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The hitchhiker's guide to becoming invasive: exotic mosquitoes spread across a US state by human transport not autonomous flight

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Abstract

Not all exotic species establish and expand aggressively (i.e. become invasive). As potential vectors of disease agents, invasive mosquitoes can have considerable impact on public health, livestock and wildlife; therefore, understanding the species characteristics and ecological circumstances promoting their invasiveness is important. The mosquito *Aedes japonicus japonicus*, originally from north-east Asia, was introduced at least two separate times to the north-eastern USA, as surmised from the initial existence of two populations with distinct nuclear and mitochondrial genetic signatures that later intermixed. Since these original introductions in the late 1990s, *Ae. j. japonicus* has expanded across 31 US states, two Canadian provinces and five European countries. Although some of the expanded range was due to other independent introductions, to understand what drove the postintroduction expansion of *Ae. j. japonicus* within the north-eastern USA, we performed a high-resolution landscape genetic analysis of 461 specimens collected across Virginia, a state south of the original introductions. All specimens were genotyped at seven pre-optimized microsatellite loci, and a subsample was sequenced at one mitochondrial locus. We concluded that throughout Virginia this species has primarily expanded in association with humans: genetic distance and distance along roads remained correlated after controlling for geographic distance, and proximity to I-95, a major interstate highway, strongly predicted nuclear ancestry. In contrast, there was very limited evidence of diffusion even at distances potentially suitable for autonomous mosquito flight. This implies that its association with humans (rather than innate species characteristics) is the single most important determinant of invasiveness in this mosquito.

Keywords: *Aedes j. japonicus*, biological invasions, human-aided dispersal, landscape genetics, multiple introductions, spatiotemporal analyses

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Introduction

Most species that are transported to a new geographic area by humans either fail to establish or establish but remain localized with minimal impact. By definition, to progress to the level of a biological invasion the geographic distribution of the exotic species must increase significantly within its new range (Richardson *et al.* 2010). Accordingly, while a few dozen mosquito species have established in areas outside their native ranges, only a handful have dramatically increased in range and abundance to become worldwide invaders with human health consequences (Juliano & Lounibos 2005). As the impact of these few species has been disproportionately large (Lounibos 2002), it is critical to understand the dispersal patterns and environmental factors

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leading to their extensive secondary (i.e. postestablishment) expansion. While much effort has been devoted to studying the contributions of phenotypic traits to invasion success, for example growth rate and competitive ability (Sakai *et al.* 2001), and also genetic factors, such as genetic variation and admixture (Dlugosch & Parker 2008), landscape genetics is a relatively recent approach uniting landscape factors with genetics to glean new insights (Lawson Handley *et al.* 2011). The study of landscape genetics considers the influence of landscape features (such as roads, rivers and mountains) on gene flow and other evolutionary processes (Manel & Holderegger 2013) and applied to species invasions, it aids in the reconstruction of invasion pathways, although studies often suffer from a lack of spatial and temporal resolution.

An excellent model system for understanding invasive species spread is the mosquito *Aedes japonicus japonicus* Theobald (Diptera: Culicidae), the most recent mosquito to become globally invasive and one for which extensive genetic tracking of its expansion is available. *Ae. j. japonicus* is a temperate species, native to northern Japan and the Korean peninsula (Tanaka *et al.* 1979). High-resolution spatial and temporal genetic analyses of early postestablishment populations have shed light on its recent expansion into North America and Europe (Fonseca *et al.* 2010; Zielke *et al.* 2014). In North America, it was first collected in Connecticut in 1997 (Andreadis *et al.* 2001) and established populations were detected in New York and New Jersey in 1998 (Peyton *et al.* 1999) and in Pennsylvania in 1999 (Fonseca *et al.* 2001). The species has subsequently spread to 31 US states, primarily in the north-east, and two Canadian provinces, as well as five countries in central Europe (Kaufman & Fonseca 2014). Until recently, the expansion of this mammalian biter known to target humans (Molaei *et al.* 2009) was primarily a nuisance concern; however, it has now become implicated as a potentially important vector of LaCrosse virus to humans (Harris *et al.* 2015; Westby *et al.* 2015). It is also extraordinarily competent for Japanese encephalitis and Rift Valley fever viruses, two emerging diseases worldwide (Takashima & Rosen 1989; Turell *et al.* 2013; Huber *et al.* 2014a).

Fonseca *et al.* (2001, 2010) demonstrated the existence of two distinct introductions of *Ae. j. japonicus* into the eastern USA, defined by nonoverlapping mitochondrial haplotypes and distinctive microsatellite allele frequencies: (i) the 'PA type' (central Pennsylvania) and (ii) the 'NY type' (New York, New Jersey and Connecticut). Detailed spatial and temporal analyses of specimens collected in later years (2002–2005) across Pennsylvania revealed reciprocal genetic exchange between the two introductions, weakening the strong association between mtDNA haplotype and nuclear signature (Fonseca *et al.* 2010).

Several landscape factors have been linked with the spatial spread of *Ae. j. japonicus* and other mosquito species, especially those in the genus *Aedes* (sensu Wilkerson *et al.* 2015). *Aedes* eggs are relatively desiccation-resistant and have delayed hatching, which means large numbers may be accidentally transported by humans when females oviposit in transient water found in lawn ornaments, plant pots, construction supplies and used tires (Kaufman & Fonseca 2014). In a similar invasive species, *Aedes albopictus,* multiple lines of evidence point towards interstate highway networks as a major expansion pathway in the USA (Moore & Mitchell 1997; Medley *et al.* 2014) primarily via the transport of eggs in used tires (Hawley *et al.* 1987). There is also evidence that adult mosquitoes can be inadvertently transported, for example in airplanes (Bataille *et al.* 2009), although this is considered a less likely means of establishment due to the small number of individuals involved (e.g. lower propagule pressure) (Lounibos 2002; Scholte *et al.* 2010). In addition to the above mechanisms of anthropogenic dispersal, it has been also proposed that *Ae. j. japonicus* adults may autonomously disperse along stream corridors, as this species often deposits eggs in rock pools along streams when available (Bevins 2007). Another landscape factor with the potential to influence *Ae. j. japonicus* expansion is temperature: this species is primarily distributed throughout cooler latitudes in both its native (Asian) and invasive (North American and European) ranges (Kaufman & Fonseca 2014). Accordingly, we have observed poor survivorship of *Ae. j. japonicus* larvae at warm temperatures in laboratory experiments (Egizi *et al.* 2014) and demonstrated a strong influence of elevation (and its proxy, temperature) on genetic patterns in Hawaii (Egizi & Fonseca 2014).

