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Research article

City-Dwellers and Country Folks: Lack of Population Differentiation Along an Urban–Rural Gradient in the Mosquito *Culex pipiens* (Diptera: Culicidae)

Ann-Christin Honnen[1,2,3,5] and Michael T. Monaghan[3,4]

[1]Swiss Tropical and Public Health Institute (Swiss TPH), 4051 Basel, Switzerland, [2]University of Basel, 4003 Basel, Switzerland, [3]Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), 12587 Berlin, Germany, [4]Berlin Center for Genomics in Biodiversity Research, 12489 Berlin, Germany, and [5]Corresponding author, e-mail: achonnen@googlemail.com

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Abstract

Mosquitoes (Diptera, Culicidae) occur in natural, urban, and peri-urban areas throughout the globe. Although the characteristics of urban and peri-urban habitats differ from those of natural habitats in many ways (e.g., fragmentation, pollution, noise, and light), few studies have examined the population connectivity of mosquitoes in urban areas. To obtain an overview of the species composition, we sampled mosquitoes from 23 sites in and around the city of Berlin, Germany. Of 23 species, five occurred in urban, 10 in peri-urban, and 20 in rural areas. *Culex pipiens* Linnaeus (Diptera: Culicidae) was the most common species collected (75% of all individuals) and occurred in all habitats. Hence this species was selected to be analysed at 10 microsatellite markers. There was no significant differentiation (F_{ST} = 0.016, P = 0.9) or isolation by distance (P = 0.06) among *Cx. pipiens* populations along an urban–rural gradient. The only significant differences detected were between *Cx. pipiens* and a laboratory population of *Cx. pipiens* f. molestus (pairwise F_{ST} = 0.114–0.148, $P \leq 0.001$ in all comparisons), suggesting that the markers chosen were suitable for the identification of population differentiation. Our results indicate that *Cx. pipiens* gene flow is widespread within and among urban, peri-urban, and rural areas and that urban habitat does not necessarily impede or enhance gene flow among these populations.

Key words: population differentiation, urbanization, citizen science, microsatellite, species inventory

Human population growth is leading to the establishment of new settlements and the expansion of existing ones. City life brings a multitude of challenges to organisms, such as noise, pollution (of air and water bodies), a scarcity of suitable breeding habitat, and artificial light (street lights and house lighting) (Longcore and Rich 2004, Grimm et al. 2008). On the other hand, the habitat structure consists of small patches of different types (housing, green areas, roads) resulting in highly structured areas with many interfaces between them (Grimm et al. 2008). These characteristics are encountered to a much lesser extent in rural areas, largely because the different land cover types occur in larger patches thereby reducing the number of interfaces relative to the number of patches. Many species prefer larger patches and are unable to sustain urban populations because of the small patch size (e.g., forest species), while others may actually thrive in the heterogeneous habitats of urban areas (e.g., orb weaving spider: Lowe et al. 2014).

Among the organisms populating urban areas are mosquitoes (Diptera:Culicidae). The challenges that urban mosquitoes need to overcome include air and water pollution (the latter primarily influencing larvae), and noise that could interfere with the detection of mates, as the recognition of conspecifics is largely driven by wing beat frequency (Gibson and Russell 2006, Robert 2009). Artificial light at night, a common feature of urban areas, can influence daily activity patterns such as host seeking and blood feeding. Chadee and Martinez (2000) showed that these behaviors were prolonged in artificial light at night in *Aedes aegypti* Linnaeus (Diptera: Culicidae), a diurnal mosquito. Given the specific set of urban habitat characteristics, it can be assumed that few species are capable of living in the city. A relatively small number of studies have assessed mosquito diversity in urban areas (Calderón-Arguedas et al. 2009, Schumann 2010, Rudolf et al. 2013, Townroe and Callaghan 2014, Wide de Valdez 2017). While the characteristics used to define 'urban' and 'rural' sites vary greatly among studies, these studies report lower species diversity compared with more rural habitats. Habitat heterogeneity seems to play an important role for mosquito diversity (Chaves et al. 2011). In a study of container-breeding species, Townroe and Callaghan (2014) reported only three species in urban (within 4 km radius of city center) experimental sites in England: *Culex pipiens* (Linneaus, 1758) (Diptera: Culicidae), *Anopheles claviger* (Meigen, 1804), and *Anopheles plumbeus* (Stephens, 1828).

Container-breeding species seem to benefit from urban habitats as there is an abundance of artificial ovipostion sites, for example, in cemeteries (Vezzani 2007, Townroe and Callaghan 2014). We are aware of only one study in Germany that assessed mosquito species diversity in a metropolitan (i.e., urban and peri-urban) area (Hamburg; Krüger et al. 2014). The aim of our study was to assess whether species in urban areas are isolated populations or if they remain connected with populations in surrounding peri-urban and rural areas. To select a species that occurs in all of these areas, we surveyed the mosquito species inventory of Berlin, the largest city in Germany (3.5 Mio. inhabitants within an 892 km^2 area; Amt für Statistik Berlin-Brandenburg, https://www.statistik-berlin-brandenburg.de) using a citizen science approach.

