***Title*:** Urban spatial heterogeneity shapes the evolution of an antiherbivore defense trait and its genes in white clover

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Abstract

Urbanization is a global threat to biodiversity due to its large impact on environmental changes. Recently, urban environmental change has been shown to impact the evolution of many species. However, much remains unknown about how urban environments influence evolutionary processes and outcomes due to the non-linearity and discontinuity of environmental variables along urban-rural gradients. Here, we focused on the evolution of hydrogen cyanide (HCN) production and its components (presence/absence of cyanogenic glycosides and the hydrolytic enzyme linamarase) in the herbaceous plant white clover (*Trifolium repens* L.), which thrive in both urban and rural areas. To comprehensively elucidate how plants evolve and adapt to heterogenous urban environments, we collected 3299 white clover plants from 122 populations throughout Sapporo, Japan. We examined the spatial variation in environmental factors, such as herbivory, sky openness, impervious surface cover, snow depth, and temperature, and how variation in these factors was related to the production of HCN, cyanogenic glycosides, and linamarase. Environmental factors showed complex spatial variation due to the heterogeneity of the urban landscape. Among these factors, herbivory, sky openness, and impervious surface cover were highly related to the frequency of plants producing HCN in populations. We also found that impervious surface cover was related to the frequency of plants producing cyanogenic glycosides, while herbivory pressure was not. As a result, the cyanogenic glycoside frequency showed a clearer trend along urban-rural gradient rather than HCN frequency, and thus, the predicted spatial distributions of HCN and cyanogenic glycosides were inconsistent. These results suggest that urban landscape heterogeneity and trait multifunctionality determines mosaic-like spatial distribution of evolutionary traits.

***Keywords*:**

urban evolution, cyanogenesis, urban-rural landscape, white clover, heterogeneity of the urban landscape, cyanogenic glycoside

1. Introduction

The threats of urbanization for biodiversity conservation have become a primary concern in the Anthropocene (McDonnell and Hahs 2015; McDonald et al. 2020). Urbanization causes dramatic environmental changes, including increases in temperature due to heat island effects, impervious surface area, habitat fragmentation, and pollution (McDonnell and Hahs 2015; Johnson and Munshi-South 2017; Rivkin et al. 2019). These environmental changes due to urbanization lead to large shifts and losses of biodiversity (McDonald et al. 2020). A component of biodiversity often overlooked is the genetic diversity and evolution within species, and recent research suggests that urbanization can significantly influence the evolution of a wide range of species across the world (Johnson and Munshi-South 2017; Szulkin et al. 2020; Diamond and Martin 2021).

Much remains to be elucidated about how urban environments influence evolutionary processes and outcomes. Previous studies on the impacts of urbanization on evolution have generally employed transects (Thompson et al. 2016; Johnson et al. 2018) or dichotomous comparisons between urban and rural areas (Barnes et al. 2011; Reid et al. 2016; Winchell et al. 2016; Yakub and Tiffin 2017). Although these approaches can examine differences in traits between urban and rural areas, they are poorly suited to revealing the specific elements of urbanization driving evolutionary shifts. This problem arises because multiple environmental factors covary along urbanization gradients. For example, both temperature and impervious surface cover increase from rural to urban areas (Alberti 2015), while vegetation cover and sky openness often decrease from rural to urban areas. However, these factors also vary non-linearly and discontinuously in different manners because of the complexity of urban development, the built environment, and human behavior (Alberti et al. 2020). For example, urbanization-induced heat island effect causes higher temperature in urban areas than rural areas (Alberti et al. 2020). However, green spaces such as parks or forests can locally reduce the temperature (Bounoua et al. 2015), leading to high variation or discontinuous spatial pattern in temperature. The complex nature of urban-rural landscapes described above are expected to create a mosaic of selection pressures on populations that conventional approaches are unlikely to adequately capture. Therefore, it is necessary to consider landscape heterogeneities and urban discontinuities to

fully understand the impacts of urbanization on various environmental factors. Furthermore, many traits are often multi-functional. For example, leaf trichomes that function in defense against herbivores also influence gas exchange, water retention, and thermoregulation (Sack and Buckley 2020). Cyanogenic glycoside in a white clover is an essential component of antiherbivore defense (Hughes 1991; Olsen et al. 2007), but they are also beneficial under drought stress, possibly because of a role in storing nitrogen necessary to repair damage (Kooyers et al. 2014). Such multi-functionality of traits, coupled with complex spatial variation in environmental gradients from urban to rural areas (hereafter urbanization gradient), could make the relationships between urbanization and trait evolution highly complex (Alberti et al. 2020). Therefore, it is important to comprehensively investigate the detailed spatial distribution of various environmental factors, trait multi-functionality, and trait variation within a species across urban-rural landscapes. Such a large-scale landscape approach will advance our understanding of evolutionary effects of urbanization.

In this study, we focused on white clover (*Trifolium repens* L.). It is a perennial herbaceous plant native to Europe that has been introduced from Europe to temperate regions throughout the world (Daday 1958; Burdon 1983; Johnson et al. 2018). White clover was first introduced to Japan from The Netherlands in 1846 (Daday 1958; Wu et al. 2021). It grows in a wide range of soil and environmental conditions including pastures, lawns, gardens, and roadsides in urban and rural environments (Burdon 1983; Johnson et al. 2018; Wu et al. 2021). White clover is self-incompatible (Burdon 1983; Johnson et al. 2018), and its flowers are borne in dense inflorescences pollinated by a variety of bee species (Burdon 1983; Kakes 1997; Larson et al. 2014; Santangelo et al. 2019). It also reproduces clonally through horizontal stems (stolons) that trail along the ground (Kemball and Marshall 1995; Thompson and Johnson 2016), and a single clone can occupy an area of up to 1 m across (Burdon 1983; Johnson et al. 2018). White clover has a Mendelian genetic polymorphism for the production of hydrogen cyanide (HCN), in which plants either produce HCN (cyanogenic) or lack the ability to produce HCN (acyanogenic) (Hughes 1991). The polymorphism is controlled by the epistatic interaction between two loci, *Ac* and *Li*, which show nearly perfect association with expression (Olsen et al. 2007; Olsen et al. 2008; Olsen and Small, 2018). The former locus *Ac* is

composed of a closely linked three-gene cluster involved in the biosynthesis of the cyanogenic glycosides lotaustralin and linamarin. The latter locus *Li* encodes the hydrolyzing enzyme linamarase. Dominant alleles at both loci confer constitutive production of the two necessary chemical precursors for HCN production (Olsen and Small, 2018; Innes et al. 2022). Cyanogenic glycosides are stored in vacuoles, whereas linamarase is found in the apoplast (Kakes 1985; Gruhnert et al. 1994). The two metabolic components form HCN (i.e., cyanogenesis) only when brought together after tissue damage. HCN is a chemical defense which is effective against herbivores (Poulton 1990; Olsen et al. 2014; Thompson and Johnson 2016), specifically against generalists (e.g. mollusks, insects, voles), which are a main causes of leaf damage (Dirzo and Harper 1982; Olsen et al. 2007; Pederson and Brink 1998). However, it also imposes ecological costs on plants (Daday 1965; Johnson et al. 2018). There is also mounting evidence that the metabolic components underlying HCN, cyanogenic glycosides may have alternative ecological functions. Cyanogenic glycosides in the white clover have been shown to contribute to drought stress tolerance (Kooyers et al. 2014). Therefore, environmental factors may select independently on each of the two individual loci as well as on cyanogenesis itself. The relative importance of different selection forces for each of the multi-level polymorphism may help to determine the spatial distribution of cyanogenesis and the frequency of the alleles of associated loci in an urban-rural landscape.

Here, we used a white clover as a model to understand how plants evolve and adapt to complex multivariate urban environmental heterogeneity within and surrounding a single city. Specifically, we address the following questions: (1) How does urbanization affect the spatial distribution and non-linearity of environmental variables across an urban-rural gradient? (2) How does complex spatial variation in multiple environmental factors simultaneously affect the evolution of cyanogenesis and its two essential alleles in the urban-rural landscape? Answering these questions will provide insights into understanding how heterogeneity in city structure and their associated environments shape the evolution of organisms.

