Chapter 19

EPIGENETICS OF COLONIZING SPECIES? A STUDY OF JAPANESE KNOTWEED IN CENTRAL EUROPE

Yuan-Ye Zhang, Madalin Parepa,*† Markus Fischer,* and Oliver Bossdorf *†*

* Institute of Plant Sciences, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

† Institute of Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 5, D-72076 Tübingen, Germany

Abstract

Some of the world's most successful invasive plants have spread across large geographic areas while retaining little or no genetic diversity. Because of this lack of heritable variation, evolutionary hypotheses are usually not invoked when attempting to explain the success of these species. However, heritable trait variation within and among invasive populations could also be created through epigenetic or other nongenetic processes, particularly in clonal invaders where somatic changes can potentially persist indefinitely. We tested this possibility in a collection of 83 genetically identical clones of the invasive Japanese knotweed *Fallopia japonica* collected across Central Europe and propagated in a common environment for several years. Using regular as well as methylation-sensitive amplified fragment length polymorphism (AFLP) markers, we found that all clones indeed belonged to the same genotype but to 27 different epigenotypes. The different knotweed clones were also phenotypically differentiated. Path analysis indicated that among-clone phenotypic variation is partly correlated with epigenetic variation, as well as both directly and indirectly (through epigenetic variation) with climates of origin. Our results thus suggest a potential role of epigenetic variation in the geographic spread and invasion success of Japanese knotweed. More generally, our study highlights the need to incorporate large-scale epigenetic screens in studies of genetically uniform clonal invaders.

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INTRODUCTION

Some of the world's most successful invasive plants are almost or entirely genetically uniform in their introduced range. Examples include the "Bermuda buttercup" (*Oxalis pes-caprae*; Ornduff 1987), Japanese knotweed (*Fallopia japonica*; Hollingsworth & Bailey 2000), alligator weed (*Alternanthera philoxeroides*; Geng *et al.* 2007), the hawkweed *Hieracium aurantiacum* (Loomis & Fishman 2009), the invasive grass *Pennisetum setaceum* (Le Roux *et al.* 2007), and the water hyacinth (*Eichhornia crassipes*; Zhang *et al.* 2010). Researchers attempting to explain the huge success of these species usually argue that they must possess some inherent ecological advantage—for example, a particularly large degree of phenotypic plasticity ("general-purpose genotype"; Baker 1965; Loomis & Fishman 2009; Oplaat & Verhoeven 2015), superior means of spreading with human help (e.g., Gravuer *et al.* 2008; Wilson *et al.* 2009), or preadaptation to disturbed habitats (Prinzing *et al.* 2002)—that is large enough to outweigh the disadvantages of genetic uniformity. Evolutionary hypotheses for invasion success (Bossdorf *et al.* 2005; Barrett *et al.* 2008; Dlugosch & Parker 2008; Prentis *et al.* 2008) are usually not invoked in these species, because these require the presence of heritable variation in the introduced range. A possibility that so far has not yet been considered is that these genetically almost or entirely uniform invaders harbor heritable variation created through epigenetic or other nongenetic mechanisms of inheritance.

Epigenetic mechanisms such as DNA methylation or histone modifications can create heritable variation in plant phenotypes, and thus maintain evolutionary potential even in the absence of DNA sequence variation (e.g., Johannes *et al.* 2009; Zhang *et al.* 2013). Studies in natural plant populations usually find that there is extensive epigenetic variation within and among natural populations, and that this epigenetic variation often exceeds and only partly correlates with DNA sequence variation (e.g., Vaughn *et al.* 2007; Richards *et al.* 2012; Schmitz *et al.* 2013; Medrano *et al.* 2014; Schulz *et al.* 2014). One possible origin for natural epigenetic variation is spontaneous epimutation, which occurs at higher frequencies than mutations of DNA sequence (Verhoeven *et al.* 2010; van der Graaf *et al.* 2015). Invasive populations that went through an extreme genetic bottleneck may thus accumulate novel epigenetic variation much more quickly than DNA sequence variation, and it is therefore possible that they are more variable at the epigenetic than at the genetic level.

The second possible origin of natural epigenetic variation is inheritance of environmentally induced epigenetic changes. Recent studies have repeatedly demonstrated that environmental differences can induce epigenetic changes, which may in turn become inherited to offspring (e.g., Verhoeven *et al.* 2010; Kou *et al.* 2011; Bilichak *et al.* 2012; Rasmann *et al.* 2012). Although there is currently much debate about the true importance of such environmentally induced transgenerational effects (Pecinka & Scheid 2012; Heard & Martienssen 2014), much of this debate is about whether epigenetic changes can be passed on to sexual offspring (i.e., whether they can be meiotically inherited). An aspect, which so far has received surprisingly little attention, is that many plants reproduce vegetatively, and that for many species this is the main mode of reproduction. For vegetative reproduction, no germline needs to be passed on, and epigenetic changes may potentially persist forever through mitotic inheritance. Hence, clonal plants should generally exhibit the greatest potential for environmentally driven epigenetic differentiation (Latzel & Klimešová 2010; Verhoeven & Preite 2013; Douhovnikoff & Dodd 2015). As all of the aforementioned genetically uniform invaders are clonal plants, it is an intriguing possibility that epigenetically based habitat adaptation—through epimutation and selection, or through persistent environmentally induced epigenetic changes—is a key mechanism explaining their huge success despite their genetic uniformity.