Population structuring depends on the dispersal capacity of individuals, the availability of suitable habitat between populations, and the presence of landscape barriers that may hinder dispersal. Service (1997) suggested that mosquito species occurring in more open habitats (i.e., meadows, rural areas) are more likely to be dispersed by wind than woodland or urban species. If this is the case, there may be reduced gene flow among urban populations of these species. Motivated flights of mosquitoes (search for resting and oviposition sites, nectar or blood sources and mates) are usually short (1–5 km, Service 1997). However, wind-assisted dispersal across very large distances is known for a number of species (*Cx.* pipiens: 850 km; for an overview see: Verdonschot and Besse-Lototskaya 2014). Thus, a highly built-up urban area may influence both motivated flight and wind-assisted dispersal, while also altering the habitat availability and suitability compared with natural landscapes.

In this study, 36 volunteers sampled mosquitoes from 23 sites in and around the city of Berlin, Germany. These sites were classified based on the predominant building type as either urban (densely built-up, with multi-storey buildings and few green patches), peri-urban (residential areas and small towns dominated by family homes with gardens and allotment gardens), or rural (farmland or unmanaged land). The first part of our study investigated the number of species per area type (urban, peri-urban, rural). The second part used genetic variation at 10 microsatellite loci to test whether urban populations of the most common species, *Cx. pipiens*, were genetically differentiated from the adjacent peri-urban and rural sites or whether ongoing gene flow prevents differentiation among area types.

Material and Methods

Sampling

Volunteers (citizen scientists) were equipped with an aspirator consisting of a flexible, transparent rubber tube (diameter: 1 cm) covered with mesh on one end, 2 ml reaction tubes (Carl Roth GmbH and Co KG, Karlsruhe, Germany) and a short manual. The volunteers independently decided when and where to sample; however, almost all samples were collected in and around their homes. This information was provided by the volunteers but kept confidential to protect their privacy. Adult mosquitoes were sampled from the beginning of May until early November of 2011 in apartment buildings, family homes, and gardens in and around the city of Berlin, Germany (Fig. 1). The only exceptions were three of the four rural areas which were sampled by the authors during sampling campaigns in September. Sampled individuals were stored at -20°C. Since the samples were mainly collected from private residences, sites were named after the municipal entity. Samples were classified by the authors as urban (nine sites), peri-urban (10 sites), or rural (four sites) based on the predominant building structure (Fig. 1A and C). Dense building

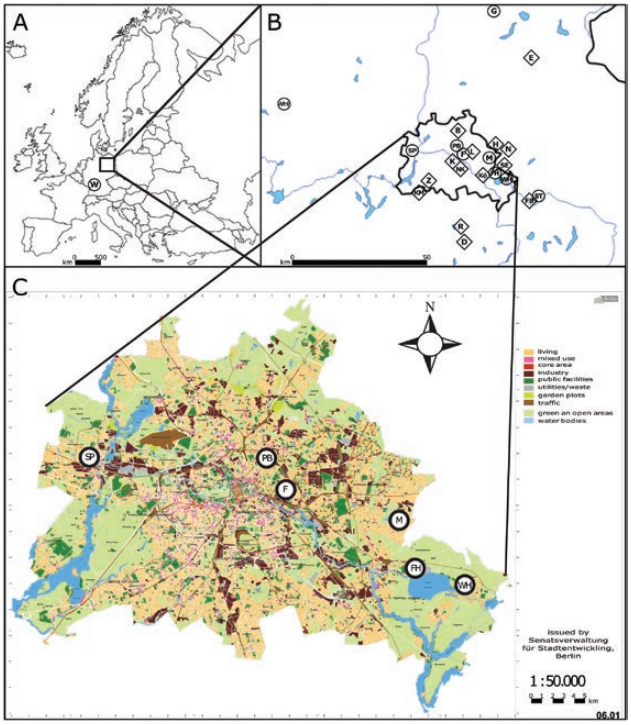

Fig. 1. (A) Map of Europe indicating the sampling area and the Weinheim sampling site (W). (B) Map of all sampling locations in and around Berlin. (C) Map of Berlin showing urban and peri-urban sample sites used for microsatellite study within city limits and the building structure. Circles indicate sites sampled for the genetic analyses and diamonds indicate site samples for species inventory only. Letters indicate sampling locations [urban: SP (Spandau), F (Friedrichshain), PB (Prenzlauer Berg), L (Lichtenberg), K (Kreuzberg), H (Hönow), NK (Neukölln), Kö (Köpenick), Z (Zehlendorf); peri-urban: FH (Friedrichshagen), M (Mahlsdorf), WH (Wilhelmshagen), B (Französisch-Buchholz) SE (Schöneiche), E (Eberswalde), N (Neuenhagen), KM (Klein Machnow), R (Rangsdorf), D (Dabendorf)]. Rural sites [four sites: ST (Störitzsee), G (Gollinsee), WHL (Westhavelland), FB (Freienbrink)]. This map was kindly provided by Berlin senate of communal development (Senatsverwaltung für Stadtentwicklung) and has been modified to specify the sampling locations.

and road infrastructure with multiple-storey houses and few green patches (mainly parks) were defined as urban. Areas with the majority of buildings consisting of family homes with gardens or allotment gardens were considered peri-urban. Rural sites were either farmland or unmanaged areas and were sampled with aspirators as well as sweep nets by the authors. Individuals were identified by author A.-C. H. to species level using the computer-based multicriterial identification program of Schaffner et al. (2001).

