2015

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Kathleen M. Kneeland
University of Nebraska-Lincoln

Steven R. Skoda
USDA-ARS-KBUSLIRL Screwworm Research Unit, Steve.Skoda@ars.usda.gov

John E. Foster
University of Nebraska-Lincoln, john.foster@unl.edu

Insect science

Insect Science (2015) 00, 1–9, DOI 10.1111/1744-7917.12219

ORIGINAL ARTICLE

Genetic variability of the stable fly assessed on a global scale using amplified fragment length polymorphism

Kathleen M. Kneeland[1], Steven R. Skoda[2] and John E. Foster[1]

[1]*Department of Entomology, 103 Entomology Hall, University of Nebraska-Lincoln, Lincoln, NE 68583 and* [2]*USDA-ARS-KBUSLIRL Screwworm Research Unit, 2700 Fredericksburg Road, Kerrville, TX 78028, USA*

Abstract The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), is a blood-feeding, economically important pest of animals and humans worldwide. Improved management strategies are essential and their development would benefit from studies on genetic diversity of stable flies. Especially if done on a global scale, such research could generate information necessary for the development and application of more efficient control methods. Herein we report on a genetic study of stable flies using amplified fragment length polymorphism, with samples of 10–40 individuals acquired from a total of 25 locations in the Nearctic, Neotropic, Palearctic, Afrotropic and Australasian biogeographical regions. We hypothesized that genetic differentiation would exist across geographical barriers. Although F_{ST} (0.33) was moderately high, the G_{ST} (0.05; representing genetic diversity between individuals) was very low; N_m values (representing gene flow) were high (9.36). The mismatch distribution and tests of neutrality suggested population expansion, with no genetic differentiation between locations. The analysis of molecular variance (AMOVA) results showed the majority of genetic diversity was within groups. The mantel test showed no correlation between geographic and genetic distance; this strongly supports the AMOVA results. These results suggest that stable flies did not show genetic differentiation but are panmictic, with no evidence of isolation by distance or across geographical barriers.

Key words AFLP; biting flies; external parasites; genetic diversity; livestock pests; *Stomoxys calcitrans*

Introduction

Stomoxys calcitrans (L.), a biting fly which parasitizes both humans and animals, is distributed worldwide except for Antarctica (Zumpt, 1973). In North America its parasitism of cattle results in >$2 billion in economic losses annually (Taylor *et al.*, 2012); as a synanthropic pest it is becoming a cause of dissention between farmers and new residents at the urban/rural interface (Thomas & Skoda, 1993). For these economic reasons, the majority of research on this pest has concentrated on its control in livestock facilities (Kneeland *et al.*, 2012).

Integrated pest management strategies implementing sanitary practices and residual insecticide applications have been successful in temporarily reducing stable fly numbers (Thomas & Skoda, 1993). But, due to insecticide resistance (Pitzer *et al.*, 2010) and dispersal capacity (Berkebile *et al.*, 1994), consistent control may not be achieved. Better understanding of stable fly population genetics may be helpful in identifying dispersal patterns as well as the genetic bases of insecticide resistance.

Amplified fragment length polymorphism (AFLP) has become recognized as a valuable molecular technique for the study of insect population genetics. It is cost effective and generates a large number of markers which can be scored using computer software. AFLP is reproducible

Correspondence: Steven R. Skoda, USDA-ARS-KBUSLIRL, Screwworm Research Unit, 2700 Fredericksburg Road, Kerrville, TX 78028, USA. Tel: +1 830 285 8318; fax: +1 830 792 0302; email: steve.skoda@ars.usda.gov

and requires only a minute amount of DNA (Bensch & Åkesson, 2005). Recently, AFLP has been employed to study the genetic variation of the fall army worm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), from North and South America (Belay *et al.*, 2012), Western bean cutworm, *Striacosta albicosta* (Smith) (Lepidoptera: Noctuidae), across the United States (Lindroth *et al.*, 2012), the bean leaf beetle, *Cerotoma trifurcata* (Forster) (Coleoptera: Chrysomelidae), throughout the Midwest United States (Tiroesele *et al.*, 2014), to compare laboratory and field samples of the spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), (Mustafa *et al.*, 2014) and stable fly across North America (Kneeland *et al.*, 2013). The benefits of AFLP make it an attractive method for analyzing a large number of samples such as in studies on a global scale.