It is important to note that besides epigenetic changes, further mechanisms can cause transgenerational phenotypic effects, including simple nutritional effects, the transmission of defense chemicals, hormones or other signaling molecules, and the vertical transmission of endophytic mutualists. All of these mechanisms are encompassed by the broader concepts of maternal environmental effects or transgenerational plasticity (Roach & Wulff 1987; Rossiter 1996; Herman & Sultan 2011). Many previous studies have demonstrated such transgenerational plasticity and its adaptive significance at the level of plant phenotype (e.g., Galloway & Etterson 2007; Whittle *et al.* 2009; Latzel *et al.* 2014). However, these efforts usually did not address the underlying physiological or epigenetic mechanisms. In any case, as for epigenetic mechanisms, clonal plants—including genetically uniform

invaders—have great potential also for other, non-epigenetic transgenerational effects.

One of the best-known cases of a genetically uniform plant invader is Japanese knotweed (*Fallopia japonica*), where a single clone has spread aggressively through a broad range of habitats in temperate Europe and North America (Beerling *et al.* 1994; Bailey & Conolly 2000; Grimsby *et al.* 2007; Gerber *et al.* 2008; Bailey *et al.* 2009). A previous study of Japanese knotweed found that invasive populations in the United States are indeed epigenetically differentiated between different habitats in the field (Richards *et al.* 2012). Here, we took advantage of an extensive live collection of invasive Japanese knotweed clones from different origins across Central Europe to test for heritable phenotypic and epigenetic variation in the species. Specifically, we asked the following questions: (1) Do genetically uniform populations of Japanese knotweed harbor significant epigenetic variation? (2) If yes, is this epigenetic variation accompanied by significant variation in phenotype? (3) What are the relationships between environmental, epigenetic, and phenotypic variation?

MATERIALS AND METHODS

Study system

Japanese knotweed (*F. japonica* (Houtt.) Ronse Decr. var. *japonica*) is a perennial member of the Polygonaceae native to Japan, Korea, and China. It is a tall and vigorous forb with a gynodioecious breeding system and the ability to reproduce and spread vegetatively through an extensive rhizome network (Smith *et al.* 2007). During the nineteenth century, the species was introduced to Europe and North America as an ornamental and forage plant (Bailey & Conolly 2000). In its introduced range, *F. japonica* invades ruderal habitats and river banks, where it often forms dense monospecific stands, decreases native biodiversity (Gerber *et al.* 2008; Aguilera *et al.* 2010), and alters nutrient cycles (Pyšek 2009). Previous research suggests that multiple factors are involved in the dominance and invasion success of *F. japonica*, its sister species *Fallopia sachalinensis*, and their hybrid *Fallopia* × *bohemica*: invasive knotweeds not only spread extremely rapidly through clonal growth (Bimová *et al.* 2003; Pyšek *et al.* 2003) but also appear to suppress native plants through allelopathy and other soil-mediated effects (Siemens & Blossey 2007; Murrell *et al.* 2011; Parepa *et al.* 2013b), and they possess a superior ability to rapidly take up nutrients and take advantage of nutrient pulses (Parepa *et al.* 2013a). The ecological impacts, together with substantial structural damage in urban areas and high removal costs make Japanese knotweed one of the most problematic plant invaders of temperate regions worldwide (Lowe *et al.* 2000). In its invasive European range, *F. japonica* appears to be genetically uniform and is represented by only a single female clone (Hollingsworth & Bailey 2000; Mandák *et al.* 2005; Krebs *et al.* 2010).

In 2005, fresh rhizomes were collected in 83 invasive populations of *F. japonica* across seven different regions in Central Europe, spanning a broad geographic and climatic range from populations near Kiel, close to the North Sea in Northern Germany, to populations in the Ticino region in Southern Switzerland (Krebs *et al.* 2010; Fig. 1a,b). In each local population, one individual clone was collected. All clones had originally been genotyped using random amplified polymorphic DNA (RAPD) markers and, together with leaf morphological characteristics and flow cytometry estimates of ploidy levels, verified to be genetically uniform *F. japonica* var. *japonica* (Krebs *et al.* 2010).

Rhizome cuttings from these invasive populations were used to establish a live collection at the Botanical Garden of the University of Marburg, Germany, where all clones were grown under the same environmental conditions for 2 years. In 2007, the collection was moved to the University of Bern, where we re-planted rhizome cuttings from all clones into 12 l pots with a 1 : 1 mixture of sand and fresh field soil (RICOTER Erdaufbereitung AG, Aarberg, Switzerland).

