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Population genetic structure and its implications for adaptive variation in memory and the hippocampus on a continental scale in food-caching black-capped chickadees

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Abstract

Food-caching birds rely on stored food to survive the winter, and spatial memory has been shown to be critical in successful cache recovery. Both spatial memory and the hippocampus, an area of the brain involved in spatial memory, exhibit significant geographic variation linked to climate-based environmental harshness and the potential reliance on food caches for survival. Such geographic variation has been suggested to have a heritable basis associated with differential selection. Here, we ask whether population genetic differentiation and potential isolation among multiple populations of food-caching black-capped chickadees is associated with differences in memory and hippocampal morphology by exploring population genetic structure within and among groups of populations that are divergent to different degrees in hippocampal morphology. Using mitochondrial DNA and 583 AFLP loci, we found that population divergence in hippocampal morphology is not significantly associated with neutral genetic divergence or geographic distance, but instead is significantly associated with differences in winter climate. These results are consistent with variation in a history of natural selection on memory and hippocampal morphology that creates and maintains differences in these traits regardless of population genetic structure and likely associated gene flow.

Keywords: adaptation, chickadee, food caching, gene flow, hippocampus, memory, population structure

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Introduction

The classic view of natural selection is that, given variation in heritable traits, strong directional selection should produce adaptive changes in these traits (Orr 2005). This appears to be the case in scatter-hoarding species, where directional selection has been suggested to produce specializations in memory and the brain. More specifically, the adaptive specialization hypothesis has been proposed to explain reported differences in the size of the hippocampus, an area of the brain involved in spatial memory, between food-caching and noncaching species (Krebs *et al.* 1989; Sherry *et al.* 1989; Pravosudov & Smulders 2010). Scatter-hoarding species cache large numbers of individual food items scattered over their home ranges and retrieve them during the winter when naturally available food is limited and unpredictable (Pravosudov & Smulders 2010). Individuals of some species, especially corvids and parids, have been reported to cache tens of thousands of food items during a single year (Praavosudov & Smulders 2010; Vander Wall 1990), which suggests a critical role of these caches in individual fitness. Successful retrieval of

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food caches should be especially important for fitness, and therefore, selection is likely to act on the mechanisms used for cache retrieval. Individuals with traits allowing for more successful cache retrieval should have a selective advantage, which should in turn result in the evolution of these traits. It has been shown that food-caching animals rely, at least in part, on spatial memory to recover previously cached food, and thus, better spatial memory should result in more successful cache recovery (Shettleworth 1995; Pravosudov & Smulders 2010). The hippocampus is involved in spatial memory function (Sherry & Vaccarino 1989; Hampton & Shettleworth 1996); hence, selection pressure on spatial memory needed to recover food caches may be expected to result in adaptive changes in hippocampal morphology and size in food-caching animals (Krebs *et al.* 1989; Sherry *et al.* 1989; Pravosudov & Smulders 2010).

We have previously examined how reliance on food caches may affect the association between spatial memory and the hippocampus by using the black-capped chickadee (*Poecile atricapillus*), a food-caching species with an extremely large range over the North American continent encompassing a great variety of environmental conditions (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Chancellor *et al.* 2011; Roth *et al.* 2011, 2012). Our previous studies were based on the hypothesis that winter climate is the main factor affecting selection on spatial memory in these small passerine birds. Colder winter temperatures demand more energy to sustain higher metabolic requirements, while both low temperatures and abundant snow fall and cover likely make naturally available food limited and unpredictable, all of which should increase dependence on cached food for survival (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Roth *et al.* 2011).

Our previous studies compared chickadees from 10 locations spread over a large continental scale (Alaska, Fairbanks (AKF), Alaska, Anchorage (AKA), British Columbia (BC), Washington State (WA), Montana (MO), Minnesota (MN), Colorado (CO), Kansas (KS), Iowa (IA) and Maine (ME), Fig. 1) and reported that chickadees from more harsh environments (AKA, AKF, ME, MN) had relatively larger hippocampi containing more hippocampal neurons compared to birds from more moderate climates (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Roth *et al.* 2011). These data on hippocampal morphology are summarized in Table 1. We also conducted a common garden experiment with birds from the two extreme populations (AKA and KS; Fig. 1) to test whether previously found differences may be due to plasticity associated with environmental influences. Our data strongly suggested the role of inheritance as the birds hand-reared in a controlled laboratory environment exhibited the same differences in spatial memory, total number of hippocampal neurons and intensity of hippocampal neurogenesis as the birds sampled from the wild (Roth *et al.* 2012).

Even though we used only the two most extreme populations for the common garden study, it is plausible to assume that the same mechanism (most likely genetically based) may be involved in producing the reported differences across all of our sampled populations and that this variation may be, at least in part, due to differential selection on spatial memory. Our data have shown that variation in the number of hippocampal neurons in black-capped chickadees has a heritable component, as birds reared and maintained in laboratory conditions and chickadees sampled directly in the wild had statistically indistinguishable numbers of hippocampal neurons despite enormous differences between natural and laboratory environments (Roth *et al.* 2012). These data suggest that a significant component of the variation in the total number of hippocampal neurons observed in 10 different populations likely has a genetic basis. Alternatively, it is also possible that the same selective agents (climate in our case) might have resulted in the evolution of the same phenotype with different underlying mechanisms (e.g. genetically based inheritance vs. experience-dependent plasticity).

