AN INVESTIGATION OF FINESCALE GENETIC STRUCTURE OF WOOD FROG (*RANA SYLVATICA*) BREEDING SITES IN A FRAGMENTED HABITAT

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ABSTRACT

AN INVESTIGATION OF FINESCALE GENETIC STRUCTURE OF WOOD FROG (*RANA SYLVATICA*) BREEDING SITES IN A FRAGMENTED HABITAT

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Habitat fragmentation is often assumed to lead to the genetic subdivision of populations. Using eight neutral microsatellite DNA markers, I tested whether recent anthropogenic habitat fragmentation in Wellington County and the Greater Toronto Area (GTA), Ontario, Canada correlated with genetic subdivision of wood frog (*Rana sylvatica*) populations. Geographic distance, road density, canopy cover, and watershed discontinuity were chosen as relevant geographic features affecting the wood frog. Of the chosen geographic features, geographic distance accounted for most of the observed variance in neutral allele frequencies; however, there was also significance for road density. Overall, there was very little genetic differentiation between the breeding sites, with the exception of one site in the GTA. This suggests that large effective population sizes, compounded with sufficient migration between sites, and little divergence time have contributed to the observed genetic homogeneity.

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INTRODUCTION

Natural or anthropogenic ecosystem modification can lead to discontinuous genetic structure across a species range (Young *et al.* 1996, Mossman and Waser 2001, Williams *et al.* 2003). Understanding the processes that contribute to genetic subdivision has long been a subject of interest, as it can have important evolutionary and ecological consequences. Small and isolated sub-populations suffer from the effect of genetic drift because of their typically small effective population size and low amounts of migration, and so are generally more at risk of extinction from stochastic processes (Wright 1931, Soulé 1986). Increases in the intensity of anthropogenic habitat fragmentation have only served to further interest in studying the process of population subdivision for conservation and management purposes, particularly, in identifying these small and isolated sub-populations. Detecting patterns of genetic population structure over short time frames and fine spatial scales is central to understanding how sudden ecosystem changes affect a species migration and dispersal abilities. As population structure is associated with both migration and dispersal, testing hypotheses about factors affecting population structure at fine scales allow investigators to make inferences about the dispersal and migration capabilities of a given species (Bohonak 1999).

Developments in both analytical and molecular techniques have fueled research in this area. Landscape genetics has elucidated the importance of geographic and environmental features in shaping population subdivision (Manel *et al.* 2003), especially natural rather than anthropogenic features, such as mountains and ridges (Funk *et al.* 2005a, Spear *et al.* 2005), snowmelt gradients (Hirao and Kudo 2004), and drainages (Antolin *et al.* 2006). Landscape genetics is popular as a theoretical construct and has

been applied mostly to coarse geographic scales. Assignment tests or assignment methods have also recently gained popularity in identifying structured populations (Manel *et al.* 2005). These methods enable researchers to ask detailed questions about specific individuals when population data is significantly structured (Funk *et al.* 2005a). They are more powerful in resolving finescale population structure than traditional measurements like *F* statistics, as they are focused exclusively on individuals, and have been widely used to estimate genetic differentiation over small spatio-temporal scales. However, their performance at finescales, where differentiation between sites is likely to be modest ($F_{ST} \leq 0.05$) yet significant, still needs exploration (Manel *et al.* 2005).

Microsatellite DNA loci have been widely used in studies of population genetics since the mid-1990s. Microsatellite DNA loci are non-coding, highly variable nucleotide repeats (2-4 base pairs in length) which occur with varying frequency in the genomes of most organisms. Assumedly neutral, they are particularly useful in paternity assays and in the detection of fine-scale population genetic differentiation and subdivision, and therefore should be best suited to the tasks of inferring recent fine scale patterns of dispersal, migration and population structure. Selkoe and Toonen (2006) proposed guidelines to counter recognized drawbacks: using a large and approximately equal number of samples for each collection site (ideally 50 or more); using a large number of loci to get a better sub-sample of the genome (seven or more); fitting the variability of loci with the temporal and spatial scale of the question, for example, studying populations at smaller scales generally require a microsatellite markers with higher mutation rates and genetic variability (employing tetranucleotide markers as opposed to dinucleotides); and characterizing the mutation model the loci are likely following are among the

recommendations for the use of microsatellite DNA loci (Selkoe and Toonen 2006). With advice being heeded in this study, microsatellite DNA loci are a powerful and useful tool for investigating patterns of migration and fine scale population structure.

Amphibians may be most susceptible to fragmentation induced population structure, as they are not typically adept dispersers and have very specific habitat requirements (Duellman 1994). Amphibians are sensitive to terrestrial, aquatic and air pollution due to their porous and absorbent skin (Duellman 1994), and have been targeted as good ecological indicators species of enivornmental quality and habitat fragmentation (Blaustein and Wake 1995; Collins and Storfer 2003). The potential utility of amphibians as genetic indicators of habitat fragmentation has been growing in popularity, specifically in areas coinciding with large mountains and refugial populations (Monsen and Blouin 2004; Funk *et al.* 2005b). However, few studies (though see Johansson *et al.* 2005) have used amphibians to test for genetic fragmentation in landscapes that have been recently altered and are almost exclusively dominated by anthropogenic features.

The North American wood frog is one of the best studied amphibian species, and has been persistently used as an indicator species of environmental quality and habitat fragmentation in many studies (Rubbo and Kiesecker 2005; Sanzo and Hecnar 2006). Wood frogs span the largest and the most continuous historic and present geographic range (10 000km^2) of any of the North American ranids (true frogs) from Alaska to the Appalachians (Berven 1990). Age of first reproduction varies throughout the species range; from 1-2 years for males/2 years females in southern Michigan (Collins 1975), 1-2 years for both males and females living at low altitudes in Maryland, and 3-4 years for both males and females living at higher altitudes in Maryland (Berven 1981). The sex-

ratio for pond breeding adults is male biased; Howard and Kluge (1985) estimated average 1.85 : 4.00 females to males over a period of three years, while Berven and Grudzien (1990) estimated an average male-biased sex ratio of ~1:2 over a period of five years for five ponds at higher altitude in Virginia, USA. Correspondingly, male mating success was measured at 39% (Howard and Kluge 1985) and ~50% (Berven and Grudzien 1990). Developmental rate also varies both altitudinally (Berven 1982) and latitudinally (Riha and Berven 1991) and may even vary at smaller geographic scales due to changing micro-climatic variables (e.g. water temperature, hydroperiod) associated with changes in amounts of canopy cover (Skelly 2004). Wood frogs tend to breed in large aggregates numbering up to the thousands over a period of ~1-2 weeks (Wells 1977, Howard and Kluge 1985), usually, but not exclusively, in small vernal pools in the early spring. Two previous studies (Newman and Squire 2001; Squire and Newman 2002) examined finescale population structure of wood frogs using five trinucleotide microsatellite DNA loci. Both were conducted at small spatial scales; pairwise geographic distance ~0-10 kms between sites. Though, differences in landscape variables were not accounted for in either study, as Newman and Squire (2001) focused on wood frog populations located within a single continuous forested habitat patch, and Squire and Newman (2002) focused on the same spatial scale of wood frog populations within a prairie pothole environment.

Here, I investigate the finescale genetic population structure of wood frogs in southwestern Ontario, an area that is heavily fragmented by human development, but historically composed of a largely continuous deciduous forested landscape (Lovett-Doust *et al.* 2003). Microsatellite DNA loci were used as genetic markers in both

traditional genetic analyses and assignment methods to test the hypothesis that fragmentation causes finescale population structure. Five broad classes of geographic features were measured to assess their effects on genetic population structure. First, I assessed the feature of pairwise geographic distance and then focused on the four remaining features: sites located on either side of a major highway, road density, canopy cover between sites, watershed discontinuity. I did not include more geographic parameters, as testing too many models and parameters leads to spurious results (Anderson and Burnham 2002).

Hypothesis/Prediction 1: If pairwise geographic distance is significantly related to observed pairwise genetic differentiation, then wood frog sites that were further away from each other would have greater values of genetic differentiation and that there would be a significant relationship of genetic isolation by distance.

Hypothesis/Prediction 2: If there was residual variation in the relationship between pairwise geographic distance values and pairwise values of genetic differentiation, then one or a combination of certain landscape features would contribute to the observed residual variation.

METHODS

Study area and sample sites

Study sites were located within Wellington county (2542km^2) and the Greater Toronto Area (GTA), Ontario, Canada (7061 km^2), two of the most populous and fastest

growing regions of Canada. Wellington County has ~ 2.7 million and the GTA has ~5.8 million human inhabitants (Government of Ontario 2006). There are a high proportion of small fragmented forest tracts (primarily deciduous), surrounded historically by agricultural land (Larson *et al.* 1999, Kerr and Cihlar 2003) that has been shifting to urban development (Caldwell 2001; Lovett-Doust *et al.* 2003).

In March 2005, potential wood frog breeding sites were chosen based on generalized habitat preferences mostly comprising forested areas with small depressions, rural roadside ditches, marshes and swamps with canopy cover. Call surveys were conducted during evenings in late March and early April 2005 to identify the potential breeding areas of wood frogs. I attempted to partition sites located between, in, or in close proximity to three of the primary urbanized areas of Wellington County: Guelph, Kitchener-Waterloo, and Cambridge (Figure 1). A total of and 399 adult individuals from nine of the Wellington sites and tadpole larvae from 50 individual egg masses was collected from one Wellington site (W2) in spring 2005, and a another 48 adult individuals were collected from site W3 in 2006 to bolster the overall sample size (Figure 1, Table 1). I had conducted previous unsuccessful population surveys early in the fall of 2004 within the GTA, and only one site (York) was used from the GTA area (Figure 1, Table1). A total of 24 adult individuals were collected and donated by Dr. Lawrence E. Licht for genotyping, from a small woodlot pond located on the York University campus in the spring of 2005 (Figure 1, Table 1).

Originally, I assumed that each collection site comprised a single deme (a randomly mating sub-population of wood frogs), and thus, that sub-populations at each site had reached genetic equilibrium between migration, mutation and drift under

Wright's island model. Each site was delineated by boundaries specific to a single woodlot, normally a road, or a minimum distance of ~1km. Several pools, marshes or swamps were typically located within a single collection site.

Adult wood frogs were caught and toe-clipped using a pair of nail clippers or surgical scissors, which were cleaned after each use by rinsing with 95% ethanol, heated with a butane lighter, and a final rinsing with ddH_20. Samples were fixed in 95% ethanol and were stored at –80°C or –20°C in lab until use. At one site (Wellington # 2), developing embryos, rather than adults, were collected. A single embryo from each egg mass was used and assumed to be an unbiased sample of the alleles of the parental generation, as only one female lays one egg mass (Howard and Kluge 1985).

York as an outlier from the Wellington collection sites

The York site represented an outlier from the rest of the dataset; as it is surrounded by urban structures that were slowly built up over the past ~40 years; the population is known to be small (Licht, pers.comm.); and it was the collection site with furthest average pairwise geographic distance values. For all non-independent statistical analyses carried out, the York site was first excluded and then included with the Wellington sites to examine whether it had undergone a reduction in neutral genetic diversity (a bottleneck) or whether it was different based on geographic distance.

Landscapes variables

Roads

The presence of roads has been correlated with observed genetic differentiation between populations for diverse animal taxa: e.g. beetles (Keller *et al.* 2005), coyotes and bobcats (Riley *et al.* 2006), and moor frogs (Vos *et al.* 2001), and to negatively affect recruitment rates of populations of spotted salamanders (*Ambystoma maculatum*) (Gibbs and Shriver 2005), a species which is similar in life-history traits to the wood frog. In New Brunswick, Canada incidence of road crossings and associated mortality of wood frogs was the highest of 10 amphibian species studied (Mazerolle 2004). I predicted that groups of sites associated with greater road density between groups, would be more genetically structured than sites grouped based on geographic distance, especially for sites on different sides of one the busiest highways in the world, the McDonald-Cartier freeway (Li *et al.* 2005).

Canopy cover

Wood frogs favour pools with more canopy cover (Skelly 2004, Gibbs *et al.* 2005), and wood frogs are assumed to have smaller offspring in uncovered anthropogenic pools, such as roadside ditches, ruts created by construction equipment, (DiMauro and Hunter 2002). I expected roads and agricultural fields to correlate with greater genetic differences among groups of sub-populations. I predicted that the amount of canopied habitat between groups of sub-populations would ameliorate those effects and that there would be less genetic structure observed between groups with large amounts of canopy cover between them.

Watershed discontinuity

Sample sites were located in four different secondary watersheds within the primary watershed of the Great Lakes: Grand River, Credit Valley, Halton, and the Toronto Region watersheds. Austin *et al.* (2004) found presence of a secondary contact between populations of bull frogs (*Rana catesbeiana*) and spring peepers (*Pseudacris crucifer*) on two different sides of the Grand River watershed. Drainage basins are among the geographic features thought to contribute to anuran population structure (Lougheed *et al.* 1999). Conversely, drainages and waterways have also been shown to facilitate past amphibian migration within southern Ontario (Smith and Green 2004). Given that southwestern Ontario is primarily flat with no drainages produced from post-glacial movement, the choice of molecular marker should only point out more recent differentiation. Based on these studies, I predicted that very little allelic variation could be attributed to sites grouped on the basis of secondary watersheds. By including watershed discontinuity in the AMOVA analysis, detection of possible earlier divergence between groups of wood frog sub-populations was excluded.

Laboratory protocols

Wood frog DNA template was isolated using a standard phenol-chloroform protocol from toe clips of adults and the tail tips of tadpole larvae collected from the Wellington #2 site. DNA was rehydrated in 100µl of 10X TE (0.01M Tris-HCl, 0.001M EDTA) buffer or ddH_20. Extracted samples used for microsatellite analyses and genotyping are listed in Table 1. For polymerase chain reactions (PCR), I used eight

tetranucleotide microsatellite loci designed for the wood frog as described by Julian and King (2003) (Table 2). I chose tetranucleotide markers because of their increased variability and their high degree of genetic variability appropriate for populations with short divergence time, compared to di-and tri-nucleotide markers (Ellegren 2004). All forward primers for each locus were labeled with fluorescent TET (6-carboxy-1,4-dichloro- 2', 4', 5', 7'-tetrachlorofluorescein) dye. PCR conditions were 94°C for 1 minute, then 30 cycles at 94°C for 45 seconds, then individual primer annealing temperatures for 45 seconds (see Table 2), 72°C for 30 seconds, and a final extension time of 72°C for 5 minutes. PCR products were stored at 4°C prior to running them on a polyacrylamide gel (details specified below). All reactions were carried out using MJ Research PTC-200 thermocyclers. Each reaction had a total volume of 25µl: 1µl of DNA template, 1X ThermoPol™ Buffer [New England Biolabs] (2.5µl), 0.2mm of each dNTP [Roche] (0.6µl), 0.4µM of each primer (0.2µl), 19.25µl of ddH_20, and 1.5U of Taq DNA Polymerase™ [New England Biolabs]. Amplified allele products for each locus for each collection site were run on 6% polyacrylamide gels (6% acrylamide (19:1), 0.5X TBE, 47% Urea). To ensure more uniform scoring between separate gels for the same locus, PCR products were run twice or several (2-5) previously amplified products were included from an earlier gel. Acrylamide gels were run at a constant 1600V, with variable wattage and milliamps. Acrylamide gels were scanned using a Hitachi FMBioII® imager (settings: Channel 2, 585nm), and were scored relative to a TAMRA™ (6-carboxytetramethylrhodamine) size standard marker (Genescan™ 350, Applied Biosystems) with ladder markings at 350, 340, 300, 250, 200, 160,150, 139, 100, and 75 bp using Hitachi FMBioII® Imaging software version 1.5.

Data Analyses

(i) Variation within collection sites

Significant departures from Hardy-Weinberg equilibrium were examined using exact tests as described by Guo and Thompson (1992), as chi-square tests can yield inconsistent results for multiple alleles in both small and large datasets (Wigginton 2005). Tests were performed in ARLEQUIN (version 3.1; Excoffier *et al.* 2005) with 1 000 000 steps in Markov chains and 100 000 burnin steps to help ensure values of standard deviation ≤ 0.01. Pairwise linkage disequilibrium between loci and probabilities for heterozygote excess, heterozygote deficiency were tested for each locus, for each collection site, and globally using GENEPOP (web version 3.4; Raymond and Rousset 2003) with Markov chain steps 10 000 000 and burnin period of 10 000 to ensure standard error values ≤ 0.01. All *P*-values for each collection sites were sequentially Bonferroni corrected for multiple tests (Rice 1989).

(ii) Differentiation between collection sites (equilibrium methods)

Weir and Cockerham's (1984) θ was used to examine multilocus pairwise genetic differentiation between the nine collection sites, which assumes mutation-drift as well as selection-gene flow equilibrium. As an estimator of the Wright's (1921) F_{ST}, θ corrects for the effects of unequal sampling of individuals within subpopulations, as well as finite subpopulation sizes. Pairwise θ values were calculated using ARLEQUIN with 10 000 permutations to ensure small values of standard deviation. θ, as an estimator of F_{ST} is superior to R_{ST} (Slatkin 1995), because of greater variance in R_{ST} when the number of

loci is small (Balloux and Goudet 2002), and the requirement that all loci fit the single stepwise mutation model (SMM) (Ohta and Kimura 1973), where it is assumed that mutation for a microsatellite loci occurs for adjacent repeat numbers (alleles) (see Appendix 1).

The relationship of pairwise estimators of F_{ST} to gene flow (migration), and by proxy, population structure, is ill-resolved and generally unrealistic, particularly when F_{ST} values are small (Appendix 2). If pairwise θ values were < 0.05 (even if they were statistically significant, $P \leq 0.05$) then that would point to little genetic population structure. If pairwise θs were ≥ 0.05 and statistically significant, then these values were deemed to be indicative of biological genetic differentiation between two sites.

To test whether pairs of sites deviated from the expectation of random distributions of individuals (i.e. panmixia), exact tests for population differentiation were carried out in ARLEQUIN (Raymond and Rousset 1995). Exact tests are designed for diploids when parentage is unknown and loci are assumedly unlinked (Goudet et al. 1996). Based on Fisher's 2x2 contingency tables, they are more powerful than similar tests of genetic differentiation when sample sizes between sites are unequal. One million Markov chain steps with 100 000 burnin steps were performed to ensure small standard error values. If there was statistical significance between two collection sites, then these sites deviated from the null hypothesis of panmixia potentially indicating that individuals at one of the two sites were experiencing assortative mating, lack of gene flow, a high degree of mutation, or a combination of these effects.

(iii) Differentiation between collection sites (non-equilibrium methods)

Given the high mutation rate of microsatellite DNA loci, large population sizes of wood frogs (thus small genetic drift), and weak or no selection on neutral microsatellite markers, much of the data were likely deviating from the assumptions of genetic equilibria. To make up for this, I used non-equilibrium analyses to estimate population parameters based on individuals and groups of sites.

