Is geographic variation within species related to macroevolutionary patterns between species?

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Abstract

The relationship between microevolution and macroevolution is a central topic in evolutionary biology. An aspect of this relationship that remains very poorly studied in modern evolutionary biology is the relationship between within-species geographic variation and among-species patterns of trait variation. Here, we tested the relationship between climate and morphology among and within species in the salamander genus *Plethodon*. We focus on a discrete colour polymorphism (presence and absence of a red dorsal stripe) that appears to be related to climatic distributions in a common, wide-ranging species (*Plethodon cinereus*). We find that this trait has been variable among (and possibly within) species for >40 million years. Furthermore, we find a strong relationship among species between climatic variation and within-species morph frequencies. These between-species patterns are similar (but not identical) to those in the broadly distributed *Plethodon cinereus*. Surprisingly, there are no significant climate–morphology relationships within most other polymorphic species, despite the strong between-species patterns. Overall, our study provides an initial exploration of how within-species geographic variation and large-scale macroevolutionary patterns of trait variation may be related.

Introduction

How are patterns of geographic variation within species related to macroevolutionary patterns of variation among species? We argue that this is a potentially important but relatively neglected topic in modern evolutionary biology. A major goal of evolutionary studies is to explain patterns of trait variation among species (macroevolutionary comparative studies), and another major focus is on processes that occur within species (microevolutionary studies). Many studies have addressed topics at the intersection of these two research areas, but without considering both geographic variation and macroevolution. For example, many studies have addressed how microevolutionary processes within species (e.g. drift, selection) might explain

variation between species, but typically treating each species as a single population (e.g. Hansen & Martins, 1996; Arnold *et al.*, 2001; Hohenlohe & Arnold, 2008). Similarly, studies have addressed the role of within-species phenotypic plasticity in between-species divergence (e.g. Emlen *et al.*, 2005; Gomez-Mestre & Buchholz, 2006), but generally have not considered geographic variation (but see below). Numerous studies have analysed the potential role of geographic variation (and local adaptation) in some modes of speciation (e.g. parapatric ecological speciation along environmental gradients: Schneider *et al.*, 1999; Rundle & Nosil, 2005; Nosil, 2012), but have not generally related these geographic patterns to larger-scale patterns of trait evolution among species. At the same time, very few studies have addressed between-species macroevolutionary patterns in within-species geographic variation (e.g. de Queiroz & Ashton, 2004; see below).

There are many possible patterns and relationships between intraspecific geographic variation and macroevolutionary trait variation. One intriguing scenario is the possibility that adaptive geographic variation

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maintains polymorphism over macroevolutionary time-scales and provides the raw material for macroevolutionary character change among species within a clade. Under this scenario, there is a relationship between a given character and one or more abiotic or biotic environmental variables that is present within species as geographic variation and is seemingly maintained over multiple speciation events and macroevolutionary timescales. However, in some species, particularly those having more extreme values for the environmental variable or occurring under a limited range of conditions, the trait may be fixed or otherwise relatively invariant. Overall, the major signatures of this scenario should include the following: (i) a relationship between the environment and geographic trait variation within one or more species that parallels the between-species relationship between environmental and trait variation and (ii) evidence that the within-species variation has been maintained over long timescales. At present, we do not know of clear examples of such a scenario. There are certainly many antecedents to the general idea that pre-existing genetic variation and patterns of variation are important in macroevolution (e.g. Schluter, 1996; Barrett & Schluter, 2008; Schluter & Conte, 2009), but these have not dealt explicitly with both intraspecific geographic variation and patterns of trait evolution among species.

We emphasize that this scenario represents only one of many possible patterns of within-species geographic variation among species and between-species trait evolution. We describe several others below. In describing these scenarios, we consider a trait that is potentially related to an environmental variable that varies geographically (e.g. temperature). First, within-species relationships between the trait and the environmental variable may be present or absent among the species in the clade, and if present, the nature of these trait–environment relationships may be similar among species across the tree or not. For example, different species may show the same trait–environment relationship, whereas others may reverse this relationship or show no relationship at all (i.e. either uniform or randomly varying across different environments). These within-species trait–environment relationships may vary in ways that are consistent with the phylogeny among species (e.g. species of the same clade share similar trait–environment relationships), vary randomly among species across the tree or show some intermediate pattern. Different patterns of geographic variation among species in a clade might arise through changes in environments and selective pressures among species, changes in levels of genetic variation among species for genes associated with the trait, or changes in the genetic or developmental basis of the trait across the tree. Trait–environment relationships among species (e.g. based on mean values among populations of each species) may then parallel those within species, may be strong but distinctly different or may be completely absent. If there is a strong trait–environment relationship among species, then variation in the trait–environment relationship within species may depend on the absolute position of each species in the overall niche space for the group. For example, species occurring in more extreme environments on the niche axis (or simply in a limited range of environments) might show limited variation in the trait, and little trait–environment relationship. In contrast, species in more intermediate environments, or occurring across a broader range of environments, might show a much stronger trait–environment relationship. These trait–environment relationships may themselves impact the niche width of the species, for example if variation in the trait allows the species to occur across a broad range of conditions, facilitating a broader species niche width and (possibly) a larger geographic range size.

To our knowledge, these potential scenarios have not been tested in any group of organisms. Nevertheless, a large body of work has tested 'ecogeographic rules' that relate environments and morphology. For example, Bergmann's rule, the tendency for body sizes to be larger in colder environments (e.g. Bergmann, 1847; Mayr, 1956), has been the focus of many studies (e.g. Ashton et al., 2000; Belk & Houston, 2002; Angilletta & Dunham, 2003; Ashton & Feldman, 2003; Adams & Church, 2008). Along the lines that we discuss, at least one study has examined whether there are large-scale phylogenetic trends in within-species patterns across major groups (e.g. de Queiroz & Ashton, 2004). Further, some studies have tested for this rule both within and between species (e.g. Adams & Church, 2008). However, to our knowledge no modern studies have attempted to relate within-species and between-species relationships between a trait and the environment. Jockusch (1997) examined variation in vertebral number within and between species in Batrachoseps salamanders (including finding parallel, within-species clines in three species), but did not include large-scale climatic data and so did not examine within- and between-species relationships between this trait and the environment. Jockusch (1997) did find a relationship between developmental temperature and vertebral number, but this plasticity was not enough to explain the geographic patterns observed.

