May 2019
No.2019-004-e

Journal of Integrated Creative Studies

Report on my visit to Kyoto University as an Advanced Futures Fellow: Evolution of cyanogenesis in *Trifolium repens* in response to spatial and temporal environmental variation in Japan

Marc T. J. Johnson
1Centre for Ecological Research, Kyoto University
[2]Department of Biology, University of Toronto Mississauga
[3]Centre for Urban Environments, University of Toronto
E-mail: marc.johnson@utoronto.ca (corresponding author's address)

Abstract. Here I report on my activity from April to June 2018 as an Advanced Futures Fellow at Kyoto University. During this time, I examined how the plant white clover (*Trifolium repens*), adapts to spatial variation at large and small spatial scales associated with environmental variation in temperature. At a large spatial scale, I sampled 25 *T. repens* populations from the South to the North of Japan, and assayed the frequency of plants that produce hydrogen cyanide (HCN), an antiherbivore defence trait that also reduces tolerance to freezing and varies due to Mendelian inheritance at two loci. I further tested for genetic clines in HCN along urban-rural gradients across three cities (Sapporo, Kyoto and Hiroshima). *Trifolium repens* exhibited a a decrease in the frequency of HCN genotypes with increasing latitude and lower temperatures. The strength of these clines were stronger than clines sampled seven decades ago, suggesting that *T. repens* has continued to adapt to large scale climatic gradients in Japan since its introduction in the 1800s. I detected a cline in HCN along the urban-rural gradient of Kyoto, whereas there were no clear genetic clines in either Sapporo or Hiroshima. My results show that *T. repens* adapts to large scale environmental gradients but seldom adapts to small scale environmental variation in Japan.

Keywords: Climate change, Cyanogenesis, Plant defence, Plant-herbivore, White clover

1. Introduction

This report documents the work I undertook during my two month stay as an Advanced Futures Fellow at Kyoto University from April -June 2018. During this time I was based on the Center for Ecological Research. In addition to the research I performed, I gave six lectures, participated in the Kyoto Forum, and interacted extensively with faculty, students and post-doctoral researchers at Kyoto University and other Japanese universities, giving them feedback on their research and ideas. My work focused on the adaptation of plants to climate, and thus this document represents a report of my preliminary findings.

Understanding how species adapt to their environment is a question of fundamental importance in biology. Evolution of species' populations along environmental gradients has long served as a model to understand how organisms adapt to spatial and temporal changes in their environment (Briggs and Walters, 1972). Spatial gradients in the environment may include changes in temperature with latitude, whereby temperature decreases at higher elevations and higher latitudes. Temporal changes may include seasonal changes or longer-term trends such as historical and contemporary climate change. Here I seek to understand how spatial and temporal variation in temperature in Japan affect the evolution of a functionally important antiherbivore defence trait in white clover (*Trifolium repens* L, Fabaceae).

White clover has long served as a model for the study of adaptation to spatial variation in temperature. This plant exhibits a genetic polymorphism for the production of the chemical defence, hydrogen cyanide (HCN), whereby plants produce either HCN (cyanogenic) or they completely lack this defence (acyanogenic). Variation in this trait is caused by Mendelian inherited allelic variation at each of two loci: CYP79D15 (hereafter referred to as the Ac locus), which produces the cyanogenic glycosides linamarin and lotaustraulin (Olsen *et al.*, 2008), and linamarase (hereafter referred to as the Li locus) which produces the enzyme that hydrolyzes the cyanogenic glycoside to produce HCN (Olsen *et al.*, 2007). Plants require at least one functional allele at both loci to produce HCN, and both loci exhibit partial or whole gene deletions that result in a recessive loss of function of that gene. If a plant is homozygous for the recessive gene deletion at either locus then the plant is acyanogenic. While HCN's primary function is involved in defence against generalist herbivores (Burdon, 1983; Hughes, 1991; Thompson and Johnson, 2016), the presence of HCN also decreases tolerance to freezing (Daday, 1954; Olsen and Ungerer, 2008; Kooyers *et al.*, 2018). Daday (1958) showed that in the native and introduced ranges of *T. repens*, plants exhibit clines in the frequency of HCN producing genotypes within populations. In the native range, he showed that the frequency of HCN producing genotypes decreases from 100% in the south of Europe (Spain), to 0% in the North and East of Europe (e.g. Norway, Russia) (Daday, 1954), where temperatures are below freezing in winter. He went on to show that there are similar but weaker clines in the non-native ranges of North America and Japan, and no clines in South America, Australia or New Zealand. Since Daday's (1958) work, *T. repens* populations have had seven decades to further adapt to environmental gradients in their introduced range, and the Earth has warmed considerably since the 1950s. These global climatic changes may have allowed clines to become steeper in the introduced range and cyanogenic plants to occur at higher latitudes.

