Clinal colour variation within a panmictic population of tree squirrels, *Tamiasciurus douglasii* (Rodentia: Sciuridae), across an ecological gradient

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Local adaptation occurs when a population in a heterogeneous environment experiences divergent ecological selection but only if selection is stronger than the homogenizing effects of gene flow. The forest environments of Oregon vary along a physical and biotic gradient from a wet, closed-canopy forest near the coast to a drier open-canopy forest eastward across the Cascade Mountains. The present study explores patterns of local adaptation in Douglas squirrels (*Tamiasciurus douglasii*) in relation to these transitions in forest structure and ecology. We test for the presence of morphological clines in relation to gene flow and, more specifically, whether any such character clines correspond with environmental clines. We sampled animals at six locations (10 specimens each) and evaluated environmental parameters across a 240-km west-to-east transect. Population structure analysis of 18 microsatellite loci indicates a single, panmictic squirrel population across the entire transect. Coalescent-based estimates show bidirectional gene flow at similar west–east intensities between squirrels in coastal and interior forests. Of the four skull traits examined, none shows a significant clinal transition. By contrast, ventral fur colour shows a strong clinal transition, from deep-orange in coastal forest to whitish–yellow in the interior forest. This pattern of phenotypic divergence coincides with the gradient in tree-canopy cover. Ventral fur colour of *T. douglasii* exemplifies a gradation of continuous phenotypic variation maintained despite ongoing gene flow in a panmictic population.   © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, **113**, 536–546.

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INTRODUCTION

Patterns of phenotypic variation within continuously distributed populations suggest that local adaptation is common in nature: as exemplified by the ecogeographical 'rules' of Gloger (1833), Bergmann (1847), and Allen (1877). Such patterns of environmentally driven phenotypic variation can be associated with spatial differences in the selective environments experienced by continuously distributed populations (Endler, 1977; Martínez *et al.*, 2014; Souto-Lima & Millien, 2014). Local adaptation is the consequence of local selection pressures that favour different traits in populations that occupy different environments. When selection is sufficiently strong, divergence can occur

even when gene flow is strong enough to homogenize the populations at neutral loci (Kawecki & Ebert, 2004). Ecological selection plays an important role in adaptation and also potentially in speciation, either because divergent selection favours the prevalence of locally beneficial alleles or because closely linked loci contribute to reproductive incompatibilities that become fixed in genetically divergent populations (Maynard Smith & Haigh, 1974; Felsenstein, 1981; Barton, 2000; Nosil, Funk & Ortiz-Barrientos, 2009; Lenormand, 2012).

Huxley (1938) coined the term 'cline' to describe geographical gradations of phenotypes within or between species, and this term can also apply to changes in allele frequencies (Slatkin, 1985). One of the most direct ways to measure the strength of selection in nature is to measure the rate of change in frequencies of genes or phenotypes across a cline

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(Slatkin, 1973). Early mathematical treatments by Fisher (1937) and Haldane (1948) showed that a balance between selection and gene flow maintains clines. Gene flow will push distinct populations towards genetic homogeneity and potentially remove clinal variation. By contrast, disruptive selection can counter the effects of gene flow and accentuate the distinction of traits or allele frequencies between populations. The tension between these evolutionary forces, as revealed by clines, generally suggests that both forces are ongoing. Therefore, studies of local adaptation provide evolutionary biologists the opportunity to understand the central issue that drives ecogeographical patterns of phenotypic variation and ultimately speciation.

A major challenge in the study of a cline is to determine whether the cline originated by primary intergradation (selection within a continuous population) or by secondary contact between genetically divergent populations (Endler, 1977). Both processes can produce similar clinal patterns over similar time periods (Barton & Hewitt, 1985). One approach for dealing with this question is to incorporate historical evidence from genetic studies to aid in the interpretation of cline formation (Thorpe, 1984; Barton & Hewitt, 1985). In the present study, we investigated a continuously distributed population across a natural ecological gradient that provides an opportunity to unravel some of these complexities.

The genus *Tamiasciurus*, consisting of the two sibling species *Tamiasciurus douglasii* and *Tamiasciurus hudsonicus*, is distributed across many coniferous forests in North America (Smith, 1970; Elliott, 1974; Lindsay, 1986; Benkman, 1995). In the Cascade Mountains of southern British Columbia and northern Washington, the two species meet along an ecological gradient over several forest types. Smith (1981) argued that these extremely different forest environments may have produced (1) interspecific divergence in fur colour that likely evolved for improved camouflage in differently illuminated canopy backgrounds and (2) interspecific divergence in skull morphology associated with bite force that is related to extracting seeds from cones that differ in hardness (serotiny). The coastal forests have primarily soft cones and interior forests primarily have hard cones.

