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Examination of nuclear loci across a zone of mitochondrial introgression between *Tamias ruficaudus* and *T. amoenus*

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The importance of hybridization in animal evolution has become controversial. Gene flow between divergent taxa can strongly influence processes of great interest to evolutionary biologists, such as speciation and adaptation. Mitochondrial DNA (mtDNA) introgression is a commonly observed, yet particularly poorly understood consequence of hybridization between divergent taxa. Several known cases of mtDNA introgression exist that are heterogeneous in time and space, and it is unknown whether this represents the influence of extrinsic factors, such as selection and demographic fluctuation, or more intrinsic ones, such as cytonuclear interactions or the mosaic evolution of reproductive isolation. It is usually assumed, on the basis of the apparent lack of hybrids at zones of contact, that this phenomenon is unaccompanied by further admixture at nuclear loci, although this assumption rarely is tested. We present an analysis of population structure and gene flow across a zone of mitochondrial introgression between 2 nonsister species of chipmunk (Sciuridae: *Tamias*). We examined patterns of variation in 11 nuclear markers (10 microsatellites and 1 sequence-based marker) and compared them with previously identified patterns of mtDNA variation. We found little evidence of nuclear gene flow but some correspondence of introgression with microsatellite population structure. This work suggests that a complex interaction of ecological and genetic factors could have structured introgression of mtDNA in this system. DOI: 10.1644/09-MAMM-A-082.1.

Key words: gene flow, hybridization, introgression, microsatellites, speciation, *Tamias*

The role of hybridization in many evolutionary processes is not well understood (Dowling and Secor 1997; Mallet 2005). Historically, a stark dichotomy has existed between the views of botanists and zoologists on this issue. Although plant hybrids, hybrid zones, and hybrid species are observed fairly commonly, animal hybrids frequently are noted for their rarity in the wild and their sterility or inviability in captivity. Additionally, foundational theory of population genetics suggests that very low levels of gene flow are sufficient to homogenize divergent populations. Thus, botanists (Anderson and Stebbins 1954; Arnold 1992; Rieseberg and Burke 2001) have a long tradition of considering the effects of hybridization in evolution, but prominent zoologists and theoreticians such as Mayr (1963) asserted that hybridization could contribute little to the evolution of animal diversity.

Recently, evidence has been increasing that hybridization in animals might be far more pervasive than previously thought (Dowling and Secor 1997; Mallet 2005). It has become apparent in studies of speciation using the genetic approach referred to as divergence population genetics (Kliman et al. 2000; Machado et al. 2002) that divergence with gene flow models of speciation (Nosil 2008; Rice and Hostert 1993) could be more appropriate than the traditional models that invoke the necessity of complete genetic isolation. Wu (2001) proposed a genic view of speciation in which accumulation of incompatibilities between populations, potentially linked to differential adaptation, is heterogeneous across the genome, leaving some segments more prone to gene flow than others. This represents a paradigm shift in studies of speciation. Further consideration not just of the traditionally examined reproductive isolating barriers but also of the genomic architecture of, and effects of selection on, divergence ultimately should be revealing (Nosil et al. 2008).

At deeper levels of divergence evidence of the frequency of hybridization is primarily in the form of numerous documented cases of mitochondrial DNA (mtDNA) introgression

between pairs of taxa considered otherwise to be good species (Good et al. 2003; Linnen and Farrell 2007; Lu et al. 2001; McGuire 2007; Ruedi et al. 1997; Shaw 2002; Sota and Vogler 2001). Although some have considered that the unlinked nature and maternal inheritance of mtDNA might make it more susceptible to introgression than other parts of the genome (Chan and Levin 2005; Funk and Omland 2003), the frequency of this phenomenon suggests that a protracted period of incomplete reproductive isolation might occur after divergence. This is supported by the additional observation of cases of temporally structured introgression events between the same taxa (Good et al. 2003; McGuire 2007). The occurrence of intermittent hybridization implies the possibility that groups of related taxa within dispersal distance of one another could be subject to introgression of neutral genetic material or potentially to selective sweeps of adaptive genes (Rieseberg et al. 2003). Alternatively, these zones of introgression could be more often artifacts of brief periods of secondary contact that end when reinforcement (e.g., selection against hybrids) leads to the cessation of further gene flow. A relative lack of studies exists to distinguish between these possibilities, measure how much cryptic gene flow actually occurs, or determine factors that might influence temporal and geographic heterogeneity in gene flow between good species. We examined nuclear loci across a zone of interspecific mtDNA introgression in a system in which mtDNA introgression has been both temporally and geographically structured.

Two western North American chipmunk species, the red-tailed chipmunk, *Tamias ruficaudus*, and the yellow-pine chipmunk, *T. amoenus* (Rodentia: Sciuridae), are thought to have exchanged mtDNA in 2 geographically discrete but widespread zones of introgression (Fig. 1). Although patterns of paraphyly between these 2 species could be the result of coalescent stochasticity, haplotypes in question are geographically structured around areas of contact, a pattern not expected under coalescence. In both cases the mtDNA introgression has been unidirectional, from *T. ruficaudus* into *T. amoenus* (Good et al. 2003, 2008). These nonsister taxa (Levenson et al. 1985; Piaggio and Spicer 2001) have overlapping cranial morphologies and, when they occur in close proximity, very similar pelage color (Levenson et al. 1985). At a broad spatial scale they have substantially overlapping distributions, with the range of *T. ruficaudus* completely enveloped within that of *T. amoenus* (which ranges from eastern Montana to western Washington and south to northern California). They generally partition habitat distinctly, however, into lower-elevation xeric ponderosa pine forest (*T. amoenus*) and higher-elevation mesic forests (*T. ruficaudus*).

The 2 zones of introgression differ in important ways. First, each introgression occurs between distinct pairs of populations (i.e., between different pairs of subspecies). One event occurred between *T. ruficaudus simulans* (TRS) and *T. amoenus canicaudus* (TACA) in northern Idaho and eastern Washington (Good et al. 2008), which resulted in the fixation of the introgressed mtDNA haplotype in the entire TACA subspecies. The other introgression zone is between *T. ruficaudus ruficaudus* (TRR) and Rocky Mountain populations of *T. amoenus luteiventris* (TALR) and is located in the Rockies of northwestern Montana, extending north into Canada along the British Columbia–Alberta border (Good et al. 2003; Fig. 1). These 4 populations each have distinct mtDNA, and each is characterized by a diagnostic bacular morphology (Demboski and Sullivan 2003; Good and Sullivan 2001; Patterson and Heaney 1987; Fig. 1). Columbia Mountain populations of *T. a. luteiventris* (TALC) share a bacular morphology with TACA and mtDNA with TALR (Good et al. 2003). The baculum, a heterotopic bone located distally in the penis (Romer and Parsons 1986), generally has been considered an important taxonomic character in chipmunks (Sutton 1995; Sutton and Patterson 2000; White 1953). Contrary to other morphological characters in chipmunks, the baculum tends to exhibit strongly discontinuous variation between taxa (White 1953). Despite intensive sampling at subspecific boundaries within *T. ruficaudus*, where gene flow is thought to be ongoing (Good and Sullivan 2001; Hird and Sullivan 2009), intermediate morphologies have not been recorded. This strongly suggests that selection plays a role in shaping and maintaining bacular morphology.