Cx. pipiens was by far the most abundant species in urban, peri-urban, and rural areas (see Results) and was used for population genetic analysis. We analysed a subset of sites, three in each category, where more than 12 individuals of *Cx. pipiens* were collected (see Table 2). There are two ecotypes of *Cx. pipiens* that are morphologically identical but ecologically different, *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus (originally described as a subspecies by Forskål, 1775). It is likely that our field-caught individuals were *Cx. pipiens* f. pipiens, because *Cx. pipiens* f. molestus is largely restricted to belowground areas (Becker et al. 2012); however, the f. molestus ecotype has been observed aboveground (Gomes et al. 2013, Di Luca et al. 2016) and the ecotypes can hybridize in areas of sympatric occurrence (Fonseca et al. 2004, Kothera et al. 2010, Gomes et al. 2013, Di Luca et al. 2016). We, therefore, included 11 individuals of

the f. molestus ecotype from a laboratory colony (LAB; Honnen et al. 2016) in the analysis to test whether any field-caught individuals would be assigned to the f. molestus ecotype by means of genetic similarity. Because the f. molestus colony was founded with individuals from the vicinity of Karlsruhe, Germany (about 525 km away from the study area), we also included six field-caught *Cx. pipiens* samples from an urban sampling site in the Karlsruhe area (W: Weinheim; Fig. 1B) in order to evaluate whether any observed genetic differences were the result of the geographic distance between sampling locations or genetic differences between ecotypes. Another common member of the *Cx. pipiens* species complex in the study area is *Culex torrentium*. Apart from male genitalia, *Cx. pipiens* can only be distinguished from *Cx. torrentium* by prealar scales which can easily rub off during handling (c.f. Danabalan et al. 2012). For individuals that were genotyped, our analysis included four microsatellite loci (discussed subsequently; Appendix Table 1 [online only]) that do not amplify in *Cx. torrentium* (Smith et al. 2005) in order to estimate the rate of *Cx. torrentium* occurrence in our samples.

DNA Extraction and Genotyping

DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturers' protocol except that the lysis step was carried out overnight. DNA was eluted to a final volume of 80 µl. Primer sequences for the 10 microsatellite loci were obtained from Fonseca et al. (1998), Keyghobadi et al. (2004), and Smith et al. (2005) (see Appendix 1 [online only]), and multiplex PCR was done using three primer mixes (see Appendix 2 [online only]). Genotyping was done on a 24-capillary 3500XL Genetic Analyzer (Applied Biosystems, Waltham, MA) and alleles were scored with GeneMapper v4.1 (Applied Biosystems).

Statistical Analysis

Prior to all analyses, the data were tested for genotyping errors (stutter bands, large-allele dropout, null alleles) using Micro-Checker 2.2.3 (Van Oosterhout et al. 2004). Because null alleles can be prevalent in mosquitoes (Lehmann et al. 1997), we calculated the null allele frequency using the EM algorithm (Dempster et al. 1977) and then calculated a second set of F_{ST} values (discussed subsequently) using ENA correction as implemented in FreeNA (Chapuis and Estoup 2007). A likelihood test of linkage disequilibrium between pairs of loci was done with dememorization of 10,000, 1,000 batches and 10,000 iterations per batch using Genepop version 4.2 (Raymond and Rousset 1995). Deviations from Hardy–Weinberg equilibrium (HWE) were calculated using Arlequin v. 3.11 (Excoffier et al. 2005).

Expected and observed heterozygosities were calculated using Arlequin. Allelic richness, a measure of allelic diversity corrected for sample sizes calculated with a rarefaction approach, was determined with FStat v. 2.9.3.2. (Goudet 2001). Allele frequencies for each locus and sampling site were also determined with FStat. The number of private alleles was obtained with CONVERT v. 1.31 (Glaubitz 2004). Global F_{ST}, pairwise F_{ST} among sites, and analysis of molecular variance (AMOVA; individual level and sampling site level) were calculated with Arlequin. Isolation by distance (IBD) was examined with Mantel tests on matrices of genetic and geographic distances using IBD v.1.52 (Bohonak 2002; http://www.bio.sdsu.edu/pub/andy/IBD.html). We used linearized F_{ST} values (F_{ST} /1-F_{ST}) and logarithmic geographic distance based on the minimum overland Euclidean distances estimated using Google Earth (7.1.2.2041).

We tested for population substructuring using the model-based Bayesian clustering analysis implemented in Structure 2.3.4 (Pritchard et al. 2000). This program groups individuals based on their genotypes into *K* clusters under the assumption that these clusters meet the conditions of Hardy–Weinberg and linkage equilibria. We allowed for admixture and correlated allele frequencies (Falush et al. 2003) and used the locprior model implemented in the software (Hubisz et al. 2009). While the 'model with prior population information' incorporated in Structure assumes that there is strong evidence for population structure and it relies on highly informative (near exact) location information, the locprior model places more weight on clustering outcomes that are correlated with location information and is thus more suitable for data with few individuals and loci with low divergence between them (Hubisz et al. 2009). We tested our data for $K = 1$ to $K = 11$ with 10 iterations per *K*. The burn-in period was set at 30,000 steps in the Markov Chain Monte-Carlo procedure followed by 50,000 replications. For each of these runs, the most probable number of *K* was evaluated with the ad-hoc statistic ΔK (Evanno et al. 2005) as implemented in the online tool Structure Harvester (Earl and von Holdt 2012). As a further test of whether field-collected specimens were indeed the f. pipiens ecotype, we conducted assignment tests using GeneClass2 version 2.0 (Piry et al. 2004) with allele-frequency-based assignment computation (Paetkau et al. 1995) and the probability criterion of Paetkau et al. (2004).

