Genetic and phenotypic variation across a hybrid zone between ecologically divergent tree squirrels (Tamiasciurus)

ANDREAS S. CHAVEZ,* CARL J. SALTZBERG† and G. J. KENAGY*
*Burke Museum and Department of Biology, University of Washington, Seattle, WA 98195, USA,
†The Evergreen State College, Olympia, WA 98505, USA

Abstract
A hybrid zone along an environmental gradient should contain a clinal pattern of genetic and phenotypic variation. This occurs because divergent selection in the two parental habitats is typically strong enough to overcome the homogenizing effects of gene flow across the environmental transition. We studied hybridization between two parapatric tree squirrels (Tamiasciurus spp.) across a forest gradient over which the two species vary in coloration, cranial morphology and body size. We sampled 397 individuals at 29 locations across a 600-km transect to seek genetic evidence for hybridization; upon confirming hybridization, we examined levels of genetic admixture in relation to maintenance of phenotypic divergence despite potentially homogenizing gene flow. Applying population assignment analyses to microsatellite data, we found that Tamiasciurus douglasii and T. hudsonicus form two distinct genetic clusters but also hybridize, mostly within transitional forest habitat. Overall, based on this nuclear analysis, 48% of the specimens were characterized as T. douglasii, 9% as hybrids and 43% as T. hudsonicus. Hybrids appeared to be reproductively viable, as evidenced by the presence of later-generation hybrid genotypes. Observed clines in ecologically important phenotypic traits—fur coloration and cranial morphology—were sharper than the cline of putatively neutral mtDNA, which suggests that divergent selection may maintain phenotypic distinctiveness. The relatively recent divergence of these two species (probably late Pleistocene), apparent lack of prezygotic isolating mechanisms and geographic coincidence of cline centres for both genetic and phenotypic variation suggest that environmental factors play a large role in maintaining the distinctiveness of these two species across the hybrid zone.

Keywords: cline, coloration, hybridization, morphology, selection, speciation, Tamiasciurus

Received 7 February 2011; revision received 26 May 2011; accepted 30 May 2011

Introduction
Geographic isolation and ecological adaptation have a powerful influence on the development of reproductive isolation in the process of speciation (Sobel et al. 2010). Limitations to gene flow are often initiated by the geographic separation of populations. Both the period of time over which populations are isolated and the evolution of divergent adaptations promote reproductive isolation through epistatic and pleiotropic effects (Dobzhansky 1937; Muller 1940, 1942; Coyne & Orr 2004). These effects can either be direct, as in the case of adaptation to a new habitat that leads to spatial isolation, or indirect, as when divergent selection for adaptive differences between populations creates divergent genomic backgrounds, which may hasten the generation of epistatic incompatibilities.

Zones of secondary contact between formerly allopatric species or divergent populations are often used as natural opportunities to study processes that are important in the early stages of speciation. The outcomes of
secondary contact vary from system to system and typically depend on the duration of isolation and whether the two species in question evolved in ecologically different environments. At one extreme, populations are reproductively isolated from one another either because they cannot interbreed or, if they do, because they are unable to produce viable or fertile offspring. At the other extreme, divergent populations may freely interbreed and eventually fuse back to one population. Many secondary contact systems fall between these scenarios and result in narrow hybrid zones. This forms a tension zone, where the width of the zone is dependent upon a balance between dispersal and selection (Barton 1983; Barton & Hewitt 1985). In that case, populations still maintain their genetic distinctiveness over most of their distribution and may proceed towards reproductive isolation, even though some gene flow or diffusion of characters may still occur (Wu 2001).

Hybrid-zone systems are also important for identifying ecologically important traits. This is especially true when a hybrid zone persists along an environmental gradient or an ecotone, because the divergent effects of natural selection are countered by the homogenizing effects of gene flow (Endler 1977; Barton & Hewitt 1985). One way to ascertain the ecological importance of traits is to compare clinal patterns of trait variation with patterns of neutrally evolving traits or markers (Gay et al. 2008). A trait or marker that is neutrally evolving is expected to have a greater cline width across the hybrid zone than a trait under strong divergent selection. Alternatively, a displaced cline may indicate that phenotypic traits of one species are beneficial to the other; thus, a hybrid zone may be important for adaptive introgression (Grant et al. 2004; Martin et al. 2006; Whitney et al. 2006; Fitzpatrick et al. 2010). Here, we examine clinal patterns of phenotypic variation in the face of hybridization between parapatric sister species of North American tree squirrels of the genus Tamiasciurus. These sibling species are good candidates for an investigation of the role of ecological factors in hybridization dynamics because they have been studied thoroughly in the field with regard to plant–animal interactions, behavioural ecology and life history evolution (Smith 1968, 1970, 1978, 1981; Benkman 1995; Boutin et al. 2006; Digweed & Rendall 2009; Sanderson & Koprowski 2009).

Tamiasciurus is represented by only two species, the Douglas squirrel (Tamiasciurus douglasii) and the red squirrel (T. hudsonicus). Previous phylogenetic inference for Tamiasciurus using mitochondrial DNA (mtDNA) showed strong support for three major clades: a single ‘western’ clade that is concordant with the taxonomic description for T. douglasii, and two other clades (‘eastern’ and ‘southwestern’) that are associated with T. hudsonicus (Arbogast et al. 2001). Low levels of mtDNA sequence divergence between these three lineages (<2.4%) suggest their divergence was relatively recent, probably during the Pleistocene. These squirrels are distributed throughout boreal and montane coniferous forests across most of North America (Fig. 1), with T. douglasii in the Pacific Coastal region and T. hudsonicus more widespread across the continent, from the southern Appalachians northward in eastern North America, throughout the Rocky Mountains, and further northward into Alaska (Steele 1998, 1999). The ranges of these two species come into contact in a narrow transitional forest region of the northern Cascade Mountains of southern British Columbia and northern Washington (Smith 1968). Tamiasciurus douglasii inhabits dense, wet coastal forests on the west side of the Cascades, whereas T. hudsonicus inhabits open, dry interior forests on the east side of the Cascades. It has been proposed that secondary contact between these species occurred following the end of the Pleistocene, because this entire region was covered by continental ice during the last glacial maximum (Smith 1981; Lindsay 1982; Arbogast et al. 2001).

