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Evolution in response to climate in the native and introduced ranges of a globally distributed plant

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The extent to which species can adapt to spatiotemporal climatic variation in their native and introduced ranges remains unresolved. To address this, we examined how clines in cyanogenesis (hydrogen cyanide [HCN] production—an antiherbivore defense associated with decreased tolerance to freezing) have shifted in response to climatic variation in space and time over a 60-year period in both the native and introduced ranges of *Trifolium repens*. HCN production is a polymorphic trait controlled by variation at two Mendelian loci (*Ac* and *Li*). Using phenotypic assays, we estimated within-population frequencies of HCN production and dominant alleles at both loci (i.e., *Ac* and *Li*) from 10,575 plants sampled from 131 populations on five continents, and then compared these frequencies to those from historical data collected in the 1950s. There were no clear relationships between changes in the frequency of HCN production, *Ac*, or *Li* and changes in temperature between contemporary and historical samples. We did detect evidence of continued evolution to temperature gradients in the introduced range, whereby the slope of contemporary clines for HCN and *Ac* in relation to winter temperature became steeper than historical clines and more similar to native clines. These results suggest that cyanogenesis clines show no clear changes through time in response to global warming, but introduced populations continue to adapt to their contemporary environments.

KEY WORDS: **Adaptation, climate change, cline, cyanogenesis, plant defense, *Trifolium repens*, white clover.**

The rate of global climate change in temperature and precipitation has been increasing over the past 100 years, with particularly rapid warming during the past four decades (IPCC 2014; Lenssen et al. 2019). These climatic changes affect the ecology and evolution of species by influencing the performance and fitness of individuals within populations. Consequently, species have three options to mitigate the effects of climate change. First, they can disperse to more suitable environments, and there is substantial evidence that many species are experiencing range shifts (Parmesan 2006; Chen et al. 2011). Second, they can acclimate to changes in climate through phenotypic plasticity in physiology and life history (Franks et al. 2014; Oostra et al. 2018). Lastly, populations may adaptively evolve to changing climatic conditions (Umina et al. 2005; Kelly et al. 2012; Franks et al. 2014; Helm et al. 2019). Compared to the roles of dispersal and plasticity, the extent to which species' populations can adapt to rapid and ongoing climate change has received less attention (Parmesan 2006; Brady et al. 2019; Capblancq et al. 2020).

In contrast to the relative paucity of evidence of evolution to temporal changes in climate, a long history of research shows that species' populations frequently have the capacity to adapt to spatial gradients in climate (Huxley 1938; Briggs and Walters

2016). In their seminal work, Clausen et al. (1940) showed that there was a genetic basis to local adaptation in morphological, physiological, and phenological traits along environmental gradients, which was established by reciprocally transplanting clones of individuals originating from different elevations from the coast of California to the Sierra Nevada. There have subsequently been numerous examples of genetic clines along climatic gradients in diverse organisms (Huey et al. 2000; Mullen and Hoekstra 2008; Züst et al. 2012). For example, *Arabidopsis thaliana* exhibits a latitudinal cline in flowering time driven by variation in alleles at the *FRIGIDA* locus and its epistatic interaction with the *FLC* locus (Caicedo et al. 2004; Stinchcombe et al. 2004). Despite many examples of spatial adaptive clines, whether individual populations along these clines can additionally adapt to rapid temporal changes in climate has been subject to much less investigation (Bradshaw and Holzapfel 2001; Umina et al. 2005; Vigouroux et al. 2011; Nevo et al. 2012; Franks et al. 2016).

Circumstantial evidence suggests that populations should be able to adapt to rapid climate change. Some of the best evidence comes from studies of how single populations evolve in response to extreme climatic events (Grant et al. 2017; Franks et al. 2018). In *Brassica rapa*, populations adapt to drought by evolving earlier flowering times after only a few generations of exposure (Franks et al. 2007), but whether such shifts play out over an entire species' range is unclear. On the Galápagos island of Daphne Major, populations of Darwin's finches have repeatedly evolved smaller or larger bodies and beaks following changes in food availability caused by interannual fluctuations in El Niño and La Niña climatic events (Grant and Grant 2002). However, other studies find evidence of maladaptation to current climates, in which local populations are not optimally adapted to their local climate (Wilczek et al. 2014; Kooyers et al. 2019; Anderson and Wadgymar 2020). For example, *A. thaliana* populations from historically warmer sites had higher average relative fitness than local genotypes at any given site across their native range (Wilczek et al. 2014). This result was associated with a shift in local climatic warming over a few decades from when the seeds were collected to when they were experimentally planted (Wilczek et al. 2014), providing evidence for an adaptation lag across the species' range. Such maladaptation may occur because both abiotic and biotic selection pressures are not shifting in synchrony with one another (CaraDonna et al. 2014; Wadgymar et al. 2017a,b). These examples highlight the challenges native species may face in rapidly adapting to changing climates. However, adaptation to climate in a species' introduced range has received less attention, and may differ from evolution in the native range if populations have undergone a bottleneck due to a founder event, had less time to adapt, or are experiencing novel environments compared to the native range (Colautti and Lau 2015).

There is a growing appreciation that adaptation in a species' introduced range can play a critical role in its persistence and ecological success (Ellstrand and Schierenbeck 2000; Vandepitte et al. 2014; Szücs et al. 2017; Bock et al. 2018, 2021). For example, adaptive changes in phenology have enabled a wetland invader, *Lythrum salicaria*, to spread northward from the eastern seaboard of the United States, to northern Ontario, Canada (Colautti and Barrett 2013). Populations of *L. salicaria* shifted toward earlier flowering dates as they moved northward, allowing them to adapt to a shorter growing season (Colautti and Barrett 2013). North American populations of *Drosophila subobscura* have evolved a parallel latitudinal cline in wing length in their introduced range two decades following introduction, which largely converges on the ancestral European cline (Huey et al. 2000), demonstrating that introduced populations can rapidly adapt to novel environments. Although clines may evolve in the introduced range, the amount of standing genetic variation and global heterogeneity in climate change will likely cause introduced populations to vary in how they evolve toward novel climatic optima. Clines in introduced areas would be expected to continue to evolve following introduction—eventually more closely resembling clines occurring in their native range. Although there are examples of adaptation to climatic gradients facilitating the expansion of species in their introduced ranges (Huey et al. 2000; Alexander et al. 2009; Samis et al. 2012; Colautti and Barrett 2013; Vandepitte et al. 2014; Szücs et al. 2017), tests of widespread species that combine historical and contemporary data would be particularly powerful to address these problems (Bradshaw and Holzapfel 2001; Grant and Grant 2002; Umina et al. 2005; Parmesan 2006; Kelly et al. 2012; Helm et al. 2019).

Although clover (*Trifolium repens* L., Fabaceae) is an ideal model to test hypotheses of adaptation to climatic variation in space and time in both a species' native and introduced ranges. *Trifolium repens* is polymorphic for an antiherbivore defense—the production of hydrogen cyanide (HCN) following tissue damage (cyanogenesis; Mirande 1912; Armstrong et al. 1913; Hughes 1991). Cyanogenesis in *T. repens* is a classic example of the maintenance of an adaptive polymorphism along an environmental gradient (Daday 1965; Kooyers and Olsen 2012, 2014). Individuals with functional (dominant) alleles at both underlying loci (hereafter *Ac* and *Li*) constitutively produce the two chemical precursors required for HCN production. However, these same individuals have reduced tolerance to abiotic stressors (e.g., freezing; Kooyers et al. 2018), potentially because of energetic costs associated with producing the cyanogenic precursors (Kakes 1989). Thus, the production of HCN represents a clear trade-off between the benefits of defense against herbivores and its cost in stressful conditions. Consistent with this expectation, the frequency of functional alleles is reduced in colder climates

(Kooyers and Olsen 2012; Santangelo et al. 2020; Daday 1954a,b, 1965). Specifically, *T. repens* displays clines whereby the frequencies of functional *Ac* and *Li* alleles decrease with increasing latitude and at higher elevation (Daday 1954a,b). For both latitudinal and elevational gradients, mean winter temperature was the best predictor of these clines, although potential biotic factors were not surveyed (Daday 1954a,b). Daday's (1954a,b, 1958) data on the evolution of clines are the first such data from plants and among the first from any organism (McConnell 1935; Dobzhansky and Queal 1938). Thus, its global distribution and long history of study make *T. repens* an excellent model system for studying adaptation to spatiotemporal climatic variation.