The 2 interspecific zones also differ in age. At the southern contact zone (between TRS and TACA), TACA mitochondrial haplotypes form a monophyletic clade nested within *T. ruficaudus* and sister to the clade that corresponds to TRS (Good et al. 2003; Fig. 1). This implies substantial age, and an estimate of the divergence time of TRS and the TACA clade dates to 3–1.5 million years ago (Good et al. 2008). This suggests an ancient, temporally discrete period of introgression with no current hybridization. Good et al. (2008) included analyses of 7 microsatellite and several nuclear sequenced-based markers that yielded no indication of nuclear genetic exchange, although mutation and drift potentially could have obscured such evidence. By contrast, the northern contact zone (between TRR and TALR) is located in a region that was unsuitable habitat during the last glacial maximum and could have been colonized only within the last few thousand years (Delcourt and Delcourt 1993; Mack et al. 1978). This zone of introgression is characterized by the sharing of identical haplotypes across the species boundary, implying that introgression has occurred recently enough that these introgressed haplotypes have not accumulated novel mutations (Good et al. 2003). Given that this zone of introgression appears so recent, we asked if hybridization might be ongoing and if the effects of interspecific gene flow are measurable at nuclear loci. We addressed this question through the analysis of population structure in the 2 species throughout the region. We compared patterns of variation between mtDNA and 11 nuclear markers (10 microsatellites and 1 sequence-based marker).

Materials and Methods

Geographic and genomic sampling.—The previously identified zone of mtDNA introgression is extensive, so to identify

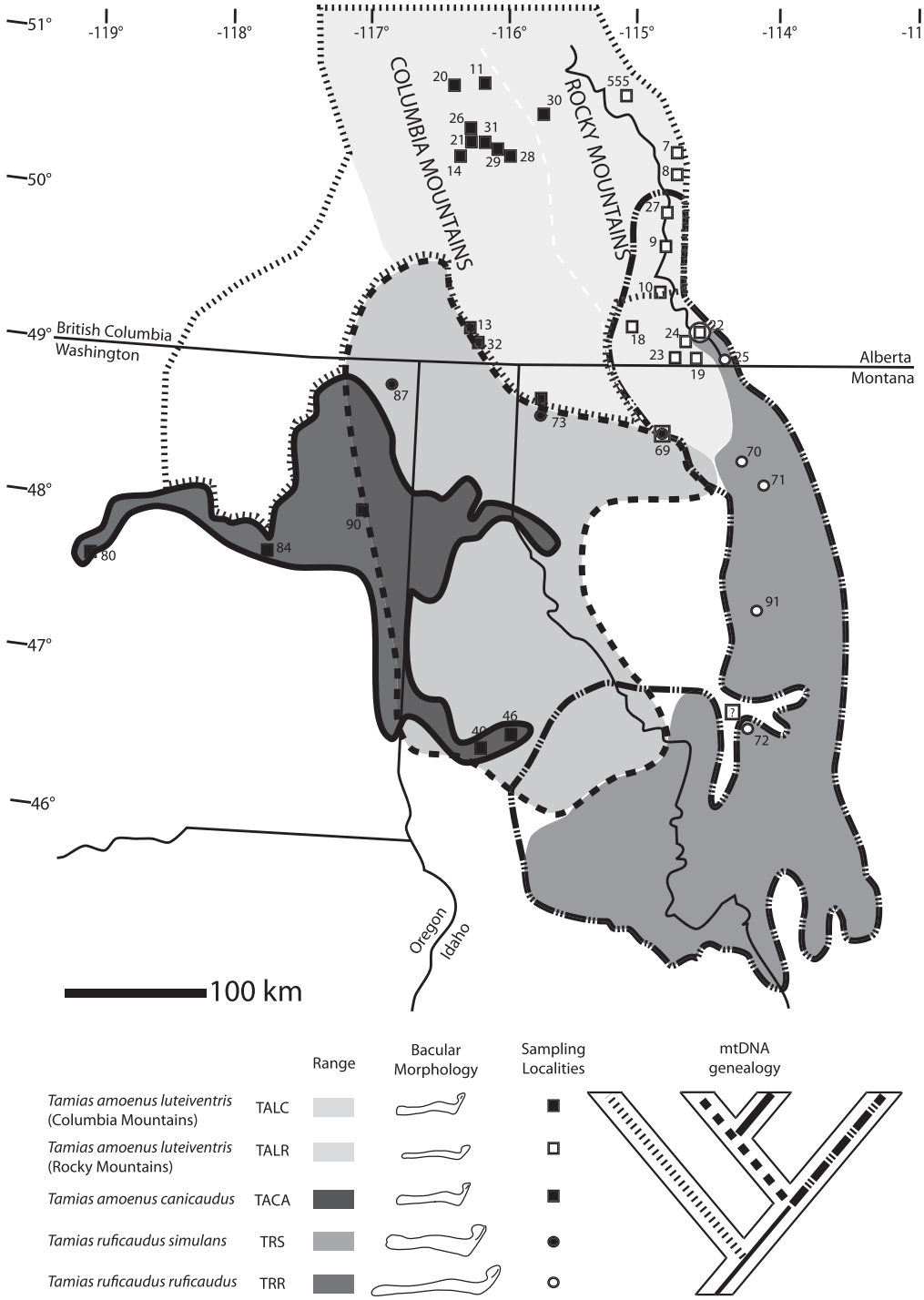

Fig. 1.—Map of sampling localities in which background shading indicates traditional subspecific boundaries, locality symbol indicates bacular morphology, and variously dashed lines indicate the extent of mitochondrial haplogroups whose relationships are indicated on the bottom right cladogram. The thin, white dashed line indicates the location of the Rocky Mountain Trench. Zones of introgression between TRR and TRS and TACA follow Good et al. (2003). See text for definition of population abbreviations.