Molecular analyses

In early 2010, we collected fresh and intact leaves from each of the 83 clones in our live collection and dried them on silica gel. From each sample, we extracted total genomic DNA using the Qiagen DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). We used AFLP markers to reexamine genotypic variation among our samples, following a modified AFLP protocol as in Zhang *et al.* (2010). To test for epigenetic variation among samples, we analyzed their DNA methylation profiles using the methylation-sensitive amplified polymorphism (MSAP) method (Reyna-López *et al.* 1997; Cervera *et al.* 2002). MSAP is a modification of the AFLP method where the frequent cutter *Mse*I is replaced by either of the two methylation-sensitive

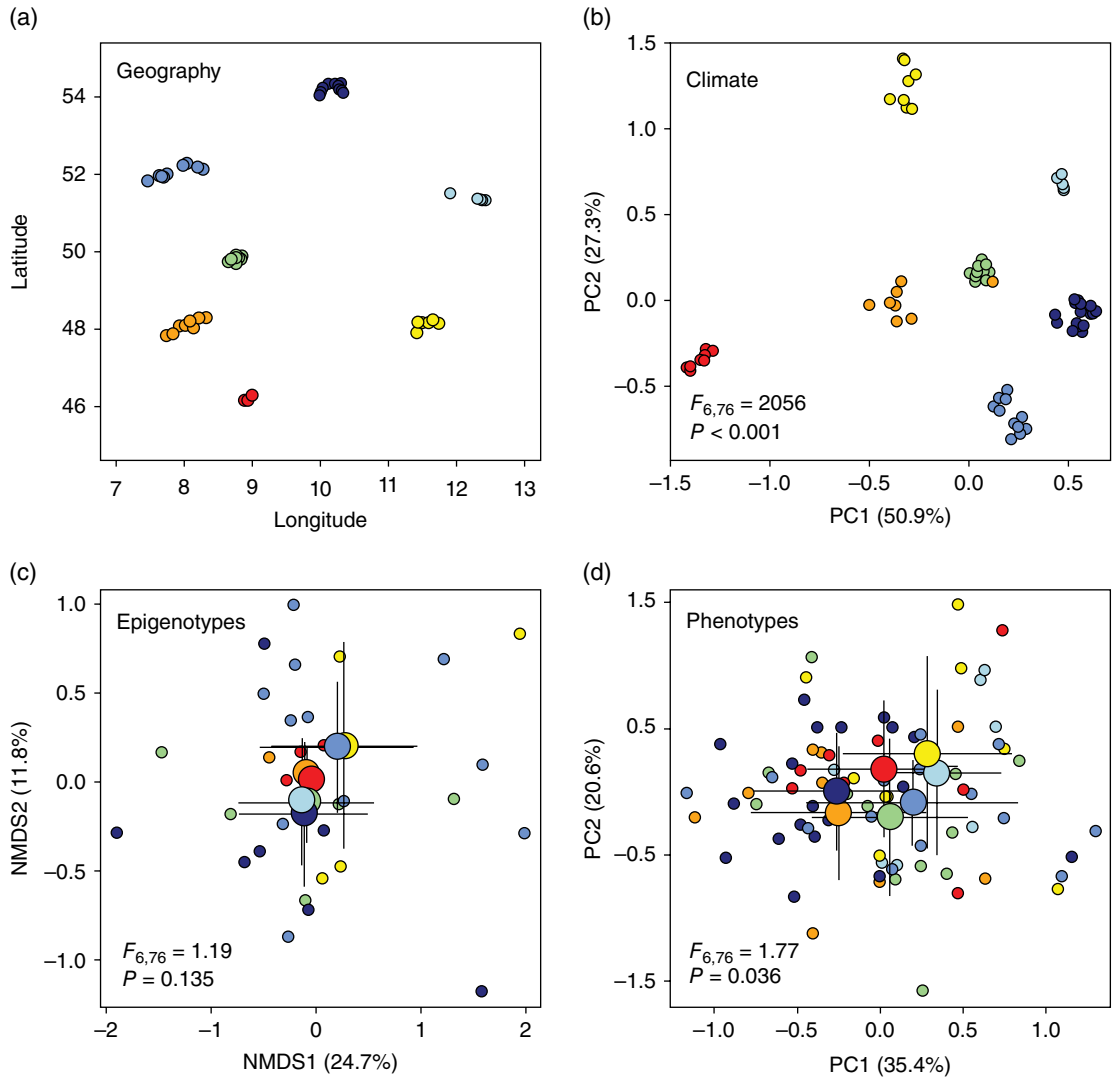

Fig. 1 Geographic (a), climatic (b), epigenetic (c), and phenotypic (d) distances among Japanese knotweed clones. Small symbols represent 83 different Central European origins of invasive Japanese knotweed (*Fallopia japonica*), with different colors for each of the seven geographic regions (see Table 1). The large symbols in the last two panels represent the regional means (±SD). Climate and phenotype plots are based on principal component analysis (PCA) of nine bioclimatic variables and seven phenotypic traits, respectively, whereas the epigenetic plot represents a non-metric multidimensional scaling (NMDS) analysis of 19 polymorphic methylation-sensitive amplified polymorphism (MSAP) markers. The test statistics are the results of multivariate tests for regional differentiation (see section "Materials and Methods" for details). (*See insert for color representation of the figure.*)

restriction enzymes *Msp*I and *Hpa*II. *Msp*I and *Hpa*II both recognize CCGG cutting site, but *Msp*I is blocked when the outer C is methylated, while *Hpa*II is blocked when either of the Cs is fully methylated. To assess the fragment sizes of AFLP and MSAP markers, we used an ABI 3730 Genetic Analyzer (Applied Biosystems, Waltham, MA) and then determined AFLP and MSAP profiles using Genemapper 3.7 (Applied Biosystems, Waltham, MA). We scored 285 AFLP markers with four primer combinations (E-ACT/M-CAG, E-ACA/M-CAC,

