Doctoral thesis

Spatial perspectives of urbanization-induced plant evolution
（都市化が駆動する植物の進化に関する空間的観点に基づいた研究）

Tomoki Ishiguro (石黒智基)

Course in Ecological Genetics, Division of Biosphere Science, Graduate School of Environment Science, Hokkaido University

北海道大学大学院環境科学院　生物科学圏専攻　生態遺伝学コース

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Chapter 1. General introduction

In the Anthropocene, the impact of urbanization on biodiversity conservation has emerged as a critical issue (McDonnell and Hahs 2015; McDonald et al. 2020). Urbanization, the process of expansion of cities, is a dominant driver of significant environmental alternations, including increased temperature due to heat island effects, more impervious surface cover, habitat fragmentation, and higher levels of pollution (Grimm et al. 2008; McDonnell and Hahs 2015; Johnson and Munshi-South 2017; Rivkin et al. 2019; Diamond and Martin 2021; Miles et al. 2021). Traditionally, many studies have focused on the effects of urbanization on the abundance or species richness of lives around cities (Grimm et al. 2008; Hahs et al. 2009; Pickett et al. 2011). In recent years, evolutionary effects of urbanization have also been recognized all over the world (Johnson and Munshi-South 2017; Szulkin et al. 2020; Diamond and Martin 2021). Researchers have shown that evolutionary processes or patterns were altered in response to urban environments. There is direct evidence for allele frequency changes across space or time, as well as heritable phenotypic changes in urban environments (Barnes et al. 2011; Reid et al. 2016; Thompson et al. 2016; Winchell et al. 2016; Johnson and Munshi-South 2017; Yakub and Tiffin 2017; Johnson et al. 2018; Fukano et al. 2020, 2023; Santangelo et al. 2022a).

Brief introduction to the background of urban evolution

Impacts of urbanization on the evolutionary processes and outcomes have been studied for a few decades. This long-standing interest started from Kettlewell's study of peppered moth, which first showed that urbanization can affect selection on populations (Kettlewell 1955; Johnson and Munshi-South 2017). Originally, peppered moths were dominant with white body color which were well camouflaged on bark lichen, and individuals with black body color were extremely rare. However, after the Industrial Revolution in England, the surfaces of the trees where peppered moths perched were covered with soot due to air pollution, and thus their white body color became more apparent to their predator, birds. As a result, the number of peppered moths with black body color increased dramatically within a few decades, and by the late 19th century, black peppered moths accounted for about 99% of the populations. Air pollution from

urban industrial activity drove the evolution of peppered moth body color change through predation pressure by birds (Kettlewell 1955; Lees and Creed 1975; Cook and Saccheri 2013; Van't Hof et al. 2016). A little later, Jared Diamond (Diamond 1986) saw a great potential for evolutionary research in cities, with features such as dense buildings, streets, and artificial green spaces that differ greatly from the natural environment (Szulkin et al. 2020). He recommended that evolutionary biologists use them as prime opportunities for studying natural selection. In the 2000s, the number of studies on the effects of urbanization-induced environmental changes on the evolution of organisms increased rapidly. Now that urbanization has been known to affect not only adaptive evolution through alternation of natural selection (Kettlewell 1955; Barnes et al. 2011; Reid et al. 2016; Thompson et al. 2016; Johnson et al. 2018; Kern and Langerhans 2018; Fukano et al. 2020, 2023; Santangelo et al. 2022a,b; Merckx et al. 2024), but also non-adaptive evolution through restricted gene flow and higher genetic drift (Crissman et al. 2010; Benjamin et al. 2016; Munshi-South et al. 2016; Lourenço et al. 2017; Kern and Langerhans 2018; Miles et al. 2018; Santangelo et al. 2018) in a wide range of taxa including fish (Benjamin et al. 2016; Reid et al. 2016; Kern and Langerhans 2018), reptiles (Winchell et al. 2016; Lourenço et al. 2017), mammals (Barnes et al. 2011; Munshi-South et al. 2016), insects (Kettlewell 1955; Crissman et al. 2010; Miles et al. 2018; Merckx et al. 2024), and plants (Thompson et al. 2016; Yakub and Tiffin 2017; Johnson et al. 2018; Santangelo et al. 2018, 2022a,b; Fukano et al. 2020, 2023). Also, evidence of the universality of urbanization-induced evolution has been rapidly accumulated across continents (Johnson and Munshi-South 2017; Rivkin et al. 2019; Santangelo et al. 2022a; Fukano et al. 2023).

Unresolved issues

Despite the significant progress made in the past few decades, our understanding toward how urban environments influence evolutionary processes and outcomes is still limited. Previous studies on urban evolution have generally applied dichotomic comparisons between urban and rural areas (Barnes et al. 2011; Benjamin et al. 2016; Reid et al. 2016; Winchell et al.

2016; Lourenço et al. 2017; Yakub and Tiffin 2017; Kern and Langerhans 2018; Miles et al. 2018) or transects (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020, 2022a; Breitbart et al. 2023). Those conventional approaches have the following three major limitations.

First, conventional approaches are poorly suited to revealing the specific elements of urbanization driving evolutionary shifts. This is because while multiple environmental factors covary along urbanization gradients, they vary non-linearly and discontinuously in different manners due to the heterogeneity of urban structures such as roads, parks, and green areas (Alberti et al. 2020; Santangelo et al. 2022b; Alberti 2023). This environmental heterogeneity can lead to different selection pressures on populations found even within a single urban region. However, conventional approaches have typically treated the urban area as a homogenous environment, and thus are unlikely to adequately capture those complex spatial variations of selection pressures on populations. Also, these relationships between traits and complex spatial variations in selection pressures suggest that clines might or might not be detectable depending on where the transects are placed. Therefore, the conventional approaches might not have detected urban evolution.

Second, conventional approaches have been performed in multiple cities and suggest that patterns of urban evolution might differ among cities (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2022a). However, conventional approaches do not take into account the influence of both macro-environmental factors (e.g., climate differences) and micro-environmental variation along urban-rural gradients in determining gene frequencies. Studies showed the relationships between gene frequency and urban-rural environmental gradients (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020, 2022a,b). Also, gene frequency varies among regions with different climate conditions (Kooyers and Olsen 2012; Wright et al. 2017; Santangelo et al. 2020, 2022a). Thus, not only the environmental variation along urban-rural gradients, but regional climate patterns may also ultimately determine the likelihood of evolution. However, the relative importance of urbanization-induced environmental changes and regional-climate-associated environmental factors on determining

gene frequency has been poorly understood. Therefore, we do not have a deep understanding of the similarity and dissimilarity of urban evolution across cities.

Third, while conventional approaches can identify trends in traits from urban to rural areas, they have not been able to elucidate the mechanisms by which natural selection changes in actual urban-rural environments. In the context of urban evolution, there has been very few field experiments based on the assessment of fitness in actual urban-rural environments (see Gorton et al. 2018; Cosentino et al. 2023). Also, very few studies have compared natural selection based on the assessment of fitness under actual urban and rural environmental conditions (see Irwin et al. 2018; Palacio and Ordano 2023). Moreover, occurrences of urban evolution suggest that there are genetic variations in functional traits within local populations. However, in the context of urban evolution, no studies have investigated the mechanism by which such genetic variations are maintained. Those genetic variations can be maintained through balancing selection, which is common and one of the important mechanisms for maintaining genetic variation within populations (Hedrick et al. 1976; Hori 1993; Hedrick 2007; Takahashi et al. 2011; Takahashi and Kawata 2013; Takahashi 2015; Sato et al. 2023). In addition, Takahashi et al. (2011) reported that balancing selection, of which negative frequency-dependent selection (NFDS) is the most representative, is involved in the maintenance and formation of clines along latitudinal gradients. This notion suggests that balancing selection is also involved in clines formation along urban-rural gradients. Without NFDS, there will be no continuous cline formation (Takahashi et al. 2011). Thus, if NFDS maintain genetic variation, the urban-rural cline is a result of interactions between NFDS and urbanization-associated environmental changes, suggesting that the pattern of natural selection itself changes between urban and rural areas. Hence, the mechanism of how natural selection (balancing selection) changes from urban to rural environments should also be investigated. However, previous studies of urbanization-induced evolution have only focused on the relationship between specific environmental changes and trait changes between urban and rural areas. Overall, to my knowledge, no studies have ever investigated whether balancing selection can maintain genetic

variations in the context of urban evolution, and whether natural selection (balancing selection) differs between urban and rural environments. Therefore, previous studies should have missed important mechanisms that can drive evolution in cities. To demonstrate those mechanisms, manipulative experiments examining balancing selection (natural selection) need to be conducted in combination with multi-site reciprocal transplanting in urban and rural environments. However, no studies have ever conducted such experiments.

Goal of this thesis

The objective of this thesis is to understand the mechanism by which plants evolve to urban environments considering spatial contexts. In order to accomplish this objective, following specific questions were addressed in this study: (1) how plants evolve to complex multivariate urban environmental heterogeneity, (2) what is the relative contribution of macro-scale environmental factors such as climate and urbanization-associated environmental changes to determine gene frequency, (3) can balancing selection maintain genetic variation, and (4) does natural selection (balancing selection) vary between actual urban and rural environments? I combined field surveys and a field experiment in urban and rural areas to answer the specific questions. White clover (*Trifolium repens* L.), which is a perennial herbaceous plant native to Europa, is distributed in 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). White clover has the following characteristics as a model plant to address my questions: (1) it can grow in a wide range of soil and environmental conditions including pastures, lawns, gardens and roadsides in urban and rural environments all over the worlds (Burdon 1983; Johnson et al. 2018; Wu et al. 2021), (2) it has a Mendelian genetic polymorphism for the production of hydrogen cyanide (HCN), and the polymorphism is controlled by the epistatic interaction between two loci, *Ac* and *Li*, which show nearly perfect association with expression (Hughes 1991; Olsen et al. 2007, 2008; Olsen and Small 2018; Innes et al. 2022), and (3) it reproduces vegetatively through stolon production (Kemball and

Marshall 1995; Thompson et al. 2016), which allow us to make clonal replicates from stolon fragments in field experiments.

Organization of the thesis

This thesis includes five chapters. Chapter 1 is the general introduction to the background of this thesis. In Chapter 2, I comprehensively elucidated how plants evolve to heterogenous urban environments through field survey, focusing on the evolution of hydrogen cyanide (HCN) production and its components (presence/absence of cyanogenic glycosides and the hydrolytic enzyme linamarase) in white clover. In Chapter 3, I comprehensively explored the differences in spatial patterns of evolutionary traits of white clover between the four cities in Hokkaido. In Chapter 4, I conducted a one-year transplant experiment to test whether balancing selection contributes to maintaining polymorphism in cyanogenesis of white clovers and whether the mode of balancing selection differs between urban and rural environments. In Chapter 5, I finally synthesized and compared my findings with other examples of urban evolution.

Chapter 2. Urban spatial heterogeneity and trait multi-functionality shapes the evolution of cyanogenesis and its genes in white clover

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 (Kettlewell 1955; Barnes et al. 2011; Munshi-South et al. 2016; Reid et al. 2016; Thompson et al. 2016; Winchell et al. 2016; Johnson and Munshi-South 2017; Yakub and Tiffin 2017; Johnson et al. 2018; 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 HCN (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, I 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, 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 (Innes et al. 2022). 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; Pederson and Brink 1998; Olsen et al. 2007). 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, I 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, I 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

My focal species was white clover, a perennial plant distributed worldwide, including in both 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. I 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). During sampling, each sample was initially placed in a zip lock, and stored in a cooler with ice. Before I conducted HCN analysis, samples had been individually transferred to microcentrifuge tubes, which were stored at -80℃.

Measurement of herbivory damage

To quantitatively assess herbivory from every plant I visually scored leaf area loss following the methods of Johnson et al. (Johnson et al. 2016). First, I 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 each population. For this assay, I followed the method of Thompson et al. (Thompson et al. 2016). After the Feigl-Anger assays, I 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 Supporting information.

Acquisition of environmental data

I 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, I 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.

I 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.net) and I 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 I considered herbivory damage to acyanogenic white clovers, which do not produce HCN, as reflecting the pure herbivory pressure at each site.

Statistical analysis

All analyses were performed in R version 4.0.5. I 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.

I used principal components analysis (PCA) and GAM to examine the spatial variation of environmental factors from urban to rural habitats. I 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.

To examine the causal relationships between various environmental factors and spatial variation in the frequency of cyanogenesis, SEM accounting for spatial autocorrelation was performed, using population-level data. In the hypothesized model, the following causal paths were assumed (figure 2-1): 1) sky openness, NDVI, impervious surface cover, temperature (LST or AMT or mean temperature in March), and snow depth directly affect the spatial variation in the frequencies of *Ac* and *Li*. 2) All of those environmental factors indirectly affect the frequencies of *Ac* and *Li* through herbivory pressure. 3) Frequencies of *Ac* and *Li* affect the frequency of cyanogenesis. 4) All of the above environmental factors affect the frequency of cyanogenesis directly and indirectly through herbivore pressure. To account for spatial autocorrelation in the model, the *gls* function including the correlation argument with coordinates of populations was used in performing the piecewiseSEM package (Lefcheck 2016). Although HCN, *Ac*, and *Li* data is originally individual-level data, environmental data is population-level data. Moreover, spatial autocorrelation should be accounted for in models. However, it is not possible to estimate complex causal paths coupling individual-level and population-level variables with spatial autocorrelation altogether in a single SEM analysis. Thus, population-level frequencies of HCN, *Ac*, and *Li* were used. Arcsine transformation was applied to herbivory pressure, and frequencies of HCN, *Ac*, and *Li* to meet normality assumption in the

SEM analysis.

In SEM models, Fisher's *C* statistic and the Akaike information criterion (AIC) were used to evaluate the overall goodness-of-fit of models. The statistical significance of Fisher's *C* can be tested 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) indicates a good model fit, with a higher *P* value generally desirable (Lefcheck 2016). I also examined the effects of the range of environmental variables on the frequencies of cyanogenesis and cyanogenesis-related alleles. For these additional analyses, NDVI, impervious surface cover, and LST were analyzed using the different values of NDVI and LST at 250 m radius from each population, and impervious surface cover at 250 m radius from each population, respectively.

Moreover, to predict the spatial distribution of traits in relation to environmental factors, I applied Bayesian inference (Gelman et al. 2013). Using the environmental factors in the best model provided by the first SEM, I 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. I checked model convergence through Rhat (Gelman et al. 2013). Finally, the average predicted values were obtained, which I 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 2-2, S2-2). Sites tended to be distributed along PC1 from the urban areas to the rural areas (figure 2-2a), 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 2-3a, c, f, g; figure S2-2a, c, f, g). Snow depth increased monotonically with distance from the city center (figure 2-3d, S2-2d). In particular, NDVI, sky openness, and herbivory pressure showed non-linear changes (figure 2-3b, e, h; figure S2-2b, 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 2-4). 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 frequency of *Ac* was negatively associated with impervious surface cover. The frequency of *Li* was directly and positively correlated with the frequency of *Ac*. Herbivory pressure was positively associated with sky openness and negatively associated with impervious surface cover. Thus, I found that herbivory mediated indirect positive and negative paths from sky openness and impervious surface cover to the frequency of cyanogenesis, respectively. There was also the negative indirect path of impervious surface cover on the frequency of cyanogenesis via the frequency of *Ac*.

Spatial variation from urban to rural areas in the frequencies of cyanogenesis, Ac, and Li

The frequencies of cyanogenesis, *Ac*, and *Li* did not increase or decrease significantly with distance from the urban center (HCN: $r^2 < 0.05$, $P = 0.87$; *Ac*: $r^2 = 0.0135$, $P = 0.201$; *Li*: $r^2 < 0.05$, $P = 0.977$). I confirmed that these results of the relationship between the frequencies of cyanogenesis, *Ac*, and *Li* and distance from the urban center did not change with the use of

GLMM assuming a binomial error distribution (figure S2-1a, b, c). However, the spatial distribution of HCN and *Ac* was clearly non-random with respect to urbanization (figure 2-5). Therefore, I explored the environmental factors that predicted spatial variation in HCN and its underlying loci. The frequency of cyanogenesis was predicted to vary in a mosaic fashion from urban to rural areas (figure 2-5a). In contrast, the frequency of *Ac* was predicted to decrease with higher impervious surface cover (i.e., in urban areas) (figure 2-5b, S2-1). Thus, the predicted spatial distributions of HCN and *Ac* were partially inconsistent, although the frequency of *Ac* and HCN were significantly associated as shown in the SEM (figure 2-4, 2-5a, 2-b, 2-S3).

4. Discussion

This study highlights that the spatial distribution of an ecologically important trait and its underlying genes are shaped by environmental heterogeneity associated with urbanization. Environmental factors showed complex spatial variation along the urbanization gradient. Among environmental factors, herbivore pressure and impervious surface cover, which are related to urban structure, explained variation in the frequency of cyanogenesis in populations. Moreover, I 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 2-2, 2-3, 2-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 2-3a, 2-b, 2-S2a). The variation in sky openness is greater near urban areas (figure 2-3e) due to microstructures in the configuration of buildings and small green areas. Although AMT (Annual Mean Temperature) decreases from urban to rural areas (figure 2-3f, figure S2-2f), 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, phosphorus, and boron concentrations than natural soils. The resource availability hypothesis proposes that, plants at poorer nutrient sites, which grow more slowly, can produce the greater amount of carbon-based defense metabolites due to a surplus of carbon (Coley et al. 1985). However, cyanogenic glycosides are nitrogen-related secondary metabolites. Therefore, in nutrient-poorer areas, maintaining nitrogen-related secondary metabolites should be much more costly. However, pollution in urban such as exhaust fumes can also increase nitrogen deposition, providing nutrients to plants (Raupp et al. 2010), and white clover has nitrogen-fixing symbionts (Dazzo and Brill 1978). In addition, Thompson et al. (2016) found that the addition of nutrients did not alter selection on HCN. Therefore, nutrient availability was

unlikely to explain spatial variation in *Ac*.

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

Third, Thompson et al. (2016) suggested that reduced snow depth in urban areas compared 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, my 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 (Johnson and Munshi-South 2017; Fenoglio et al. 2020). Muratet and 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 abundance 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 my study, I found a

large amount of variation in herbivory pressure even at the same distance from urban center across study sites (figure 2-3h). 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 2-5), and this notion is consistent with a study of Santangelo et al. (Santangelo et al. 2020). However, herbivory pressure is also an important selective force for cyanogenesis (figure 2-4). 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 2-5, figure S2-3). I 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 2-5a) 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. My 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 my study site, this notion is unlikely to explain the pattern because there were many large and dense patches in urban areas and many small patches in a crop field in rural areas. Moreover, Santangelo et al. (2018) also showed that while clines in HCN can evolve by drift, clines at individual alleles (i.e., *Ac* and *Li*) cannot consistently evolve due to drift. Future

molecular works will be necessary to confirm this expectation. This study also illustrates that genetic hierarchy and multifunctionality of polymorphic traits, and the multiple layers of urbanization-induced environmental changes have a critical role for shaping spatial variation in genotypic frequencies. In conclusion, the urban-rural landscape approach, which considers the complexity of multivariate environmental heterogeneity, provides great insights toward an understanding of the complex evolutionary processes in urban environments. The approach used in this study can be applied to future research to advance our understanding of the evolution of organisms in urban environments.

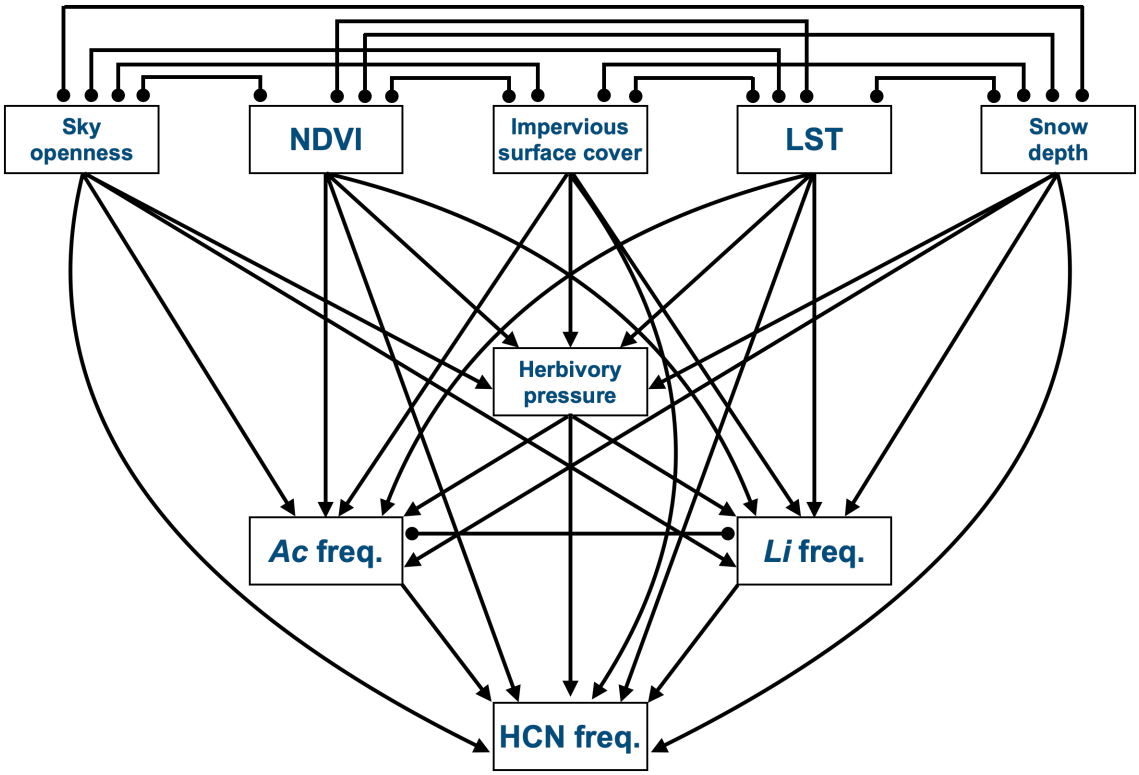


Figure 2-1. The hypothetical structural equation model of urbanization-driven environmental changes and evolutionary changes in the frequency of *Ac, Li,* and HCN. Lines with circular ends show correlations.

