UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mating System and Evolutionary Genetics of an Invasive African Drosophilid: *Zaprionus indianus*

A Thesis submitted in partial satisfaction of the requirements for the degree
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in

Biology

by

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The Thesis of Giovanni Hanna is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Mating System and Evolutionary Genetics of an Invasive African Drosophilid: *Zaprionus indianus*

by

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Master of Science in Biology

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Zaprionus indianus is an invasive African drosophilid to the Americas and a generalist that poses major economic threats to figs. It was first introduced to Brazil in 1998 and quickly found its way into the literature with reports of its citing in South, Central, and North America. Little work has been done, however, to understand the invasive ability of this fly. Using laboratory and wild populations of *Z. indianus*, I studied the mating system and conducted a preliminary study on the evolutionary genetics of this species. Results from my studies on its mating system

have shown that males experience a delay in sexual maturity that may contribute to inbreeding avoidance, that males seem to hedge their bets by allocating their sperm across several matings, and that individuals from both sexes remate frequently which may ultimately result in an increase in genetic diversity of the colonizing population. Interestingly, the demographic studies show low genetic diversity of this fly worldwide, a continuous population decline starting long before the colonization of *Z. indianus* of the Americas, and support for a multiple introduction scenario in Mexico from three very different source populations.

1. Chapter 1:

1.1. Introduction

Invasive species are recognized as a leading threat to biodiversity as well as an increasing economic concern (1, 2). Despite the importance of these problems, specific attributes responsible for the establishment and spread of invaders remain unknown (3, 4). In particular, studies that examine the biology of invasive *Drosophila* species in laboratory and introduced environments are surprisingly rare, despite the potential insights that can be gained from such comparisons. Here I examine the mating system as well as the evolutionary and ecological genetics of the invasive fig fruit fly, *Zaprionus indianus*, in the laboratory and in an introduced environment, to explore possible reasons for its success as an invader.

The fig fruit fly is an Afrotropical drosophilid that has recently expanded its geographical range into India (5), the Palearctic region (6), and the Americas (7, 8). It belongs to the drosophilid genus *Zaprionus,* and despite its name, belongs to the African subgenus *Zaprionus sensu stricto*, which includes another 45 species restricted to Africa (9). In 1998, *Z. indianus* was detected for the first time in the Americas in São Paulo City, São Paulo, Brazil (7), and Vilela proposed that it might have inadvertently been introduced by air travel due to the increasing number of flights from several African countries to São Paulo City. Since then, *Z. indianus* has succeeded in becoming abundant in almost every country in South America and was detected in Chiapas, Mexico in 2002 (10), Florida, U.S.A in 2005 (8), and California, U.S.A in 2006 (10).

Although this fly has mostly been associated with tropical environments with its population peaking during the hot and humid season (11, 13), it also has been detected in relatively large numbers in the desert environments of Mexico, Iraq, and Egypt (10, 12, 13). Once established, the fig fruit fly does not seem to displace most native species (11, 14), but was still given pest status in 2005 by the Florida Department of Agriculture & Consumer Service (15) because of its ability to infest ripe figs. It is a generalist that is known to use 74 species in 31 plant families in Africa as hosts (21) and has recently been shown to use over 40 more fruit hosts in Florida and Brazil (7, 9, 11, 14-19), with females even using cactus fruit and rotten cactus tissue as oviposition sites (20).

Thermal tolerance studies in *Z. indianus* and have shown that males grown at 15°C in the laboratory were 100% sterile, a lower limit temperature difference of three degrees when compared to temperature tolerance in *D. melanogaster.* On the higher temperature side, however, males were similar in tolerance to *D. melanogaster*, becoming sterile at 30°C. At both temperature extremes, however, females continued to lay eggs although at a lower rate than at more moderate temperatures. The higher sensitivity to cold in males of *Z. indianus* might explain why this species remains restricted to tropical and subtropical climates (6, 22).

With details of the mating system of this fly absent from the literature, I decided to address this topic by determining many of the mating system characters of *Z. indianus* using laboratory and wild populations, and compare these to those of other classified species. A second aim of my study was to investigate the genetic structure, demographic history, and phylogenetic relationship of the recently established Mexican

metapopulation of *Z. indianus* to other world populations, using DNA sequence data from a single mitochondrial marker, a segment of cytochrome c oxidase subunit I (COI), a data set that comprises Chapter 2 of this thesis. Here I discuss potential male and female reproductive strategies and how these strategies may relate to invasive capabilities of *Z. indianus.*

1.2. Materials and Methods

1.2.1. Stocks and culture maintenance

Laboratory-reared flies came from a stock established combining five males and five females from each of 10 isofemales lines collected by John Pool in Yokadouma, Cameroon, in March 2004. The lines had been maintained at the *Drosophila* Species Stock Center (University of California San Diego, La Jolla, CA). Flies were reared under uncrowded conditions on banana medium in 200-ml bottles with live yeast at 22 ± 1°C at an approximate 12L:12D photoperiodic cycle. For all laboratory studies, adult males and females were collected as virgins within 24 h of eclosion under light CO_2 and were stored separately at 10 flies/vial on banana food vials seeded with live yeast.

1.2.2. Age at reproductive maturity

The age at which flies engage in copulation was determined separately for each sex. One virgin test individual was placed in a yeasted vial with two virgins of the opposite sex that were 10-12 days old, and known to be reproductively mature based on previous behavioral observations made in the laboratory. Vials were observed for a 2 h period in the morning, copulations were noted, and inseminated females from all treatments were saved in order to determine gonadal maturity of the sex under study. Gonadal maturity of the virgin flies of different ages was inferred if at least one offspring was produced from the copulation.

1.2.3. Progeny production from a single mating

Sexually mature flies (six days of age) of each sex were paired and when a copulation was observed, the female was separated and transferred to fresh, yeasted food vials every three days in order to determine the number of progeny a single mating produced.

1.2.4. Female remating latency

To determine female remating latency, six-day-old virgin females were paired with 6-day-old virgin males in fresh vials. Mated females were transferred to new vials containing virgin males immediately (n=30), 1-h (N = 20), 4-h (N = 20), 7-h (N = 20), 10-h (N = 20) and 24-h (N = 20) after the first mating. These females were observed for a period of 0.5 hr at each of these time periods. Females that did not remate during a 10-h period were retested the following morning.

1.2.5. Remating and its influence on female fertility

Females that were inseminated twice (N= 25) were individually placed in banana vials and transferred to fresh vials every 48 h until they stopped laying fertilized eggs. Progeny emerging from each vial were counted and the number was compared to the number of progeny from females mating just once.

1.2.6. Progeny counts from wild-caught females

Wild-caught females were aspirated from platters containing rotting fruits at a rural site in Alamos, Sonora, Mexico (27° 0'59.32"N, 108°56'49.99"W) in November 2011. Styrofoam bowls were set out at the site and contained a mixture of oranges,

banana, and mangoes, and the edges of the bowls were circled with Vaseline shmutz to prevent ants from getting into the fruits. Aspirated females were immediately isolated into individual yeasted banana vials and transferred once to fresh vials after 3 days. Females were discarded after they ceased ovipositing in the vials.

1.2.7. Progeny counts from the mating of older virgin females

A trait that could potentially assist invasiveness of a species would be the ability to reproduce at advanced age. To examine this possibility for *Z. indianus*, flies were kept alive for over three months. Virgin females of 70 (n=30) and 100 days of age (n=21) were individually paired with two virgin, but sexually mature males of 10 days of age in fresh food vials. After copulations took place, inseminated females were immediately transferred to individual fresh vials seeded with yeast and transferred every 48 h until they stopped laying fertilized eggs. The progeny produced by each mating was recorded.

1.2.8. Male remating and its influence on male fertility

Because flies of both sexes were determined to be reproductively mature at six days of age, subsequent experiments utilized flies of this age. Six-day-old virgin males were paired with six-day-old virgin females in fresh food vials. Mated males were transferred repeatedly to new vials containing two virgin females during a 2 h period. The number of times each male mated was recorded and all inseminated females were saved and transferred every 48 h, to avoid larval crowding, to fresh, yeasted vials. The progeny number produced by each mating was recorded to determine if progeny production changed with repeated male mating.