The single species *T. douglasii* in western and central Oregon is distributed across a similar forest gradient (Franklin & Dyrness, 1973; Homer, Fry & Barnes, 2012) (Fig. 1) where it shows morphological variation in coat colour and skull morphology (Verts & Carraway, 1998) that may resemble that of the interspecific morphological variation between *T. douglasii* and *T. hudsonicus*. The *T. douglasii* found across the Oregon forest gradient should be

useful for studying the contrasting roles of selection and gene flow within a single species. In the present study, we combine cline analyses of fur colour and skull morphology with estimates of population structure and gene flow across several forest types. First, we estimate the number of *T. douglasii* populations and rates of gene flow along a west-to-east sampling transect consisting of six major localities, 10 specimens per locality, and a genetic analysis based on 18 microsatellite loci. We then compare the genetic patterns with morphological traits to test the hypothesis that clinal morphological variation corresponds statistically with transitions in forest canopy.

MATERIAL AND METHODS

We collected 60 specimens of *Tamiasciurus douglasii* from six localities, separated by a mean of 40 km, along a total 240-km west-to-east transect across Oregon (Fig. 1, Table 1). Each locality included 10 specimens collected within a 15-km radius and represented a single forest type. We designed our linear survey transect to span a gradient of the varied forest types in which *T. douglasii* resides, from wet, closed-canopy coastal forest to dry, open-canopy interior forest. Localities 1–3 are in the Coastal Douglas-fir zone (*Pseudotsuga menziesii* var. *menziesii*). Locality 4 is in the subalpine forest zone, and localities 5 and 6 are in the Interior Pine Forest zone (*Pinus ponderosa*/*Pinus contorta*) (Fig. 1) (Franklin & Dyrness, 1973; Kiilsgaard & Barrett, 1999; Homer *et al.*, 2012). All squirrel specimens are accessioned at the Burke Museum, University of Washington.

PERCENTAGE CANOPY OPENNESS

To document variation in openness of forest canopy across the ecological gradient, we acquired data on percentage tree-canopy cover at each sampling locality from digitized images in the National Land Cover Database (NLCD) (Homer *et al.*, 2012) using ARCGIS (ESRI, Inc.). The NLCD is a Landsat-based, 30-m resolution, land-cover database for the U.S.A. The percentage tree-canopy cover quantifies per-pixel tree-canopy fraction as a continuous variable from 1% to 100%. We used a quadrat-sampling method to obtain a mean percentage cover value for each sampling location. Percentage cover was based on 10 randomly selected points within a 0.08 km^2 area, which is approximately equivalent to the size of 10 Douglas squirrel territories (Smith, 1968). For our analyses, we converted the percentage canopy cover to percentage canopy openness by subtracting each value from 100%.
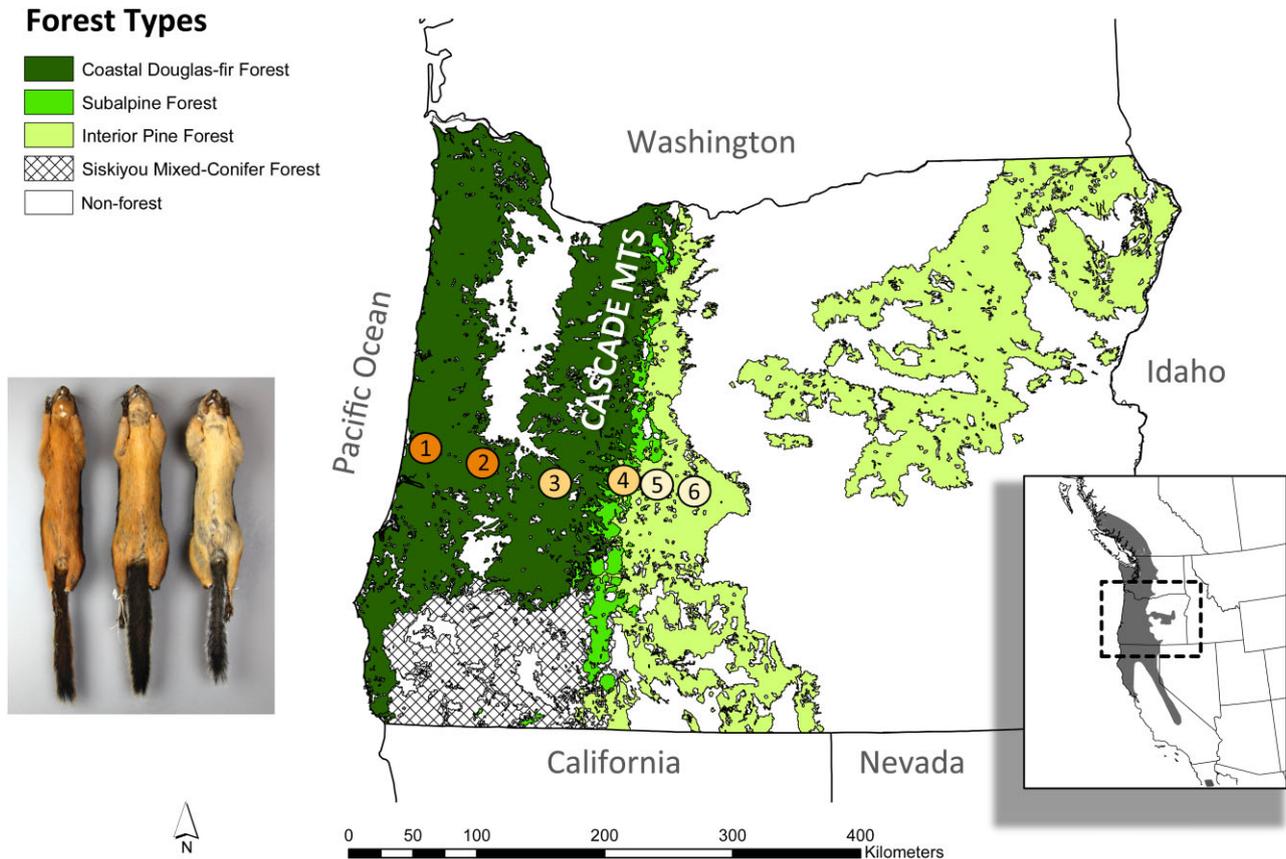
Forest Types