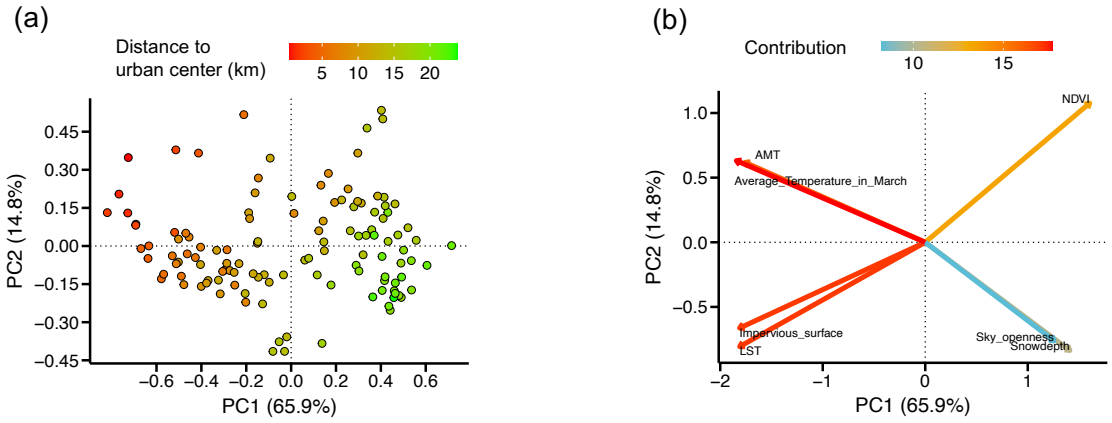


Figure 2-2. Visualization of spatial variation in environmental factors across the urban and rural gradient. (a) Environmental differences among sites, colored according to the distance from the city center. (b) The eigenvectors for environmental variables, colored according to their contribution to 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.

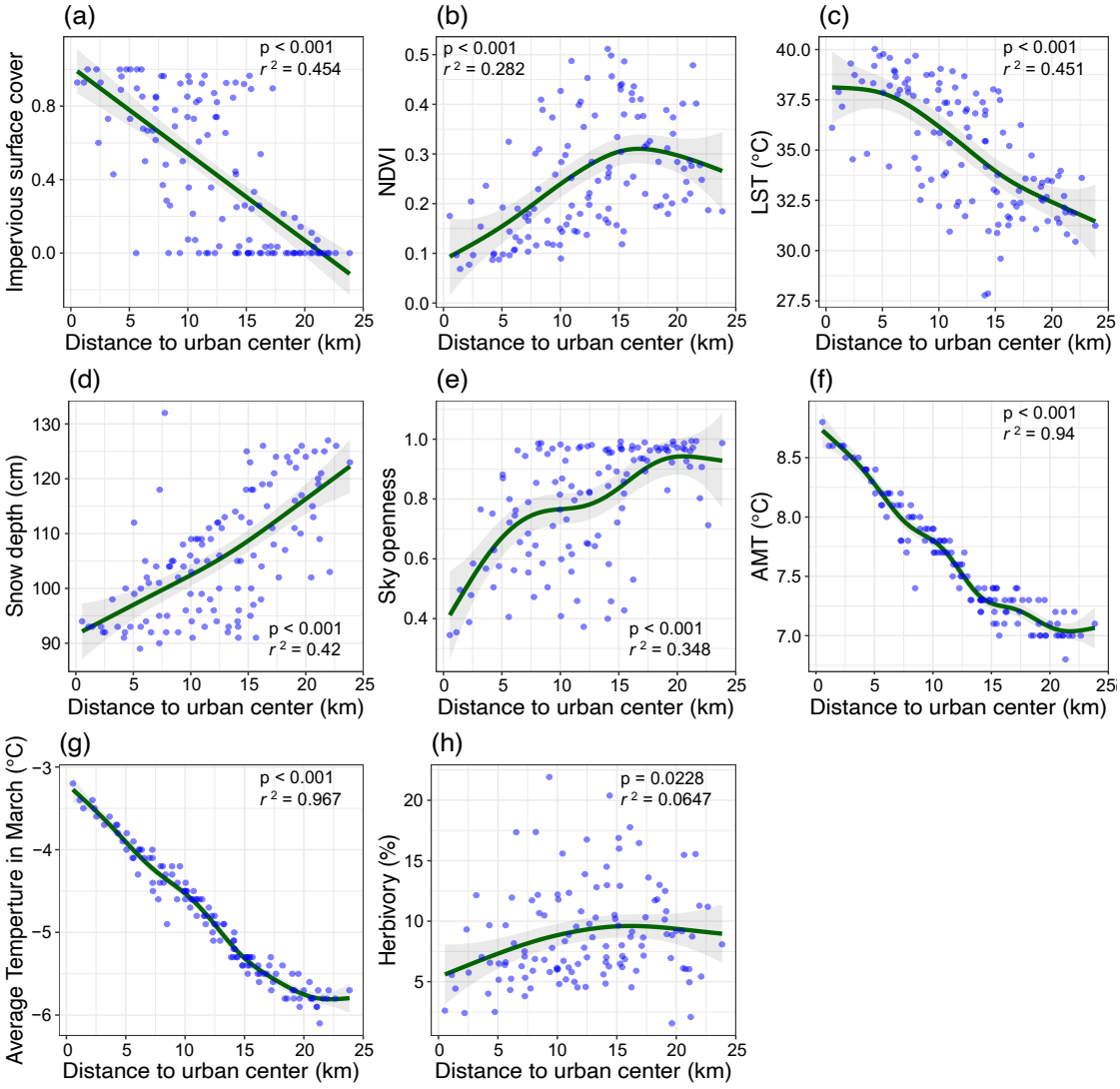


Figure 2-3. Relationships between the distance to the urban center and environmental variables. Different spatial ranges for 250 m radius (a, b, c) and point estimates of the center of each site (d, e, f, g, h). *P* values were obtained by a GAM. NDVI, LST, and AMT represent Normalized Difference Vegetation Index, land surface temperature, and annual mean temperature, respectively.

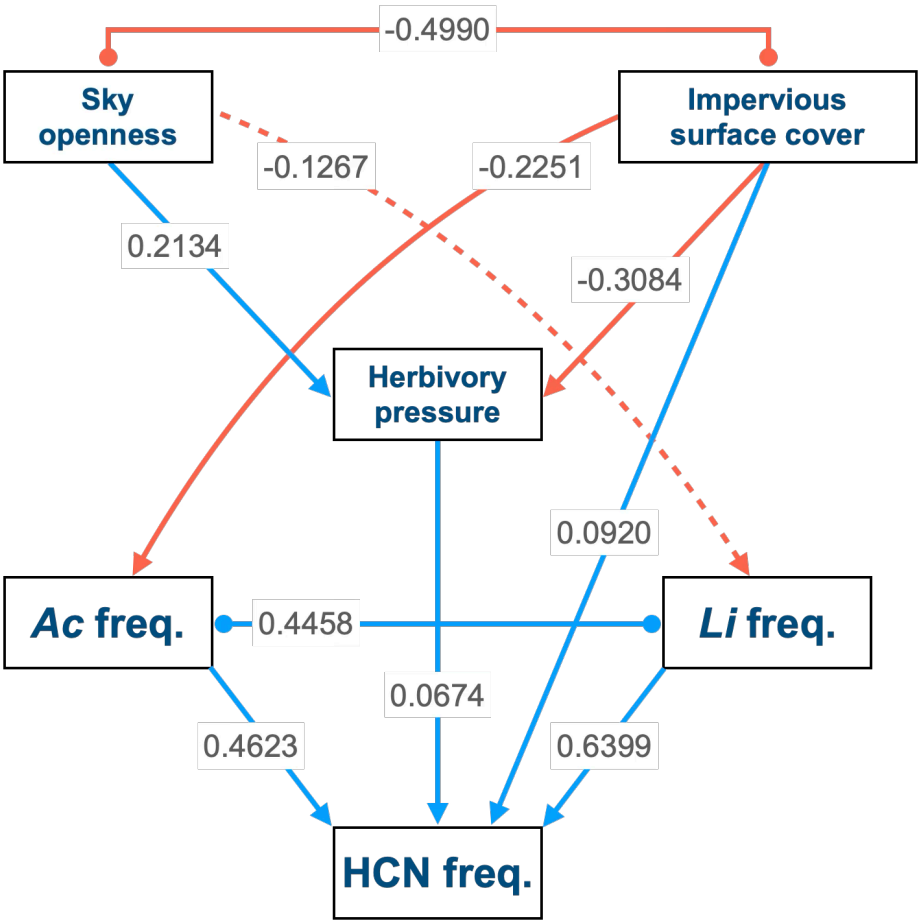
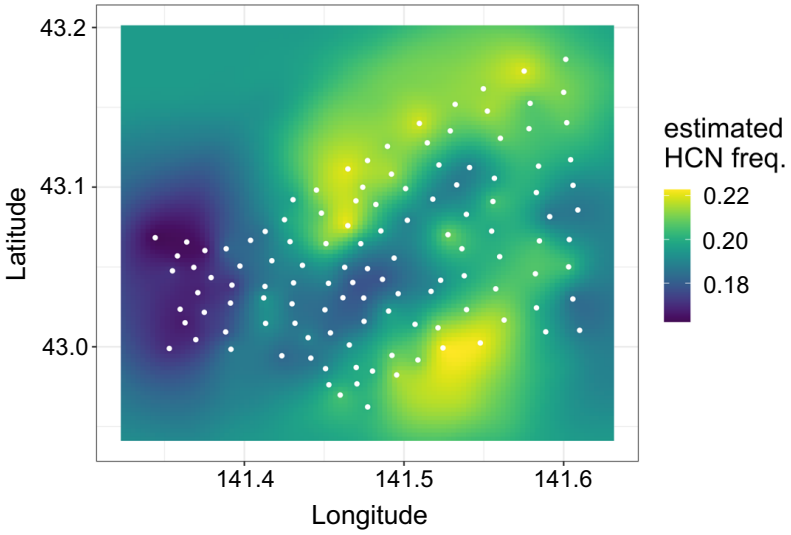
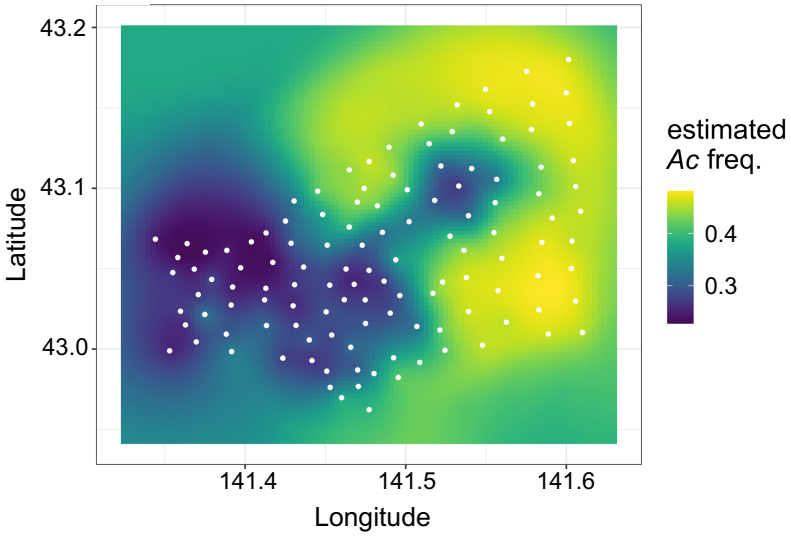


Figure 2-4. The best model of the structural equation model of urbanization-driven environmental changes and the frequency of *Ac, Li,* and HCN. Blue and red arrows show positive and negative effects, respectively. Bold arrows show the paths with significant coefficients ($P < 0.05$) and dashed arrows show non-significance ($P > 0.05$). Lines with circular ends show correlations. Standardized path coefficients are shown.

(a)


43.2
43.1
43.0
Latitude
141.4
141.5
141.6
Longitude
estimated
HCN freq.
0.22
0.20
0.18

(b)


43.2
43.1
43.0
Latitude
141.4
141.5
141.6
Longitude
estimated
Ac freq.
0.4
0.3

(c)

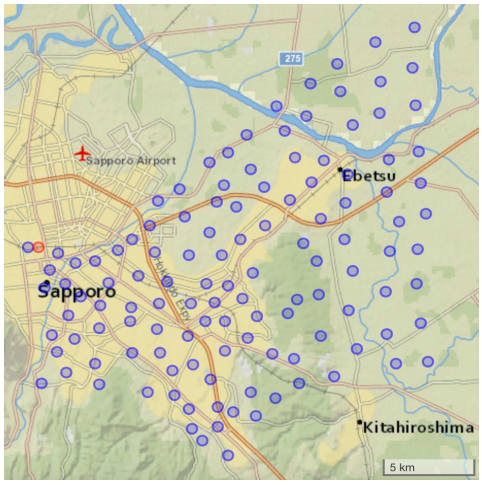

275
Sapporo Airport
Ebetsu
Sapporo
Kitahiroshima
5 km

Figure 2-5. HCN frequency prediction model (a) and *Ac* frequency prediction model (b). Simplified topographic map of Sapporo and its suburb and rural areas. I 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

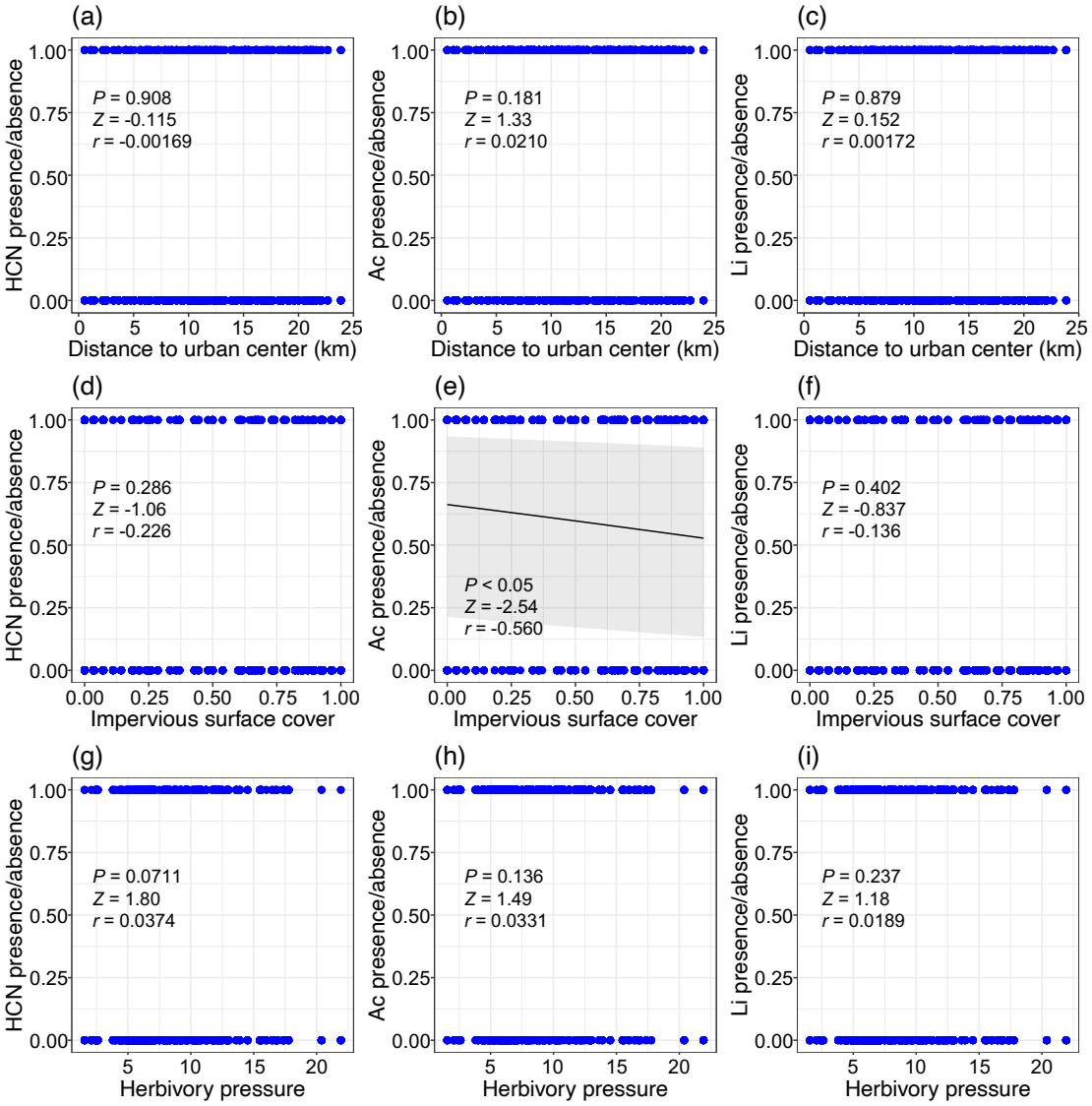
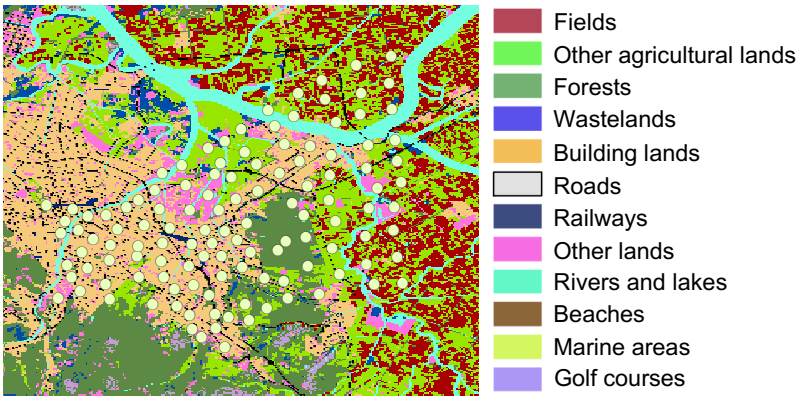
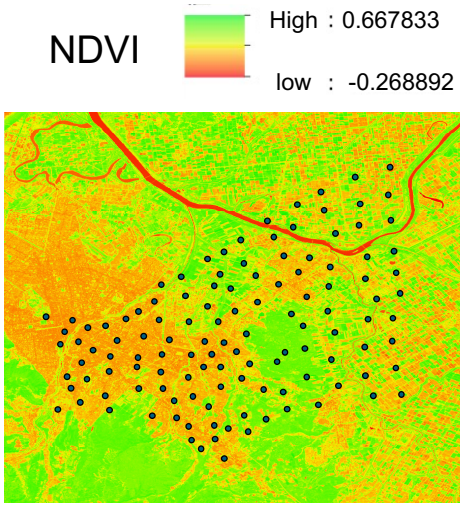


Figure S2-1: Relationships between the distance to the urban center and the frequencies of HCN (a), *Ac* (b), and *Li* (c), between impervious surface rate for 250 m radius from each site and the frequencies of HCN (d), *Ac* (e), and *Li* (f), and between the herbivory and the frequencies of HCN (g), *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 frequencies of HCN, *Ac*, and *Li*, respectively. The results of these analyses were not changed except the relationship between the frequencies of HCN and the herbivory ($P = 0.0457$, $r^2 = 0.0328$).