1.3. Results

1.3.1. Age at reproductive maturity

The ages at which males and females first engage in copulation are reported in Table 1. Males appear to mature approximately one day later than females. Approximately half of the females mated by three days of age, while only about 25% of the males mated at this age. By the time the flies were five days old, however, the percentage of males mating had caught up to that of the females. Young males that did not engage in copulation were clearly sexually immature since they also did not even court females.

1.3.2. Progeny production from a single mating

Productivity from a single mating in sexually mature flies of *Z. indianus* is found in Figure 1. Progeny numbers ranged from 17 to 68 (n = 43 females) with a mean and standard error of 39.0 ± 1.85 progeny.

1.3.3. Female remating latency

In the laboratory, the time to remating tests clearly show that females only mate once per day. None of the once-inseminated females (n = 20) engaged in copulation for a second time during the day of copulation. When mated females were courted, all rejected any male attempts to copulate. Twenty-four hours later, however, all inseminated females had remated.

1.3.4. Remating and its influence on female productivity

The number of progeny produced following one remating of mature females is found in Figure 1. Productivity from two matings (n = 25) ranged from 39 to 82 progeny with a mean and standard error of 60.3 ± 2.47 progeny, an increase in productivity by slightly more than 50% ($F = 48.04$, $P < 0.0001$) relative to females mated once in the laboratory.

1.3.5. Progeny counts from wild-caught females

The number of progeny produced from wild-caught females is also shown in Figure 1. 100% of the females (n = 7) caught in the wild laid fertilized eggs in the food vials, and productivity ranged from 25 to 78 with a mean and standard error of 51.57 ± 7.56 progeny, a value intermediate between what was observed in once and twice-mated laboratory females. Table 2 compares average progeny numbers produced from the wild-caught females to that produced by once and twice-mated females in the laboratory, with $F = 21.5$ and $P < 0.0001$, suggesting that females can carry sperm from more than one male in nature.

1.3.6. Progeny counts from the mating of older virgin females

Although 100% of the virgin females of 70 and 100 days of age engaged in copulation, many of the females from both age groups produced no progeny from the matings: 22/30 of the 70 day old females produced an average of 24.0 ± 3.2 progeny while the rest of the test females of this age produced none, whereas only 11/21 of the

100 day old females produced 12.3 ± 3.4 progeny from the matings and the rest produced none.

1.3.7. Male remating and its influence on male fertility

Males were observed to remate up to 10 times when continually supplied with virgin females during a 2-h period in the laboratory (n = 23). Average progeny numbers remained fairly constant across the first five matings (Figure 2) with 96.4% of the variability explained by differences within these mating orders (Table 3). Progeny numbers then decrease from 36.4 ± 2.83 (n = 8) in the fifth mating to 31.9 ± 2.30 (n = 16), 28.5 ± 4.30 (n = 13), and 18.0 ± 6.3 (n = 7) in the sixth, seventh, and eighth matings, respectively. Only three males mated nine times and two of these mated ten times in the 2 h period, accounting for the large standard error bar and range observed in the graph at these mating orders.

1.4. Discussion

1.4.1. Male reproductive strategies

The maturation experiment revealed that males mature sexually one day later than females, a delay that can contribute to inbreeding avoidance. This delay is not unique to males in *Z. indianus*: Markow has shown that 60% of the 42 species examined in her study show similar and even more extreme delays in male maturation (23). Such is the case for species such as *D. pachea, D. bifurca, and D. kanekoi*, where males mature 10 to 15 days later than females (24). However, the longevity data for *Z. indianus* (25) coupled with the maturity data suggest that males in the laboratory spend less than 10% of their adult lives in an immature state, and fertility data from my study shows that females are still capable of oogenesis and insemination at 100 days of age (Figure 3), suggesting the existence of plentiful mating opportunities for males in their lifetimes. Thus, a short delay of one day might be a relatively inexpensive way to avoid inbreeding in *Z. indianus*, with females maturing earlier than males and thereby decreasing the probability of mating with a sibling. This hypothesis is supported further by the fact that more than 80% of individuals eclosing the first day are females in this species, with the sex ratio shifting towards almost total male bias by the end of adult eclosion from a fresh batch of pupae in the laboratory (Hanna, laboratory observations). Although studies have not been performed on the timing of dispersal of males and females, data from population and evolutionary genetic studies point to extensive and rapid dispersal of this species (Chapter 2; Hanna and Pfeiler, manuscript in preparation), and if early dispersal is for virgin females, it would provide further evidence of inbreeding avoidance.

Male *Z. indianus* allocate fairly equal amounts of sperm across the first 5 matings with sperm counts decreasing thereafter (Figure 2, Table 3). This pattern of sperm allocation across matings is not unique to *Z. indianus* and has been documented in other species such as *Drosophila mojavensis* (26) and *Drosophila pachea* (27). I hypothesize that the ejaculate allocation behavior of male *Z. indianus* species may be a diversified bet-hedging strategy (28) that is maintained because of the propensity of females to remate. With females remating daily and males able to allocate almost equal amounts of sperm across several females, this strategy, in time, would favor phenotypes with low variances in reproductive success over alternatives with higher variances and potentially higher mean fitnesses (29; 30), an observation that I confirm in *Z. indianus* with its production of 39 ± 1.85 progeny from a single mating.

Another possible reason for the delay in male maturity in this fly is the production of large sperm. In their study of 42 species of *Drosophila*, Pitnick et al. show that larger sperm are more energetically costly to produce than shorter ones, and suggest that the post-eclosion maturation time of males may represent the time required to develop the large testes needed to manufacture sperm of a given length (24). In *Drosophila*, rapid female remating has been shown to be associated with exaggerated ejaculate traits in the form of sperm gigantism, seminal nutrient donations, or both (31). Given that sperm is 5.1 mm in length in *Z. indianus* (32), or a little more than 2.5 times the length of that in *Drosophila melanogaster* (33), it can therefore be considered semi-gigantic according to the sperm grouping standards established by Markow (31). It remains to be determined if

many of the seminal substances contained in the ejaculate are incorporated by females into their somatic and ovarian tissues.

1.4.2. Female reproductive strategies

Several observations made in laboratory and wild populations of *Z. indianus* suggest that females are sperm-limited. Compared to other *Drosophila* species in which female remating frequency has been determined, *Z. indianus* females remate quite frequently, a behavior that is not very surprising given that they produce much fewer progeny from one copulation compared to females of other species (Figure 4). Females in this species mate once per day in the laboratory and are considered rapidly remating according to a grouping system established by Markow (31). Further support for the one day delay in female stems from my observations in laboratory and wild populations of this fly, finding that recently-inseminated females display a body rocking movement in refusal of later male attempts to copulate, a behavior that seems to be unique to females of the genus *Zaprionus* (34). In addition, dissection of the post mating female reproductive tract provides evidence of an "insemination reaction" or the appearance of an opaque mass that prevents the female from remating temporarily (35). In the laboratory, females will continue to lay unfertilized eggs throughout most of their life when provided with a protein supplement such as yeast in their diet, a behavior that is not seen in all fruit flies. In addition, I found that females can increase their productivity by 50% by remating (Table 2) and based on progeny numbers, females seem to carry sperm from at least 2 males at a given time in the wild (Figure 1). Females in natural populations are expected to suffer a reduction in fitness when sperm is unavailable.

