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RESEARCH ARTICLE

Why sampling scheme matters: the effect of sampling scheme on landscape genetic results

Michael K. Schwartz · Kevin S. McKelvey

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Abstract There has been a recent trend in genetic studies of wild populations where researchers have changed their sampling schemes from sampling pre-defined populations to sampling individuals uniformly across landscapes. This reflects the fact that many species under study are continuously distributed rather than clumped into obvious "populations". Once individual samples are collected, many landscape genetic studies use clustering algorithms and multilocus genetic data to group samples into subpopulations. After clusters are derived, landscape features that may be acting as barriers are examined and described. In theory, if populations were evenly sampled, this course of action should reliably identify population structure. However, genetic gradients and irregularly collected samples may impact the composition and location of clusters. We built genetic models where individual genotypes were either randomly distributed across a landscape or contained gradients created by neighbor mating for multiple generations. We investigated the influence of six different sampling protocols on population clustering using program STRUCTURE, the most commonly used model-based clustering method for multilocus genotype data. For models where individuals (and their alleles) were randomly distributed across a landscape, STRUCTURE correctly predicted that only one population was being sampled. However, when gradients created by neighbor mating existed, STRUCTURE detected multiple, but different numbers of clusters, depending on sampling protocols. We recommend testing for fine scale autocorrelation patterns prior to sample clustering, as the scale of the autocorrelation appears to influence the results. Further, we recommend that researchers pay attention to the impacts that sampling may have on subsequent population and landscape genetic results.

Keywords Landscape genetics · Microsatellite · Population structure · Sample design · Sampling

Introduction

Within continuously distributed populations, mating with proximal individuals (hereafter neighbor mating) will lead to local patterns of genetic autocorrelation producing gradients across a landscape and isolation-by-distance patterns (Kimura and Weiss 1964; Malecot 1973; Morton 1973, Sokal and Oden 1978a,b; Barbujani 1987). Neighbor mating will lead to patterns of close relatedness at fine scales and, conversely, larger gradients of change in gene frequencies at larger scales. Additionally, in natural populations, habitat fragmentation and the presence of isolating barriers may separate populations into discrete groups which gradually drift apart and evolve independently. Because these two phenomena (neighbor mating and isolation by barriers) occur simultaneously within populations, interpretation problems can arise when samples from an isolation by distance gradient are grouped and compared (Manel et al. 2003; Musiani et al. 2007); inappropriate grouping can lead to incorrect conclusions as to the presence, location, and nature of putative barriers.

Landscape genetics has been described as the amalgamation of population genetics and landscape ecology

M. K. Schwartz (✉) · K. S. McKelvey
USDA Forest Service, Rocky Mountain Research Station,
800 E. Beckwith Ave., Missoula, MT 59801, USA
e-mail: mkschwartz@fs.fed.us

(Manel et al. 2003; Holderegger and Wagner 2006). The major theme of this field has been to understand how landscape features influence spatial structure either by examining predefined groups of samples, or using clustering programs with spatially references samples that have associated multilocus data to identify populations. Although other landscape genetic approaches exist (e. g. Double et al. 2005; Cushman et al. 2006; Storfer et al. 2007), the most common way to conduct individual-based landscape genetic analyses is to sample individuals diffusely across a landscape, detect discontinuities among mulitlocus genotypes using various clustering algorithms, and correlate these discontinuities with landscape features (e.g, Piertney et al. 1998; Manel et al. 2003). The recent increase in published papers that use a landscape genetics approach (Holderegger and Wagner 2006) likely reflects two facts: first many natural populations are not clumped into well defined spatially obvious populations and instead are more continuously distributed across a landscape and, second, the wealth of highly variable molecular markers available for many species now provides sufficient power to use a range of clustering methods (Barbujani and Sokal 1989; Bertorelle and Barbujani 1995; Epperson and Li 1996; Pritchard et al. 2000; Corander et al. 2004).

Individual based analyses allow the data to determine appropriate groupings through the use of clustering statistics: within-data patterns indicate what the optimal number of clusters is and where the appropriate cluster boundaries are. As with any clustering algorithm, however, underlying assumptions are that there are biologically meaningful clusters to be discovered and that structural elements not associated with clustering, such as gradients, are small relative to the elements that contribute to clustering.

The most common tool used to statistically cluster populations and define population substructure in the field of molecular ecology is program STRUCTURE (Pritchard et al. 2000, Falush et al. 2003, 2007), although other approaches and programs exist (e.g., PARTITION, Dawson and Belkhir 2001; BAPS2, Corander et al. 2004; GENELAND, Guillot et al. 2005a, b; 2D LSA in GENALEX, Double et al. 2005, Peakall and Smouse 2006; HMRF models, François et al 2006; TESS Chen et al. 2007). Unlike STRUCTURE, some of these impose additional spatial constraints on the derived solutions. STRUCTURE assumes that collected samples represent K populations; and uses a Markov Chain Monte Carlo (MCMC) method to assign individual multi-locus genotypes to populations, minimizing Hardy–Weinberg deviations and linkage disequilibrium. In most cases in the molecular ecology literature K is unknown, and STRUCTURE computes the probability of the clusters for all values $1 \leq K \leq N$, where N is an arbitrary integer, generally <8 in published studies (Cegelski et al. 2003; Natoli et al. 2005; Jorde et al. 2007). The K with the highest likelihood is most supported, and samples are subsequently divided into clusters based on their assignment (Pritchard et al. 2000; Falush et al. 2003). However, the authors of STRUCTURE state that this approach "merely provides an ad hoc approximation" of the number of clusters and that "the biological interpretation of K may not be straightforward" (Pritchard et al. 2007).