Tamiasciurus douglasii and T. hudsonicus are phenotypically distinct in coloration and skull morphology (Smith 1981; Lindsay 1982). Tamiasciurus douglasii is darker dorsally and has an orange ventral colour and is slightly smaller than T. hudsonicus, which has a white ventral colour. Smith (1981) argued that phenotypic differences in coloration and skull morphology result from local adaptation to variation in forest environments. For instance, the lighter ventral coloration of T. hudsonicus is an adaptation for background matching in an open canopy forest to reduce detection by predators. In addition, the stronger jaw musculature of T. hudsonicus is an adaptation for opening harder, closed pine cones that have evolved in an environment with a high frequency of fires. In the transitional forest habitat where these species are sympatric, individuals with intermediate coloration and vocalization frequencies have been recorded and are thought to be hybrids (Smith 1968; Stevens & Nellis 1974). However, Lindsay (1982) argued that these two species are reproductively isolated from one another and that character convergence in the transitional forest habitat is an alternative explanation for the evolution of intermediate traits.

The goal of our study was to characterize environmental, genetic and phenotypic variation across the secondary contact zone between these two squirrel taxa to assess whether species boundaries are being maintained and, if so, how. First, we undertook a population genetic analysis using microsatellite DNA to confirm the presence of hybridization. We also sought to verify
the reproductive viability of hybrids by assessing the generation of hybrids with a multilocus analysis. Finally, we examined patterns of clinal variation in ventral coloration, cranial morphology, mtDNA clade assignment and microsatellite genotype assignment in relation to the forest transition across the hybrid zone. Among these patterns, we tested the general hypothesis that phenotypic traits under strong divergent selection should exhibit the sharpest cline patterns.

Materials and methods

Sampling

We sampled 397 museum specimens of *Tamiasciurus douglasii* and *T. hudsonicus* from 29 localities along a nearly 600-km west-to-east study area across the northern Cascade Mountains region of northern Washington, southern British Columbia, and northern Idaho (Fig. 1; Table S1, Supporting information). These specimens were collected between 1920 and 1991, with a majority (60%) collected in the region of sympathy in two major sampling periods: 1963–1965 by Smith (1968) and 1971–1973 by Stevens & Nellis (1974). All the specimens are accessioned at the Burke Museum, University of Washington (UWBM), and the University of Kansas Natural History Museum (KU).

Habitat associations across study area

Smith (1968) described the association of coniferous tree species and forest types with both *Tamiasciurus* species and the putative hybrids in the northern Cascade Mountains. *Tamiasciurus douglasii* is found on the western side of the north Cascade Mountains and mostly in western hemlock (*Tsuga heterophylla*) and Pacific silver fir (*Abies amabilis*) vegetation zones, whereas *T. hudsonicus* is found on the east side and mostly in ponderosa pine (*Pinus ponderosa*) and interior Douglas-fir (*Pseudotsuga menziesii*) vegetation zones. The squirrel species are sympatric near the crest of the northern Cascade Mountains in an approximately 25-km wide transitional forest zone that includes a subalpine fir (*Abies lasiocarpa*) vegetation zone on the west side of the Cascade crest and an interior Douglas-fir vegetation zone also on the west side. These forest zones have been most recently defined by the Washington and Idaho GAP Analysis Programs (http://wdfw.wa.gov/conservation/gap/, http://www.wildlife.uidaho.edu/idgap/index.htm) and the British Columbia Biogeoclimatic Ecosystem Classification (BEC) and Ecology Research program (http://www.for.gov.bc.ca/hre/becweb/index.html) and are also in accord with Franklin & Dyrness (1973).

We designated the associated forests types according to the above habitat classification system for all 29
GENETIC AND PHENOTYPIC VARIATION ACROSS A HYBRID ZONE 3353

sampling localities by overlaying the centre-most location of all individuals at each locality on current ecoregion maps (above URL’s). We collapsed several specific forest types from these maps into the following three general forest zones from west to east: wet coastal forest, transitional forest and dry interior forest. Localities 1–9 were in the wet coastal forest zone, 10–18 in the transitional forest zone and 19–29 in the dry interior forest zone.

DNA extraction

We extracted genomic DNA from a 1.5 × 1.5 mm snippet of footpad tissue from each museum study specimen. Skin snippets were cleansed in an ethanol wash every 3 h for a 24-h period to remove salts and polymerase chain reaction (PCR) inhibitors that may have been inadvertently added during the preservation process of museum skins (Mullen & Hoekstra 2008). Following the washes, we used the prescribed protocol within the DNeasy Tissue kit (Qiagen, Valencia, CA, USA) to extract genomic DNA. We undertook several steps during this process to avoid and detect any possible contamination. First, we used a new sterilized razor blade for snipping each sample. Second, we performed ethanol washes in a separate room from where PCR amplifications were performed. We also included negative extractions and PCR controls in the genotyping process. Finally, we repeated DNA extractions from 20 individuals using skin snippets from another part of the study specimen and compared their microsatellite genotype profiles with original samples.

Microsatellite amplifications and screening

We genotyped all 397 specimens at nine polymorphic microsatellite loci originally identified in T. hudsonicus by Gunn et al. (2005): Thu03, Thu08, Thu14, Thu21, Thu23, Thu25, Thu31, Thu41 and Thu42. Each 3.32-µL reaction mixture contained 1.0 µL of nuclease-free H2O, 0.5 µL of 10× Bovine Serum Albumin (BSA), 0.5 µL of 10× PCR buffer, 4.5 mM MgCl2, 0.286 mM of each dNTP, 0.75 µM of each primer, 0.31 U of JumpStart Taq DNA polymerase (Sigma, St. Louis, MO, USA) and 1.68 µL of genomic DNA. We used a touchdown PCR protocol consisting of a denaturing step at 94 °C for 3 min; followed by eight cycles (with a decreasing 1 °C annealing temperature after 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; and with a final extension period of 72 °C for 45 min. We diluted PCR amplification products by 1:10 with nuclease-free water. Individuals were genotyped on an ABI 3730 Genetic Analyzer in a 17-µL multiplex sample (three primer-pair set) containing 3 µL of diluted PCR products (1 µL from each primer pair), 13.896 µL Hi-Di (ABI) and 0.104 µL 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, stuttering or null alleles, which may be elevated when using museum specimens that putatively contain partially degraded DNA. Micro-checker investigates the presence of null alleles when the combined probability test shows an overall significant excess of homozygotes evenly distributed across homozygote classes. Our results detected no null alleles in any of the nine microsatellite loci.

