Electronic Supplementary Material – Johnson et al., Urban evolution in *Trifolium repens*

Text S1. Supplementary materials and methods

(a) HCN assay

Upon collection, each sample was initially placed in a sealed plastic bag, and stored in a cooler with ice. Prior to HCN analysis and DNA extraction, samples were individually transferred to microcentrifuge tubes, which were stored at -80 ˚C.

Each plant was assayed for the presence or absence of HCN using the Feigl-Anger assay method of Gleadow et al. ("Experiment 1" protocol) [1], which was modified by Thompson et al. [2] to accomodate a 96-well plate format. We made Feigl-Anger test papers, which turn blue in the presence of HCN. Test papers were made by dipping Whatman 3 (GE Healthcare Life Sciences, Amersham, United Kingdom) filter papers into a solution containing copper ethyl acetate (0.5% W/V) and tetrabase (0.5% W/V) (product #: M44451, Sigma-Aldrich, St. Louis, MO, USA) dissolved in chloroform, followed by air drying. Copper ethyl acetate was made fresh from cupric sulphate (product #:12849, Sigma-Aldrich), sodium acetate (product #: S2889, Sigma-Aldrich) and ethylacetoacetate (product #:688983, Sigma-Aldrich), as described by Gleadow et al. [1]. To assay HCN, plants were removed from the -80˚C freezer and one leaf (1-2 cm in diameter) from each plant was placed in every other well of a 96-well plate (product #: 781600, BrandTech Scientific, Germany) (48 leaves per plate). 80 µL of ddH_20 was added to each well and leaves were macerated using pipette tips. The test paper was firmly secured over the 96-well plate and placed in a 37 ˚C incubator for 3 h. After incubation, the test paper was examined and plants were scored for the presence (blue colour) or absence (uncoloured) of HCN.

(b) DNA extraction and microsatellite genotyping

Genomic DNA was extracted using a CTAB-chloroform extraction protocol adapted to a 96-well plate format following the protocol of Agrawal et al. [3]. One to two leaves from a single plant were placed into each 1.2 mL cluster tube (product #: 4408, Corning Life Sciences, Corning, NY, USA) containing three stainless steel beads (3mm, OPS diagnostics, Lebanon, NJ, USA). Samples were freeze-dried (Christ Epsilon 2-6D LSCplus, Martin Christ, Gefriertrocknungsanlagen, Germany) for 48 h, and homogenized (PowerGen High-Throughput Homogenizer, Fisher Scientific, USA) for 2-3 min at full speed (1600 rpm). The ground tissue was extracted in 500 µL of CTAB buffer (50 mL 1M Tris pH-8.0, 20 mL 0.5M EDTA pH-8.0, 41.5 g NaCl, 0.22 g L-ascorbic acid, 10 g CTAB with 500 mL ddH_20) and 1 µL of β-mercaptoethanol, which was maintained in a water bath at 60 ˚C for 20 minutes. Plates were then centrifuged at maximum speed and 275 µL of the supernatant was transferred to a new cluster tube. An equivalent volume of chloroform was added to each tube and the plate was inverted for up to 30 seconds. The plate was centrifuged at maximum speed for 20 min and 150 µL of the upper aqueous layer was transferred to a new 96-well assay block (product #: 07200723, Corning Life Sciences, Salt Lake City, UT, USA). DNA was precipitated by adding 150 µL of isopropanol to each sample, followed by inverting the plate several times and storing it in a -20 ˚C freezer for 20 min. The plate was subsequently centrifuged at maximum speed for 20 min, the isopropanol was poured off, followed by the addition of 200 µL of 70% ethanol to each sample and incubation at 37 ˚C overnight. After drying, the pellet was dissolved and resuspended in 50 µL of TE buffer pH 8 (1M Tris-HCl, 0.5M EDTA, ddH_20). The DNA concentration and purity of select random samples were quantified using a ND1000 spectrometer (ThermoFisher Scientific Inc., Wilmington, DE, USA). After quantification, 20 to 50 ng/µL of working stock of DNA was prepared and stored at -20 ˚C until further use for genotyping. An exception to the

methods described above was for the city of Guelph, in which samples were extracted individually using the same CTAB protocol.

Microsatellite loci were amplified using multiplexed PCR reactions. These loci were selected based on whether they were known to be polymorphic and exhibit diploid inheritance. Twenty primer pairs were designed with fluorescently labeled (PET, NED, VIC, and FAM flourophores) forward and unlabeled reverse primers (Integrated DNA Technologies, Coralville, IA, USA). Primers for these loci were taken from several sources as shown in table S2 [4-6].