Here, we examine the relationship between variation in a morphological trait and variation in the environment within and between species of Plethodon salamanders. Two discrete colour morphs occur in several species of Plethodon (Petranka, 1998): a striped morph (with a distinct mid-dorsal stripe that is typically red or similarly coloured) and an unstriped morph (lacking the mid-dorsal stripe and with mid-dorsal coloration that is typically grey as on the flanks). Some species are polymorphic and include both colour morphs, whereas

others appear to be fixed for one morph or the other (Petranka, 1998). Most of our knowledge about the biology of this polymorphism comes from studies of *Plethodon cinereus*, one of the most common vertebrates in eastern North America (Petranka, 1998). In *P. cinereus*, the colour morphs appear to be genetically heritable and not phenotypically plastic (Highton, 1975). The function of the stripe is unclear, but may be associated with frequency-dependent selection by predators (Fitzpatrick *et al.*, 2009). One of the most intriguing aspects of this polymorphism is that different colour morphs seem to be associated with different ecological distributions. In *P. cinereus*, striped individuals seem to occur at higher frequency in cooler environments (i.e. higher latitudes and elevation) and appear to be less heat- and drought-tolerant and more cold-tolerant than unstriped individuals (e.g. Lotter & Scott, 1977; Moreno, 1989; Petranka, 1998; Gibbs & Karraker, 2006; Anthony *et al.*, 2008; Noël & Lapointe, 2010; but see Petruzzi *et al.*, 2006; Moore & Ouellet, 2014). The two colour morphs are sympatric through much of the range of *P. cinereus* (Petranka, 1998). On Long Island, New York, however, colour morphs are parapatrically distributed (but separated by polymorphic populations; Williams *et al.*, 1968), and differences in morph frequencies and environments are strongly related to genetic divergence (Fisher-Reid *et al.*, 2013). This pattern raises the possibility that climatic-niche differences between colour morphs may be important in speciation, especially because several species in the genus are fixed for different morphs (Petranka, 1998). Colour morphs on Long Island are also associated with variation in costal groove number (Williams *et al.*, 1968; Fisher-Reid *et al.*, 2013): costal grooves indicate divisions between body segments on the trunk, and the number of costal grooves is related to the number of trunk vertebrae (e.g. Jockusch, 1997). However, the possible relationships between colour morphs and costal groove number have not been tested more widely across species. Overall, there are many intriguing but fragmentary patterns in *P. cinereus* and related species that suggest the possibility of relationships between morphology and climate both between and within species.

In this study, we explore how within-species geographic variation may be related to between-species, macroevolutionary patterns of trait variation, and how morphology–environment relationships within species vary among species at a macroevolutionary scale. Specifically, we analyse data on colour morph frequencies and climate within and between *Plethodon* species to address two main questions. First, are there relationships between colour morphs and climate within polymorphic *Plethodon* species, and how have these relationships evolved among species? Second, are there relationships between morphology and climate between species that parallel the within-species patterns? We also address several related questions, such as how

climate–morphology relationships within species might influence the climatic-niche widths of species and geographic range sizes, and whether the coupling of costal groove numbers, climate and morph frequencies observed within species (e.g. Fisher-Reid *et al.*, 2013) also occurs among species.

Materials and methods

Phylogeny

We used the time-calibrated, multilocus, near-comprehensive phylogeny of *Plethodon* species from Kozak *et al.* (2009), based on the plethodontid root age of 61 Myr (Fig. 1). Although this root age for Plethodontidae is somewhat younger than in some other studies (e.g. Roelants *et al.*, 2007; Vieites *et al.*, 2011; Pyron, 2014), the estimated age of *Plethodon* itself (~42 Myr) is similar (~45 Myr) to that estimated by Zheng *et al.* (2011), a study using multiple nuclear genes across all salamanders. Furthermore, using a tree with an older plethodontid root age (69 Myr) yields the same age for *Plethodon* (~42 Myr; Kozak *et al.*, 2009). Fisher-Reid & Wiens (2011) estimated a phylogeny of *Plethodon* using more loci than Kozak *et al.* (2009), but the tree was very similar in overall topology, was not time-calibrated and lacked some species (i.e. *P. neomexicanus*). Other plethodontid phylogenies also included fewer *Plethodon* species and so were not used (e.g. Vieites *et al.*, 2011).

The time-calibrated tree for *Plethodon* was then pruned to include only those species showing the striped–unstriped colour polymorphism or that were fixed for one morph or the other. In short, *Plethodon* consists of four named, well-supported clades (Western *Plethodon*, *cinereus* group, *wehrlei-welleri* group and *glutinosus* group; Wiens *et al.*, 2006; Kozak *et al.*, 2009; Fisher-Reid & Wiens, 2011). Western *Plethodon* occur in north-western North America, whereas the other three clades occur in broad-scale sympatry in eastern North America (Petranka, 1998). Both striped and unstriped colour morphs are present in the first three groups, which include the two groups closest to the *Plethodon* root. Species of the *glutinosus* group are more difficult to assign to one of these two morphs, typically having dark dorsal coloration with white spots (Petranka, 1998). Therefore, our main analyses excluded this relatively derived and recent group. However, we also conducted a set of analyses including this group (see Results). We also excluded the spotted *P. punctatus* (*wehrlei-welleri* group), although its inclusion has relatively little impact (see Results). Overall, our analyses were based primarily on the 22 species that could be readily classified as either striped, unstriped or polymorphic based on the descriptions in Petranka (1998) and AmphibiaWeb (2015; Fig. 1).
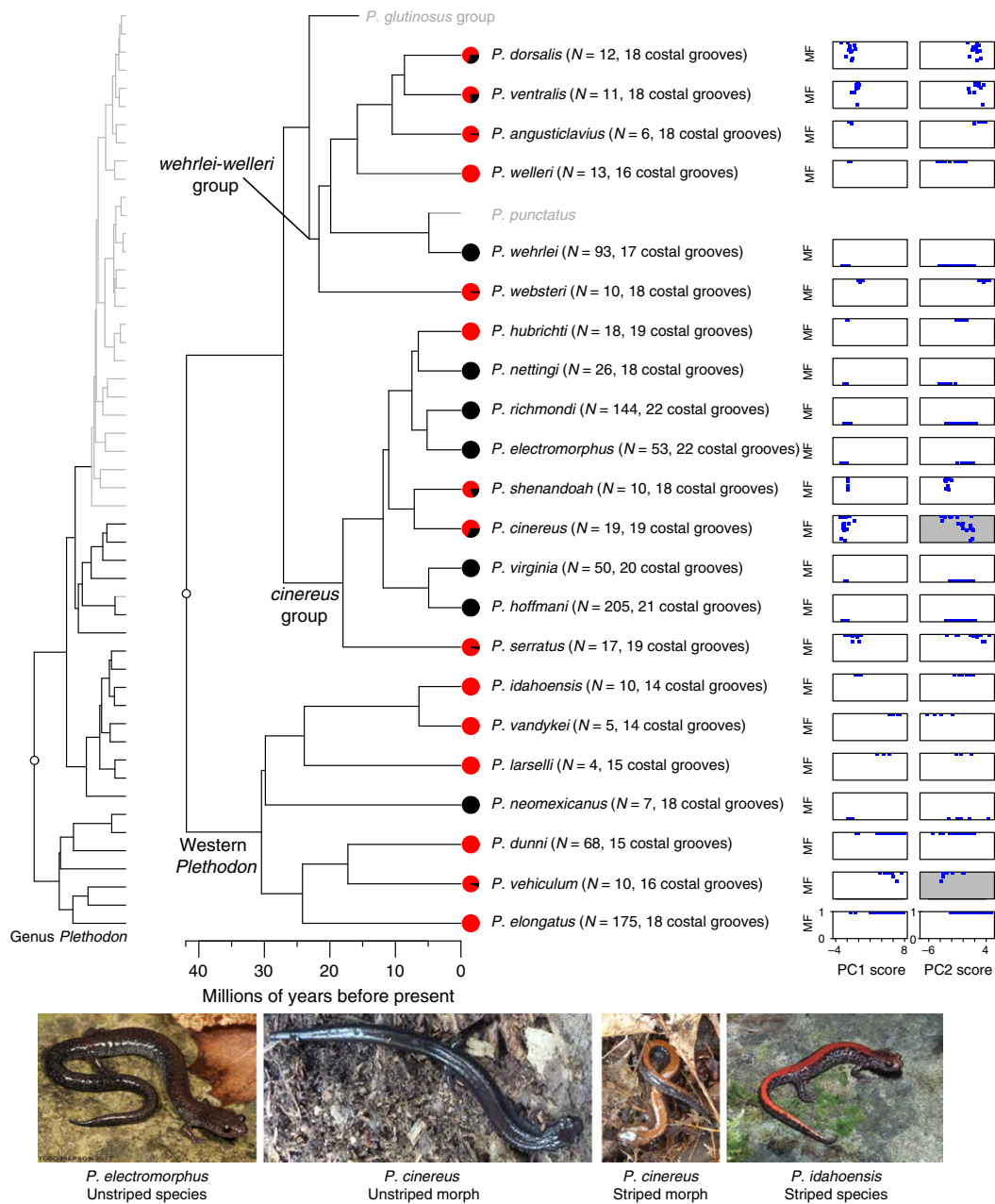
Fig. 1 Time-calibrated phylogeny of genus *Plethodon* (left) and the subset of species that are the focus of this study (right), showing morph frequencies, costal groove numbers and within-species relationships between climatic variables (PC1 and PC2) and morph frequencies. In each graph, the y-axes depict morph frequency (MF), ranging from 0 (all unstriped) to 100% (all striped), and the x-axes depict PC1 or PC2 scores. The PC1 x-axis ranges from −4 to 8, and the PC2 x-axis ranges from −7 to 5. Significant relationships are indicated with grey boxes. Pie charts display mean morph frequency (red = striped; black = unstriped) across the sampled localities for each species (Table 2), and the median costal groove number is listed. *N* is the number of localities used for climatic data for each species, and also the number of localities used to estimate morph frequencies for polymorphic species. See Fig. S2 for zoomed-in graphs for the eight polymorphic species. Pictured are the two colour morphs of *P. cinereus*, and representative monomorphic species (striped: *P. idahoensis*; unstriped: *P. electromorphus*). Photographs used with permission from M.C. Fisher-Reid (*P. cinereus*); W. Leonard (*P. idahoensis*); and T. Pierson (*P. electromorphus*).