Population structure and hybridization assignment

To determine the most probable number of genetic clusters that characterizes this secondary contact zone, we analysed our genotype data with two Bayesian assignment methods. STRUCTURE 2.3.3 is a model-based Markov chain Monte Carlo (MCMC) approach that clusters individuals to minimize Hardy-Weinberg disequilibrium and gametic phase disequilibrium between loci within groups (Pritchard et al. 2000). This method is useful for studying population structure in contact zones because it allows for the presence of admixed individuals in the sample. STRUCTURE requires a user-defined number of populations (K) to test for the true populations numbers. We set the model parameters to admixture with correlated allele frequencies among populations and performed 10 replicate runs for each value of K ranging from 1 to 10 with a burn-in of 2.5 × 104 followed by 1.25 × 105 repetitions. Each run estimated the ‘log probability of data’ (L(K)). We then estimated the number of clusters (K) based on these likelihood values using the ad-hoc metric (ΔK) developed by Evanno et al. (2005). We chose this measure because Evanno et al. revealed with simulated data that STRUCTURE overestimates the numbers of populations in contact zone systems. This method is based on the rate of change in the log probability of data between successive K values. We also used STRUCTURAMA 2.0 to estimate the number of discrete genetic clusters (Huelsenbeck and Andolfatto 2007). In contrast to STRUCTURE, this program does not require a user-defined number of populations. The assignment of individuals to clusters and the number of clusters are treated as random variables under a Dirichlet process prior. We ran multiple analyses with different prior mean-numbers of populations [E(K) = 2, 5, 10] to explore whether the results remained consistent despite different priors. All MCMC analyses were run for 1 × 106 generations with a sample frequency of 1000 and the first 100 observations discarded as burn-in.

© 2011 Blackwell Publishing Ltd
After determining the numbers of genetic clusters in the northern Cascade Mountains study area, we used STRUCTURE to estimate individual admixture proportions, i.e., the estimated proportion of an individual’s genotype originating from each of the parental populations. Following Väihä & Primmer (2006), we categorized individuals into three clusters, using a range of q-values between 0 and 0.10 as pure T. hudsonicus, 0.90–1.0 as pure T. douglasii and 0.10–0.90 for admixed individuals. In addition, we used the program NEWHYBRIDS 1.1 to identify hybrid individuals (Anderson & Thompson 2002). This program is a more specific Bayesian method for identifying hybrids and can be used to identify individual assignment to various hybrid categories (F1, F2, backcross, pure, etc.). Unlike Structure, which treats q as a random continuous variable, NEWHYBRIDS treats q as a discrete variable with up to six genotype frequency classes. NEWHYBRIDS uses a MCMC sampling approach to acquire estimates from the posterior distribution that reflect the level of certainty that an individual belongs to a certain hybrid class. We also included 16 individuals located 250–500 km away from the study area as reference samples representing pure genotypes of each species (Table S1, Supporting information). We performed 1 × 10⁶ MCMC sweeps with a burn-in of 1 × 10⁵. We ran the analyses separately using the Uniform and Jeffreys-like priors because the Uniform prior can under-emphasize the influence of alleles that are rare in populations. However, neither prior should heavily influence the results of the analysis. A posterior probability value of 0.5 for the membership in a class was used as a threshold for assigning individuals to a specific class.

To assess the power of NEWHYBRIDS for detecting later-generation hybrids from our empirical data set, we performed assignment tests on a simulated data set containing individuals with known hybrid identities and belonging different hybrid classes. Using HYBRIDLAB 1.0 (Nielsen et al. 2006) with this data set, we generated ten simulated hybrids in each of the four hybrid classes: F1, F2 and F1 backcrosses to each species. Next, we combined these 40 simulated hybrids into a data set containing empirical data from 50 T. douglasii and 50 T. hudsonicus individuals that were identified to be pure from our STRUCTURE results, as well as eight T. douglasii and eight T. hudsonicus individuals located outside of the study area that were presumed to be pure representatives of each species to set as extra prior information for the analysis. We ran the simulated data set in NEWHYBRIDS under the same settings and analysed them separately under the Uniform and Jeffreys-like priors. Our results demonstrated that both analyses using different prior distributions performed well at inferring individuals belonging to the F1 hybrid class. The analysis with the Uniform prior distribution accurately assigned all simulated F1 individuals to the F1 hybrid class, whereas the analysis with the Jeffreys-like priors distribution correctly assigned nine out of ten F1 individuals to the F1 hybrid class. However, our results were not as strong for the assignment of later-generation hybrids to the right classes. The analysis using the Jeffreys-like priors correctly assigned 6 out of 10 simulated F2 individuals to the F2 hybrid class, and the analysis using Uniform priors correctly assigned only one out of ten to the right class. Neither analysis was able to accurately assign simulated F1 backcrosses to the right hybrid class. The inability of NEWHYBRIDS to correctly assign later-generation hybrids class is likely due to a lack of sufficient loci (see Väihä & Primmer 2006). Therefore, our confidence in interpreting the NEWHYBRIDS results from the empirical data set is strong for inferring the correct assignment of F1 hybrids, but weak for the correct assignment of F2 hybrids and F1 backcrosses.

Mitochondrial DNA sequencing and analysis

We chose the mitochondrial DNA control region for simply determining individual assignment to mtDNA clades. The more geographically extensive work of Arbogast et al. (2001) with cytochrome-b has previously established mtDNA phylogenetic relationships. We amplified a 312- to 328-bp sequence of the control region using PCR with primers OSU5020L and OSU5021H (Wilson et al. 2005). We used a modified protocol to better amplify sequences because of possible degradation of DNA from museum specimens. The PCR was performed in 15-µL reaction volumes containing 4.74 µL of nuclease-free H2O, 1.5 µL of 10 × BSA, 1.5 µL of 10 × PCR buffer, 3 mM MgCl₂, 0.19 mM of each dNTP, 0.66 µM of each primer, 0.9375 U of JumpStart Taq DNA polymerase (Sigma) and 1.8 µL of genomic DNA. We performed PCR amplifications in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The cycle conditions included a denaturing step at 94 °C (3 min), followed by 35 cycles (45 s at 94 °C, 30 s at 54 °C and 1 min at 72 °C) with a final extension period of 30 min at 72 °C. We treated all PCR products with ExoSapIT (USB Corp.) to remove unincorporated nucleotides and primers. PCR samples were run on either ABI 3100 or 3730 × 1 genetic analyzers (Applied Biosystems Inc.) with manual editing and alignment performed using SEQUENCER 4.6 (Gene Codes Corp.). Sequences were deposited in GenBank under accession numbers JF303085–JF303476 and JF308196–JF308209.

