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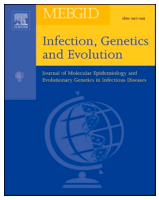

Origins of high latitude introductions of *Aedes aegypti* to Nebraska and Utah during 2019

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ABSTRACT

Aedes aegypti (L.), the yellow fever mosquito, is also an important vector of dengue and Zika viruses, and an invasive species in North America. *Aedes aegypti* inhabits tropical and sub-tropical areas of the world and in North America is primarily distributed throughout the southern US states and Mexico. The northern range of *Ae. aegypti* is limited by cold winter months and establishment in these areas has been mostly unsuccessful. However, frequent introductions of *Ae. aegypti* to temperate, non-endemic areas during the warmer months can lead to seasonal activity and disease outbreaks. Two *Ae. aegypti* incursions were reported in the late summer of 2019 into York, Nebraska and Moab, Utah. These states had no history of established populations of this mosquito and no evidence of previous seasonal activity. We genotyped a subset of individuals from each location at 12 microsatellite loci and ~ 14,000 single nucleotide polymorphic markers to determine their genetic affinities to other populations worldwide and investigate their potential source of introduction. Our results support a single origin for each of the introductions from different sources. *Aedes aegypti* from Utah likely derived from Tucson, Arizona, or a nearby location. Nebraska specimen results were not as conclusive, but point to an origin from southcentral or southeastern US. In addition to an effective, efficient, and sustainable control of invasive mosquitoes, such as *Ae. aegypti*, identifying the potential routes of introduction will be key to prevent future incursions and assess their potential health threat based on the ability of the source population to transmit a particular virus and its insecticide resistance profile, which may complicate vector control.

1. Introduction

Aedes aegypti (L.), the yellow fever mosquito, is an invasive species in North America with a long history of transmitting viruses to humans. Likely originating on islands off the east coast of Africa, *Ae. aegypti* entered sub-Saharan Africa about 50,000–85,000 years ago (Soghigian et al., 2020). In the 16th century, the species was introduced to the New World via the European slave trade in water barrels intended to collect rainwater on the docks and ships during transatlantic voyages (Spielman and D' Antonio, 2001; Gloria-Soria et al., 2016; Crawford et al., 2017; Kotsakiozi et al., 2018a; Powell et al., 2018). Since then, *Ae. aegypti* has spread around the globe to sub-tropical and tropical areas on every

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continent except Antarctica and is now endemic in South America and parts of temperate North America (Johnson et al., 2017). *Aedes aegypti* distribution is temperature-restricted as this species is not able to survive long cold periods, i.e., temperate winters (Farajollahi and Crans, 2012). In his authoritative book, Christophers (Christophers, 1960) sets the distribution limits of year-around activity to localities with a mean temperature of 10 °C or higher, corresponding to 30-35° N and S latitudes in the coldest month.

While the above applies to established populations of *Ae. aegypti,* the species can temporarily colonize localities at higher latitudes in summers, sometimes causing disease epidemics. For example, the city of Philadelphia in the United States of America (US) at latitude 40° N had severe outbreaks of yellow fever in 1793, causing the newly formed US Congress to adjourn and flee the city (Foster et al., 1998). Outbreaks of yellow fever also occurred in northern port cities of Europe like Saint-Nazaire, France (1861) and Swansea, UK (1865) in the 19th century, associated with the arrival of infected sailors and the introduction of *Ae. aegypti*, which thrived locally in the summer months (Chantemesse and Borel, 1905; Schaffner and Mathis, 2014). Reports of *Ae. aegypti* across the US from 1995 to 2016, which include those outside its permanent range, contain records as far north as Chicago, Detroit, and southern New Hampshire (43° N latitude) (Hahn et al., 2016; Hahn et al., 2017). Likewise, in Europe, *Ae. aegypti* has been intercepted as far north as the Netherlands (Brown et al., 2011a; Ibañez-Justicia et al., 2017) and England (Dallimore et al., 2017). These records indicate that *Ae. aegypti*'s association with humans allows the species to "hitchhike" considerable distances in a short time. Given *Ae. aegypti*'s importance as a vector of human viruses, documenting where the new temporary introductions originate is relevant for preventing future incursions and to assess potential health threats. For example: Did the incursion originate from an area known to have insecticide resistance that could compromise standard vector control approaches? Did it originate from an area with ongoing disease epidemics indicating the vector population is capable of virus transmission? And did the incursion stem from a location with consistent travel/trade where increased border inspections or quarantines could limit future introductions?

Two *Ae. aegypti* incursions were reported in the late summer of 2019 into US states with no history of established populations and no evidence of seasonal activity of the species: Nebraska [NE] and Utah [UT]. On 27 August 2019, the Nebraska Department of Health and Human Services (NDHHS) collected five mosquitoes morphologically and genetically identified as *Ae. aegypti* during routine surveillance from York, NE. This detection was the first reported record of this invasive mosquito in the state of Nebraska. Enhanced surveillance after the initial detection collected additional 118 *Ae. aegypti* (87 adult females; 31 adult males). An NDHHS epidemiological investigation to determine possible routes of introduction of *Ae. aegypti* into York reported residents' travel histories to areas south of Nebraska. This included eight southern US states: Arizona, Arkansas, Florida, Louisiana, Missouri, New Mexico, Oklahoma, and Texas, as well as to destinations in the Caribbean, Mexico, and South America. On 29 August 2019, during surveillance for *Culex* larvae in Moab, UT, staff of the Moab Mosquito Abatement District (MMAD) collected two larval specimens not recognized as one of the common mosquito species inhabiting the area. Further identification of specimens using taxonomical keys indicated that the specimens were likely *Ae. aegypti*. In Utah, *Ae. aegypti* had been previously detected during the summer of 2013 near Leeds, in the southwest corner of the state (S. Amodt personal communication), but has not been detected in the state since. Following the initial 2019 detection in Moab, surveillance using adult mosquito traps collected additional adult *Ae. aegypti* and more larval specimens were also found during inspections of nearby properties.

