MITOCHONDRIAL DNA SUGGESTS RECENT ORIGINS OF SUBSPECIES OF THE SHARP-SHINNED HAWK AND GREAT BLUE HERON ENDEMIC TO COASTAL BRITISH COLUMBIA AND SOUTHEAST ALASKA

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ABSTRACT: Genetic studies of subspecies endemic to Haida Gwaii (Queen Charlotte Islands) in British Columbia and the Alexander Archipelago of southeast Alaska have frequently found patterns of genetic differentiation corresponding to these phenotypically based taxa. Divergence and speciation are common among island populations of birds, and evidence suggests this region has fostered such divergence during previous glacial maxima. We examined divergence in the mitochondrial gene for NADH dehydrogenase subunit 2 (ND2, a marker used in other studies of regional endemism) in two additional coastal subspecies endemic to this region, of the Sharp-shinned Hawk (*Accipiter striatus perobscurus*) and Great Blue Heron (*Ardea herodias fannini*). In both the hawk and heron genetic diversity in ND2 was remarkably low in contrast to that in mitochondrial genes in other species with regional endemics. In both *Accipiter striatus perobscurus* and *Ardea herodias fannini* we found only the haplotype most common in continental populations. We found low but significant divergence in frequencies of haplotypes of ND2 between *A. s. perobscurus* and continental populations of the Sharp-shinned Hawk and no significant population divergence in the Great Blue Heron. In contrast with other regional endemics that do show signals of having persisted through at least one past Ice Age in an unglaciated refugium, these subspecies may have arisen relatively recently, with their adaptation to the local environment leading to darker coloration paralleling that of the region's older endemics. Alternatively, species-wide selective sweeps of mitochondrial DNA prior to divergence of these taxa may have rendered this genetic marker less useful for tracking divergence arising in a refugium.

The Pleistocene epoch that began about 2.6 million years ago was characterized by dramatic fluctuations in Earth's climate (Berger 1984). Global cooling on a 100,000-year cycle caused a series of glaciations that had a profound effect on the distribution of species (Avise and Walker 1998). Genetic evidence has suggested that isolation during Pleistocene glacial cycles promoted divergence and speciation in habitats fragmented by the advance and retreat of continental ice sheets (Weir and Schluter 2004). Many phylogenetic and population genetic studies have focused on Haida Gwaii (Queen Charlotte Islands), British Columbia, and the surrounding region because of the number of endemic taxa from this region that have been described for many classes of organisms, including birds (Topp and Winker 2008), plants (Ogilvie 1989), insects (Kavanaugh 1989), and mammals (Fleming and Cook 2002). Many of these studies have supported the

Figure 1. Ventral and dorsal views of adult males of the darker *Accipiter striatus perobscurus* (A, UAM 28334) and the paler *A. s. striatus* (B, UAM 34176). For scale, the museum labels are 19 mm wide.

hypothesis of an unglaciated refugium in the Haida Gwaii area for mammals, insects, plants, reptiles, gastropods, fish, and birds (Schafer et al. 2010, table 2; Pruett et al. 2013, table 4; Klicka et al. 2011, Graham and Burg 2012, Lait et al. 2012, Burg et al. 2014, Withrow et al. 2014). Although Haida Gwaii has been a focal area for many of these studies, the evidence for the refugium is regional, as the ranges of some of the taxa centered on Haida Gwaii extend beyond these islands (possibly as a result of post-glacial expansion). It is also not possible at present to determine exactly where the putative refugium was located.

Past shifts in spatial distribution and population size can leave distinct patterns in the genetic makeup of populations and species. Populations that became isolated in ice-free refugia during glacial cycles and remain reproductively segregated from immigrants should be genetically distinct via complete or nearly complete lineage sorting (Nei 1975, Pruett 2013). Rapid range expansion following glacial retreat is expected to reduce genetic diversity as alleles are lost and homogeneity increases (Ibrahim et al. 1996, Hewitt 1996, 2000), as seen in populations that occurred on the northern

Figure 2. Ventral and dorsal views of the darker *Ardea herodias fannini* (A, UAM 7767) and the paler *A. h. herodias* (B, UWBM 30074). For scale, the museum labels are 19 mm wide.

edges of refugia (Hewitt 2001). Higher genetic diversity is expected in those refugial populations that acted as the source of expansion for the founding populations (Hewitt 1996).