We used both maximum likelihood (ML) and Bayesian Inference (BI) methods to infer phylogenetic relationships of our mtDNA data set. We estimated the
The BI phylogenetic reconstructions were performed using MrBayes 3.1 (Ronquist & Huelsenbeck 2003) with 100 bootstrap replicates to evaluate nodal support for phylogenetic clades (Felsenstein 2003) using Markov Chain Monte Carlo (MCMC) sampling. Two different runs (each with one cold and three heated chains) were analysed for 2 \times 10^6 generations with trees sampled every 100 generations, which was when the average standard deviation of the split frequencies became <0.10. The first 25% of sampled trees were discarded as burn-in after visual inspection using TRACER 1.5 (Rambaut & Drummond 2007) revealed that these initial samples had not reached stationarity. We sequenced one specimen from each of the three categories: (i) pure T. hudsonicus (N = 185), (ii) admixed ancestry between T. douglasi and T. hudsonicus (N = 35) and (iii) pure T. hudsonicus (N = 168).

Analyses of cranial morphology

To examine differences in cranial morphology that may have resulted from either adaptive genetic divergence or phenotypic plasticity in response to different forest environments, we examined three cranial characters that seemed likely to serve important roles in modulating jaw strength and function (Smith 1981). Linear measurements were made to the nearest one hundredth millimetre using digital calipers (Mitutoyo Corp., Japan) as follows: (i) sagittal crest, measured as distance between the temporal lines; (ii) the angular moment arm (AMA), measured as distance between the mandibular notch to the angular process; (iii) coronoid moment arm (CMA), measured as distance between the coronoid processes and the mandibular condyle.

Morphological traits typically scale with body size, which can obscure interesting differences in traits among species that differ in body size (Reist 1986). Because T. hudsonicus is slightly larger in body length than T. douglasi (Smith 1981), differences in cranial traits between the two species can be confounded by differences in overall body length. Accordingly, we used an Analysis of Covariance (ANCOVA) to eliminate the effects of body size (Berner 2011). For this analysis, we used body length (taken as total length minus tail length as measured at the original preparation of each museum specimen) as a covariate in the model. Each measurement was divided by the grand mean to preserve between-group differences (Berner 2011). None of the three traits showed sexual dimorphism, and therefore, sexes were pooled for each species (Fig. S1, Supporting information). Species were binned into three microsatellite genotype groups (pure T. douglasi, admixed and pure T. hudsonicus) as assigned by our STRUCTURE analyses. These genotype groups were used as the factor variable in our ANCOVA model. For each trait, species always produced a significant effect at explaining the variance among data. However, the species-by-body-length interaction was never significant, which allowed us to reanalyse each ANCOVA without the interaction. We used the residuals from this model as our size-corrected data for the cline analyses. All analyses were carried out in JMP 7 (SAS Institute, Cary, NC, USA).

Clinal analysis

To estimate the relationship between spatial position and clinal variation of genetic and phenotypic data, we
fitted maximum likelihood clines to geographic variation of mtDNA haplotype assignments, ventral colour scores, principal component scores for cranial morphology and microsatellite genotype assignments 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). For the mtDNA data, we compared allele frequency clines with different numbers of parameters: a simple sigmoid model (two parameters), an asymmetric model (four parameters) and a three-parts model with different positions of tails (six parameters). We treated microsatellite genotype assignment, ventral colour and cranial morphology as quantitative characters and compared five different candidate models to find the best-fitting curve: bimodal without introgression, bimodal, trimodal without introgression, trimodal and unimodal. Bimodal distributions are characteristic of hybrid zones with very limited hybridization or introgression because of high dispersal of parental genotypes and very high selection against hybrids or high assortative mating. Most hybrid zones are characteristic of trimodal distributions, which can be described as having a pattern somewhere between a unimodal and a bimodal distribution (Gay et al. 2008). In this case, hybrids form a well identifiable group with intermediate allele frequencies and often high phenotypic variation because of varying levels of introgression. Unimodal distributions are characteristic of situations where intermediate hybrid genotypes predominate (hybrid swarm) or of relatively weak disruptive selection (Jiggins & Mallet 2000). We first analysed each character independently and used the Akaike Information Criterion (AIC) to rank the candidate models. We estimated cline width by measuring the geographic distance between 20% and 80% of the parental frequencies (Endler 1977). We performed pairwise comparisons of cline coincidence (centre) and concordance (width) of each character with the geographical cline of the mtDNA clade assignment by constraining each character to have the same centre or slope as the mtDNA cline. We also performed a comparison of cline coincidence and concordance all three cranial characters, coloration and microsatellite genotype assignment. We used the AIC to select the best model out of the constrained and unconstrained models. Evidence ratios were 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 1–0, with 1 representing T. douglasii-like character and 0 representing T. hudsonicus-like character.

For these cline analyses across the three forest zones, we reduced the spatial complexity of the 29 localities (Fig. 1) to a one-dimensional axis (transect) that follows a west-to-east orientation (Fig. 3). The topographical complexity of the hybrid zone in the northern Cascade Mountains presented a challenge in the transformation of the study area into a simple one-dimensional transect because of the uneven boundaries of the forest zones along the mountain axis. Therefore, the distance values in our cline analyses (cline width and centre) should be interpreted as relative values, rather than true distances. We binned each locality into one of three forest zones and measured straight west-to-east distances (km) from each locality to the boundary of the transitional forest zone. In the wet coastal forest zone, localities #1–9 were measured to the westernmost boundary of the transitional forest zone, and in the dry interior forest zone, localities #19–29 were measured to the easternmost transitional forest boundary. For localities within the transitional forest zone (10–18), we measured a straight-line distance of each locality to the midpoint of this zone. To obtain a total distance across the entire transect, we assigned a distance value of zero to the westernmost locality (#1) and adjusted the remaining locality distances relative to this locality, resulting in a total distance of 467 km. The geographic coordinates of each specimen and each locality (measured as the centre-most individual of all individuals at each locality) are given in Table S1 (Supporting information), along with the distance in km of each locality along the entire 467-km transect (as shown on the x-axis of Fig. 3). In addition, not all samples from each locality were from exactly the same location. For localities close to the contact zone, we used watershed boundaries to delineate the spatial extent of the locality. For localities at or near the ends of the transect, we grouped samples from greater distances apart because we presumed these contained pure values for each species.

Results

Population genetic structure and genotypic identification of hybrids

All our analyses of genetic population structure indicate the existence of two population groups in the northern Cascade Mountains that correspond with the taxonomic descriptions of T. douglasii and T. hudsonicus. Bayesian clustering analyses using microsatellite genotype data revealed two such population groupings. The ΔK statistic of Evanno et al. (2005) based on STRUCTURE likelihood estimates also showed that two genetic clusters best
characterized this multilocus data set (Table 1). All three STRUCTURAMA analyses using the Dirichlet process prior with population values of 2, 5 and 10 revealed that the highest posterior probability for the number of populations was also two (Table 1).