Using program STRUCTURE to illuminate various ecological and evolutionary patterns and processes has been extremely popular in molecular ecology and landscape genetics; the initial paper describing the method has been cited 1494 times in scientific journals as of January 11, 2008 and over 400 times in the previous 6 months (ISI Web of Knowledge v 3.0), far more frequently than any of the other clustering programs specific to population genetics. These applications range from evaluating the spread of West Nile Virus by *Culex pipiens* mosquitoes (Fonseca et al. 2004), to understanding the patterns and causes of human population substructure (Rosenberg et al. 2002, 2003, 2005; Parra et al. 2003). In the molecular ecology literature, STRUCTURE results have been used to better understand the ecology, habitat structure, and natural and anthropogenic features that impact substructure and dispersal of many taxa including marine mammals (Natoli et al. 2005; Jorde et al. 2007), amphibians (Funk et al. 2005), flowering plants (Tero et al. 2003), and insects (Repaci et al. 2006). STRUCTURE has also been used to inform management and conservation decisions. For instance, STRUCTURE has been used to provide advice on how best to partition the wolverine (*Gulo gulo*) harvest in Montana (Cegelski et al. 2003, 2006) and to mitigate the influence of roads and natural barriers on bobcats (*Lynx rufus*) in Michigan (Millions and Swanson 2007) and California (Riley et al. 2006).

There have been several well-constructed evaluations of STRUCTURE's ability to correctly delineate the number of clusters represented in a dataset (Evanno et al. 2005; Latch et al. 2006; Latch and Rhodes 2006). Latch et al. (2006), testing subpopulations with no internal structure and no migration, concluded that STRUCTURE performed exceedingly well at assigning populations given low levels of differentiation among groups (i.e., $F_{ST} = 0.03$). However, they noted that F_{ST} must be at least 0.05 to reach a 97% assignment accuracy rate (Latch et al. 2006). Alternatively, Evanno et al. (2005) found that in most of their simulations, representing three different migration models, the traditional metric of "log probability of data" was not maximized at the correct number of subpopulations (K). Evanno et al. (2005) created an ad hoc statistic: ΔK, the second order rate of change of the likelihood function with respect to K. This statistic was able to accurately reflect the true number of clusters, but because it was a second order statistic could never evaluate $K = 1$ (Evanno et al. 2005).

To date none of the clustering programs (including STRUCTURE), have been adequately tested in situations where significant genetic gradients exist within a continuous population (but see Witherspoon et al. 2006 for limited simulations). Further, there has not been an evaluation on the effects of irregular sampling on optimal cluster determinations. Rosenberg et al. (2005) tested STRUCTURE with respect to its performance to detect clusters versus clines on multi-locus genotype data from human populations sampled worldwide. They showed via subsampling methods, that clusters of humans were not a consequence of uneven sampling along genetic clines as had been suggested by Serre and Paabo (2004). In fact, their data suggest that less uniformly distributed samples produced lower "clusteredness" than more uniformly sampled distributions (Rosenberg et al. 2005). However, while this demonstrated that the derived clusters were not sampling artifacts, it does not provide a direct test of the behavior of STRUCTURE when confronted with continuous gradients and clumped samples.

Our goal in this paper was to test the influence of sampling schemes on the behavior of the most commonly used clustering method in a simulated continuous population characterized by gradients associated with neighbor mating.

Methods

To evaluate the effect of sampling and gradients on program STRUCTURE we simulated a population of 10,000 organisms arranged as a square of 100 × 100 territories. All individuals were given 2 alleles per locus for 15 independent diploid, co-dominant loci, each with 7 alleles per locus. We assigned alleles to each locus by randomly drawing individual alleles numbered between 1 and 7 in the following proportions 1 = 0.38, 2 = 0.24, 3 = 0.17, 4 = 0.08, 5 = 0.05, 6 = 0.04, and 7 = 0.03, similar to microsatellite allele distributions associated with several wildlife species that we currently study (e.g., Schwartz et al. 2003, 2004, 2006). The initial population showed no spatial autocorrelation.

Neighbor mating can create a gradient across space such that the population would be structured in an isolation-by-distance manner. To create the neighbor mating distribution, all 10,000 individuals mated simultaneously (although to avoid edge effects, all analyses were conducted on the interior 2,500 individuals; 50 × 50 territories). We randomly chose one of each individual's eight neighbors for them to mate with (organisms at the edges of the surfaces could only mate with 1 of 5 partners and those in the corners could only mate with 1 of 3 neighbors). Offspring received alleles from both parents assuming that all loci were independent and, at each locus, each allele was chosen with equal probability. Offspring replaced the individual causing full population turnover each generation. Since each individual produced 1 offspring, population size was constant. We initially ran this procedure for 20 generations to produce gradients.

We were interested in the effects of sampling schemes on population clustering when the population was randomly mating and when there was an underlying neighbor mating scheme. Therefore, we established a series of six different sampling schemes reflective of how samples are collected in the conservation genetics and molecular ecology literature (Fig. 1a–f). The sampling schemes used were as follows: (A) Trapper sampling—360 individual samples were drawn randomly across the entire simulated landscape (2,500 available individuals). (B) Research sampling—360 individuals were drawn in 10 clusters of 36, which is similar to sampling schemes where researchers collect most, or all individuals, from a small portion of the landscape and compare these samples to other landscape patches (e.g., many small mammal trapping grid studies such as Mossman and Waser 2001, Burton et al. 2002). (C) Corner sampling—similar to research sampling, two distinct areas in a landscape are compared to estimate gene flow or substructure. However, with corner sampling only a subset of individuals are captured within each sampling area. This type of sampling is common with ungulates, marine fish, and carnivores (e.g., Pardini et al. 2001; Schwartz et al. 2006; Hicks et al. 2007). (D) Line transect sampling—10 equally spaced lines of 36 individuals were sampled following the study design of many small mammal, forest carnivore, and plant studies (e.g., Gamache et al. 2003). (E) Multi-generation trapper sampling—120 individuals were sampled in generations t_{18}–t_{20} for a total of 360 individuals. Studies of rare carnivores often require multiple generations of trapper samples to accumulate to estimate gene flow. Examples of this type of sampling occur in Cegelski et al. (2003). (F) Mixed sampling—this is probably the most common type of sampling for carnivores and marine mammals, where samples are obtained from hunters, harvesters, and trappers, and through research effort (e.g., Schwartz et al. 2003). Here we have one block of 36 individuals (6 × 6 cells in the simulation) collected in one generation (t_{18}) and 324 individuals captured diffusely across the study area and over multiple generations (t_{18}–t_{20}).