Recent work also shows that *T. repens* can adapt to the much smaller spatial gradients in temperature caused by cities and surrounding rural areas. In several North American cities, HCN frequencies are low in downtown regions of cities and higher in suburban and rural areas (Thompson *et al.*, 2016; Johnson *et al.*, 2018). These genetic clines appear to be caused by a gradient in colder minimum winter temperatures in cities. Since urban areas have convergent features throughout the world, it might be the case the plants like *T. repens* consistently adapt to cities throughout their range, but this possibility of global parallel evolution to urban environments has not been tested.

Here I test the hypothesis that the HCN polymorphism in *T. repens* has adapted to spatial and temporal climatic temperature gradients in Japan. I asked three specific questions. First, does *T. repens* exhibit a cline in the frequency of HCN genotypes with either latitude or winter temperatures in Japan? Second, has the strength of this cline changed through time, and has the frequency of HCN genotypes increased in northern populations? And third, does HCN exhibit genetic clines along urban-rural gradients in large Japanese cities, as has been seen in North America. The results reported here contribute to our understanding of how plants adapt to climatic variation. The results presented in this report are preliminary and conclusions should be viewed as tentative and incomplete. The full works will be published separately as part of the M.Sc. thesis of Simon Innes, and as part of the Global Urban Evolution Project (www.globalurbanevolution.com)

2. Material and Methods

The material obtained to answer our research questions were collected at two spatial scales: 1) broad geographic samples from across Japan, which answered questions 1 and 2; and, 2) smaller scale samples within individual cities. My description of the methods for sampling at each of these scales follows.

2.1. Contemporary and historical samples of *T. repens* HCN frequencies across Japan

To address questions 1 and 2, we sampled 25 populations of *T. repens* from the South (Nishi-Oyama: 31.17523 N, 130.59019E) to the North of Japan (Sapporo: 43.10601 N, 141.40253 E) (Figure 1). From each population, we collected seeds from a minimum of 20 plants. These seeds were then grown from seed in a growth chamber at the University of Toronto Mississauga and phenotyped by S. Innes for the presence or absence of HCN using a modified version of the Feigl-Anger assay (Gleadow *et al.*, 2011) as described in (Thompson and Johnson, 2016). The presence of HCN indicates plants that have at least one functional allele at both Ac and Li. We are presently determining the frequency of the dominant and recessive alleles at both loci individually. In total, we assayed 903 plants with an average of 36 plants per population. To obtain historical data from Japan, we extracted the data presented in Appendix I of Daday (1958) for HCN phenotype, geospatial coordinates and temperature from each of the seven populations sampled from Japan in the 1950s. Using these data we used linear regression and analysis of variance (ANOVA) implemented in R to determine how the frequency of cyanogenic genotypes within populations changed with latitude and winter temperature for both the historical and contemporary data sets.