To initially qualitatively assess population subdivision coinciding with canopy cover, watershed discontinuity, distance, and roads, I used locus by locus analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) using ARLEQUIN. Locus-by-locus AMOVA is particularly useful when the sample sizes for collection sites are small (Michalakis and Excoffier 1996; Yang 1998), as it compensates for missing data by analysing each locus independently, using permutation tests at four hierarchical levels: among groups (specified by original hypotheses and predictions) (see Tables 6 and 7, and Figures 2-11); within groups among collection sites; among individuals within collection sites; and among sites among groups. Five groupings were analysed, first without the outlying York site, and then with York. The groupings were based on landscape and geographic features set out prior to collection: sites grouped by road density with minimum pairwise geographic distance between sites grouped together ≤10km; sites grouped on the basis of which side of the M-C 401 highway they were located; site with furthest average pairwise straightline geographic distance; sites grouped with maximum canopy cover with minimum pairwise geographic distance between sites grouped together ≤10km; and sites grouped together according to their secondary watershed.

All landscape variables were measured from maps created using ESRI® ArcMap™ 9.1 ArcEditor, using geospatial data from the Ontario Ministry of Natural

Resources Land Cover Database derived from digital, multispectral LANDSAT Thematic Mapper data collected between the years 1991-2002 (Figures 2-11). The original 28 detailed landcover classes for this dataset were simplified to include just four landcover types: water cover, agricultural land, canopy cover, and developed land (Figure 1). Road cover geospatial data was from the Ontario Fundamental Data Set obtained through the Ontario Geospatial Data Exchange, with all types of roads quantified as line features between the years 1977-1996.

To detect finer scale structure between sites, assignment methods were performed using software package STRUCTURE (version 2.1; Pritchard *et al.* 2000). Such methods have the potential to assist in detecting fine scale genetic structure, and may be helpful when the biological system shows little genetic differentiation as its focus is on individual organisms. This is in contrast to traditional equilibrium methods of analyses which automatically assign individuals to the presumed sub-populations where they were collected. Furthermore, STRUCTURE has been useful in detecting population structure in other species of ranids of differing vagilities, at both large and smaller geographic scales (Zeisset and Beebee 2001; Funk *et al.* 2005a). STRUCTURE uses a Bayesian clustering approach to estimate the number of sub-populations (K), and assigns individuals to one or more of these putative sub-populations based on their genotypes. Five independent runs were performed for each K-value to verify convergence; estimates for K ranged from 1-3 putative sub-populations. For the preliminary analyses, no prior information was specified about the site of origin for any of the 396 individuals. Correlated allele frequencies and genetic admixture models were used, as I assumed that divergence time between all of the collection sites was minimal, and that in recent

history, there was likely some degree of genetic exchange between breeding aggregates. Further specified STRUCTURE analyses were performed under the groupings of sites that were statistically significant in the AMOVA analyses. All Markov chains consisted of 100 000 burnin steps, and 1 000 000 additional iterations to ensure convergence between independent runs. The *K* and *lnP(X|K)* were averaged from the five independent runs and the posterior probability, *lnP(K|X),* was calculated using Bayes' rule. STRUCTURE assumes that all loci conform to Hardy-Weinberg Equilibrium, are freely recombining, and not under any specific model of mutation for microsatellite DNA loci. This last assumption may be unrealistic as microsatellites typically have high rates of mutation or rates of mutation may be different for each locus (Ellegren 2004). For the AMOVA analyses that were statistically significant, it was expected that STRUCTURE runs would identify the specific individuals and genoytpes contributing to differences in distributions of allele frequencies. Furthermore, that they would complement the statistically significant AMOVA analyses with low *ln* likelihood values, $\geq$ -3000, associated with high posterior probabilities, ~1.0.

(iv) Likelihood of single stepwise mutation model (SMM) for microsatellites and estimation of effective population size for all sites

Mutation is a major source of genetic variability in microsatellite DNA loci with mutation rates (μ) ranging from 10^{-2}-10^{-6} (Ellegren 2004). Examining mutation models of each locus is important for further analyses of any sites (e.g. York) that might have experienced a reduction in allelic diversity due to lack of migration, as mutation is then one of the only existing sources creating new neutral genetic diversity. Analyses in

population genetics generally assume a single stepwise mutation model (SMM; Ohta and Kimura 1973) for microsatellite DNA loci evolution. For loci that follow the SMM this results in an approximately normal distribution of microsatellite allele frequencies (Appendix 1). Although even when microsatellite allele frequency histograms appear normally distributed, the model has been shown to produce spurious allelic distributions (Valdes *et al.* 1993). To test whether loci fell under the SMM, each locus was examined under a maximum likelihood approach using the program MISAT (Nielsen 1997). MISAT estimates the parameters Θ and p, where $\Theta = 4N_e\mu$, N_e is the effective population size, μ is the mutation rate of each microsatellite DNA locus, and p is the proportion of multi-step mutations, which indicates whether microsatellite loci were better fit to a generalized stepwise mutation model (GSM) than the SMM. For testing the GSM, the estimate(s) for minimum and maximum proportion of multi-step mutations were set at $p = 0.01$, and $p = 0.5$, respectively, and the initial value for $p = 0.02$ for all 8 loci. The initial value for Θ was taken using the method of moments estimate (Waples 1989). All Markov chains were run for a minimum 1 000 000 iterations to ensure accurate estimation of log L (Θ). For testing the SMM, the initial estimates(s) for Θ was taken again by using the methods moments estimate, all Markov chains were run for a minimum 100 000 iterations.

The parameter for effective population (N_e) size contributes to the amount of observed neutral genetic diversity and indirectly, the amount of migration between sample sites. Effective population sizes of each site and for the entire region were roughly estimated based on the proportion excess of heterozygotes compared to those in Hardy-Weinberg equilibrium at each sampling site (Pudovkin *et al.* 1996; Luikart and

Cornuet 1999). The program N_eESTIMATOR (Peel *et al.* 2004) was used to do this for the overall population, n=396, and for 24 samples (bootstrapped) from each collection site, based on the lowest sample size, the York site. If N_e sizes were large or infinite, then this would be indicative of a large amount of neutral genetic variation coming from within the collection sites themselves.

(v) Isolation by distance – Mantel test

A regression analysis of Rousset's (1997) $\theta / (1 - \theta)$ values of genetic distance against geographic distance (in kms) was performed to determine if there was a significant relationship between the two dissimilarity matrices. Mantel tests were carried out with 30 000 permutations using ISOLATION BY DISTANCE WEB SERVICE (IBDWS) (version 2.6; Jensen *et al.* 2005) to ensure small values of standard error. Relationships between logarithmic genetic distance and logarithmic geographic distance were also included in the analysis to normalize the distribution of values in the data. I performed two sets of isolation by distance analyses, one with the York site included, and one set without that site. If the Wellington sites showed a significant IBD relationship ($P \leq 0.05$), then it would indicate that wood frogs were limited in their dispersal based on increasing geographic distance between sites. If the York site showed a significant IBD relationship, then any of its genetic differences could potentially be attributed due to its having the largest average pairwise distance of any of the sites included in the analysis. Geographic straight-line distances between collection sites were estimated using latitude/longitude distance calculator available from: (http://jan.ucc.nau.edu/~cvm/latlongdist.html) and are presented in Appendix 3. As per

Berven and Grudzien's (1990) finding that wood frog dispersal should be severely limited outside a 1km radius, we included the lowest pairwise distance value between sites W#3 and W#4 which was ~1km.

(vi) Reduction in genetic variability for the York site

To estimate whether the York site had undergone a significant reduction in the number of alleles, the Garza-Williamson (G-W) statistic (Garza and Williamson 2001) was calculated for each locus and each collection site using ARLEQUIN. The allelic richness for each locus at each collection site was also estimated using FSTAT (version 2.9.3.2; Goudet 2001). Allelic richness is defined as the number of alleles within a collection site standardized for the lowest number of samples between collection sites; for my dataset the number was standardized to 24 diploid individuals, for the site that contained the smallest sample size of individuals. Locus *RsyD33* had low numbers of alleles per site, and therefore was not included in this analysis. I then compared samples from all Wellington collection sites to the samples from the York site using a simple one-way analysis of variance (ANOVA) in SPSS version 12 for Windows (SPSS Inc., 2003). If York wood frogs had experienced a reduction in neutral genetic diversity (a bottleneck) associated with little migration, then York values of allelic richness would be significantly different than values of allelic richness of the combined Wellington county sites.

RESULTS

(i)Variation within collection sites

Seven of eight examined loci were polymorphic for the eight Wellington sites with number of alleles (N_A) ranging between five and 41. For the same seven loci at the York site, N_A ranged from two to-15. Locus *RsyD33* had N_A ranging from 1-2 (100bp-104bp), with one collection site, Wellington #3 having a private third allele product of 124bp. Private alleles for each locus and collection site are listed in Appendix 4.

Following sequential Bonferroni correction ($P \leq 0.00625$ for Wellington #2-8, $P \leq 0.00714$ for Wellington #1 and York), all 78 exact tests conformed to Hardy-Weinberg equilibrium (Table 3). Significant values for heterozygote deficiency were detected for locus *RsyD88* for sites W#2 ($P = 0.0003$, S.E. = 0.0001), W#3 ($P = 0.0059$, S.E. = 0.0004), and W#8 ($P = 0.0035$, S.E. = 0.0002). There were no significant values of heterozygote excess for any locus or any site. No comparisons between pairs of loci for each collection site or overall sites yielded a statistically significant *P*-value, indicating all loci were unlinked with each other (Table 4).

(ii) Differentiation between collection sites (genetic equilibrium assumed)

For 36 pairwise tests of genetic differentiation (θs), 15 (42%) tests were significant for $P \leq 0.001$ level, 10 (28%) tests were significant for $P \leq 0.01$ level, seven tests (19%) $P \leq 0.05$ level, and 4 (11%) tests were not significant (Table 5). Larger pairwise differences were detected for the York site, ranging from 0.0891-0.13113; all values were significant ($P \leq 0.001$). All 36 exact tests of population differentiation for diploid individuals yielded no significant *P*-values.

(iii) Differentiation between collection sites (non-equilibrium results)

Locus-by-locus hierarchical AMOVA analysis showed that, when York site was not included, the largest percentage of variation in distributions of allele frequencies was attributed to the among populations among groups component (mean of 96.76%; Tables 6 and 7). Small, but significant values of variance for the among groups component occurred for two groupings: road crossings with distance considered (0.71%, $P \leq 0.01$), and sites grouped according to which side of the M-C 401 freeway they were located (0.50%, $P \leq 0.05$). When the York site was included in AMOVAs, the largest percentage of variation for the among groups component of variance was attributed to the grouping with the site with furthest geographic distance (York), (9.3%, $P \leq 0.0001$).

The first runs of STRUCTURE with no prior population information included produced very low mean values of $lnP(X|K)$. K=1 had the highest value of the three scenarios, and posterior probabilities, $P(K|X)$ was the highest for K=1 model (Table 8, Fig. 12), suggesting the sampling sites were not subdivided, even with the York site included. Further runs of structure with sites separated on the basis of statistical significance from the AMOVA analyses were inconclusive and failed to provide reliable information on membership of specific individuals. Furthermore, likelihood values from these analyses were even lower than those provided for the first scenario with unspecified prior population information.

(iv) Likelihood of single stepwise mutation model (SMM) for microsatellites and estimation of effective population size for sample sites

Five of the eight loci (*RsyC52*, *RsyC41*, *RsyD33*, *RsyC23*, and *RsyC11*) used in the analysis produced allelic distributions that did not conform to a normal gamma

distribution (Figures 13 and 14). Likelihood-ratio tests for comparison of microsatellite mutational models revealed that *RsyC63, RsyD40, RsyC23,* and *RsyC11* conformed more to the SMM, and *RsyD88, RsyC52, RsyC41,* and *RsyC11* conformed more to a GSM (Table 9). Estimates of *Ne* and *D*, the mean proportion of heterozygote excess for each site from the bootstrap value are provided in Table 10.

(v) Mantel test

The analysis of genetic isolation by distance revealed no significant relationship between geographic distance and genetic distance when the York site was excluded. When the York site was included, there were significant relationships between the genetic distance and the log geographic distance ($r = 0.5905$, one sided $p \leq 0.0038$), the log genetic distance and the geographic distance ($r = 0.6613$, one sided $p \leq 0.0403$), and the log genetic distance and the log geographic distance ($r = 0.5982$, one sided $p \leq 0.013$) (Figures. 15 and 16, Table 11).

(vi) Reduction in genetic variability for York site

Measures of the Garza-Williamson statistic and allelic richness (Tables 12 and 13) suggested that the York site may have experienced a bottleneck. However, the one-way simple between groups ANOVA and post-hoc tests revealed no significant statistical difference between measures of allelic richness for Wellington and York sites ($P= 0.684$, d.f.= 8) (Figures 17 and 18).

DISCUSSION

Wood frog population structure

Nearly all of my analyses based on microsatellite DNA allele frequencies point to very little genetic population structure between wood frog breeding sites in Wellington County. The low pairwise θ values ($\leq$0.05); no significant differences in the exact tests of population differentiation; in the AMOVAs most of the variation was accounted for by the among populations among groups component; no IBD relationship (Mantel tests) for Wellington sites; finally, no population subdivision revealed during runs of STRUCTURE are all indicative of panmixia and likely a sufficient degree of migration between sites. The HWE analyses, also confirm that there was primarily panmixia within sites. Heterozygote deficiency was only detected for three sites: W#2, W#3 and W#8. W#2 was the only larval site analysed within the data set and because of potential bigamy and polygamy for males (Howard and Kluge 1985), it is possible that some of the larvae from different egg masses had the same fathers and the same alleles, as many of the egg masses we collected were directly connected to each other. The heterozygote deficiency observed at W#3, could be explained by my having collected and included separate adult individuals from this site in two separate years (2005 and 2006). It is possible that a biased sample of the offspring of the 2005 set of collected adult wood frogs were collected in 2006, and these offspring contributed a proportion of paternal and maternal alleles identical to the breeding adult individuals of 2005. Only 32 individuals were collected from W#8 and, by chance may have consisted of a collection of a non-random sample of homozygotes, as genotyping errors were ruled out by running two independent gels. It is unlikely that the deficiencies of heterozygotes for W#2, W#3 and W#8 can be attributed to Wahlund's effect as the pairwise θ values for these sites were small (Table

5) and that there was no significant values for exact tests of population differentiation between wood frog sites in Wellington County. These results were surprising and contrary to original predictions that wood frogs, because of their specific habitat requirements and other biological attributes (Berven 1990; Duellman 1994; Skelly 2004; Rubbo and Kiesecker 2005), would exhibit substantial population structure.

There were exceptions to the general finding of little population structure between sites. One exception was the pairwise θ values between the collection sites, which indicate small but statistically significant values of genetic differentiation. This suggests a non-random sampling of alleles between sites, and in particular, for the York site which had the highest set of pairwise θ values (Table 5). This exception could be an artifact of the unrealistic assumptions of Wright's Island model, i.e. mutation-drift and gene flow-selection equilibria, which were likely violated. First, because the tetranucleotide microsatellite DNA loci that I used in my analysis typically have high mutation rates (Ellegren 2004), and this is likely outweighing the effect of neutral genetic drift. Additionally, the evolutionary time for expected divergence between sites is likely short (>200 years) and microsatellite markers are selectively neutral, as such, selection is unlikely to be equal to or to outweigh migration events of the recent past. The STRUCTURE analysis, which does not require these assumptions of genetic equilibria, revealed no population subdivision, although the AMOVAs suggested small but significant variation mostly due to road density. The significant genetic IBD relationship for wood frogs revealed by the Mantel tests when the York was included could be artificial, as the range of pairwise distance values for York were substantially higher than the rest of the Wellington sites (Appendix 3). In two other independent studies of wood

frog population structure, both Newman and Squire (2001) and Squire and Newman (2002) concluded that wood frogs showed little fine-scale genetic differentiation between ponds separated by pairwise distances less than 10km; small but significant differentiation was only apparent among an outlier pond that was located 20km away. Despite the fact that my study sites were located in highly fragmented landscape, even over geographic distances (>30km) where an increase in population structure was expected, there was no significant relationship between geographic distance and genetic distance for the Wellington sites. When the York site was excluded, there were no significant genetic IBD relationships for the remaining Wellington county wood frog sites. This relationship could also be indicative of a distance threshold, where genetic differences only become significant after the average pairwise distance exceeds ~ 50 km. This conclusion is further substantiated by the AMOVA analysis grouping the site with furthest pairwise geographic distance (York) separately from the other Wellington county sites.

The single most important contributing factor to the lack of population structure of wood frogs in Wellington County is likely the large effective population size. Previous studies have demonstrated that even with high mortality in the pre-metamorphic stages and annual fluctuations in population size, there was still a considerable number of emerging juveniles (Hereid and Kinney 1966; Howard and Kluge 1985; Berven 1990). Further, the indirect estimates of N_e were infinite for five of the collection sites, and the sites with smaller N_e values (W#5, W#6, W#7 and York, see Table 10) would still possess enough individuals to offset the effects of genetic drift supposing there was sufficient migration from other neighboring ponds.

Large N_e sizes may be positively correlated with large amounts of juvenile age-specific dispersal and migration, as intra-specific competition for resources may be higher in these years, resulting in more juveniles dispersing to neighbouring ponds contributing to the high amount of observed neutral genetic heterozygosity. In addition, the entire study region was relatively flat and unlikely to impose much of a barrier to dispersing individuals. Numerous studies have shown that mountain ridges form effective physical barriers to migration and contribute to extreme subdivision of temperate amphibian populations (e.g. Monsen and Blouin 2004; Funk et al. 2005a; Spear et al. 2005). Indirect estimates of migration rates between sites would be unreliable due to the extremely low F_{st} values (< 0.05) and the fact that the data do not conform to genetic equilibrium expectations (Whitlock and McCauley 1999) (see Appendix 2). It is clear that the rate is sufficiently high to homogenize the populations in Wellington County. This result is contradictory to the finding of Berven and Grudzien's (1990) study of wood frogs populations in a mountainous region. Their direct estimates of migration using mark-recapture between sites showed that straight-line dispersal distance of wood frogs is as small as 1.5km. The disagreement between my study and Berven and Grudzien's (1990) could be partially explained by the presence of mountain ridges and the different elevations of their sample sites in the Appalachians. Altitude was not considered for all of my sites, as none of the landscape was higher than 250m above sea level, and none were lower than 200m above sea level. Furthermore, human settlement in the study area has only been about 200 years, and it is not wholly surprising that no subdivision among the current populations has materialized.

Another, perhaps more important, reason for lack of population structure is that the dispersal and migratory ability of animals may be far beyond human perception. From the classical example of the Giant Galapagos Tortoise (Beheregaray *et al.* 2004) to several recent cases in anurans (Smith and Green 2004; Funk *et al.* 2005b), some animal species have demonstrated surprising ability to disperse from one region to another. The negative influence of roads on the variance of allele frequencies between groups of sites means that there was likely some homogenizing effect of migration and thus dispersal between sites.

The York site showcased the neutral genetic resiliency of wood frogs to urban development. Although it has a small estimated N_e (15.8), and likely little migration from other populations for at least the past 50 years, there was virtually no population structure or indication of a reduction in neutral genetic diversity (a bottleneck). This could potentially be due to a lower sample size than the rest of the sample sites, or alternatively, that the effective population size alone has been sufficient enough over time to maintain neutral genetic diversity within the York site.