PCR reactions were prepared as 10 µL reactions using the Type-It Microsatellite PCR kit Qiagen, Maryland, USA) on an Eppendorf Mastercycler pro S (Eppendorf, Hamburg, Germany). PCR was performed in 96-well plates (product #: 89049-178, VWR, Radnor, PA, USA) using a master mix of 5 µL of 2X Multiplex PCR Mastermix (HotStarTaq *Plus* DNA Polymerase, PCR buffer with 6mM of $MgCl_2$, and dNTPs), 1 µL of 10X primer mix, 3 µL of RNase-free water and 1 µL of genomic DNA. The 10X primer mix was created by adding 10 µL of each forward and reverse 100 µM primer stock, brought up to a final volume of 500 µL with TE buffer. PCR reactions were organized into four multiplex sets based on the primers' T_m and allowed for multiplexing of up to 6 loci. These four sets included four 10X primer mixes for (1) F05, B11, C10, X31, H04, G02; (2) C03, C02, B08, X16, H09, A05; (3) D12, E05, B04, G05, X34; (4) C06, H06, H11 (see table S3). Sets of primers were amplified under varying PCR touchdown conditions and annealing temperatures were adjusted to optimize PCR conditions. The PCR conditions were determined from Kölliker et al. [5] and adapted to follow the Type-It kit's user manual. For each multiplexed PCR reaction, the thermocycler touchdown profile included an initial step of 5 min at 95 ˚C to activate the HotstartTaq, 10 cycles of denaturation at 95 ˚C for 30 s, annealing at 55 ˚C for 90 s, extension at 72 ˚C for 30 s with a reduction of annealing

69 temperature of 1 ˚C by every cycle, followed by 20 cycles of 30 s at 95 ˚C, 90 s at 45 ˚C, 30 s at
70 72 ˚C, and a final extension time of 30 min at 60 ˚C. This profile was used for primers: F05, B11,
71 C10, X31, H04, G02, D12, E05, B04, G05, X34. A similar thermocycler touchdown profile was
72 used for primers: C03, C02, B08, X16, H09, A05, C06, H06 and H11. For this profile, there was
73 an initial annealing temperature of 60 ˚C and a final annealing temperature of 50 ˚C. A subset of
74 samples from each PCR 96-well plate were run on a 1% agarose gel to confirm successful
75 amplification. Following successful electrophoresis, the PCR products were sent for genotyping
76 on an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA) using an internal LIZ-
77 500 size standard. Genotyping was performed at The Centre for Applied Genomics (Sick Kids
78 Hospital, Toronto, Canada) and the Centre for the Analysis of Genome Evolution and Function
79 (University of Toronto, Toronto, Canada)
80 GeneMarker 1.97 (SoftGenetics, State College, PA, USA) was used to manually identify
81 alleles and genotype all individuals. Markers F05 and X34 were excluded from analyses due to
82 inconsistent amplification and difficulties with calling alleles, respectively. We used
83 MicroChecker 2.2.3 [7] to determine whether null alleles, scoring error or large-allele dropout
84 was present at loci. Arlequin 3.5.2.2 [8] was used to examine if loci deviated from Hardy-
85 Weinberg equilibrium (HWE) [8]. The markers X16 and D12 were excluded from analyses due
86 to consistent deviations from HWE and a high (>35%) frequency of null alleles. The remaining
87 16 loci occasionally showed deviation from HWE in specific populations, but such deviations
88 were inconsistent among populations and loci were in equilibrium in most populations. Linkage
89 disequilibrium between each pair of loci was calculated in FSTAT 2.9.3 [9] based on 2400
90 permutations, and accompanied by a Bonferonni correction for multiple tests at a significance of

0.05. No loci were in linkage disequilibrium. Population genetic analyses were performed using the microsatellite genotype dataset based on the 16 loci.

(c) Statistical analyses

(i) Does *T. repens* evolve parallel clines in HCN in response to urbanization (Question 1)?

We tested for parallel clines in HCN using linear mixed effects models implemented using the *lmer* function in the *lme4* 3.4.2 [10] and *lmerTest* 2.0-33 [11] packages. Populations were treated as the unit of replication and HCN frequency within each *T. repens* population was the response variable, which we fitted to the following model: HCN frequency = Intercept + Distance + *City* + *Distance* × *City* + *Error*. Terms in normal font are fixed effects and were tested as *F*-tests with denominator degrees-of-freedom adjusted using the Satterthwaite correction using the *anova* statement and the *REML = FALSE* option in the *lmer* function. Terms in italics are random effects and were tested using likelihood ratio-tests fit to a χ^2 distribution with one degree-of-freedom using the *rand* statement and the *REML = TRUE* option. The *p*-values of random effects were divided by 2 since they were one-tailed tests [12]. The effect of Distance was a continuous fixed effect variable that described the distance from the city centre. Since equal halves of each transect were in urban and rural areas, yet transects varied in length, we standardized the length of each city's transect to a minimum value of 0 (city centre) and a maximum value of 1 (furthest rural population). Qualitatively identical results and conclusions were found if we used non-standardized distance (electronic supplementary material, table S2). The effect of *City* described variation in HCN frequency among the 20 cities sampled, which we treated as a random effect because the cities represented a sample of all possible cities that could have been sampled, and because we were not interested in how specific cities differed from one another. The interaction *Distance* × *City* was a random effect that tested whether the slope of urban-rural clines in HCN

frequency varied among cities. The residuals were normally distributed and showed no evidence of heteroscedasticity.