Morphological data

We tested the hypothesis that morph frequencies and climate were related (within and between species) based on a combination of existing data from the literature and climatic databases, as well as new data from museum specimens. Species known to be monomorphic were assigned morph frequencies of 100% (all striped) or 0% (all unstriped) based on literature descriptions (Petranka, 1998; AmphibiaWeb 2015). For these species, we used all available georeferenced localities from Kozak & Wiens (2010) for climatic data. Petranka (1998) noted that *P. hoffmani* and *P. virginia* are predominantly unstriped, but do contain some striped individuals. However, the striped morph is extremely rare in these species, and no striped individuals for either species are present in the U.S. National Museum (USNM hereafter) collection (A. Wynn, pers. comm.), the world's largest collection of *Plethodon* specimens (~216 000 specimens; HerpNet.org). Importantly, the USNM contains 89.5% of listed *P. hoffmani* specimens (1745/1949 records) and 99.8% of *P. virginia* specimens (460/461 records; HerpNet.org). Therefore, these two species were treated as having frequencies of 0% striped. However, if the true frequency is actually slightly higher than 0%, this should have virtually no impact on our analyses.

For species known to be polymorphic, we obtained climatic and morphological data to test the relationship between environment and morph frequencies among populations. We estimated morph frequencies (% striped) for georeferenced localities (i.e. localities with latitude and longitude) by examining preserved specimens at the USNM and scoring individuals for colour morph. Our observations of many living and preserved *Plethodon* species suggest that standard alcohol preservation does not hinder correct assignment of preserved specimens to colour morphs. From the USNM database, we selected localities for each polymorphic species. Localities were chosen that were as follows: (i) georeferenced and (ii) had 4 or more individuals. When a given locality was selected, up to 85 specimens from that locality were scored (mean: 31 individuals). Whenever possible, all individuals from a locality were scored. However, several localities had hundreds of individuals or were stored with hundreds of individuals from multiple localities, and in these instances, only the first 30–50 individuals from the selected locality were scored (i.e. the first specimens removed and sorted from a given jar for a given locality; jars appeared to be well mixed and unbiased with regard to morph frequencies).

Most species were represented by at least ten moderately well-sampled (>15 individuals) localities at the USNM, which broadly cover the geographic ranges of these species, and so all of these localities were included. For *P. shenandoah*, all of our sampled localities are necessarily very close together geographically, as this species is known only from three isolated mountaintops within 10 km of each other (Hammerson & Mitchell, 2004). Additionally, for two species, *P. cinereus* and *P. serratus*, there were more localities available than we could reasonably sample, and so we systematically subsampled localities based on the details of the geographic range of each species. *Plethodon serratus* is known from four disjunct regions (Petranka, 1998), and we sampled a total of 17 localities representing all four regions. *Plethodon cinereus* is widely distributed in north-eastern North America (Petranka, 1998). We selected at least one locality from every state represented in the USNM collection, plus a high- and low-elevation locality in the very well-sampled states (Pennsylvania, Maryland, Virginia, West Virginia) to better represent the range of relevant climatic variation, for a total of 19 localities. We initially excluded *P. cinereus* localities from Long Island and the Delaware–Maryland–Virginia (Delmarva) Peninsula, as these two coastal regions exhibit climatic and morph frequency patterns that are very different from mainland *P. cinereus* (Williams *et al.*, 1968; Highton, 1977; Fisher-Reid *et al.*, 2013). We then tested the effect of including these regions by conducting supplementary within-species analyses, which included two localities each from both Long Island and Delmarva (one 100% striped and one 100% unstriped). We note that simply including every known locality in these small, densely sampled regions might lead to artifactual results on the species-wide relationship between morph frequencies and climate, and so we instead attempted to structure our sampling of localities geographically.

Overall, each of the 8 polymorphic species was scored for 6–19 localities (mean: 12), with 4–85 individuals per locality (mean: 31), and a total of almost 3000 specimens examined. Morph frequencies and geographic coordinates for each of the 95 museum-based localities are provided in Appendix S1. Colour morph data for each individually scored USNM specimen are provided in Appendix S2. Three individuals of *P. ventralis* appeared to be a solid red colour but completely lacked a dorsal stripe. These were counted as unstriped individuals. In contrast, the erythristic morph of *P. cinereus* is also completely red in colour, but the outline of a distinct stripe is visible in both live and preserved specimens (M. C. Fisher-Reid, pers. observ.). These decisions should have little impact on the overall estimates of morph frequencies in these populations and species.