As part of the ongoing collaboration with Mosquito Control Districts and Public Health Departments in the US, a subset of specimens from both York, NE and Moab, UT was sent for genetic analysis to the Powell Laboratory at Yale University. Over the past 15 years, the Powell Laboratory has amassed a worldwide genetic database of *Ae. aegypti* that clearly shows that populations occupying different geographic regions have distinct genetic signatures (Gloria-Soria et al., 2016). This database combines allele frequencies at 12 microsatellite loci from ~12,000 mosquitoes from over 350 geographically distinct population samples and ~ 20,000 single nucleotide polymorphic markers (SNPs) from 5420 mosquitoes from 260 population-samples and continues to grow. These databases have been used to infer the origin of the introduction of *Ae. aegypti* into the Netherlands (Brown et al., 2011a), California (Gloria-Soria et al., 2014; Pless et al., 2017), Las Vegas (Pless and Raman, 2018), and Washington DC (Gloria-Soria et al., 2018). Here we perform comparable analyses for recent (2019) introductions of *Ae. aegypti* to York, NE and Moab, UT to identify potential source areas and infer the number of introductions.

2. Material and methods

2.1. Collections

2.1.1. Nebraska

Local health department staff set three routine mosquito traps at biweekly intervals during the 2019 surveillance season (26 June – 28 September 2019) within York, NE. Traps used for routine collections were CDC miniature light traps (Model 512; John W. Hock Company, Gainesville, FL) baited with dry ice, set in the late afternoon or early evening, and retrieved the next morning. After the first detection of *Ae. aegypti* on 27 August 2019, enhanced surveillance was implemented starting on 6 September 2019 followed by several additional rounds of trapping on 7 September, 25 September, and 9 October. During the first round of enhanced surveillance, ten traps (5 CDC light traps, 5 Biogents Sentinel [BG] traps - Biogents AG, Regensburg, Germany) were set on four adjacent properties to the residence where *Ae. aegypti* was first detected. In the second round, trapping was expanded to 14 mosquito traps (9 CDC light raps, 5 BG traps) that were distributed throughout the residential neighborhood. The third and fourth rounds consisted of 25 BG traps distributed in the neighborhood. All traps were baited with dry ice while BG traps were also baited with the BG lure - combination of ammonia, lactic acid, and caproic acid designed to mimic human skin odors (Farajollahi et al., 2009). Traps were set mid-morning (BG traps) or late afternoon (CDC light traps). Both trap types were then collected the following morning where live mosquitoes were immobilized and killed via freezing and then shipped overnight on dry ice to the NDHHS Mosquito Identification Laboratory for morphological identification (Donahue et al., 2021). Traps continued to be positive for *Ae. aegypti* until trapping stopped due to colder weather conditions that developed. Traps were positive on the following dates: 06 September 2019, 07 September 2019, 17 September 2019, 18 September 2019, 25 September 2019, and 09 October 2019. Twenty-four mosquitoes collected from eight different traps were sent in September 2019 to the Powell lab at Yale University for further genetic testing (2 males and 22 females).

2.1.2. Utah

Following the detection of two *Ae. aegypti* larva during MMAD routine larval surveillance on 29 August 2019, adult surveillance was performed using BG traps baited with dry ice, with an additional five BG traps also baited with the BG lure. Traps were deployed in the vicinity of the larval samples on multiple nights starting on 30 August 2019, allowed to operate overnight, and collected the next day for enumeration and identification. Further trapping was performed in transects stemming from the initial infestation site to determine the extent of the *Ae. aegypti* distribution and the location of higher population densities. Trap collection nets were transported to the laboratory where the nets were placed into a low-temperature freezer to kill the mosquitoes. The mosquitoes were then identified using standard dichotomous keys (Darsie Jr and Ward, 1981). Larval samples were also collected during

door-to-door residential surveys using standard one-pint dippers or dumping of small containers holding water into plastic zip-lock bags. Larval specimens were transported back to the laboratory and identified alive under 10× magnification (Farajollahi and Price, 2013). Larvae were then placed into rearing jars and allowed to mature into adults for confirmation. Adults were then killed by placing the tops of the emergence jars into a freezer. The adults from the BGS traps and from emerged larval collections were stored in a − 20 °C freezer until shipment for genetic analysis. Surveillance was continued until traps were negative for three consecutive weeks and colder weather conditions limited mosquito activity. Positive traps for *Ae. aegypti* were detected on 3, 4, 6, 7, 8, 15, and 21 September 2019. All traps were negative thereafter. Twenty-three mosquito females collected between September 7–11 across 13 traps were shipped overnight on dry ice to the Powell Laboratory at Yale University in December 2019.

2.2. DNA extraction and genotyping

DNA was extracted from 29 and 23 samples from Nebraska and Utah, respectively, using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, with an additional RNAse A (Qiagen, Hilden, Germany) step. Samples were stored at −20 °C until further use.

2.2.1. Microsatellites

Twenty-nine individual mosquitos from Nebraska and 23 from Utah were genotyped as described in Gloria-Soria et al. (2016) (Gloria-Soria et al., 2016). The microsatellite loci analyzed were: A1, B2, B3, A9 (trinucleotide repeats), and AC2, CT2, AG2, AC4, AC1, AC5, AG1, and AG4 (di-nucleotide repeats) (Slotman et al., 2007; Brown et al., 2011b). Polymerase chain reactions were conducted as 10 μl reactions using the Type-it Microsatellite PCR Master Mix (Qiagen, Hilden, Germany), 25 nM of each forward primer, 250 nM of each reverse primer, and 500 nM of a fluorescently labeled M13 primer. Thermocycler conditions were: 94 °C x 10′, 35 x (94 °C x 30″, 54 °C x 30″, 72 °C x 30″), and 72 °C x 5′. The resulting products were processed for fragment analysis at the DNA Analysis Facility at Science Hill at Yale University, using GS 500 Rox internal size standard (Applied Biosystems, Waltham MA, USA). Microsatellite alleles were scored using GeneMapper v4.0 (Applied Biosystems Waltham MA, USA).