potentially geographically broad effects of nuclear introgression we collected at several distinct localities where both species should occur syntopically or in close proximity (Good et al. 2003; Nagorsen et al. 2002; Fig. 1; Appendices I and II). To place observed genetic variation in context we also incorporated samples from both previous and unpublished studies, some of which were distant from the contact zone (Demboski and Sullivan 2003; Good et al. 2003, 2008; Schulte-Hostedde et al. 2001). Samples outside the contact zone included *T. a. canicaudus*, *T. a. luteiventris* from the Columbia Mountains, and northern populations of *T. r. simulans*. In total we genotyped 220 individuals from 30 localities (Appendix I); 172 individuals are vouchered specimens. Several specimens with known bacular morphol-

ogy were available for nearly every sampling locality, confirming the identities of species present. Samples were collected by various individuals from 1999 to 2007. Although this represents a substantial time period over which allelic frequencies could change, for the geographic scale at which the following analyses will be conducted we do not expect local fluctuations to impact greatly our ultimate conclusions.

Most individuals were genotyped at a total of 12 loci: 10 nuclear microsatellites, 631 base pairs (bp) of the mitochondrial gene cytochrome *b* (*Cytb*), and 1,104 bp of primarily noncoding sequence from intron 1 of the nuclear gene acrosin (*Acr*), an acrosomal binding protein. Acrosin is a sperm-binding protein that has been shown to evolve rapidly and contain useful phylogenetic information (Gatesy and Swanson 2007). Animal-use protocols were approved by the University of Idaho Institutional Animal Care and Use Committee (protocol UIACUC-2005-40), and the work was done following guidelines of the American Society of Mammalogists (Gannon et al. 2007).

Extraction, polymerase chain reaction, sequencing, and fragment analysis.—Genomic DNA was extracted from tissue (either heart, lungs, kidney, liver, or ear clips) using one of the following protocols: the Phenol:Chloroform:IAA/Chloroform: IAA (PCI/CI) ''hot'' method (Sambrook and Russell 2001), a modified dodecyl–trimethyl ammonium bromide/cetyltrimethyl ammonium bromide protocol (Gustincich et al. 1991), or QIAGEN DNeasy extractions kits (QIAGEN, Valencia, California). For the broader survey of variation in *T. amoenus* 7 microsatellite loci, corresponding to EuAmMS 26, EuAmMS 35, EuAmMS 37 EuAmMS 86, EuAmMS 108, EuAmMS 114, and EuAmMS 142, from Schulte-Hostedde et al. (2000) were amplified via polymerase chain reaction using dye-labeled primers and reaction conditions described therein. For the analysis of taxa directly relevant to the contact zone, 10 microsatellite loci (further adding EuAmMS 41, EuAmMS 94, and EuAmMS 138) were amplified. Resulting fragments were analyzed on an ABI PRISM 3100 capillary sequencer (Applied Biosystems, Inc., Foster City, California). Individual genotypes were then assessed using GENEMAPPER version 3.7 (Applied Biosystems, Inc.).

The fragment of *Cytb* was amplified using primers *Cytb* A and *Cytb* Vc according to reaction conditions described in Good et al. (2003), and *Acr* was amplified according to Good et al. (2008). The resulting polymerase chain reaction products were purified using QIAquick PCR Purification Kits (QIAGEN) and then sequenced on the ABI PRISM 3100 using BigDye Terminator chemistry (Applied Biosystems, Inc.). Chromatograms were aligned and edited using CodonCode ALIGNER (CodonCode Corp., Dedham, Massachusetts). *Acr* was characterized by very low heterozygosity, so heterozygous sites (as inferred by the presence of double peaks of approximately equal height in chromatograms) were coded as ambiguities and gametic phase was not determined.

Microsatellite analyses.—We evaluated deviation from Hardy–Weinberg equilibrium, calculated pairwise F_{ST} and R_{ST}, estimated actual and expected heterozygosity, and tested for differentiation between both sampling localities and the previously named a priori population groupings using GENEPOP version 3.4 (Raymond and Rousset 1995). F_{ST}, also called the fixation index (Weir and Cockerham 1984), and R_{ST} (Slatkin 1995) are measures of the amount of genetic diversity apportioned among populations. The measures differ in that F_{ST} assumes an infinite alleles mutation model, but R_{ST} assumes a stepwise mutation model. We examined our microsatellite data for the presence of null alleles and other possible sources of genotype error within sampling localities using the program MICRO-CHECKER (Van Oosterhout et al. 2004).

To place the variation within *T. amoenus* in context we combined data from Good et al. (2008) and individuals genotyped for this study into a data set of *T. amoenus* that spans southern British Columbia, western Montana, northern Idaho, and eastern Washington. Only 7 loci were available from Good et al. (2008), so for this analysis we combined only those 7 loci from our data rather than treating the other 3 as missing in data from Good et al. (2008). We then used STRUCTURE (Pritchard et al. 2000) to assign individuals to populations and estimate admixture. STRUCTURE implements a Bayesian clustering algorithm that assigns individuals to 1 of k populations, allowing for admixture, without a priori knowledge of source population. The variable k is user-defined, and we evaluated 5 replicates of each value of k from 1 to 12. We allowed a burn-in of 25,000 generations and sampled from the subsequent 125,000. We evaluated the optimal value of k in 2 ways. First, we followed Evanno et al. (2005) in calculating Δk, which is the 2nd-order rate of change of the probability of the data given k—that is, P(D|k)—with respect to k, divided by the *SD* across replicates. We took the highest value of Δk to indicate the highest level of population structure. This essentially evaluates the point at which the increase in P(D|k) slows the most with respect to increasing k. Second, we used STRUCTURAMA (Huelsenbeck and Andolfatto 2007), which infers population structure under a Dirichlet process model, treating k as a random variable. We ran the Markov chain Monte Carlo for 100,000 generations, sampling every 25 generations and discarding the first 100 samples as burn-in. We performed the analysis with the prior expected number of populations set to both 2 and 4.