Results

Mosquito Species Inventory

A total of 686 individuals were caught, of which 555 could be morphologically identified to species. We found a total of 23 species from six genera: five (22%) species in urban, 10 (43%) in peri-urban, and 20 (87%) in rural areas. By far, the most common species was *Cx. pipiens* (75% of all identified individuals) (Table 1). The species found in urban Berlin were *Cx. pipiens* (171 individuals), *Culex territans* (Walker, 1856) (1), *Aedes vexans* (Meigen 1830) (1), *Culiseta annulata* (Schrank 1776) (2), and *Coquillettidia richiardii* (Ficalbi, 1889) (4) (Table 1). All other species were found either in peri-urban or rural areas (Fig. 1A, Table 1).

Genetic Diversity

The microchecker analysis found signs of stutter bands at six loci and these electropherograms were re-examined by eye by two different individuals. In the case of ambiguous scoring, the sample was excluded from further analysis. Large-allele drop out was not observed at any of the loci. However, signs of null alleles were detected at all but two loci (CxqGT6b, CxqGT4). A separate examination of each sampling site found that the number of sites displaying null alleles at a given locus ranged from 0 to 6. There was no linkage between any two pairs of loci (Bonferroni-adjusted $P > 0.001$) hence all loci were used in subsequent analyses. We were able to obtain genotypes for a total of 176 individuals, ranging from 12 to 25 individuals per site. There were fewer individuals genotyped from the laboratory colony (LAB; 11) and from Weinheim (W; six) (Table 2). Expected and observed heterozygosity differed significantly at eight out of 10 loci, indicating that the assumptions underlying HWE were violated in the overall data set. When this analysis was repeated excluding the f. molestus data, six out of 10 loci were not in HWE. This may have occurred for several reasons. There may be several populations (e.g., urban, peri-urban, rural) in the overall data set and mating may not be random. Small sample sizes also may influence the outcome through the sampling of many alleles in a relatively small number of individuals. Null alleles could also lead

Table 1. Species inventory of the study area

Category	Site	Site code	Aedes		Culex			Culiseta					Ochlerotatus										Coquillettidia	Anopheles		No. of specimens
			cinerus	vexans	pipiens	territans	modestus	alaskaensis	annulata	fumipennis	morsitans	orchoptera	flavescens	cyprius	cantans	caspius	excrucians	geniculatus	leucomelas	punctor	pullatus	intrudens	richiardii	maculipennis s.l.	plumbeus	
Urban	Friedrichshain	F			61				1																	62
	Hönow	H			5																					5
	Köpenick	Kö			7				1																	8
	Kreuzberg	K			8																					8
	Lichtenberg	L			1																					1
	Neukölln	NK			3																					3
	Prenzlauer Berg	PB		1	23	1																				25
	Spandau	SP			28																					28
	Zehlendorf	Z																					4			4
Total urban				1	136	1			2														4			144
Peri-urban	Dabendorf	D			1																					1
	Eberswalde	E		6	6									1				1		1		1w				16
	Französisch-Buchholz	B		1	14					1																16
	Friedrichshagen	FH		2	85				4				2													93
	Klein Machnow	KM																1								1
	Mahlsdorf	M		9	35				1																	45
	Neuenhagen	N			13																					13
	Rangsdorf	R			3				1				1													5
	Schöneiche	SE		6	7				2							1										16
	Wilhelmshagen	WH		1	23																					24
Total peri-urban				25	187				8	1			3	1		1		2		1		1				230
Rural areas	Freienbrink	FB	1	2				3			2	3	4													15
	Kleiner Gollinsee	G			22	2	2		2																1	29
	Störitzsee	ST	1		41			1	1		1	1					1					1				48
	Westhavelland	WHL	2	7	31	2	1				2		30	2	3			1	2		2		2	2		89
Total rural			4	9	94	4	3	4	3		5	4	34	2	3		1	1	2		2	1	2	2	1	181
Grand total individuals			4	35	417	5	3	4	13	1	5	4	37	3	3	1	1	3	2	1	2	2	6	2	1	555
Total sites			3	9	20	3	2	2	8	1	3	2	4	2	1	1	1	3	1	1	1	2	2	1	1	

Cells contain the number of specimens collected at the given site from May to November 2011. The two bottom rows report the sum total of individuals from each species, and the total number of sites in which a given species was found.

to HWE violation but the global F_{ST} corrected for null alleles (ENA) was 0.016 (95% CI: 0.010–0.024) and thus only marginally lower than the uncorrected F_{ST}: 0.017 (CI: 0.010–0.026). We, therefore, concluded that null alleles did not have a significant impact on the results (see Appendix Table 7 [online only] for the results of null alleles and Appendix Table 8 [online only] for F_{ST} per sampling site).

