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Population genetics reveal *Myotis keenii* (Keen's myotis) and *Myotis evotis* (long-eared myotis) to be a single species

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Abstract: Recognizing delineations of gene flow among groups of animals can be challenging, but necessary for conservation and management. Of particular importance is the identification of species boundaries. Several physical and genetic traits have been used with mixed success to distinguish *Myotis keenii* (Merriam, 1895) (Keen's myotis) and *Myotis evotis* (H. Allen, 1864) (long-eared myotis), but it is unclear whether species distinction is biologically warranted. We generated 12-14 microsatellite loci genotypes for 275 long-eared Myotis representing 4 species -- *M*. *keenii*, *M. evotis, Myotis septentrionalis* (Trouessart, 1897) (northern myotis), and *Myotis thysanodes* Miller, 1897 (fringed myotis) -- from across northwestern North America, and 23 *Myotis lucifugus* (Le Conte, 1831) (little brown myotis) as outgroup. Population genetics analyses revealed four well defined groups (species): *M. septentrionalis, M. thysanodes, M. lucifugus* and a single group comprising *M. keenii* and *M. evotis*. We document high rates of gene flow within *M. evotis*/*keenii*. Cytochrome *b* gene (mtDNA) sequencing failed to resolve morphologically identifiable species. We highlight the importance of geographically thorough investigation of genetic connectivity (nuclear markers) when assessing taxonomic status of closely related groups. We document a morphometric cline within *M. evotis*/*keenii* that may in part explain earlier analyses that led to the description of the smaller-bodied *M. keenii* (type locality Haida Gwaii). We conclude that *M. keenii* does not qualify as a genetic or biological species.

Key words: *Myotis keenii* (Keen's myotis), *Myotis evotis* (long-eared myotis), *Myotis thysanodes* (fringed myotis), *Myotis septentrionalis* (northern myotis), *Myotis lucifugus* (little brown myotis), microsatellites, taxonomy

Introduction

In animals whose morphological evolution is constrained, convergence of traits has made taxonomy difficult (Lowe et al. 2004). Microchiropteran bats are a classic example of shared ecological constraints, with flight and nocturnal behaviour limiting morphological differentiation (Norberg 1994; Ruedi and Mayer 2001; Kawai et al. 2003), and phenotypic plasticity potentially confounding morphological traits that are often used to differentiate species (Miller and Allen 1928; van Zyll de Jong 1985). Genetic tools may help to overcome these challenges (e.g. Barratt et al. 1997). Baker and Bradley (2006) define a genetic species as a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups. Unlike conventional systematics approaches, the methods of population genetics assess whether, and to what extent, the populations under study are connected by gene flow. Nuclear markers are required as mitochondrial (mtDNA) is a single locus generally transferred from females to offspring, and thus may not provide a precise or accurate picture of contemporary gene flow (Avise 2004).

Four species of long-eared myotis bats are recognized in northwestern North America based on morphological traits and geographic location: *Myotis keenii* (Merriam, 1895) (Keen's myotis), *Myotis septentrionalis* (Trouessart, 1897) (northern myotis), *Myotis evotis* (H. Allen, 1864) (long-eared myotis) and *Myotis thysanodes* Miller, 1897 (fringed myotis) (Wilson and Reeder 2005). *M. evotis*, *M. thysanodes* and *M. septentrionalis* are widespread across western North America; *M. keenii* is restricted to Pacific coastal regions. *M. keenii* was first described to science as *Vespertilio subulatus keenii* by Merriam (1895) based on 3 specimens from Masset, Haida Gwaii (formerly Queen Charlotte Islands). Subsequently Miller and Allen (1928) classified

this taxon as conspecific with *M. septentrionalis* with *M. keenii keenii* being treated as the Pacific coastal subspecies and *M. keenii septentrionalis* the eastern and northwestern subspecies. Based on cranio-dental traits, ear size, forearm length, metacarpal lengths and pelage traits, van Zyll de Jong (1979) and van Zyll de Jong and Nagorsen (1994) elevated both taxa to species-rank, and considered the monotypic *M. keenii* to be sister species of *M. evotis*. *M. keenii* and *M. evotis* are notoriously difficult to discriminate in the hand. Van Zyll de Jong and Nagorsen (1994) noted intermediate forms in what was assumed to be a hybridization zone; they concluded that molecular data might be needed to resolve the systematics of these groups. Due to its restricted range, *M. keenii* has been placed on a number of conservation lists of a species of concern (e.g. G3-Vulnerable, NatureServe 2018) including in Washington where it is a state candidate species for possible listing as endangered, threatened or sensitive (Hayes and Wiles 2013), and in British Columbia where it was "red-listed" to denote potential threatened or endangered status (Firman and Barclay 1993). The initial goal of our study was to determine field protocol to differentiate *M. keenii* from the widerspread more common *M. evotis*, but preliminary morphological and acoustic data from across BC (unpublished) brought into question the validity of two distinct species.

A phylogeographic analysis of the mitochondrial cytochrome *b* (*Cyt b*) gene across the range of these long-eared Myotis species (Dewey 2006) weakly supported *M. keenii* as a distinct species, and subsequent analyses adding 6 anonymous nuclear loci produced multiple possible Myotis species trees for *M. keenii, M. evotis, M. thysanodes,* and *M. lucifugus* (Carstens and Dewey 2010). Stemming from the suggestion by Carstens and Dewey (2010) of gene flow among *M. keenii, M. evotis*, and *M. thysanodes*, Morales et al. (2017) examined 'species-with-

gene-flow' models of phylogeny, concluding gene flow occurred among these taxa during divergence prior to the Pleistocene. Until Platt et al. (2018) and Morales et al. (2017), phylogenies of Myotis were based on mtDNA (Dewey 2006) or combinations of mtDNA and nuclear sequences (Stadelmann et al. 2007; Carstens and Dewey 2010), in which mtDNA may have swamped potentially conflicting signals from nuclear markers (Platt et al. 2018). Platt et al.'s (2018) separate analyses of mitochondrial versus nuclear sequences revealed consistently conflicting gene trees between these markers, concluding that bifurcating phylogenies based on a single gene (ie. mtDNA-based species trees) are less likely to reflect true Myotis radiation than phylogenetic networks based on multiple nuclear loci. As has been repeatedly discovered in other taxa, conclusions based solely on mtDNA sequence data can be erroneous or misleading, with examination of nuclear DNA producing different, usually more biologically-reflective, conclusions (e.g. Cronin et al. 1991; Worthington-Wilmer et al. 1994 vs. 1999; Talbot and Shields 1996 vs. Paetkau et al. 1998; Elias et al. 2007). Here we focus not on historical isolation and patterns of evolution within lineages, but examine current patterns of gene flow upon which to base management and conservation decisions. Our study is the first to examine contemporary gene flow of *M. keenii*, using population genetics techniques.

We sequenced *Cyt b* to provide reference to previous work only, but focussed our investigation on microsatellite genotypes to assess nuclear gene flow amongst the 4 nominate species of long-eared Myotis in northwestern North America (*Myotis keenii*, *Myotis septentrionalis*, *Myotis evotis*, and *Myotis thysanodes*), along with *Myotis lucifugus* (Le Conte, 1831) (little brown myotis), which is morphologically distinct, and in some molecular genetic studies has been suggested to be closely related to the long-eared species (Dewey 2006;

Stadelmann et al. 2010, but see Platt et al. 2018). The descriptions of *M. keenii* by van Zyll de Jong (1979) and van Zyll de Jong and Nagorsen (1994) relied largely on morphometrics, including ear and forearm lengths, traits found to help differentiate *M. keenii* from *M. evotis*, so we also examine these morphometrics.

Materials and methods

Sampling

We captured bats using mist nets. We measured the forearm (to nearest 0.1 mm) and pinnae and tragus lengths (to nearest 0.5 mm), described fur colour and looked for the presence of fringe of hair on the tail membrane to aid with species identification as per Nagorsen and Brigham (1993). We took tissue samples from bat wings using a 2mm disposable biopsy punch, and we affixed each wing punch to an index card using scotch tape. In one case, we swabbed an individual's wing to pick up epithelial cells instead of taking a wing biopsy (Player et al. 2017) due to the approaching winter when a biopsy wound was unlikely to heal. This swab was stored in a paper envelope. We conducted all genetic sampling in 2006 – 2014. We genetically sampled bats of five species (*M. lucifugus, M. evotis, M. septentrionalis*, *M. keenii, M. thysanodes*) across their ranges in western North America: British Columbia (all species), Alberta (*M. evotis, M. septentrionalis*), Washington Olympic Peninsula (*M. keenii* and *M. evotis*), Alaska (*M. keenii*), and Flathead Indian Reservation Montana (*M. thysanodes*) (Fig. 1). *Myotis keenii* and *M. evotis* are thought to be sympatric in western British Columbia (van Zyll de Jong 1984) and western Washington (Harvey et al. 2011).