For each of the six sampling schemes we evaluated STRUCTURE at t_0 (random mating) and at t_{20} (or t_{18}–t_{20} for Multi-generation trapper sampling and Mixed sampling) when there was an underlying neighbor mating scheme. We chose those STRUCTURE parameters most commonly used in the literature, recommended by the manual, and used in other STRUCTURE evaluations

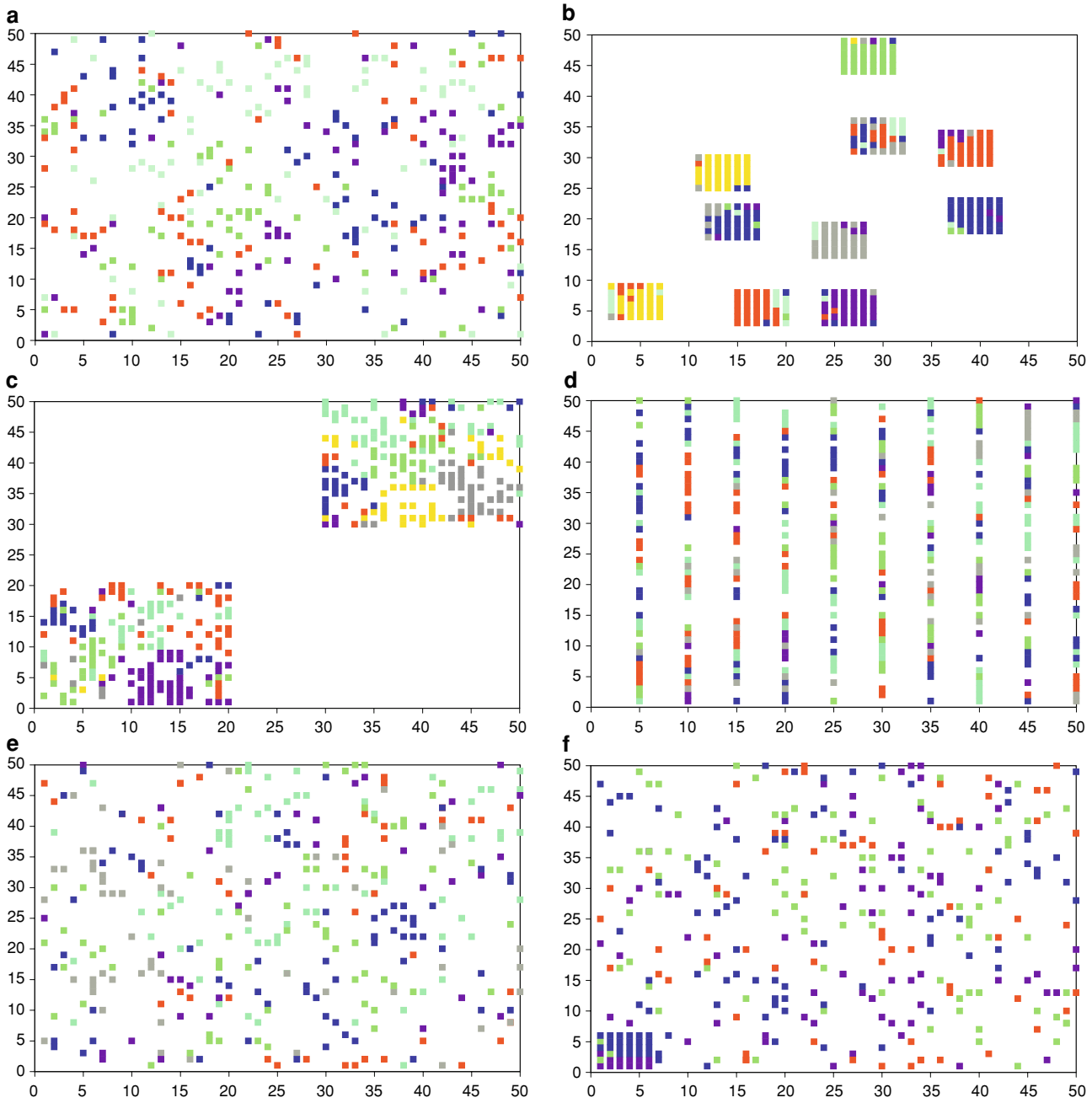

Fig. 1 Schematic of the sampling schemes used in this exercise. These schemes were established to mimic real sampling schemes used in the published literature. The colored squares represent the clusters that STRUCTURE assigned individuals (based on maximum Q per individual) for the iteration with the best supported K per sampling scheme in generation t_{20}. The values of the *X* and *Y* axis are cell references in our 50 × 50 grid. In sampling schemes 1E and 1F some points represent more than one sample, as samples were drawn over multiple generations

(Falush et al. 2003; Evanno et al. 2005; Pritchard et al. 2007). Thus, we chose the admixture model and the option of having allele frequencies correlated between populations (recommended by Falush et al. 2003) for detecting subtle population substructure. We used a burn-in period of 10,000, consistent with simulations conducted by Evanno et al. (2005). We then ran each iteration for 500,000 MCMC repetitions. It has been reported that different iterations can produce different likelihood values (Evanno et al. 2005), thus for each of the 12 data sets we conducted 20 independent iterations (similar to Evanno et al. 2005) in order to quantify the variation in log-likelihood for each K. All iterations were tested for K = 1–7, a common set of K used in the published literature.

The most supported K maximized LnP(D) (also called L(K) in Evanno et al. 2005), which is the log-likelihood of the data at each step of the MCMC minus half the variance averaged across the 20 iterations. Additionally, we tested Evanno et al.'s (2005) ad hoc test statistic ΔK. To assess the spatial arrangement of the derived clusters, we color-coded the location of all samples by cluster for each sampling scheme; for this assessment, for each of the six sampling schemes we chose the iteration with the highest likelihood to graph. We also examined the estimated membership fractions (Q) for each of the most supported simulations. Specifically, we calculated the random expectation of Q if membership was equally divided; the mean, median, max and min Q; and the index "clusteredness" (see Rosenberg et al. 2005).