Our goal was to follow up on the genetic analyses performed shortly after the introduction of *Ae. j. japonicus* into the eastern USA using high-resolution spatial genetics. Given the extensive mixing between the two genetic strains already apparent in 2002–2005 across Pennsylvania (Fonseca *et al.* 2010), we expected that individuals of mixed heritage would have dispersed south and proliferated, obscuring the signal of these two introductions in the years since. To test this hypothesis, we evaluated spatial genetic patterns of *Ae. j. japonicus* in 2011 across the US state of Virginia, a state both south of the initial introduction (e.g. well into the area of secondary expansion) and offering comparable size and scope to the high-resolution analyses conducted previously (Fonseca *et al.* 2010). Furthermore, we examined the correlation between genetic signatures (nuclear and mitochondrial) and landscape features such as stream corridors, highways, elevation, climate

and location of preferred larval habitat (e.g. used tires) in an effort to understand the dispersal pathways of this mosquito species.

Methods

Sample collection and extraction

Specimens of *Ae. j. japonicus* were collected in June–August 2011 during a surveillance campaign that targeted 89 independent cities and counties in Virginia with no prior collection record of this species (J. Kiser and C. Abadam, unpublished data). Larvae were collected from artificial containers (one container per site) using 30-mL plastic pipettes or larval dippers (Clarke Dipper, Clarke, Roselle, IL), then placed on ice until they were identified to species following Farajollahi & Price (2013). A total of 1564 *Ae. j. japonicus* larvae were identified from collections at 118 of 163 sites, comprising 79 of the 89 jurisdictions sampled. There were 10 counties in Virginia where no *Ae. j. japonicus* larvae were detected despite considerable sampling effort, all of them at low elevations along the coast (J. Kiser and C. Abadam, unpublished data).

Between 1 and 9 specimens from each collection site were preserved in a 2-mL screw-capped vial with 95% ethyl alcohol. When possible, different larval instars were selected from a site to decrease as much as possible the likelihood of oversampling siblings. Before DNA extraction, specimens were removed from the vials and placed on a clean paper towel allowing a few moments for any residual ethanol to evaporate, then processed in Qiagen DNeasy blood and tissue 96-well plate kits (Qiagen, Valencia, CA). A total of 461 specimens from 100 different collection sites, comprising 74 independent jurisdictions, were used for genetic analysis (Fig. 1A, and Table S1, Supporting information).

Microsatellite genotyping

Specimens were genotyped at seven microsatellite loci (Widdel *et al.* 2005) using the modified primer OJ5R3 (instead of OJ5R) to reduce null alleles, following the protocols in Fonseca *et al.* (2010). Microsatellite PCR products were sized on an ABI 3130XL Genetic Analyser 16 Capillary machine (Applied Biosystems, Foster City, CA). Alleles were scored in GENE MAPPER 3.5 (Applied Biosystems) using bins optimized on worldwide populations of this species (Huber *et al*. 2014b).

Mitochondrial sequencing

While microsatellites are both highly variable and cost-effective DNA markers, unfortunately there are currently only seven microsatellite loci optimized for *Ae. j. japonicus* that show dependable Mendelian

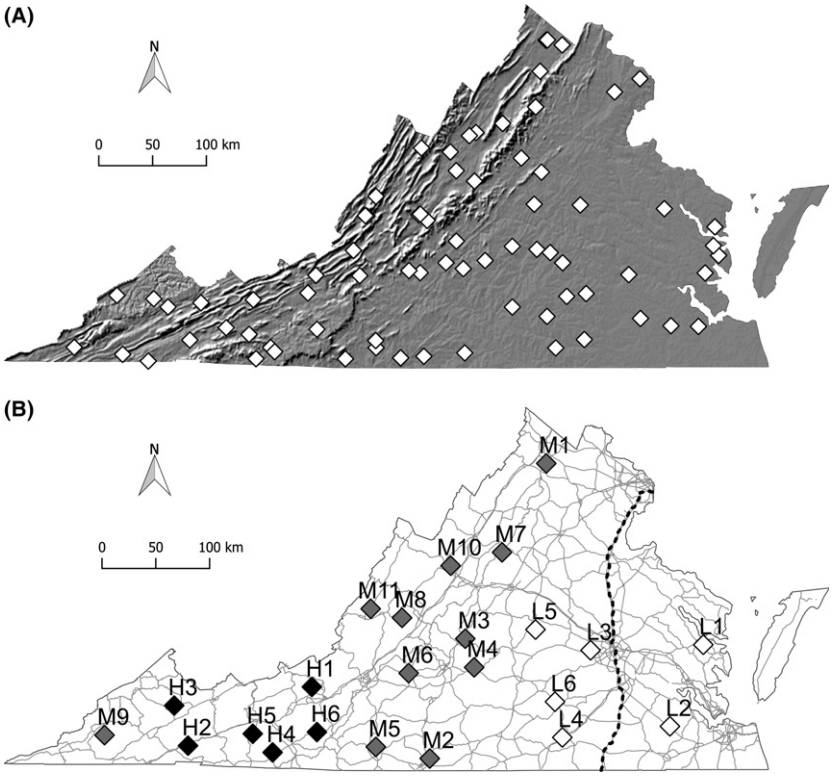

Fig. 1 (A) Plot of all collection sites (white diamonds) of *Ae. j. japonicus* in Virginia for specimens used in genetic analysis. Base map is SRTM30_Plus (global bathymetry and elevation data at 30 arc seconds, Becker *et al.* 2009) downloaded with hillshading using DEM Explorer (Han *et al.* 2012). (B) Plot of population group mid-points by elevation. Each group is composed of samples from neighbouring counties that add up to equal a sample size of at least 16 (see Table 1 for groupings). Elevation categories are as follows: black = high (500+m), grey = mid (200–500 m), white = low (<200 m). Light grey lines denote the state's larger roads and highways, and the path of Interstate-95 is highlighted with a dotted black line.

inheritance (Widdel *et al.* 2005). Thus, we also sequenced a mitochondrial locus, allowing a more comprehensive picture of genetic patterns due to the differing mutation rates and modes of inheritance of nuclear and mitochondrial DNA (Sunnucks 2000). To maximize information per individual while minimizing cost, all specimens were genotyped at the seven microsatellite loci, but only a subset of 96 were sequenced at the mtDNA locus (Table S2, Supporting information).

To subsample specimens for mitochondrial sequencing, we used a random number generator to select 32 specimens from each of three elevation categories (Low = below 200 m, Mid = 200–500 m, High = above 500 m). Equal representation across elevations was chosen as we have previously identified elevation as a major influence on genetic diversity in this species (Egizi & Fonseca 2014). The randomly selected specimens were distributed throughout the sampled areas of Virginia (compare Fig. 1A to Fig. S1, Supporting information). From each of the 96 specimens, we sequenced a portion of the sodium dehydrogenase subunit 4 (ND4) locus, known to be variable in this mosquito species (Fonseca *et al.* 2010) using the same protocol as in Egizi & Fonseca (2014); Zielke *et al.* (2014). PCR products were cleaned with ExoSap-IT (Affymetrix, Santa Clara, CA), cycle sequenced and then sequenced on an ABI 3730XL Genetic Analyser 96 capillary machine (Applied Biosystems, Foster City, CA). The sequences were assembled and cleaned in SEQUENCHER 4.10.1 (GeneCodes Co., Ann Arbor, MI).

