

## Survey of the Genetic Diversity of *Phormia regina* (Diptera: Calliphoridae) Using Amplified Fragment Length Polymorphisms

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**ABSTRACT** There is very little information concerning carrion fly population genetic structure. We generated amplified fragment length polymorphism (AFLP) profiles for the common blowfly, *Phormia regina* (Meigen), from sites spanning the contiguous United States. Analysis of molecular variance (AMOVA) based on 232 loci found significant variation ( $\Phi_{SC} = 23\%$ ) among discrete samples (those collected at a bait in one location over a short period of time). Samples collected in the same location but at different times were also distinct. When samples were pooled into geographic regions (east, central, west), the variation was negligible ( $\Phi_{CT} = 0\%$ ). A Mantel test found only a very weak correlation between individual genetic and geographic distances. Relative relatedness coefficients based on shared allele proportions indicated individual samples were likely to contain close relatives. *P. regina* arriving at an individual carcass typically represent a nonrandom sample of the population despite a lack of geographic structure. A female blow fly produces hundreds of offspring at one time; therefore, newly emerged siblings may respond in concert to an odor plume. These results may be of interest to forensic entomologists, many of whom use a laboratory colony founded from a small sample for the growth studies that support casework. Discrepancies between published growth curves may reflect such random differences in the founding individuals.

**KEY WORDS** forensic entomology, genotype, blow fly, inbreeding, genetic assignment

Blow flies (Calliphoridae) are ubiquitous and prominent decomposers (Putnam 1978), and a few species are specialized vertebrate parasites (Spradbery 1994). The study of calliphorids has made fundamental contributions to many areas of biology. Perhaps the most notable studies have been in ecology (Ullyett 1950, Nicholson 1958, Denno and Cothran 1976, Ives 1991), physiology (Wigglesworth 1936, Dethier 1955, Fraenkel and Zdarek 1970, Stoffolano 1976, Saunders 1987), human and livestock health (Zumpt 1965, Greenberg 1973, Hall and Wall 1995), and forensic investigation (Smith 1986, Byrd and Castner 2001, Greenberg and Kunich 2002).

It is relatively easy to observe adults or larvae at the carcass or live host but more difficult elsewhere. The adults are strong fliers (Norris 1965), and mark and recapture experiments suggested that marked individuals initially dispersed randomly (MacLeod and Donnelly 1957, 1958, 1962).

The models used for forensic analysis, mostly consisting of laboratory developmental data and succession patterns from animal carcasses (Kamal 1958, Greenberg 1991, Anderson 2000, Byrd and Allen 2001, Grassberger and Reiter 2001), originate from a small

number of geographic locations. Although some of these publications seem to show biological differences between regional populations of the same species, differences in experimental methods make it difficult to interpret these apparent conflicts (Tarone and Foran 2006). Although studies of other insects found developmental variability in different genetic backgrounds (Parsch et al. 2000, Blanckenhorn 2002), only two published studies directly examined calliphorid growth rates as a function of geographic source. Cyr (1993) failed to find regional variation in *Phormia regina* (Meigen) from across the United States, whereas Tarone (2007) described a significant difference in growth rates between California and either Michigan or West Virginia *Lucilia sericata* (Meigen).

A variety of approaches have been used for calliphorid population genetic questions. Morphological and biochemical measurements of *Lucilia cuprina* (Wiedemann) showed differentiation between adjacent populations on sheep and on garbage dumps (Clarke and McKenzie 1987). Mitochondrial haplotype surveys detected no regional variation on a continent-wide scale for some common blow fly species (Hall et al. 2001, Lyra et al. 2005, Boehme 2006). Individual *L. sericata* in southern England could be distinguished, even within a single sheep strike, based on randomly amplified polymorphic DNA (RAPD) profiles (Stevens and Wall 1995, 1996). Microsatellite