In our current study, we ask whether population genetic differentiation and potential isolation among populations is associated with large differences in hippocampal morphology. It has often been suggested that genetic isolation with resulting restriction of gene flow is important for the evolution of adaptive traits (e.g. Puechmaille *et al.* 2011), but it is also important to understand whether strong selection for particular traits may produce adaptive changes in the presence of gene flow in otherwise genetically undifferentiated populations (Lenormand 2002). Therefore, we asked whether chickadee populations with large differences in hippocampal attributes are genetically differentiated at neutral genetic loci and whether hippocampal differences are only observed among populations that are geographically and genetically isolated. If patterns of genetic differentiation at neutral loci are associated with patterns of phenotypic differentiation, this may suggest that either isolation is necessary for adaptive divergence or phenotypic differences might be a product of genetic drift rather than of natural selection. Finding different or contrasting patterns of genetic and phenotypic differentiation, on the other hand, would suggest that the observed phenotypic differences could be a product of natural selection acting in the face of gene flow. These conclusions would be further strengthened if environ-

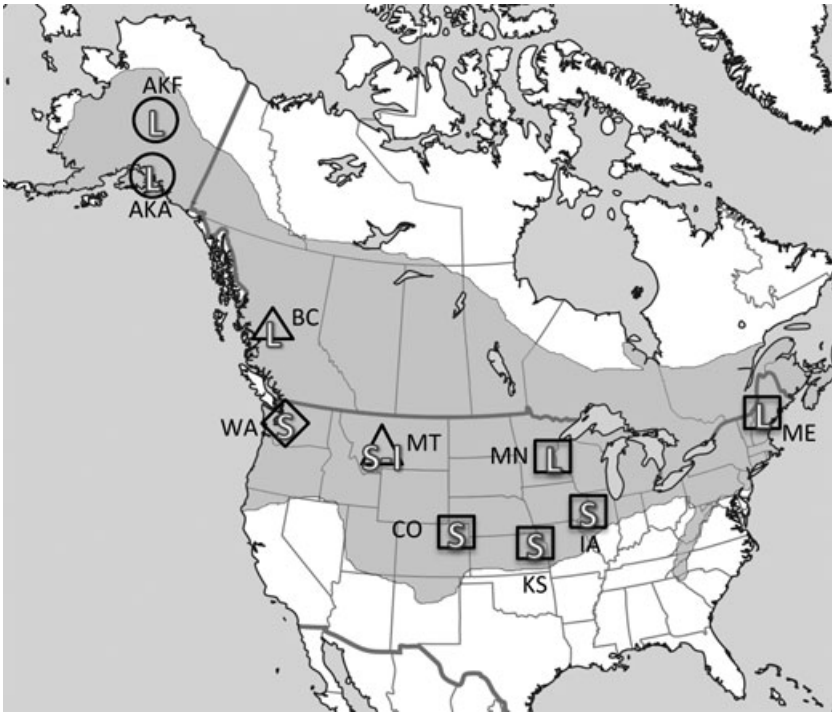

Fig. 1 A map of sampling locations (AKF, Alaska Fairbanks; AKA, Alaska Anchorage; BC, British Columbia, Prince George; MT, Montana, Missoula; WA, Washington, Seattle; CO, Colorado, Fort Collins; Kansas, Manhattan; IA, Iowa, Mt. Vernon; MN, Minnesota, Minneapolis; ME, Maine, Presque Isle). Unique shapes identify genetically distinct populations defined by STRUCTURE analysis. Letters within each shape correspond to the coarse comparative grouping based on hippocampal data: L, generally larger hippocampus with more neurons; S, generally smaller hippocampus with fewer neurons; I, somewhat intermediate values between L and S (based on Roth & Pravosudov 2009; Roth *et al.* 2011).

mental differences (winter climate), rather than genetic divergence at neutral markers, predicted the observed phenotypic differences.

We used mitochondrial DNA sequences (mtDNA; Avise 2000) and amplified fragment length polymorphism (AFLP; Vos *et al.* 1995; Pritchard *et al.* 2000; Falush *et al.* 2007) to test for genetic differentiation among the chickadee groups used in our previous studies of spatial memory and the hippocampus. Mitochondrial data have been widely used in phylogeographic studies and provide a useful comparison as a maternally inherited locus with a smaller effective population size compared to nuclear loci (Avise 2000). AFLPs have been used to estimate population structure because they can assay hundreds of anonymous markers sampled from throughout the genome, the vast majority of which are likely to be neutral (Bensch & Åkesson 2005).

The overall goal of our study was to investigate whether significant population divergence in hippocampal morphology is associated with genetic differentiation at neutral markers or whether divergence in hippocampal morphology is independent of genetic structure or geographic distance, but rather associated with differences in winter climate, which is expected to provide strong selection pressure on memory and the hippocampus.

Materials and methods

Samples

We used the following numbers of birds from the 10 sampling locations used in our previous studies of the hippocampus: Maine—12; Alaska, Fairbanks—34; AKA—34; British Columbia—37; Montana—40; Washington—33; Minnesota—12; Iowa—12; KS—24; and Colorado—19. Samples contained all of the birds that were those used for previous brain analyses including hippocampal volume and the total number of hippocampal neurons (Table 1, Fig. 1; Roth & Pravosudov 2009; Roth *et al.* 2011) plus additional birds in most populations. It is important to note that there were no significant differences between males and females in any of the measured brain parameters (Roth & Pravosudov 2009; Roth *et al.* 2011). DNA was extracted from blood using the mouse tail protocol on an AutoGenprep 965 extraction system (Autogen). DNA concentration and purity were assessed using a NanoDrop ND-1000 spectrophotometer.

