Title: Global urbanization drives adaptation in the plant white clover

Authors: The Global Urban Evolution Project

(Full author list and affiliations given on pp. 24-30)

*Corresponding author. Email: **marc.johnson@utoronto.ca**

Abstract: Urbanization dramatically transforms environments in ways that alter the evolution of life. We examined whether urban environmental change drives parallel evolution by sampling 110,019 white clover plants from 6,169 populations in 160 climatically diverse cities from all inhabited continents. Plants were assayed for hydrogen cyanide, a Mendelian antiherbivore defence that also affects tolerance to abiotic stressors. Urban-rural gradients were associated with the evolution of phenotypic clines for hydrogen cyanide in 47% of cities throughout the world. Variation in the strength of clines among cities was explained by environmental changes in drought stress and vegetation cover that varied among cities. Sequencing 2,074 genomes from 26 cities revealed that the evolution of urban-rural clines was best explained by adaptive evolution. Our results demonstrate that urban environmental change is leading to adaptation at a global scale.

Once sentence summary: Convergent urban environmental change at a global scale drives adaptation in a cosmopolitan plant

Main Text: Urbanization is a driver of both environmental and evolutionary change. Towns and cities are rapidly expanding throughout the world to accommodate human population growth. These urban areas represent novel ecosystems, in which urban development alters multiple environmental factors (*1*). Recent research shows that urban environmental change can influence four evolutionary processes: mutation, genetic drift, gene flow, and adaptation due to natural selection (*2, 3*). Despite numerous examples of how urbanization affects genetic drift and gene flow (*4, 5*), the effects of urbanization on adaptive evolution have received less attention (*6-8*). Adaptation to urban environments can impact species' conservation (*9*), the spread of pests and disease (*2*), eco-evolutionary feedbacks (*10*), as well as urban planning and human society (*11*). However, the few examples of adaptation to urban environments focus on just one or a small number of cities in a single region (*2*). It is therefore unclear whether populations can adapt to urban habitats in similar ways across cities throughout the world.

Parallel adaptive evolution is most likely when populations experience similar environmental selective pressures on the same genes or phenotypes (*12, 13*). For urbanization to drive parallel evolution, urban areas must converge in environmental features that affect an organism's fitness. Urbanization can lead to similar environmental changes across cities (*14*), but whether urban environmental convergence causes parallel evolution has never been examined at a global scale.

Here we test how global urbanization affects environmental change and evolution in a cosmopolitan plant species, white clover (*Trifolium repens* L., Fabaceae). White clover populations are polymorphic for the production of hydrogen cyanide (HCN), an antiherbivore chemical defence controlled by two genes (*15*). At least one functional allele at each of two unlinked loci (*Ac* and *Li*) are required to produce HCN following tissue damage, while plants that are homozygous for gene deletions (*ac* and *li* alleles) at either locus lack HCN (*16, 17*).

Notably, these deletions occur throughout the world, resulting in standing genetic variation on which selection can act (*18*). Previous work showed that herbivores select for the production of HCN, and abiotic stressors (e.g., freezing and drought) influence the costs and benefits of the metabolic components underlying the defence (*19, 20*). Variation in these environmental factors is credited with driving the evolution of clines in HCN production at continental and regional scales (*21, 22*), including in response to urban environments (*23-25*). Thus, HCN production could evolve in response to urbanization if there are urban-rural gradients in herbivory, winter temperature, or drought.

We examined global urban environmental and evolutionary change across the diverse climates that white clover inhabits. To this end, we created the Global Urban Evolution Project – the largest spatially replicated test of urban adaptation and parallel evolution in natural populations. The present study builds on our previous work (*23-25*) by sampling cities globally across diverse climates in both the native (Europe and western Asia) and introduced ranges, by quantifying many environmental factors from each population, and by integrating evolutionary genomic analyses using whole genome sequence data. This project spanned 160 cities across 26 countries (Fig. 1, *15*) in white clover's native (Europe and western Asia) and introduced ranges (Fig. 1, Fig. S1). From these cities, we phenotyped 110,019 plants from 6,169 sampling sites (hereafter "populations", Table S1). Populations within each city were sampled along an urban-rural transect, with half of each transect in urban and suburban areas (i.e., areas with high building density), and the other half in rural areas (Fig. 2E-G) (*15*).

Across 160 cities, we tested whether urban white clover habitat converged to be more similar and less variable in their environments compared to rural habitats (*15*). Urban and rural habitats significantly diverged (H_0: $\text{urban}_{\text{mean}} = \text{rural}_{\text{mean}}$, $P_{\text{bootstrapped}} < 0.01$, Fig. 2A) along two principal component axes that accounted for 65% of the variation in the multivariate environments between the two habitats across cities. Urban locations consistently had more impervious surface, higher summer temperatures and less vegetation than rural populations (Fig. 2B, Fig. S2). The remaining environmental variables changed along urban-rural gradients in many cities, but these changes were less consistent in direction among cities (Fig. S2, Table S2). Although urban and rural environments diverged on average, urban-rural changes in the environment were not always parallel (H_0: parallel urban-rural changes among cities, $P_{\text{bootstrapped}} < 0.01$, Fig. 2A). Additionally, environmental variance among urban populations was lower than the environmental variance among rural populations ($F_{9,1570} = 31.76$, $P < 0.001$, Fig. S3). Together these results show that on average urbanization leads to similar and less variable environmental conditions in some factors (e.g., impervious surface, summer temperature, summer vegetation), but not in others (e.g. potential evapotranspiration, snow cover, winter vegetation), which could lead to variation in the degree of parallel evolution.