Costal groove number is another morphological trait that has been suggested to vary with climate in certain salamanders; however, this has not been explicitly tested (e.g. Highton, 1977; Jockusch, 1997; but see Fisher-Reid *et al.*, 2013 for an analysis on Long Island). We obtained data for each species on median costal groove number from the literature (Highton, 1962,

1997, 1999; Petranka, 1998). Unlike colour morph, variation in costal grooves has a more obvious adaptive explanation, with more costal grooves hypothesized to be beneficial for fossorial species (Jockusch, 1997). Many salamanders burrow underground when surface environmental conditions are unfavourable (too hot or too dry; Heatwole, 1960). We acknowledge that comparing costal groove numbers and morph frequencies among localities within species would also be interesting (as in Fisher-Reid et al., 2013), but this is outside the scope of the current study.

Climatic data

For the 14 monomorphic species (Fig. 1), we used data from 19 climatic variables previously extracted by Kozak & Wiens (2010) from the WorldClim data set at ~1 km^2 scale (Hijmans et al., 2005). These data included 871 georeferenced localities (range: 4–205 localities/species, mean: 62 localities/species). For the eight polymorphic species, we extracted these same 19 climate variables for only those 95 georeferenced localities for which we estimated morph frequencies from USNM specimens (range: 6–19 localities/species, mean: 12 localities/species).

In order to verify that we had large enough samples for polymorphic species to represent their realized climatic niche, we compared the climatic-niche breadths estimated here with the climatic-niche breadths estimated by Fisher-Reid et al. (2012), which are more extensively sampled (i.e. based on locality data only, with no morph frequencies). Comparisons were made based on niche breadths estimated from principal component analysis (PCA) of the climatic variables for the two PCs that explain more variation than expected by chance (see below for details). These two estimates of niche breadth were very strongly correlated for PC1 ($r_s = 0.929$, $P = 0.002$), with no obvious outliers. There was also a significant correlation for PC2 ($r_s = 0.738$, $P = 0.046$). However, P. vehiculum had a much larger PC2 climatic-niche breadth in the Fisher-Reid et al. (2012) analysis (breadth = 7.9; 91 localities) than in the present study (breadth = 4.4; 10 localities). This does not present a problem for our between-species analyses, however, as nearly all of the individuals we sampled at the USNM for P. vehiculum were striped (mean striped morph frequency among populations = 95%). Thus, this species is effectively monomorphic for our analyses, and PC2 did not show a significant relationship with morph frequencies among species. It may instead present a problem for our within-species analyses, as the limited range of climatic variation among populations might prevent finding a significant relationship between climate and morph frequencies. However, we did find a significant relationship between PC2 and morph frequency in P. vehiculum (see Results). This demonstrates that the more limited

sampling of localities for niche breadth in this species was not problematic overall.

Statistical analyses

To reduce the complex climatic data (19 variables), we performed a PCA on the correlation matrix of the climate data for all 22 species across all 966 localities using JMP version 10.0.2 (SAS Institute Inc.; Table 1, Fig. 2). All subsequent statistical analyses were conducted in R version 3.0.2 (R Development Core Team 2013). PC1–3 account for >90% of the climatic variation among species; however, we only used PC1–2 (83.4% of the variation) in subsequent analyses, because only these two axes account for more variation than expected by chance (according to the broken-stick distribution; Jackson, 1993). Following Fisher-Reid et al. (2012), we estimated the mean climatic-niche values (among localities) and climatic-niche breadth (maximum minus minimum values among localities) for each species for PC1–2 and seven univariate temperature and precipitation variables. These seven variables are thought to be potentially important in determining the large-scale distribution of salamander species

Table 1 Factor loadings, eigenvalues and percentage variation explained for the first two axes of the principal components analysis on the 19 climatic variables.

Climatic variables	PC1	PC2
BIO1: Annual mean temperature	0.24	**0.71**
BIO2: Mean diurnal range (mean of monthly [maximum temperature − minimum temperature])	−0.20	**0.78**
BIO3: Isothermality (BIO2/BIO7 × 100)	**0.93**	0.14
BIO4: Temperature seasonality (SD × 100)	**−0.97**	0.11
BIO5: Maximum temperature of warmest month	−0.43	**0.88**
BIO6: Mean temperature of coldest month	**0.93**	0.22
BIO7: Temperature annual range (BIO5–BIO6)	**−0.91**	0.32
BIO8: Mean temperature of wettest quarter	**−0.79**	−0.06
BIO9: Mean temperature of driest quarter	**0.79**	0.39
BIO10: Mean temperature of warmest quarter	**−0.61**	**0.66**
BIO11: Mean temperature of coldest quarter	**0.86**	0.32
BIO12: Annual precipitation	**0.78**	−0.41
BIO13: Precipitation of the wettest month	**0.96**	−0.15
BIO14: Precipitation of the driest month	**−0.85**	−0.30
BIO15: Precipitation seasonality (coefficient of variation)	**0.94**	0.18
BIO16: Precipitation of the wettest quarter	**0.96**	−0.17
BIO17: Precipitation of the driest quarter	**−0.80**	−0.33
BIO18: Precipitation of the warmest quarter	**−0.87**	−0.30
BIO19: Precipitation of the coldest quarter	**0.97**	−0.13
Eigenvalue	12.53	3.31
% Variation	65.93	17.43

PC1–PC2 account for 83.4% of the variation among species and account for significantly more variation than expected by chance according to a broken-stick distribution. Bolded values load strongly (absolute value > 0.9 for PC1, >0.6 for PC2) onto a given PC axis.
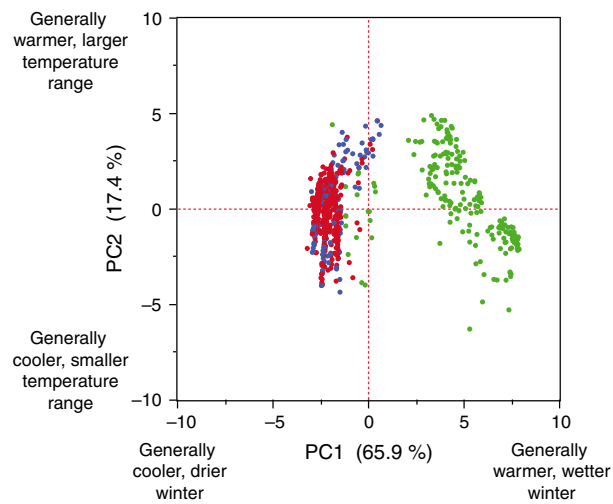
Fig. 2 PCA of climatic niche for 22 *Plethodon* species that show clear presence or absence of red dorsal stripe (excluding *P. glutinosus* group and *P. punctatus*). Green = Western *Plethodon* species. Blue = *P. wehrlei-welleri* group (not including *P. punctatus*). Red = *P. cinereus* group.

(Fisher-Reid *et al.*, 2012) and include annual mean temperature (BIO1), yearly temperature extremes and range (BIO5, BIO6, BIO7), total annual precipitation (BIO12), and measures of temperature and precipitation seasonality (BIO4, BIO15). Variable loadings on these PCs are summarized in the Results and in Table 1.