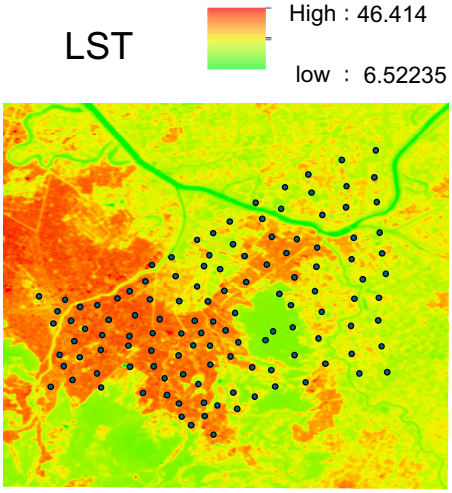
(a)


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

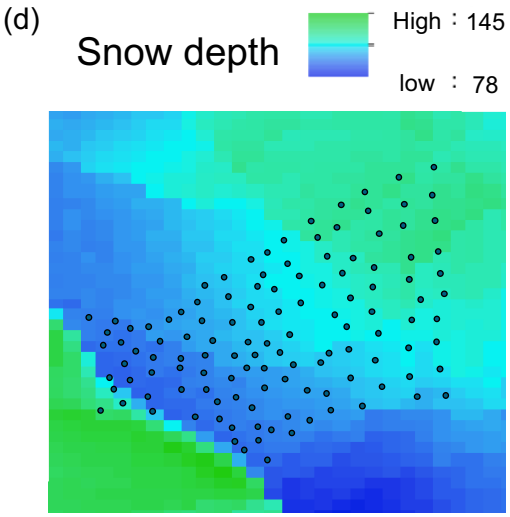
(b)


NDVI
High : 0.667833
low : -0.268892

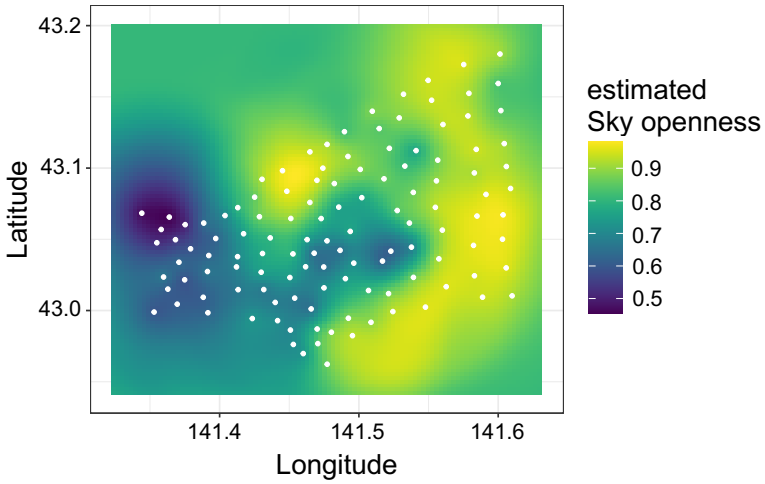
(c)


LST
High : 46.414
low : 6.52235

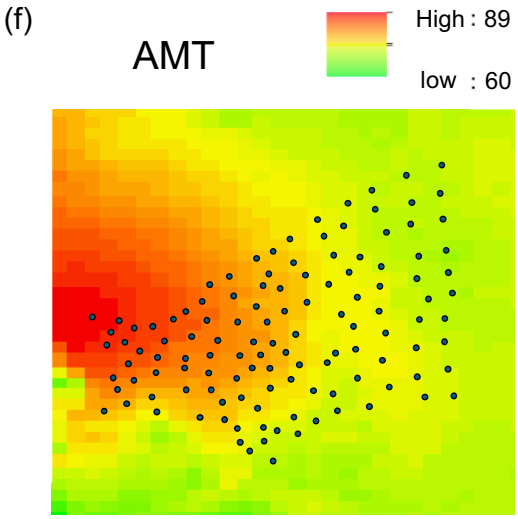
(d)


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

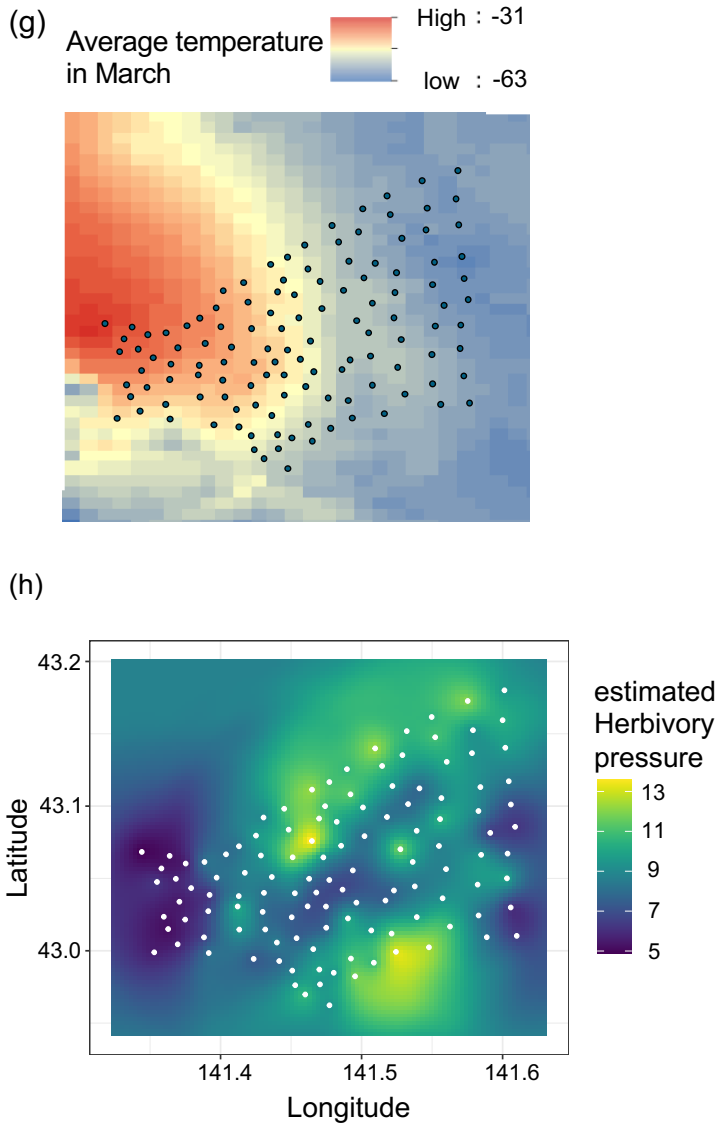


Figure S2-2: Heat maps showing spatial changes in environments from urban areas to rural areas. (a) Land use in the vicinity of Sapporo. Impervious surface cover included Building lands, Roads, and Railways. Sky openness prediction model (e) and herbivory pressure prediction model (h). White or black points denote sites of sampling.

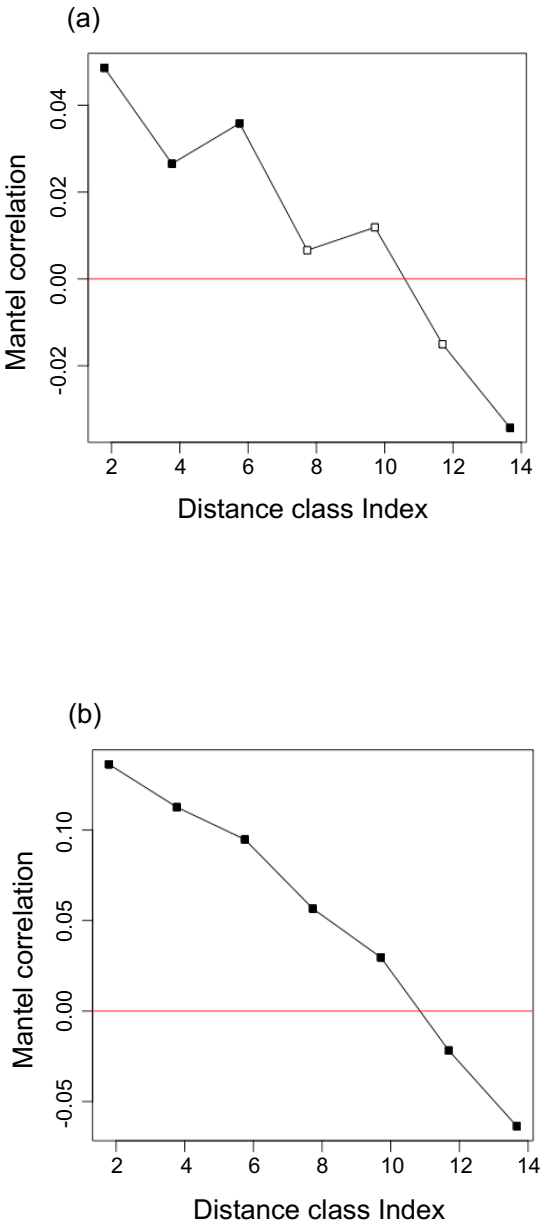


Figure S2-3: Mantel correlograms for the predicted frequencies of (a) HCN and (b) *Ac*. A correlogram is a graph in which spatial correlation values are plotted as the geographic distance classes among all study sites. X-axis shows between-site distance classes (km) and Y-axis indicates Mantel correlation 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 I 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. (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. (2011), incubated at 37℃ for 3 hours. The reactions were recorded to determine the presence (blue color) or absence (uncolored) 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 (30 μl) and 70 μl of ion-exchanged water was used to determine the presence or absence of the *Ac* locus. A total of 80 μl of 10 mM cyanogenic glycoside linamarin (Toronto Research Chemicals) solution (30 μl) and 50 μl ion-exchanged water was used to determine the presence or absence of the *Li* locus.

Chapter 3: Relative effects of environmental variations in climates and urban-rural gradients on determining different gene frequency among cities

1. Introduction

Urbanization is progressing all over the world and causing environmental changes such as the heat island effect, pollution, and habitat fragmentation, leading to a significant impact on biodiversity and the ecology of living organisms (Grimm et al. 2008; McDonnell and Hahs 2015; Rivkin et al. 2019; McDonald et al. 2020). In recent years, it has also been reported that environmental changes associated with urbanization also impact the evolution of organisms in various taxonomic groups all over the world, indicating universality in urban evolution (Johnson and Munshi-South 2017; Rivkin et al. 2019; Santangelo et al. 2022a; Fukano et al. 2023). On the other hand, it has been suggested that patterns of urban evolution might differ among cities. Previous studies investigating similarities and dissimilarities among cities have performed qualitative comparisons based on a dichotomous approach comparing traits between urban and rural areas (Winchell et al. 2016; Yakub and Tiffin 2017) and a transect approach comparing the slope of gene frequency with distance from the urban centers among cities (Thompson et al. 2016; Santangelo et al. 2020, 2022a). However, these approaches have two problems: 1) ignoring the influence of micro-urban structure (Chapter 2; Ishiguro et al. 2024) and 2) do not take into account the influence of both macro-environmental factors (e.g., climate differences) and environmental variation along urban-rural gradients in determining gene frequencies. Hence, the similarities of urban evolution across cities remain poorly understood.

While the similarities of urban evolution across cities have been still unclear, a few studies compared multiple cities to reveal general and specific patterns of urban evolution. For example, Thompson et al. (2016) detected evolutionary clines in three of the four cities studied. These urban areas in the three cities were more exposed to cold winter air than rural areas because of less snow cover. The authors discussed that this weather condition could have led to the formation of a cline in cyanogenesis of white clover. In contrast, the city where clines were not detected had higher snow depths in both urban and rural areas. Thus, regional climates may influence the likelihood of urban evolution (Thompson et al. 2016). On the other hand, other studies have reported that gene frequency largely varies among different climate regions

(Kooyers and Olsen 2012; Wright et al. 2017; Santangelo et al. 2020, 2022a). However, how those regional climates and urban-rural environments determine urban evolution has been poorly understood. In particular, 1) the relative evolutionary impacts of macro-scale environmental differences such as climate and micro-scale urbanization-induced environmental changes, and 2) how those macro- and micro-scale environmental changes collectively determine gene frequencies remain unclear.

Moreover, the absence of observed clines does not necessarily mean that urban evolution does not occur. In Chapter 2, my landscape approach showed that environmental changes due to urbanization (e.g., impervious surface rate and sky openness) led to urban organism's evolution, while there were no clear clines of genetic frequency along the distance from urban to rural areas. It should also be noted that the detection of clines can depend on transect positions due to complex environmental heterogeneity from urban to rural areas (also see Thompson et al. 2016; Santangelo et al. 2022b). Therefore, in order to elucidate similarities and dissimilarities in evolutionary patterns among cities and climates, it is necessary to compare the spatial distribution patterns of evolutionary traits at a broad landscape scale with a mechanistic modeling approach rather than cline-based comparisons in multiple cities. In other words, the landscape approach implemented in Chapter 2 should be applied to multiple cities.

In this study, I aim to examine the similarity and dissimilarity of urban evolution among four cities in Hokkaido with different climatic conditions, focusing on the cyanogenesis of white clover. For this purpose, I adopted a landscape approach, which can comprehensively investigate spatial variation in environmental factors and the frequency of cyanogenesis from urban to rural areas. In particular, I address the following aspects: 1) the differences in environmental spatial variation among cities; 2) spatial distribution of gene frequencies: elucidating relationships between the distance from the urban centers and spatial structure of gene frequency; 3) SEM modeling considering latitude and longitude to quantitatively evaluate the effects of large-scale environmental differences and micro-scale urban-rural environmental variations on gene frequency.

2. Materials & Methods

Study system

I used white clover (*Trifolium repens* L., Fabaceae) as a model to address my research purposes. It is a perennial herbaceous plant that reproduces vegetatively through stolons and sexually through self-incompatible dense inflorescences with clusters of flowers (Burdon 1983; Kakes 1997; Johnson et al. 2018; Santangelo et al. 2019). It is native to Eurasia and introduced globally to temperate environments (Daday 1958; Burdon 1983; Johnson et al. 2018). It is typically found in pastures, lawns, and roadsides in cities all over the world (Burdon 1983; Johnson et al. 2018; Wu et al. 2021), providing us with an ideal system to compare evolutionary patterns in response to urbanization.

White clover exhibits a Mendelian-inherited polymorphism for the production of hydrogen cyanide (HCN) (Hughes 1991). HCN can serve as a potent chemical antiherbivore defense, specifically effective against generalists such as mollusks, insects, and voles (Dirzo and Harper 1982; Poulton 1990; Pederson and Brink 1998; Olsen et al. 2007, 2014). Cyanogenesis is often polymorphic in many populations; cyanogenic genotypes produce HCN following tissue damage, whereas HCN is absent from acyanogenic genotypes (Hughes 1991). The polymorphism in cyanogenesis of white clover is caused 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 locus *Ac*, composed of a closely linked three-gene cluster, is required for the production of the cyanogenic glycosides (linamarin and lotaustralin), and the locus *Li* encodes the hydrolyzing enzyme, linamarase (Olsen and Small 2018, Innes et al. 2022). Dominant alleles at both loci are required to produce HCN (Olsen et al. 2014). Therefore, only *AcLi* genotype can produce HCN (i.e., cyanogenic), whereas the other three genotypes (*Acli*, *acLi*, and *acli*) cannot (i.e., acyanogenic). As described in Chapter 2, there is also accumulating evidence that the cyanogenic glycosides may have ecological costs by contributing to drought stress tolerance (Kooyers et al. 2014) and reducing freezing tolerance

(Kooyers et al. 2018). Therefore, environmental factors may select independently each of the two individual loci as well as cyanogenesis itself.

The island of Hokkaido is located in the northernmost in the Japanese archipelago within the cool-temperate climate zones (Igarashi et al. 2011). The lowland of Hokkaido is dominated by agricultural land such as cultivated land area and farmland, and interspersed with cities and towns. I selected 4 distanced cities (Sapporo, Hakodate, Kushiro, Asahikawa) (figure 3-1a), which are considered to incorporate both climatic variation (e.g., snow fall and temperature) and variance in urban structure that can influence the evolution of plants (Thompson et al. 2016; Alberti et al. 2020; Ishiguro et al. 2024). Thus, comparing the fine-scale distribution of evolutionary traits such as cyanogenesis, *Ac*, and *Li* among the cities in different climate conditions will capture the mechanisms by which make difference in evolutionary patterns in the cities.

Measurement of herbivory damage

Following the method of Johnson et al. (2016), I measured herbivory damage by visually quantifying the proportion of leaf area consumed. Initially, I selected the three largest, fully expanded leaves from each individual. Subsequently, I assessed the extent of herbivory damage using a six-ranking system: 0) no herbivory damage, 1) 1–10%, 2) 11–25%, 3) 26–50%, 4) 51–75%, and 5) 76–100% damage. The median value within each rank's range was employed to calculate the herbivory. Then, these values were averaged over the three leaves from each individual plant (Johnson et al. 2016). Visual estimates of herbivory in this system yield results consistent with those obtained from imaging software (https://imagej.net) and are sufficiently precise to the quantification of herbivory (Chapter 2; Ishiguro et al. 2024).

Sampling and Hydrogen cyanide, cyanogenic glycosides, linamarase assays

I conducted sampling at Sapporo in 2020 (Chapter 2; Ishiguro et al. 2024), and at the other three cities in 2022. I sampled 122, 40, 31, and 41 sites (hereafter ‘populations’), covering

the urban and rural areas around Sapporo, Hakodate, Kushiro, and Asahikawa, respectively (figure 3-1). In total, I sampled 234 populations including roadsides, parks, green spaces, cemeteries, footpaths, roadside ditches, and residential lawns. I collected on average 24 (range 15-36) individuals per population for a total of 5589 plants, and obtained GPS coordinates for each population. To avoid sampling the same genet, plant samples were collected ensuring at least 2 m between adjacent plants (Thompson et al. 2016).

Each sampled white clover plant was assayed using the Feigl–Anger assays (Feigl and Anger 1966) following the methods of Thompson et al. (2016) to determine the frequency of plants producing HCN within populations. For white clover plants that tested HCN-absent, I then used modified Feigl–Anger assays to determine the presence of *Ac* or *Li*, using different leaves of the same individuals. Detailed methods for the assays are provided in Chapter 2. Feigl-Anger assays yield consistent results with PCR assays for the presence/absence of *Ac* and *Li* (Johnson et al. 2018). Thus, this method was sufficiently accurate for screening cyanogenesis and its associated genes.

Acquisition of environmental data

I collected environmental data at each site to quantify environmental differences in each city as potential explanatory factors for the differences in evolutionary patterns of cyanogenesis and related genes among cities. I obtained environmental data for Sapporo from Ishiguro et al. (2024). For the other three cities, I obtained environmental data following the procedure below. For normalized difference vegetation index (NDVI) (Pettorelli et al. 2005), Landsat-8 (https://doi.org/10.5066/P975CC9B) satellite images taken in the most recent 5-years image available were acquired. NDVI is an indicator of the amount of green vegetation present. Likewise, for land surface temperature (hereafter 'LST'), satellite images were taken from Landsat-8 in the most recent 5-year image available (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 NDVI and LST were used in analyses.

Annual mean temperature (hereafter ‘AMT’), the monthly daytime mean temperature in March, and annual maximum snow depth in average (hereafter ‘snow depth’) were obtained from the Ministry of Land, Infrastructure, Transport and Tourism (https://nlftp.mlit.go.jp/ksj/index.html). These estimates were calculated as the standard value for each 1-km mesh (tertiary mesh‐the minimum distance available) based on the observed values for the past 30 years.

I determined the percentage of impervious surface within a 250 m radius of each population through the land use data obtained from the Ministry of Land, Infrastructure, Transport and Tourism (https://nlftp.mlit.go.jp/ksj/index.html). The impervious surface cover indicates the percentage of artificial ground surfaces such as buildings, concrete, and asphalt. Therefore, it serves as a major indicator of urbanization.

I also measured sky openness as the ratio of the percentage of the sky visible at each site. To obtain these data, I used a fisheye lens and followed the methods of Ishiguro et al. (2024) (Chapter 2). The sky openness is an indicator of how much sunlight white clover receives. I used the rate of herbivory damage to acyanogenic white clover (hereafter ‘herbivory pressure’) as an indicator of herbivory pressure because I considered herbivory damage to acyanogenic white clovers, which do not produce HCN, reflecting the pure herbivory pressure at each population.

Finally, I calculated the standardized distance between each population and the urban center since the sampling areas varied among the cities. I standardized the length of each city’s distance between each population and urban center to a minimum value of 0 (city center) and a maximum of 1 (furthest rural population).

Statistical analyses

I used generalized linear mixed models (GLMM) with a binomial distribution to examine spatial variation patterns in the frequency of cyanogenesis versus standardized distance

across the cities. I then used principal components analysis (PCA) to examine the spatial variation of environmental factors from urban to rural habitats in the four cities.

To examine the causal relationships between various environmental factors and spatial variation in the frequency of cyanogenesis, SEM accounting for spatial autocorrelation was performed, using population-level data. In the hypothesized model, the following causal paths were assumed (figure 3-2): 1) sky openness, NDVI, impervious surface cover, temperature (LST or AMT or mean temperature in March), snow depth, longitude, and latitude directly affect the spatial variation in the frequencies of *Ac* and *Li*. 2) All of those environmental factors indirectly affect the frequencies of *Ac* and *Li* through herbivory pressure. 3) Frequencies of *Ac* and *Li* affect the frequency of cyanogenesis. 4) All of the above environmental factors affect the frequency of cyanogenesis directly and indirectly through herbivore pressure. Due to the too much-assumed causal relationships, it is not possible to make a model in which longitude and latitude directly affect the gene frequencies and indirectly affect the gene frequencies through all other environmental factors and herbivory. Therefore, the causal relationships between longitude (or latitude) and other environmental data were assumed through the correlation between them. To account for spatial autocorrelation in the model, the lme function including the correlation argument with coordinates of populations was used in performing the 'piecewiseSEM' package (Lefcheck 2016). The city ID was incorporated as a random effect in order not to treat multiple populations in a city as independent samplings. Although HCN, *Ac* and *Li* data is originally individual-level data, environmental data is population-level data. Moreover, spatial autocorrelation should be accounted for in models. However, it is not possible to estimate complex causal paths coupling individual-level and population-level variables with spatial autocorrelation altogether in a single SEM analysis. Thus, population-level frequencies of HCN, *Ac* and *Li* were used. Arcsine transformation was applied to herbivory pressure, and frequencies of HCN, *Ac* and *Li* to meet normality assumption in the SEM analysis.