We wanted to determine if the patterns of local autocorrelation created by our neighbor mating simulations were realistic. We therefore compared the spatial autocorrelation patterns associated with simulated neighbor mating to those found in a large-scale intensive sample of individual black bears (*Ursus americanus*) on a grid established in North Idaho (data described in detail in Schwartz et al. 2006, Cushman et al. 2006). This dataset was used because in the bear work we did not sample based on groups, but rather evenly at 1.6 km intervals across two mountain ranges (3,000 km^2 area). The black bear dataset consisted of a 9-locus microsatellite genotype from 146 individuals sampled using non-invasive genetic snares. Landscape resistance modeling using these data suggested that genetic structure was primarily related to gradients of landcover and elevation, although distance was also a significant factor (Cushman et al. 2006). Spatial autocorrelation analyses were conducted using GenAlEx Version 6 (Peakall and Smouse 2006) with even distance classes of size 2 (25 classes for the simulated data and 32 classes for the bear data), 99 permutations and 100 bootstraps to produce confidence intervals around the null hypothesis of a random distribution and around the correlation coefficient.

In the cases of research and corner sampling, where samples were spatially clumped, we compared traditional F_{ST} estimates between each of the sampled blocks (not the STRUCTURE derived F_{ST} results) in generation t_{20} using program FSTAT 2.9.3.2 (Goudet 1995). Finally, we explored the influence that the number of generations had on our results by running additional simulations for 50, 100, and 250 generations and comparing autocorrelation plots.

Results

For each of the random mating simulations (t_0) the average LnP(D) over 20 iterations was maximized at K = 1 indicating that only one cluster existed (Table 1). Alternatively, the neighbor mating simulations supported anywhere from 5 to 7 clusters, depending on the sampling scheme (Fig. 2a–f, Table 1). The trapper sampling showed a near linear increase in LnP(D) from K = 1 to K = 5 before declining (Fig. 2a), whereas the multi-generation trapper sampling and line transect sampling showed an increase in LnP(D) from K = 1 to K = 6 before declining (Fig. 2d, e). The research sampling and corner sampling showed an increase in LnP(D) from K = 1–7 (Fig. 2b, c). The most complex sampling scheme, the mixed sampling showed an increase to K = 4 before declining and subsequently increasing. In all cases, mean F_{ST} derived by STRUCTURE among clusters was large (>0.058; Table 2).

Table 1 Most supported K value from program STRUCTURE, suggesting the number of clusters present in the dataset

Sampling scheme	Random mating	Neighbor mating LnP(D) criteria	Neighbor mating ΔK criteria
Trapper	1	5	3
Research	1	7	2
Corner	1	7	2
Line transect	1	6	2
Multi-generation	1	6	4
Mixed	1	7[a]	2

[a] In the mixed sampling there was a peak at K = 4 and K = 7. Many researchers would have selected K = 4

We color coded individuals based on the cluster to which they were assigned, given the most supported STRUCTURE iteration per sampling scheme in the most supported K, after neighbor mating occurred (Fig. 1). No obvious spatial clustering was evident for clusters delineated by trapper, corner, line transect and multi-generation sampling. However, for the smaller sample blocks in research and mixed sampling, genetic and spatial clusters were highly correlated ($\chi^2 = 1248.99$, 63 d.f., $P < 0.0001$ for the Research sampling, comparing cluster assigned individuals in blocks to random expectations; Fig. 1b).

The ΔK method (Evanno et al. 2005) of evaluating the number of clusters suggested K = 2 for research sampling, corner sampling, line transect sampling and mixed sampling (Fig. 3; K = 2 is the lowest possible number of clusters definable using this method). For the other sampling patterns, trapper and multi-generation trapper sampling, K = 3 and K = 4 were most supported, respectively (Fig. 3a and e, Table 1).

We were interested in determining if there were signals in the estimated membership fractions (Q) of each individual that could inform us as to the underlying neighbor mating distribution. Mean Q and clusteredness, which are highly correlated statistics (0.9 in this study), varied substantially between sampling schemes, despite the underlying surface