Having examined higher-level population structure in *T. amoenus*, we repeated this process of using STRUCTURE and STRUCTURAMA to identify populations that are relevant to the analysis of the contact zone. We examined coancestry coefficients produced by STRUCTURE to determine whether admixed individuals are present at the contact zone between TRR and *T. amoenus luteiventris (TAL)*. We included individuals of TRR, TRS, and individuals from TAL from both the Rocky (TALR) and Columbia (TALC) mountains. We treated individuals with >10% coancestry between *T. ruficaudus* and *T. amoenus* as being potentially admixed, a standard applied by previous hybrid zone studies using STRUCTURE (Gay et al. 2007). However, because STRUCTURE is not designed specifically to detect recent hybridization, we used the program NEWHYBRIDS (Anderson and Thompson

2002), which employs a Bayesian algorithm that assigns individuals to 1 of 6 hybrid classes without a priori knowledge of population structure: parental 1, parental 2, F1, F2, backcross 1, and backcross 2.

Phylogenetic analyses.—Gene trees for both *Acr* and *Cytb* were estimated in a similar fashion. To expedite analyses we removed redundant haplotypes using MacClade version 4.06 (Maddison and Maddison 2003). For each locus we selected models of sequence evolution using DT-ModSel (Minin et al. 2003) and performed iterative maximum-likelihood searches (Sullivan et al. 2005) using PAUP* 4.0b (Swofford 2000). Searches consisted of 1,000 random addition sequence replicates followed by tree-bisection-reconnection branch swapping. We estimated nodal support in 2 ways. Using PAUP* we conducted 500 bootstrap replicates (Felsenstein 1985) with 1 tree held at a time (MAXTREES = 1). We also used MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) to estimate the posterior probability of each node. We used 2 independent runs of 4 Metropolis-coupled Markov chains each to generate a sample from the posterior distribution of trees. We ran the chains until the standard deviation of split frequencies was 0.01, discarding the first 25% of the sample as burn-in. We then combined the sample of trees from each run and constructed a majority rule tree to obtain partition frequencies.

Results

Microsatellite diversity.—Basic diversity statistics reported were calculated within aggregate groups delimited by subspecies or bacular morphology or both (Table 1), although they also were calculated per sampling locality when sample size permitted. All loci were polymorphic within groups, with an average of 14.8 alleles per locus, ranging from 11 to 25. Observed (H_O) and expected (H_E) heterozygosities ranged from 0.44 to 0.69 and 0.43 to 0.74, respectively. Tests for deviation from Hardy–Weinberg equilibrium within sampling localities and by locus yielded 9 instances of significant deviation ($P < 0.05$) in 204 tests. Tests for linkage disequilibrium between pairs of loci across all populations yielded no significant instances of linkage disequilibrium. Deviations from Hardy–Weinberg equilibrium calculated by sampling locality were not clustered by locus or population. For loci where deviations between H_E and H_O were observed in multiple aggregate populations (such as EuAmMS142 within populations of *T. amoenus*), those deviations generally were insignificant when calculated by sampling locality. Given that our aggregate populations span large geographic distances, observed deviations are more consistent with Wahlund effects derived from population structure that is unaccounted for. The tests of data quality using MICRO-CHECKER indicated no evidence of genotyping error due to stutter or allelic dropout. Three instances of possible null alleles were found, but these were not clustered by taxon, sampling locality, or locus. Therefore, we do not regard these data as strongly violating the major assumptions of the methods we

Table 1.—Microsatellite diversity by subspecies or bacular morphology, or both, of *Tamias ruficaudus* and *T. amoenus*, including number of alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and *P*-value (only values < 0.05 shown) for Hardy–Weinberg exact tests.

Locus	*T. r. simulans*				*T. r. ruficaudus*				*T. a. luteiventris* (Rocky Mountains)				*T. a. luteiventris* (Columbia Mountains)				Total across populations[a]				*T. a. canicaudus*[b]			
	A	H_O	H_E	*P*	A	H_O	H_E	*P*	A	H_O	H_E	*P*	A	H_O	H_E	*P*	A	H_O	H_E	*P*	A	H_O	H_E	*P*
EuAmMS26	4	0.29	0.31		8	0.76	0.76		10	0.65	0.65		7	0.49	0.49		13	0.55	0.55		9	0.54	0.70	<0.01
EuAmMS35	5	0.78	0.72		6	0.58	0.66		10	0.73	0.69		6	0.67	0.69		12	0.69	0.69		10	0.77	0.84	
EuAmMS37	9	0.66	0.62		8	0.68	0.71		7	0.35	0.31		2	0.07	0.07		14	0.44	0.43		10	0.64	0.77	<0.01
EuAmMS41	4	0.53	0.55		8	0.60	0.56		6	0.69	0.72		6	0.51	0.61		11	0.58	0.61					
EuAmMS86	5	0.56	0.52		8	0.73	0.79		9	0.45	0.54		8	0.75	0.76		16	0.62	0.65		7	0.71	0.78	
EuAmMS94	7	0.75	0.75		4	0.44	0.45		7	0.25	0.35	<0.01	10	0.71	0.78		14	0.54	0.58	0.03				
EuAmMS108	7	0.71	0.65		7	0.50	0.60		9	0.57	0.69	<0.01	4	0.24	0.27		15	0.51	0.55	0.01	7	0.67	0.74	
EuAmMS114	8	0.71	0.78		9	0.74	0.75		8	0.66	0.69		9	0.62	0.71		14	0.68	0.73	0.03	4	0.29	0.57	<0.01
EuAmMS138	7	0.78	0.73		4	0.42	0.41		8	0.79	0.78		10	0.64	0.69		14	0.66	0.65					
EuAmMS142	11	0.74	0.68		10	0.68	0.75		13	0.70	0.83	<0.01	9	0.49	0.68	<0.01	25	0.65	0.74	<0.01	10	0.79	0.84	<0.01
$\bar{X}$ across loci	6.7	0.65	0.63		7.2	0.61	0.64		8.7	0.58	0.63	<0.01	7.1	0.52	0.57	0.03	14.80	0.59	0.62	<0.01	8.1	0.63	0.75	
SE	0.72	0.05	0.04		0.63	0.04	0.04		0.63	0.06	0.06		0.84	0.07	0.07		1.22	0.03	0.03		0.85	0.07	0.04	

[a] Includes data from *T. ruficaudus* and *T. a. luteiventris* only.
[b] Data from Good et al. (2008).