For this study, we asked two questions: In the context of other avian species studied in this region, do genetic data reflect observed patterns of phenotypic divergence in two subspecies of birds endemic to the coast of northwestern North America? And do these two populations share a pattern of genetic divergence consistent with the Haida Gwaii region's serving as a refugium during the Pleistocene, as many other regional endemics do? Specifically, we examined the northwestern coastal subspecies of the Sharp-shinned Hawk (*Accipiter striatus perobscurus*) and Great Blue Heron (*Ardea herodias fannini*). Both of these are examples of regional endemic populations that have undergone phenotypic differentiation from the widespread continental populations sufficient to be recognized as subspecies (Snyder 1938, Chapman 1901, Dickerman 2004a, b, c). *Accipiter striatus perobscurus* occurs in the breeding season from southeastern Alaska along the adjacent coast of British Columbia to Vancouver Island and

winters from Haida Gwaii south to Santa Barbara, California (Dickerman 2004c). In comparison to the continental *A. s. velox*, *Accipiter striatus perobscurus* is darker (Figure 1), with shorter wings and tail and longer but thin tarsi (Dickerman 2004c). *Ardea herodis fannini* is a taxon of special concern in Canada (COSEWIC 2008) and a year-round resident in southeastern Alaska and south through Haida Gwaii, with nesting recorded northwest to Prince William Sound (Dickerman 2004b). This is the range as restricted in the most recent critical revision (Dickerman 2004b). It is not the range currently considered by wildlife managers for this subspecies (Environment Canada 2016), but their decision (COSEWIC 2008) was based on AOU (1983) and Payne (1979), neither of which represents the critical revision of the topic that Dickerman (2004b) presented (and the American Ornithologists' Union has not critically evaluated subspecies since 1957). *Ardea herodias fannini* has plumage distinctively darker gray than that of the mainland subspecies (Figure 2), as well as significantly shorter exposed culmen and tarsi (Dickerman 2004a, b). Both *Accipiter striatus perobscurus* and *Ardea herodias fannini* parallel other subspecies of birds endemic to this region of temperate rainforest and cloudy skies in being darker than other populations of their species.

METHODS

To evaluate the genetic distinctiveness of *Accipiter striatus perobscurus* and *Ardea herodias fannini*, we sequenced the mitochondrial gene for NADH dehydrogenase subunit 2 (ND2) and compared the sequences with those of *Accipiter striatus velox* and *Ardea herodias herodias* and *A. h. wardi*, widespread in mainland North America. We chose this marker to enable comparisons among similar studies of other birds endemic to the region (e.g., Pruett et al. 2013, Withrow et al. 2014). ND2 is a mitochondrial marker commonly used in genetic studies of birds and has proven widely informative. Zink et al. (2005) suggested that it is evolving approximately neutrally, neither favored nor disfavored by natural selection, and on the basis of this assumption we can estimate population parameters such as genetic diversity and demographic history with reasonable confidence (Lovette 2004). Although variation in the sequence of mitochondrial genes is not expected to be coupled with genetic differentiation resulting from selection of the external phenotype (e.g., plumage), it can provide a deeper understanding of the evolutionary history of intraspecific variation (e.g., Topp and Winker 2008).

From the University of Alaska Museum (UAM), the University of Washington Burke Museum (UWBM), and the Museum of Southwestern Biology (MSB:Bird:; Appendix), we obtained tissue samples from 25 specimens of *Accipiter striatus*, 14 of *A. s. perobscurus*, and 11 of *A. s. velox*. We obtained samples from 36 specimens of *Ardea herodias*, 22 of *A. h. fannini*, 5 of *A. h. herodias*, and 9 of *A. h. wardi*. We selected specimens' collection localities to maximize geographic coverage of both species' ranges (Figure 3). Following the manufacturer's protocol (Qiagen, Valencia, CA), we extracted DNA from frozen tissues and nine heron eggshells by using a DNeasy Tissue Kit.