(ii) Does the strength of urban-rural clines in HCN vary with city size?

To test question 2, the strength of urban-rural clines in HCN was regressed against city area using multiple linear regression implemented with the *lm* function in R. The strength of urban-rural clines was quantified as the slope of the relationship between HCN frequency and distance within each city individually. City area was square-root transformed to improve normality.

We used 12 analyses to identify the best predictors of: (i) cline strength and (ii) the average HCN frequency within each city. The full model for both sets of analyses was: Response variable = Intercept + Plant Density + City Area + Human Population Size + Human Population Density + *Error*. City area was transformed as described above, and plant density, human population size and human population density were log-transformed to improve normality. The best model was determined using a backwards stepwise regression procedure based on optimizing the model's Akaike Information Criterion (AIC) using the *stepAIC* function.

(iii) Do urban populations have less genetic diversity than rural populations?

The effects of urbanization on genetic diversity within eight cities that varied in size were examined using linear mixed effects models that were similar in structure to those described above under (i). Genetic diversity within populations was quantified using four different statistics: observed heterozygosity (H_O), expected heterozygosity (H_E), allelic richness (A_R) and the inbreeding coefficients (F_{IS}). H_O and H_E were calculated using GenAlEx 6.503 [13], and A_R and F_{IS} were calculated using FSTAT [9]. Each measure of genetic diversity was calculated for each locus individually and then averaged across loci to estimate the population mean genetic diversity. Mean genetic diversity per city was then calculated by averaging across the 15

populations within a city and these data are summarized in the electronic supplementary material (table S4).

(iv) Are urban populations genetically differentiated from rural populations, after accounting for isolation-by-distance?

To assess how spatial distance and urbanization affected pairwise population genetic differentiation, we used Redundancy Analysis (RDA) and Mantel correlograms [14], with separate analyses performed on each individual city. Genetic differentiation between populations within cities was quantified as F_{ST} using GenAlEx 6.503 [13].

Isolation-by-distance (IBD) was tested in two ways. First, we performed a RDA of the allele frequencies for each city regressed against the distance of each population from the urban centre using the *rda* function in the *vegan* package [15]. Significance was determined using 10000 permutations implemented using the *anova.cca* function. Second, to test how IBD may be scale-dependent, we performed a multivariate Mantel correlogram [14], implemented using the *mantel.correlog* function in the *vegan* package, with significance of Mantel's r assessed using 10000 permutations of the data while correcting for multiple tests. Mantel correlograms test whether the strength of genetic differentiation between populations varies at different distance classes between populations.

The effect of urbanization on genetic differentiation was assessed separately for each city using a partial RDA that controlled for IBD [14]. This analysis involved first conditioning the RDA model on distances between populations, followed by testing whether the residual variation in genetic differentiation between populations could be explained by whether populations were located in urban versus rural areas, with statistical significance assessed as described above. This partialling of the data allowed us to assess the effects of urbanization after accounting for IBD in

each city. We subsequently substituted the binary urban/rural explanatory factor for % impervious surface and the number of buildings, which allowed us to assess whether these descriptors of urbanization produced similar results as the categorical effect of urbanization. Mantel tests were also performed and gave similar results for the effects of IBD and urbanization, so we only provide the RDA and Mantel correlogram results here because of the lower statistical power associated with Mantel tests [16].

Finally, we explored the population genetic structure of *T. repens* among populations and cities using Discriminant Analysis of Principal Components (DAPC) [17] and STRUCTURE [18]. We first performed discriminant analysis of principal components (DAPC) to understand how populations were structured between and within cities [17] implemented in the R package adegenet 2.1.0 [19]. The number of genetic clusters across all individuals sampled was determined using the *find.clusters* function, which determined the optimal number of clusters according to the minimum Bayesian Information Criterion (BIC). DAPC was then performed to assess whether cities, and urban and rural populations associated with each city were consistently genetically differentiated from one another.