Table 1 Morphological and brain measurements from chickadees from all sampled locations (mean and SE, $n = 12$ for each location)

Location	Body Mass (g)	Wing length (mm)	Brain mass (g)	Absolute hippocampal volume (mm^3)	Absolute number of hippocampal neurons ($\times 10^6$)	Telencephalon volume (mm^3)	Relative hippocampal volume (mm^3)*	Relative number of hippocampal neurons ($\times 10^6$)*	Dates collected
Alaska Fairbanks	11.37 (0.17)	65.88 (0.70)	0.77 (0.01)	28.15 (0.87)	1.952 (0.161)	494.26 (15.55)	28.38 (0.85)	2.030 (0.128)	18–20 September 2007
British Columbia	10.87 (0.17)	67.05 (0.64)	0.80 (0.02)	26.60 (0.82)	1.877 (0.126)	502.61 (21.94)	27.04 (0.98)	2.013 (0.148)	29 September –1 October 2007
Montana	11.42 (0.30)	65.83 (0.58)	0.73 (0.02)	25.07 (0.77)	1.735 (0.104)	446.90 (21.90)	25.19 (0.85)	1.745 (0.128)	10 October 2007
Colorado	11.65 (0.19)	67.19 (0.42)	0.76 (0.01)	25.03 (0.87)	1.727 (0.115)	485.82 (11.84)	25.04 (0.79)	1.736 (0.120)	15–17 October 2007
Kansas	12.84 (0.24)	69.15 (0.73)	0.74 (0.02)	23.15 (0.63)	1.578 (0.098)	459.10 (14.91)	22.50 (1.01)	1.393 (0.153)	21–23 October 2007
Alaska Anchorage	11.10 (0.16)	66.04 (0.76)	0.80 (0.02)	27.20 (0.64)	2.582 (0.065)	511.41 (18.36)	27.38 (0.73)	2.603 (0.094)	18–20 September 2008
Washington	11.19 (0.24)	61.69 (0.63)	0.70 (0.01)	22.65 (0.66)	1.719 (0.105)	430.25 (14.03)	22.33 (0.74)	1.680 (0.095)	22 October 2008
Maine	11.60 (0.23)	67.50 (0.48)	0.78 (0.02)	26.08 (0.99)	2.537 (0.106)	497.13 (22.85)	26.17 (0.72)	2.548 (0.092)	28 September 2008
Minnesota	11.64 (0.25)	65.54 (0.61)	0.78 (0.01)	25.91 (0.72)	2.560 (0.092)	497.20 (13.34)	26.00 (0.72)	2.570 (0.092)	9 October 2008
Iowa	12.11 (0.26)	65.08 (0.75)	0.74 (0.02)	24.06 (0.45)	1.609 (0.076)	482.53 (19.88)	24.06 (0.72)	1.609 (0.091)	13–16 October 2008

*Least-square means (based on Roth & Pravosudov 2009; Roth *et al.* 2011).

Mitochondrial DNA

A portion of the mitochondrial control region was amplified by PCR using HCRCBox and LmochCR1 primers (Grava *et al.* 2012). Amplified DNA was sequenced by cycle sequencing using an ABI 3130xl sequencer. Chromatograms were visually inspected using MEGA v. 4.0.2 (Tamura *et al.* 2007). Mitochondrial haplotype diversity was estimated in DNASP v.5 (Librado & Rozas 2009). To investigate the distribution of genetic variation among populations, pairwise Φ_{ST} values were calculated using ARLEQUIN v. 3.5.1.2 (Excoffier & Lischer 2010) and significance was determined with 10 000 permutations. Φ_{ST} values are similar to *F*-statistics, except that the latter account for variation in allele frequencies, while the former encompass variation in frequencies as well as sequence divergence between haplotypes (i.e. all alleles are not equidistant). Insertion/deletions were treated as a fifth state. To correct for multiple tests, a Benjamini–Hochberg correction (Benjamini & Yekutieli 2001) was applied. To visualize the relationships among mitochondrial sequences, a haplotype network was constructed using TCS v.1.21 using the default setting of 95% confidence limits (Clement *et al.* 2000).

AFLP generation and scoring

AFLPs were generated following the protocol of Vos *et al.* (1995) as modified for vertebrates by Kingston & Rosel (2004). Sixteen selective PCR primer pairs were used, consisting of all combinations of two fluorescently labelled EcoR1 + ANN primers and eight Taq1 + ANN primers. Fragment profiles were generated by capillary electrophoresis on an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems). Fragment scoring was made using GENEMAPPER 4.0 software (Applied Biosystems). All samples were scored concurrently and blindly for each selective primer pair. AFLP fragments were grouped in 1-bp bins ranging in size from 90 to 500 bp. The scoring protocol developed by Kingston & Rosel (2004) was used to minimize potential noise associated with underamplification of large fragments or uneven amplification among samples. A threshold of 100 fluorescence units was set as the minimum amplitude for fluorescence peaks to qualify as potential marker loci, while baseline fluorescence was generally below 25 units. The presence of false-negative peaks (<100 fluorescence units) in a bin with scorable peaks from other individuals resulted in the rejection of the marker. For each primer pair, the fragment length of the largest monomorphic marker was taken as the upper size limit for scorable loci to prevent scoring problems resulting from PCR drop off with fragment length.