All 10 microsatellite loci were polymorphic and the number of alleles per locus ranged from 4 to 49. Allelic richness per locus and sampling location ranged from 1.73 to 7.19. The total number of alleles per site (A) ranged from 94 to 143 and mean allelic richness (A_R) ranged from 3.5 to 5.3 (based on a minimum sample size of four individuals). Fewer alleles were observed in the f. molestus ecotype colony population (LAB; A = 48) and the Weinheim (W) site in southern Germany (W: A = 56). The inbreeding coefficient (F_{IS}) per site ranged from 0.152 to 0.285 (for detailed data per site and locus see Appendix Table 3 [online only]), with the lowest value found at site W and the highest in the f. molestus population (LAB). The number of private alleles ranged from 2 (F, SP, LAB) to 10 (G) (see Appendix Table 6 [online only]).

Population Structure

Pairwise F_{ST} values indicated no significant differences among any of the sites in and around Berlin (Table 3). The only significant values were found when comparing the *Cx. pipiens* f. molestus laboratory population (LAB) with all others (F_{ST} = 0.114 to 0.148; Table 3). When LAB was excluded in a second analysis, there were no significant pairwise F_{ST} values. Pooling sites into urban (F, SP, PB), peri-urban (FH, WH, M), and rural (G, ST, WHL) habitat areas resulted in low and non-significant pairwise F_{ST} values among the groups (data not shown). The analysis of molecular variance was not significant between sampling sites (F_{ST} = 0.0158, df = 10, sum of squares [SS] = 65.209, variance components [Va] = 0.0619, P = 0.945 + -0.002) or among urban, peri-urban, and rural groups (F_{CT} = 0.0004, SS = 9.277, Va = 0.0016, df = 2, P = 0.307 + -0.004). There was no significant IBD (P_{Itailed} = 0.0599, R^2 = 0.201), although there was a trend towards greater differentiation with increasing distance (Fig. 3).

Using Bayesian clustering, the most probable number of clusters detected was two (ΔK = 41.37; see Appendix Fig. 4a [online only]) and this clearly separated individuals from the LAB population of the f. molestus ecotype from all others (Fig. 2a). One LAB individual had a high proportion of the alternate genotype, and a few individuals from other populations had a low proportion of the LAB genotype (Fig. 2a). Repeating the analyses without the LAB individuals resulted in three clusters (ΔK = 3.16; see Appendix Fig. 4b [online only]). There was no clear pattern of structuring among sampling sites (Fig. 2b). No *Cx. pipiens* f. pipiens individual was assigned to the f. molestus group in the assignment tests (probabilities ranging between 0.0 and 1.2%; see Appendix Table 9A [online only]). We cross-checked this result by assigning the f. molestus samples to all other sampling sites as well. The probabilities ranged between 0.0 and 54.9 % (18 out of 110 comparisons showed probabilities >10%; Appendix Table 9B [online only]).

Table 2. Subset of samples used for the microsatellite analyses

	Site code	No. of individuals genotyped
Urban	F	18
	PB	18
	SP	12
Peri-urban	FH	18
	M	17
	WH	20
Rural	ST	25
	G	17
	WHL	14
Molestus	LAB	11
Pipiens	W	6

Molestus denotes samples of *Cx. pipiens* f. molestus from a laboratory colony (LAB) that were included to verify that all study samples belonged to the *Cx. pipiens* f. pipiens ecotype; Pipiens denotes *Cx. pipiens* f. pipiens samples collected from the same geographical region (referred to as W) as the colony founding individuals. Site codes are given in Table 1.

Discussion

Species Inventory of the City of Berlin

Fewer species were collected in the urban area of Berlin (Germany) compared with surrounding peri-urban and rural areas, a finding similar to a study in southern England (Townroe and Callaghan 2014). All five species we detected have previously been found in an urban environment (e.g., Krüger et al. 2014). Because sampling was done as a citizen science project, sampling time of day and season were chosen by the volunteers and do not necessarily reflect scientifically desired patterns. The advantage of such a scheme is that sampling locations are chosen upon the sight of a mosquito and rather than whether a site is thought to provide suitable mosquito habitat. This was an important aspect of our study design as previous studies of urban mosquito diversity have deliberately picked sights that were likely to have mosquitoes. For example, cemeteries are often sampled (Vezzani 2007,

Table 3. Pairwise F_{ST} values for the comparison of sites (below diagonal) and P-values (above diagonal)

	F	FH	G	M	PB	LAB	SP	ST	WHL	WH	W
F	*	0.707	0.907	0.802	0.983	**0.000**	0.992	0.904	0.277	0.937	0.284
FH	0.002	*	0.493	0.198	0.666	**0.000**	0.435	0.466	0.243	0.766	0.394
G	-0.002	0.004	*	0.116	0.250	**0.000**	0.980	0.517	0.445	0.537	0.284
M	0.000	0.009	0.011	*	0.455	**0.000**	0.170	0.314	0.177	0.611	0.035
PB	-0.004	0.003	0.008	0.005	*	**0.000**	0.686	0.949	0.286	0.854	0.280
LAB	**0.132**	**0.125**	**0.144**	**0.132**	**0.117**	*	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
SP	-0.007	0.007	-0.005	0.012	0.003	**0.137**	*	0.600	0.835	0.796	0.232
ST	-0.001	0.004	0.004	0.006	-0.002	**0.128**	0.004	*	0.134	0.755	0.155
WHL	0.010	0.011	0.007	0.012	0.010	**0.148**	0.001	0.012	*	0.573	0.136
WH	-0.002	0.001	0.004	0.003	0.001	**0.114**	0.002	0.001	0.005	*	0.146
W	0.016	0.012	0.014	0.030	0.016	**0.142**	0.019	0.019	0.028	0.023	*

Significance level was 0.001 after Bonferroni-correction and significant values are indicated in bold. Site codes are given in Table 1.