Overall, my results have several important implications. Firstly, wood frogs, as well as many other anurans, have often been labeled as poor dispersers because of their very specific habitat requirements, and therefore, as good indicator species for detecting habitat fragmentation between ponds (Rubbo and Kiesecker 2005). The results of this study suggest otherwise. Secondly, the negative effect on of human altered landscapes on neutral genetic diversity of many species may not be as severe as perceived, especially over short time periods.

Anthropogenic landscape features

Of the four types of landscape features examined, only road density revealed significant but small contribution to population subdivision. The among groups component of AMOVA analyses, detected coarse patterns of genetic variation for groupings based on number of road crossings (with and without distance considered), and based on breeding sites located on both sides of the M-C 401 freeway. These results were not surprising since roads have been recognized of having large impacts on population structure even within a very short timeframe. For example, Keller *et al.* (2005), on a timescale of ~45 years, detected a reduction in effective population size (N_e) between two local populations in the flightless ground beetle (*Carabus violaceus*) since the construction of a major highway in 1969 near Bern, Switzerland. Also, Riley *et al.* (2006) examined coyote and bobcat populations from north to south across a large freeway in California constructed in 1949, using both direct observation of radio-collared individuals and indirect estimate using microsatellite DNA markers. They found that northern populations showed a significant difference in population structure from southern populations across the freeway.

Additionally, although AMOVA analyses revealed significant structure for some of the groupings (Table 6 and 7), assignment methods were unable to further resolve this relationship under the same groupings of sites. This is in agreement with Manel *et al.* (2005) who suggested that data sets with low amounts of genetic differentiation, where most F_{ST} values are ≤ 0.05, may not lend themselves accurately to assignment test analyses. From my data, I suggest that the use of assignment methods is better reserved for data that show overall high preliminary degree of genetic differentiation.

Microsatellite DNA as a tool in population genetics

Undoubtedly, microsatellite DNA loci are powerful genetic markers for detecting fine scale neutral genetic population structure. However, caution should be exercised in the explanation and interpretation of these data. Explicit and implicit assumptions surrounding microsatellite DNA are often ignored or unexplained by those who use them. First, the assumption that microsatellite DNA markers are neutral means that investigators are limited in regards to interpreting the overall evolutionary significance of the microsatellite DNA data. Neutral markers cannot yield accurate information about the adaptive or evolutionary potential of a population, as the relationship between quantitative genetic diversity and neutral genetic diversity is not straightforward; this is reviewed thoroughly in Holderegger *et al.* (2006). This does not negate the value of using neutral genetic data to address ecological or conservation minded questions merely those questions about the actual fitness of a population cannot be addressed directly with microsatellites. Information about the genetic population structure of an organism may be used to formulate more specific hypotheses about population demography (Spielman *et al* 2004). Second, microsatellite DNA markers have very high rates of mutation, and this may create more artificial allelic diversity by mutation than would be present by the process of allelic identity by descent alone. Investigators would do well to ensure that rates of mutation for each locus are examined, especially before attempting to use more contemporary analytical techniques like assignment tests where no mutation models are specified for individual loci.

Concluding Remarks

Researchers should be very careful about selecting their biological model system for the detection of population structure. Wood frogs in southwestern Ontario may not be the best model organisms for showing recent genetic population structure in conjunction with current patterns of anthropogenic habitat fragmentation. Large effective population size of individuals at each breeding site may overshadow the potential genetic structure that might otherwise occur in other animal species with smaller effective population sizes, like large mammals. With large effective population sizes, even with wood frogs displaying high philopatry to breeding sites (Berven and Grudzien 1990), enough juveniles may migrate to other breeding sites to keep discrete breeding patches cohesive and continuous in their allele frequencies as was observed in Newman and Squire (2001) and Squire and Newman (2002). On the other hand, some other species, such as large mammals, possess intrinsic traits and requirements that probably already contribute to large degree of population structure in absence of habitat fragmentation (Cardillo *et al.* 2005). This may at least partially contribute to the significant population structure detected in coyotes and bobcats by Riley *et al.* (2006). Abundance, low vagility, short generation times, and non-overlapping generations for easier estimation of population genetic parameters (N_e and gene flow) would be a few characteristics for an ideal model organism in studying population structure.

The lack of population subdivision of the wood frog in southwestern Ontario is surprising; and is contradictory to almost every one of my original perceptions about the species and the landscape. In light of these results, it is perhaps our own assumptions that need to be revised. The dispersal capability of many species may be far beyond human

ability to appreciate, and their neutral genetic resilience to habitat fragmentation may also be much higher than originally expected.

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Table 1. Summary of wood frog samples collected, extracted and genotyped in 2005, with one site, Wellington #3 having been resampled in 2006. Wellington #9 and Wellington #10 were not suitably amplified due to contamination.

Name of collection site	Number of samples collected	Number extracted	Number genotyped	GPS location
Wellington #1	34	34	34	N 43°28.56 W 079°59.56
Wellington #2	50	48	45	N 43°25.23 W 080°36.54
Wellington #3 (2005)	54	48	48	N 43°28.07 W 080°13.39
Wellington #3 (2006)	78	78	48	N 43°28.07 W 080°13.39
Wellington #4	44	44	44	N 43°27.57 W 080°12.59
Wellington #5	29	29	29	N 43°27.42 W 080°01.48
Wellington #6	54	54	48	N 43°18.19 W 080°22.28
Wellington #7	50	50	46	N 43°32.18 W 080°08.54
Wellington #8	32	32	32	N 43°32.18 W 080° 06.18
York	24	24	24	N 43°46.17 W 079°29.48
Wellington #9	50	50	**0**	N 43°27.47 W 080°15.26
Wellington #10	52	52	**0**	N 43°15.17 W 080°20.17

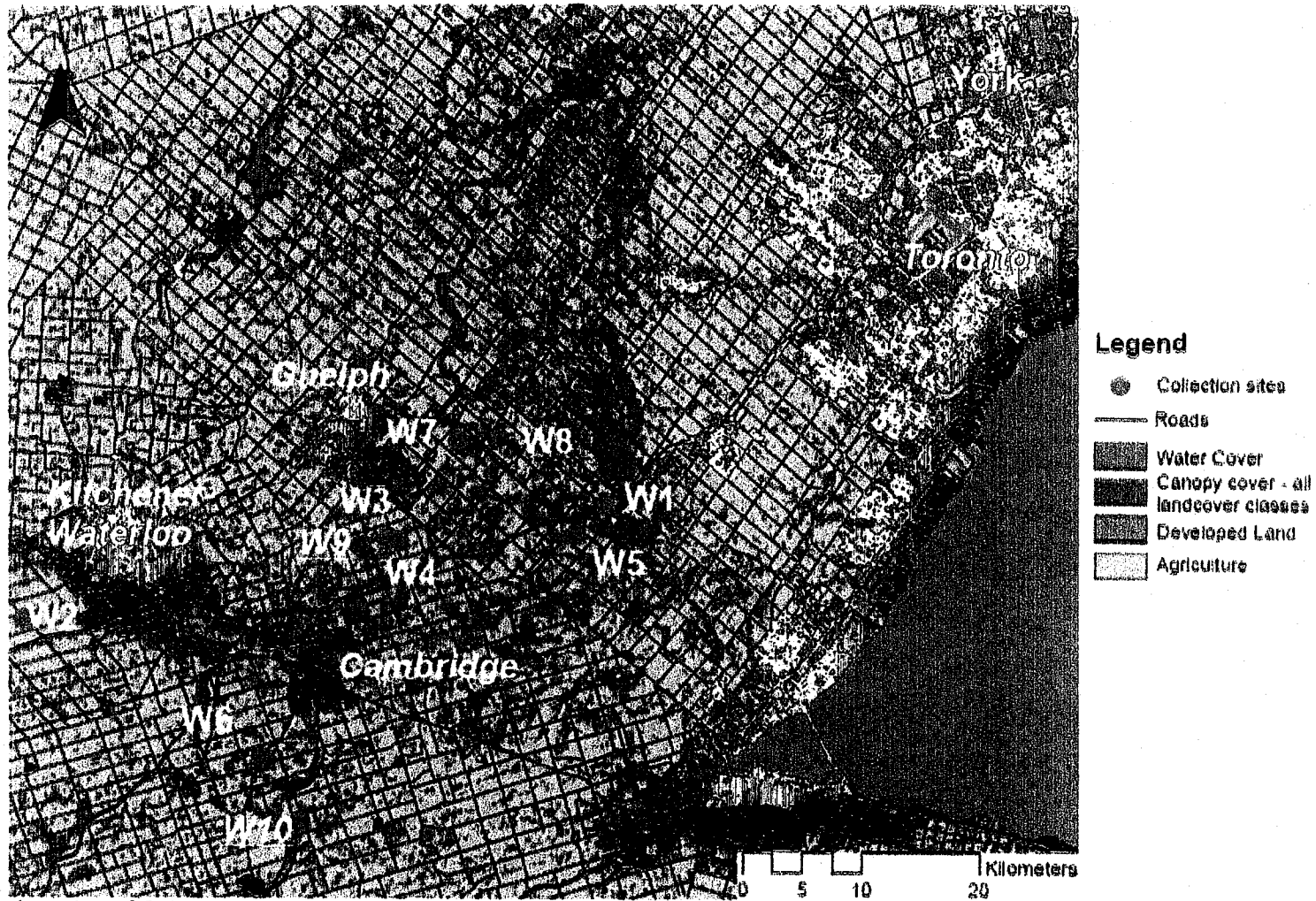


Figure 1. Location of 2005 wood frog (*Rana sylvatica*) collection sites, sites in white bold typeface denote successfully genotyped populations, white italicized typeface denotes the major urban areas, and italicized, underlined typeface denotes collection sites that were not successfully extracted or genotyped.

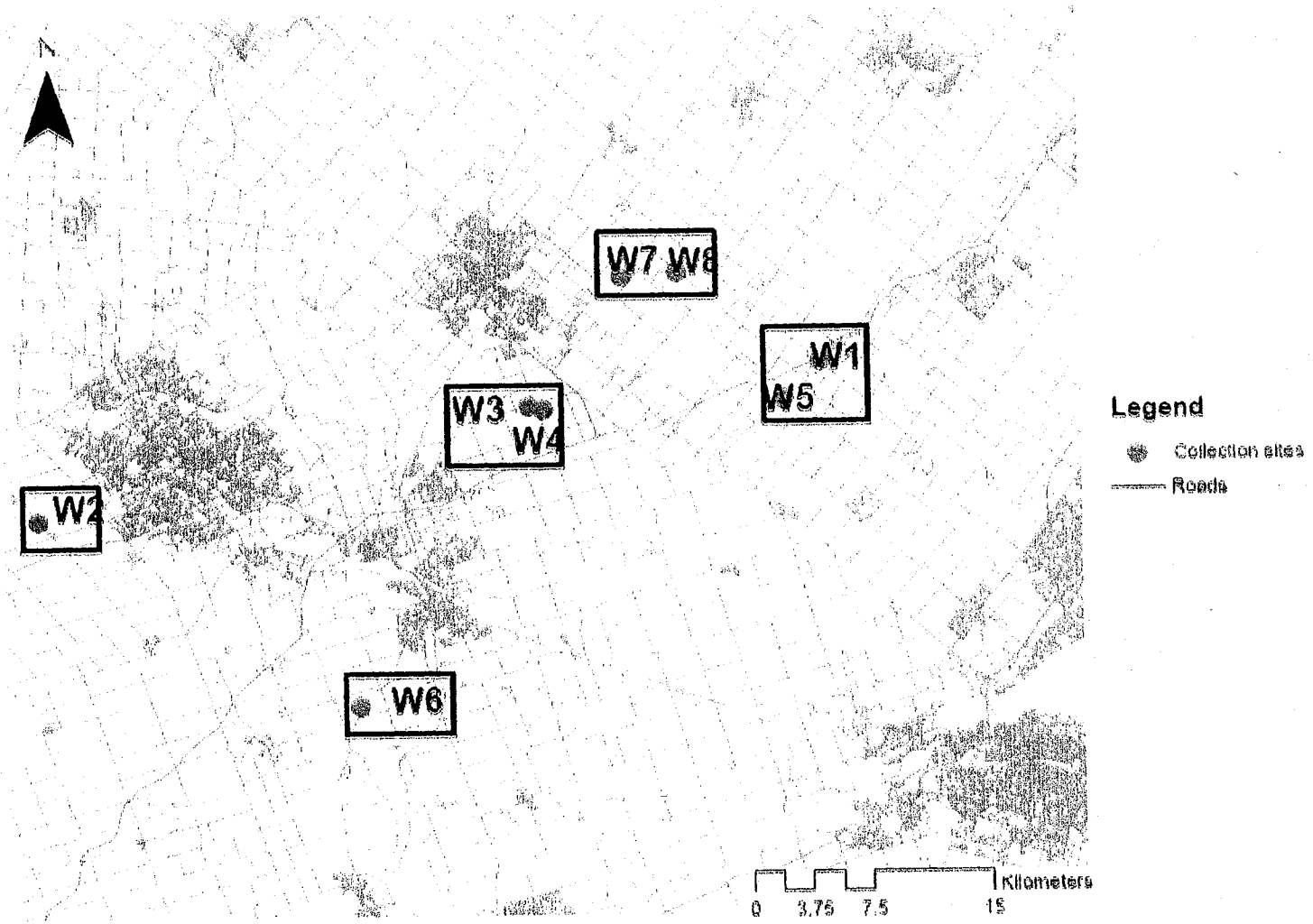


Figure 2. Groupings of Wellington sites for AMOVA analysis showing groups based on sites separated by lowest values of road density.

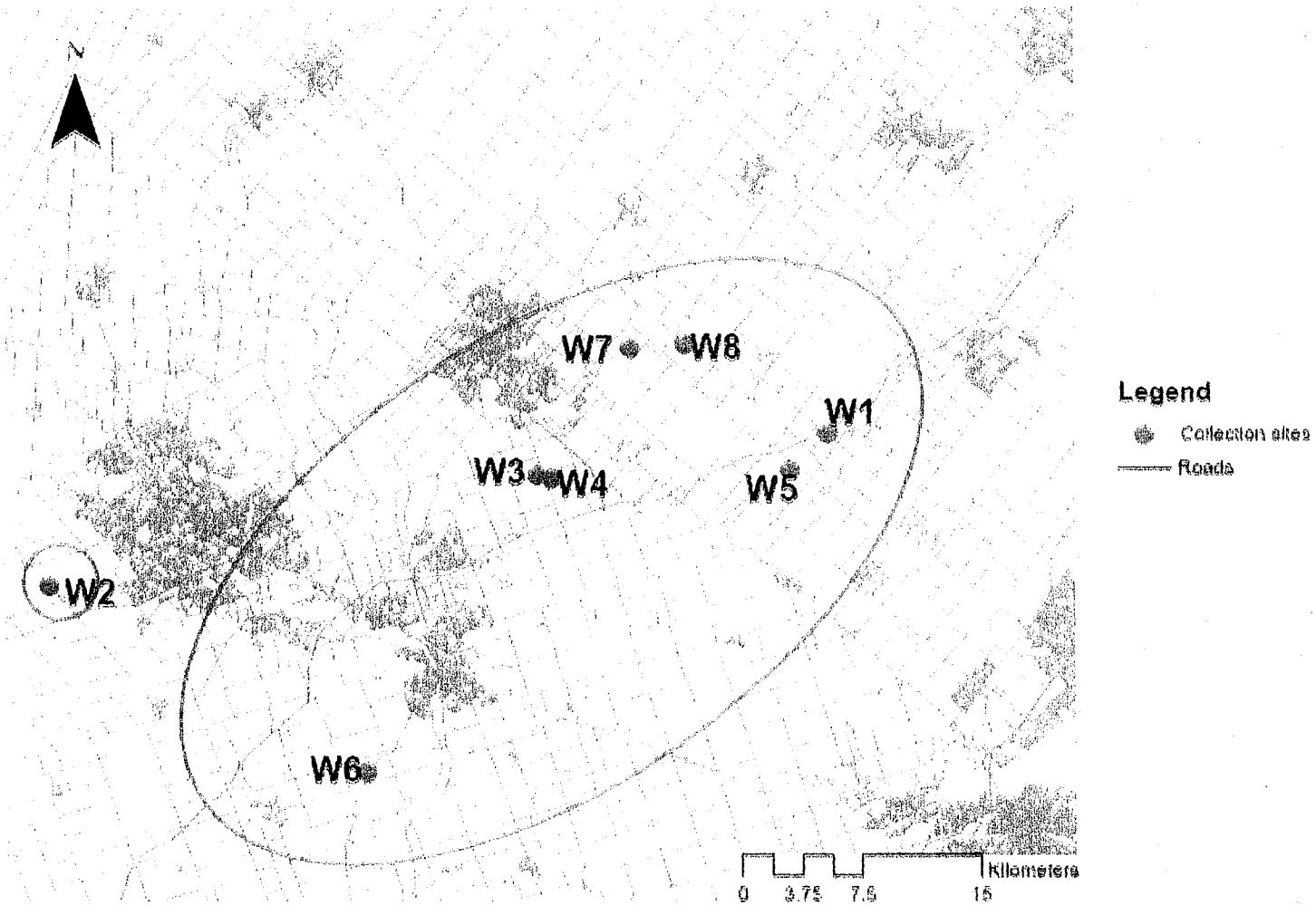


Figure 3. Groupings of Wellington sites for AMOVA analysis showing groups based on site with furthest average pairwise straightline distance.