We tested the within-species relationships between morph frequency and climate using Spearman's rank correlation. We used this nonparametric method given that these data are non-normally distributed, after testing for normality for each variable with a Shapiro–Wilk test. For each of these eight polymorphic species (*P. angusticlavius, P. cinereus, P. dorsalis, P. serratus, P. shenandoah, P. vehiculum, P. ventralis* and *P. websteri*), we separately tested for a relationship between morph frequency at each locality and each of the nine climate variables listed above (PC1, PC2 and the seven univariate variables). In theory, analyses within species should account for phylogenetic nonindependence of populations, just as comparative studies do between species (e.g. Jockusch, 1997; Zamudio, 1998). However, phylogenetic information is limited within most of these species. Furthermore, as most species showed no relationship between morph frequencies and climate among populations (see Results), it seems unlikely that these analyses are generally overestimating relationships due to phylogenetic nonindependence.

To explore the between-species relationships between morph frequencies, costal groove numbers and climate, we used phylogenetic generalized least squares (PGLS; Martins & Hansen, 1997). These analyses were implemented using the R package *caper* version 0.5.2 (Orme *et al.*, 2013). For each of the nine climate variables

listed above, we compared mean values among localities for each species to either mean morph frequency (mean frequency among the sampled populations of each species) or median costal groove number. We also compared mean morph frequency to median costal groove number.

Prior to conducting PGLS analyses, we compared the fit of four evolutionary models to the data using the fitContinuous function in the R package *geiger* version 2.0.1 (Harmon *et al.*, 2008). We estimated the likelihood value for each variable for each model and compared their relative fit using the Akaike information criterion (AIC). An AIC difference of 4 or greater was considered strong support for a given model (Burnham & Anderson, 2002). The four models compared were Brownian motion (BM; strong phylogenetic signal (λ), i.e. $\lambda = 1$; Pagel, 1999), Ornstein-Uhlenbeck (OU; equivalent to stabilizing selection with a single optimum), white noise (WN: no phylogenetic signal; i.e. $\lambda = 0$) and estimated lambda (EL; similar to Brownian motion but with λ estimated, intermediate between 0 and 1). Different models were supported for different variables (Appendix S3), but in almost every case, the preferred model did not have a significantly better fit than the EL model (AIC difference <4). The only exception was BIO7, for which the AIC difference between the EL model and the preferred model (OU) was 6.08. Therefore, we present PGLS results from the EL model as our primary results, but we also show that other models give similar results (Appendix S3). Note that the BM and WN models are effectively just special cases of the EL model and that the purpose of PGLS is to account for different levels of phylogenetic signal.

To test the prediction that polymorphic species have wider climatic-niche breadths than monomorphic species, we generated an index of polymorphism (hereafter, IP) and tested for a relationship between this index and the nine climatic-niche breadth variables described above. This index ranges from 0 to 50 and is estimated as:

$$IP = 50 - |\,50 - |\,100 - \% \text{ striped }|\,|.$$

To obtain a species-level value, the index is estimated using the mean morph frequency among populations for a given species. Thus, both types of monomorphic species would have IP values of 0, and the more polymorphic a species is (i.e. closer to 50:50 striped: unstriped within populations and among populations), the closer the IP value is to 50. The relationship between IP and climatic-niche breadth was explored using PGLS as described above.

We also used PGLS to test whether species that are more polymorphic or show a stronger relationship between climate and morph frequency have larger geographic range sizes or climatic-niche breadths. We obtained data on species' range sizes compiled by

Rödder *et al.* (2009; data originally from www.red list.org; Appendix S4). First, we tested for a relationship between species' index of polymorphism (IP) and their geographic range sizes. Second, we tested whether the r_s-values from the within-species correlations between climate (PC1 and PC2) and morph frequency were correlated with geographic range size or climatic-niche breadth. We used the absolute value of r_s for these analyses, as we are interested in the strength of the relationship between climate and morph frequency (i.e. the magnitude of r_s), not the direction. Monomorphic species were assigned an r_s-value of 0.

We were also interested in whether climate–morphology relationships evolved concordantly with the phylogeny. The most direct test of this question is to analyse the r_s-values on the phylogeny, using the evolutionary model-fitting analyses described above. Specifically, supporting the EL or BM models over WN would support the idea that the climate–morphology relationship is concordant with the phylogeny (rather than evolving randomly across the tree). Support for the OU model would support conservatism, but not necessarily concordance with the phylogeny. For these analyses, we used raw r_s-values rather than the absolute values, to reflect both the strength and direction of the relationship between morphology and climate (i.e. positive or negative).

Finally, we visualized patterns of morph frequency evolution across the phylogeny. To do this, we initially conducted maximum likelihood ancestral reconstruction of morph frequencies on the pruned, 22-species phylogeny. Morph frequency was reconstructed as both a discrete trait (with possible states being polymorphic, striped and unstriped) and a continuous trait (mean % striped among sampled populations).

For the discrete analysis, we used the Mk one-rate model in Mesquite version 2.75, as this is the most straightforward model for three-state characters (Maddison & Maddison, 2011). However, two important disadvantages of this approach are that it (i) does not incorporate frequency information within species and (ii) does not allow for straightforward ordering of the character states (i.e. presumably the striped and unstriped states should go through an intermediate polymorphic state).

For the continuous analysis, we used the command 'ace()' in the R package *ape* (Paradis *et al.*, 2004). As the morph frequency data fit an OU model best (see description of model testing above; Appendix S3), we first transformed the tree using 'rescale()' in *geiger* (Harmon *et al.*, 2008) and used the transformed tree and estimated α-value (a measure of phylogenetic constraint) for the reconstruction. Although the continuous approach should (in theory) be superior for reconstructing ancestral trait frequencies (which are continuous), this approach may also be biased towards reconstructing ancestral species as being polymorphic. Therefore, we

considered results from both approaches. We note that both reconstructions should be treated with considerable caution.

We also conducted these reconstructions after including the species of the *glutinosus* group (and *P. punctatus*). Species were coded based on Petranka (1998) and the numerous photographs of each species available from AmphibiaWeb (2015). Most species were spotted, and thus, we added a fourth colour category. The *glutinosus* group also contains two species that appear to be striped (i.e. *P. yonahlossee* and *P. petraeus*), and some that are both unstriped and unspotted (e.g. *P. montanus*; Appendix S4).

Results

For each species, the mean values and climatic-niche breadth for PC1–2 are summarized in Table 2, along with summaries of species' overall morph frequencies. Estimates of the mean value and niche breadth for the seven univariate climatic variables for each species are summarized in Appendix S4. Phylogenetic relationships among species, morph frequencies, costal groove numbers and climate–morph frequency correlations are summarized in Fig. 1.

Significant within-species relationships between morph frequency and climate were uncommon among

Table 2 Morph frequency and climatic-niche variables for 22 *Plethodon* species.