AFLP data were analysed using the Bayesian population clustering program STRUCTURE (v. 2.3.2) (Pritchard *et al.* 2000; Falush *et al.* 2007). Prior to running STRUCTURE, we visually examined the data for statistical or artefactual outliers using nonmetric multidimensional scaling (NMDS) and detected no problematic individuals. In STRUCTURE, we ran an admixture model with correlated alleles 10 times for each value of k (from 2 to 15) with burn-in of 100 000 steps followed by sampling of 1 000 000 steps. To determine the most informative value of k, we used the *ad hoc* statistic Δk following Evanno *et al.* (2005). In addition, we also compared variance at each k to test whether an increase in variance occurs after the k with the largest Δk. After determining the most effective level of k for our data, we re-ran STRUCTURE using the USEPOPINFO option to incorporate sampling locations as prior information, which has been suggested as a method for inferring direct gene flow in recent generations subsequent to the identification of population structure (Pritchard *et al.* 2000; Falush *et al.* 2007). We also examined STRUCTURE results for the k-values below the largest Δk value.

Finally, the program AFLP-SURV was used with default settings to generate pairwise F_{st} values between all populations (Vekemans *et al.* 2002), which were used in multiple Mantel tests as described below.

Comparisons between genetic distances and phenotypic traits

Mantel and partial Mantel tests have often been used to investigate the relationships among populations associated with genetic distance, geographic distance and phenotypic or ecological traits (Legendre & Legendre 1998). More recently, a multiple regression technique has been proposed by Lichstein (2007), which allows for the simultaneous consideration of multiple predictor variables while retaining the permutations appropriate for the nonindependence of distance matrices observed in a spatial framework. We used this approach as implemented in the R package *ecodist* to model differences in hippocampal morphology among populations as predicted by genetic divergence (F_{st} values derived from AFLPs), geographic distance and differences in winter climate (winter temperature). Temperature was averaged (prior to the creation of a distance matrix based) over winter months (November–March) tabulated for the period 1971–2000 (Roth & Pravosudov 2009; Roth *et al.* 2011). For hippocampal traits, we used both population mean values for relative hippocampal volume and the total number of hippocampal neurons (Table 1). The data were z-transformed to generate standardized values before the creation of pairwise differences used in multiple Mantel analysis. For both hippocampal parameters and winter temperature averaged over four winter months, distance matrices included in multiple Mantel tests were based on Euclidean distances, calculated with the *dist* function in *R*.

Results

Mitochondrial DNA

A total of 46 variable sites, including two insertion/deletion events, defined 51 haplotypes in a 675-bp sequence of the mitochondrial control region in 146 birds (Alaska, Fairbanks—12, AKA—12, British Columbia—12, Washington—11, Montana—22, Colorado—19, Iowa—12, KS—23, Minnesota—12, Maine—11). Twenty-one of the haplotypes were found in more than one individual, and ten of the shared haplotypes were restricted to a single sampling site. Haplotype diversity was high, ranging from 0.46 to 0.93, and was generally higher in the eastern populations (Kansan, Minnesota and Maine). The number of unique haplotypes, those found in a single bird, was lower in the west than in the east. Pairwise Φ_{ST} values ranged from 0.027 to 0.656. The Φ_{ST} values were significant for 37 of the 45 pairwise tests indicating genetic differences among populations (Table 2). AKA and Alaska, Fairbanks were not significantly different from each other, but were significantly different from all the other sampled populations, and had lower haplotype diversity. Similarly, Washington was significantly different from all other populations and had low haplotype diversity. Colorado had high haplotype diversity and was significantly different from all other sampled populations, although it did share haplotypes with birds from adjacent sampling sites in Montana and KS. The generally high level of differentiation in mitochondrial sequences (Table 2) can also be seen in the haplotype network (Fig. 2). Few haplotypes are found in more than one or even two populations, and the distribution of haplotypes shows some geographic structure (note the clustering, for example, of Alaskan sequences). The distribution of haplotypes is indicative of both gene flow and a lack of differentiation in some areas. Note, for example, some of the more interior haplotypes in the network that are shared among multiple populations, such as the single haplotype that is found in Iowa, KS, Minnesota and Maine (Fig. 2B).

AFLP

The AFLP analyses were based on samples from 257 birds and generated 583 loci (0.16% of missing data). There were 167.03 (mean with SD = 6.33) bands present

Table 2 mtDNA pairwise Φ_{ST} values (below diagonal) and P values (above)

	AKF	AKA	BC	WA	MT	CO	IA	KS	MN	ME
AKF	*	0.276	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
AKA	0.027	*	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001
BC	**0.615**	**0.518**	*	<0.0001	0.017	<0.0001	0.045	<0.0001	0.104	0.082
WA	**0.450**	**0.289**	**0.425**	*	0.001	<0.0001	<0.0001	0.003	0.0004	<0.0001
MT	**0.438**	**0.344**	**0.118**	**0.244**	*	0.028	0.104	0.002	0.105	0.002
CO	**0.489**	**0.375**	**0.288**	**0.282**	**0.066**	*	0.001	0.035	0.007	<0.0001
IA	**0.610**	**0.495**	0.108	**0.375**	0.048	**0.143**	*	0.0004	0.112	0.027
KS	**0.487**	**0.366**	**0.285**	**0.193**	**0.114**	**0.059**	**0.195**	*	0.048	<0.0001
MN	**0.530**	**0.417**	0.056	**0.287**	0.043	**0.092**	0.055	0.070	*	0.021
ME	**0.656**	**0.562**	0.074	**0.470**	**0.157**	**0.303**	**0.109**	**0.299**	**0.090**	*

AKA, Alaska Anchorage; AKF, Alaska Fairbanks.
Values significant after correction for multiple tests (P_{crit} = 0.0452) are indicated in boldface.

per individual, and we have found in total 547 polymorphic bands and 36 fixed bands.