Latitudinal clines in cyanogenesis are known to occur in both *T. repens*' native and introduced ranges. *Trifolium repens* is native to Europe and western Asia and was introduced throughout the world for forage and fertilizer between the mid-1700s and early 1900s (Daday 1958; Burdon 1983; Kjærgaard 2003). A strong latitudinal cline was found in *T. repens*' native range (Daday 1954a), with cyanogenic genotypes fixed (i.e., 100% of plants produced HCN) in populations around the Mediterranean, and acyanogenic genotypes fixed (i.e., 0% of plants producing HCN) in populations from northern Europe. Similar clines have also been documented in its introduced range, but these clines were historically weaker or even absent (Daday 1958; Ganders 1990; Kooyers and Olsen 2012, 2013). Cyanogenesis clines have also evolved along urbanization gradients (Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020), demonstrating the ability of *T. repens* to evolve to novel climatic gradients at varying spatial scales (Wright et al. 2018). In the absence of climate change, cyanogenesis clines are expected to continue to become steeper to more closely resemble clines in the native range, but despite *T. repens*' ability to evolve in response to novel climatic gradients, whether cyanogenesis clines can evolve in response to recent temporal changes in climate is unclear (IPCC 2014; Lenssen et al. 2019). If cyanogenesis clines are slowly evolving in response to climate change, our study time period may be too short to capture this adaptation. The evolution of cyanogenesis may also be driven by biotic factors that may not keep pace with climate change, or its evolution may be driven by multiple forces (i.e., a combination of biotic and abiotic factors) that are changing simultaneously (CaraDonna et al. 2014). Additionally, temporal heterogeneity in selective pressures would slow the formation of clines. The white clover system provides the opportunity to understand evolution in response to climate change in a naturally replicated framework using geographically disparate introduction events as replicates of the evolutionary process.

Here, we seek to understand how *T. repens* evolves in response to spatial and temporal variation in climate in its native and introduced ranges. To address this objective, we first compared the frequencies of alleles at two loci (*Ac* and *Li*) underlying the production of HCN between collections separated by six decades. This comparison allowed us to address the hypothesis that *T. repens* has rapidly adapted to global climate change. If the cyanogenesis polymorphism has been important for the adaptation of *T. repens* to climate change, then we predict that the frequencies of *Ac* and *Li* alleles would increase in populations that have experienced increases in mean winter temperature. Specifically, if warmer winter temperatures relax selection against dominant alleles, or if warmer temperatures lead to higher herbivory or otherwise increase selection for the cyanogenic phenotype, then the frequencies of *Ac* and *Li* in these populations should also increase (Fig. 1a). However, if cyanogenesis is not important for the adaptation of populations to climate change, then dominant allele frequencies are expected to remain relatively unchanged through time. Second, to dissect the possible role of biotic interactions in the evolution of HCN production across *T. repens*' native range, we asked: Do cyanogenesis clines correspond to levels of herbivore damage? If HCN production is more often driven by biotic factors rather than environmental stressors, then we might expect to see an increase in the frequency of HCN production where herbivory is highest (e.g., increased herbivory at lower latitudes; Coley and Aide 1991; Anstett et al. 2016; Kooyers et al. 2017). By contrast, if abiotic stressors solely determine the frequency of HCN production within populations, then we expect to see no change in herbivory across latitudes. Third, we leverage our comparison of cyanogenesis clines between the native and introduced ranges, to ask: Have *T. repens* clines in introduced regions evolved to be more similar to clines in the native range? If introduced populations have become better adapted to spatial climatic gradients in the introduced range, then we expect the slopes of clines in relation to winter temperature to become steeper through time and more similar to those of the native range (Fig. 1b). If introduced populations are not continuing to adapt in their respective ranges, then we expect that clines have remained relatively unchanged through time.

Methods

STUDY SYSTEM

Trifolium repens is an allotetraploid perennial herbaceous legume that originated from the hybridization of two diploid progenitors during the last glacial maximum (Williams et al. 2012; Griffiths et al. 2019). It thrives in regularly grazed pastures and mowed lawns by spreading clonally via stolons (Burdon 1983). Its flowers are borne on dense inflorescences that are pollinated by bees. Plants are outcrossing due to gametophytic self-incompatibility (Atwood 1940; Burdon 1983; Casey et al. 2010), leading to high

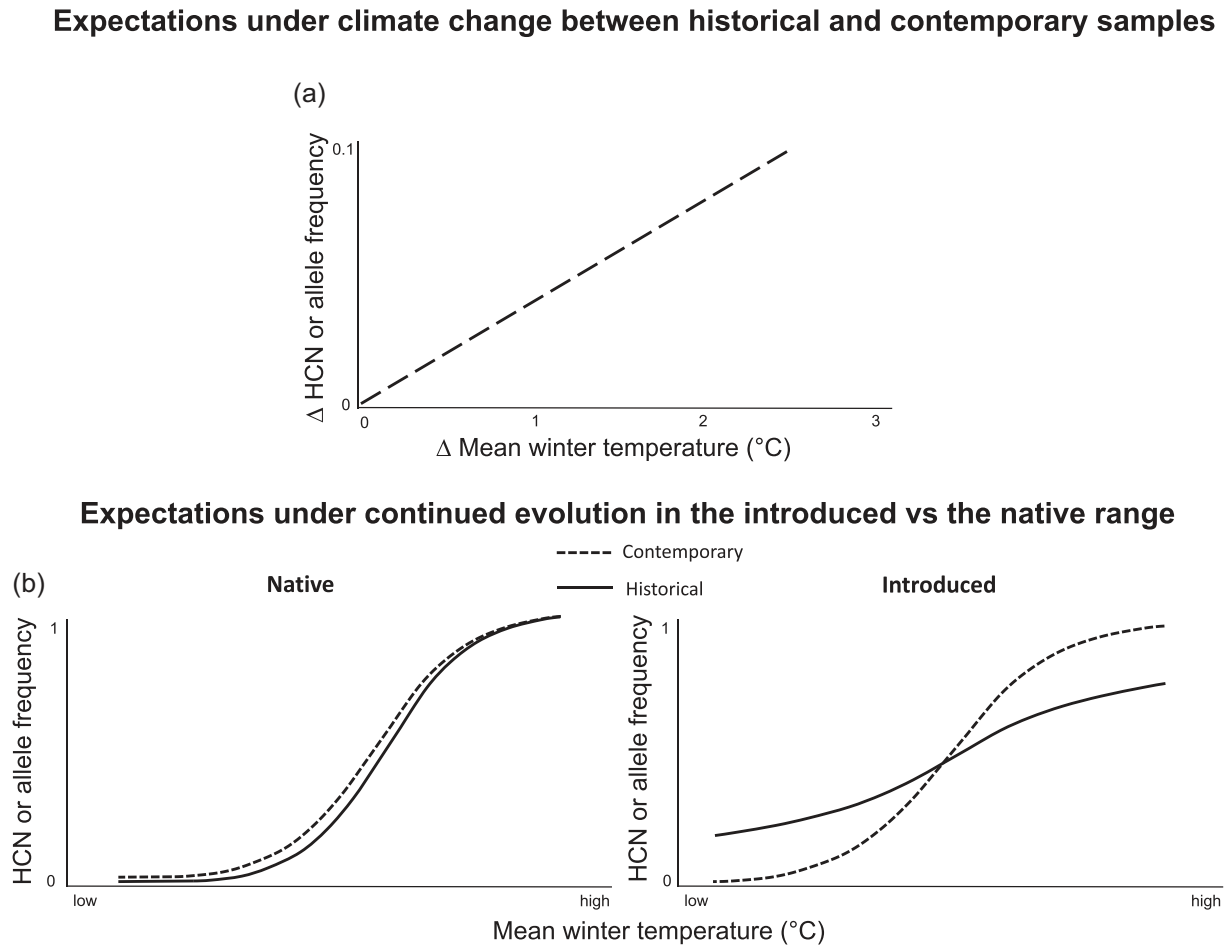

Figure 1. **Predictions of how HCN production or dominant (*Ac* or *Li*) allele frequencies have changed through time in *Trifolium repens*. (a) Predicted changes in the relationship between frequencies of HCN production, *Ac*, or *Li* versus changes in mean winter temperature through time between recent and historical populations if they are evolving in response to climate change. Frequencies of HCN production, *Ac*, or *Li* within populations are expected to increase as a function of changes in temperature through time similarly to how cyanogenesis varies across space (dashed line). (b) Predicted changes in the slopes of the relationship between HCN production, *Ac*, or *Li* with a gradient in mean winter temperature between recent and historical populations in the introduced versus the native ranges of *T. repens*. Clines are expected to steepen through time if *T. repens* is continuing to adapt to spatial temperature gradients in the introduced range, but the clines will remain relatively unchanged in the native range if native populations are at an evolutionary equilibrium.**

levels of standing genetic variation across both its native and introduced ranges (Kooyers and Olsen 2013, 2014).