To further explore the genetic structure of urban and rural populations within cities, we used the Bayesian-clustering software STRUCTURE 2.3.4 [18]. For defining K within individual cities, a series of 10 independent runs per pre-defined K (ranging from 1 to 15) was conducted using the admixture model with correlated allele frequencies and sampling locations as a defined prior. Markov chain Monte Carlo (MCMC) simulations were run with 100,000 burn-in iterations followed by a final 500,000 steps. The number of genetic clusters within cities was determined following the methods of Evanno et al [20], which identifies the number of clusters that maximize ΔK divided by the standard deviation.

Table S1. Characteristics of each city sampled for HCN frequency and population genetic variation in south-central Ontario. All cities were sampled for HCN frequency, and we studied molecular genetic variation within cities denoted by *. For each city, we indicate the latitude and longitude of the population closest to the city centre, the number of populations sampled (N), the human population size, city area (km^2), population density (people/km^2), number of dwellings, and dwelling density (dwellings/km^2). The city of Waterloo also included Kitchener. All data describing city characteristics were taken from Statistics Canada's 2016 Census data [21]. The single exception to this was the area of the city of New Tecumseth, which in the census data included the entire region of New Tecumseth, which is mostly rural; we calculated the area of this city using Google Earth Pro's polygon tool.

city	latitude, longitude	N	population	area	population density	dwellings	dwelling density
Acton*	43.630°, -80.042°	20	9462	7.8	1213	3577	459
Angus	44.323°, -79.879°	20	12640	14.35	881	4272	298
Barrie	44.388°, -79.688°	25	141343	99.00	1428	54227	548
Bradford	44.111°, -79.575°	20	29862	11.94	2501	9960	834
Brantford*	43.139°, -80.269°	27	97496	72.44	1346	40732	562
Cobourg	43.959°, -78.164°	27	19440	22.36	869	8958	401
Elmira*	43.603°, -80.559°	20	10161	7.87	1292	3872	492
Everett*	44.191°, -79.490°	20	1670	1.58	1055	561	355
Fergus*	43.707°, -80.378°	20	20767	17.66	1176	8249	467
Georgetown	43.654°, -79.911°	20	42123	24.03	1753	14679	611
Guelph*	43.544°, -80.251°	20	131794	87.22	1511	55927	641
London	42.984°, -81.243°	40	383822	420.35	913	175558	418
New Tecumseth	44.153°, -79.869°	20	34242	20.36	125	13191	648
Orangeville	43.918°, -80.095°	30	28900	15.61	1852	10696	685
Port Hope*	43.948°, -78.294°	25	12587	12.67	994	5638	445
St. Thomas	42.779°, -81.183°	26	41813	25.97	1610	18157	699
Stratford	43.374°, -80.979°	25	31465	28.28	1113	14302	506
Waterloo*	43.464°, -80.52°	30	233222	136.77	1705	95495	698
Whitchurch-Stouffville	43.971°, -79.245°	25	32634	14.05	2323	10668	759
Woodstock	43.131°, -80.747°	30	40902	48.97	835	17530	358

Table S2. Relationship between HCN frequency, non-standardized distance and city. Urbanization was characterized by the distance (D) in km of each population from the urban centre. Populations were sampled along an urban-rural transect that captured continuous variation in urban development (from heavily urbanized to rural). City was represented by 20 cities that varied in size. An *F*-test was used to test the significance of the fixed effect of distance. A likelihood ratio-test was used to test the significance of the random effects of city and the city x distance interaction, in which significance was tested according to a χ^2 distribution with one degree-of-freedom.

effect	d.f.	F/χ^2	*p*
distance (D)	1, 469	4.96	0.026
city (C)	1	26.40	<0.001
D x C	1	0	0.500

Table S3. Primers used to amplify microsatellite loci to characterize population genetic structure along urban-rural gradients in eight cities. For each primer we include the abbreviated name, fluorophore attached to the forward primer, the forward and reverse primer sequences, and the original source of the primer sequence and microsatellite characterization.