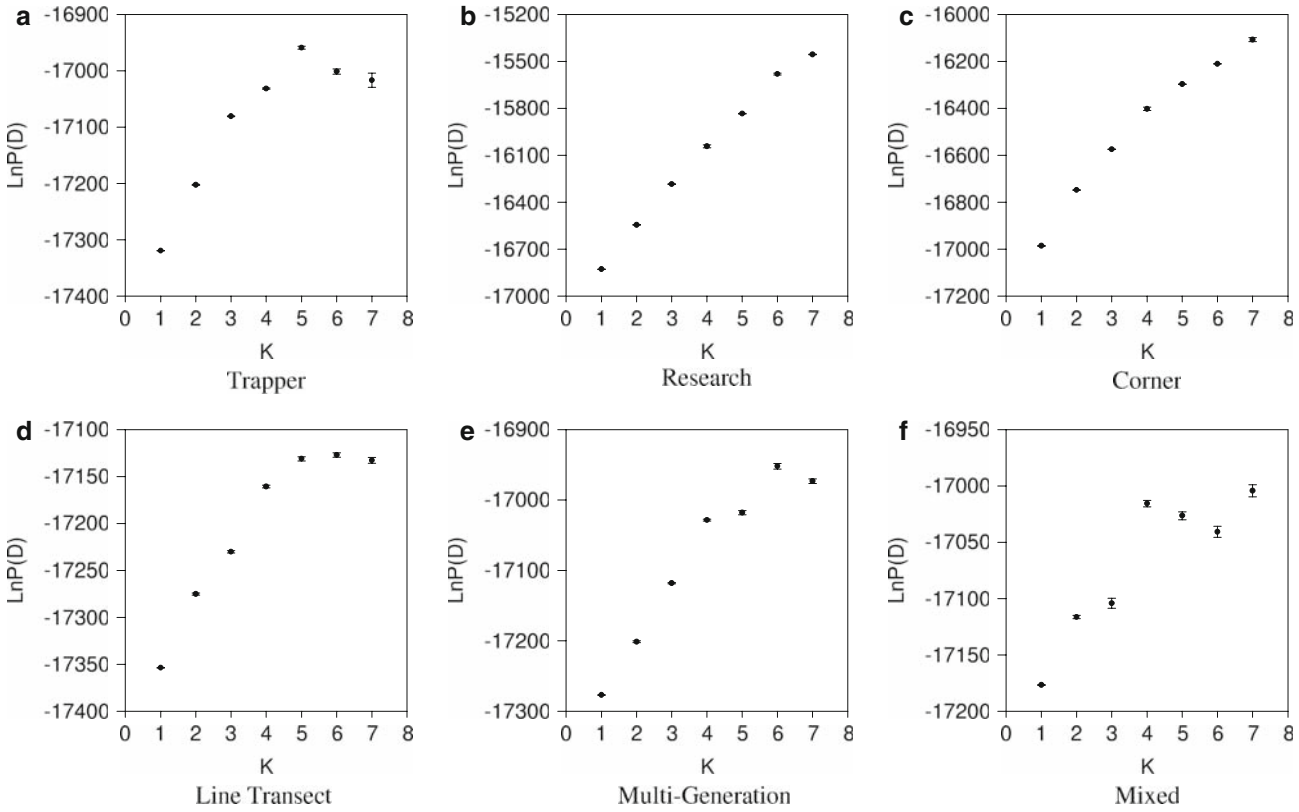

Fig. 2 Plot of the STRUCTURE simulations for K = 1–7 after neighbor mating for 18–20 generations (depending on the simulation; see text for details). K is the number of subpopulations evaluated by program STRUCTURE. The error bars are the standard error across the 20 independent iterations of STRUCTURE. The least negative LnP(D) value is the grouping most supported by program STRUCTURE

Table 2 Mean F_{ST} per group for each of the most supported number of clusters generated by STRUCTURE after 20 generations of neighbor mating (18–20 generations in the multi-generation trapping and mixed sampling schemes)

Cluster	Trapper (K = 5)	Research (K = 7)	Corner (K = 7)	Line transect (K = 6)	Multi-generation (K = 6)	Mixed (K = 7)
1	0.074	0.120	0.083	0.070	0.059	0.080
2	0.068	0.126	0.090	0.070	0.058	0.085
3	0.076	0.108	0.094	0.070	0.060	0.083
4	0.072	0.107	0.088	0.070	0.061	0.086
5	0.067	0.133	0.087	0.071	0.058	0.081
6	–	0.113	0.088	0.070	0.059	0.085
7	–	0.130	0.091	–	–	0.087

being constant (Range of mean Q = 0.333–0.741; Table 3). For the most supported K, Mean Q was always substantially larger than the random expectation (Table 3).

For the two sampling schemes where samples were collected in discrete blocks (Corner sampling and research sampling) we calculated traditional F-statistics among sample blocks (not STRUCTURE delineated clusters) for the neighbor mating simulations. F_{ST} was 0.010 (95% CI 0.006–0.014) for the Corner sampling and 0.086 (95% CI 0.071–0.101) for the research sampling; F_{IS} was 0.151 (95% CI 0.130–0.174) for the corner sampling and 0.101 (95% CI: 0.084–0.119) for the research sampling. Pairwise F_{ST} ranged from 0.048 to 0.136 between blocks in the research sampling scheme. The correlation between genetic distance and geographic distance was positive ($R_{xy} = 0.118$), but the relationship was not significant (Mantel test: $Z = 179.88$, $P = 0.24$).

There was no spatial autocorrelation for the trapper sampling (i.e., random sampling) in generation t_0, as expected (Fig. 4a). However, after running these data for 20 generations under our neighbor mating scheme, significant spatial autocorrelation could be detected up to 6 cells away

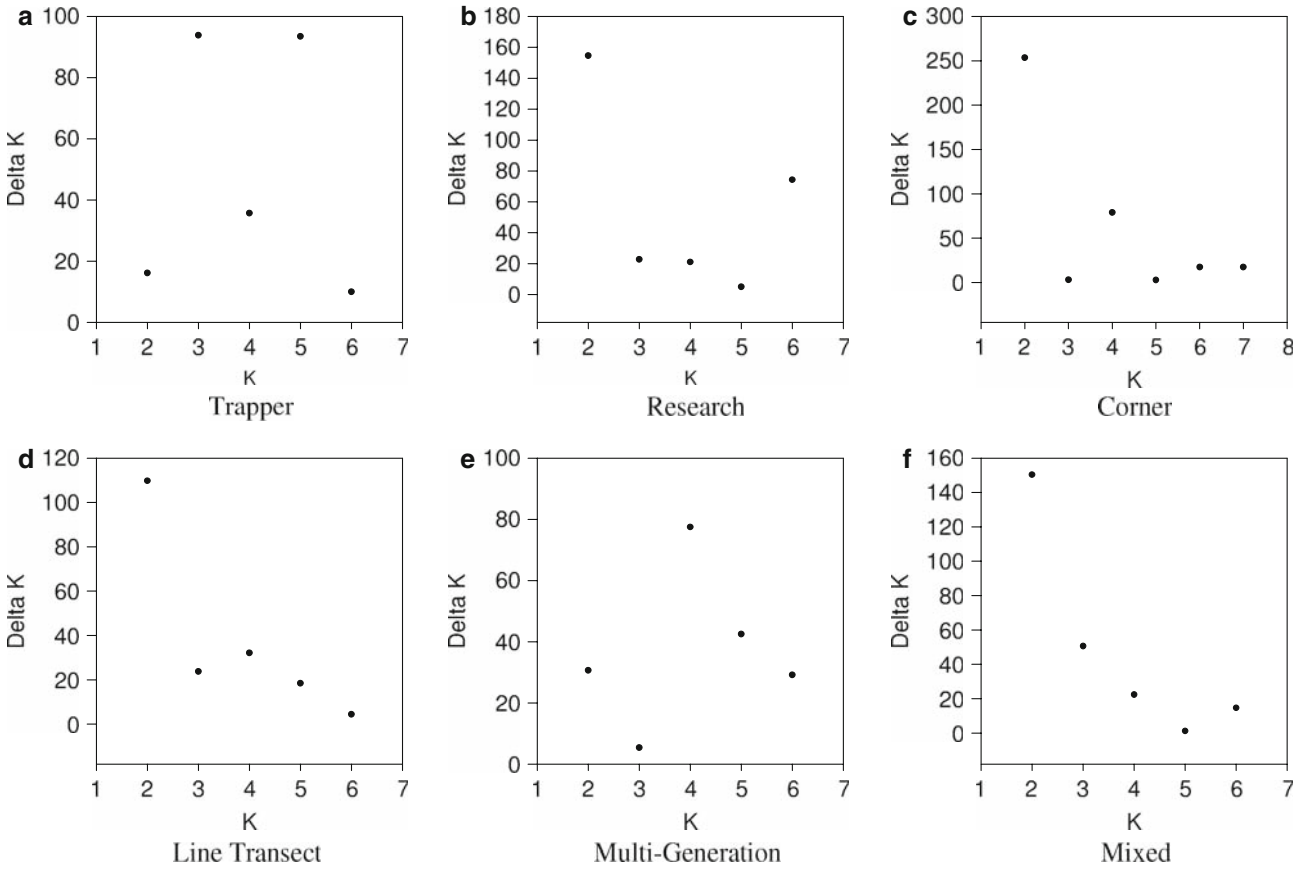

Fig. 3 Results of the STRUCTURE simulations using the Evanno ΔK method to evaluate the most supported grouping (K)