Species	Morph frequency (% striped)	Mean PC1	Mean PC2	PC1 Breadth	PC2 Breadth
P. angusticlavius	98.7	−1.384	3.164	0.624	2.099
P. cinereus	69.3	−2.315	−0.424	2.385	5.585
P. dorsalis	70.1	−1.502	2.203	2.375	2.169
P. dunni	100.0	5.661	−1.028	8.241	7.339
P. electromorphus	0.0	−2.428	0.757	1.014	2.720
P. elongatus	100.0	5.433	1.003	9.290	7.062
P. hoffmani	0.0	−2.451	0.089	1.019	5.040
P. hubrichti	100.0	−1.984	−0.451	0.048	1.847
P. idahoensis	100.0	−0.011	0.529	0.958	3.416
P. larselli	100.0	4.285	−0.049	2.102	2.726
P. neomexicanus	0.0	−1.244	0.149	0.830	6.795
P. nettingi	0.0	−2.377	−3.132	0.360	2.788
P. richmondi	0.0	−1.689	−0.186	1.138	5.243
P. serratus	96.5	−0.785	1.891	2.858	7.416
P. shenandoah	80.7	−1.912	−2.634	0.063	1.233
P. vandykei	100.0	6.131	−4.091	1.902	4.551
P. vehiculum	95.2	5.741	−2.128	3.723	3.908
P. ventralis	76.5	−0.355	2.439	1.423	2.553
P. virginia	0.0	−2.204	−0.581	0.331	4.076
P. websteri	98.7	0.257	3.660	0.810	1.851
P. wehrlei	0.0	−2.435	−0.692	1.249	6.034
P. welleri	100.0	−1.507	−1.935	0.252	4.858

Morph frequency is the frequency of the striped morph, averaged across sampled populations for polymorphic species.

the eight polymorphic species (Fig. 1). *Plethodon cinereus* and *P. vehiculum* both showed significant correlations between morph frequency and PC2, but in opposite directions (Table 3). Given the variable loadings on PC2 (Table 1), populations of *P. cinereus* with a higher proportion of striped individuals occur in colder climates with a smaller diurnal temperature range relative to those with more unstriped individuals. In contrast, striped populations of *P. vehiculum* are found in warmer climates with a larger diurnal range. *Plethodon cinereus* shows several additional significant correlations in several raw BIO variables (Table 3), and these largely mirror those suggested by previous studies (see Introduction). Our study is consistent with earlier studies in that mainland populations of *P. cinereus* in colder climates with greater precipitation have higher proportions of striped individuals (Table 3). Inclusion of Long Island and Delmarva *P. cinereus* localities has minimal

impact on the species-level results and data (Appendix S5).

There are several significant relationships between striped morph frequency and climate among species (see Appendix S6 for full results; all results are from PGLS). For temperature-related variables, these relationships are in the opposite direction found for *P. cinereus* (and in the same direction as *P. vehiculum*), with predominantly striped species in warmer and wetter climates. Within-species striped morph frequency shows a highly significant positive relationship with mean PC1 among species ($r^2 = 0.316$, $P = 0.007$; Fig. 3). Sixteen of 19 climatic variables load strongly (absolute value ≥0.6) onto PC1, and eight of these have magnitudes >0.9 (Table 1). These eight variables suggest that largely striped *Plethodon* species occur in localities that have greater precipitation in the coldest quarter, wettest month, and wettest quarter, greater isothermality, greater precipitation seasonality, warmer minimum temperatures in the coldest month, lower temperature seasonality, and smaller temperature ranges compared to unstriped *Plethodon* species. This pattern seems to largely reflect the climatic distributions of predominantly striped species living in the wet Pacific Northwest region of North America (Fig. 2). There was no significant relationship between morph frequency and PC2 among species ($r^2 = 0.016$, $P = 0.569$). Frequency of the striped morph (mean among populations) also shows significant relationships among species with several raw BIO variables (all of which load strongly on PC1). These results are summarized in Appendix S6.

Inclusion of the *glutinosus* group and *P. punctatus* disrupts these significant patterns, however (Appendix S7). Inclusion of *P. punctatus* alone, coded as unstriped, yields the same results as presented above (Appendix S7). Thus, the significant relationship between colour morphs breaks down when the *glutinosus* group is included, seemingly because these species occur in many different climates (see Fig. S1), but are predominantly spotted (rather than striped or unstriped).

Among the 22 focal species, median costal groove number also shows significant relationships with climate and morph frequencies. There is a significant positive relationship between costal groove number and PC2 ($r^2 = 0.264$, $P = 0.014$), annual mean temperature (BIO1; $r^2 = 0.192$, $P = 0.041$), and maximum temperature of the warmest month (BIO5; $r^2 = 0.241$, $P = 0.020$). Thus, species with more costal grooves are found in warmer climates. Costal groove number is also strongly negatively related to morph frequency ($r^2 = 0.392$, $P = 0.002$; Fig. 4), such that striped species have fewer costal grooves than unstriped species.

The index of polymorphism was not significantly related to any measures of climatic-niche breadth (i.e. for PC1, PC2 or mean climatic-niche breadth; Appendix S6). There is a significant negative relationship between IP and geographic range size, suggesting that more
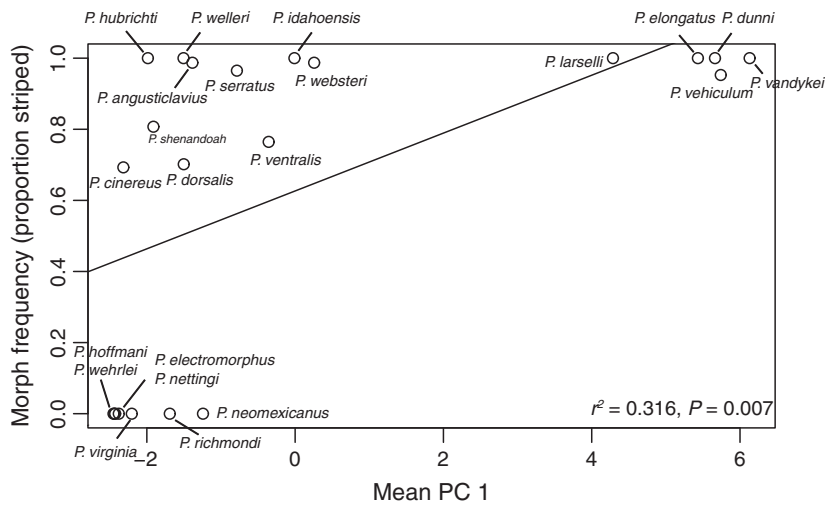
Table 3 Significant correlation results between locality morph frequency and climate variables for polymorphic species (*Plethodon cinereus* and *P. vehiculum*).

Species	Variable	Correlation	Interpretation
P. cinereus	PC2	$r_s = -0.71$, $P = 0.0006$	Higher proportion striped individuals where PC2 is lower (colder climates with smaller diurnal range and a colder warmest quarter)
P. vehiculum	PC2	$r_s = 0.70$, $P = 0.0240$	Higher proportion striped individuals where PC2 is higher (warmer climates with larger diurnal range and a warmer warmest quarter)
P. cinereus	BIO1 – annual mean temperature	$r_s = -0.67$, $P = 0.0019$	Higher proportion striped individuals in colder climates
P. cinereus	BIO5 – maximum temperature of warmest month	$r_s = -0.73$, $P = 0.0004$	Higher proportion striped individuals in locations where maximum temperatures are cooler
P. cinereus	BIO6 – minimum temperature of coldest month	$r_s = -0.60$, $P = 0.0066$	Higher proportion striped individuals in colder locations
P. cinereus	BIO12 – annual precipitation	$r_s = 0.47$, $P = 0.0425$	Higher proportion striped individuals in wetter locations

r_s = Spearman rank correlation (for non-normal data, Shapiro–Wilk test).