2.2.2. SNP genotyping

Twelve individuals from Utah and 24 from Nebraska were genotyped using the *Ae. aegypti* Axiom_aegypti1 SNP chip (Life Technologies Corporation, Carlsbad CA, USA, CAT#550481; (Evans et al., 2015)) at the University of North Carolina Functional Genomics Core, Chapel Hill following the Axiom® 2.0 Assay Automated Workflow User Guide (Life Technologies. Carlsbad, CA, USA). The Beckman Coulter Biomek FXP Automated Laboratory Workstation (Beckman Coulter Life Sciences. Indianapolis, IN, USA) and Affymetrix Gene Titan MC Instrument (Life Technologies. Carlsbad, CA, USA) were used for all sample preparation, hybridization, ligation, washing, staining and scanning of the samples. Briefly, 200 ng of gDNA is amplified for 23 h at 37 °C using Module 1 of the Axiom 2.0 Reagent Kit (Thermo Fisher Scientific. Vilnius, Lithuania). After amplification, the samples are fragmented using Module 2 of the Axiom Reagent Kit (Thermo Fisher Scientific. Vilnius, Lithuania). The fragmented DNA is precipitated overnight at −20 °C. Precipitated DNA is then centrifuged for 40 min at 4 °C at 3200 x g (4000 RPM) in an Eppendorf 5810R centrifuge (Eppendorf AG. Hamburg, Germany). Resuspension and hybridization preparation of the samples are carried out using Module 2 of the Axiom Reagent Kit. Following preparation of the hybridization plate, the samples are denatured and transferred to a GeneTitan hybridization tray. The Axiom array plate and hybridization tray are then loaded onto the GeneTitan MC Instrument. The samples hybridize on the GeneTitan for 23.5 h. Following hybridization, ligation and stain trays are prepared and loaded onto the GeneTitan MC Instrument. Ligation, washing, staining and scanning of the array plate is carried out on GeneTitan MC Instrument. Initial sample and array quality are assessed using the Thermo Fisher Axiom Analysis Suite software v. 5.1 (Affymetrix, Inc., Santa Clara, CA, USA). Generating SNP genotyping calls is also carried out using the same software. One Utah individual was excluded from the analysis due to low genotyping quality. Additionally, data from previously described samples from populations of *Ae. aegypti* collected worldwide were also used (see Table S1 for details).

2.3. Analyses

Individuals from Nebraska and Utah were analyzed together with previously genotyped individuals across *Ae. aegypti* distribution (Table S1).

2.3.1. SNP calling and filtering

Loci that fail to genotype at 80% or more of the individuals from the global dataset were filtered out from the 22,849 loci obtained from the SNP-chip that met Mendelian expectations, using the –geno 0.2 option in PLINK 1.9 ((Chang et al., 2015); *www.cog-genomics.org/plink/1.9/*). Subsequently, individuals missing more than 5% of the remaining SNPs were removed with the –mind 0.05 option. The data was further filtered for minimum allele frequency of 1% (MAF; –maf, 0.01) and linkage disequilibrium (LD; –indep-pairwise 50 10 0.3). The final dataset had 13,692 SNPs and 688 individuals from across *Ae. aegypti* distribution.

2.3.2. Genetic diversity

Average observed (H_o) and expected (H_e) heterozygosities were estimated from the microsatellite dataset that included all populations from the Americas in GenoDive 3.04 (Meirmans and van Tienderen, 2004). Allelic richness (AR) was calculated in HPRARE (Kalinowski, 2005), which uses rarefaction to correct for unequal sample sizes (N = 30 genes). Estimates of genetic diversity were not calculated from the SNP dataset to avoid influence of possible ascertainment bias arising from the selection of SNPs included in the *Ae. aegypti* Axiom_aegypti1 SNP chip on these parameters.

2.3.3. Population structure

We first used the larger microsatellite dataset, which provides a greater global representation of *Ae. aegypti* populations, to determine the subspecies of the introductions and their main genetic affinities. Genetic clustering at the global level was evaluated with a multivariate approach, using Discriminant Analysis of Principal Components (DAPC (Jombart et al., 2010)) on microsatellite allele frequencies from populations representing *Ae. aegypti* world distribution and plotted with the ADEGENET package (Jombart, 2008) in R v. 3.2.2. (R Core Team, 2020). Population structure was further evaluated via the Bayesian clustering method implemented by the software STRUCTURE v. 2.3 (Pritchard et al., 2000) on the microsatellite dataset of populations outside Africa. STRUCTURE identifies genetic clusters and assigns individuals to these clusters with no a priori information of sample location. The most likely number of clusters (K) was determined by conducting 20 independent runs from each K = 1 to 6. Each run assumed an admixture model and correlated allele frequencies using a burn-in value of 100,000 iterations followed by 500,000 repetitions. The optimal number of K clusters was determined following the guidelines of Prichard et al. (Pritchard et al., 2000) and the Delta K method (Evanno et al., 2005), as implemented by STRUCTURE HARVESTER (Earl and VonHoldt, 2012). Results were plotted with the program DISTRUCT v.1.1 (Rosenberg, 2004). Principal component analysis was performed on the SNP global dataset, as well as on the Out-of-Africa (N = 643) and the Americas (N = 499) subsets using PLINK 1.9 (Chang et al., 2015) –pca command and plotted in R (R Core Team, 2020). Relatedness among individuals within York, NE and Moab, UT was determined by estimating kinship coefficients in VCFtools v.0.1.14 (Danecek et al., 2011) with the –relatedness2 command and the