The assignment tests from STRUCTURE revealed the presence of hybrid individuals in our study area. About 9% (37 of 397) of all individuals from the entire study transect showed an admixed ancestry. Pure T. douglasii are represented by 48% (190 of 397) of all individuals and pure T. hudsonicus by 43% (170 of 397). Of all 37 admixed individuals, 28 (76%) were located in the transitional forest zone. These 28 individuals also represented 20% of all individuals from the transitional forest zone. Results from NEWHYBRIDS indicated the presence of later-generation hybrids and thus reproductive viability among hybrids. From the analysis using the Jeffreys-like priors, we could infer that all hybrids belonged to the F2 hybrid class. However, because our analyses using simulated hybrids were only correct 60% of the time in identifying hybrids to the F2 generation, it is possible that these hybrids actually belonged to other hybrid classes. Even so, we are confident that they were not F1 hybrids, because our analyses with simulated hybrids showed a strong ability to correctly identify F1 hybrids.

**Relationship between mtDNA haplotype and microsatellite genotype**

Phylogenetic inferences of mitochondrial sequence variation using Bayesian analyses (Fig. 2) and maximum likelihood (not shown) both revealed two major clades.

**Table 1** Estimates of population number (K) from STRUCTURE and STRUCTURAMA analyses using nine microsatellite loci

<table>
<thead>
<tr>
<th>No. of populations</th>
<th>STRUCTURE ad-hoc statistic</th>
<th>STRUCTURAMA posterior probability distributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>ΔK</td>
<td>E(K) = 2</td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>0.09</td>
</tr>
<tr>
<td>2</td>
<td>103.53</td>
<td>0.91</td>
</tr>
<tr>
<td>3</td>
<td>1.99</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>5.71</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>0.63</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>0.24</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>4.46</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>2.01</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>0.21</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>6.69</td>
<td>—</td>
</tr>
</tbody>
</table>

The number of populations with the highest ΔK value estimated from STRUCTURE and the highest posterior probability estimated from STRUCTURAMA is K = 2 (shown in bold).

© 2011 Blackwell Publishing Ltd
cline were estimated as 155 and 27 km, respectively. The three-part model with six parameters showed the best fit for the clinal pattern of geographic variation in mtDNA clade assignments (Table 3). The geographic cline for microsatellite genotype assignment (Fig. 3b) was steep, and all pure genotypes of each species were found only in their respective forest zone or in the transitional forest zone, but never in the forest zone inhabited by the other species. Furthermore, about 75% of the admixed genotypes were found in the transitional forest zone. Maximum likelihood estimates of the centre and width of this cline were 150 and 14 km, respectively. The trimodal distribution showed the best fit of the clinal pattern for microsatellite genotype assignment (Table 3), which is characteristic of hybrid zones that form a well identifiable group with intermediate allele frequencies and high variance because of varying levels of introgression. The geographic cline for fur coloration (Fig. 3c) was very steep and revealed that the typical fur colour of each species was found only in its

Fig. 2 Bayesian gene tree of 392 samples inferred for *Tamiasciurus douglasii* and *T. hudsonicus* across the northern Cascade Mountains study area (Table S1, Supporting information), based on 327 base pairs of the mitochondrial control region. Bayesian posterior probabilities are shown below each major node and maximum likelihood bootstrap values above. The haplotypes in the *T. hudsonicus* clade are labelled 'Th' and numbered 1–39, and the haplotypes in the *T. douglasii* clade are labelled 'Td' and numbered 1–36. Stars represent 17 haplotypes containing 97 individuals assigned as either admixed genotype or a mismatch between microsatellite genotype assignment and mtDNA haplotype assignment. Outgroup sequence is *T. hudsonicus mogollonensis* from Arizona (T.h.m.).
Discussion

Identification of hybrids and their forest association

We have shown that Tamiasciurus douglasii and T. hudsonicus form distinct genetic clusters and hybridize in a secondary contact zone in the northern Cascade Mountains. Our genetic data suggest that the intermediate morphological phenotypes and behaviours previously observed by Smith (1968) and Stevens & Nellis (1974) resulted from this hybridization. We also found that most of the hybrids occurred in a relatively narrow band of ecotonal habitat. Although both species are ecologically dependent on coniferous forest habitat for food and shelter, each occupies a different forest type: T. douglasii in wet, western coastal forests and T. hudsonicus in dry, eastern interior forests. Smith (1968, 1970, 1978, 1981) found divergence of feeding efficiency, life history strategies, coloration and vocalization across this east-west cline of forest environments. The transitional forest ecotone where we identified most of the hybrids is located slightly to the west of the crest of the Cascade Mountains and includes both a subalpine forest and an atypical, high-altitude Douglas-fir forest community. This special Douglas-fir community is unusual because it contains a complex and highly diverse mixture of coniferous tree species that are otherwise typical of both eastern dry forests (lodgepole and ponderosa pines) and western wet forests (western hemlock and western red cedar). The great breadth of the Cascade Mountain range in this region has created an exceptional rain shadow effect on the west side of the Cascade crest, which provides locally drier environmental conditions that support this unique forest assemblage (Franklin & Dyrness 1973). Moreover, this region is also near the area of contact between two varieties of Douglas-fir trees, Pseudotsuga menziesii var. menziesii (coastal Douglas-fir) and P. menziesii var. glauca (interior Douglas-fir) which possess adaptive differences in phenology and growth rate (St. Clair et al. 2005). A recent phylogeographic analysis of these two tree varieties reveals that they represent divergent lineages of mtDNA and chloroplast-DNA that show genetic introgression and likely moved into secondary contact in the Cascade Mountains during the Holocene (Gugger et al. 2010). Douglas-fir is used extensively by both T. douglasii and T. hudsonicus for both food and shelter, and therefore, it is likely that the postglacial secondary contact of these coniferous tree lineages facilitated secondary contact of the squirrel lineages.