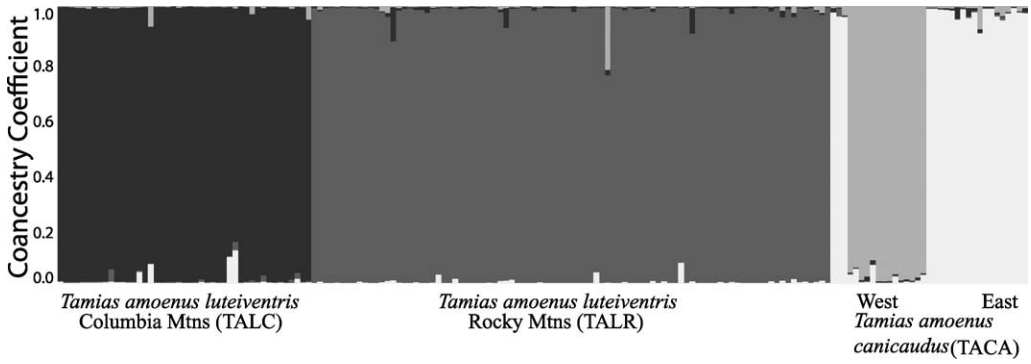

Fig. 2.—Plot of population assignments and coancestry coefficients ($k = 4$) generated by Structure (Pritchard et al. 2000) for *Tamias amoenus*. Columns represent individuals in the analysis. Shading represents assignment by Structure to a population. The size of the shaded portion of each column represents the posterior mean estimate of the proportion of that individual's microsatellite genotype derived from that population. Individuals are sorted 1st by a priori population designation (according to bacular morphology or traditional subspecies) and then by sampling locality.

use to infer population structure and admixture. Tests of both genic and genotypic differentiation strongly rejected ($P < 0.01$) the null hypothesis of no differentiation between populations at all loci, indicating that substantial genetic structure exists in these data.

Population structure and hybridization.—Analysis of microsatellite population structure in *T. amoenus* using Structure inferred geographically coherent clusters for $k = 2$ to $k = 4$; for none of these was there a high frequency of admixed individuals (>10% coancestry with another population). At $k = 2$ individuals clustered into groups consistent with species boundaries as defined by bacular morphology (i.e., TACA + TALC and TALR). At $k = 3$ each of the named taxa formed clusters (i.e., TACA, TALC, and TALR each formed a cluster), and at $k = 4$ TACA split into an eastern and a western cluster. A plot of individual assignments and coancestry for $k = 4$ are presented in Fig. 2. The results of the test of Evanno et al. (2005) identified $k = 2$ as the highest level of structure, whereas $k = 4$ had the highest posterior probability (estimated with Structurama), with $k = 5$ receiving some probability even though Structure seemingly could not parse individuals into that many populations in a geographically sensible manner. Pairwise F_{ST} and R_{ST} for populations defined at $k = 4$ (Table 2) ranged from 0.241 to 0.369 and 0.643 to 0.855, respectively.

The analysis of population structure at the contact zone (including 10 loci for TRS and TRR and excluding TACA) yielded geographically cohesive clusters from $k = 2$ to $k = 4$. At $k = 2$ clusters are either TRS + TRR and TALR + TALC (2 runs) or TALC + TRS + TRR and TALR (3 runs). At $k = 3$ clusters are TALR, TALC, and TRR + TRS (4 runs), or TALR, TRR, and TRS + TALC are combined (1 run). At $k = 4$, TALR, TALC, TRR, and TRS each form their own cluster (Fig. 3A). The test of Evanno et al. (2005) indicated $k = 4$ as the highest level of population structure, and Structurama also placed >98% posterior probability on $k = 4$. Pairwise F_{ST} and R_{ST} for $k = 4$ (Table 3) ranged from 0.251 to 0.343 and 0.267 to 0.686, respectively. No substantial admixture was inferred by Structure at any value of k with the exception of 1 TALR individual from site 19, which it consistently assigned ~10% coancestry with *T. ruficaudus* (either TRR or TRS).

We used NewHybrids to evaluate the possibility of hybridization at the contact zone. Because NewHybrids considers hybridization between 2 populations, we restricted our analysis to the 2 populations identified by Structure that are relevant to the mtDNA introgression, TALR, and TRR. NewHybrids corroborated the assignments made by Structure and correctly associated members of each population. Most individuals received >99% posterior probability as belonging to 1 of the 2 parental classes, and the single potentially admixed TALR individual from site 19 was assigned to the parental class of that population with 100% posterior probability, suggesting that it is not a hybrid (Fig. 3B).

Given the consistent indication in Structure of admixture of a single individual, we examined its microsatellite genotype. Two alleles at 2 different loci (EuAmMS108 and EuAmMS142) in its genotype are at extremely low frequency in TALR (0.3% and 0.2%) but at very high frequency in TRR (21.5% and 42.6%). Given that this individual was sampled at site 19, potentially in sympatry with undetected TRR, it could represent a backcross hybrid (according to description of Nagorsen et al. [2002] of TRR habitat in British Columbia).

Phylogenetic analyses.—For *Cytb* we recovered 32 haplotypes with 78 parsimony-informative sites, and for *Acr* we recovered 11 haplotypes with 15 parsimony-informative sites (Table 4). DT-ModSel selected the models HKY and GTR + I for *Acr* and *Cytb*, respectively. Both searches yielded single

Table 2.—Pairwise estimates of F_{ST} (below diagonal) and R_{ST} (above diagonal) for comparisons between populations of *Tamias amoenus* defined by Structure (Pritchard et al. 2000) for $k = 4$. See text for definitions of population abbreviations.

	TALC	TALR	TACA east	TACA west
TALC	—	0.629	0.772	0.704
TALR	0.367	—	0.855	0.785
TACA east	0.292	0.320	—	0.643
TACA west	0.369	0.338	0.241	—