Assignment of samples to population groups

Because most collection sites contained only a handful of larvae (mean ± SD = 4.8 ± 2.0), we grouped nearby sites together to form 'population groups' of at least 16 specimens for analysis. Several collection sites that had a small number of specimens and were not in close proximity to other sites were included in individual-based analyses but excluded from the subset of analyses relying on population groups (namely Craig, Patrick, Powhatan, and Page counties and the cities of Manassas Park and Falls Church) (Table S1, Supporting information). Using this method, a total of 23 population groups were identified comprising 447 specimens, which were used in subsequent analyses (Table 1). While grouping together sites with different genetic signatures removes their individual signal, it also creates increased within-population variability, obscuring differences between groups and reducing our chance of finding population structure where it does not exist (type I statistical error). However, because this grouping strategy could make it difficult to detect very localized patterns of dispersal (e.g. <30 km), we also performed analyses on individual genotypes without combining specimens into groups.

We defined the latitude and longitude coordinates of each population group as the mid-point of the GPS coordinates of all sample sites within the group, calculated using the Geographic Midpoint Calculator (http://www.geomidpoint.com/). Sites within the same population group were on average 35.2 km apart, and the largest distance between any two sites within the same group was 80 km. The average distance between population group mid-points was 195.6 ± 47.8 km (mean ± SD). The two closest groups were H4 and H5 (24.8 km), and the two furthest were L1 and M9 (549.4 km). The elevation of each population group was calculated as the average elevation of all sample sites within the group. Differences in elevation between the lowest and highest elevation sites (min–max) within each group are given in Table 1. Population groups were named by elevation category: locations collected between 0 and 200 m above sea level (L = low elevation), those collected between 200–500 m (M = mid-elevation), and those collected above 500 m (H = high elevation) (Fig. 1B, Table 1).

Calculation of landscape variables

Air temperature information for each population group was obtained from the NOAA National Climatic Data Center Mapping Tool (http://gis.ncdc.noaa.gov/map/viewer/) using the nearest weather station to the group mid-point. Temperature variables noted were mean annual precipitation and mean annual temperature (in °C). A list of potential used tire vendors in Virginia and their latitude and longitude coordinates were obtained from a Places search in GOOGLE EARTH Version 7.1 (Google Inc., Mountain View, CA) with the terms 'used tires in Virginia'. Each potential vendor was researched via the Internet to confirm they sold used tires. Vendors who could not be confirmed were excluded. A total of 133 used tire vendors were identified, and the distance from each population group to the closest tire vendor was calculated using the Near tool in the Analysis Toolbox in ARCMAP Version 10.2 (ESRI, Redlands, CA). The distance from the centre of each population group to the nearest major highway (class 1 or class 2 roads only), as well as to Interstate 95 specifically, was also calculated using the Near tool in ARCMAP Version 10.2 (ESRI). Interstate 95 (I-95) is a major multilane highway stretching 1917 miles along the US east coast from Maine to Florida. According to the I-95 Corridor Coalition (2015), it is heavily trafficked with an average of 72 000 vehicles per day making up over a third (35%) of the nation's vehicle miles travelled (VMT).

Table 1 Population groupings of at least 16 individuals, following Fig. 1B

Group	Counties included	*N*	Latitude	Longitude	Elevation	Elev range	*I*	H_O	uHe	Anc.	Hwy	Avg. T
L1	Essex, Gloucester, Lancaster, Mathews, Middlesex	16	37.56871	−76.53217	20.79	3.4–26.8	1.100	0.539	0.570	0.733	6.32	58.0
L2	Isle of Wight, Southampton, Sussex	16	36.904929	−76.87305	26.92	11.3–36.6	0.948	0.518	0.542	0.797	1.00	58.4
L3	Amelia, City of Hopewell, Louisa	25	37.523738	−77.68407	80.67	12.5–123.8	0.984	0.533	0.528	0.675	1.58	56.5
L4	Brunswick, Lunenburg, Mecklenburg	20	36.814244	−77.97274	118.67	92.4–142.0	0.941	0.479	0.490	0.777	0.06	58.1
L5	Cumberland, Fluvanna	17	37.691306	−78.24706	130.53	99.4–149.1	0.960	0.546	0.527	0.730	5.28	58.0
L6	Charlotte, Dinwiddie, Nottoway	16	37.105488	−78.04771	144.58	101.2–203.9	1.038	0.536	0.562	0.666	1.54	56.7
M1	City of Winchester, Clarke, Frederick, Rappahannock, Warren	24	39.022386	−78.13708	202.48	169.5–235.3	1.002	0.536	0.539	0.720	1.80	54.6
M2	City of Danville, Halifax, Pittsylvania	17	36.64191	−79.32815	202.59	136.5–267.0	1.175	0.617	0.623	0.549	3.25	58.5
M3	Amherst, Buckingham, City of Buena Vista	20	37.617623	−78.96479	209.86	151.2–247.8	0.981	0.511	0.532	0.809	2.96	56.4
M4	Appomattox, Campbell	16	37.385002	−78.87407	228.45	212.8–244.1	1.094	0.554	0.607	0.538	2.86	56.0
M5	City of Martinsville, Henry	23	36.736302	−79.87902	256.41	214.9–243.8	1.108	0.542	0.587	0.600	5.16	55.5
M6	City of Bedford, City of Lynchburg, City of Salem, Bedford Co	20	37.337952	−79.54375	276.91	211.8–341.7	1.112	0.617	0.604	0.337	0.18	56.1
M7	City of Harrisonburg, Greene, Orange, Rockingham	20	38.313187	−78.58897	301.18	151.5–446.5	0.936	0.545	0.515	0.800	5.80	55.6
M8	Alleghany, City of Lexington, Rockbridge	16	37.786407	−79.61116	329.99	295.7–388.6	1.221	0.648	0.649	0.317	6.04	54.9
M9	Dickenson, Lee, Scott	20	36.832948	−82.65212	454.97	403.3–497.7	1.058	0.621	0.596	0.286	8.53	55.1
M10	Augusta, Highland, Staunton, Waynesboro	23	38.206013	−79.11453	481.66	406.6–629.1	1.085	0.559	0.582	0.650	0.50	53.6
M11	Bath, City of Covington	18	37.859177	−79.93192	483.79	385.6–708.4	1.257	0.667	0.651	0.274	1.42	54.6
H1	Giles, Pulaski	19	37.226357	−80.53314	572.49	563.9–585.2	1.046	0.608	0.589	0.428	7.62	51.6
H2	City of Bristol, Smyth, Washington	20	36.746573	−81.7987	612.24	541.3–713.5	1.115	0.612	0.594	0.299	1.93	53.5
H3	Buchanan, Russell, Tazewell	21	37.074195	−81.94018	642.01	452.9–880.9	1.110	0.571	0.597	0.423	7.31	52.4
H4	Carroll, City of Galax	19	36.691337	−80.93403	744.86	686.1–776.6	1.080	0.635	0.596	0.292	2.63	51.8
H5	Bland, Grayson, Wythe	25	36.844113	−81.13685	763.78	692.2–788.2	1.140	0.595	0.599	0.358	3.13	51.1
H6	Floyd	16	36.85804	−80.48116	837.29	836.4–838.2	1.122	0.643	0.604	0.396	0.03	51.5

N = sample size; *I* = Shannon's information index; H_o = observed heterozygosity; uHe = unbiased expected heterozygosity; Anc. = average proportion PA ancestry (NY = 1 – PA); Hwy = distance (km) from nearest major (class 1 or 2) highway; Avg. T = mean annual temperature. Elevation is averaged across sample sites within a group; Elev range = min–max site elevations within a group (in meters). H_o and uHe are averaged over 7 nuclear loci.