In SEM models, Fisher's *C* statistic and the Akaike information criterion (AIC) were used to evaluate the overall goodness-of-fit of models. The statistical significance of Fisher's *C*

can be tested 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) indicates a good model fit, with a higher P value generally desirable (Lefcheck 2016). I also examined the effects of the range of environmental variables on the frequencies of cyanogenesis and the cyanogenesis-related alleles. For these additional analyses, NDVI, impervious surface cover, and LST were analyzed using the different values of NDVI and LST at 250 m radius from each population, and impervious surface cover at 250 m radius from each population, respectively.

Moreover, to illustrate the spatial distribution of traits in relation to environmental factors, I applied Bayesian inference (Gelman et al. 2013). Using the data of cyanogenesis frequency acquired through field survey, I built a spatial random field model to explore the fine-scale distribution of cyanogenesis frequency while accounting for spatial autocorrelation. Finally, I created a spatial distribution map using the Kriging method, for the frequency of the cyanogenesis and the cyanogenesis-related locus.

All analyses were performed in R ver. 4.3.2 (www.r-project.org). I used the packages 'piecewiseSEM' (Lefcheck 2016) and 'nlme' (Pinheiro et al. 2021) for structural equation modeling (SEM). Kriging was performed using the packages of 'gstat', 'sp' and 'maptools'.

3. Results

Environmental changes in multiple cities

Sites tended to be distributed along PC2 from the urban areas to the rural areas (figure 3-3a), with higher impervious surface cover and AMT values in urban areas, and higher values of NDVI and sky openness in rural areas (contribution: PC1 = 43.3%, PC2 = 30.4%). However, other environmental factors such as mean temperature in March and snow depth tended to vary more along PC1 than PC2. Therefore, the environmental factors varying along PC2 consistently vary along an urban gradient in the four cities, while environmental factors varying along PC1 do not consistently vary in the four cities (figure 3-3b).

Effects of urban environmental changes on gene frequencies across multiple cities

The best model included longitude, impervious surface cover, AMT, latitude, herbivory pressure, the frequency of *Ac*, and the frequency of *Li* (AIC = 63.287, Fisher's C = 9.287, *P* value for Fisher's *C* = 0.979, figure 3-4). The frequency of cyanogenesis within populations directly increased with the frequency of *Ac*, the frequency of *Li*, AMT, and latitude. Herbivory pressure marginally increased the frequency of cyanogenesis (Standardized coefficient = 0.0452, *P* = 0.0646). The frequency of *Ac* was negatively associated with longitude, impervious surface cover, and latitude. The frequency of *Li* was negatively associated with latitude. Also, the frequency of *Li* was directly and positively correlated with the frequency of *Ac*. Herbivory pressure was negatively associated with longitude and impervious surface cover. Longitude and latitude were negatively associated with AMT. Also, in the PCA analyses, AMT and impervious surface cover vary with the distance from the urban centers, suggesting urbanization-induced environmental changes affected gene frequency (figure 3-3, 3-4). Although the coefficients of macro-scale environmental differences (longitude and latitude) are larger, the coefficients of micro-scale urbanization-induced environmental variations (impervious surface cover and AMT) are of a similar order. Overall, the best model showed both micro-scale urbanization-induced environmental changes and macro-scale environmental differences affected the spatial distribution of evolutionary traits across the cities.

Spatial variation from urban to rural areas in the frequencies of cyanogenesis and Ac across cities

I did not find significant spatial changes in the frequency of cyanogenesis with distance from the urban center across the cities, except for Asahikawa (Sapporo: r = -0.0405; Z = -0.115; P = 0.908; Hakodate: r = -0.147; Z = -0.325; P = 0.745; Kushiro: r = -0.0478; Z = -0.071; P = 0.943; Asahikawa: r = 1.26; Z = 2.04; $P < 0.05$). In Asahikawa, the frequency of cyanogenesis increased with distance from the urban center. Moreover, I did not find significant spatial changes in the frequency of *Ac* with distance from the urban center across the cities (Sapporo: r

= 0.500; Z = 1.33; p = 0.181; Hakodate: r = 0.402; Z = 0.551; P = 0.582; Kushiro: r = 0.379; Z = 0.686; P = 0.492; Asahikawa: r = 1.01; Z = 1.95; P = 0.0507) (figure 3-5). Then, I explored and compared the spatial variation in HCN in the four cities. Although three of the four cities showed a mosaic-like distribution of cyanogenesis from urban to rural areas, there was a common trend with lower HCN frequency in urban areas and higher HCN frequency in rural areas (figure 3-6). The same trend was observed in *Ac* frequency, with lower *Ac* frequency in urban areas and higher *Ac* frequency in rural areas (figure 3-7).

4. Discussion

This study highlighted the similarity of urban evolution mechanisms across different cities. Environmental factors such as impervious surface cover and sky openness consistently have common spatial patterns along urban-rural gradients, but environmental factors such as mean temperature in March and snow depth varied widely among cities. I found that both micro-scale urbanization-induced environmental changes and macro-scale environmental differences determine the gene frequency of each population.

Environmental changes from urban to rural areas in cities

It is necessary to consider the common and different patterns in environmental changes due to urbanization among cities to understand the difference in urban evolutionary patterns. This study found which environmental factors generally covaried with urbanization across different cities. Impervious surface cover, AMT, NDVI, and sky openness were commonly associated with urban-rural changes. In contrast, others (i.e., mean temperature in March, snow depth, and LST) were largely varied with geographic variations among cities rather than urban-rural changes. The mean temperature in March, snow depth, and LST are likely to be highly related to winter environmental conditions. In other words, differences in impervious surface cover, AMT, NDVI, and sky openness were larger in urban-rural ranges than those among the focal four cities. The differences in winter climate conditions were likely to be largely regulated

by geographic locations rather than urban-rural ranges. Although many factors covaried with urban-rural landscapes, this result indicates which factors most contributed to urban-rural environmental variation.

Relative effects of environmental change due to urbanization and climate on herbivore pressure, cyanogenesis, and its genes

My SEM model highlighted the consistent environmental effects on gene frequency across the cities. Even with the macro-scale environmental differences of latitude and longitude included, pathways were detected from impervious surface cover and AMT, which are components of urbanization, to herbivory, *Ac,* and HCN. This means that there are characteristic and consistent urbanization-associated environmental changes that are driving urban evolution in parallel across cities. In addition, the macro-scale environmental differences in latitude and longitude were also associated with gene frequency and herbivory. Relatively, the coefficients of macro-scale environmental differences were slightly larger than urbanization-induced environmental changes. These results suggest that macro-scale environments primarily affect gene frequency, and secondly micro-scale urban-rural environmental variation shapes gene frequency, driving urban evolution across different cities. To my knowledge, no other study has shown the relative effects of macro-scale environment and urban-rural environmental variations on evolution.

The best model indicates abiotic factors in urbanization gradients and longitude influenced biotic herbivore pressure, which marginally increased cyanogenic frequency. The negative association of impervious surface cover with herbivory pressure was consistent with the results of Chapter 2 (figure 2-4). Thus, the reasons described in Chapter 2: 1) The number of herbivores may decrease with urbanization; 2) high impervious surface cover in the surrounding area may inhibit the colonization of herbivores, can explain the negative association of impervious surface cover with herbivory. In contrast, the negative association of longitude with herbivory pressure may be due to the climatic shift with longitude. Several studies reported that

abundance and diversity of plants and animals including insects decrease at higher latitudes (Gaston 2000; Kozlov et al. 2013; Gao et al. 2018). Higher latitudes tend to have lower temperatures and precipitation. These climatic conditions can be harsh for many organisms, limiting the survival and reproduction of species. In Hokkaido Island, longitude is likely to represent the climatic change more than latitude, at least among my study regions, and the number of herbivores is expected to decrease in the eastern part of Hokkaido due to lower temperature and precipitation.

This study also showed relationships between multiple environmental factors along the urbanization gradients on cyanogenesis and its associated genes. The negative association of impervious surface cover with *Ac* allele was consistent with the results of Chapter 2 (figure 2-4). As discussed in Chapter 2, the following two reasons could explain that impervious surface cover reduces *Ac* frequency: 1) higher drought stress in rural areas (decreased impervious surface cover) can favor *Ac* allele with drought tolerance; 2) higher freezing due to reduced snow cover in urban areas can impose selection on *Ac*, with which white clovers are less likely to survive freezing than cyanotypes not producing cyanogenic glycosides. In addition, the negative association of longitude with *Ac* can also be explained by the reduced freezing tolerance of *Ac* (Kooyers et al. 2018). In eastern Hokkaido, especially in Kushiro, snow cover is extremely low and thus plants are directly exposed to cold winter air. Therefore, white clovers with non-*Ac* allele can be preferred. Although latitude was positively associated with HCN frequency, latitude was negatively associated with the frequency of *Ac* and *Li*, indirectly had negative association with HCN frequency. The coefficients of the indirect effects from latitude to HCN frequency through *Ac* and *Li* are larger than the direct effects from latitude to HCN frequency. Thus, HCN frequency could decrease with higher latitude, and this notion is consistent with previous studies (Kooyers and Olsen 2012; Santangelo et al. 2020).

The spatial distribution of evolutionary traits across cities

This study highlighted that comparing the spatial distribution patterns of evolutionary

traits at a broad landscape scale with a mechanistic modeling approach is much more effective in elucidating similarities and dissimilarities in evolutionary patterns among cities and climates rather than traditional cline-based comparisons in multiple cities. My results showed that even with the complex spatial distribution of evolutionary traits across cities, the landscape approach applied to multiple cities successfully revealed the consistent environmental effects on gene frequency across the cities, while the conventional cline-based approach did not detect the relationship between urbanization and spatial distribution of evolutionary traits (figure 3-4, 3-5). Thus, I emphasize that my landscape approach is much more beneficial for urban evolution research than conventional approaches.

This study also demonstrates that differences in both macro-scale climate and micro-scale urban structure among cities may be responsible for differences in urban evolutionary patterns. Through applying the landscape approach and structural equation modeling to multiple cities, I have identified the environmental factors that most contribute to environmental changes along urbanization gradients among various environmental factors that are also related to macro-scale regional environmental differences. Moreover, I clarified that those environmental factors, relating to both macro-scale regional and micro-scale urban-rural environmental variation, can commonly drive urban evolution in multiple cities. Furthermore, I identified the relative effects of macro-scale and micro-scale environmental variations on determining gene frequency within populations. While macro-scale factors are essential to determine gene frequency, urban-rural environmental variation also contributes in a similar order of effect size. Future research should conduct more landscape approaches in multiple cities with different climate conditions.

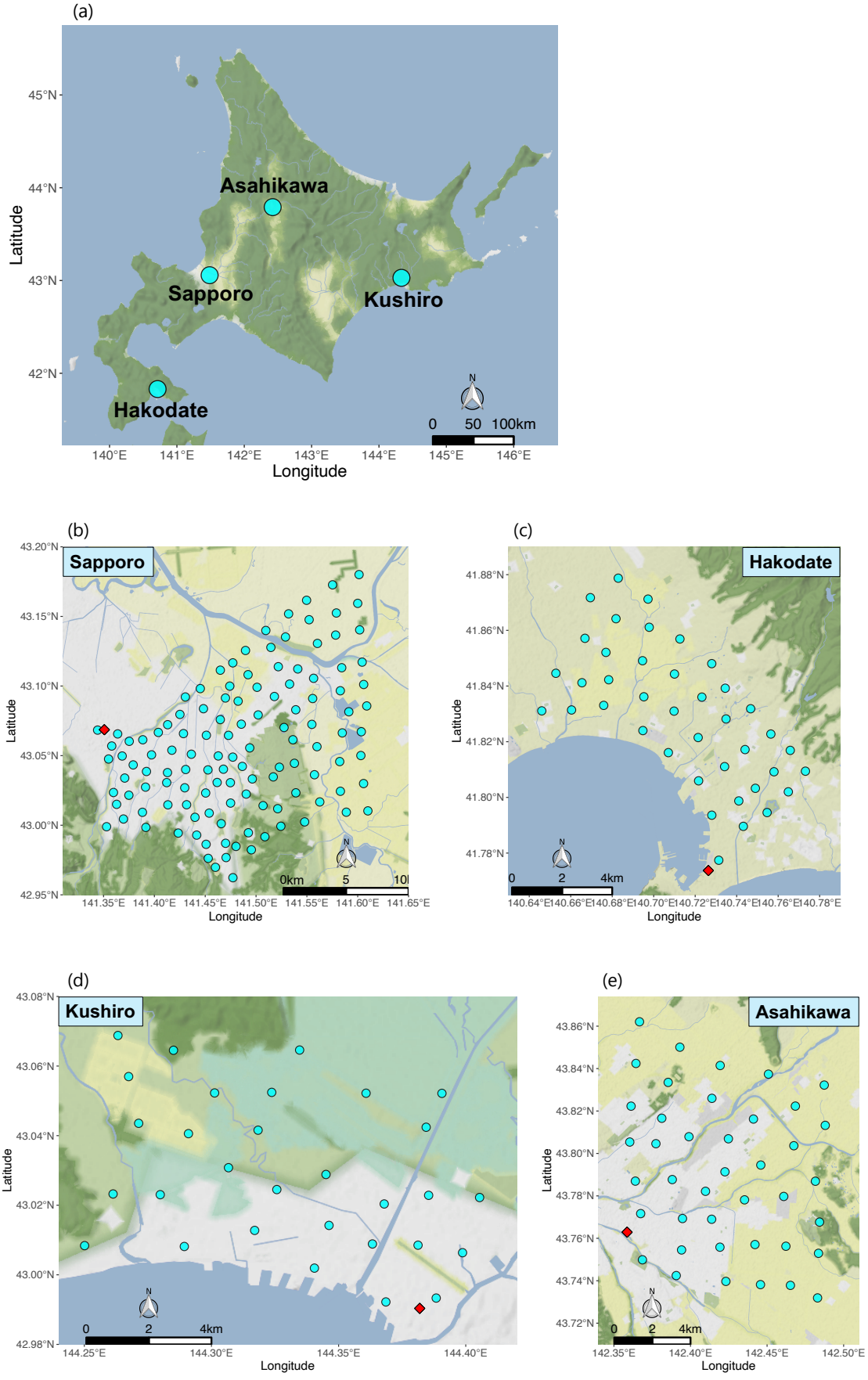

(a)
Asahikawa
Sapporo
Kushiro
Hakodate
45°N
44°N
43°N
42°N
Latitude
140°E
141°E
142°E
143°E
144°E
145°E
146°E
Longitude
0 50 100km
(b)
Sapporo
43.20°N
43.15°N
43.10°N
43.05°N
43.00°N
42.95°N
Latitude
141.35°E
141.40°E
141.45°E
141.50°E
141.55°E
141.60°E
141.65°E
Longitude
0km 5
(c)
Hakodate
41.88°N
41.86°N
41.84°N
41.82°N
41.80°N
41.78°N
Latitude
140.64°E
140.66°E
140.68°E
140.70°E
140.72°E
140.74°E
140.76°E
140.78°E
Longitude
0 2 4km
(d)
Kushiro
43.08°N
43.06°N
43.04°N
43.02°N
43.00°N
42.98°N
Latitude
144.25°E
144.30°E
144.35°E
144.40°E
Longitude
0 2 4km
(e)
Asahikawa
43.86°N
43.84°N
43.82°N
43.80°N
43.78°N
43.76°N
43.74°N
43.72°N
Latitude
142.35°E
142.40°E
142.45°E
142.50°E
Longitude
0 2 4km

Figure 3-1. Maps of the four cities in Hokkaido (a). Simplified topographic map of Sapporo (b), Hakodate (c), Kushiro (d), and Asahikawa (e), and their suburb and rural areas. I defined the urban centers as the Sapporo Station (43°04'07.1"N, 141°21'02.9"E), Hakodate station (41°46’25.4"N, 140°43'34.9"E), Kushiro station (42°59'25.3"N, 144°22'54.8"E), and Asahikawa station (43°45'46.4"N, 142°21'31"E) of Sapporo, Hakodate, Kushiro, and Asahikawa, respectively. The urban centers are shown as red points. Cyan points denote sampling sites.

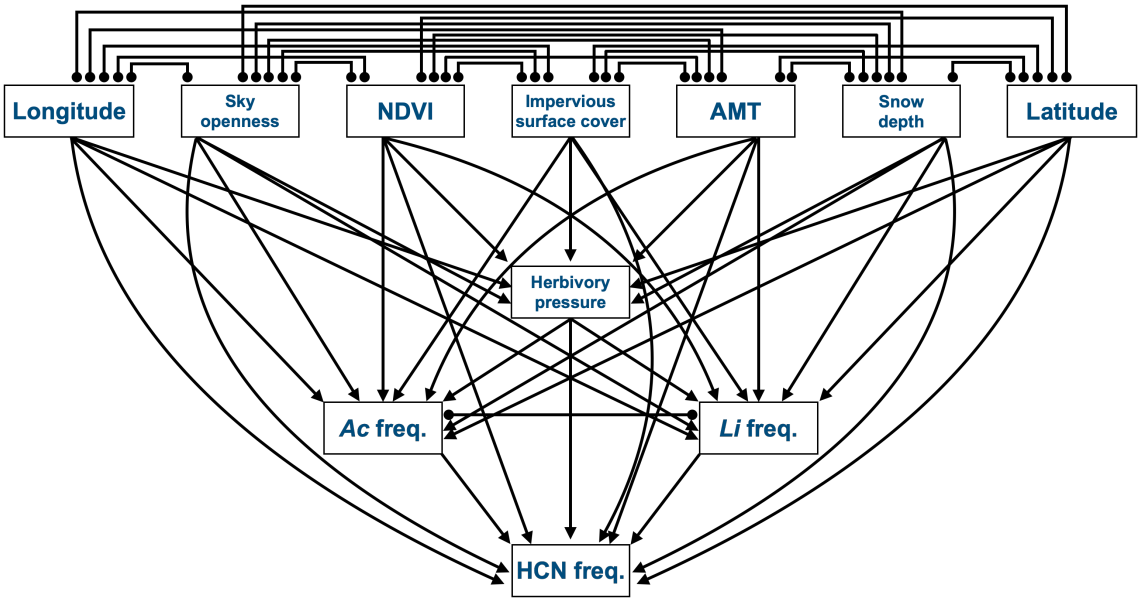


Figure 3-2. The hypothetical structural equation model of micro-scale urbanization-driven environmental changes, micro-scale climate-associated environmental changes, and evolutionary changes in the frequency of *Ac, Li,* and HCN. Lines with circular ends show correlations.

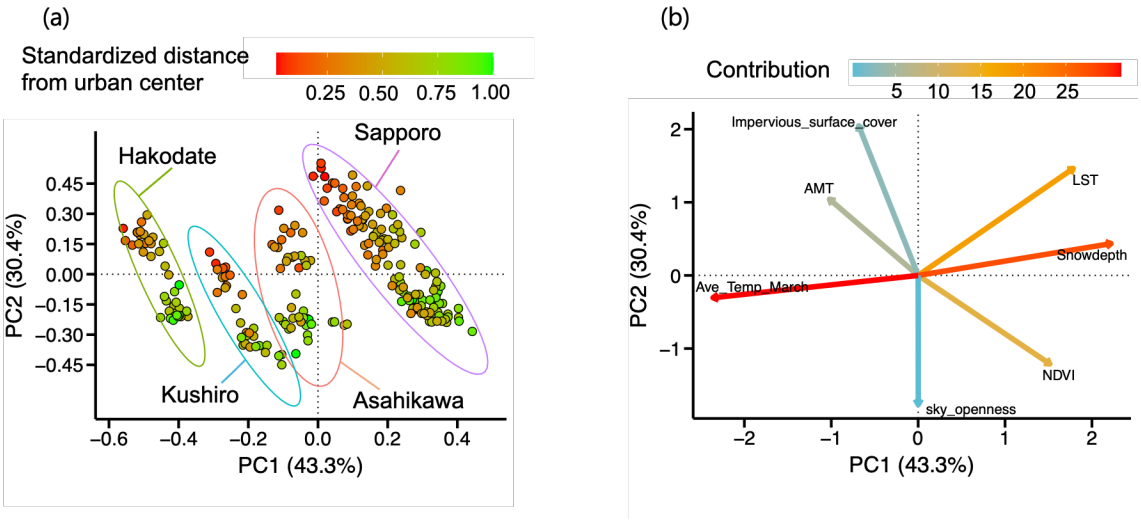


Figure 3-3. Visualization of spatial variation in environmental factors across the urban and rural gradients across the four cities. (a) Environmental differences among sites, colored according to the distance from the city center. (b) The eigenvectors for environmental variables, colored according to 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.