Fig. 3 Relationship between mean PC1 and species morph frequency (mean of localities for polymorphic species). Striped *Plethodon* species have higher PC1 values than unstriped *Plethodon* species, indicating that striped species are generally found in warmer and wetter climates than unstriped species. Raw data values are plotted, but analyses are based on PGLS.
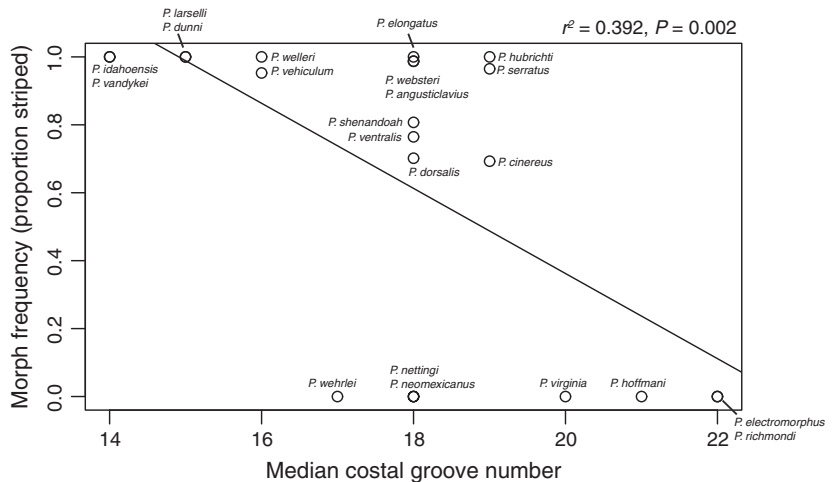

Fig. 4 Relationship between species' median costal groove number and striped morph frequency. Striped *Plethodon* species have fewer costal grooves than unstriped *Plethodon* species. Raw data values are plotted, but analyses are based on PGLS.

polymorphic species do have larger range sizes ($r^2 = 0.354$, $P = 0.003$). There is also a significant positive relationship between the strength of the within-species correlation between morph frequency and PC2 and species geographic range size ($r^2 = 0.358$, $P = 0.003$). There are no significant relationships between the strength of the correlation between morph frequency and PC1, nor between the strength of the within-species correlation between climate and niche breadth (Appendix S6).

Our results also show that there are no phylogenetic trends in the within-species relationship between climate and morph frequencies among species across the tree. Thus, there is no phylogenetic signal for r_s-values for either PC1 or PC2 (i.e. white noise is the best-fitting model and the estimated λ is zero; Appendix S3).

Reconstructions of ancestral morph frequencies across the tree estimated the most recent common ancestor of extant *Plethodon* as 71% striped (Fig. S2A), with similar values for the ancestors of all three groups (Western

Plethodon: 72%, *cinereus* group: 64% and *wehrlei-welleri* group: 70%). This result implies that the stripe has been maintained as a within-species polymorphism for 42 million years (from the age of the ancestor of the genus to the multiple polymorphic species extant today). Importantly, consistent with this idea, all three of these basal clades of *Plethodon* contain species that are polymorphic, striped and unstriped (suggesting that each clade evolved from a single ancestral species with both striped and unstriped states and that was therefore polymorphic). Thus, the striped and unstriped morphs appear to have been present for over 40 million years, with fixation of each morph in at least one species in each of the three relevant clades, and at least one species in each clade remaining polymorphic.

Discrete ancestral reconstruction of morph frequency categories shows the most recent common ancestor of *Plethodon* as ambiguous (nearly equal proportional likelihoods for striped, unstriped and polymorphic states; Fig. S2B). This approach also gives ambiguous

reconstructions for all three species groups, but is not inconsistent with the idea that the striped/unstriped polymorphism is ancestral for *Plethodon* and for all three species groups (Western *Plethodon*, *cinereus* group and *wehrlei-welleri* group), with the striped morph more likely for Western *Plethodon* and unstriped more likely for the *cinereus* group. Addition of *P. punctatus* and the *glutinosus* group in these analyses yields similar results for both continuous and discrete ancestral reconstructions (Fig. S2C–D, respectively).

Discussion

A major challenge in evolutionary biology is to reconcile patterns of trait variation within and between species, but few modern studies have attempted to do this. Here, we examine patterns of within-species trait variation across a phylogeny and test whether geographic variation within species is related to macroevolutionary patterns of trait variation among species. Specifically, we examine relationships between colour morph frequencies and climate within and between salamander species in a phylogenetic context.

One possible scenario by which within-species variation may relate to macroevolutionary patterns is if within-species relationships between environmental variation across space and trait variation among populations help maintain within-species polymorphisms over macroevolutionary timescales. These polymorphisms may then become fixed for alternate character states in species that occur in more extreme environments. Our results for *Plethodon* salamanders show mixed support for this scenario. In support of this scenario, we find that the average morph frequencies of species are strongly related to the overall climate where each species occurs (at least among the three basal clades of *Plethodon*; Fig 3.). Thus, species in wetter and warmer climates have higher frequencies of the striped morph, and species in drier and colder environments have higher frequencies of the unstriped morph. This pattern alone implies that occurrence in intermediate environments might have helped maintain polymorphism in this trait, and spread of species into more extreme environments helped drive the macroevolutionary fixation of alternate states in different lineages. Furthermore, examining the striped–unstriped polymorphism across a time-calibrated phylogeny as a continuous variable suggests the possibility that this trait has been maintained in a polymorphic condition for ~42 million years (i.e. polymorphic in the ancestor of *Plethodon,* and in the ancestor of all four species groups of *Plethodon*). Reconstructing the polymorphism as a discrete variable yields more ambiguous, but not inconsistent, results. Regardless of these ancestral reconstructions, the striped and unstriped conditions are clearly present in species in all four species groups. One potential explanation for this pattern is that each group therefore had a polymorphic ancestor, as opposed to evolving the different conditions independently through parallel evolution, or acquiring them through introgression (the latter possibility seems unlikely given the current absence of hybridization between species groups; summarized in Wiens *et al.*, 2006).

The relationships between climate and morphology are more complicated at the within-species scale, however. First, 6 of the 8 polymorphic species did not show significant within-species patterns of geographic variation in morph frequencies that were related to the climatic variables (Fig. 1). Second, the two species having within-species climate–morph relationships (*P. cinereus* and *P. vehiculum*) do not show a perfect match with the between-species patterns. Specifically, both the between-species patterns and those in *P. cinereus* show higher striped frequencies in wetter climates, but *P. vehiculum* does not. Further, *P. cinereus* shows higher striped frequencies in cooler climates, whereas the between-species patterns and *P. vehiculum* show higher striped frequencies in warmer climates.

Considering these patterns of geographic variation in the light of the phylogeny (Fig. 1) suggests that significant within-species relationships between climate and colour morph frequency evolved independently in species in two different clades (Western *Plethodon* and *cinereus* group), both clades in which most species are fixed for one morph or the other. Intriguingly, a strong relationship between climate and morph frequency did not evolve in the *wehrlei-welleri* group, the only group in which the majority of species are polymorphic (Fig. 1).