Previous research on the global population structure of stable flies used direct sequencing of mitochondrial and nuclear genes (Marquez *et al.*, 2007; Dsouli-Aymes *et al.*, 2011). Using AFLP, an analysis of restriction fragments of total genomic DNA rather than specific genes, more information can be gained due to the large number of loci generated. We hypothesized that genetic differentiation will exist across geographical barriers, as suggested by Dsouli-Aymes *et al.* (2011) and Marquez *et al.* (2007). If accepted, this would suggest the occurrence of isolated demes, which would facilitate locating the source of resistance genes or dispersal patterns. The alternative hypothesis, finding high genetic variation between individuals and a great deal of gene flow between locations, would support the results of Jones *et al.* (1991) and Szalanski *et al.* (1996), indicating that stable flies are panmictic.

Materials and methods

Samples

Stable fly samples from the Western Hemisphere were the same as those used in previous research (Kneeland *et al.*, 2013). Samples were supplied by colleagues around the world (Table 1); different collection methods were used, and some specimens had been preserved from previous research. However, all samples were preserved in 95% ethanol which was poured off prior to shipping. Ethanol was refreshed upon arrival and samples were stored at 4°C until DNA extraction. Sample sizes ranged from 20 to 40 individuals except Western Washington with 10 samples; for some analyses these samples were combined with Eastern Washington (Table 1).

DNA extraction

DNA was extracted using the CTAB (Hexadecyltrimethylammonium bromide) method (Doyle & Doyle, 1987) with modifications. The procedures for extraction (which included homogenization of the thorax, addition, and incubation of Proteinase K and RNaseA, and centrifugation followed by supernatant transfer and cold storage) and quantification of DNA from individuals for each sample location using a spectrophotometer were the same as previously reported (Kneeland *et al.*, 2013).

AFLP-PCR

AFLP-PCR was performed using a protocol commonly used in our laboratory (Lindroth *et al.*, 2012; Kneeland *et al.*, 2012, 2013), modified from Vos *et al.* (1995), and included: (i) restriction of the DNA with *Mse*I and *Eco*RI restriction enzymes; (ii) adapter ligation with *Mse*I and *Eco*RI adapters; (iii) preamplification; and (iv) selective amplification with 4 combinations of *Mse*I and *Eco*RI primers (Table 2). Afterwards, 1.5 μL of product from each selective amplification, resulting from specific primer combinations as determined from preliminary tests and optimization, was electrophoresed on KB^{plus} 6.5% polyacrylamide gel in the GeneReadIR 4200 DNA analyzer (LI-COR, Lincoln, NE, USA) for 2 h to separate the DNA markers. The gel image was saved for scoring and further analysis.

The optimized PCR mix consisted of 1.2 μL 10× PCR buffer, 0.72 μL $MgCl_2$, 0.09 μL Amplitaq 360 DNA polymerase (Applied Biosystems, Grand Island, NY, USA), 0.4 μL of 10 mmol/L dNTP mix, 0.75 μL of 5.0 μmol/L M-primer, 0.3 μL of 1 μmol/L E-primer, and 6.79 μL dH_2O. The selective mix (10 μL) and 2 μL of diluted template DNA were transferred to 0.2 mL PCR tubes. The selective amplification PCR program consisted of 1 cycle of 94 ºC for 30 sec, 65 ºC for 30 sec, and 72 ºC for 1 min; 12 cycles of 94 ºC for 30 sec, 65 ºC for 30 sec (with the 65 ºC annealing temperature decreasing by 0.7 ºC each cycle to a final temperature of 56 ºC) and 72 ºC for 1 min, 23 cycles of 94 ºC for 30 sec, 56 ºC for 30 sec, and 72 ºC for 1 min; 4 ºC hold. Following selective amplification, samples were denatured by adding 2.5-μL blue stop solution (LICOR Biosciences, Lincoln, NE, USA) to each tube and incubating at 95 ºC for 3 min; samples were then ready for electrophoresis.