Ancestry of Virginia specimens

To determine the ancestry of Virginia *Ae. j. japonicus* specimens (whether they have a NY or PA nuclear signature, or a mixture of both), we performed a STRUCTURE analysis (Pritchard *et al.* 2000), including reference specimens from the original collections in 1999 (New York and New Jersey) and 2000 (Pennsylvania) (*N* = 25 of each), along with all 461 genotyped Virginia specimens. STRUCTURE uses a Bayesian approach to assign multilocus genotypes into one or more ancestral clusters (Pritchard *et al.* 2000), and its calculations are independent of any *a priori* grouping. The model with admixture was run with a burn-in period of 10 000 followed by 10 000 reps. We determined the most likely number of clusters using STRUCTURE HARVESTER (Earl & vonHoldt 2012) to

implement the method of Evanno *et al.* (2005). STRUCTURE also gives scores for each individual (*Q* scores) that estimate the proportion of its ancestry originating from each cluster (Pritchard *et al.* 2000). In our analysis, these *Q* scores signify the proportion of each individual's nuclear ancestry derived from the NY type ('NY ancestry') and the proportion similar to the PA type ('PA ancestry') (most likely $K = 2$). We calculated the average proportion NY and PA ancestry for each collection site and population group by averaging *Q* scores across individuals.

To examine in detail the contributions of landscape variables to patterns in proportion ancestry (NY vs. PA), a beta-regression was conducted using the BETAREG package in R (Cribari-Neto & Zeileis 2010). This approach fits a nonlinear model using the beta-distribution and a logit link and is suitable when the response variable is (0,1) bounded as is the case with proportions (Ferrari & Cribari-Neto 2004). Model selection was evaluated using the function dredge() in package MUMIN (Barton 2015) to evaluate all possible models and rank them by AICc. AICc is a second-order derivation of AIC (Akaike's information criterion, which estimates the relative distance between true and approximating models) and is preferred for small sample sizes (Burnham & Anderson 2002). For each of the top ten models ranked by AICc in dredge(), the BIC (Bayes information criterion) was also calculated. In contrast to AIC and AICc, the BIC roughly corresponds to the posterior probability of a candidate model and is not a distance estimate (Burnham & Anderson 2002). To compare the top ten models, Δ_i was calculated as the difference between each model's AICc and the lowest AICc value ($\Delta_i = AICc_i - AIC_{min}$). Models with $\Delta_i < 2$ were considered to have substantial support (Burnham & Anderson 2002). Diagnostic plots were used to evaluate the fit of the most highly ranked models (Cribari-Neto & Zeileis 2010).

Each of the two original introductions identified by Fonseca *et al.* (2010) had a characteristic nuclear microsatellite genotype and associated set of ND4 haplotypes ('mitotypes') (Fonseca *et al.* 2010). In Virginia, we examined the correspondence between ND4 haplotype and nuclear cluster assignment (NY vs. PA) using both an analysis of molecular variance (AMOVA) and chi-square test. Each of the 96 ND4-haplotyped individuals was sorted by nuclear ancestry (based on which cluster contributed >50% of their ancestry). Then, an AMOVA was run using GENALEX 6.501 (Peakall & Smouse 2006) to determine whether mitotype frequencies differed between the NY and PA nuclear groups. For the chi-square test, we used those same nuclear groups and defined mitochondrial groups based on the NY vs. PA-associated mitotypes reported in Fonseca *et al.* (2010) (H1 = NY, H9/H12 = PA), then tested whether individuals are more likely to have matching nuclear and mitochondrial designations or more likely to have mixes.

Effects of landscape features on genetic structure

To examine measures of genetic diversity in relation to landscape factors using the population groupings, allelic richness (A_R) was calculated in FSTAT 1.2 and standardized to the smallest population size ($N = 16$) using rarefaction (Goudet 1995) for each group. Shannon's information index (*I*) and observed and unbiased expected heterozygosity were calculated in GENALEX 6.501 (Peakall & Smouse 2012). We tested for significant values of Spearman rank order correlations between genetic variables (allelic richness, Shannon's *I*, observed heterozygosity and unbiased expected heterozygosity) and landscape variables (latitude, longitude, elevation, annual precipitation, average annual temperature, distance from used tire vendors, distance from I-95 and distance from any nearest highway using the rcorr() command in the HMISC package in R (R Core Team 2013). Spearman's rho, a nonparametric method, was chosen due to several of the genetic variables failing to meet criteria of normality despite transformation. A multiple test correction was performed using the p.adjust() command with option 'BH' to control the false-discovery rate as in Benjamini & Hochberg (1995) and adjusted *P* values are reported in the results.

To examine the relationship between pairwise genetic distance (nuclear distance, calculated from the microsatellite data) and multiple landscape features, we created several distance matrices: an elevation distance matrix was calculated by subtracting the difference in elevation between pairs of population groups. A geographic distance matrix was calculated in GENALEX 6.501 (Peakall & Smouse 2006). Matrices of distance along roads and distance along streams were calculated using the Network Analyst toolbox in ARCMAP Version 10.2 (ESRI, Redlands, California). This tool maps each input point (e.g. GPS coordinates) to the nearest place on the network (whether roads or streams) and then calculates the distance between. In this analysis, input points were group mid-points. For the analysis of distance along streams, all pairs of populations that were not in the same watershed (i.e. not connected by streams) had to be excluded, leaving only 75 of 529 possible pairs. The genetic distance matrix used in these analyses was made from pairwise F_{ST} values calculated in FSTAT 1.2 (Goudet 1995) (Table S3, Supporting information).

The genetic distance matrix (pairwise F_{ST} values) was tested against the landscape distance matrices (straight geographic, elevation, roads and streams) in Mantel

tests in FSTAT 1.2 (Goudet 1995). Each distance matrix was first tested individually and then in a partial Mantel to control for straight geographic distance. As Mantel tests can perform poorly in situations with high spatial autocorrelation (Guillot & Rousset 2013), we also performed a multiple regression on distance matrices (MRDM) which has been proven reliable in several landscape genetics scenarios (e.g. Balkenhol *et al.* 2009; Prunier *et al.* 2015). Further, we calculated commonality coefficients for each MRDM model, a variance partitioning procedure that disentangles the individual vs. shared contribution of each predictor variable to R^2, useful when the predictors are themselves correlated (Ray-Mukherjee *et al.* 2014) as is the case when comparing spatial distance matrices. This was performed using the R packages ECODIST (Goslee & Urban 2007) and YHAT (Nimon *et al.* 2013) following the CAonDM script provided by Prunier *et al.* (2015) in Dryad.