[INSERT FIGURE 1]

2.2 Sampling urban-rural clines in HCN within cities

To answer question 3, we sampled plants along urban-rural clines in each of three large Japanese cities: Sapporo, Kyoto and Hiroshima (Figure 1). From each city we sampled plants from 44-45 populations along an urban-rural clines, as shown in Figures 2. These clines started in the urban centre of each city, continued through the suburbs and then several kilometres into rural areas outside of the city. From each city we sampled 10-20 plants separated by at least 3m, for a total of 874 plants from Sapporo, and 994 plants from each of Kyoto and Hiroshima. From each plant we collected 3-4 leaves, which were stored in 2 mL microcentrifuge tubes in a -80C freezer until the HCN assay was performed as before. We then used linear regression to determine whether HCN frequency within each population was related to the relative distance from the urban centre. Distance from the urban centre was used as a metric of urbanization, which is well correlated with the percentage of impervious surface in each of the three cities sampled. Distance was relativized based on the Euclidean distance from the urban center, with 0 corresponding to the urban center of a city, and a distance of 1 representing the most distance rural site; typically 30-40 km from the downtown region (Figure 2).

[INSERT FIGURE 2]

3. Results

3.1 Latitudinal clines in HCN

The frequency of HCN within populations declined at higher latitudes and increased with higher winter temperatures. Correspondingly, the frequency of HCN was significantly related to latitude (Slope = -0.045, P = 0.005, R^2 = 0.29) and winter temperatures in January (Slope = 0.037, P = 0.014, R^2 = 0.24).

The historical frequency of HCN within populations showed no significant relationship with either latitude (Slope = -0.006, P = 0.75, R^2 = 0.02) or temperature (Slope = 0.008, P = 0.59, R^2 = 0.016). When the contemporary and historical data for HCN frequency is included in a single model, there is a significant effect of latitude ($F_{1,28}$ = 8.76, P = 0.006), but no difference in average HCN frequency between the two samples (contemporary vs historical) ($F_{1,28}$ = 2.16, P = 0.152), and there was no interaction between latitude and when plants were sampled ($F_{1,28}$ =1.71, P = 0.20; Figure 3). A visual inspection of Figure 3 suggests that the slope of HCN versus latitude is steeper in the contemporary sample compared to the historical sample, with higher frequencies of HCN in the South and roughly equivalent frequencies in the North.

[INSERT FIGURE 3]

3.2 Urban rural clines in HCN

Of the three cities sampled, a significant cline in HCN versus distance from the city center was observed in only a single city (Figure 4). In Kyoto, there was a significant negative relationship between HCN frequency and distance from the city centre (Slope = -0.193, P = 0.045, R^2 = 0.092; Figure 4B), indicating that the frequency of HCN was higher in the city than in the surrounding rural areas. No relationship was found in either Hiroshima (Slope = -0.01, P = 0.931, R^2 = 0; Figure 4C) or Sapporo (Slope = 0.111, P = 0.2, R^2 = 0.039; Figure 4A).

[INSERT FIGURE 4]

4. Discussion

The results collected during my Advanced Futures Fellowship show that *T. repens* has evolved clear clines in the frequency of HCN from the South to the North of Japan, which is associated with a large gradient in temperature. Of the three cities examined, only one city showed a significant cline in HCN. I discuss the importance of these results in relationship to adaptation to spatial and temporal variation in environments.

4.1 Contemporary and historical clines in HCN across Japan

The contemporary sample of *T. repens* across Japan showed a clear signature of an adaptive cline in genotype frequency. Consistent with Daday's (Daday, 1954; Daday, 1958), the frequency of HCN in populations declined from the South to the North of Japan. The percentage of HCN within populations was predicted to increase by 3.7% for every degree of warming in January, which is consistent with colder temperatures selecting against cyanogenic genotypes. Based on the equation of the best fitting regression line, 63% of all plants were cyanogenic at the very south of Kyushu, and 0% of plants were predicted to be cyanogenic (i.e. 100% acyanogenic) at the very northern tip of Hokkaido.