We made field identifications to species level for each of the long-eared Myotis species when possible. We attempted to differentiate *M. keenii* from *M. evotis* by (1). Location – all long-eared Myotis from Alaska and Haida Gwaii were assumed to be *M. keenii* based on previously described species range (van Zyll de Jong 1985, Nagorsen and Brigham 1993, van Zyll de Jong and Nagorsen 1994, Naughton 2012); (2). Morphology – in western areas where *M. keenii* or *M. evotis* have been previously thought sympatric (Hazelton, Lillooet, Vancouver Island, Washington Olympic Peninsula, Skagit, Bella Coola) we used distinct shoulder patches, and long black ear pinnae (extending >5mm beyond the nose when laid forward) to identify *M. evotis*. If species-level identification could not be made, the sample was labelled 'LEMY', long-eared Myotis, which could be *M. keenii*, or *M. evotis* (*M. keenii/evotis*), and in Hazelton could also include *M. septentrionalis* given the similarity of all 3 of these species in this northern site (van Zyll de Jong 1985).

Measurements of forearms and ear lengths were compared using *t*-tests with means expressed as ± standard error. All procedures were done in accordance with guidelines developed by the Canadian Council on Animal Care, under a British Columbia provincial wildlife handling and animal care permits VI08-44304, VI07-34454, VI10-62230, and CB09-59777.

Genetic Processing

We extracted DNA from wing tissue using QIAGEN DNeasy Blood and Tissue kits, and following QIAGEN's instructions for tissue. For the single swab sample, we clipped a ~ 5 mm disc of polyester for extraction.

We tested 20 microsatellite markers, with the goal of identifying a set that amplified strongly, without interference from non-specific peaks, and that appeared variable across our study groups. These 20 markers included 9 that were first identified in *Myotis sodalis* Miller and Allen, 1928 (Indiana myotis) (Trujillo and Amelon 2009; Oyler-McCance and Fike 2011), 6 from *M. lucifugus* (Burns *et al.* 2012), 4 from *Myotis grisescens* A.H. Howell, 1909 (gray myotis) (Lindsay *et al.* 2013), and 1 from *Myotis myotis* (Borkhausen, 1797) (mouse-eared myotis) (Castella and Ruedi 2000). We reviewed the primer design for each marker and modified several primers, either to reduce the length of the amplified product or to reduce the amount of repetitive sequence between primer binding site and microsatellite. We identified 14 markers for use in our study (Supplementary Table S1): MS3D02, MS3F05, MS3E02 (adapted from Trujillo and Amelon 2009), F19 (adapted from Castella and Ruedi 2000), Mluc1, Mluc5, Mluc11, Mluc21, Mluc25 (adapted from Burns et al. 2012), IBatCA11, IBatCA38, IBatCA47, IBatM7 (adapted from Oyler-McCance and Fike 2011), MYGR.2-19 (adapted from Lindsay et al. 2013). Genotypes were error-checked using the protocol of Paetkau (2003), including review of each electronic scoring call by two technicians.

Our initial efforts to sequence the mitochondrial *Cyt b* gene used primers L15162 and H15915 (Irwin et al. 1991), which amplify a region of ~ 750 base pairs, with some length variation between haplotypes. We were unable to amplify from some coastal specimens of *M. keenii* with these primers, likely because of polymorphism in one of the primer binding sites. We therefore used the successful sequences that we obtained with the original primers to design a new pair of primers, targeting sites that appeared to be conserved in British Columbia long-eared bats (forward: 5'-TTACTAATCTACTCTCCGCAATCC-3', reverse: 5'-

GTAGAGAATTGATGCTAATTGTCC-3'). These primers amplified a region 160 bp shorter than the originals, reducing the sequence reads to ~ 590 bp.

Genetic Analyses

We tested the *M. evotis/keenii* microsatellite dataset for conformance to Hardy-Weinberg (H-W) assumptions of random mating using the probability test within GENEPOP 3.1d (Raymond and Rousset 1995). Any locus within each population that failed the H-W test was tested for a deficit of heterozygotes using a global test (Rousset and Raymond 1995). All loci were also tested for linkage disequilibrium using a probability test (Garnier-Gere and Dillman 1992). Critical values for these tests were adjusted for the experiment-wise error rate using the Dunn-Sidak method (Sokal and Rohlf 1995). These tests were performed within GENEPOP 3.1d (Raymond and Rousset 1995).

Our population genetics analysis of the microsatellite data started with a genotype-based principle components algorithm in GENETIX (Belkhir 1999). We performed a nested series of clustering analyses, starting with the entire dataset, and then removing the most distinct groups to facilitate better resolution of groups that were less well resolved in earlier iterations. At the coarsest level this analysis confirmed differences between well-established species. At the finest level it elucidated relationships within and among the closest related species.

We then used a Monte Carlo Markov Chain (MCMC) algorithm in STRUCTURE to assess the number of distinct genetic groups within the entire microsatellite dataset and a subset of the closest related species as determined in GENETIX and STRUCTURE (*M. thysanodes*, *M.*

evotis, and *M. keenii*), to estimate the number of distinct group (*k*) and the proportional ancestry of each individual in each identified group (STRUCTURE; Pritchard et al. 2000). Once again, these analyses assessed 2 levels of biological organization, with some groups in our dataset expected to experience little or no current or recent gene flow (i.e. species), and other groups representing sampling areas within an interconnected population system with ongoing gene flow. In the case of *M. evotis* and *M. keenii*, the goal of our analysis was to assess which of these paradigms best described the relationship of these groups. To assess the number of species in the dataset and not make a priori assumptions of gene flow, we parameterized STRUCTURE to allow for correlated allele frequencies between populations, and mixed individual ancestry (admixture), as would be expected in the presence of ongoing gene flow, a conservative approach to identifying taxonomic groupings. STRUCTURE asks the user to set the number of populations into which the dataset should be divided (*k*), and produces estimates of proportional ancestry for each individual in each of the *k* populations produced by a given MCMC. We performed runs across the range of $k = 1 – 5$, 20 repeats at each value of *k*. We used 100,000 burn-in cycles and collected probabilities over the next 400,000 cycles.

We then used Evanno's criterion ΔK (Evanno et al. 2005) to objectively derive the uppermost hierarchical value of *k*. Because of uneven sample sizes, skewed heavily to *M. evotis/keenii*, and the known tendency for uneven sample sizes to lead to a downward-biased estimate of *k* (Puechmaille 2016), we additionally ran STRUCTURE using a subsampled dataset, reducing the number of *M. evotis/keenii* samples at heavily sampled sites to be similar to

sample sizes attained for the other species. We thus present STRUCTURE results for two datasets: full and subsampled[1].

We examined geographic pattern of genetic distances (F_{ST}, GENEPOP 3.1; Raymond and Rousset 1995) within the combined *M. evotis*/*M. keenii* dataset. Genetic distances between study sites were plotted on a map. We examined *Cyt b* haplotype relationships and distances were calculated using the method of Jukes and Cantor (1969) contained in the DNADIST program (PHYLIP 3.6; Felsenstein 2005).

Results

Samples

We genetically sampled 281 individuals: *M. keenii* (22 in Haida Gwaii archipelago including at the type locality Graham Island, and 28 from southeast Alaska), *M. evotis* (37 from BC interior and Alberta), *M. septentrionalis* (26 from northern Alberta and Hazelton, BC), *M. keenii/M. evotis/M. septentrionalis* (108 from western BC and Washington), *M. thysanodes* (35 from Okanagan, BC and 1 from northwestern Montana), and *M. lucifugus* (11 from western BC [Haida Gwaii, Hazelton, Bella Coola, Okanagan] and 13 from eastern BC [Kootenay, Flathead]) as the related species outgroup. We measured both forearm lengths and ear pinnae lengths of 229 *M. evotis/keenii,* and forearm lengths of 826 *M. lucifugus.*

Microsatellites

[1] Each dataset including sample details can be found in Supplementary Table S2 in Dryad doi:10.5061/dryad.h9b3d30

We produced 12-14 locus genotypes for 275 individuals: 23 *M. lucifugus,* 58 *M. septentrionalis*, 33 *M. thysanodes*, 50 *M. evotis* and 50 *M. keenii* from outside the recognized range overlap for *M. evotis* and *M. keenii,* and 61 *M. evotis/keenii* from within the overlap range of these species. We affirmed the assumption of random mating within all sampling sites of *M. evotis/keenii* and concluded that null alleles were not a problem in this dataset; only 1 of 98 tests had a *p* value smaller than the Dunn-Sidak experiment-wise error correction (Sokal and Rohlf 1995). We found no evidence of linkage disequilibrium.