Cyanogenesis in *T. repens* is a polymorphic trait regulated by independently segregating polymorphisms at the *Ac* and *Li* loci (Mirande 1912; Armstrong et al. 1913; Corkill 1942; Atwood and Sullivan 1943). Expression of this polymorphism is controlled by the epistatic interaction between these two loci. The first locus encodes a closely linked three-gene cluster for the biosynthesis of the cyanogenic glucosides lotaustralin and linamarin (Melville and Doak 1940; Olsen et al. 2008; Olsen and Small 2018)—we denote this locus *Ac* based on its historical use in the literature (Corkill 1942; Daday 1954a; Hughes 1991). The second locus (*Li*) encodes the hydrolyzing enzyme linamarase (Coop 1940; Olsen et al. 2007), which cleaves sugar moieties from lotaustralin and linamarin leading to the production of HCN. Because cyanogenic glucosides are stored in vacuoles, whereas linamarase is found in the apoplast (Kakes 1985; Gruhnert et al. 1994), HCN is only produced following tissue lysis when cyanogenic glucosides and linamarase have the opportunity to interact (Hughes 1991). HCN interrupts the electron transport chain in cellular respiration by inhibiting cytochrome-c oxidase (Antonini et al. 1971), making it a potent antiherbivore defense (Angeesing 1974; Dirzo and Harper 1982; Thompson and Johnson 2016; Santangelo et al. 2019). The presence of a single dominant allele at both loci (i.e., *Ac*–*Li*–) confers a plant with the cyanogenic phenotype (cyanotype AcLi: Corkill 1942). Individuals homozygous for gene deletions at either locus are acyanogenic (cyanotypes Acli, acLi, and acli; Olsen et al. 2007, 2008, 2013).

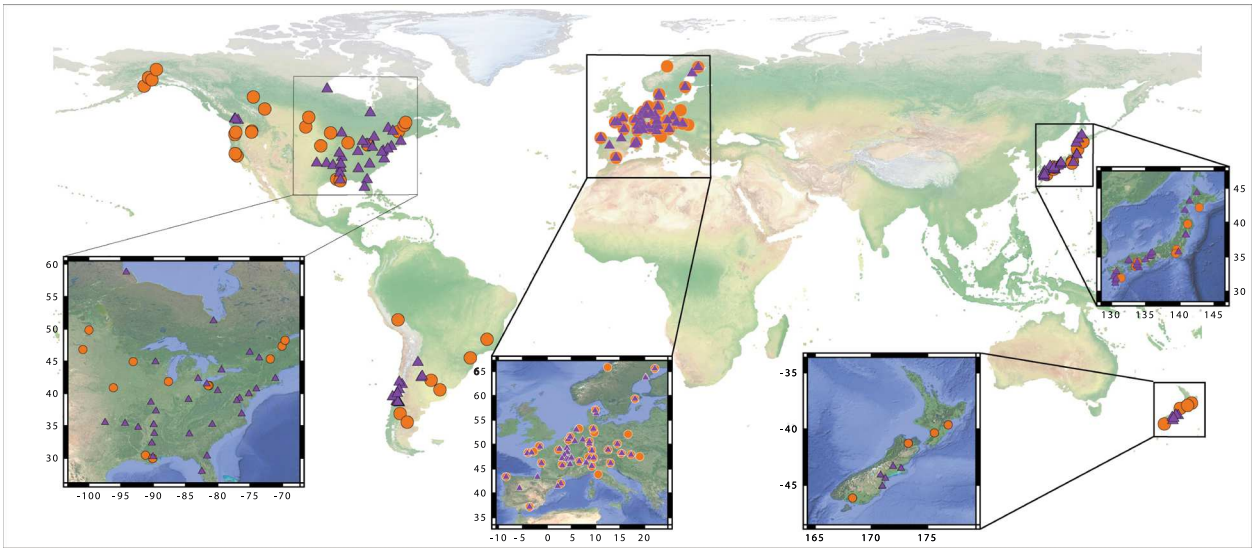

Figure 2. **Map of global distribution of historical and contemporary sampling locations. Contemporary populations (purple triangles) are overlaid onto historical sampling locations (orange circles) obtained from the appendices of the work of Daday (1954a, 1958) in both the native (shapes outlined in white) and the introduced (shapes outlined in black) ranges of *Trifolium repens*.**

CALCULATION OF ALLELE FREQUENCIES

The classic studies of Hunor Daday served as the source for all historical allele frequencies for this project (Daday 1954a, 1958). We extracted allele frequency data from the appendices of his 1954a and 1958 papers, for all countries shown in Figure 2. In regions where historical sampling was sparse (e.g., New Zealand and South America), we chose to keep locations from outside of our contemporary sampling to increase statistical power in these sampling regions. We excluded historical samples from the United Kingdom and Ireland because there were many populations ($n = 12$) not represented in our sampling, which could have biased our results. Regardless, our results are qualitatively identical even if these samples are included (Table S1). Contemporary samples were taken from the native and introduced ranges of *T. repens* between 2008 and 2018 (Table S2). In *T. repens*' native range, samples were collected in summer 2018 from sites spanning continental Europe, from the south of Spain to the north of Sweden, and west from the Atlantic coast to eastern Europe (Fig. 2). At each site, ~20 ripe infructescences were collected, ensuring that plants were at least 3 m apart to avoid sampling the same clone. Seeds were returned to the University of Toronto, Canada, scarified with sandpaper, and germinated in 100-mL pots filled with Pro-Mix LP15 (Premier Tech, Rivière-du-Loup, Canada) potting soil. Plants were grown in Conviron MTPS (Winnipeg, Canada) environmental chambers at 350 μmol of light with an 18-hour day: 6-hour night cycle at 25°C. Each individual received three to five pellets of Nutricote Total 14-13-13 (Arysta, New York, NY, USA) once its first true leaf unfurled. Tissue was collected for cyanogenesis assays when plants had produced at least three to four medium-sized leaves.

Samples from the introduced range were obtained from North America, South America, Japan, and New Zealand between 2008 and 2018 with the aim of resampling the geographic range investigated by Daday (1958) in each region. In North America, samples were taken from the Pacific Northwest, and the central and eastern half of Canada and the United States, extending south to Louisiana and Florida, and north to the coast of Hudson's Bay in Manitoba and Ontario (Fig. 2). In South America, sampling was done from south-central Chile to northern Argentina, west to the Pacific coast, and east to central Argentina (Fig. 2). In Japan, samples were taken from the southern tip of Kyushu to northern Hokkaido (Fig. 2). Finally, in New Zealand samples were taken from Otago in the south to Pegasus Bay on the South Island (Fig. 2). A combination of stolon cuttings and mature infructescences were collected from each population, maintaining at least 3 m between plants. When stolon cuttings were collected, tissue was immediately stored in a −80°C freezer and then assayed. When infructescences were collected, seeds were removed, germinated, and grown to the three- to four-leaf stage. From these plants, we collected, stored, and assayed fresh tissue in an identical manner as described above for stolons collected from natural populations. A total of 6947 stolons were collected from 35 populations originating from North America and New Zealand (mean ± SE: 212 ± 32.7, range = 31−1032). A total of 3628 individuals from 96 populations originating from North America, South America, Japan, and Europe (mean ± SE: 37 ± 1.18, range = 9−54) were grown from seed. In all ranges, sampling of rural sites was prioritized where possible since recent research shows that cyanogenesis frequencies evolve in response to urbanization

(Thompson et al. 2016; Johnson et al. 2018; Santangelo et al. 2020).