E-ACG/M-CAA, and E-AGC/M-CAC), and 324 MSAP markers with four primer combinations (E-ACG/H-AAT, E-ACG/H-CAT, E-AGC/H-AAT, and E-AAC/H-CAC) with either *Msp*I or *Hpa*II digestion.

To assess error rates in AFLP and MSAP fingerprinting, we repeated both methods on 23 samples (Bonin *et al.* 2004; Pompanon *et al.* 2005). Error rates were 0.02, 0.03, and 0.31% per sample and locus for AFLP, MSAP with *Msp*I, and MSAP with *Hpa*II, respectively, resulting in an average of 0.5, 0.1, and 1.0 errors per multilocus fingerprint. Compared to previous studies, these error rates were rather low, indicating that our peak scoring and (epi-)genotyping were reasonably robust. The higher error rate of the *Hpa*II restriction enzyme likely resulted from the greater levels of polymorphism detected with this enzyme than with *Msp*I (see later text). In molecular marker studies, error rates often increase with the number of polymorphic loci studied (Arnaud-Haond *et al.* 2007), probably because a larger number of loci more likely includes highly polymorphic—and more error-prone—individual loci.

We assigned the 83 knotweed clones to genotypes and epigenotypes, based on their pair-wise sample mismatch distances for AFLP markers, MSAP (*Msp*I), and MSAP (*Hpa*II) markers, respectively, using GenoDive (Meirmans & van Tienderen 2004). Since for all of these markers scoring errors cannot be ruled out and may create a peak at lower genetic or epigenetic distances (Meirmans & van Tienderen 2004; Arnaud-Haond *et al.* 2007), we used a mismatch threshold for assigning two samples to different genotypes or epigenotypes. Based on the estimated 1.0 errors per multilocus epigenotype for *Hpa*II, we allowed one mismatch between samples for this restriction enzyme. As this was already the lowest possible threshold, we used the same for AFLP and *Msp*I data, even though these markers had lower error rates. Thus, samples were generally assigned to different genotypes or epigenotypes if they differed in at least two AFLP or MSAP markers.

To characterize epigenetic diversity across samples, as well as within and among the different regions, we calculated the total numbers of polymorphic MSAP loci for the *Msp*I cutter, the *Hpa*II cutter, or both combined, separately for each region, or across all samples. Finally, we explored the epigenetic similarity of different origins using non-metric multidimensional scaling (NMDS; *vegan* package in R; Oksanen *et al.* 2015) of multi-locus MSAP profiles, using only the 19 loci with levels of polymorphism above 0.05. To test for regional differentiation, we used the *adonis* function in *vegan*, which hierarchically partitions the variance in distance matrices, in our case a Euclidean matrix based on the first and second NMDS axes.

Phenotypic variation

To test for phenotypic variation among the 83 knotweed clones, we set up a 2-year common garden experiment in spring 2008. We planted rhizome cuttings of 8 cm length, each with two intact nodes, into 61 pots filled with a 1 : 1 mixture of sand and fresh field soil, at 5 cm below the soil surface. Since there was some variation in the thickness of the planted rhizomes, we recorded the diameter of each. We planted five replicate pots for each clone. The 415 pots were arranged in five blocks in an experimental garden at the University of Bern, with one replicate of each clone in each block, and complete randomization within blocks. In the fall of 2008, we measured the leaf chlorophyll content of each clone, using a chlorophyll meter (SPAD-502, Konika Minolta, Osaka, Japan). We then cut all aboveground biomass, determined the total leaf area of each plant using a leaf area meter (LI-3100, Li-Cor, Lincoln, NE), dried and weighed leaves and remaining biomass, and determined the specific leaf area (SLA) of each plant as the ratio of total leaf area to total leaf dry weight, as well as its total dry aboveground biomass. In the spring of 2009, all clones resprouted from the rhizomes. To avoid nutrient depletion, we added slow-release fertilizer to each pot once. In the fall of 2009, we measured chlorophyll content and SLA, as described earlier, and then harvested the entire plants, including roots and rhizomes, which were carefully washed and separated. All plant parts were dried and weighed.

We analyzed the phenotype data with analysis of covariance, with initial size as a covariate, and experimental block, region of origin, and clone nested within region as categorical factors. As initial size, we used the rhizome volume, calculated from length and diameter measurements. We did these analyses for seven phenotypic traits: SLA in 2008 and 2009, leaf chlorophyll content in 2008 and 2009, total aboveground dry biomass in 2008, dry rhizome biomass in 2009, and total belowground (rhizome + root) biomass in 2009.