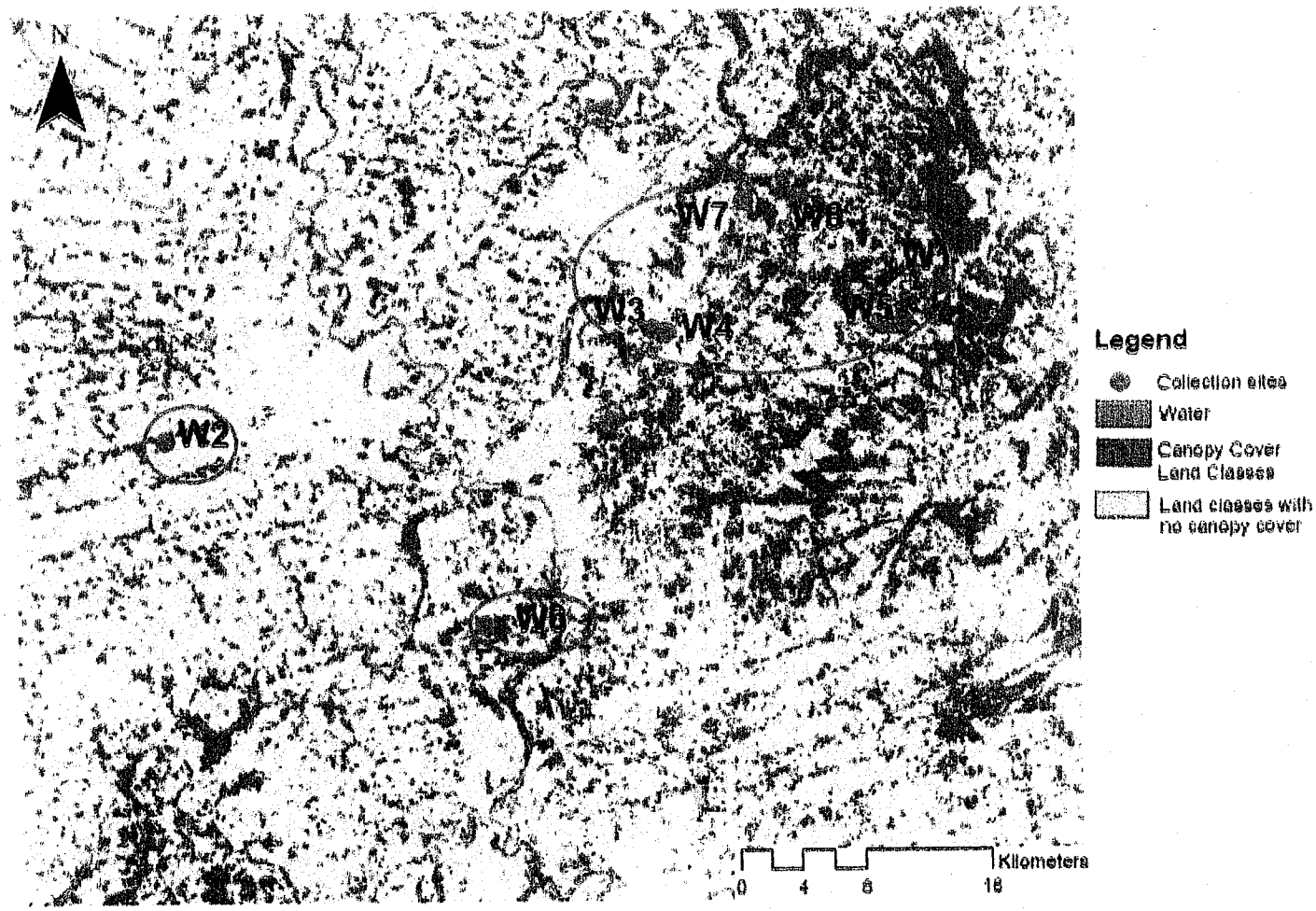


Figure 4. Groupings of Wellington sites for AMOVA analysis showing groups based on maximum values of canopy cover between sites.

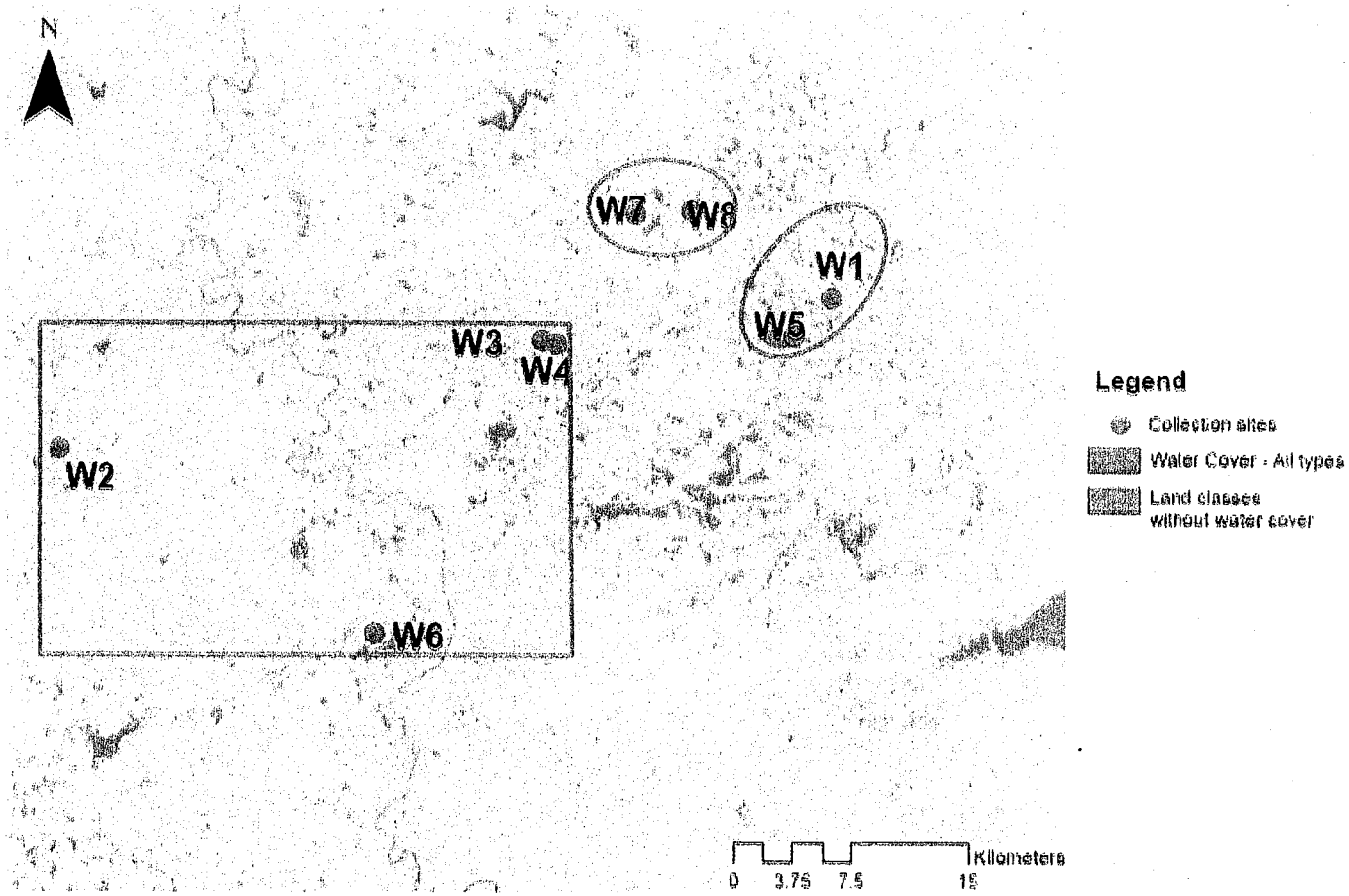


Figure 5. Groupings of Wellington sites for AMOVA analysis showing groups based on watershed discontinuity.

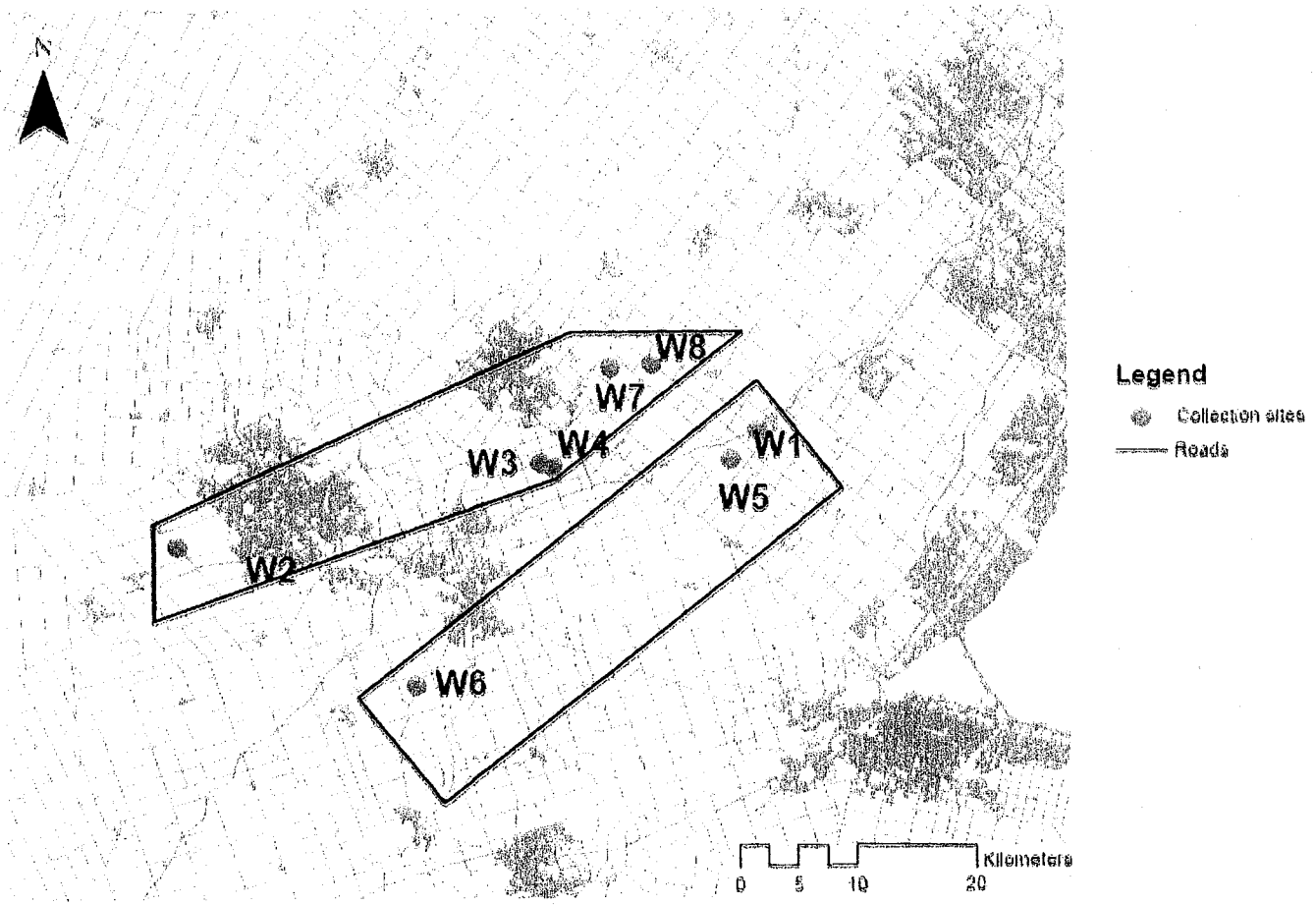


Figure 6. Groupings of Wellington sites for AMOVA analysis showing groups based on location with respect to the M-C Freeway.

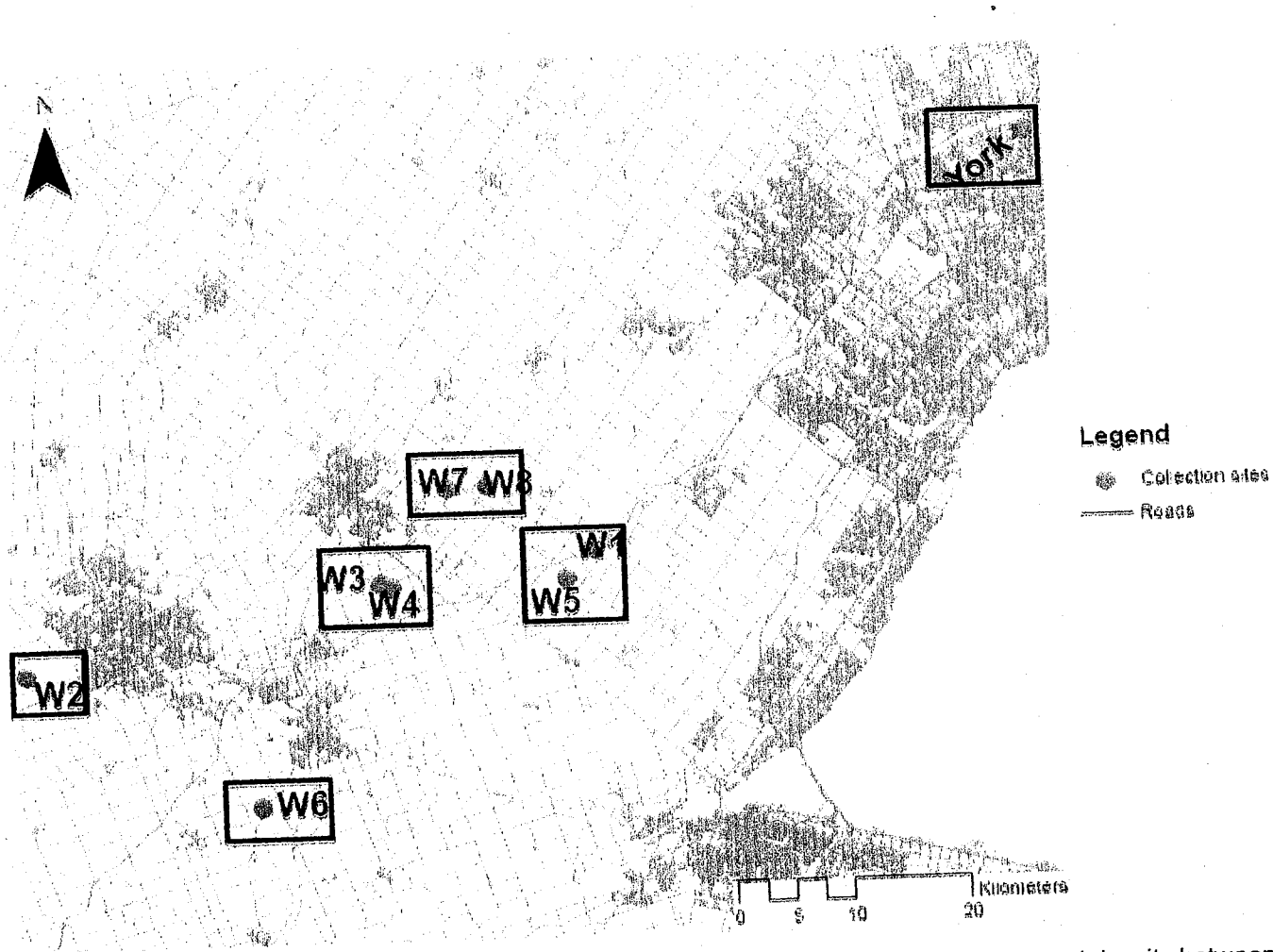


Figure 7. Groupings of Wellington sites and York site for AMOVA analysis showing groups based on lowest road density between sites.

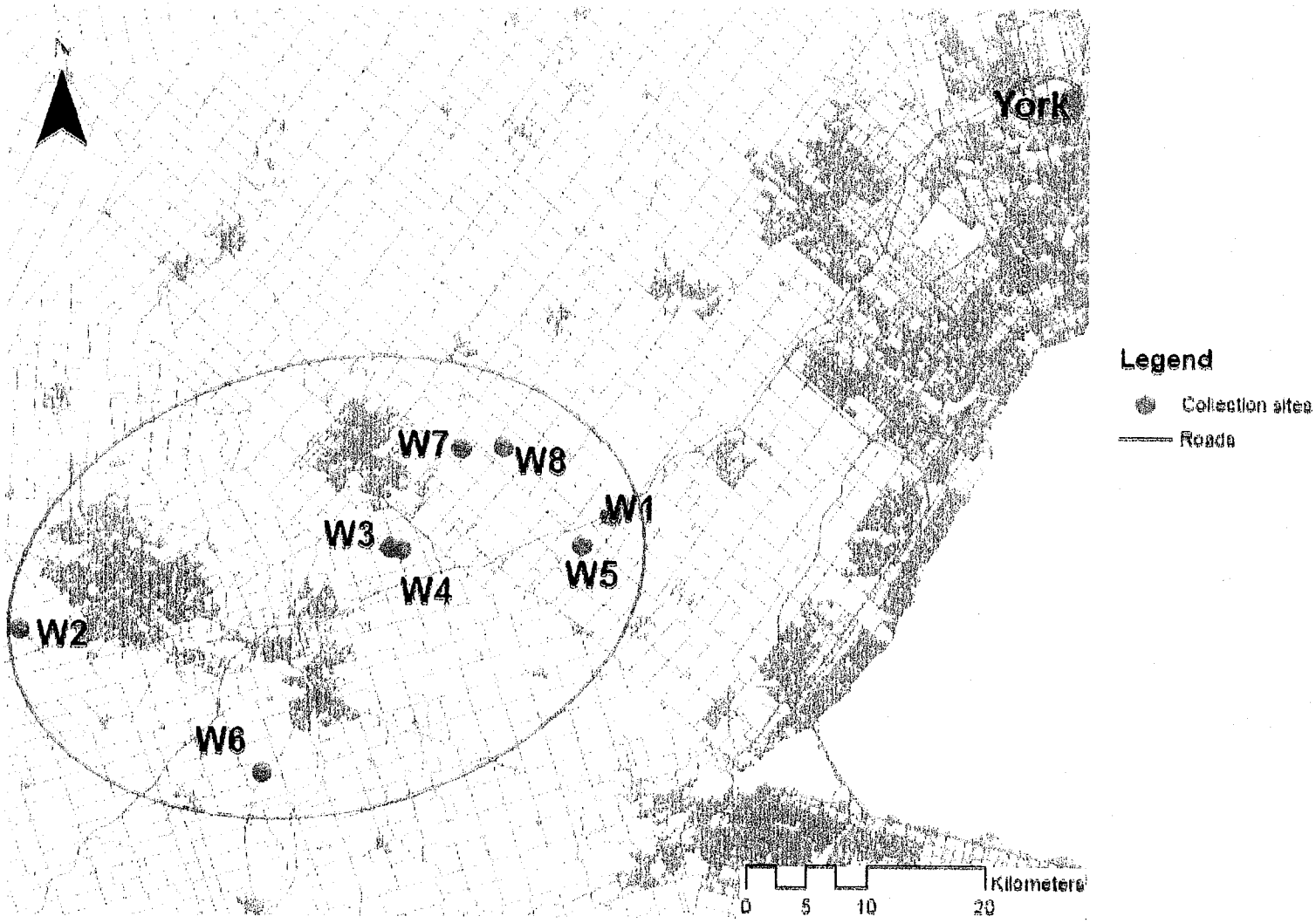


Figure 8. Groupings of Wellington sites and York site for AMOVA analysis showing groups based on site with furthest average pairwise straightline distance.