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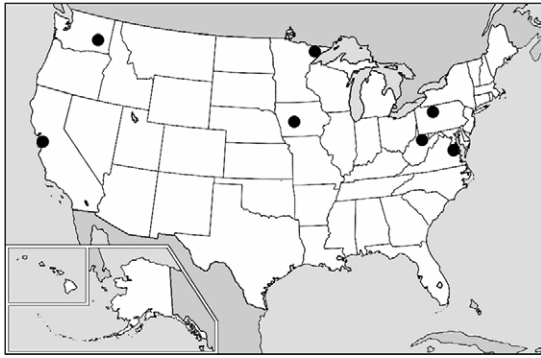


Fig. 1. Map of United States (<http://wikimedia.org>) showing sample collection locations. Each circle represents a single sample collection with the exception of WV (six samples collected) and WA (four total samples collected).

typing methods developed for the parasitic screw-worm fly *Cochliomyia hominivorax* (Coquerel) failed to find significant population structuring across Uruguay, reinforcing the idea of panmixis (Torres et al. 2004, 2007). Microsatellite typing methods were described for the carrion feeding *L. illustris* (Meigen) and *L. sericata* (Florin and Gyllenstrand 2002) in northern Europe. However, we found that, with our North American calliphorid specimens, these methods either failed to amplify a locus or an amplified locus was monomorphic (J.D.W., unpublished data).

Amplified fragment length polymorphisms (AFLP) (Vos et al. 1995) provide an alternative to the labor-intensive and costly development of microsatellite methods for new species. This polymerase chain reaction (PCR)-based method for detecting restriction site polymorphisms can be applied directly to new species in the absence of prior genomic information. Additionally, the large number of loci potentially produced can be used to infer population genetic structure (Reineke et al. 1999, Cervera et al. 2000, Timmermans et al. 2005, Nunez et al. 2006).

We report here the first nuclear DNA population genetic survey of a North American blow fly, based on the widespread and common species *Phormia regina*. *P. regina* has been implicated as a veterinary pest (Knipling and Rainwater 1937, Deonier 1942), but its greatest applied importance is as one of the most commonly used indicators of the postmortem interval (PMI) for North American death investigations (N. H. Haskell, personal communication).

**Materials and Methods**

**Wild Fly Sampling.** Adult *P. regina* for a population survey were collected by hand net at a decayed meat bait. A wild “sample” consisted of individuals captured at one place during a maximum 30-min collection period (Fig. 1; Table 1). Ten individuals from each sample were stored at  $-20^{\circ}\text{C}$  until processed for genetic analysis.

**Laboratory-bred Flies.** Once it became apparent that wild flies in a sample were highly related (see

Table 1. Fly collection locations and dates

| Sample name | County         | Location            | Date                   |
|-------------|----------------|---------------------|------------------------|
| CA          | Alameda        | Berkeley, CA        | 11 Nov. 1997           |
| IA          | Madison        | Winterset, IA       | 4 July 2004            |
| MN          | St. Louis      | Orr, MN             | 15 May 2001            |
| PA          | Bradford       | Bradford, PA        | 25 Aug. 2006           |
| VA          | Prince William | Quantico, VA        | 18 May 1999            |
| WA1         | Whitman        | Lyle Grove, WA      | 9 Oct. 2006            |
| WA2         | Benton         | Prosser, WA         | 10 Oct. 2007           |
| WA3         | Columbia       | Tucannon River, WA  | 9 Oct. 2006            |
| WA4         | Whitman        | Lyle Grove, WA      | 18 July 1999           |
| WV1         | Monongalia     | Morgantown, WV      | 1 May 2005             |
| WV2         | Monongalia     | Morgantown, WV      | 20 May 2006            |
| WV3         | Monongalia     | Morgantown, WV      | 27 May 2005            |
| WV4         | Monongalia     | Morgantown, WV      | 9 Aug. 2006            |
| WV5         | Monongalia     | Coopers Rock, WV    | 21 Aug. 2006           |
| WV6         | Preston        | Bruceeton Mills, WV | 17 Aug. 2006           |
| Pr1         | Monongalia     | Morgantown, WV      | Summer 2007 lab colony |

All samples were stored at a minimum of  $-20^{\circ}\text{C}$  until used for DNA extraction.