name	fluorophore	forward	reverse	source
G05	NED	TGCTGAGCGAATGTAAGT	CGAAACAAATTGGGTTGA	[4]
E05	6-FAM	CAAGCCCGAACCTGTTTGCTAT	TCGATCTAGTCTTGAACTTTCCTT	[4]
H06	PET	TGAAATTGTTAGGTCGGA	TGACCATAACTATTGGAAAC	[4]
F05	PET	TGTAATTTGAGGAGTGGAAGCCGG	ACTGAACGCAATGTGATCCAAGGG	[4]
B11	PET	ATCTAACCGTATAAACACTGTG	GCTTTGTTGGATACATTGAG	[4]
H09	NED	GAACACTACCCATAACATAAAG	CTAGTATCATCTAAACCCTTGA	[4]
C06	PET	AAATGATAAGCTCATATCCAGCAA	TAGTATGGATTGCCATTGAGATTT	[4]
G02	NED	ATATCTAACCGTATAAACACTGTG	AAATGCTTTGTTGGATACATTGAG	[4]
H11	VIC	AGAAAGGTGAATGATGAAA	TCTAATTCTTCCAATAGGG	[5]
B08	VIC	TTTTGCTAATAAGTAATGCTGC	GGACATTATGCAATGGTGAG	[5]
C02	VIC	AAATAAAACCACAAGTAACTAG	TATAGGTGATTTGAAATGGC	[5]
C03	PET	TATGCTGGTAGATAAACTTAAA	TGCTCTGGAGATTGATGG	[5]
X31	6-FAM	TCTGTTTTGTTGGCCATGC	TTGCAAAGTGTTTGGAAGGA	[5]
X34	NED	TGACAGAAGACCTGATGTACCG	TTCCACTCTTAGCATCAACTGG	[5]
X16	6-FAM	AAGTGTTGGACAAGGAAACTAGG	TCTCTAGATCACCGGCATTG	[5]
A05	NED	CAGTAAAGGAATCTGTTCAAACT	AAACACCAATCAGACCGAAA	[5]
D12	VIC	CTGTAATTCAGGAAGAAAGCAAGG	ATAAGCATTTGTAGTAATCCACT	[6]
C10	VIC	GTACCTGGAAATGTTGATT	GAGCAGCCATGACCTCTG	[6]
B04	6-FAM	AACATGACTCTGTCCTTGTACC	GACATCAGAACTTCTACCTTCG	[6]
H04	6-FAM	ATCAGTCAGAAATCCGTGGGC	TCGACGCGGAATTGGATAAG	[6]

Table S4. Effects of urbanization and cities on genetic diversity. Genetic diversity was quantified as observed heterozygosity (H_O), expected heterozygosity (H_E), allelic richness (A_R), and the inbreeding coefficient (F_{IS}) from each population. Urbanization was characterized by the distance (D) of populations from the nearest urban centre, which captured variation in the degree of urban development (see methods). City was represented by eight different cities that varied in area and other characteristics (electronic supplementary material, table S1). An *F*-test was used to assess the effect of the fixed effect of distance. A likelihood ratio test assessed the significance of the random effects of city and the city × distance interaction, in which significance was tested according to a χ^2 distribution with one degree-of-freedom, and the p-value halved because it was a one-tailed test.

	H_O		H_E		A_R		F_{IS}	
effect	F/χ^2	*p*	F/χ^2	*p*	F/χ^2	*p*	F/χ^2	*p*
distance (D)	<0.001	0.994	0.03	0.872	1.92	0.169	0.12	0.735
city (C)	15.92	**<0.001**	16.10	**<0.001**	227.40	**<0.001**	6.84	**0.009**
C x D	3.25	**0.035**	1.00	0.50	1.07	0.150	2.77	0.598

Table S5. Summary statistics for measures of genetic diversity in urban and rural environments in each of eight cities. We present the mean ± standard error for observed heterozygosity (H_O), expected heterozygosity (H_E), allelic richness (A_R), and the inbreeding coefficient (F_{IS}), which were averaged across urban and rural populations separately. Summary statistics are based on the mean diversity across all 16 microsatellite loci.

	H_O		H_E		A_R		F_{IS}	
city	urban	rural	urban	rural	urban	rural	urban	rural
Acton	0.546±0.078	0.583±0.075	0.647±0.071	0.660±0.059	3.217±0.152	3.289±0.138	0.211±0.087	0.173±0.086
Brantford	0.619±0.076	0.584±0.078	0.673±0.048	0.673±0.080	4.973±0.179	4.758±0.275	0.134±0.089	0.185±0.071
Elmira	0.621±0.098	0.583±0.073	0.650±0.083	0.665±0.060	3.660±0.217	3.748±0.189	0.098±0.121	0.179±0.096
Everett	0.585±0.064	0.624±0.096	0.656±0.061	0.655±0.052	4.239±0.189	4.23±0.155	0.162±0.076	0.101±0.114
Fergus	0.622±0.096	0.573±0.057	0.713±0.057	0.675±0.065	5.533±0.246	5.148±0.163	0.181±0.104	0.202±0.085
Guelph	0.587±0.080	0.584±0.089	0.688±0.057	0.661±0.084	3.396±0.120	3.297±0.197	0.200±0.101	0.166±0.120
Port Hope	0.533±0.053	0.539±0.098	0.639±0.045	0.638±0.098	4.378±0.161	4.222±0.245	0.218±0.070	0.202±0.157
Waterloo	0.609±0.064	0.635±0.090	0.671±0.044	0.685±0.062	4.133±0.118	4.153±0.192	0.147±0.068	0.127±0.089

Table S6. Correlations between genetic diversity statistics and characteristics of cities. For all correlations, city was treated as the unit of replication ($N = 8$).