2. Materials & Methods

Study system

Our focal species was white clover, a perennial plant distributed worldwide, including in urban and rural habitats. In 2020, between 12 June and 25 September, a total of 122 sites (hereafter "populations") were selected, covering an area of ca. 300 square kilometers in the urban and rural areas around Sapporo, Hokkaido. Sapporo is the fifth largest city in Japan with a population of over 1.9 million and has heavy snow fall (4.79 m per year). The habitats of the populations included parks, green spaces, cemeteries, footpaths, and roadsides. We collected on average 27 (±3 SD) individuals per site for a total of 3299 plants, and obtained GPS coordinates for each population. To avoid duplicate sampling of clones, samples were collected with a minimum of 2 m spacing (Thompson et al. 2016).

Measurement of herbivory damage

To quantitatively assess herbivory from every plant we visually scored leaf area loss following the methods of Johnson et al. (2016). First, we picked the three largest leaves from each individual, and then, evaluated the herbivory damage with the following six ranks; 0: 0% herbivory damage, 1: 1%~10%, 2: 11~25%, 3: 26%~50%, 4: 51~75%, 5: 76~100%. The median value of the range for each rank was used to calculate herbivory damage and the values were averaged across three leaves for each individual (Johnson et al. 2016). In preliminary experiments, visually estimating herbivory damage yielded results consistent with those derived from analyses using Image J (https://imagej.nih.gov/ij/) (Paired t-test, $P = 0.159$; Pearson correlation between visual and ImageJ values, $r = 0.95$, $P < 0.01$, n = 20).

The presence or absence of hydrogen cyanide, cyanogenic glycosides and linamarase

Feigl-Anger assays (Feigl and Anger 1966) were performed to determine the frequency of white clover plants producing HCN within in each population. For this assay, we followed the method of Thompson et al. (2016). After the Feigl-Anger assays, we then used modified Feigl-Anger assays by independently performing them for each allele controlling the production of cyanogenic glycosides

(*Ac – dominant functional allele present, ac – recessive gene deletion*) and linamarase (*Li – dominant functional allele present, li – recessive gene deletion*), using different leaves of the same individuals. The presence/absence of *Ac* and *Li* examined through this assay reflects genetic differences because these are Mendelian inherited traits controlled by a single locus with two alleles (Olsen et al. 2007; Thompson et al. 2016). Detailed methods for the assays are provided in the electronic supplementary material, text S1.

Acquisition of environmental data

We collected environmental data at each site to investigate how environmental factors associated with urban to rural habitats affect spatial variation in the frequency of cyanogenic white clover and related alleles in the population. For Normalized Difference Vegetation Index (NDVI) (Pettorelli et al. 2005), an indicator of the amount of green vegetation present, satellite images taken in June 2010 were acquired from ALOS (Daichi) (https://www.eorc.jaxa.jp/ALOS/jp/dataset/ori_j.htm). For land surface temperature (hereafter "LST"), satellite images were taken from Landsat-8 in May (2015, 2016, 2017, 2019, 2020; https://doi.org/10.5066/P975CC9B). Estimates of both NDVI and LST were calculated from the images using ArcGIS at a radius of 250 m and a 5-year average value of LST was used in analyses.

Annual mean temperature (hereafter "AMT"), the monthly daytime mean temperature in March, and maximum snow depth (hereafter "snow depth") were obtained from the Ministry of Land, Infrastructure, Transport and Tourism (https://nlftp.mlit.go.jp/ksj/index.html). In Sapporo, usually, ground surface is completely covered with snow from December to mid-March. Therefore, we regarded March, when the snow melted and plants were exposed to the air, as the most relevant month for the freezing tolerance of white clovers. These estimates were calculated as the standard value for each 1km mesh (tertiary mesh – the minimum distance available) based on the observed values for the past 30 years.

To calculate impervious surface cover (at a radius of 250 m around each site), land use data was obtained from the Ministry of Land, Infrastructure, Transport and Tourism

(https://nlftp.mlit.go.jp/ksj/index.html). Impervious surface cover indicates the percentage of artificial ground surfaces, including buildings, concrete and asphalt. Therefore, it serves as a major indicator of urbanization.

We measured sky openness as the ratio of the percentage of the sky visible at each site. These data were obtained by taking three digital photos of the sky with a fisheye lens from approximately 30 cm above the ground at the same time of plant sampling. Each sampling site (i.e., the range of sampled clovers of a population) was divided into three sections and a photo was taken at each centroid of each section. Averaging the sky openness values of three photos is relevant to represent the sky openness environment experienced by a sampled clover population. Image J was used to separate the images into the sky, structures, and canopy (https://imagej.nih.gov/ij/) and we calculated the average of the three images for each population. The sky openness is an indicator related to how much sunlight white clover receives.

The rate of herbivory damage to acyanogenic white clover (hereafter "herbivory pressure") was used as a measure of herbivory pressure because we considered herbivory damage to acyanogenic white clovers, which do not have antiherbivore defense traits (cyanogenesis), as reflecting the pure herbivory pressure at each site.

Statistical analysis

All analyses were performed in R version 4.0.5. We used the packages mgcv (Wood 2017) for generalized additive models (GAM), piecewiseSEM (Lefcheck 2016) and nlme (Pinheiro et al. 2021) for structural equation modeling (SEM). Bayesian inference was performed using glmmfiels (Anderson and Ward 2019), and Kriging was performed using the packages of gstat, sp, and maptools.

We used principal components analysis (PCA) and GAM to examine the spatial variation of environmental factors from urban to rural habitats. We also performed generalized linear mixed models (GLMM) with a binomial distribution (i.e., binomial regression) to explore how the frequencies of HCN, *Ac*, and *Li* changed among populations and with how these frequencies change along the distance from urban center.

188 To examine the causal relationships between various environmental factors and spatial
189 variation in the frequency of cyanogenesis, SEM accounting for spatial autocorrelation was
190 performed, using population-level data. In the hypothesized model, the following causal paths were
191 assumed (figure 1): 1) sky openness, NDVI, impervious surface cover, temperature (LST or AMT or
192 mean temperature in March), and snow depth directly affect the spatial variation in the frequencies
193 of *Ac* and *Li*. 2) All of those environmental factors indirectly affect the frequencies of *Ac* and *Li*
194 through herbivory pressure. 3) Frequencies of *Ac* and *Li* affect the frequency of cyanogenesis. 4) All
195 of the above environmental factors affect the frequency of cyanogenesis directly and indirectly
196 through herbivore pressure. To account for spatial autocorrelation in the model, the *gls* function
197 including the correlation argument with coordinates of populations was used in performing the
198 piecewiseSEM package (Lefcheck 2016). Although HCN, *Ac*, and *Li* data is originally individual-
199 level data, environmental data is population-level data. Moreover, spatial autocorrelation should be
200 accounted for in models. However, it is not possible to estimate complex causal paths coupling
201 individual-level and population-level variables with spatial autocorrelation altogether in a single SEM
202 analysis. Thus, population-level frequencies of HCN, *Ac*, and *Li* were used. Arcsine transformation
203 was applied to herbivory pressure, and frequencies of HCN, *Ac*, and *Li* to meet normality assumption
204 in the SEM analysis.
205 In SEM models, Fisher's *C* statistic and the Akaike information criterion (AIC) were used to
206 evaluate the overall goodness-of-fit of models. The statistical significance of Fisher's *C* can be tested
207 by the χ^2 test, in which a *P* value of > 0.05 (rather than < 0.05 as is typically the case with *P* values)
208 indicates a good model fit, with a higher *P* value generally desirable (Lefcheck 2016). We also
209 examined the effects of the range of environmental variables on the frequencies of cyanogenesis and
210 cyanogenesis-related alleles. For these additional analyses, NDVI, impervious surface cover, and LST
211 were analyzed using the different values of NDVI and LST at 250 m radius from each population,
212 and impervious surface cover at 250 m radius from each population, respectively.
213 Moreover, to predict the spatial distribution of traits in relation to environmental factors, we

applied Bayesian inference (Gelman et al. 2013). Using the environmental factors in the best model provided by the first SEM, we built a spatial random field model to explore effects of environmental factors on cyanogenesis frequency while accounting for spatial autocorrelation. Bayesian inference was performed with non-informative priors, four MCMC chains, 250,000 burn-in values, and 250,000 iterations. We checked model convergence through Rhat (Gelman et al. 2013). Finally, the average predicted values were obtained, which we used to create a spatial prediction using the Kriging method, for the frequency of the cyanogenesis and the cyanogenesis-related locus.