Based on data from seven populations sampled in Japan, Hunor Daday concluded that *T. repens* had evolved a cline in one of the two loci (Ac) in response to the temperature gradient that occurs in this country. However, this conclusion was not based on any statistics, but instead relied on a qualitative assessment of frequencies at Ac and Li in relation to winter temperature and latitude. While he did not specifically look at the frequency of HCN plants within populations, it is surprising that we found no relationship between HCN and latitude or temperature in his sample. Moreover, I tested the correlation between the frequency of Ac and temperature in his dataset and found it to be non-significant (r = 0.21, P = 0.64), suggesting that Daday's conclusions about an adaptive cline in Japan were unfounded. Based on this comparison of the contemporary and historical samples of *T. repens* in Japan, it would appear that populations in Japan have become better adapted to spatial variation in climate since the 1950s. Since the lines cross at higher latitudes (Figure 3), it indicates that cyanogenic genotypes have not shifted further north, as might be predicted as a response to climate change. However, when we test these observations explicitly, we find no difference in the relationship between HCN frequency vs latitude between the two dataset. Given the relatively small number of populations sampled by Daday, it is difficult to discern whether this lack of an interaction reflects the low statistical power inherent to Daday's sampling of just seven populations, or whether there has not been sufficient time for adequate evolution change since the 1950s. Unfortunately, these two possibilities cannot be disentangled, but we are looking to other countries to see if this can be resolved from a larger global sample.

4.2 Adaptation of *T. repens* to cities in Japan

Based on earlier results from North America (Thompson *et al.*, 2016; Johnson *et al.*, 2018), we hypothesized that cities in Japan would exhibit urban-rural clines in the frequency of HCN, with the lowest level of HCN in urban locations. Contrary to our prediction, we did not detect any cline in two cities (Hiroshima and Sapporo) and a weak cline in the opposite direction to what was predicted in Kyoto (Figure 4). These results have several possible interpretations. First, it is possible that there is something unique to North American cities to make it more likely for *T. repens* to adapt to those cities. Although North American cities are on average younger than Japanese cities, this possibility seems unlikely since we have now seen clines in other cities throughout the world, including other Asian cities included in the Global Urban Evolution project. The other possible explanation for the cline in Kyoto, is that all rural samples were sampled from an elevation that was ca. 70m higher. However, given that HCN was on average 19% lower in the rural compared to the urban area, and the temperature difference between these habitats is expected to be <0.5C, then this degree of temperature difference cannot account for the large difference in HCN. Perhaps the most likely explanation is that the herbivore communities were higher in the city than the rural area, a possibility that remains to be tested.

4.3 Conclusions

Based on my study of plant adaptation across Japan, I can make several tentative conclusions about the evolution of HCN within *T. repens* in response to spatial and temporal variation in the environment. First, it is clear that *T. repens* has adapted to latitudinal variation in climate since its introduction to Japan in the 1800s. Second, circumstantial evidence suggests that seven decades since Daday's work has allowed populations to adapt more to climate than was observed in the 1950s, although this conclusion remains tentative and is not entirely supported by all analyses. Finally, we found limited evidence for adaptation of *T. repens* to cities, with the one significant cline being opposite to what has been observed in North American cities. These results suggest that climate over large spatial scales is most important in driving the evolution of adaptations of *T. repens* to temperature gradients in Japan.

5. Acknowledgements

I have many people to thank for this project and my time in Japan. Dr. Masotoshi Murase and Dr. Takayuki Ohgushi were both generous and kind in their invitation to me and for being gracious hosts. I thoroughly enjoyed my time as an Advanced Futures Visiting Fellow, which led to productive research, discussions and collaborations. I thank the scientists in the Center for Ecological Research for hosting my stay, including Hirokazu Toju, who allowed me to use his lab and equipment. The International House residence provided us with generous space for my accommodations. Dr. Shunsuke Utsumi hosted my visit to Sapporo University and he and his lab worked with me to sample and then assay the urban population. Reagan, Mae and Oscar Johnson collected many of the samples used in this study. All assays unrelated to cities in this report were performed by Simon Innes, a M.Sc. student in my lab. James Santangelo performed all analyses related to global clines.