We next tested whether convergent urban environmental change causes parallel evolution in an ecologically important trait of white clover. We examined evolution in response to urbanization by testing for a relationship between HCN production and distance to the urban center (i.e., an “HCN cline”), as well as other metrics of urbanization (*15*). Our model explained 28% of the variation in the frequency of HCN production within populations (Table S3). Across 160 cities, distance from the city center was positively related to the frequency of HCN-producing plants (distance: $\chi^2_{df=1} = 12.35$, $P < 0.001$). The probability that a plant produced HCN increased by 44% on average from the center of an urban area to the furthest rural population (Fig. 2C, D). However, cities varied in the strength and direction of clines (distance × city interaction: $\chi^2_{df=1} = 1001$, $P < 0.001$, Fig. 2C, D). Overall, 47% of cities exhibited a significant ($P < 0.05$) cline (*15*), with 39% of cities (62 of 160) showing a positive cline in which HCN production was less common in urban than rural populations, and 8% of cities (13 of 160) had negative clines (Fig. 2, Table S4). Positive and negative clines occurred in both the native and introduced ranges, with the former being more prevalent among continents and across diverse climates (Fig. 1).

Given the prevalence of HCN clines at a global scale, we sought to identify the evolutionary processes driving variation in the strength and direction of clines. In addition to natural selection, non-adaptive evolution can lead to the evolution of clines (*26*). Importantly, the epistatic genetic architecture of HCN production makes the loss of the trait more likely with increased genetic drift (*26*). Therefore, the prevalence of positive clines could reflect stronger drift in urban populations (*4, 5*). To examine whether urban populations exhibited stronger drift, we estimated pairwise nucleotide diversity (π) of putatively neutral sites using whole genome sequence data from ~80 individuals per city, with samples equally split between urban and rural habitats across 26 cities (N = 2,074, *15*). These cities were selected to capture variation in the strength and direction of clines, geography, and climate (Fig. 1) (*15*).

Genetic diversity was not consistently different between urban and rural habitats and did not explain variation in the slope of HCN clines along urban-rural gradients. On average, urban and rural habitats did not differ in neutral genetic diversity ($F_{1, 25} = 0.028$, $P = 0.87$; Fig. 3A). Furthermore, the difference in π between urban and rural habitats within a city was not strongly related to the slope of HCN clines ($F_{1, 24} = 0.25$, $P = 0.62$; Fig. 3B, Fig. S4), and urban-rural differences in genetic diversity were similar between cities with and without clines ($F_{1, 24} = 0.017$, $P = 0.90$).

Variation in the strength of genetic differentiation and gene flow between urban and rural habitats can influence the ability of populations to adapt to urban environments (*27*). To test the association between genetic differentiation and the evolution of HCN clines we estimated population genetic differentiation between urban and rural populations using both F_{ST} and principal components analysis (PCA) (Fig. S5), in addition to urban-rural admixture (Fig. S6) (*15*). Urban-rural F_{ST} was low (mean = 0.012 ± 0.002 [SE]) and did not differ significantly between cities with and without clines ($F_{1, 24} = 1.47$, $P = 0.24$; Fig. 3C, Fig. S4). Neither F_{ST} ($F_{1, 24} = 1.42$, $P = 0.25$; Fig. 3D) nor urban-rural differentiation measured using PCA ($F_{1, 24} = 1.10$, $P = 0.31$, Fig. S5) predicted the strength of clines in HCN production. The absence of strong differentiation was associated with extensive admixture between urban and rural populations (Fig. S6). Since genetic differentiation is consistently low and gene flow appears to be high among urban and rural populations, the repeated evolution of clines suggests strong selection on HCN production along urban-rural gradients. This conclusion is further supported by direct tests of selection on the *Ac* and *Li* loci, as well as HCN production, in which differentiation (using a statistic equivalent to F_{ST}) between urban and rural populations was stronger than expected under neutral evolution in cities with HCN clines compared to cities without clines (Figs. 3E, 3F, *15*).

Multiple environmental stressors are known to influence the evolution of HCN production at continental scales (*20-22, 28*), so we asked: What environmental factors explain variation in the evolution of HCN production along urban-rural gradients? Environmental factors related to drought and vegetation cover were the strongest predictors of variation in HCN clines, accounting for 11.3% of the variation in the strength of clines (Table S5, Table S6). Change in potential evapotranspiration (PET) along urban-rural gradients was one of the most consistent predictors of evolution in HCN production (Table S5); the frequency of HCN production tended to be higher in rural than urban populations where PET was also greater in rural habitats (Fig. 4A,C; Fig. S7). Since high PET can lead to plant water stress under low soil moisture, this result is consistent with drought selecting for higher HCN production, a pattern also observed at continental scales (*21*). The effect of PET on the evolution of HCN production only occurs when the amount of vegetation in and around cities is low (Fig. 4A). When vegetation cover is relatively high (and impervious surface is low) along the whole urban-rural transect, HCN clines tend to be positive regardless of variation in PET (Fig. 4A-C). Importantly, the amount of vegetation is positively correlated with invertebrate herbivore biomass and diversity (*29*), which can select for increased HCN production (*20*). When combined with the observation that herbivores are often less abundant in urban habitats (*30*), our evidence suggests that herbivores are selecting for greater HCN production in rural than urban areas. The positive association between urban-rural changes in vegetation and the positive slope of HCN clines in some cities further supports this interpretation (Fig. 4D). Put simply, herbivory seems to select for higher HCN production in rural areas, but in the absence of strong pressure by herbivores (i.e., when there is less vegetation across the whole gradient), drought is the main selective agent. Contrary to previous findings, urban-rural changes in temperature did not explain changes in HCN production (*24*). Overall, these results suggest that biotic and abiotic factors influence the evolution of urban-rural HCN clines, but the particular factors that drive the evolution of clines vary among cities.