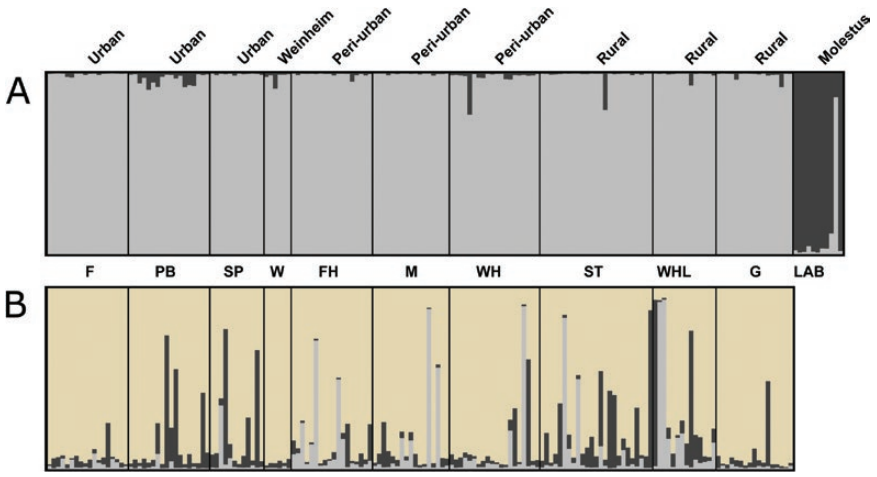

Fig. 2. (A) Bayesian clustering of *Cx. pipiens* populations from Berlin and surrounding areas, from southern Germany (W) and from the *Cx. pipiens* f. molestus laboratory colony (LAB), resulting in two clusters ($K = 2$). (B) The same analysis run without LAB resulted in three clusters ($K = 3$). Each individual is represented by a single bar; the colors denote the proportion of the genotype belonging to the respective cluster.

Rudolf et al. 2013, Krüger et al. 2014). The aim of our study was not to obtain an exhaustive catalogue of species diversity but to determine which species occur in the core area of a large, densely built-up city, and whether populations show signs of 'urbanization', differentiating them from conspecific rural or natural populations.

Citizen science is increasingly used for different questions, owing to recently developed technologies (Bonney et al. 2009, 2014). Citizen scientist projects related to mosquito surveillance have been employed across Europe (for a review see Kampen et al. 2015). A project in Germany ('Mückenatlas') asking volunteers to collect and send mosquito specimens, together with a form containing basic sampling information, has thus far recovered 39 out of 50 described species (Kampen et al 2015). It is, therefore, feasible to obtain an overview of the species inventory at a given place. Kampen et al. (2015) concluded that all citizen science projects benefit the scientific community by helping to design more targeted sampling efforts to answer specific questions. Therefore, this tool was an appropriate choice to get an overview of the mosquito diversity as well as a method to collect samples at the same time. A limitation to this approach was that rural sites lacked citizen scientists and hence were sampled during single visits using sweep nets in order to obtain sufficient numbers of mosquitoes in a short time. This may have introduced a bias such that the more targeted approach (sweep-netting) may have led to the capture of more species, influencing the conclusion that species diversity is higher in rural areas. We cannot reject this possibility, but we believe that this bias remained small for two reasons. First, volunteers were able to collect 10 different species using handheld aspirators, showing an increase in numbers from urban to peri-urban sites. Second, the authors sampled one rural site (ST) with handheld aspirators and captured a comparable number of species, although fewer individuals per species. While we might have missed rare species, this collection still represents a robust estimate for more abundant species.

There were five cases of a single record in the species inventory where we cannot rule out misidentification (peri-urban: *Culiseta fumipennis, Ochlerotatus caspius, Ochlerotatus punctor*; rural: *Ochlerotatus excrucians, An. plumbeus*). *Cs. fumipennis* and *Ochlerotatus pullatus* (found two times in WHL) have not been previously recorded in Brandenburg, although both species occur in Germany (Schumann 2010). These specimens may have been carried long distances by wind, or simply misidentified.