The frequency of dominant *Ac* and *Li* alleles within each population was inferred from cyanogenesis phenotyping with Feigl-Anger assays (Feigl and Anger 1966; Gleadow et al. 2011), using an optimized method from Thompson et al. (2016). Briefly, leaf tissue from individuals was placed into every second well of 96-well plates, leaving empty wells between each sample to prevent bleeding on the assay paper between plants. We added 80 μL of H_2O to each well and macerated the tissue within them. A precut filter paper saturated, then dried, with Feigl-Anger reagent was then placed over each 96-well plate and placed in an incubator at 37°C. After 3 hours, individuals were scored for the presence of HCN by visual inspection of a blue color on the filter paper (cyanotype AcLi). For all acyanogenic individuals, successive tests were conducted by adding 20 μL of H_2O and 80 μL of 0.2 EU/mL linamarase (LGC Standards CDX-00012238-100, Teddington, UK), or 50 μL of H_2O and 30 μL of 10 mM linamarin (Sigma-Aldrich 68264, St-Louis, MO, USA) to identify Acli and acLi cyanotypes, respectively. If an individual failed to produce any positive score after all three assays, its genotype was deemed homozygous recessive at both loci (i.e., acli cyanotype). Previous studies that have compared cyanotype based on Feigl-Anger assays with genotypes using PCR based assays have found near perfect correspondence between the two (Olsen et al. 2007, 2008; Kooyers and Olsen 2012; Thompson et al. 2016). Daday used a comparable but different assay method, using picric acid (Corkill 1940), to assess the presence of dominant alleles. Although both Feigl-Anger and picric acid give comparable results (Hughes 1991), the former is preferred because picric acid is potentially explosive. We compared both tests on a subset of samples (107 individuals) and found 96.3% of plants showed concordant results between methods (Text S1). Because cyanogenesis is controlled by the presence of a single dominant allele at both loci underlying this trait (*Ac* and *Li*), the only genotypes we could determine with certainty were the homozygous recessives *acac* and *lili* (i.e., from Acli, acLi, and acli cyanotypes). Therefore, we calculated allele frequencies by first determining the frequency of the homozygous recessive genotypes (*acac* and *lili*) within populations, and then assumed loci were in Hardy-Weinberg equilibrium to determine the frequencies of *Ac* and *Li*. This assumption is reasonable given the obligately outcrossing mating system, the random (nonassortative) movement of pollinators among cyanotypes, and the typically large population sizes of *T. repens* (Burdon 1983; Lachance 2009; Santangelo et al. 2019). In total, 10,575 individuals were assayed as the contemporary sample from 131 populations spanning five continents (mean number of individuals per population ± SE: 80.7 ± 10.7, range = 9−1032).

HERBIVORY MEASUREMENTS

Herbivory was measured in the native range of *T. repens* during 2018 to assess how the frequency of HCN production within populations is related to the amount of leaf tissue eaten by herbivores. We focused our estimates of herbivory on native populations for logistical reasons, and because we expect that the frequency of HCN production, *Ac*, and *Li* is most likely to be in equilibrium due to a long history of selection by herbivores and temperature in the native range. Estimates of consumed leaf tissue (hereafter herbivory) were visually scored to the nearest percentage of leaf damage, which is an accurate method for quantifying herbivory that is comparable to digital methods (Johnson et al. 2016). At each European site, one haphazardly selected leaf from each of ∼20 individuals spaced at least 3 m apart was scored for leaf damage to the nearest 1%. Plants used for herbivory estimates were haphazardly selected from the same populations used for the collection of infructescences. Individuals were not scored if leaf damage was found to be the result of mowing (i.e., leaf damage showed a dried and frayed edge). Plants were scored from July 7 to August 28 of 2018, beginning with the southernmost sites in Spain. Sampling progressed northward and eastward before ending in northern Sweden. This approach of sampling in a northward direction followed the methods of Anstett et al. (2014), and allowed us to ensure that plants experienced a roughly comparable amount of the growing season. To control for confounding differences in sampling date, we used growing degree days (GDD) experienced by each population in 2018 as a covariate in our statistical analyses of herbivory (see STATISTICAL ANALYSES). GDD is a metric of thermal accumulation through time and is frequently used to predict plant development and insect population growth (McMaster and Smika 1988). We calculated GDD based on the thermal accumulation above 5°C with an upper threshold of 30°C during 2018 until the date of sampling (Mence 1964; McMaster and Wilhelm 1997), extracted from the E-OBS daily gridded temperature dataset (Cornes et al. 2018). In total, 974 individuals from 50 populations were scored for herbivory (mean number of individuals per population ± SE: 19.5 ± 2.13, range = 10−20).

CLIMATE DATA

Cyanogenesis clines have repeatedly evolved in response to environmental gradients (Daday 1954a,b, 1958; Ganders 1990; Kooyers and Olsen 2012, 2013). We extracted a number of climatic variables to investigate their possible role in generating the observed clines. All climate data were extracted from the CRU TS version 4.02 dataset and managed using the *raster* package in R version 3.5.2 (Climatic Research Unit, University of East Anglia; R Core Team 2018; Hijmans 2019; Harris et al. 2020). These data are monthly means for a range of climatic variables (e.g., mean temperatures, precipitation, etc.) at 0.5° resolution

covering all land areas except for Antarctica. Data were extracted for each population using corresponding longitudes and latitudes. Time calibrated 5-year averages were used for each time of collection by taking the average of mean winter temperature (January and July for northern and southern hemispheres, respectively) from 1950 to 1954 and 2013 to 2017 for our historical and contemporary data, respectively. Calibrating average mean winter temperature in this way allowed us to account for temporal changes in temperature due to climate change within each population when testing for continued adaptation to spatial gradients in temperature in the introduced range (question 3). Mean monthly precipitation and potential evapotranspiration were also extracted and averaged from 1950 to 1954 and from 2013 to 2017 for historical and contemporary populations, respectively, and were used to create an aridity index (precipitation/potential evapotranspiration). Extracting temperature data for each population from the time at which it was sampled allowed us to test our research question about whether *T. repens* has continued to adapt to spatial gradients in temperature in the introduced range while controlling for climate change through time (also see STATISTICAL ANALYSES).

STATISTICAL ANALYSES

Evolution in response to climate change

To test whether cyanotype frequencies changed through time in response to climate change, we used general linear models that examined whether changes in the frequency of HCN production, *Ac*, and *Li* within populations were correlated with changes in mean winter temperature over time. Differences in frequencies of HCN production, *Ac*, and *Li* as well as differences in mean winter temperature were calculated for historical and contemporary populations within 50 km of each other. This test was performed in the native range and repeated with the addition of populations from the introduced range. Contemporary populations were averaged if multiple populations were within 50 km of a historical sampling location. Historical populations fixed for HCN production, *Ac*, or *Li* were excluded from these analyses. These criteria yielded 27 pairs of sampling locations for HCN production and *Li*, and 21 pairs for *Ac* in the native range. When the introduced range was also considered, an additional five pairs for HCN production and *Ac* and four for *Li* were added to the analysis. We focused our main analysis on the native range because allele frequencies in these populations are most likely at an evolutionary equilibrium, but we present results for both the native and introduced ranges in the Supporting Information for completeness (Fig. S1; Table S3). If the evolution of cyanogenesis tracked and kept pace with climate change, we expected a positive relationship between changes in cyanogenesis frequencies and mean winter temperature through time similar to the relationship that exists across latitudinal gradients (Fig. 1a).