	Ho		He		A_R		F_{IS}	
predictors	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
plant density	-0.532	0.175	-0.623	0.099	0.108	0.799	0.241	0.566
mean HCN	0.140	0.733	0.479	0.230	0.182	0.666	0.160	0.705
$slope_{HCN\ vs\ distance}$	-0.239	0.569	0.381	0.352	0.290	0.486	0.551	0.157
city area	0.400	0.327	0.533	0.173	0.029	0.947	-0.136	0.749
% impervious surface	-0.112	0.791	0.021	0.960	-0.114	0.787	0.189	0.655
no. buildings	0.526	0.181	0.397	0.330	-0.166	0.694	-0.376	0.358
human population size	0.296	0.476	0.557	0.152	0.046	0.914	0.015	0.972
population density	0.602	0.115	0.560	0.149	-0.238	0.570	-0.367	0.367

Table S7. Estimates of pairwise F_{ST} between populations and their statistical significance in eight cities. Genetic variation within populations was characterized using 16 polymorphic microsatellite markers. F_{ST} was calculated between every population within a city and statistical significance was tested using 9999 randomized permutations using GenAlEx 6.503. *p*-values could not be tested in Elmira because of missing data.

(Table is provided as an Excel table)

Table S8. Results from redundancy analysis (RDA) testing the effects of distance and urbanization on genetic differentiation between populations. A significant effect of distance indicates that genetic differentiation increases with increasing distance between populations (i.e. isolation-by-distance). The effect of urbanization tests whether urban-rural genetic differentiation was greater than urban-urban or rural-rural differentiation among urban populations, after accounting for isolation-by-distance.

	distance		**urban vs rural**	
city	***p***	***R^2***	***p***	***R^2***
Acton	**<0.001**	**0.23**	0.118	0.08
Brantford	0.586	0.07	0.165	0.08
Elmira	0.104	0.09	0.422	0.07
Everett	0.117	0.09	0.509	0.07
Fergus	**0.002**	**0.20**	**0.010**	**0.11**
Guelph	**0.013**	**0.12**	0.200	0.08
Port Hope	0.525	0.09	**0.033**	**0.11**
Waterloo	0.624	0.07	0.544	0.07

Table S9. Results from multivariate Mantel correlograms in each of eight cities. At each distance class, Mantel's r was calculated to determine whether populations are more (positive values) or less similar (negative values), compared to other distance classes. Statistical significance was assessed using 10000 randomized permutations of the data. In five of the eight cities, populations are significantly more similar to one another at one or both of the two smallest distance classes, whereas Mantel r was typically non-significant at larger distance classes.

city	distance class	N	Mantel r	*p*
Acton	0.461	50	**0.311**	**0.001**
	1.062	38	**0.222**	**0.007**
	1.663	34	**0.204**	**0.013**
	2.264	24	0.052	0.333
Brantford	0.761	42	0.029	0.363
	1.802	40	-0.030	0.718
	2.843	40	0.194	0.054
	3.884	26	**-0.223**	**0.035**
Elmira	0.375	50	0.056	0.240
	0.825	36	0.010	0.480
	1.275	38	0.038	0.720
	1.725	26	0.046	0.960
	2.175	22	0.021	1.000
Everett	0.391	48	0.061	0.255
	0.934	24	0.018	0.510
	1.476	54	0.108	0.524
	2.019	18	-0.086	0.698
Fergus	0.574	44	**0.242**	**0.014**
	1.583	46	0.102	0.146
	2.592	22	-0.022	0.391
	3.601	26	-0.072	0.489
Guelph	0.906	42	**0.225**	**0.004**
	1.977	40	**0.151**	**0.038**
	3.048	30	-0.021	0.386
	4.119	26	0.020	0.771
	5.191	28	0.018	1.000
Port Hope	0.407	46	**0.247**	**0.003**
	1.001	36	-0.072	0.189
	1.594	38	-0.082	0.378
	2.188	20	-0.027	0.568
Waterloo	0.822	46	-0.115	0.065
	1.826	38	**0.217**	**0.023**
	2.829	30	-0.105	0.140
	3.833	32	-0.176	0.108

Table S10. Average pairwise estimates of F_{ST} between populations. For each city, we show the mean pairwise F_{ST} between all populations, between urban and rural populations (urban-rural), among urban populations (urban-urban), and among rural populations (rural-rural).

city	all	urban-rural	urban-urban	rural-rural
Acton	0.062	0.068	0.054	0.058
Brantford	0.045	0.046	0.044	0.045
Elmira	0.051	0.052	0.051	0.048
Everett	0.057	0.057	0.063	0.046
Fergus	0.058	0.066	0.046	0.049
Guelph	0.059	0.059	0.052	0.066
Port Hope	0.057	0.059	0.053	0.066
Waterloo	0.044	0.044	0.044	0.043
overall	**0.054**	**0.056**	**0.051**	**0.053**