Our results have general implications for understanding how environmental change affects adaptation in a widespread species. Parallel evolution is a hallmark of natural selection because it suggests that adaptation proceeds in a repeatable way when populations face similar environments (*12, 13*). However, departures from parallel evolution are common, and a major goal of recent research involves quantifying how ecological and evolutionary factors interact to influence variation in adaptive responses to similar environments (*12*). Our results suggest that variation in the strength and direction of HCN clines is driven by variation in biotic and abiotic factors among cities. Variation in additional unmeasured factors (e.g., gene flow from agricultural varieties, pollution, etc.) might further explain variation in the strength of clines, and future work will seek to explore such mechanisms. Our study highlights the power of using globally replicated urban environments to understand the presence, causes and consequences of (non)parallel evolution.

Urbanization is increasingly transforming rural and natural environments into unique ecosystems that Earth's biodiversity has never experienced, and the change is altering the evolution of life on our planet. By performing a field study of urban environmental and evolutionary change across 160 cities distributed across all inhabited continents, our results show that urbanization leads to environmental change that can drive rapid adaptive evolution throughout the world. If adaptation to urban environments is common, as suggested by our results, then this could have multiple effects on populations and ecosystems. This knowledge could help conserve some of Earth's most vulnerable species (*9*), mitigate the impacts of pests (*2*), improve human well-being (*8, 11*), and contribute to understanding fundamental eco-evolutionary processes (*10*).

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Supplementary Materials

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References (31-125)

Authors: James S. Santangelo[1,2]†, Robert W. Ness[1,2]†, Beata Cohan[1]†, Connor R. Fitzpatrick[3]†, Simon G. Innes[4,1]†, Sophie Koch[1]†, Lindsay S. Miles[1,2]†, Samreen Munim[5,1]†, Pedro R. Peres-Neto[6]†, Cindy Prashad[1]†, Alex T. Tong[1]†, Windsor E. Aguirre[7], Philips O. Akinwole[8], Marina Alberti[9], Jackie Álvarez[10], Jill T. Anderson[11], Joseph J. Anderson[12], Yoshino Ando[13], Nigel R. Andrew[14], Fabio Angeoletto[15], Daniel N. Anstett[16], Julia Anstett[17,18], Felipe Aoki-Gonçalves[19], A.Z. Andis Arietta[20], Mary T.K. Arroyo[21,22], Emily J. Austen[23], Fernanda Baena-Díaz[24], Cory A. Barker[25], Howard A. Baylis[26], Julia M. Beliz[27,28], Alfonso Benitez-Mora[29], David Bickford[30], Gabriela Biedebach[30], Gwylim S. Blackburn[31], Mannfred M. A. Boehm[16], Stephen P. Bonser[32], Dries Bonte[33], Jesse R. Bragger[34], Cristina Branquinho[35], Kristien I. Brans[36], Jorge C. Bresciano[37], Peta D. Brom[38], Anna Bucharova[39], Briana Burt[40], James F. Cahill[41], Katelyn D. Campbell[25], Elizabeth J. Carlen[42], Diego Carmona[43], Maria Clara Castellanos[44], Giada Centenaro[45], Izan Chalen[10,46], Jaime A. Chaves[10,47], Mariana Chávez-Pesqueira[48], Xiao-Yong Chen[49,50], Angela M. Chilton[51], Kristina M. Chomiak[40], Diego F. Cisneros-Heredia[10,46], Ibrahim K. Cisse[40], Aimée T. Classen[52], Mattheau S. Comerford[53], Camila Cordoba Fradinger[54], Hannah Corney[55], Andrew J. Crawford[56], Kerri M. Crawford[57], Maxime Dahirel[58], Santiago David[59], Robert De Haan[60], Nicholas J. Deacon[61], Clare Dean[62], Ek del-Val[63], Eleftherios K. Deligiannis[64], Derek Denney[11], Margarete A. Dettlaff[41], Michelle F. DiLeo[65], Yuan-Yuan Ding[49], Moisés E. Domínguez-López[66,67], Davide M. Dominoni[68], Savannah L. Draud[69], Karen Dyson[9], Jacintha Ellers[70], Carlos I. Espinosa[71], Liliana Essi[72], Mohsen Falahati-Anbaran[73,74], Jéssica C. F. Falcão[75], Hayden T. Fargo[1], Mark D. E. Fellowes[76], Raina M. Fitzpatrick[77], Leah E. Flaherty[78], Pádraic J. Flood[79], María F. Flores[22], Juan Fornoni[80], Amy G. Foster[81], Christopher J. Frost[82], Tracy L. Fuentes[9], Justin R. Fulkerson[83], Edeline Gagnon[84,85], Frauke Garbsch[81], Colin J. Garroway[86], Aleeza C. Gerstein[87], Mischa M. Giasson[88], E. Binney Girdler[89], Spyros Gkelis[64], William Godsoe[90], Anneke M. Golemiec[5], Mireille Golemiec[1], César González-Lagos[29,91], Amanda J. Gorton[92], Kiyoko M. Gotanda[93,26], Gustaf Granath[12], Stephan Greiner[81], Joanna S. Griffiths[94], Filipa Grilo[35], Pedro E. Gundel[95,54], Benjamin Hamilton[40], Joyce M. Hardin[69], Tianhua He[96,97], Stephen B. Heard[88], André F. Henriques[35], Melissa Hernández-Poveda[56], Molly C. Hetherington-Rauth[1], Sarah J. Hill[14], Dieter F. Hochuli[98], Kathryn A. Hodgins[99], Glen R. Hood[100], Gareth R. Hopkins[101], Katherine A. Hovanes[102], Ava R. Howard[101], Sierra C. Hubbard[69], Carlos N. Ibarra-Cerdeña[103], Carlos Iñiguez-Armijos[71], Paola Jara-Arancio[104,105], Benjamin J. M. Jarrett[106,26], Manon Jeannot[107], Vania Jiménez-Lobato[108], Mae Johnson[109], Oscar Johnson[110], Philip P. Johnson[111], Reagan Johnson[112], Matthew P. Josephson[113], Meen Chel Jung[9], Michael G. Just[114], Aapo Kahilainen[65], Otto S. Kailing[115], Eunice Kariñho-Betancourt[116], Regina Karousou[64], Lauren A. Kirn[99], Anna Kirschbaum[117], Anna-Liisa Laine[118,65], Jalene M. LaMontagne[7,119], Christian Lampei[39], Carlos Lara[120], Erica L. Larson[121], Adrián Lázaro-Lobo[122], Jennifer H. Le[123], Deleon S. Leandro[124], Christopher Lee[99], Yunting Lei[125], Carolina A. León[29], Manuel E. Lequerica Tamara[98], Danica C. Levesque[126], Wan-Jin Liao[127], Megan Ljubotina[41], Hannah Locke[57], Martin T. Lockett[128], Tiffany C. Longo[34], Jeremy T. Lundholm[55], Thomas MacGillavry[68], Christopher R. Mackin[44], Alex R. Mahmoud[27], Isaac A. Manju[101], Janine Mariën[70], D. Nayeli Martínez[63,129], Marina Martínez-Bartolomé[130,122], Emily K. Meineke[131], Wendy Mendoza-Arroyo[116], Thomas J. S. Merritt[126], Lila Elizabeth L. Merritt[126], Giuditta Migiani[68], Emily S. Minor[111], Nora Mitchell[132,133], Mitra Mohammadi Bazargani[134], Angela T. Moles[32], Julia D. Monk[20], Christopher M. Moore[135], Paula A. Morales-Morales[136], Brook T. Moyers[137,138], Miriam Muñoz-Rojas[51,139], Jason Munshi-South[42], Shannon M. Murphy[121], Maureen M. Murúa[140], Melisa Neila[29], Ourania Nikolaidis[123], Iva Njunjić[141], Peter Nosko[142], Juan Núñez-Farfán[80], Takayuki Ohgushi[143], Kenneth M. Olsen[27], Øystein H. Opedal[106], Cristina