Data analysis

Gels were scored for presence or absence of bands using SAGA Generation 2 software (LICOR Biosciences,

Table 1 Number of individuals used, geographical coordinates and elevation of stable fly collection sites representing 5 global regions.

Location, city, state	No. of individuals	Coordinates†	Elevation
Afrotropical region			
Makokou, Gabon	40	0.532158N, 12.829655E	480 m (1574.8 ft)
Plaine des Cafres, La Reunion Is.	36	-21.2192N, 55.5590E	1293 m (4242.1ft)
Australasian region			
Gatton, Qld,		-27.333158N, -152.163266W	107 m (351 ft)
Pinjarra Hills, Qld	23	-27.322043N, -152.535131W	36.6 m (120 ft)
Nearctic region			
Lethbridge, Alberta, Canada	40	49.413656N, 112.503062W	893.0 m (2930 ft)
Russell, Ontario, Canada	40	45.152633N, 75.213009W	68.9 m (226 ft)
Jonesboro, Arkansas	24	35.502237N, 90.421540W	97.2 m (319 ft)
Jasper Co., Indiana	38	39.031979N, 88.052427W	165.8 m (544 ft)
Manhattan, Kansas	40	39.110099N, 96.341801W	361.2 m (1185 ft)
Medicine Lake, Montana	39	47.585650N, 107.563472W	865.0 m (2838 ft)
Lincoln, Nebraska	38	40.495959N, 96.393773W	353.6 m (1160 ft)
Raleigh, North Carolina	25	35.494691N, 78.430072W	109.7 m (360 ft)
Kerrville, Texas	23	30.0428N, 99.3854W	536.8 m (1761 ft)
Washington 1 (eastern)	28		
Frazier (Moxee)		46.528205N, 120.374317W	347.8 m (1141 ft)
Ferguson (Moxee)		46.528205N, 120.374317W	347.8 m (1141 ft)
Russell (Prosser)		46.133357N, 119.442946W	205.7 m (675 ft)
Stark		46.506087N, 120.193906W	449.3 m (1474 ft)
Washington 2 (western)	10		
Carstens		48.4877N, 121.703W	211.2 m (693 ft)
Silvana		48.241N, 122.369W	30.8 m (101 ft)
Neotropical region			
Potrerillos Arriba, Chiriqui, Panama 40	40	8.404593N, 82.285030W	869.3 m (2852 ft)
Dolega, Chiriqui, Panama	40	8.344668N, 82.252513W	285.3 m (936 ft)
Palearctic region			
Fuglebjerg, Denmark	40	55.316263N, 11.494282W	21.3 m (70 ft)
Næstved, Denmark	38	55.189888N, 11.799569W	14.9 m (49 ft)
Roskilde, Denmark	40	55.550185N, 11.94664W	41.1 m (135 ft)
Hyllinge, Denmark	40	55.69786N, 11.848336W	17.4 m (57 ft)
Hyllinge, Denmark	40	55.705953N, 11.925521W	17.4 m (57 ft)
Lynge, Denmark	40	55.846009N, 12.251469W	47.9 m (157 ft)
Slangerup, Denmark	40	55.834287N, 12.155846W	27.7 m (91 ft)
Le Faut, near Seyne-les-Alpes, France 40	40	44.341025N, 6.406077W	1340 m (4396.3 ft)
Gharb area north of Kenitra, Morocco 40	40	34.687207N, 6.005123W	30 m (98.4 ft)

†Locations south of the equator are represented by a "-" to conserve the N–W designation.

Lincoln, NE, USA); reports were generated as binary matrices (1s and 0s).