6. References

Briggs, D. and Walters, S. M., *Plant Variation and Evolution*, McGraw-Hill Book Company, New York, 1972

Burdon, J., *Trifolium repens* L. *J. Ecol.*, **71**, 307-330, 1983

Daday, H., Gene frequencies in wild populations of *Trifolium repens*. *Heredity*, **8**, 377-384, 1954

Daday, H. Gene frequencies in wild populations of *Trifolium repens* L. *Heredity*, **12**, 169-184, 1958

Gleadow, R., Bjarnhold, N., Jørgensen, K., Fox, J. and Miller, R. In *Research Methods in Plant Sciences, Volume 1: Soil Allelochemicals,* Eds. Narwal, S., Szajdak, L. and Sampietro, D., Studium Press, Houston, TX, 2011

Hughes, M., The cyanogenic polymorphism in *Trifolium repens* L. (white clover). *Heredity*, **66**, 105-115, 1991

Johnson, M.T.J., Prashad, C.M., Lavoignat, M. and Saini, H. S., Contrasting the effects of natural selection, genetic drift and gene flow on urban evolution in white clover (*Trifolium repens*). *Proc. R. Soc. B*, **285**, 20181019, 2018

Kooyers, N.J., Hartman Bakken, B., Ungerer, M. and Olsen, K. M., Freeze-induced cyanide toxicity does not maintain the cyanogenesis polymorphism in white clover (*Trifolium repens*). *Am. J. Bot.*, **105**, 1-8, 2018

Olsen, K. M., Hsu, S. and Small, L. L., Evidence on the molecular basis of the Ac/ac adaptive cyanogenesis polymorphism in white clover (*Trifolium repens* L.). *Genetics*, **179**, 517-526, 2008

Olsen, K. M., Sutherland, B. L. and Small, L. L., Molecular evolution of the *Li/li* chemical defence polymorphism in white clover (*Trifolium repens* L.). *Molecular Ecology*, **16**, 4180-4193, 2007

Olsen, K.M. and Ungerer, M.C., Freezing tolerance and cyanogenesis in white clover (*Trifolium repens* L. Fabaceae). *Int. J. Plant Sci.*, **169**, 1141-1147, 2008

Thompson, K.A. and Johnson, M.T.J., Antiherbivore defenses alter natural selection on plant reproductive traits. *Evolution*, **70**, 796-810, 2016

Thompson, K. A., and Renaudin, M. and Johnson, M.T.J., Urbanization drives the evolution of parallel clines in plant populations. *Proc. R. Soc. B*, **283**, 20162180, 2016