Ornelas[144], Amy L. Parachnowitsch[88,12], Aaron S. Paratore[40], Angela M. Parody-Merino[37], Juraj Paule[145], Octávio S. Paulo[35], João Carlos Pena[146], Vera W. Pfeiffer[147], Pedro Pinho[35], Anthony Piot[31], Ilga M. Porth[31], Nicholas Poulos[148], Adriana Puentes[149], Jiao Qu[33], Estela Quintero-Vallejo[150], Steve M. Raciti[151], Joost A. M. Raeymaekers[152], Krista M. Raveala[65], Diana J. Rennison[153], Milton C. Ribeiro[146], Jonathan L. Richardson[154], Gonzalo Rivas-Torres[10,155], Benjamin J. Rivera[89], Adam B. Roddy[156], Erika Rodriguez-Muñoz[56], José Raúl Román[157], Laura S. Rossi[142], Jennifer K. Rowntree[62], Travis J. Ryan[158], Santiago Salinas[89], Nathan J. Sanders[52], Luis Y. Santiago-Rosario[159], Amy M. Savage[123], J.F. Scheepens[160,117], Menno Schilthuizen[161], Adam C. Schneider[69,1], Tiffany Scholier[149,162], Jared L. Scott[163], Summer A. Shaheed[34], Richard P. Shefferson[164], Caralee A. Shepard[69], Jacqui A. Shykoff[165], Georgianna Silveira[166], Alexis D. Smith[111], Lizet Solis-Gabriel[63], Antonella Soro[167], Katie V. Spellman[168,144], Kaitlin Stack Whitney[169], Indra Starke-Ottich[145], Jörg G. Stephan[170,149], Jessica D. Stephens[171], Justyna Szulc[172], Marta Szulkin[172], Ayco J. M. Tack[45], Ítalo Tamburrino[22], Tayler D. Tate[101], Emmanuel Tergemina[79], Panagiotis Theodorou[167], Ken A. Thompson[59,173], Caragh G. Threlfall[98], Robin M. Tinghitella[121], Lilibeth Toledo-Chelala[63], Xin Tong[49], Léa Uroy[58,174], Shunsuke Utsumi[13], Martijn L. Vandegehuchte[107,33], Acer VanWallendael[175], Paula M. Vidal[22], Susana M. Wadgymar[176], Ai-Ying Wang[127], Nian Wang[177], Montana L. Warbrick[142], Kenneth D. Whitney[132], Miriam Wiesmeier[178], J. Tristian Wiles[69], Jianqiang Wu[125], Zoe A. Xirocostas[32], Zhaogui Yan[177], Jiahe Yao[179], Jeremy B. Yoder[148], Owen Yoshida[55], Jingxiong Zhang[125], Zhigang Zhao[179], Carly D. Ziter[6], Matthew P. Zuellig[180], Rebecca A. Zufall[57], Juan E. Zurita[10], Sharon E. Zytynska[178,181], Marc T.J. Johnson[1,2]*†