By the polymerase chain reaction, we amplified 1024 and 987 base pairs

of ND2 from *Accipiter striatus* and *Ardea herodias,* respectively, using ND2 primers L5215 (Hackett 1996) and H6313 (Johnson and Sorenson 1998). For the reaction, we used 0.8 µL of each primer at 10-mM concentration, 0.5 µL of a 10 mM solution of deoxynucleotide triphosphate (dNTP), 0.13 µL of Taq DNA polymerase, 1.6 µL of 25 mM $MgCl_2$, 5 µL of 5X Taq Buffer (Promega, Madison, WI), 14.5 µL water, and 2 µL of extracted DNA template for a total reaction volume of 25 µL. The thermal regime started with 2 min at 94° C, followed by 39 cycles of 94° C for 30 seconds, 52° C for 1 min, 72° C for 2 min, and a final elongation step at 72° C for 5 min. The cleanup and sequencing were done at the High-Throughput Genomics Unit (University of Washington, Seattle), by means of an ExoSAP cleaning process and cycle sequencing with BigDye chemistry on an ABI 3730XL high-throughput capillary sequencer (Applied Biosystems, Foster City, CA). Cycle-sequencing amplifications were done with the primers for sequencing. Sequences were aligned and edited with Sequencher version 4.7 (Gene Codes, Ann Arbor, MI).

To generate median-joining networks illustrating the frequencies of ND2 haplotypes from each species we used Network version 4.6.1.3 (Bandelt et al. 1999). To test the neutrality of mutations in the gene and change in population size we calculated Fu and Li's D^* and F^* statistics (Fu and Li 1993), Tajima's D (Tajima 1989), and R_2 values (Ramos-Onsins and Rozas 2002) with DnaSP version 5 (Librado and Rozas 2009). Following post-glacial expansion, the sizes of many populations in this region may be expected to have increased. To determine whether the increases in population sizes we estimated were significant, we ran simulations of coalescence with 50,000 replicates and calculated 95% confidence intervals on the basis of a model of population size being constant (Librado and Rozas 2009). We ran each simulation three times for confirmation. We used Arlequin version 3.5.1.2 (Excoffier et al. 1992) to calculate pairwise F_{ST} values for ND2 sequences of the two pairs of subspecies with 10,100 permutations and to determine whether these estimates differed from zero.

RESULTS

Accipiter striatus

We obtained 1024 base pairs of ND2 data from the 25 specimens of *Accipiter striatus* sampled at eight locations ranging from the interior of Alaska to New York (Figure 3). We found variation in four of these 1024 sites, representing five unique haplotypes (Figure 4). These haplotypes differed by one or two base pairs, and all specimens of *A. s. perobscurus* sampled shared the most common haplotype found in *A. s. velox* (Figure 3). Two haplotypes represent transitions from adenine to guanine in the third codon position, and two represent transitions from adenine to guanine in the second codon position.

We estimated the degree of population expansion and genetic structure for all samples pooled (i.e., at the species level). Past population expansion was indicated by strongly significant values for R_2 ($P < 0.0001$; Table 1). Fu and Li's F^* and D^* differed significantly from zero ($P < 0.001$). Tajima's

Figure 3. Locations of samples used in this study. Numbers specify number of individuals sampled within each circle. Patterns represent different subspecies.

D was negative but was not significant ($P = 0.54$; Table 1). Despite low genetic diversity, we found a low but significantly different level of population structure between the two subspecies (average pairwise difference; $P = 0.024$; Table 1).

Figure 3 (*continued*)

Ardea herodias

We obtained 987 base pairs of ND2 data from the 36 specimens of *Ardea herodias* sampled from nine locations ranging from Kodiak Island to the Texas coast (Figure 3). We found variation at two of the 987 sites, corresponding to three unique haplotypes differing by one to two base pairs. The haplotype network illustrates low divergence among haplotypes and