- Coastal Douglas-fir Forest
- Subalpine Forest
- Interior Pine Forest
- Siskiyou Mixed-Conifer Forest
- Non-forest

Figure 1. Map of the 240-km study transect in Oregon across a gradient of forest types (Franklin & Dyrness, 1973; Homer *et al*., 2012). Coloured circles show the six *Tamiasciurus douglasii* sampling localities (Table 1). Photo inset shows variation in ventral fur colour of representative study specimens. The forest types represented in our samples are wet Coastal Douglas-fir Forest (dark green tone; localities 1, 2, and 3); transitional Subalpine Forest (medium green tone; locality 4); and dry Interior Pine Forest (light green tone; localities 5 and 6). Map inset shows in gray the entire geographical range of *T. douglasii* from British Columbia to California, including the relict population in Baja California.

MOLECULAR METHODS

We extracted whole genomic DNA from liver tissue using the prescribed protocol of DNeasy Tissue Kit (Qiagen). We genotyped all 60 specimens at 18 polymorphic microsatellite loci originally developed for *T. hudsonicus*: Thu03, Thu08, Thu14, Thu21, Thu23, Thu25, Thu31, Thu32, Thu33, Thu37, Thu38, Thu40, Thu41, Thu42, Thu49, Thu50, Thu55, and Thu59 (Gunn *et al*., 2005). Polymerase chain reactions (PCRs) were carried out in 4.0-µL reaction volumes that included 2.03 µL of nuclease-free H_2O, 0.2 mg mL^{-1} of bovine serum albumin, 0.33 × PCR buffer, 0.83 mM of $MgCl_2$, 0.067 mM of each dNTP, 0.167 µM of each primer, and 0.35 U of Taq DNA polymerase and 30 ng of genomic DNA template. We used a touchdown PCR protocol consisting of a denaturing step at 94 °C for 3 min; followed by eight cycles (annealing temperature decreased by 1 °C each cycle) of 94 °C for 15 s, 68 °C for 15 s, and 72 °C for 30 s; followed by 20 cycles of 94 °C for 45 s, 59 °C for

15 s, and 72 °C for 30 s; with a final extension period of 72 °C for 45 min. We diluted PCR amplicons by 1 : 15 with nuclease-free water prior to fragment analysis on an ABI 3730 Genetic Analyzer (Applied Biosystems). Individual samples were multiplexed in three primer-pair sets in a 17-µL volume consisting of 3 µL of diluted PCR products (1 µL from each primer pair), 13.896 µL Hi-Di (ABI), and 0.104 lL GeneScan ROX400HD size standard. Allele sizes were visualized and scored using GeneMapper (ABI). We examined the data in MICRO-CHECKER (Van Oosterhout *et al*., 2004) to assess genotyping errors, such as allelic dropouts and stuttering or null alleles. Our results detected no null alleles in any of the 18 microsatellite loci.