Those mating system characteristics I have examined allow me to predict that a female-biased operational sex ratio (OSR), or at least a less male-biased OSR compared to *D. melanogaster*, may exist in *Z. indianus*. The OSR, or the number of receptive females relative to the number of sexually mature males (36), is a reliable indicator of the time and space in which sexual selection takes place and provides insight to the intensity of male-male competition over females in the wild. My prediction is based upon (a) females having to remate frequently in this species because of their sperm limitation, making them sexually receptive every day and thus allowing for many mating opportunities for the males (b) males maturing sexually after females, (c) absence of sexual dimorphism in this species, (d) absence of male secondary sexual characters and special behavioral tactics (Hanna, personal observations), and (e) presence of sperm gigantism (32), which is a trait associated with delayed male sexual maturation (24). These mating system features suggest that male-male competition should be low in natural populations of this species relative to species such as *D. melanogaster*. This suggestion is further supported by the fact that most females were still fertile at 70 days of age and many still fertile at 100 days of age in the laboratory (Figure 3). While the age attained by these flies in nature is not known, if the flies live several months, many copulations could occur in a lifetime. Regardless, because females appear to mate often, sexual selection is expected to favor ejaculate characters useful in postmating competition as opposed to the precopulatory male behaviors or traits.

1.4.3. Invasive abilities

As mentioned in the introduction, *Z. indianus* is a fairly new drosophilid in the Americas. Although females in this species seem unable to oviposit in immature fruits with intact skin, they are considered an invasive species in the Americas because of their oviposition behavior in and around the ostiole of ripe figs (37), as well as their generalist feeding lifestyle. Invasive species are usually characterized by rapid growth and reproduction, high dispersal abilities, ecological competence, generalist lifestyles, phenotypic plasticity, and association with humans. While many fruit flies share similarities with *Z. indianus* with respect to their mating system, long age and maintained fertility of females at 100 days of age could be a reason allowing for their invasiveness. However, a lack of knowledge of average lifespan in most *Drosophila* in the wild leaves this observation an open question. Although I suggest that the delay in male maturity might contribute to inbreeding avoidance, this hypothesis could further be tested by studying the effect of inbreeding on reproductive fitness in this species and by examining the dispersal behavior of females upon eclosion. If my prediction of a female-biased OSR holds true for populations in the wild, the bet-hedging strategy observed in males and the frequent remating in females would clearly allow for genetic variability of future generations, and ultimately adaptability of the species to new resources, habitats, and environments.

1.5. Figures

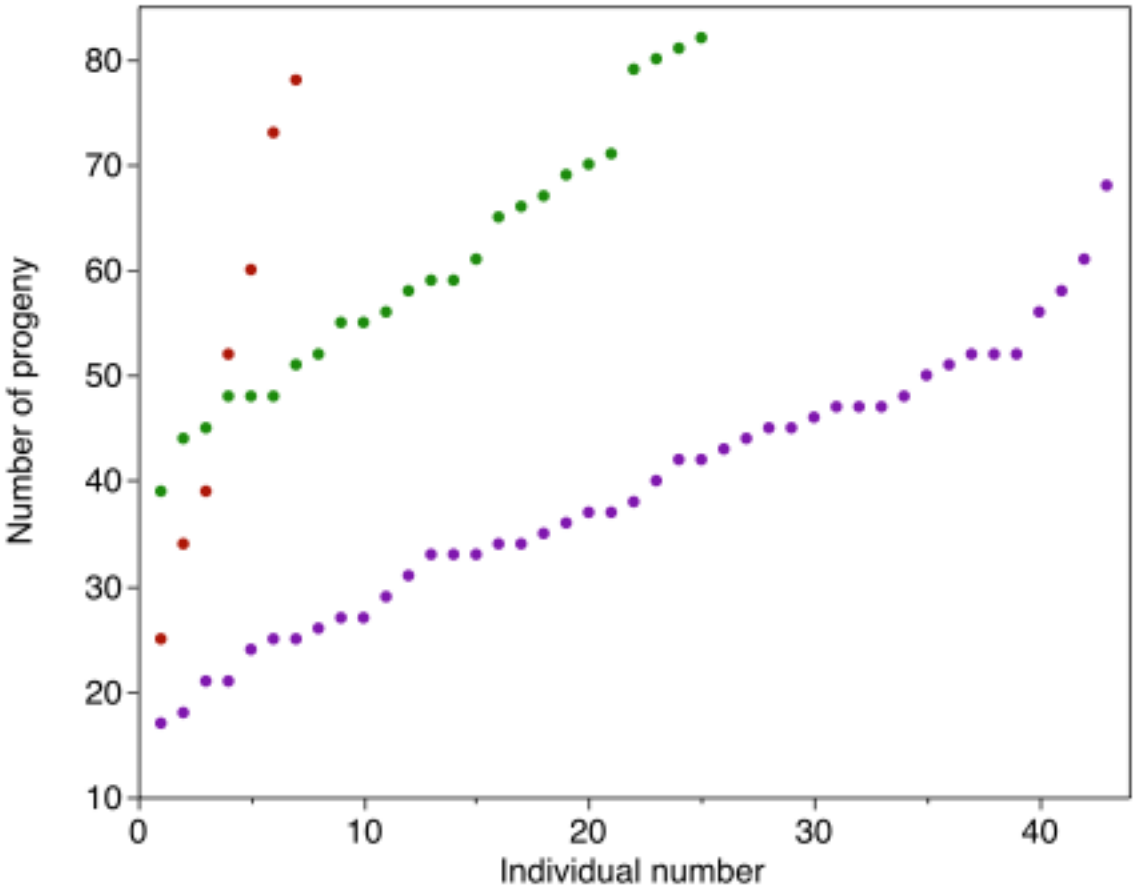

Figure 1: Productivity of females mated once in the laboratory (n = 43, purple markers), twice in the laboratory (n = 25, green markers), and caught from the wild (n = 7, red markers)

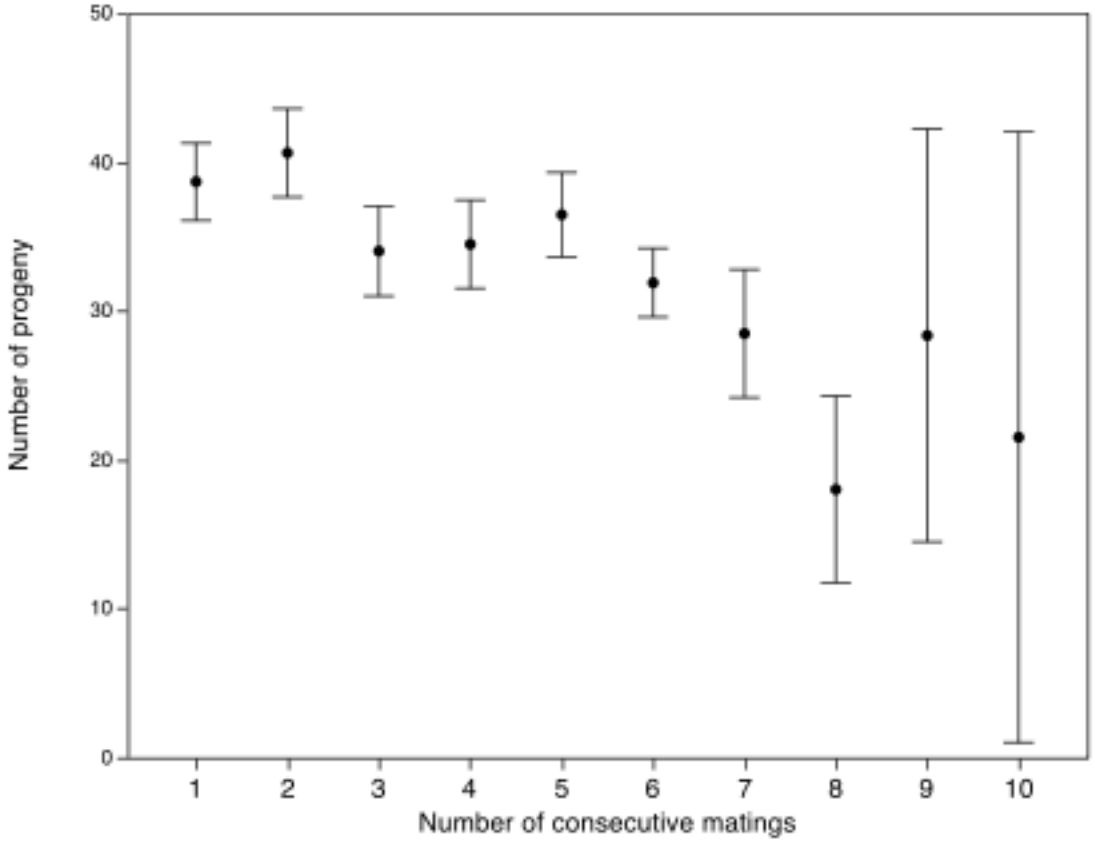

Figure 2: Average productivity of 23 males on their first through tenth consecutive matings. Note that only two males mated 10 times and thus the large range observed at this mating order.