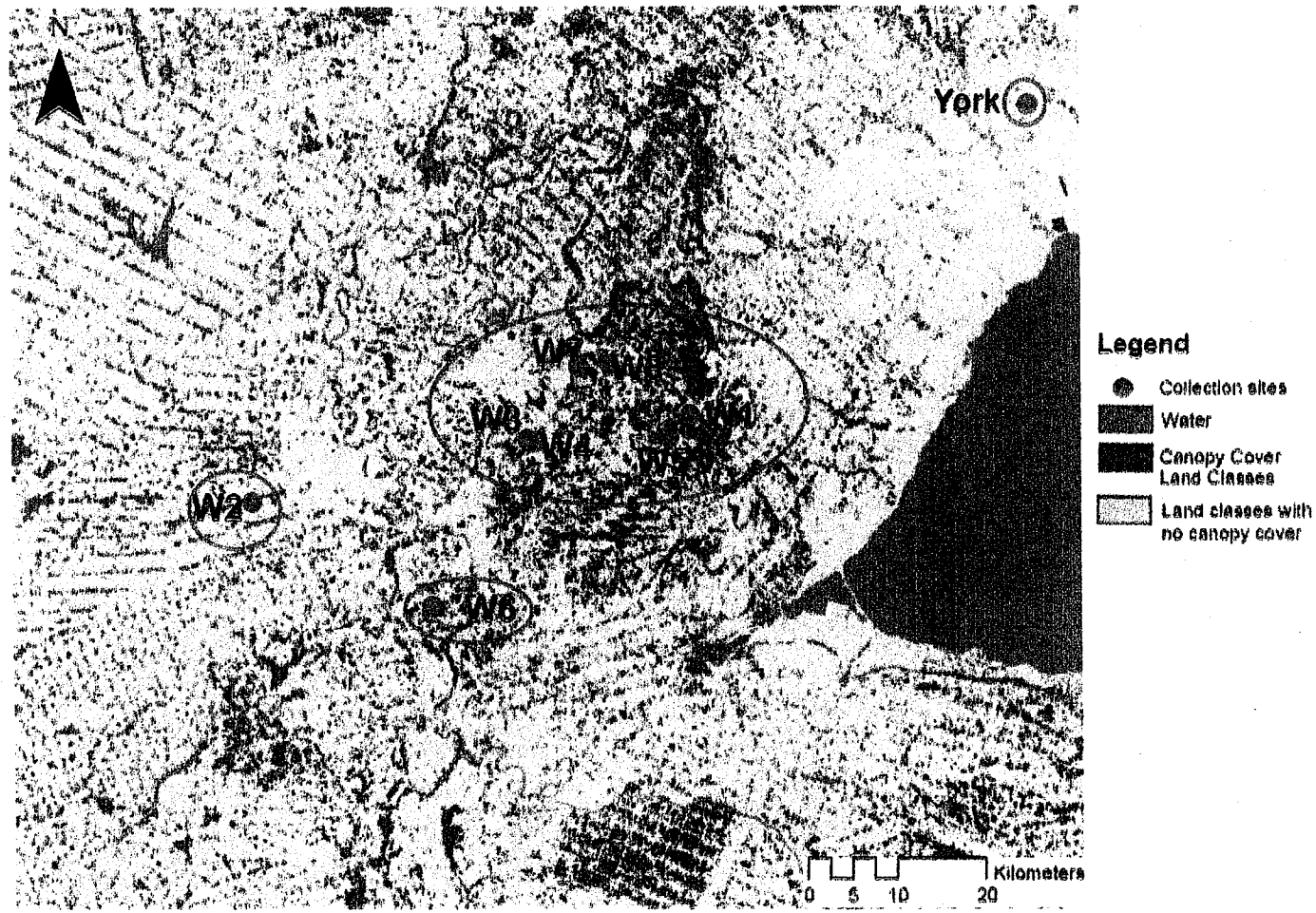


Figure 9. Groupings of Wellington sites and York site for AMOVA analysis showing groups based on maximum values of canopy cover between sites.

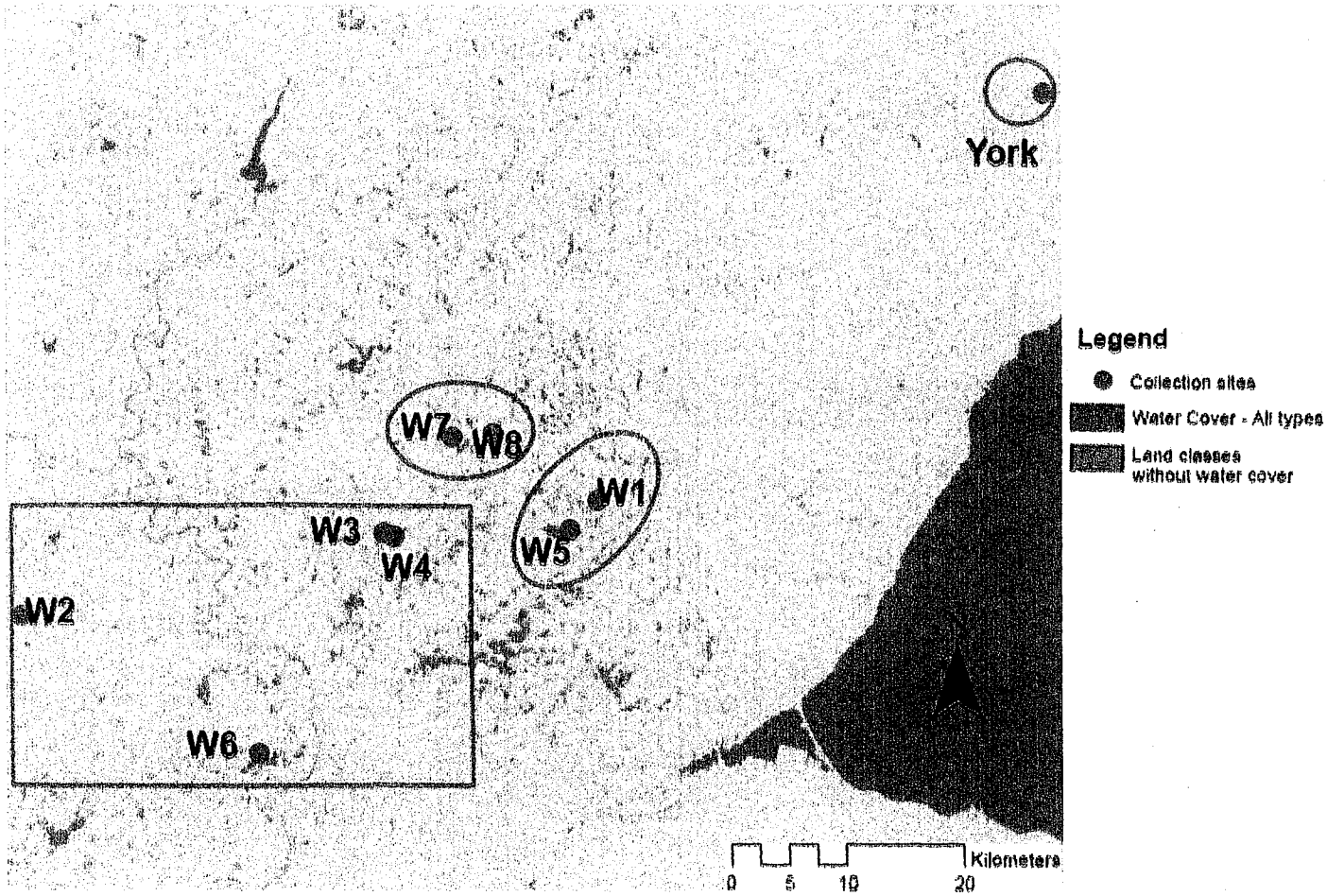


Figure 10. Groupings of Wellington sites and York site for AMOVA analysis showing groups based on watershed discontinuity.

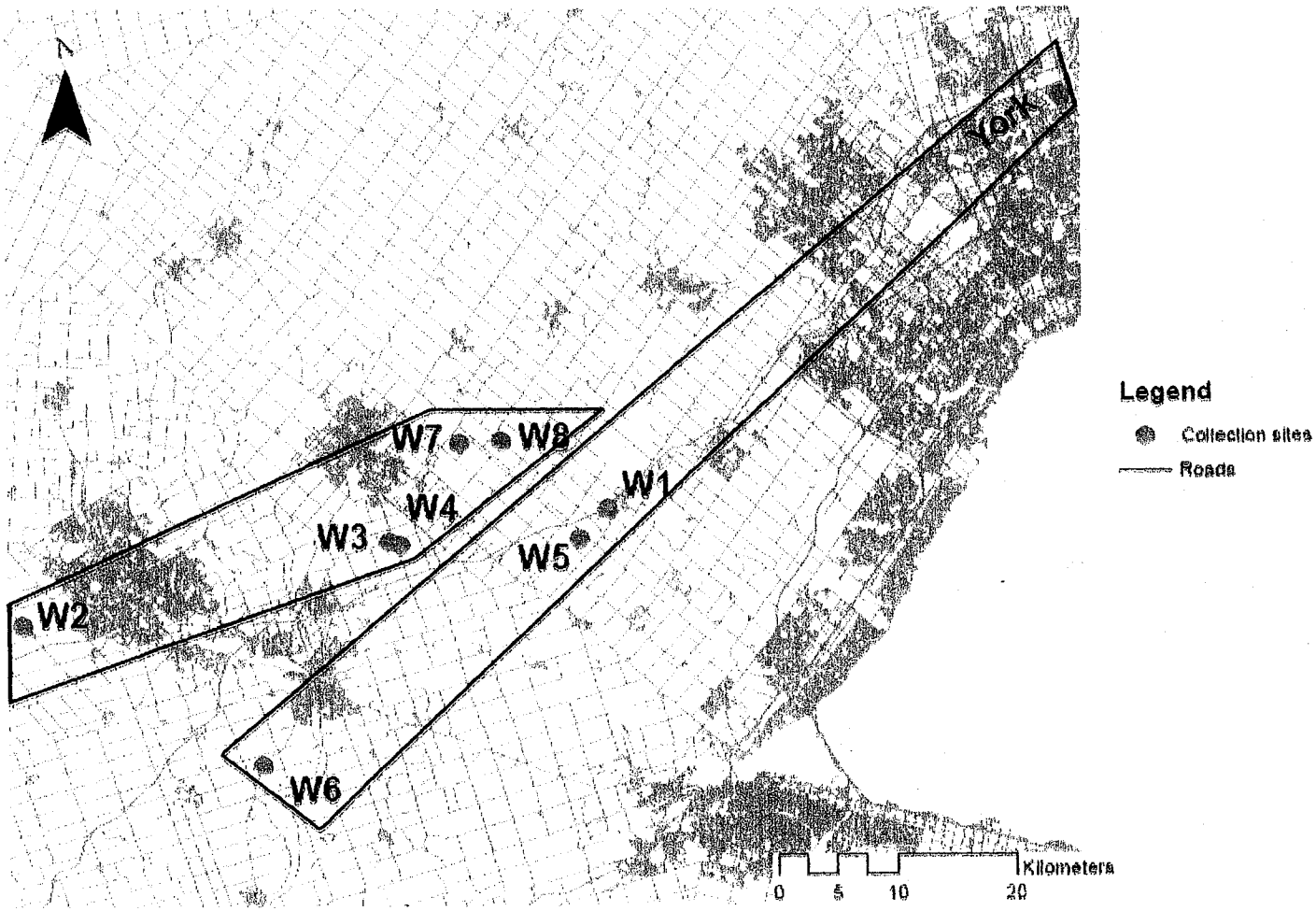


Figure 11. Groupings of Wellington sites and York site for AMOVA analysis showing groups based on location with respect to M-C Freeway.

Table 2. Primer sequences (F = forward, R = reverse), allele sizes (bp), annealing temperatures (°C) and the total number of alleles observed per locus for the eight microsatellite DNA loci developed by Julian and King (2003) for *Rana sylvatica*.

Locus	Primer sequences (5'-3')	Product size range (bp)	Annealing Temp (°C)	Total Number of Alleles for each locus
RsyC11	F: TTACTTTCAGTTTCAAAAGGCAG R: TACACAGTGCTTCACAAGTTCC	110-206	57.0	25
RsyC23	F: AGGGCATTATTACATTTTGGTC R: AGGAAATTACAGAGGACTGTGG	214-250	54.0	10
RsyC41	F: GTCAAAACACAGATGCACAATC R: ACAAAACAGGAATCGGTCATAC	88-140	54.0	10
RsyC52	F: CCATACAACCGTGATTACAAAG R: ATATACCACCCTTCCAGAGATG	134-234	54.0	21
RsyC63	F: CAGAAAATTGCCGAAAAGG R: TGGGCTTAAGAAACAAAAGAAC	130-298	54.0	41
RsyD33	F: CAGAGTGACACCAAAATTTACC R: TACTAGATCCACGTGTAGCACC	100-105	54.0	2
RsyD40	F: TGATTGATTGTTCACTATTGGG R: AAGTAGATTATGTGCTGCAAACTG	167-235	54.0	17
RsyD88	F: TCAATCCATCAGTCTGTCTGTC R: GGATTTTGTAAAGAATGCTCCTC	142-214	52.0	19

Table 3. Genetic variability at eight microsatellite loci for nine sampling sites: N_A = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} values, and N = number of samples used from each sampling site.

Collection site		RsyD88	RsyC63	RsyC52	RsyC41	RsyD40	RsyD33	RsyC23	RsyC11	Average (by site)
Well#1	N_A	12	27	10	6	14	1	6	12	11
N=34	H_O	0.912	0.940	0.515	0.788	0.970	-	0.667	0.794	0.798
	H_E	0.888	0.957	0.598	0.707	0.916	-	0.718	0.815	0.800
	F_{IS}	-0.028	0.018	0.140	-0.116	-0.060	-	0.072	0.026	0.007
Well#2	N_A	13	29	12	5	13	2	8	17	12.4
N=43	H_O	0.744	1.00	0.698	0.907	0.902	0.023	0.698	0.791	0.720
	H_E	0.909	0.969	0.652	0.761	0.914	0.023	0.688	0.844	0.720
	F_{IS}	0.183*	-0.032	-0.071	-0.194	0.013	-	-0.014	0.064	-0.007
Well#3	N_A	14	35	19	7	18	3	7	23	15.8
N=96	H_O	0.780	0.945	0.713	0.745	0.883	0.052	0.479	0.830	0.678
	H_E	0.857	0.952	0.736	0.705	0.899	0.051	0.571	0.827	0.700
	F_{IS}	0.090**	0.007	0.031	-0.056	0.018	-0.017	0.162	-0.003	0.029
Well#4	N_A	12	28	11	8	15	2	8	15	12.4
N=44	H_O	0.818	0.930	0.773	0.698	0.858	0.068	0.524	0.762	0.679
	H_E	0.897	0.955	0.701	0.739	0.901	0.108	0.626	0.816	0.718
	F_{IS}	0.088	0.026	-0.104	0.056	0.048	0.374	0.164	0.067	0.090
Well#5	N_A	10	19	6	7	6	2	6	11	8.4
N=29	H_O	0.821	1.000	0.357	0.690	0.964	0.148	0.759	0.931	0.709
	H_E	0.867	0.942	0.412	0.708	0.910	0.140	0.739	0.811	0.691
	F_{IS}	0.053	-0.063	0.135	0.027	-0.061	-0.061	-0.028	-0.152	-0.019
Well#6	N_A	13	27	11	4	14	2	7	19	12.1
N=48	H_O	0.750	0.891	0.617	0.667	0.979	0.062	0.646	0.833	0.681
	H_E	0.903	0.946	0.578	0.705	0.913	0.061	0.701	0.854	0.708
	F_{IS}	0.171	0.058	-0.068	0.054	-0.074	-0.022	0.079	0.024	0.028
Well#7	N_A	13	25	13	6	16	2	5	16	12
N=46	H_O	0.800	0.973	0.600	0.696	0.911	0.087	0.652	0.889	0.701
	H_E	0.898	0.952	0.632	0.715	0.906	0.084	0.591	0.821	0.700
	F_{IS}	0.111	-0.022	0.051	0.027	-0.006	-0.034	-0.106	-0.084	-0.008
Well#8	N_A	11	26	12	7	15	2	6	17	12
N=32	H_O	0.688	0.968	0.594	0.688	0.934	0.032	0.594	0.938	0.680
	H_E	0.872	0.953	0.620	0.696	0.912	0.032	0.666	0.900	0.707
	F_{IS}	0.214*	-0.016	0.043	0.013	-0.028	-	0.110	-0.042	0.042
York	N_A	6	15	7	3	7	1	3	5	5.9
N=24	H_O	0.792	0.917	0.625	0.583	0.792	-	0.167	0.750	0.661
	H_E	0.778	0.900	0.617	0.434	0.785	-	0.196	0.723	0.633
	F_{IS}	-0.019	-0.019	-0.013	-0.356	-0.008	-	0.152	-0.039	-0.043
Average (by locus)	N_A	10.1	25.7	11.2	5.9	13.1	1.9	6.2	15	-
	H_O	0.790	0.952	0.610	0.718	0.910	0.067	0.576	0.835	-
	H_E	0.874	0.947	0.616	0.686	0.895	0.083	0.611	0.823	-
	F_{IS}	0.096	-0.005	0.016	-0.061	-0.018	0.048	0.066	-0.015	-

'*' denotes $P \leq 0.00714$, significant deficit of heterozygotes for 7 tests; '**' denotes $P \leq 0.00625$ significant deficit of heterozygotes for 8 tests.

Table 4. Pairwise linkage disequilibrium χ^2 tests for all eight microsatellite DNA loci for *Rana sylvatica* (Julian and King 2003).

Locus Pair	χ^2	d.f.	P-value
Loc88 & Loc63	4.281	16	0.998
Loc88 & Loc52	19.627	16	0.237
Loc63 & Loc52	5.370	14	0.980
Loc88 & Loc41	12.126	18	0.841
Loc63 & Loc41	13.362	16	0.646
Loc52 & Loc41	10.674	16	0.824
Loc88 & Loc40	8.441	18	0.971
Loc63 & Loc40	7.832	16	0.954
Loc52 & Loc40	6.129	16	0.987
Loc41 & Loc40	20.002	18	0.333
Loc88 & Loc33	10.835	14	0.699
Loc63 & Loc33	1.809	12	1.000
Loc52 & Loc33	8.841	14	0.841
Loc41 & Loc33	11.121	14	0.676
Loc40 & Loc33	6.278	14	0.959
Loc88 & Loc23	14.599	18	0.689
Loc63 & Loc23	5.309	16	0.994
Loc52 & Loc23	25.644	16	0.059
Loc41 & Loc23	11.253	18	0.883
Loc40 & Loc23	16.947	18	0.527
Loc33 & Loc23	7.814	14	0.899
Loc88 & Loc11	19.524	18	0.360
Loc63 & Loc11	1.286	16	1.000
Loc52 & Loc11	24.609	16	0.077
Loc41 & Loc11	15.102	18	0.655
Loc40 & Loc11	7.277	18	0.988
Loc33 & Loc11	6.462	14	0.953
Loc23 & Loc11	18.945	18	0.395

Table 5. Pairwise θs (below diagonal) and generalized probability that allele frequency distributions are the same between sampling sites when all loci (including RsyC63) are combined, when missing data = 0.10 ***P<0.001, **P<0.01, *P<0.05, and NS= not significant

	Well. #1	Well. #2	Well. #3	Well. #4	Well. #5	Well. #6	Well. #7	Well. #8	York
Well. #1	-	**	***	**	**	NS	*	*	***
Well. #2	0.00905	-	***	NS	***	**	*	*	***
Well. #3	0.02022	0.00846	-	*	***	***	***	**	***
Well. #4	0.01057	0.00325	0.00475	-	**	***	*	***	***
Well. #5	0.01230	0.01565	0.01989	0.01307	-	***	**	**	***
Well. #6	0.00439	0.00801	0.02230	0.01599	0.01716	-	*	NS	***
Well. #7	0.00540	0.00532	0.01207	0.00576	0.01474	0.00513	-	NS	***
Well. #8	0.00748	0.00611	0.01358	0.01230	0.01160	0.00503	-0.00143	-	***
York	0.11867	0.10752	0.13113	0.12018	0.12512	0.09631	0.09689	0.08910	-

Table 6. Locus by locus AMOVA for only the Wellington sites, 10000 iterations, 20000 bootstraps. Allowed level of missing data is set at 0.10 and locus *RsyC63* is included, reference figures are provided for each grouping.