POPULATION STRUCTURE

We used STRUCTURE, version 2.3.4 (Pritchard, Stephens & Donnelly, 2000) with data from the 18

Table 1. Specimens ($N = 60$) of *Tamiasciurus douglasii* collected from Oregon, with locality numbers (Fig. 1), county, locality description, latitude and longitude, and museum number

Location number	County	Locality	Latitude (decimal degrees)	Longitude (decimal degrees)	UWBM
1	Lane	Dunes City	43.8802	−124.1516	82028
1	Douglas	Dunes City	43.8994	−123.8637	82029-31
1	Lane	Dunes City	43.9223	−123.9449	82032-33
1	Douglas	Dunes City	43.8936	−123.8711	82034-37
2	Lane	Siuslaw Falls County Park	43.8606	−123.3609	82038-46
2	Lane	Siuslaw Falls County Park	43.8236	−123.3242	82047
3	Lane	Oakridge	43.7251	−122.6956	82048-49
3	Lane	Oakridge	43.6966	−122.6654	82050-51
3	Lane	Oakridge	43.7258	−122.7016	82052
3	Lane	Oakridge	43.6970	−122.7194	82053
3	Lane	Oakridge	43.6677	−122.6103	82054-57
4	Deschutes	Waldo Lake	43.7465	−121.9464	82071-78, 82080-81
5	Deschutes	Wickiup Reservoir	43.7431	−121.6903	82061-70
6	Deschutes	Newberry Crater	43.7070	−121.3268	81487-89, 81490-3, 82058-60

UWBM, University of Washington, Burke Museum.

microsatellite loci to assess whether population structure is associated with forest type. This analysis returns the most probable number of genetic clusters that characterizes samples from this transect and assigns individuals to these populations. STRUCTURE is a model-based algorithm method that uses Bayesian statistics and Markov chain Monte Carlo (MCMC) to make cluster assignments using genetic data. We used the 'admixture' model with correlated allele frequencies among populations and performed five replicate runs for each value of K ranging from 1 to 9 with a burn-in of 1.0×10^5 followed by 5.0×10^5 repetitions. Each run estimated the 'log probability of data' [L(K)]. We recorded the ln Prob(Data) for each run and averaged the ln Prob(Data) across runs for each value of K. To select the most appropriate number of K from our data, we chose the smallest value of K associated with the peak in the Ln P(D) as suggested by Pritchard, Wen & Falush, (2007). We evaluated runs for adequate mixing by evaluating whether point estimates of the likelihood were consistent among the five independent runs after an initial burn in of 100 000 generations.

GENE FLOW BETWEEN COASTAL AND INTERIOR POPULATIONS

To understand whether gene flow across the study transect is sufficient to homogenize variation in neutral morphological traits, we estimated gene flow rates and directionality using IMA2 (Hey, 2010). IMA2 uses a coalescent-based model of isolation-with-migration under a Bayesian framework to co-estimate the multilocus effective population sizes (present and ancestral), divergence times, and migration rates (Nielsen & Wakeley, 2001; Hey & Nielsen, 2004, 2007). To improve computational efficiency of our multilocus genotype dataset consisting of 18 microsatellite loci, we analyzed a subset of the 60 collected individuals. The sampling design included 18 individuals from localities 1, 2, and 3 (coastal) and 18 individuals from localities 5 and 6 (interior). We assumed that all microsatellite alleles evolved under a stepwise mutation model and set the inheritance scalars at 1 for all loci.

We performed several exploratory runs in IMA2 using 'MCMC mode' to determine the values for the most efficient swapping of MCMC chains and also to allow for appropriate prior settings for population parameters (i.e. t [divergence time], Θ [theta], and m [migration rate]). We then performed nine independent runs with different starting seeds in 'MCMC mode' to sample genealogies and obtain model parameter estimates. We determined that a sufficient burn-in period of sampled genealogies was achieved after burn-in trend plots had reached a plateau. We assessed convergence by checking for consistent parameter values from multiple long runs, monitoring effective sample size values, and trendlines. Next, we used sampled genealogies from the 'MCMC mode' in a new analysis 'Load Geneologies Mode' to statistically evaluate whether the fully parameterized migration and population size model ranked as a better model than 25 simpler nested models with fewer migration and

population size scenarios using Akaike Information Criterion (AIC) (Table 1) (Carstens, Stoute & Reid, 2009). We also calculated two related information theoretical statistics to provide objective measures of model support: Akaike weights (ω_i), which is the normalized relative likelihoods of the model, and the evidence ratio ($E_{min/i} = \omega_{min}/\omega_i$), which compares each model to the best model.