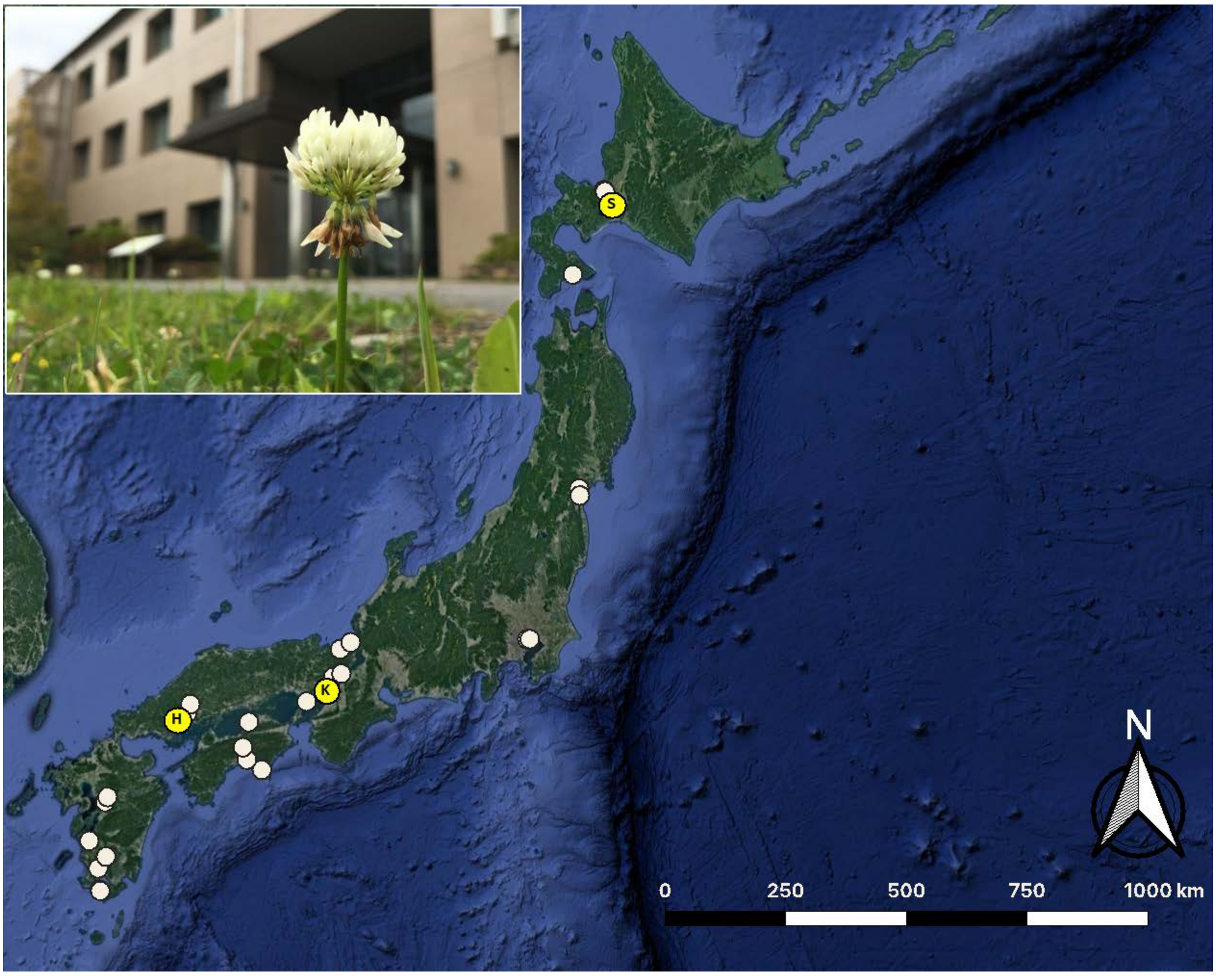

Figure 1. Map of Japan showing sampling locations of *T. repens* populations along latitudinal gradient (white circles), as well as the cities sampled (yellow circles) when testing for urban-rural clines in HCN within populations. The three cities included Sapporo (S), Kyoto (K) and Hiroshima (H). Inset shows *T. repens* growing outside of the Center for Ecology at Kyoto University.