Results), full siblings were generated in the laboratory for comparison. Ten individuals were reared from a single clutch of eggs. Both parents were from the first generation produced by flies collected in Morgantown, WV.

**DNA Extractions.** Thoracic tissue (Sperling et al. 1994) was extracted using a DNeasy Kit (Qiagen, Valencia, CA) from each fly according to the manufacturers’ protocol. DNA samples were stored at  $-20^{\circ}\text{C}$  until further use.

**AFLP Amplifications.** All primers and adaptors were purchased from Integrated DNA Technologies (Coralville, IA). Genomic DNA digests were done using the following: 2 U *Pst*I (Promega, Madison, WI), 2 U *Eco*RI (Promega), 5  $\mu\text{l}$  10 $\times$  Buffer H (Promega), 0.5  $\mu\text{l}$  bovine serum albumin (BSA; Promega), 35  $\mu\text{l}$  genomic DNA, and PCR water for a total volume of 50  $\mu\text{l}$ . Digests were done at  $37^{\circ}\text{C}$  for 3 h, followed by denaturing of the enzymes at  $70^{\circ}\text{C}$  for 15 min. Double-stranded AFLP adaptors were made according to the following: 3  $\mu\text{g}$  of each adaptor (Table 2), 6  $\mu\text{l}$  10 $\times$  Buffer H (Promega), and water to a total volume of 120  $\mu\text{l}$ . Each solution was heated to  $65^{\circ}\text{C}$  for 10 min,  $37^{\circ}\text{C}$  for 10 min, and  $25^{\circ}\text{C}$  for 10 min and stored at  $-20^{\circ}\text{C}$ . Ligation of the adaptors to the digested DNA was done using the following: 1  $\mu\text{l}$  of each adaptor, 1  $\mu\text{l}$  of DNA Ligase buffer 1U T4 DNA Ligase (Pro-

Table 2. Primer and adaptor sequences

| Name                   | Sequence (5’-3’)                            |
|------------------------|---|
| <i>Eco</i> RI adaptors | CTCCTAGACTGCGGTACC AATTGGT<br>ACGCAGTCTAC   |
| <i>Pst</i> I adaptors  | CTCCGTAGACTGCGGTACATGCA TGT<br>ACGCAGTCTACG |
| <i>Eco</i> RI+A        | GACTGCGTACCAATTCA                           |
| <i>Pst</i> I+A         | GACTGCGTACATGCAGACA                         |
| <i>Pst</i> I+AAAC      | GACTGCGTACATGCAGACAAC                       |
| <i>Pst</i> I+ACC       | GACTGCGTACATGCAGACACC                       |
| <i>Pst</i> I+AGT       | GACTGCGTACATGCAGACAGT                       |
| <i>Pst</i> I+ATC       | GACTGCGTACATGCAGACAT                        |

**Table 3.** *Phormia regina* AMOVAs (% = percentage of molecular variance attributed to each source category)

| Source                | df  | SS      | MS   | Estimated variance | Percentage | Statistic   | Value  | P     |
|-----------------------|-----|---------|------|--------------------|------------|-------------|--------|-------|
| Among regions         | 2   | 188.4   | 94.2 | 0.00               | 0%         | $\Phi_{CT}$ | -0.002 | 0.65  |
| Among samples/regions | 12  | 1,159.5 | 96.6 | 7.22               | 23%        | $\Phi_{SC}$ | 0.23   | 0.001 |
| Within samples        | 135 | 3,295.0 | 24.4 | 24.4               | 77%        | $\Phi_{ST}$ | 0.23   | 0.001 |
| Total                 | 149 | 4,642.9 |      | 31.62              | 100%       |             |        |       |