Herbivory

To test the effects of latitude on herbivory across *T. repens*' native range, we used a general linear model to understand how herbivory changes between populations with respect to latitude while accounting for the frequency of HCN production in each population. This was done by fitting herbivory to the following model:

$$\begin{aligned}\text{Herbivory} = {} & \text{intercept} + \text{HCN frequency} + \text{latitude} + \text{GDD} \\ & + \text{HCN frequency} \times \text{latitude} + \text{error}.\end{aligned}$$

In our model, "HCN frequency" tested how changes in herbivory were predicted by the frequency of cyanogenesis within populations, "latitude" tested how changes in herbivory were predicted by latitude, and the interaction of "HCN frequency × latitude" tested how changes in herbivory are explained by latitude, given frequencies of cyanogenesis. "GDD" was included as a covariate to test for the potential effect of different sampling dates on our herbivory estimates (see HERBIVORY MEASUREMENTS; Table S4). A type-III sums-of-squares ANOVA in R was used to evaluate how herbivory changes with respect to latitude at varying frequencies of cyanogenesis. Mean herbivory for each population was $\log(x + 1)$ transformed to improve normality and homogeneity of variance in the residuals.

Continued adaptation to temperature gradients in the introduced range

To test whether *T. repens* has continued to adapt to spatial variation in temperature in the introduced range since the 1950s, we used a generalized linear model (GLM) that examined how the frequency of HCN production, *Ac*, and *Li* changed with mean winter temperature through time. This was done by fitting each response variable (HCN production, *Ac* allele or *Li* allele frequencies) individually to the following model:

$$\begin{aligned}\text{Response variable} = {} & \text{intercept} + \text{mean winter temp.} + \text{origin} \\ & + \text{time sampled} + \text{mean winter temp.} \\ & \times \text{time sampled} + \text{origin} \times \text{time sampled} \\ & + \text{mean winter temp.} \times \text{origin} \\ & + \text{mean winter temp.} \times \text{origin} \\ & \times \text{time sampled} + \text{error}.\end{aligned}$$

In our model, "mean winter temp." tested how our response variables (i.e., HCN phenotype and allele frequencies) are predicted by changes in mean winter temperature across space, "origin" tested how the mean of response variables differed between the native and introduced ranges, and "time sampled" (a categorical variable of historical vs. contemporary sampling)

tested differences in the mean of responses variables through time. The interaction of "mean winter temp. × time sampled" tested how response variables changed with mean winter temperature through time. The interaction of "origin × time sampled" tested whether the native or introduced ranges experienced greater shifts through time in their mean response variables relative to one another. Finally, the three-way interaction "mean winter temp. × origin × time sampled" assessed whether a change in latitudinal clines through time depended on the range in which it occurred. To observe continued evolution in the introduced range, we expected the relationship between cyanogenesis frequencies and mean winter temperature to increase in more recently sampled populations from the introduced range but not the native range (Fig. 1b). GLMs were first fit to binomial distributions, but thereafter fit to quasibinomial distributions because our data were underdispersed ($\Phi < 1$). Statistical significance was measured with a type-III analysis of deviance approach and fit to an *F*-distribution using the *car* package in R (Tjur 1998; Fox and Weisberg 2011; R Core Team 2018).

Additional GLMs using aridity rather than mean winter temperature as an environmental variable did not show any change in aridity through time, or any association between aridity and cyanogenesis or underlying allele frequencies (Text S3). All analyses, data manipulation, and visualization were facilitated with the *interactions* and *tidyverse* packages in R (Wickham 2017; R Core Team 2018; Long 2019).

Results

EFFECTS OF CLIMATE CHANGE ON CYANOGENESIS

Populations of *T. repens* showed no evidence of evolution of cyanogenesis in response to climate change. Changes in frequencies of HCN production, *Ac*, and *Li* were not associated with changes in mean winter temperature through time in *T. repens*' native range (Δ mean winter temp.: $F_{1,25} = 0.92$, $P_{\text{HCN}} = 0.346$; $F_{1,19} = 3.20$, $P_{Ac} = 0.089$; $F_{1,25} = 0.06$, $P_{Li} = 0.806$; Fig 3; Table S3) or in both its native and introduced ranges (Δ mean winter temp.: $F_{1,30} = 0.03$, $P_{\text{HCN}} = 0.856$; $F_{1,24} = 0.55$, $P_{Ac} = 0.466$; $F_{1,29} = 0.23$, $P_{Li} = 0.635$; Fig S1; Table S3). Despite observing an average increase of 1.84°C (SE: 0.096) between contemporary and historical sites within 50 km of each other in the native range, and 1.41°C (SE: 0.238) in both the native and introduced ranges, changes in frequencies of HCN production, *Ac*, and *Li* did not match the predicted magnitude or direction of changes in mean winter temperature through time (Fig. 1a).

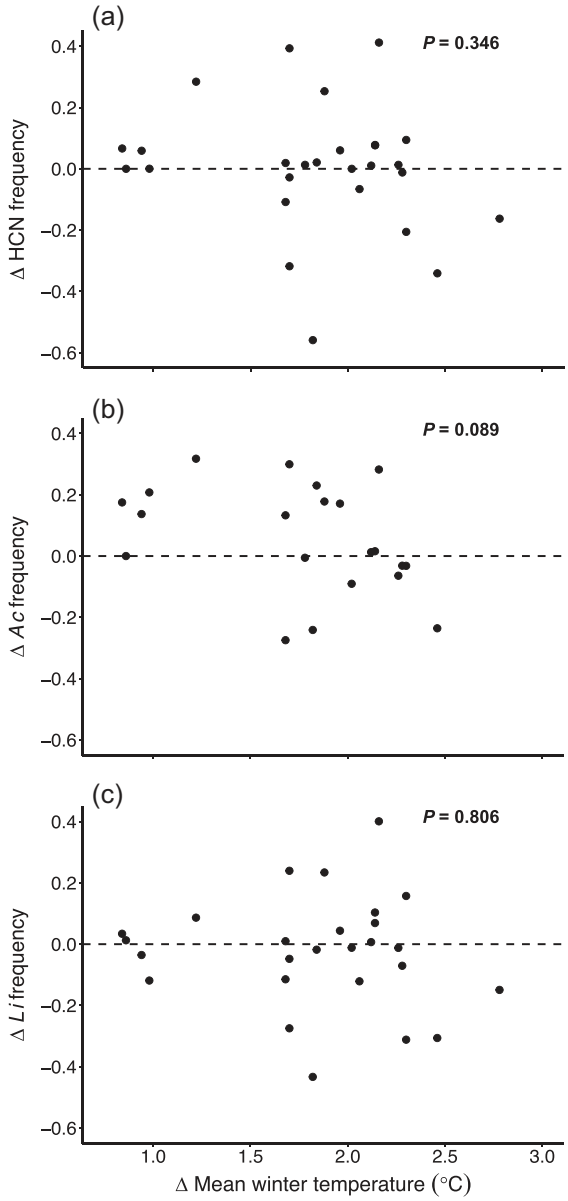

Figure 3. A test for the evolution of cyanogenesis in response to climate change. Changes in mean frequencies of (a) HCN production, (b) *Ac*, and (c) *Li* against changes in mean winter temperature test whether cyanogenesis and its underlying loci evolve in response to climate change. Cyanogenesis, *Ac*, and *Li* frequencies did not track changes in mean winter temperatures through time as would be expected if climate change was influencing their evolution. *P*-values for linear regression results are plotted in each panel. Results for both the native and introduced ranges can be found in the Supporting Information (Fig. S1).

EFFECTS OF LATITUDE AND CYANOGENESIS ON HERBIVORY

Populations of *T. repens* showed a strong latitudinal cline in herbivory, but this cline was dependent on cyanogenesis frequencies. Herbivory significantly increased with decreasing frequencies of HCN production ($F_{1,44} = 7.87$, $P = 0.008$; Fig. 4). From southern Spain to northern Sweden, herbivory increased from 2% to 12% of leaf tissue removed in *T. repens*' native range. The trend in herbivory was also affected by changes in the frequency of HCN

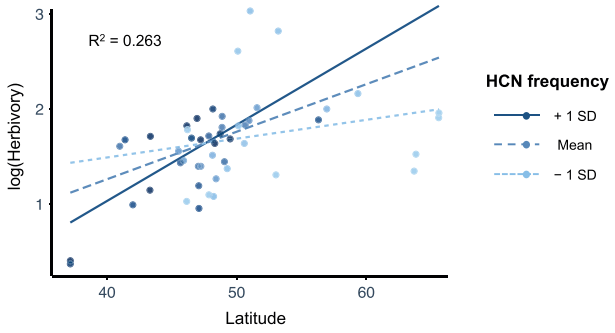

Figure 4. Changes in herbivory along latitudinal gradients in *Trifolium repens*' native range. Herbivory significantly increased in populations with lower frequencies of HCN production ($F_{1,44} = 7.782$, $P = 0.008$), and was strongly influenced by changes in the frequency of HCN production across latitudes ($F_{1,44} = 7.917$, $P = 0.007$). The R^2_{adj} is also reported for this model.

production across latitudes (HCN frequency × latitude interaction; $F_{1,44} = 7.92$, $P = 0.007$), in which decreasing frequencies of HCN production within populations decreased the effect of latitude on herbivory (Fig. 4). Additionally, differences in lengths of growing season associated with sampling time, calculated as GDD up to the date of sampling, had no effect on herbivory ($F_{1,44} = 0.165$, $P = 0.687$; Table S4).