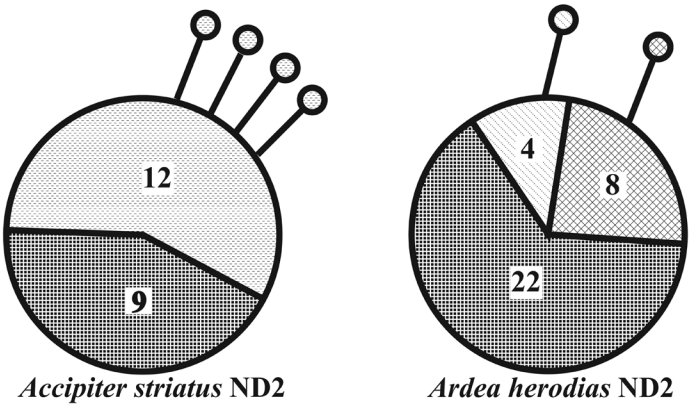

Figure 4. Networks representing the haplotypes of ND2 in 25 specimens of *Accipiter striatus* and 36 of *Ardea herodias*. Black, subspecies *Accipiter s. perobscurus* and *Ardea h. fannini*, subspecies endemic to coastal British Columbia and southeast Alaska; shading, other populations, *Accipiter s. velox* (dashed lines), *Ardea h. herodias* (diagonal shading), and *A. h. wardi* (cross hatching). Numbers correspond to number of individuals of each haplotype; each small circle represents one individual.

no difference in structure between *A. h. fannini* and *A. h. herodias* or *A. h. wardi* (Figure 4). The two alternative continental haplotypes represent transitions between cytosine and thymine in the third codon position.

Again, we estimated the degree of population expansion and genetic structure of all samples pooled (i.e., at the species level). Past population expansion was indicated by strongly significant values for R_2 ($P < 0.0001$; Table 1). Fu and Li's F^* and D^* differed significantly from zero (F^*, $P = 0.005$; D^*, $P = 0.003$). Tajima's D was positive but not significant ($P = 0.83$; Table 1). Samples of *A. h. fannini* did not differ significantly from samples of other subspecies of the Great Blue Heron, as indicated by a low and not significant value of F_{ST} ($P = 0.071$; Table 1). As before, to obtain the best comparisons

Table 1 Statistics Summarizing Patterns of Variation in the Mitochondrial Gene ND2 in *Accipiter striatus* and *Ardea herodias*

Statistic[a]	*Accipiter striatus*	*Ardea herodias*
n	25	36
Nucleotide diversity (π)	0.0003	0.0001
Haplotype diversity (H)	0.300	0.110
Tajima's D (D_T)	–0.0189	0.0001
Fu and Li's F^*	–0.0024***	–0.0007**
Fu and Li's D^*	–0.0157***	–0.0015**
R_2 (Ramos-Onsins and Rozas 2002)	0.1622****	0.1480****
F_{ST}[b]	0.0228*	0.0031

[a]Levels of significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

[b]Average pairwise divergence between subspecies endemic to coastal northwestern North America and populations elsewhere across the North American continent.

possible among lineages, given heterogeneity in sampling, we compared the regional endemic to all continental populations combined. Exploring further within *Ardea herodias* also showed no significant differences in values of F_{ST} between any pair of subspecies in our samples.

DISCUSSION

Genetic Diversity

Our results revealed remarkably low genetic diversity in both *Accipiter striatus* and *Ardea herodias*, in contrast to other birds with regional endemic subspecies (Table 2). Furthermore, no individual of either of our focal regional endemic subspecies had a unique haplotype. This result is consistent with neither of these subspecies' having persisted in a refugium in the Haida Gwaii region during the last glacial maximum, but rather having colonized post-glacially (Hewitt 1996, Pruett et al. 2013).Similarly, Pruett et al. (2013) observed similar patterns of low genetic diversity and a lack of unique haplotypes in some other birds of Haida Gwaii such as the Red-breasted Sapsucker (*Sphyrapicus ruber*), and Swainson's Thrush (*Catharus ustulatus*); in each case, they attributed the low diversity to post-glacial colonization approximately 13,000–19,000 years before present. In contrast, Withrow and Winker (2014) found that the island subspecies of the Northern Saw-whet Owl (*Aegolius acadicus brooksi*) diverged significantly from the subspecies of the mainland (*A. a. acadicus*) in both the nuclear and mitochondrial genomes (amplified fragment length polymorphisms and ND2). In a similar study analyzing the mitochondrial gene for cytochrome *b* in the Hairy Woodpecker (*Picoides villosus*), Steller's Jay (*Cyanocitta stelleri*), and Pine Grosbeak (*Pinicola enucleator*), Topp and Winker (2008) also found distinct differentiation in the genes they tested between Haida Gwaii populations and those of the mainland. These contrasting results reflect how species' different life-history strategies and histories of colonization may provide conflicting evidence for an unglaciated refugium on Haida Gwaii.