3. Results

Environmental changes from urban to rural areas

The environmental factors showed non-uniform changes from urban to rural areas (figure 2, S2). Sites tended to be distributed along PC1 from the urban areas to the rural areas (figure 2a), with higher impervious surface cover and LST values in urban areas, and higher values of NDVI in rural areas (contribution: PC1 = 65.9%, PC2 = 14.8%). However, other environmental factors such as sky openness and snow depth tended to vary more along PC2 than PC1. Therefore, across the entire sampling area, the environmental factors vary in a dissimilar manner. Impervious surface cover, LST, AMT, and the monthly mean temperature in March decreased monotonically with distance from the city center (figure 3a, c, f, g; figure S2a, c, f, g). Snow depth increased monotonically with distance from the city center (figure 3d, S2d). In particular, NDVI, sky openness, and herbivory pressure showed non-linear changes (figure 3b, e, h; figure S2b, e, h).

Effects of environmental change due to urbanization on adaptive traits considering the genetic hierarchy of cyanogenesis

The best model included sky openness, impervious surface cover, herbivory pressure, the frequency of *Ac*, and the frequency of *Li* (AIC = 52.359, Fisher's $C = 12.359$, P value for Fisher's $C = 0.262$; figure 4). The frequency of cyanogenesis within populations directly increased with the frequency of *Ac*, the frequency of *Li*, herbivory pressure, and the impervious surface cover. The

241 frequency of *Ac* was negatively associated with impervious surface cover. The frequency of *Li* was
242 directly and positively correlated with the frequency of *Ac*. Herbivory pressure was positively
243 associated with sky openness and negatively associated with impervious surface cover. Thus, we
244 found that herbivory mediated indirect positive and negative paths from sky openness and impervious
245 surface cover to the frequency of cyanogenesis, respectively. There was also the negative indirect
246 path of impervious surface cover on the frequency of cyanogenesis via the frequency of *Ac*.
247
248 *Spatial variation from urban to rural areas in the frequencies of cyanogenesis, Ac, and Li*
249 The frequencies of cyanogenesis, *Ac*, and *Li* did not increase or decrease significantly with
250 distance from the urban center (HCN: $r^2 < 0.05$, $P = 0.87$; *Ac*: $r^2 = 0.0135$, $P = 0.201$; *Li*: $r^2 < 0.05$, P
251 $= 0.977$). We confirmed that these results of the relationship between the frequencies of cyanogenesis,
252 *Ac*, and *Li* and distance from the urban center did not change with the use of GLMM assuming a
253 binomial error distribution (figure S1a, b, c). However, the spatial distribution of HCN and *Ac* was
254 clearly non-random with respect to urbanization (figure 5). Therefore, we explored the environmental
255 factors that predicted spatial variation in HCN and its underlying loci. The frequency of cyanogenesis
256 was predicted to vary in a mosaic fashion from urban to rural areas (figure 5a). In contrast, the
257 frequency of *Ac* was predicted to decrease with higher impervious surface cover (i.e., in urban areas),
258 and to increase at lower impervious surface cover (figure 5b, S1). Thus, the predicted spatial
259 distributions of HCN and *Ac* were partially inconsistent, although the frequency of *Ac* and HCN were
260 significantly associated as shown in the SEM (figure 4, 5a, b, S3).
261
262 **4. Discussion**
263 This study highlights that the spatial distribution of an ecologically important trait and its
264 underlying genes are shaped by environmental heterogeneity associated with urbanization.
265 Environmental factors showed complex spatial variation along the urbanization gradient. Among
266 environmental factors, herbivore pressure and impervious surface cover, which are related to urban
267 structure, explained variation in the frequency of cyanogenesis in populations. Moreover, we found

that impervious surface cover was associated with the frequency of *Ac* alleles in the population, while herbivory pressure was not. The gradient in impervious surface cover due to urbanization appears to drive the evolution of cyanogenic glycoside production, which is a key component of cyanogenesis.

Environmental changes from urban to rural areas

To understand the impacts of urbanization on various environmental factors, it is necessary to consider landscape complexity and urban discontinuities (Alberti et al. 2020; Santangelo et al. 2022b). This study revealed that the heterogeneity of the urban landscape results in non-linear environmental gradients between urban and rural habitats. The various environmental characteristics change greatly from urban to rural areas in contrasting ways (figure 2, 3, S2), each contributing to environmental heterogeneity. There are remnant forests in both urban and rural areas, which would cause the variation in impervious surface cover and NDVI (figure 3a, b, S2a). The variation in sky openness is greater near urban areas (figure 3e) due to microstructures in the configuration of buildings and small green areas. Although AMT (Annual Mean Temperature) decreases from urban to rural areas (figure 3f, figure S2f), which may be due to the heat island effect (Alberti et al. 2020), parks and green spaces can locally reduce temperature (Bounoua et al. 2015). These non-uniform changes in environmental factors due to urban heterogeneity suggest that the multiple layers of urban-induced environmental changes can have complex and non-linear impacts on evolutionary traits. The heterogeneity of the urban-rural landscape must be considered to fully understand the environmental changes due to urbanization.

Effects of environmental change due to urbanization on cyanogenic glycosides

This study clarified the relationship between multiple environmental factors along the urbanization gradients on cyanogenesis and its associated genes. There are three possibilities for the impervious surface cover to reduce the frequency of the functional *Ac* allele, which is required to produce cyanogenic glycosides: 1) soil nutrient status, 2) drought stress, and 3) snow and freezing. First, Li et al. (2013) reported that urban soils had lower soil organic matter, available nitrogen,

295 phosphorus, and boron concentrations than natural soils. The resource availability hypothesis
296 proposes that, plants at poorer nutrient sites, which grow more slowly, can produce the greater amount
297 of carbon-based defense metabolites due to a surplus of carbon (Coley et al. 1985). However,
298 cyanogenic glycosides are nitrogen-related secondary metabolites. Therefore, in nutrient-poorer areas,
299 maintaining nitrogen-related secondary metabolites should be much more costly. However, pollution
300 in urban such as exhaust fumes can also increase nitrogen deposition, providing nutrients to plants
301 (Raupp et al. 2010), and white clover has nitrogen-fixing symbionts (Dazzo and Brill 1978). In
302 addition, Thompson et al. (2016) found that the addition of nutrients did not alter selection on HCN.
303 Therefore, nutrient availability was unlikely to explain spatial variation in *Ac*.

304 Second, Kooyers and Olsen (2013) showed that aridity may be an important agent of
305 selection that favors cyanogenic glycosides in drier areas (but see Foulds and Grime 1972). Kooyers
306 et al. (2014) reported that cyanogenic glycoside-producing white clover had relatively higher fitness
307 in treatments simulating moderate and persistent drought stress, and suggested it affects the cline
308 formation of HCN production along the drought gradient. Unger (1999) also investigated the water
309 vapor pressure inside and outside the city in Szeged, Hungary, suggesting that cultivation of wheat
310 and corn would make the soil in rural areas drier than the ground surface in urban areas, where it was
311 watered in parks and private gardens and shaded by buildings. In fact, rural areas around Sapporo,
312 especially the eastern part of the sampling region, are likely to be drier than urban areas due to massive
313 wheat cultivation. Santangelo et al. (2022a) reported that drought stress tended to select for higher
314 HCN production in some rural areas, which involves higher *Ac* production. Thus, drought stress can
315 select for *Ac*. However, Santangelo et al. (2022a) also reported that urban areas had higher aridity on
316 average than rural areas across 160 cities, and a transect located in northern Sapporo followed this
317 pattern in the study by Santangelo et al. (2022a). Because that transect in northern Sapporo and our
318 study regions in this study were not overlapping, drought stress in urbanization gradients may be
319 inconsistent even in the single city.