Groups	Category of groupings	Reference map	Number of groups	Variance components	Percentage of variation	P-value
W1 and 5, W3 and 4, W7 and 8, W2, W6	Road crossings	Figure 2	5	Among groups	0.70993	0.00743
				Within groups among sites	0.52660	0.00119
				Among individuals within sites	2.05070	0.00673
				Among populations among groups	96.71277	0.00040
W(1 and 3-8), W2	Site with furthest average pairwise geographic distance	Figure 3	2	Among groups	-0.45373	NS=0.95
				Within groups among sites	1.27246	≤0.0000
				Among individuals within sites	2.05937	1
				Among populations among groups	97.12189	0.00505
						0.0001
W(1,3,4,5, 7,and 8) , W6, W2	Maximum canopy cover	Figure 4	3	Among groups	-0.07070	NS=0.58
				Within groups among sites	1.19150	≤0.0000
				Among individuals within sites	2.05310	1
				Among populations among groups	96.82611	0.00644
						0.0001
W1 and 5, W(2, 3, 4, 6), W 7 and 8	Sites in different secondary watersheds	Figure 5	3	Among groups	0.26284	NS=0.16
				Within groups among sites	0.98997	≤0.0000
				Among individuals within sites	2.05036	1
				Among populations among groups	96.69683	0.00653
						0.0001
W(1, 5, and 6), W (2,3,4,7 and 8)	Sites grouped on different sides of the 401	Figure 6	2	Among groups	0.50457	0.02158
				Within groups among sites	0.90634	≤0.0000
				Among individuals within sites	2.04708	1
				Among populations among groups	96.54201	0.00683
						0.0002

Table 8. Bayesian inference of the number of populations of wood frogs based on 9 collection sites in Wellington county and York, using the Bayesian clustering method of Pritchard *et al.* (2000).

Group	K	Mean lnP(X|K)	P(X|K)
Wellington #1-8, and York	1	-12060	~1.0
	2	-12534	~0.0
	3	-12774	~0.0

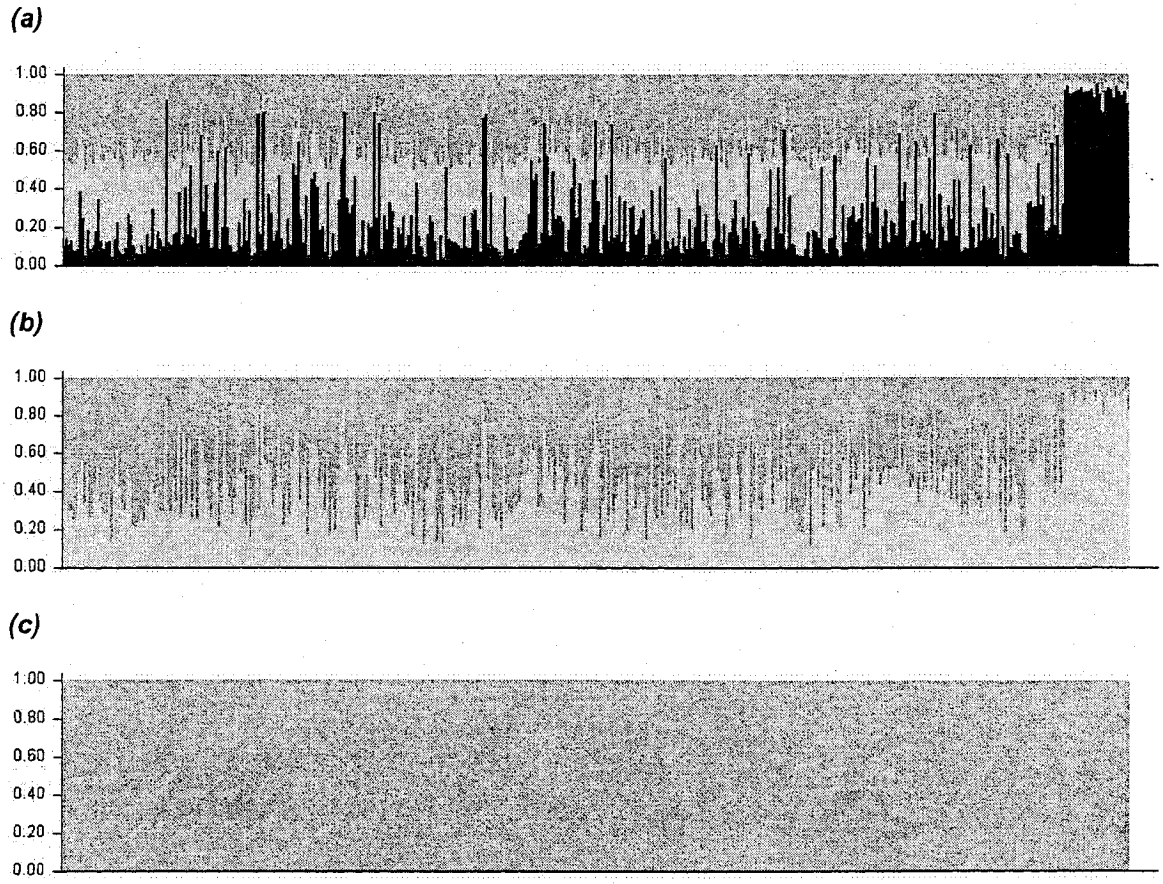


Figure 12. Representative bar plots obtained from genetic assignment tests from the program STRUCTURE (Pritchard *et al.* 2000): (a) K=3, (b) K=2, (c) K=1, where no putative population information is specified. Each vertical bar on the x-axis represents one individual. The scale of 0.00-1.00 on the y-axis represents the proportion of genetic membership to a cluster. For sub-figures (a) and (b), the last portion of the plot is representative of the York site individuals.

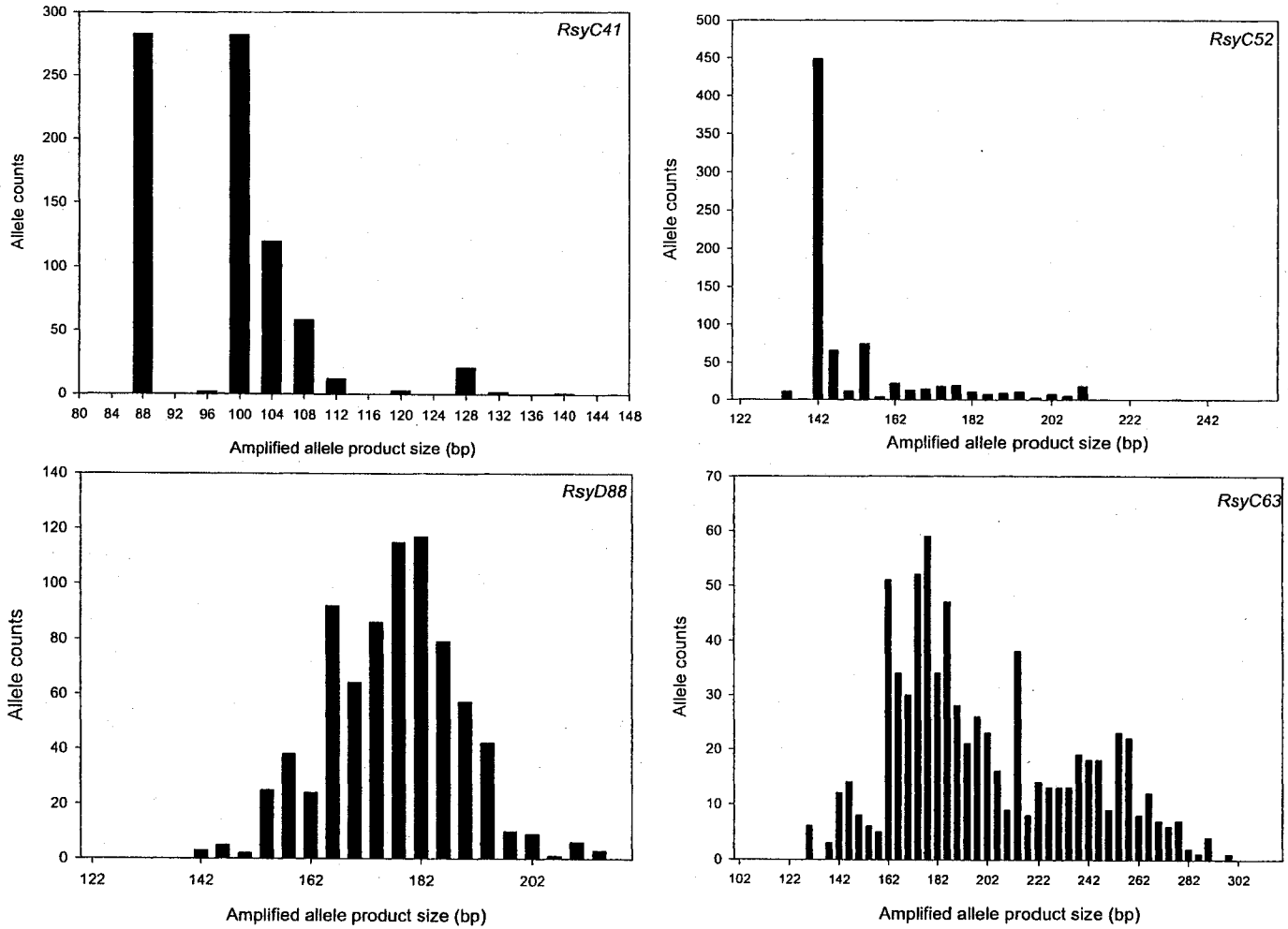


Figure 13. Histograms of microsatellite amplified allele products for loci *RsyC41, RsyC52, RsyD88, RsyC63* for all 9 wood frog *(Rana sylvatica)* collection sites.

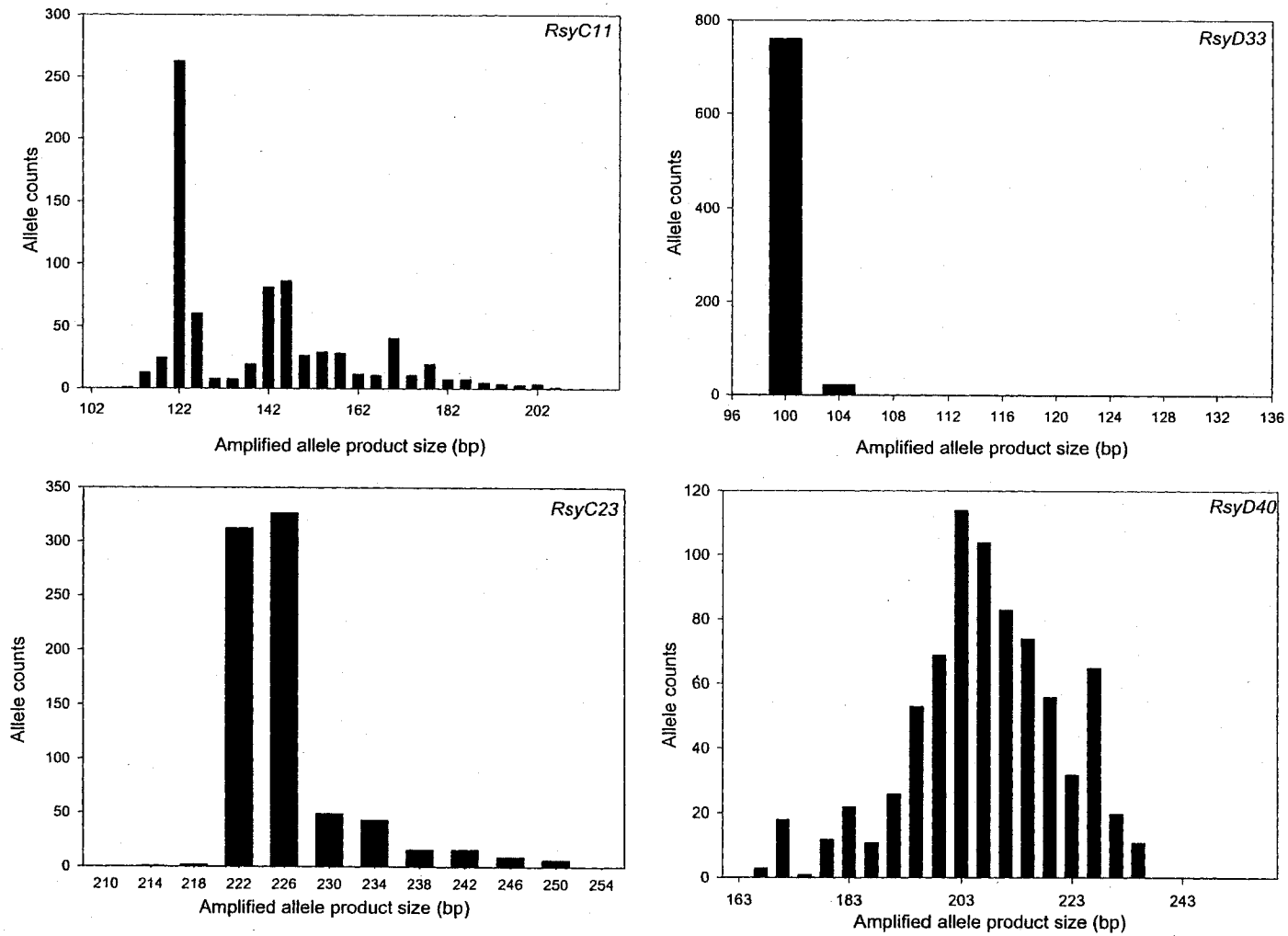


Figure 14. Histograms of microsatellite amplified allele products for loci *RsyC11, RsyC23, RsyD33, RsyD40* for all 9 wood frog (*Rana sylvatica)* collection sites.

Table 9. Results of likelihood-ratio tests comparing the fit of two microsatellite mutational models (GSM and SMM) to the data.
‡ One model fit the data significantly better than the other if twice the difference in log likelihood values was 3.84 (χ^2, d.f.=1).
*P = 0.05; **P =0.005.

Locus name	SMM Θ	SMM Log L (Θ)	GSM Θ	GSM estimated proportions of multi-step mutations (p)	GSM Log L (Θ)	Log L difference‡	Best model
RsyD88	30.450527	-84.850970	33.814248	0.034500	-76.699386	8.151584**	GSM
RsyC63	171.581727	-309.304871	187.032214	0.034500	-336.359475	27.054604**	SMM
RsyC52	18.920563	-122.319958	22.626653	0.059000	-117.713824	4.606134**	GSM
RsyC41	7.148274	-65.641507	2.118007	0.353000	-54.833028	10.808479**	GSM
RsyD40	33.541026	-80.999770	53.525400	0.010000	-83.439490	2.43979*	SMM
RsyD33	0.582375	-22.527081	0.361073	0.426500	-13.473247	9.053834**	GSM
RsyC23	7.823970	-46.980690	10.194330	0.020000	-48.058824	1.078134	SMM
RsyC11	48.902655	-133.299474	58.581306	0.010000	-136.065717	2.766243*	SMM

Table 10. Summary of N_e (effective population size) and *D* (proportion of heterozygote excess averaged over all loci) estimates for each sample site bootstrapped for 24 individuals based on collection site with lowest sample size, York (n=24).

Sampling site	Estimate of D	Ne estimate
Wellington #1	0.2088	Infinite
Wellington #2	-0.4725	Infinite
Wellington #3	0.1755	Infinite
Wellington #4	-0.9461	Infinite
Wellington #5	0.7545	10.4
Wellington #6	-0.9354	41.8
Wellington #7	-0.3470	11.1
Wellington #8	0.5679	Infinite
York	0.2874	15.8
Overall	-0.0862	Infinite

Table 11. Summary of all linear and logarithmic regressions, Mantel tests associated *P*-values, and Pearson's correlation coefficient for geographic distances and θ/(1-θ) genetic distances, based on 30 000 randomizations using IBDWS (Jensen *et al.* 2005). Bold typeface indicates a significant Mantel test associated *P*-value, $P \leq 0.05$.

Type of regression	Loci excluded	Sites excluded	Pearson's r	P-value
Linear (geographic distance and genetic distance)	63	York	-0.1601	0.6710
Log genetic distance	63	York	-0.1482	0.6812
Log geographic distance	63	York	0.0292	0.4356
Log (geographic and genetic distance)	63	York	0.0432	0.4039
Linear (geographic distance and genetic distance)	-	-	0.7774	0.0682
Log genetic distance	-	-	0.6613	**0.0403**
Log geographic distance	-	-	0.5905	**0.0379**
Log (geographic and genetic distance)	-	-	0.5982	**0.0130**
Linear (geographic distance and genetic distance)	63	-	0.7734	0.0723
Log genetic distance	63	-	0.6679	0.0734
Log geographic distance	63	-	0.5811	**0.0485**
Log (geographic and genetic distance)	63	-	0.5350	**0.0486**
Linear (geographic distance and genetic distance)	-	York	-0.0526	0.5266
Log genetic distance	-	York	0.1147	0.3423
Log geographic distance	-	York	0.1347	0.2965
Log (geographic and genetic distance)	-	York	0.2752	0.0974

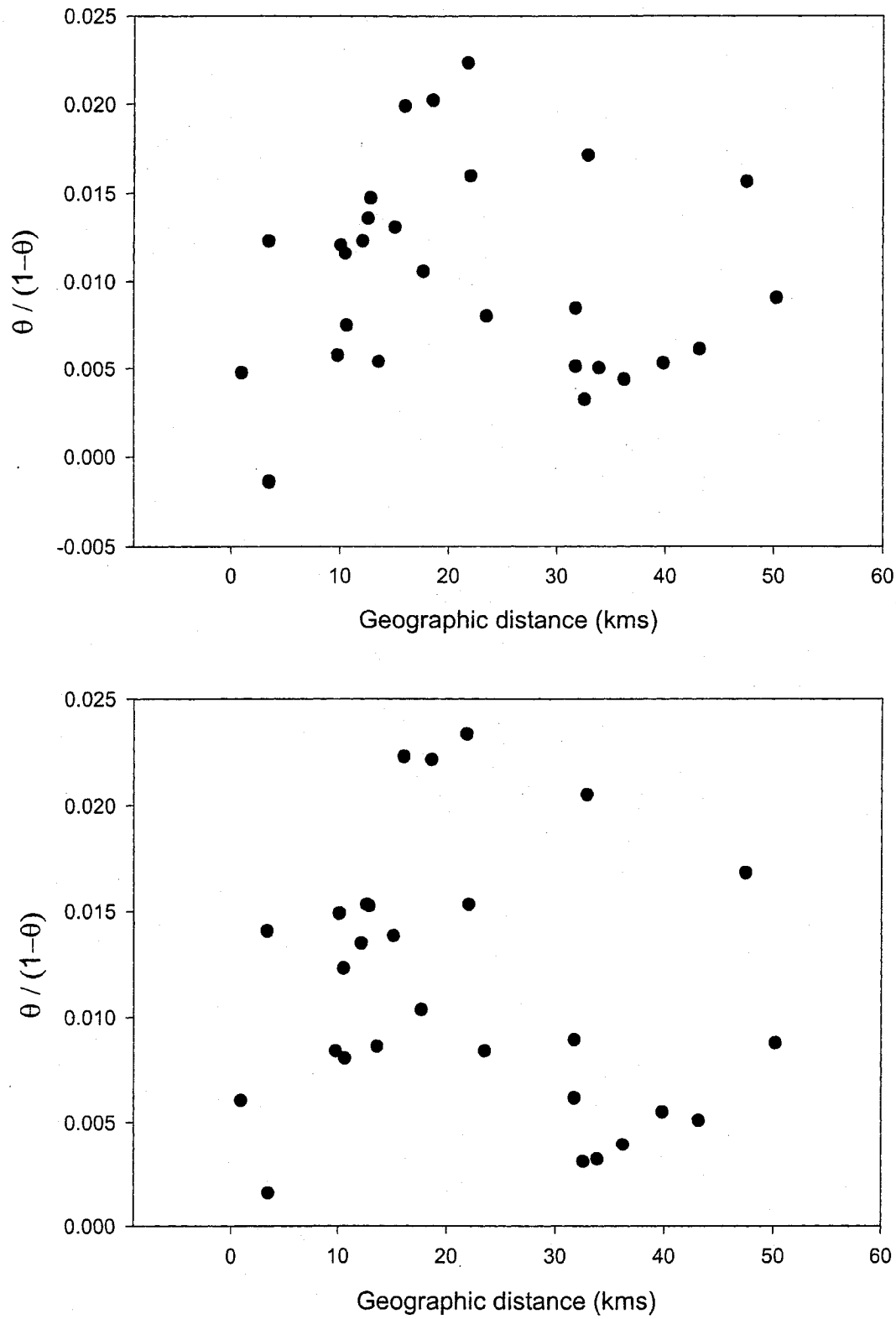


Figure 15. Correlation of θ/(1- θ) Rousset's (1997) genetic distance with geographic distance between pairs of wood frog sampling sites from Wellington county, above figure includes locus RsyC63, lower does not.