To explore overall phenotypic similarities among different origins, we conducted a principal component analysis (PCA; *vegan* package in R) using all seven phenotypic traits, and we then analyzed the Euclidean distance matrix based on the first and second PC with the

adonis function in R to test for regional differentiation in multivariate phenotypes.

Relationships between environmental, epigenetic, and phenotypic variation

To quantify climatic variation, we used long-term (1950–2000) data from the WorldClim database (www.worldclim.org). Specifically, we used nine bioclimatic variables that we considered to be most important to describe mean climate as well as climatic variability: annual mean temperature, total annual precipitation, the minima and maxima of temperature and precipitation (temperature of the coldest/hottest month and precipitation of the driest/wettest month), seasonality (=standard deviation) of temperature and precipitation, and isothermality (=temperature mean diurnal range/annual temperature range). To explore regional climate differences, we conducted a PCA and tested for regional differences as with the phenotypic data, using a Euclidean distance matrix based on the first two climate PCs.

To examine the relationships between environmental, epigenetic, and phenotypic variation, we used structural equation modeling (SEM), which related the multivariate MSAP and phenotypic data with climatic data from the origins of the knotweed clones. We used the *lavaan* package in R (Rosseel 2012) to test for the effects of climate (nine variables) and epigenetic variation (first two NMDS axes) on phenotypic variation (seven traits), also allowing indirect effects of climate on phenotype through epigenetic variation. We started with a full model where each climate variable had a direct effect on each phenotypic trait as well as the two epigenetic variables, and also each of the two epigenetic variables affected each phenotypic trait. We then removed climate variables that did not show any significant correlation with epigenotype or phenotype, as well as phenotypic traits that were not significantly affected by any of the climate or epigenetic variables, to obtain the most parsimonious structural equation model.

RESULTS

Molecular variation

We found significant epigenetic variation, but only very little genetic variation among the 83 knotweed clones. A small fraction of AFLP markers (12 out of 285, 4.2%) were polymorphic, with mostly one, and rarely two or three mismatches between samples. GenoDive assigned all samples to one clone, that is, for samples that differed by two or three loci all one-mismatch intermediates existed, so that all were assigned to the same clone. MSAP markers, in contrast, were more variable, particularly when the restriction enzyme *Hpa*II was used. With the *Msp*I cutter, 7% of the MSAP loci (24 out of 324) were polymorphic, but with *Hpa*II there were 25% polymorphic loci (81 out of 324; Table 1). With one mismatch allowed between samples, the 83 samples were assigned to 6 or 27 unique epigenotypes using the *Msp*I and *Hpa*II cutter, respectively. Because of the low levels of polymorphism in the *Msp*I data, adding the *Msp*I data to the *Hpa*II data hardly altered the total the number of polymorphic loci or epigenotypes identified, and did not affect the conclusions drawn. Therefore, we only present the MSAP analyses based on the *Hpa*II cutter. Out of the 27 epigenotypes identified, one epigenotype accounted for about 2/3 of our samples. This epigenotype was present in all of the seven regions, and dominant (>50% of the samples) in five of them, whereas most of the other epigenotypes were rare and occurred in only one or two regions, with one or two origins per region (Table 1). The analysis of the NMDS-based distance matrix did not find significant regional differentiation in multilocus MSAP epigenotypes (Fig. 1c).

Phenotypic variation

Many of the phenotypic traits measured were strongly affected by the spatial block in our experimental garden and, to a lesser degree, by the initial sizes of the planted rhizomes (Table 2). In several of the traits, we found significant differences between geographic regions, or among origins (=clones) within regions (Table 2). There were significant region effects on SLA in 2008, the chlorophyll content in 2009, and in the final total belowground biomass. For instance, plants from the regions of Kiel and Freiburg consistently produced more belowground biomass, but had lower SLA, than the plants from Leipzig and München (Fig. 2). These findings for individual phenotypic traits were corroborated by the analysis of multivariate phenotypic distances, which also indicated significant regional differentiation in phenotype (Fig. 1d). Besides regional differences, we also found significant

Table 1 Results of MSAP marker analysis, using the *Hpa*II restriction enzyme, that quantify DNA methylation variation in common-garden offspring of invasive Japanese knotweed from 83 Central European origins

Region	Number of samples*	Polymorphic loci	% of loci polymorphic	Number of epigenotypes	Epigenotypes†
Kiel	20	42	13%	10	e1(10×), e2, e3(2×), e4, e5, e6, e7, e8, e9, e10
Osnabrück	16	37	11%	8	e1(9×), e11, e12, e13, e14, e15, e16, e17
Leipzig	7	8	2%	2	e1(6×), e18
Darmstadt	13	23	7%	5	e1(9×), e2, e19, e20, e21
München	8	34	10%	6	e1(3×), e22, e23, e24, e25, e26
Freiburg	10	7	2%	1	e1(10×)
Ticino	9	5	2%	3	e1(7×), e4, e27
Total	83	81	25%	27	

*Within a region of origin, each sample represents a geographically distinct location.
†Different epigenotypes are denoted by e1–e27. If an epigenotype was found multiple times within a region, this is indicated in brackets.