Genetix -- In our highest level PCA we included samples from all species (Fig. 2A). This analysis revealed 3 clear clusters corresponding to *M. septentrionalis*, *M*. *lucifugus*, and a broad cluster that included *M. thysanodes*, *M. keenii*, *M. evotis* and most 'LEMY' samples; 32 LEMY were revealed through this and other analyses (e.g., *Cyt b* sequencing) as misclassified *M. septentrionalis*. Repeating the PCA after removing *M. septentrionalis* (including misclassified LEMY) and *M. lucifugus,* produced clear separation of *M. thysanodes* from the *M. evotis/keenii* cluster (Fig. 2B). By contrast, when we removed *M. thysanodes*, limiting the PCA to the *M. evotis/keenii* group, samples from coastal (largely thought to be *M. keenii*, especially in the northwestern sites) and interior (*M. evotis*) regions formed a series of broadly overlapping clusters (Fig. 2C).

STRUCTURE – No admixture and uncorrelated allele frequencies are expected among well-defined species; however, to be conservative in our analyses, we allowed the possibility of gene flow among all individuals, thus admixture and correlated allele frequencies were assumed. Using the full dataset, the most probable clustering of the data using Evanno's Δ*K* was *k* = 2 groups (*M. evotis/keenii* and *M. thysanodes* as one group and *M. lucifugus* and *M.*

septentrionalis as a second group), with *k* = 4 a close second. However, because our sampling of *M. evotis/keenii* was substantially larger (*n* = 161) compared to *M. thysanodes* (33), *M. lucifugus* (23) and *M. septentrionalis* (58), and because skewed sample sizes can lead to an underestimate of *k* (Puechmaille 2016), we re-ran this analysis with a subsampled dataset as recommended by Puechmaille (2016). We randomly removed ~75% of the individuals only at sites that had more than 15 samples of *M. evotis/keenii* (*n* = 63), retaining the geographic spread of our sampling and reran the STRUCTURE analysis with the same settings, followed by calculation of Δ*K*. This yielded *k* = 4 as the highest number of clusters (species) in the dataset, corresponding to four species: *M. lucifugus*, *M. septentrionalis*, *M. thysanodes*, and the combined *M. evotis/keenii* (Fig. 3). We re-ran this analysis with just *M. thysanodes*, *M. evotis* and *M. keenii* samples (the latter two subsampled) and confirmed *k* = 2 as the highest probable number of clusters corresponding to two species: *M. thysanodes* and one species cluster of all *Myotis evotis/keenii* individuals (Fig. 4).

Estimates of F_{ST} between sampling sites for all *M. evotis/keenii* are generally low (≤ 0.06), including across the Coast Mountain Range and are particularly low across the Rocky Mountain Range (Fig. 5). This is further support for *M. evotis*/keenii as one interbreeding group over a large geographic area and is in contrast to F_{ST} values between *M. septentrionalis*, *M. lucifugus*, *M. thysanodes* and *M. evotis/keenii*: *M. septentrionalis – M. thysanodes* 0.178, *M. septentrionalis - M. lucifugus* 0.111, *M. thysanodes – M. lucifugus* 0.179; *M. evotis*/keenii to *M. septentrionalis*, *M. lucifugus* and *M. thysanodes* are 0.129, 0.107, and 0.154, respectively.

mtDNA Sequences

We sequenced up to 750 base pairs (minimum 339) of the *Cty b* gene for 73 individuals, identifying 14 haplotypes representing all putative species (Table 1). Overall, 'ke' haplotypes differed from 'lu', 'se', 'ev', and 'th' haplotypes by 32, 78, 7 and 15 base pairs, respectively. Of 49 samples identified as "LEMY" or *M. evotis* or *M. keenii* in the field, 37 (representing 4 haplotypes) and 10 (2 haplotypes) individuals matched sequences identified in Genbank as *M. keenii* (97-100% sequence identity) and *M. evotis* (99-100%), respectively, with 2 being *M. septentrionalis*. All sequenced *M. evotis/keenii* sampled west of the Coast Mountains had "ke" haplotypes, all "ev" haplotypes were found to the east, and samples from within the Coast Mountain range (Skagit and Hazelton) were mixed "ev" and "ke". One *M. thysanodes* from the eastern side of the Coast Mountains carried a "ke" haplotype, inconsistent with the species identification made in the field, and later confirmed though nuclear genetic analysis as *M. thysanodes* (above). In fact, all 6 *M. thysanodes* identified in the field through diagnostic fringed uropatagium, forearm and ear length (Nagorsen and Brigham 1994) carried mitochondrial haplotypes associated with *M. keenii* (1; 99%) or *M. evotis* (5; 94 – 100%) using BLAST searches of Genbank. All 8 samples identified as *M. lucifugus* in the field carried *Cyt b* haplotypes that matched to *M. lucifugus* records in Genbank (94-100%). We identified 8 *M. septentrionalis* through haplotypes. Four were from northern Alberta and were assumed to be *M. septentrionalis* based on field identifications. There is an error rate of 17% in that area differentiating *M. septentrionalis* from *M. lucifugus* by morphology in the field (Grindal et al. 2011). The other 4 samples that were mitochondrially identified as *M. septentrionalis* were "LEMY" from Hazelton, where no attempt was made in the field to differentiate this species from *M. evotis* and *M. keenii* (van Zyll de Jong 1985). All bats with *M. septentrionalis*

mitochondrial haplotypes were also identified as *M. septentrionalis* by nuclear genetic analysis (Fig. 4). We obtained distances using the F84 model (Kishino and Hasegawa 1989; Felsenstein and Churchill 1996) contained in DNADIST of PHYLIP, and used neighbour-joining (Saitou and Nei 1987) to generate a phylogenetic tree (Supplementary Fig. S1).

Morphology

We examined the geographic pattern of ear pinnae lengths of *M. evotis*/keenii to latitude and longitude (combined into single variable latitude*longitude, Lat*Long; Fig. 6A). We found that both ear lengths (18.8 ± 0.1 mm in south/east; 16.7 ± 0.1 mm in north/west) and forearm lengths (Fig. 6B; 38.0 ± 0.1 mm in south/east; 36.3 ± 0.1 mm in north/west) are shorter in the north/west sampling areas (Alaska, Haida Gwaii, Hazelton; >7000 Lat*Long) compared to all other locations (south/east; <6200 Lat*Long; ear length *t*-test, $t = 17.0$, df = 227, $p << 0.001$; forearm length *t*-test, $t = 10.3$, df = 150, $p << 0.001$). This same pattern of shorter north/west forearm lengths was also observed in the other geographically wide-ranging species in our study, *M. lucifugus* (south/east 37.22 ± 0.05 mm; north/west 36.84 ± 0.07 mm; *t*-test, $t = 4.38$, df = 625, $p << 0.001$).

Discussion

Until the advent of molecular techniques, taxonomy of Myotis bats relied on often minor morphological differences in characters to identify species. Genetic analyses have redefined some bat species classifications (e.g., Hoofer and Van Den Bussche 2003; Racey et al. 2007) and helped to clarify others (e.g., Lausen et al. 2008). In this investigation, we present

population genetics evidence that long-eared myotis groups previously thought to comprise two species, *M. evotis* and *M. keenii*, are connected by a rate of gene flow that is too high and too widespread to support their recognition as different species. We have documented what is not a hybrid zone between two species, but extensive gene flow across the ranges of what has been previously considered *M. keenii* and *M. evotis* distributions in the Pacific northwest. This supports ours and others' (Cowan and Guiguet 1965; Davis 1967; van Zyll de Jong and Nagorsen 1994) findings that differentiating these species morphologically in the field across the Pacific northwest where they are sympatric is challenging. Our large sample size (161 individuals) from across the entire range of *M. keenii* and as far east as Alberta for *M. evotis*, provided a thorough examination of gene flow. Estimates of F_{ST} between coastal and interior populations ranged as low as 0.02, with differences between populations thus accounting for ~ $1/10^{th}$ as much of the variance in allele frequencies seen in recently fractured populations of a mammal after only a few generations of anthropogenic habitat fragmentation (e.g., Proctor et al. 2012). At it's extreme, individuals in areas considered 'pure' *M. keenii* (Alaska, Haida Gwaii) compared to 'pure' *M. evotis* on the prairies of Alberta on the eastern side of the Rocky Mountain Continental Divide (distance >1600 km) had an F_{ST} of only 0.06. This is in contrast to interspecies F_{ST} values over our study area ranging from 0.107 – 0.179 (Fig. 5).

Supporting previous genetic studies and known morphological differences, we confirmed *M. septentrionalis* and *M. lucifugus* to each be genetically distinct with both nuclear and mitochondrial markers. Our population genetic data showed that while closely related to *M. keenii* and *M. evotis*, *M. thysanodes* breeds as a distinct group. This is in contrast to Morales et al. (2017) who, based on species tree models of nuclear ultra-conserved element sequences

from 34 *M. evotis* and 20 *M. thysanodes* from as far south as New Mexico, and ~12 *M. keenii*, concluded that evolutionary divergence with gene flow among all 3 species occurred as much as 66,782 generations ago. Morales et al. (2017) concluded that *M. keenii* split off from *M. evotis* and *M. thysanodes*, and all three 'diverged with gene flow.' This is in contrast to Platt et al. (2018) who, using more than three times as many ultra-conserved nuclear elements but only one individual of each of the three species, determined that *M. evotis* split from *M. thysanodes* and *M. keenii*.