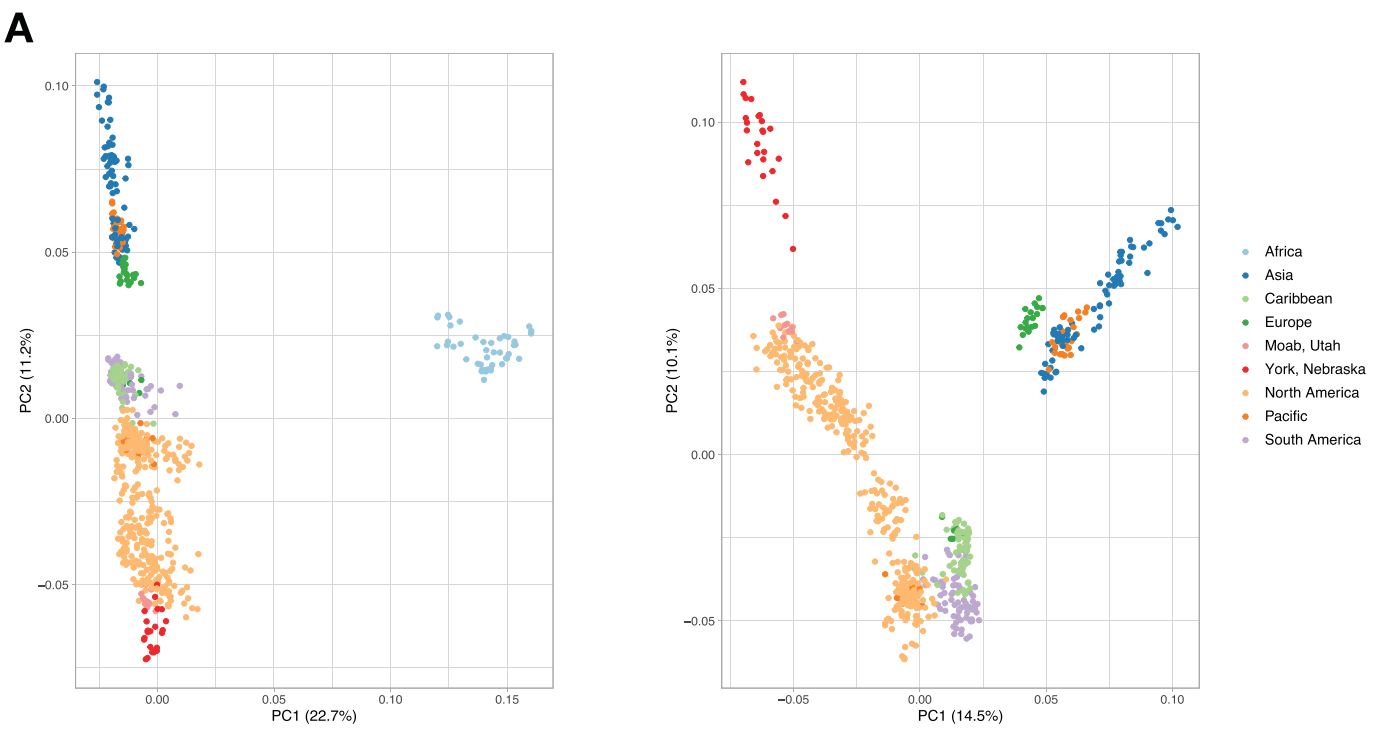

Fig. 1. Principal component (PC) analysis of *Aedes aegypti* populations across the species distribution range using 13,692 SNPs. A) global dataset, and B) Out-of-Africa dataset. Colors indicate different geographic regions, except for the populations from York, Nebraska and Moab, Utah; which are represented by their own color.

population dataset that had not been filtered for LD, as suggested by Manichaikul et al. (Manichaikul et al., 2010). Coefficients above 0.354 are considered duplicates or monozygotic twins, coefficients ranging from 0.177 and 0.354 are first-degree relationships (parent-progeny or full-siblings), and values ranging from 0.0884 to 0.177 correspond to 2nd-degree relationships (cousins, half-siblings, etc.) (Manichaikul et al., 2010).

2.3.4. Genetic affinities

A first genetic group assignment based on microsatellite genotypes was performed using DAPC (Jombart, 2008; Jombart and Ahmed, 2011), by positioning the Nebraska and Utah individuals onto the discriminant functions generated from the Out-of-Africa dataset and specifying three genetic clusters. Subsequently, genetic assignment tests of the Nebraska and Utah *Ae. aegypti* against a dataset that included all populations from the Americas were performed in GeneClass2 (Piry et al., 2004) using SNP data. Previous studies have shown higher accuracy of this assignment method using SNPs rather than microsatellites (Gloria-Soria et al., 2018; Kotsakiozi et al., 2018b). Ten independent runs were conducted with sets of 3500 SNPs drawn at random using the command –thin-count 3500 from PLINK 1.9. ((Chang et al., 2015); *www.cog-genomics.org/plink/1.9/*), and the Bayesian criteria for likelihood estimation to determine the population-assignment ranking (Rannala and Mountain, 1997). Self-assignment tests on the SNP reference dataset resulted in all individuals assigned to the expected geographic location. Additionally, we inferred the original geographic location of the introduced individuals in Locator (Battey et al., 2020) using a bootstrap of 10. Locator implements a deep learning approach on unphased genotype data to predict location based on a training set, without assuming any explicit model of variation over the landscape. Maps were generated with the maps package (Becker and Wilks, 2018) in R v. 3.2.2. (R Core Team, 2020) and the s.class function from ADEGENET (Jombart, 2008) to represent all bootstrap results and the inertia ellipses per individual. Inertia ellipses are meant to highlight the geographic locations assigned to an individual across the 10 bootstrap iterations and show the area to which most of the iterations were projected; they do not represent a confidence interval.

A phylogenetic tree was constructed from the Americas' dataset using a maximum likelihood approach in IQ-TREE v.1.6.12 (Nguyen et al., 2015). Branch support was evaluated with 5000 replicates for the ultrafast bootstrap approximation [−bb 5000] (Hoang et al., 2018) and 1000 replicates for the SH-like approximate likelihood ratio test [−alrt 1000] (Guindon et al., 2010). Nine *Ae. aegypti formosus* individuals, a closely related subspecies restricted to Africa, were included as outgroups (Gabon, Cameroon, and South Africa). The best-fitting substitution model was determined by the software using a model finder algorithm that resembles jModelTest/ProtTest (−m TEST option). The resulting tree was plotted with FigTree v. 1.4.4 (*http://tree.bio.ed.ac.uk/software/figtree/*). A population maximum likelihood tree of the Americas was built in TreeMix (Pickrell and Pritchard, 2012) using the same African outgroups as the individual-based phylogeny. TreeMix uses allele frequencies to construct the tree and tests for evidence of admixture (gene flow) between diverged populations, which would violate the assumptions of a phylogeny. Five trees were built, without migration events and allowing one to four events (−m 4). Support was evaluated using 100 rounds of bootstrapping. Trees were summarized in Beast2 TreeAnnotator (Bouckaert et al., 2019) and plotted with the function provided by TreeMix for R (Pickrell and Pritchard, 2012).