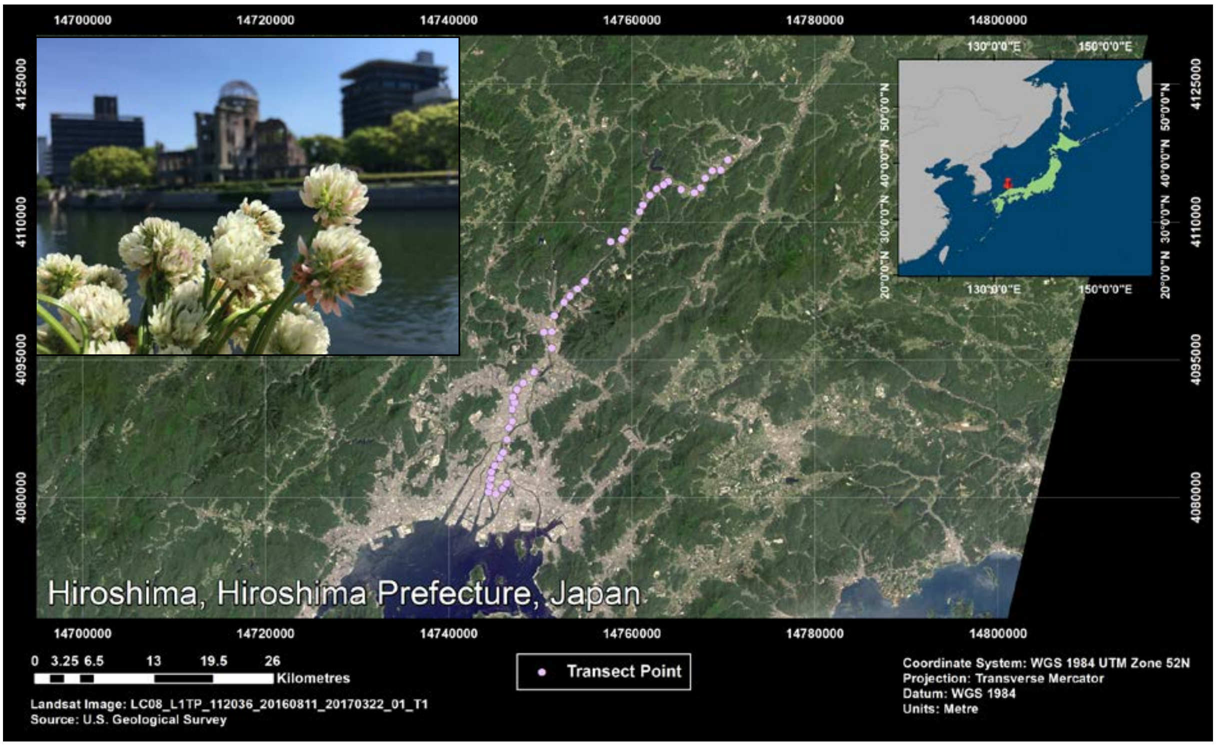

Figure 2. Sampling sites in Hiroshima city, shown as light purple circles. We tested for a cline in HCN frequency along the urban-rural cline. Insets show white clover plants collected from Peace Memorial Park, Hiroshima, and the location of the city within Japan.