Table 3 Summary statistics of the estimated membership fractions (Q) across 20 iterations of each sampling scheme

	Rand exp.	Mean Q	Median Q	Max Q	Min Q	Clusteredness
Trapper	0.200	0.567	0.556	0.917	0.245	0.492
Research	0.143	0.741	0.799	0.967	0.207	0.721
Corner	0.143	0.596	0.575	0.939	0.224	0.568
Line transect	0.167	0.333	0.272	0.912	0.195	0.253
Multi-generation	0.167	0.452	0.373	0.920	0.205	0.412
Mixed	0.250	0.453	0.419	0.882	0.270	0.317

Random exp. is 1/K and clusteredness is a measure of the averaged clumping of individuals, which is the extent that individuals are estimated to belong to 1 cluster versus a combination of clusters (following the formula in Rosenberg et al. 2005)

(first 3 bins; Fig. 4b). Similarly, our black bear data showed a steady decline in spatial autocorrelation for the first 10 km (first 5 bins), then either no or negative autocorrelation as distance increased (Fig. 4c). The magnitude of autocorrelation was similar between bears (max r = 0.19, 95% CI Lower = 0.15, Upper = 0.23) and the simulated data (max r = 0.27, 95% Lower = 0.25, CI Upper = 0.29) suggesting that our simulations were at least plausible.

Increasing the number of generations produced marginal increases in spatial autocorrelation as well as concomitant increases in the distance at which spatial autocorrelation was significant (Fig. 5). However, these increases were small and followed an asymptotic relationship (Fig. 6) suggesting that our results were relatively insensitive to the number generations in which neighbor mating occurred prior to applying STRUCTURE.

Discussion

Before discussing the results, it should be noted that the STRUCTURE instruction manual states that, "Isolation by

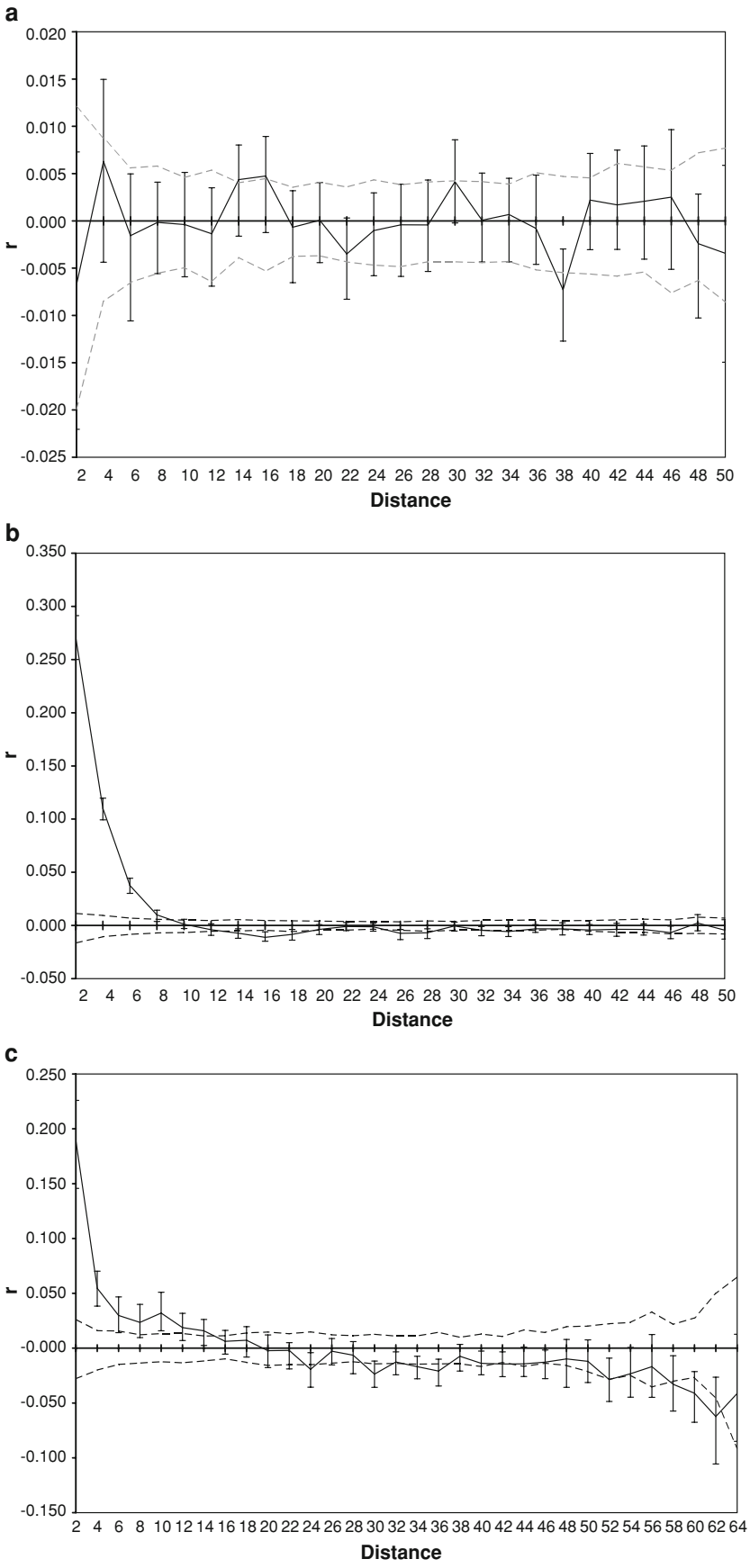

Fig. 4 Spatial autocorrelation correlogram plots of (**a**) the trapper sampling (random model) at t_0, (**b**) the trapper sampling at t_{20}, after the neighbor mating gradient was established, and (**c**) the black bears (*Ursus americanus*) dataset from North Idaho (9 locus microsatellite dataset). The *y* axis is the genetic correlation coefficient (*r*) as computed by Peakall and Smouse (2006) as a function of distance. The 95% confidence interval (dashed lines) around the null hypothesis of a random distribution and the bootstrapped 95% confidence error bars around *r* are also displayed