Table S11. Results from simple linear regressions of HCN frequency versus distance from the urban centre performed on each of the 20 cities sampled. For each regression, the intercept, slope and standard error of the slope or shown for the best fitting line. The *t*-value and *p*-value correspond to the statistical test of whether the slope is significantly different from 0.

city	intercept	slope	std. err.	t-value	*p*
Acton	0.366	0.024	0.108	0.220	0.828
Angus	0.331	0.021	0.145	0.146	0.886
Barrie	0.269	0.085	0.086	0.989	0.333
Bradford	0.268	-0.011	0.078	-0.143	0.888
Brantford	0.280	0.095	0.094	1.011	0.322
Cobourg	0.211	-0.012	0.102	-0.119	0.906
Elmira	0.414	-0.084	0.125	-0.674	0.509
Everett	0.223	0.128	0.084	1.513	0.148
Fergus	0.355	0.280	0.089	3.148	0.006
Georgetown	0.298	0.118	0.118	0.999	0.331
Guelph	0.173	0.305	0.095	3.207	0.005
London	0.298	-0.020	0.073	-0.268	0.790
New Tecumseth	0.353	-0.083	0.104	-0.798	0.435
Orangeville	0.333	0.061	0.099	0.621	0.540
Port Hope	0.198	0.141	0.084	1.683	0.106
Saint Thomas	0.385	-0.034	0.110	-0.306	0.763
Stratford	0.343	0.100	0.084	1.191	0.246
Waterloo	0.302	-0.055	0.103	-0.532	0.599
Whitchurch-Stouffville	0.289	0.043	0.086	0.497	0.624
Woodstock	0.367	0.082	0.090	0.907	0.372
Mean	0.303	0.059	0.098		

Figure S1. The relationship between allelic richness (A_R) and % impervious surface. For both A_R and % impervious surface, we removed variation due to the effects of city and the number of buildings, and then plotted the residual values.