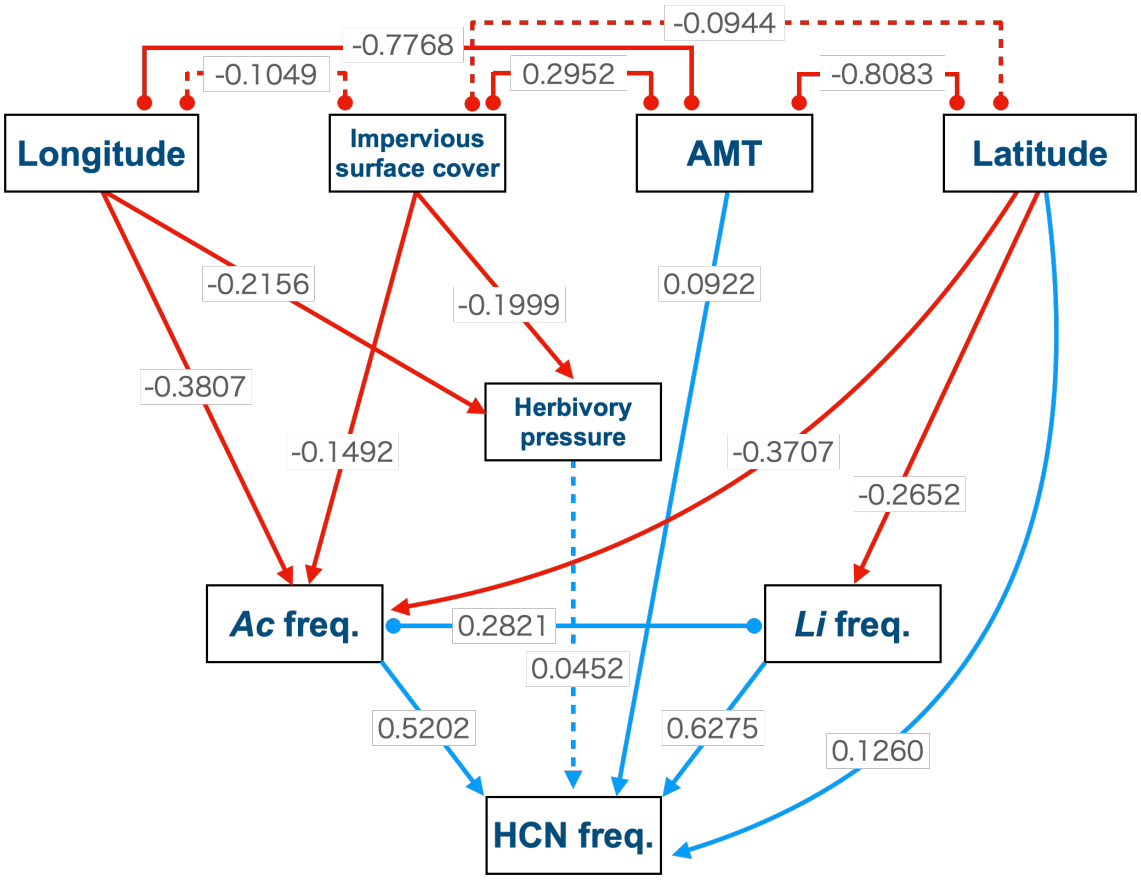


Figure 3-4. The best model of the structural equation model of micro-scale urbanization-driven environmental changes, micro-scale climate-associated environmental changes, and the frequency of *Ac, Li,* and HCN. Blue and red arrows show positive and negative effects, respectively. Bold arrows show the paths with significant coefficients ($P < 0.05$) and dashed arrows show non-significance ($P > 0.05$). Lines with circular ends show correlations. Standardized path coefficients are shown.

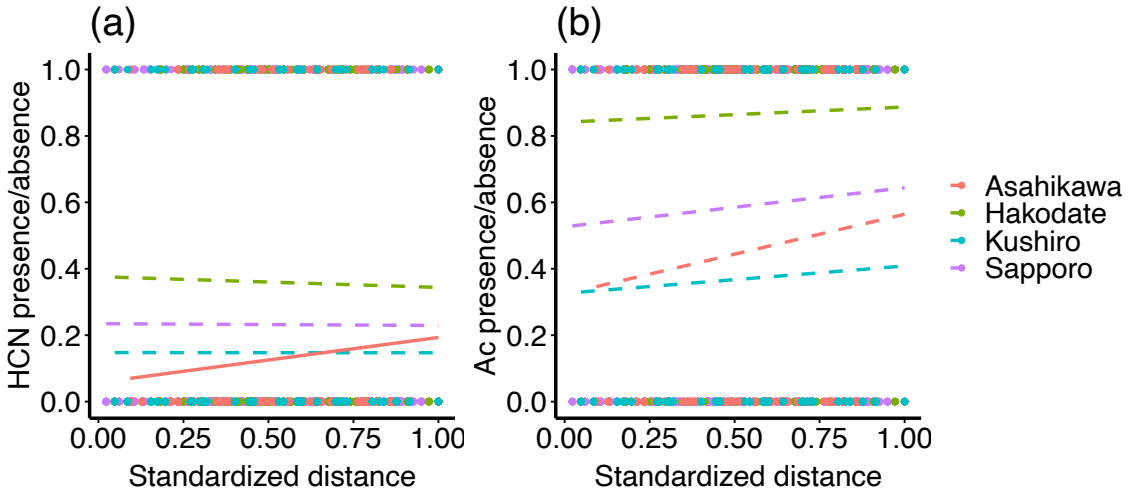


Figure 3-5. Relationships between the standardized distance to the urban centers and the frequencies of (a) HCN, and (b) *Ac* among the cities. The solid line shows a significant relationship between the standardized distance to the urban centers and the gene frequency ($P < 0.05$), and dashed lines show non-significance ($P > 0.05$).

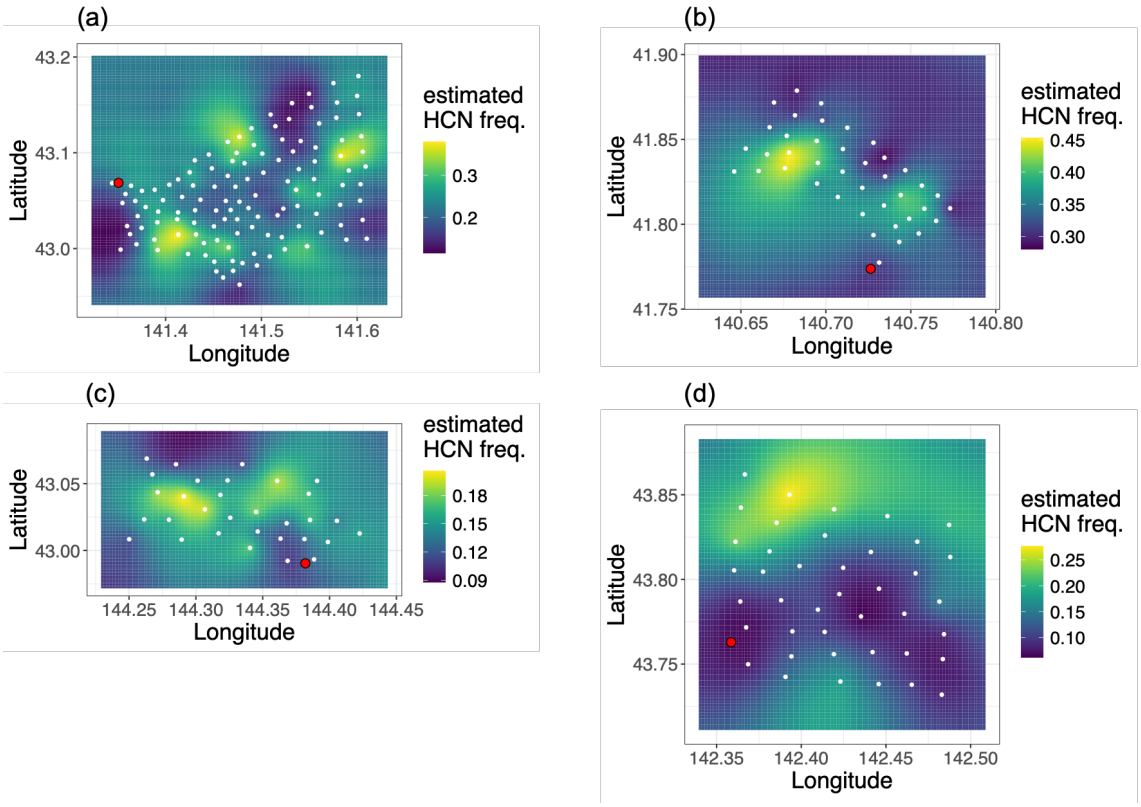


Figure 3-6: Heat maps showing spatial changes in HCN frequency from urban areas to rural areas. Spatial changes in HCN frequency in Sapporo (a), Hakodate (b), Kushiro (c), and Asahikawa (d), respectively. White points denote sampling sites and red points denote the urban centers.

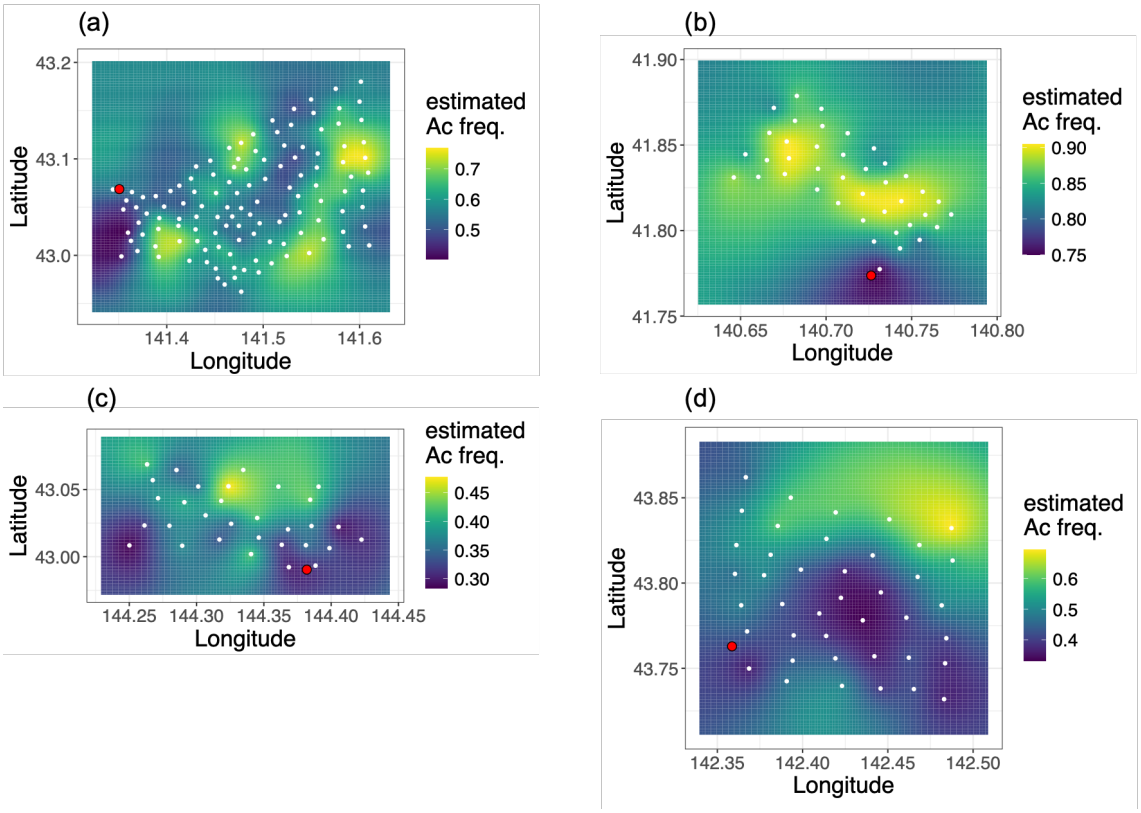


Figure 3-7: Heat maps showing spatial changes in *Ac* frequency from urban areas to rural areas. Spatial changes in *Ac* frequency in Sapporo (a), Hakodate (b), Kushiro (c), and Asahikawa (d), respectively. White points denote sampling sites and red points denote the urban centers.

Chapter 4: Urban-rural herbivory contrast mediates the mode shift of frequency-dependent selection on cyanogenesis in white clover

1. Introduction

Urban areas have been increasing worldwide with growing populations, accounting for approximately 3% of land surface area (Seto et al. 2010; Zhou et al. 2015; Johnson et al. 2018). Urbanization causes dramatic environmental changes, including the heat island effect, habitat fragmentation, and air and water pollution (McDonnell and Hahs 2015; Bai et al. 2017; Johnson and Munshi-South 2017; Rivkin et al. 2019). These urbanization-induced environmental changes have a profound impact on biodiversity and the ecology of organisms (McKinney 2006; Bai et al. 2017; McDonald et al. 2020). Recently, urbanization has also been shown to affect evolution of organisms in various taxonomic groups (McDonnell and Hahs 2015; Johnson and Munshi-South 2017; Rivkin et al. 2019).

Previous studies on the impacts of urbanization on evolution have examined whether there are continuous changes in gene frequency (i.e., clines) associated with environmental gradients from urban to rural areas (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020, 2022a; Breitbart et al. 2023). For example, white clover (*Trifolium repens* L.) has genetic polymorphisms in cyanogenesis, in which cyanogenic plants can produce the chemical substance hydrogen cyanide (HCN) and acyanogenic plants cannot (Daday 1958; Hughes 1991; Olsen and Small 2018). Santangelo et al. (2022a) reported that the cyanogenic plant frequency within a population (HCN frequency) of white clovers formed clines, increasing with the distance from urban centers around the world. These results suggest that urban environments select for acyanogenic and rural environments select for cyanogenic plants (also see Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020). Occurrences of these urban evolutions mean that genetic variation in functional traits has been maintained within local populations. However, to my knowledge, no studies have investigated the mechanism by which such genetic variation is maintained in the context of urban evolution.

One of the important mechanisms maintaining genetic variation is “balancing selection,” of which negative frequency-dependent selection (NFDS) is the most representative. NFDS can maintain genetic variation within populations by favoring rare alleles over common alleles and

has been demonstrated both in theory and in the fields (Ayala and Campbell 1974; Hori 1993; Hedrick 2007; Takahashi et al. 2011; Takahashi and Kawata 2013; Takahashi 2015; Sato and Kudoh 2017; Sato et al. 2023). It is well known that genetic polymorphism in cyanogenesis is maintained in a white clover population at various environments, independent of urbanization (Daday 1954, 1958, 1965; Hughes 1991; Kooyers and Olsen 2012). Although NFDS can occur in plant chemical traits (Lankau and Strauss 2007; Evans et al. 2016), it has not been revealed how the polymorphism in cyanogenesis is maintained in white clover. Possible mechanisms by which cyanogenic and acyanogenic white clover exhibit NFDS can include: 1) herbivory; 2) competition through secondary metabolites. First, HCN produced by cyanogenic plants can serve as an antiherbivore defense and be effective against herbivores such as generalist insects and animals (Poulton 1990; Olsen et al. 2007, 2014; Thompson and Johnson 2016). Because herbivores such as mollusks and Dermaptera prefer acyanogenic plants to cyanogenic plants (Dirzo and Harper 1982; Compton and Jones 1985), if cyanogenic plants are relatively rare, they may be more likely to avoid herbivory damage (but see Hughes 1991). Second, secondary metabolites may alter competitive interaction outcomes among plant individuals in a frequency-dependent manner. For example, there are genetic variations in the content of the glucosinolate, sinigrin, in *Brassica nigra* (Lankau and Strauss 2007). *Brassica nigra* with high sinigrin can dominate by excluding other plant species due to strong interspecific competition mediated by effects on soil microorganisms. When *B. nigra* is a dominant species, producing high levels of sinigrin is costly and thus makes *B. nigra* disadvantageous as to intraspecific competition, which favors low-sinigrin *B. nigra*. When low-sinigrin *B. nigra* become dominant, other plant species become established because they cannot exclude other plant species, and high-sinigrin *B. nigra*, which are stronger at interspecific competition, are favored. These results suggest that negative frequency-dependent dynamics might occur in *B. nigra* between individuals advantageous at interspecific competition and individuals advantageous at intraspecific competition with respect to sinigrin content variations.

In addition, as noted above, cyanogenesis in white clovers often forms a cline with

urbanization (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020, 2022a,b). The maintenance and formation of clines is likely to be a gradual shift in the equilibrium point of polymorphisms (the point at which the fitness of each phenotype is equal). However, for polymorphisms in non-quantitative traits, the absence of NFDS leads to no continuous clines formation because the most advantageous phenotype will be completely dominant in the region (Takahashi et al. 2011). Thus, if polymorphism in cyanogenesis is maintained by NFDS in white clover, the urban-rural cline is a result of interactions between NFDS and urbanization-induced environmental changes, suggesting that the pattern of natural selection changes between urban and rural areas. To examine this hypothesis, manipulative field experiments examining frequency-dependent selection need to be conducted in combination with multi-site reciprocal transplanting in actual urban and rural environments. No studies have achieved such a reciprocal experiment in urban evolution studies to date, and that hypothesis has yet to be examined. It should also be noted that very few field experiments have been based on the assessment of fitness in actual urban-rural environments (see Gorton et al. 2018; Cosentino et al. 2023).

This study aimed to elucidate the mechanisms that maintain universal white clover genetic polymorphism and that change gene frequencies between urban and rural areas, especially focusing on balancing selection (especially NFDS) on cyanogenesis in white clovers. I conducted field transplanting experiments using clones of white clover (*Trifolium repens* L.) obtained from both urban and rural populations. I classified the transplanting sites into urban and rural environments based on differences in the degree of forest cover. Specifically, I address the following research questions: Q1) can balancing selection (NFDS) maintain genetic variation in cyanogenesis of white clovers, and Q2) how does the modes of balancing selection (NFDS) change between urban and rural areas?

2. Materials & Methods

Study System

The white clover, *Trifolium repens* L, is a perennial herbaceous plant, which is native to Europe that has been introduced to temperate regions worldwide (Daday 1958; Burdon 1983; Johnson et al. 2018). A white clover was first introduced to Japan from the Netherlands in 1846 (Daday 1958; Wu et al. 2021). It occurs in a wide range of soil conditions such as pastures, lawns, meadows, and roadsides in urban and rural environments (Burdon 1983; Johnson et al. 2018; Wu et al. 2021). White clovers reproduce vegetatively through stolon production, allowing us to make clonal replicates from stolon fragments.

There is a Mendelian polymorphism in white clovers for the production of hydrogen cyanide (HCN), which is effective against herbivores (Poulton 1990; Olsen et al. 2014; Thompson and Johnson 2016). Cyanogenic (HCN present) and acyanogenic (HCN absent) cyanotypes co-occur in many populations in most temperate environments (Daday 1958; Santangelo et al. 2019). The cyanogenesis polymorphism of white clover is regulated 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). Dominant alleles at both loci confer constitutive production of these two precursors (Olsen and Small, 2018; Innes et al. 2022). Thus, only plants with dominant alleles at both loci (i.e., *AcLi*) produce HCN.

Plant Collection and Genotyping

From June 20, 2022 to July 6, 2022, I collected white clover stolon cuttings from four urban and seven rural populations within 23 km of the city center of Sapporo, Hokkaido (figure 1a). The urban populations were collected in the city habitats including parks and roadsides. The rural populations included footpaths, green spaces, and the edges of forests. Stolons were collected with a minimum of 2 m spacing to avoid repeated sampling of the same genet (Thompson et al. 2016). Soon after I brought stolons to the laboratory, I made four replicate cuttings from each individual. Each of stolon cuttings had length of 7 cm and the same number of nodes. Stolon cuttings were incubated in plug trays (3.6 cm × 4.0 cm, with a depth of 7.0 cm

for each cell) filled with commercial compost soil, and established in a greenhouse until transplanting. I had 65 genotypes from urban populations, and 80 genotypes from rural populations for the transplant experiments (figure 4-1b).

To determine the chemical phenotype (i.e., cyanogenic or acyanogenic) of each white clover plant genotype, I performed Feigl–Anger assays (Feigl and Anger 1966). Feigl–Anger assays determine whether HCN is present or not in a sample by a color change reaction (Gleadow et al. 2011). For this assay, I followed the method of Thompson et al. (2016). Detailed methods for the assays are described in the Supporting information. The number of genotypes of the experimental plants in *AcLi* loci was as follows. Urban genotypes: cyanogenic *AcLi*, 19; acyanogenic, 46; rural genotypes: cyanogenic *AcLi*, 21; acyanogenic, 59.

Reciprocal Transplant Experiment Establishment

I conducted my transplant experiment at the four elementary schools near Sapporo city with permissions. In this experiment, I especially focused on plant cyanotype (i.e., cyanogenic or acyanogenic) and plant origin (urban-origin or rural-origin) to detect the response to selection generated by the frequency of cyanogenic plants around individuals. The schools were located in the urban and rural areas in the vicinity of Sapporo, Hokkaido, Japan from July 2022 through June 2023 (figure 4-1a, b). Before transplanting into the field, established cuttings in a greenhouse were moved to deep-bottomed plastic containers (80 cm × 50 cm, with a height of 20 cm) filled with the same commercial soil used in the initial stolon incubation. Hereafter, I refer to the plastic container as “container”. I made fifteen 15 cm × 15 cm partitioned quadrates within each container using commercial corrugated plastic, and placed one individual plant at the center of each quadrate. 10 containers were placed at each of the four elementary schools, and 580 genotypes were established in total (10 containers × 15 genotypes× 4 elementary schools – 20 vacant spaces = 580 plants) (figure 4-1c). The position of plants within each container was randomized so that I could use the container as a random effect in the statistical

analyses. Also, the number of cyanogenic plants "*AcLi*" around each individual was randomly manipulated so that I can detect frequency-dependent effects of cyanogenesis on fitness.