In both species we studied we found genetic diversity to be greater in continental populations, which is consistent with expectations of greater genetic diversity in larger populations (Hartl and Clark 1989). Pearlstine (2004) found low genetic diversity in *Accipiter striatus* across North America in the mitochondrial genes ND2 and COI. A comparable study of mitochondrial genetic diversity of *Ardea herodias* has yet to be undertaken.

Population Expansion

Fu and Li's $F^*$and D^* differed significantly from zero in both *Accipiter striatus* and *Ardea herodias*, suggesting either a departure from selective neutrality or population expansion. This pattern is highlighted by strongly significant values of R_2, suggesting a population expansion in both species. Tajima's D, which is less sensitive to population expansion than Fu and Li's $F^*$and D^* (Ramos-Onsins and Rozas 2002), was not significant in either species, but negative for the Sharp-shinned Hawk and positive for the Great Blue Heron. This suggests a stronger signal for expansion in the hawk, an expansion possibly more recent than that of the heron. These results are

Table 2 Diversity of Haplotypes in Mitochondrial DNA in Birds with Subspecies Endemic to Haida Gwaii or the Perhumid Rainforest Zone of North America

		Haida Gwaii/Perhumid Zone		Mainland					
Species	Gene	Number of haplotypes	Number of haplotypes/ number of samples	Number of haplotypes	Number of haplotypes/ number of samples	π^a	θ^b	H^c	Reference
Northern Saw-whet Owl	ND2 cyt b	1[d]	0.10	2	0.10	0.00055	1.943	0.499	Topp and Winker 2008
Northern Saw-whet Owl	cyt b	3[d]	0.13	6	0.30	0.00043	4.886	0.333	Withrow et al. 2014
Hairy Woodpecker	cyt b	3[d]	1.00	9	0.60	0.00492	10.389	0.922	Topp and Winker 2008
Hairy Woodpecker	cyt b	3[d]	0.75	13	0.59	0.00508	6.111	0.916	Pruett et al. 2013
Swainson's Thrush	cyt b	4	0.40	20	0.74	0.00941	17.514	0.878	Pruett et al. 2013
Sooty Grouse	cyt b	2	0.22	4	0.25	0.00023	2.880	0.230	Pruett et al. 2013
Steller's Jay	cyt b	2[d]	0.18	15	0.93	0.00241	18.296	0.869	Topp and Winker 2008
Steller's Jay		2[d]	0.18	21	0.72	0.00278	19.512	0.926	Pruett et al. 2013
Pine Grosbeak	cyt b	7[d]	0.50	15	0.93	0.00697	12.519	0.917	Topp and Winker 2008
Great Blue Heron	ND2	1	0.04	3	0.21	0.0001	1.944	0.110	This study
Sharp-shinned Hawk	ND2	1	0.11	5	0.31	0.0003	3.840	0.300	This study

[a]Nucleotide diversity.
[b]Waterson's theta.
[c]Haplotype diversity.
[d]Signal of a past refugium.

consistent with those of Hull and Girman (2004), who found a signature of rapid expansion in western populations of the Sharp-shinned Hawk in response to the retreat of the ice sheet at the end of the last glacial maximum.

Genetic Divergence

Despite low genetic diversity in the sequence of ND2, *Accipiter striatus perobscurus* did differ significantly from *A. s. velox* in the distribution of haplotypes of this gene. Additional sampling of continental populations of *Ardea herodias*, if it yields more haplotypes, might also reveal significant differentiation between these populations.

It is likely that Haida Gwaii was one of several ice-free areas that persisted along the northwest coast of North America during the last glacial maximum about 26,500 to 19,000 years before present (Hetherington et al. 2003). This type of biogeographic history suggests two possible explanations for our results. One scenario is that *Accipiter striatus perobscurus* and *Ardea herodias fannini* split from continental populations more recently than have other regional endemics, then expanded rapidly. This would account for the lack of divergence of these taxa in ND2 despite their morphological distinctiveness. In this case, their dark coloration arose as adaptation to the humid, cloudy environment, paralleling darker plumage colors found among the older avian regional endemic subspecies, but possibly during colonization after the glaciers last retreated. Another possibility is that the common haplotypes observed are positively selected (or that such selection has occurred elsewhere in the linked mitochondrial genome or on the W chromosome; Smeds et al. 2015), reducing the genetic variation in the two species as the result of a strong selective sweep prior to divergence. In such a case differentiation in the mitochondrial genome would be difficult to identify.

