found in *B. dorsalis* were unique, and none were shared by a majority of individuals. Moreover, the haplotype network showed that many intermediate haplotypes are missing. This could be explained if the fly has been repeatedly introduced from many parts of its native range, putting together highly divergent haplotypes that evolved allopatrically. If the fly is naturally present in Yunnan, the occurrence of many missing haplotypes shows that a larger sample size will be necessary to properly estimate the haplotype diversity. A complete phylogeographic study of the species, with populations sampled from its entire distribution, will be necessary to fully understand the origins and the diversity of the Yunnan populations.

The analyses of genetic structure of the oriental fruit fly in Yunnan consistently show that the population from Ruili is significantly differentiated from the others (pairwise *F*st ranging from 0.15 to 0.18, and average genetic distances being higher than for all other population comparisons). Moreover, no signif-

icant pattern of isolation by distance appears when RL individuals are excluded. These results show that gene flow is reduced between the westernmost sampled location and the other populations. Yunnan is a mountainous province. Several ranges, namely Santai, Nu, Bangma, Laobie, Wuliang, and Ailao, run parallel through the region from northwest to southeast (Yunnan Statistical Bureau 2002). Three rivers, namely the Nujiang, Lanchangjiang, and Jingshajiang, lie between these mountain ranges, creating deep valleys. Ruili is isolated from Jinghong, Huanian, Kunming, and Hekou by the high mountains mentioned above. These mountains appear to form natural geographic barriers that prevent the fly's dispersion and limit gene flow.

However, mean distance between haplotypes within Ruili is quite high, reaching 0.0139, i.e., as high as the mean distances between Ruili and the other four populations (Table 3). A close examination of the haplotypes found in Ruili show that four of them (corresponding to five individuals) are highly divergent from the other haplotypes found in Yunnan and form two population-specific branches in the haplotype network. The other two haplotypes found in Ruili cluster with haplotypes from the other populations. This evidence suggests that Ruili is a contact zone between flies of different origins. It is possible that the most divergent haplotypes are more common in neighboring regions such as Burma. Once again, it will be necessary to sample the whole geographic range to fully understand the relationships between Yunnan populations.

While the overall genetic diversity is high, the populations from Yunnan (apart from Ruili) do not show any clear pattern of geographic structuring. Within-population variation is as high as between-population differences (Tables 3 and 4), and populations >380 km distant (Jinhong and Kunming) are not significantly differentiated. The evidence suggests that high levels of gene flow occur between distantly related populations, either because of high dispersal ability of the fly or recurrent insect movements caused by human activity, which is consistent with the hypothesis of multiple introductions in Yunnan. The limited levels of commercial transport of fruits also may contribute to the homogenization of the pest populations. It is interesting to note that the population of Kunming, Huanian, and Jinghong are not differentiated, even though two relatively high mountain ranges (Ailao and Wuliang) separate Jinghong from the other populations. Some passes in the mountain ranges could permit fly migration. However, air currents originating in the Bengal fjord dominate this area, flowing from southwest to northeast (Chao 1987). H.Y. (unpublished data) found that the fruit fly was able to disperse >250 km with the wind. It is thus plausible that the high gene flows measured between Jinghong and Huanian are partly caused by passive wind dispersion of the fly. Hekou and Kunming are significantly divergent although they are also ≈390 km apart, most probably because the direction of the main air currents do not encourage fly dissemination between the two populations. No significant pattern of isolation by distance was detected between KM, HN, HK, and JH, which is consistent with what is expected for a species having high dispersion capacities (Peterson and Denno 1998). We therefore suggest that air current plays an important role in the spreading of the pest populations and thus influences interpopulation gene exchange. Our results show that the Yunnan flies exhibit a high level of genetic diversity and that many haplotypes are missing in our samples. A larger-scale study and a bigger sample size per population are now needed to disentangle the question of the origins of the oriental fruit flies in Yunnan and to precisely determine the levels of gene flow between populations, which are currently obscured by the high number of private haplotypes.

Kunming differs from the other four populations in that the local occurrence of the fly is seasonal, probably because of unsuitable temperatures between November and April (Ye 2001). In addition, host fruits are absent in winter in the Kunming area. Ecological observations suggest that the insects cannot survive the winter there and that the zone is recolonized annually from nearby, year-round populations. Our results show a very close genetic relationship between Kunming and Huanian, where the fly is constantly present. These two populations are the most closely related, sharing three haplotypes out of nine total haplotypes present in the two populations. However, if the Kunming population becomes extinct each November and is recolonized in the spring, we would expect to observe a founder effect with reduced local genetic diversity, unless a large number of founders is involved each year. However, our results show that genetic diversity is less in Huanian than in Kunming. One possibility is that Kunming flies originate from several surrounding southwestern locations and are dispersed over long distances through air currents and fruit exchange. Ecological investigations as well as multilocus genetic studies (Davies et al. 1999) are now needed to infer the source populations.

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