STRUCTURE analysis of AFLP loci showed that ln(likelihood) continuously increased as k increased, consistent with some genetic structure among our study populations. Using Δk estimation, we determined that four clusters provided the best fit to the data: (i) an Alaska population that includes birds from both Anchorage and Fairbanks; (ii) a British Columbia and Montana population; (iii) a Washington population; and (iv) a population cluster including Maine, Minnesota, Iowa, KS and Colorado (Fig. 2A). All individuals in Alaska, Washington, KS and Iowa were assigned to their respective populations with very few individuals having small probabilities of being assigned to any other population. A few more birds assigned to the British Columbia–Montana cluster had some probability of being assigned to either Alaska, Washington or the Maine–Minnesota–Iowa– KS –Colorado cluster. Finally, a few birds from Maine, Minnesota and Colorado had some probabilities of being assigned to the British Columbia–Montana (mainly), Washington or Alaska populations. Nonetheless, all four populations showed robust genetic structure suggesting significant genetic differentiation (Fig. 2A). Variance for the mean value of ln likelihood among all runs was low for k ranging from 2 to 4 (SD = 0.3, 0.7, 0.5, respectively), but rising dramatically at k = 5 (SD = 61.2), which also supports our conclusion that the data are well partitioned into four genetically differentiated clusters.

Although the best fit was predicted with k = 4, the results of STRUCTURE analyses at lower values of k are also supportive of the separation of the two Alaska populations as well as Washington, British Columbia and Montana into different clusters. At k = 2, STRUCTURE separates both Alaska groups and all other locations into two separate clusters, and at k = 3, Alaska Anchorage and Alaska Fairbanks form one cluster, Washington, British Columbia and Montana form the second cluster, and all other locations (Colorado, KS, Iowa, Minnesota, Maine) form the final third cluster.

After establishing that all of our birds most likely form four genetically distinct populations, we used the USEPOPINFO option (Pritchard *et al.* 2000; Falush *et al.* 2007) to investigate the probability that some of the individuals in these four clusters may have an immigrant mixed ancestry in the last three generations (set in the program by the GENSBACK option), which would be suggestive of direct gene flow between these populations. We used GENSBACK = 3 generations and MIGRPRIOR = 0.05 (0.95 probability that the individual has pure ancestry from the assigned population). This model showed that no individuals in any of the four designated populations have a significant probability of being of mixed immigrant ancestry during the last three generations, suggesting low or potentially no direct gene flow between these four populations, or at least between the exact locations representing these populations (Fig. 2A).

Because the inference of no genetic structure among eastern populations is important for the conclusions that we draw, we conducted an additional, focused analysis of five eastern groups predicted to form the same genetically undifferentiated cluster (Iowa, Minnesota, Maine, KS and Colorado) to test whether a separate analysis of just these groups might detect a subtle genetic structure that may have been missed in the previous larger analysis including all 10 groups. STRUCTURE predicted no genetic structure within these groups at all tested values of k (2 through 7), with all birds having high probabilities to form only a single genetically distinct group. These results support the main model, which placed these five groups into a single genetically undifferentiated cluster. It is important to note that the geographic distance between some of the eastern populations is much larger than that between, for exam-