[1] Department of Biology, University of Toronto Mississauga; Mississauga, ON, Canada.
[2] Centre for Urban Environments, University of Toronto Mississauga; Mississauga, ON, Canada.
[3] Department of Biology, University of North Carolina; Chapel Hill, NC, USA.
[4] Department of Biology, University of Louisiana; Lafayette, LA, USA.
[5] Department of Biology, Queen's University; Kingston, ON, Canada.
[6] Department of Biology, Concordia University; Montreal, QC, Canada.
[7] Department of Biological Sciences, DePaul University; Chicago, IL, USA.
[8] Department of Biology, DePauw University; Greencastle, IN, USA.
[9] Department of Urban Design and Planning, University of Washington; Seattle, WA, USA.
[10] Colegio de Ciencias Biológicas y Ambientales, Universidad San Francisco de Quito USFQ; Quito, Ecuador.
[11] Department of Genetics, University of Georgia; Athens, GA, USA.
[12] Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University; Uppsala, Sweden.
[13] Field Science Center for Northern Biosphere, Hokkaido University; Sapporo, Hokkaido, Japan.
[14] Natural History Museum, Zoology, University of New England; Armidale, NSW, Australia.
[15] Programa de Pós-Graduação em Geografia da UFMT, campus de Rondonópolis; Cuiabá, Brazil.
[16] Department of Botany and Biodiversity Research Centre, University of British Columbia; Vancouver, BC, Canada.
[17] Graduate Program in Genome Sciences and Technology, Genome Sciences Centre, University of British Columbia, Vancouver, British Columbia, Canada.
[18] Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada.

[19]Red de Biología Evolutiva, Instituto de Ecología, A. C.; Xalapa, Mexico.
[20]School of the Environment, Yale University; New Haven, CT, USA.
[21]Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.
[22]Instituto de Ecología y Biodiversidad, Universidad de Chile; Santiago, Chile.
[23]Department of Biology, Mount Allison University; Sackville, NB, Canada.
[24]Red de Ecoetología, Instituto de Ecología A. C.; Xalapa, Mexico.
[25]Department of Biology, University of Ottawa; Ottawa, ON, Canada.
[26]Department of Zoology, University of Cambridge; Cambridge, UK.
[27]Department of Biology, Washington University in St. Louis; St. Louis, MO, USA.
[28]Department of Biology, University of Miami; Miami, FL, USA.
[29]Centro de Investigación en Recursos Naturales y Sustentabilidad (CIRENYS), Universidad Bernardo O'Higgins; Santiago, Chile.
[30]Department of Biology, University of La Verne; La Verne, CA, USA.
[31]Département des sciences du bois et de la forêt, Université Laval; Quebec, QC, Canada.
[32]Evolution & Ecology Research Centre, School of Biological, Earth and Environmental Sciences, UNSW Sydney; Sydney, NSW, Australia.
[33]Department of Biology, Ghent University; Ghent, Belgium.
[34]Department of Biology, Monmouth University; West Long Branch, NJ, USA.
[35]Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa; Campo Grande, Lisboa, Portugal.
[36]Department of Biology, KU Leuven; Leuven, Belgium.
[37]School of Agriculture and Environment, Wildlife and Ecology group, Massey University; Palmerston North, Manawatu, New Zealand.
[38]Department of Biological Sciences, University of Cape Town; Cape Town, South Africa.
[39]Institute of Landscape Ecology, University of Münster; Münster, Germany.
[40]Gosnell School of Life Sciences, Rochester Institute of Technology; Rochester, NY, USA.
[41]Department of Biological Sciences, University of Alberta; Edmonton, AB, Canada.
[42]Louis Calder Center and Department of Biological Sciences, Fordham University; Armonk, NY, USA.
[43]Departamento de Ecología Tropical, Universidad Autónoma de Yucatán; Mérida, Yucatán, México.
[44]School of Life Sciences, University of Sussex; Brighton, UK.
[45]Department of Ecology, Environment and Plant Sciences, Stockholm University; Stockholm, Sweden.
[46]iBIOTROP Instituto de Biodiversidad Tropical, Universidad San Francisco de Quito; Quito, Ecuador.
[47]Department of Biology, San Francisco State University, San Francisco, CA, USA.
[48]Unidad de Recursos Naturales, Centro de Investigación Científica de Yucatán AC; Mérida, Yucatán, México.
[49]School of Ecological and Environmental Sciences, East China Normal University; Shanghai, China.
[50]Shanghai Engineering Research Center of Sustainable Plant Innovation, Shanghai 200231, China.
[51]Centre for Ecosystem Science, School of Biological, Earth and Environmental Sciences, UNSW Sydney; Sydney, NSW, Australia.