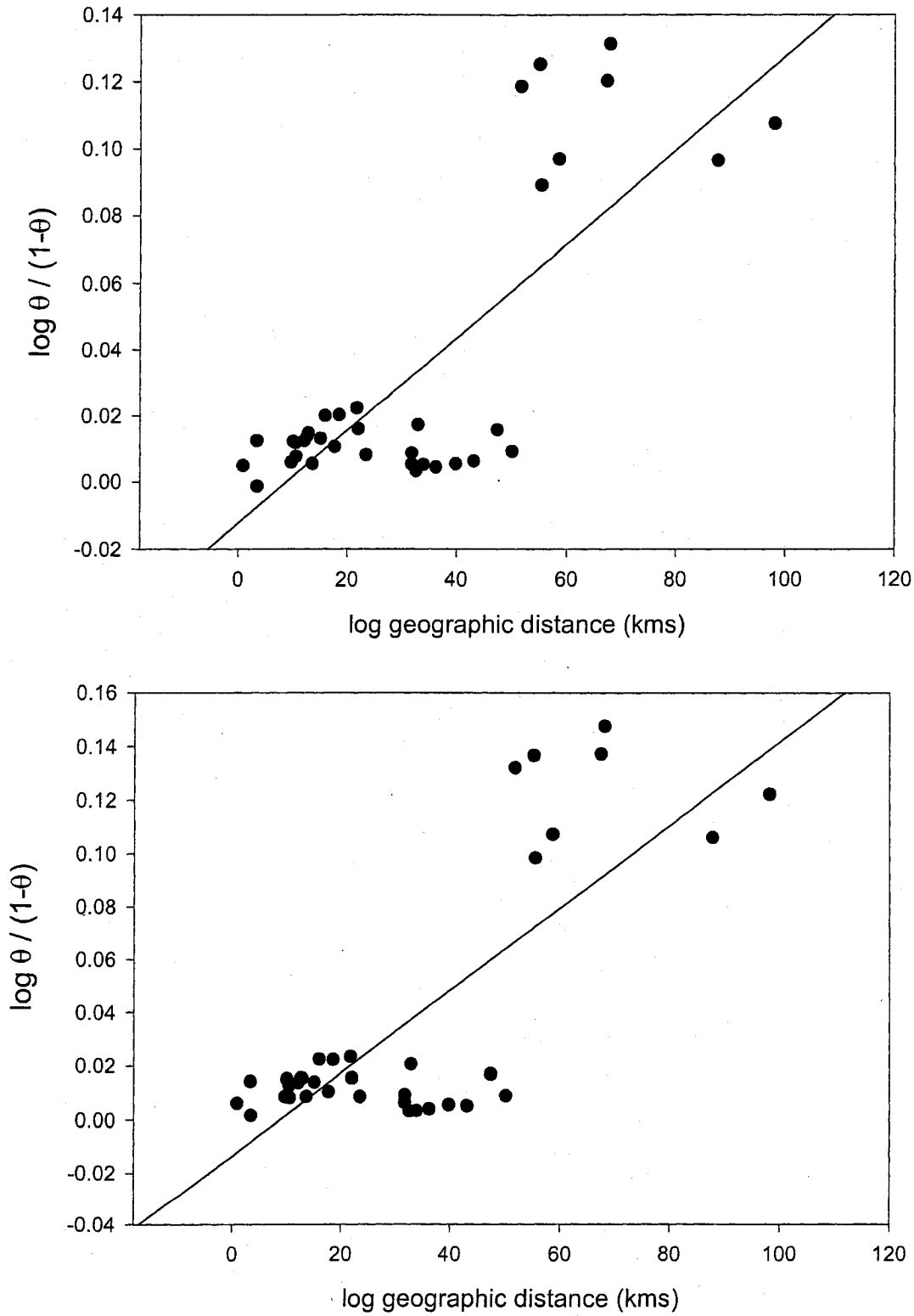


Figure 16. Correlation of log θ/(1- θ) Rousset's (1997) log genetic distance with geographic distance between pairs of wood frog sampling sites from Wellington county and the York site, above figure includes RsyC63, lower figure does not.

Table 12. Garza-Williamson (2001) index summarizing the potential reduction of alleles due to drift, mutation and lack of migration.

Locus	W1	W2	W3	W4	W5	W6	W7	W8	York	Mean	S.D.
RsyD88	0.70588	0.81250	0.93333	0.92308	0.62500	0.86667	1.00000	1.00000	0.85714	0.85818	0.1201
RsyC63	0.72973	0.78378	0.85366	0.73684	0.46341	0.75000	0.67568	0.66667	0.48387	0.68263	0.1234
RsyC52	0.52632	0.60000	0.95000	0.55000	0.75000	0.42308	0.81250	0.66667	0.35000	0.6254	0.17967
RsyC41	0.54545	0.45455	0.50000	0.66667	0.63636	0.66667	0.54545	0.58333	0.60000	0.57761	0.06919
RsyD40	0.87500	0.81250	1.00000	0.88235	0.76471	0.93333	0.94118	0.93750	0.77778	0.88048	0.07646
RsyD33	1.00000	1.00000	0.42857	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.93651	0.17958
RsyC23	0.85714	0.88889	1.00000	1.00000	1.00000	0.87500	1.00000	0.75000	0.75000	0.90234	0.09862
RsyC11	0.63158	0.73913	0.95833	0.83333	0.50000	1.00000	0.69565	0.89474	0.38462	0.73749	0.19593
Mean	0.73389	0.76142	0.82799	0.82403	0.71744	0.81434	0.83381	0.81236	0.65043	0.77508	0.05993
S.D.	0.15644	0.15796	0.21503	0.15112	0.19036	0.18322	0.16661	0.15478	0.21859	0.17712	0.02461

Table 13. Allelic richness per locus and sampling site, values bootstrapped and averaged, based on a minimum sample size of 24 diploid individuals (York site).

Loci	*W1*	*W2*	*W3*	*W4*	*W5*	*W6*	*W7*	*W8*	*York*	*Mean*
RsyD88	11.271	12.431	11.215	11.194	9.835	12.379	11.514	10.613	6.000	12.427
RsyC63	23.433	25.028	22.342	22.162	18.678	20.869	21.754	23.176	15.000	24.053
RsyC52	8.959	9.460	11.992	9.042	5.429	8.630	9.896	10.655	7.000	11.098
RsyC41	5.911	4.994	5.340	6.481	6.483	3.997	5.490	6.190	3.000	5.723
RsyD40	13.270	12.296	13.535	13.259	12.782	12.871	13.832	14.040	7.000	13.571
RsyC23	5.656	6.869	5.361	6.800	5.972	5.876	4.394	5.878	3.000	6.114
RsyC11	11.020	13.374	14.448	12.335	10.444	14.826	12.649	15.525	5.000	14.398

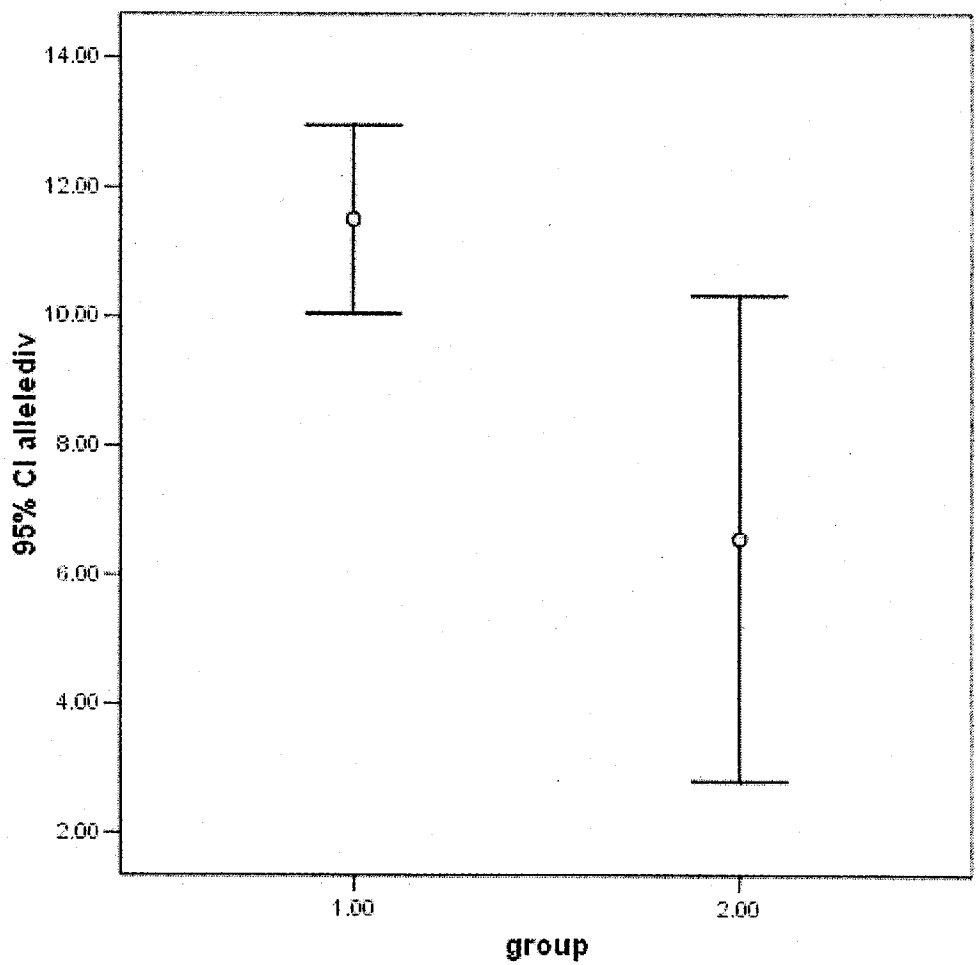


Figure 17. One-way simple ANOVA plot of the comparison between allelic richness (allelediv) values for the York group (Group 2) and the Wellington sites (Group 1) with 95% confidence intervals, using SPSS version 12 for Windows (SPSS 2003).

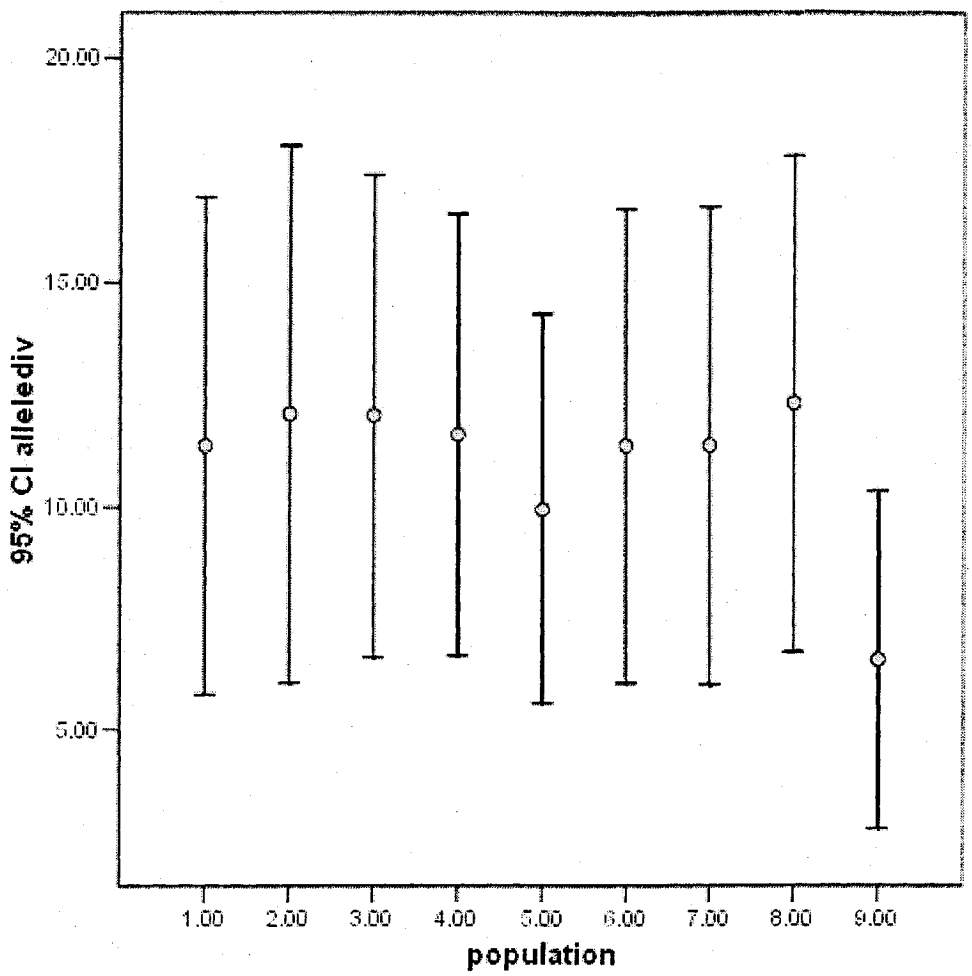
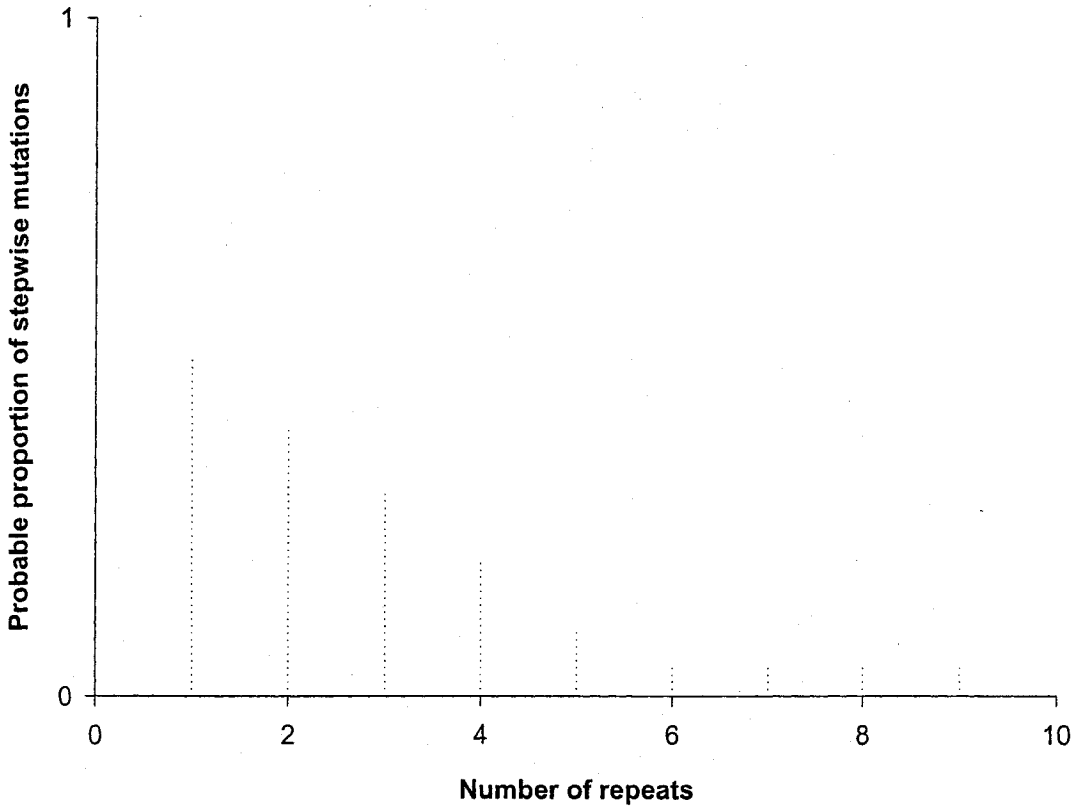
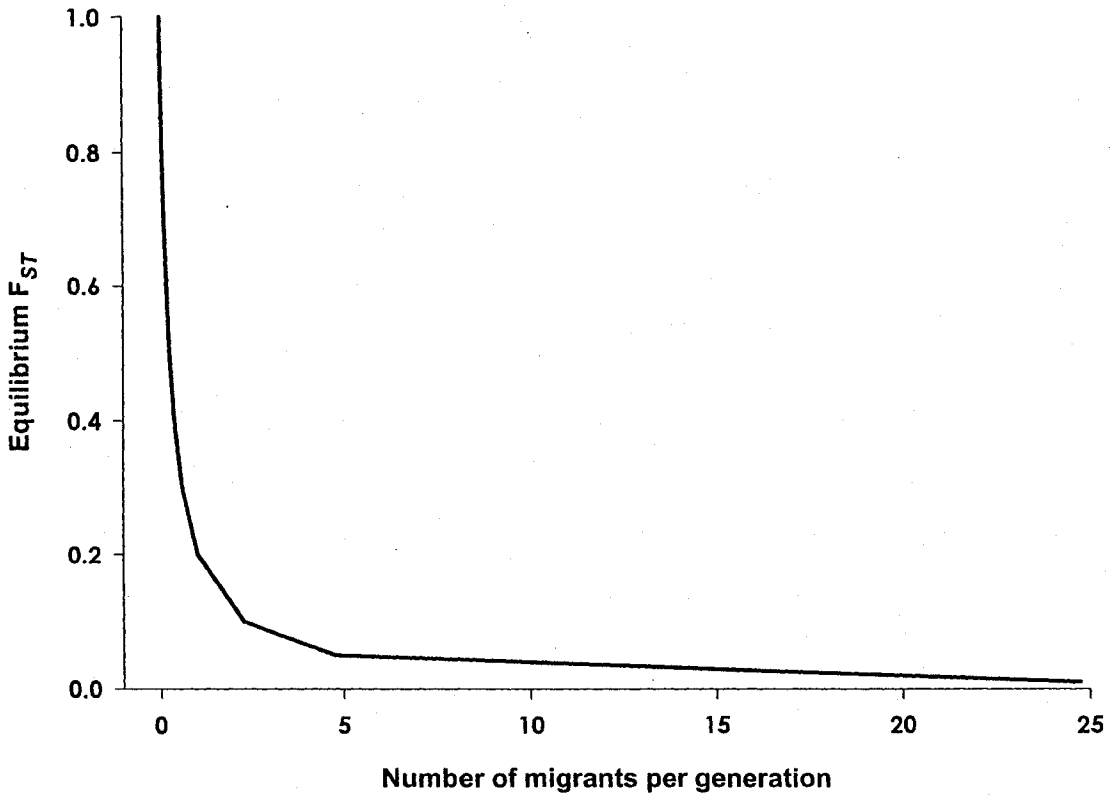


Figure 18. Summary of post-hoc tests for comparison of the allelic richness (allelediv) for all nine wood frog (*Rana sylvatica*) collection sites performed in SPSS version 12 for WINDOWS (SPSS 2003).