Spatial autocorrelation tests were performed in GENALEX 6.501 (Peakall & Smouse 2012) using the multilocus approach developed by Smouse & Peakall (1999). In this method, an autocorrelation coefficient *r* is calculated using the genetic covariance between pairs of individuals within each geographic distance class. Then, the significance of *r* is tested both by bootstrapping (which generates a confidence interval) and a nonparametric permutation technique (which generates a *P* value) (Smouse & Peakall 1999). This method therefore allows an examination of the spatial extent over which individuals tend to be genetically similar, independent of population groupings. Because the pairwise covariance used to calculate *r* measures the extent to which two individuals vary in the same direction from a genetic centroid (Smouse & Peakall 1999), this analysis was performed separately for the two genetic clusters identified by STRUCTURE (roughly corresponding to the western vs. eastern parts of the state, e.g. see Fig. 2) using distance classes of 5 km.

The program GENETICSTUDIO (Dyer 2009) was used to create a Population Graph, a visual way of displaying genetic structure using a graph theory approach. The nodes of the graph represent populations, whose sizes are proportional to within-population variation, while the edges connect pairs of populations and represent significant covariance between them (reflecting gene flow) (Dyer & Nason 2004). The sum of all edge lengths equals the among-population variation component of genetic variance calculated in an AMOVA (σ^2_A), and therefore, Population Graphs can be considered an extension of an AMOVA test that visualizes how the variation is apportioned rather than just testing whether it exists (Dyer & Nason 2004). We also used the Graph component of GENETICSTUDIO to identify compressed and extended edges in the Population Graph with a chi-square test. Compressed edges are genetically more different than expected, given their geographical distance, suggesting a barrier to gene flow between those two nodes. Extended edges are more similar than expected given their distance, suggesting possible long-distance dispersal (Garrick *et al.* 2009).

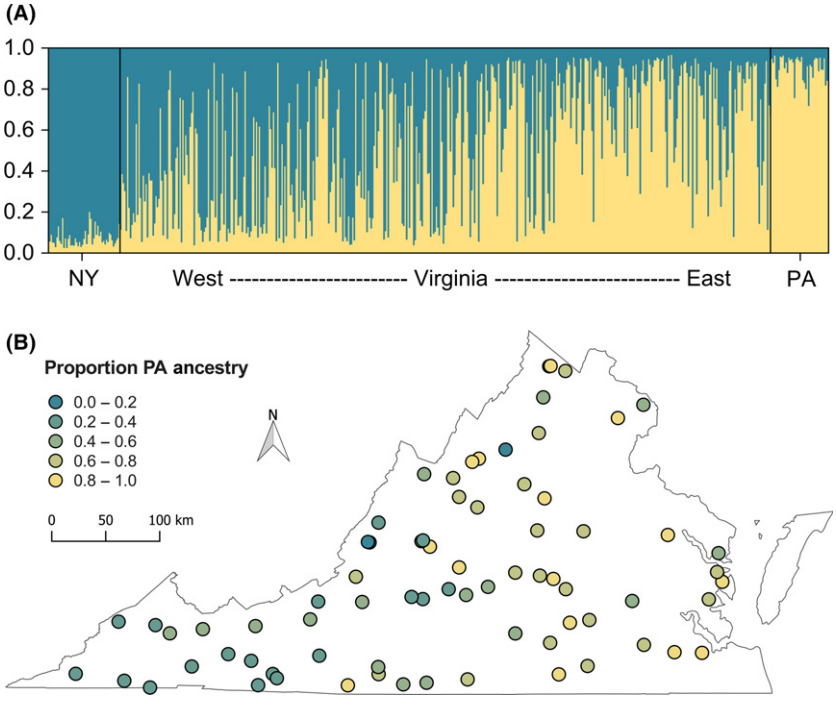

Fig. 2 (A) STRUCTURE graph displaying proportion nuclear ancestry of PA type (Yellow) and NY type (Blue) in a representative sample of the original New York and Pennsylvania introductions (collected in 1999 and 2000) and across modern day Virginia (collected in 2011). Virginia specimens are ordered by longitude. (B) Plot of *Ae. j. japonicus* collection sites in Virginia shaded by average proportion PA nuclear ancestry (calculated from STRUCTURE Q-scores) within each collection site. NY ancestry = 1- PA ancestry. Locations correspond to Fig. 1A.

Results

Ancestry of Virginia specimens

Unexpectedly given the prevalence of mixed genotypes already present further north (in New York, New Jersey and Pennsylvania) during 2002–2005 (Fonseca *et al.* 2010), there was still a strong gradient in NY vs. PA ancestry across Virginia in 2011. The eastern part of the state had a greater proportion PA ancestry while the western part had greater NY ancestry (Fig. 2, also see Fig. S2, Supporting information). Despite a strong correlation with multiple landscape variables, including longitude, temperature, elevation and distance from I-95 (Table S4, Supporting information) in the regression analysis containing all predictors (with PA ancestry as the response), only distance from I-95 was significant and had the largest estimate for beta (Table 2). The top two models ranked by AICc (with Δ_i <2, see Table S5, Supporting information) were Model 1 (distance from I-95+ highway distance) and Model 2 (distance from I-95 alone) (Table 2). Interestingly, while Models 1 and 2 had nearly identical AICc and BIC rankings, indicating similar fit, examination of residuals plots for Model 2 revealed the presence of a lurking variable unaccounted for in the model. This trend was not present in the residuals plots for Model 1, suggesting that the missing variable was highway distance and that Model 1 was the best fit overall.

Similarly, PA mitotypes (H9 & H12) just like PA genotypes were more common in the eastern part of the state, and the NY mitotype (H1) in the west (Fig. 3). Overall, mitochondrial–nuclear associations from the original two introductions (Fonseca *et al.* 2010) were broadly maintained (Fig. 4). A chi-square testing this association (e.g. that a specimen with NY nuclear genotype was equally likely to have either a NY or PA mitotype) was significant both when genotypes were defined as >50% ancestry ($X^2 = 8.97$, $P < 0.01$) and when genotypes were defined more stringently, as >75% ancestry ($X^2 = 6.59$, $P < 0.05$). This result was echoed in an AMOVA test, where specimens with a NY genotype had significantly different mitotype frequencies than specimens with a PA genotype ($\Phi_{PT} = 0.07$; $P = 0.007$). Interestingly, introgression of the PA mitotype into NY genotypes was greater than the reverse, an asymmetry that may have been driven by a number of specimens in the western part of the state with NY nuclear ancestry but an H9 mitotype (Figs 3 and 4).