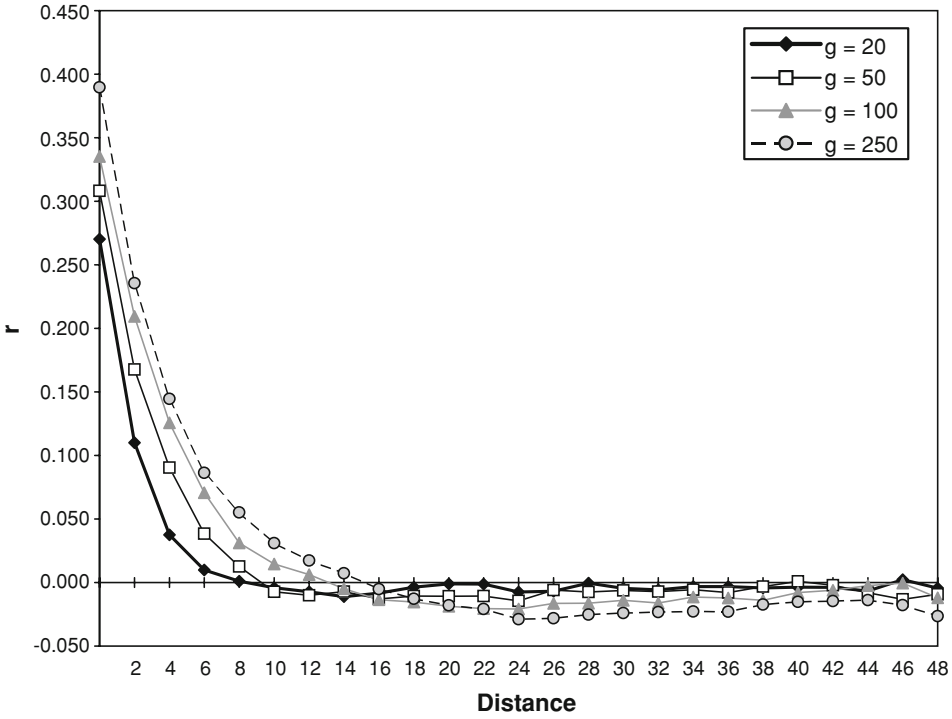

Fig. 5 The effect of number of generations (g) on spatial autocorrelation magnitude (r) and distance in these simulations, suggesting the plausibility of these simulations

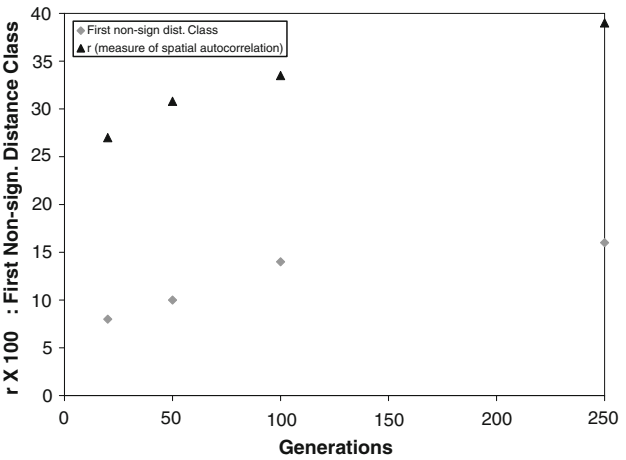

Fig. 6 The relationship between generation length and autocorrelation magnitude (r; times 100 for visual purposes) and distance of the autocorrelation effect. Both relationships are asymptotic

distance refers to the idea that individuals may be spatially distributed across some region, with local dispersal….The underlying STRUCTURE model is not well suited to data from this kind of scenario….In such situations, interpreting the results may be challenging" (Pritchard et al. 2007). Thus, while Pritchard et al. (2007) speak to the limits of their program, their advice appears to be ignored in many landscape genetic papers. Specifically, the practice of using STRUCTURE to determine the best supported K presupposes no a priori knowledge as to what factors (e.g., distance, ecological barriers, etc.) structure populations. Thus, although STRUCTURE and other similar programs allow priors to be incorporated to the analysis, due to the manner in which they are commonly used, they seldom are.

Our principle findings are consistent with Pritchard et al.'s (2007) advice. STRUCTURE does identify existing structural patterns in our data. For example, consider Fig. 1b showing the Research sampling where STRUCTURE suggests that there are seven populations, when in fact there is one population with neighbor mating forming local areas of relatedness. Because the local areas of relatedness were consistent with sampling patterns, there was a high degree of association between spatial blocks and STRUCTURE clusters. This is a valid analysis of the existing population sub-structure, but without additional information interpretation is problematic.

We might, for example, believe that we were observing a highly fragmented population consisting of many largely independent and well defined subpopulations, but with some level low levels of migration. Confidence in our result would be further bolstered by the relatively large pairwise F_{ST} results associated with the defined clusters. These patterns, in turn, could be correlated with various putative barriers such as roads, valleys, high ridges, and human settlements leading to the telling of a variety of compelling, but erroneous, stories. Further, consider the 37 samples identified by green blocks in Fig. 1b. Thirty-two green blocks are found in one cluster at the top, center of the simulated landscape. The remainder are scattered throughout the plot. We could claim that the top center block is an isolated population that produces a few dispersers (5 found in 3 other blocks) and maintains its genetic

variation through low levels of immigration from four different blocks (producing the yellow, grey, maroon and blue blocks). If these data were used to inform conservation decisions, one might allocate scarce resources to the top center block to bolster productivity or increase connectivity.