3. Results

Overall genetic diversity estimated from microsatellites was lower in the introduced collections than in other populations in the continent. Observed heterozygosity (Ho) was 0.4195 in York, NE and 0.3953 in Moab, UT, compared to an average of 0.5200 ± 0.0811 across populations in the Americas, with no statistical difference between these introduced populations and the rest of the Americas dataset (Kruskal-Wallis chi-squared = 3.5889, df = 1, p-value = 0.0581). Estimated allele richness (AR) in the York, NE collection was 2.17 and 2.44 in the Moab, UT collection, lower than the average of 3.58 ± 0.5492 (2.25–4.69)

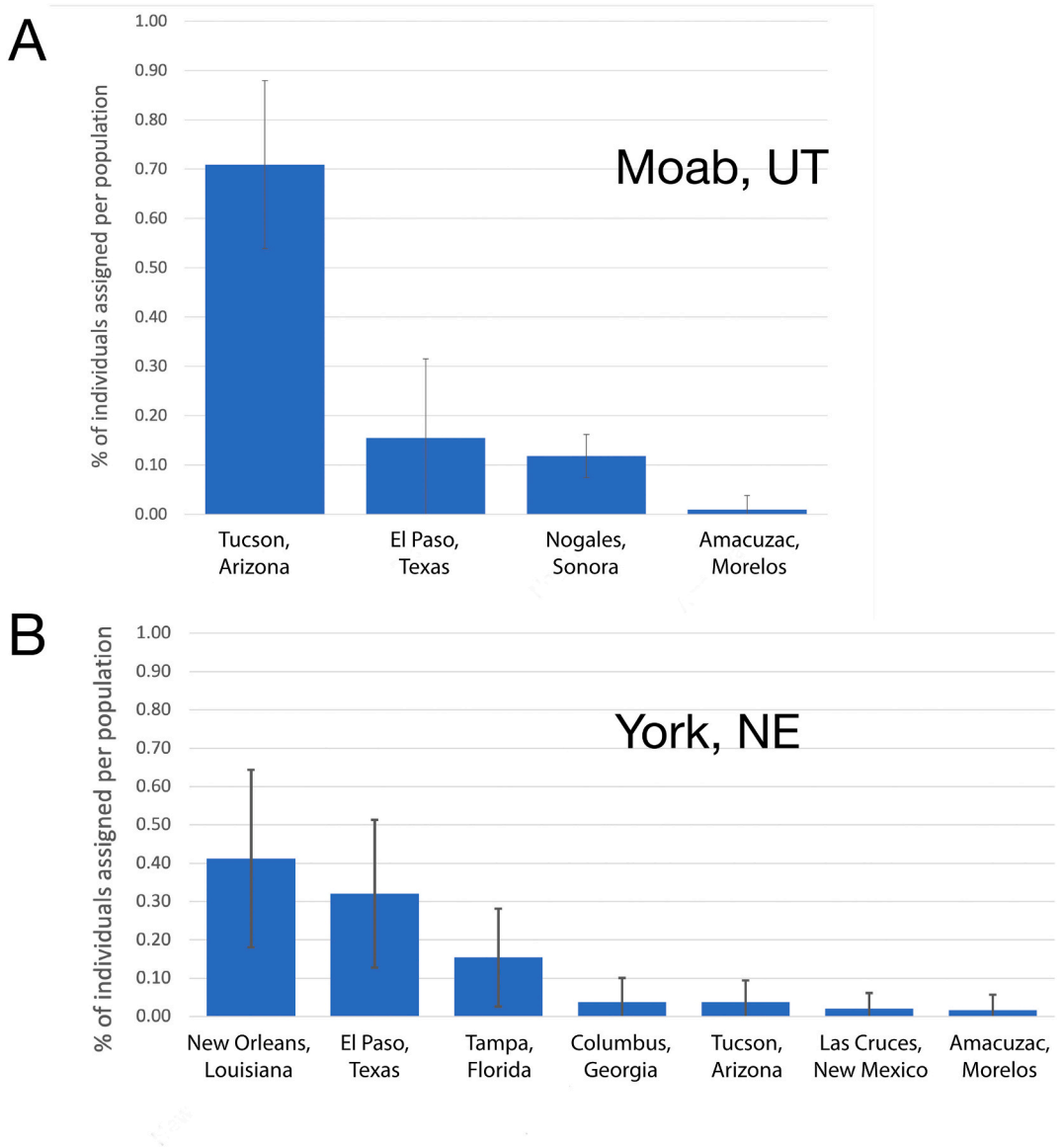

Fig. 2. Percentage of individuals from A) Moab, Utah and B) York, Nebraska, assigned with the highest score to each of the reference populations depicted in the X-axis. Each column represents the average fraction of individuals assigned across 10 independent replicas using 3500 randomly selected SNP markers. Error bars are the standard deviation. Only populations to which at least one individual was assigned are shown. Assignments were performed using Bayesian criteria for likelihood estimation with GeneClass2 (Piry et al., 2004).

from the Americas (Kruskal-Wallis chi-squared = 5.3202, df = 1, p-value = 0.0211; Table S2).

DAPC on the global microsatellite data indicates that the introductions from Nebraska and Utah clustered with *Ae. aegypti* populations outside Africa, corresponding to the subspecies *Ae. aegypti aegypti* (Fig. S1). Bayesian clustering analysis identified K = 3 as the optimal number of genetic clusters present in the Out-of-Africa microsatellite dataset, separating North and Central America, South America, and Asia; grouping both York NE and Moab, UT within the North American group (Fig. S2). This was consistent with the population assignment performed with DAPC, considering K = 3 clusters, where both York, NE and Moab, UT were assigned to a genetic group containing most North American populations (Fig. S3). Likewise, the PCA on the SNP global dataset supports that York, NE and Moab, UT collections belong to the *Ae. aegypti aegypti* cluster, which includes all Out-of-Africa populations (Fig. 1A). Further analysis on the Out-of-Africa populations, shows the first PC separating populations from Asia and the Pacific from those in the American continent. In the PCA from Out-of-Africa populations, the second PC separates York, NE from the rest of the American continent (Fig. 1B).