Effect of landscape features on genetic structure

Shannon's *I*, a measure of within site group genetic diversity, was positively correlated with elevation ($r = 0.54$, $P = 0.0209$) and distance from I-95 ($r = 0.62$, $P = 0.0060$) and negatively correlated with longitude ($r = -0.57$, $P = 0.0132$). Although not significant, there was also a negative relationship between Shannon's *I*

Table 2 Beta-regression results for the model with all possible explanatory variables (Full Model) as well as the top two models (Models 1 and 2) ranked highest by model selection criteria (see text). Response variable is proportion PA ancestry, calculated from STRUCTURE Q-scores. Pseudo R^2 = squared correlation between linear predictors and the link-transformed response (Ferrari & Cribari-Neto 2004)

	Pseudo R^2	AICc	BIC	Predictor	Estimate	SE	Z value	Pr(>|z|)
Full model	0.7399	−6.394	−13.37	(Intercept)	0.18208	0.08658	2.103	**0.0355**
				Lat	0.05319	0.12256	0.434	0.6643
				Long	0.09916	0.39111	0.254	0.7999
				TireDist	0.02055	0.10601	0.194	0.8463
				DistHwy	0.21103	0.13176	1.602	0.1092
				Dist95	−0.95565	0.41889	−2.281	**0.0225**
				Elev	0.03784	0.39631	0.095	0.9239
				AvgT	−0.11806	0.30339	−0.389	0.6972
				AnnPrec	0.17737	0.12663	1.401	0.1613
Model 1	0.7176	−32.509	−30.19	(Intercept)	0.17824	0.09001	1.98	**0.0477**
				Dist95	−0.75825	0.10454	−7.253	**4.08E-13**
				DistHwy	0.19737	0.1013	1.948	0.0514
Model 2	0.6689	−31.929	−29.79	(Intercept)	0.16979	0.09635	1.762	0.078
				Dist95	−0.68122	0.10341	−6.587	**4.48E-11**

AICc = corrected Akaike's information criterion. BIC = Bayes information criterion. Predictor abbreviations: Lat = latitude; Long = longitude; TireDist = distance to the nearest used tire vendor; DistHwy = distance to the nearest class 1 or 2 highway; Dist95 = distance from Interstate 95; Elev = elevation; AvgT = average annual temperature; AnnPrec =average annual precipitation. Estimates are maximum-likelihood estimates of standardized beta-coefficients. Z and *P* values are for a Wald's test to determine whether the estimate of beta is significantly different from zero. Significant *P* values are bolded.

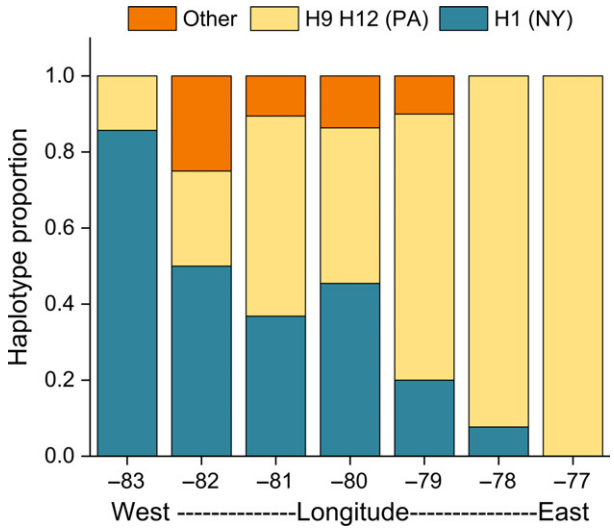

Fig. 3 Bar graph of the proportion of mitochondrial haplotypes ordered by longitude ($N = 96$). *X*-axis is labelled with the end-point of each bin. PA haplotypes are H9 and H12, the NY haplotype is H1, and other haplotypes (not associated with either original introduction) are H3, H4, H6 and H11 (see Fonseca *et al.* 2001, 2010).

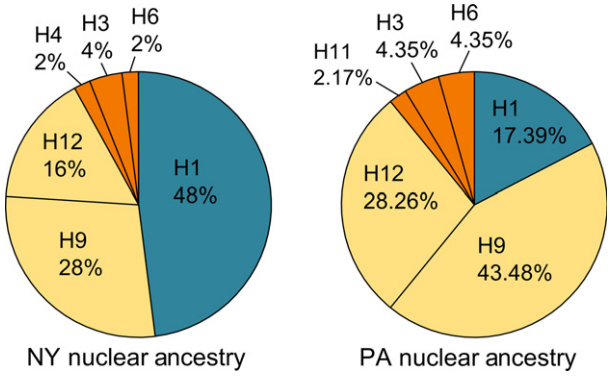

Fig. 4 Comparison of nuclear ancestry (defined by STRUCTURE Q-scores >0.50 for each cluster) vs. mitochondrial haplotypes in Virginia populations showing a greater introgression of PA haplotypes into NY nuclear genotypes than the reverse. Colour designations for haplotypes follow Fig. 3. Prior to mixing between the NY and PA introductions, haplotypes H9 and H12 were exclusively found in PA nuclear genotypes, while haplotype H1 was exclusively found in NY nuclear genotypes.

and average temperature ($r = -0.42$, $P = 0.1014$). Observed heterozygosity showed a similar pattern, positive correlation with elevation and distance from I-95 but negative correlation with longitude and temperature (Table S4, Supporting information) indicative of a general east–west gradient in genetic diversity. There were no significant correlations between genetic diversity variables and latitude, tire distance or distance from the nearest highway. There were also no significant correlations between landscape variables and mean allelic richness standardized to the smallest sample size ($N = 16$).

Mantel tests at the larger spatial scale, across all population groups, revealed that isolation by geographic distance explains approximately 25% of the variation in F_{ST} for the whole pairwise matrix ($r = 0.496$, $P = 0.001$). Both elevational separation and distance along roads show a much smaller value for r once geographic distance is accounted for ($r = 0.376$, $P = 0.001$ vs. partial $r = 0.119$, $P = 0.057$ for elevation; $r = 0.507$, $P = 0.001$ vs. partial $r = 0.142$, $P = 0.023$ for roads) although in the case of roads, it was still significant, indicating that roads have an additive effect on genetic connectivity that cannot be explained by geographic distance alone. In the subset of pairwise comparisons within watersheds, streams showed a small but significant correlation with F_{ST} ($r = 0.417$, $P = 0.041$) but this ceased to be significant once geographic distance was controlled for (partial $r = 0.282$, $P = 0.164$). These Mantel findings were supported by the MRDM analysis with commonality coefficients (Table 3) which found that the road matrix coefficient remained a significant predictor in a model accounting for geographic distance, and further, that roads uniquely contributed 7.54% towards the R^2 on top of what it shared with geographic distance. MRDM analyses also showed a lack of significant (and unique) contribution by stream networks and elevation distance matrices (Table 3). Spatial autocorrelation analyses comparing within eastern (PA) vs. western (NY) genetic clusters detected a significant positive autocorrelation in only the first distance class (≤5 km) for both ($r_{NY} = 0.07$, $r_{PA} = 0.05$, both $P = 0.001$).

The Population Graph shows a well-connected topology (Fig. 5A), suggesting there are no populations or groups of populations that are highly isolated from gene flow. The least-connected population is M10, which shares significant covariance with only 2 nodes, L2 and M4. Interestingly, these are not its closest neighbours (Fig. 1B). There is further evidence for widespread long-distance dispersal from the analysis shown in Fig. 5B, where extended edges stretch between low- and high-elevation populations, spanning the state. By contrast, compressed edges between pairs of populations in the mountainous west side of the state indicate possible barriers to gene flow (Fig. 3B, Fig. 1A,B).