320 Third, Thompson et al. (2016) suggested that reduced snow depth in urban areas compared
321 with non-urban areas and low winter temperatures would play a role in exerting selection against

HCN. Kooyers et al. (2018) reported that white clovers producing cyanogenic glycosides (*AcLi* and *Acli*) were significantly less likely to survive freezing than cyanotypes not producing cyanogenic glycosides. In this study, increased impervious surface cover was correlated with lower snow depth and higher LST (for snow depth: r= -0.46, $P < 0.01$; for LST: $r = 0.90$, $P < 0.01$). This suggests that impervious surface causes snow to melt, and lower temperatures impose costs on lower freezing tolerance of plants with *Ac*. The reason why impervious surface cover explained *Ac* frequency variation rather than snow depth and LST could be that impervious surface cover imposes selection on *Ac* through not only freezing but also drought stress. It should also be noted that these environmental factors did not strongly associate with cyanogenesis or *Li*. Overall, our results support that environmental factors influenced by impervious surface cover would impose selection on the functions of *Ac* as stress tolerance/susceptibility.

Effects of environmental change due to urbanization on herbivore pressure and cyanogenesis

The best model indicates abiotic factors in urbanization gradients influenced biotic herbivore pressure, which was positively related with cyanogenesis frequency. The plant apparency hypothesis (Feeny 1976) may be relevant for the positive effect of sky openness on herbivory pressure. Plant apparency is the probability of a plant being found by herbivores, and high accessibility by herbivores can lead to high apparency and resultant high herbivore abundance. Several recent studies supported that apparent plants suffer greater herbivory than unapparent plants (Zverev et al. 2017; Rossetti et al. 2019; Martini et al. 2021). Therefore, white clover at sites where sky openness is greater may be more easily found and attacked by herbivores, which can select for cyanogenesis.

On the other hand, the following two reasons can explain the negative effects of impervious surface cover on herbivory pressure. 1) The number of herbivores may decrease with urbanization; 2) high impervious surface cover in the surrounding area may inhibit the colonization of herbivores. In general, populations of insects and other organisms could decrease as urbanization progresses (Miles et al 2019). Muratet & Fontaine (2015) also reported that the application of insecticides and herbicides in urban areas reduced the population abundances of butterflies and other insects. However,

the relationship between urbanization and herbivory pressure is inconsistent (Raupp et al. 2010). Raupp et al. (2010) illustrated that the abuncance of several arthropod species, such as gall insects and leaf-miners, could increase with urbanization. These species-specific sensitivities to urbanization may result in the inconsistent patterns of herbivory pressure. In our study, we found a large amount of variation in herbivory pressure even at the same distance from urban center across study sites (figure 3h). Therefore, it is unrealistic to say the number of herbivores consistently decreases with urbanization. The increase in impervious surface cover with urbanization would fragment habitats and inhibit herbivore movement (Raupp et al. 2010; Johnson and Munshi-South 2017). Therefore, greater impervious surface cover may prevent herbivores from colonizing white clover patches, resulting in decreased herbivore pressure. The latter explanation, that high impervious cover inhibits the colonization of herbivores, is likely to be more plausible. However, the former and latter explanations are not mutually exclusive. In addition, it should also be noted that impervious cover can correlate with other unmeasured environmental factors that may influence herbivory pressure. Thus, future studies should investigate the mechanisms by which impervious surface cover is associated with herbivory.

Spatial distribution of hydrogen cyanide production and its components along an urbanization gradient

As mentioned above, natural selection based on stress tolerance/susceptibility function (i.e., drought and freezing) is likely to be strongly associated with frequencies of *Ac*. Direct selection on *Ac* by these factors is likely to be stronger rather than selection on cyanogenesis as an antiherbivory defense along the urbanization gradients.

Thus, selection on *Ac* may primarily generate HCN frequency clines in the urbanization gradients as a general trend (figure 5), and this notion is consistent with a study of Santangelo et al. (2020). However, herbivory pressure is also an important selective force for cyanogenesis (figure 4). Herbivory pressure is more variable than other abiotic environments due to impervious surface cover, sky openness, and other unmeasured factors. This notion can explain the fundamental difference

between the predicted spatial distributions of frequencies of *Ac* and cyanogenesis (figure 5, figure S3). We argue that the relative importance of different urban-rural environmental factors for the fitness consequences governs the spatial distribution of cyanogenesis and its component cyanogenic glycoside. In other words, when freezing stress is more critical, HCN frequency decreases along with *Ac* decline. When drought stress is critical, requirements of *Ac* increase but cyanogenic glycosides should be maintained without the use for HCN production following herbivory. When herbivory pressure is more critical, selective pressure is exerted toward the direction of simultaneous production of both cyanogenic glycosides and linamarase.

Furthermore, this study has implications for phenotypic evolution of urban-rural clines. The predicted map of frequencies of cyanogenesis (figure 5a) suggests that an urban-rural cline in cyanogenesis can be detected, depending on the direction and distance of a transect. This expectation is consistent with the study of Santangelo et al. (2022b) which reported intraurban environmental heterogeneity can drive fine-scale adaptive clines within cities. Among-city variation in the presence/absence of clines may be due to heterogeneity within a single city as well as differences in environmental conditions among cities. Even in a city where no cline is detected, a cline may be observed depending on how a transect is placed or how contrasting urban and rural sampled. Thus, conventional approaches may fail to detect effects of urbanization if they do not account for spatial heterogeneity appropriately.

This study demonstrates the importance of considering multivariate environmental heterogeneity in studying the evolution of organisms in urban environments. The urban-rural landscape approach allowed us to identify clear associations between environmental factors and traits of white clover. Our results provide evidence that the complexity of urbanization-induced environmental changes, such as impervious surface cover, sky openness, and herbivory pressure, play a critical role in shaping spatial variation in genotypic frequencies. The previous study by Santangelo et al. (2018) cautioned that stronger genetic drift in urban areas due to smaller population sizes and fragmentation can lead to lower frequencies of cyanogenesis in urban areas. However, in our study site, this notion is unlikely to explain the pattern because there were many large and dense patches in