Morphological identification of mosquitoes can be problematic, but we believe this did not significantly affect our results or contributes to inflated diversity estimates. We employed a computer-based identification key that allows for the use of multiple characters for species identification (Schaffner et al. 2001). An advantage of this approach is that the absence of a single character does not hinder identification, as can be the case in most dichotomous keys. One particular challenge in our data is that *Cx. torrentium* and *Cx. pipiens* can only be distinguished morphologically with confidence in males (Weitzel et al. 2011). Because the distribution of these two species overlaps in central Europe (Hesson et al. 2014), including in urban habitats (Rudolf et al. 2013), we included microsatellite markers in our population genetic analysis that amplify in *Cx. pipiens* and not in *Cx. torrentium* (i.e., CxpGT4/EMMA, CxpGT20, CxpGT46, CxpGT53; Smith et al. 2005). Out of 20 samples from Weinheim (W), there was not a single individual where genotyping failed at all four diagnostic loci. However, there were 14 individuals that consistently failed to amplify at the loci CxpGT53 and CxpGT20. These were excluded from further analyses as we could not rule out that these individuals might have been *Cx. torrentium*. All analyses were, therefore, performed using six samples as described in the Materials and Methods and Results sections. We observed this pattern only for the samples from Weinheim. Because not all samples were genotyped, it is likely that some *Cx. torrentium* individuals may have gone undetected in the species inventory, leading to an overestimation of *Cx. pipiens* abundance. Rudolf et al. (2013) carried out a Germany-wide surveillance of species of the *Cx. pipiens* complex. Their sampling site 'Oder valley' was closest to our study sites. They found ~20% of the species pools tested to be mixed, i.e., pools with *Cx. torrentium* and *Cx. pipiens* (total number of individuals collected: 272). The remaining 80% were single-species pools of *Cx. pipiens*. This comparison only provides a rough estimate of the distribution of the two species in the study area; however, it supports the fact that *Cx. pipiens* is the dominant species and that *Cx. torrentium* was comparatively rare. The fact that there were no genotyping errors at the diagnostic loci (i.e., loci not amplifying in *Cx. torrentium*) in the samples collected by volunteers also suggests that the proportion of *Cx. torrentium* individuals in the study area was small compared with *Cx. pipiens*. We conclude that even if not all identifications were correct, the influence on our results is likely

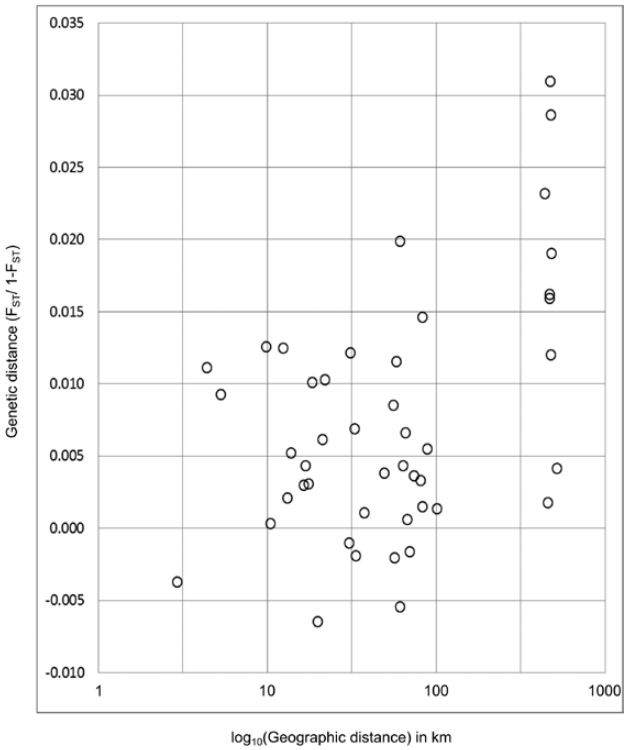

Fig. 3. Results of the test for isolation-by-distance (IBD) among groups using Mantel tests on matrices of genetic and geographic distances with IBD v.1.52 (Bohonak 2002; http://www.bio.sdsu.edu/pub/andy/IBD.html). We used linearized FST-values calculated as FST/(1-FST) and logarithmic geographic distance matrices based on the minimum overland Euclidean distances between each pair of sampling sites (dots) estimated using Google Earth for the test for IBD. The correlation was run with 100,000 randomizations ($Z = 0.6576$). To visualize the distance, we give kilometers as units for physical on the x-axis.

to be small, with no effect on our conclusion that *Cx. pipiens* was the most abundant species.

One possible reason for our finding may be that there are differences in habitat use, competitive ability, or both, in urban areas between *Cx. pipiens* and *Cx. torrentium*. Townroe and Callaghan (2014) did not detect any *Cx. torrentium* in urban sites. Rudolf et al. (2013) reported them in two metropolitan areas in Germany, but they sampled either very small towns surrounded by large open agricultural areas or, in the case of Hamburg, green and moderately built-up areas adjacent to or outside the city borders. Most of these sampling sites would be classified as peri-urban and not urban in our study. Patch size and habitat heterogeneity are important factors influencing species diversity. For example, *Anopheles maculatus* s.s. and *Anopheles minimus* s.l., prefer large forested areas and were found to decrease in abundance in more fragmented habitats (Overgaard et al. 2003). In contrast, Chaves et al. (2011) reported more species in more heterogeneous habitat in urban areas. Most species in the latter study occurred in peri-urban, green areas, although the differences in numbers were small among collection sites (Chaves et al. 2011). *Cx. torrentium* has been described as strictly ornithophilic (Becker et al. 2012), it may thus be more prevalent in green, open areas (peri-urban or rural) following its preferred host. Taken together, there is some evidence that *Cx. torrentium* seems to prefer more open areas over more built-up areas, and *Cx. pipiens* occurs in both.