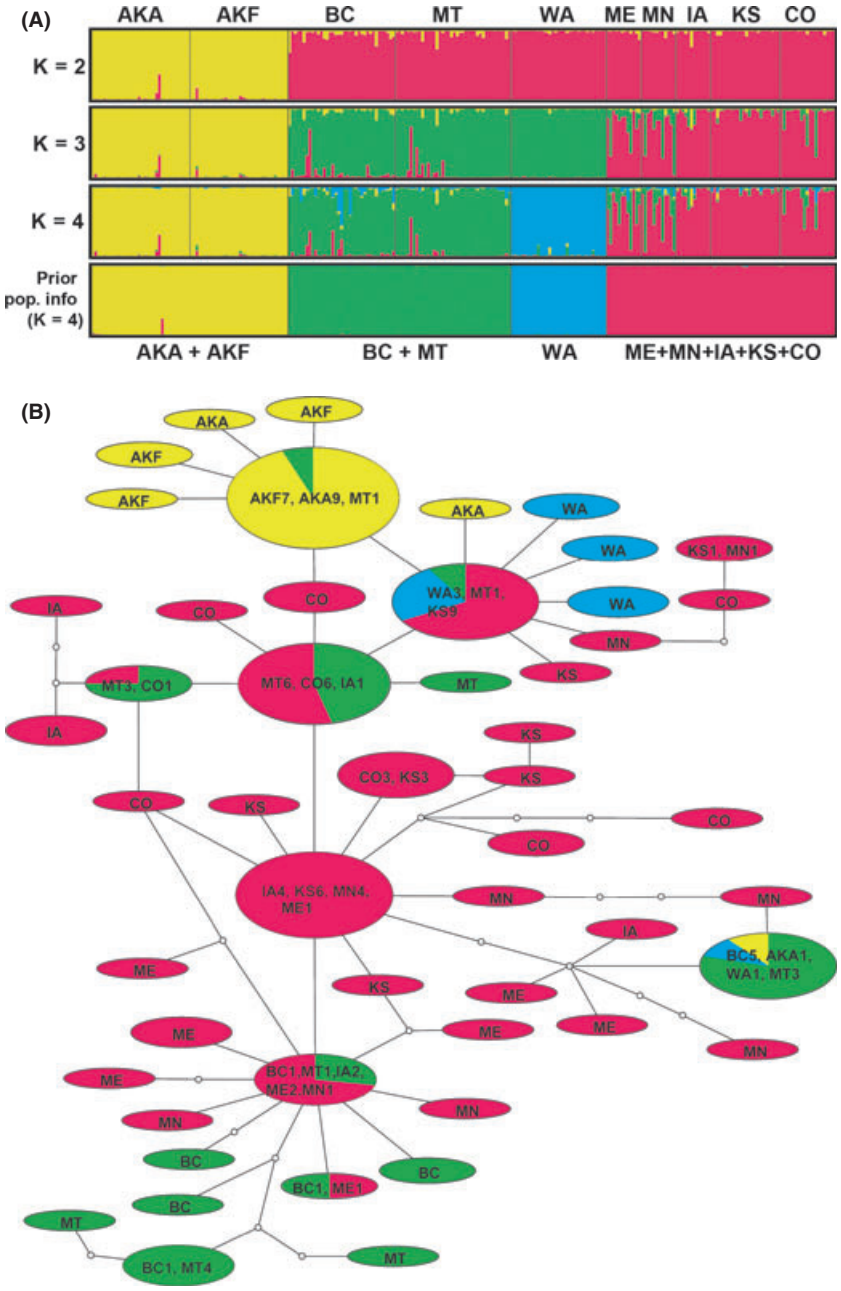

Fig. 2 (A) Population assignment of chickadees from 10 geographic locations (Fig. 1) based on STRUCTURE analysis of AFLP at $k = 2$, 3 and 4 (top three panels) and likely ancestry of individuals in four identified populations at $k = 4$, estimated using USEPOPINFO option in STRUCTURE (bottom panel). (B) Haplotype distribution about 10 geographic locations. Numbers after locations refer to how many individuals share that haplotype (e.g. MT3, three birds from MT have the same haplotype). Small white circles represent inferred haplotypes. The size of the ovals is proportional to the number of individuals possessing a given haplotype. Colours of all clusters and haplotypes match the colours of the STRUCTURE-assigned populations at $k = 4$.

ple, Iowa, Minnesota and Montana, but the Montana population has been detected as genetically distinct from both Iowa and Minnesota.

Finally, a multiple Mantel test significantly predicted differences in hippocampal morphology among populations based on the inclusion of winter climate (temperature), geographic distance and pairwise F_{st} values derived from AFLP variation; results from the multiple Mantel test were as follows: $R^2 = 0.31$, $F = 6.19$, and $P = 0.008$. Among the included variables, only winter climate was a significant, individual predictor of population differences in hippocampal morphology (Fig. 3);

in contrast, both the effects of geographic distance and F_{st} values were nonsignificant (Fig. 3). Although for simplicity we have focused on a single, multiple Mantel test involving three predictor variables (Lichstein 2007), we also explored pairwise analyses between each predictor variable and population differences in hippocampal morphology. Also, we investigated partial Mantel tests between population differences in hippocampal morphology and both differences in winter climate and AFLP F_{st} values, while accounting for geographic distances. In all of these cases, winter climate emerges clearly as being most strongly and significantly associated with population divergence in hippocampal morphology ($P < 0.05$), while the effects of geographic distance and neutral genetic divergence remain nonsignificant.

Comparing mtDNA and AFLP results

AFLP and mtDNA analyses reached generally similar conclusions in that they both separated both Alaska populations and Washington into genetically distinct populations, and both groups of analyses found evidence consistent with gene flow among the eastern populations. This can be seen in the Φ_{ST} values (Table 1) and also in the mitochondrial genealogy: AKA and Alaska Fairbanks haplotypes in particular were mostly unique (one haplotype was shared with Montana, and one was shared with Montana, Washington and British Columbia birds) and occupy a peripheral position in the network, suggesting a more derived position relative to the lower, continental populations (which are also likely larger populations, given the higher haplotype diversity and greater number of birds with unique haplotypes). Iowa and Minnesota birds were also predicted to be genetically similar by both analyses. Unlike the results from AFLPs, mtDNA analyses made more significant distinctions among some of the other groups. For example, Colorado birds were significantly different from birds from all other locations (Table 1). KS birds were similar to the birds from Minnesota, but were significantly different from birds from all other locations. Chickadees from British Columbia were similar to chickadees from Iowa, Minnesota and Maine, but different from all other locations. Finally, Montana chickadees were similar to chickadees from Iowa and Minnesota, but distinct from chickadees from all other locations. One specific difference between the AFLP and the mtDNA analyses concerned birds from British Columbia and Montana: STRUCTURE was unable to detect differences between birds from the two sampling sites, while the Φ_{ST} analyses of mitochondrial variation showed significant differences between these two groups (unique haplotypes in both areas are also evident in the haplotype network, Fig. 2B). Overall, however, a pairwise Mantel test using Φ_{ST} values from mtDNA analysis and F_{st} from AFLP analysis showed a significant association (correlation coefficient = 0.76, $P = 0.006$).