403 urban areas and many small patches in a crop field in rural areas. Moreover, Santangelo et al. (2018)
404 also showed that while clines in HCN can evolve by drift, clines at individual alleles (i.e. *Ac* and *Li*)
405 cannot consistently evolve due to drift. Future molecular works will be necessary to confirm this
406 expectation. This study also illustrates that genetic hierarchy and multifunctionality of polymorphic
407 traits, and the multiple layers of urbanization-induced environmental changes have a critical role for
408 shaping spatial variation in genotypic frequencies. In conclusion, the urban-rural landscape approach,
409 which considers the complexity of multivariate environmental heterogeneity, provides great insights
410 toward an understanding of the complex evolutionary processes in urban environments. The approach
411 used in this study can be applied to future research to advance our understanding of the evolution of
412 organisms in urban environments.
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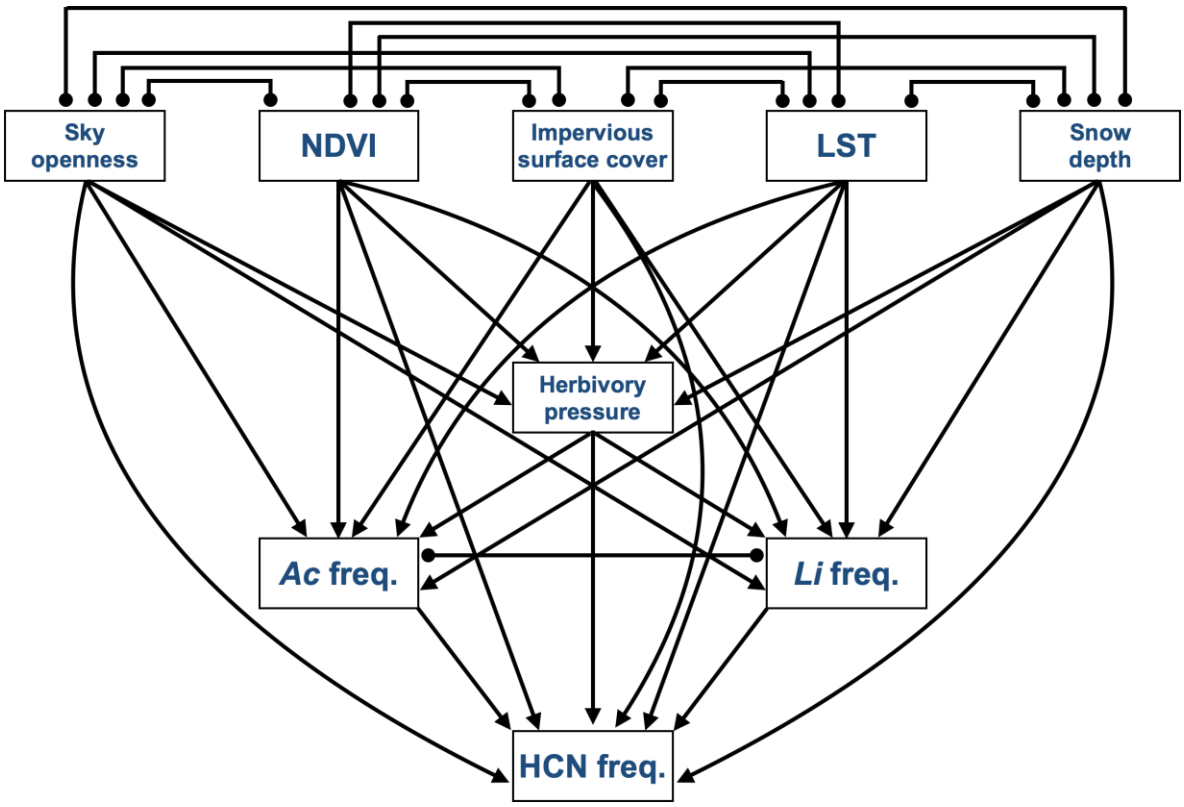
607

608 **Figure legends**

609



610

611 **Figure 1.** The hypothetical structural equation model of urbanization-driven environmental changes

612 and evolutionary changes in the frequency of *Ac, Li,* and HCN. Lines with circular ends show

613 correlations.

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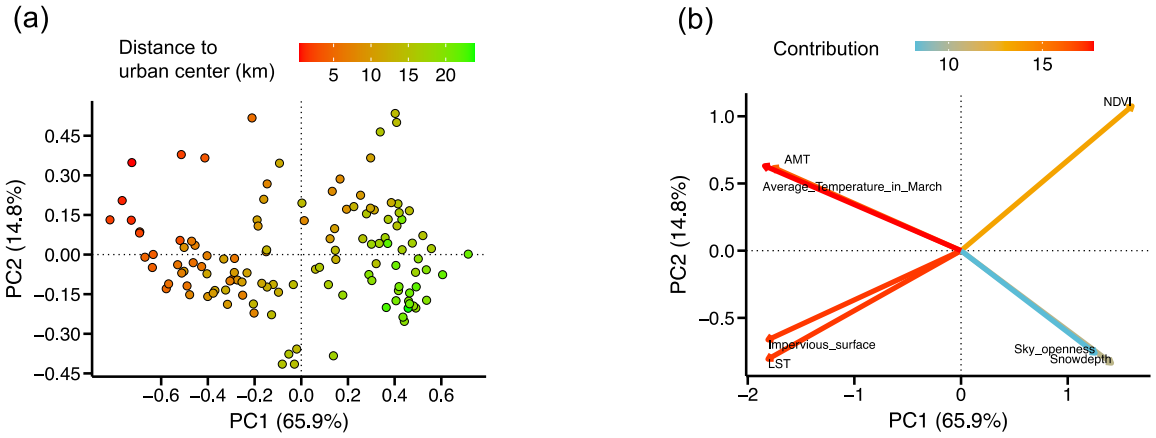

615

616 **Figure 2.** Visualization of spatial variation in environmental factors across the urban and rural

gradient. (a) Environmental differences among sites, colored according to the distance from the city center. (b) The eigenvectors for environmental variables, colored according to their contribution to PC1. The environmental variables included LST, NDVI, impervious surface cover, AMT, average temperature in March, snow depth, and sky openness. NDVI, LST, and AMT represent Normalized Difference Vegetation Index, land surface temperature, and annual mean temperature, respectively.

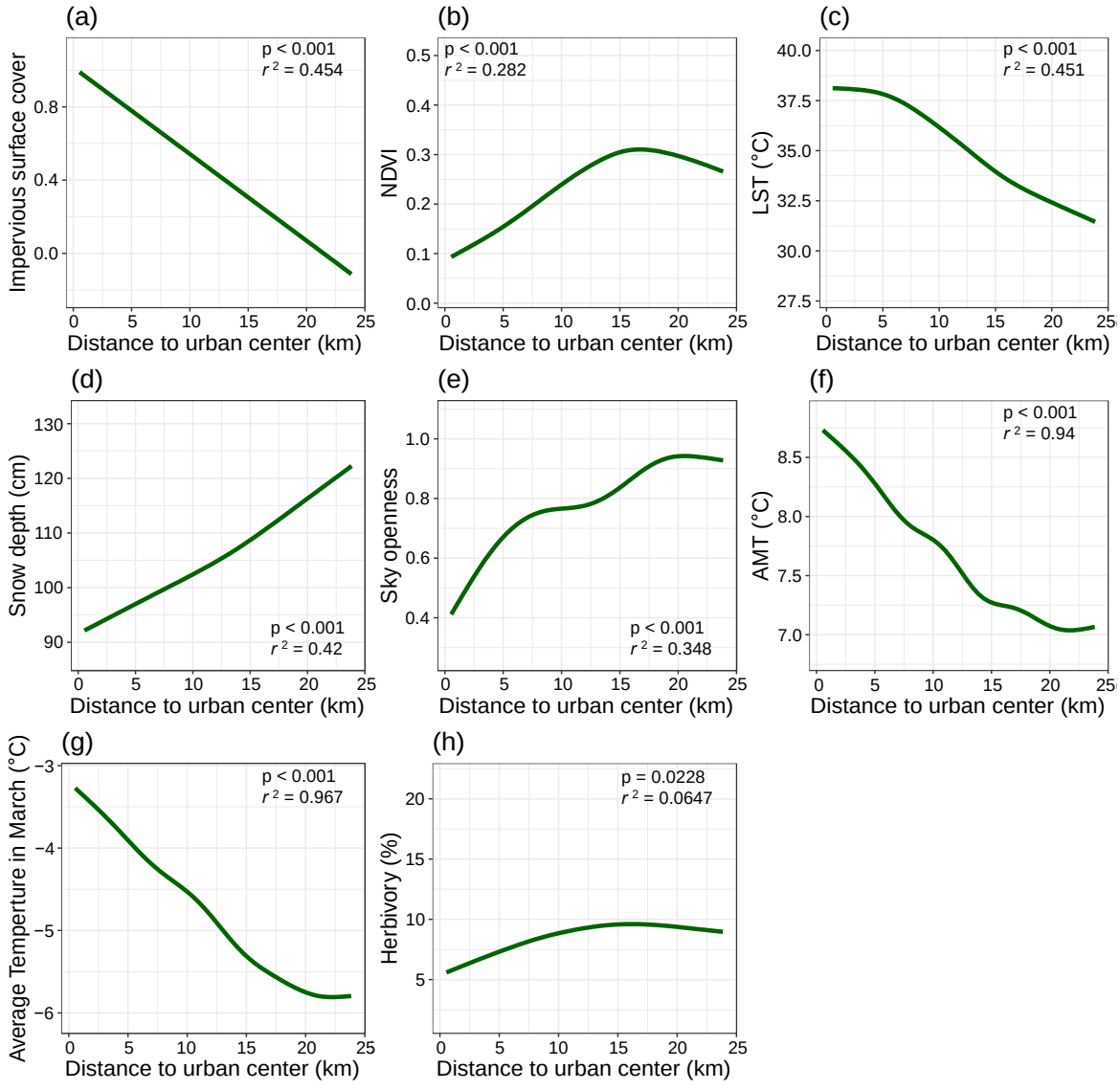

Figure 3. Relationships between the distance to the urban center and environmental variables. Different spatial ranges for 250 m radius (a, b, c) and point estimates of the center of each site (d, e, f, g, h, i). *P* values were obtained by a GAM. NDVI, LST, and AMT represent Normalized Difference