[52]Department of Ecology and Evolutionary Biology, University of Michigan; Ann Arbor, MI, USA.
[53]Department of Biosciences, Rice University; Houston, TX, USA.
[54]IFEVA, Universidad de Buenos Aires, CONICET, Facultad de Agronomía; Buenos Aires, Argentina.
[55]Biology Department, Saint Mary's University; Halifax, NS, Canada.
[56]Department of Biological Sciences, Universidad de los Andes; Bogotá, Colombia.
[57]Department of Biology and Biochemistry, University of Houston; Houston, TX, USA.
[58]ECOBIO (Ecosystèmes, biodiversité, évolution), Université de Rennes; Rennes, France.
[59]Department of Zoology and Biodiversity Research Centre, University of British Columbia; Vancouver, BC, Canada.
[60]Department of Environmental Studies, Dordt University; Sioux Center, IA, USA.
[61]Department of Biology, Minneapolis Community and Technical College; Minneapolis, MN, USA.
[62]Department of Natural Sciences, Ecology and Environment Research Centre, Manchester Metropolitan University; Manchester, UK.
[63]Instituto de Investigaciones en Ecosistemas y Sustentabilidad, UNAM; Morelia, Mexico.
[64]Department of Botany, School of Biology, Aristotle University of Thessaloniki; Thessaloniki, Greece.
[65]Faculty of Biological and Environmental Science, Organismal & Evolutionary Biology Research Programme, University of Helsinki; Helsinki, Finland.
[66]Corporación Científica Ingeobosque; Medellín, Antioquia, Colombia.
[67]GTA Colombia S.A.S. Envigado, Antioquia, Colombia.
[68]Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow; Glasgow, Scotland, UK.
[69]Department of Biology, Hendrix College; Conway, AR, USA.
[70]Department of Ecological Science, Vrije Universiteit Amsterdam; Amsterdam, The Netherlands.
[71]Departamento de Ciencias Biológicas y Agropecuarias, Universidad Técnica Particular de Loja; Loja, Ecuador.
[72]Departamento de Biologia, Universidade Federal de Santa Maria (UFSM); Santa Maria, Rio Grande do Sul, Brazil.
[73]Department of Plant Sciences, School of Biology, College of Science, University of Tehran; Tehran, Iran.
[74]NTNU University Museum, Norwegian University of Science and Technology, 7491 Trondheim, Norway.
[75]Red de Estudios Moleculares Avanzados, Instituto de Ecología A. C.; Xalapa, Mexico.
[76]School of Biological Sciences, University of Reading; Whiteknights Park, Reading, Berkshire, UK.
[77]Department of Biology, Northern Arizona University; Flagstaff, AZ, USA.
[78]Department of Biological Sciences, MacEwan University; Edmonton, AB, Canada.
[79]Max Planck Institute for Plant Breeding Research; Cologne, Germany.
[80]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México; Ciudad de México, México.
[81]Max Planck Institute of Molecular Plant Physiology; Potsdam-Golm, Germany.
[82]BIO5 Institute, University of Arizona; Tucson, AZ, USA.

[83]Alaska Center for Conservation Science, University of Alaska Anchorage; Anchorage, AK, USA.
[84]Tropical Diversity, Royal Botanical Garden of Edinburgh; Edinburgh, UK.
[85]Département de biologie, Université de Moncton; Moncton, New Brunswick, Canada.
[86]Department of Biological Sciences, University of Manitoba; Winnipeg, MB, Canada.
[87]Departments of Microbiology & Statistics, University of Manitoba; Winnipeg, MB, Canada.
[88]Department of Biology, University of New Brunswick; Fredericton, NB, Canada.
[89]Department of Biology, Kalamazoo College; Kalamazoo, MI, USA.
[90]BioProtection Research Centre, Lincoln University; Lincoln, Canterbury, New Zealand.
[91]Departamento de Ciencias, Facultad de Artes Liberales, Universidad Adolfo Ibáñez, Santiago, Chile.
[92]Department of Ecology, Evolution, and Behaviour University of Minnesota; Minneapolis, MN, USA.
[93]Department of Biological Sciences, Brock University; St. Catharines, Ontario, Canada.
[94]Department of Environmental Toxicology, University of California; Davis, CA, USA.
[95]ICB - University of Talca, Chile.
[96]School of Molecular and Life Science, Curtin University; Perth, Australia.
[97]College of Science, Health, Engineering and Education, Murdoch University, Murdoch, WA, Australia.
[98]School of Life and Environmental Sciences, The University of Sydney; Sydney, NSW, Australia.
[99]School of Biological Sciences, Monash University; Melbourne, VIC, Australia.
[100]Department of Biological Sciences, Wayne State University; Detroit, MI, USA.
[101]Department of Biology, Western Oregon University; Monmouth, OR, USA.
[102]School of Natural Resources and the Environment, University of Arizona; Tucson, AZ, USA.
[103]Departamento de Ecología Humana, Cinvestav Mérida; Yucatan, México.
[104]Departamento de Ciencias Biológicas y Departamento de Ecología y Biodiversidad, Facultad de Ciencias de la Vida, Universidad Andrés Bello; Santiago, Chile.
[105]Institute of Ecology and Biodiversity (IEB), Chile.
[106]Department of Biology, Lund University; Lund, Sweden.
[107]Department of Biology, Norwegian University of Science and Technology; Trondheim, Norway.
[108]Escuela Superiro de Desarrollo Sustentable, Universidad Autónoma de Guerrero -CONACYT; Las Tunas, Mexico.
[109]Clarkson Secondary School, Peel District School Board; Mississauga, ON, Canada.
[110]Homelands Sr. Public School, Peel District School Board; Mississauga, ON, Canada.
[111]Department of Biological Sciences, University of Illinois at Chicago; Chicago, IL, USA.
[112]St. James Catholic Global Learning Centre, Dufferin-Peel Catholic District School Board; Mississauga ON, Canada.
[113]Department of Biosciences, University of Calgary; Calgary, AB, Canada.
[114]Ecological Processes Branch, U.S. Army ERDC-CERL; Champaign, IL, USA.
[115]Department of Biology, Oberlin College; Oberlin, OH, USA.
[116]Escuela Nacional de Estudios Superiores Unidad Morelia, UNAM; Morelia, Mexico.
[117]Institute of Evolution and Ecology, University of Tübingen; Tübingen, Germany.
[118]Department of Evolutionary Biology and Environmental Studies, University of Zurich; Winterthurerstrasse, Zurich, Switzerland.