Table 2 Analysis of variance of phenotypic variation in common-garden offspring from 83 origins of the invasive Japanese knotweed

Phenotypic traits	Block (d.f. = 4)		Initial size (d.f. = 1)		Region (d.f. = 6)		Origin (region) (d.f. = 76)	
	F-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value
SLA (2008)	184.27	**<0.001**	24.40	**<0.000**	2.35	**0.031**	1.42	**0.020**
SLA (2009)	11.56	**0.001**	1.79	0.182	0.52	0.795	0.93	0.651
Chlorophyll (2008)	180.10	**<0.001**	4.75	**0.030**	0.50	0.808	0.96	0.563
Chlorophyll (2009)	151.93	**<0.001**	1.06	**0.304**	2.42	**0.027**	0.94	0.619
Aboveground mass (2008)	44.50	**<0.000**	17.41	**<0.000**	0.82	0.555	0.92	0.668
Rhizome mass (2009)	0.04	0.839	37.29	**<0.000**	1.76	0.107	1.40	**0.024**
Total belowground mass (2009)	1.29	0.257	0.46	0.499	2.57	**0.019**	1.12	0.247

The plants originated from seven different regions in Central Europe. Initial size is the estimated volume of the planted rhizomes. Significant *P*-values are in bold.
d.f., degrees of freedom and the residual d.f. = 323; SLA, specific leaf area.

differences among origins within regions for SLA 2008 and final rhizome mass (Table 2).

Relationships between environmental, epigenetic, and phenotypic variation

The most parsimonious SEM linking environmental, epigenetic, and phenotypic variation contained only five of the nine tested climate variables and five of the seven measured phenotypic traits (Fig. 3). The fit of the model was good (RMSEA = 0.000, $P = 0.680$). Interestingly, variables describing climate means were dropped from the model, and those maintained in the model were all related to climate variability and extremes, indicating that these may be most relevant for explaining variability of Japanese knotweed. The model indicated that phenotypic variation among clones of Japanese knotweed is related to both environmental and epigenetic variation, with two groups of traits: (1) variation in SLA was significantly related to climate of origin but especially to the epigenetic variation among clones. Some of these strong epigenetic influences appeared to be climate-related, whereas

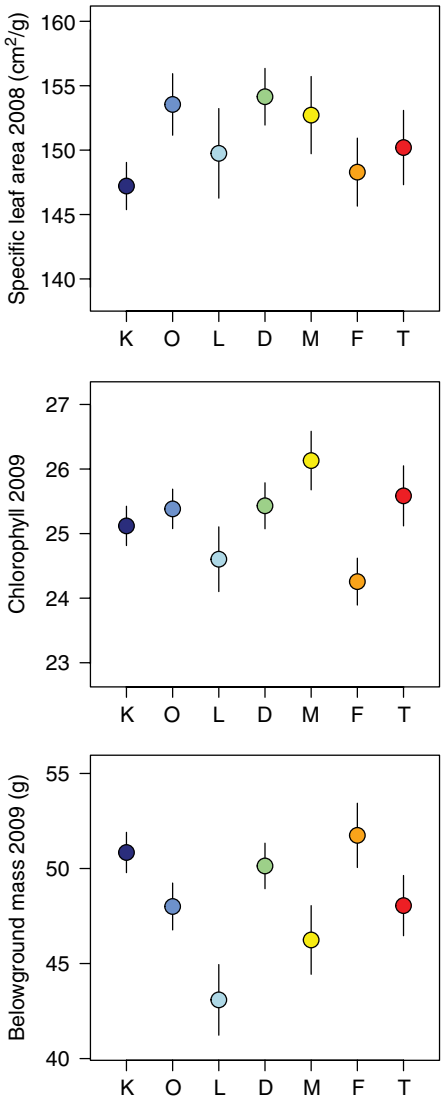

Fig. 2 Regional variation in phenotype among invasive Japanese knotweed clones. The data (means ± SD) are from common-garden progeny of 83 Central European origins of *Fallopia japonica* distributed across seven geographic regions: K = Kiel, O = Osnabrück, L = Leipzig, D = Darmstadt, M = München, F = Freiburg, and T = Ticino. The colors are as in Fig. 1. (*See insert for color representation of the figure.*)

others were independent of climate; (2) variation in above- and belowground biomass traits was significantly correlated with several of the climate variables, but not with epigenetic variation. The strongest patterns were found for belowground biomass, which was significantly related to four of the five climate variables, in particular precipitation variability.

DISCUSSION

Some invasive plants appear to colonize large ranges without any genetic diversity. This is surprising, given the importance of genetic diversity for adaptation and thus, presumably, range expansion. Here, we find that genetically uniform clones of Japanese knotweed, one of the world's most successful plant invaders (Lowe *et al.* 2000), harbor substantial epigenetic and phenotypic variation, and that this variation is associated with climate of origin and thus possibly involved in habitat adaptation.