Population Genetics of Cx. pipiens

The most common species found in the first part of our study was *Cx. pipiens*, and we investigated its population genetic structure by means of microsatellite analysis in order to infer the levels of gene flow and population connectivity along an urban–rural gradient. There was no clear differentiation into single populations or among urban, peri-urban, or rural groups when pooled, suggesting that all individuals were derived from a single population with continuous gene flow through overlapping ranges. This may in part be attributed to the low number of markers used, although previous studies have successfully detected differentiation on a small geographical scale with a comparable set of 11 microsatellite markers (Hemme et al. 2010). The f. pipiens and f. molestus (11 individuals) were clearly differentiated based on F_{ST} values and Bayesian clustering. Our laboratory colony was established from wild-caught individuals from Karlsruhe in Southern Germany, ~525 km away from the study area. This geographical distance may have contributed to the pattern of differentiation found between f. molestus and f. pipiens. In order to confirm that the observed pattern was due to genetic differences and not geographic origin, we incorporated samples from Weinheim, a town close to Karlsruhe (ca. 70 km apart). Those samples were significantly different from f. molestus but not from f. pipiens. Moreover, we did not detect isolation-by-distance in our data set; suggesting that the observed pattern was caused by ecotypes and not by distance.

Our finding of widespread gene flow in *Cx. pipiens* is in line with the findings of Kothera et al. (2010) who reported no differentiation between *pipiens* from New York and Chicago, United States. An earlier study by Huang et al. (2008) found no genetic differentiation across similar geographical scales (max. distance between sampling sites 444 km). Huang et al. (2008) also included a comparison of urban and rural populations of *Cx. pipiens* in their study thus corroborating our findings. Motivated flight in mosquitoes is usually restricted to distances of <5 km, independent of the flying ability of

the species (Service 1997). Verdonschot and Besse-Lototskaya (2014) gave an overview of studies of maximum average flight distances for 105 species in seven genera. They obtained an average flight distance for *Culex* mosquitoes (in mark-recapture studies) of 921 m per day (SD 613.1 m) and estimated average maximum dispersal distance of 9,695 m for *Cx. pipiens* (Verdonschot and Besse-Lototskaya 2014). Therefore, the range analysed here (max. distance between sampling sites 100.82 km), could potentially allow for the differentiation between populations. Long distances can only be covered by wind-assisted dispersal or transportation by humans, but these flights are not controlled by the mosquito itself (Service 1997, Egizi et al. 2016). This phenomenon may have contributed to the observed genetic homogeneity and the lack of strong pattern of IBD. Distances covered intentionally are highly influenced by weather conditions and availability of feeding ground and breeding sites, i.e., if there are suitable sites nearby dispersal may be limited (Service 1997, Verdonschot and Besse-Lototskaya 2014). Based on an average maximum dispersal of 9,695 m for *Cx. pipiens*, and given the prevalence of natural water bodies in Berlin and the surrounding federal state of Brandenburg, it is reasonable to assume that long-distance dispersal is not the most prevalent way of dispersal in the population and thus differentiation is generally possible. The lack thereof suggests that a multitude of breeding grounds exist (Vezzani 2007, Townroe and Callaghan 2014) that facilitate stepping-stone gene flow on a large scale.

The rationale for incorporating f. molestus samples into the structure analysis was to enable us to confirm morphological identification of the f. pipiens individuals and to detect the occurrence of hybrids from the field-collected samples. Together with the results from the assignment tests, this indicates that we had no misidentified f. pipiens or f. molestus individuals in the data set. Interestingly, one individual from the f. molestus population was grouped into the f. pipiens cluster (Fig. 2A). We note that this individual was sampled from a strictly autogenous laboratory colony that had no connection with other mosquito populations. We conclude that even with multiple genetic markers, it remains difficult to identify members of the ecotypes unambiguously. Recent work reports that hybrids are common when the two forms occur in sympatry (Rudolf et al. 2013, Di Luca et al. 2016). Danabalan et al. (2012) also reported an incongruence of assigning the individuals to their respective ecotype using different assays. This highlights two major difficulties that currently remain unresolved. First, confident identification of the ecotype is not yet possible, even using multiple assays or multiple markers or both. Second, there is a need to evaluate the assumption that occurrence of the molestus form is restricted to hypogean habitats in temperate climates. The molestus form is not able to diapause in winter (Becker et al. 2012); however, we cannot exclude that favorable conditions in summer may lead to the formation of temporary (i.e., seasonal) aboveground populations. This may, in turn, facilitate hybridization between the two ecotypes. A study conducted in The Netherlands found that f. molestus and f. pipiens were both frequently found to feed indoors and also to hybridize (rates from 6 to 15%) (Vogels et al. 2015). Aboveground breeding sites of f. molestus were also detected during larval sampling (Vogels et al. 2015). This suggests that f. molestus is not restricted to hypogean habitats but instead flies aboveground in search for hosts and sometimes even breeding sites. Future studies aiming to differentiate between ecotypes or to test whether city life favors particular phenotypes or genotypes should focus on approaches that either use loci linked to phenotypic differences (candidate genes) or genome scans that obtain a better resolution by increasing the number of markers.

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