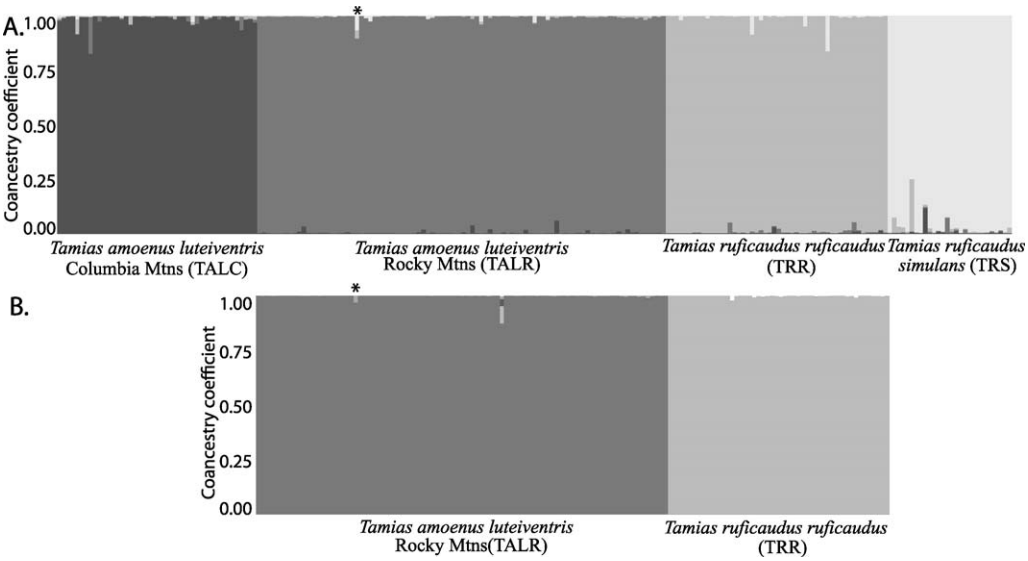

Fig. 3.—A) Plot of population assignment and coancestry coefficients generated by Structure (Pritchard et al. 2000) for populations relevant to the northern contact zone. Columns represent individuals in the analysis. Shading represents assignment by Structure to a population. The size of the shaded portion of each column represents the posterior mean estimate of the proportion of that individual's microsatellite genotype derived from that population. Individuals are sorted 1st by a priori population designation (according to bacular morphology or traditional subspecies) and then by sampling locality. B) Plot of posterior probability of assignment to 1 of 6 hybrid classes by NewHybrids (Anderson and Thompson 2002). The asterisks on the plots represent a single individual of possible hybrid origin.

maximum-likelihood trees (Fig. 4). The nuclear locus *Acr* yielded a well-supported genealogy (Fig. 4A) consistent with microsatellite species-level assignments and lacking evidence of introgression as previously observed in the mtDNA. Additionally, *Acr* appeared to reflect the population assignments within *T. amoenus* by Structure, because all of the TALR individuals grouped together with 99% posterior probability and the TALC individuals all had an identical, unique haplotype. Support for this observation is weak, however, because we sequenced only a few TALC individuals at *Acr*, and intraspecific variation in the gene fragment is low. The mtDNA tree indicated the previously identified pattern of unidirectional introgression from TRR into TALR (Fig. 4B). In contrast to *Acr* and the microsatellites, however, *Cytb* shows little divergence across the TALR–TALC boundary. The most common haplotype is shared in both populations. Sequences were deposited in GenBank under accession numbers FJ804225–FJ804409.

Discussion

In this study we examined variation in a suite of nuclear markers in the context of a previously identified zone of mtDNA introgression between *T. amoenus* and *T. ruficaudus*. In the area around the contact zone nuclear genetic variation is strongly partitioned, with high pairwise F_{ST} values between groups defined by bacular morphology, and that variation in mtDNA is incongruent. In addition to the TRR haplotypes shared across the species boundary, the most common *T. a. luteiventris* haplotype is shared across the TALR–TALC boundary, masking substantial divergence at microsatellite markers (F_{ST} = 0.342). With the exception of 1 potential hybrid individual, the results suggest little ongoing hybridization. Examination of the data also suggests that the extent of mtDNA introgression in this area is correlated with the underlying nuclear genetic structure. Introgressed haplotypes are fixed within most areas of sympatry for TALR, and for a substantial distance away from the contact zone, but they are not found in TALC. The exception, however, is that not all

Table 3.—Pairwise estimates of F_{ST} (below diagonal) and R_{ST} (above diagonal) for comparisons between contact-zone populations of *Tamias amoenus* and *T. ruficaudus* defined by Structure (Pritchard et al. 2000) for k = 4. See text for definitions of population abbreviations.

	TALC	TALR	TRR	TRS
TALC	—	0.652	0.666	0.668
TALR	0.342	—	0.686	0.666
TRR	0.340	0.300	—	0.267
TRS	0.343	0.321	0.251	—

Table 4.—Nucleotide variation by locus for contact-zone populations of *Tamias amoenus* and *Tamias ruficaudus*, including sample size (n), number of unique haplotypes (nHaps), number of parsimony-informative sites (#PI), and number of variable sites (#var).

Locus	Length (bp)	Gene region[a]	n	nHaps	#PI	#var
Cytb	631	5′ region	167	32	78	143
Acr	1,104	ex1–ex2	121	11	15	77

[a] All flanking exon sequences are partial.