Kinship estimates using the SNP dataset suggest that over half (53%) of the pairwise relationships among Moab, UT individuals are of 1st degree, and 29% of 2nd degree. In York, NE 34% of pairwise relationships were estimated to be 1st degree relationships and 26% of 2nd degree (Table S3). Because these collections are comprised of a small sample of highly related individuals, the microsatellite allele frequencies used for population genetic analyses may not accurately represent that of their population of origin. However, this should not affect the individual-based analyses and this problem should be minimized in the data derived from the SNP chip, because of a much larger number of markers.

Individual genetic assignment tests (GeneClass2) using ten different subsets of 3500 SNPs, assigned the majority of Moab, UT individuals (96 ± 9%) to Tucson, AZ (Fig. 2A). Individuals from York, NE were assigned to more than one population, with the larger percentage of individuals assigned to New Orleans, LA (41 ± 23%) and El Paso, TX (32 ± 19%),

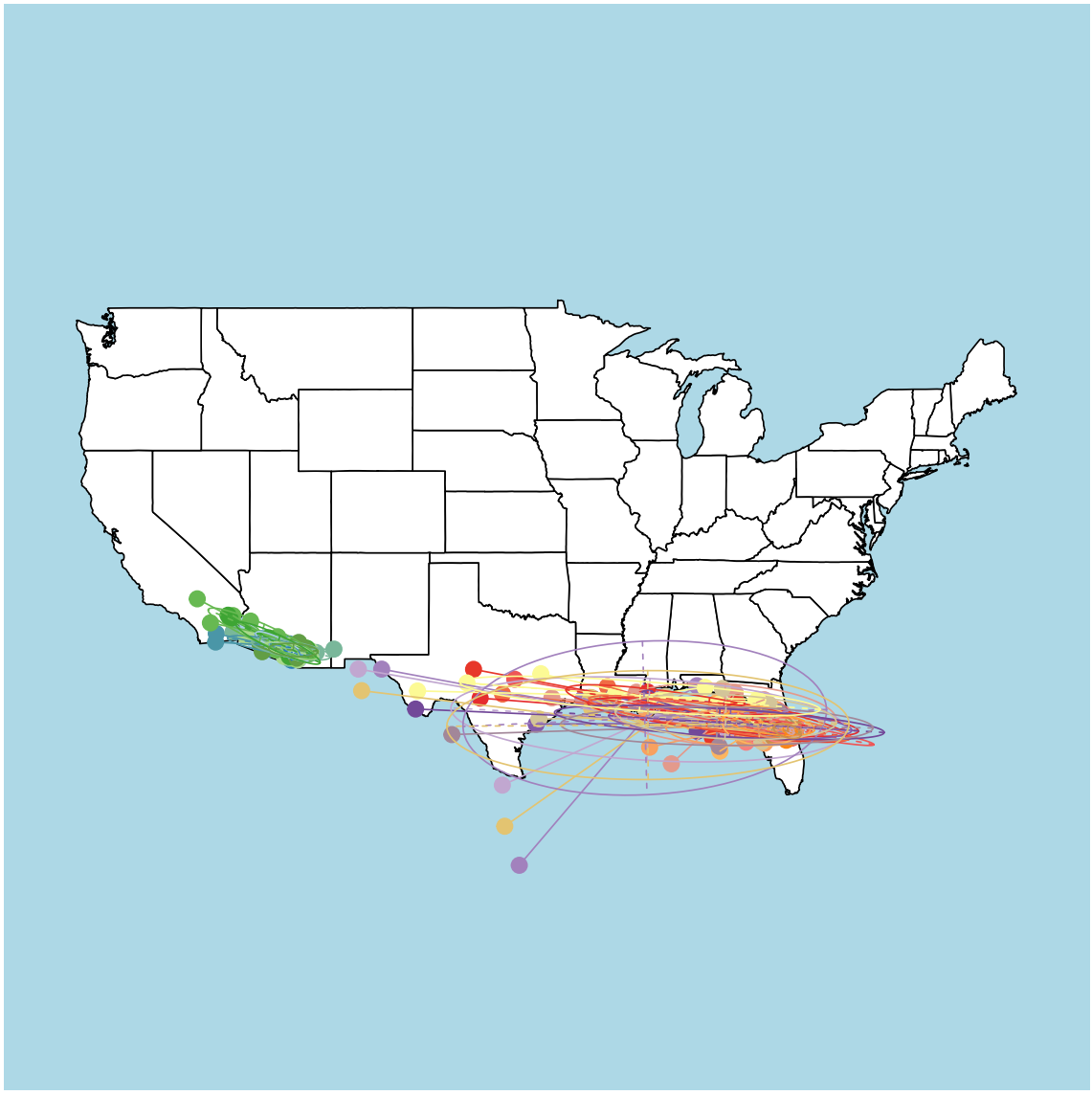

Fig. 3. Geographic assignment of individuals from Moab, Utah (blue/green shades) and York, Nebraska (yellow/red/purple shades) as determined in Locator (Battey et al., 2020) using the Americas dataset as reference and 13,692 SNPs. Each individual is represented by 10 points of the same color (10 bootstraps), and the inertia ellipses show the area where most of the points were projected for a particular individual. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

followed by Tampa, FL (15 ± 13%); see Fig. 2B. Geographic assignment (Locator) of individuals from Moab, UT places them in the region between Southern California and Arizona (Fig. 3). York, NE individuals appear more broadly distributed, spanning New Mexico, Texas, Louisiana, and Florida (Fig. 3). This is consistent with the phylogenetic tree (Fig. S4) and the population tree (Fig. 4), where Moab, UT is placed in a well-supported clade with Tucson, AZ, and southern California. The position of York, NE in both trees is unclear since clade support is low. The phylogenetic tree supports a monophyletic origin for both the Moab, UT and the York, NE introductions (Fig. S4). Admixture analyses in Treemix did not find evidence of geneflow involving either Moab, UT or York, NE, there is only evidence of possible admixture between Florida populations, Louisiana, and a Brazil population, with African *Ae. aegypti* (Fig. S5). Accounting for these admixture events did not change the relationships around Moab, UT and York, NE (Fig. S5).