Discussion

Across Virginia, there was a significantly greater proportion of PA ancestry and associated mitotypes ('PA type') in the east, at lower elevations, vs. a greater proportion of NY ancestry and its associated mitotype ('NY type') at higher elevations in the west. We found that

Table 3 Results from multiple regression on distance matrices (MRDM) analysis with commonality coefficients conducted using the CAonDM() script by Prunier *et al.* (2015) on response variable pairwise F_{ST}. Plotted residuals met assumptions of normality, and therefore, option 1 was selected (linear regression)

Model	Model R^2	Model *P*	Predictor	Beta	*P*	U (%)	C (%)	T
Roads	0.266	0.0001	Roads	1.499	**0.0283**	0.0200 (7.54)	0.2369 (89.12)	0.2569
			GD	−0.997	0.1422	0.0089 (3.33)	0.2369 (89.12)	0.2458
Elevation	0.260	0.0001	Elevation	0.1436	0.1120	0.0142 (5.46)	0.1268 (48.78)	0.1410
			GD	0.4156	**0.0001**	0.1190 (45.76)	0.1268 (48.78)	0.2458
Streams	0.253	0.0001	Streams	−0.089	0.1351	0.0069 (2.74)	0.0017 (0.067)	0.0086
			GD	0.5263	**0.0001**	0.2441 (96.59)	0.0017 (0.067)	0.2458

GD stands for 'geographic distance' based on coordinates. *P* values result from a significance test with 10 000 permutations.
U = unique contribution of each predictor, C = common contribution (shared among predictors), and T = total contribution of each predictor to the model fit. Significant *P* values are bolded.

the nuclear/mitochondrial associations from the original introductions have been largely maintained in Virginia despite the fact that admixture between the two was detectable as early as 2002 in more northern states (Fonseca *et al.* 2010). These results suggest at least two separate introductions into Virginia of populations broadly similar to the original PA and NY types, and subsequent expansion and mixing along a contact zone within Virginia, contrary to our original expectation that individuals with mixed NY-PA heritage simply diffused south. The former scenario of separate introductions is further supported by the fact that *Ae. j. japonicus* was first detected in Virginia in 2000 in the east (Harrison *et al.* 2002) and early populations had a strong PA signature (D.M. Fonseca, unpublished data). The east–west gradient in genetic diversity (lower diversity in the eastern part of the state) also supports this conclusion, as the original population that established in Pennsylvania had significantly lower genetic diversity than the New York population (Fonseca *et al.* 2010).

Model selection algorithms consistently chose models containing distance from I-95 as best explaining PA ancestry, suggesting a possible avenue for its expansion (Pennsylvania and Virginia are connected by this highway). Because I-95 is itself correlated with longitude, temperature and elevation, we must be cautious in our interpretation of its significance; however, it remains plausible as an expansion pathway given that other expansions of invasive insects have been linked to this

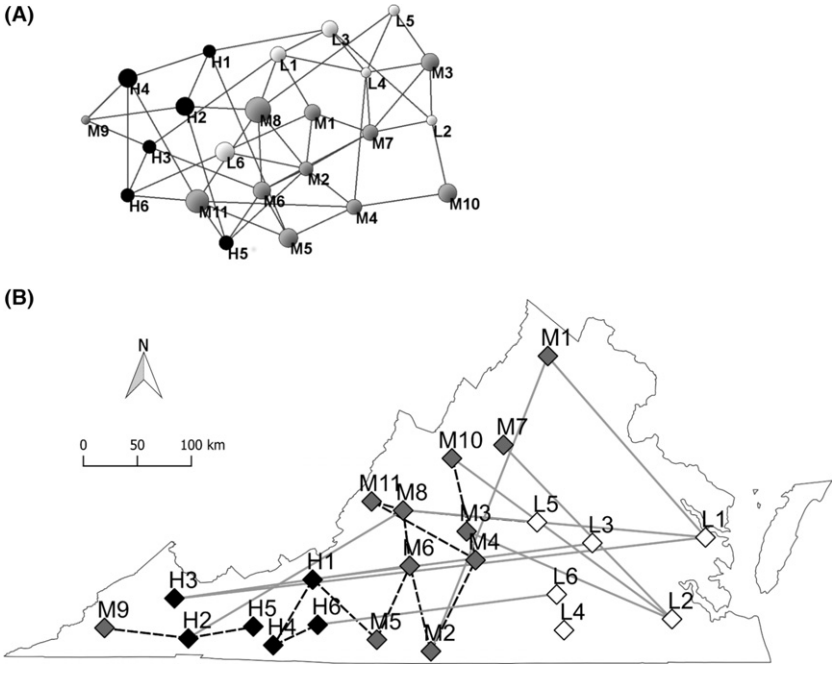

Fig. 5 (A) Network graph of population groupings of *Aedes j. japonicus* in Virginia. Nodes are coloured by elevation (black = high, grey = mid, white = low), and node size is proportional to within-population variation. Lines connecting nodes are called edges and represent significant covariance between the two nodes, such as gene flow between populations. (B) Map of Virginia and population grouping mid-points with compressed (black dashed lines) and extended (grey solid lines) edges. Compressed edges indicate significantly less genetic similarity than expected given their geographic distance, while extended edges indicate greater similarity than expected given their distance.

highly trafficked corridor in Virginia (e.g. Fall canker-worm, Asaro & Chamberlin 2015). Additionally, within Pennsylvania the PA *Ae. j. japonicus* type was originally associated with military bases – populations from different bases being more similar to each other than to populations just outside the base (Fonseca *et al.* 2010) – and all five of Virginia's largest military bases are at low elevations, in the east and close to I-95. By contrast, the mode and pathways of introduction of the NY type into Virginia are less clear: this type was originally most common in New York, New Jersey and Connecticut, three coastal states, so its presence in the western mountains of Virginia was unanticipated. However, there is some evidence for a long-distance translocation of individuals with NY ancestry into eastern Ohio, as early as 2000 (Fonseca *et al.* 2001). This part of Ohio is connected to western Virginia by several highways (such as Interstate 77), so the NY type could have arrived in Virginia after travelling first to Ohio. The asymmetric introgression of PA haplotypes (specifically, H9) into NY nuclear genotypes could imply that the NY type came to Virginia some years after the PA type, once mixing had begun farther north. This is supported by the fact that in 2002–2003, the NY type from Ohio had begun mixing with PA types in western Pennsylvania (Fonseca *et al.* 2010). However, asymmetric introgression of mitochondrial haplotypes can result from a multitude of factors, including disparate population sizes (Toews & Brelsford 2012), and the presence of compressed edges in the western part of the state suggests populations with NY nuclear ancestry may be more isolated from each other (i.e. smaller effective population size). Finally, although we did not find an association between genetic diversity and the distance from used tire vendors this could be due to our failure to identify all possible locations of used tire imports (such as those discarded in dumps and waste piles). The association of *Ae. j. japonicus* with tires is well supported by other lines of evidence (Bartlett-Healy *et al.* 2012; Kampen & Werner 2014).