627 Vegetation Index, land surface temperature, and annual mean temperature, respectively.

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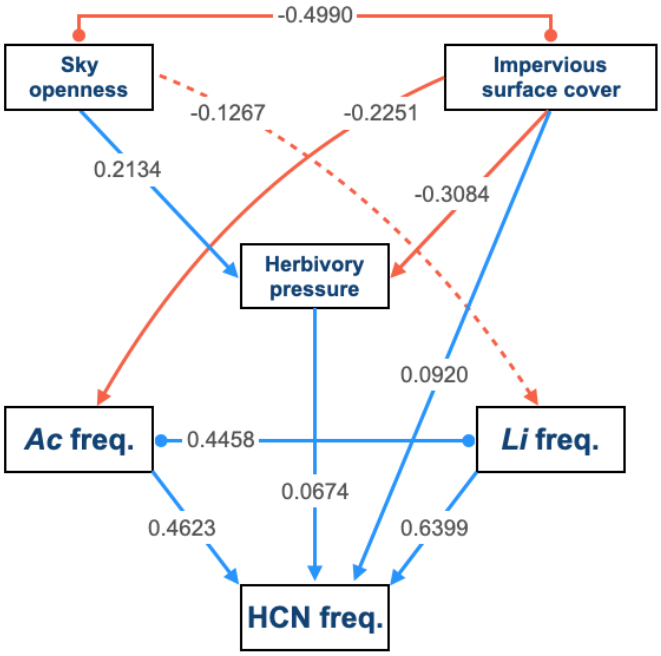
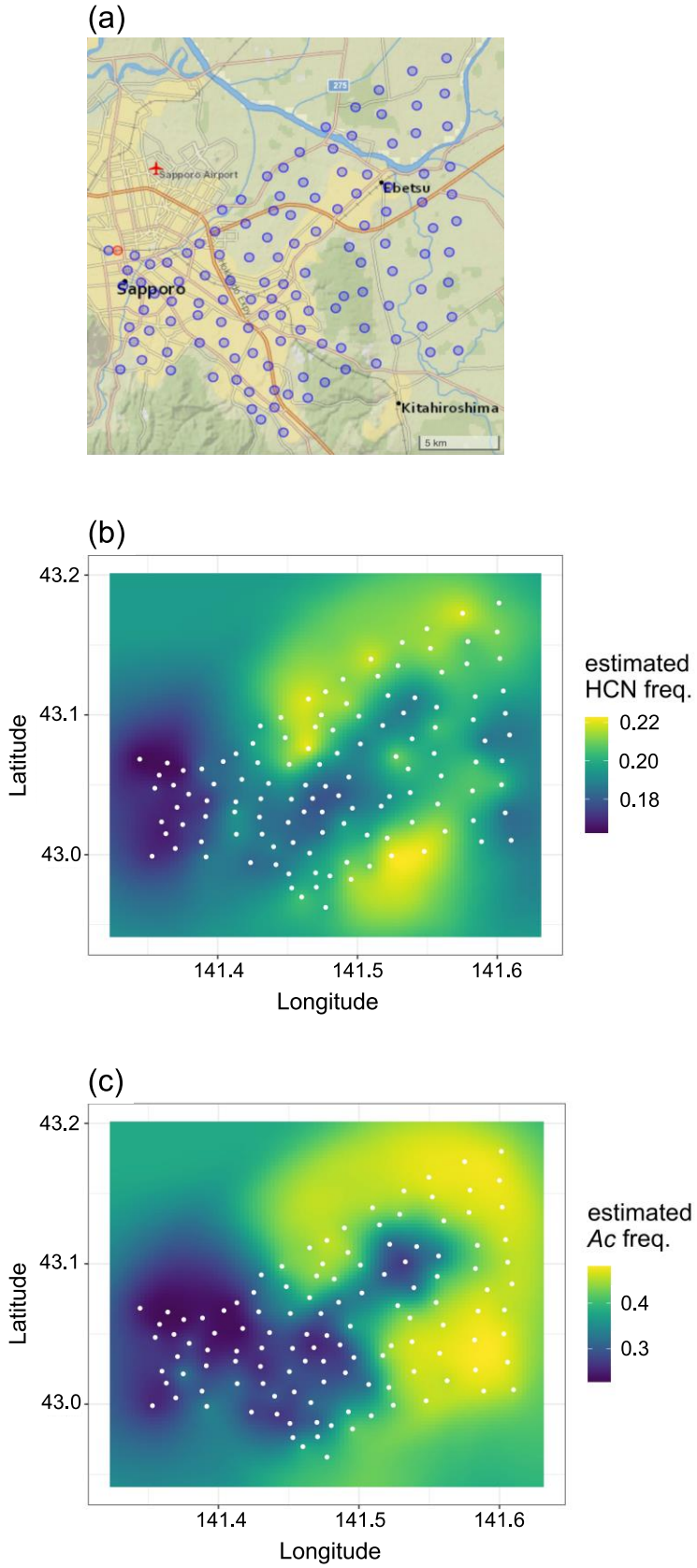


629

630 **Figure 4.** The best model of the structural equation model of urbanization-driven environmental
631 changes and the frequency of *Ac, Li,* and HCN. Blue and red arrows show positive and negative
632 effects, respectively. Bold arrows show the paths with significant coefficients ($P < 0.05$) and dashed
633 arrows show non-significance ($P > 0.05$). Lines with circular ends show correlations. Standardized
634 path coefficients are shown.



635

636 **Figure 5.** HCN frequency prediction model (a) and *Ac* frequency prediction model (b). Simplified

topographic map of Sapporo and its suburb and rural areas. We defined the urban center of Sapporo as the Sapporo Station (43°04'07.1"N, 141°21'02.9"E), shown as a red point. (c). White or purple points denote sampling sites.

Supplementary Information for: Urban complexity determines the spatial pattern of evolution in antiherbivore traits of white clover