Overall, we acknowledge that the initial scenario we described (i.e. long-term maintenance of polymorphism linked to within-species trait–environment relationships) may not apply in this system and that climate-related geographic variation within species may be unimportant in the macroevolution of this trait. However, many other factors may be involved. For example, many species, despite being polymorphic, have frequencies that are nevertheless dominated by one morph or another (i.e. *P. angusticlavius, P. serratus, P. vehiculum* and *P. websteri* are polymorphic but are each >95% striped; Table 2). Thus, even if clades are ancestrally polymorphic, occurrence in less intermediate environments may drive polymorphic species towards fixation and greatly reduce within-species trait–climate relationships. Furthermore, in the wide-ranging *P. cinereus*, the ecological distributions of the colour morphs seem to change across the geographic range. For example, on the mainland, there are higher striped frequencies in cooler climates (this study), but on Long Island, higher unstriped frequencies are associated with cooler climates (Fisher-Reid *et al.*, 2013), as we find at the macroevolutionary scale in this study. Thus, despite the significant macroevolutionary trend, there may be considerable variation in the climate–morphology relationship among populations within species, which may help maintain polymorphism but obfuscate specieswide

relationships between morphology and climate. Furthermore, in *P. cinereus*, the different ecological preferences of the colour morphs can potentially contribute to their coexistence locally. Within a single site, striped morphs may be active at cooler temperatures and unstriped morphs may be more active under hotter and drier conditions (e.g. Moreno, 1989; Fisher-Reid *et al.*, 2013). This pattern is intriguing given the parallels to the macroevolutionary patterns, but may also tend to reduce geographic variation in morph frequencies and reduce climate–morph relationships (i.e. if both morphs can occur almost everywhere in a species range). Another important possibility is that the colour morphs are decoupled from climate within some species, despite the relationships between climate and morph frequencies across species.

An obvious question that these patterns raise is whether the colour morphs are directly related to physiological tolerances. An earlier study within *P. cinereus* suggested that there were physiological differences even between sympatric morphs (Moreno, 1989), but a later study showed that the relationship between metabolic rate and temperature between morphs was quite variable among populations (Petruzzi *et al.*, 2006). Our analyses here do not incorporate data from physiology or microclimate. However, analyses across plethodontids suggest that field body temperatures are closely related to macroscale climatic variables (Kozak & Wiens, 2007). Similarly, our analyses of microclimatic distributions of these morphs on Long Island suggest that these data support inferences from macroscale climatic data (Fisher-Reid *et al.*, 2013). Finally, we note that data on physiological tolerances and microclimate are important and interesting, but for our purposes in this study, they are primarily important in explaining the large-scale climatic distributions that we analyse directly here.

There are two other interesting results of our study. First, we show that species with stronger relationships between climate (PC2) and morph frequencies have larger geographic ranges. This finding raises the possibility that the presence of two morphs within a species, each with somewhat different ecological preferences, facilitates having a larger geographic range size. However, it is also possible that a large range size facilitates the evolution of a strong within-species climate–morphology relationship. These hypotheses are difficult to distinguish at present. Interestingly, we note that species with stronger trait–climate relationships do not have wider climatic niches, although it seems that a broad climatic niche might facilitate a larger range size or the evolution of strong trait–climate relationships.

Second, we found a strong, negative relationship between costal groove number and morph frequency between species, similar to the relationship documented within *P. cinereus* on Long Island (Williams *et al.*, 1968; Fisher-Reid *et al.*, 2013), and the weaker pattern on the Delmarva Peninsula (Highton, 1977; see caveats in his

Discussion). We also found strong relationships between costal groove numbers and climate. Costal groove numbers may be related to burrowing behaviour, with burrowers having more costal grooves (i.e. more vertebrae) than nonburrowers (Jockusch, 1997). Terrestrial salamanders burrow when conditions at the surface are unsuitable, particularly when conditions are too dry (Heatwole, 1960). Our results also suggest that unstriped *P. cinereus* and unstriped species, which have more costal grooves, are found in drier environments than striped *P. cinereus* and striped species, which have fewer costal grooves. Overall, these results suggest that both traits might show interesting parallels in their trait–climate relationships both within and between species. However, further investigation of within-species variation in costal groove numbers within *Plethodon* species in relation to climatic variables is needed.

Our results support the idea that the striped–unstriped polymorphism may be important for speciation in this system, albeit indirectly. On Long Island, populations of pure unstriped morphs of *P. cinereus* on eastern Long Island are differentiated from adjacent striped and polymorphic populations in their climatic distributions, microsatellites, a mitochondrial gene and costal groove numbers (Fisher-Reid *et al.*, 2013). In two studies in Ohio, 71–72% of male–female pairs in polymorphic populations of *P. cinereus* were same-colour pairs, suggesting potential for assortative mating by colour (Anthony *et al.*, 2008; Acord *et al.*, 2013). Here, we find that species with different colour morph frequencies do tend to occur in different climatic regimes. Furthermore, we find that the general associations between colour morphs and climate and costal groove number at fine spatial scales on Long Island also occur across species. We do not suggest that colour polymorphism itself plays a role in speciation in this case (e.g. as in many cases where colour polymorphism is related to sexual selection; review in Gray & McKinnon, 2006). Instead, we argue that the colour polymorphism (and costal groove number) is associated with potentially divergent ecological preferences and/or physiologies that might contribute to isolation between incipient species, as suggested by the significant macroevolutionary relationships between climatic variables and these morphological traits.

Our study raises many questions for future research on this system. What is the nature of the relationship between colour morphs and climatic distributions in *Plethodon*? Are there ecological reasons why certain colour morphs are favoured in some climates? Is there genetic linkage or pleiotropy between colour morphs and physiological tolerances? Are the different ecological preferences in sympatric colour morphs found in *P. cinereus* also found in sympatric populations in other polymorphic species?

In this study, we provide an analysis of how within-species geographic variation (i.e. relationships between

climatic variables and a morphological trait) and between-species patterns of character evolution may be related. Our results show that there can be parallels, but that there is also considerable variation in the strength and details of the within-species climate–trait relationships among species. These patterns may have important implications for other topics, such as maintaining trait variation over tens of millions of years and species geographic range sizes. Understanding the generality of these patterns will require similar studies in other groups of organisms.

Acknowledgments

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 USNM locality information.

Appendix S2 USNM specimen information.

Appendix S3 Results of PGLS model fit and model testing.

Appendix S4 Summary data used in primary analyses of 22 focal species and secondary analyses including *P. glutinosus*-group.

Appendix S5 Supplementary methods and results for inclusion of Long Island and Delmarva *P. cinereus.*

Appendix S6 PGLS results.

Appendix S7 Supplementary methods and results for inclusion of all *Plethodon* species.

Figure S1 PCA of climatic niche for 51 *Plethodon* species.

Figure S2 Ancestral reconstruction results.

Figure S3 Time-calibrated phylogeny with morph frequency climate relationships for eight polymorphic species only.