While Morales et al. (2017) suggested these species may experience little present gene flow, slightly different analyses of these same long-eared samples with other *Myotis* species (Morales and Carstens 2018) led to the conclusion that "gene flow in North American *Myotis* bats occurs at interspecific levels whenever species come into sympatry, regardless of whether they are sister species or not" (2nd page). Small sample sizes of *M. keenii*, and *M. evotis*, limited geographic sampling biased to overlap zones between *M. evotis* and *M. thysanodes*, a priori assumptions in all models of three taxonomically distinct species, necessary subsampling of data, and use of ultra-conserved elements (vs. microsatellites with high mutation rates and proven use for assessment of recent population sub-structure; Goldstein and Schlotterer 1999) may have contributed to Morales et al. (2017) not detecting the current and extensive gene flow that we document in the Pacific northwest over the entire range of *M. keenii*.

An analogous situation exists with *M. lucifugus*: using similar methods of Morales et al. (2017), Morales and Carsten's (2018) winning species tree model suggest divergence with gene flow among all subspecies, which they elevate to species level and conclude are non-sister lineages. This is in contrast to the findings of Lausen et al. (2008) who present morphological,

behavioural and genetic evidence that two of these subspecies (*M. l. lucifugus* and *M. l. carissima*) are taxonomically synonymous and recommend that the *carissima* subspecies designation be dropped due to complete sympatry with *M. l. lucifugus* (Dewey 2006) and thus widespread interbreeding, and inability to differentiate 'subspecies' based on any of the original defining traits (Simmons 2005). This widespread sympatry and interbreeding is supported by Morales and Carstens (2018), but their argument for species designation is one of semantics and a long-standing debate: Morales and Carstens (2018) uses the phylogenetic species concept (reviewed in Wheeler and Meier 2000), defining species as monophyletic lineages based on historical divergence, whereas our study and Lausen et al. (2008) use the genetic species concept (Baker and Bradley 2006) requiring genetic isolation (but not necessarily reproductive isolation). While we do not wish to entertain here a continuation of the debate between systematists and conservation biologists (e.g., Goldstein et al. 2000) recall that our study arose out of the need to differentiate *M. keenii* from *M. evotis*, given the extreme differences in their potential status listings (vulnerable vs least concern globally, respectively; NatureServe 2018), *M. keenii*'s data deficient status nationally (COSEWIC 2003), and the absence of reliable genetic, morphological, or geographic boundaries to differentiate the two species. Extensive range-wide gene flow among 'phylogenetic species' does not lend itself to species diagnosability, and therefore erodes conservation programs that depend on differentiable attributes in present-day individuals.

Our microsatellite results clearly delineate *M. thysanodes* as a distinct species and are congruent with field experience, where morphological traits differentiate *M. thysanodes*. This is, not surprisingly, in contrast with our mtDNA sequences revealing identical haplotypes

between *M. thysanodes* and the *M. keenii/evotis* group. Taxonomic confusion resulting from analysis of mtDNA has been reported in other species of bats (e.g., Lausen et al. 2008) and other taxa (e.g., Paetkau et al. 1998), stemming from its single locus, maternally-inherited, non-recombining nature. In conclusion, our microsatellite results clearly resolve distinct breeding groups that align with identification based on field morphometrics: *M. septentrionalis*, *M. lucifugus*, and *M. thysanodes* as distinct species, and *M. evotis* and *M. keenii* as one species, aligning with the lack of morphological distinctness (Nagorsen and Brigham 1993). Some admixture seen in the STRUCTURE assignment of individuals at $k = 2$ (Fig. 4), may provide support for Morales et al. (2017) who suggested some degree of evolutionary gene flow has occurred between *M. thysanodes* and *M. evotis/keenii*.

A gradual accumulation of genetic differentiation over geographic space that we observed in the *M. evotis/keenii* cluster from west to east (Fig. 2C) suggests a semi-continuous population system connected by gene flow throughout (population structure). This was not strong enough population substructure to be detected using ΔK (Evanno 2005) but it does suggest gene flow in an isolation by distance pattern that may be mediated largely by males, as evidenced by the lack of common mitochondrial haploytpes in coastal and interior populations, and the ~ 1% divergence of those regional haplotype groups. Our F_{ST} data also suggest that genetic connectivity varies across the study region, rather than conforming to a strict 'isolation by distance' model. This pattern may reflect the complex landscape forms that facilitate and limit gene flow such as high elevation mountains and low-lying river valleys. For example, F_{ST} = 0.02 between Juneau, Alaska and southern Vancouver Island (~ 1,300 km separation along the western coast), but 0.03 between Vancouver Island and the eastern side of the Coast Mountain

range (Skagit; ~ 200 km separation over a mountain range). However, all of these values are small, and sampling on the northeast side of the Coast Mountains too limited to identify specific landscape features as limiting to gene flow.

While our analysis of nuclear markers clearly delineated 4 species -- *M. thysanodes*, *M. evotis*/*keenii*, *M. lucifugus* and *M. septentrionalis* – the mitochondrial *Cyt b* gene presented a confusing mix of *M. evotis*, *M. keenii*, and *M. thysanodes*. Using mtDNA, Dewey (2006), Carstens and Dewey (2010), Stadelmann et al. (2010), and Platt et al. (2018) present multiple species trees that include some *M. lucifugus* subspecies and *M. evotis/keenii/thysanodes*. Platt et al. (2018) shows complete discordance between mtDNA (single locus) and nuclear-derived (thousands of loci) species trees. These results provide cautionary examples of the risks of using any single marker to study relationships (Platt et al. 2018), particularly if that single marker only records the gene flow mediated by one sex (e.g. Cronin et al. 1991; Worthington-Wilmer et al. 1994 vs. 1999; Talbot and Shields 1996 vs. Paetkau et al. 1998; Elias et al. 2007; Lausen et al. 2008;). Additionally, the discordance of mtDNA with morphological and geographic divisions among well-defined species (e.g. *Sorex* spp., Demboski and Cook 2001; *Eptesicus* spp., Mayer and von Helversen 2001; butterflies, Elias et al. 2007), together with its inability to represent contemporary gene flow, serves caution to those interpreting mtDNA sequence divergence in a taxonomic context.

Examination of *M. evotis* and *M. keenii* based solely on nuclear DNA includes Platt et al. (2018) and this study. The former included nuclear sequencing of one individual per species (1 *M. evotis* from Mexico and 1 *M. keenii* from Alaska). Our study using population genetics techniques to thoroughly examine more than 160 *M. evotis/keenii* individuals from across northwestern North America (Alaska, BC, Washington and Alberta), is the first of its kind and has shown widespread contemporary gene flow

among *M. evotis/keenii* requiring a restructuring of taxonomic classification to best reflect biologically relevant processes for conservation and management purposes.

Myotis keenii has undergone much taxonomic revision. Initially considered to consist of two subspecies – *M. k. keenii* and *M. k. septentrionalis* (Fitch and Shump 1979) – differences in crania between the two groups (Genoways and Jones 1969) led van Zyll de Jong (1979) to investigate cranial, dental and external characters of specimens from across Canada, leading him to recognize *M. septentrionalis* as a distinct species. A more thorough geographic sampling of *M. keenii* by van Zyll de Jong and Nagorsen (1994) was stimulated by remarks (e.g., Cowan and Guiguet 1965; Davis 1967) on the difficulty of differentiating *M. keenii* from *M. evotis* in areas of sympatry. Samples in this 1994 study spanned a wide area (Alaska, BC, Washington, Oregon, and Canadian prairie provinces), but there were substantial gaps in sampling. A priori reference groups were Haida Gwaii (Queen Charlotte Islands) for *M. keenii*, and east of the Coast Mountains for *M. evotis*. The presumed overlap zone for the two species was the coastal strip west of the Coast Mountains, including Vancouver Island.