Supplementary Figures

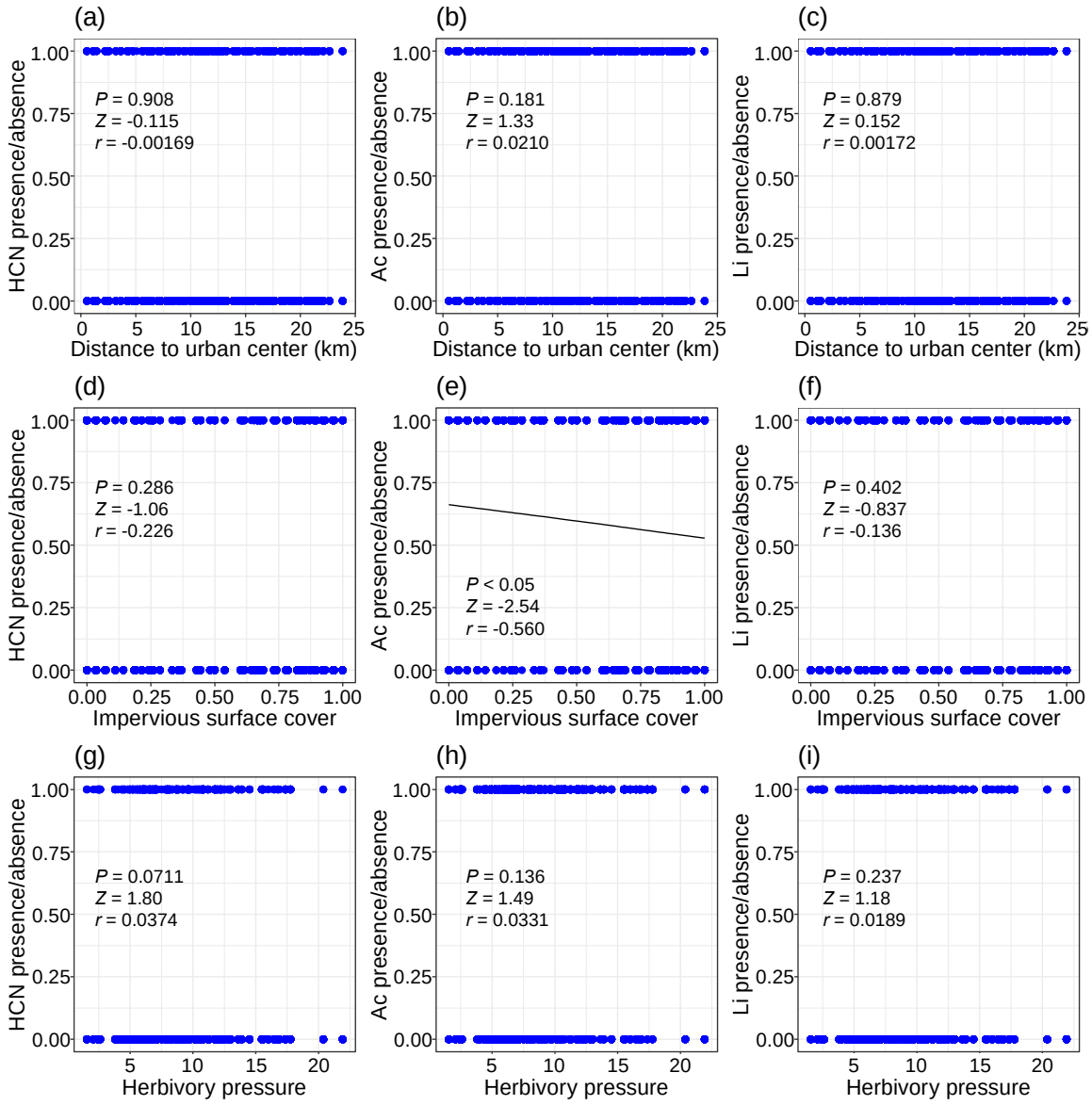
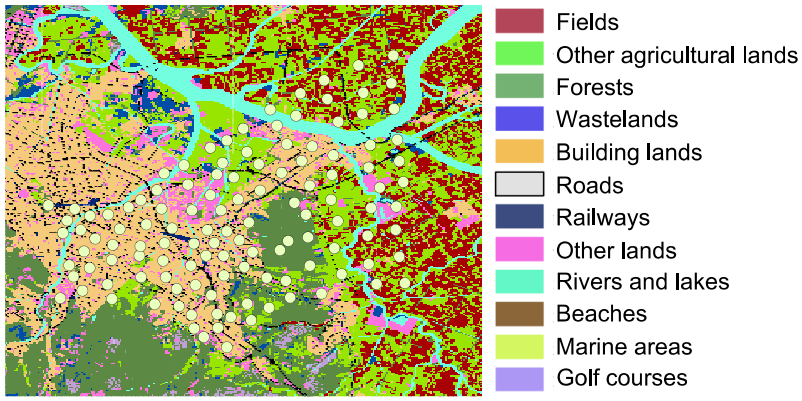


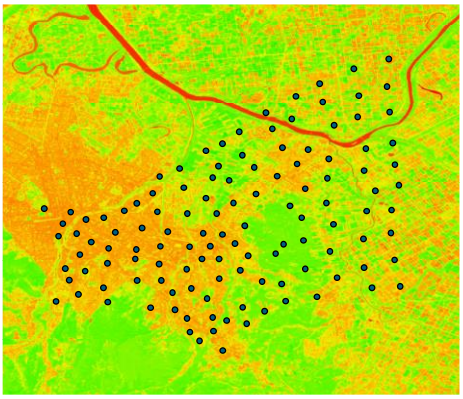
Figure S1: Relationships between the distance to the urban center and the frequencies of HCN (a), *Ac* (b), and *Li* (c), between impervious surface rate for 250 m radius from each site and the frequencies of HCN (d), *Ac* (e), and *Li* (f), and between the herbivory and the frequencies of HCN (g), *Ac* (h), and *Li* (i), respectively. *P* and *Z* values were obtained by wald test. Regression analysis with arcsine transformation was also performed. Arcsine transformation was applied to herbivory pressure, and

650 frequencies of HCN, *Ac*, and *Li*, respectively. The results of these analyses were not changed except
651 the relationship between the frequencies of HCN and the herbivory ($P = 0.0457$, $r^2 = 0.0328$).

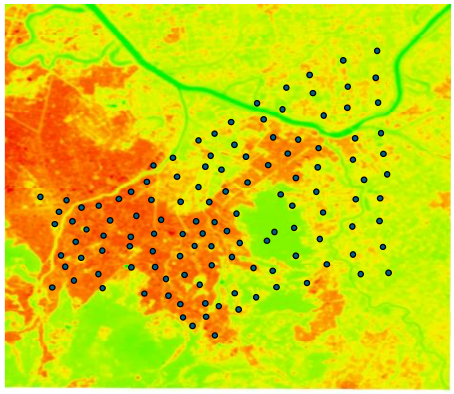
(a)


Fields
Other agricultural lands
Forests
Wastelands
Building lands
Roads
Railways
Other lands
Rivers and lakes
Beaches
Marine areas
Golf courses

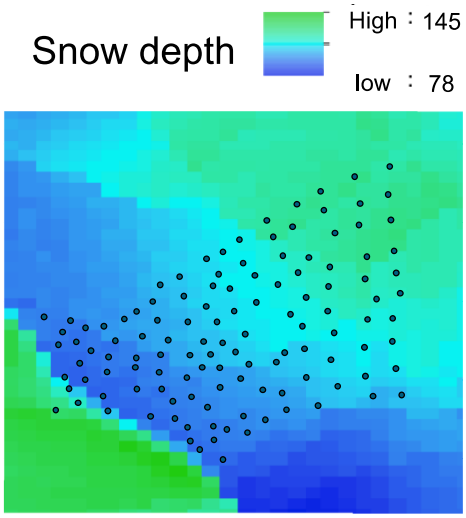
(b)


NDVI
High : 0.667833
low : -0.268892

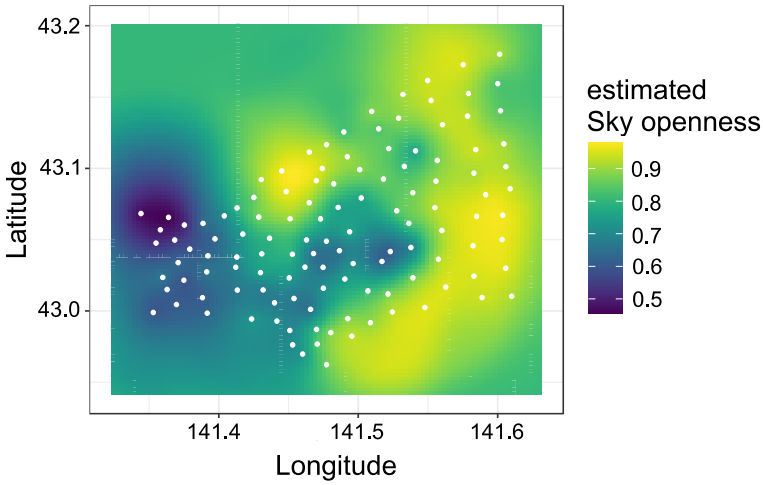
(c)


LST
High : 46.414
low : 6.52235

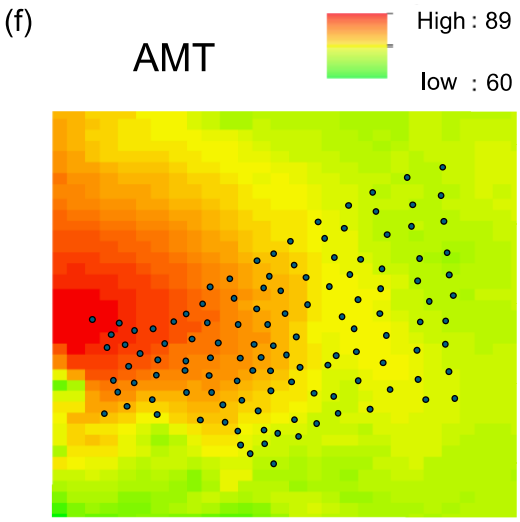
(d)