Given that mtDNA is a maternally inherited marker, sex-bias in dispersal patterns may potentially affect the results of the analyses. Unfortunately, no detailed information exists about sex-specific dispersal in the black-capped chickadee, but it is likely that, as in most bird species, it is female-biased. However, significant similarity of the mtDNA and AFLP analyses suggests that our results are fairly robust.

Discussion

The population genetic results reported here show genetically structured chickadee populations through-

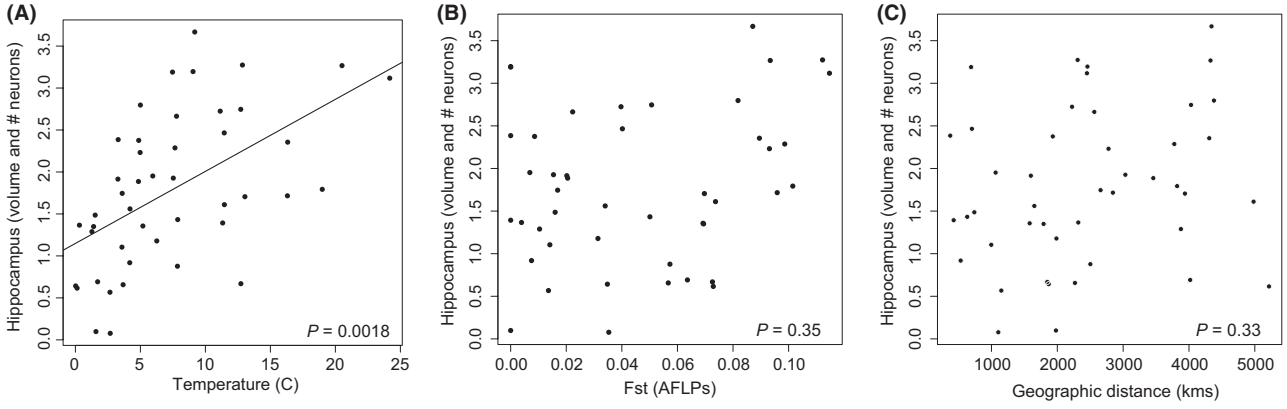

Fig. 3 Associations among pairwise population differences in hippocampal morphology (volume and the total number of neurons, z-transformed data) and (A) pairwise differences in winter climate (ambient mean daily temperature averaged over winter months—November–March), (B) pairwise genetic divergence (AFLP F_{st}) and (C) pairwise geographic distance. The P values associated with each graph are from a multiple Mantel test involving all three variables (see text for details).

out North America. However, while some populations with known differences in hippocampal morphology have been found to be genetically distinct, others appear to be genetically undifferentiated, suggesting relatively unrestricted gene flow. Overall, population divergence in phenotypic traits (hippocampal morphology) is not associated with genetic differentiation at neutral markers, which is consistent with the possibility that, at least in some populations, these differences have evolved in response to natural selection and potentially in the face of gene flow. Pairwise analyses of population genetic divergence, geographic distance and winter climate further support this conclusion by showing that only winter climate is significantly associated with population phenotypic divergence in hippocampal morphology.

Analyses of AFLPs identified four regional genetic clusters (Fig. 2A) as having the highest likelihood for explaining the variation revealed in our data. In particular, birds from the two Alaska populations and Washington were quite distinct, both from each other and from other populations. Specimens from British Columbia and Montana were identified as deriving from one population that was also fairly distinct from all other areas, even though the Montana population contains a large diversity of mitochondrial haplotypes. Importantly, the variation in mitochondrial DNA was broadly consistent with a picture of population structure and some level of population isolation: many populations contained unique haplotypes (Fig. 2B) and differences inferred by Φ_{ST} analyses found the majority of populations to be distinct, even within clusters identified by the AFLP analyses.

Significant genetic differentiation suggests that the gene flow between Alaska, Washington, British Columbia–Montana and the rest of the locations may be limited relative to the gene flow within each of these groups. At the same time, our nuclear data strongly suggest that gene flow is comparatively unrestricted between Iowa, Maine, Minnesota, KS and likely Colorado. We stress that these inferences should be considered in a comparative context: that is, Maine and Minnesota have, for example, shared a more recent demographic history with each other than either of those areas has with Alaska or Washington. AFLP variation includes many loci randomly distributed throughout the genome, the vast majority of which are expected to be neutral and so differences in genetic structure likely reflect changes due to genetic drift. It is important to note that our sample sizes for Maine, Minnesota and Iowa were slightly lower (12) than those for other populations and that these low sample sizes could theoretically be responsible for our failure to find significant genetic differentiation between these populations. We, however, do not think this is the case, as some of the populations in the same genetically undifferentiated cluster (Colorado and KS) have larger sample sizes, and all of these populations were significantly different from Montana, British Columbia, Washington and Alaska populations.

We cannot exclude the possibility that some of the loci sampled may not be neutral and, in fact, we would hypothesize that there should be significant differences in some genetic regions involved in hippocampal functions. However, those unidentified regions associated with brain function (however important biologically) must comprise a relatively small proportion of the total genome and would therefore be highly unlikely to be included in our AFLP data set. Moreover, even if functional genetic regions associated with the hippocampus were included, differences in these genes would produce a population genetic structure that parallels the differences in memory and hippocampal morphology among our sampling locations. Our results, however, showed that differences in hippocampal morphology were significantly associated with differences in winter climate, but not with genetic divergence at neutral loci or geographic distance (Fig. 3).