Our AFLP analysis of the 83 clones of Japanese knotweed indicated that all of them belonged to the same genetic clone, or were at least nearly identical, which is consistent with the results of several previous molecular studies of European populations (Hollingsworth & Bailey 2000; Mandák *et al.* 2005; Krebs *et al.* 2010). In contrast, MSAP markers detected substantial DNA methylation variation and assigned our 83 samples to 27 different epigenotypes. Although we cannot entirely rule out DNA sequence variation in our samples—rare somatic mutations cannot be distinguished from scoring errors by the AFLP method (Douhovnikoff & Dodd 2003; Bonin *et al.* 2004; Meirmans & van Tienderen 2004)—our results indicate that epigenetic variation is at least an order of magnitude larger than genetic variation among Central European origins of Japanese knotweed. As all Japanese knotweed in Europe is descended from a single clone (Bailey & Conolly 2000), there are two possible explanations: (1) spontaneous epimutations have accumulated much faster than sequence mutations during the more than 150 years since the species was introduced, or (2) environmental differences in the source populations induced epigenetic changes that have persisted through several years of vegetative propagation in a common environment.

If the observed epigenetic variation is the result of spontaneous epimutation, it could be largely neutral, without any functional and ecological significance. DNA methylation changes are expected to have the greatest effects when they occur close to transposable elements or in the regulatory regions of genes, but they are thought to be of little functional significance in the

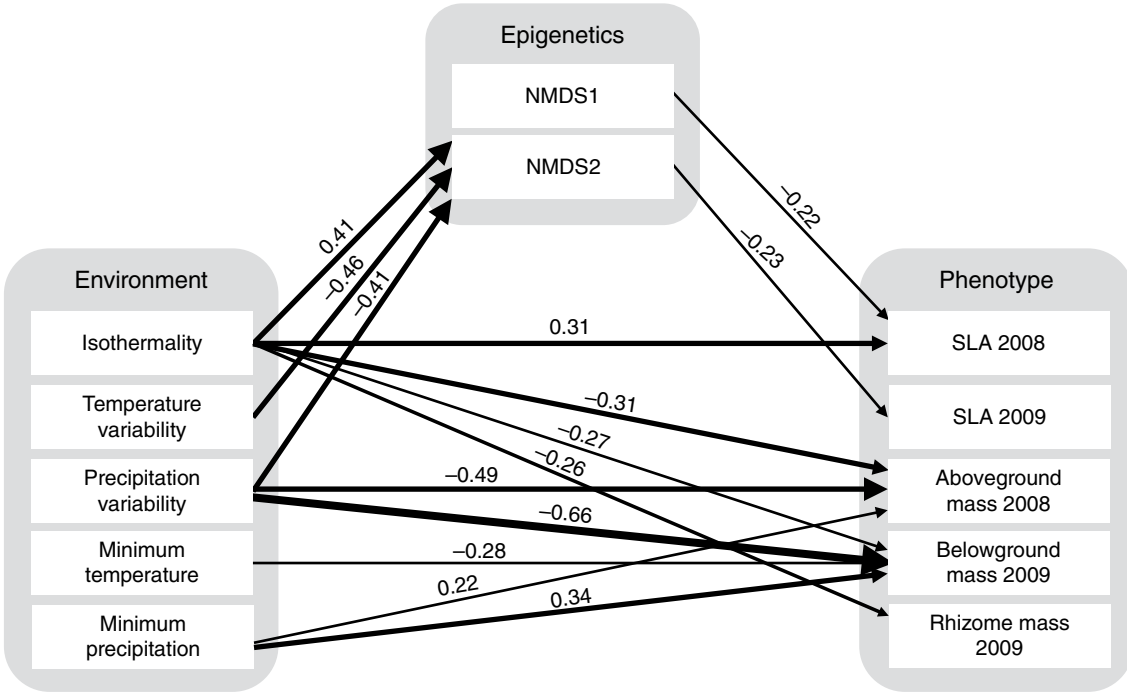

Fig. 3 Relationships between environmental, epigenetic, and phenotypic variation among Japanese knotweed clones. The data describe 83 Central European origins of the invasive Japanese knotweed (*Fallopia japonica*). The epigenetic variables are the first two axes from a non-metric multidimensional (NMDS) analysis of 19 polymorphic MSAP (=DNA methylation) markers. The phenotyping of different clones was after several years of propagation in a common environment. The arrows indicate significant correlations between variables, with arrow thickness scaled to the effect size (values on arrows).

coding region of genes (Mirouze *et al.* 2009; Becker *et al.* 2011; van der Graaf *et al.* 2015). If epigenetic variation is largely due to epimutation, we should find many private epigenotypes that occur only in single locations. This is indeed what we find. Many of the identified epigenotypes are rare, which suggests that even if part of the epigenetic variation in Japanese knotweed is nonrandom, epimutation rates may be high and add enough "noise" for clones to be assigned to different epigenotypes even if they share a common epigenetic response to the environment.