Hybrid viability

Our genetic evidence based on microsatellite data demonstrated that hybrids are reproductively viable and able to backcross with both parental species. The assignment of all hybrids to a later-generation hybrid class indicates that hybrids must have been reproductively viable to successfully breed beyond the F₁ generation. Hybrid viability is further supported by the fact that 19% of the pure T. douglasii specimens (based on microsatellite genotype assignment) and 15% of the pure T. hudsonicus specimens possessed a mtDNA haplotype belonging to the opposite species, which could only occur through multiple generations of hybrid backcrossing with both
parental forms. This bidirectional pattern of mtDNA introgression suggests that species discrimination in mating, whether through male–male competition or female mate choice, is not strong enough to maintain complete reproductive isolation in *Tamiasciurus*. In other mammalian taxa that hybridize, it has been suggested that prezygotic isolating mechanisms such as variation in bacular morphology and aggressive mating behaviour have caused asymmetric introgression patterns, i.e., mtDNA capture (Macholán et al. 2007; Good et al. 2008). However, unlike most members of the squirrel family (Wade & Gilbert 1940), male *Tamiasciurus* possess a minute os penis, or baculum, that is considered vestigial (Layne 1952), and thus, this structure may not play an important role in reproductive isolation. Furthermore, interspecific copulations may be facilitated by a lack of overt mate choice by females, which would result in multi-male mating; this situation has been shown to occur in *Tamiasciurus* populations (Arbetan 1993; Lane et al. 2007; Bonanno & Schulte-Hostedde 2009). On the other hand, multi-male mating in *Tamiasciurus* may lead to sperm competition (Bonanno & Schulte-Hostedde 2009), which could promote interspecific assortative mating in a hybrid zone such as ours.

Fig. 3 Clinal patterns of variation among individuals at 29 localities in the three forest zones across the west-to-east study transect. Derivation of the 467-km transect (x-axis) is described in Methods and materials, and the distance of each locality is given in Table S1 (Supporting information). Dotted vertical lines represent limits between the three forest zones. The area west (left) of the dotted lines represents the wet coastal forest zone, between the lines is the transitional forest zone, and the area east (right) of the dotted lines represents the dry interior forest zone. (a) Proportion of individuals belonging to the two mtDNA clades, where 1.0 = *Tamiasciurus douglasii* and 0.0 = *T. hudsonicus*. (b) Genotype assignment proportions (q-values) based on microsatellite data of 397 individuals as determined by STRUCTURE. We have categorized these values as pure *T. douglasii* genotype (upper 10% shaded; extreme = 1.0), pure *T. hudsonicus* genotype (lower 10% shaded; extreme = 0.0) and admixed genotype (middle 80%). Only 37 of the 397 individuals (9%) were admixed. (c) Ventral colour scores of 388 individuals, ranging over four shades from darkest (1.0) to lightest (0.0). (d) Sagittal crest residual values for 207 individuals. (e) Angular moment arm (AMA) residual values for 192 individuals. (f) Coronoid moment arm (CMA) residual values for 192 individuals. The values for all three cranial characters (d-f) represent size-corrected values determined by the ANCOVA.
Phenotypic variation

The trimodal cline model for fur coloration supports a scenario in which hybridization has produced intermediate phenotypes, but in which these phenotypes do not spread outside the contact zone because of strong selection against them. The sharp cline for this trait and its position within the ecotonal forest zone suggest that divergent selection might be acting strongly on this variable and perhaps heritable trait. Fur coloration is ecologically important in mammals and under strong selection for cryptic protection from predators (Powell 1982; Kiltie 1992; Stoner et al. 2003; Hoekstra et al. 2005; Mullen & Hoekstra 2008). Most studies examining selection on fur colour have focused on dorsal pelage and its match to ground colour; however, the arboreal lifestyle of tree squirrels also makes their underside coloration a target of selection. Smith (1981) studied the ecology of both species of Tamiasciurus in this region and argued that reduced light intensity in the dense canopy forest (of the west) should favour a darker ventral fur colour for protection against avian predators from the side or below, whereas the brighter background sky in the more open canopy forest to the east should favour lighter ventral fur colour. It would be interesting to assess with experimental selection studies whether these divergent phenotypes are being selected for greater matching to their respective forest environments (Kiltie 1992; Vignieri et al. 2010). It could also be argued that these species have not interbred for long enough for these traits to spread sufficiently across the hybrid zone. However, if this were the case, then we might not have documented relatively deep introgression of the neutral mtDNA marker across the hybrid zone in both directions. Thus, we conclude that ventral fur colour appears to be an ecologically important trait.

Table 3 Comparison of hybrid-zone models using the Akaike Information Criteria (AIC) for clinal variation in microsatellite genotype assignment, mtDNA clade assignment, ventral colour score and three cranial features

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameters</th>
<th>$\text{AIC}_c$</th>
<th>$\Delta\text{AIC}$</th>
<th>$\text{AIC}$ weights</th>
<th>Evidence ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA clade assignment</td>
<td>3-Part</td>
<td>6</td>
<td>380.0937</td>
<td>0</td>
<td>0.8018</td>
</tr>
<tr>
<td>Asymmetric</td>
<td>4</td>
<td>382.8901</td>
<td>2.7964</td>
<td>0.1981</td>
<td>4.0</td>
</tr>
<tr>
<td>Simple sigmoid</td>
<td>2</td>
<td>397.1475</td>
<td>17.1170</td>
<td>~0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Simple sigmoid</td>
<td>2</td>
<td>397.1475</td>
<td>17.1170</td>
<td>~0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Microsatellite genotype assignment</td>
<td>Trimodal</td>
<td>18</td>
<td>1747.1802</td>
<td>0</td>
<td>0.7575</td>
</tr>
<tr>
<td>Trimodal No Introgression</td>
<td>16</td>
<td>1749.4579</td>
<td>2.2778</td>
<td>0.2425</td>
<td>3.1</td>
</tr>
<tr>
<td>Bimodal</td>
<td>12</td>
<td>2737.7096</td>
<td>990.5294</td>
<td>~0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal No Introgression</td>
<td>10</td>
<td>2764.0177</td>
<td>1016.8375</td>
<td>~0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Ventral colour score</td>
<td>Trimodal</td>
<td>18</td>
<td>1747.1802</td>
<td>0</td>
<td>0.7575</td>
</tr>
<tr>
<td>Trimodal No Introgression</td>
<td>16</td>
<td>1749.4579</td>
<td>2.2778</td>
<td>0.2425</td>
<td>3.1</td>
</tr>
<tr>
<td>Bimodal</td>
<td>12</td>
<td>2737.7096</td>
<td>990.5294</td>
<td>~0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal No Introgression</td>
<td>10</td>
<td>2764.0177</td>
<td>1016.8375</td>
<td>~0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Sagittal crest width</td>
<td>Trimodal</td>
<td>18</td>
<td>279.2470</td>
<td>9.4975</td>
<td>0.0074</td>
</tr>
<tr>
<td>Trimodal No Introgression</td>
<td>16</td>
<td>283.8755</td>
<td>5.2807</td>
<td>0.0611</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal</td>
<td>12</td>
<td>283.4637</td>
<td>5.2807</td>
<td>0.0611</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal No Introgression</td>
<td>10</td>
<td>288.7445</td>
<td>0</td>
<td>0.8564</td>
<td>—</td>
</tr>
<tr>
<td>Coronoid moment arm</td>
<td>Trimodal</td>
<td>18</td>
<td>117.5428</td>
<td>0</td>
<td>0.9883</td>
</tr>
<tr>
<td>Trimodal No Introgression</td>
<td>16</td>
<td>105.5160</td>
<td>12.0269</td>
<td>0.0024</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal</td>
<td>12</td>
<td>105.4318</td>
<td>12.1110</td>
<td>0.0024</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal No Introgression</td>
<td>10</td>
<td>95.4318</td>
<td>22.1110</td>
<td>0.0024</td>
<td>&gt;10</td>
</tr>
<tr>
<td>AMA</td>
<td>Trimodal</td>
<td>18</td>
<td>109.8733</td>
<td>0</td>
<td>0.9883</td>
</tr>
<tr>
<td>Trimodal No Introgression</td>
<td>16</td>
<td>104.9091</td>
<td>4.9642</td>
<td>0.0836</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal</td>
<td>12</td>
<td>104.5430</td>
<td>5.3303</td>
<td>0.0696</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bimodal No Introgression</td>
<td>10</td>
<td>93.2671</td>
<td>16.6061</td>
<td>0.0024</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