Arlequin software (Excoffier *et al.*, 2005) was used to generate the Analysis of Molecular Variance (AMOVA; used to calculate the amount of genetic variation allocated among groups, among subpopulations within groups, and within subpopulations), Tajima's D and Fu's FS tests of neutrality, the mismatch distribution and the Mantel test. Popgene (Yeh & Boyle, 1997) was used to determine Nei's genetic diversity (*G*-statistics). The coefficient of variation was calculated in the Bootsie program (Payne *et al.*, 2014) wherein the sampling variance of AFLP marker data is estimated via a bootstrap procedure; markers are resampled with replacement and compared (Tivang *et al.*, 1994). Phylip (Felsenstein, 1989) was used for bootstrapping and generating the dendrogram using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Graphs and figures were edited with MEGA5 (Tamura

Table 2 Nucleotide sequences of adapters, preamplification primers and selective primers used with DNA from stable fly samples. *Eco*RI selective primers (E-AAC and E-ACA) were tagged with fluorescent dye.

Primer ID	Primer type	Sequence[†]
*Eco*RI-1 (forward)	Adapter	5′-CTCGTAGACTGCGTACC-3′
*Eco*RI-2 (reverse)	Adapter	5′-AATTGGTACGCAGTCTAC-3′
*Mse*I-1 (forward)	Adapter	5′-GACGATGAGTCCTGAG-3′
*Mse*I-2 (reverse)	Adapter	5′-TACTCAGGACTCAT-3′
E (N+0)	Preamp primer	5′-GACTGCGTACCAATTC-3′
M (N+1)	Preamp primer	5′-GATGAGTCCTGAGTAAC-3′
M-CAC	Selective primer	5′-GATGAGTCCTGAGTAACAC-3′
M-CTA	Selective primer	5′-GATGAGTCCTGAGTAACTA-3′
M-CTC	Selective primer	5′-GATGAGTCCTGAGTAACTC-3′
E-AAC	Selective primer	5′-GACTGCGTACCAATTCAAC-3′
E-ACA	Selective primer	5′-GACTGCGTACCAATTCACA-3′

[†]Sequences were originally described by Vos *et al.* (1995).

Table 3 The Analysis of Molecular Variance (AMOVA) results for genetic variation of stable flies collected from 5 global regions.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	5	7133.472	3.12833 Va	3.52
Among subpopulations within groups	20	19964.642	26.92663 Vb	30.28
Within subpopulations	885	52090.915	58.85979 Vc	66.20
Total	910	79189.029	88.91475	
Fixation indices				
F_{SC}: 0.31388				
F_{ST}: 0.33802				
F_{CT}: 0.03518				

Significance tests (1023 permutations).
Vc and F_{ST}: P(rand. value $<$ obs. value) $=$ 0.00000
P(rand. value $=$ obs. value) $=$ 0.00000
$P = 0.00000 + (-0.00000)$
Vb and F_{SC}: P(rand. value $>$ obs. value) $=$ 0.00000
P(rand. value $=$ obs. value) $=$ 0.00000
$P = 0.00000 + (-0.00000)$
Va and F_{CT}: P(rand. value $>$ obs. value) $=$ 0.00293
P(rand. value $=$ obs. value) $=$ 0.00000
$P = 0.00293 + (-0.00164)$

et al., 2007) and SigmaPlot (Systat software Inc., Chicago, IL, USA).

Results

There were 882 individuals from 25 locations used for these analyses, an average of 35 flies per location. Using 4 primer pairs (Table 2), the AFLP technique generated 387 loci per individual. The Bootsie analysis (no. of markers vs. % coefficient of variation) indicated that 96% of the variation in the stable fly population can be accounted for by the number of markers used.