As is made clear by the above discussion, we found that *Ae. j. japonicus* routinely capitalizes on human transport to travel large distances quickly, which would not be possible for them to do autonomously. It is well known that introductions of invasive mosquitoes into new locations are associated with human traffic, primarily cargo ships (Lounibos 2002). However, our analysis reveals that *Ae. j. japonicus* has continued to exploit humans as their main mode of travel on a more local scale, during their secondary expansion: proximity along roads explained additional variation in genetic distance not explained by geographic distance alone. Additionally, populations on opposite sides of the state (well beyond the dispersal capability of mosquitoes) were more similar than expected by chance (Fig. 5). Despite the possibility of streams acting as dispersal corridors for adults of this species (Bevins 2007), we found no effect of stream distance on genetic distance. While our ability to detect autonomous dispersal may have been limited in some of the analyses by our use of population groupings, autocorrelation performed on individual specimens did detect evidence of diffusion but only at very short distances (<5 km). Adults of other invasive *Aedes* species have also been found to be weak flyers with self-powered abilities of a kilometre or less based on mark–recapture studies (Niebylski & Craig 1994; Russell *et al.* 2005; Marini *et al.* 2010). A closely monitored population of *Ae. j. japonicus* that established in Belgium in 2002 was unable to expand beyond one tire recycling centre in over 10 years (Damiens *et al.* 2014). While the presence of compressed edges in the Appalachian region indicates the mountains are acting as a barrier to gene flow, they may be primarily because they act as barriers to human vehicular traffic.

Our results strongly indicate that the main mode of expansion of *Ae. j. japonicus* has been human-mediated. Many studies of mosquito population genetics have invoked human-aided long-distance dispersal (LDD) as an explanation for low genetic differentiation between geographically distant populations (Lehmann *et al.* 1996; Huber *et al.* 2004; Brown *et al.* 2011; Brelsfoard & Dobson 2012; Damal *et al.* 2013) although fewer have explicitly tested the effects of human transportation on the spread of an invasive species (but see Fonzi *et al.* 2015 regarding boat traffic among islands). Medley *et al.* (2014) studied the effects of land cover types on the US expansion of another aggressive invader, *Aedes albopictus*, using nine microsatellite loci. They examined patterns across a much larger spatial scale than ours (across its entire central-eastern distribution, 1–3 sample points per state) and found the best-fit models for genetic distance at broad spatial scales contained an array of landscape factors including highways. At smaller scales, agricultural landscapes and forests appeared to limit gene flow leading to the conclusion that *Ae. albopictus* disperse autonomously at these smaller scales. However, due to the scale of their study we note that these 'smaller' spatial scales would still require mosquitoes to self-transport hundreds of kilometres across states, and we argue that large swathes of agricultural lands and forests also act as a barrier to vehicular traffic, reconciling their results with ours.

Interestingly, contrary to our previous findings of the strong influence of elevation (probably due to the effects of temperature) on *Ae. j. japonicus* genetic patterns in Hawaii (Egizi & Fonseca 2014), we did not find a strong elevational pattern in Virginia that could not also be explained by other factors (like LDD +

admixture, see above). PA ancestry was better explained by a sample's proximity to I-95 than by elevation or temperature, and elevation was not better at explaining genetic distance than geographic distance alone. However, given the demonstrated negative effects of high temperature on this species' survival (Scott 2003; Egizi *et al.* 2014), we are hesitant to rule out any effect of elevation on this species in Virginia. For example, it is possible that the east–west gradient we observed could actually reflect differential responses to temperature among the two genetic types (e.g. both types were introduced at low elevations multiple times but PA alone was able to establish). Alternatively, it is possible that the relatively weak elevational gradient in Virginia (compared to the steep flanks of the Hawaiian volcanoes, where we did see an effect of elevation), coupled with much greater gene flow from nearby states and fewer generations per year (due to winter) have resulted in overall weaker selective pressures in Virginia compared with Hawaii, despite the introduction to Hawaii likely being more recent. Common garden experiments profiling the temperature tolerances of low- vs. high-elevation populations in Hawaii and Virginia can help to untangle these hypotheses of local adaptation and differential selection.

Overall, we conclude that *Ae. j. japonicus* has been able to achieve a rapid and extensive secondary expansion primarily through exploitation of human-assisted transportation across multiple spatial scales, both within and across states. By contrast, its autonomous dispersal capabilities appear localized and limited as evidenced by its failure to expand in Belgium (Damiens *et al.* 2014). We propose that a pattern of human-enabled secondary expansion even over short distances may be a common thread in successful mosquito invasions. The idea that an invasive mosquito may be mostly or entirely reliant on humans for its secondary dispersal stands in contrast to several definitions of the term 'invasive species' that presuppose a species capable of expanding by its own agency postestablishment (Ehrlich 1986; Falk-Petersen *et al.* 2006; Simberloff 2010). Importantly, we argue that movement by/with humans can shape a species' expansion in ways that autonomous spread cannot; specifically, it can lead to large-scale admixture between locally adapted populations and potentially greater evolutionary flexibility (genetic diversity and novel genotypes) on which natural selection can act (Suarez & Tsutsui 2008; Schierenbeck & Ellstrand 2009). Selection could then favour traits that enhance a species' ability to invade (Keller & Taylor 2010) and, in the case of mosquitoes, traits that alter their ability to harbour pathogens and interact with hosts. In this manner, human transport could continue to play a pivotal role even postestablishment, by altering vectorial capacity and thus creating the potential for vectorborne disease outbreaks. This result has far-reaching implications for disease prevention, and may require a paradigm shift from vector control as a responsibility of communities towards individual responsibility (such as taking care to disinsect water-holding containers prior to long-distance transport).

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A.E. and D.M.F. jointly designed the project and wrote the manuscript; A.E. conducted molecular laboratory work

and landscape analyses; J.K. and C.A. collected all Virginia specimens and provided feedback on the manuscript.

Data accessibility

Microsatellite genotype data are available in Dryad:-doi:10.5061/dryad.sd3b0/2. The sequence for haplotype H1 is available in GenBank (Accession No. AF305879.1), and the polymorphisms used to define all other haplotypes (relative to H1) are the same as in Fig. 3 of Fonseca *et al.* (2001).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Complete list of locations where *Ae. j. japonicus* larvae were collected in 2011

Table S2 Individual specimens sequenced with ND4 primers to determine mitochondrial haplotype

Table S3 Pairwise F_{ST} values calculated in FSTAT 1.2 (Goudet 1995)

Table S4 Pairwise Spearman correlations for all variables calculated in R using the rcorr() command in HMISC

Table S5 Results of dredge() model selection algorithm in MUMIN package (Barton 2015) for top 10 beta regression models, ranked by AICc, plus full and intercept-only models

Fig. S1 Map of specimens selected for ND4 sequencing in Virginia, plotted by haplotype (one point per individual).

Fig. S2 Linear regression of Longitude on PA ancestry across individuals.