Appendix 1. Generalization of a microsatellite DNA locus that has undergone stepwise mutation, and the probable proportion number of repeats created in replication slippage with no DNA repair during a mutational event.



Appendix 2. Generalized relationship of number of migrants (*Nm*) to decreasing fixation index F_{ST} values among demes, assuming genetic equilibrium under Wright's island model of migration, where $F_{ST} \sim 1/(4N_m + 1$



Appendix 3. Pairwise geographic straight-line distance (kms) (below diagonal) between wood frog (*Rana sylvatica*) collection sites, calculated using latitude/longitude distance calculator available from: http://jan.ucc.nau.edu/~cvm/latlongdist.html.

	W1	W2	W3	W4	W5	W6	W7	W8	York
W1	-								
W2	50.22	-							
W3	18.53	31.73	-						
W4	17.66	32.57	0.95	-					
W5	3.40	47.48	15.98	15.07	-				
W6	36.21	23.47	21.72	21.98	32.86	-			
W7	13.59	39.82	10.05	9.76	12.81	31.74	-		
W8	10.60	43.15	12.57	12.08	10.47	33.88	3.50	-	
York	51.71	98.14	67.86	67.24	55.10	87.80	58.55	55.44	-

Appendix 4. Raw allele frequency data for wood frog (*Rana sylvatica*) for each locus and each collection site, unique alleles are in **bold** typeface and identified with an *.

Locus	Allele	Population Well #1	Well #2	Well #3	Well #4	Well #5	Well #6	Well #7	Well #8	York
RsyD88										
	142	**0.0441***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	146	0.0147	0.0000	0.0055	0.0341	0.0000	0.0000	0.0000	0.0000	0.0000
	150	0.0000	0.0000	0.0000	0.0000	0.0179	0.0000	0.0111	0.0000	0.0000
	154	0.0000	0.0581	0.0330	0.0227	0.0000	0.0417	0.0111	0.0156	0.1250
	158	0.0000	0.0581	0.0604	0.0114	0.0000	0.0313	0.1444	0.0469	0.0417
	162	0.0000	0.0233	0.0275	0.0455	0.0357	0.0313	0.0333	0.0781	0.0000
	166	0.0441	0.0465	0.1868	0.1705	0.2500	0.0417	0.0778	0.0781	0.1250
	170	0.0588	0.1047	0.0275	0.0795	0.0893	0.0313	0.0556	0.1406	0.3542
	174	0.1765	0.0814	0.0495	0.1364	0.1250	0.1250	0.1333	0.0313	0.2708
	178	0.1324	0.1628	0.1593	0.1136	0.1071	0.1667	0.1444	0.2188	0.0833
	182	0.1029	0.1163	0.2582	0.0909	0.1964	0.0833	0.1333	0.2188	0.0000
	186	0.0588	0.1395	0.0934	0.1591	0.0714	0.1563	0.0889	0.0781	0.0000
	190	0.1912	0.1047	0.0495	0.0682	0.0536	0.1146	0.0222	0.0625	0.0000
	194	0.1324	0.0465	0.0220	0.0682	0.0000	0.0625	0.1222	0.0313	0.0000
	198	0.0294	0.0233	0.0220	0.0000	0.0000	0.0000	0.0222	0.0000	0.0000
	202	0.0000	0.0000	0.0055	0.0000	0.0000	0.0833	0.0000	0.0000	0.0000
	206	**0.0147***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	210	0.0000	0.0000	0.0000	0.0000	0.0536	0.0313	0.0000	0.0000	0.0000
	214	0.0000	**0.0349***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
RsyC63										
	130	0.0000	0.0250	0.0055	0.0349	0.0000	0.0000	0.0000	0.0000	0.0000
	138	0.0152	0.0000	0.0000	0.0000	0.0200	0.0000	0.0000	0.0161	0.0000
	142	0.0152	0.0250	0.0220	0.0349	0.0200	0.0000	0.0000	0.0161	0.0000
	146	0.0455	0.0000	0.0165	0.0000	0.0000	0.0217	0.0270	0.0161	0.0625
	150	0.0000	0.0250	0.0110	0.0000	0.0000	0.0109	0.0000	0.0161	0.0417
	154	0.0152	0.0000	0.0000	0.0233	0.0000	0.0109	0.0135	0.0000	0.0208
	158	0.0000	0.0000	0.0055	0.0233	0.0000	0.0000	0.0135	0.0161	0.0000
	162	0.0606	0.0750	0.0934	0.0581	0.1400	0.1196	0.0000	0.0161	0.0000
	166	0.0455	0.0500	0.0275	0.0465	0.1000	0.0870	0.0405	0.0323	0.0000

170	0.0000	0.0250	0.0549	0.0465	0.0600	0.0326	0.0676	0.0323	0.0208
174	0.0303	0.0500	0.1044	0.0930	0.0400	0.0109	0.0676	0.1290	0.0625
178	0.1212	0.0000	0.0659	0.0814	0.0600	0.0652	0.1486	0.1290	0.0833
182	0.0606	0.0500	0.0220	0.0000	0.0800	0.1087	0.0541	0.0645	0.0000
186	0.1061	0.0250	0.0989	0.0814	0.0600	0.0326	0.0541	0.0484	0.0000
190	0.0152	0.0375	0.0495	0.0465	0.0200	0.0109	0.0676	0.0645	0.0000
194	0.0455	0.0625	0.0165	0.0116	0.0000	0.0435	0.0270	0.0484	0.0000
198	0.0000	0.0125	0.0220	0.0116	0.0200	0.0978	0.0405	0.0323	0.1042
202	0.0000	0.0375	0.0385	0.0930	0.0000	0.0217	0.0270	0.0000	0.0208
206	0.0758	0.0125	0.0055	0.0116	0.0400	0.0217	0.0541	0.0000	0.0000
210	0.0152	0.0250	0.0110	0.0000	0.0000	0.0217	0.0270	0.0000	0.0000
214	0.0000	0.0750	0.0440	0.0814	0.0000	0.0326	0.0135	0.0161	0.2500
218	0.0000	0.0125	0.0275	0.0116	0.0200	0.0000	0.0000	0.0000	0.0000
222	0.0152	0.0375	0.0165	0.0000	0.0000	0.0217	0.0000	0.0161	0.0833
226	0.0152	0.0250	0.0165	0.0349	0.0200	0.0217	0.0135	0.0000	0.0000
230	0.0000	0.0000	0.0220	0.0116	0.1000	0.0000	0.0270	0.0161	0.0000
234	0.0152	0.0500	0.0165	0.0000	0.0000	0.0109	0.0135	0.0000	0.0625
238	0.0152	0.0500	0.0275	0.0233	0.0000	0.0109	0.0000	0.0000	0.1250
242	0.0303	0.0500	0.0000	0.0116	0.0600	0.0543	0.0270	0.0161	0.0000
246	0.0606	0.0250	0.0165	0.0000	0.0000	0.0543	0.0270	0.0323	0.0000
250	0.0152	0.0125	0.0055	0.0116	0.0000	0.0109	0.0000	0.0484	0.0208
254	0.0303	0.0250	0.0055	0.0000	0.1000	0.0435	0.0676	0.0484	0.0208
258	0.0000	0.0250	0.0604	0.0349	0.0200	0.0000	0.0270	0.0484	0.0000
262	0.0303	0.0250	0.0055	0.0233	0.0000	0.0000	0.0000	0.0161	0.0000
266	0.0000	0.0250	0.0275	0.0116	0.0000	0.0000	0.0405	0.0000	0.0208
270	0.0152	0.0000	0.0110	0.0233	0.0000	0.0000	0.0000	0.0323	0.0000
274	0.0303	0.0250	0.0055	0.0116	0.0000	0.0000	0.0000	0.0000	0.0000
278	0.0303	0.0000	0.0165	0.0116	0.0000	0.0109	0.0000	0.0000	0.0000
282	**0.0303***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
286	0.0000	0.0000	0.0000	0.0000	0.0000	**0.0109***	0.0000	0.0000	0.0000
290	0.0000	0.0000	0.0055	0.0000	0.0000	0.0000	0.0135	0.0323	0.0000
298	0.0000	0.0000	0.0000	0.0000	**0.0200***	0.0000	0.0000	0.0000	0.0000

RsyC52										
	134	0.0303	0.0233	0.0106	0.0341	0.0000	0.0106	0.0000	0.0000	0.0208
	138	0.0000	0.0000	**0.0053***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	142	0.6212	0.5698	0.4840	0.5114	0.7500	0.6383	0.5889	0.6094	0.6042
	146	0.1212	0.1047	0.0532	0.1477	0.0179	0.0957	0.1333	0.0625	0.0000
	150	0.0000	0.0116	0.0266	0.0000	0.0000	0.0532	0.0111	0.0000	0.0000
	154	0.0606	0.1047	0.1436	0.1250	0.1786	0.0106	0.0667	0.0469	0.0833
	158	0.0000	0.0000	0.0053	0.0114	0.0000	0.0213	0.0000	0.0000	0.0000
	162	0.0000	0.0814	0.0426	0.0000	0.0179	0.0000	0.0333	0.0469	0.0000
	166	0.0303	0.0116	0.0213	0.0227	0.0179	0.0000	0.0222	0.0156	0.0000
	170	0.0606	0.0233	0.0053	0.0227	0.0179	0.0000	0.0556	0.0000	0.0000
	174	0.0152	0.0233	0.0585	0.0000	0.0000	0.0319	0.0111	0.0000	0.0000
	178	0.0152	0.0000	0.0426	0.0000	0.0000	0.0532	0.0222	0.0625	0.0000
	182	0.0303	0.0000	0.0106	0.0000	0.0000	0.0000	0.0111	0.0625	0.0417
	186	0.0000	0.0233	0.0000	0.0341	0.0000	0.0000	0.0222	0.0156	0.0000
	190	0.0000	0.0000	0.0106	0.0000	0.0000	0.0000	0.0111	0.0156	0.1042
	194	0.0000	0.0000	0.0106	0.0114	0.0000	0.0638	0.0000	0.0313	0.0000
	198	0.0000	0.0000	0.0106	0.0000	0.0000	0.0106	0.0000	0.0000	0.0000
	202	0.0000	0.0116	0.0213	0.0114	0.0000	0.0000	0.0111	0.0156	0.0000
	206	0.0152	0.0000	0.0053	0.0000	0.0000	0.0000	0.0000	0.0000	0.0625
	210	0.0000	0.0116	0.0319	0.0682	0.0000	0.0000	0.0000	0.0156	0.0833
	234	0.0000	0.0000	0.0000	0.0000	0.0000	**0.0106***	0.0000	0.0000	0.0000
RsyC41										
	88	0.4545	0.2907	0.3670	0.3721	0.4655	0.3021	0.3478	0.4063	0.2708
	96	0.0000	0.0000	0.0000	0.0116	0.0172	0.0000	0.0000	0.0000	0.0000
	100	0.2727	0.3140	0.3723	0.3023	0.2069	0.3958	0.3804	0.3438	0.7083
	104	0.0909	0.2209	0.1223	0.1744	0.1897	0.2188	0.1413	0.1719	0.0208
	108	0.1061	0.0698	0.0957	0.0930	0.0862	0.0833	0.0543	0.0156	0.0000
	112	0.0455	0.0000	0.0053	0.0116	0.0000	0.0000	0.0652	0.0156	0.0000
	120	0.0000	0.0000	0.0000	0.0000	0.0172	0.0000	0.0000	0.0313	0.0000
	128	0.0303	0.1047	0.0319	0.0233	0.0172	0.0000	0.0109	0.0000	0.0000
	132	0.0000	0.0000	0.0000	0.0116	0.0000	0.0000	0.0000	0.0156	0.0000
	140	0.0000	0.0000	**0.0053***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

RsyD40									
	167	0.0000	0.0122	0.0106	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	171	0.0152	0.0000	0.0319	0.0357	0.0893	0.0000	0.0111	0.0313	0.0000
	175	0.0000	0.0000	0.0053	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	179	0.0000	0.0000	0.0213	0.0476	0.0000	0.0104	0.0222	0.0156	0.0000
	183	0.0303	0.0976	0.0160	0.0714	0.0000	0.0000	0.0222	0.0156	0.0000
	187	0.0152	0.0244	0.0053	0.0119	0.0357	0.0208	0.0111	0.0156	0.0000
	191	0.0758	0.0488	0.0266	0.0000	0.0357	0.0729	0.0111	0.0313	0.0000
	195	0.0606	0.0610	0.0904	0.0595	0.0357	0.0729	0.0556	0.1094	0.0208
	199	0.0758	0.0366	0.1223	0.0357	0.0893	0.1354	0.0889	0.0781	0.0833
	203	0.1212	0.1463	0.2128	0.1548	0.0536	0.1458	0.1222	0.1563	0.0625
	207	0.1667	0.0732	0.1064	0.2024	0.1250	0.1458	0.2000	0.1719	0.0000
	211	0.0909	0.0976	0.0957	0.1071	0.1964	0.0625	0.1111	0.0625	0.2292
	215	0.1061	0.1341	0.0585	0.1071	0.1071	0.0729	0.1111	0.1094	0.1250
	219	0.1212	0.1098	0.0691	0.0357	0.0714	0.0833	0.0333	0.0469	0.1042
	223	0.0455	0.0610	0.0532	0.0238	0.0357	0.0208	0.0333	0.0781	0.0000
	227	0.0303	0.0976	0.0532	0.0833	0.1071	0.0417	0.0778	0.0469	0.3750
	231	0.0455	0.0000	0.0160	0.0119	0.0000	0.0625	0.0556	0.0313	0.0000
	235	0.0000	0.0000	0.0053	0.0119	0.0179	0.0521	0.0333	0.0000	0.0000
RsyD33									
	100	1.0000	0.9884	0.9740	0.9432	0.9259	0.9688	0.9565	0.9839	1.0000
	104	0.0000	0.0116	0.0208	0.0568	0.0741	0.0313	0.0435	0.0161	0.0000
	124	0.0000	0.0000	**0.0052***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
RsyC23									
	214	0.0000	0.0000	0.0000	0.0000	0.0000	**0.0104***	0.0000	0.0000	0.0000
	218	0.0000	0.0116	0.0000	0.0119	0.0000	0.0000	0.0000	0.0000	0.0000
	222	0.3788	0.3372	0.2819	0.2738	0.3621	0.4375	0.5000	0.4844	0.8958
	226	0.3485	0.4419	0.5904	0.5476	0.3448	0.3021	0.4022	0.3125	0.0625
	230	0.1364	0.0116	0.0426	0.0357	0.1034	0.1250	0.0543	0.0781	0.0000
	234	0.0909	0.0698	0.0213	0.0595	0.0862	0.0833	0.0326	0.0625	0.0417
	238	0.0303	0.0000	0.0160	0.0357	0.0690	0.0313	0.0109	0.0000	0.0000
	242	0.0000	0.0233	0.0426	0.0119	0.0345	0.0104	0.0000	0.0313	0.0000
	246	0.0152	0.0581	0.0053	0.0238	0.0000	0.0000	0.0000	0.0000	0.0000

RsyC11	**250**	0.0000	0.0465	0.0000	0.0000	0.0000	0.0000	0.0000	0.0313	0.0000
	110	0.0000	0.0000	**0.0053***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	114	0.0000	0.0000	0.0053	0.0119	0.1207	0.0104	0.0333	0.0000	0.0000
	118	0.0000	0.0814	0.0266	0.0119	0.0000	0.0208	0.0667	0.0625	0.0000
	122	0.3824	0.3372	0.3670	0.3810	0.3793	0.3333	0.3667	0.2500	0.0833
	126	0.1176	0.0581	0.0426	0.1310	0.0000	0.1042	0.0111	0.0938	0.2500
	130	0.0441	0.0000	0.0106	0.0119	0.0000	0.0104	0.0111	0.0000	0.0000
	134	0.0441	0.0116	0.0160	0.0000	0.0000	0.0104	0.0000	0.0000	0.0000
	138	0.0882	0.0000	0.0160	0.0238	0.0517	0.0313	0.0000	0.0469	0.0000
	142	0.1324	0.1395	0.1277	0.0476	0.1034	0.1042	0.1111	0.1094	0.0000
	146	0.0147	0.1163	0.1223	0.1310	0.1552	0.0313	0.1667	0.1094	0.1667
	150	0.0000	0.0465	0.0479	0.0357	0.0172	0.0625	0.0111	0.0469	0.0000
	154	0.0441	0.0581	0.0479	0.0595	0.0000	0.0313	0.0444	0.0156	0.0000
	158	0.0588	0.0116	0.0160	0.0000	0.0517	0.1042	0.0444	0.0156	0.0625
	162	0.0147	0.0349	0.0213	0.0000	0.0172	0.0104	0.0000	0.0313	0.0000
	166	0.0000	0.0116	0.0106	0.0119	0.0517	0.0208	0.0000	0.0313	0.0000
	170	0.0000	0.0116	0.0319	0.0476	0.0000	0.0417	0.0111	0.0625	0.4375
	174	0.0441	0.0116	0.0106	0.0119	0.0000	0.0208	0.0111	0.0156	0.0000
	178	0.0000	0.0233	0.0213	0.0595	0.0172	0.0208	0.0444	0.0313	0.0000
	182	0.0000	0.0000	0.0000	0.0238	0.0000	0.0208	0.0333	0.0156	0.0000
	186	0.0000	0.0233	0.0053	0.0000	0.0000	0.0104	0.0111	0.0469	0.0000
	190	0.0000	0.0000	0.0213	0.0000	0.0000	0.0000	0.0000	0.0156	0.0000
	194	0.0147	0.0000	0.0160	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	198	0.0000	0.0000	0.0053	0.0000	0.0345	0.0000	0.0000	0.0000	0.0000
	202	0.0000	0.0116	0.0053	0.0000	0.0000	0.0000	0.0222	0.0000	0.0000
	206	0.0000	**0.0116***	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000