Analysis of molecular variance and Nei's genetic diversity

Results indicated that the majority of the variation in the stable fly samples occurred within subpopulations (66.20%), with 30.28% of the variation occurring among sub-populations within groups (Table 3). There was a very low percentage (3.52%) of variation among groups. The

Table 4 Analysis of Nei's genetic diversity in subdivided demes of stable flies collected from 5 global regions.

	h*	H_t	H_s	G_{st}	N_m
Total	0.4278	0.4288	0.3046	0.0507	9.3580
SD	0.0871	0.0076	0.0067		

h*, Nei's gene diversity; H_t, total diversity; H_s, diversity within demes; G_{st}, diversity among demes; N_m, estimate of gene flow based on G_{st}.

F_{ST} value (0.338) suggested some differentiation, but the G_{ST} (0.051) and N_m (9.358) indicated a large amount of gene flow between locations (Table 4).

Tests of neutrality and mismatch distribution

Tajima's D neutrality test showed no significant positive deviations from zero for D; Fu's FS results were large negative values, 28% had significant *P* values (<0.02) and 48% were approaching significance (<0.05) (Table 5). In the mismatch distribution test for demographic expansion, there were no significant SSD values and all samples had a very low raggedness index; these results are indicative of a population undergoing expansion (Table 6).

Mantel test

The genetic distance matrix was used as the Y matrix and geographical distance was used for X1. Therefore, the mean value of Y is the mean genetic differences found between the samples, and mean value X1 is the mean distance in km between collection locations. The **Z** statistic represents the Hadamard product of the 2 matrices ($\mathbf{Z_{XY}} = \mathbf{X} * \mathbf{Y} = \sum_{i=1}^{N} \cdot \sum_{j=1}^{i} \cdot x_{ij}\, y_{ij}$), where **Y** was held constant. Determination of **Y** by **X**1 indicates the percentage influence the geographical location had on the genetic difference, and the value here (0.003993) was very low (Excoffier *et al.*, 2005). This and the low correlation coefficient (0.063186) indicate that there was no correlation between geographic location and genetic distance in these samples (Table 7); these results are supported by the shotgun pattern of the scatter plot (Fig. 1).

Dendrograms

The UPGMA method used for constructing the dendrogram, which assumes that evolutionary (mutational) rates are equal for each group (Michener & Sokal, 1957), provided a dendrogram that split the samples into 2 major clades: the Arkansas samples were separate from both clades (Fig. 2). However, there is no apparent differentiation of biogeographical regions. Both clades contain Palearctic and Nearctic samples. The Australasian sample was in the first clade. The second clade contained the Neotropical and Afrotropical regions, but they were dispersed among the Palearctic and Nearctic locations.

Table 5 Tajima's D and Fu's FS tests of neutrality of stable flies collected from 5 global regions.

Location	Tajima's D	*P* value	Fu's FS	*P* value
Afrotropical region				
Gabon	1.2701	0.90700	-6.77236	0.00800†
La Reunion	1.6676	0.91700	-4.68662	0.03500
Australasian region				
Australia	0.6666	0.77200	-1.28857	0.14400
Nearctic region				
Alberta	1.7096	0.94000	-5.73634	0.02300
Arkansas	0.9190	0.83500	-2.31784	0.07600
Indiana	1.7257	0.92600	-5.55184	0.02900
Kansas	1.3179	0.90000	-5.83173	0.02200
Montana	1.6209	0.93000	-5.66537	0.02700
NCarolina	1.6328	0.92900	-7.59943	0.01400†
Nebraska	1.5266	0.89400	-5.70206	0.02700
Ontario	1.7248	0.94900	-5.73629	0.02800
Texas	0.6608	0.77400	-2.64343	0.07100
Washington E	1.3703	0.91200	-3.04760	0.08700
Washington W	0.8734	0.81600	0.64121	0.42600
Neotropical region				
Panama 1	1.9909	0.95300	-5.31491	0.02800
Panama 2	1.6731	0.92500	-5.58761	0.02600
Palearctic region				
Denmark 1	0.7698	0.79000	-6.64507	0.02100
Denmark 2	1.0483	0.88800	-7.18014	0.01000†
Denmark 3	0.9235	0.84700	-6.91942	0.00800†
Denmark 4	0.4248	0.75300	-7.49868	0.01200†
Denmark 5	2.7279	0.98800	-4.38613	0.06200
Denmark 6	2.2898	0.97300	-4.76219	0.04700
Denmark 7	1.5933	0.93200	-5.92560	0.01800†
France	1.7218	0.94700	-5.90426	0.03100
Morocco	1.0836	0.87800	-7.52242	0.01400†

†Fu's FS *P*-values are considered significant at <0.02 (Fu, 1997).