4. Discussion

Genetic analysis of the 2019 *Ae. aegypti* collections from York, NE and Moab, UT are consistent with recent introductions of a relatively small number of propagules. Genetic clustering and assignment suggest that the introductions to Nebraska and Utah did not originate from the same source. The two collections display lower genetic diversity relative to established populations in the Americas and a high degree of kinship, which suggests the introduction of a small number of individuals.

While collections at each locality are monophyletic (Fig. S4), their putative source is better supported in the case of Utah than for Nebraska. The origin of *Ae. aegypti* from Moab, UT is most likely Tucson, AZ (Fig. 2A) or a nearby locality (Fig. 3). In contrast, our results cannot identify the source of *Ae. aegypti* from York, NE. Assignment tests and the low support of the sister clades to Nebraska suggest that it may have originated further east than those in Utah (Figs. 2B and 3, and Fig. S4), but the origin could not be further delineated. The phylogenetic tree supports a single source of these samples, plausibly from a southcentral or southeastern US population not represented in our reference dataset. Finally, a possibility exists that this collection represents an established cryptic population that had not been detected and has sufficiently diverged from its original source. However, this possibility is unlikely

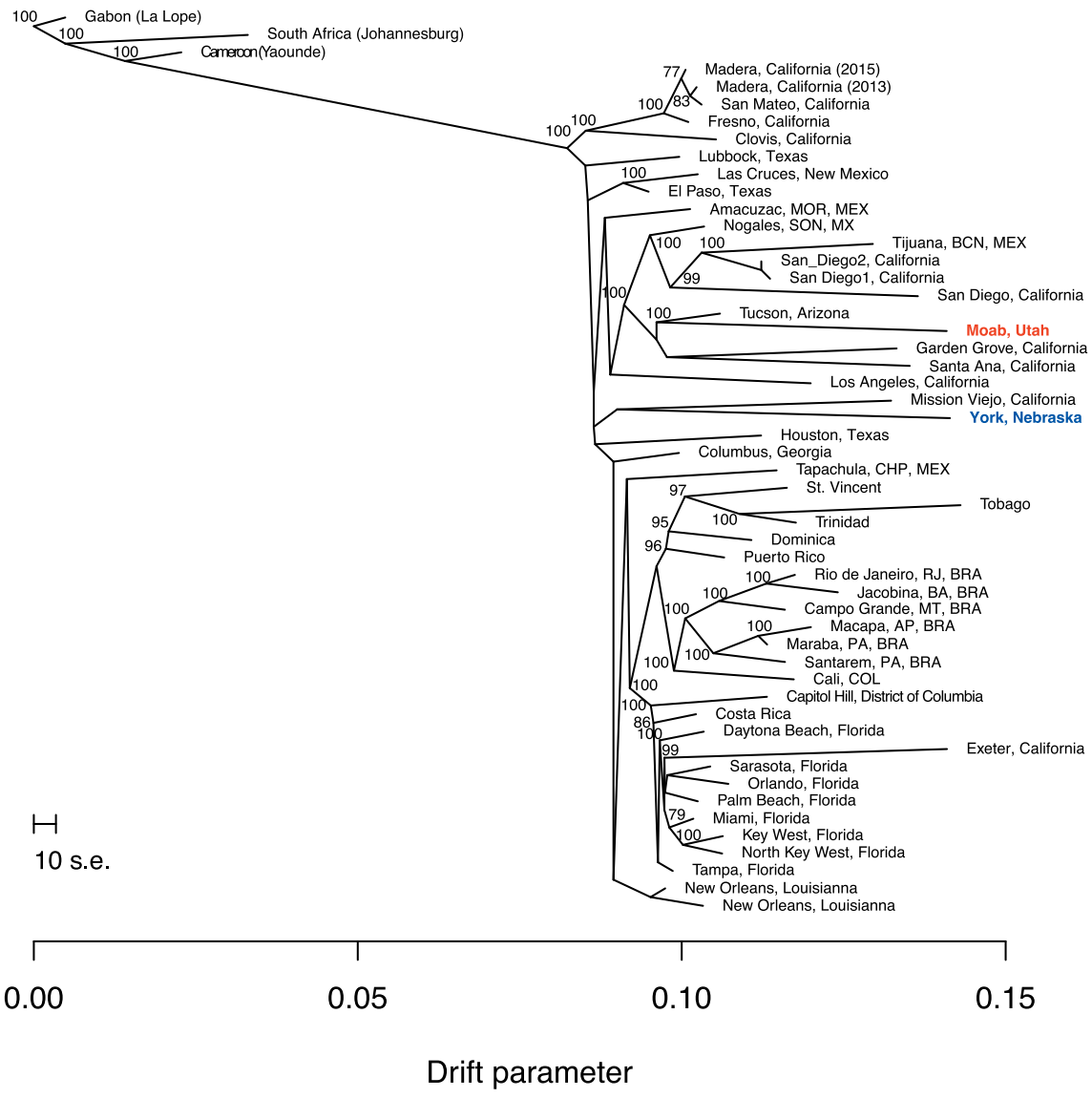

Fig. 4. Maximum likelihood population tree from the Americas. The tree was built from 21,249 SNPs using TreeMix without migration events (Piry et al., 2004), and is rooted using three African populations. Moab, Utah (red) and York, Nebraska (blue) are highlighted. Numbers next to nodes are bootstrap support values ($N = 100$). Only support values above 75 are shown. The scale bar shows ten times the average standard error of the entries in the sample covariance matrix, representing the amount of genetic drift along each population. A tree allowing up to four migration events is shown in Fig. S5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

due to the comprehensive NDHHS surveillance program which had not previously detected *Ae. aegypti* in York or elsewhere in the state and the morphological distinctiveness of *Ae. aegypti* from all other mosquitoes native to this region.