For comparisons of morphology among the reference groups, van Zyll de Jong and Nagorsen (1994) used ear length, forearm length, lengths of third and fifth metacarpals, and length of the tibia, finding that all five external morphological measurements and 12 cranial measurements were significantly smaller in the specimens of *M. keenii* compared to specimens of *M. evotis*. Measurements for *M. keenii* (Haida Gwaii) were compared to *M. evotis* from Oregon, Washington, southern BC, and the Canadian prairies. All specimens of *M. evotis* were from locations further south than the *M. keenii* samples, raising the possibility that the clines in body size (ear length and forearm) that we describe in our study underpinned the findings of

van Zyll de Jong and Nagorsen (1994) that *M. keenii* was smaller than *M. evotis*. Van Zyll de Jong and Nagorsen (1994) assigned intermediate specimens to a putative hybrid zone between *M. keenii* and *M. evotis*, but we propose that these intermediate forms represented different points along morphological clines in body size. Although large geographic gaps separated his 6 samples from the western United States, a principal component analysis by Manning (1993) demonstrated possible clines of decreasing cranial size and increasing pelage darkness from east to west. Similar to the forearm size cline in *M. evotis/keenii*, we found that forearm lengths of the other wide-ranging bat species in our study, *M. lucifugus*, are shorter in the northwest part of our study region (Alaska, Haida Gwaii and Hazelton) compared to southern/eastern conspecifics. That similar morphometric patterns are seen in *M. evotis/keenii* and *M. lucifugus* suggests that common ecological factors may drive the regional variation in bat body size. Van Zyll de Jong and Nagorsen (1994) report darker fur in coastal *M. evotis* versus more inland samples, a pattern we also observed in our *M. evotis/keenii* samples. Using reflectance measurements from the mid-dorsal region of study skins, Manning (1993) demonstrated that *M. evotis* populations from coastal Oregon and Washington were darker than populations east of the coastal ranges in the United States. This pattern of darker pelage in western coastal regions occurs in other mammals (Nagorsen 2002) and may support Gloger's Rule. Environmental influences on pelage colour and morphology have added to the confusion around the taxonomy of other bat species (e.g., *M. daubentonii*- Bogdanowicz 1990) and latitudinal influence over forearm length has been reported in other bat studies (e.g., *M. daubentonii*- Bogdanowicz 1990; *M. lucifugus*- Lausen et al. 2008). Van Zyll de Jong and Nagorsen (1994) hypothesized that "an ecological factor (or factors) associated with the coast

rain forest ecosystem has prevented the species from expanding beyond it" (p. 1076). Our morphological analysis supports this, but our genetic analysis does not, suggesting that the morphological features thought to differentiate *M. keenii* from *M. evotis* reflect ecological or environmental induction differences. This highlights the importance of thorough geographic sampling and examination of potential clines when investigating species boundaries.

We conclude that *M. keenii* does not qualify as a genetic (Baker and Bradley 2006) or biological species (Mayr 1969) and this taxon should be synonymized with *M*. *evotis*. According to the taxonomy of Manning (1993) based on size and pelage colouration, populations formerly classified as *M*. *keenii* fall within the range of *M*. *evotis pacificus* a smaller darker northwest coastal subspecies distributed from northern California to British Columbia. This has been distinguished from the larger paler *M. e. chrysonatus* with an extensive range inland across southern B.C., Alberta and the western United States (Manning 1993). Four additional subspecies with restricted ranges in the southwestern United States and Mexico were recognized by Manning (1993) and although there have been questions raised (e.g., van Zyll de Jong and Nagorsen 1994, Alvarez-Castafieda and Bogan 1997), these 6 nominate subspecies are currently recognized by authorities such as Wilson and Reeder (2005). Because of the extensive gene flow and clinal variation demonstrated in our study we suggest a review of subspecies distinctions across the full range of *M*. *evotis* may be warranted. Because we sampled across the northern distribution of *M. evotis* which did not include the type locality for this species (Monterey, California), we suggest a geographically broader population genetic study could be informative.

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Table 1. Haplotypes of *Cyt b* sequences. Genetic species identification listed here reflects the closest match to existing GenBank sequences with the BLAST search tool (similarity in brackets). Submitted sequences' accession numbers listed from this project.

Genetic Species Assignment	Number of individuals sequenced (Field ID and Locations)	Haplotype and Range of Number of Base Pairs	Base Pair differences	Accession Numbers
M. keenii (97-100%)	1 – *M. thysanodes* (Okanagan) 25 – *M. keenii* (Alaska, Skagit, Vancouver Island, Haida Gwaii) 9 – *M. keenii*/evotis (Hazelton, Washington, Skagit, Vancouver Island) 3 – *M. evotis* (Skagit)	ke1, ke1a, ke1b, ke1c; 339 – 748 bp	1 bp between ke1 and each of ke1a, ke1b, ke1c	MH012242, MH012243, MH012244, MH012245
M. evotis (99-100%)	7 – *M. evotis* (southern Alberta, Okanagan, Kootenay, Hazelton, Skagit) 3 – *M. evotis*/keenii (Hazelton, Skagit) 1 – *M. thysanodes* (Okanagan) 1 – *M. thysanodes/evotis* (Okanagan)	ev1, ev1a; 561 – 723 bp	ev1 - ke1 by 6 bp; ev1 - ev1a by 1 bp.	MH012246, MH012247
M. septentrionalis (99-100%)	4 – *M. septentrionalis* (Alberta) 4 – LEMY (Hazelton)	se1, se1b	se1 - ke1 by 78 bp; se1-se1b differ by 2 bp.	MH012253, MH012254
M. lucifugus (94-100%)	8 – *M. lucifugus* (Hazelton, Bella Coola,	lu1, lu1a, lu2, lu3; 590-750 bp	differences: lu1-2 by 19 bp; lu2-3 by	MH012249, MH012250,

specifically: *M. l. alascensis* (Hazelton, Bella Coola), *M. l. lucifugus* (Okanagan, Kootenay), *M. l. relictus* (Kootenay, Flathead)	Okanagan, Kootenay, Flathead)		31 bp; lu1-3 by 10 bp; lu1 - lu1a by 1 bp; lu1 – ke1 by 23 bp; lu2 – ke1 by 32 bp; lu3 – ke1 by 13 bp.	MH012251, MH012252
Special Cases:				
M. thysanodes / *M. evotis* (100%)	2 – *M. evotis* (Alberta)	ev2 561-749 bp	ev2 - ev 1 by 4 bp	MH012248
M. evotis (98-99%)	5 – *M. thysanodes* (Okanagan, Lillooet, Montana)	th1 511 – 719 bp	th1 – ke1 by 15 bp	MH012255

Fig. 1. Genetic sampling locations. V. Island is Vancouver Island, Olympic Pen. is Olympic Peninsula. Sites where *M. evotis/keenii* were sampled are denoted with an underline if they have been considered to be part of *M. keenii* range only, an asterisk if considered to be outside of *M. keenii* range, and no denotation if the sites were considered within the overlap of both species' distributions.

Fig. 2. Cluster results from GENETIX for (A) *Myotis septentrionalis* (solid triangles), *Myotis thysanodes* (X), *Myotis lucifugus* (solid circles) and the *Myotis evotis*/*keenii* group (open circles and open squares), (B) *Myotis thysanodes* (X), Myotis coastal long-eareds (open squares) and interior long-eareds (open circles) and (C) *Myotis evotis/keenii* group. All sampling locations included: Coastal (X); southeast Alaska, Haida Gwaii, Vancouver Island, WA (Olympic Peninsula), interior west (open circles; Hazelton, Lillooet, Okanagan, Skagit), and the interior east (open squares; Kootenays, Flathead, and southwest Alberta).

Fig. 3. A) Evanno's criterion Δ*K* applied to STRUCTURE output to assess the number of groups (*k*) in the data for all genotyped individuals representing 5 putative species: *Myotis septentrionalis, M. thysanodes, M. lucifugus, M. keenii*, and *M. evotis*. Heavily sampled sites for the latter two species were subsampled to reduce bias in the estimation of *k* (Puechmaille 2016), which was found to be 4. B) STRUCTURE barplot for group membership of individuals in the 4 species groupings shows a small level of admixture for some individuals.

Fig. 4. Evanno's Δ*K* method applied to STRUCTURE output determined *k* = 2 for dataset containing *M. thysanodes* and the subsampled dataset of *M. evotis* and *M. keenii* (A). These two clusters correspond to two species: *M. thysanodes* and *M.evotis/keenii* with a small degree of admixture between species for some individuals (B).

Fig. 5. Map of *M. evotis/keenii* sampling locations with pairwise F_{ST} scores. Higher F_{ST} values correspond to east-west comparisons, with minimal values in the north-south dimension. Glaciers are represented

by texture on map. Relatively low F_{ST} values support *M. evotis* and *M. keenii* as one interbreeding group (i.e. one species) across the study area.

Fig. 6. Ear pinnae (A) and forearm (B) lengths of *M. evotis/keenii* regressed against latitude*longitude.