The best model for each cline is in bold. Evidence ratios (AIC weight of the best model divided by the AIC weight of the listed model) for each character show the relative likelihood of the best model being correct.
found different clinal distributional patterns that are indicative of moderate to strong selection strength acting on these phenotypes. Both species of squirrels consume seeds from cones, as a primary food resource, after they have mechanically removed the scales from the cones (Smith 1968). *Tamiasciurus hudsonicus* lives in a dry forest environment where several species of coniferous trees have evolved harder and thicker cone scale tissue than the conifer species in the wetter forests where *T. douglasii* lives (Smith 1968). For example, lodgepole pine (*Pinus contorta*), an important food resource within the range of *T. hudsonicus*, has evolved fire-mediated serotiny in its cones, which requires greater jaw force of squirrels to open than for cones of any of the tree species west of the Cascade Mountains. Mammals can produce different amounts of jaw force by modulations of their temporal and masseter jaw musculature (Turnbull 1970). Studies on jaw structure and feeding efficiency in *Tamiasciurus* have shown that *T. hudsonicus* is more powerful and faster than *T. douglasii* at chewing through harder cones (Smith 1970, 1981). The presence of a sagittal crest in mammals generally indicates strong jaw muscles. The sagittal crest develops through the convergence of the temporal lines on the parietal bone and serves primarily as the origin of the temporalis muscle, one of the main chewing muscles. *Tamiasciurus hudsonicus* generally possess a large temporal muscle and a distinct sagittal crest, making them more efficient at

<table>
<thead>
<tr>
<th>Character</th>
<th>Hybrid-zone models</th>
<th>Parameters</th>
<th>$\Delta_{\text{AIC}}$</th>
<th>$\Delta_{\text{AIC}}$ weights</th>
<th>Evidence ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral colour</td>
<td>Centre Constraint</td>
<td>23</td>
<td>-3634.152</td>
<td>41.454</td>
<td>0.000</td>
</tr>
<tr>
<td>Slope Constraint</td>
<td>22</td>
<td>23</td>
<td>-811.489</td>
<td>2684.116</td>
<td>0.000</td>
</tr>
<tr>
<td>Slope and Centre Constraints</td>
<td>24</td>
<td>22</td>
<td>-1056.912</td>
<td>2618.694</td>
<td>0.000</td>
</tr>
<tr>
<td>Sagittal crest width</td>
<td>Centre Constraint</td>
<td>15</td>
<td>102.586</td>
<td>7.342</td>
<td>0.025</td>
</tr>
<tr>
<td>Slope Constraint</td>
<td>15</td>
<td>15</td>
<td>218.959</td>
<td>123.715</td>
<td>~0</td>
</tr>
<tr>
<td>Slope and Centre Constraints</td>
<td>14</td>
<td>14</td>
<td>217.741</td>
<td>122.497</td>
<td>~0</td>
</tr>
<tr>
<td>Unconstrained</td>
<td>16</td>
<td>24</td>
<td>95.244</td>
<td>0</td>
<td>0.975</td>
</tr>
<tr>
<td>Coronoid moment arm</td>
<td>Centre Constraint</td>
<td>23</td>
<td>296.423</td>
<td>26.910</td>
<td>~0</td>
</tr>
<tr>
<td>Slope Constraint</td>
<td>23</td>
<td>23</td>
<td>404.442</td>
<td>134.929</td>
<td>~0</td>
</tr>
<tr>
<td>Slope and Centre Constraints</td>
<td>22</td>
<td>22</td>
<td>402.978</td>
<td>133.465</td>
<td>~0</td>
</tr>
<tr>
<td>Unconstrained</td>
<td>24</td>
<td>24</td>
<td>269.513</td>
<td>0</td>
<td>~1</td>
</tr>
<tr>
<td>AMA</td>
<td>Centre Constraint</td>
<td>23</td>
<td>299.834</td>
<td>18.349</td>
<td>~0</td>
</tr>
<tr>
<td>Slope Constraint</td>
<td>23</td>
<td>23</td>
<td>411.934</td>
<td>130.448</td>
<td>~0</td>
</tr>
<tr>
<td>Slope and Centre Constraints</td>
<td>22</td>
<td>22</td>
<td>411.974</td>
<td>130.488</td>
<td>~0</td>
</tr>
<tr>
<td>Unconstrained</td>
<td>24</td>
<td>24</td>
<td>281.486</td>
<td>0</td>
<td>~1</td>
</tr>
<tr>
<td>Microsatellite genotype assignment</td>
<td>Centre Constraint</td>
<td>23</td>
<td>1904.322</td>
<td>55.876</td>
<td>~0</td>
</tr>
<tr>
<td>Slope Constraint</td>
<td>21</td>
<td>21</td>
<td>3728.995</td>
<td>1870.549</td>
<td>~0</td>
</tr>
<tr>
<td>Slope and Centre Constraints</td>
<td>20</td>
<td>20</td>
<td>3733.619</td>
<td>1875.172</td>
<td>~0</td>
</tr>
<tr>
<td>Unconstrained</td>
<td>22</td>
<td>22</td>
<td>1858.446</td>
<td>0</td>
<td>~1</td>
</tr>
</tbody>
</table>

The best model for each cline is in bold. Evidence ratios (AIC weight of the best model divided by the AIC weight of the listed model) for each character show the relative likelihood of the best model being correct.