P for  $\Phi_{SC}$ ,  $\Phi_{CT}$ , and  $\Phi_{ST}$  are based on 999 permutations across the full data set.

mega), and water to total 10  $\mu$ l. This was added directly to the 50  $\mu$ l of digested DNA and held at room temperature for 3 h with gentle agitation every hour. Two microliters of ligated DNA was added to the following preselective amplification: 10  $\mu$ l 2 $\times$  Promega Master PCR Mix (Promega), 1  $\mu$ l of 25 pg/ $\mu$ l *EcoRI*+A primer, 1  $\mu$ l of 25 pg/ $\mu$ l *PstI*+A primer, and 6  $\mu$ l water. The thermal cycler program included an initial denaturation at 94°C for 2 min and 26 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Each amplified product was diluted with 100  $\mu$ l water, and 1  $\mu$ l was used in the following selective amplification: 10  $\mu$ l 2 $\times$  Promega Master PCR Mix, 6  $\mu$ l water, 1.5  $\mu$ l *EcoRI* FAM-labeled primer (Applied Biosystems, Foster City, CA), and 1.5  $\mu$ l of one of four *PstI* selective primers (Table 2) using the following thermal cycler conditions: an initial denaturation at 94°C for 2 min, followed by 12 cycles of touchdown PCR with 94°C for 30 s, 65–56°C for 30 s, and 72°C for 1 min, followed by 27 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

**Sample Preparation and Electrophoresis.** PCR product (0.5  $\mu$ l) was added to 9.7  $\mu$ l HiDi formamide (Applied Biosystems) and 0.3  $\mu$ l LIZ600 size standard (Applied Biosystems) and run on a 3130xl Genetic Analyzer (Applied Biosystems) using the following conditions on a 50-cm 16-capillary array: oven temperature of 60°C, injection voltage of 1.6 kV, injection time of 15 s, and run voltage of 15 kV for a total run time of 30 min.

**Data Analysis.** Raw data from the genetic analyzer was imported into GeneMapper v 4.0 (Applied Biosystems). AFLP default settings were used with the exceptions of: peak detector setting for blue was set to 500 rfu, a global southern method was used for sizing, and the analysis range was from 90 to 500 bp. Once all samples were analyzed in GeneMapper, each primer set genotype was exported to Excel (Microsoft, Redman, WA). At the time of this analysis, the number of allele sizes we observed exceeded the maximum number of columns in an Excel file. Therefore, the data were cropped by using only alleles that were observed in either >6.25% (a modification of criteria used by Gompert et al. 2006) or <90% (to avoid mostly monomorphic loci) of individuals. This resulted in the scoring of 232 polymorphic alleles from the four primer combinations.

**Population Differentiation.** Analysis of molecular variance (AMOVA) was performed using GenAlEx 6.1 (Peakall and Smouse 2006). The hierarchical test

shows the partitioning of molecular variance within samples, among samples, and among regions to determine the overall structure of the genotypes. Geographic regions were arbitrarily defined as west (WA1, WA2, WA3, WA4 and CA samples; see Table 1 for sample locations), central (MN and IA), and east (PA, VA, WV1-6). In addition to determining the degree of variation partitioning, GenAlEx (Peakall and Smouse 2006) was used to perform a Mantel Test of genetic distances (calculated from pairwise  $\Phi_{ST}$ ) and Euclidean geographic distances to determine whether a relationship exists between the two. SAS-JMP (SAS Institute, Cary, NC) was used to determine the statistical significance of the relationship between the pairwise comparisons of distances.

**Relatedness.** Relatedness calculations were done using SPAGeDi, a program for the spatial pattern analysis of genetic diversity (Hardy and Vekemans 2002). The software is capable of treating the data without spatial information to calculate estimates of genetic differentiation of pairwise statistics between individuals. A relatedness coefficient is defined as the proportion of alleles shared between individuals, and these coefficients are dependent on reference allele frequencies that were calculated according to Hardy (2003). Within the confines of this program, an inbreeding coefficient must be assumed, and in this case, it was assumed to be 0, and the software is designed to be robust to moderate violations of this assumption.