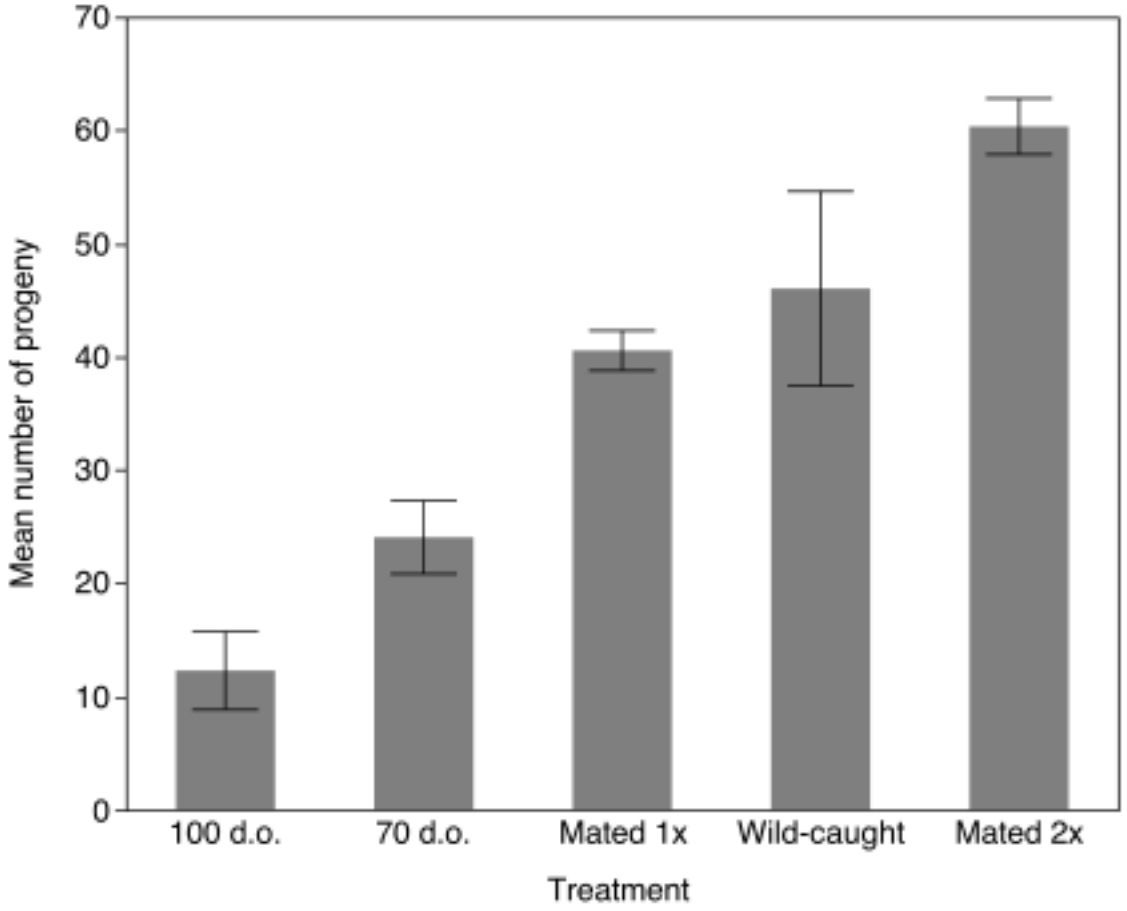

Figure 3: Mean progeny numbers produced from the mating once of females of 100 days of age, 70 days of age, and 10 days of age to sexually mature males of 10 day of age, as well as mean progeny produced by wild-caught females and females of 10 days of age mated twice to different virgin males.

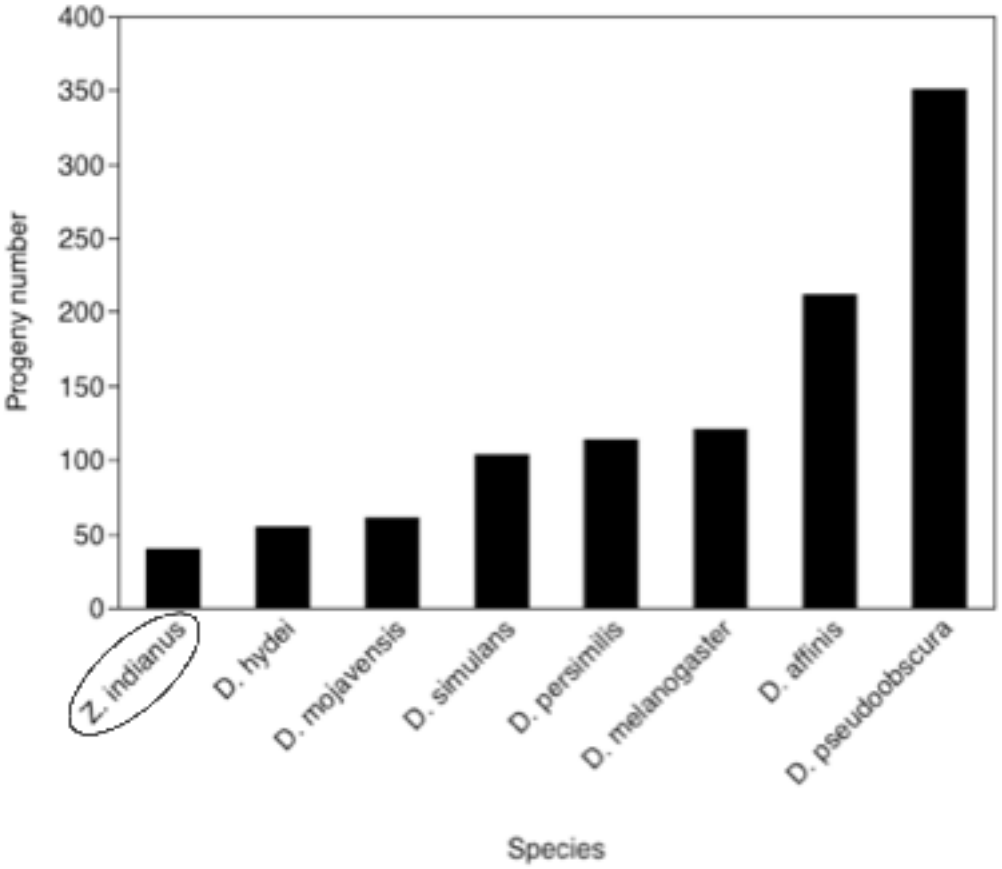

Figure 4: Average productivity of different species of Drosophilids from one mating in the laboratory.

1.6. Tables

Table 1: Ages at reproductive maturity in days for males and females in *Z. indianus*.

Sex	Age (days)	n mated/ n observed	Percent
Males	1	0/15	0
	2	1/30	3.3
	3	6/26	23
	4	21/29	72
	5	23/25	92
	6	25/25	100
Females	1	0/15	0
	2	10/40	25
	3	12/25	48
	4	20/24	83
	5	23/25	92
	6	25/25	100

Table 2: ANOVA of treatment differences in progeny of females mated once, twice, and wild-caught females in *Zaprionus indianus*.