CONTINUED ADAPTATION TO TEMPERATURE GRADIENTS IN THE INTRODUCED RANGE

We detected varying levels of evidence of continued evolution of clines in frequencies of HCN production, *Ac*, and *Li* in the introduced range of *T. repens*. In both the historical and contemporary samples, clines in the frequencies of HCN production, *Ac*, and *Li* significantly increased with increasing mean winter temperature (mean winter temp.: $P_{HCN} < 0.001$, $P_{Ac} < 0.001$, $P_{Li} < 0.001$; Fig. 5; Table 1), and this effect was weaker in the introduced range (mean winter temp. × origin: $P_{HCN} < 0.001$, $P_{Ac} < 0.001$, $P_{Li} < 0.001$; Fig. 5d–f; Table 1). On average, the frequency of HCN production increased by 8% and 5% per 1°C in the historical and contemporary samples of the native range, and by 1% and 2% per 1°C in the introduced range, respectively. *Ac* increased by 8% and 5% per 1°C in the historical and contemporary samples of the native range, and by 1% and 2% per 1°C in the introduced range, respectively. Lastly, *Li* increased by 6% and 4% per 1°C in the historical and contemporary samples of the native range, and by 1% and 2% per 1°C in the introduced range, respectively.

Statistical support for changes in the slopes of these clines through time was clearest for *Ac*, marginally nonsignificant for HCN production, and weakest for *Li* (mean winter temp. × time sampled × origin: $P_{Ac} = 0.019$, $P_{HCN} = 0.060$, $P_{Li} = 0.177$; Table 1), suggesting that clines are becoming steeper for at least the dominant *Ac* allele in the introduced range of *T. repens* and more closely matching clines in the native range (Fig. 5e), although HCN production and *Li* are also trending in that direction (Fig. 5d, f). Clines for the frequencies of HCN production, *Ac*, and *Li* did not significantly change through time across both the native and introduced ranges (mean winter temp. × time sampled: $P_{HCN} = 0.552$, $P_{Ac} = 0.056$, $P_{Li} = 0.868$; Table 1), further suggesting that this change appears to be largely driven by changes in slope in the introduced range. We repeated these analyses with just our European and North American data; however, these results are less clear and are included in the Supporting Information for completeness (Table S5).

Discussion

We tested for the evolution of cyanogenesis and the polymorphic loci that control this trait in *T. repens* in response to global climate change, as well as evidence of continued evolution of cyanogenesis in its introduced range. Three results are most important for answering our research questions. First, cyanogenesis frequencies have not changed over the past 60 years in either the native or introduced ranges, in a way that would be expected if they had evolved in response to ongoing climate change. Second, contrary to our predictions, herbivory was greatest in native populations at high latitudes, where the frequency of HCN production was lowest, which is consistent with a defensive role of HCN production at lower latitudes. Additionally, given the low frequency of HCN production in these populations, our results also suggest that costs associated with producing the metabolic components of cyanogenesis in cold climates could impose stronger selection than the selective benefits of this defense trait against herbivores. Third, in the introduced range of *T. repens*, the steepness of cyanogenesis clines in response to winter temperature has increased through time (Fig. 5d, e). These results lead us to conclude that despite recent global warming, *T. repens* shows no clear evidence of adaptation in cyanogenesis to climate change. However, populations from the introduced range appear to be continuing to adapt in response to environmental gradients. We discuss how our results and conclusions provide insight into how species evolve in response to spatiotemporal variation in climate.

EVOLUTION IN RESPONSE TO CLIMATE CHANGE

To assess the evolution of cyanogenesis in response to climate change, we tested for differences in the frequency of cyanogenesis in relation to changes in mean winter temperature through time, between the 1950s and contemporary time (2008−2017). As outlined in the introduction (Fig. 1a), we expected changes in the frequencies of HCN production, *Ac*, and *Li* to match increases in mean winter temperature if populations of *T. repens* evolved quickly in a manner consistent with adaptation to increasing temperatures.