Discussion

These results indicate a large amount of gene flow between locations on a global scale. Comparable results have been reported at a local scale (Szalanski *et al.*, 1996; Gilles *et al.*, 2007). Dsouli-Aymes *et al.* (2011) showed

Table 6 Results of the mismatch distribution test for demographic expansion of stable flies collected from 5 global regions.

Location	SSD	*P* value	Raggedness index	*P* value
Afrotropical region				
Gabon	0.0038	0.57	0.0023	0.90
La Reunion	0.0107	0.06	0.0036	0.72
Australasian region				
Australia	0.0088	0.71	0.0104	0.71
Nearctic region				
Arkansas	0.0062	0.75	0.0072	0.67
Indiana	0.0027	0.84	0.0016	0.98
Kansas	0.0022	0.90	0.0023	0.91
Montana	0.0023	0.78	0.0024	0.75
N Carolina	0.0013	0.75	0.0024	0.65
Nebraska	0.0041	0.62	0.0022	0.97
Ontario	0.0014	0.90	0.0021	0.90
Texas	0.0158	0.12	0.0076	0.60
Washington E	0.0132	0.14	0.0054	0.74
Washington W	0.0228	0.66	0.0290	0.84
Neotropical region				
Panama 1	0.0036	0.80	0.0022	0.97
Panama 2	0.0038	0.55	0.0031	0.65
Palearctic region				
Denmark 1	0.0018	0.99	0.0019	0.93
Denmark 2	0.0036	0.63	0.0025	0.88
Denmark 3	0.0138	0.06	0.0023	0.99
Denmark 4	0.0035	0.63	0.0023	0.90
Denmark 5	0.0017	0.78	0.0024	0.68
Denmark 6	0.0014	0.76	0.0021	0.81
Denmark 7	0.0117	0.08	0.0023	0.99
France	0.0035	0.70	0.0019	1.00
Morocco	0.0015	0.82	0.0028	0.52

Note: This analysis is based on the distribution of differences in pairs of haplotypes. The small raggedness index suggests population expansion which is supported by the low (insignificant) SSD values. The demographic expansion test is based on the sudden expansion model.

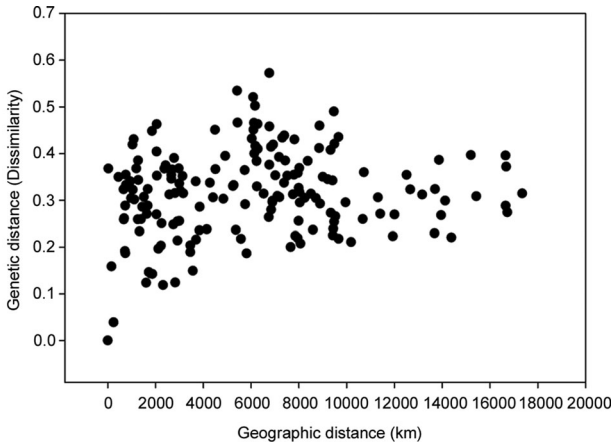

Fig. 1 Scatter plot of Mantel test created in Sigma Plot. The shotgun pattern of scatter indicates no correlation between stable fly genetic distances and geographic locations.