How these 2019 introductions arrived to York, NE and Moab, UT is unknown. Both locations are outside the mosquito flight range, thought to be a few hundred meters at most (Harrington et al., 2005), so active dispersal is unlikely. However, the species can be transported passively via desiccation-resistant eggs which are deposited in artificial containers commonly transported by humans. Containers that have been associated with *Ae. aegypti* eggs include tires, potted plants, plant saucers, children's toys, buckets, urban trash, and many other items commonly found in backyards and along inhabited properties (Focks et al., 1981; Yee, 2008; Chen et al., 2009; Mukhtar et al., 2018). Inhabitants from the area in York, NE where *Ae. aegypti* was found had travel histories that included states where *Ae. aegypti* is established: Arizona, Arkansas, Florida, Louisiana, Missouri, New Mexico, Oklahoma, and Texas. Dispersal via passenger vehicles could have brought the mosquitoes into the state. During the NDHHS epidemiological investigation residents also reported the recent purchase of tropical plants, known to be responsible for introducing another important mosquito vector, *Ae. albopictus,* into the US, the Netherlands, and Belgium (Madon et al., 2003; Scholte and Takken, 2007; Demeulemeester et al., 2014).

Major highways connect Moab, UT to California and Arizona, which have established populations of *Ae. aegypti*, for example Interstates I-10 and I-15. Although the population of Moab is only a little over 5000 inhabitants, there were over 1.6 million visitors to the region in 2019, drawn to Arches and Canyonlands National Parks (National Park Service, 2021). Tourists to this area regularly use recreational vehicles, campers, and/or trailers in addition to passenger cars and trucks, which can provide refuge for mosquitoes like *Ae. aegypti*. We also cannot rule out the possibility of adult mosquito dispersal via human-assisted mechanisms such as movement of goods for commercial activities. These observations reinforce the need for close monitoring of tourist

hotspots and/or border towns for *Ae. aegypti* and other harmful pests.

Regardless of how the mosquitoes traveled to Nebraska and Utah, we document herein *Ae. aegypti* detections beyond what was previously considered its suitable range. The cold winters in York, NE and Moab, UT may prevent the mosquitoes from surviving in these cities year-round and becoming permanently established (Farajollahi and Crans, 2012). However, the microclimates of individual properties, such as greenhouses, septic tanks, storm sewers, or underground tunnels (Barrera et al., 2008; Manrique-Saide et al., 2012; Lima et al., 2016), might create habitats for this species to survive harsh winter conditions even if the general landscape is inhospitable. A clear example is the establishment of *Ae. aegypti* in Washington DC (Gloria-Soria et al., 2018; Lima et al., 2016). Additionally, climate change is also likely making higher latitudes favorable for tropical/subtropical species like *Ae. aegypti (*Kraemer et al., 2019).

Efficient, efficacious, and aggressive integrated abatement efforts are the best defense to slow down and eradicate invasive species, such as *Ae. aegypti,* in newly introduced areas for the protection of public health and continued enhancement of quality of life. Early detection is key to successfully prevent an invasion from becoming established. In Moab, UT, during the initial infestations in 2019, door-to-door and area-wide surveillance and control efforts were undertaken in transects stemming from the initial area of detection. These efforts followed guidelines set forth by Faraji and Unlu (2016) (Faraji and Unlu, 2016) and the best management practices published by the American Mosquito Control Association in collaboration with the CDC (AMCA 2017 (AMCA (American Mosquito Control Association), 2017)). In short, all residential parcels and open public lands were surveilled for larval and adult mosquitoes. Larval source reduction was conducted in container habitats that could be removed (ex: used tires and disposable artificial containers), while larvicide applications were conducted in habitats that were permanent fixtures (ex: bird baths, animal watering troughs, small ponds). Barrier residual applications using synthetic pyrethroids were conducted within positive parcels and adjacent areas, while truck-mounted ultra-low volume applications of pyrethroid adulticides were conducted at night to further reduce adult mosquito populations. Surveillance and control efforts were maintained through the month of September and were effective in elimination of *Ae. aegypti* from the area, as no additional specimens were collected the remainder of the season. This was further corroborated the following year during 2020, as no adult or larval specimens of *Ae. aegypti* were detected in Moab, despite trapping efforts utilizing carbon dioxide and BGS traps. At the time of detection in 2019, the city of York, NE did not have mosquito control capabilities and in consultation with the CDC's entomologist it was determined that a door-to-door campaign in the affected neighborhood was the appropriate response, since it was late in the mosquito season and the cold weather was approaching. Both in Moab, UT and York, NE, door-to-door campaigns were also used for public education and community outreach to assist mosquito abatement personnel with further reduction of standing water and disposal of artificial water-holding containers. During these visits, York, NE handed out mosquito larvicide kits and conducted a survey to gather information on residents' travel history and recent purchases of tropical plants. Trapping efforts in 2020 and 2021 in York did not reveal additional detections.

Timely and aggressive vector control measures such as those implemented by NDHHS and MMAD upon the *Ae. aegypti* detections in 2019 are needed. Continuing education of mosquito control personnel for those outside the current range of these species will further support the success of surveillance and control activities. Public education will also be needed in tourist hotspots like Moab, UT and near other National Parks to let visitors know of ways they can help prevent the spread of invasive mosquitoes. Finally, understanding potential routes of introduction and phylogenetic relationships will be key to the restriction and elimination of future introductions. Given the impact that *Ae. aegypti* can have on human quality of life, efficient surveillance and control measures should be of paramount importance.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2022.105333.

Data reporting

Raw allele frequencies of data published for first time in this study are available through VectorBase.org (Giraldo-Calderón et al., 2022), Population Biology Project ID: VBP0000813. Data from the reference panel are available at VectorBase.org, Population Biology Project IDs: VBP0000138, VBP0000176, VBP0000177, VBP0000295, VBP0000269, VBP0000715; from the European Molecular Biology Laboratory−European-Bioinformatics Institute BioSamples group SAMEG188691; and from the references listed in Table S1.

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Declaration of Competing Interest

The authors declare no declare no competing interests.

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