If, in contrast, the observed epigenetic variation is the result of natural selection or environmental induction, then it should be accompanied by systematic variation in phenotype. In our common garden experiment, we found significant variation in several phenotypic traits among the different clones representing different populations and regions of origin. Besides differences in belowground biomass and rhizome production, the clones significantly differed in two key traits of plant ecological strategy: leaf chlorophyll content and SLA. Leaf chlorophyll is closely related to leaf nitrogen, photosynthetic capacity, and nutrient allocation strategy, whereas SLA is thought to capture one of the main general axes of plant strategy and life history variation (Westoby *et al.* 2002; Wright *et al.* 2004), with high SLA related to fast growth and competitive ability in nutrient-rich environments, and low SLA reflecting a slower growth strategy in less favorable environments. SLA has frequently been found to be positively correlated with plant invasiveness (e.g., Grotkopp *et al.* 2002; Hamilton *et al.* 2005; Grotkopp & Rejmánek 2007).

In summary, the 83 investigated genetically identical knotweed clones were not only epigenetically variable, but they also showed significant variation in several key phenotypic traits related to habitat adaptation. It is important to stress that we measured both

epigenetic and phenotypic variation in a common garden, so the observed variation did not reflect phenotypic plasticity, but it was stable variation that persisted for several years and across several vegetative generations, despite identical environmental conditions.

To further corroborate the ecological significance of the observed epigenetic and phenotypic variation, we used a structural equation model that linked the two kinds of variation with the climatic variation of the 83 origins. We found that with regard to their epigenetic and climatic correlates, the studied phenotypic traits fell into three groups: (1) SLA was strongly correlated with epigenetic variation and to a lesser degree with climatic variation. Our data thus support the idea that for this important plant strategy trait different epigenetically based phenotypes of knotweed may have been selected in different areas of the invasive European range. (2) Biomass traits were significantly correlated with climate of origin, but not with epigenotype. Here, the persistent phenotypic differences may also reflect some aspect of habitat adaptation, for instance, greater root production in resource-limited habitats (Poorter & Nagel 2000). The underlying mechanisms may either be non-epigenetic such as long-term nutritional or physiological effects (Herman & Sultan 2011; Latzel *et al.* 2014), or the low-resolution MSAP method did not capture the relevant epigenetic variation. (3) Leaf chlorophyll content was neither correlated with epigenetic variation nor with any of the climatic variables, despite significant regional differentiation detected in the analyses of variance. Here, our analysis most likely did not include any of the environmental factors that are relevant for this functional trait.

Altogether, the SEM analysis supports the idea that at least part of the observed phenotypic variation is functionally related to epigenetic variation, and that the observed epigenetic variation cannot entirely result from epimutation. Both kinds of variation thus appear to be ecologically relevant and ultimately driven by environmental differences among habitats of origin. A more thorough understanding of these functional relationships would require (a) more detailed small-scale environmental information from all source populations, in particular about light, soil, and nutrient conditions, and (b) higher-resolution epigenomic data. Both were beyond the scope of our study, and in particular the latter is currently still not feasible in a polyploid species with a large genome such as Japanese knotweed.

There is one previous study on epigenetic variation in invasive knotweed. Richards *et al.* (2012) also used AFLP and MSAP markers to analyze 16 invasive *Fallopia* populations on Long Island, NY. In contrast to our study, however, their samples contained different knotweed species and hybrids, including multiple genotypes of *F. japonica*. Consistent with our study, they found that epigenetic variation greatly exceeded genetic variation, and that plants with the same AFLP haplotypes differed at the level of DNA methylation. Although the same authors also showed that genetically identical plants from different populations strongly differed in phenotype (Richards *et al.* 2008), they did not link epigenetic and phenotypic variation, and their study design did not allow testing for environmental correlates. We are aware of only one other epigenetic study of an invasive plant: Gao *et al.* (2010) analyzed epigenetic variation, again using MSAP, in three (almost) genetically uniform populations of the invasive alligator weed (*A. philoxeroides*) in China. They, too, found that epigenetic variation was much greater than DNA sequence variation, and that populations from different origins maintained part of their epigenetic variation in a common environment.

Our study demonstrates that genetically uniform clones of Japanese knotweed are epigenetically and phenotypically variable across Central Europe, and that this variation is related to the environment of origin. A plausible interpretation is that Japanese knotweed, despite its genetic uniformity, has adapted to different habitats through epigenetic or other nongenetic means. It is an intriguing possibility that similar results might be found in other successful clonal invaders, and we clearly need more studies that thoroughly address these questions across a larger range of clonal invaders.

Epigenetic variation is generally thought to be more dynamic than genetic variation, and theoretical models have shown that epigenetic inheritance may be adaptive particularly in changing environments (e.g., Geoghegan & Spencer 2012; Klironomos *et al.* 2013). If epigenetically based adaptation is possible, it might play a particularly important role in biological invasions, and it might be "game-changing" for genetically uniform clonal invaders. Clearly, we need to study not only the genetics, but also the epigenetics of colonizing species.

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