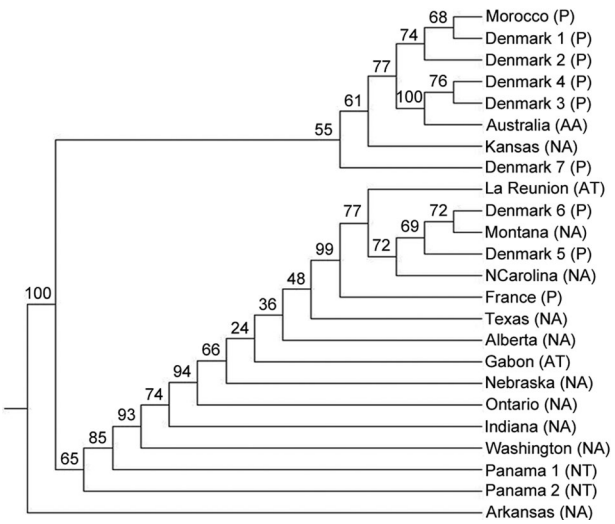

Fig. 2 Dendrogram of stable fly samples from all 5 biogeographical regions in this study. (Initials following location names denote biogeographical region: AT, Afrotropical; AA, Australasian; NA, Nearctic; NT, Neotropical; P, Palearctic).

opposing results, with the majority of the variation in their samples occurring among groups (85.14% for COI, 71.73% for CytB, and 77.06% for NDI-16S). However, our results support the findings of Marquez *et al.* (2007) and Dsouli-Aymes *et al.* (2011) in many respects. Both authors suggested an Afrotropical origin of stable flies, which our data support. They suggested isolation of the Oriental region, which we cannot confirm as we did not obtain samples from the Oriental region. We included samples from Australia, which the other studies lacked, but no differentiation was found between Australia and the other regions.

The low G_{ST} (0.051), high N_m (9.358) and results from AMOVA support a high level of gene flow between locations; we do note that 30.28% of the total variation among subpopulations within groups may indicate some genetic differentiation within some regions that were sampled. The F_{ST} (0.338) is higher than the G_{ST}, and suggests some genetic differentiation. Nei's genetic diversity (G_{ST}) is comparable to Wright's F_{ST} wherein if there are only 2 alleles they produce equivalent values. F_{ST} was developed for study of populations with only 2 alleles at a locus, whereas G_{ST} measures the degree of differentiation

in multiple populations, with multiple alleles at each locus; therefore G_{ST} may be a more accurate representation of gene differentiation.

Tajima's D and Fu's FS tests of neutrality and the mismatch distribution suggest that these subpopulations may have undergone (or are undergoing) population expansion. Strong positive FS results indicate population subdivisions or bottlenecks whereas strong negative results suggest population expansion (Fu, 1997). According to Tajima (1989), negative D values suggest recent bottlenecks or large insertion–deletion polymorphisms, while positive D values suggest restriction site or small insertion–deletion polymorphisms. Our results of Tajima's D are high, with *P* values approaching 1, and support population expansion. Considering that the AFLP technique produces restriction site fragments for analysis, the large D values could be due to restriction site polymorphisms. The results herein of Fu's FS are significantly negative (≤ 0.02) or approaching significance (<0.05), also indicating population expansion. In the mismatch distribution, significant SSD values infer deviation from the sudden expansion model. A small raggedness index (<0.05) suggests population expansion whereas larger raggedness values (>0.05) suggest stationary populations or bottlenecks (Rogers & Harpending, 1992; Cordaux *et al.*, 2003). The raggedness index values of this work are low for all of the locations, indicative of population expansion. These results support the hypothesis that stable flies originated in one region and populations expanded over time. They may also be an indication that, rather than extensive gene flow, with the recent population expansion there has not been enough time for genetic differentiation.

The low correlation coefficient (0.063186) and the shotgun pattern of the scatter plot resulting from our data when using the Mantel test indicated no correlation between genetic distance and geographical distance in our samples. This supports acceptance of our alternative hypothesis of high genetic variation between individuals and a great deal of gene flow between locations, as proposed by Jones *et al.* (1991) and Szalanski *et al.* (1996).