In both the Sharp-shinned Hawk and Great Blue Heron, the subspecies endemic to the northwest coast show an apparent mismatch in divergence between phenotypic variation, governed by nuclear genes, and variation in mitochondrial DNA. Additional sampling and sequence data on both subspecies are warranted for further study of their population structure and diversity.

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APPENDIX

Specimens used in this study, with identifiers (UAM, University of Alaska Museum; UWBM, University of Washington Burke Museum; MSB, Museum of Southwestern Biology, University of New Mexico), voucher numbers, and GenBank accession numbers.

Species	Voucher number	Locality	GenBank accession number
Ardea herodias fannini			
British Columbia			
	UAM22572	Queen Charlotte Island	KX083585
	UAM34646	Queen Charlotte Island	KX083587
	UAM34655	Queen Charlotte Island	KX083599
	UAM34656	Queen Charlotte Island	KX083589
	UAM34659	Queen Charlotte Island	KX083590
	UAM34647	Queen Charlotte Island	KX083591
	UAM34648	Queen Charlotte Island	KX083592
	UAM34649	Queen Charlotte Island	KX083593
	UAM34652	Queen Charlotte Island	KX083594
	UAM34653	Queen Charlotte Island	KX083595
	UAM22573	Moresby Island	KX083600
	UAM22600	Graham Island	KX083598
	UAM24728	Vancouver Island	KX083611
	UAM24726	Vancouver Island	KX083612
	UAM24727	Vancouver Island	KX083613
Alaska			
	UAM7767	Juneau	KX083610
	UAM25904	Juneau	KX083597
	UAM25905	Juneau	KX083596
	UAM13500	Ketchikan	KX083614

(*Continued*)

APPENDIX (*continued*)

	UAM18947	Ketchikan	KX083617
	UAM18137	Ketchikan	KX083599
	UAM20826	Kodiak Island	KX083616
Ardea herodias herodias			
	UAM14169	Minnesota	KX083615
	UWBM66270	Everett, Washington	KX083618
	UWBM74070	Bow, Washington	KX083619
	UWBM77579	Kingston, Washington	KX083620
	UWBM80456	Hoquiam, Washington	KX083586
Ardea herodias wardi			
New Mexico			
	MSB:Bird:20590	Guadalupe County	KX083606
	MSB:Bird:20432	Sandoval County	KX083607
	MSB:Bird:22800	Bernalillo County	KX083601
	MSB:Bird:44245	Bernalillo County	KX083603
	MSB:Bird:23064	San Miguel County	KX083602
	MSB:Bird:22225	Sierra County	KX083608
	MSB:Bird:22224	Sierra County	KX083609
Texas			
	MSB:Bird:18304	Matagorda County	KX083604
	MSB:Bird:18344	Refugio County	KX083605
Accipiter striatus perobscurus			
British Columbia			
	UAM8083	Queen Charlotte Island	KX083635
	UAM27278	Queen Charlotte Island	KX083629
	UAM28334	Queen Charlotte Island	KX083630
	UAM28335	Queen Charlotte Island	KX083621
	UAM28336	Queen Charlotte Island	KX083622
	UAM28337	Queen Charlotte Island	KX083624
	UAM28338	Queen Charlotte Island	KX083625
	UAM28339	Queen Charlotte Island	KX083623
	UAM8998	Graham Island	KX083631
Alaska			
	UAM28340	Juneau	KX083626
	UAM29602	Juneau	KX083639
	UAM25662	Juneau	KX083640
	UAM23815	Juneau	KX083641
	UAM27052	Ketchikan	KX083642
Accipiter striatus velox			
Alaska			
	UAM13481	Ketchikan	KX083633
	UAM26110	White Pass	KX083627
	UAM29603	Dyea	KX083628
	UAM11257	Kodiak	KX083632
	UAM18494	Fairbanks	KX083634
	UAM22264	Fairbanks	KX083636
	UAM22474	Fairbanks	KX083637
	UAM22165	Fairbanks	KX083638
	UAM9372	Fairbanks	KX083643
Other regions			
	UAM29639	New York	KX083645
	UAM15081	Montana	KX083644