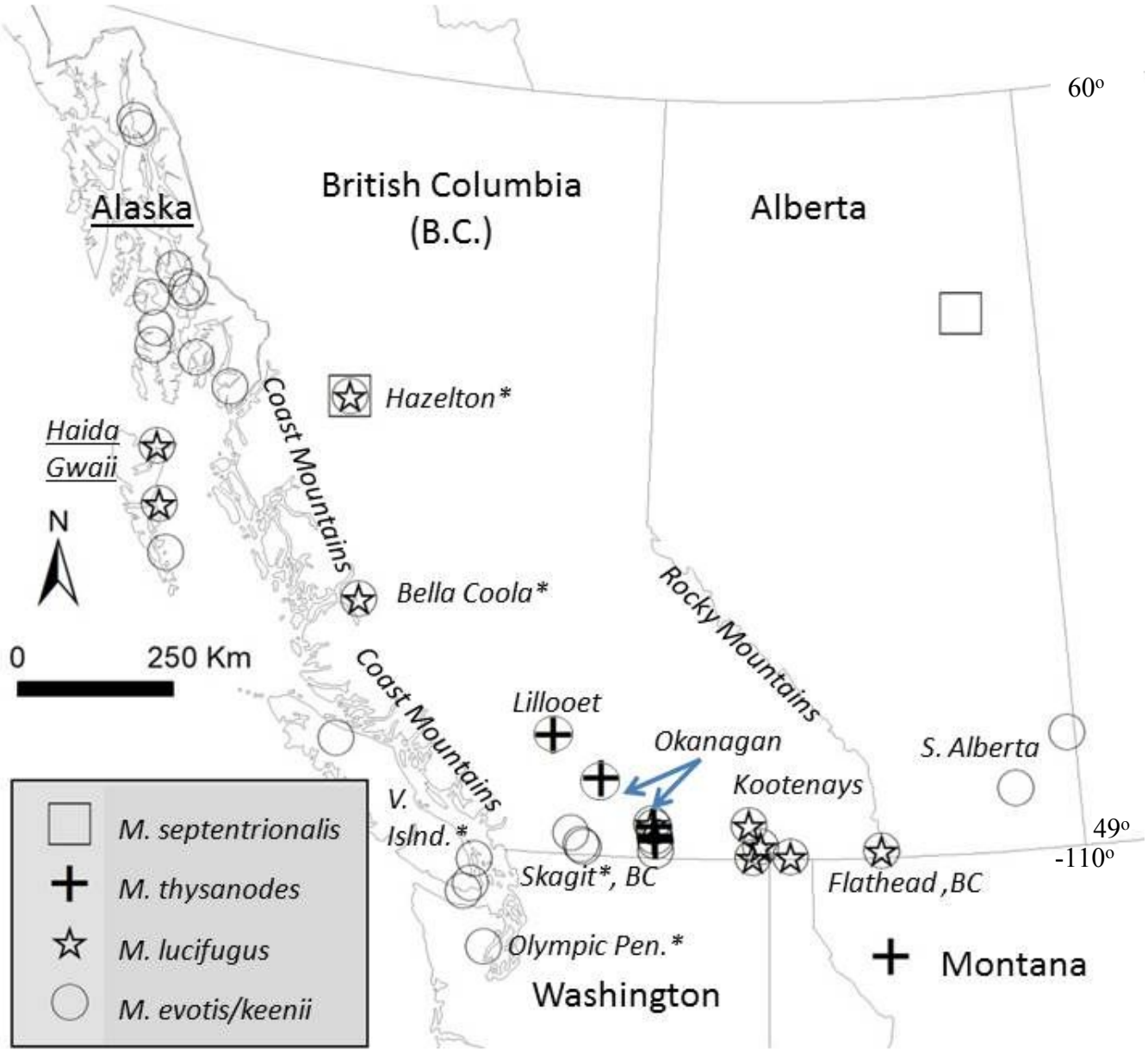

60º
British Columbia
(B.C.)
Alberta
Alaska
Hazelton*
Haida
Gwaii
N
Coast Mountains
Bella Coola*
Rocky Mountains
0
250 Km
Coast Mountains
Lillooet
Okanagan
S. Alberta
Kootenays
V.
Islnd.*
49º
-110º
Skagit*, BC
Flathead ,BC
Olympic Pen.*
Montana
Washington
M. septentrionalis
M. thysanodes
M. lucifugus
M. evotis/keenii

Figure 1.

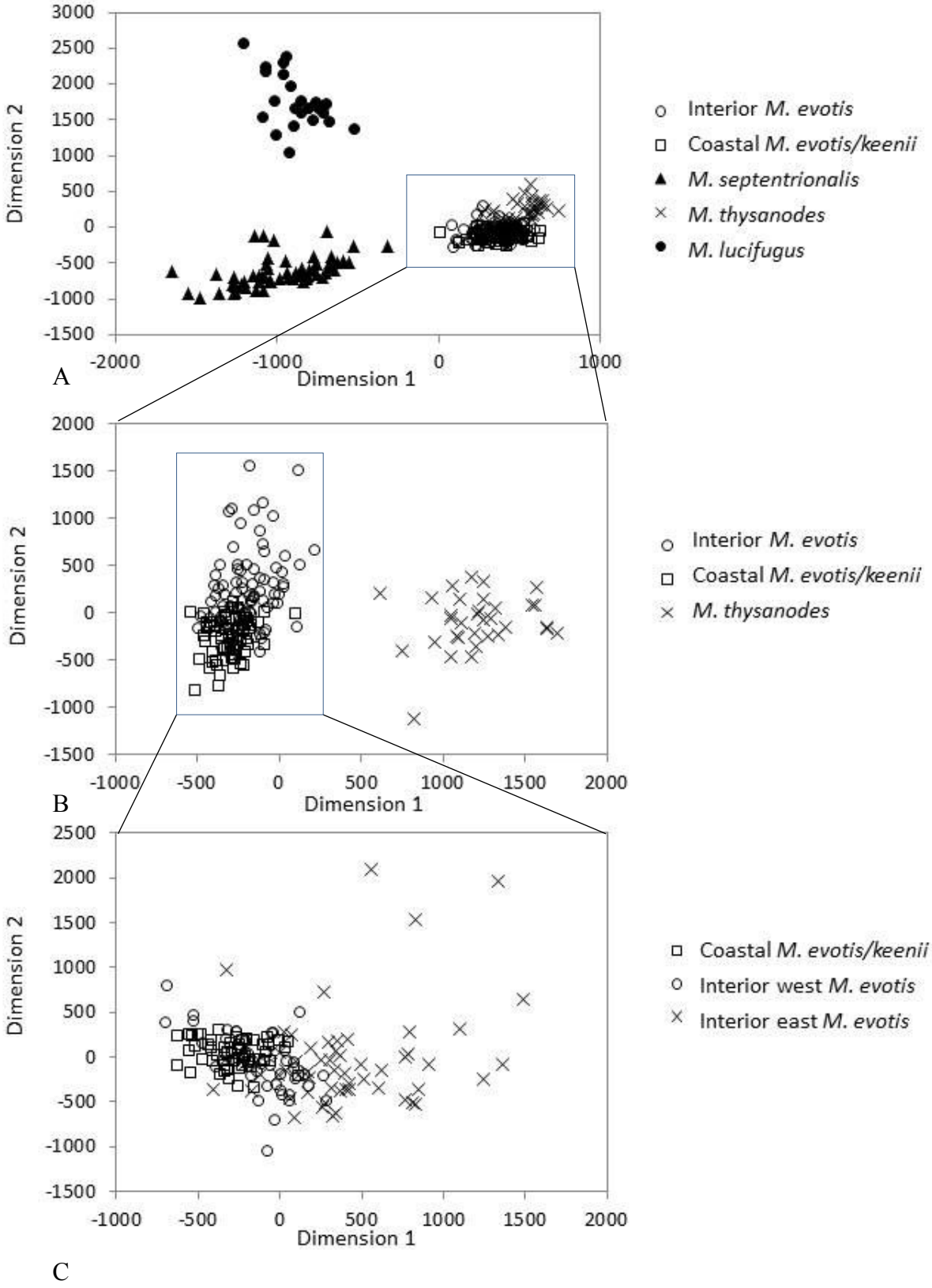
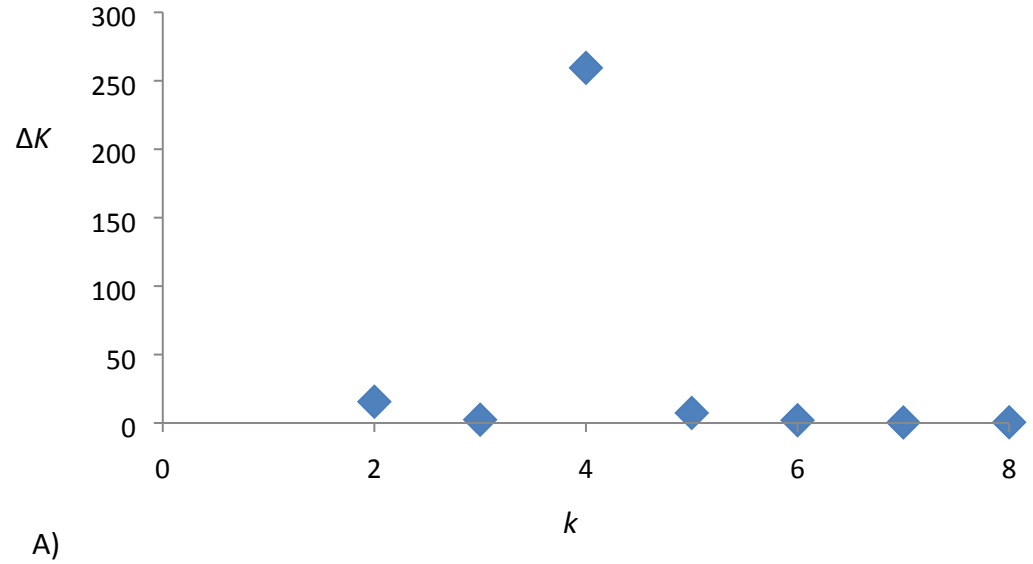
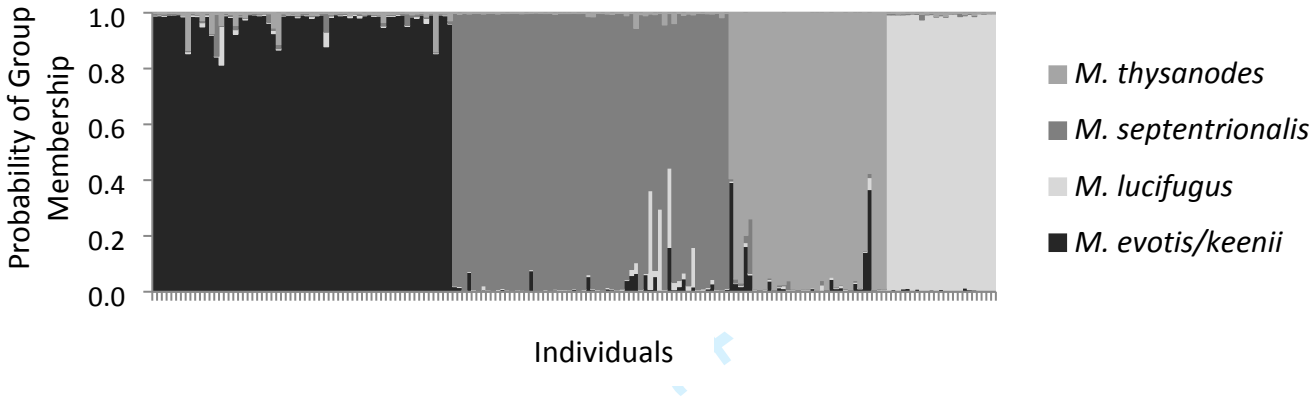