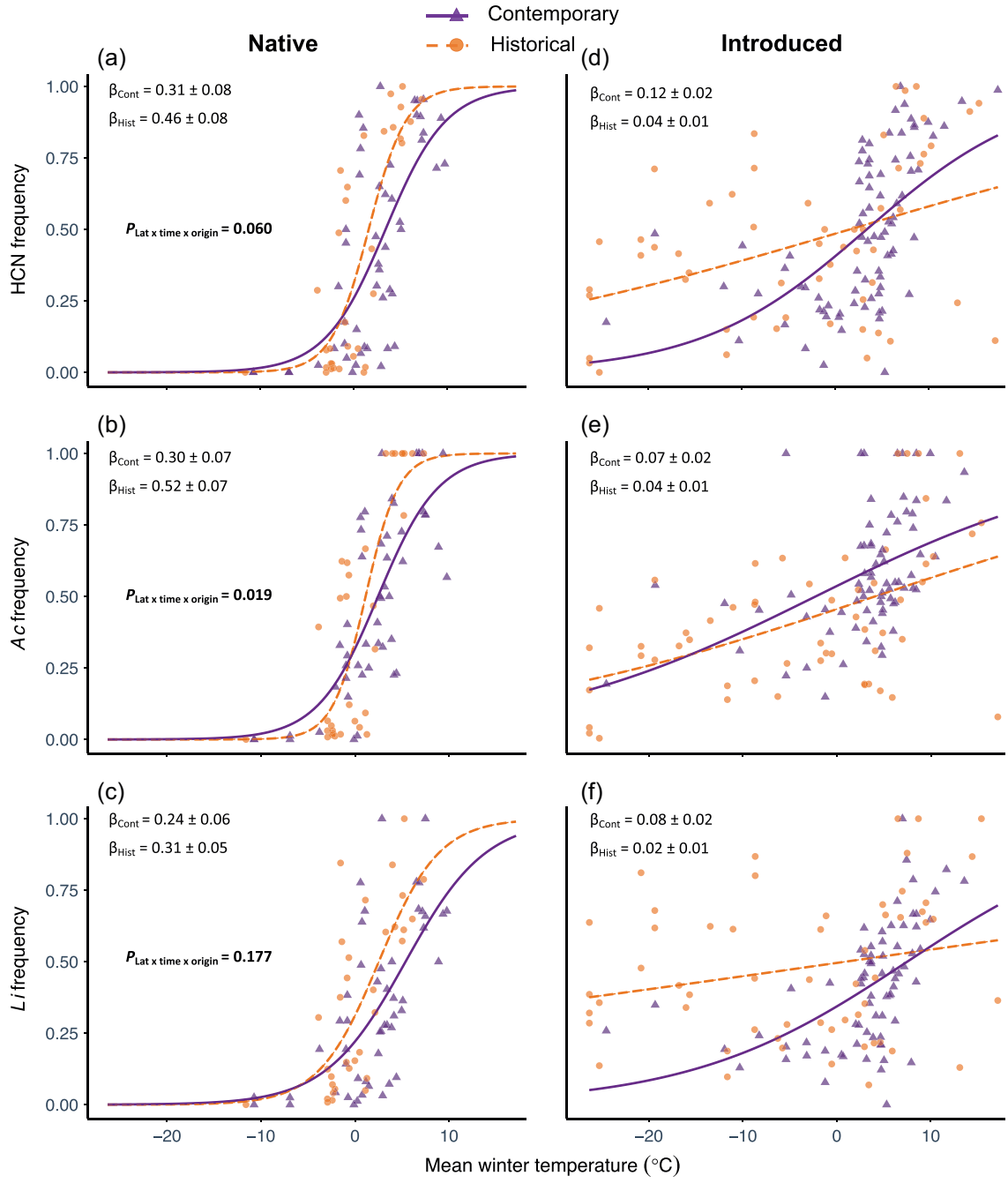

Figure 5. A test for continued evolution to spatial gradients in temperature within *Trifolium repens*' native and introduced ranges. Changes in the slopes of the frequencies of (a, d) HCN production, (b, e) *Ac*, and (c, f) *Li* versus mean winter temperature (°C) through time (i.e., mean winter temperature × time sampled interaction) would be consistent with continued evolution to spatial variation in temperature within *T. repens*' native (a, b, c) and introduced ranges (d, e, f). To further illustrate the interaction (or lack thereof), we add the slope ($\beta \pm$ SE) values for each regression line to the figure, extracted using *sim_slopes* from the *interactions* R package (Long 2019). For the frequencies of HCN production and *Ac*, clines steepened more through time in the introduced than native range (significant mean winter temp. × time sampled × origin interaction), which was expected if *T. repens* continued to adapt to spatial climatic gradients in its introduced range. GLM results are presented in Table 1.

Table 1. Summary of results from the models testing changes in the frequencies of cyanogenesis (HCN production), *Ac*, and *Li* as a function of mean winter temperature (°C) through time in the native and introduced ranges of *Trifolium repens*.

Response	SS	*F*	*P*
a) HCN frequency			
Mean winter temp.	2310.9	107.802	**<0.001**
Time sampled	107.2	5.001	**0.026**
Origin	169.7	7.918	**0.005**
Mean winter temp. × Time sampled	7.6	0.356	0.552
Mean winter temp. × Origin	885.5	41.307	**<0.001**
Time sampled × Origin	0.1	0.005	0.944
Mean winter temp. × Time sampled × Origin	76.9	3.587	0.060
b) *Ac* frequency			
Mean winter temp.	2250.3	137.635	**<0.001**
Time sampled	1.1	0.067	0.797
Origin	177.5	10.856	**0.001**
Mean winter temp. × Time sampled	60.5	3.701	0.056
Mean winter temp. × Origin	1168.9	71.491	**<0.001**
Time sampled × Origin	9.2	0.561	0.455
Mean winter temp. × Time sampled × Origin	91.4	5.592	**0.019**
c) *Li* frequency			
Mean winter temp.	1237.2	78.426	**<0.001**
Time sampled	163.8	10.381	**0.001**
Origin	250.6	15.882	**<0.001**
Mean winter temp. × Time sampled	0.4	0.028	0.868
Mean winter temp. × Origin	535.0	33.916	**<0.001**
Time sampled × Origin	0.9	0.059	0.808
Mean winter temp. × Time sampled × Origin	28.9	1.835	0.177

***Note*: Mean winter temperature was determined as the 5-year average in the years preceding each collection period for January and July in northern and southern hemispheres, respectively. Time sampled contrasted samples collected in the 1950s versus and the 2010s. Origin refers to whether populations were collected from the native or introduced range. The columns report the sums of squares (SS), the *F*-statistic (*F*), and the *P*-value (*P*). Significant values at $P < 0.05$ are bolded.**

However, our results showed no association between changes in cyanogenesis and changes in temperature, which suggests that *T. repens* has not evolved at the cyanogenesis loci in response to climate change since the 1950s.

A comparison of the changes in the frequencies of HCN production and its underlying alleles across space versus through time further suggests that populations did not track temporal changes in climate change. On average, across all contemporary populations we observed a 3.78% increase in the frequency of HCN production, a 3.41% increase in *Ac*, and a 2.81% increase in *Li*, with each 1°C change across the latitudinal gradient. Over the 60 years between our historical and contemporary samples, we observed a 2.34°C degree increase, which would translate to an 8.84% increase in the frequency of HCN production, a 7.98% increase in *Ac*, and a 6.58% increase in *Li*, if changes in the frequencies of HCN production and dominant alleles through time tracked changes in temperature in space in an equivalent way (Fig. 1a). The average change between the pairs of historical and contemporary populations used to test for a response to climate change was 0.19%, 5.64%, and −5.41% for frequencies of HCN production, *Ac*, and *Li*, respectively, which is smaller than expected from the change in space and not associated with changes in temperature (Fig. 3).

Several possible explanations could account for this lack of response in cyanogenesis to climate change. First, even if climate change has affected *T. repens*, it is possible populations have had insufficient time to adapt to this change as populations often exhibit temporal lags in adaptation (Wilczek et al. 2014; Kooyers et al. 2019; Anderson and Wadgymar 2020). Second, a lack of gene flow from populations from high-temperature areas may also limit *T. repens*' ability to adapt to changing climates (Bontrager and Angert 2019). These scenarios would be evidence for temporal or spatial lags in adaptation to climate change (Wilczek et al. 2014), but we view these latter two scenarios as unlikely. The lack of population structure in *T. repens* (Kooyers and Olsen 2012), combined with the rapid evolution of clines documented in North America and along urbanization gradients (Kooyers and Olsen 2012; Thompson et al. 2016;

Santangelo et al. 2020), suggests high levels of gene flow between populations, extensive standing genetic variation, and the capacity to rapidly adapt to changes in the environment. Third, if herbivores are the primary driver of clines, herbivore communities may have been unable to track changing climates, leading to limited changes in selection on cyanogenesis (CaraDonna et al. 2014). However, given that we observed the highest levels of herbivory at high latitudes in the native range, this explanation also seems unlikely. Three other more likely scenarios are possible. Traits and their underlying genes unrelated to HCN production may have evolved as adaptations in response to climate change, which we did not assess in this study (Wright et al. 2018). Second, *T. repens* may exhibit phenotypic plasticity that allows it to tolerate global warming without severe fitness costs. Third, there may be temporal fluctuations in selection whereby cyanogenesis frequencies in populations may not be responding rapidly because of increased variance in environmental variables. None of these explanations are mutually exclusive, and it is likely that there are multiple factors that explain this lack of an evolutionary response (Wright et al. 2021). There could also be other environmental factors driving the evolution of cyanogenesis clines; however, our expectation that cyanogenesis frequencies would respond to climate change was not unreasonable given that this trait is known to evolve along climatic gradients—specifically in response to differences in winter temperature (Daday 1954a,b, 1958; Kooyers and Olsen 2012; Thompson et al. 2016; Santangelo et al. 2020).

Even though we predicted that cyanogenesis clines would evolve in response to climate change, there remains little evidence that species adapt to gradual changes in climate, although there is considerable evidence they can adapt to rapid and extreme climatic events (Grant et al. 2017). In *Boechera stricta*, marginal increases in spring temperatures from 1973 to 2011 contributed to a 0.2–0.5 day per generation acceleration in flowering time (Anderson et al. 2012). In *A. thaliana*, local genotypes show higher relative fitness across multiple reciprocal transplant sites (Wilczek et al. 2014), suggesting that populations can adapt to spatial variation in climate given sufficient time. However, individuals from historically warmer sites had higher average fitness across all transplant sites (Wilczek et al. 2014). This suggests that although *Arabidopsis* populations are adapting to their home ranges, populations may be experiencing an adaptation lag due to shifting local optima. Our results suggest *T. repens* may be experiencing an adaptation lag, but reciprocal transplants are needed to test this hypothesis. Many more studies have documented evolutionary responses to rapid and extreme climatic events. Extreme droughts have contributed to the onset of earlier flowering in relatively short periods of time in *B. rapa* and wild cereal populations (Franks et al. 2007; Nevo et al. 2012), and have influenced the evolution of body size as well as beak morphology in Galápagos finches (Grant and Grant 2002). Such extreme events will likely affect southern populations of *T. repens* more strongly than northern populations, such that the environments of southern populations may become too arid to support local populations, and only northward migration will rescue them.