Of course, we cannot put a date on divergence in hippocampal traits among different populations; thus, we do not know whether hippocampal changes arose in the face of contemporary gene flow (e.g. among populations within the red cluster, Fig. 2A) or whether they arose at an earlier time (while populations were more isolated) and persist today despite the gene flow. However, both interpretations are equally interesting and suggestive of natural selection affecting the variation in ecological traits that has been observed.

Our data suggest that winter climate is the overarching factor associated with selection on hippocampal structure supporting the evolution of enhanced spatial memory, as hypothesized by previous studies in this system, and it seems likely that these traits respond to climate-based selection independent of gene flow. Chickadees from the two Alaska populations have larger hippocampi containing more neurons compared to the birds from almost all other locations, except Maine and Minnesota and marginally British Columbia (Roth & Pravosudov 2009; Roth *et al.* 2011), and they also appear to be genetically distinct from all other populations (Fig. 1). On the other hand, chickadees from Minnesota and Maine have larger hippocampi containing more neurons compared to the birds from Iowa and KS (Roth & Pravosudov 2009; Roth *et al.* 2011), yet all four locations form a single genetically distinct population and likely share extensive gene flow, which is additionally supported by the mtDNA

analysis. Finally, chickadees from Washington are genetically distinct from all other populations, yet their hippocampal morphology is similar to that in birds from KS and Iowa, which share somewhat similar environment. A Mantel test in a multiple regression framework solidified these conclusions by showing that population divergence in hippocampal morphology is significantly associated with differences in winter climate, but not in genetic divergence or geographic distance (Fig. 3).

We note that we have investigated population genetic structure, but have not explicitly addressed the possibility for genetic clines associated with, for example, isolation by distance. This approach was necessitated by our sampling scheme, which was in turn driven by previous experimental work. Thus, the results presented here do not rule out the possibility of more subtle clinal variation within, for example, the eastern populations. A recent simulation study (Chen *et al.* 2007) concluded that STRUCTURE is highly capable of detecting clinal variation in contrast to previous suggestions to the contrary. In addition, our secondary analysis with STRUCTURE that focused only on the eastern populations (the red group in Fig. 2) while excluding the other populations was consistent with the hypothesis that these populations form a single, large gene pool. Moreover, mtDNA results also suggest relatively less genetic differentiation at least between some of the populations showing the largest hippocampal differences (Minnesota and Iowa). In fact, birds from Minnesota and Iowa share haplotypes suggesting gene flow between these populations, either contemporary or in the recent past. In addition, STRUCTURE did detect genetic differentiation between Montana and Minnesota and Iowa and the geographic distance between them is smaller than between Iowa, Minnesota and Maine, which have all been assigned to a single population.

Our previous work suggested that the differences in hippocampal morphology between the Alaska and KS populations, which are genetically differentiated, are not experience-based but rather inherited (Roth *et al.* 2012). In addition, our previous comparison of laboratory-reared and maintained and wild chickadees strongly suggested that the variance in at least the total number of hippocampal neurons has a heritable basis rather than being caused by environment-dependent experiences (Roth *et al.* 2012). If we assume that the relationship between the hippocampus morphology and climate is the result of selection for spatial memory in all populations, then combined, these data suggest that strong selection on memory and hippocampal morphology imposed by winter climate severity may produce adaptive changes in these traits regardless of genetic differentiation and potential isolation among populations and hence likely regardless of the relative amount of gene flow (and this inference holds even if traits arose at an earlier time, as discussed above). This would suggest that differences in memory and hippocampal morphology might evolve independently in different populations as a function of climate-induced selection on memory.

Alternatively, it may also be possible that the mechanisms generating and maintaining significant variation in memory and hippocampal morphology may be different between genetically differentiated and undifferentiated populations. While differences in hippocampal morphology between genetically differentiated populations may have a heritable basis as a result of selection, similar differences between relatively undifferentiated populations (such as those hippocampal differences separating Iowa and Minnesota) may be maintained by experience-dependent plasticity. These possibilities cannot be addressed with the data in hand and must await the collection of further experimental data to differentiate between potential mechanisms. In addition to experience-dependent plasticity, the observed differences among some of the populations may also be, at least in part, due to parental and epigenetic effects. The population genetic results that we have presented here lay the foundation for future studies that should focus specifically on genetically undifferentiated populations in close geographic proximity in order to investigate whether variation in memory and hippocampal morphology may be maintained by different mechanisms, and how these mechanisms might interact with differing levels of gene flow.

Beyond the identification of population structure in the context of divergence in hippocampal traits, the mitochondrial results point towards a complex biogeographic history for the black-capped chickadee in North America that is beyond the scope of this study. For example, Alaska has low diversity and may have been the result of a founder event, which could explain the structure of the haplotype network with geographically proximate populations (e.g. Alaska and British Columbia) not directly linked. In general, the presence of glacial refugia during Pleistocene climatic cycles might likely be reflected in the divergence of populations from Alaska, northwestern areas and central continental populations (Hewitt 2000). However, more geographically intensive sampling is needed to make inferences about biogeographic history; instead, our purpose here has been to place previous behavioural and morphological work in a population genetic context to set the stage for future investigations into natural selection and diversification in memory-related traits.

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Data accessibility

mtRNA: GenBank accessions JX164148–JX164198.

AFLP: Dryad accession doi:10.5061/dryad.5p6b1.

GenBank sequences: supplemental material available with the online version of this article.

Supporting information

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

Appendix S1 mtDNA GenBank sequences.

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