Source of Variation	Sum of Squares	df	Mean Squares	F-ratio	P
Treatment	7328.0	2	3664.0	21.5	<0.0001
Error	12273.2	72	170.4		
Total	19601.1	74			

Table 3: ANOVA of treatment differences in progeny of the first 5 successive matings in males of *Z. indianus* to different virgin females.

Source of Variation	Sum of Squares	df	Mean Squares	F-ratio	P
Treatment	698.6	4	174.6	0.97	0.43
Error	18634.4	103	180.9		
Total	19333.0	107			

2. Chapter 2:

2.1. Introduction

There are over 2000 *Drosophila* species whose distributions range from narrowly restricted, single island endemics to panmictic, cosmopolitan taxa (38). One species, *Zaprionus indianus*, has recently expanded its geographical range from Africa into India, the Palearctic region, and the Americas. In 1998, *Z. indianus* was detected for the first time in the Americas in São Paulo City, São Paulo, Brazil, and Vilela proposed that it might have inadvertently been introduced by air travel due to the increasing number of flights from several African countries to São Paulo City (7). Since then, *Z. indianus* has succeeded in becoming abundant in almost every country in South America and was detected in Chiapas, Mexico in 2002 (10), Florida, U.S.A in 2003 (8), and California, U.S.A in 2006 (10). There are few records of this fly in countries north of Brazil and south of Mexico due to lack of collection in that region, with one report of this fly in Uruguay (22) and one in Argentina (39).

Introduced species offer an exciting opportunity for research in ecological and evolutionary genetics. Since only a few studies were undertaken in the Americas with respect to these kinds of questions, I decided to investigate the demographic history and phylogenetic relationship of the recently established metapopulation of *Z. indianus* in Mexico using DNA sequence data from a single mitochondrial marker, a segment of cytochrome c oxidase subunit I (COI). This preliminary study shows low genetic diversity of this fly worldwide, a continuous population decline starting long before the

colonization of this fly of the Americas, and supports a multiple introduction scenario in Mexico from three very different source populations.

2.2. Materials and Methods

2.2.1. Sampling

A total of 19 individuals of the Drosophilid *Zaprionus indianus* were collected in November 2011 from baits containing a mixture of rotting bananas, oranges, and mangoes, setup at a rural site in the town of Alamos, Sonora, Mexico. Six individulals were collected in January 2012 by Dr. Dan Lindsley in Oaxaca, Mexico. Also included in the study were Genbank sequences from a previous study (40) with 20 sequences for *Z. indianus* (Accession No. EF632353-72), two sequences for Z. africanus (Accession No. EF632373-4), one sequence for Z. gabonicus (Accession No. EF632375), one sequence for Z. megalorchis (Accession No. EF632376), and one sequence for Z. tuberculatus (Accession No. FJ948781).

2.2.2. DNA extraction and amplification

Total genomic DNA was extracted from individual flies using the DNeasyTM (QIAGEN Inc., Valencia, CA) protocol. The polymerase chain reaction (PCR) was used to amplify a segment of the COI gene with primers LCO1490f (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198r (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') using standard PCR conditions (41). All PCR cycling conditions included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s of denaturation, 45 °C for 1 min of annealing, and 72 °C for 1 min of extension, with a final extension of 7 min at 72 °C. Verification of successful amplification was assessed by agarose gel electrophoresis.

Sequencing reactions were performed on an Applied Biosystems (Foster City, CA) ABI 3730XL DNA sequencer at the Eurofins DNA Sequencing Facility, Petaluma, California, using the amplifying primers. Sequences were proofread and aligned in Sequencher 4.1 (GeneCodes Corp.) followed by manual editing. Sequences were trimmed to remove ambiguous sites, resulting in a final segment of 658 bp. Aligned sequences were translated in MEGA version 3.1 (42) using the invertebrate mitochondrial genetic code; no stop codons or indels were found. Calculations of genetic distances among sequences [K2P distances (43)] were carried out in MEGA. Calculations of genetic diversity indices were performed in DnaSP version 4.10 (44).

2.2.3. Population genetic analyses

An analysis of molecular variance (AMOVA, 45) performed in ARLEQUIN version 3.1 (46) was used to test for population structure in *Z. indianus* for the individuals sampled from Alamos (N = 19) and Oaxaca (N = 6), Mexico.

2.2.4. Phylogenetic analyses

Relationships among COI haplotypes from the entire data set were initially assessed with the neighbor-joining (NJ) algorithm of Saitou and Nei (47) carried out in MEGA using K2P distances. The Mexican haplotypes partitioned into three monophyletic clades. Because of a lack of population structure detected at the localities of Alamos and Oaxaca, Mexico, all analyses of demographic history were conducted on the metapopulation combining the individuals from both localities.

Bayesian analyses were implemented in MrBayes version 3.1 (48). Two separate runs were conducted with identical results. The model of nucleotide substitution that best fit the data set, determined with Modeltest 3.7 (49) using the Akaike Information Criterion, was GTR+G. Bayesian analyses were run under the parameters of this model (nst = ‘‘6”; rates = ‘‘gamma”) for 5,000,000 generations, sampled every 250th generation (20,000 trees sampled), using the default random tree option to begin the analysis. Clade support, expressed as posterior probabilities, was estimated utilizing a Markov chain Monte Carlo (MCMC) algorithm. Log-likelihood values from four simultaneous MCMC chains (three hot and one cold) stabilized at about 3500 generations. The first 14 trees, therefore, were discarded from the analysis (burnin = 14). Another drosophilid, *Drosophila mojavensis* (Genbank Accession No. DQ383715), was used as the outgroup.

2.2.5. Demographic analyses

Statistical tests designed to assess whether nucleotide polymorphisms deviate from expectations under neutral theory [Tajima’s D (50) and Fu’s FS (51)] were carried out in ARLEQUIN. The positive values for Fu’s F_S seen from individuals sampled in Alamos, Oaxaca, as well as the pooled number of individuals suggested that the metapopulation that exists in Mexico had experienced a recent population bottleneck or overdominant selection. I further explored the demographic history of this metapopulation, utilizing three different tests of the sequence data: (a) analysis of the distribution of pairwise sequence differences (mismatch distribution; 52) performed in ARLEQUIN; (b) Bayesian skyline analysis implemented in BEAST version 1.2 (53); and (c) estimation of changes in population size carried out in FLUCTUATE version 1.4 (54).

For populations that have undergone an historical expansion, plots of the distribution of pairwise differences among haplotypes are expected to be unimodal, whereas populations in equilibrium generally show a multimodal distribution (52). Under the sudden expansion model the parameters generated are τ, the time to the population expansion (=2ut, where u is the mutation rate for the entire gene segment and t is the number of generations since the expansion), and the mutation parameters θ_0 and θ_1, where $\theta_0 = 2uN_0$, and $\theta_1 = 2uN_1$ (N_0 and N_1 are the population sizes before and after the expansion, respectively) (55). The significance of the estimated parameters is obtained by calculating the sum of square deviations (SSD) statistic and the raggedness statistic (rg; 52), and their corresponding P values (46). The sudden expansion model is rejected when $P < 0.05$.

The Bayesian skyline analysis utilizes MCMC sampling of sequence data to estimate a posterior distribution of effective population size through time (53). Bayesian skyline analyses were run under the conditions of the GTR + G model (four gamma categories). The mean mutation rate per site per generation (l) was set at 1.15×10^{-8}. I arrived at this rate by assuming (i) an average pairwise sequence divergence rate of 2.3% per million years (Brower, 1994) and (ii) a generation time of one year. The number of grouped intervals (m) was set to ten. Five million iterations of the MCMC chains were run, sampling every1000 iterations; the first 500,000 chains were discarded as burnin. The Bayesian skyline plots were generated with TRACER version 1.2.1 (53).

The FLUCTUATE program provides an estimate of long-term female effective population size (N_{ef}) and evaluates whether N_{ef} has changed or remained stable over time

(54). The simultaneous maximum-likelihood estimates of the mutation parameter θ (where $\theta = 2N_{ef}\mu$) and the exponential population growth parameter (g) were obtained from a final extended run of ten short chains of 100,000 steps each and two long chains of 200,000 steps each, sampling every 20th step. Initial estimates of θ were based on number of segregating sites (56), with the random tree default setting selected for the starting genealogy.