ANALYSIS OF POPULATION DEMOGRAPHY

Clinal patterns of allele frequencies sometimes arise at the geographical range margins of rapidly expanding populations (Hallatschek & Nelson, 2008; Excoffier, Foll & Petit, 2009). This is caused by increases in rare allelic variants to high frequencies at the geographical range margins of a rapidly expanding population. This phenomenon, called 'surfing,' can produce clinal patterns of allelic variation that can be mistakenly interpreted as disruptive selection. Klopfstein, Currat & Excoffier (2006) found that 'surfing' will occur more often in smaller populations with limited dispersal abilities that are undergoing rapid range expansion. To assess whether rapid demographic expansion can explain clinal patterns of phenotypic traits, we assessed evidence for historical fluctuations in population size using data from the 18 microsatellite loci with LAMARC, version 2.1.8 (Kuhner, 2006). LAMARC estimates a population-growth parameter, g, by using the coalescent and a MCMC genealogy sampler. The exponential growth parameter, g, can be defined by the equation $\theta_{t(time\ before\ present)} = \theta_{t(present\ time)}^{-gt}$ where the time parameter t is measured in units of mutations. Positive values of g indicate a growing population and negative values represent shrinking populations. A value for g of zero represents constant size. We used the 99% confidence intervals for g to test for significant difference from zero. For each maximum likelihood run, we used 20 short chains of 10 000 steps and two long chains with 200 000 steps. The number of short chains was based on when parameter estimates appeared to stabilize. We used a Brownian motion model for the mutation model, which is a statistical approximation of the stepwise model. Each run was replicated three times with different starting seeds and with three simultaneous heating searches. We determined adequacy of parameter settings by evaluating whether independent runs gave similar results.

COLOUR SCORING

The most visually striking difference between costal and interior *T. douglasii* is their ventral fur colour. The ventral region of these squirrels changes from dark orange to whitish–yellow from west to east along the study transect. We measured spectral reflectance of the mid-ventral region from prepared museum specimens using a USB2000 (Ocean Optics) spectrophotometer with a dual deuterium and halogen light source. The probe was held at a 45° angle to the surface of the body for three independent measurements that were subsequently averaged for analysis. Spectral reflectance wavelengths were recorded using SPECTRASUITE (Ocean Optics). We analyzed the degree of CIE (International Commission on Illumination) whiteness in the ventral region from our reflectance measurements. Changes in CIE whiteness are an appropriate colourimetric parameter for the present study because the measures correspond with ventral fur colour variation along the study transect.

ANALYSIS OF SKULL MORPHOLOGY

We examined three skull characters that are reasonable reflections of bite force and masticatory function (Smith, 1981), and one trait not known to be directly related to bite force (width of foramen magnum). Linear measurements were made to the nearest 0.01 mm using digital calipers (Mitutoyo Corp.) for the skull traits: (1) sagittal crest, measured as distance between the temporal lines; (2) the angular moment arm, measured as distance between the mandibular notch and the angular process; (3) coronoid moment arm, measured as distance between the coronoid processes and the mandibular condyle; and (4) foramen magnum, measured as the transverse distance across the foramen magnum.

Morphological traits typically scale with body size, which can obscure differences in traits among populations that differ in body size (Reist, 1986). Because the body size of *T. douglasii* is known to be larger in eastern Oregon than in western Oregon (Verts & Carraway, 1998), differences in size of skull features between populations could be confounded by these known differences in overall body size. Accordingly, we made ratios of each skull character to total skull length. Because we have previously shown that none of these traits showed sexual dimorphism (Chavez, Saltzberg & Kenagy, 2011), we pooled both males and females in this analysis.

CLINE ANALYSIS

To estimate the relationship between spatial position on our transect and clinal variation of genetic and morphological data, we fitted maximum likelihood clines to geographical variation of spectral reflectance of the ventral region and four skull characters using CFIT-7 (Gay *et al.*, 2008). CFIT uses a simulated annealing function that includes Metropolis algorithms to fit three-part clines that include a

central sigmoid part and two exponential tails (Szymura & Barton, 1986). We treated ventral colour and skull morphology as quantitative characters for this analysis and compared three different candidate models to find the best-fitting curve: unimodal, bimodal, and trimodal. Unimodal distributions are characteristic of situations where intermediate phenotypes predominate, indicating relatively weak disruptive selection (Jiggins & Mallet, 2000). Bimodal distributions are characteristic of very high selection against intermediate phenotypes. Trimodal distributions can be interpreted as having a pattern somewhere between unimodal and bimodal distributions (Gay *et al.*, 2008).