<table>
<thead>
<tr>
<th>Hybrid-zone models</th>
<th>Parameters</th>
<th>$\Delta_{\text{AIC}}$</th>
<th>$\Delta_{\text{AIC}}$ weights</th>
<th>Evidence ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centre Constraint</td>
<td>78</td>
<td>-3095.712</td>
<td>48.048</td>
<td>~0</td>
</tr>
<tr>
<td>Slope Constraint</td>
<td>78</td>
<td>-3111.901</td>
<td>31.859</td>
<td>~0</td>
</tr>
<tr>
<td>Slope and Centre Constraints</td>
<td>74</td>
<td>-3143.760</td>
<td>0</td>
<td>~1</td>
</tr>
<tr>
<td>Unconstrained</td>
<td>82</td>
<td>-2997.069</td>
<td>34.907</td>
<td>~0</td>
</tr>
</tbody>
</table>

The best model is in bold.
chewing harder cones than T. douglasii, in which these characters are less pronounced (Smith 1981). Our demonstration of a bimodal clinal pattern of sagittal crest width and cline centre in the transitional forest zone suggests that this trait is ecologically important and that it is responding to transitional variation in conifer species. Another important component of the temporalis muscle complex is the coronoid process, which is the insertion point for the temporalis muscle. The force applied along the temporal muscle is applied along the CMA, which is related to the length between the coronoid process and the mandibular condyle. Smith (1981) found that CMA is positively correlated with size of the temporal muscle in Tamiasciurus and accordingly is larger in T. hudsonicus than in T. douglasii. The angular moment arm (AMA) is related to the force applied along the masseter muscle and is another important trait involved in bite force in mammals. Both CMA and AMA exhibit trimodal clinal patterns and cline centres located within the transitional forest zone, which suggests that these traits are under moderately strong selection across the hybrid zone. All of these cranial features are subject to ontogenetic effects caused by environmental variation. However, it is beyond the scope of this study to ascertain the degree to which phenotypic variation is affected by underlying genetic variation versus phenotypic plasticity.

The occurrence of similar cline centres and widths of all phenotypic characters and the microsatellite genotype assignment within the narrow transitional forest zone demonstrates that ecological selection is possibly maintaining the geographic position of this hybrid-zone system. This could be due to the fact that selection is acting not only against a particular trait, but perhaps further against a correlated set of traits and associated loci. Strong statistical associations (linkage disequilibria) between genes, chromosomes and morphological characters are generated by the continual diffusion of the combination of parental genes into the centre of a hybrid zone (Barton & Hewitt 1989). Therefore, any disruptive selection that prohibits a particular trait from permeating through a hybrid zone may also be prohibiting other traits from permeating. If selection is strong, linkage disequilibrium between parental alleles becomes even stronger and pulls clines together (Slatkin 1975; Barton 2001).

Conclusion

The montane regions of northwestern North America contain 'suture zones' for many pairs of terrestrial vertebrate species (Remington 1968; Swenson & Howard 2005). Pleistocene cycles of glaciation and associated historic north-south habitat shifts, together with the existence of prominent north-south montane axes (Cascade and Rocky Mountains), have played a major role in the vicariance and subsequent secondary contact of populations (Brunsfeld et al. 2001; Shafer et al. 2010). During colder intervals, populations were forced into separate refugia (often eastward and westward), which resulted in allopatric divergence. The warming climate of the Holocene facilitated the spread of populations out of refugia and in some cases into secondary contact with previously segregated populations. The outcome of secondary contact varies among major vertebrate taxa, although most show limited hybridization in narrow regions of contact. These outcomes include directional asymmetries in introgression because of premating behaviours (Krosby & Rowher 2009), limited hybridization because of postmating factors (Irwin et al. 2009), and ancient, rather than contemporary hybridization (Good et al. 2008). Several wide-ranging boreal mammals have similar distributions and apparent secondary contact zones similar to those of Tamiasciurus (Arbogast & Kenagy 2001), but hybridization has not been investigated in most of these taxa (Runck et al. 2009). The low mtDNA sequence divergence (1.0–2.4%) between major Tamiasciurus lineages suggests that their divergence is very recent, perhaps dating only to the late Pleistocene (Arbogast et al. 2001). The recency of this divergence may explain why these two forms can still interbreed; perhaps not enough epistatic incompatibilities have developed between loci. This temporal perspective also provides a compelling argument that diversifying selection on the observed colour phenotypes and skull morphology phenotypes in Tamiasciurus has been strong and that recently divergent lineages can remain separate units despite introgression.

Acknowledgements

This project was funded by the Burke Museum Mammal Program and a Genome Training Grant from the Department of Genome Sciences at the University of Washington. We thank the University of Kansas, Museum of Natural History, for access to specimens. We also thank Sylvia Yang for assistance with the curve-fitting model; and we thank Joe Felsenstein, Toby Bradshaw, Sievert Rohwer, Stevan Springer and Adam Leaché’s Lab Group for comments on the manuscript.

References


Gunn MR, Dawson DA, Leiveston A et al. (2005) Isolation of 18 polymorphic microsatellite loci from the North American red squirrel, Tamiasciurus hudsonicus (Sciuridae, Rodentia), and their cross-utility in other species. Molecular Ecology Notes, 5, 650–653.


Muller HJ (1942) Isolating mechanisms, evolution and temperature. Biological Symposium, 6, 71–125.


Genetics, 155, 945–959.


Canadian Journal of Zoology, 64, 1363–1368.


A.S.C. is a PhD Candidate whose research interests include applying molecular and morphological approaches to the understanding the ecological and evolutionary history of mammal populations. C.J.S. is an undergraduate at Evergreen State College and is interested in the morphological evolution of mammals. G.J.K. is Curator of Mammals, Emeritus, at the Burke Museum, where he works with graduate students interested in collections-based research on the historical biogeography and evolution of contemporary mammal populations.

Data accessibility

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Localities and other identifying information for the northern Cascade Mountains study area of 397 museum specimens of *Tamiasciurus douglasii* and *T. hudsonicus* used in this study.

Fig S1 Results from the ANCOVA showing effect tests for each of the three cranial characters.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.