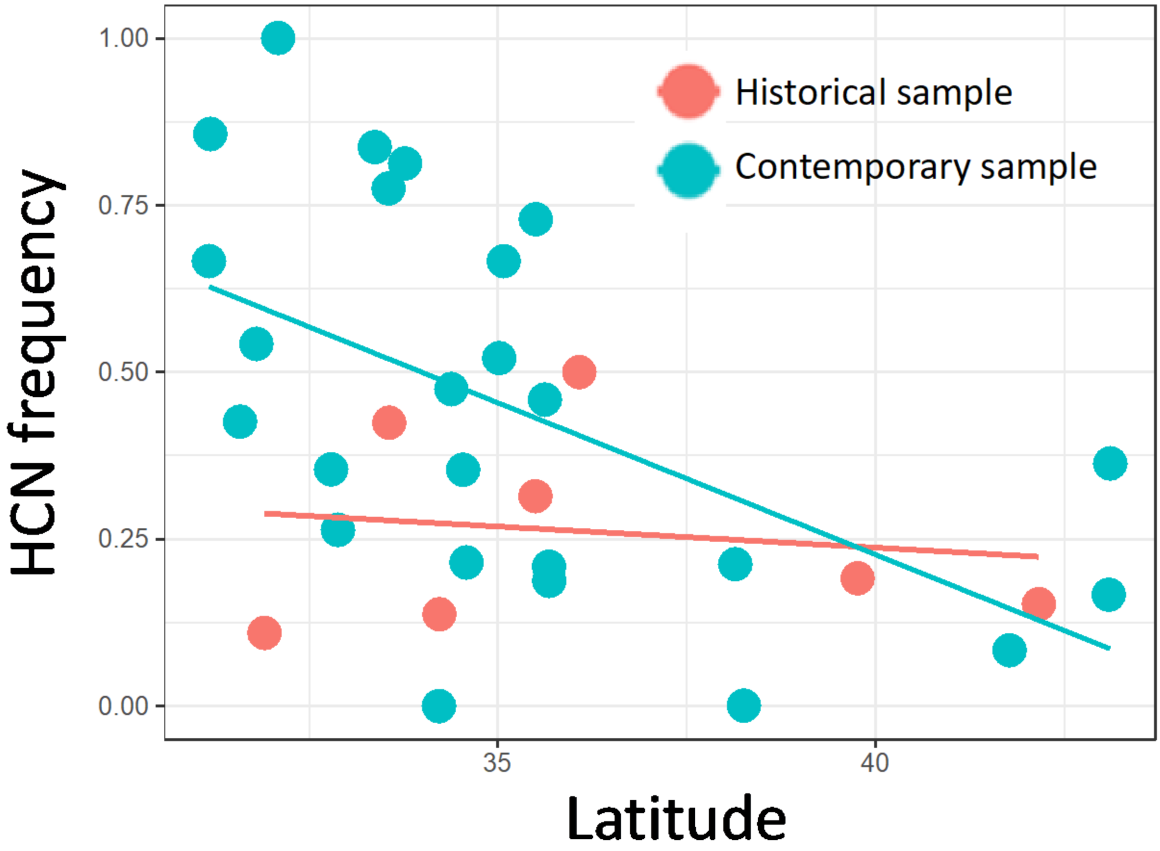

Figure 3. Relationship between the frequency of HCN within populations and latitude from my sample in 2018 (contemporary) and Daday's sample from the 1950s (historical).

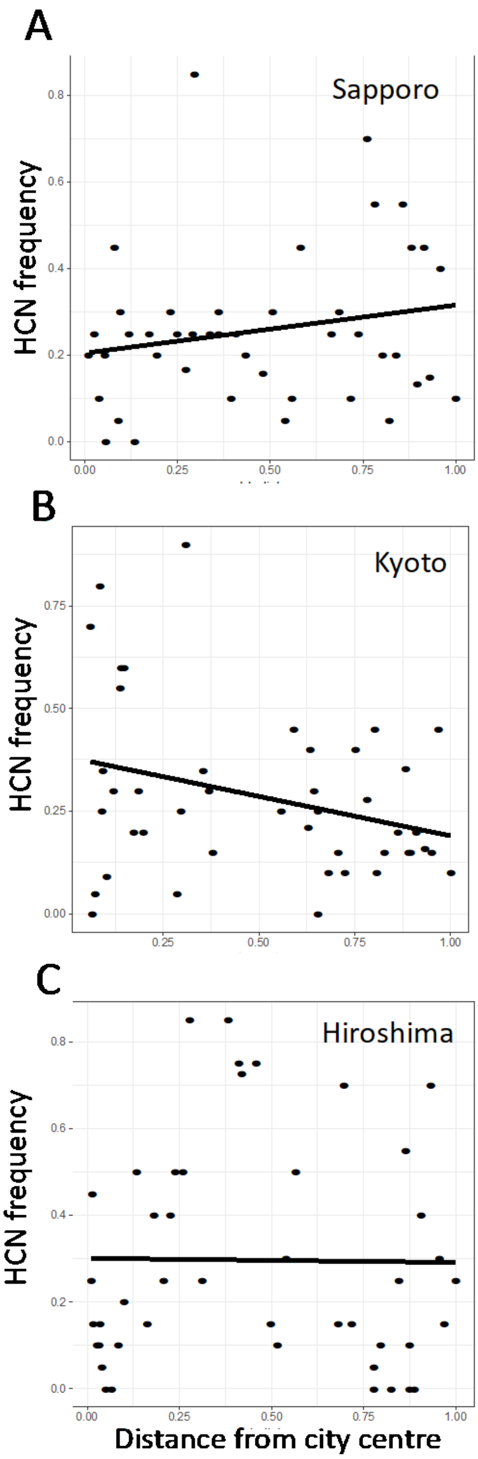

Figure 4. Relationship between the frequency of HCN producing plants within populations along urban-rural clines in (A) Sapporo, (B) Kyoto, and (C) Hiroshima. Each point represents a population and the line show the best-fitting linear regression to the data. Distance from the urban center are standardized with 0 representing the urban center and 1 representing the most rural location.