Dimension 2
Dimension 1
Interior M. evotis
Coastal M. evotis/keenii
M. septentrionalis
M. thysanodes
M. lucifugus
A
Interior M. evotis
Coastal M. evotis/keenii
M. thysanodes
B
Coastal M. evotis/keenii
Interior west M. evotis
Interior east M. evotis
C

Figure 2.


300
250
200
150
100
50
0
ΔK
0
2
4
6
8
k

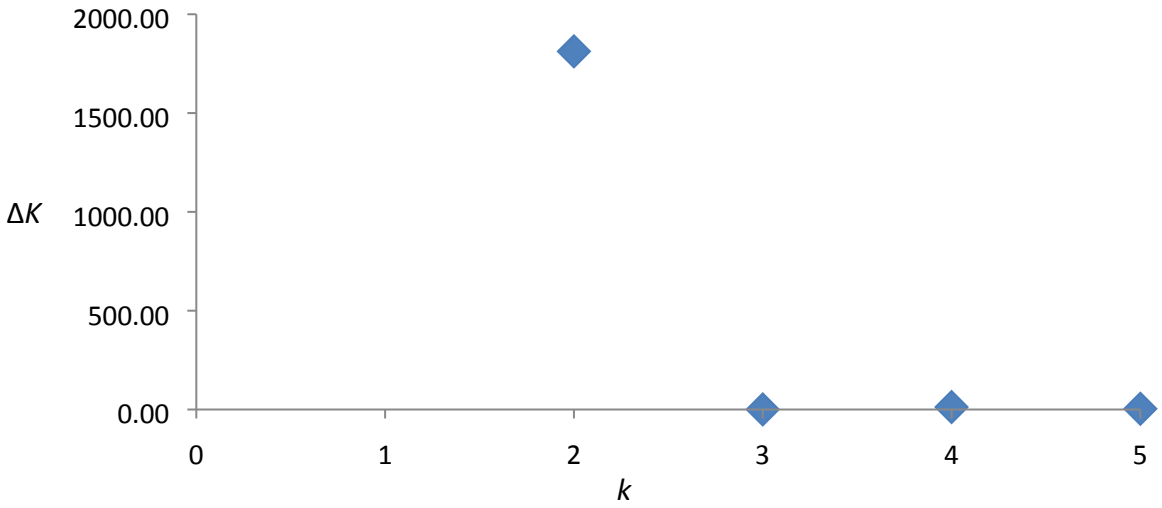
A)


Probability of Group Membership
1.0
0.8
0.6
0.4
0.2
0.0
Individuals
M. thysanodes
M. septentrionalis
M. lucifugus
M. evotis/keenii

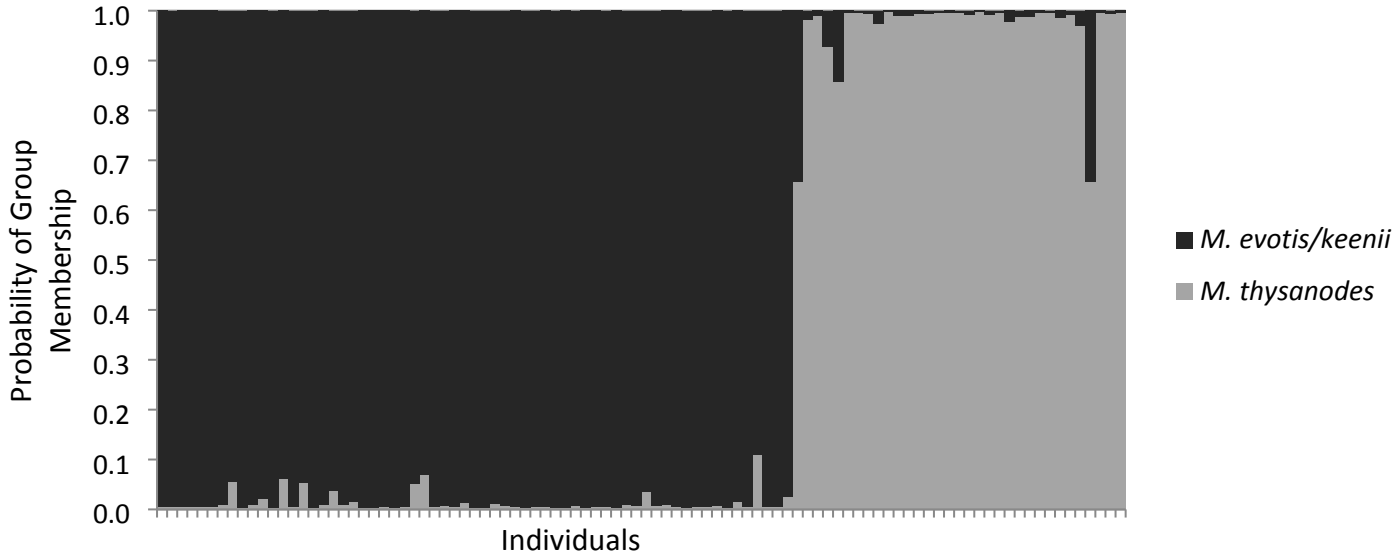
B)

Figure 3.

k


2000.00
1500.00
1000.00
500.00
0.00
ΔK
0
1
2
3
4
5
k

A)


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Probability of Group Membership
Individuals
M. evotis/keenii
M. thysanodes

B)

Figure 4.