Our simulated neighbor mating, where mating is always with one of eight nearest neighbors, is only a model. However, many natural mating structures can and do produce similar patterns of local autocorrelation, as demonstrated by our empirical black bear dataset (Fig. 4c). Patterns of local autocorrelation will lead samples to appear strongly clustered if sampling occurs at similar or smaller scales than the autocorrelation. In our simulations, the Research sampling areas are 6 × 6—small enough that all organisms sampled in an area are more closely related than would be expected in a panmictic population (Fig. 4). Therefore, in the research sampling simulations STRUCTURE grouped individuals into areas that made geographic sense and were visually appealing. However, when sampling areas were larger than the correlogram plot asymptote, assignment patterns made little intuitive or geographical sense.

In many field studies that use microsatellite data to investigate population structure, sample collection is ad hoc. Samples are frequently obtained from trappers (Kyle and Strobeck 2002; Cegelski et al. 2003), individual research efforts (Tallmon et al. 2002), or combinations of the two (Schwartz et al. 2003). If patterns of local autocorrelation exist in the population, then divergent spatial and temporal scales associated with these ad hoc samples can produce misleading patterns. For example, in our combined research/trapping simulation (mixed sampling), the "research" area looks different and unique in an otherwise pattern-less landscape (Fig. 1f). This, however, is an artifact of the research area being on the same scale as the autocorrelation pattern rather than any unique properties associated with the sampling area.

Our results presented here should be expanded upon with additional simulations. For example, we did not increase or decrease the number of loci. Instead we used a number commonly used in many wildlife and conservation genetics studies. Additional simulations could also choose to use higher numbers of bi-allelic markers to simulate single nucleotide polymorphisms (SNPs) or other types of genetic markers. Futhermore, sample size and the interaction of sample size with number of loci could be modeled. We did, however, ensure that our results were not an artifact of a single realization by running each sampling scenario for three iterations on two additional neighbor mating surfaces. In 3 cases (random, line transect, and multi-generational), the most supported K varied one group from the reported averages: 6 and 4 for random (reported = 5); 7 and 6 for line transect (reported = 6) 7 and 5 for multi-generational (reported = 6). In no cases was K = 1.

Recommendations and conclusions

Based on these simulations, we believe that the practice of applying STRUCTURE (and likely any other clustering program) to ad hoc data, finding the most likely K, and subsequently constructing narratives to explain the derived patterns could lead to erroneous conclusions regarding the role of landscape features on genetic structure. Furthermore, implementing management policies based on incorrect inferences from these patterns could be hazardous. Prior to applying clustering algorithms to samples, some understandings concerning patterns of within-population genetic correlation must be derived. We therefore recommend that, prior to analyzing population structure, one determines the patterns of local autocorrelation and then carefully considers how these patterns may influence results. This is particularly true when applying powerful pattern recognition programs like STRUCTURE. Examining the patterns of local relatedness can be accomplished using individual based models through commonly applied autocorrelation analyses, Mantel tests, isolation by distance plots, or through examining relatedness or kinship (using CERVUS (Kalinowski et al. 2007) or other similar programs) between spatially close individuals. Examining each individual's estimated proportion of population membership (Q in program STRUCTURE) over several iterations, to look for patterns that indicate isolation by distance is structuring individuals (see Rosenberg et al. 2002), however, does not appear to be completely reliable given our simulations. In the line transect and mixed sampling schemes mean Q, median Q, clusteredness, and the ratio of mean Q to random expectations were all low, which may serve as a signal that the underlying surface is due to isolation by distance through neighbor mating. However, even in these sampling schemes median Q was nearly twice the random expectation with the maximum Q being near unity. For research sampling, median Q was 0.799 and clusteredness was 0.721 (Table 3) which, given the number of loci and sample size would be considered strong support for clustering (see Rosenberg et al. 2002, 2005).

Determining the presence of local autocorrelation (likely to confuse clustering programs such as STRUCTURE) is not adequately addressed by standard pair-wise F_{ST} tests for isolation by distance between study areas or groups of samples (Fig. 5). In our simulations and the Idaho black bear data, spatial autocorrelation occurred at very fine scales, below the level of the study area. While F_{ST} was significant between research sampling locations,

and STRUCTURE's clustering results were visually compelling, the pairwise F_{ST} between the research sampling areas was not significantly related to geographic distance. Thus, tests for isolation by distance between study areas would be rejected, erroneously reinforcing the idea that barriers were leading to the observed structures. We therefore suggest sampling on a fine-grained grid, relative to the species life history, and evaluating spatial autocorrelation within each study area using correlogram plots similar to Fig. 4.

The effects of spatial autocorrelation on group statistics are not limited to clustering programs like STRUCTURE. If local autocorrelation exists, Pairwise F_{ST} values will depend strongly on the relationship between the scale of the sampling frame and the semivariogram asymptote. If the sampling scale is smaller than the asymptote, then the autocorrelation will decrease within-group variance and reinforce between-group patterns. If larger than the asymptote, fine-scale autocorrelation will increase within-group variation thereby decreasing the strength of between group patterns. For example, in the research sampling, where the sampling is at a scale equal to the autocorrelation, F_{ST} (between group variance) was significantly different than zero ($F_{ST} = 0.086$), whereas it was much lower in the Corner sampling ($F_{ST} = 0.010$) where the sampling was at a scale larger than the local population autocorrelation.

Lately, there has been a trend to use genetic clustering programs that impose a geographic constraint on the results (e.g., BAPS2, Corander et al. 2004; GENELAND, Guillot et al. 2005a, b; HMRF models, François et al. 2006). Further simulations should be conducted to determine if spatial constraints improve clustering results when samples are drawn unevenly and when autocorrelation patterns exist.

While other fields of the natural sciences (e.g., ecology, geology) have focused intensively on study design and the effect of spatial structure in biasing results (Sokal et al. 1998; Legendre et al. 2002, 2004); we believe the field of molecular ecology has paid less attention to these sampling issues. Our simulations demonstrate that sampling can have a large effect on subsequent results in a common research situation (isolation by distance and patchy sampling) and likely have major impacts on the interpretation of data from wild populations. None of the results from our various sampling approaches suggest a single biological population, sampling had strong effects on the number (Figs. 2, 3); spatial pattern (Fig. 1) and strength of membership (Table 3), and hence both the assumed reliability of the clusters and their biological meaning. At times it won't be possible to collect samples in a perfect grid pattern, and we do not suggest that this is strictly required. However, we recommend that researchers pay more attention to the impacts that sampling schemes may have on their results, especially as they examine questions related to substructure.

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