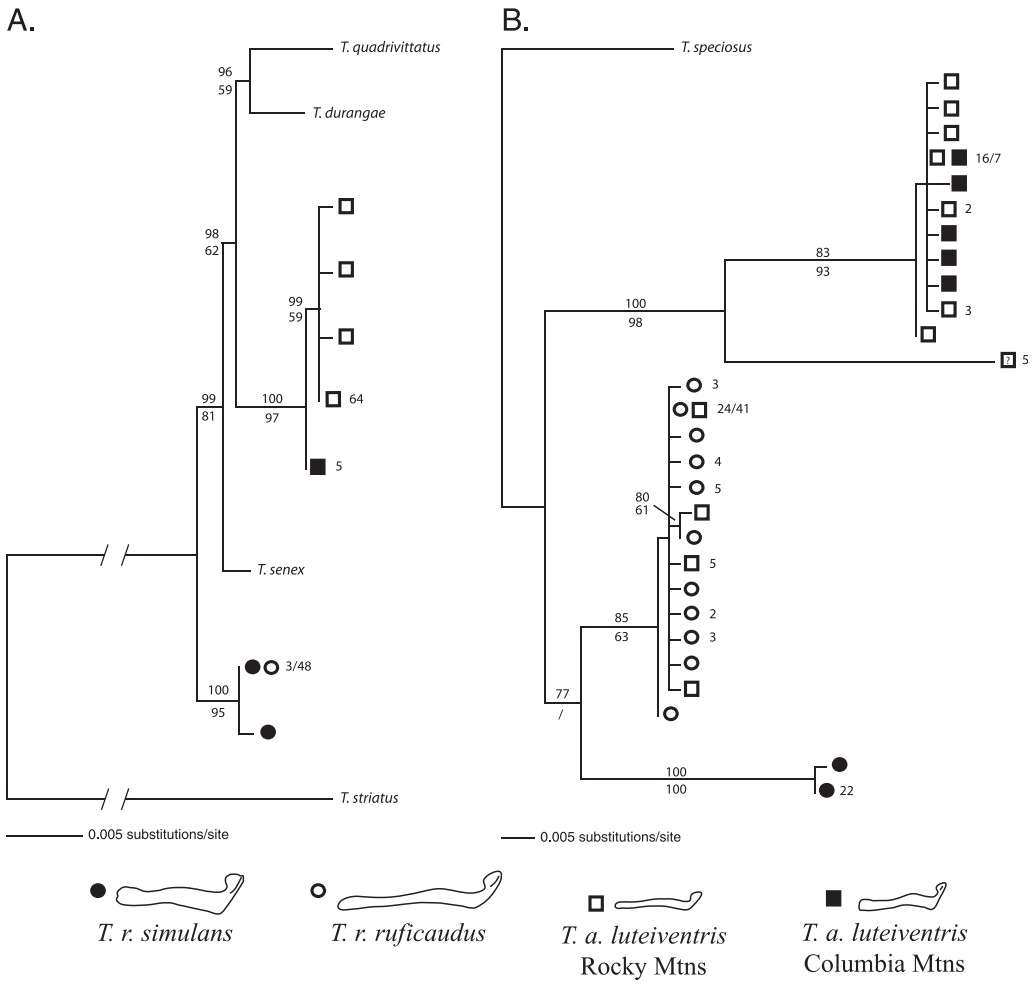

Fig. 4.—Maximum-likelihood topologies for acrosin (*Acr*) and cytochrome *b* (*Cytb*) found using PAUP* (Swofford 2000). Support values are Bayesian posterior probabilities (as percentage, above branch) and maximum-likelihood bootstrap percentages (below branch). Loci are A) *Acr* and B) *Cytb*. Symbols and numbers to the right of branches represent bacular morphology and sample size for given haplotypes.

sampling sites with individuals that could be considered sympatric with TRR contain introgressed alleles. Specifically, at site 72, outside of Missoula, Montana, 5 *T. amoenus* and 4 *T. ruficaudus* were sampled. These individuals of *T. amoenus* grouped with TALR in all analyses. These individuals also are part of the traditional subspecies *T. a. luteiventris*, yet have a distinctly divergent *T. amoenus* mtDNA type (Demboski and Sullivan 2003). Unfortunately, bacular morphology for individuals at this site is unknown. Introgressed TRR haplotypes also have been observed in TRS in northwestern Montana (Good et al. 2003; Fig. 1). This has created an unusual circumstance in which TRR haplotypes have introgressed in parallel through 2 distinct populations, but more extensively in the heterospecific one than in the conspecific. At site 69 TRS individuals carrying their native mtDNA type are in contact with TALR individuals carrying the TRR mtDNA type.

This work raises some questions about the relative roles of selection, drift, mate choice, reproductive isolation, and extrinsic barriers to gene flow in governing the extent, location, and timing of introgressive hybridization. The broad geographic extent of alleles in TALR could have 2 potential explanations. The 1st is that TRR mtDNA poses some selective advantage, and thus it currently is sweeping to fixation. The 2nd is that during a population bottleneck it drifted to high frequency and became widespread following reexpansion. These explanations may be difficult to distinguish. Although evidence is mounting that nonneutral mtDNA evolution may be common (Bazin et al. 2006), it is also likely that the populations under consideration have experienced drastic fluctuations in size. Most of the zone of mtDNA introgression was glaciated during the last glacial maximum (Delcourt and Delcourt 1993; Mack et al. 1978). Limits to the spatial distribution of introgressed mtDNA also could have multiple explanations. It seems possible, given the strong divergence in bacular morphology and microsatellite variation between TALR and TALC, that these populations represent partially reproductively isolated entities. Under a selective advantage hypothesis, the absence of introgressed haplotypes in TALC might be a result of a barrier to gene flow that prevents the introduction of beneficial alleles. Under neutrality, both TAL populations could have experienced postglacial

expansion, resulting in a narrow contact zone that has not undergone substantial admixture.

By contrast, at the southernmost scope of our sampling, no strong microsatellite or bacular differentiation is seen in TRR (Good et al. 2003), or microsatellite differentiation in TALR (bacular morphology is unknown), yet introgression is not observed despite opportunities for contact. This suggests 2 possibilities. The 1st is that if TRR mtDNA has a selective benefit, it is environmentally dependent, leading to heterogeneity of introgression. The 2nd is that hybridization itself is environmentally mediated. Chan and Levin (2005) proposed a model of cytoplasmic DNA introgression that might describe this situation accurately. Their analysis suggested that mtDNA introgression from a rare species to a more common relative can occur easily if in the absence of conspecific males, females of the rare species become more likely to accept heterospecific mates. TRR reaches its northernmost range limit on the British Columbia–Alberta border and for much of its range in Canada inhabits only a small elevational range (1,900–2,000 m), whereas in Idaho and Montana *T. ruficaudus* is known from elevations as low as 560 m (Nagorsen et al. 2002). Given the reduced quantity of apparently suitable habitat, the possibility that TRR in Canada also exists at reduced densities, especially compared to the abundant TALR, seems very plausible. Additionally, *Tamias* tends to breed with multiple mates, and multiple paternity is high (Schulte-Hostedde et al. 2003). Taken in combination, this is highly suggestive of a mechanism that could cause spatial heterogeneity in introgression between these taxa.

Although our understanding of the causes and consequences of gene flow between *T. amoenus* and *T. ruficaudus* is incomplete, the spatial structuring of introgressed haplotypes and the large areas of contact between the species suggests a complex interplay of ecological and genetic factors. A better understanding of these processes will require more-intensive sampling at population boundaries, a mitochondrial genomic approach capable of addressing hypotheses about the role of natural selection, and a distributional modeling approach.

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APPENDIX I

Collection localities (Loc), sample sizes (*n*) and types of data collected: microsatellite (μ), mitochondrial DNA (mtDNA), acrosin (*Acr*), and bacular morphology (bac). For bac, y = yes, n = no. ''Per bac. morph.'' indicates the total number of individuals per bacular morphology.