The dendrogram is indicative of a panmictic population, also supporting our alternative hypothesis, with multiple introductions into the Western Hemisphere and no apparent isolation by distance or geographical barriers. When a dendrogram is generated with only the Nearctic and Neotropical samples (data not shown; see Kneeland *et al.*, 2013), there is a logical pairing of sample groups from similar locations; the same applies for a dendrogram generated from the sample groups from the Eastern Hemisphere (data not shown). However, when all samples were analyzed together the Palearctic and Afrotropical regions were interspersed between the others; we interpret this as indication that the stable fly origins were from these regions. Five of the Denmark samples group together in the first clade, which suggests some isolation (this may represent a large portion of the 30.28% variation of subpopulations within groups as indicated in the results from the AMOVA); the remaining 2 Denmark samples join the second clade adjacent to Montana and North Carolina, which are closely related in the dendrogram of samples from the Western Hemisphere (Kneeland *et al.*, 2013). Australia and Morocco are associated with the Denmark locations in the first clade. Nebraska and Kansas, neighbors both geographically and in the dendrogram for the Western Hemisphere (along with Arkansas; Kneeland *et al.*, 2013), appear in different clades in the current dendrogram. The seemingly haphazard grouping occurred, most probably, because variability within each location supersedes any patterns of relationship between locations.

The mixing of stable fly samples from La Reunion and Gabon into the Western Hemisphere branch of the dendrogram supports the hypothesis by Dsouli-Aymes *et al.* (2011) that there were multiple introductions of stable flies into the Western Hemisphere from the Palearctic and Afro-tropical regions. Human migration and colonization undoubtedly contributed to the expansion and distribution of the stable fly as well as the high level of gene flow on this global scale. Because *Stomoxys calcitrans* is a synanthropic livestock pest, and considering the dynamic movement of humans and livestock for agricultural and recreational purposes, it is not unexpected that the stable fly population is dynamic, with substantial genetic variation and gene flow globally.

In conclusion, results herein are consistent throughout the different analyses. They support, despite the different analysis techniques, the hypothesis of Marquez *et al.* (2007) and Dsouli-Aymes *et al.* (2011) that stable flies originated in the Eastern Hemisphere. We suggest that, except for the Oriental region from which we had no samples, the stable fly is a panmictic population with gene flow occurring unimpeded across geographical barriers. Therefore, we reject our hypothesis of genetic differentiation across geographic barriers in favor of our alternative hypothesis of high genetic variation and gene flow between locations as proposed by Jones *et al.* (1991) and Szalanski *et al.* (1996). Our results revealed information on the origin of stable flies which will be useful for integrated pest management programs as strategies for stable fly control should be equally effective in all locations. This study did not address the origin and dispersal of stable flies in local areas, but our results increased the knowledge base to facilitate further research in that area. Larger scale global projects are necessary to provide

insight into stable fly population dynamics both on the local scale and globally, enabling the development of more efficient pest management strategies.

Acknowledgments

Many thanks to the following individuals for supplying stable flies for this project: Henrik Skovgaard, University of Aarhus, Copenhagen, Denmark; Gerard Duvallet, Université Paul Valéry-Montpellier 3, Montpellier, France; Geoff Brown, Department of Employment, Economic Development and Innovation, Queensland, Australia; Kevin Floate, Agriculture and Agri-Food Canada, Lethbridge, Alberta; Steven Mihok, Russell, Ontario; Holly Ferguson, WSU Extension, IAREC, Prosser, WA; Gregory Johnson and Kristina Hale, Montana State University, Bozeman, MT; Wes Watson, North Carolina State University, Raleigh, NC; Ludek Zurek, Kansas State University, Manhattan, KS; Tanja McKay, Arkansas State University, State University, AR; Ralph E. Williams, Purdue University, West Lafayette, IN; Dennis Berkebile, USDA-ARS, Lincoln, NE. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. USDA is an equal opportunity provider and employer.

Disclosure

All authors are without conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject of this manuscript.

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Accepted March 4, 2015