2.3. Results

2.3.1. Genetic diversity and population genetics

Genetic diversity indices for *Z. indianus* are shown in Table 4. Values for both haplotype diversity (h) and nucleotide diversity (π) were greater in Oaxaca than in Alamos. For both regions, however, nucleotide diversity was low (π = 0.00747-0.01003) and haplotype diversity was relatively high (h = 0.591-0.600). Overall values for the combined regions are also given in Table 4 for comparison. Fu's F was not significant in both individual and combined regions, and Tajima's D was only significant for individuals sampled in Oaxaca. Relative rates tests (Tajima, 1993) were not significant in *Z. indianus*, indicating that a molecular clock could not be rejected.

The AMOVA conducted on populations from Alamos and Oaxaca (Table 5) revealed a lack of population structure (F_{ST} = 0.057, P = 0.30) between these localities, with 94.32% of the variation existing within populations of *Z. indianus* in Alamos and Oaxaca and 5.68% among them.

2.3.2. Phylogenetic relationships

Initial NJ analyses of *Z. indianus* COI sequences showed that the Mexican haplotypes resolve into 3 clades (Figure 5) that are part of 5 clades worldwide (Figure 6). Clade I was comprised of individuals from Western Africa, Western Europe, and Eastern USA. Clade II was comprised of individuals from the Eastern Mediterranean. Clade III was comprised of individuals from Central Africa and South America. Individuals from all three clades were found in Mexico. Mean genetic distance between clades I, II, and III

was 1.1% with a maximum K2P distance of 1.7%. The mean genetic distance between world clades was 1% with a maximum K2P distance of 2.3%. Maximum parsimony (not shown) and Bayesian analyses (Figure 7) confirmed the partitioning of clades I, II, and III, but support for the split was weaker in the Maximum parsimony tree.

2.3.3. Historical demography

Because of the lack of population structure between Alamos and Oaxaca in Mexico ($F_{ST} = 0.057$, $P = 0.30$), individuals from both localities were combined for the tests of demographic history. A plot of the distribution of pairwise differences among COI haplotypes in Mexico (Figure 8) conformed to expectation for populations that have been stable in time. The mismatch distribution test statistics SSD and rg were small and not statistically significant (Table 6), indicating that the sudden expansion model could not be rejected.

Results of analyses of COI sequence data using FLUCTUATE were consistent with those of the mismatch distribution. The exponential population growth parameter (g) was negative and significantly different from zero (Table 7), indicating a population decline. The Bayesian skyline plot (Figure 9) showing the estimated changes in median N_{ef} over time was in agreement with results from the mismatch distribution and FLUCTUATE, and supported a continuous decline in population size beginning ~870,000 years ago.

2.4. Discussion

2.4.1. Demographic history

Results of different tests of demographic history were generally congruent and suggested that *Z. indianus* has been experiencing a continuous population decline since ~ 870,000 years ago. Although values for the sum of square deviations (SSD) and raggedness (rg) statistics are not significant and thus a sudden expansion model cannot be rejected, the Bayesian analysis and FLUCTUATE results clearly suggest a population decline. The Bayesian skyline plot (Figure 9) shows that the decline began ~ 870,000 years ago with an initial population size of ~ 400,000 individuals, and continued to decline till this day to a size of ~ 87,000 individuals.

As described earlier, I have assumed a standard 2.3% molecular clock and a generation time of one year in the tests of demographic history. Although generation times of *Z. indianus* in the wild are not known, it is unlikely that one generation is only produced each year. A current study of the mating system of *Z. indianus* supports rapid remating frequencies in both sexes and suggests that multiple generations are produced every year (Hanna and Markow, unpublished manuscript). Because of the various assumptions associated with the estimation of μ, the mean mutation rate per site per generation for *Z. indianus*, the time axis in the Bayesian skyline plot (Figure 9) should only be considered a rough estimate. But regardless of the number of generations per year, it is apparent that *Z. indianus* experienced a worldwide decline rather than a local one. This fly was never documented in the Americas prior to 1998 (7); it is therefore impossible for the decline

observed in the Bayesian skyline plot to represent the population of this fly in Mexico, but instead refers to a global behavior exhibited by this fly.

2.4.2. Colonization history of the Americas

Two possible scenarios are proposed for how *Z. indianus* colonized the Americas. The first one suggests that a large propagule carrying most of the genetic variation was introduced only once into Brazil in 1998, with following generations of this fly beginning their colonizing route North and reaching the U.S. in 2003. This scenario has been supported by chromosomal (57) and allozymic studies (58). It would also assume that *Z. indianus* individuals from Fayum Egypt, Haifa Israel, or possibly other countries in the Eastern Mediterranean and North Africa would disperse West into Central or North-West Africa, be carried by air travel into São Paulo, Brazil, as proposed by Vilela (7), then colonize the Americas by passive or active dispersal.

The second scenario suggests that multiple propagules were introduced at different times in the Americas and that different waves of introductions took place during the colonization of this fly of Mexico. Although dispersal has not been studied in this species, the speed of colonization of South, Central, and North America suggest rapid dispersal. To my knowledge, the only other demographic study of this fly was undertaken in Egypt in 2009 where the authors support that Egyptian populations are descendent of two allochronic events of colonization: one from an older northward range expansion from Africa via the Nile valley, and a more recent one from Asia via fruit trade.

2.4.3. Conclusions

The decline in population size suggested by the different demographic history tests is very interesting. Invasive species are usually expected to show high levels of genetic diversity facilitating their adaptation to new environments. However, a low number of founders carrying the appropriate genetic variability in Brazil might have been enough for this fly to colonize the Americas. At this point, sampling several populations of this fly is necessary to provide insight to the degree of gene flow between populations, population structure, as well as verify the decline in population size suggested by this study.

2.5. Figures

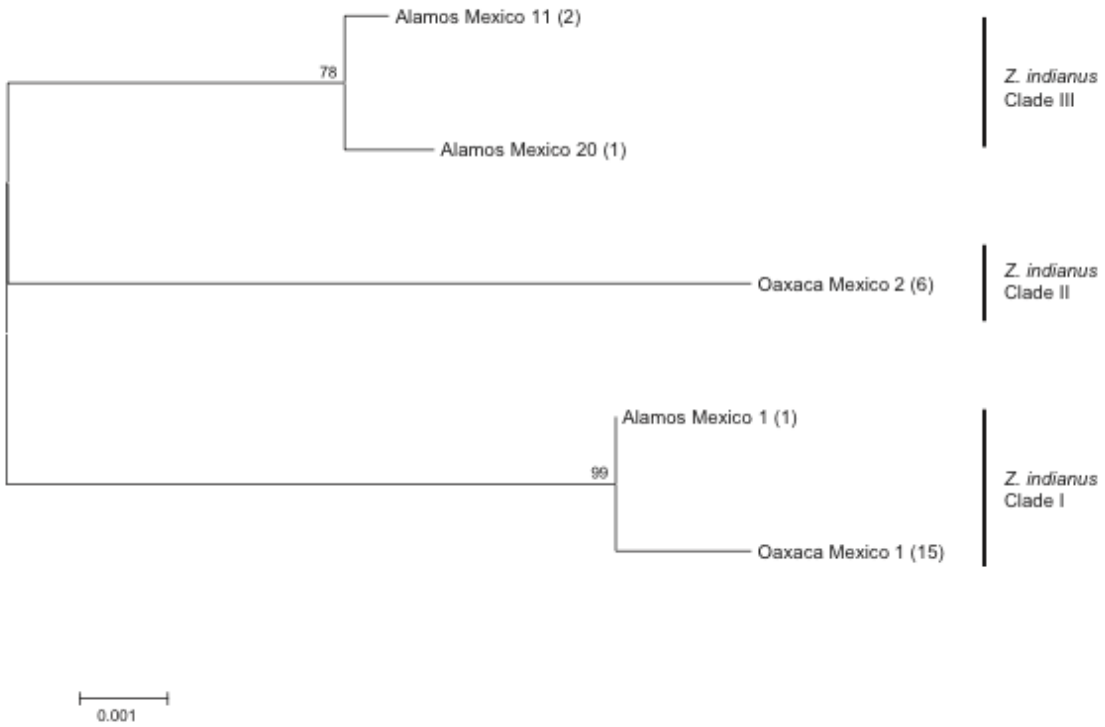

Figure 5: Unrooted neighbor-joining tree obtained using each of the five COI haplotypes found in the drosophilid *Zaprionus indianus* from Alamos and Oaxaca, Mexico. Clade support values are shown on branches. Branch terminals are labeled with locality and sample identification number. The pooled number of individuals with the same haplotype from both localities is given in parentheses.