On 28 and 29 of July in 2022, I moved the containers to the four elementary schools: two in areas near forest stands (SPR and KM) and two in areas far from forest stands (FKZ and NM) (figure 4-1a). I measured the % degree of a forest cover within a 100 m square of each elementary school using the polygon area tool in Google map (Google, Inc., Mountain View, CA, USA). The degree of forest cover of SPR, KM, FKZ, and NM were 33%, 54%, 4%, and 1%, respectively. Hereafter, SPR and KM are referred to as "forest sites" and FKZ and NM are non-forest sites. The forest sites would represent a rural environment whereas the non-forest sites would represent an urban green environment because all four sites were at large elementary school areas, and their environmental condition was very alike except for the degree of forest cover regardless of whether sites were in urban areas or rural areas. Additionally, rural environments generally have greater vegetation cover compared to urban environments (Fukano et al. 2020; Santangelo et al. 2022a; Ishiguro et al. 2024). Therefore, the difference in the degree of forest cover among sites would represent the environmental condition rather than whether they are just located in urban areas or rural areas. Immediately after the transplantation, plants were thoroughly watered, after which they were left exposed to local environmental conditions. However, I watered SPR, FKZ, and KM on 17 of May and NM on 18 of May in 2023 due to extreme drought conditions.

Trait Measurements

Growth and Survival

All data collection was performed blind with respect to the cyanotype, genotype, and population origin of each plant. I examined not only the survival but also biomass (above-ground biomass, below-ground biomass) as fitness measures for white clover because white clover exhibits extensive clonal growth as a perennial (Murray-Stoker and Johnson 2024). I dug up all plants on 14 of June in 2023. I first washed soil and debris from all plants, and then split

them into above-ground and below-ground plant tissues. Then, I dried above- and below-ground tissues at 70 °C for 72 h in a dry-chamber, respectively. Plants with no harvested above-ground and below-ground biomass were regarded as dead individuals. Specifically, I calculated the frequency of cyanogenic plants around every plant as a measure of the frequency dependence of cyanogenesis (hereafter "neighbor HCN frequency"). My ability to accurately evaluate sexual fitness was hindered because only a subset of the plants bloomed, and I could not collect enough seeds. However, white clovers reproduce vegetatively through the dense stolons along the ground surface (Kemball and Marshall 1995; Thompson and Johnson 2016) and therefore biomass will be an effective measure of plant fitness.

Herbivory

I quantified herbivore damage on leaves for each plant twice during the experiment (early September, early October) using a protocol of Johnson et al. (2016). To measure herbivory, I selected a maximum of three fully expanded non-senescing leaves on each plant and visually estimated the proportion of tissue consumed by herbivores in six ranks as 1) 1–10%, 2) 11–25%, 3) 26‑50%, 4) 51‑75% or 5) >75%. The median value of the range for each rank was used to calculate herbivory and the values were averaged across three leaves for each individual (Johnson et al. 2016). In my previous study, visual estimates of herbivory in this system yield results consistent with those obtained from imaging software (https://imagej.nih.gov/ij) and are sufficiently precise to quantification of herbivory (Chapter 2; Ishiguro et al. 2024).

Statistical Analyses

To compare the difference in herbivory damages between the forest sites and the non-forest sites, I performed linear mixed effect models (LMMs). The fixed effects were origin type (whether a plant was obtained in urban environments or rural environments), place type

(whether a plant was placed at a forest site or a non-forest site), and the cyanotype (cyanogenic or acyanogenic). The interaction between the three of them was also incorporated. I also performed LMMs without the third-order interaction (i.e., LMMs with the three fixed effects and the interaction between every two of origin type and place type and cyanotype), and LMMs without any interactions. The population where I took plants and container ID were included as random effects through the three models.

To test NFDS, the neighbor HCN frequency was calculated as follows at first; (the number of cyanogenic plants - the number of cyanogenic plants) / (the number of cyanogenic plants + the number of cyanogenic plants). At this point, the values range from -1 to 1. Hence, I added 1 to the values and then divided it by 2 so that the value of the neighbor HCN frequency ranges from 0 to 1. I used generalized linear mixed models (GLMMs) and linear mixed effect models (LMMs) to test whether plant survival and biomass depend on the neighbor HCN frequency, respectively. The fixed effects were the neighbor HCN frequency, origin type, place type, and the cyanotype. I also incorporated interactions between the neighbor HCN frequency or place type or cyanotype. The population where I took plants and plate ID were incorporated as random effects. I log-transformed the biomass value throughout the analyses.

All statistical analyses were performed in R ver. 4.3.2 (www.r-project.org). I used the package “lme4” (Bates et al. 2015) for the linear mixed-effect model and generalized linear mixed model, “ggeffects” (Lüdecke 2018) and “insight” (Lüdecke et al. 2019) for calculating regression line for figures through the predicted values from the linear mixed-effect model and generalized linear mixed model. Likelihood ratio test through ANOVA was performed using the package “lmerTest” (Kuznetsova et al. 2017) and “pbkrtest” (Halekoh and Højsgaard 2014) to determine whether an explanatory variable of linear mixed-effect model and generalized linear mixed model has a significant effect.

3. Results

Differences in herbivory between sites

The herbivory was significantly affected only by the explanatory variable of whether the site was a forest site or non-forest site (table 4-1, figure 4-2). Cyanotypes (i.e., cyanogenic or acyanogenic), origin types (urban-origin or rural-origin), and their interactions did not significantly influence the herbivory (table 4-1, figure 4-2a). The herbivory was significantly higher at the forest sites than the herbivory at the non-forest sites (table 4-1, figure 4-2b).

Mode-shift of frequency-dependence between forest and non-forest sites

Survival and belowground biomass depended on the neighbor HCN frequency, as confirmed by a significant interaction between the site type and the neighbor HCN frequency and origin type (table 4-2, figure 4-3a, b, e, f). Also, the interaction between the site type and the neighbor HCN frequency and cyanotype affected the survival (table 4-2, figure 4-4a, b).

At the forest sites, the survival of urban-origin plants increases as the neighbor HCN frequency increases, and the survival of rural-origin plants decreases as the neighbor HCN frequency increases (figure 4-3a). In contrast, at the non-forest sites, the survival of urban-origin plants decreases as the neighbor HCN frequency increases, and the survival of rural-origin plants slightly increases as the neighbor HCN frequency increases (figure 4-3b). These patterns are consistent with those of the belowground biomass (figure 4-3e, f). In brief, the NFDS mode changed between the forest sites and non-forest sites.

At the forest sites, the cyanogenic plants exhibited NFDS. The survival of both cyanogenic and acyanogenic plants decreases as the neighbor HCN frequency increases, but cyanogenic plants exhibit a steeper regression curve (figure 4-4a). Contrary to that, at the non-forest sites, the cyanogenic plants exhibited positive frequency-dependent selection. The survival of both cyanogenic and acyanogenic plants increases as the neighbor HCN frequency increases, and the cyanogenic plants exhibit a steeper regression line (figure 4-4b). These results are supported by the results, in which each genotype showed the similar trends (figure 4-5).

4. Discussion

My field experiment empirically highlights that the mode of frequency-dependent natural selection changes between forest sites (rural environments) and non-forest sites (urban environments). Specifically, NFDS occurred in forest sites with higher herbivory pressure, and positive frequency-dependent selection (PFDS) occurred in non-forest sites with lower herbivory pressure. These results suggest that genetic variations are maintained by balancing selection (NFDS) in rural environments. However, urbanization breaks the balancing selection, leading to a loss of genetic variations. In the following discussion, I argue that hypothetical trade-offs between competition, HCN tolerance, and HCN production can explain the frequency-dependent selection and its mode shift. Based on the results, I added a schematic illustration of results summary (figure 4-6).

Effects of environmental differences on herbivory pressure

This study empirically demonstrated that herbivory pressure was higher in rural environments than in urban environments. The reason for the increased herbivory pressure in rural environments could be the increased number of herbivores in rural environments. Typically, green spaces can serve as sources of herbivores (Buczkowski and Richmond 2012; Bode and Maciejewski 2014). It is also known that green spaces and grasslands are turned into asphalt as urbanization progresses, and the abundance and diversity of organisms decrease (Buczkowski and Richmond 2012; Miles et al. 2019; McDonald et al. 2020; Piano et al. 2020). Thus, increased vegetation cover in rural environments can lead to higher herbivory pressure. These results are consistent with the results in Chapter 2.

Interestingly, there was no difference in herbivory between cyanogenic individuals, which are generally considered to have antiherbivory defense traits, and acyanogenic individuals, which do not have antiherbivory defense traits. One possible explanation is species-specific herbivory. In previous experiments, using cyanogenic and acyanogenic plants, herbivores such as mollusks and Dermaptera preferred acyanogenic plants to cyanogenic plants (Dirzo and Harper 1982; Compton and Jones 1985). However, weevils, and Lepidoptera such as

Polyommatus icarus, showed no consistent preference (Dirzo and Harper 1982; Compton and Jones 1985). My results are also consistent with previous studies that showed no significant differences in herbivory between cyanogenic and acyanogenic white clover in the field (Wright et al. 2017). In addition, mobile herbivores could recolonize onto the cyanogenic plant after HCN diffusion even though HCN released from a cyanogenic plant temporally repels herbivores. I also argue that the above problems were the reason why previous studies have not successfully provided evidence of NFDS in white clover from a herbivory perspective.

No significant differences in herbivory due to origin differences can be explained by the following reason. Differences in herbivory pressure between urban and rural environments lead to variations in tolerance to HCN generated by surrounding cyanogenic plants rather than variations in tolerance to the herbivory itself. Specifically, urban-origin plants evolved to urban environments are more tolerant to HCN, whereas rural-origin plants evolved to rural environments are less tolerant to HCN. The detailed mechanism is explained in the next section.

Frequency-dependent dynamics due to the differences in plant origin environments

This study empirically showed that white clovers from different origin environments (obtained in urban environments or rural environments) exhibited the opposite pattern of frequency-dependence for neighbor HCN frequency between forest sites and non-forest sites.

At the forest sites, which represent rural environments, herbivory pressure was higher than in the non-forest sites (table 4-1, figure 4-2, 4-6). Cyanogenic plants produce HCN when their tissues are damaged by herbivory (Hughes 1991), and HCN can be frequently produced in environments with high herbivory pressure. Because cyanogenic and acyanogenic plants equally receive herbivory (table 4-1, figure 4-2), cyanogenic plants are also frequently damaged under higher herbivory pressure, and thus the surrounding individuals can be exposed to released HCN. As HCN is toxic to plants as well as animals and microorganisms (Krasuska et al. 2024), differences in tolerance to HCN toxicity can lead to frequency-dependent dynamics. Cyanide-resistant respiration is ubiquitous in plants and plays an important role in HCN tolerance that

maintains homeostasis under high concentrations of HCN (Thiers et al. 2023). Kuo et al. (2023) reported that both cyanogenic and acyanogenic white clovers have cyanide-resistant respiration and there was genetic variation in HCN tolerance. Furthermore, the cyanide-resistant respiration pathway dramatically reduces ATP production, resulting in a decrease in plant growth (Moore et al. 1978; Hirose et al. 1989). Hence, HCN tolerance can be antagonistic with competitive growth (i.e., trade-off between HCN tolerance and competition). Because rural environments have more vegetation cover than urban environments (Fukano et al. 2020; Santangelo et al. 2022a; Ishiguro et al. 2024) and are considered to be highly competitive, rural-origin plants are expected to be more competitive than urban-origin plants. In fact, Fukano et al. (2020) reported that plants from rural populations have higher growth rates and are more tolerant to competition compared to plants from urban populations. Overall, more competitive rural-origin plants are expected to be less tolerant to HCN, whereas urban-origin plants are expected to be less competitive but more tolerant to HCN. Therefore, when the neighbor HCN frequency is low, there is very little HCN released in the surrounding area, and the rural-origin plant, which is less HCN tolerant but more competitive, has an advantage over the urban-origin plant. In contrast, when the neighbor HCN frequency is higher, HCN releasing occurs in the surrounding area and thus the urban-origin plant with high HCN tolerance has an advantage over the rural-origin plant (figure 4-3a, e, 4-6).

At the non-forest sites, which represent urban environments, herbivory pressure was much lower than in the forest sites (table 4-1, figure 4-2, 4-6). Thus, the amount of HCN released from cyanogenic plants should be lower than forest sites. However, plants are exposed to a small amount of HCN when neighbor HCN frequency is high because herbivory pressure is low but not zero. In this case, cyanide-resistant respiration, which is less efficient than normal respiration is activated for low amounts of HCN. Thus, urban-origin plants, which are with higher HCN tolerance would be at a competitive disadvantage. When neighbor HCN frequency is low at this low herbivory environment, cyanide-resistant respiration cannot be triggered because plants receive very low herbivory and are not exposed to HCN from surrounding

cyanogenic plants. In this situation, urban-origin plants were shown to have an advantage over rural-origin plants (figure 4-3b, f, 4-6).

Mode-shift of frequency-dependent selection between forest sites and non-forest sites

This study highlighted that the mode of frequency-dependent natural selection on cyanogenesis changed between urban and rural environments (i.e., forest sites and non-forest sites). The following reasons would explain the mechanisms of the mode-shift of frequency-dependent selection. At the forest sites, cyanogenic plants and acyanogenic plants showed patterns similar to rural-origin plants and urban-origin plants, respectively (figure 4-3a, e, 4-4a). Fitness of cyanogenic plants (or rural-origin plants) was higher than acyanogenic plants (or urban-origin plants) when neighbor HCN frequency was low. In contrast, fitness of acyanogenic plants (or urban-origin plants) was higher than cyanogenic plants (or rural-origin plants) when neighbor HCN frequency was high. This might be related to trade-offs between producing HCN, tolerance to HCN, and competition. Since producing HCN requires both cyanogenic glycosides and hydrolytic enzymes (Hughes 1991; Olsen and Small 2018), cyanogenic plants incur more costs compared to acyanogenic plants. Being tolerant to HCN is also costly because it involves metabolic pathways such as AOX and β-Cas (Kuo et al. 2023). Then, there can be a trade-off between producing HCN and tolerance to HCN. In fact, fitness components of the HCN-producing type actually decreased in HCN-tolerant-type advantageous conditions (i.e., a higher neighbor HCN frequency at forest sites). In addition, as noted above, HCN tolerance and competition are thought to be in a trade-off relationship. Thus, producing HCN means lower HCN tolerance, leading to highly competitive, and non-producing HCN means higher HCN tolerance, leading to lower competition. Therefore, when the neighbor HCN frequency is low and plants are not exposed to HCN emission, cyanogenic plants with less HCN tolerance but more competitive are advantageous over acyanogenic plants. When the neighbor HCN frequency is high and plants are exposed to HCN, acyanogenic plants that do not produce HCN but are more tolerant to HCN have an advantage over cyanogenic plants with lower HCN

tolerance (figure 4-4a, 4-6).

At the non-forest sites, cyanogenic plants and acyanogenic plants also showed patterns similar to rural-origin plants and urban-origin plants, respectively (figure 4-3b, f, 4-4b). However, the result exhibited positive frequency-dependent selection (PFDS). In this low herbivory environment, when the neighbor HCN frequency becomes higher, acyanogenic plants are exposed to a small amount of HCN, triggering cyanide-resistant respiration. Because cyanide-resistant respiration is less effective than normal respiration, acyanogenic plants are disadvantageous over cyanogenic plants with less HCN tolerance. When neighbor HCN frequency is low, plants receive little herbivory damage and HCN is not released from any plants. In this case, the result suggests that acyanogenic plants are advantageous over cyanogenic plants (figure 4-4b, 4-6).

This study has implications for a new mechanism for the formation of evolutionary clines, which are the continuous variations of traits from urban to rural areas. Previous studies on urban evolution have typically focused on a relationship between traits and specific environmental factors which are thought to be selective pressure along urban-rural gradient (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020, 2022a,b). This study is the first to empirically demonstrate that NFDS, occurs mediated through high herbivory pressure in rural environments. Thus, genetic variation in cyanogenesis is maintained in rural environments through NFDS, which can result in a consistently higher HCN frequency. In contrast, urbanization breaks this balancing selection and leads to PFDS through lower herbivory pressure. Given this PFDS in urban, HCN frequency can be fixed at either 0 or 1. However, acyanogenic plants are advantageous over cyanogenic plants with lower herbivory pressure and lower neighbor HCN frequency (fig 4-4b, 4-6). Because typical urban sites have much lower herbivory than my experimental, non-forest sites, relative advantages of acyanogenic plants would be greater than this experimental result. Moreover, in actual urban areas, there is less vegetation cover and competition is less likely to occur than in rural areas (Fukano et al. 2020), which should favor less competitive types (i.e., HCN-tolerant, non-HCN producing, urban type).

Thus, even under PFDS, acyanogenic plants could be more frequently dominant in urban environments. However, sometimes there were some populations with high HCN frequency even in urban areas (Ishiguro et al. 2024; Chapter 2; Chapter 3), suggesting PFDS can also lead to HCN dominance in urban areas occasionally.

Interestingly, the SEM model in Chapters 2 and 3 consistently showed that herbivory pressure was positively associated with HCN frequency. As described above, high herbivory pressure in rural environments causes NFDS through increasing HCN emission from neighbors, resulting in the maintenance of polymorphism in cyanogenesis and relatively high HCN frequency. Also, low herbivory pressure through urbanization causes PFDS but can lead to acyanogenic dominance in urban environments. My results indicate that herbivory does not directly impose selection for HCN along urbanization gradients, but mediates the mode shift of frequency-dependent selection. This can explain how herbivory pressure associates with HCN frequency variation even though there are no clear differences in herbivory between cyanogenic and acyanogenic plants as previous studies have shown (Hughes 1991; Wright et al. 2017).

Overall, I provided evidence of herbivory-mediated regulation of NFDS and PFDS imposed by neighboring plants. I emphasize that this notion is also consistent with my Chapters 2 and 3, in which HCN frequency was associated with herbivory pressure. While greater herbivory pressure in rural regions perpetually maintains high HCN frequency through NFDS, less herbivory in urban regions can lead to low HCN frequency with PFDS and less competitive conditions. Future urban evolution studies should pay attention to frequency-dependent selection and its mode shifts from urban to rural environments.

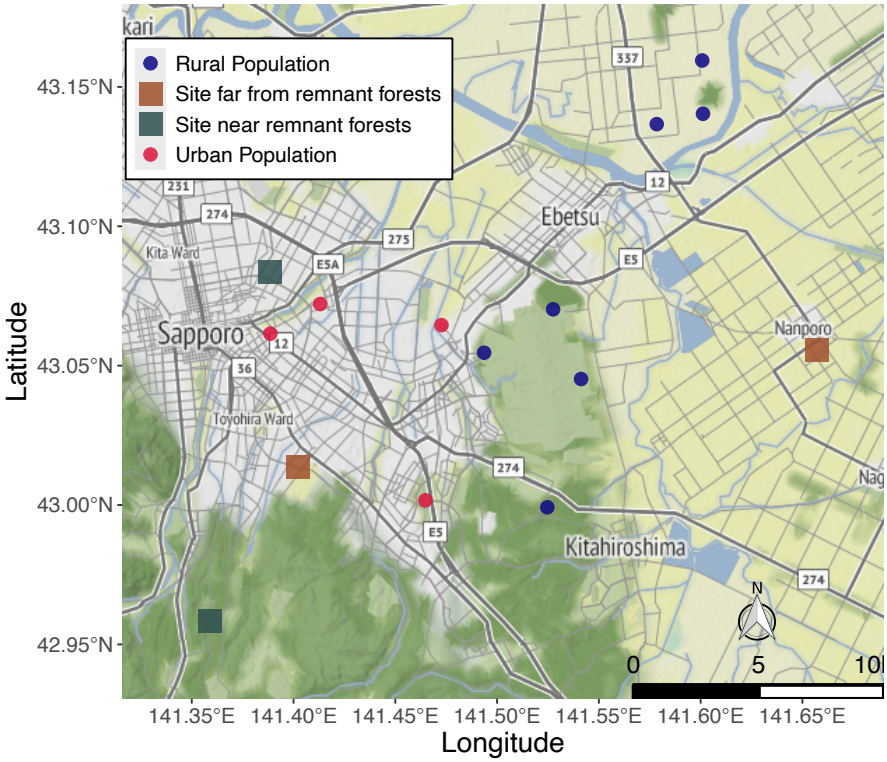
Table 4-1. Summary of the linear mixed-effects models testing the effects of cyanotype, origin type, site type, and their interactions on herbivory. I also provide the numerator degrees of freedom (NumDF), denominator degrees of freedom (estimated using the Kenward-Roger method; DenDF), F-values calculated from Type III ANOVA, and *P* values for each fixed effect. Bolded terms are significant at $P < 0.05$.