			Data type				Locality information		
Loc[a]	Taxon[b]	*n*	μ	mtDNA	*Acr*	bac	Latitude	Longitude	Description
7	TALR	1	1	1	1	n	50.333	−114.617	Alberta, Canada, Kananaskis Country, 2 km S Baril Creek on Hwy 940
8	TALR	1	1	1	—	n	50.217	−114.583	Alberta, Canada, Kananaskis Country, Wilkinson Creek
9	TALR	2	2	2	2	y	49.782	−114.682	British Columbia, Canada, 5 km W Racehorse Pass
10	TALR	2	2	2	—	n	49.582	−114.642	British Columbia, Canada Andy Good Creek, below Mt. Ptolemy
11	TALC	1	1	1	—	y	50.762	−116.239	British Columbia, Canada, Brisco, km 19–20 Red Rock Rd.
13	TALC	1	1	1	—	y	49.167	−116.65	British Columbia, Canada, Creston Valley, West side, Topaz Creek Forestry Rd.
14	TALC	2	2	2	—	y	50.438	−116.37	British Columbia, Canada, Delphine Mine Trail
18	TALR	2	2	2	2	y	49.23	−114.814	British Columbia, Canada, Jcn Lodgepole Rd. and Windfall Mt. Rd.
19	TALR	19	19	19	18	y	49.036	−114.354	British Columbia, Canada, Kishinena Creek, km 99 Kishinena Rd.
20	TALC	1	1	—	—	y	50.719	−116.545	British Columbia, Canada, Lead Queen Mt., east side
21	TALC	9	9	2	—	y	50.467	−116.308	British Columbia, Canada, Paradise Mine Rd.
22	TALR/TRR	20	20	20	15	y	49.256	−114.402	British Columbia, Canada, Rainy Ridge vicinity, Middle Kootenay Pass, Middlepass Creek
23	TALR	11	11	11	9	y	49.104	−114.454	British Columbia, Canada, Sage Creek, 3.5 km from Flathead Rd.
24	TALR	3	3	3	3	y	49.157	−114.267	British Columbia, Canada, Sage Creek, 21 km from Flathead Rd.
25	TRR	1	1	1	1	n	49.018	−114.078	British Columbia, Canada, Source of Akamina Creek, 30 km W Wall Lake
26	TALR	1	1	—	—	y	50.476	−116.307	British Columbia, Canada, Spring Creek Mine Site
27	TALR	8	8	8	8	y	50.102	−114.745	British Columbia, Canada, Todhunter Creek headwaters
28	TALC	11	11	—	2	y	50.403	−116.186	British Columbia, Hopeful Creek vicinity
29	TALC	8	8	—	—	y	50.422	−116.222	British Columbia, Canada, Mount Brewer vicinity
30	TALC	3	3	—	1	n	50.541	−115.993	British Columbia, Canada, Stoddart Creek, north ridge
31	TALC	1	1	—	—	y	50.448	−116.256	British Columbia, Canada, Toby Creek, 1.5 km SW Panorama Ski Area
32	TALC	2	2	2	—	y	49.158	−116.533	British Columbia, Canada, Wynndel, Darcie Shephard Farm
69	TALR/TRS	19	18	19	19	y	48.42	−114.825	Montana, Flathead Co. 25 km W Whitefish, 2 km S Good Creek
70	TRR	10	10	10	10	y	48.393	−113.945	Montana, Flathead Co. 8 km E Hungry Horse, Desert Mt.
71	TRR	14	14	14	13	n	48.28	−113.61	Montana, Flathead Co., near Essex along Highway 2
72	TA/TRR	9	9	9	—	y	46.586	−114.578	Montana, Granite Co., Brewster Creek, Sliderock Mt.
73	TALC/TRS	11	11	11	4	y	48.343	−115.601	Montana, Lincoln Co., 6.7 km S Libby, Flower Creek
87	TRS	15	15	6	2	y	48.85	−117.18	Washington, Pend Oreille Co., Sullivan Creek drainage
91	TRR	7	7	3	3	n	47.404	−113.726	Montana, Missoula Co., Lindbergh Lake
555	TALR	25	25	8	8	n	—	—	Alberta, Canada, Kananaskis Country
per bac. morph.	TALR	93	92	75	64				
	TALC	45	45	14	4				
	TRS	32	32	23	16				
	TRR	50	50	46	37				
	Total	220	219	158	121				

[a] Localities numbered following Good et al. (2003).
[b] TRS = *Tamias ruficaudus simulans*, TRR = *T. r. ruficaudus*, TALC = *Tamia amoenus luteiventris* Columbia Mountains, TALR = *T. a. luteiventris* Rocky Mountains.

Appendix II

List of specimen numbers by locations. Location numbers correspond to those listed in Appendix I (following Good et al. 2003).

7: PMA2
8: PMA1
9: RBCM19903, RBCM19904
10: RBCM19888, RBCM19889
11: RBCM19684
13: R20037
14: RBCM19927, RBCM19928
18: RBCM19905 BCM19921
19: NMR33 NMR34 NMR35 NMR36 NMR37 NMR38 NMR39 RBCM19669 RBCM19670 RBCM19674 RBCM19675 SMH27 SMH28 SMH29 SMH30 SMH31 SMH32 SMH33 SMH34
20: RBCM19783
21: RBCM19922 RBCM19923
22: RBCM19877 RBCM19879 RBCM19881 RBCM19882 RBCM19886 RBCM19910 RBCM19875 RBCM19880 RBCM19884 RBCM19885 RBCM19887 RBCM19906 RBCM19907 RBCM19914 RBCM19915 RBCM19916 RBCM19917 RBCM19918 RBCM19919 RBCM19920
23: NMR30 NMR31 NMR32 RBCM19664 RBCM19665 RBCM19680 SMH22 SMH23 SMH24 SMH25 SMH26
24: RBCM19679 RBCM19681 RBCM19682
25: RBCM19683
26: RBCM19742
27: RBCM19894 RBCM19895 RBCM19896 RBCM19897 RBCM19898 RBCM19899 RBCM19900 RBCM19901
28: RBCM19767 RBCM19771
29: RBCM19752 RBCM19753 RBCM19756 RBCM19757 RBCM19759 RBCM19763 RBCM19764 RBCM19766
30: RBCM19748
31: RBCM19750
32: RBCM19650 RBCM19651
69: JMG107 JMG109 JMG110 JMG111 JMG112 JMG115 NMR50 NMR51 JMG108 JMG113 JMG114 JMG116 JMG117 JMG118 JMG119 NMR52 NMR53 NMR54 SMH38
70: JMG134 JMG135 JMG136 JMG137 JMG138 JMG139 JMG140 JMG141 JMG142 JMG159
71: JRD026 NMR40 NMR41 NMR42 NMR43 NMR44 NMR45 NMR46 NMR47 NMR471 NMR48 NMR49 SMH35 SMH36 SMH37
72: F126103 F126104 F126105 F126106 F126107 F126129 F126130 F126131 F126133
73: JMS198 JMS196 JMS198 JMS219 JMS220 JMS221 JMS199 JMS216 JMS217 JMS218 SMH19 SMH20
87: CMNH81574 JMS205 JMS206 JMS207 JMS208 JMS209
91: NMR55 NMR56 NMR57
555: S1879 S1880 S1881 S1885 S1886 S1887 S1888 S1894