Finally we examined whether all characters shared the same cline centre (coincidence) and same cline width (slope concordance) using four models of constraint: (1) the same cline centre; (2) same cline slope; (3) same cline centre and slope; or (4) centre and slope left unconstrained. For this analysis, we performed two tests to see whether ventral fur colour was similar or not with all skull characters by testing two sets of models including fur colour and skull characters together and another with only skull characters together. Finally, we tested another two sets of models to see whether forest canopy openness fit clinal patterns of ventral colour and all skull colours. All model testing was analyzed with AIC to rank candidate models. Evidence ratios were also provided for each AIC model comparison to show the relative likelihood of the best model being correct when compared against other models. Different starting positions and an optimal number of chains were used for each analysis to ensure that the algorithms used in CFIT were adequately exploring parameter space. Data for each character were transformed to a scale of 0–1, with 0 representing coastal *T. douglasii* populations and 1 representing interior populations.

RESULTS

POPULATION STRUCTURE AND ASSIGNMENT

STRUCTURE analysis revealed the presence of a single ancestral population of Douglas squirrels across the entire forest gradient transect. This means that any clinal variation in morphology across our transect would have originated *in situ*, rather than from secondary contact of two ancestral populations.

GENE FLOW

IMA2 analysis of gene flow indicates equal rates of bidirectional gene flow across the study transect (Table 2). This finding demonstrates that any observed morphological divergence along the transect would be occurring in the presence of gene flow.

Table 2. Ranked results of the top five out of 25 nested population/migration models in 'Load Genealogies Mode' in IMA2

Model description	POP CO	POP IN	POP AN	MIGR IN to CO	MIGR CO to IN	Log (P)	K	AIC	Δ_i	ω_i	$E_{min/i}$
POP : Coastal = interior/MIGR : coastal = interior	0.4	(0.4)	10.0	20.2	(20.2)	−3.4	3	13.60	0.00	0.21	1.0
POP : Each different/MIGR : coastal to interior only	0.4	0.2	10.0	(0.0)	20.5	−2.4	4	14.00	0.41	0.17	1.2
POP : Coastal = interior/MIGR : interior to coastal only	4.5	(4.5)	10.0	6.5	(0.0)	−3.7	3	14.11	0.52	0.16	1.3
POP : Coastal = interior/MIGR : both directions	0.4	(0.4)	10.0	22.6	17.8	−2.9	4	15.15	1.55	0.10	2.2
POP : Coastal = interior/MIGR : coastal to Interior only	1.4	(1.4)	10.0	(0.0)	4.1	−4.4	3	15.58	1.98	0.08	2.7

The 25 models include different population size (POP) scenarios for coastal (CO), interior (IN), and ancestral (AN) populations, as well as different migration (MIGR) scenarios between coastal and interior populations. Joint parameter estimates for each population and migration direction are shown, including values that were fixed (in brackets) for each listed model.
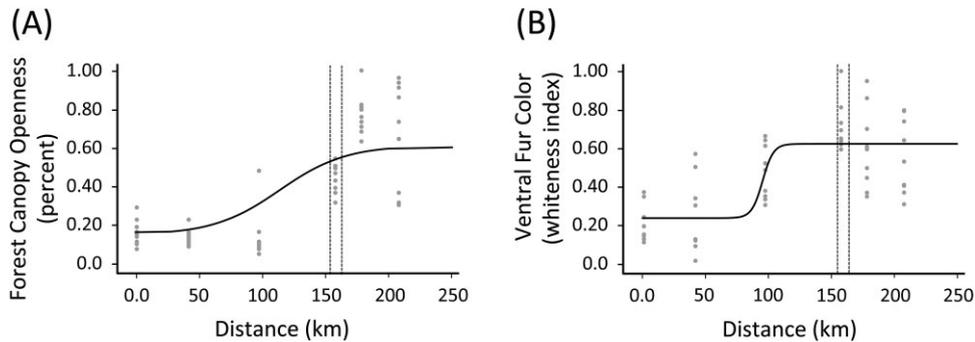
Figure 2. Clinal patterns of variation at localities 1–6 across the gradient of three forest types. The two vertical lines delimit the three forest types: wet Coastal Douglas-fir Forest to the left (west), transitional Subalpine Forest between the lines, and dry Interior Pine Forest to the right (east). The x-axis shows the distance along the study transect beginning from the westernmost locality. A, percentage tree-canopy openness of 10 randomly sampled points at each sampling locality. B, ventral colour score (whiteness index) from spectrophotometer measurements of 60 individuals (10 per locality).