[119]Urban Wildlife Institute, Department of Conservation and Science, Lincoln Park Zoo, Chicago, IL, USA.
[120]Departamento de Ecología, Universidad Católica de la Santísima Concepción; Concepción, Chile.
[121]Department of Biological Sciences, University of Denver; Denver, CO, USA.
[122]Department of Biological Sciences, Mississippi State University; Starkville, MS, USA.
[123]Department of Biology, Center for Computational & Integrative Biology, Rutgers University-Camden; Camden, NJ, USA.
[124]Programa de Pós-Graduação em Geografia da UFMT, campus de Rondonópolis; Brasil.
[125]Kunming Institute of Botany, Chinese Academy of Sciences; Kunming, Yunnan, China.
[126]Department of Chemistry & Biochemistry, Laurentian University; Sudbury, ON, Canada.
[127]Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University; Beijing, China.
[128]School of BioSciences, University of Melbourne; Melbourne, VIC, Australia.
[129]Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Coyoacán, Mexico City, 04510, Mexico.
[130]Department of Biological Sciences, Auburn University; Auburn, AL, USA.
[131]Department of Entomology and Nematology, University of California; Davis, CA, USA.
[132]Department of Biology, University of New Mexico; Albuquerque, NM, USA.
[133]Department of Biology, University of Wisconsin - Eau Claire; Eau Claire, WI 54701.
[134]Agriculture Institute, Iranian Research Organization for Science and Technology (IROST); Tehran, Iran.
[135]Department of Biology, Colby College; Waterville, ME, USA.
[136]Instituto de Biología, Universidad de Antioquia, Medellín, Colombia.
[137]Department of Biology, University of Massachusetts Boston; Boston, MA, USA.
[138]Agricultural Biology, Colorado State University, Fort Collins, CO, USA.
[139]Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Av. Reina Mercedes s/n, 41012 Sevilla, Spain.
[140]Facultad de Estudios Interdisciplinarios, Centro GEMA- Genómica, Ecología y Medio Ambiente, Universidad Mayor; Santiago, Chile.
[141]Evolutionary Ecology Group, Naturalis Biodiversity Center; Leiden, The Netherlands.
[142]Department of Biology and Chemistry, Nipissing University; North Bay, ON, Canada.
[143]Center for Ecological Research, Kyoto University; Otsu, Shiga, Japan.
[144]Bonanza Creek Long Term Ecological Research Program, University of Alaska Fairbanks; Fairbanks, AK, USA.
[145]Department of Botany and Molecular Evolution, Senckenberg Research Institute and Natural History Museum Frankfurt; Frankfurt am Main, Germany.
[146]Departamento de Biodiversidade, Instituto de Biociências, Univ Estadual Paulista - UNESP; Rio Claro, São Paulo, Brazil.
[147]Nelson Institute for Environmental Studies, University of Wisconsin-Madison; Madison, WI, USA.
[148]Department of Biology, California State University, Northridge; Los Angeles, CA, USA.
[149]Department of Ecology, Swedish University of Agricultural Sciences; Uppsala, Sweden.
[150]Facultad de Ciencias y Biotecnologia, Universidad CES; Medellín, Colombia.
[151]Department of Biology, Hofstra University; Long Island, NY, USA.
[152]Faculty of Biosciences and Aquaculture, Nord University; Bodø, Norway.