## Results

**Population Genetic Analyses.** AMOVAs are reported in Table 3. The  $\Phi$  statistic is analogous to the *F* statistic for determining the partitioning of genetic variation (Wright 1969). The greatest proportion of genetic variation resided among individuals ( $\Phi_{ST} = 77\%$ ,  $P = 0.001$ ). Although a moderately high proportion resided among samples ( $\Phi_{SC} = 23\%$ ,  $P = 0.001$ ), this was not an effect of geographic region ( $\Phi_{CT} = 0\%$ ,  $P = 0.65$ ). Therefore, an individual collected in the west shared no greater proportion of alleles with other individuals from west samples than it did with individuals collected from elsewhere. This analysis provides an estimate of the degree of nonrandom genetic

**Table 4.** Summary of Mantel test results

| SSx          | SSy               | SPxy          | Rxy   | P    |
|--------------|-------------------|---------------|-------|------|
| 1,765,654.45 | 21,376,411,721.25 | 27,148,019.49 | 0.140 | 0.01 |

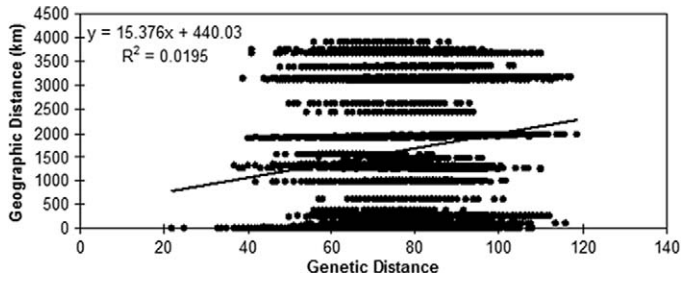


Fig. 2. Relationship between genetic and geographic distances for *P. regina*.

structure present in a single sample of blow flies. Even samples collected from roughly the same geographic location, but at a different time (WV samples) or separated by a relatively small distance (WA samples) showed genetic variation among them. A regression analysis of geographic and genetic distances found only a very weak positive relationship (Table 4; Fig. 2;  $R^2 = 0.0195$ ,  $F = 222.5$ ,  $P < 0.0001$ ).

**Relative Relatedness Coefficients.** A series of pairwise comparisons were made among all individuals in the dataset and were used to determine the degree of relatedness for all pairwise comparisons among individuals (Fig. 3). SPAGeDIs calculations were made using the entire dataset as a reference of allele frequencies; therefore, the estimates are relative. These calculations were based on an assumed inbreeding coefficient of zero. If this is an underestimate of the true inbreeding coefficient, the result was an underestimation of the spatial structure (Hardy 2003). Hardy (2003) showed, however, that the analysis is

robust to an incorrect estimate of inbreeding. The most extreme such error possible, assuming  $F_1$  (inbreeding coefficient) of 0 when it actually is  $F_1 = 1$ , leads to a bias in relative relatedness calculations of no more than 40%. On average across the entire dataset, relatedness coefficients among individuals from a single sample were 0.30 (range, 0.06–0.72). Carrion flies attracted to a bait during a brief period of time share a higher proportion of alleles than would be expected if individuals were selected at random from the population.

**Discussion**

The *P. regina* population within the study area seems to be panmictic. However, adults coming to bait over a short period of time were highly related. Mean relatedness within a sample was always positive and in some cases even greater than that of a sample of laboratory-generated full siblings (Fig. 3). A plausible