Table 3. Comparison of four cline models using the Akaike information criteria (AIC) for clinal variation in ventral fur colour

Phenotypic trait	Model	Parameters	Likelihood	AIC$_c$	Δ_{AIC}	AIC weights
Ventral fur colour	**Unimodal**	**7**	**24.35**	**−32.55**	**0.00**	**0.86**
	Trimodal	14	30.21	−23.08	9.47	0.01
	Bimodal	8	23.78	−28.73	3.82	0.13
Tree canopy openness and ventral fur colour	**Centre constraint**	**20**	**88.10**	**−127.72**	**0.00**	**1.00**
	Slope constraint	20	79.63	−110.78	16.94	~0
	Slope and centre constraints	19	77.59	−109.59	18.13	~0
	Unconstrained	21	79.63	−107.83	19.89	~0

Trait comparison for coincidence (same cline centre) and concordance (same cline width) models between clines of tree-canopy openness and fur colour using the AIC. The best models are shown in bold.

HISTORICAL POPULATION DEMOGRAPHY

The results of our LAMARC analysis indicate only a slight decline in historical population size, which excludes the possibility that rapid population expansion would have produced any clinal variation in morphology. The maximum likelihood estimate of population growth (g) was small and negative (−0.053), with a 99% certainty of being nonzero.

CLINAL PATTERNS OF MORPHOLOGICAL PHENOTYPIC VARIATION VERSUS FOREST CANOPY COVER

Tree-canopy cover varied clinally from closed canopy in the west to more open canopy in the east across our sampling transect (Fig. 2A). Ventral fur colour showed clinal variation from dark in the west to light in the east (Fig. 2B). We found that fur colour and canopy cover had the same centre point (spatial coincidence, as indicated by AIC ranking) but not the same cline width (concordance) (Table 3). None of the skull traits

showed clinal patterns across the ecological gradient, suggesting that they have not been under strong divergent selection across the ecological gradient (see Supporting Information, Fig. S1, Table S1).

DISCUSSION

CLINAL VARIATION IN VENTRAL FUR COLOUR

We were able to demonstrate a cline in ventral fur colour of *T. douglasii* within a single, panmictic population. This observation provides compelling evidence that local selection on fur colour has been sufficient to overcome homogenization by gene flow. We found only a slight decline in historic population size, suggesting that our estimates are not vulnerable to allele surfing artefacts (Klopfstein *et al.*, 2006; Hallatschek & Nelson, 2008; Excoffier *et al.*, 2009). Population structure estimates suggest that the clinal pattern of intraspecific fur coloration has resulted from primary intergradation because *T. douglasii* in the study area

has only a single population-of-origin. Furthermore, a range-wide phylogenetic analysis of *T. douglasii* using nuclear intronic loci showed no significant population structure across the entire range of the species, much less in Oregon (Chavez *et al.*, 2014). Of the morphological traits measured, only fur colour appears to form a character cline. The spatial coincidence of cline centre points for tree-canopy cover and ventral fur colour (Fig. 2) suggests that the change in fur colour along the transect is related to selective pressures associated with forest structure, possibly the effect of ambient light on camouflage. Whatever the selection pressure, the same pattern of ventral coloration that evolved between *T. douglasii* and *T. hudsonicus* has evolved independently within *T. douglasii*, suggesting that these different ventral colours may be locally adaptive.

LOCAL SELECTION ON VENTRAL FUR COLOUR

Selection on ventral fur colour may be associated with predator avoidance in arboreal mammals (Kiltie, 1989; Bradley & Mundy, 2008; Kamilar & Bradley, 2011). Unlike ground-dwelling rodents that have evolved protective colour polymorphisms in their dorsal regions (Benson, 1933; Hoekstra, Drumm & Nachman, 2004; Steiner, Weber & Hoekstra, 2007), the ventral region of tree squirrels appears to be an important target of natural selection as a result of the arboreal behaviour of tree squirrels. Tree squirrels often feed and rest in an upright posture in the forest canopy, which exposes their ventral region to detection by predators. Forest raptors, such as the northern goshawk (*Accipiter gentilis*), are major predators on *Tamiasciurus* and are able to visually detect and hunt squirrels through complex canopy structures (Kenward, 1982; Beier & Drennan, 1997). In light of these predatory pressures, natural selection should favour ventral coloration that best matches the overall microhabitat (e.g. tree-bark, needles, sky light) of the canopy background. We suggest that selection should favour darker ventral coloration in coastal forests because the denser canopy structure creates a low-light background that makes darker squirrels more inconspicuous to predators. By contrast, selection should favour lighter ventral coloration in the interior forest because this matches the brighter background of the open canopy. Smith (1981) made similar arguments for the importance of background matching and divergent interspecific selection in the zone of secondary contact between *T. douglasii* and *T. hudsonicus* in the northern Cascade Mountains of British Columbia. Additionally, extensive research has demonstrated that fur coloration has important thermoregulatory effects on the absorption of solar radiation in mammals (Walsberg, 1983).