Response	Predicor	p-value for lmer	NumDF	DenDF	F-value	p-value
Herbivory	Cyanotype	0.128	1	136.604	2.077	0.152
	Origin type	0.387	1	9.084	0.314	0.589
	Site type	**0.013**	**1**	**53.325**	**8.579**	**0.005**
	Cyanotype × Origin type	0.628	1	135.162	0.086	0.770
	Cyanotype × Site type	0.421	1	431.424	0.474	0.492
	Origin type × Site type	0.330	1	428.256	2.004	0.158
	Cyanotype × Origin type × Site type	0.697	1	424.446	0.151	0.698
Herbivory	Cyanotype	0.128	1	136.241	2.089	0.151
	Origin type	0.289	1	9.070	0.299	0.597
	Site type	**0.006**	**1**	**53.391**	**8.613**	**0.005**
	Cyanotype × Origin type	0.767	1	135.133	0.081	0.776
	Cyanotype × Site type	0.464	1	432.700	0.536	0.464
	Origin type × Site type	0.165	1	430.017	1.927	0.166
Herbivory	Cyanotype	0.117	1	171.672	2.290	0.132
	Origin type	0.618	1	7.211	0.273	0.617
	Site type	**0.004**	**1**	**37.042**	**9.559**	**0.004**

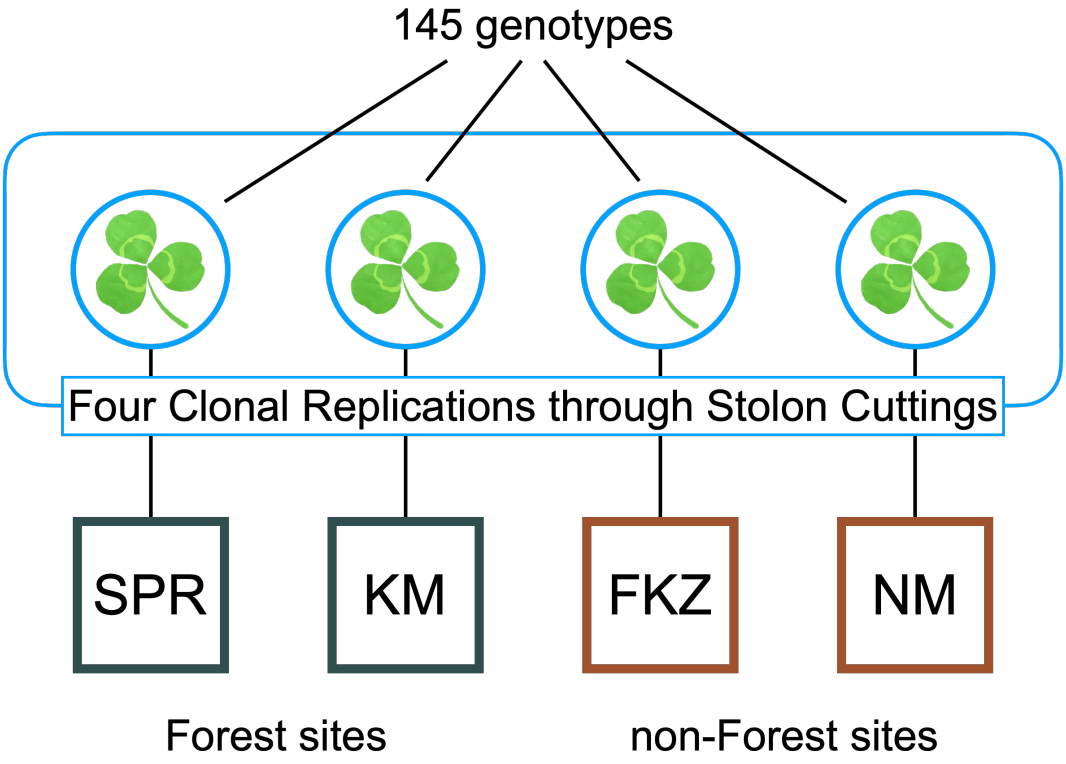
Table 4-2. Summary of the generalized linear mixed-effects model and the linear mixed-effects models testing the effects of cyanotype, origin type, site type, and their interactions on fitness measurement. I also provide χ^2 statistics, degrees-of-freedom calculated from type II Wald chi-square tests, and *P* values for each fixed effect in the generalized linear mixed-effects model. The numerator degrees of freedom (NumDF), denominator degrees of freedom (estimated using the Kenward-Roger method; DenDF), F-values calculated from Type III ANOVA, and *P* values were provided for each fixed effect in the linear mixed-effects models. Bolded terms are significant at $P < 0.05$.

Response	Predicor	p-value for glmer	χ2	Df	p-value	
Survival	Neighbor HCN freq.	0.132	**4.370**	**1**	**0.037**	
	Origin type	**0.036**	2.546	1	0.111	
	Site type	0.363	0.099	1	0.753	
	Cyanotype	0.157	0.062	1	0.803	
	Neighbor HCN freq. × Origin type	0.094	0.008	1	0.929	
	Neighbor HCN freq. × Site type	0.256	1.196	1	0.274	
	Origin type × Site type	**0.041**	0.011	1	0.918	
	Cyanotype × Neighbor HCN freq.	**0.010**	0.608	1	0.435	
	Cyanotype × Site type	0.093	1.149	1	0.284	
	Neighbor HCN freq. × Origin type × Site type	**0.016**	**5.814**	**1**	**0.016**	
	Neighbor HCN freq. × Cyanotype × Site type	**0.003**	**8.820**	**1**	**0.003**	
Response	**Predicor**	**p-value for lmer**	**NumDF**	**DenDF**	**F-value**	**p-value**
Aboveground biomass	Neighbor HCN freq.	0.447	1	383.189	0.238	0.626
	Origin type	0.502	1	33.640	0.073	0.789
	Site type	0.478	1	145.187	0.044	0.835
	Cyanotype	0.364	1	281.054	1.550	0.214
	Neighbor HCN freq. × Origin type	0.212	1	393.469	0.052	0.819
	Neighbor HCN freq. × Site type	**0.037**	1	391.641	1.379	0.241
	Origin type × Site type	0.212	1	403.361	1.549	0.214
	Cyanotype × Neighbor HCN freq.	0.547	1	408.961	0.688	0.407
	Cyanotype × Site type	0.941	1	412.430	0.005	0.942
	Neighbor HCN freq. × Origin type × Site type	0.104	1	411.974	2.613	0.107
	Neighbor HCN freq. × Cyanotype × Site type	0.948	1	409.768	0.004	0.948
Belowground biomass	Neighbor HCN freq.	0.308	**1**	**408.262**	**0.001**	**0.976**
	Origin type	0.287	1	27.929	0.120	0.731
	Site type	0.190	**1**	**112.893**	**0.003**	**0.955**
	Cyanotype	0.546	1	327.601	0.900	0.343
	Neighbor HCN freq. × Origin type	0.152	1	395.029	0.084	0.772
	Neighbor HCN freq. × Site type	**0.013**	1	411.609	1.589	0.208
	Origin type × Site type	**0.048**	**1**	**389.938**	**3.920**	**0.048**
	Cyanotype × Neighbor HCN freq.	0.429	1	411.321	0.775	0.379
	Cyanotype × Site type	0.867	1	405.459	0.028	0.868
	Neighbor HCN freq. × Origin type × Site type	**0.016**	**1**	**399.831**	**5.809**	**0.016**
	Neighbor HCN freq. × Cyanotype × Site type	0.759	1	411.078	0.092	0.761

(a)


Rural Population
Site far from remnant forests
Site near remnant forests
Urban Population
Sapporo
Ebetsu
Nanporo
Kitahiroshima
Kita Ward
Toyohira Ward
43.15°N
43.10°N
43.05°N
43.00°N
42.95°N
Latitude
141.35°E
141.40°E
141.45°E
141.50°E
141.55°E
141.60°E
141.65°E
Longitude
N
0
5

(b)


145 genotypes
Four Clonal Replications through Stolon Cuttings
SPR
KM
FKZ
NM
Forest sites
non-Forest sites

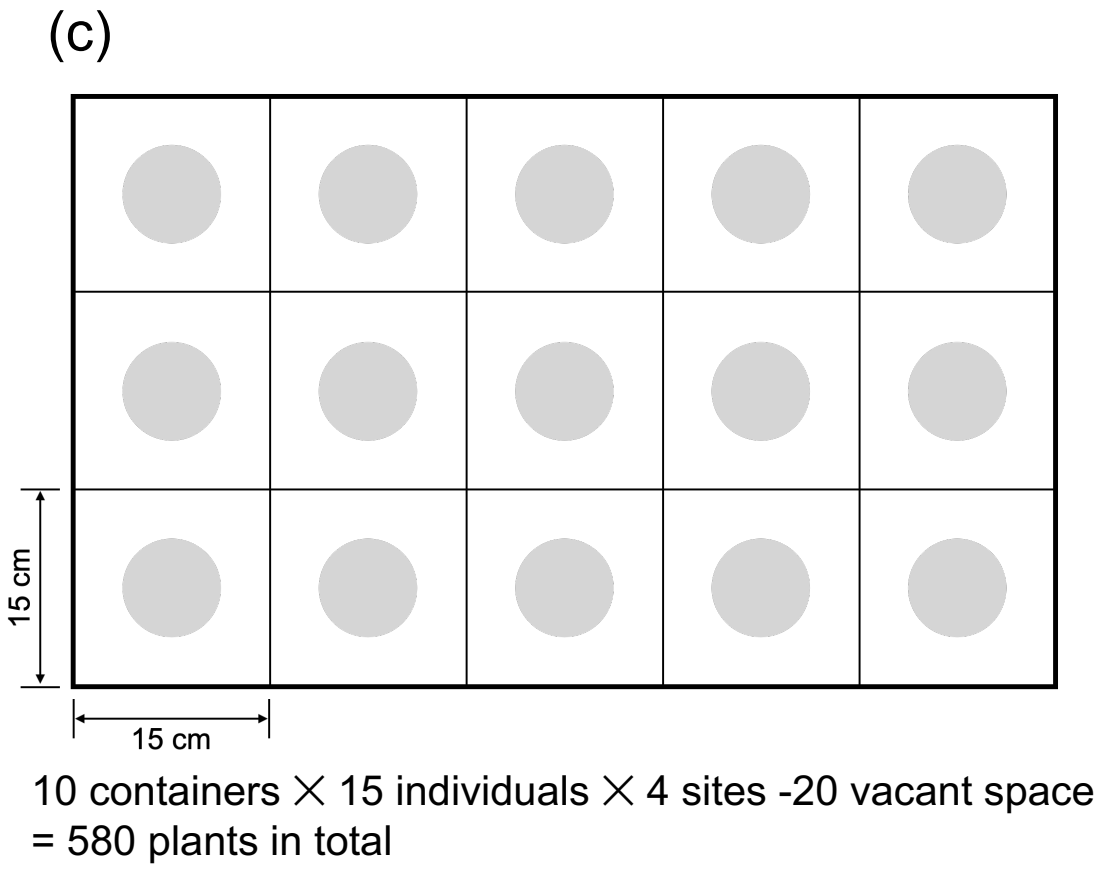


Figure 4-1. Simplified topographic map of experimental sites and sampling populations. Square points denote sites and circle points denote sampling populations (a). A schematic diagram of the experimental design (b, c). Totally 145 genotypes were collected from urban and rural populations. Four clonal replications were made through collecting each of the 145 genotypes and placed at each experimental site (b). All plants were set at the center of partitioned quadrate (gray circle) within containers placed at the four sites (c).

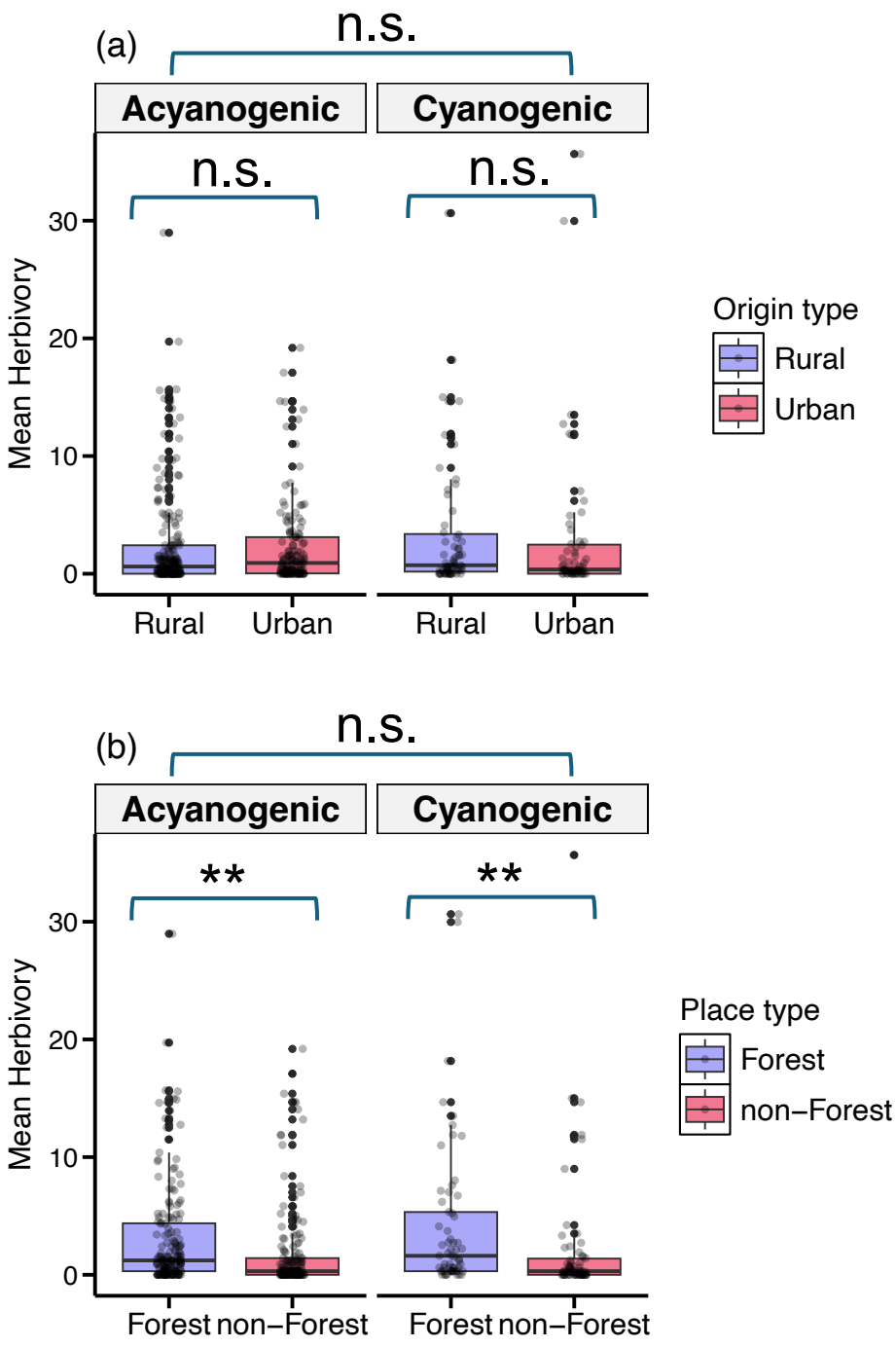


Figure 4-2. (a) Herbivory difference between cyanogenic and acyanogenic and between rural-origin plants and urban-origin plants. (b) Herbivory difference between cyanogenic and acyanogenic and between forest sites and non-forest sites.

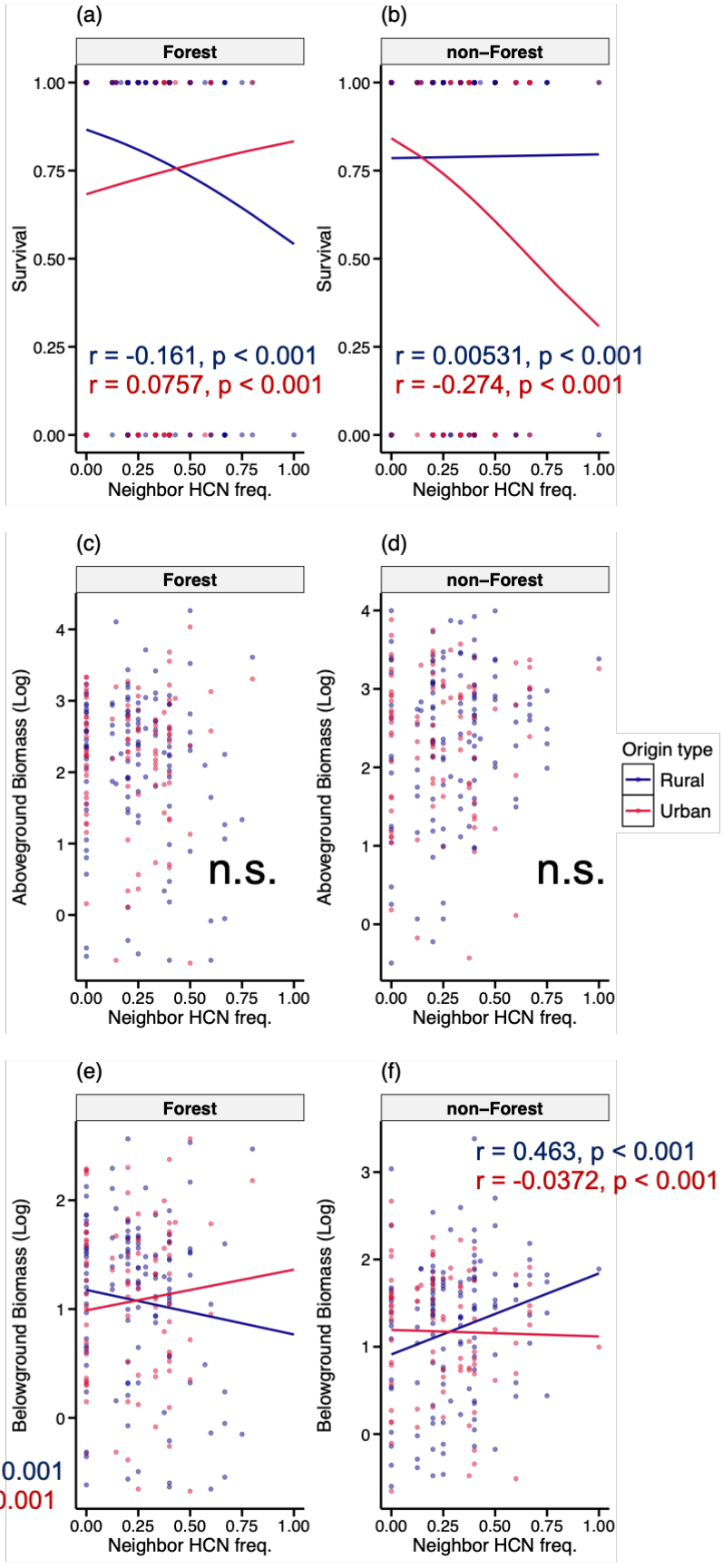

(a)
Forest
(b)
non–Forest
Survival
1.00
0.75
0.50
0.25
0.00
r = -0.161, p < 0.001
r = 0.0757, p < 0.001
r = 0.00531, p < 0.001
r = -0.274, p < 0.001
0.00 0.25 0.50 0.75 1.00
Neighbor HCN freq.
(c)
(d)
Aboveground Biomass (Log)
n.s.
Origin type
Rural
Urban
(e)
(f)
Belowground Biomass (Log)
r = 0.463, p < 0.001
r = -0.0372, p < 0.001
r = -0.204, p < 0.001
r = 0.187, p < 0.001

Figure 4-3. Frequency-dependent fitness variation for survival (a, b), aboveground biomass (c, d), and belowground biomass (e, f) in the forest sites and the non-forest sites. Red lines indicate the fitness of urban-origin plant individuals and blue lines indicate the fitness of rural-origin plant individuals.