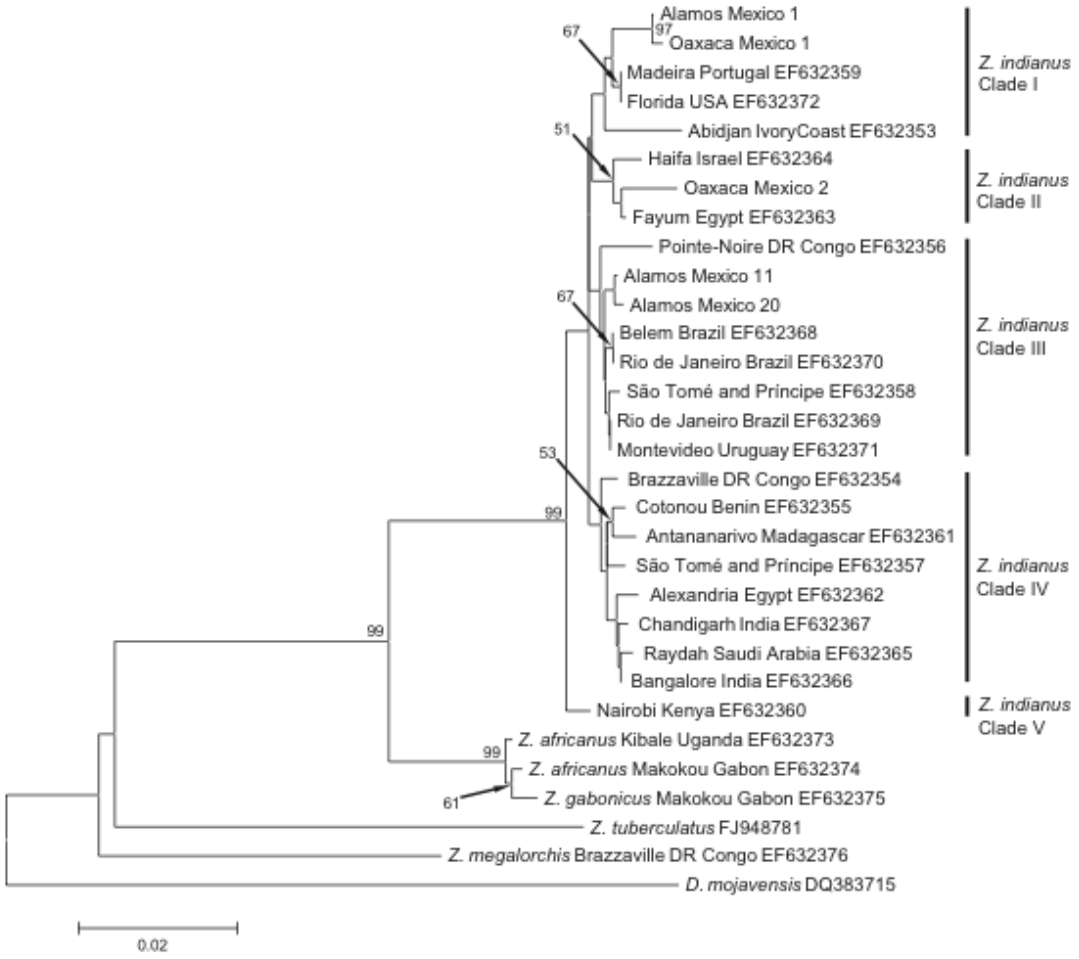

Figure 6: Neighbor-joining tree obtained using each of the COI haplotypes found in the drosophilid *Zaprionus indianus* from Alamos and Oaxaca, Mexico, and other world localities obtained from Genbank. Clade support values are shown on branches, and values with <50% support were removed. Branch terminals are labeled with locality and sample identification number.

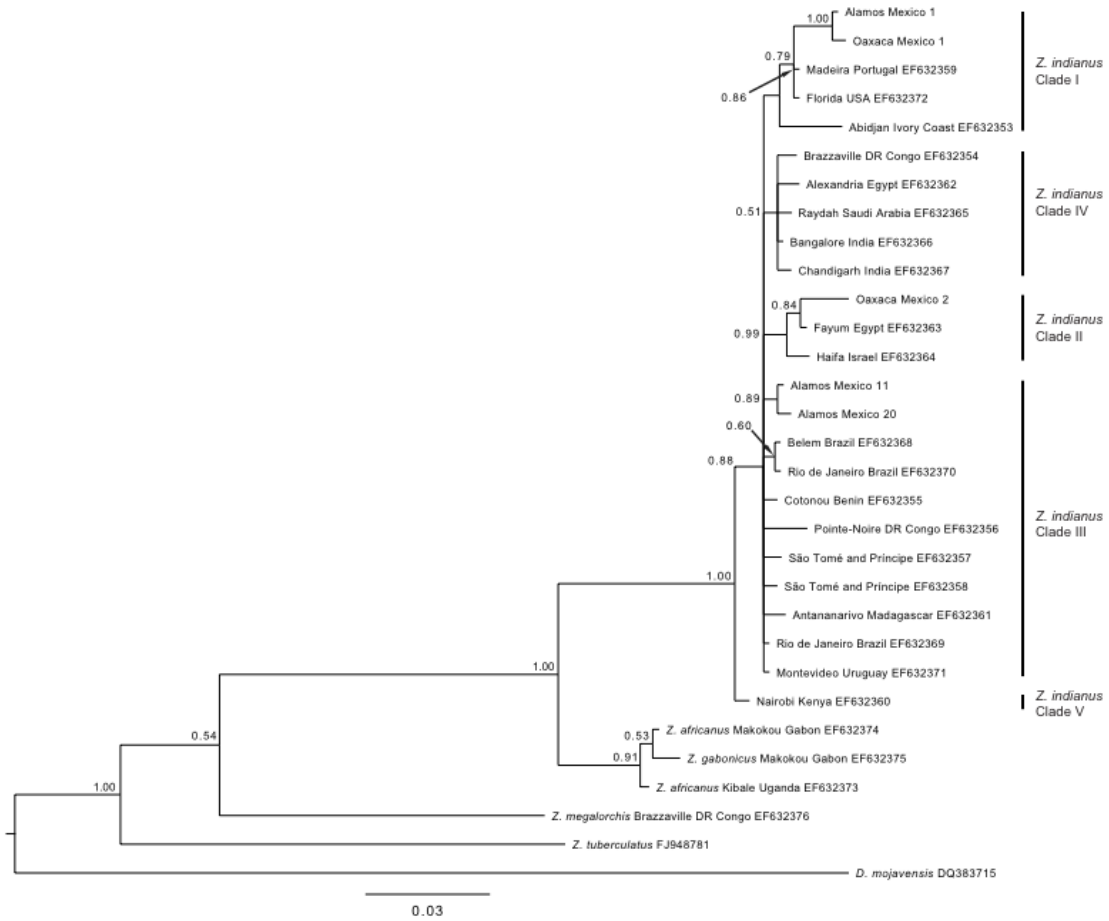

Figure 7: Bayesian 50% majority rule consensus tree showing relationships among haplotypes in *Zaprionus indianus* collected from Alamos and Oaxaca, Mexico and other world localities obtained from Genbank, and based on analysis of a 658 bp segment of the COI gene. The tree was rooted with the drosophilids *Z. megalorchis, Z. tuberculatus, and D. mojavensis*. Clade support expressed as posterior probabilities is shown above branches. Scale shows substitutions per site. Branch terminals are labeled with locality and sample identification number.