Snow depth
High : 145
low : 78

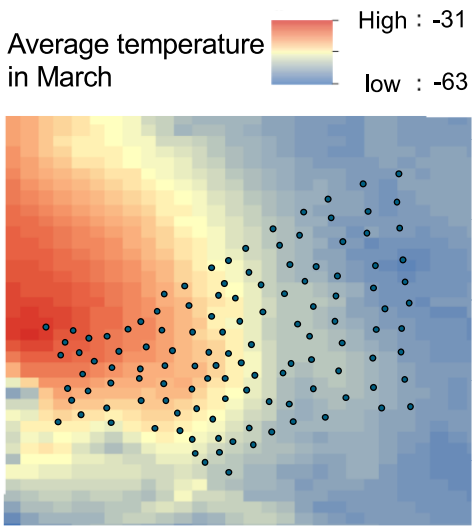
(e)


43.2
43.1
43.0
Latitude
141.4
141.5
141.6
Longitude
estimated
Sky openness
0.9
0.8
0.7
0.6
0.5

(f)


AMT
High : 89
low : 60

(g)


Average temperature
in March
High : -31
low : -63

(h)

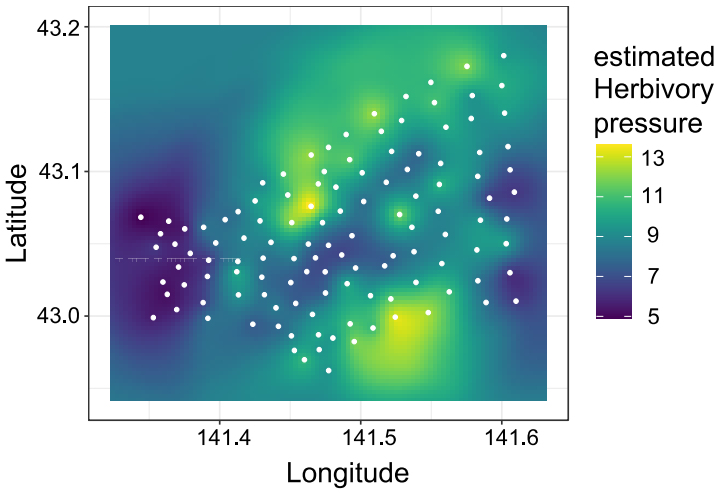

43.2
43.1
43.0
Latitude
141.4
141.5
141.6
Longitude
estimated
Herbivory
pressure
13
11
9
7
5

Figure S2: Heat maps showing spatial changes in environments from urban areas to rural areas. (a) Land use in the vicinity of Sapporo. Impervious surface cover included Building lands, Roads, and Railways. Sky openness prediction model (e) and herbivory pressure prediction model (h). White or black points denote sites of sampling.

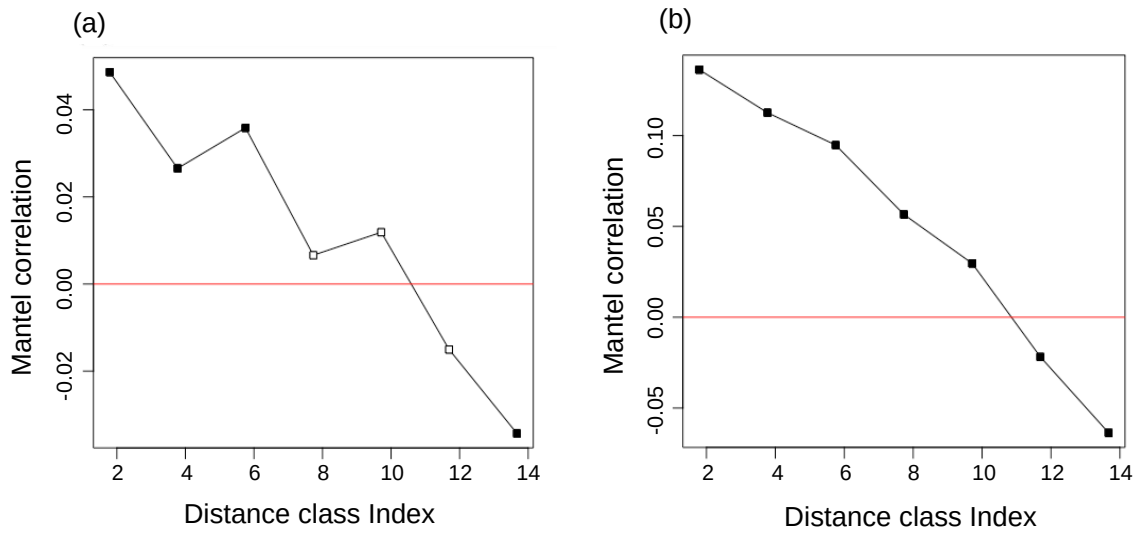


Figure S3: Mantel correlograms for the predicted frequencies of (a) HCN and (b) *Ac*. A correlogram is a graph in which spatial correlation values are plotted as the geographic distance classes among all study sites. X-axis shows between-site distance classes (km) and Y-axis indicates Mantel correlation coefficients for distance classes. Black squares represent that coefficients were significantly different from zero at the 0.05 level after Holm multiple comparison adjustment. The general decay trends of coefficients with distance classes were found for both correlograms, indicating spatial correlations in HCN and *Ac*. However, the changes in the coefficients in HCN are not monotonic and the coefficients are not significantly different from zero in the middle distance classes, whereas the coefficients in *Ac* show a clear, monotonic decay with statistical significance. In addition, overall Mantel correlation coefficient between the predicted HCN frequency and geographical distance are much lower than that between the predicted *Ac* frequency and geographical distance (HCN; $r = 0.09133$, $P < 0.01$, *Ac*; $r = 0.3158$, $P < 0.001$). Thus, spatial distribution patterns of HCN and *Ac* frequencies are significantly different.

Supplementary Text

Supplementary text S1: HCN assay

During sampling, each sample was initially placed in a zip lock, and stored in a cooler with ice. Before we conducted HCN analysis, samples had been individually transferred to microcentrifuge tubes, which were stored at -80.

The Feigl-Anger assay (Feigl and Anger 1966) was performed to determine the frequency of white clover cyanogenesis and its associated loci (*Ac/ac*, *Li/li*) in each population. For this assay, I used 48 wells, half of the 96-well microplate to reduce contamination and false positives in the detection of HCN following the methods of Thompson et al. (Thompson et al. 2016). One whole leaf was transferred to each well for each individual. If only small whole leaves were available, two whole leaves were used. Each well was filled with 80 μL of distilled water. Using a pipette tip, crush and soften the tissue in the wells until they were sufficiently macerated. Then, the plates were covered with Feigl-Anger test papers made by myself following the protocols of Gleadow et al. (Gleadow et al. 2011), incubated at 37°C for 3 hours. The reactions were recorded to determine the presence (blue colour) or absence (uncoloured) of HCN. After the Feigl-Anger assays, I further did a modified Feigl-Anger assay to determine the presence or absence of each locus related to HCN. In short, a total of 100 μl of 10 mM hydrolytic enzyme linamarase (FUJIFILM Wako Pure Chemical Corporation) solution (30ul) and 70ul of ion exchange water was used to determine the presence or absence of the *Ac* locus. A total of 80ul of 10mM cyanogenic glycoside linamarin (Toronto Research Chemicals) solution (30ul) and 50ul ion exchange water was used to determine the presence or absence of the *Li* locus.

700 **References**

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702 spot-test reagent for hydrogen cyanide and cyanogen. - Analyst 91: 282–284.

703 Gleadow, R. et al. 2011. Cyanogenic glycosides. - Research methods in plant sciences 1: 283–310.

704 Thompson, K. A. et al. 2016. Urbanization drives the evolution of parallel clines in plant populations.
705 - Proc. Biol. Sci. in press.