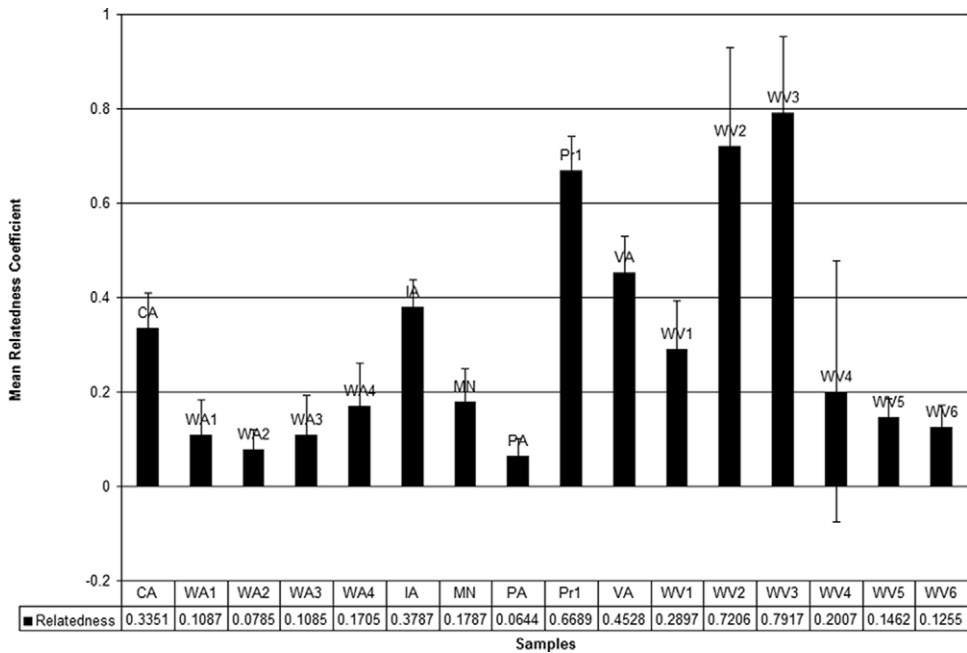


Fig. 3. Relative relatedness of *P. regina* adults attracted to a bait. Bars show mean and SD of pairwise comparisons within samples. Bar labels refer to locations given in Table 1. Sample Pr1 represents full siblings produced in the laboratory.

explanation for this pattern is that *P. regina* siblings arrive together at a carcass. A gravid female deposits up to 300 eggs at one time (Yin and Stoffolano 1998), which must lead to large numbers of full siblings emerging as adults in the same place at the same time. Perhaps such individuals respond to the same carrion odor plume.

Of course it is not at all unusual for siblings of many animal species to begin life in close proximity and then to disperse (Wilcock et al. 2005). What is not yet clear for *P. regina* is whether or not dispersal is a function of age or reproductive status. Adult females visit carrion for protein before developing eggs, so it is possible that the relatedness we observed for samples are not characteristic of gravid females arriving simultaneously at a corpse.

These results have practical implications for at least two aspects of forensic entomology. First, a laboratory colony, such as is commonly used to produce the growth models for postmortem interval estimation (Kamal 1958, Greenberg 1991, Anderson 2000, Byrd and Allen 2001, Grassberger and Reiter 2001) could be inbred from its founding if it is started from a single collection, even if a large number of founding individuals were included. Some growth-rate studies of the same species by different authors seem to disagree. Forensic entomologists have considered whether or not this could be the effect of experimental conditions or regional genetic variation (Wells and Kurahashi 1994, Tarone 2007). To these we add a potential genetic random sampling effect.

Second, if the adult flies depositing eggs on a corpse are as related as those we observed attracted to a bait, it might be possible to use *P. regina* AFLP data to reconstruct the postmortem movement of a corpse. A larva may be left behind at the original location when a corpse is moved (Wells et al. 2001). By using methods of source population assignment (Wasser et al. 2004, De Riek et al. 2007), an investigator could associate such a larva to the source population (that of the corpse).

We did not, however, determine the reproductive status of the flies we genotyped. Therefore, we do not know if the high proportion of alleles shared by individuals attracted to a bait would be found in individuals likely to oviposit at about the same time on the same corpse. This will be the subject of future research, as will be the effect of other factors, such as season and habitat, on blow fly population genetic structure.

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