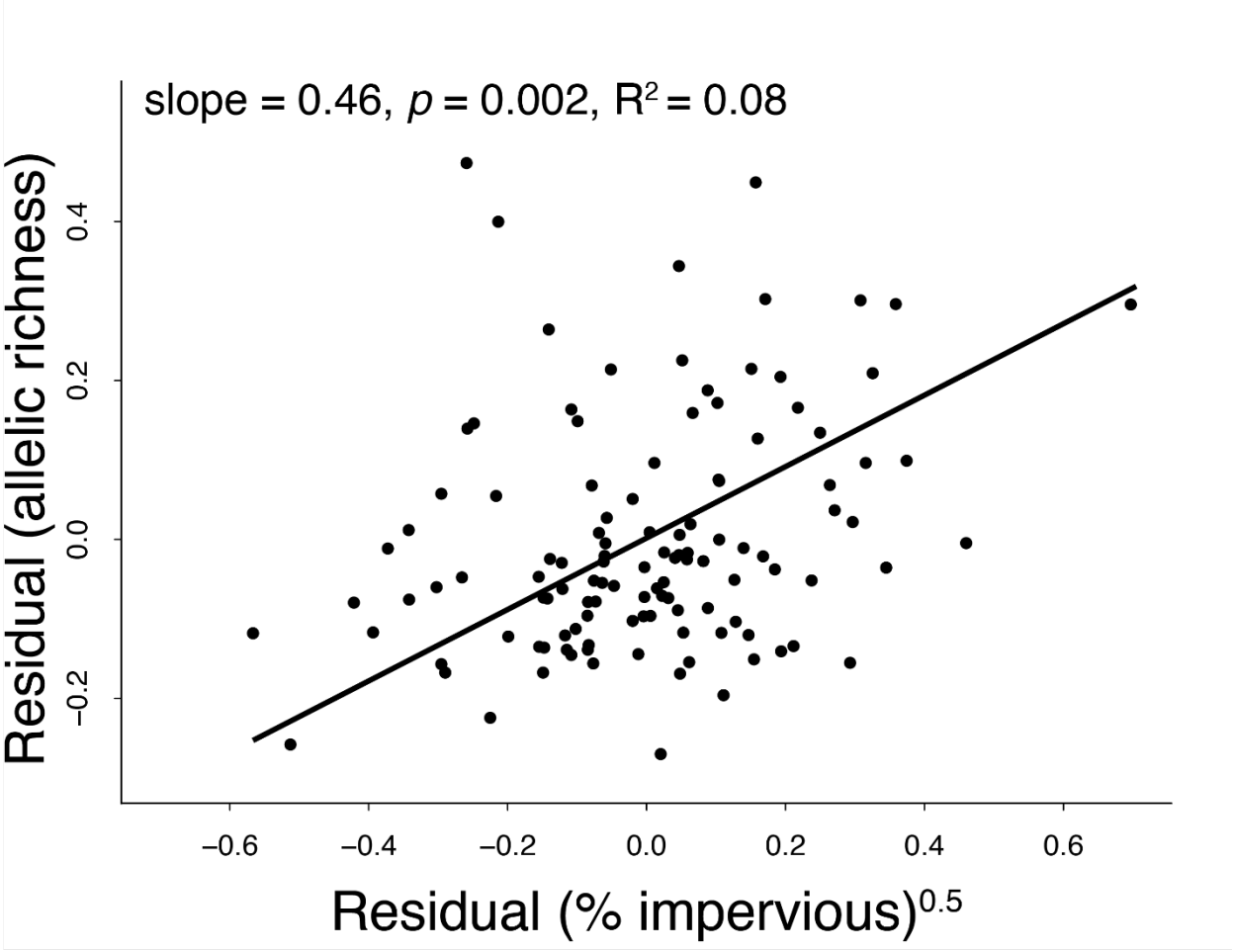
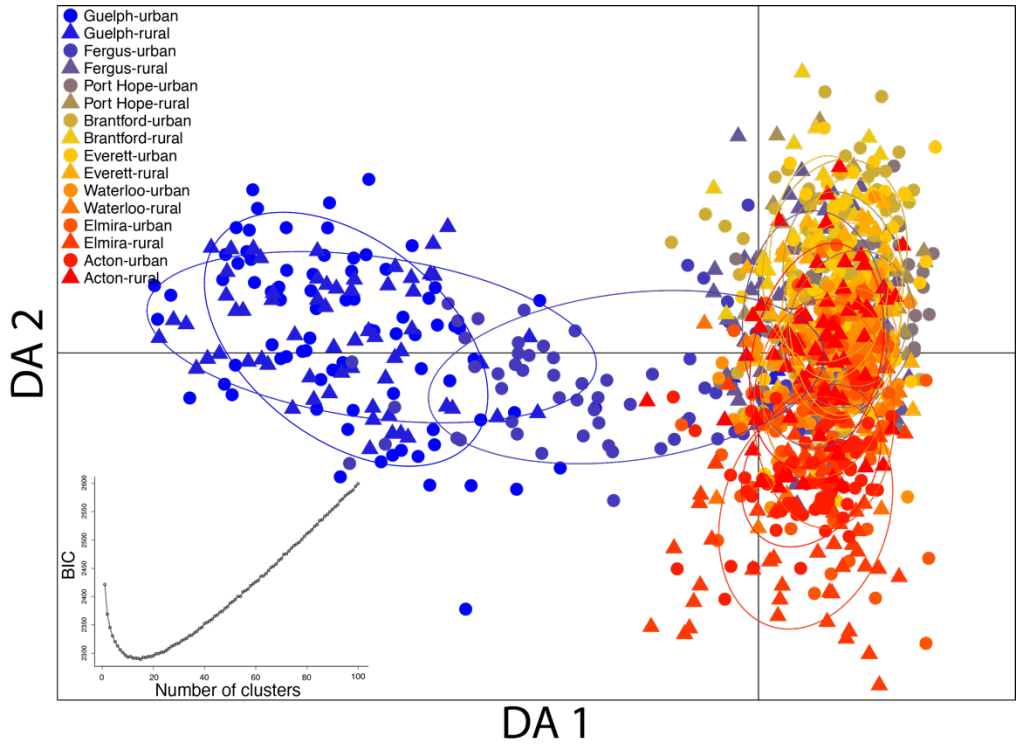


Figure S2. Discriminant analysis of principal components (DAPC) of molecular genetic variation across individuals from urban and rural populations among the eight cities. The raw data were based on the 16 microsatellite loci amplified from all 1200 individuals. City and urban/rural environments were identified as strata in the analysis. DAPC analysis were performed with n.pca=100 and n.da=20. We show an ordination of the first two discriminant axes (DA), which explained 66% of the variance in population genetic structure (DA1 = 56.9%, DA2 = 9.5%). Guelph populations were genetically distinct from most other cities, with broad overlap between urban and rural populations. The genetic structure of Fergus urban populations was intermediate between Guelph and other cities, and clearly differentiated from Fergus rural populations. All other cities were broadly overlapping with one another, with some variation along DA 2, and no clear differences between urban and rural populations. Inset: The optimal number of genetic clusters among all individuals was determined using the *findclusters* function with n.pca = 100 and n.da=20. This resulted in an optimal number of ca. 10 genetic clusters.



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