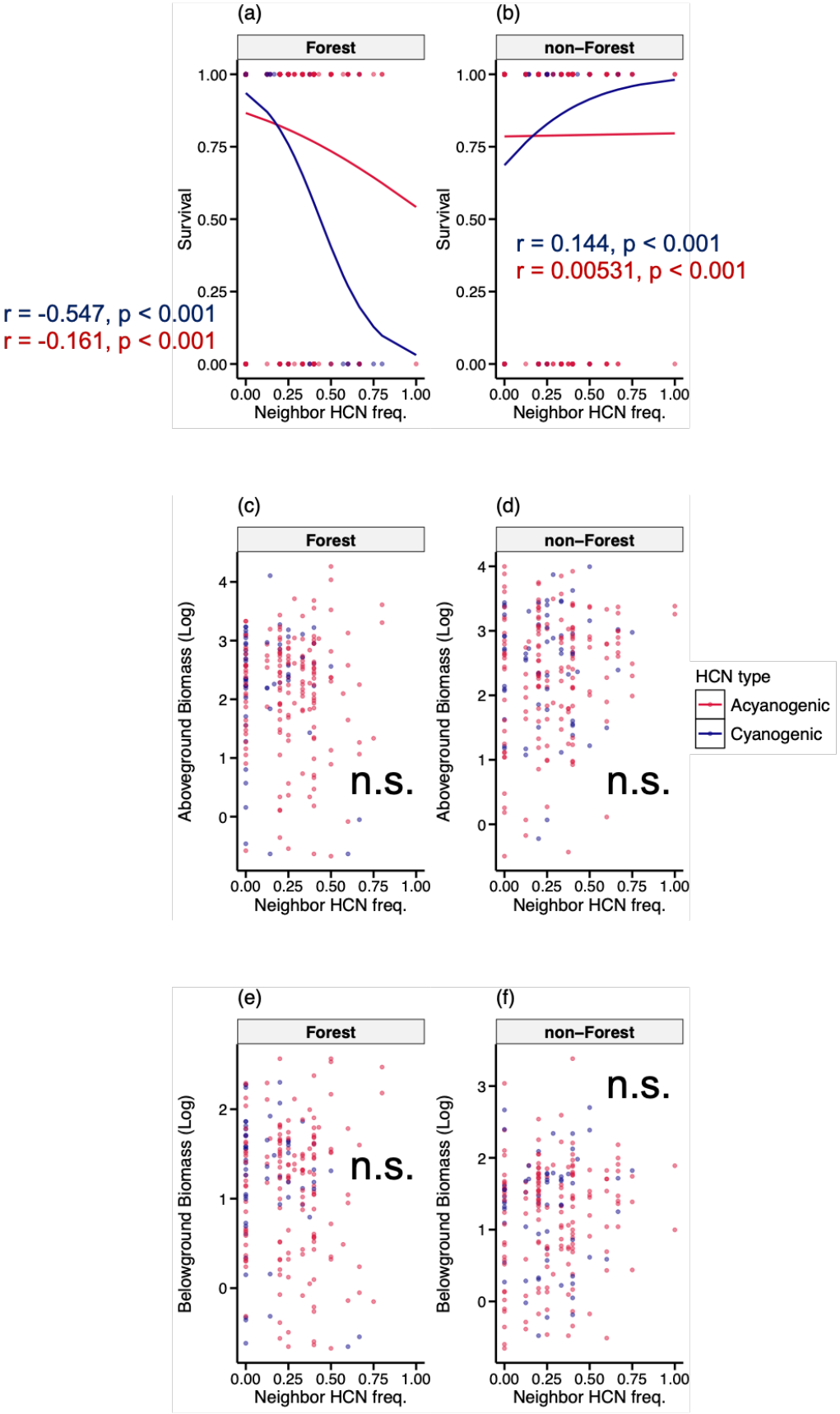

(a)
Forest
(b)
non−Forest
Survival
Neighbor HCN freq.
r = -0.547, p < 0.001
r = -0.161, p < 0.001
r = 0.144, p < 0.001
r = 0.00531, p < 0.001
(c)
(d)
Aboveground Biomass (Log)
n.s.
HCN type
Acyanogenic
Cyanogenic
(e)
(f)
Belowground Biomass (Log)

Figure 4-4. Frequency-dependent fitness variation for survival (a, b), aboveground biomass (c, d), and belowground biomass (e, f) in the forest sites and the non-forest sites. Red lines indicate the fitness of acyanogenic plant individuals and blue lines indicate the fitness of cyanogenic plant individuals.

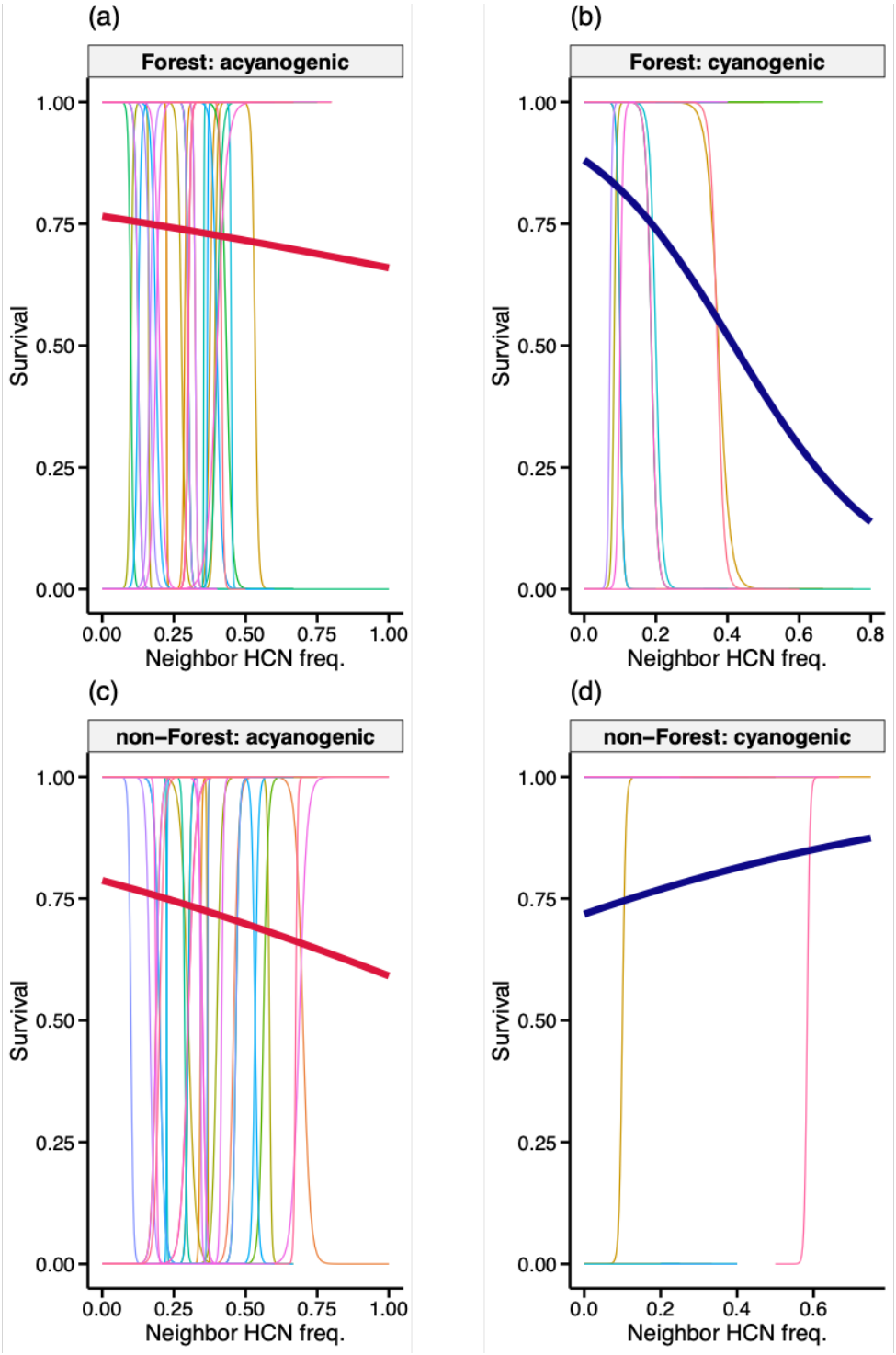


Figure 4-5. Frequency-dependent survival variation of each genotype in the forest sites (a, b) and the non-forest sites (c, d). Each curve shows the logistic regression curve for survival of each genotype, and bold lines show the mean slope of the logistic regression curves. Red curves indicate the mean survival of acyanogenic genotypes (a, c) and blue curves indicate the mean

survival of cyanogenic genotypes (b, d).

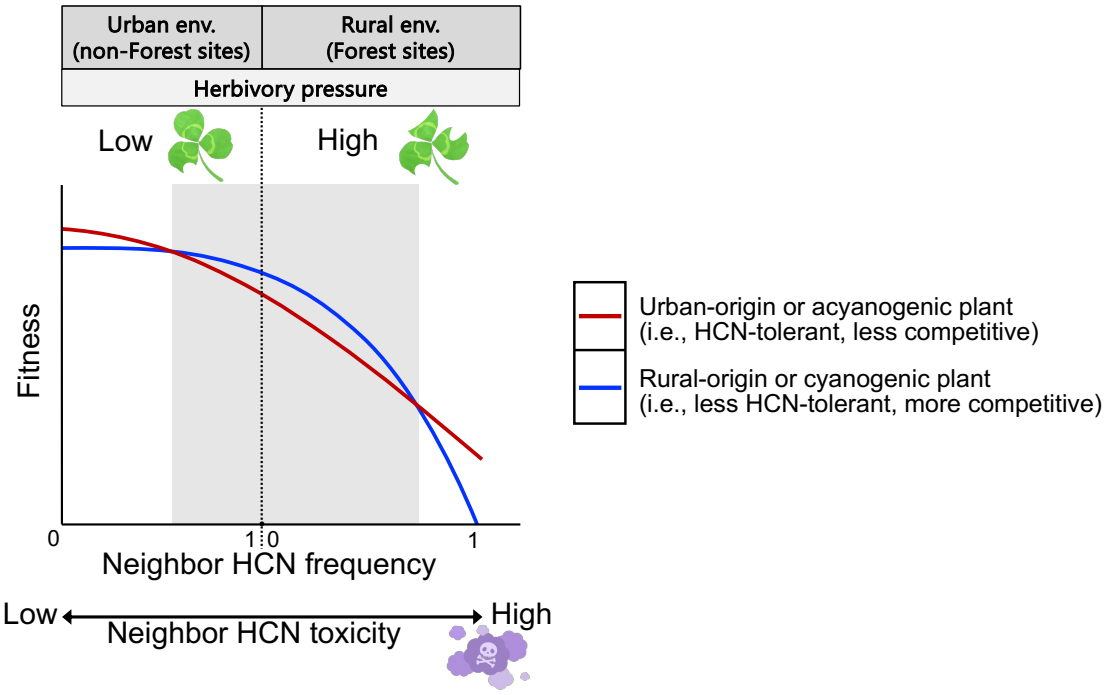


Figure 4-6. A schematic illustration of results summary. Red line represents fitness of urban-origin HCN-tolerant (or acyanogenic) plants (i.e., less competitive). Blue line represents rural-origin less HCN-tolerant (or cyanogenic) plants (i.e., more competitive). The white areas indicate HCN-tolerant (or less competitive) plants are advantageous over less HCN-tolerant (or more competitive) plants. The gray area indicates less HCN-tolerant (or more competitive) plants are advantageous over HCN-tolerant (or less competitive) plants.

Chapter 5: General discussion

This thesis clearly demonstrated the significance of considering spatial contexts on the urban evolution of white clovers. In this Chapter, I first summarize the results obtained from my studies, and then synthesize and discuss my findings with other examples of urbanization-induced evolution. Finally, I discuss future directions for a better understanding of urbanization-induced evolution.

In Chapter 2, I investigated how white clover evolved to complex multivariate urban environmental heterogeneity, focusing on the evolution of hydrogen cyanide (HCN) and its components (presence/absence of cyanogenic glycosides and the hydrolytic enzyme linamarase). I adopted a landscape approach, which enables us to identify what specific factors are driving urban evolution through comprehensively analyzing the relationship between the detailed spatial distribution of various heterogenous environmental factors and spatial variation of focal traits in a broad range of urban-rural areas. I collected 3299 white clover plants from 122 populations in the vicinity of Sapporo. Then, I 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 and its components. As a result, frequency of *Ac*, allele controlling the production of cyanogenic glycoside, showed a clear trend along urban–rural gradient and HCN frequency showed a mosaic-like spatial distribution. Thus, the predicted spatial distributions of HCN and *Ac* were inconsistent. These findings suggested that urban environmental heterogeneity and multi-functionality of cyanogenic glycoside shape a mosaic-like spatial distribution of evolutionary traits.

In Chapter 3, to understand similarities and dissimilarities in evolutionary patterns across multiple cities, I expanded the landscape approach as Chapter 2 for different cities in Hokkaido. A total of 5589 white clover plants from 234 populations were analyzed, including 40 populations in Hakodate, 41 in Asahikawa, and 31 in Kushiro, in addition to the 122 Sapporo population. I comprehensively investigated and compared the spatial distribution of evolutionary traits of white clovers in the four cities. These four cities have different climate

conditions. As a result, both macro-scale climate and micro-scale urban-rural environmental variation determine gene frequency within populations in multiple cities. Relatively, the coefficients of macro-scale environmental differences are a little larger than urbanization-induced environmental changes, but in a similar order. These results suggest that macro-scale environment first changes gene frequency within populations, and then micro-scale urban-rural environmental variation additively determines gene frequency, driving evolution.

In Chapter 4, I conducted a field transplant experiment in actual urban and rural environments to empirically test whether balancing selection contributes to maintaining polymorphism in cyanogenesis of white clovers, and whether the mode of natural selection differs between urban and rural environments. In particular, I focused on frequency-dependent selection on cyanogenesis and examined the effects on fitness measurement (survival rate and biomass) by randomly manipulating the frequency of surrounding plants producing HCN (cyanogenic) around each individual. As a result, I found that negative frequency-dependent selection (NFDS) occurred in rural environments mediated by higher herbivory pressure, while positive frequency-dependent selection (PFDS) occurred in urban environments mediated by lower herbivory pressure. These results empirically highlight the mode of natural selection changes between urban and rural environments. In other words, genetic variations are maintained by balancing selection (NFDS) in rural environments, whereas urbanization breaks this balancing selection, leading to a loss of genetic variations and predominance of acyanogenic plants in urban environments.

Cline formation along urban-rural gradient

As described in the general introduction (Chapter 1), urbanization-induced evolution has been observed all over the world. Many conventional studies have performed a transect approach, which tests whether focal genetic traits show clear relationships with the distance from the urban center. Especially in white clovers, several studies showed that the frequency of cyanogenic plants increased with distance from urban centers in some cities but there were no

clines in other cities (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2022a). However, the mechanisms by which those trait clines are shaped (or not shaped) have been poorly understood. This thesis has several implications for urban–rural clines in trait evolution. The predicted map of the frequency of cyanogenesis in Chapter 2 suggests that an urban–rural cline in cyanogenesis can be observed, but it will largely depend on the direction and distance of a transect. This is due to the environmental heterogeneity of urban landscape. As noted in Chapter 2, this result supports the study of Santangelo et al. (2022b) which reported intraurban environmental heterogeneity can drive fine-scale adaptive clines within cities. This suggestion was also supported by the results of Chapter 3, which showed the similar pattern to Chapter 2. Hence, these findings suggest that the presence or absence of a cline in a city is unlikely to be an appropriate measure as the degree of urban evolution. A cline could not be observed, depending on the placement of the transect or the contrast between urban and rural sampling places. Therefore, conventional studies could have failed to detect evolutionary effects of urbanization because those studies ignored environmental heterogeneity in urban landscape (Alberti 2015, 2023; Alberti et al. 2020; Santangelo et al. 2022b; Ishiguro et al. 2024; Chapter 2). In contrast, my landscape approach provided a great insight into the spatial structure of urban evolution within and across cities. Furthermore, through the landscape approach for the heterogeneous urban-rural environments, I can estimate which factors were most relevant for driving urban evolution (e.g., impervious surface ratio and its associated herbivory as Chapter 2 and Chapter 3). Therefore, I emphasize that my landscape approach is much more fruitful for urban evolution research than conventional approaches.

Furthermore, conventional studies only focus on the relationships between traits and specific environmental variation that is thought to be selective pressure along urban-rural gradient (e.g., temperature; Chapter 1). However, my findings of Chapter 4 suggest a novel mechanism by which an urban-rural evolutionary cline is formed. The mode shift of natural selection can contribute to the formation of urban evolution cline as follows. NFDS in rural environments can maintain genetic polymorphisms. In contrast, PFDS in urban environments

leads to a loss of polymorphism and a unidirectional evolution toward acyanogenesis (Chapter 4). These findings suggest that mode-shift of natural selection between urban and rural environments can also shape traits cline along urban-rural gradients. In Chapter 2, the SEM model showed that urbanization-associated herbivory directory increases HCN frequency. Then, the results in Chapter 4 clarified that herbivory does not directory select HCN as an antiherbivory defense trait, but mediate frequency-dependent selection, leading to differences in HCN frequency between urban and rural areas.

Overall, these results suggest that considering not only the non-linear and multivariate environmental changes in complex urban landscapes, but also the mode-shift of natural selection between urban and rural environments would be beneficial to understand the patterns and mechanisms of cline formation along urban-rural gradients.

Spatial perspective of urban-induced evolution

As urban areas cover 3% of land surface with drastic environmental changes and are still growing, the effects of urbanization on evolution are now a subject of great interest (McDonnell and Hahs 2015; Johnson and Munshi-South 2017; Rivkin et al. 2019; Alberti 2023). I found that impervious surface cover and associated herbivory pressure, which vary along the urban-rural environmental gradients, are important factors driving urban evolution (Chapter 2; 3). These environmental factors drive urban-rural trait variations in parallel across multiple cities (Chapter 3).

Furthermore, evolution can be driven within a very local scale (within a few kilometers) if there is an environmental contrast in forest cover, in other words, a contrast in impervious surface cover (Chapter 4). This notion is supported by several recent studies showing that urbanization-induced evolution can occur even within a few kilometers (Santangelo et al. 2022b; Fukano et al. 2023). Moreover, I have empirically demonstrated that environmental contrasts can even change the mode of natural selection (Chapter 4). Urban areas include various types of environments such as roads, parks, and green areas, shaping

environmental contrasts (Alberti et al. 2020; Santangelo et al. 2022b). Therefore, urban-rural landscape heterogeneity, which makes environmental contrasts even in a single urban area, has significant consequences for the formation of spatial evolutionary patterns. Also, the landscape approach at multiple cities provided insight into the relative contribution of macro-scale climate-associated environmental variation and micro-scale urban-rural environmental variation to determine gene frequencies within populations (Chapter 3). While macro-scale environmental factors are essential to determine gene frequency, urban-rural environmental variation also contributes to determining gene frequency in a similar order of effect size. However, conventional studies have mainly focused on evolution at a micro-scale at which they only investigate the relationship between trait variation and urban-rural environmental gradients in a city or cities (Munshi-South et al. 2016; Winchell et al. 2016; Yakub and Tiffin 2017; Johnson et al. 2018; Theodorou et al. 2021; Breitbart et al. 2023). Thus, only focusing on a specific spatial scale such as a micro-scale can mask the evolutionary dynamics or mechanisms at other scales. Therefore, integrated multiscale surveys are needed. However, studies across multiple spatial scales are still lacking, thus the differences in evolutionary mechanisms by different spatial scales has been unexplored. The approaches used in this study can be utilized to enhance our understanding of the evolution of organisms in urban environments.

Trait multifunctionality, genetic correlation, and trade-offs

Many traits are often multi-functional. For example, Sage (2004) reported that C_4 photosynthesis is related to tolerance of high temperatures, mild or moderate drought, soil salinity, and low CO_2. Also, leaf trichomes of plants not only contribute critically to physical defense against herbivory but also environmental tolerance by influencing light relations, gas exchange, and water repellency (Sack and Buckley 2020). Moreover, the relative importance of each function of a multi-functional trait depends on the surrounding environment (Sack and Buckley 2020). In Chapter 2, it has been suggested that multifunctionality of cyanogenic glycoside, a component of HCN, had an important role in shaping the evolution of cyanogenesis

in a heterogenous urban-rural landscape. In Chapter 4, frequency-dependent natural selection and its mode shift between urban and rural areas suggest that there are genetic trade-offs between competition, HCN tolerance, and HCN production. These results suggest that genetic correlations/trade-offs among multiple traits and the multifunctionality of a single trait have a critical role in evolution. However, this point of view has been ignored in the context of urban evolution. Therefore, addressing genetic correlations and genetic trade-offs among multiple traits and the multifunctionality of a single trait will greatly advance our understanding of the mechanisms of urban evolution.

Future direction

This thesis has provided insight into where and how evolution occurs in complex urban landscapes in multiple spatial contexts. However, this thesis only focused on selection, and did not empirically investigate the effects of gene flow and genetic drift on the evolution. As described in Chapter 1, there are a lot of studies that showed the restricted gene flow and stronger genetic drift in urban areas also lead to evolution. However, most studies investigated the effects of urbanization on selection, genetic drift, and gene flow individually. Therefore, the relative importance of natural selection, genetic drift, and gene flow in shaping the urban evolution is poorly understood (Johnson et al. 2018). With the growing number of molecular techniques become available these days, integrating the effects of natural selection, gene flow, and genetic drift will be valuable to understand evolutionary processes and outcomes due to urbanization. However, it is still difficult to find the contribution of gene flow and selection on fine spatiotemporal scales involved in urbanization-induced evolution from genomic information.

Another direction is that I should investigate ecological consequences of evolution in urban areas. Historically, researchers have studied ecological and evolutionary processes separately (Jewell and Bell 2023). Recently, under both laboratory and field conditions, studies showed evolution can occur on ecological timescales, and ecological and evolutionary processes

can have reciprocal interactions (Post and Palkovacs 2009; Utsumi 2015; Hendry 2019; Fukano et al. 2022). For example, Fukano et al. (2022) showed that the evolution of competitive traits in *Eleusine indica* can influence the community assembly by changing interspecific competitive interactions. Also, evolution-induced ecological changes such as changes in community assembly can feedback to the evolution of a species in the community (Utsumi 2015). Hence, investigating ecological consequences of urbanization-induced evolution will lead us to understand not only evolutionary processes and outcomes, but also the contemporary dynamics in urban ecosystems. However, understanding of ecological consequences of urban evolution is still limited (Alberti et al. 2020; Alberti 2023). Further studies incorporating those points of view will lead us to a better understanding of the mechanisms by which lives adapt and evolve in complex urban ecosystems.

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