[153]Division of Biological Sciences, University of California San Diego; San Diego, CA, USA.
[154]Department of Biology, University of Richmond; Richmond, VA, USA.
[155]Estación de Biodiversidad Tiputini, Colegio de Ciencias Biológicas y Ambientales, Universidad San Francisco de Quito USFQ, Quito, Ecuador.
[156]Department of Biological Sciences, Institute of Environment, Florida International University; Miami, FL, USA.
[157]Agronomy Department, University of Almería; Almería, Spain.
[158]Department of Biological Sciences and Center for Urban Ecology and Sustainability, Butler University; Indianapolis, IN, USA.
[159]Department of Biological Sciences, Louisiana State University; Baton Rouge, LA, USA.
[160]Faculty of Biological Sciences, Goethe University Frankfurt; Frankfurt am Main, Germany.
[161]Institute of Biology Leiden, Leiden University; Leiden, The Netherlands.
[162]Department of Biological and Environmental Science, University of Jyväskylä; Jyväskylä, Finland.
[163]Department of Biology, University of Louisville; Louisville, KY, USA.
[164]Organization for Programs on Environmental Science, University of Tokyo; Tokyo, Japan.
[165]Université Paris-Saclay, CNRS, AgroParisTech, Ecologie Systématique et Evolution, 91405, Orsay, France.
[166]Department of Biology, Providence College; Providence, RI, USA.
[167]General Zoology, Institute for Biology, Martin Luther University Halle-Wittenberg; Halle, Germany.
[168]International Arctic Research Center, University of Alaska Fairbanks; Fairbanks, AK, USA.
[169]Science, Technology and Society Department, Rochester Institute of Technology; Rochester, NY, USA.
[170]SLU Swedish Species Information Centre, Swedish University of Agricultural Sciences; Uppsala, Sweden.
[171]Department of Biology, Westfield State University; Westfield, MA, USA.
[172]Centre of New Technologies, University of Warsaw; Warsaw, Poland.
[173]Department of Biology, Stanford University, Stanford, CA, USA.
[174]UMR 0980 BAGAP, Agrocampus Ouest-ESA-INRA, Rennes, France.
[175]Plant Biology Department, Michigan State University; East Lansing, MI, USA.
[176]Biology Department, Davidson College; Davidson, NC, USA.
[177]College of Horticulture and Forestry Sciences/ Hubei Engineering Technology Research Center for Forestry Information, Huazhong Agricultural University, Wuhan, China; Hubei, China.
[178]School of Life Sciences, Technical University of Munich; Munich, Germany.
[179]School of Life Sciences, Lanzhou University; Lanzhou, China.
[180]Institute of Ecology and Evolution, University of Bern; Bern, Switzerland.
[181]Department of Evolution, Ecology and Behaviour, University of Liverpool; Liverpool, UK.

† These authors were members of the lead team
***Corresponding author. Email: marc.johnson@utoronto.ca**

FIGURE LEGENDS

Fig. 1. Cities sampled for urban environmental and evolutionary change. Blue dots indicate cities with positive clines for hydrogen cyanide (HCN) production along urban-rural gradients (HCN_{urban}<HCN_{rural}). Red dots show negative clines (HCN_{urban}>HCN_{rural}). Grey dots indicate cities without a cline. Plants from the 26 cities outlined in black underwent whole genome sequencing. Inset: White clover and a honeybee.

Fig. 2. Urban environmental and evolutionary change across cities. (A) Principal component analysis showing environmental differences between urban (orange dots) and rural (green dots) habitats; ovals represent 95% CI. Lines connect urban and rural habitats from the same city. (**B**) The eigenvectors for environmental variables, coloured according to their contribution to PC2. The environmental variables included vegetation in winter ($NDVI_{winter}$) and summer ($NDVI_{summer}$), snow accumulation (NDSI), surface temperature in winter (LST_{winter}) and summer (LST_{summer}), aridity index (AI), potential evapotranspiration (PET), impervious surface (GMIS) and elevation (DEM). (**C**) Histogram of the slopes from binomial regressions of the relationship between HCN production within populations and distance from the city center. Distance was standardized to vary between 0 (urban center) and 1 (furthest rural population) in each city, so that cities that varied in size were compared on the same scale. The dashed vertical line corresponds to the mean slope across cities and overlap between bars showing cities with significant (blue and red) and non-significant clines (grey) are shown as muted colours. (**D**) The relationship between HCN production within populations and distance for each city; colours correspond to panel C. The black line shows the positive main effect of distance across cities ($P < 0.001$). (**E-G**) Examples of transects, with the orange lines showing the urban boundary, pie charts (jittered to reduce overlap) showing the proportion of HCN+ plants coloured in yellow.

(**H-J**) Frequency of HCN production versus distance for the cities shown in E-G. The line shows the regression line ± 95% CI.

Fig. 3. Genetic diversity and differentiation within and between urban and rural habitats. (**A**) Mean (± SE) pairwise nucleotide diversity (π) for urban (orange) and rural (green) plants across cities. (**B**) The relationship between the slope of HCN clines versus the difference in nucleotide diversity between habitats, where each point is a city. (**C**) Histogram showing the distribution of genetic differentiation (F_{ST}) between urban and rural habitats for each city, coloured with respect to the significance of HCN clines. (**D**) Relationship between the absolute value of the slope of HCN clines versus F_{ST}. (**E**) Percentage of cities in which differentiation between urban and rural habitats at *Ac* or *Li* exceeds neutral expectation in cities with or without significant HCN clines (*15*). (**F**) Percentage of cities with differentiation in HCN production between urban and rural habitats that exceeds neutral expectation in cities with or without significant HCN clines (*15*). *P*-values in E and F correspond to χ^2-test for independence.

Fig. 4. Environmental predictors of urban-rural clines in HCN production. (**A**) Change in potential evapotranspiration along urban-rural gradients (PET_{β}) interacts with the regional amount of summer vegetation (i.e., $NDVI_{summer\_mean}$) to explain variation in the slopes of HCN clines. (**B**) The relationship between the slopes of HCN clines and the regional amount of winter vegetation ($NDVI_{winter\_mean}$). (**C**) PET_{β} interacts with the regional amounts of impervious surface ($GMIS_{mean}$) to predict the slope of HCN clines. (**D**) Change in summer vegetation along urban-rural gradients ($NDVI_{summer\_\beta}$) interacts with regional aridity (AI_{mean}) to explain variation in the slope of HCN clines. Acronyms as in Fig. 2.