LATITUDINAL GRADIENTS IN HERBIVORY

We detected increased herbivory at higher latitudes in *T. repens*' native range (Fig. 4). At first pass, this result is inconsistent with the notion that latitudinal clines in herbivory explain the evolution of cyanogenesis clines, and instead implies that other factors related to the costs of the defense or production of its metabolic components are the primary driver of clines. Although higher herbivory is typically expected at lower latitudes (Kozlov 2008; Adams and Zhang 2009; Johnson and Rasmann 2011; Hiura and Nakamura 2013; Anstett et al. 2014; Kim 2014), the counter-gradient in herbivory is partially explained by the presence of a strong cyanogenesis cline in *T. repens*' native range (Daday 1954a). Examining the interaction between HCN frequency within populations and latitude reveals that the strength of the cline in herbivory is strongest among those populations that represent the highest levels of HCN production (i.e., +1 SD), and weakest among populations that have the lowest frequency of HCN production (i.e., −1 SD). This implies that the spatial gradient in the frequency of HCN production may be partially explained by latitudinal clines in herbivory. In a similar way, higher levels of defense were observed in southern populations of *Oenothera biennis* (Anstett et al. 2015), which appear to cause decreasing herbivory by some insect species at lower latitudes (Anstett et al. 2014, 2015). Thus, across the entire cline of *T. repens*, it appears that biogeographic gradients in herbivory and environmental stressors (e.g., cold winter temperatures) together maintain the cline in cyanogenesis due to a trade-off between the benefits and costs of HCN production and its underlying metabolic components (Kooyers et al. 2018; Santangelo et al. 2020; Daday 1954a, 1958). We suggest that, at lower latitudes, higher frequencies of HCN production benefit plants by deterring herbivores while there is likely little cost of the cyanogenesis loci due to environmental stressors. At increasingly higher latitudes, costs associated with producing the biochemical components necessary for cyanogenesis likely increase because of decreased tolerance to freezing, and this cost could impose stronger selection than the selective benefits of HCN production against herbivores. This hypothesis is consistent with previous results showing a defensive role of HCN production (Dirzo and Harper 1982; Hughes 1991; Thompson and Johnson 2016; Santangelo et al. 2019), whereas at increasingly higher latitudes cyanogenic individuals have a decreased tolerance to freezing (Daday 1965; Kooyers et al. 2018). However, experiments using manipulations of herbivore pressure would be needed to confirm this hypothesis.

EVOLUTION OF INTRODUCED SPECIES

Our results suggest that *T. repens* adapted quickly to spatial gradients in climate following its initial introduction to each continent, and that the slopes of clines at the *Ac* locus have continued to steepen in the introduced range during the past six decades. Historically, clines in the introduced range of *T. repens* were either weaker or absent when compared to clines in the native range (Daday 1958). As outlined in the introduction, we expected that cyanogenesis clines along temperature gradients would steepen through time if introduced populations were continuing to adapt to climatic gradients (Fig. 1b), so that they would more closely match those observed in the native range (Daday 1954a).

We observed some evidence of continued evolution of cyanogenesis at one of its underlying loci (*Ac*), in the introduced range of *T. repens* (Fig. 5e). Clines in the native range have remained relatively unchanged (Fig. 5a–c), whereas clines for the frequency of HCN production and especially the dominant *Ac* allele have become steeper in the introduced range since the 1950s (Fig. 5d, e). In fact, the average rate of increase in the frequency of HCN production, *Ac*, and *Li* across temperature gradients has doubled in the introduced range of *T. repens* since it was first sampled in the 1950s (Daday 1958), although this change was only statistically significant for *Ac*.

Our work adds to the current body of evidence that shows introduced species can adapt and thrive in nonnative environments. Parallel clines between a species' native and introduced ranges have been observed in multiple species (Ganders 1990; Huey et al. 2000; Maron et al. 2004; Alexander et al. 2009; Kooyers and Olsen 2012, 2013; Samis et al. 2012), providing evidence of repeated rapid adaptation to climatic gradients (Huey et al. 2000; Kooyers and Olsen 2012; Samis et al. 2012; Szücs et al. 2017). For example, rapid evolution of flowering time in introduced North American populations of *L. salicaria* facilitated its invasion into more northern climates (Colautti and Barrett 2013). Similarly, *Hypericum perforatum* evolved parallel adaptive clines between its native and introduced ranges (Maron et al. 2004). These results are similar to the case of *T. repens*, which forms parallel clines in cyanogenesis relative to temperature in both its native and introduced ranges (Kooyers and Olsen 2012, 2013; Kooyers et al. 2014; Daday 1954a, 1958). However, despite our evidence of continued adaptation in HCN production and at the *Ac* locus, these clines have not yet evolved to be as steep in the introduced ranges as in the native range. One explanation for this difference between the native and introduced ranges is that *T. repens* has adapted to spatial climatic gradients in traits unrelated to HCN production (Wright et al. 2018). A second explanation is that the combination of selection pressures on cyanogenesis is different in each range, such as the benefits of HCN production being less in the introduced range. A third nonmutually exclusive explanation is that this adaptive evolution is ongoing. A combination of reciprocal transplant and herbivore manipulation experiments, combined with simulation modeling that establishes null hypotheses of how cyanogenesis responds to different selection pressures, as well as population genomics approaches that reveal regions of the genome experiencing selection could help to determine the genetic and phenotypic mechanisms by which *T. repens* evolves adaptive clines in the introduced range.

Conclusions

This study shows that although species readily adapt to spatial gradients in climatic conditions, they may not rapidly respond to more gradual temporal changes in climate, or they may respond differently in geographically disparate areas where the benefits and costs of specific loci may change. It is plausible that *T. repens* is experiencing a lag in adaptation and that responses to climate change have not yet manifested themselves, especially because our study organism is a clonal perennial. It is also likely that environmental heterogeneity across sampling ranges, time since colonization, and ongoing gene flow among continents are affecting the evolution of introduced populations. Future studies should aim to experimentally validate the hypothesized mechanisms maintaining the cyanogenesis polymorphism within populations and creating cyanogenesis clines across spatially heterogeneous environments.

AUTHOR CONTRIBUTIONS

All authors designed the project. SGI, JSS, NJK, and MTJJ collected samples or contributed data. SGI conducted the analyses and wrote the first draft. All authors edited subsequent drafts and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

All code and data used throughout this manuscript are available on the GitHub page for SGI (https://github.com/innessim/MSc_project.git). Data have been permanently archived in DRYAD (https://doi.org/10.5061/dryad.76hdr7szt).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary information

Text S1. Cyanogenesis is an individual plant's ability to produce HCN following tissue damage. The presence of hydrogen cyanide (HCN) in plant tissue can be detected by more than one method. Cyanogenic and acyanogenic individuals can be phenotyped using Picric Acid or Feigl-Anger assays since they both use a reagent which changes colour in the presence of HCN (Corkill 1940; Daday 1954; Feigl and Anger 1966).

Text S2. We tested for the effects of drought on cyanogenesis since previous investigations revealed the importance of this trait in stress tolerance (Siegień and Bogatek 2006), and in predicting cyanogenesis clines (Kooyers and Olsen 2013; Kooyers et al. 2014). First, changes in drought through time were assessed with a general linear model examining the association between mean aridity (the quotient of precipitation and potential evapotranspiration) to latitude and the time of sampling. The interpretation of the model was similar to those described above.

Table S1. Summary of results from the model testing for changes in the frequencies of HCN, *Ac*, and *Li* as a function of mean winter temperature (°C) through time including historical populations from the United Kingdom and Ireland.

Table S2. Historical and contemporary populations used in our analyses.

Table S3. Historical and contemporary populations used to test for evolution in response to climate

Table S4. Summary of results from the model testing changes in herbivory along latitudinal gradients in *Trifolium repens'* native range.

Table S5. Summary of results from the models testing for changes in the frequencies of cyanogenesis (HCN production), *Ac*, and *Li* as a function of latitude through time including only European and North American populations.

Figure S1. A test for the evolution of cyanogenesis in response to climate change in both the native and introduced ranges of *Trifolium repens*. Changes in mean frequencies of (A) HCN production, (B) *Ac*, and (C) *Li* against changes in mean winter temperature through time provided evidence of evolution in response to climate change. In all cases, cyanogenesis frequencies did not track changes in mean winter temperatures through time as would be expected if climate change was influencing the evolution of this trait (Fig. 1a). *P*-values (*P*) for linear regression results are plotted in each panel.