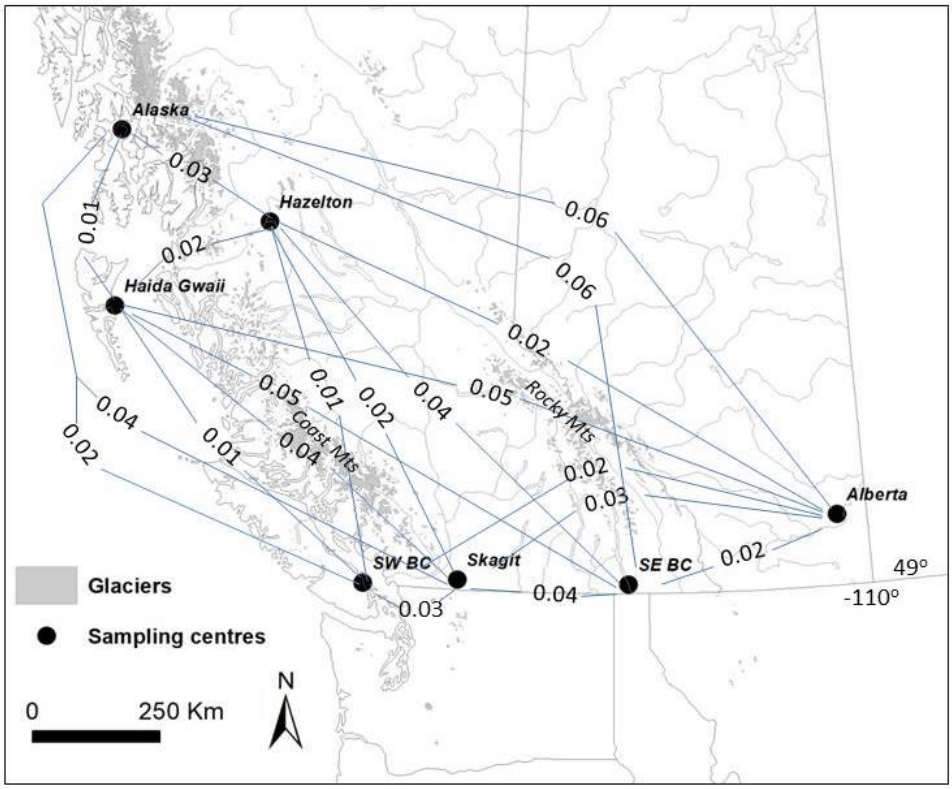
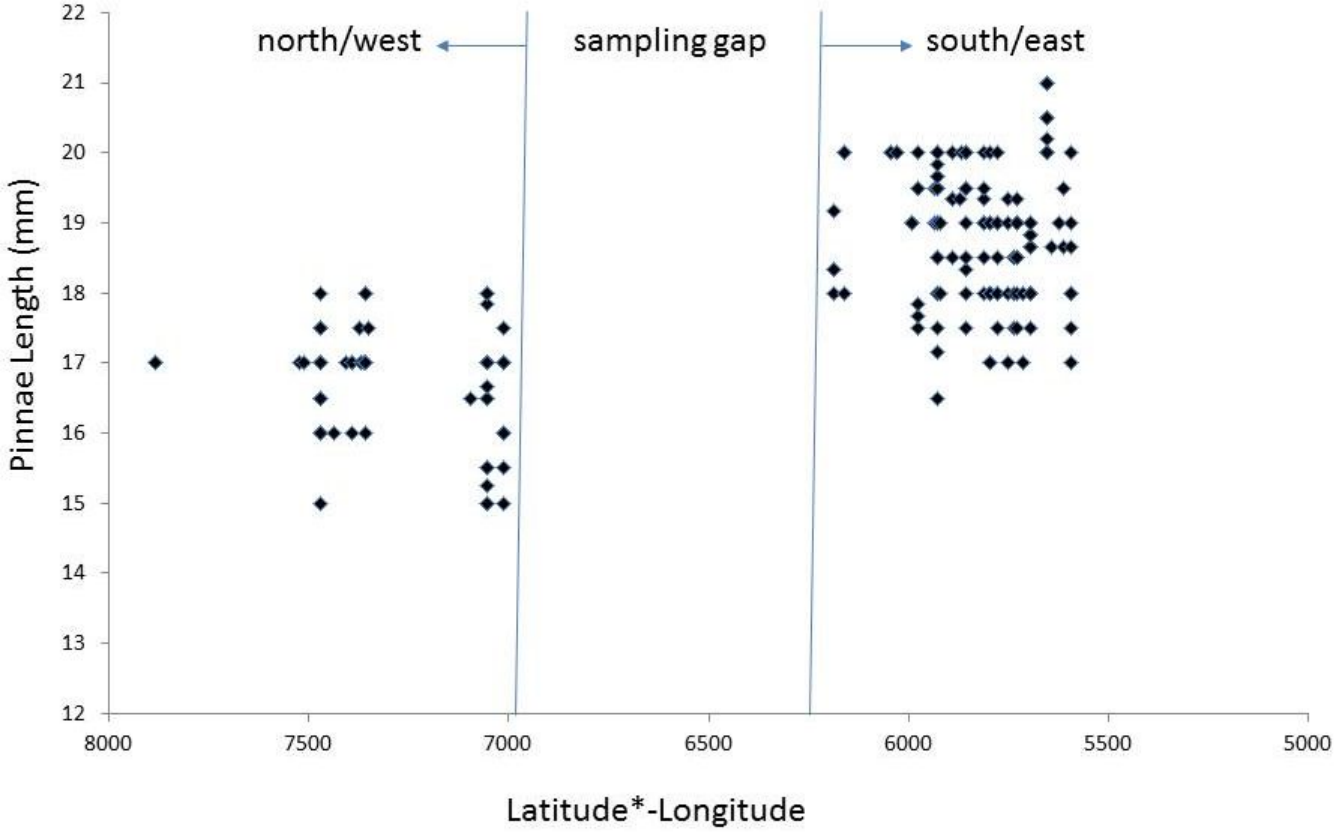
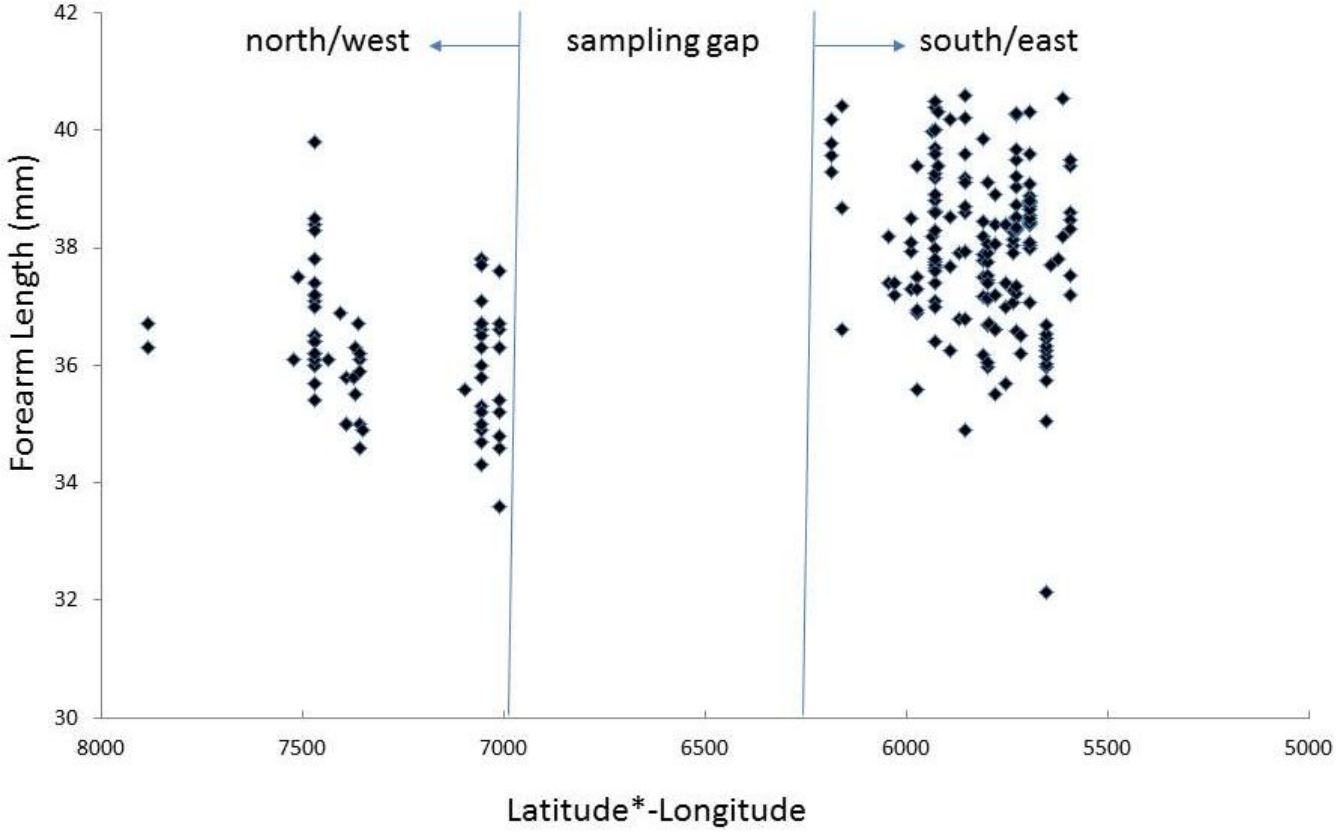


Figure 5


north/west
sampling gap
south/east
Pinnae Length (mm)
22
21
20
19
18
17
16
15
14
13
12
8000
7500
7000
6500
6000
5500
5000
Latitude*-Longitude

A)


north/west
sampling gap
south/east
Forearm Length (mm)
42
40
38
36
34
32
30
8000
7500
7000
6500
6000
5500
5000
Latitude*-Longitude

B)

Figure 6.