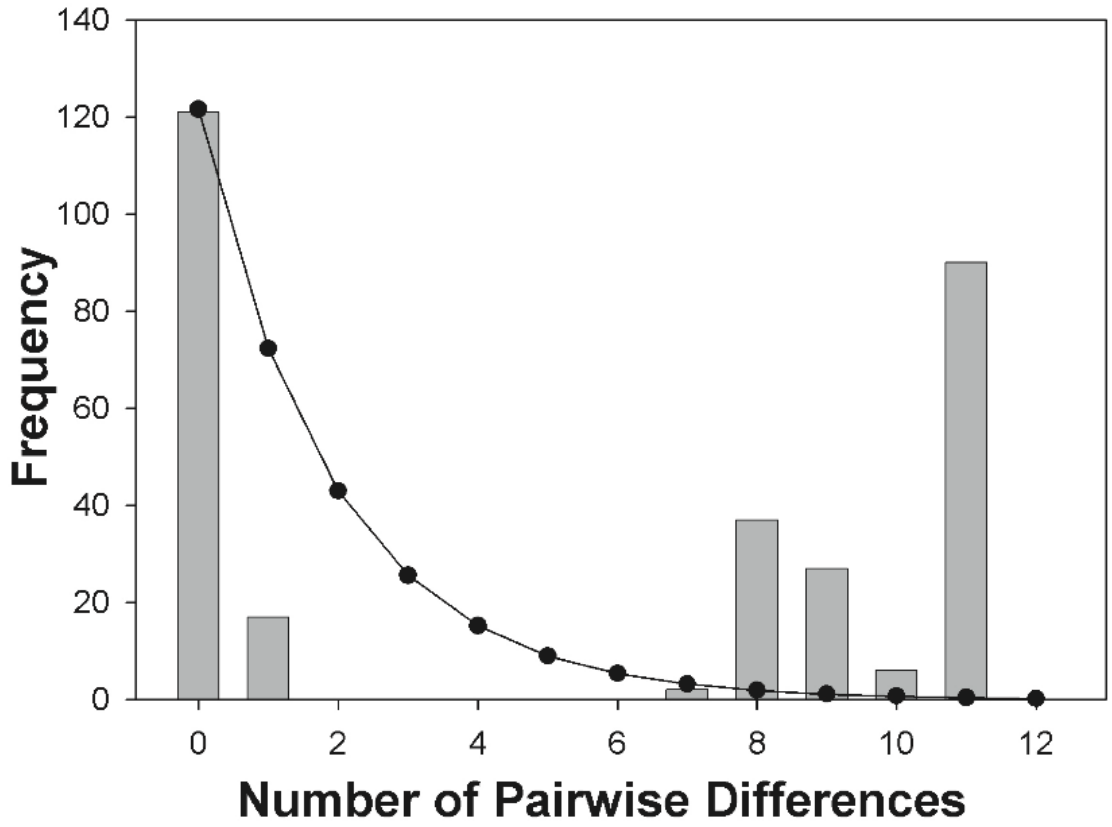

Figure 8: Distribution of pairwise differences among COI haplotypes (mismatch distribution) in the drosophilid *Zaprionus indianus* individuals sampled from Mexico (vertical bars). The solid line represents the expected distributions exhibited by stable populations. The multimodal distribution of observed pairwise differences expected for populations which have been relatively stable is seen in the plot.

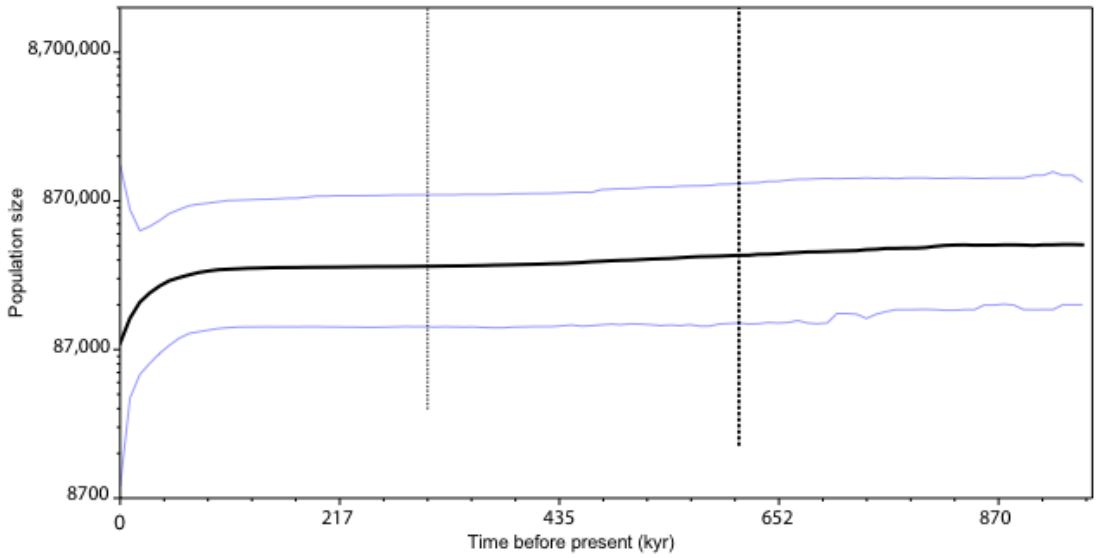

Figure 9: Bayesian skyline plot showing change in effective female population size (Nef) over time for the drosophilid *Zaprionus indianus*. Population size is given on a logarithmic scale. A value of 1.15×10^{-8} for μ, the mean mutation rate per site per generation, was assumed. The thick solid line represents the median estimates of population size; the thin solid lines show the 95% HPD (highest posterior density) intervals.

2.6. Tables

Table 4: Summary of genetic diversity indices and results of neutrality tests (Tajima's D and Fu's F_s) in the COI gene segment in the Drosophild *Zaprionus indianus*.

Locality	N	L	k	K	h (±SD)	π (±SD)	Tajima's D	Fu's F_S
Alamos & Oaxaca	25	658	14	5	0.597 ± 0.090	0.00821 ± 0.00127	1.58	5.245
Alamos	19	658	14	5	0.591 ± 0.118	0.00747 ± 0.00166	0.839	3.71
Oaxaca	6	658	11	2	0.600 ± 0.129	0.01003 ± 0.00216	2.23*	6.46

N, number of sequences; L, sequence length (bp), k, number of variable sites: K, number of haplotypes; h, haplotype diversity; π, nucleotide diversity.
* Significant at the 0.05 level.

Table 5: Analysis of molecular variance (AMOVA) for populations of *Zaprionus indianus* collected from two localities: Alamos and Oaxaca, in Mexico.

Source of Variation	df	Sum of Squares	Variance Components	% of Variation
Among Populations	1	4.089	0.15898 Va	5.68
Within Populations	23	60.711	2.63959 Vb	94.32
Total	24	64.8	2.79857	

Table 6: Results of the mismatch distribution of COI sequences of the drosophilid *Zaprionus indianus* from the pooled number of individuals (N = 25) caught in Alamos and Oaxaca, Mexico.

τ (95% CI)	θ_0	θ_1	SSD	rg
10.4 (0.000, 96.635)	0.000	1.466	0.176 (P = 0.113)	0.311 (P = 0.186)

Table 7: Effective female population size (N_{ef}) and exponential growth rate (g) in the drosophilid Zaprionus indianus calculated with FLUCTUATE.

No. of COI sequences	θ	N_{ef}	g (1/μ generations)
25	0.003452 (±0.00140)	1.50×10^5	-1.94 (±1.79)

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