LACK OF CLINAL VARIATION IN SKULL MORPHOLOGY

Somewhat unexpectedly, none of the skull traits associated with bite force exhibited clinal transitions despite strong differences in cone hardness between coastal and interior conifer species. A possible selective factor that could lead to phenotypic variation in biting ability is the transition within forests of the Pacific Northwest in the hardness (serotiny) of coniferous tree cones, which vary from softer cone species in the west to harder cone species in the east (Lindsay, 1986; Schoennagel, Turner & Romme, 2003). Wheatley (2007) also found a lack of association between body size and cone hardness within *T. hudsonicus* in mixed forests in Canada (Wheatley, 2007). By contrast to these negative intraspecific findings, significant interspecific differences do exist in skull morphology between *T. douglasii* and *T. hudsonicus* across their hybrid zone in an ecologically similar forest system in the northern Cascade Mountains of British Columbia and Washington (Smith, 1981; Chavez *et al.*, 2011). We surmise that the present study did not reveal similar correlations between skull morphology and cone hardness because the homogenizing effects of gene flow are stronger within this single species than between species. Furthermore, skull morphology of tree squirrels has been reported to be conserved over evolutionary time (Emry & Thorington, 1984); thus, pleiotropy may constrain adaptation of skull morphology in *T. douglasii*. Perhaps counter-selective pressures, such as an expanded brain case and forward positioning of the eyes for rapid arboreal locomotion, limit the extent to which, or rate at which, a squirrel skull can be modified by selection (Swiderski & Zelditch, 2010). Thus, although the present study adds new information that major morphological change has not evolved in response to differences in cone hardness, it is possible that further morphological analysis (e.g. finite element analysis) would be able to detect finer-scale changes (Richmond *et al.*, 2005).

GENETICS AND THE EVOLUTION OF PIGMENTATION VERSUS SKULL MORPHOLOGY

It is also possible that fur colour can respond to selection more rapidly than skull morphology as a result of underlying differences in the genetic architecture of these different types of traits. Of the large number of pigmentation genes underlying fur coloration, a few genes of major effect have been identified as contributors to colour variation in mammals (Hubbard *et al.*, 2010). These genes include the melanocortin-1 receptor and agouti-signalling protein, which are both important in melanin synthesis (Hoekstra, 2006). Evolution may be rapid in these traits because of their simple genetic architecture

(Hubbard *et al*., 2010) compared to that for skull character architecture, which has a complex quantitative genetic basis (Cheverud *et al*., 1997; Leamy, Routman & Cheverud, 1999). Ventral fur colour may have evolved from being originally orange to later becoming white in *T. douglasii*, a transition that could have resulted from a simple loss of gene function (Olson, 1999; Springer, Crespi & Swanson, 2011). Losses of function can result from many different mutations that achieve a similar phenotypic effect. Therefore, mutations that result in a loss of function may segregate at higher frequency, or originate more often, than appropriate variation in a quantitative trait. This would leave genetically simple loss of function traits capable of rapid evolution given an appropriate selection pressure. For example, many of the celebrated examples of adaptive ecological differences between closely-related species are the result of loss of functional mutations (Bradshaw & Schemske, 2003; Shapiro *et al*., 2004; Protas *et al*., 2006).

Studies of phenotypic divergence in the face of gene flow provide important examples of adaptation, and the selective agents responsible for these phenotypic differences can be presumed to be currently active and identifiable. The present study examines patterns of local adaptation within a Douglas squirrel population across an ecological gradient. Future studies of local adaptation in this species could also investigate the processes that cause divergent selection (Vignieri, Larson & Hoekstra, 2010) or the degree to which phenotypic plasticity and genetic architecture influence the expression and evolution of morphological traits. Additionally, direct measures of selection on these traits in each environment (e.g. by common-garden or reciprocal-transplant experiments) would be of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Clinal patterns of variation at localities 1–6 across the gradient of three Forest Types, plotted as in Figure 2. A, ratio of angular moment arm to skull length for 38 individuals. B, ratio of coronoid moment arm to skull length for 37 individuals. C, inverse ratio of sagittal crest width to skull length for 37 individuals. D, ratio of foramen magnum to skull length for 34 individuals.

Table S1. Comparison of cline models using the Akaike information criteria (AIC) for clinal variation of four skull traits. Trait comparison for coincidence (same cline centre) and concordance (same cline width) models between clines of all four skull traits together and between tree-canopy openness and all four skull traits using the AIC. The best models are shown in bold.